University of Southampton

UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH & LIFE SCIENCES

School of Biological Sciences

Parasitoid fitness and Cry1Ab.

Does the insecticidal protein Cry1Ab derived from *Bacillus thuringiensis* affect the beneficial parasitoid *Cotesia marginiventris*?

by

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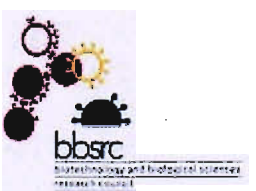

syngenta

Paracelsus; also known as the father of toxicology wrote,

**"All substances are poisons:
there are none which is not a poison.
The right dose differentiates a poison
and a remedy"**

Paracelsus (1493-1541)

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES
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Doctor of Philosophy

PARASITOID FITNESS AND CRY1AB - DOES THE INSECTICIDAL PROTEIN CRY1AB DERIVED FROM *BACILLUS THURINGIENSIS* AFFECT THE BENEFICIAL PARASITOID *COTESIA MARGINIVENTRIS*?

By Susan Kay Smith

Transgenic insecticidal crops are in widespread use throughout world agriculture and have the potential to pose a risk to non-target organisms. Any novel transgenic crop requires rigorous testing prior to commercialisation using an ecological risk assessment framework. The aim of this study is to help inform this process using the parasitoid *Cotesia marginiventris*, the host *Spodoptera littoralis,* purified Cry1Ab insecticidal protein and Bt176 transgenic maize. Laboratory worst case scenario experiments were carried out to establish the host-mediated, direct and transgenerational effects of Cry1Ab on the beneficial parasitoid *C. marginiventris*

ELSIA analysis was undertaken to establish the expression levels of Cry1Ab in Bt176 transgenic maize, mean expression levels were 11μg g^{-1} and ranged between 7.5 and 15.3μg g^{-1} fresh weight dependant on the age and leaf stage of the plant.

To assess the direct toxicity of the Cry1Ab toxin activated Cry1Ab was diluted to 1mg ml^{-1} and subcutaneously injected into *S. littoralis* hosts parasitized by *C. marginiventris* avoiding contact with the host gut where pathogenicity occurs. In feeding trials to assess host mediated effects activated Cry1Ab was added to artificial diet at concentrations of 100, 20, 10, 2, 1 and 0μg g^{-1} Cry1Ab. *C. marginiventris* larvae were raised on *S. littoralis* larvae fed with one of the respective artificial diet treatments above, Bt176 transgenic maize or its near isoline. The objectives of these feeding studies was to establish any indirect or direct toxicity of the Cry1Ab protein on *C.*

Marginiventris, and the host mediated dose at which any effects could be seen compared to plant expression levels.

Under direct exposure in injecting trials, parasitoid tibia length and sex ratio showed no significant difference between *C. marginiventris* injected with Cry1Ab or distilled water. Mean pupation was significantly longer in Cry1Ab injected treatment (9.5 days) when compared to the injected control group (9.1 days). *C. marginiventris* raised on *S. littoralis* hosts ingesting the toxin at 100µg g^{-1} Cry1Ab showed a significantly extended pupation time (11.1 days) when compared to controls (9.5 days), mean tibia length was also significantly shorter at this concentration. The negative effects on pupation time and tibia length were therefore only measurable at 5-10 times plant expression levels. ELISA analysis confirmed that *S. littoralis* hosts ingested high amounts of Cry1Ab whilst feeding on both plant tissue and artificial diet treatments. However, whilst high concentrations of Cry1Ab were found in *S. littoralis* larvae, frass and parasitoid cocoons none was detectable in parasitoid adults. Therefore the toxin is not accumulating up the food chain and appears to be secreted by *C. marginiventris* juveniles prior to adult emergence.

Further laboratory feeding experiments were carried out to establish the host mediated effects of Cry1Ab on *C. marginiventris* attack rate, host preference, parasitism success, emergence success and tibia length when reared on *S. littoralis* hosts ingesting 200, 20, 0µg g^{-1} Cry1Ab, Bt176 transgenic maize or its near isoline. Host preference of adult parasitoids was also assessed to establish if adults showed any avoidance behaviour toward hosts reared on Bt176 maize, no avoidance behaviour was found. There was no significant difference between attack rate, proportion of attacks resulting in cocoon formation or proportion of adults emerging between the treatments. Tibia length was significantly shorter in the Bt176, 200 and 20µg g^{-1} Cry1Ab treatments when compared to the near isoline and control artificial diet treatments. However, there was a trend towards reduced parasitism and emergence success following exposure to the Cry1Ab toxin. *C. marginiventris* larvae were exposed to the toxin via *S. littoralis* hosts at 100µg g^{-1} over two generations. Following two generations of exposure to the toxin pupation time was significantly longer (10.4 days) when compared to controls (10.0 days). Neither tibia length, parasitism or emergence success were significantly different following exposure over two generations.

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Definitions and abbreviations.

Acute tests short term tests usually carried out at relatively high concentration often used to determine the dose at which 50% of test organisms die (see LC_{50} below).

Assessment Endpoint an explicit expression of the environmental value to be protected and its attributes for example a beneficially insect and its fecundity and recruitment.

Bacillus thuringiensis (Bt) soil dwelling bacterium from which insecticidal proteins can be derived.

Chronic tests longer term terms which look at no lethal effects of ecological significance for example reproductive capacity and growth.

Cry proteins crystalline insecticidal protein genes derived from *Bacillus thuringiensis*.

ECB – European Corn Borer

ERA – Ecological Risk Assessment

Exposure how likely the organism of interest is to come into contact with the stressor.

Hazard how toxic a substance is to the organism of interest.

Hazard Quotient (HQ) ratio of the exposure dose divided by minimum dose above which adverse effects can be seen, allowing an assessment of risk to be made.

Lethal Concentration 50 (LC_{50}) test concentration at which 50% of the test organisms suffer mortality.

Measurement Endpoint ecological attributes that are effected by the contaminant are unambiguous and readily measurable these must be closely related to the assessment endpoint for example mortality and pupation time.

No observable effect concentration (NOEC) is the greatest concentration or amount of a substance, found by experimentation or observation, which causes no detectable adverse alteration of morphology, functional capacity, growth, development or life span of the organism under defined conditions.

Non-target organism any organism affected by the gene product but for which the product was not designed.

PEP - Phosphoenolpyruvate

Risk a combination of the effects of hazard and exposure.

Sub-lethal effects those effects that have ecological significance for example reproductive capacity and growth but are not fatal.

Type 1 error a truly non-significant result deemed significant by a statistical test.
Type 2 error a truly significant result deemed non-significant by a statistical test.

USEPA United States Environmental Protection Agency.

i. Aims and Objectives

i. Aims

The aim of this study was to place host mediated and direct exposure experiments within an ecological risk assessment (ERA) framework to enhance and inform the decision making process already in place and avoid where appropriate unnecessary, costly and time-consuming testing at a higher tier. Avoiding higher tier testing when appropriate may be prudent as with increasing complexity comes increased cost and with increasing realism comes decreasing statistical power (Romeis et al., 2006a).

ii. Objectives

1 Biological variation.

Establish the natural variation within the system at the first and second trophic levels to allow consumption levels of the toxin to be evaluated.

Chapter 2.

2 Bt176 Cry1Ab expression levels

Establish the level and variation of Cry1Ab expression in Bt176 to allow an assessment to be made of how any effects of direct or host mediated toxicity compare to field exposure levels.

Chapter 3

3 Direct and Host mediated toxicity

Assess the impact of direct and indirect effects of the toxin i.e. those due to sub-optimal host and those due to the direct toxicity (hazard) of the toxin.

Chapter 4

4 Effect on successive generations

Establish the cumulative effect of the Cry1Ab toxin on parasitoid fitness following exposure to the purified over several generations and any host avoidance behaviour.

Chapter 5

5 Trigger values

Establish the level of Cry1Ab exposure at which effects occur in the parasitoid and relate these to levels which may trigger higher tier testing.

This objective incorporates work from several chapters and is discussed and summarized in **Chapter 6**

Chapter 1

Introduction

An overview of the risk assessment process, Cry1Ab toxin, genetically modified maize and non-target insects.

Project rational and summary.

1. 1 Introduction

Life on Earth depends on the effective functioning of a huge variety of many interrelated and interconnected ecological systems (Lovei and Arpaia, 2005). Therefore any loss or perturbation to ecological systems due to the introduction of a stressor in the environment, such as genetically modified (GM) crops requires careful study to ensure there is no loss of biodiversity at a local, regional or global scale. However, as with any new or emerging technology there may be an inherent or unknown risk, the risk assessment process seeks to quantify and clarify these risks.

The genetic modification of crops may provide a way of helping meet the specific needs of the world population as it continues to expand and provide solutions to problems linked to drought (Daniell, 2002), the production of affordable, transportable pharmaceuticals (Daniell et al., 2001; Daniell et al., 2002; Dunwell, 1999), as well as its more conventional role in protecting against crop losses to pathogen (Daniell et al., 2002) and insect attack (Fearing et al., 1997). Improvements in crop yield may also reduce the pressure on "wilderness" being converted to agricultural land where population increases continue (Atkinson et al., 2001). The use of genetically modified plants resistant to insect attack also has the potential to offer reduced pesticide use (Lovei and Arpaia, 2005) and ease of pest control due to the systemic, season long expression of plant incorporated insecticidal genes.

This concept of the human manipulation and alteration of crops is not novel, however what has changed is the process by which this manipulation occurs which has altered radically over the last few decades, along with the phenotypic traits that can be introduced. The manipulation of crops to improve their yield or resistance to pests has probably occurred since agriculture first appeared via the selective breeding of the most robust or high quality plants. Maize is a particularly striking example of this transformation with the modern variety of maize almost unrecognisable from its ancestral form with the modern form having approximately a 100 times greater yield (Belzile, 2002).

1.1.1 What is Environmental Risk Assessment and why is it important?

Transgenic crops or genetically modified (GM) crops as they were formerly referred it (James, 2005) offer a huge potential to advance crop production methods however potential risks must be measured and quantified where possible. Therefore, it is vital to have a robust and rigorous process that allows these potential risks to be assessed and monitored. In very simple terms Environmental Risk Assessment is a process that allows informed decision making by estimating the potential harm new and/or novel substances may cause when released into the environment (Maltby, 2006). Ecological risk assessment (ERA) is the component of environmental risk assessment which assesses these effects on non-anthropic communities and ecosystems (Maltby, 2006). It is an important tool in regulating novel technologies such as genetically modified (GM) crops by providing a scientific and clearly defined process to allow the regulation and registration of such technologies (Maltby, 2006).

Within a risk assessment framework the assessment of any effect comprises of three phases these being; 1 – Problem formulation, 2 – Analysis and 3 – Risk characterisation (Iscan, 2004; Maltby, 2006). Problem formulation identifies potential hazards and formulates the study plan whereas the analysis phase assesses the exposure to and effects of the stressor whilst finally the risk characterisation draws together the potential exposure and effects from the analysis phase to characterise the risk (Iscan, 2004; Maltby, 2006).

The characterisation of risk is therefore made up of two components these being "hazard" and "exposure"(Conner et al., 2003; Maltby, 2006; Raybould, 2004) and it is important to differentiate between these two components as there is no risk from a highly hazardous (toxic) substance if the study organism never comes into contact with it (exposure) (Maltby, 2006). The hazard can be more fully defined as "the intrinsic ability of the technology to cause harm" (Raybould, 2004), for example direct toxicity and exposure; how likely that harm is to occur (Raybould, 2004) i.e. is the organism likely to come into contact with the hazard . The experimental element of risk assessment can be used to define the potential hazard and this is the region of ecological risk assessment this study considers. To establish the potential for exposure to the hazard requires a detailed understanding of the biology and ecology of the system and this is outside the remit of this study.

1.1.1.i What is ERA trying to protect? Endpoints.

In carrying out a risk assessment it is vital to determine robust and measurable parameters on which to assess the potential risk, these are know as endpoints. There are generally recognised as being two types of endpoints: assessment endpoints and measurement endpoints (USEPA., 1994). Measurement endpoints as defined by the EPA (1994) are "ecological attributes that are adversely effected by contaminants and readily measurable.....and closely related to the assessment endpoint". An "assessment endpoint is an explicit expression of the environmental value to be protected, operationally defined as an ecological entity and its attributes" (USEPA., 1998) an ecological entity may be for example in important insect species and its attributes fecundity and recruitment (USEPA., 2003).

Measurement endpoints may be lethal or sub lethal effects or acute or chronic tests (USEPA., 2003). An example of sub lethal effects may be reduced growth, impaired reproductive capacity and/or behavioural changes (USEPA., 1994). Acute toxicity tests are short term tests at relatively high concentration and may determine the lethal concentration at which 50% of organisms die (LC_{50}), this will specify both the concentration and duration at which lethality occurs (USEPA., 1994). Conversely chronic tests can assess sub lethal effects which have ecological significance such as reduced growth that may for example lead to reduced reproductive capacity (USEPA., 1994).

The formulation of assessment endpoints require the input from a range of stakeholders including scientists as ecosystems are complex both temporally and spatially and it is not possible for all organisms to be the subject of an ecological risk assessment.

The assessment endpoint should be explicitly linked with the risk management goal ((USEPA., 2003) figure 1), for example conserving natural biological control agents. These overall assessment endpoints then further need to be linked to measures which can be made at an organism level, such as in the case of invertebrates, survival, fecundity and growth (USEPA., 2003). The effect of the stressor on these measurement endpoints can then be assessed under laboratory conditions to evaluate the impact on the assessment endpoint. Therefore all endpoints "must be unambiguous, susceptible to the stressor and accessible to prediction" (Poppy, 2003).

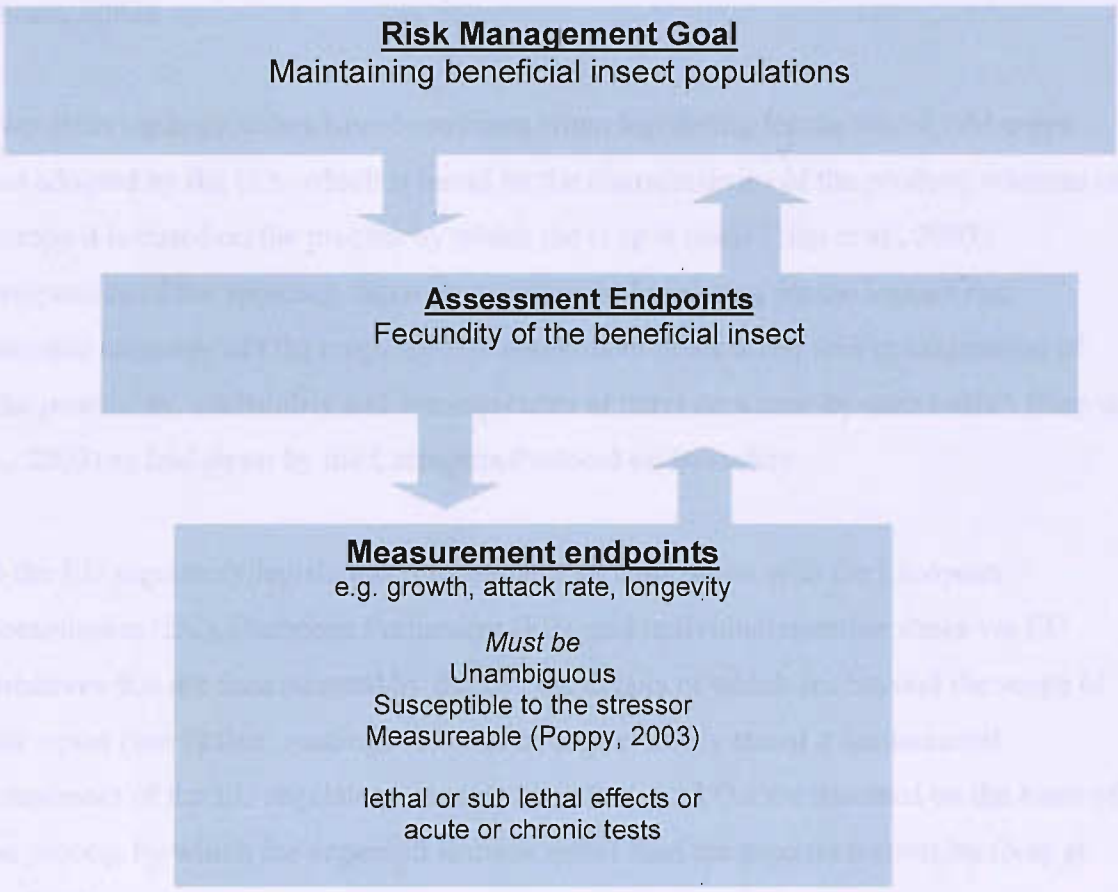


Figure 1.1 – Ecological risk assessment – the relationship between risk management goal, assessment endpoints and measurement endpoints.

1.1.1.ii Risk assessment - the Regulatory Approach

The risk assessment process is an integral part of European and world legislation for the introduction of genetically modified crops with the Cartagena protocol on Biosafety providing a generalised framework for the implementation of the legislatory framework within individual countries (Nap et al., 2003). The protocol expressly states that risk assessment relating to genetically modified organisms (G.M.O.s) should be undertaken on a case by case basis, using sound science and a transparent process, to identify and evaluate the potential adverse effects of G.M.O.s in the environment (http://www.unep.ch/biosafety/development/devdocuments/capbdgbrochEN.pdf, dated March, 2002). However, the protocol leaves individual nations to implement compliant

legislation, therefore the United Nations Environment Program was set up to assist individual countries signed up to the protocol in formulating their regulatory framework (http://www.unep.ch/biosafety/development/devdocuments/capbdgbrochEN.pdf, dated March, 2002).

Two differing approaches have been taken when legislating for the use of GM crops, that adopted by the U.S. which is based on the characteristics of the product, whereas in Europe it is based on the process by which the crop is made (Nap et al., 2003). Irrespective of the approach taken by countries in legislating for the impact and potential concerns of GM crops the risk assessment process requires consideration of "the possibility, probability and consequences of harm on a case-by-case basis" (Nap et al., 2003) as laid down by the Cartagena Protocol on Biosafety.

In the EU regulatory legislation is formulated in association with the European Commission (EC), European Parliament (EP), and individual member states via EU directives that are then adopted by the EP, the details of which are beyond the scope of this report (see further reading). However, as previously stated a fundamental component of the EU regulatory framework is that G.M.O.s are assessed on the basis of the process by which the organism is made rather than the product it contains (Nap et al., 2003).

Due to the explicit nature of the Cartagena Protocol setting down the use of risk assessment within the legislation for GM crops this project uses concepts taken from ecological risk assessment which are further underpinned by ecotoxicology. Ecotoxicology is the study of the "harmful effects of chemicals (toxicology) within the context of ecology" (Walker et al., 2001), whereas ecological risk assessment is a process of evaluating the likely outcome of harmful or undesirable ecological effects occurring (USEPA., 2003). A risk assessment therefore provides a basis to make an informed decision on the potential hazard or harm a substance may cause.

The concepts of familiarity and the precautionary principle guide ERA in its regulatory framework (Conner et al., 2003). The precautionary principle being part of the Cartagena protocol on Biosafety and also the basis of EU regulation (Conner et al., 2003). The precautionary principle is laid down in the Cartagena Protocol on Biosafety

and in essence states that "lack of scientific certainty.....and taking into account risks to human health, shall not prevent a decision being made, as appropriate" regarding the proposed G.M.O. (SCBD, 2000). However it has been argued that the lack of clarity in the definition can lead to the interpretation of "if in doubt do nothing" (SCBD, 2000).

1.1.1.iii The Tiered Testing Approach.

Risk assessment is based on a tiered approach with the level of realism at each tier increasing, for example a tier 1 test is considered to be the worse case scenario and undertaken exclusively in laboratory conditions for example studies carried out by Losey, et al, (1999) using pollen and monarch butterflies, tier 2 testing may involve more realism by using manipulated populations but in a controlled environment and tier 3 testing may occur in the field reflecting the natural environment. Both components of risk i.e. hazard and exposure can be evaluated at each level (Romeis et al., 2006a).

Tier 1 tests are highly controlled laboratory tests that indicate hazard and exposure in a controlled replicable manner. These tests indicate harmful effects in a variety of conditions and indicate if higher tier testing is required (White Paper on Tier-Based Testing for the Effects of Proteinaceous Insecticidal Plant-Incorporated Protectants on Non-Target Arthropods for Regulatory Risk Assessments http://www.epa.gov/oppbppd1/biopesticides/pips/tier-based-testing.pdf dated 6 April, 2007). This system of testing helps avoid the need for continuous data collection by indicating if higher tier testing is required and avoiding unnecessary testing (White Paper on Tier-Based Testing for the Effects of Proteinaceous Insecticidal Plant-Incorporated Protectants on Non-Target Arthropods for Regulatory Risk Assessments http://www.epa.gov/oppbppd1/biopesticides/pips/tier-based-testing.pdf dated 6 April, 2007). Higher tier tests can indicate if any risk identified in tier 1 tests is replicated in the field via field routes of exposure (Romeis et al., 2006a). As the level of realism increases so does the complexity and cost however, this increase in the level of realism and subsequent increase in experimental complexity can reduce the level of statistical power (Romeis et al., 2006a).

In cases were sufficient breath of knowledge and test data exist it may be possible to assess any potential risk without the need for further testing (Romeis et al., 2006a) however this would clearly not be the case with novel lines. Therefore, used correctly this process can reduce cost by preventing unnecessary testing.

However, the consideration of the acceptability of any risk identified by this process is not an intrinsic part of the risk assessment process rather a consideration for politicians and society (Conner et al., 2003) along with ethical and economic considerations. This final step being part of the broader risk management and risk decision process (Johnson et al., 2007).

1.1.2 Non-target organisms

Simply put a non-target organism is any organism that is affected by the gene product but for which the product was not designed. In the case of transgenic maize containing the Cry1Ab insecticidal protein the target is the lepidopteran pest *Ostrinia nubilalis* (European corn borer) (Romeis et al., 2006b) and an example of a non-target arthropod the hymenopteran parasitoid wasp *Cotesia marginiventris*.

In order to undertake a meaningful and rigorous risk assessment of non-target organisms assessors require a clear understanding of what contitutes a non-target and what requires protecting. Without this basic early consideration it is not possible to develop and test clear hypotheses. However, it is also not possible or practicable to test all non-target organisms therefore it is necessary to "select appropriate species to serve as surrogates for ecologically and economically important non-target organisms that can be tested under worst-case conditions in the laboratory" [(Romeis et al., 2006a) from Barrett et al. 1994]..

To assess the safety of genetically modified (GM) crops containing insecticidal gene products it is vital that the effects on non-target organisms are assessed using a systematic and comparable methodology as the role of non-target organisms is a vital part of biological control (Romeis et al., 2004). The potential impact of genetically modified crops on non-target organisms is not only via direct toxicity but also indirectly via changes made to farming practices as a result of the introduction of these crops ((Hails, 1999) also see (Firbank, 2003; Firbank et al., 1999; Hawes et al., 2003; Perry et al., 2003)) or due to changes in prey quality (sub-optimal host quality) (Meissle et al., 2005) (Vojtech et al., 2005). Therefore, it is vital to understand the effects on non-target organisms at all trophic levels. The unintentional removal or reduction of these organisms could lead to reduced natural control of pest species for example parasitoids at the third trophic level as these act as a natural control of pest species.

The risk assessment of non-target organisms takes a comparative approach in that any effects are compared against the baseline taken from the non-transgenic near-isogenic counterpart that is considered to have no unacceptable adverse effects (Raybould et al., 2007). When testing non-target organisms it is also important to consider sensitivity to the stressor and the life stage of the individual (Raybould et al., 2007).

Insecticides derived from the *Bacillus thuringiensis* (Bt) bacterium are a widely used transgene product and have been in long term use as foliar insecticides (Hilbeck et al., 1998b). Bt based foliar insecticides are generally considered to have little or no effect on the natural enemies of pest species, however it is vital to assess the effects of PIP GM crop varieties containing the *Bt* toxin as early findings are based on studies designed to test for the undesirable effects of *B. thuringiensis* acting as foliar insecticides (Hilbeck et al., 1998b).

There are two very distinct differences between the two methods of introduction of Bt toxin into the environmental. Firstly, commercially available GM plants expressing Bt genes do so throughout the plant (depending on the promoters used) and during most of the growing season until the plant senesces (Hilbeck et al., 1998a) whereas foliar insecticidal applications are made at discrete intervals and are effected by environmental conditions such as ultra violet light and rain (Navon, 2000). Therefore the period of exposure non-target organisms are subject to is extended in agricultural systems using GM crops. Secondly, *Bt* genes expressed in transgenic crop varieties may also be expressed in a truncated activated form which differs from the inactive crystal form present in foliar insecticides (Hilbeck et al., 1998b) therefore gut lysis may not be required to attain toxicity (see section 3.3) as is the case with transgenic maize Bt176.

Despite these differences and potential risks and hazards it is important to bear in mind that the global loss of crops to insect pests is approximately 14% (Hilder and Boulter, 1999) therefore there is a real need to reduce this damage whilst avoiding problems associated with insect resistance (Navon, 2000). Also, the damage caused by insects is not linked solely to herbivory but insects can also act as vectors for disease (Magg et al., 2001) providing further impetus for long term sustainable solutions to combating crop pests.

1.1.3 Bacillus thuringiensis *(Bt)*

The insecticidal proteins δ-endotoxins (Cry proteins) derived from the *Bacillus thuringiensis* (*Bt*) bacterium are probably some of the most widely used PIPs in commercialisation (Hilbeck et al., 1998, Hilder and Boulter, 1999, Peferoen, 1997, Mendelsohn et al., 2003, (Romeis et al., 2006b). The *Bacillus thuringiensis* bacterium is a naturally occurring soil bacterium that has insecticidal properties against a range of

insects (de Maagd et al., 2001; Kumar and Venkateswerlu, 1998; Mendelsohn et al., 2003). Microbial sprays containing Bt products have been widely used in agriculture since the WWII and are generally regarded are environmentally friendly and safe (Romeis et al., 2006b). Products containing *Bt* have been used in agriculture for several decades (Hilbeck et al., 1998b), with crop plants expressing the *Bt* gene commercialised in the U.S. since the mid-1990s (Mendelsohn et al., 2003), (Romeis et al., 2006b).

The introduction and expression of genes encoding Bt Cry proteins into plants was probably one of the first major projects undertaken by the plant biotechnology industry (Peferoen, 1997). Some of the most widely used commercial transgenic crops are modified to contain this insecticidal protein (Hilbeck et al., 1998b; Mendelsohn et al., 2003). The introduction of the *Cry* toxin genes has helped reduce the time and effort put into the application of synthetic pesticides and therefore reduce the cost (Peferoen, 1997) involved in these processes.

In 1983 the first partial sequence of the *Bt* crystal protein gene was published (Peferoen, 1997) from Hofte and Whiteley, 1989) and by 1997 over 100 crystal protein gene sequences had been published (Peferoen, 1997). By 1995 commercial potato varieties engineered with *Bt* for resistance to the Colorado potato beetle where available followed in 1996 by *Bt* cotton; resistant to the tobacco budworm and cotton bollworm and *Bt* corn hybrids resistant to the European corn borer (Peferoen, 1997). Between 1996 and 2005 transgenic crops expressing herbicide tolerant genes were the most widely used, followed by those expressing insect resistant traits and stacked genes for two traits (James, 2005). By 2005 18% of the 90.0 million hectares of transgenic crops were Bt expressing crops, with herbicide tolerant soybean, maize, oil rapeseed and cotton accounting for 71% (James, 2005). Potato plants expressing Cry3Aa originally available from 1996 were subsequently withdrawn from the market in 2001 (Romeis et al., 2006b).

1.1.3.i Crystalline (Cry) Proteins

The *Bacillus thuringiensis* species produce proteinaceous, crystalline inclusions known as Cry proteins or δ-endotoxins, during sporulation (de Maagd et al., 2001; Garcia-Robles et al., 2001; Hilder and Boulter, 1999) (Figure 1.1)) which can be lethal to insect

larvae when ingested (Kumar and Venkateswerlu, 1998). Each Cry toxin has a defined specificity usually to a few species within a particular insect order and with a limited number showing toxic effects to nematodes (de Maagd et al., 2001) from Marroquin, 2000).

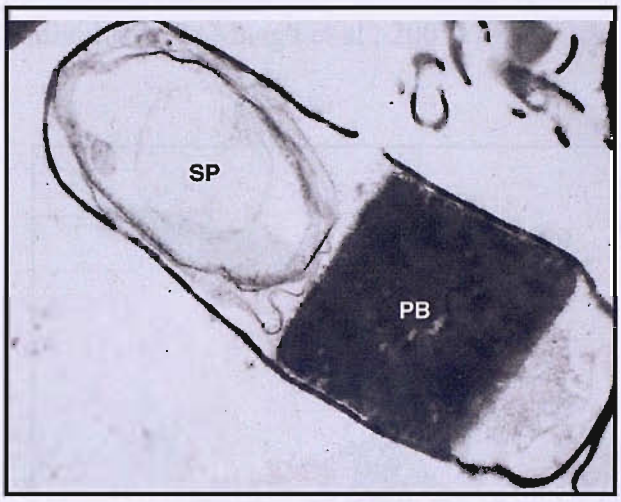


Figure 1.2 - Transmission electron micrograph of a sporulating *Bacillus thuringiensis* cell. δ-Endotoxins are produced as regularly shaped crystals (PB; protein body) next to a spore (SP). The vegetative cell wall eventually breaks to release the spore and crystal. The cell shown is approximately 2 μm long. (de Maagd et al., 2001)

A given strain of *Bacillus thuringiensis* will normally synthesize between 1 and 5 toxins within one or several crystals (de Maagd et al., 2001). These proteins form a large family of related proteins (Sanchis et al., 1994). These inclusions contain a variety of polypeptides one of which is a 130-140kDa inactive, protoxin (Kumar and Venkateswerlu, 1998). This protoxin is activated proteolytically in the insect's midgut into the active 60-70kDa toxin (figure 2 (Hilder and Boulter, 1999; Kumar and Venkateswerlu, 1998). This activated toxin can also be obtained *in vitro* by solubilizing the crystals at an alkaline pH and incubating with trypsin, pepsin or exudates from the larval gut (Kumar and Venkateswerlu, 1998).

The pathogenicity of the activated toxin occurs via receptors on the brush border membrane of the larval mid gut where ion-selective channels or non-selective pores are opened or formed (figure 1.2) these pores disrupt the osmotic balance across the insect

gut membrane (de Maagd et al., 2001; Hilder and Boulter, 1999; Sanchis et al., 1994). The disruption of the osmotic balance causes swelling and ultimately lysis of the gut epithelial cells (Hilder and Boulter, 1999; Sanchis et al., 1994) leading to death of the insect, usually occurring in 1-3 days from starvation and/or sceptaemia (Hilder and Boulter, 1999). A lack of binding or reduced binding to receptors can lead to resistant individuals (de Maagd et al., 2001).

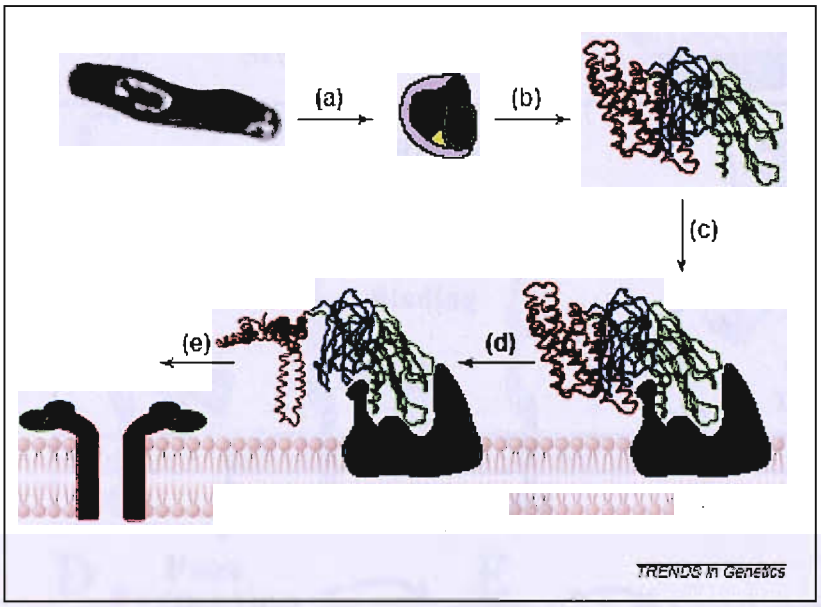


Figure 1.3 – Mode of action of Cry toxin on (a) ingestion the toxin is dissolved by insect gut juices (b) the C and N-terminals of the protein are cleaved, (c) toxin is now in its active form and binds to the gut receptors, (d) structural rearrangement of the toxin allows insertion into the membrane (e) pore is formed. (de Maagd et al., 2001).

Prior to absorption and digestion in the mid gut plant material is broken down in the foregut into small pieces with the foregut also acting as a sieve retaining larger particles and stopping them entering the mid gut (figure 1.3 (Whalon and Wingerd, 2003)), therefore, for Bt to be an effective pathogen it must be sufficiently small to allow movement into the midgut (Whalon and Wingerd, 2003).

The insertion of the Cry toxin into the membrane leads to two distinct and ultimately fatal processes, firstly the pores are K^+ selective ion channels disrupting the K^+ gradient and causing and increase of K^+ in the hemolymph ((Whalon and Wingerd, 2003) figure 1.4). Secondly, the pH balance is disrupted in the gut, with a corresponding increase in hemolymph pH ((Whalon and Wingerd, 2003) figure 1.4). These effects lead to "gut

paralysis and feeding inhibition and subsequently starvation" (Whalon and Wingerd, 2003).

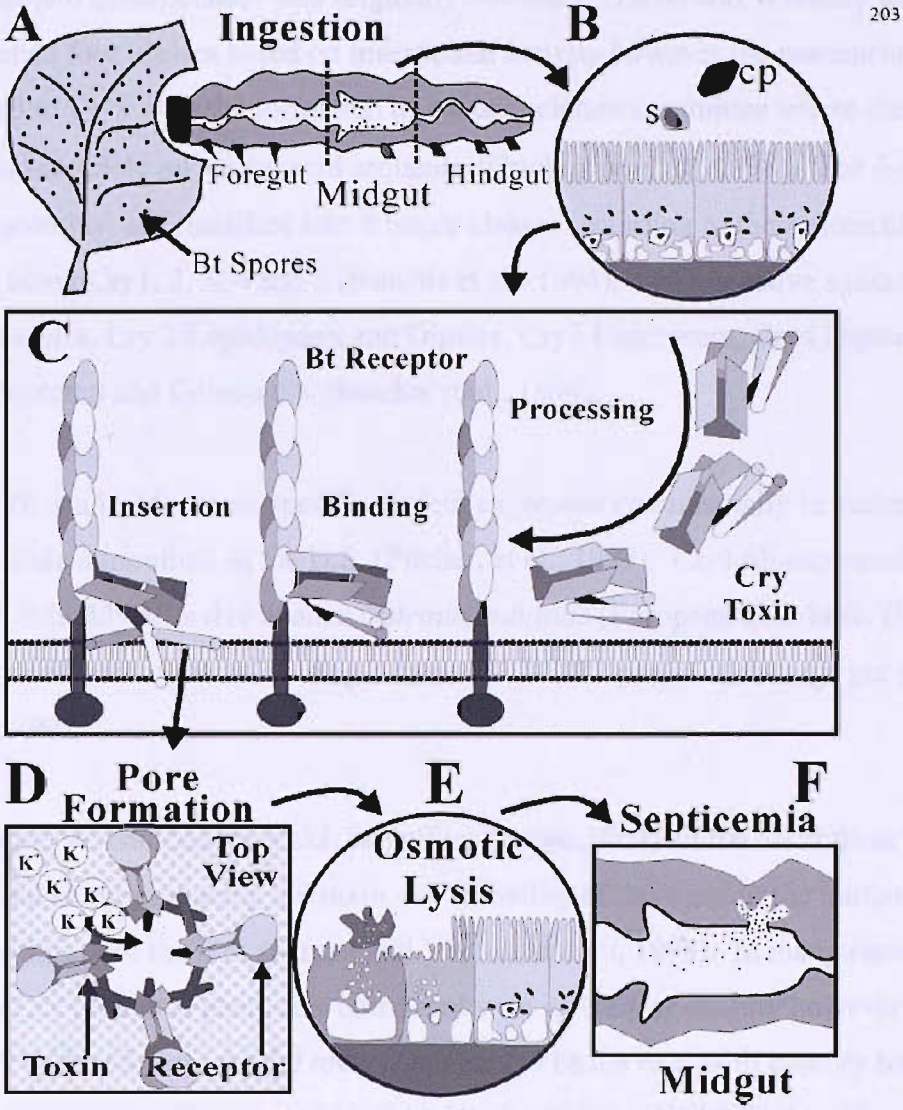


Figure 1.4 – Mode of action of Cry protein in lepidopteran larval gut. A: Ingestion, B: Endotoxins enter insect midgut, C: Cry toxins are proteolytically processed into their active form in the midgut, the activated toxin binds to epithelial cell receptors. D: Pore formation occurs leads to cell lysis. F: Midgut membranes are badly damaged and death follows due to starvation and/or septicaemia. Taken from (Whalon and Wingerd, 2003).

The highly alkaline environment in Lepidoptera and Diptera gut allows solublization and therefore activation of the toxin however the weakly acidic gut of Coleoptera may

explain why they are only susceptible following *in vitro* solubilization (de Maagd et al., 2001).

Cry protein nomenclature was originally devised by Hofte and Whiteley in 1989 and contained four classes based on insecticidal activity however the nomenclature was revised in 1993 with the formation of a nomenclature committee where classification was based solely on amino acid sequence (Crickmore et al., 1998). The δ-endotoxins (Cry proteins) are classified into 5 major classes according to their molecular structure these being Cry1, 2, 3, 4 and 5 (Sanchis et al., 1994). Cry1 is active against Lepidoptera, Cry 2 Lepidoptera and Diptera, Cry3 Coleoptera, Cry4 Diptera and Cry5 Lepidopteran and Coleoptera (Sanchis et al., 1994).

Cry1Ab is a lepidopteran specific protein expressed commercially in maize and used in large scale agriculture in the U.S. (Pilcher, et al., 1997). Cry1Ab expressed in maize plants is highly effective against *Ostrinia nubilalis* (European corn borer (ECB)) an introduced pest of maize causing between $37-$172 per ha of damage per year (Pilcher et al., 1997).

There are several factors which can affect the specificity of the toxin these being the bacterial strain producing the toxin, the solubility of the toxin in the midgut and the susceptibility of the host (Kumar and Venkateswerlu, 1998). In many cases the toxicity of the Cry protein is positively correlated with its binding affinity however in the case of Cry 1Ab and Cry 1Ac the reverse appears to be the case with toxicity being inversely related to binding (Kumar and Venkateswerlu, 1998). Whilst the specificity of binding may be an important indicator of toxicity differential processing by gut proteases is also a determinant (Kumar and Venkateswerlu, 1998); the number and affinity of binding sites also appears important for specificity and potency (Sanchis et al., 1994).

1.1.4 Genetically modified maize variety - Bt176

Whilst the use of chemical foliar insecticides provides some protection against the loss of maize yield due to the ECB they only have a narrow application window and do not provide complete cover due to the feeding habit and behaviour of the ECB juveniles (Koziel et al., 1993). Genetically modified maize plants provide a commercially viable

alternative giving season round and constitutive expression of the *Bt* toxin without the logistical difficulties of foliar application. Therefore one of the primary goals in developing transgenic maize lines containing the *Bt* endotoxins was to tackle this veracious pest (Archer et al., 2000).

The transgenic Bt-maize line event 176 was developed specifically to resist attack from the ECB which is a major agricultural pest of maize (http://www.agbios.com/dbase.php?action=Submit&evidx=31 last updated 31/1/06). Event 176 contains the *cryIA(b)* gene under the control of two promoters, one regulating *Bt* gene expression in green plant tissue (the maize PEPC promoter) and the other gene expression in pollen (Fearing et al., 1997; Magg et al., 2001). Both promoters contain the synthetic *cryIA(b)* gene encoding the truncated and therefore activated Cry1A(b) protein (Fearing et al., 1997). Event 176 also contains a selectable marker which confers resistance to the herbicide phosphinothrincin (Koziel et al., 1993) and has a single site of insert for the transgene and a few copies of the gene (Koziel et al 1993).

The gene is a synthetic version of the native gene containing increased G-C levels from 38% to 65% allowing high levels of toxin expression in the maize plants (Koziel et al., 1993). However the synthetic gene retains 65% homology with the native gene utilizing 648 of the 1155 native gene base pairs yet still producing the same active toxin as the native gene when cleaved in the insect gut (Koziel et al., 1993). Under testing Cry 1Ab protein levels in leaf tissue were found to be in the region of 1,500 ng Cry 1A(b)/mg soluble protein, however levels of 4,000 ng Cry 1A(b)/mg soluble protein where found (Koziel et al., 1993). Therefore Event 176 provides a very effective tool against ECB infestation even at very high levels of infection (Koziel et al., 1993).

Bt176 was tested in a range of conditions and found to have no competitive advantage other than those intended e.g. ECB resistance and herbicide tolerance to glufosinate, additionally there have been no reported cases of maize surviving as a weed therefore weediness and invasion of natural habitats is not likely (http://www.agbios.com/dbase.php?action=Submit&evidx=31 last updated 31/1/06). Acute oral toxicity tests on mice and bobwhite quail also showed no mortality as

expected as in mammalian simulations Cry1Ab was shown to degrade rapidly (http://www.agbios.com/dbase.php?action=Submit&evidx=31 last updated 31/1/06).

1.1.5 Spodoptera littoralis *(Boisduval) (Lepidoptera: Noctuidae)*

The second trophic level in this study used as host and directly exposed to the *Bt* toxin is the lepidopteran moth *Spodoptera littoralis*. *Spodoptera littoralis* is commonly known as the cotton worm, African cotton leafworm or Egyptian cotton leafworm (Hilbeck et al., 1999). *S. littoralis* is a polyphagous species (De Leo and Gallerani, 2002; Mazier et al., 1997) which attacks Solanaceae and Brassicaceae species (Krishnan and Kodric, 2005). In some regions *S. littoralis* is a major pest of cotton (Mazier et al., 1997) and therefore of agronomic importance. As *S. littoralis* larvae feed in a chewing manner it has the potential to incorporate insecticidal toxins whilst feeding as juveniles (Meissle et al., 2003).

In regions of Egypt *S. littoralis* is a serious cotton pest (Hillbeck, 1999). When fully developed the mature larvae are 35-45mm in length and have dark black/brown colouration (figure 1.5c). Larvae are initially gregarious becoming solitary (www.inra.fr) and when reared at 25°C take approximately 15 days to complete development from egg (figure 1.4a) to pupa (figure 1.5d), with the adult moth (figure 1.5e) emerging in less than a week. *S. littoralis* is a non-target pest of the *B. thuringiensis* toxin (Hilbeck et al., 1999) and is therefore often used to assess the impact of the toxin on non-target organisms and also as a second trophic level in tri-trophic interaction research. *S. littoralis* is relatively insensitive to Cry1Aa, Cry1Ab and Cry1Ac δ -endotoxins but is susceptible to Cry1C and Cry1E (Keller et al., 1996).

The 1st and 2nd instar larvae of *S. littoralis* are highly susceptible to Cry1C and Cry1E whereas there is considerable resistance in the 3rd to 6th instar, this phenomenon appears to be the case in many lepidopteran species (Keller et al., 1996). The reduced sensitivity in advanced instars is linked to high proteolytic activity in these instars which leads to the complete degradation of Cry proteins (Keller et al., 1996).

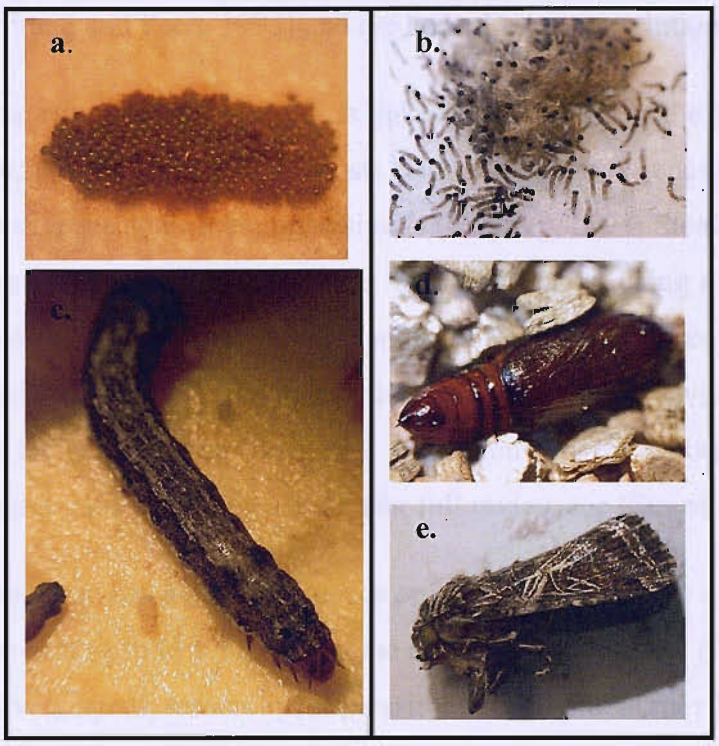


Figure 1.5 - Life stages of *Spodoptera littoralis* (a) egg cluster laid on netting, (b) newly emerged first instar larvae, (c) mature larvae feeding on artificial diet, (d) pupa on vermiculite substrate, (e) adult.

1.1.6 Parasitoid Biology

The insect order Hymenoptera comprises over 200,000 species of ants, bees, wasps and sawflies (van Wilgenburg et al., 2006). The Hymenopteran parasitoids wasps are a vital component of natural biological control in an agricultural setting, have been and continue to be an area of active research at the third trophic level as is the case in this study.

A parasitoid is defined by the feeding habit of its larva, feeding exclusively on the body of another arthropod (the host) ultimately killing it (Gullan and Cranston, 2005) page 6)). Parasitoids can be divided into two categories according to their feeding behaviour (Godfray, 1994; Stettler et al., 1998), those which feed from inside their hosts being endoparasitoids overcoming the host immune system (Stettler et al., 1998) and those which live externally but have their mouthpart buried within the host and are referred to as ectoparasitoids (Godfray, 1994). Therefore endoparasitoids generally have a

narrower host range as they have to directly contend with the hosts immune system (Strand and Pech, 1995) and are locked into an evolutionary arms race with their host.

The survival of parasitoids is linked to the ability of the larva to cope with the host's immune system, with the hosts cellular response of encapsulation playing a major role in defence against endoparasitoids (Godfray, 1994; Stettler et al., 1998). Parasitoids are able to overcome the hosts immune response using a variety of mechanisms such as polydnaviruses, venom, ovarian and the egg surface (see (Stettler et al., 1998; Strand and Pech, 1995). The mechanism of encapsulation having three phases 1 - recognition of the parasitoid as non-self, 2 – adhesion between hemocytes when forming the capsule and 3 – killing the parasitoid (for full review see (Strand and Pech, 1995).

Host size has a major influence on parasitoid fitness as it determines the maximum amount of food available for the developing parasitoid (Godfray, 1994). When the host is very small, resources for parasitoid development may be so scarce that the parasitoid fails to mature and dies (Godfray, 1994). If resources are sufficient enough to prevent death, malformation may occur, therefore host condition, independent of size and age can influence parasitoid fitness (Godfray, 1994). Hind tibia length (figure 1.6) is often used as a measure of adult fitness as it has been shown to be positively correlated to a variety of fitness indicators (Sagarra et al., 2001) Zhou et al., 2007). Host condition may have both positive and negative consequences such as reduced nutrition but weakened cellular defence (Godfray, 1994). Hymenopteran insect parasitoids also have a genetic system which allows females to determine the sex of offspring. Therefore, sex ratio may also be affected by host size with males being laid in small or poor quality hosts (Murdoch et al., 1992).

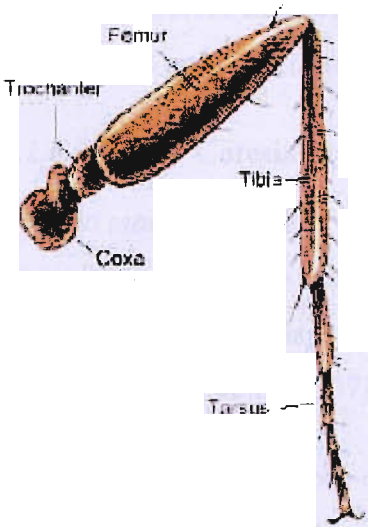


Figure 1.6 – Generalized anatomy of parasitoid leg (http://www.einsteins-emporium.com/life/animal-info/insects/insect_anatomy_leg.htm).

1.1.6.i Sex determination

Hymenopteran insect parasitoids have a haplodiploid genetic system which allows adult females to determine the sex of each offspring (Van Baaren et al., 1999) (Murdoch et al., 1992) (King, 1987). Fertilized (diploid) eggs develop into females and unfertilized (haploid) eggs into males (Van Baaren et al., 1999) (Zhou et al., 2007) (van Wilgenburg et al., 2006). Arrhentoky is the most usual form of reproduction where female eggs receive sperm from a reservoir in the spermatheca whereas male eggs develop parthenogenically and are not fertilized (Murdoch et al., 1992) (van Wilgenburg et al., 2006).

In this haplodiploid system Hymenoptera sex is determined by a single locus with females being heterozygous and males hemizygous (van Wilgenburg et al., 2006) if the population undergoes inbreeding which can occur as a result of collection and laboratory rearing diploid sterile homozygous males are produced and put a genetic burden on the population (van Wilgenburg et al., 2006) (Zhou et al., 2007). Some diploid males may produce diploid sperm which lead to triploid (sterile) progeny (van Wilgenburg et al., 2006). This system is referred to as single-locus complementary sex determination (sl-CSD) and is thought to be the mechanism that regulates sex determination in all Hymenopteran insects (Hopper et al., 2007) (van Wilgenburg et al., 2006; Zhou et al., 2007). Females wasps are also able to recognise the different size of

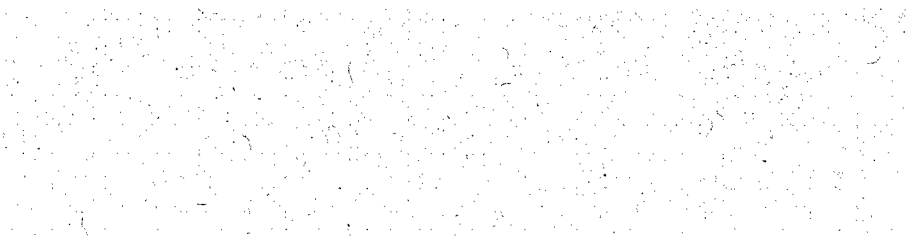
host and therefore in a host community of different size able to preferentially oviposit female eggs into larger hosts (Murdoch et al., 1992; Ueno, 1998).

1.1.6.ii Cotesia marginiventris

Cotesia marginiventris is a solitary endoparasitoid (Jalali et al., 1987; Tillman, 2001) of many lepidopteran hosts in a variety of agricultural settings (Tillman, 2001). This genus of parasitoid wasps (Hymenoptera: Braconidae) is widely used for biological control (Hopper et al., 2007).

C. marginiventris are a pro-ovogenic parasitoid with the highest number of eggs available 2 days after eclosion (Baur and Boethel, 2003). Pro-ovogenic parasitoids emerge with a fixed complement of eggs therefore host food can be used for either an energy source or nutrient source to mature eggs (Godfray, 1994). In contrast synovigenic parasitoids mature eggs throughout the adult life cycle (Godfray, 1994) and therefore may limit the number of oviposition attacks a parasitoid can carry out in a given time.

C. marginiventris adults preferentially oviposit in early instar hosts, generally 1st to 3rd instar (Jalali et al., 1987; Yeargan and Braman, 1989), with the highest oviposition success being in larvae of 3-4 day old (Jalali et al., 1987) i.e. 1st and 2nd instar. Mature larvae (figure 1.7a) emerge from a small hole in the side of the host and immediately begin spinning a cocoon (figure 1.7b.), with adults emerging 3-4 days later (figure 1.7c.).



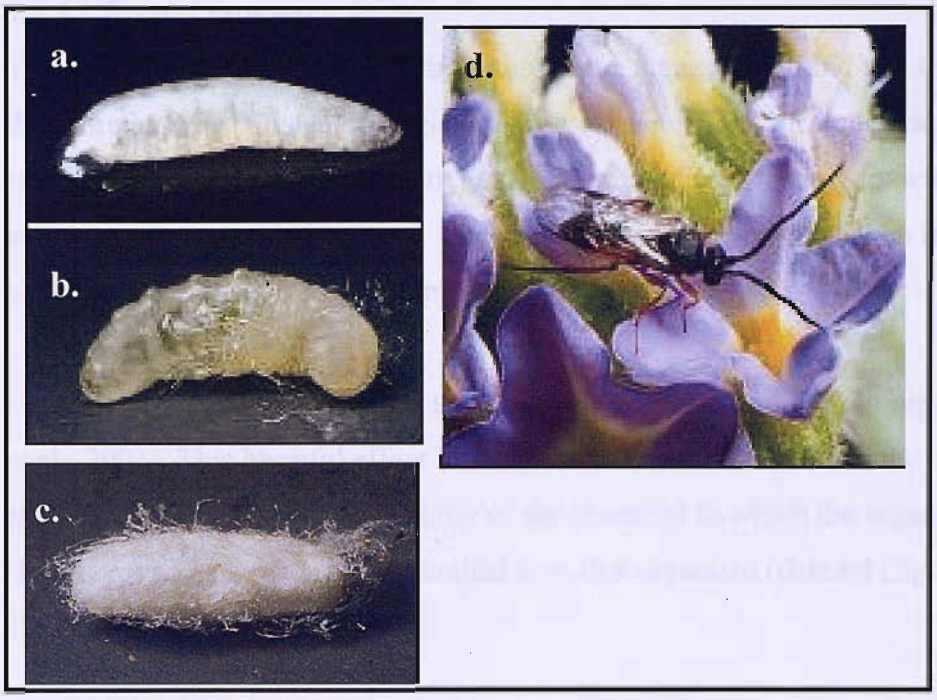


Figure 1.7 – The life stages of the endoparasitoid *Cotesia marginiventris* (a) early stage larvae, (b) mature larvae, (c) cocoon and (d) adult. http://edis.ifas.ufl.edu/IN280 (updated 9/11/05).

1.1.7 Project rational

This project can be summarized in terms of risk management and risk assessment and is therefore based within an ecological risk assessment (ERA) framework. ERA utilizes concepts such as risk management goal, endpoints, tiered testing, toxicity, exposure, and dose response relationships. The project also uses concepts taken from ecotoxicology testing that underpin risk assessment.

Ecotoxicology is the measure of the harmful effects of chemicals to organisms (Walker et al., 2001). This harmful effect is composed of two measures and the relationship between them, these are the quantity of the chemical to which the organism is exposed (exposure) and how toxic the chemical is to that organism ((hazard (figure 1.8) section 1.1.1) (Walker et al., 2001).

Within this project the risk management goal is to maintain parasitoid populations by avoiding detrimental impacts on biological control agents either via direct or prey mediated effects. The issue of direct toxicity as opposed to prey mediated effects has not been fully resolved and therefore it is important to address this specifically which was an important component of this research project.

In order to assess the risk management goal and the effects of Cry1Ab within an ERA framework this project utilises assessment endpoints i.e. assessing the impact of GM maize on a specific parasitoid population and these are linked to measurement endpoints (section 1.1.1.i). Measurement endpoints can then be assessed experimentally under laboratory conditions e.g. pupation time, sex ratio, tibia length.

Ultimately the project aims to contribute new knowledge that relates changes identified in laboratory bioassays (measurement endpoints) to those at the population level (assessment endpoints). The link between measurement and assessment endpoints is made possible by setting the project within the ecological risk assessment format discussed above.

Figure 1.8 – Relationship between risk, hazard and exposure.

However it is beyond the scope of this project to carry out a full study of the feeding behaviour of *S. littoralis* in the field exposed to *Bt* maize therefore any estimates are based on consumption rates in the laboratory and levels of expression of the Cry1Ab toxin in maize i.e. no choice trials. Therefore this study does not account for feeding on a complex diet that may be experienced in a field population of *Spodoptera littoralis*.

1.1.8 Thesis Chapter Summary

This thesis sets out a series of experimental data designed to assess the potential environmental impact of genetically modified maize (Bt176) at the third trophic level. The thesis gives an overview of the project, including the project rational, the experimental work undertaken and a general discussion of the implications of its findings. It is not designed to be a comprehensive risk assessment of GM maize and a specific parasitoid but draws generic conclusions regarding the direct and host mediated effects of GM maize and how this can inform the ecological risk assessment process.

Chapter 1 - rationale behind the project giving an overview of what it is designed to achieve and how this relates to ecological risk assessment, hazard and exposure. This is followed by a general introduction to the area of study and pertinent background information.

Chapter 2 - baseline data from studies carried out at the first and second trophic levels i.e. Bt176 transgenic maize and *Spodoptera littoralis* assessing the natural variation of the experimental system.

Chapter 3 - experimental data gained from Cry1Ab stability trials of the uncleaved crystal protein and the truncated toxin; using immunoassay (western blot analysis) and biological activity assays using *Ostrinia nubilalis*.

Chapter 4 – experimental data of the direct and indirect (host mediated) effects of exposure to the Cry1Ab toxin at the third trophic level (*Cotesia marginiventris*). Biochemical analysis of movement through the trophic levels is also included using ELISA analysis.

Chapter 5 – experimental data of the potential cumulative effects of the Cry1Ab toxin after exposure of two generations of *Cotesia marginiventris* to the Cry1Ab toxin.

Chapter 6 – General discussion and conclusions of the implications of the study as a whole and how this can inform the ecological risk assessment process.

Chapter 2

Sub-lethal and lethal effects of Bt176 transgenic maize on the herbivore *Spodoptera littoralis*.

2.1 Introduction

Maize, *Zea mays* L. is one of the three major world crops along with rice and wheat (Wisniewski et al., 2002). The European corn borer (ECB), *Ostrinia nubilalis* is a major pest of maize both in North America and Europe (Fearing et al., 1997) causing significant crop loss if left uncontrolled. Therefore there is a need to protect this major food and fodder crop from insect attack whilst ensuring natural enemies of this pests are not negatively impacted, as in the case of the European corn borer (ECB) natural enemies have a significant impact on pest mortality (Wisniewski et al., 2002).

In the United States 25% of the maize grown is genetically modified to express the crystalline (Cry) protein produced by the *Bacillus thuringiensis* bacterium (Wisniewski et al., 2002) to protect against ECB attack (Fearing et al., 1997; Koziel et al., 1993). The ECB is particularly hard to control with conventional pesticides due to its feeding habit of boring into plant stems (Fearing et al., 1997) protecting it from coming into contact with externally applied pesticide.

Bacillus thuringiensis bacteria produce insecticidal crystal proteins within the cytoplasm (Bravo, et al. 2002) these large protoxins are inactive until proteolytically cleaved in the insect gut (Hofte and Whiteley, 1989; Koziel et al., 1993) approximately half of the protein is removed from the C terminus and 25-30 amino acids from the N-terminal (Bravo, et al. 2002) resulting in the fully activated protein. The activated protein binds to receptors in the insect mid gut before insertion into the membrane and pore formation causing cell lysis (Koziel et al., 1993), cessation of feeding and mortality.

To allow stable and sufficient expression of the toxin in plants the truncated, rather than the native version of the gene, is expressed (Koziel et al., 1993) as the C-terminal extension is believed play a role in crystal formation but not required for toxicity ((Bravo et al., 2002) from Park 2000). However the small peptide N-terminal sequence remains in place, this short sequence may play a role in pore formation therefore lack of processing of the sequence may lead to reduced pathogenicty of the toxin (Bravo et al., 2002).

Event 176 encodes the truncated Cry1Ab protein resulting in the production of an amino acid sequence that is identical to the N-terminal 648 amino acids of the 1155 amino acid full-length native Cry1Ab protein produced by the bacteria (Fearing et al., 1997; Koziel et al., 1993) . Event 176 is under the control of the phosphoenolpyruvate (PEP) carboxylase promoter, which is expressed in green tissue along with a pollen specific promoter (Fearing et al., 1997; Koziel et al., 1993). Expression levels of the truncated toxin vary dependant on season, age and growth stage of the plant, with typical levels of expression of around 13,000 ng g^{-1} fresh weight, with the LC_{50} for ECB neonates being 20-30 ng/g diet (Koziel et al., 1993). This relationship between the dose of a toxicant and the response of the living system is central in the study of toxicology and ecotoxicology (Calow, 1994) and is an underlying principle in this study.

Therefore, given the high rate of continuous, constitutive expression it is vital to understand the potential hazards and therefore risks posed by GM crops specifically those containing Cry1Ab. Within classical pesticide risk assessment for example guidelines laid down for testing pesticides advocate 1st tier; worse case scenario testing that determines the lethal rate 50 i.e. the application rate which causes 50% mortality in the target population, as the testing endpoint. This procedure also uses a hazard quotient (HQ) to establish a trigger value above which further testing is required or below which the hazard is deemed acceptable (Candolfi et al., 2001) this procedure however takes little account of the likely exposure of the test organism.

In this study *Spodoptera littoralis* are used at the second trophic level in worst case scenario testing as they are a non-target herbivore and only slightly susceptible to Cry1Ab (Hilbeck et al., 1998a; Keller et al., 1996). Proteolysis by gut juices in *S. littoralis* late instars (3rd-5th) lead to almost complete degradation of Cry1Ab therefore any sensitivity to the toxin tends to be apparent in early instars where proteolysis is less active (Keller et al., 1996). Therefore, *S. littoralis* is a powerful tool in tri-trophic studies and also shares similar morphology and behaviour to *O. nubilalis* the target of Cry1Ab incorporated plant protectant in the field.

The data reported here has four main objectives the primary objective being 1) to establish natural mortality to allow for corrections not due to perturbation to host diet

and subsequently to 2) establish the total toxin load *S .littoralis* could be subject to over their juvenile life cycle. 3) To compare mass and consumption of *S. littoralis* when on artificial diet, Bt176 genetically modified (GM) maize and its isoline, thereby establishing the natural variation in the system and 4) to assess variability of expression in Bt176 genetically modified maize. This provides the baseline data for the future dose response trials using purified Bt toxin.

2.2 Materials and Methods

2.2.1 Maize plants

Transgenic maize (*Zea mays L.*) plants, (Syngenta event Bt-176) expressing the Cry1Ab protein and the untransformed near isoline control cultivar were used in all bioassays. Individual maize plants where cultivated in a greenhouse at 25°C ±5°C and at a relative humidity of 50%± 20%, in 1.0l pots 13cm diameter, 12cm high. New plants were sown weekly and used for experiments between 3-5 weeks old (V5-V7) as *S. littoralis* are known to be primarily a pest of young plants (Dutton et al., 2005).

2.2.2 Insects

Bioassays were maintained in a cooled incubator (AC/013, Thermo-Electron, Basingstoke, UK.) at 25°C ±1.5°C, RH 60% ±20% and a photoperiod of L:D 14:10H. *Spodoptera littoralis* were maintained for bioassay from eggs supplied by Syngenta, Bracknell, U.K. Eggs were supplied on netting (oviposition substrate) at the commencement of each experiment. On receipt *Spodoptera littoralis* eggs were left on netting and placed in 7l (30x30x20cm) plastic containers with ventilation containing an excess of artificial diet (Beet Armyworm diet F9220B Bio-Serve, Frenchtown, NJ 08825 (see appendix 1 for full ingredients and preparation details)), transgenic or isoline maize to emerge. Plant material was changed daily. For bioassays all neonate larvae were left to develop for 4 days into 2nd instar larvae before being disturbed allowing the larvae to become robust enough to withstand weighing and general handling. After 4 days larvae were transferred into individual 250ml pots (Roundstone, Catering Equipment Ltd, Melksham, Wiltshire, UK) with the appropriate diet materials. At 7 days old the larvae and diet were weighed daily to establish larval mass and consumption rate of diet. Where larvae were not weighed daily a mean of the previous and current measurements were used to establish daily consumption rate and mass.

2.2.3 Diet consumption, development and survival

2.2.3.i Survival of early instar larvae.

To establish survival of 1st and 2nd instar larvae and therefore the proportion of larvae available for parasitism newly hatched neonate larvae were removed from large containers and placed in groups of 20 individuals in 250ml plastic pots (Roundstone

Catering Equipment Ltd, Melksham, Wiltshire, UK). Fresh maize leaves were added daily to the Bt176 or control plants. In the artificial diet condition new diet was added at the commencement of the experiment. At day 7 after hatch survival was recorded. Each condition was repeated 3 times with 5 pots of 20 individuals in each replicate, totalling 300 individuals in each condition.

2.2.3.ii Effects of artificial diet on Spodoptera littoralis.

Seven days after emerging 2nd instar larvae weighing between 0.01 and 0.02g were removed and transferred to individual 250ml clear, circular pots (Roundstone Catering Ltd., Melksham, U.K.) with ventilation. Artificial diet approximately 4 x 3 x 2cm in size and weighing between 6.0 and 9.0g was also added to the pots. The diet was changed in all pots 10 days after inclusion to avoid excessive drying out and general unpalatability, vermiculite was also added to a depth of approximately 2cm on day 10. The vermiculite layer acted as a pupation substrate.

Larvae and diet were weighed every 24 hours until pupation. Pupae were then weighed 2 days after formation to ensure a robust cuticle.

2.2.3.iii Effect of maize diet on Spodoptera littoralis.

Seven days after emerging 2nd instar larvae raised on control maize weighing between 0.01 and 0.02g and larvae weighing between 0.001 and 0.005g raised on Bt maize were removed and transferred to individual 250ml clear, circular pots (Roundstone Catering Ltd., Melksham, U.K.) with ventilation. Leaves were excised from the plants, the 3rd, 4th or 5th leaf from the base of the plants only were used, as preliminary trials noted that irrespective of transgene concentration *S. littoralis* larvae did not feed above the 5th leaf (Dutton et al. 2005). All leaf sections were placed in a bag and chosen at random, folded and added to individual larval pots after weighing and changed every 48 hours.

Larvae and diet were weighed every 24 hours until pupation and maize leaves were changed daily. Vermiculite was added to each pot to a depth of approximately 2cm on day 14 of the experiment to act as a pupation substrate. Pupae were weighed 2 days after formation to ensure a robust cuticle.

2.2.4 Cry1A(b) protein quantification.

Detection and quantitative determination of the amount of Cry1A(b) protein expressed in the experimental transgenic maize line Bt176 (Syngenta) was carried out using enzyme-linked immunosorbant assays ((ELISA) Pathoscreen kit (Agdia, USA). Seven plants from each condition were selected at 5, 4 and 3 weeks old these being growth stage V6, V5, V4 respectively. Three leaves were excised from each plant these being leaf 3, 4 and 5 from the base of the plant excluding the 3 week old plant which only had the 3rd and 4th leaves removed due to only being at growth stage V4.

Five, 5mm diameter leaf discs were removed from each leaf and placed into a 1.5ml eppendorf tube with 300µl of 5% mPBS Tween buffer solution. Two stainless steel ball bearings were added to each sample eppendorf. The samples were then loaded into a macerator and agitated for 1.5minutes then rotated and agitated for a further 1.5 minutes. Samples were centrifuged at 13 rpm for 5 minutes to spin down any remaining plants tissue. All samples where then maintained on ice prior to analysis.

2.2.4.i ELISA analysis

ELISA analysis was carried out using a Cry1Ab/1Ac ELISA Pathoscreen kit (Agdia, USA). Samples were diluted to 1/100 to be within optimal range of sensitivity compared to the standards. Samples were assayed according to the standard protocol of the kit and plates read at 450nm (Anthos reader 2001, Anthos labtech instruments).

2.2.5 Statistical Analysis

All statistical analysis was carried out using SPSS for windows version 14. All measurement data of mass and consumption was transformed using log transformation as the data did not conform to a normal distribution. Repeated measures data i.e. mass and consumption was analysed using GLM, repeated measures and a post hoc Tukey test.

2.3.1 Effects of Bt maize on Spodoptera littoralis

2.3.1.i Survival of first and second instar larvae.

Survival of first and second instar larvae was assessed from hatching to 6 days old i.e. prior to commencement of consumption and mass trials, to establish natural mortality not due to perturbation to diet. There was no significant difference between survival of neonates raised on either artificial diet, Bt176 transgenic maize or its isoline (Kruskal-Wallis, $p>0.05$, $K=0.56$, d.f. 2). Over 3 replicate trials ($n=5$) totalling 300 individuals 82% of individuals raised on artificial diet survived to day 6, 81% and 85% of those raised on Bt176 and its isoline respectively survived.

2.3.1.ii Survival of S. littoralis *juveniles*

The survival of *S. littoralis* juveniles was further assessed to establish mortality throughout the juvenile phase. Larvae reared on artificial diet, Bt176 transgenic maize and its isoline was assessed using pupation as the concluding point of the trial.

Sixteen days after emerging all surviving larvae reared on artificial diet had pupated and the survival rate including neonate survival was more than 80% (figure 2.1), mean days to pupation was 15 days. Similarly at day 22 after emergence there was only 20% mortality in larvae reared on either transgenic and isoline maize. However, at day 22 mortality increased sharply in both maize conditions this corresponded to a decrease in body mass and consumption rate. At day 30 no larvae reared on either maize line had pupated (figure 2.1).

The survival probability of larvae did not differ between either the Bt176 group or the isoline control ($x^2=0.717$, d.f.=1, $p=0.397$). The survival probability of the artificial diet group was not included in statistical analysis as at day 16 all remaining larvae reared on artificial diet had pupated whereas the survival of the larvae reared on Bt176 and isoline maize was in sharp decline steeply at day 22 with only 1 larvae in each group surviving until pupation i.e. less than 1% survival (figure 2.1).

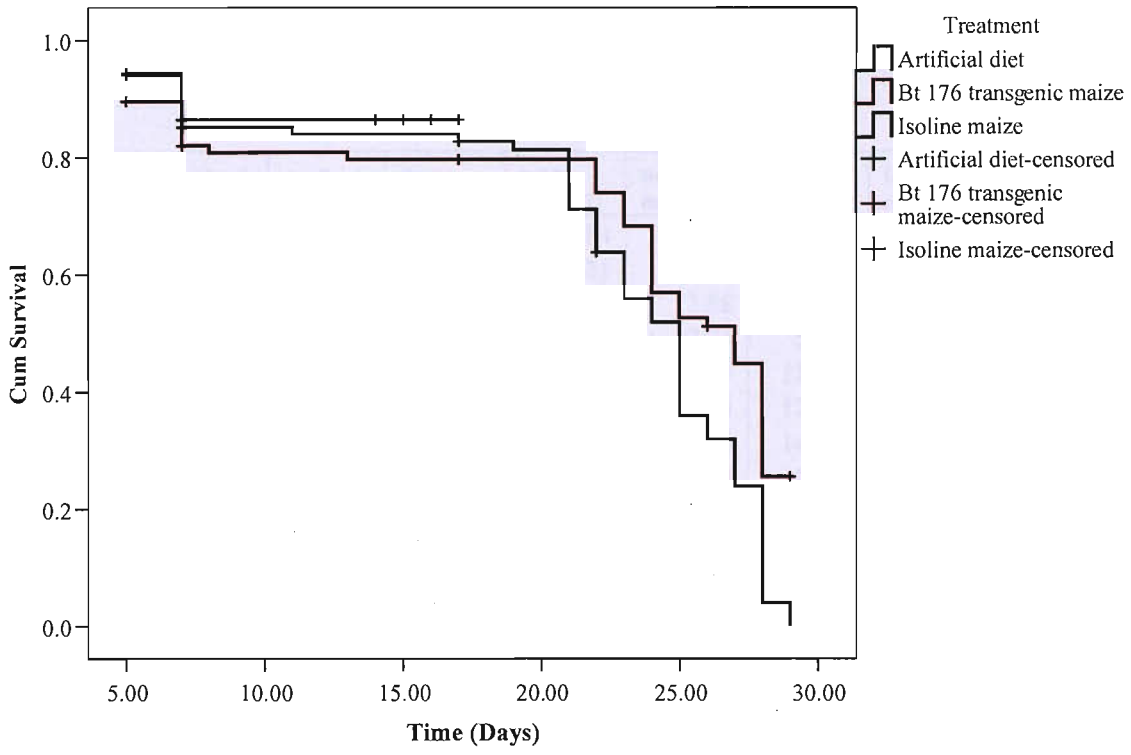


Figure 2.1 – The survival probability of *Spodoptera littoralis* juveniles reared on (a) artificial diet (green), (b) Bt176 transgenic maize (red) and (c) isoline maize (black).

2.3.2 Sub-lethal effects instars 3-5.

The sub-lethal effect of the different diet regimes was monitored to establish the effect of diet at the second trophic level. Larval mass (figure 2.2) and consumption (figure 2.3) were measured for the first 17 days of the *S. littoralis* juvenile life cycle as this period is far in excess of the parasitism window for *Cotesia marginiventris* adults, *S. littoralis* larvae being too large and aggressive to be parasitized towards the end of the second instar (5-9 days dependent on diet).

Trials were conducted to establish how much natural variation occurred both between and within replicates and between conditions. At day 10 the mean larval mass in artificial trial 1 was 48% greater than trial 2 in the same condition (figure 2.2). Figure 2.2 illustrates the substantial amount of variation between all trials irrespective of condition.

At day 10 artificial diet replicates differed significantly ($F_{1,48} = 1123.86$, $p<0.0001$), as did the Bt176 genetically modified maize replicates ($F_{1,56} = 275.89$, $p<0.0001$) and the isoline replicates ($F_{1,56}=59.23$, $p<0.0001$) therefore data from each replicate could not be pooled, this variation was not consistent across the groups or trials.

Day	Artificial diet (1)	Artificial diet (2)	Artificial diet (3)	Bt176 maize (1)	Bt176 maize (2)	Bt176 maize (3)	Isoline maize (1)	Isoline maize (2)	Isoline maize (3)
7	21.73	18.38	12.16	4.78	2.84	1.25	6.16	1.60	5.61
8	55.79	24.84	31.16	8.21	9.21	1.86	10.83	37.00	9.67
9	75.20	63.63	57.02	11.44	13.98	2.88	33.12	44.00	18.04
10	164.30	85.48	95.02	34.50	33.33	7.74	51.40	73.00	38.73
11	229.48	191.05	190.95	47.16	59.00	10.50	110.01	108.00	46.01
12	442.26	310.73	385.41	77.10	72.00	20.28	127.21	110.00	72.58
13	655.04	513.47	675.63	138.11	116.00	30.06	142.52	128.00	72.58
14	841.21	829.94	965.85	135.84	119.00	54.93	160.37	160.00	109.36
15	804.22	1074.99	1059.41	156.15	132.00	96.04	206.62	183.00	119.31
16	927.79	1053.47		239.82	160.00	108.50	314.60	203.00	146.37
17		368.75		334.35	171.00	123.38	355.24	204.00	205.80

Figure 2.2 – Mean larval mass (mg) of *Spodoptera littoralis* from 7-17 days after hatch feed on artificial diet (Beet armyworm diet), Bt176 transgenic maize and its isoline (replicate group).

As data could not be pooled individual replicates were compared at day 7, 10 and 14 to establish if diet type had a significant effect on larval mass at these arbitrary time intervals. At day 7 in trial 1 the Bt176 and isoline maize reared larvae were not significantly different ($p=0.242$). Whereas in trial 2 the artificial diet and isoline diet reared larvae were not significantly different ($p=0.07$). At day 10 trials 1 and 2 showed a significant difference between Bt176 and both the artificial diet and isoline maize however these similarities did not continue in trial 3. By day 14 larval mass was significantly different in all trails and conditions. (See appendix 2 for full table of significance levels).

2.3.3 Consumption levels instars 3-5.

Spodoptera littoralis larval consumption was monitored from day 8 to 17 to establish the likely toxin load juvenile parasitoids may be subject to following oviposition. This represents a period in excess of the time scale juvenile *C. marginiventris* are maturing within the host, as preliminary trials showed that parasitoids reared on hosts raised on artificial diet emerge from the host and spin cocoons 9-11 days after parasitism i.e. at 13 – 15 days host development.

Larval consumption in the late second to early third instar (day 8-9) often showed zero consumption (figure 2.3) this represents the lack of sensitivity of the test rather than zero consumption. The reduction in consumption at day 15 and 16 in artificial diet treatment (figure 2.3) reflects the cessation of feeding witnessed prior to pupation and also reflects a reducing sample size therefore a few individual having a disproportionate effect on the dataset.

Day	Artificial diet (1)	Artificial diet (2)	Artificial diet (3)	Bt176 maize (1)	Bt176 maize (2)	Bt176 maize (3)	Isoline maize (1)	Isoline maize (2)	Isoline maize (3)
8	0.085	0.000	0.043	0.000	0.044	0.000	0.052	0.000	0.037
9	0.055	0.038	0.037	0.138	0.043	0.011	0.023	0.042	0.000
10	0.186	0.004	0.109	0.030	0.073	0.037	0.153	0.081	0.082
11	0.142	0.191	0.119	0.060	0.136	0.043	0.008	0.060	0.043
12	0.180	0.171	0.332	0.030	0.020	0.108	0.020	0.000	0.107
13	0.326	0.164	0.242	0.198	0.148	0.172	0.097	0.000	0.172
14	0.629	0.611	1.229	0.307	0.058	0.110	0.158	0.084	0.110
15	0.008	0.459	0.152	0.138	0.075	0.015	0.315	0.070	0.015
16	0.017	0.197		0.162	0.070	0.101	0.098	0.060	0.101
17		0.000		0.177	0.026	0.123	0.181	0.046	0.123

Figure 2.3 – Consumption (g day^{-1}) of *S. littoralis* between day 8 and 17 after hatch when reared on artificial diet, Bt176 transgenic maize and its isoline (replicate groups).

The cumulative consumption of artificial diet (1) 1.628g , artificial diet (2) 1.835g, artificial diet (3) 2.263g, Bt176 (2) 1.061g, Bt176 (3) 1.18g, isoline (2) 0.58g and isoline (3) 1.356g. Artificial diet cumulative consumption represents the total consumption up to pupation whereas cumulative consumption in Bt176 and isoline represents the point at which consumption was consistently negative due to over 95%

mortality in each treatment and also the point past which parasitoid larvae would have emerged and commenced cocoon spinning.

2.3.4 Cry1Ab expression levels during maize development

Cry1Ab levels where determined over a range of plant ages and developmental stages. Green leaf tissue was sampled from plants aged 3, 4 and 5 weeks old and leaves at stage V3, V4 and V5 as *Spodoptera littoralis* juveniles are known to feed only on young plant and leaf tissue (Dutton et al., 2005).

Cry1Ab expression levels varied over the age and leaf stage of the plants, with plants at 5 weeks old showing the most consistent expression levels at between 10.0 and 12.4 μg g^{-1} and the lowest expression levels in the 3 week old plant at stage V4 leaf of 7.5 μg g^{-1} and the highest level of 15.3μg g^{-1} in the 4 week old plant at stage V3 (figure 2.4).

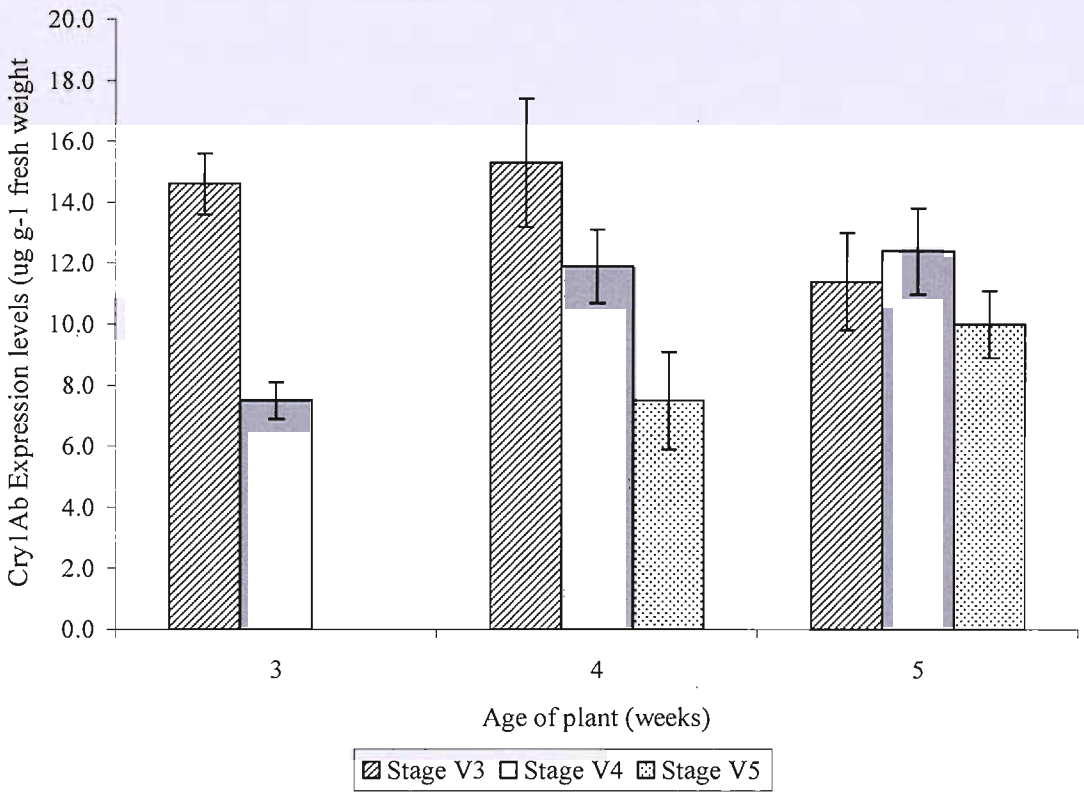


Figure 2.4 – Mean Cry1Ab expression levels in Bt176 transgenic maize line (μg g^{-1} fresh weight) in plants aged 3, 4 and 5 weeks at leaf stage V3, V4, V5.

The mean expression levels in Bt176 maize was 11µg g^{-1} fresh weight therefore given the consumption levels host reared on an artificial diet in trial 1 would have had a total toxin load of 20µg during their juvenile life cycle, insects reared on Bt176 11µg and those reared on the isoline maize 5µg, in replicate 2 the total toxin load would have been 24 µg, 14µg and 15µg respectively.

2.4.1 S. littoralis *juvenile larval development*

The main aim of this baseline data trial was to establish the amount of natural variation in this system. The variation in larval mass and consumption varies greatly not only with time but also with cohort trial. In order to establish if further testing needs to be carried out at a higher tier any value triggering higher tier testing needs to reflect or compensate for this natural variation not only within subject groups but also between replicate trials. Taking account of this variation will allow confident predictions regarding potential toxicity to be made, particularly as any effect seen at the third trophic level may be host quality mediated rather than due to the direct toxicity of the Cry1Ab toxin (Couty et al., 2001b; Vojtech et al., 2005).

It is also important to notice the significant difference between the effects of the artificial diet, Bt176 and isoline maize diets. Larvae raised on artificial diet were at least twice the mass of those raised on Bt176 maize at day 10 for example however there was only a 15% reduction in the mass of larvae raised on artificial diet and those raised on the isoline maize at day 10 in trial 2 (appendix 2). It is clear from this and other studies that *Spodoptera littoralis* raised on GM maize are adversely affected by the transgene product (Dutton et al., 2005; Vojtech et al., 2005) and this effect is more than the slight effect suggested by Keller (1996).

There was a significant difference between Bt176 larval mass and isoline larval mass in all but two trials on day 7 and day 14 with larval mass of individuals raised on Bt176 being consistently less than those raised on its isoline. Conversely, some trials showed no significant difference between larval mass of individuals raised on artificial diet and isoline maize e.g. trail 2, day 7 ($p=.073$), trial 1, day 10 ($p=0.151$) and trial 2, day 10 ($p=0.360$). This data clearly shows that Bt176 is a suboptimal diet when compared to the artificial diet. The isoline maize also produces less robust individuals making the artificial diet an ideal tool for use in a replicable bioassay, although still subject to considerable biological variation.

The reduced fitness of individuals raised on either maize type is clearly reflected in levels of mortality post day 22, at this point over 80% of individuals raised on artificial

diet had survived and pupated whereas survival of individuals raised on maize was in steep decline. These individuals then showed reduced feeding and body mass with less than 1% surviving to pupation. This lack of fitness may reflect the lack of diet choice whereas in a field environment *S. littoralis* juveniles may have a range of diets improving pupation success. However there is clear evidence that non-target organisms do take up Cry1Ab in a field setting (Harwood et al., 2005) therefore do forage on crops containing this transgene product rather than displaying avoidance behaviour. Studies also show that *S. littoralis* do ingest high levels of Cry1Ab when reared in laboratory conditions (Vojtech et al., 2005) and in dietary choice trials exposing *Poecilus cupreus* larvae to prey raised of Bt containing and non Bt containing diet there was no evidence of larvae avoiding prey containing Bt (Meissle et al., 2005) .

However, whilst larvae did not simply avoid consuming Bt176 maize the level of consumption in each trial and condition did vary considerably. Individuals raised on artificial diet consumed at least 17% (mean cumulative diet consumed over juvenile life cycle 1.9g) more diet than those raised on either form of maize with larvae raised on Bt176 consuming 1.12g over their juvenile life cycle and those raised on its isoline 0.968g. In classical pesticide toxicology where the hazard quotient (HQ) is used to assess the safety of a substance (HQ being a comparison of the estimated dose (intake) relative to a reference dose (known) of the toxicity of the substance (USEPA., 1994)) the variation in consumption has implications for setting any values that trigger higher tier testing as if the intake (dose) differs widely from the challenge dose then the subsequent dose relative to body weight will differ across challenge doses and any trigger value will not be an accurate reflection of toxicity.

The Environmental Panel of Advisory Committee on Pesticides (ACP) (Candolfi et al., 2001) advised a 50% effect level as an appropriate trigger value for higher tier testing but noted the lack of sensitivity of experimental design which is also reflected in the trials reported here in the level of variation seen. It has also been reported that in chemical risk assessment uncertainty can be of several orders of magnitude, this is particularly apparent when extrapolating the effects of high doses to those at low doses (Hill and Sendashonga, 2003). Due to the inherent uncertainty of the risk assessment process it is common for several data sources to be used to make an assessment of risk for example case-specific toxicity tests, community structure and species abundance

(Hill and Sendashonga, 2003). Therefore, whilst methodologies need to be replicable and generic it may still be necessary to draw conclusions regarding risk on a case by case basis.

2.4.2 Bt176 transgenic maize *Cry1Ab expression*

To enable worst case scenario testing to be carried out using a dose response immunoassay by ELISA testing was used to evaluate expression levels of the toxin in Bt176 plants. Expression levels varied with age and leaf stage with leaf stage V3 at week 4 having the highest levels of expression at 15.3 $\mu g\ g^{-1}$ fresh weight. Mean expression levels of 11$\mu g\ g^{-1}$ fresh weight are in the same region as those reported by Koziel et al. (1993) in the same line of 13$\mu g\ g^{-1}$ fresh weight. Expression levels decreased consistently with increased leaf stage (V3: 13.3, V4: 10.6 and V5: 8.8$\mu g\ g^{-1}$ fresh weight) irrespective of plant age. Variation of expression is also evident dependent on the section of leaf sampled with the middle section of leaf appearing to have consistent and high levels of expression when compared to the growing and point area of the leaf (Dutton et al., 2005) therefore samples in this trial were collected near the middle section of the leaf.

Given the consumption levels the maximum toxin load individuals raised on Bt176 were subject to was approximately 14μg over their juvenile life cycle. Assuming a mean toxin load equivalent to Bt176 the maximum toxin load juveniles raised on artificial diet would be subject to is 24μg. However, this is not the total load that parasitoid juveniles are likely to be exposed to as they parasitize hosts between 5-9 days old and emerge and spin cocoons when hosts are 12-14 days old, in aphids only $1/10^{th}$ of the GNA seen in the aphid host was passed to the parasitoid (Couty et al., 2001a). Also whilst beneficial insects appear to suffer negative effects from consuming (Hilbeck et al., 1998a) transgenic maize Cry1Ab is not accumulated through the food chain, with no residues found in parasitoid adults and only traces in cocoon silk with the toxin appearing to be excreted on pupation (Couty et al., 2001b; Vojtech et al., 2005).

This study illustrated the degree of natural variation that can occur within an experimental system irrespective diet treatments. This was evident in both larval mass but particularly in consumption data which often recorded as zero. Whilst consumption occurred this zero consumption rating was in part due to mean measures of natural

dehydration of both plant material and artificial diet which negated any positive consumption by the larvae when developing through very early instars. The issue of dehydration of the diet was largely rectified in later experimental designs by placing the diet in small individual containers and allowing larvae to feed on the surface of the diet.

Chapter 3

Stability of Cry1Ab incorporated into artificial diet and biological activity against the susceptible herbivore *Ostrinia nubilalis*.

3.1 Introduction

Bacillus thuringiensis (Bt) is a gram-positive soil bacterium that is characterized by its ability to produce crystalline inclusions (Cry proteins) during sporulation (Hofte and Whiteley, 1989; Koziel et al., 1993). These inclusions have a range of toxicity; most show toxicity to lepidopteran larvae, with some causing toxicity in Diptera and coleopteran larvae (de Maagd et al., 2001; Hofte and Whiteley, 1989) and a small number in nemotodes (de Maagd et al., 2001). As defined by Crickmore et al (1998) a Cry protein is a "parasporal inclusion (crystal) protein from *B. thuringiensis* that exhibits some experimentally verifiable toxic effect to a target organism, or any protein that has an obvious sequence similarity to a known Cry protein."

The insecticidal properties of the crystal proteins produced by the *Bacillus thuringiensis* bacterium and high levels of production have lead to considerable interest in these proteins (Crickmore et al., 1998), their amenability to *in vitro* production typically in *E. coli* also makes them a powerful tool for assessing the dose at which changes in insect behaviour and/or morphology become apparent. As illustrated in this study the protein can be incorporated into artificial diet at highly specific doses whilst maintaining biological activity.

The mode of action of the inclusions occurs in the larval mid gut where the toxin is cleaved, leaving an active fragment of between 27 and 140 kDa (Hofte and Whiteley, 1989) dependent on the specific toxin, as the bacterium generally produces between 1-5 different Cry toxins (de Maagd et al., 2001). Pathogenicity occurs in the larval mid gut where epithelial cells produce and secrete digestive enzymes and where digestion occurs, it is also the point in the gut where absorption of the products produced by digestive processes occurs (Gullan and Cranston, 2005).

The high pH (10-11) in the lepidopteran gut and the presence of digestive enzymes allows the inactive protoxin to be processed i.e. cleaved into its activated form (Hofte and Whiteley, 1989; Whalon and Wingerd, 2003), without this high pH the Cry1Ab protein would not maintain its pathogenicity. This proteolytic cleavage allows the toxin to interact with epithelial cells and bind to its receptor ((Whalon and Wingerd, 2003). It

is this irreversible insertion into the membrane following cleavage that leads to its pathogenic effects.

The nomenclature for the *Bacillus thuringiensis* crystal proteins is constantly under review and updated, there are currently 19 listed and identified Cry1Ab toxins and 49 Cry toxins (Crickmore et al., 2005). Whilst there is variation between the genetic sequences of the different classes of Cry toxins there is also high levels of conservation with 3 dimensional structures in Cry1, Cry2 and Cry3 sharing a high degree of structural similarity (de Maagd et al., 2001). Also proteins with the same primary rank tend to affect the same insect order whereas changes at secondary and tertiary rank level may indicate changes to potency and targeting within that insect order (Crickmore et al., 1998).

The inclusion of the genes coding for Cry toxins into agricultural crop plants can greatly reduce the amount of economic loss due to damage by herbivorous insects, with the European corn borer (ECB), *Ostrinia nubilalis* being a particularly graphic example of the success of including these plant protection products. The ECB is a serious economic pest of maize in both America and Europe with yield losses as great as 7% per borer per plant possible (Koziel et al., 1993). Due to the behaviour of the larvae in boring into the plant at the third instar stage they are particularly difficult to control with conventional pesticide applications (Koziel et al., 1993).

However, whilst the inclusion of these gene products is of great economic benefit there is a potential for exposure of the toxin to non-target organisms, including those which play a beneficial role in regulating pest populations. This project aims to assess the impact of the Cry1Ab insecticidal toxin, on a non-target parasitoid via inclusion of the purified toxin incorporated into artificial diet via a lepidopteran host that can proteolytically process the Bt diet.

In order to conduct these trials it is necessary to establish the stability and biological activity of the toxin in the experimental system. As a highly susceptible pest of the Cry1Ab protein the ECB was used in these trials to establish the stability of the protein when incorporated into the artificial diet and maintained at 25°C during consumption. The protein was also assessed biochemically to ensure it was not cleaved into

biologically inactive fragments. Therefore, this chapter discusses the results of these stability and biological activity trials.

3.2.1 Cry1Ab δ-endotoxin purification

Bacillius thuringiensis crystals (Cry1Ab7 (appendix 3) where prepared from *E. coli* (Neil Crickmore, University of Sussex, Brighton, UK). 500ml of broth was inoculated with the culture and an appropriate antibiotic. The broth was incubated at 37°C or 30°C for 2-3 days. Following incubation the culture was centrifuged in a JLA8 rotor (1000ml pot) at 6,500rpm for 10minutes. Each 500ml pellet was then resuspended in 30ml TES buffer (appendix 3) in a Falcon tube. Lysozyme was added to a concentration of 2mg/ml (i.e. 60mg per 30ml) and then incubated at RT in 'Ferris Wheel' for 3-4 hours. Following ferris wheel incubation the sample was sonicated four times for 1 minute on full power and then transferred to an Oakridge tube and centrifuged at 12,000 rpm for 10 minutes. The pellet was resuspended using a spatula in 30ml 0.5M NaCl, sonicated briefly and then centrifuged at12,000 rpm for a further 10 minutes.

At this point the pellet was washed as required until the sample turned to a white colour. At each step sonicating the sample aided the washing process. The pellet was washed in two washing buffers these being ice-cold 0.5M NaCl followed by ice-cold distilled water. The final pellet was resuspended in 25ml ice-cold water and 2µl removed and run on an SDS-PAGE electrophoresis gel to gauge purity and concentration.

Truncated Cry1Ab1 (appendix 4) was prepared and supplied by Dr William Moar (University of Auburn, USA). The toxin was eluted directly off a HPLC column as a single peak and then desalted, therefore attaining close to 100% purity.

3.2.2 Cry1Ab heat stability and visualization in artificial diet

Beet armyworm artificial diet was prepared following the standard protocol (appendix 1) prior to the addition of dried artificial diet the agarose gel was allowed to cool to 70°C, 60°C and 50°C once the gel reached the required temperature 20µg g^{-1} of uncleaved or truncated Cry1Ab was added, immediately after the addition of the toxin the requisite amount of dried artificial diet was added and mixed thoroughly. Samples were prepared in 1.5ml eppendorf tubes using a microspatular and stored at 1-5°C prior to use. Samples were visualized using western blot analysis and GelCode Blue staining.

3.2.3 Generic methodology for biochemical assays.

3.2.3.i SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE) of protein analysis

SDS-PAGE (gel type NuPAGE 4-12% Bis-Tris 1.0) was used for the visualization of proteins and facilitation of GelCode Blue staining, transfer onto PVDF membrane and Amido black staining for excising bands for protein N-terminal sequencing (as manufacturers guidelines (Invitrogen NuPAGE NP0323 gel and Invitrogen NP001 NuPAGE MOPS running buffer, Invitrogen life technologies, 1600 Faraday Ave, Carlsbad, CA 92008)). This methodology was not used for establishing concentration of the Cry1Ab sample directly after washing.

Artificial diet based samples were heated at 100°C for 5 minutes agitated by hand and then heated for a further 5 minutes. Non-artificial diet based samples were heated for 5 minutes at 100°C. The comb and white strip were removed from NuPAGE Novex Bis-Tris gels (Invitrogen life Technologies, Carlsbad, CA 92008) and rinsed in deionised water. Novex gel tanks were assembled. 500ml of 1 x NuPAGE MOPS (Invitrogen NP001) running buffer was mixed using Milli-Q water. The upper tank was filled with buffer and the remainder of the buffer added to the lower tank. Gels were run at 200Volts for 50 minutes. On completion of run cycle gels were removed and opened at the edges using a gel knife.

3.2.3.iii Gel code blue staining

Following electrophoresis the gel was placed in a clean tray and rinsed 3 times for 5 minutes, with 100-200ml of ultra pure water. Following washing 20ml of Gelcode Blue stain was added and incubated with gentle shaking for 1 hour.

3.2.3.vi Cry1Ab Western blot analysis

Samples were run on SDS-PAGE gel (NuPAGE 4-12% Bis-Tris 1.0, Invitrogen in MOPS running buffer (section 3.2.3.i). The gel was blotted onto 0.2um nitrocellulose membrane using Xcell II Mini-gel blot module (Invitrogen) and transfer buffer (Invitrogen #LC3675) containing 20% methanol. The transfer was undertaken at 30V for 60 minutes.

The blot was transferred to a Falcon tube and blocked overnight in 25ml, 5% Marvel in PBS, 0.1% Tween 20. The primary antibody: Anti-Cry1Ab Rabbit 4, bleed 4 at

1:10,000 dilution in PBS, 0.1% Tween 20 was added and incubated for 1 hour. Following incubation the blot was washed 3 for 10 minutes in 10ml PBS, 0.1% Tween 20. The secondary antibody: Anti-rabbit IgG, peroxidise-labelled (GE Healthcare NA934VS) at 1:10,000 dilution in PBS, 0.1% Tween 20 was added and incubated for 1 hour, then washed 3 for 10 minutes in 10ml PBS, 0.1% Tween 20.

Detection was carried out using Roche LumiLight reagents (Roche Applied Science (2.5ml mixed reagent/blot)) for 5 minutes. The film was then exposed for 2 minutes prior to processing.

3.2.4 N-terminal sequencing and Amido black staining

Truncated Cry1Ab1 samples solublized in distilled water were run on SDS-PAGE gel (section 3.2.i) for N-terminal sequencing and transferred onto PDVF membrane (GE Healthcare (Hybond P #RPN303F)) and transferred into a semi dry chamber. 0.1% solution of Amido black 10B was prepared in 40% methanol and 1% acetic acid. The PVDF blot was agitated in the dye for 1 minute. The blot was destained using water. The water was changed until the sample was sufficiently destained (however this dye can not be over destained). The selected bands of interest were excised for sequencing (Procise CLC494 protein sequencer, Applied Biosystems).

3.2.5 Biological activity bioassay

As a susceptible pest of Cry1Ab *Ostrinia nubilalis*, European Corn Borer (ECB) was assayed to assess the biological activity of the full length uncleaved Cry1Ab1 and the truncated version of the toxin Cry1Ab7 when incorporated into artificial diet, both the mass and survival of ECB juveniles was assessed.

The uncleaved (Cry1Ab7 (appendix 4) and truncated (Cry1Ab1 (appendix 4)) version of the toxin was incorporated in Armyworm Diet, (Bioserv. F9219B) at concentrations of 100ng g^{-1}, 50ng g^{-1} and 10 ng g^{-1} and control diet was also made containing no toxin. The diet was prepared following the recipe in appendix 1, however the agar was allowed to cool to 65°C at which point the appropriate concentration of toxin was added following suspension in distilled water and sonication, the dried artificial diet was added after the solubilized toxin and mixed thoroughly. Between 2-3ml of diet was added to 25mm diameter, capped LDPE containers (Fisher Scientific, Loughborough, UK) and

allowed to cool. On cooling all diet samples where frozen at -20°C until experimental use.

ECB eggs masses were received from (Garst seed Co., Slater, IA 50244, USA) at the commencement of the experiment. Eggs were allowed to hatch into first instar larvae. 5 first instar larvae where added to each LDPE container containing diet, with a perforated cap for ventilation. Larvae were maintained at 25°C ± 1°C, 60% RH and photoperiod 16:8 light: dark. At 5 days old half of the pots were removed and ECB larvae weighed and mortality recorded. At 12 days the remaining larvae were removed, weighed and mortality recorded. In total 25 larvae were added to each of five LDPE pots in each treatment totalling 175 individual larvae.

3.3.2 Cry1Ab heat stability and visualization

The truncated Cry1Ab1 and uncleaved Cry1Ab7 toxin was incorporated into Armyworm artificial diet (F9220B Bioserv) at 50°C, 60°C and 70°C to establish the stability of the forms of toxin in the diet and at a range of incorporation temperatures. Prior to commencing this trial a sample of Cry1Ab7 uncleaved toxin was coloured using household food dye and mixed following the same protocol for heat stability trials. The uncleaved Cry1Ab7 took up the dye colour readily and tests showed that the crystals mixed evenly throughout the diet sample irrespective of the quantity of diet prepared.

Using SDS-PAGE gel code blue analysis the toxin incorporated in the diet was not detectable at any of the incorporation temperatures or in any of the toxin forms (figure 3.1). Furthermore the truncated Cry1Ab1 form solubilised in distilled water (figure 3.1 lane 6) was also undetected. The only band detected is at approximately 95kDa (figure 3.1, lane 8) from the Cry1Ab7 form solubilized in water.

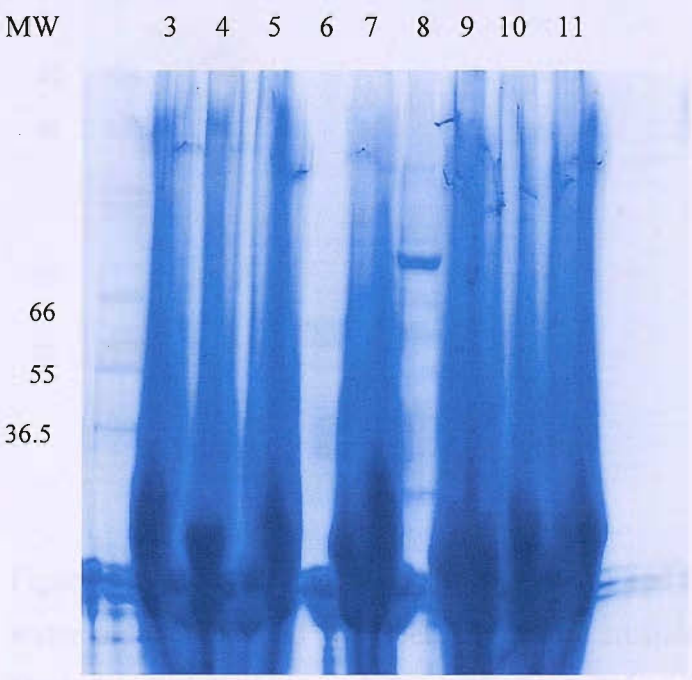


Figure 3.1 – SDS-PAGE gel code blue stain of Cry1Ab1 and Cry1Ab7 diet incorporated toxin. Cry1Ab1 diet incorporated toxin at lane (3) 50°C, (4) 60° and (5) 70°C. Lane (6) Cry1Ab1 solubilized in water, (7) control diet no toxin. Cry1Ab7 diet incorporated toxin, lane (8) dissolved in water, (9) 50°C, (10) 60° and (11) 70°C. Molecular weight (kDa) marker (MW) Mark 12 (Invitogen, Corporation, USA.)

Cry1Ab1 and Cry1Ab7 samples were further visualised using western blot analysis Lumilight reagents (Roche applied Science). As with Gelcode blue visualisation Cry1Ab samples incorporated into artificial diet were not detectable (figure 3.2 lanes 4-9)). However, both Cry1Ab samples dissolved in water were visualized with Western blot analysis. The visualized Cry1Ab7 sample at 120kDa represents the uncleaved protoxin and the two clearly defined bands in the Cry1Ab1 sample at between 60-70kDa represent the N-terminally activated (lower band) and unprocessed (upper band) form of the toxin (figure 3.2)

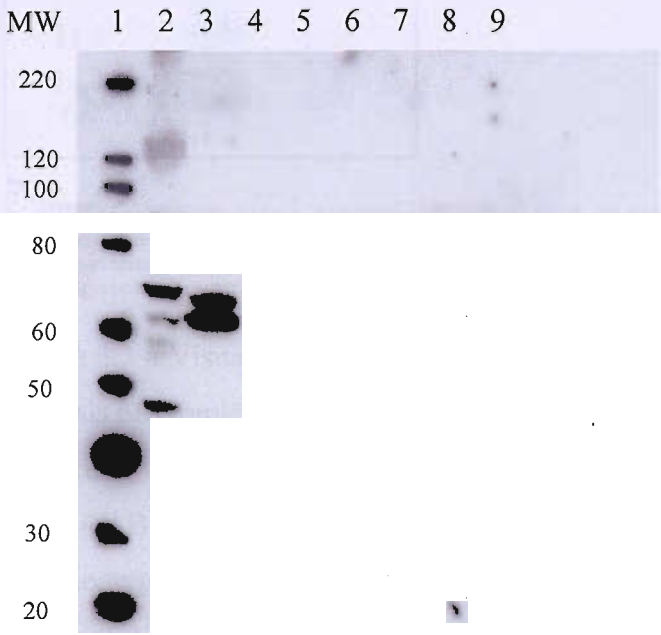
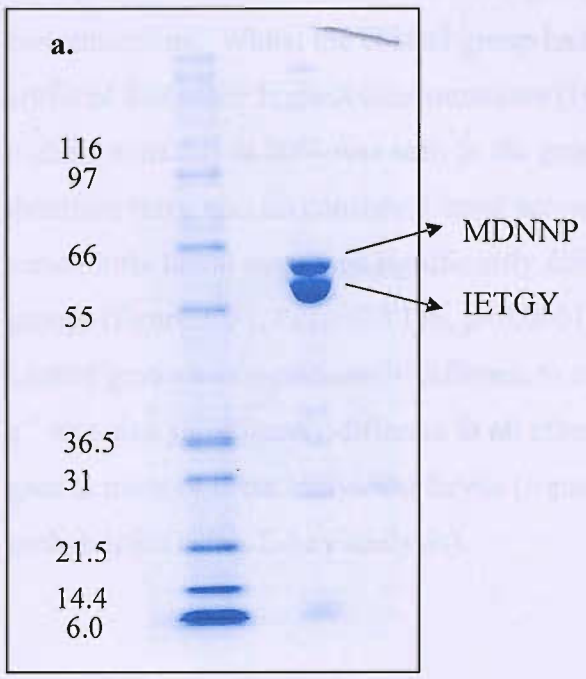


Figure 3.2 - Western blot analysis of Cry1Ab1 and Cry1Ab7 samples dissolved in water and incorporated into Beet Armyworm artificial diet, lane 2 – Cry1Ab7 and lane 3 Cry1Ab1, remaining lanes 3-9 contain Cry1Ab incorporated in artificial diet.

The N-terminal processing elucidated in the Cry1Ab1 Western blot sample (figure 3.2, lane 3) was confirmed by N-terminal sequencing with two clearly defined sequences

one showing cleavage of the first 28 N-terminal amino acids ((N-terminal processed) lower band(figure 3.3))), the region linked with toxin potency (Bravo et al., 2002) and the other lower band unprocessed (figure 3.3).



b.

```
1 mdnnpninec ipynclsnpe vevlggerie tgytpidisl sltqfllsef vpgagfvlgl   = Cry1Ab1
1 mdnnpninec ipynclsnpe vevlggerie tgytpidisl sltqfllsef vpgagfvlgl   = Cry1Ab2
1 mdnnpninec ipynclsnpe vevlggerie tgytpidisl sltqfllsef vpgagfvlgl   = Cry1Ab3
1 mdnnpninec ipynclsnpe vevlggerie tgytpidisl sltqfllsef vpgagfvlgl   = Cry1Ab19
```

Figure 3.3 – Visualization of processing of Cry1Ab1 truncated protein (a) SDS-PAGE analysis of sample solubilized in distilled water, stained with Gel-code blue and Mark12 molecular weight markers (b) amino acid sequence from N-terminal sequencing (Applied Biosystems PROCISE-cLC Sequencer) of first five residues of Cry1Ab1 sample, region highlighted in red indicates sampled sequence.

3.3.2 Stability of Cry1Ab incorporated into artificial diet

3.3.2.ii Biological assay

Whilst Western blot analysis shows some solubility and stability in water it does not clearly confirm biological activity therefore, the stability of the both Cry1Ab forms was assessed using *Ostrinia nubilalis*, European corn borer (ECB) which is known to be highly susceptible to the Cry1Ab toxin. The two forms of the toxin were incorporated into Beet armyworm diet, at doses more than three times the LC_{50} (20-30ng g^{-1}(Koziel

et al., 1993)) at 100ng g^{-1}, over twice the LC_{50} 50ng g^{-1} and less then half the LC_{50} at 10 ng g^{-1}. Mortality and mass was noted at 5 and 12 days development.

At day 5 mortality varied little between groups exposed to the various toxin concentrations. Whilst the control group had 4% mortality, the larvae reared on artificial diet at the highest concentrations (100ng g^{-1}) only had 6% mortality. The highest mortality at 20% was seen in the group at 50ng g^{-1} and 10ng g^{-1} activated toxin therefore there was no consistent trend across the groups. However, whilst mortality varied little larval mass was significantly different between the control and all other groups (figure 3.1 (, $F_{6,141}$=77.176, $p<0.0001$)). Tukey post hoc test showed that the control group was significantly different to all other groups, the uncleaved form at 10ng g^{-1} was also significantly different to all other groups, as illustrated by the significantly greater mass of these individual larvae (figure 3.1 (see appendix 5 for full list of probabilities using Tukey analysis).

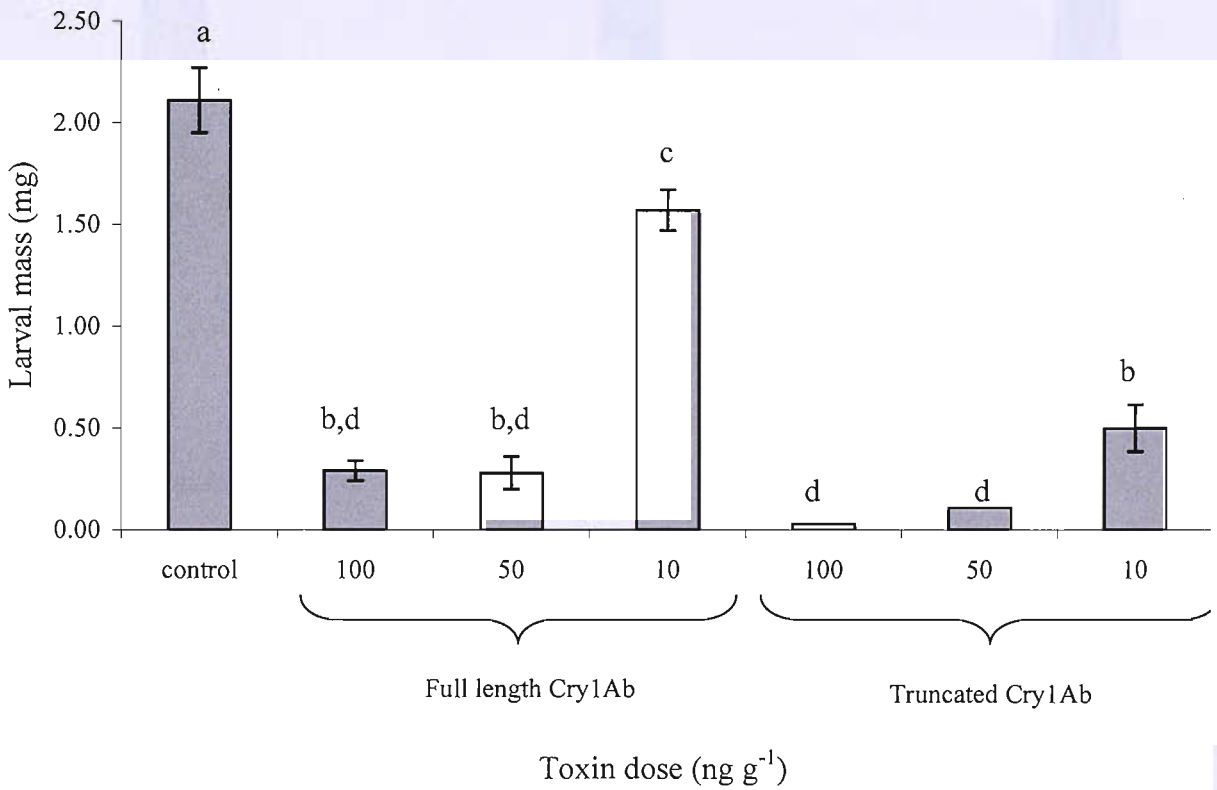


Figure 3.4 – Laval mass of *Ostrinia nubilalis* after 5 days development exposed to (a) uncleaved full length Cry1Ab (b) truncated Cry1Ab at 100, 50 and 10 ng g^{-1}.

At day 12 there was 100% mortality of larvae reared on artificial diet at 100 ng g^{-1} and 50 ng g^{-1} in the truncated form. The uncleaved form had 40% and 36% mortality at 100 ng g^{-1} and 50 ng g^{-1} respectively with the truncated and uncleaved at 10 ng g^{-1} having 12% mortality, the control group having 28% mortality. However, larval mass differed significantly between all groups (figure 3.2 ($F_{4, 88}$=81.921, p<0.001). Tukey post hoc testing showing that all groups where significantly different (figure 3.2 (full details of significance levels given in appendix 6).

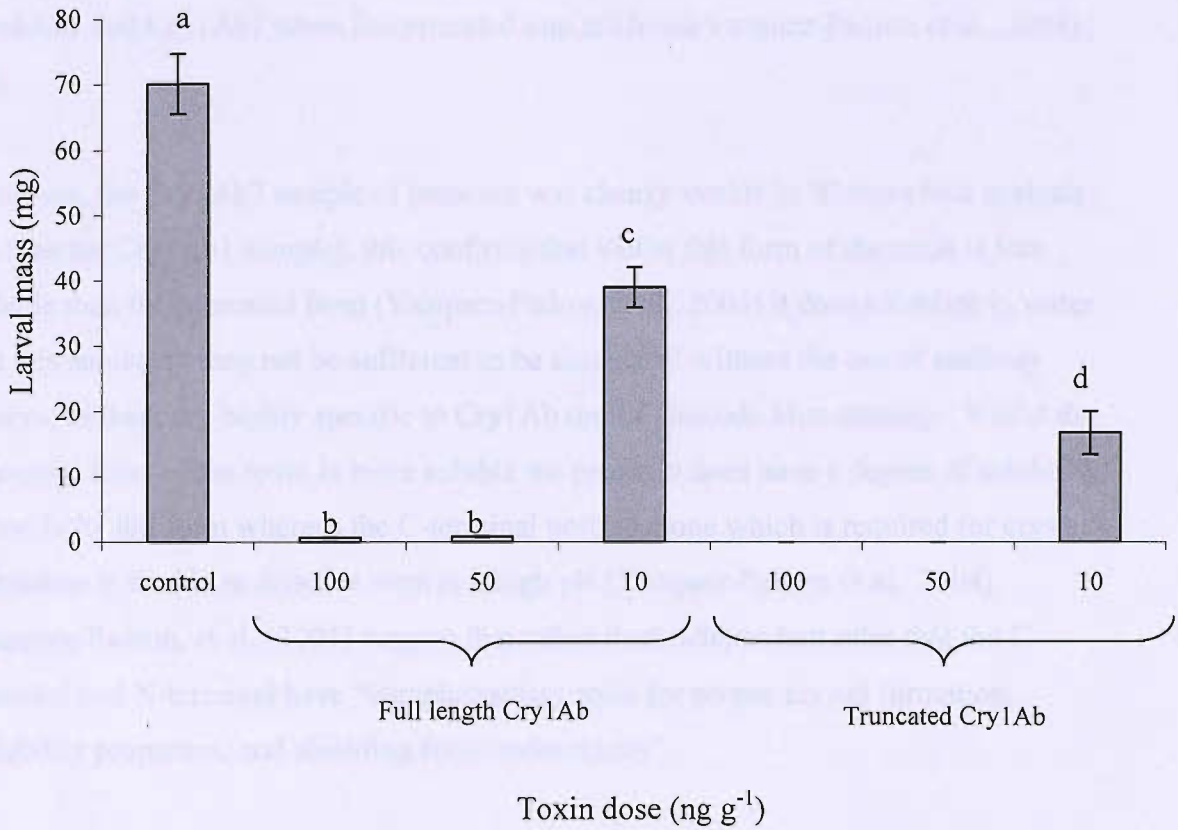


Figure 3.5 – Laval mass of *Ostrinia nubilalis* after 12 days development exposed to (a) uncleaved full length Cry1Ab (b) truncated Cry1Ab at 100, 50 and 10 ng g^{-1}.

Whilst mortality may not be a conclusive indicator of biological activity in this experimental model the significantly reduced larval mass at all doses and toxin forms including that at only half the LC_{50} show that the toxin is not only biologically active but retains this activity over the length of the trial as no larval recovery was seen post 5 day monitoring.

3.4 Discussion

3.4.1 Cry1Ab heat stability and solubility

Western blot analysis of artificial diet samples prepared at a 50°C, 60°C and 70°C proved inconclusive regarding the stability of the toxin in the diet however it is unclear if this is due to detection difficulties or inactivation of the toxin therefore biological assays were also performed to address this ambiguity and establish biological activity of Cry1Ab1 and Cry1Ab7 when incorporated into artificial(Vazquez-Padron et al., 2004) diet.

However, the Cry1Ab7 sample of protoxin was clearly visible in Western blot analysis (as was the Cry1Ab1 sample), this confirms that whilst this form of the toxin is less soluble than the truncated form (Vazquez-Padron et al., 2004) it does solublize in water but this solubility may not be sufficient to be elucidated without the use of antibody probes, as these are highly specific to Cry1Ab unlike Gelcode blue staining. Whilst the truncated form of the toxin is more soluble the protoxin does have a degree of solubility when in its full form whereas the C-terminal portion alone which is required for crystal formation is unable to dissolve even at a high pH (Vazquez-Padron et al., 2004). Vazquez-Padron, et al. (2004) suggest that rather than independent roles that the C-terminal and N-terminal have "complementary roles for proper crystal formation, solubility properties, and shielding from endotoxicity".

The apparent partial solubility of the protoxin in water allows this form of the toxin to be added to artificial diet samples when dissolved in this way following sonication. Preliminary trials with dyed Cry1Ab7 samples also confirmed that this form of the toxin does distribute freely within the diet however is subject to some "clumping" of crystals.

Although, both the Western blot analysis and Gelcode blue staining do not confirm if the toxin samples are biologically active or heat stable the methodology does point to a high degree of heat stability. The Western blot analysis requires the samples to be heated to 100°C for 5 minutes following this process both forms of the toxin where still visible on the Western blot analysis. Therefore, it is likely that the amount of extraneous protein in the artificial diet masks any Cry1Ab detection (figure 3.1) and/or

that the concentration of the Cry1Ab in the diet samples was below the detection level of the assay. Samples prepared with the toxin dissolved in water were over 10 times more concentrated than those in the artificial diet samples.

3.4.2 Biological activity

Whilst, it is clear that the protoxin dissolves less freely in water than the truncated form (Vazquez-Padron et al., 2004) the effect on biological activity when processed in the insect gut may be limited. Experimental data from ECB dose response showed little difference between variation between samples of the protoxin incorporated diet and the truncated toxin suggest that the larvae are freely coming into contact with the toxin and that it is evenly distributed throughout the diet sample . However the greater mass seen in individuals reared on the Cry1Ab7 samples compared to those raised on the truncated form at both days 5 and 12 suggest a reduced potency of the toxin in its full length form, what is unclear is if this reduced potency is linked to reduced dissolving or due to the effect of incomplete processing or binding in the larvae gut.

3.4.3 N-terminal sequencing and Cry1Ab1 cleavage

To establish Cry1Ab1 toxin processing the two distinct bands elucidated in the Cry1Ab1 sample were dissolved in water and separated and visualized using Western blot analysis before excising for N-terminal sequencing. The bands represent the truncated form of the toxin that is cleaved from its native 1155 amino acids to 648 amino acids (approximately 67kDa (Bravo et al., 2002; Koziel et al., 1993)). Further processing then takes place to remove 25-30 amino acids from the N-terminal (Bravo et al., 2002). The lower band (figure 3.3) represents this N-terminal processed region which is involved in insertion to the larval membrane and pore formation (Bravo et al., 2002). In the unprocessed form the development of *E. coli* was unaffected whereas in the processed form development was severely affected (Bravo et al., 2002) therefore this N-terminal processing clearly has a role in potency . Additionally, in *Manduca sexta* exposure to the unprocessed form no pores were formed and membrane depolarization did not occur (Bravo et al., 2002). Therefore, the presence of two forms of the Cry1Ab1 toxin in this sample are likely to affect potency in this system, given the similar concentration of each form this may only deliver half the expected dose when administered avoiding the gut and further processing (direct effect).

Whilst, differential processing at the N-terminal region may affect potency of the sample a further consideration is the differential potency of the two forms of Cry1Ab these being the truncated form Cry1Ab1 and the protoxin form being Cry1Ab7.

Prior to cleavage both forms are 1155 amino acids in length and have 99% sequence similarity ((http://www.ncbi.nlm.nih.gov/ 5/05/06) appendix 4). There are six changes in the amino acid sequences four of which occur in the processed N-terminal sequence ((http://www.ncbi.nlm.nih.gov/ 5/05/06) appendix 4). Given that toxins assigned a different quaternary rank can be identical (quaternary ranks assigned to each independently sequenced toxin gene) and that quaternary rank distinguishes 95% sequence similarity (Crickmore et al., 2005) it is unlikely species specificity or potency will be affected by these changes.

Chapter 4

The direct and host mediated effects of Cry1Ab toxin on the parasitoid wasp *Cotesia marginiventris*.

4.1. Introduction

Crops expressing the Cry1Ab insecticidal protein derived from *Bacillus thuringiensis* (Bt) are in widespread use in agriculture consequently there is the potential for non-target organisms to come into contact with this toxin. Given the ubiquitous use of the protein and the likelihood of exposure to non-target organisms it is prudent to establish if non-targets are detrimentally affected by the toxin and also to ensure that there is a robust structure in place to clarify any risk whether this risk is related to hazard or exposure. This is particularly important for non-target organisms such as parasitoid wasps that can act as biological control agents. This is particularly relevant to *Cotesia marginiventris* used in this study as it is a solitary endoparasitoid (Jalali et al., 1987; Tillman, 2001) of many lepidopteran hosts in a variety of agricultural settings (Tillman, 2001) therefore is likely to be exposed to the endotoxin via the host.

Genetically modified Bt crops express the transgenic protein throughout the growing session and in relatively high concentrations therefore non-target organisms can show sub lethal effects of exposure to Bt insecticidal proteins (Hilbeck et al., 1999). This is in contrast to conventional pesticides containing Bt proteins which are sprayed at discrete intervals and subject to degradation by UV light and rain. Also plant expressed forms of *B. thuringiensis* proteins are expressed mostly in their truncated fully activated form (Koziel et al., 1993) whereas conventional pesticides containing Bt require further processing in the insect gut to attain their active conformation.

The insecticidal Cry proteins derived from the *Bacillus thuringiensis* (*Bt*) bacterium are probably some of the most widely used transgenic crops in commercialisation (Hilbeck et al., 1998, Hilder and Boulter, 1999, Peferoen, 1997, Mendelsohn et al., 2003), and have insecticidal properties against a range of insects (de Maagd et al., 2001; Kumar and Venkateswerlu, 1998; Mendelsohn et al., 2003). Transgenic crops have been in commercialisation for the past 10 years and in 2005 the global area of transgenic crops was 90 million hectares (James, 2005). Transgenic crops were planted by 8.5 million farmers in 21 countries (James, 2005).

The pathogenicity of the activated toxin occurs via receptors on the brush border membrane of the larval mid gut where ion-selective channels or non-selective pores are opened or formed disrupting the osmotic balance across the insect gut membrane (de Maagd et al., 2001; Hilder and Boulter, 1999; Sanchis et al., 1994). The disruption of the osmotic balance causes swelling and ultimately lysis of the gut epithelial cells (Hilder and Boulter, 1999; Sanchis et al., 1994) leading to death of the insect. This is an important methodological consideration as avoiding contact with the host gut allows parasitoids to be directly exposed to the toxin whilst avoiding some of the suboptimallity seen in hosts ingesting Cry1Ab incorporated into artificial diet.

Ecological risk assessment (ERA) is an important tool in regulating novel technologies such as genetically modified (GM) crops and quantifying any risk. ERA provides a scientific and clearly defined process to allow the regulation and registration of such technologies (Maltby, 2006). The quantification of risk requires the assessment of two components these being "hazard" and "exposure"(Conner et al., 2003; Maltby, 2006; Raybould, 2004), it is important to differentiate between these two components as there is no risk from a highly toxic substance if the study organism never comes into contact with it (exposure) (Maltby, 2006). Hazard can be more fully defined as "the intrinsic ability of the technology to cause harm" (Raybould, 2004) for example direct toxicity and exposure; how likely that harm is to occur (Raybould, 2004) i.e. is the organism likely to come into contact with the hazard . The experimental element of risk assessment can be used to define the potential hazard and this is the region of ecological risk assessment this study considers.

In carrying out a risk assessment it is vital to determine robust and measurable parameters on which to assess the potential risk, these are know as endpoints. There are generally recognised as being two types of endpoints: assessment endpoints and measurement endpoints (USEPA., 1994). Measurement endpoints as defined by the EPA (1994) are "ecological attributes that are adversely effected by contaminants and readily measurable.....and closely related to the assessment endpoint". An "assessment endpoint is an explicit expression of the environmental value to be protected, operationally defined as an ecological entity and its attributes" (USEPA., 1998) an ecological entity may be for example in important insect species and its attributes; fecundity and recruitment (USEPA., 2003).

Measurement endpoints may be lethal or sub lethal effects or acute or chronic tests (USEPA., 2003). An example of sub lethal effects may be reduced growth, impaired reproductive capacity and/or behavioural changes (USEPA., 1994). Acute toxicity tests are short term tests at relatively high concentration and may determine the lethal concentration at which 50% of organisms die (LC_{50}), this will specify both the concentration and duration at which lethality occurs (USEPA., 1994). Conversely chronic tests can assess sub lethal effects which have ecological significance such as reduced growth that may for example lead to reduced reproductive capacity (USEPA., 1994).

This study considers both acute toxicity in the form of a single large dose and chronic exposure over the maturation time of the parasitoid larvae within the host. The aim of the study was to establish the hazard to the non-target parasitoid wasps *Cotesia marginiventris* from the Cry1Ab toxin and importantly if this hazard was due to direct toxicity of the Cry1Ab protein or due to suboptimality of the host. Previous studies have shown adverse effects on mortality and development time to parasitoids when exposed to the Cry1Ab toxin (Hilbeck et al., 1999) (Vojtech et al., 2005) (Hilbeck et al., 1998a) (Ramirez-Romero et al., 2007) but have not resolved the question of direct or suboptimal host mediated effects.

4.2 Materials and Methods

4.2.1 Maize plants

Transgenic maize (*Zea mays L.*) plants, (Syngenta event Bt-176) expressing the Cry1Ab protein and the untransformed control cultivar were used in all bioassays. Individual maize plants where cultivated in a greenhouse at 25°C ±5°C and at a relative humidity of 50%± 20%, in 1.0l, 13cm diameter, 12cm high pots, 3 seeds were sown into each pot. Plants were cultivated in Vapagro modular compost and no fertilizer was applied. New plants were sown weekly and used for experiments between 3-5 weeks old (V5-V7) as *S. littoralis* are known to be primarily a pest of young plants (Dutton et al., 2005).

4.2.2 Insects

All insect cultures and bioassays were maintained at 25°C ±1.0°C, RH 60% ±20% and a photoperiod of L:D 14:10H in a cooled incubator ((AC/013, Thermo-Electron, Basingstoke, UK.).

Spodoptera littoralis were used as a non-target herbivore as it is only partially susceptible to the Cry1Ab protein produced by *Bacillus thuringiensis* (Sneh et al., 1981). *S. littoralis* were maintained for bioassay from eggs supplied by Syngenta, Bracknell, U.K., eggs were supplied each week on netting (oviposition substrate). On receipt *Spodoptera littoralis* eggs clusters were left on the netting and placed in 250ml pots (Roundstone, Catering Equipment Ltd, Melksham, Wiltshire, UK) with ventilation containing an excess of artificial diet (Beet Armyworm diet F9220B Bio-Serve, Frenchtown, NJ 08825 (see appendix 1 for full ingredients and preparation details)) was added to each pot.

Cocoons of the endoparasitoid *Cotesia marginiventris* were obtained from the Laboratoire d'Entomologie Evolutive, Institut de Zoologie, Université de Neuchâtel, Switzerland. *Cotesia marginiventris* adults were maintained in 30x30x30cm Perspex cages at standardized conditions. Adults were reared on a 20% honey/water solution soaked cotton wool which was changed every 48 hours.

For general culturing 30-35 second instar *Spodoptera littoralis* juveniles were removed from large emergence groups and placed in 250ml pots (Roundstone, Catering

Equipment Ltd, Melksham, Wiltshire, UK) with ventilation containing an excess of artificial diet (Beet Armyworm diet F9220B Bio-Serve, Frenchtown, NJ 08825). *S. littoralis* juveniles were confined with 2 mated *C. marginiventris* females (2-6 days old); pots also contained a small ball of damp cotton wool dipped in honey. Pots were observed until at least one female had attacked a host. The *C. marginiventris/S. littoralis* groups were then left to parasitize for 24 hours as this was observed to be the time over which the highest parasitism occurred (Tillman, 2001). After 24 hours the adult *C. marginiventris* and honey solution were removed. *C. marginiventris* juveniles took between 8-12 days to emerge during which time the pots were monitored and any large unparasitized *S. littoralis* were removed and fresh artificial diet added as required.

4.2.3 Direct exposure – Injecting of S. littoralis *juveniles*

Second instar *Spodoptera littoralis* juveniles were weighed and those weighing between 0.5 and 1.1mg (i.e. 1st and 2nd instar (Vojtech et al., 2005)) were removed from large emergence groups and placed in fresh 250ml pots (Roundstone, Catering Equipment Ltd, Melksham, Wiltshire, UK) with ventilation containing an excess of artificial diet (Beet Armyworm diet F9220B Bio-Serve, Frenchtown, NJ 08825) in groups of 35 individuals, 7 groups in total were parasitized for each bioassay replicate. Two mated *C. marginiventris* females (2-6 day old) were added, pots also contained a small ball of damp cotton wool dipped in honey. Pots were observed until at least one female had attacked a host. The C. *marginiventris/S. littoralis* groups were then left to parasitize for 24 hours.

S. littoralis juveniles were raised and parasitized as for the artificial diet bioassays. Following parasitism *S. littoralis* juveniles were maintained in groups of 35 in 250ml pots. 4 days after parasitism juveniles were weighed and divided at random into one of the experimental groups. 20 juveniles were assigned to each group these being water injection, Cry1Ab injection, ice exposure control and no injection control. Following weighing juveniles were maintained in groups of 10 in plastic pots (60mm diameter x 30mm deep) with an excess of artificial diet.

Cleaved, lyphosized Cry1Ab was diluted to 1mg ml^{-1} using room temperature distilled water. The solution was divided into 400µl aliquots, red dye was added at 0.1% v/v and

stored immediately at -20 °C for injecting. Red dye was also added to distilled water at 0.1% and stored at -20 °C for injecting in 1ml aliquots.

1 mm glass rods were pulled at an initial pull of 9 and furnace strength 8 (Micro-electrode puller, C.F.P.). Cleaved, lyphosized Cry1Ab was diluted to 1mg ml^{-1} containing 0.1% red dye and distilled water containing 0.1% Scarlet dye (containing E110 Sunset Yellow) to facilitate visualization of injecting. Each needle was loaded with 9µl of either distilled water or solubilized Cry1Ab solution.

S. littoralis juveniles were placed on a ridged solid agarose mould in a 90mm diameter Petri dish. The Petri dish was held on top a 250ml pot containing ice to chill the mould. The larvae were injected once they had stopped moving. Ice control larvae were held on the chilled mould until they stopped moving then removed. An un-injected and un-cooled control was also carried out.

S. littoralis juveniles were injected for 1 second at 2.0psi using pure nitrogen, oxygen free gas at 40psi using a micro-injector PL1-100, (Medical Systems corp., Greenvale, NY11548) between the 1st and 2nd pair of abdominal prolegs. The area of the sphere of delivered volume was measured. Using the area of a sphere equation the volume of liquid delivered in each injection was calculated as 0.5µl. Following injection juveniles were maintained in groups of 10 in 60mm diameter pots containing an excess of artificial diet. *S. littoralis* juveniles were weighed at 7 days following parasitism. Pupation time and adult tibia length were recorded. In total 20 larvae were exposed to each treatment over 10 replicates totalling 800 larvae.

All *S. littoralis* larvae were stored on death or emergence of the parasitoid and *C. marginiventris* cocoons and adults were stored on completion of the experiment for ELISA analysis at -20 °C.

4.2.4 Biological activity bioassay – Plutella xylostella

As an insect highly susceptible to Cry1Ab *Plutella xylostella* were exposed to aliquots of both solubilized Cry1Ab and dye marked distilled water after they had undergone freezing and thawing and use for injecting to confirm biological activity.

Plutella xylostella larvae were obtained from a stock culture at the University of Southampton, Southampton, England. *P. xylostella* were maintained at 23°C ±1°C, 70% RH and 8:16 light: dark. Chinese cabbage plants were cultivated in Vapagro modular compost in 0.25l pots at 25°C ±5°C and at a relative humidity of 50%± 20% fertilizer was not applied. New plants were sown as required for culture and experimental work and harvested at 4- 6 weeks old for experimental work.

Non-senescesing leaves were removed from the base of Chinese cabbage plants and placed in a 250ml pots. A small ball of damp cotton wool was placed around the cut area of the leaf to maintain leaf turgor. Leaves were painted with the 300µl of the remaining solution of each solubilized Cry1Ab or 300µl of distilled water and red dye aliquot. Leaves where then allowed to dry. When dry 10, 3rd or 4th instar *P. xylostella* were placed on each leaf and mortality monitored and recorded until all remaining larvae had pupated.

4.2.5 Prey mediated effects - Artificial diet dose response bioassay

Second instar *Spodoptera littoralis* juveniles and weighing between 0.5 and 1.1mg were removed from large emergence groups and placed in fresh 250ml pots (Roundstone, Catering Equipment Ltd, Melksham, Wiltshire, UK) with ventilation containing an excess of artificial diet (Beet Armyworm diet F9220B Bio-Serve, Frenchtown, NJ 08825) in groups of 35 individuals, 7 groups in total were parasitized for each bioassay replicate. Two mated *C. marginiventris* females (2-6 day old) were added, pots also contained a small ball of damp cotton wool dipped in honey. Pots were observed until at least one female had attacked a host. The C. *marginiventris/S. littoralis* groups were then left to parasitize for 24 hours.

Cleaved (fully active), lyphosized Cry1Ab was diluted to 1mg ml^{-1} using room temperature distilled water, following dilution the solution was divided into aliquots sufficient to make 1 litre of artificial diet at concentrations of 1, 2, 10, 20, 100µg g^{-1} Cry1Ab and used immediately.

Artificial diet Beet Armyworm diet F9220B Bio-Serve, Frenchtown, NJ 08825was prepared following manufactures instruction (see appendix 1). Prior to the addition of dried artificial diet the agarose gel was allowed to cool to 65°C, as toxin stability

analysis had shown the toxin to maintain biological activity at this temperature (see Chapter 3). Once the gel reached 65°C the required volume of diluted Cry1Ab was added, immediately after the addition of the toxin the dried artificial diet was added and mixed thoroughly to obtain the following concentrations 1, 2, 10, 20, 100 and 0µg g^{-1} (control) Cry1Ab. 2-3ml of artificial diet was then added to 25mm diameter, LPDE containers (Fisher scientific, UK).

Following the parasitism period *S. littoralis* juveniles were removed and assigned at random to one of the experimental conditions. 3 juveniles were placed in a 25mm diameter, LPDE container (Fisher scientific, UK) containing 2-3ml of artificial diet containing 1, 2, 10, 20, 100 or 0 µg g^{-1} (control) Cry1Ab. Each experimental trial contained 10 pots in each condition with a total of 30 individuals and 3 replicates were carried out for each condition totalling 720 juveniles. 30 individuals in 3 replicates were also exposed to Bt176 and its isoline maize. For exposure to plant material *S. littoralis* juveniles were placed in plastic pots (60mm diameter x 30mm deep) following parasitism in groups of 3, excised pieces of leaf plant material was added to the pots and changed every 48 hours in both Bt176 and control maize.

S. littoralis juveniles were weighed at 4 and 7 days post parasitism at which time the artificial diet was also weighed. Larval frass was removed at day 7 and stored at -20°C for further analysis. Pupation time, eclosion time (pupation to adult emergence), time to adult starvation and adult *C. marginiventris* tibia length were recorded.

All *S. littoralis* larvae were stored on death after emergence of the parasitoid and *C. marginiventris* cocoons and adults were stored on completion of the experiment for ELISA analysis all samples were stored at -20 °C prior to testing.

4.2.6 ELISA analysis

Cry1Ab concentrations in insect material were quantified using a commercial Cry1Ab/Cry1Ac ELISA Pathoscreen kit (Agdia, USA).). All frozen samples were weighed and number of individuals in each samples recorded prior to testing. Samples were then added to a 1.5ml centrifuge tube and mixed with 1 x mPBS Tween buffer solution at a ratio of 1:5 w/v for *S. littoralis* larvae and frass, 1:20 for *C. marginiventris* cocoons and adults. This was the minimum buffer volume to provide a minimum 100µl

sample for ELISA testing. *Spodoptera littoralis* larvae and frass samples were further diluted by x10 and x 100 to be within optimal range of sensitivity compared to the standards. Two stainless steel ball bearings were added to each sample eppendorf. The samples were loaded into a macerator and agitated for 1.5 minutes then rotated and agitated for a further 1.5 minutes. Samples were centrifuged at 13,000 rpm for 5 minutes to spin down any remaining tissue. All samples where then maintained on ice prior to analysis.

Following centrifugation 100µl of supernatant was dispensed into each well of a 96-well microplate provided with the kit and an ELISA test conducted. Samples were assayed according to the standard protocol of the kit and plates read at 450nm (Anthos reader 2001, Anthos labtech instruments).

Corresponding tissue blanks (samples from control treatments) were subtracted from all sample treatments and limit of detection was determined by interpolation from Cry1Ab standard curve at 3 standard deviation from tissue blank means (as manufactures instructions). Any samples between 0.26 and 0.07 were therefore reported as traces.

4.2.7 Statistical Analysis

All statistical analysis was carried out using SPSS for windows version 14, excluding sex ratio analysis, which was analysed using Microsoft Excel for windows. All weight and tibia length data was transformed using logarithmic transformation in order to conform to a normal distribution of one-way ANOVA. Tukey post hoc test was used following one-way ANOVA if a significant result was obtained.

4.3.1 Direct effects – Injecting trials

***4.3.1.i Spodoptera littoralis* mass.** The mass of *S. littoralis* larvae was monitored prior to and following the injection process to assess any detrimental effects to the host. *S. littoralis* larvae were weighed before injection (4 days after parasitism) to ensure only larvae within a range of mass of 0.5 and 1.1 mg were used for injecting. Host were then weighed 3 days after being injected i.e. 7 days following parasitism. Larvae subjected to Cry1Ab injecting had significantly reduced mass when compared to the uninjected control ($F_{3,431} = 2.954$, $P = 0.031$). However, larvae injected with distilled water and treated to chilling (ice control) were not significantly different to Cry1Ab or injected controls ((figure 4.1) $F_{3,431}=2.954$, $P=0.50$).

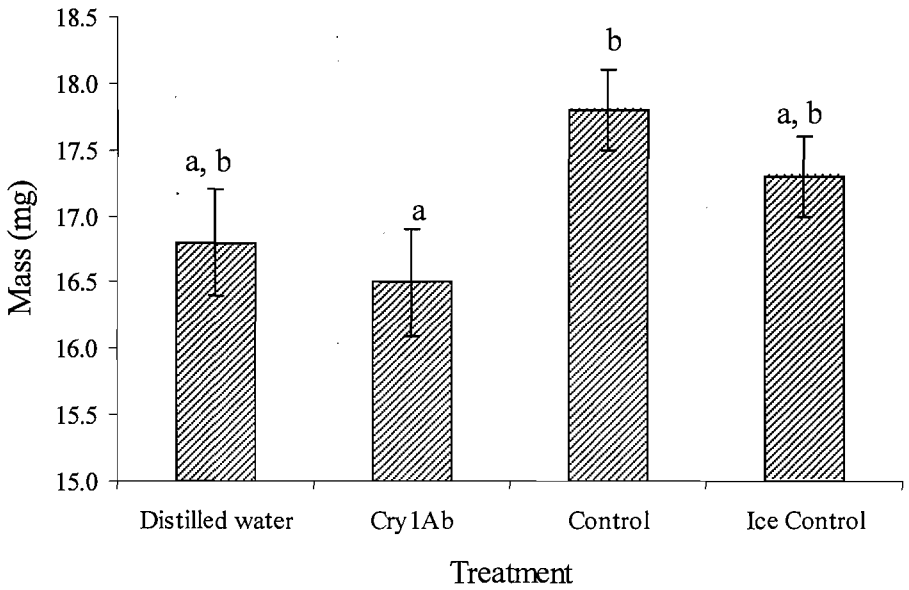


Figure 4.1 – Mean larval mass of *Spodoptera littoralis* larvae at 3 days after injecting (7 days after parasitism). Columns with different letters represent treatment means that are significantly different at $P = 0.029$.

4.3.1.ii Spodoptera littoralis *mortality*. Host mortality was significantly higher in both distilled water and Cry1Ab injection treatments when compared to both uninjected

control conditions ($x^2 = 51.49$, d.f. = 3, $P<0.001$). Cumulative mortality of Cry1Ab and distilled water injection treatments was 62.5% and 56% respectively whereas both uninjected control conditions was between 32% and 35% (figure 4.2). These rates of mortality include instances where hosts were parasitized but parasitoids failed to emerge. The majority of mortality in the injected conditions occurred in the 24 hours immediately following injecting with 50% mortality in Cry1Ab injected hosts and 41% mortality in distilled water injected controls. Whereas at this time point in uninjected controls (day 8 after parasitism) mortality was less than 10% (figure 4.1).

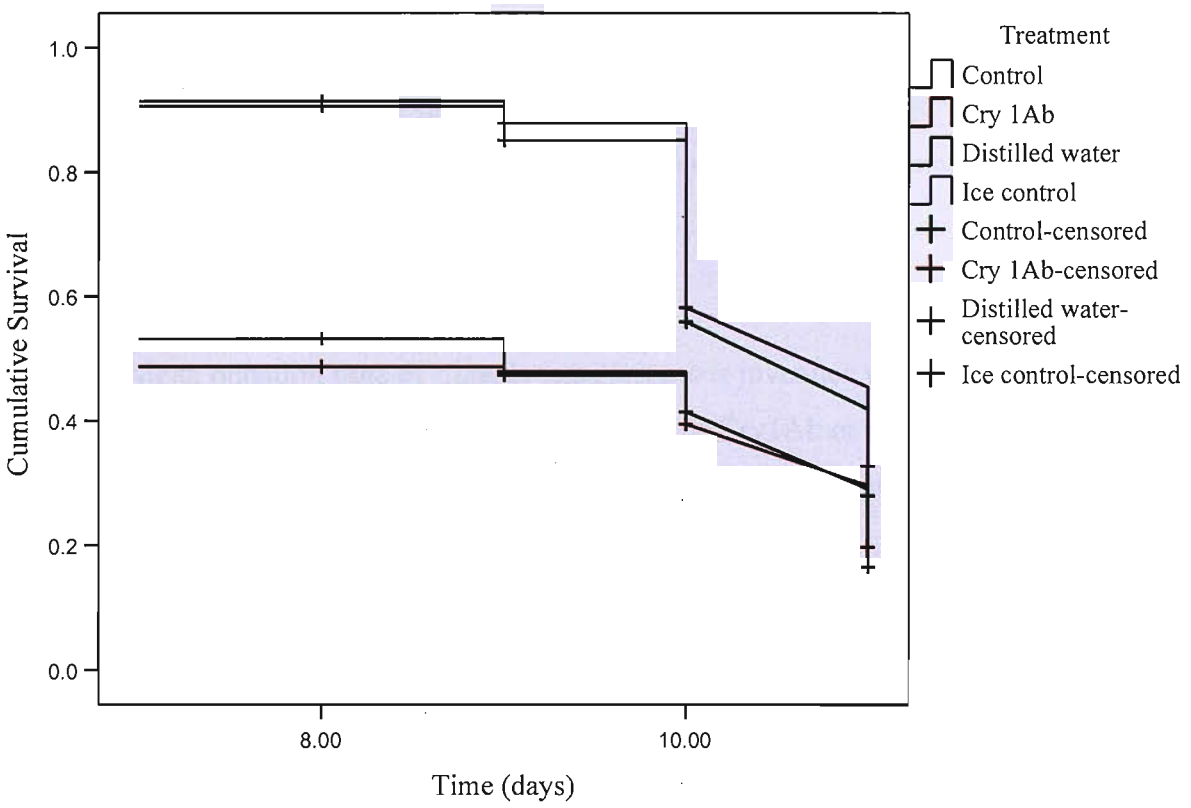


Figure 4.2 – *Spodoptera littoralis* host mortality following parasitism by *Cotesia marginiventris* parasitoids and Cry1Ab and distilled water injection treatment and controls.

4.3.1.iv Cotesia marginiventris *pupation time* – Pupation time was significantly longer in *C. marginiventris* larvae raised in Cry1Ab injected hosts when compared to those injected with the distilled water control (($F_{1,100}$ = 6.961, P = 0.01) figure 4.3). Parasitoids reared on Cry1Ab injected host took 9.5 days to pupate compared to a mean of 9.1 days in distilled water injected hosts. There was no significant difference between pupation time in the other treatments with pupation taking a mean of 9.1 – 9.2 days (figure 4.3) $F_{3,285}$=3.365, P>0.05)).

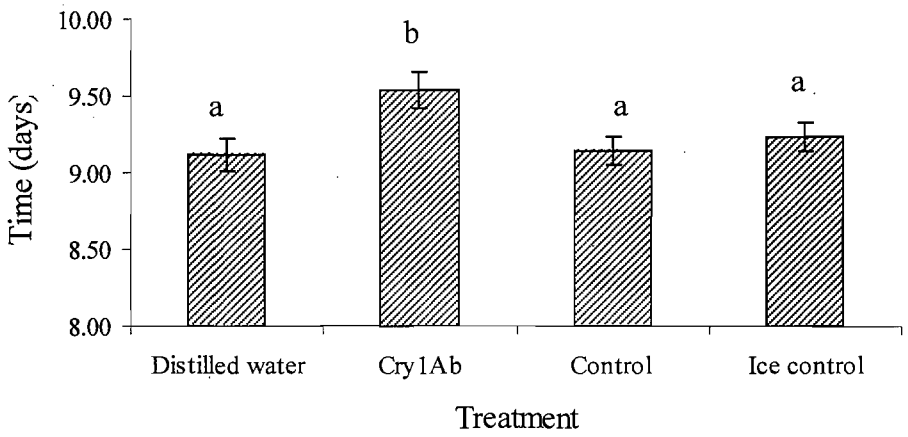


Figure 4.3 – Mean pupation time of *Cotesia marginiventris* juveniles when raised in *Spodoptera littoralis* hosts injected with distilled water or Cry1Ab or no treatment controls. Columns with different letters represent treatment means that are significantly different at P < 0.05.

4.3.1.v Cotesia marginiventris *adults, tibia length*. Tibia length was used as a measure of adult size (Blanckenhorn et al., 2003) (Ellers and van Alphen, 2002) (Van Emden and Kifle, 2002) and as an indicator of fitness (Sagarra et al., 2001) when comparing adult parasitoids. Tibia length did not differ significantly between the distilled water injection control and Cry1Ab injection ($F_{3,331}$ = 4.682, P>0.05), however, Cry1Ab injected treatment of *C. marginiventris* had a significantly shorter tibia than uninjected controls at a mean of 0.825mm and 0.844mm respectively ($F_{3,331}$ = 4.682, P=0.004). Distilled water injected controls were also significantly shorter than uninjected controls ($F_{3,331}$ = 4.682, P=0.033 (figure 4.4)).

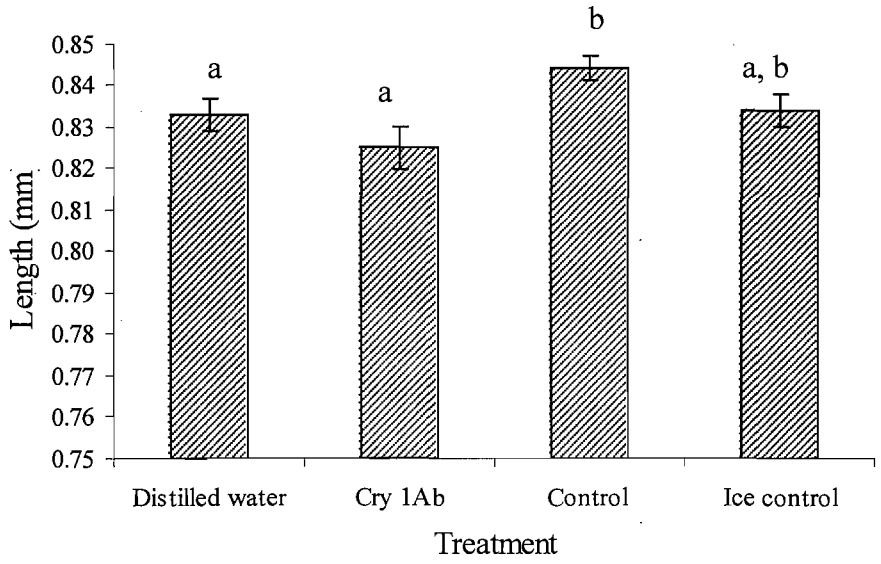


Figure 4.4 – Mean tibia length of *Cotesia marginiventris* adults exposed to Cry1Ab toxin via microinjected *Spodoptera littoralis* host. Columns with different letters represent treatment means that are significantly different at $P < 0.05$.

4.3.1.v **C. marginiventris *emergence rates*** - Emergence rates varied little with treatment with a range between 82% emergence in Cry1Ab injected group and 90% in both no treatment control and ice control and 88% in the distilled water control ($F_{3,35}=0.618$, $P=0.608$)

4.3.1.vi **C. marginiventris *sex ratio*** - Sex ratio did not differ significantly between any of the treatments ($G=0$, d.f $=3$, $P>0.05$). In the distilled water injection, the ice control and no injection control treatments mean sex ratio was identical at 46% females and 54% males (figure 4.5). Whilst, there were fewer females in the Cry1Ab injected population at 38% female this was not significantly lower than the other treatments (figure 4.5).

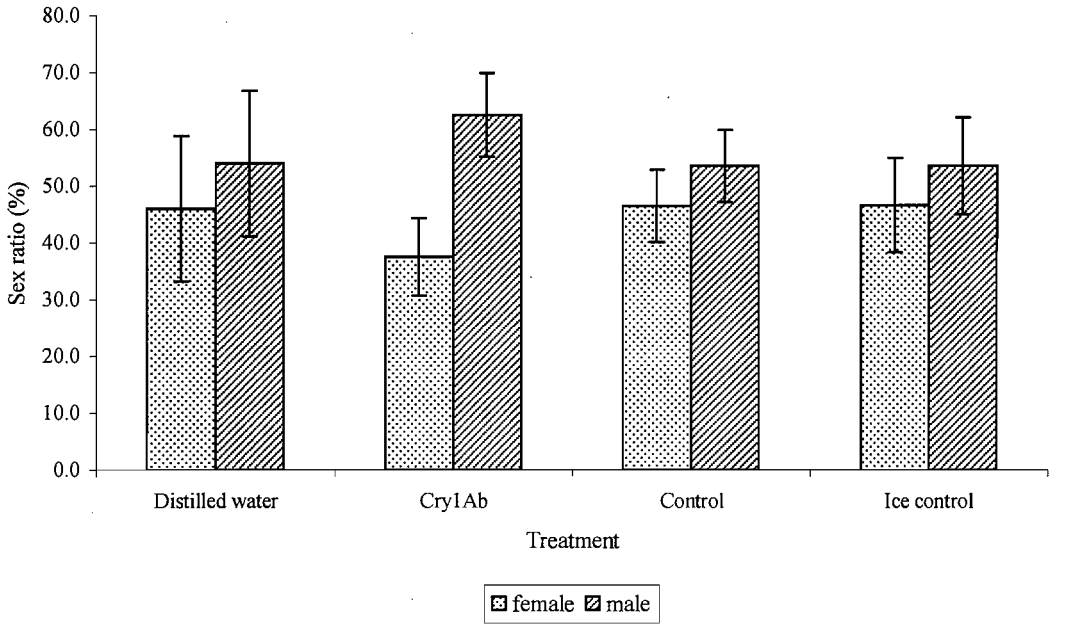


Figure 4.5 – Sex ratio of *C. marginiventris* adults raised on *S. littoralis* host injected with Cry1Ab, distilled water or control treatment (± S.E.).

4.3.2 **Plutella xylostella *Positive control*** – As an insect highly susceptible to the Cry1Ab toxin *Plutella xylostella* larvae were used as a positive control to determine the biological activity of injection aliquots following freezing and use for injecting trials. All aliquots used for injecting were tested for biological activity, 3 showed 100% mortality within 48 hours and the remaining aliquots 80% mortality. Figure 4.6 shows a clear lack of feeding in the Cry1Ab treatment condition and dead larvae (circled red (figure 4.6a)) and the control treatment with pupating larvae (circled red (figure 4.6b)). Distilled water controls containing colouring were also used and showed between 20-50% mortality between point of exposure and pupation (approximately 4 days).

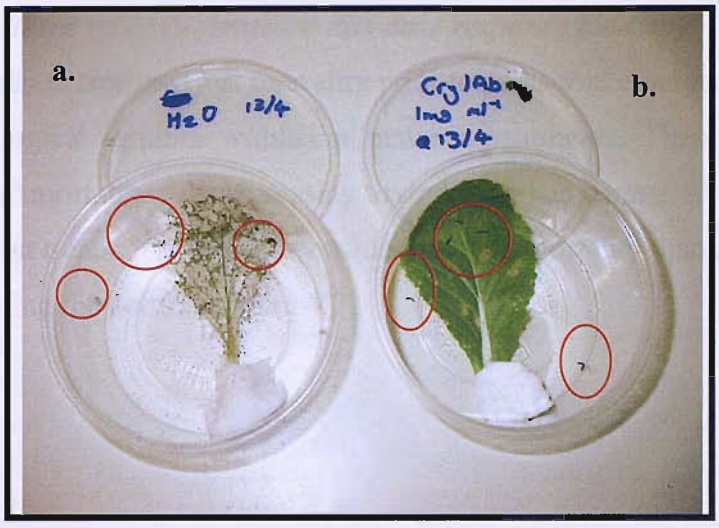


Figure 4.6 – Positive control using the susceptible moth larvae of *P. xylostella* to assess the biological activity of (a) distilled water and (b) Cry1Ab aliquots used for direct toxicity injecting trials.

4.3.3 Host mediated effects - Artificial diet dose response bioassay

4.3.3.i* S. littoralis *mortality. Host mortality prior to parasitoid emergence showed considerable biological variation within and between treatments. There was no clear pattern of reduced mortality with increasing toxin load when compared to controls. However, the plant treatments showed considerably less mortality than all artificial diet treatments including the control (figure 4.7).

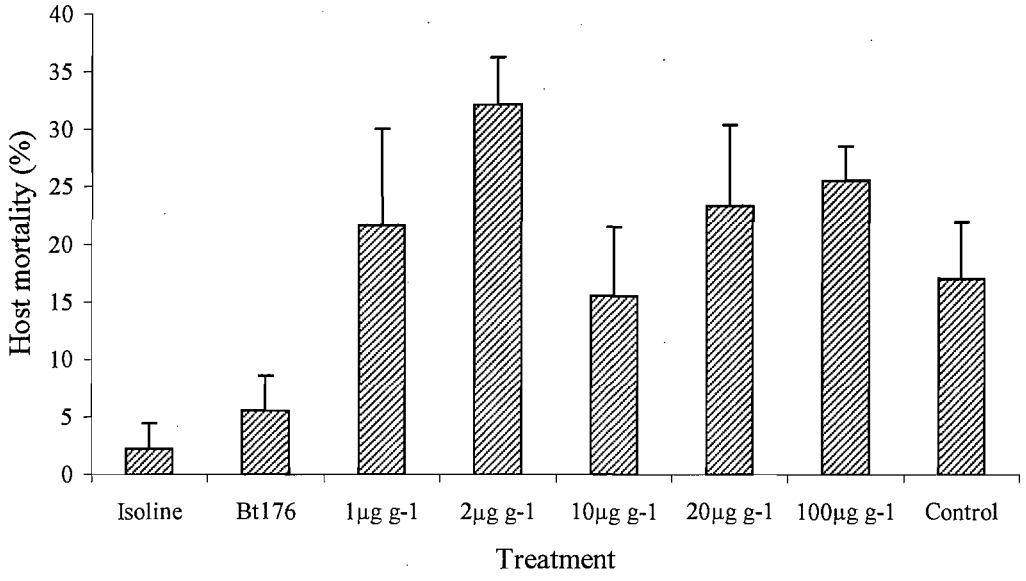


Figure 4.7 – Mortality of *S. littoralis* hosts following parasitism and prior to parasitoid emergence, reared on artificial diet containing 1, 2, 10, 20, 100µg g^{-1} Cry1Ab, Bt176 transgenic maize and its near isoline (±SE).

4.3.3.ii Spodoptera littoralis *mass* – Cry1Ab treated *Spodoptera littoralis* hosts showed significantly reduced mass when reared on 10, 20, 100µg g^{-1} incorporated artificial diet and Bt176 when compared to the control ($F_{7,532}$=17.285, P<0.05), the lowest mass being at 100µg g^{-1} (figure 4.8). 10 and 20µg g^{-1} represent the lowest and highest plant expression levels. The mass of larvae reared on artificial diet at 1 and 2 µg g^{-1} and the isoline were not significantly different to the control ($F_{7,532}$=17.285, P>0.05). This trend continued at day 7 after parasitism with larval mass of hosts reared at 20 and 100µg g^{-1} significantly lower than controls ($F_{7,486}$=24.624, P<0.05), however in some conditions the larvae recovered as larval

mass at 10μg g^{-1} and on Bt176 transgenic maize was not significantly different to controls ($F_{7,486}$=24.624, P>0.05 (figure 4.9)).

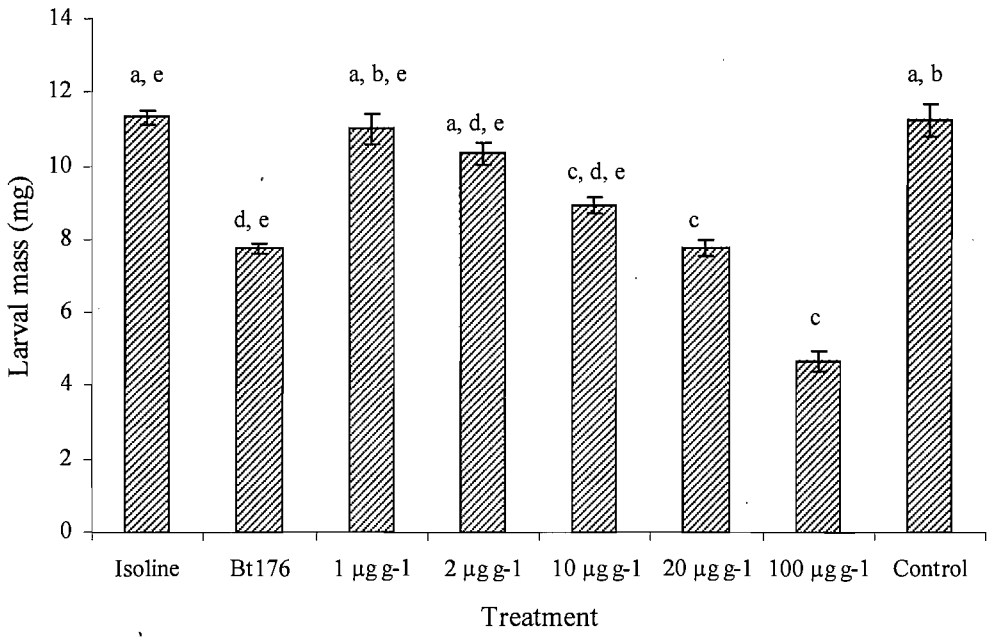


Figure 4.8 – Larval mass of *Spodoptera littoralis* hosts reared on artificial diet containing 1, 2, 10, 20, 100 μg g^{-1} Cry1Ab, Bt176 transgenic maize or its isoline (±SE) 4 days post parasitism. Columns with different letters represent treatment means that are significantly different at P < 0.05.

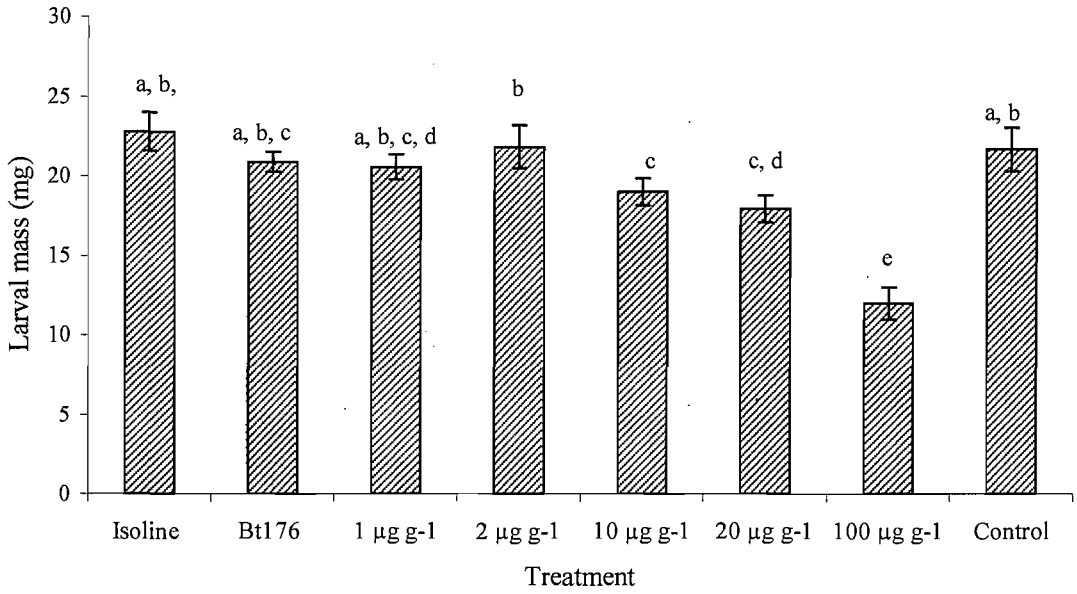


Figure 4.9 – Larval mass of *S. littoralis* hosts reared on artificial diet containing 1, 2, 10, 20, 100 μg g^{-1} Cry1Ab, Bt176 transgenic maize or its isoline (±SE) 7 days post parasitism. Columns with different letters represent treatment means that are significantly different at $P < 0.05$.

4.3.3.iii ***Cotesia marginiventris Pupation time*** – *C. marginiventris* pupation time was significantly longer in the 100μg g^{-1} treatment when compared to the control ($F_{7,320}$=26.09, p<0.001). Pupation took a mean of 11.1 days compared to 9.5 days in control also there was little biological variation either between or within treatments (figure 4.10). Conversely pupation of parasitoids reared in host raised on both plant treatments was significantly shorter than controls ($F_{7,320}$=26.09, p<0.001) taking on average just 8.6 days in both plant treatments. The remaining treatments ranged between 9.8 to 10 days (figure 4.10).

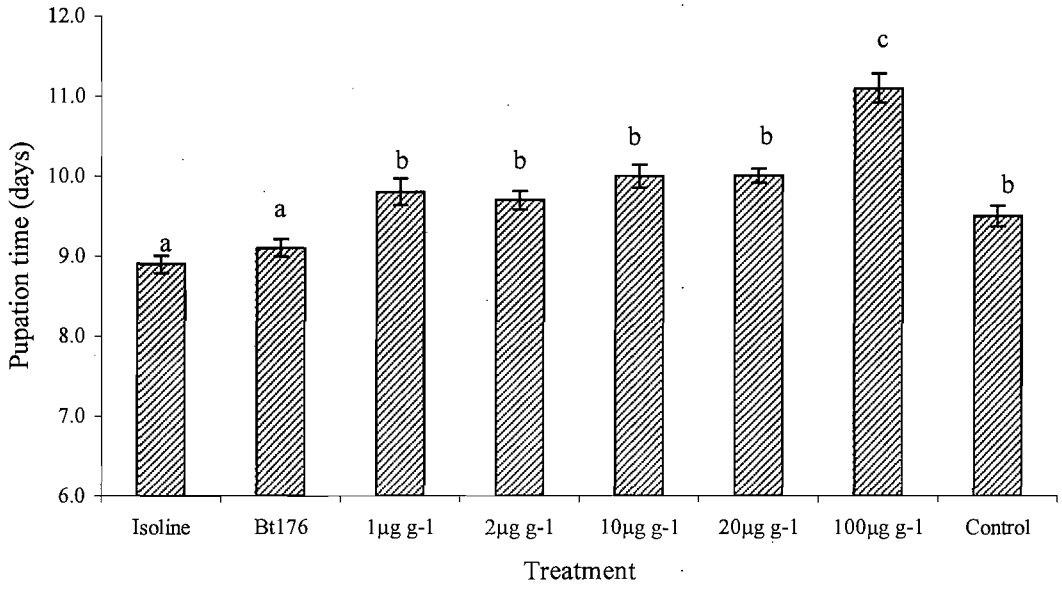


Figure 4.10 – Pupation time of *Cotesia marginiventris* adults raised on *Spodoptera littoralis* hosts raised on artificial diet containing 1, 2, 10, 20, 100 μg g^{-1} Cry1Ab, Bt176 transgenic maize or its isoline (±SE). Columns with different letters represent treatment means that are significantly different at $P < 0.001$.

4.3.3.iv ***Cotesia marginiventris*** **Tibia length** – Tibia length was used as an indicator of fitness and size of adults. Tibia length was only significantly reduced at 100 μg g^{-1} Cry1Ab when compared to the control ($F_{1,62}$=11.124, p<0.001 (figure 4.11)). Tibia length was 0.842mm in parasitoid adults raised on hosts ingesting 100 μg g^{-1} Cry1Ab artificial diet compared to a mean of 0.867mm in the control group (figure 4.11).

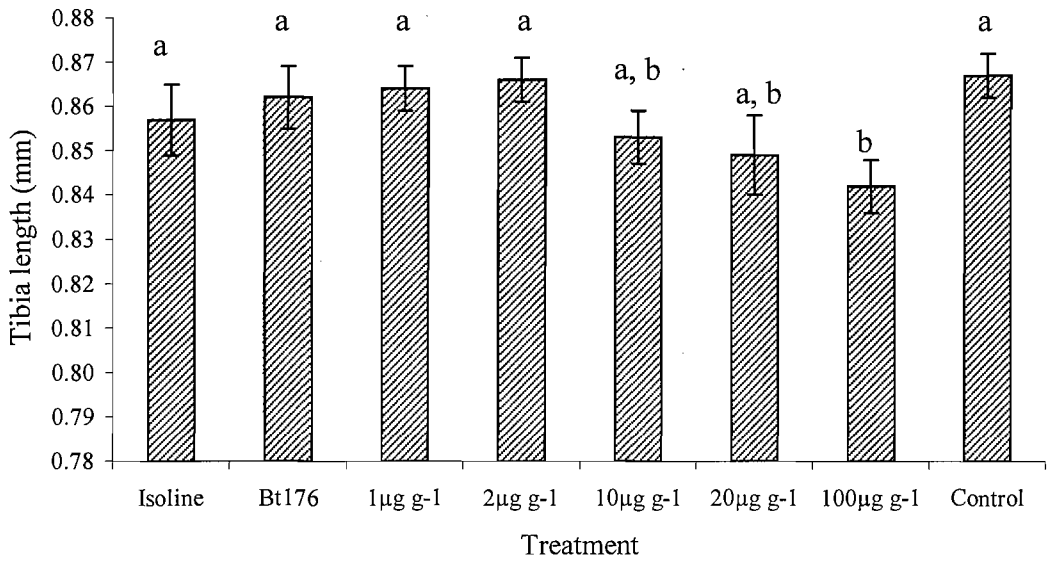


Figure 4.11 – Mean tibia length of *C. marginiventris* adults raised on *Spodoptera littoralis* hosts raised on artificial diet containing 1, 2, 10, 20, 100 μg g^{-1} Cry1Ab, Bt176 transgenic maize or its isoline (±SE). Columns with different letters represent treatment means that are significantly different at $P < 0.05$.

4.3.3.v Cotesia marginiventris Eclosion time (pupation to adult emergence) – Time to eclosion did not follow the same pattern as tibia length and pupation as there was no significant difference between any of the artificial diet conditions ($F_{7,302}$=2.046, P>0.05) with eclosion time ranging between 4.5 and 4.8 days (figure 4.12). However, eclosion time was significantly longer in both plant conditions when compared to the control ($F_{2,106}$=3.837, P=0.025). Eclosion time was 4.9 days in the isoline condition and 5.0 days in the Bt176 condition compared to 4.6 days in the control group (figure 4.12).

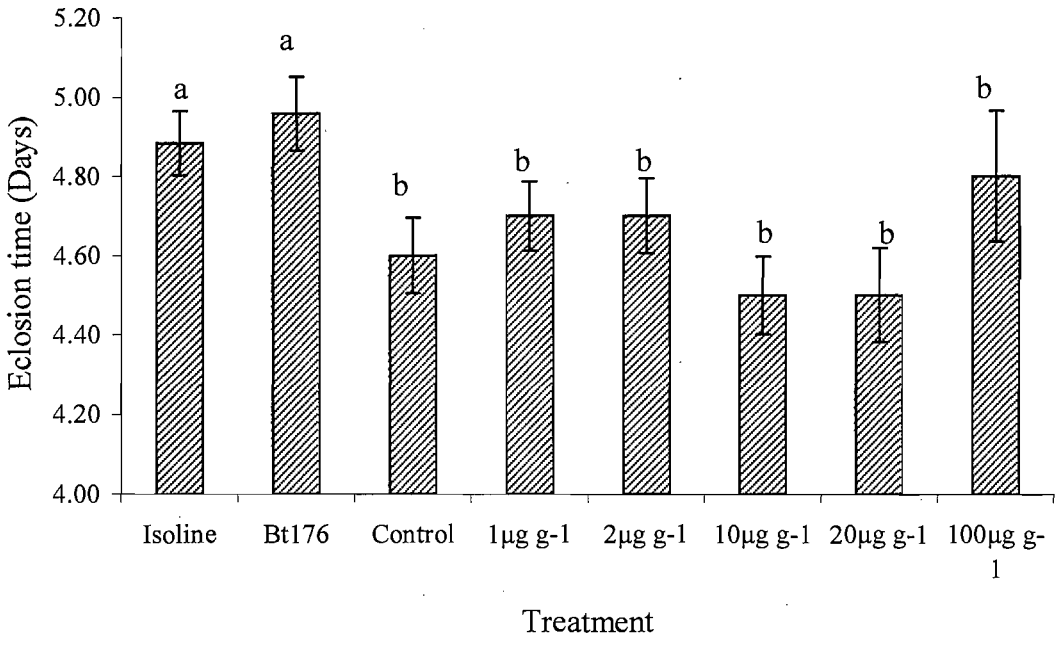


Figure 4.12 – Mean eclosion time of adult parasitoids *C. marginiventris* from pupation to emergence adults raised on *Spodoptera littoralis* hosts raised on artificial diet containing 1, 2, 10, 20, 100 µg g^{-1} Cry1Ab, Bt176 transgenic maize or its isoline (±SE). Columns with different letters represent treatment means that are significantly different at $P < 0.05$.

Adult longevity was also measured in the form of a starvation trial of adult parasitoids. Adult longevity varied little with a mean range of 3 to 3.5 days in all conditions.

4.3.3.vi **Cotesia marginiventris *Sex ratio*** – Whilst the lowest proportion of males were found in the Bt176 replicates at a mean of 54% and the highest proportion of males in the 2 µg g^{-1} Cry1Ab treatment at 72% there was no significant difference between the sex ratio and controls ($G=6.85$, d.f.=7, $P>0.05$) however the amount of variation between trials in the Bt176 treatment should be noted (figure 4.13).

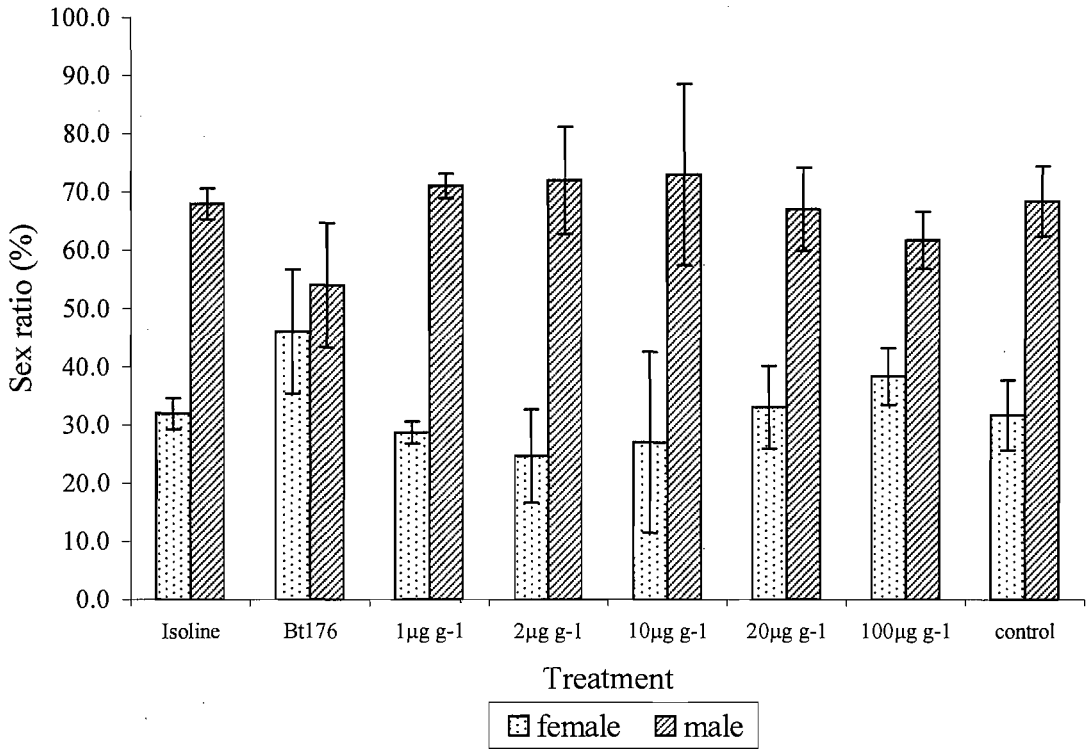


Figure 4.13 – Sex ratio of *C. marginiventris* adults raised on *S. littoralis* hosts reared on artificial diet containing 1, 2, 10, 20, 100 µg g^{-1} Cry1Ab, Bt176 transgenic maize or its isoline (±SE).

4.3.4 Cry1Ab movement through the trophic levels – All host larvae were collected and frozen following parasitoid emergence to assess toxin concentration. As would be expected the concentration of Cry1Ab detected in larvae increased considerably as the concentration within the diet increased, with *S. littoralis* host containing between 0.021 and 1.872 µg g^{-1} Cry1Ab toxin at 1 and 100 µg g^{-1} Cry1Ab artificial diet treatment respectively (figure 4.14).

Large quantities of the Cry1Ab toxin were removed from the host system during feeding. Levels in frass were measured at 7 days after parasitism with a mean of 13.60µg g^{-1} of Cry1Ab expelled. However, the toxin did move through the trophic levels at increasing levels dependant on the ingested diet concentration. The toxin was not detectable in the parasitoid cocoons until host were reared on 10µg g^{-1} Cry1Ab and with the highest amounts of toxin in the 100µg g^{-1} Cry1Ab treatment at 1.9µg g^{-1} Cry1Ab (figure 4.14).

All adult parasitoid samples from all replicates were pooled and no Cry1Ab was detected in adult *C. marginiventris* (figure 4.14).

Sample	No. of individuals (replicates)	Fresh weight homogenized tissue (mg: range)	Cry1Ab per fresh weigh ($\mu g\ g^{-1}$; Mean $\pm$SE)
S. littoralis larvae			
1 $\mu g\ g^{-1}$	9-15 (3)	105-172	0.021 (0.003)
2 $\mu g\ g^{-1}$	10-15 (3)	123-141	0.038 (0.007)
10 $\mu g\ g^{-1}$	10-15 (3)	140-162	0.095 (0.012)
20 $\mu g\ g^{-1}$	10-15 (3)	112-175	0.676 (0.103)
100 $\mu g\ g^{-1}$	15-19 (2)	85-143	1.872 (0.444)
Control	10-15 (2)	92-163	0.000 (0.000)
Isoline maize	10 (3)	102-138	0.000 (0.000)
Bt176 maize	10 (2)	92-106	0.058 (0.184)
S. littoralis frass			
1 $\mu g\ g^{-1}$	n/a (2)	210-250	0.026 (0.097)
2 $\mu g\ g^{-1}$	n/a (2)	286-290	0.040 (0.007)
10 $\mu g\ g^{-1}$	n/a (2)	212-224	0.099 (0.018)
20 $\mu g\ g^{-1}$	n/a (2)	183-207	1.091 (0.003)
100 $\mu g\ g^{-1}$	n/a (2)	152-160	13.60 (2.486)
Control	n/a (2)	173-284	0.000 (0.000)
Isoline maize	n/a (2)	145-212	0.000 (0.000)
Bt176 maize	n/a (2)	159-161	1.387 (0.004)
C. marginiventris cocoons			
1 $\mu g\ g^{-1}$	11-19 (2)	8-15	Not detectable
2 $\mu g\ g^{-1}$	17-19 (2)	16-17	Trace
10 $\mu g\ g^{-1}$	17-19 (2)	13-14	0.702 (0.245)
20 $\mu g\ g^{-1}$	14-15 (2)	10-11	0.281 (0.284)
100 $\mu g\ g^{-1}$	11-18 (2)	7-18	1.957 (1.763)
Control	13-17 (2)	10-17	0.000 (0.000)
Isoline maize	22-27 (2)	15-20	0.000 (0.000)
Bt176 maize	21-22 (2)	15-18	0.428 (0.428)
C. marginiventris adults			
1 $\mu g\ g^{-1}$	38 (1)	15	Not detectable
2 $\mu g\ g^{-1}$	40 (1)	20	Not detectable
10 $\mu g\ g^{-1}$	43 (1)	17	Not detectable
20 $\mu g\ g^{-1}$	38 (1)	14	Not detectable
100 $\mu g\ g^{-1}$	35 (1)	11	Not detectable
Control	34 (1)	16	Not detectable
Isoline maize	48 (1)	19	Not detectable
Bt176 maize	41 (1)	14	Not detectable

Figure 4.14. Results of ELISA analysis of (a) the herbivore *S. littoralis* (larvae and frass and (b) the parasitoid *C. marginiventris* (cocoons and adults) reared on *S. littoralis* larvae raised on artificial diet containing 1, 2, 10, 20, 100$\mu g\ g^{-1}$ Cry1Ab, transgenic

maize Bt176 or its isoline control. The number of replicates and individuals in each replicate showing the mean concentration of Cry1Ab ($\mu g\ g^{-1}$ fresh weight).

4.4 Discussion

The results presented in this study suggest that there may be some direct toxicity to *Cotesia marginiventris* parasitoids when exposed to a very high level single dose of the Cry1Ab toxin. *C. marginiventris* were directly exposed to the Cry1Ab toxin via injection into the host haemolymph avoiding the host gut and indirectly through host mediated exposure in the form of Cry1Ab incorporated into artificial diet.

Larval mass was monitored in both direct exposure experiments and host mediated exposure to provide an indicator of host quality given that smaller hosts represent reduced resources for the parasitoid and that females ovipositing in hosts are able to accurately assess host quality (Ode and Heinz, 2002). When compared at 7 days after parasitism (3 days after injection) to the uninjected control, hosts injected with the Cry1Ab protein had significantly reduced mass however they were not significantly lighter than either the distilled water injected controls or the ice controls. The ice control group accounted for 2.5% of the 7% mean reduced mass seen between the uninjected control and Cry1Ab injected treatment.

In the host mediated exposure at 10μg g^{-1} and 20μg g^{-1} host mass was reduced by 12.5% and 17.5% respectively when compared to controls and by 45% in the 100μg g^{-1} treatment. The quantity of Cry1Ab injected equates to approximately 5 days consumption at a dose of between 10–20μg g^{-1} (i.e. low-high end of plant expression levels) in a single dose. This dose was calculated measuring the sphere of the ball injected and then calculating the volume of Cry1Ab within the sphere this being 0.5μl Cry1Ab in each single injection. The mean quantity of artificial diet consumed in this period was then calculated (1.25 g Cry1Ab consumed in 5 days at 10μg g^{-1} Cry1Ab). In this study (table 1) less than 2% of the Cry1Ab was retained within the host which would give an equivalent of 5 days consumption in a single dose at 200μg g^{-1}. However a more conservative estimate is taken from Raybould et al. (2007) with an estimated environmental concentration (EEC) of 0.1 times the concentration of ingested Cry1Ab giving the injection estimate of equivalence of 5 days consumption at 10–20μg g^{-1}. All conversions were conservative therefore less diet may be consumed in this period which in effect increases the relative potency of the injection. These calculations also show the inherent difficultly when extrapolating direct toxicity to indirect toxicity in the field.

Therefore, the surviving hosts were considerably less stressed than following continued exposure at comparable doses and over 30% of the reduction in mass is explained by the experimental procedure (ice control) and not as a result of host exposure to the toxin. Further more in the host mediated exposure groups larvae already show significantly reduced mass at day 4 before the direct exposure group have been subject to the toxin.

There was no significant difference between sex ratios in control groups when compared to other treatments in either direct or indirect exposure treatments. This confirms that preferential sex allocation biased towards more females was not occurring. This is as expected as the individuals within each group were homogenous at the point of parasitism and females had not experienced large hosts that can lead to production of more males when smaller hosts are encountered (King, 1994a) (King, 1994b) (Ode and Heinz, 2002). Therefore, any negative impacts are a result of post parasitism exposure to the toxin and not related to host quality at the time of parasitism.

Whilst the injection technique led to less stressed individual hosts mortality was considerably higher in injected treatments than non-injected controls as a direct result of the injection process, with over 40% of host mortality occurring in the injected treatments 24-48 hours after injection. The levels of mortality in host mediated exposure was highly variable and showed no pattern of increasing mortality with increasing toxin exposure. Both the transgenic and isoline plant groups showed the lowest mortality levels at below 10% however earlier studies have shown that survival of juveniles on plant material does not relate to a high survival probability and pupation of unparasitized larvae (see chapter 2). Host mortality in host mediated exposure was constantly lower than in direct exposure experiments with the highest levels of mortality less than 35%.

Tibia length and pupation time were used as measurement endpoints of the effects of the direct toxicity of Cry1Ab. Tibia length was not significantly different between Cry1Ab and distilled water injected treatments however tibia length was significantly shorter in both injected controls compared to uninjected controls this finding may be indicative of the differences in host quality between these two treatments discussed earlier.

There was a significant difference in pupation time between the Cry1Ab treatments and all other conditions. The increase in pupation time may be due to non specific binding which can occur when an excess of ligand is added to the system (Mendel and Mendel, 1985) as in this case where a single large dose was administered greatly in excess of that which the parasitoid would be exposed via host ingested toxin. To date Cry1Ab is found to be specific to Lepidoptera, although the presence of a direct effect may suggest receptors are yet to be identified. Ramirez-Romero (2007) suggest a direct effect of the toxin delivered by Bt-maize on *C. marginiventris* however their study is carried out using Cry1Ab incorporated into artificial and Bt-maize plant tissue.

It is important to note that in real terms this extension in pupation time is a mean of 0.5 days and that the challenge dose was equivalent to 5 days consumption in the range of plant expression levels. Therefore to establish how this effect translates to the route of exposure seen in the field a dose response experiment was undertaken using a wider range of measurement endpoints. The inclusion of a greater range of endpoints was to facilitate the elucidation of the sensitivity of tibia length and pupation time as sensitive measures of stress in the parasitoid.

Pupation time was significantly longer in host mediated exposure at 100 $\mu g\ g^{-1}$ Cry1Ab when compared to the control treatment. Pupation took a mean of 11.1 days compared to 9.5 days in the control. Sanders et al. 2007 found no extension of development time in juvenile *Campoletis sonorensis* parasitoids despite the subsequent adults being 15-30% smaller than those reared on control maize hybrid. Tibia length (indicating reduced size) was also significantly different in this group being 0.842mm in the Cry1Ab fed group and 0.867mm in the control group however this change is considerably less than that reported by Sanders et al (2007) and this may reflect the different feeding regimes of *C. marginiventris* and *C. sonorensis* i.e. *C. sonorensis* consume the whole host including the gut whereas *C. marginiventris* do not consume the gut. Also, this negative impact was only seen at the highest level of exposure i.e. 100$\mu g\ g^{-1}$ Cry1Ab which is 5-10 times greater than plants expression levels. At this concentration the partially resistant *S. littoralis* hosts suffer significant negative effects in terms of reduced mass and is therefore approaching the maximum limit of host mediated exposure. As previous studies have shown that at 182.6$\mu g\ g^{-1}$ Cry1Ab was

highly toxic to a *Spodopteran* host and not amenable to experimentation (Ramirez-Romero et al., 2007).

Eclosion time from pupation to adult emergence showed no differences between artificial diet treatments and controls ranging from 4.5-4.8 days however it was significantly longer in plant treatments. Adult longevity was also measured in the form of a starvation trial and was highly consistent between both treatments and individual replicates at between 3.0 and 3.5 days and therefore appears to be an insensitive measure to parasitoid stress.

ELISA analysis was undertaken to establish how the toxin was transferred between the trophic levels. Large amount of toxin were identified by ELSIA analysis in hosts, frass and cocoons of artificial diet feed treatments and not in *C. marginiventris* adults. Cry1Ab was not detected in injected treatments this may simply reflect the comparatively small acute dose delivered by this technique compared to the long term chronic exposure seen in larvae reared on artificial diet.

The lack of toxin in adult *C. marginiventris* is consistent with other studies that whilst identifying small quantities in cocoons and significant quantities in host larvae failed to find any in adult parasitoids (Sanders et al., 2007; Vojtech et al., 2005). Interestingly Sander et al (2007) also found detectable levels of Cry1Ab in *Campoletis sonorensis* pupae reared on Bt maize fed *Spodoptera frugiperda* but not in the parasitoid adults. *C. sonorensis* parasitoids consume the entire host prior to pupation (Wilson and Ridgway, 1975) in contrast *C. marginiventris* that do not consume the gut of the host and therefore any transfer of the toxin to parasitoid would be most likely to be evident in parasitoids consuming the entire host.

The use of high doses of toxin in this study and the presence of the toxin in the parasitoid cocoons confirms that the toxin readily moves through the trophic levels and into the parasitoid but that the toxin is then removed from the system and is not accumulated in adult parasitoids. This removal of the toxin may occur via the meconial pellets that are excreted at the end of larval development (Viggiani, 1984). However, many parasitoids obtain protein or energy from feeding on the host haemolymph (Murdoch et al., 1992) and this may also explain the extension of pupation time seen in

the direct exposure group as the Cry1Ab toxin was deposited directly into the haemolymph.

The prey mediated and direct exposure studies outlined here provide an indication that pupation time and tibia length are both practical and measurable endpoints, with pupation time being a very sensitive measure whereas starvation time and eclosion time proved less sensitive to stress and the use of starvation time particularly can lead to unnecessarily lengthy experimental times. Crucially, pupation time and tibia length are both unambiguous and susceptible to the stressor these are key criteria when assessing the suitability of measurement endpoints (Poppy, 2003). Also, the use of purified forms of plant incorporated protectants such as Cry1Ab allows rigorous testing of potential hazard as it allows highly controlled exposure (Raybould et al., 2007) and eliminates the natural variation seen when using plant material.

This study also demonstrates the need to relate any direct toxicity to field levels as whilst a hazard may exist in terms of direct toxicity the dose is sufficiently large as to not be encountered in a field environment. It also illustrates the potential need for a case-by-case analysis of non-target organisms as Romeis et al (2004) showed that *Chrysoperla carnae* (Stephens) larvae directly exposed to toxin at doses of 10,000 times that it would ingest via Bt fed host prey showed no direct effects and therefore concluded that any negative effects reported were as a result of sub-optimal prey.

Chapter 5

The host mediated effects of Cry1Ab toxin on the parasitoid wasp *Cotesia marginiventris* (Hymenoptera: Braconidae): host preference and the effect of Cry1Ab over successive generations.

5.1 Introduction

The insect order Hymenoptera comprises over 200,000 species of ants, bees, wasps and sawflies (van Wilgenburg et al., 2006). Within this order the Hymenopteran parasitoid wasps are a vital component of biological control and therefore have been extensively studied to establish the impact of GM crops introduced into an agriculture setting. *Cotesia marginiventris* is a solitary endoparasitoid (Jalali et al., 1987; Tillman, 2001) of many lepidopteran agricultural pests (Tillman, 2001) with this genus of parasitoid wasps (Hymenoptera: Braconidae) being widely used for biological control (Hopper et al., 2007).

C. marginiventris are pro-ovogenic parasitoids with the highest number of eggs available soon after eclosion (Baur and Boethel, 2003). Pro-ovogenic parasitoids emerge with a fixed complement of eggs therefore host food can be used as either an energy source or nutrient source to mature eggs (Godfray, 1994). In contrast synovigenic parasitoids mature eggs throughout the adult life cycle (Godfray, 1994) (Rivero-Lynch and Godfray, 1997) and therefore may limit the number of oviposition attacks a parasitoid can carry out in a given time whereas there is the potential for pro-ovigenic parasitoids to be egg limited prior to death (Collier et al., 1994).

Host size has a major influence on parasitoid fitness as it determines the maximum amount of food available for the developing parasitoid (Godfray, 1994). When the host is very small, resources for parasitoid development may be so scarce that the parasitoid fails to mature and dies (Godfray, 1994). If resources are just plentiful enough to prevent death, malformation may occur, therefore host condition, independent of size and age can influence parasitoid fitness (Godfray, 1994). However, host condition may have both positive and negative consequences such as reduced nutrition for the developing parasitoid but weakened host cellular defence (Godfray, 1994) reducing the likelihood of encapsulation.

Hind tibia length is often used as a measure of adult fitness as it has been shown to be positively correlated to a variety of fitness indicators (Sagarra et al., 2001) Zhou et al., 2007). Variation in adult size can influence reproductive success by increasing longevity, fecundity and search efficiency (Harvey et al., 1994 and references therein).

Hymenopteran parasitoids also have a haplodiploid genetic system which allows females to determine the sex of offspring (King, 1987) (Murdoch et al., 1992) (Van Baaren et al., 1999) with fertilized (diploid) eggs developing into females and unfertilized (haploid) eggs into males (Tagawa, 2000) (Van Baaren et al., 1999) (Zhou et al., 2007) (van Wilgenburg et al., 2006).

Therefore, sex ratio may be affected by host size with males being laid in small or poor quality hosts (Murdoch et al., 1992). A sharp threshold in host size above which the parent should lay only females and below which only males has been predicted however the position appears more complex with the threshold dependant on the distribution of host size rather than absolute measures of host size (Murdoch et al., 1992).

Given the importance of these parasitoids in an agricultural context and the likelihood of them being exposed to the Cry1Ab toxin via lepidopteran hosts the aim of this study was to address any cumulative effects that may occur due to exposure to the Cry1Ab insecticidal protein over successive generations. Any effects of successive generations of exposure may have immediate and evolutionary implications for example changes in sex ratio and host selection.

Previous study (chapter 4) has shown that there are potential direct and host mediated effects of the Cry1Ab on parasitoid fitness (Meissle et al., 2003) (Ramirez-Romero et al., 2007) (Couty et al., 2001a). Given that the aim of ecological risk assessment (ERA) is the establish both hazard and exposure (Conner et al., 2003) (Maltby, 2006) (Raybould, 2004) and some direct effect of Cry1Ab has been identified (chapter 4 section 3.1) it would be prudent to introduce more realism into the experimental system. Therefore, within the ecological risk assessment framework it was important to establish if these effects were cumulative over several generations by exposing the parasitoid to the toxin over more than one generation. The assessment of any cumulative effects also allows a more robust assessment of risk to be made (chapter 6 figure 6.1).

The success of the parasitoid involves not only host selection but successful oviposition, larval development and emergence from the cocoon (Blande et al., 2004). Therefore,

attack rate, parasitism success, cocoon formation and adult emergence where investigated along with pupation time, sex ratio and tibia length.

5.2.1 *Maize plants*

Transgenic maize (*Zea mays L.*) plants, (Syngenta event Bt-176) expressing the Cry1Ab protein and the untransformed control cultivar were used in all bioassays. Individual maize plants where cultivated in a greenhouse at 25°C ±5°C and at a relative humidity of 50%± 20%, in 1.0l, 13cm diameter, 12cm high pots, 3 seeds were sown into each pot. Plants were cultivated in Vapagro modular compost and no fertilizer was applied. New plants were sown weekly and used for experiments between 3-5 weeks old (V5-V7) as *S. littoralis* are known to be primarily a pest of young leaves (Dutton et al., 2005).

5.2.2 *Insects*

All insect cultures and bioassays were maintained at 25°C ±1.0°C, RH 60% ±20% and a photoperiod of L:D 14:10H in a cooled incubator (AC/013, Thermo-Electron, Basingstoke, UK.).

Spodoptera littoralis were used as a non-target herbivore as it is only partially susceptible *to the Cry1Ab protein produced by Bacillus thuringiensis* (Sneh et al., 1981). *S. littoralis* were maintained for bioassay from eggs supplied by Syngenta, Bracknell, U.K., eggs were supplied each week on netting (oviposition substrate). On receipt *Spodoptera littoralis* eggs clusters were left on the netting and placed in 250ml pots (Roundstone, Catering Equipment Ltd, Melksham, Wiltshire, UK) with ventilation containing an excess of artificial diet (Beet Armyworm diet F9220B Bio-Serve, Frenchtown, NJ 08825 (see appendix 1 for full ingredients and preparation details)) was added to each pot.

Cocoons of the endoparasitoid *Cotesia marginiventris* were obtained from the Laboratoire d'Entomologie Evolutive, Institut de Zoologie, Université de Neuchâtel, Switzerland. *Cotesia marginiventris* adults were maintained in 30x30x30cm Perspex cages at standardized conditions. Adults were reared on a 20% honey/water solution soaked cotton wool which was changed every 48 hours.
For general culturing 30-35 second instar *Spodoptera littoralis* juveniles were removed from large emergence groups and placed in 250ml pots (Roundstone, Catering

Equipment Ltd, Melksham, Wiltshire, UK) with ventilation containing an excess of artificial diet (Beet Armyworm diet F9220B Bio-Serve, Frenchtown, NJ 08825). *S. littoralis* juveniles were confined with 2 mated *C. marginiventris* females (2-6 days old); pots also contained a small ball of damp cotton wool dipped in honey. Pots were observed until at least one female had attacked a host. The *C. marginiventris/S. littoralis* groups were then left to parasitize for 24 hours as this was observed to be the time over which the highest parasitism occurred (Tillman, 2001). After 24 hours the adult *C. marginiventris* and honey solution were removed. *C. marginiventris* juveniles took between 8-12 days to emerge during which time the pots were monitored and any large unparasitized *S. littoralis* were removed and fresh artificial diet added as required.

5.2.3 Host preference bioassays

5.2.3.i Artificial diet preparation

Uncleaved, full-length, lyphosized Cry1Ab diluted was to 0.5mg ml^{-1} using room temperature distilled water, following dilution the solution was divided into aliquots sufficient to make 1 litre of artificial diet at a concentration of 200 and 20µg g^{-1} Cry1Ab and control diet containing no Cry1Ab and used immediately.

Artificial diet Beet Armyworm diet F9220B Bio-Serve, Frenchtown, NJ 08825 was prepared following manufactures instruction (see appendix 1). Prior to the addition of dried artificial diet the agarose gel was allowed to cool to 65°C, as toxin stability analysis had shown the toxin to maintain biological activity at this temperature (see Chapter 3). Once the gel reached 65°C the required volume of diluted Cry1Ab was added, immediately after the addition of the toxin the dried artificial diet was added and mixed thoroughly to obtain the following the appropriate concentration and control. 2-3ml of artificial diet was then added to 25mm diameter, LPDE containers (Fisher scientific, UK).

5.2.3 Host Preparation

Five artificial diet containers from each diet where removed from the freezer on the day hosts were due to be introduced to it in order to allow sufficient time for the diet to completely thaw. Fifteen, 2nd instar hosts were then placed into each of the diet containers creating a total of 75 hosts on each diet. Before the hosts where put onto the diet, any excess moisture was soaked up by using a paper as to prevent the hosts from drowning once placed into the container. The artificial diet containers were placed into the incubator (as standard conditions) and left for three days to allow the hosts to feed and develop on the diet. For the plant diets (Isoline and Bt.176), the 3rd-5th leaves on the plant were removed, and then placed in equal quantities into 2 cylindrical plastic pots (height; 45mm, diameter; 100mm) along with 20 hosts, so that for each of the two diets there were 2 pots, containing approximately 2 leaves, with 20 hosts in each.

5.2.4 Insect bioassay - No-choice trials

After 3 days on the respective artificial diet and plant treatments the *S. littoralis* larvae were removed form the incubator. Forty *S. littoralis* larvae from each diet treatment were removed at random from the containers, divided equally, and placed into two of the attack arenas (cylindrical plastic pot, height; 25mm, diameter; 100mm Roundstone, Catering Equipment Ltd, Melksham, Wiltshire, UK). The hosts' weight was recorded before separation into the arenas.

A single, mated, female *C. marginiventris* parasitoid was then removed from the laboratory culture (2-6 days old) and placed into one of the attack arenas. The parasitoid was monitored for 10 minutes upon introduction to the arena, the time of the first attack and the total number of attacks were recorded. After 10 minutes the parasitoid was removed to prevent any further attacks, and the hosts were transferred to a larger plastic container (height; 45mm, diameter; 100mm) which contained control artificial diet, and was returned to the incubator. A new female parasitoid was introduced to each of the attack arenas containing the hosts. Ten replicates for each treatment were carried out totalling 1000 individual host larvae and 50 *C. marginiventris* females. The host weight upon its placement into the attack arena was recorded.

5.2.4.i Survivorship of developing parasitoid

As well as the number of attacks being recorded, the number of parasitoid larvae emerging and spinning cocoons, and the number of cocoons resulting in emerging adults were also recorded to allow mortality or survivorship at each stage to be calculated.

5.2.4.ii Parasitoid fitness

Adult parasitoids emerging were assessed for fitness by their hind tibias, legs were removed using a pair of dissection scissors and the hind tibia measured under a binocular dissection microscope.

5.2.5 Insect bioassay - Host preference; two-way choice conditions

The host and diet preparation for the two-way choice test was as previously described for plant material to compare isoline and Bt176 diets. To eliminate any possible effect of host size on parasitoid selection host development was monitored and once they reached the size range of 0.5-1.1mg they were removed for experimental trials (Vojtech et al., 2005). 20 hosts were used for each replicate and treatment and placed in arenas as in no-choice insect bioassays. To distinguish between the two host types, those from one diet were marked under a dissection microscope with a 0.6mm *stylo* pen. A control was carried out to ensure that marking had no effect on the parasitoids choice of host, (as described below for choice trials) using marked and unmarked host larvae raised on artificial diet containing no Cry1Ab. As an extra precaution the hosts being marked were alternated with each replicate, i.e. for replicate 1 the hosts raised on the Isoline maize diet were marked, then for replicate 2 those raised on the Bt.176 transgenic maize were marked. The 10 marked and 10 unmarked hosts from the two diets were placed into an attack area, along with a female parasitoid. The number of attacks on the marked and unmarked hosts was recorded over a 10 minute period.

5.2.6 Cumulative effects

5.2.6.i Prey mediated effects – F_1 generation

Second instar *Spodoptera littoralis* juveniles weighing between 0.5 and 1.1mg (Vojtech et al., 2005) were removed from large emergence groups and placed in fresh 250ml pots (Roundstone, Catering Equipment Ltd, Melksham, Wiltshire, UK) with ventilation containing an excess of artificial diet (Beet Armyworm diet F9220B Bio-Serve, Frenchtown, NJ 08825) in groups of 35 individuals. Seven groups in total were parasitized for each bioassay replicate. Two mated *C. marginiventris* females (2-6 day old) were added, pots also contained a small ball of damp cotton wool dipped in honey. Pots were observed until at least one female had attacked a host. The *C. marginiventris/S. littoralis* groups were then left to parasitize for 24 hours (Tillman, 2001).

Uncleaved, full-length, lyphosized Cry1Ab was diluted to 1mg ml^{-1} using room temperature distilled water, following dilution the solution was divided into aliquots sufficient to make 1 litre of artificial diet at a concentration of 100µg g^{-1} Cry1Ab and control diet containing no Cry1Ab and used immediately.

Artificial diet Beet Armyworm diet F9220B Bio-Serve, Frenchtown, NJ 08825 was prepared following manufactures instruction (see appendix 1). Prior to the addition of dried artificial diet the agarose gel was allowed to cool to 65°C, as toxin stability analysis had shown the toxin to maintain biological activity at this temperature (see Chapter 3). Once the gel reached 65°C the required volume of diluted Cry1Ab was added, immediately after the addition of the toxin the dried artificial diet was added and mixed thoroughly to obtain the following the 100 µg g^{-1} Cry1Ab concentration and control. 2-3ml of artificial diet was then added to 25mm diameter, LPDE containers (Fisher scientific, UK).

Following the parasitism period *S. littoralis* juveniles were removed and assigned at random to one of the experimental conditions. 3 juveniles were places in a 25mm diameter, LPDE container (Fisher scientific, UK) containing 2-3ml of artificial diet containing 100 or 0 µg g^{-1} (control) Cry1Ab. Each experimental trial contained 10 pots in each condition with a total of 30 individuals and 3 replicates were carried out for each condition over two generation totalling 810 juveniles.

Larvae where weighed at 4 and 7 days after parasitism. Pupation time, sex ratio and tibia length of the resulting generation was recorded.

5.2.6.ii Prey mediated effects – F_2 generation

Second instar *Spodoptera littoralis* juveniles and weighing between 0.5 and 1.1mg were removed from large emergence groups and placed in fresh 250ml pots (Roundstone, Catering Equipment Ltd, Melksham, Wiltshire, UK) with ventilation containing an excess of artificial diet (Beet Armyworm diet F9220B Bio-Serve, Frenchtown, NJ 08825) in groups of 20 individuals. Five groups in total were parasitized for each treatment using one mated *C. marginiventris* female (2-6 day old), pots also contained a small ball of damp cotton wool dipped in honey from each condition. Pots were observed until the female had attacked a host. The C. *marginiventris/S. littoralis* groups were then left to parasitize for 24 hours.

Following the parasitism period *S. littoralis* juveniles were removed and assigned to the appropriate experimental condition these being control to control, 100µg g^{-1} to 100µg g^{-1} and 100µg g^{-1} to control diet formulation, 3 juveniles were placed in a 25mm diameter, LPDE container (Fisher scientific, UK) containing 2-3ml of artificial diet containing 100 or 0 µg g^{-1} (control) Cry1Ab. Ten pots were used in each replicate therefore 90 larvae were trialled in each treatment.

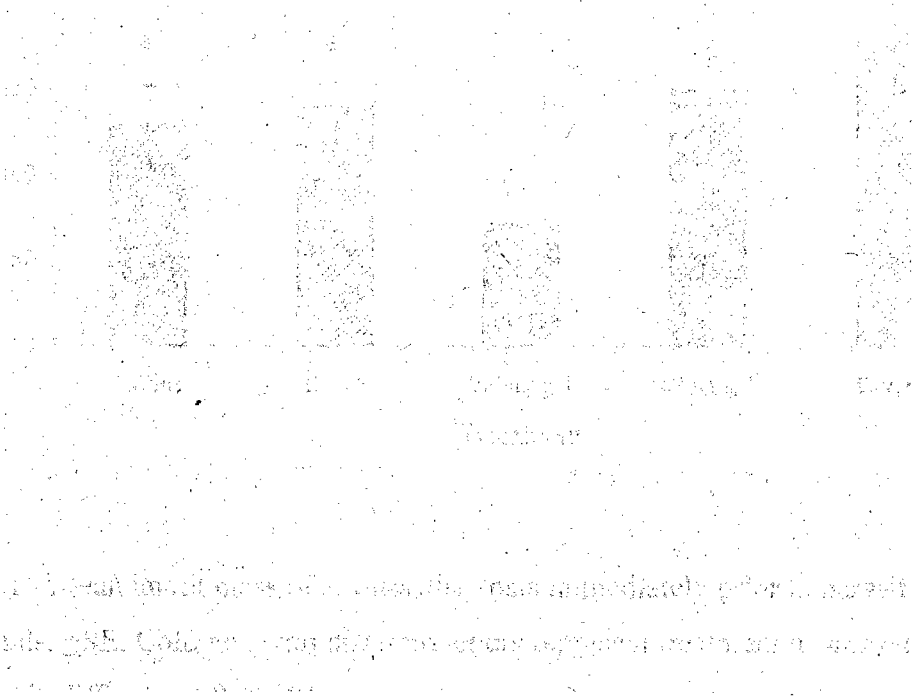
Host mass was recorded at 4 and 7 days after pupation. Pupation time, tibia length and sex ratio of the resultant generation of *C. marginiventris* was recorded.

5.2.6.iii F1 and F2 attack rate bioassay.

A further 5 groups of 20 second instar *S. littoralis* host weighing between 0.5 and 1.1 mg were added to 100 ml Roundstone pots for each treatment and 1 mated *C. marginiventris* female added to each time pot. No artificial diet or honey solution was added to pots. *C. marginiventris* females were observed for 10 minutes. The time to first attack, number of attacks and the subsequent emergence rate was recorded. Following the observation period *S. littoralis* hosts were maintained in 250ml Roundstone pots with an excess of artificial diet until adult parasitoid emergence. This process was repeated for the subsequent generation of parasitoid adults.

5.2.7 Statistical analysis

All statistical analysis was carried out using SPSS v. 14 (excluding sex ratio analysis). All weight and tibia length data was transformed using logarithmic transformation in order to conform to a normal distribution. The proportion of attacks resulting in a cocoon formation and proportion of emerging *C. marginiventris* adults was transformed using Arcsine transformation in order to conform to a normal distribution and satisfy the assumptions of one-way ANOVA. As count data, all attack rate data was analysed using the non-parametric Kruskal-Wallis test when the number of groups exceed 2 and post-hoc as required the Mann-Whitney *U* Test. The Mann-Whitney *U* Test was used when only 2 groups required analysis. Sex ratio data was analysed using the G-test and calculated using Microsoft Excel for windows.



5.3.1 Insect bioassay – Single generation effects

5.3.1.i Host larval mass at time of parasitism

S. littoralis hosts were allowed to develop for 3-4 days into second instar larvae on their respective diet or plants treatments prior to parasitism. Therefore, host weight at time of parasitism in the no-choice host preference study was not controlled to establish if any trend in attack rate occurred due to host mass. As would be expected host larvae reared on control diet containing no Cry1Ab toxin were the heaviest (chapters 2 and 3) at a mean of 178mg whilst those reared on artificial diet containing 200µg g^{-1} Cry1Ab were the lightest at 71mg (figure 5.3.1). The larval mass of hosts raised on the control diet was significantly greater than all other treatments including larvae reared on isoline plant material ($F_{4,952}$=25.563, P<0.001) whilst those reared on artificial diet at 200µg g^{-1} were significantly lighter ($F_{4,952}$=25.563, P<0.001 (figure 5.1)).

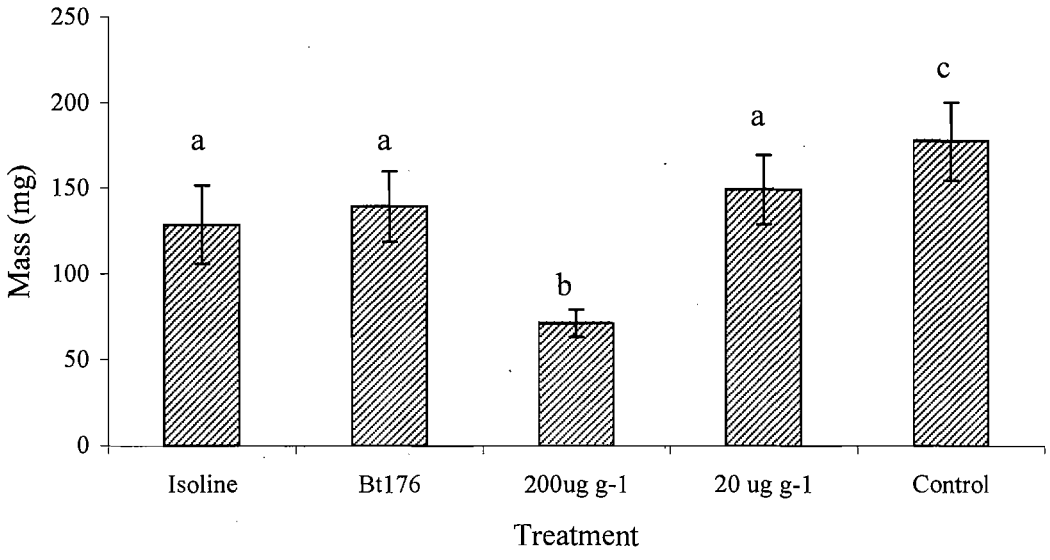


Figure 5.1 - Mean larval mass of *S. littoralis* hosts immediately prior to parasitism in no choice trials. ±SE. Columns with different letters represent treatment means that are significantly different at P<0.001.

5.3.1.ii Regression analysis of host weight with attack rate (no-choice conditions)

Regression analysis was carried out to assess whether there was a correlation between average host size and attack rate. Figure 5.2 indicates a negative correlation between weight and rate of attack by the adult parasitoids, i.e. attack rate decreased with an increasing host weight, however this trend was not significant ($F_{1,46}$=3.79, P>0.05), and therefore attack rate did not co-vary significantly with host weight.

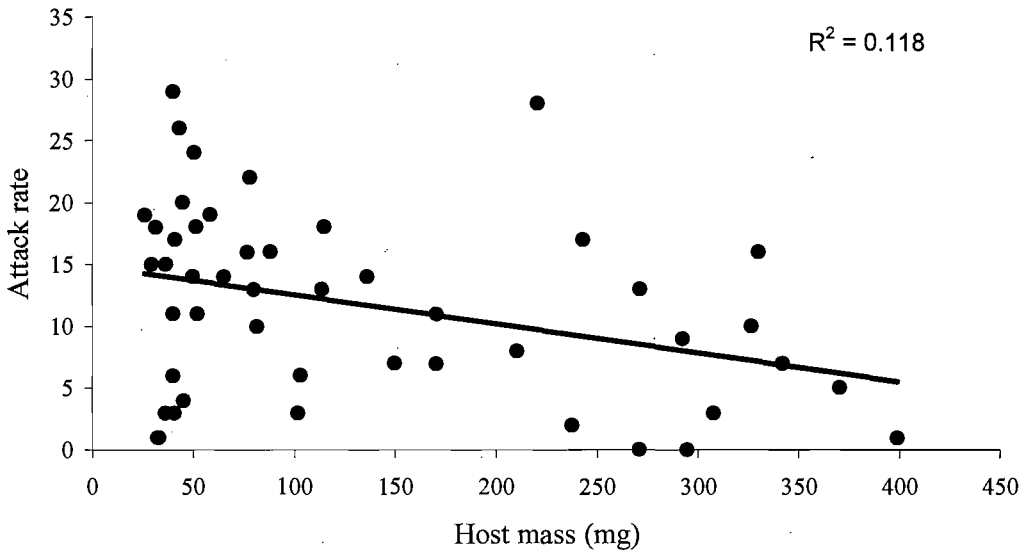


Figure 5.2 – Regression analysis of attack rate when compared to larval mass of *Spodoptera littoralis* raised on artificial diet of 20, 200 or 0µg g^{-1} Cry1Ab and Bt176 plant material and its isoline.

5.3.1.iii Host preference under no-choice conditions

Female *C. marginiventris* showed no significant preference for *S. littoralis* hosts reared on either transgenic maize Bt176, its isoline or artificial diet containing 200, 20 or 0µg g^{-1} Cry1Ab (χ^2=6.467, d.f.=4, P=0.167). Hosts reared on the isoline plant material had the highest attack rate (14.7 ± 2.89SE.) and those reared on the Bt176 transgenic maize the lowest ((7.5 ± 2.33SE) figure 5.3).

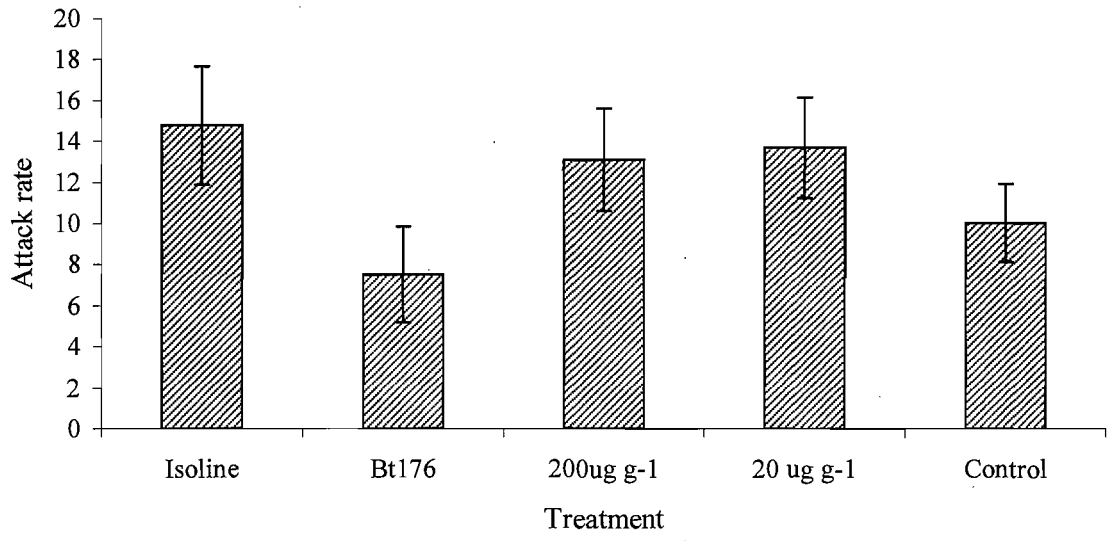


Figure 5.3 - Mean number of attacks over 10 minutes by *C. marginiventris* on *S. littoralis* larvae hosts reared on different diet treatments. ±SE. Treatments are not significantly different P>0.05.

5.3.1.iv Survivorship of developing parasitoids

The results from the two survivorship and mortality measures, i.e. the number of attacks resulting in the emergence of parasitoid larvae which subsequently forms a cocoon, and the number of cocoons that result in the emergence of an adult parasitoid are represented in figures 5.4 and 5.5 respectively. The results suggest a trend in which the hosts reared on artificial diet containing 20 and 200µg g^{-1} Cry1Ab toxin have a higher mortality of parasitoid between the cocoon and adult emergence stage at 17.2% and 21.4% of attacks resulting in a cocoon compared to 42.5% in the control treatment, however this trend was not statistically significant ($F_{4,23} = 1.228$, P>0.05).

There was less variation both within and between treatments in the proportion of cocoons resulting in emerging adult parasitoids compared to the amount of variation in the number of attacks resulting in cocoon formation. As with the proportion of attacks resulting in cocoon formation there was no significant difference between the host treatments when comparing adult emergence rates ($F_{4,41} = 1.048$, P>0.05). Therefore despite differences in larval mass the survival and mortality of the developing

parasitoids was not affected by the host larvae being reared on diets containing the Cry1Ab toxin or Cry1Ab incorporated in the transgenic maize.

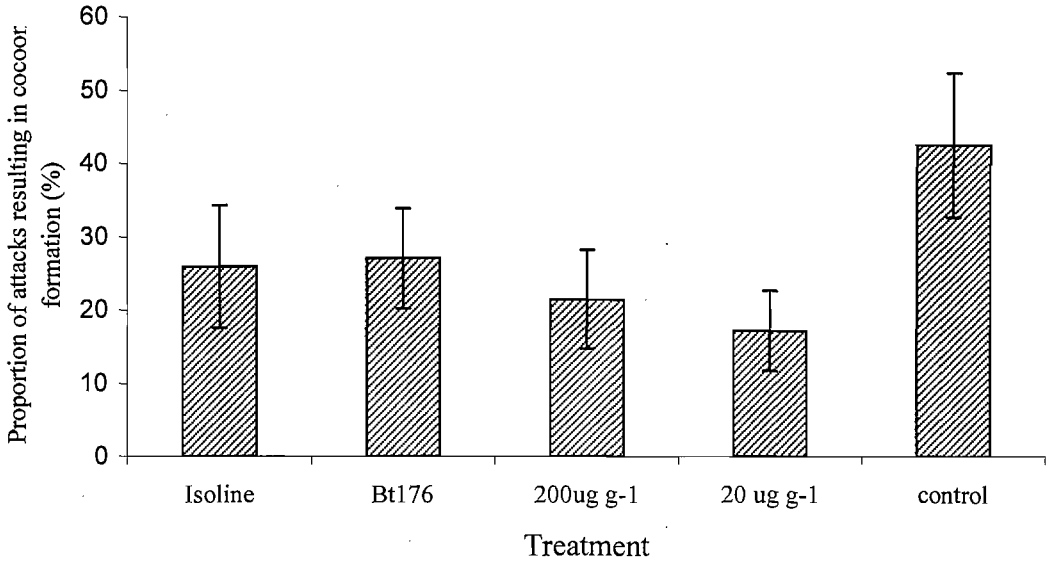


Figure 5.4 - Proportion of all attacks resulting in parasitoid cocoon formation, i.e. survivorship to cocoon stage in each treatment (+S.E.). Treatments are not significantly different P>0.05.

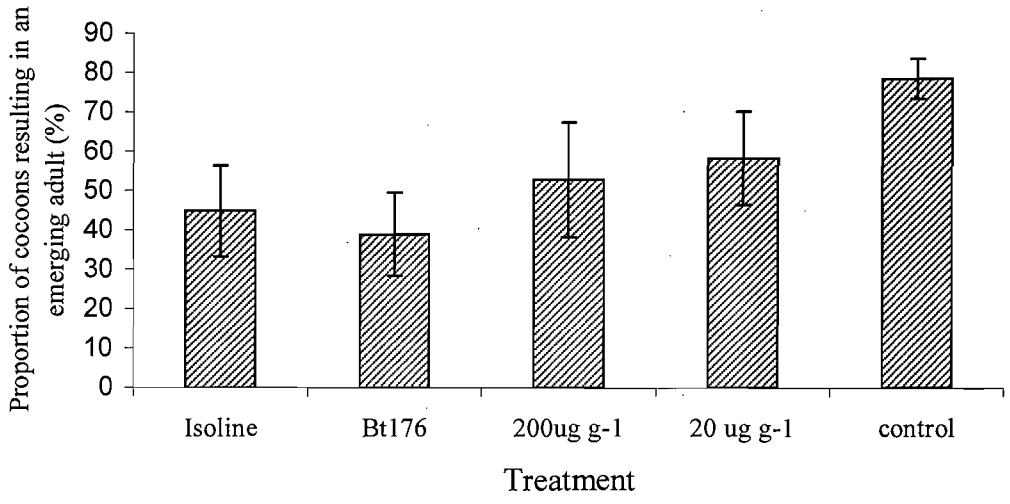


Figure 5.5 - Proportion of cocoons resulting in an emerging adult *C. marginiventris* +S.E). Treatments are not significantly different P>0.05.

5.3.1.v Fitness of emerging parasitoids – tibia length

Tibia length of the emerging adult parasitoid was used as an indicator of fitness of adult parasitoids and as a measure of size. Tibia lengths were significantly shorter in adults reared on hosts consuming artificial diet containing 20 and 200μg g^{-1} Cry1Ab and Bt176 transgenic maize ($F_{4,95} = 7.222$, $P<0.05$) when compared to both the isoline treatment and the control artificial diet containing no Cry1Ab (figure 5.3.6). Mean tibia length was shortest in the 200μg g^{-1} Cry1Ab and Bt176 fed groups at 0.79mm and longest in the isoline groups at 0.84mm (figure 5.6).

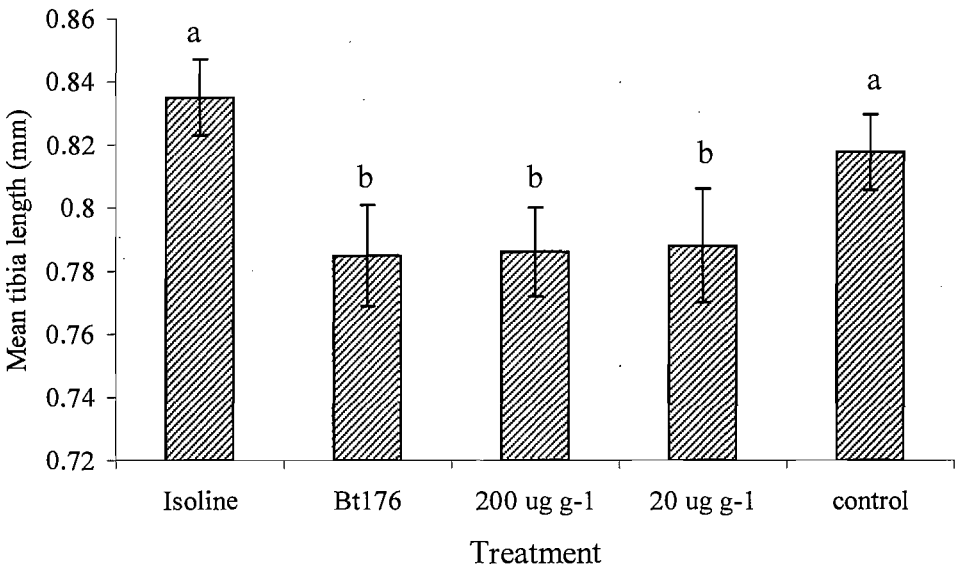


Figure 5.6 - Mean tibia length of emerging parasitoids raised on *S. littoralis* hosts reared on isoline, Bt176 plant material or artificial diet containing 200, 20 or 0μg g^{-1} Cry1Ab ±SE.). Columns with different letters represent treatment means that are significantly different at $P<0.05$.

5.3.1.vi Host preference two-way choice conditions – Bt176 and isoline maize

Adult *C. marginiventris* female attack behaviour was observed for a period of 10 minutes to establish if there was any significant difference in the number of attacks on *S. littoralis* hosts raised on transgenic maize Bt176 or its isoline. In order to distinguish between the two groups they were marked for visual identification. A control experiment was carried out of 3 replicate groups to ensure the marking process did not affect attack rate. Whilst unmarked hosts were attacked at a slightly higher rate than

marked hosts 6.0 (±SE;1.10) and 5.9 (±SE;0.98) respectively there was no significant difference between the two control groups (χ^2=48.5, d.f.=19, P=0.912). Therefore, the marking method did not influence parasitoid choice of host (figure 5.7)

Results from the host preference experiments in which the parasitoid was given a two-way choice of hosts reared on either isoline or transgenic Bt.176 plants indicate that hosts reared on the isoline maize suffered more attacks on average (6.4 ± SE;0.94) than those reared on the transgenic Bt.176 (6.2 ± SE;1.17) however this was not significantly different ((χ^2=48.0, d.f.=19, P=0.912) figure 5.3.7).

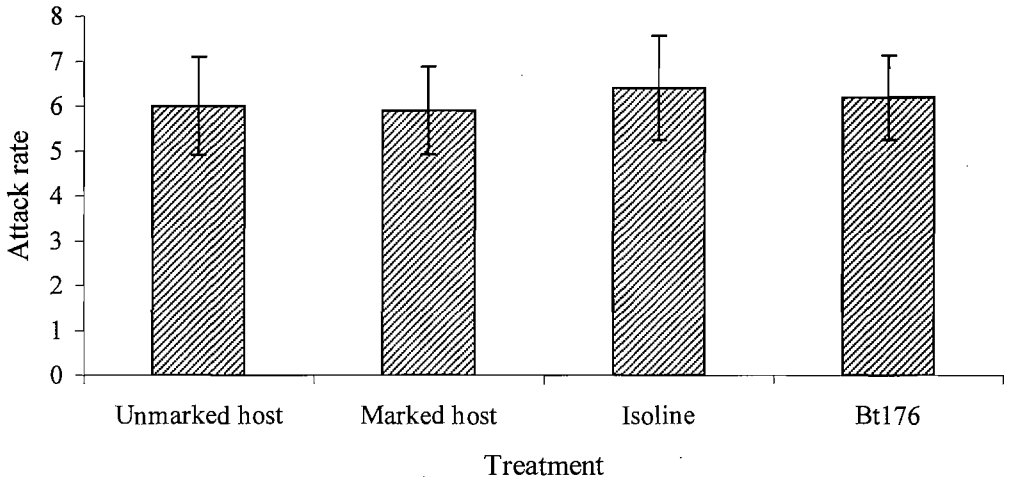


Figure 5.7 - Mean number of attacks by *C. marginiventris* over 10 minutes on *S. littoralis* hosts unmarked and marked, reared on isoline maize or transgenic Bt176. Treatments are not significantly different P>0.05.

In the two-way choice experiments weight of the host was controlled to eliminate any possible influence on attack rate. One-way ANOVA was carried out on individual replicates revealing no significant difference between the weights of the hosts raised on the two diets or in the marked or unmarked treatments (see appendix 7).

5.3.2 Insect bioassay - Cumulative generational effects

5.3.2.i Host mass

At day 4 following parasitism *S. littoralis* hosts were significantly smaller in the 100µg g^{-1} Cry1Ab treatment (7.5mg) when compared to controls (($F_{1,267}$=109.227, P<0.001) 10.3mg), this trend continued at day 7 ($F_{1,271}$=35.090,P<0.001) with hosts reared on control diet having a mean weight of 23mg compared to 17mg when raised on artificial diet containing 100µg g^{-1} Cry1Ab (figure 5.8).

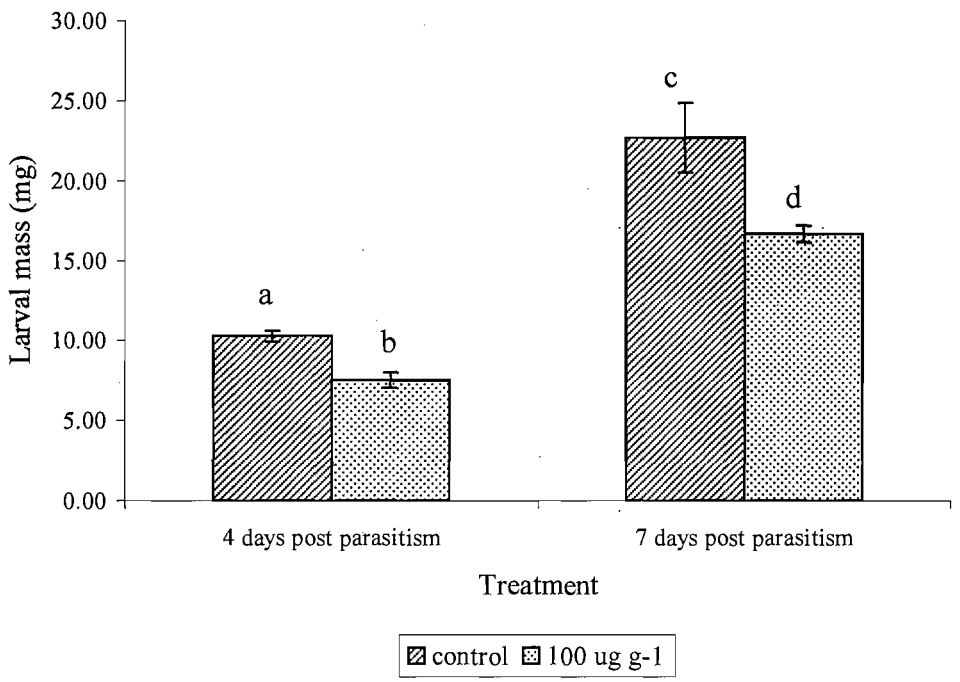


Figure 5.8 – Larval mass of parasitized *Spodoptera littoralis* larvae raised on control artificial diet containing no Cry1Ab and artificial diet containing 100µg g^{-1} Cry1Ab at 4 and 7 days post parasitism (+SE.). Columns with different letters represent treatment means that are significantly different at P<0.001.

Host larvae reared on artificial diet containing 100µg g^{-1} Cry1Ab were also significantly smaller ($F_{2,231}$=13.927, P<0.001) in experimental groups used for F_2 *C. marginiventris* adults. However as expected there was no significant difference in the two control groups (($F_{2,231}$=13.927, P=0.888) figure 5.9).

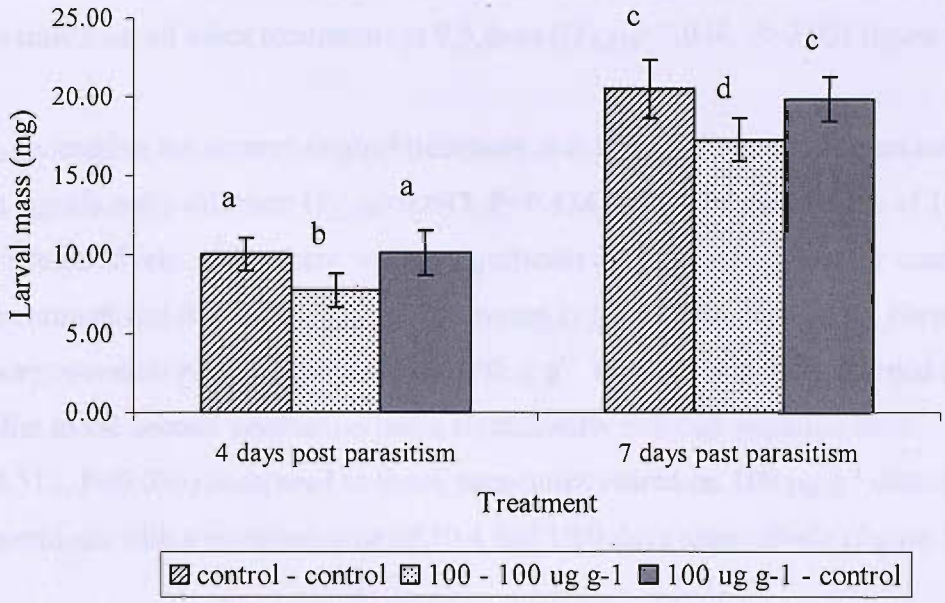


Figure 5.9 – Larval mass of parasitized *S. littoralis* larvae raised on control artificial diet containing no Cry1Ab parasitized with *Cotesia marginiventris* raised on control diet, artificial diet containing 100 g g-1 Cry1Ab parasitized with *C. marginiventris* raised on artificial diet containing 100 g g-1 Cry1Ab and artificial diet containing no Cry1Ab parasitized with *C. marginiventris* raised on artificial diet containing 100µg g^{-1} Cry1Ab at 4 and 7 days post parasitism. Columns with different letter represent treatment means that are significantly different at $P<0.001$.

5.3.2.ii Pupation time

C. marginiventris pupation time was longer in treatments using artificial diet containing 100µg g^{-1} Cry1Ab in both first and second generation treatments when compared to their respective controls (figure 5.10).

In the F_1 generation *C. marginiventris* parasitoids raised on a diet of 100µg g^{-1} Cry1Ab took on average 9.9 days to pupate compared to 9.5 days in the control treatment ($F_{1,147}=7.204$, $P=0.008$). The F_2 generation in all treatments took longer to pupate than in the first generation however this was not significant when comparing the 100ug g^{-1} treatment in the F_1 generation (9.9 days) and the control-control treatment in the F_2 generation (($F_{4,336}=7.048$, $P=0.98$) 10.1 days) and the 100 µg g^{-1} Cry1Ab –control treatment in the F_2 generation (($F_{4,336}=7.048$, $P=0.667$) 10.0 days). The F_1 *C.*

marginiventris generation raised on *S. littoralis* on control diet had significantly shorter pupation time than all other treatments at 9.5 days (($F_{4,336}$=7.048, P>0.05) figure 5.12).

In the F_2 generation the control-control treatment and 100μg g^{-1} – control treatment were not significantly different ($F_{1,109}$=0.643, P=0.424) with a pupation time of 10.1 and 10.0 days respectively. Also there was no significant difference between the control-control treatment and the 100-100 μg g^{-1} treatment ($F_{1,102}$=1.381, P=0.243). However the *C. marginiventris* parasitoids raised on 100μg g^{-1} Cry1Ab and then returned to the control diet in the second generation had a significantly reduced pupation time ($F_{1,111}$=4.512, P=0.036) compared to those parasitoids reared on 100 μg g^{-1} diet over both generations with a pupation time of 10.4 and 10.0 days respectively (figure 5.10).

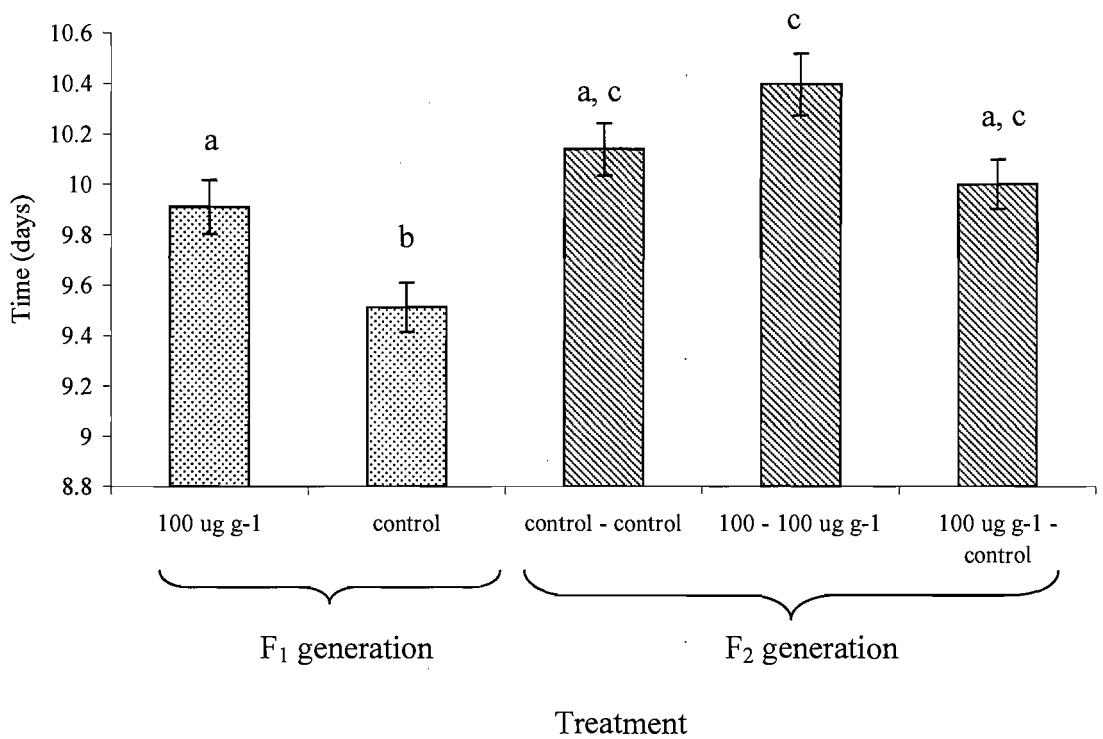


Figure 5.10 – Pupation time of *Cotesia marginiventris* larvae in the first and second generations following exposure to the Cry1Ab toxin (±SE). Columns with different letter represent treatment means that are significantly different at P<0.05.

5.3.2.iii Tibia length

In the first generation of *C. marginiventris* adults reared on 100 μg g^{-1} Cry1Ab tibia length was reduced when compared to controls at 0.831mm however this was not significantly different to the control group at 0.844mm ($F_{4,234}$=1.040, P=0.387). This continued into the second generation with adults raised in hosts reared on 100μg g^{-1} Cry1Ab having the shortest tibia length at 0.838mm compared to 0.850mm in both the control groups (Figure 5.11). However, as in the first generation this reduction was not significantly different ($F_{4,234}$=1.040, P=0.387).

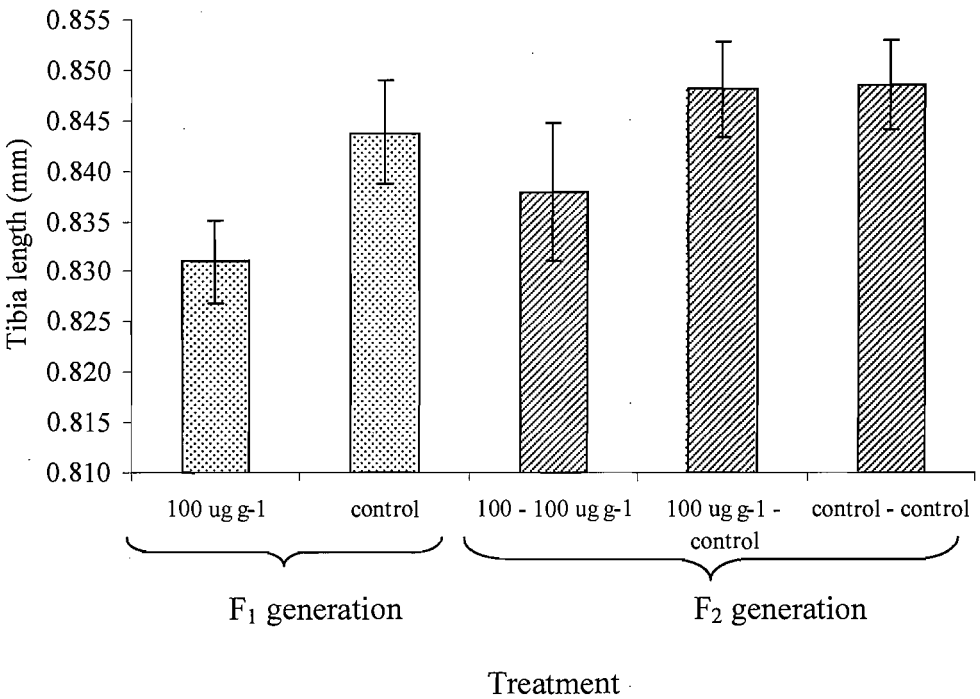


Figure 5.11 – Mean tibia length of adult parasitoids following exposure to the Cry1Ab toxin in the F_1 and F_2 generation (±S.E). Treatments are not significantly different P>0.05.

5.3.2.iv Adult fitness bioassays over successive generations

Following exposure to the Cry1Ab toxin or control adult parasitoids were assessed for their fitness following the treatment in the first and second generations. Attack rate was assessed to establish if adults exposed to the different treatments attacked more or less than when compared to controls. Parasitism rate was also monitored as a function of attack rate to assess the proportion attacks resulting in cocoon formation and then the

proportion of these cocoons that resulted in an adult parasitoid. Finally sex ratio was monitored to establish if exposure to the toxin resulted in a male biased population.

5.3.2.v Attack rate

After emerging females were removed from each replicate and treatment in the first and second generation of *C. marginiventris* adults. There was a significant difference between the number of attacks with the highest mean attack rate (χ^2=10.347, d.f.=4, P=0.35) in the 100µg g^{-1} Cry1Ab – 100µg g^{-1} Cry1Ab group after 2 generations of exposure to the toxin with an attack rate of 2.1 attacks min^{-1}. Conversely, the lowest attack rate was in the second generation parasitoid adults that had no exposure to the toxin in either generation at 1.4 attacks min^{-1} (figure 5.12).

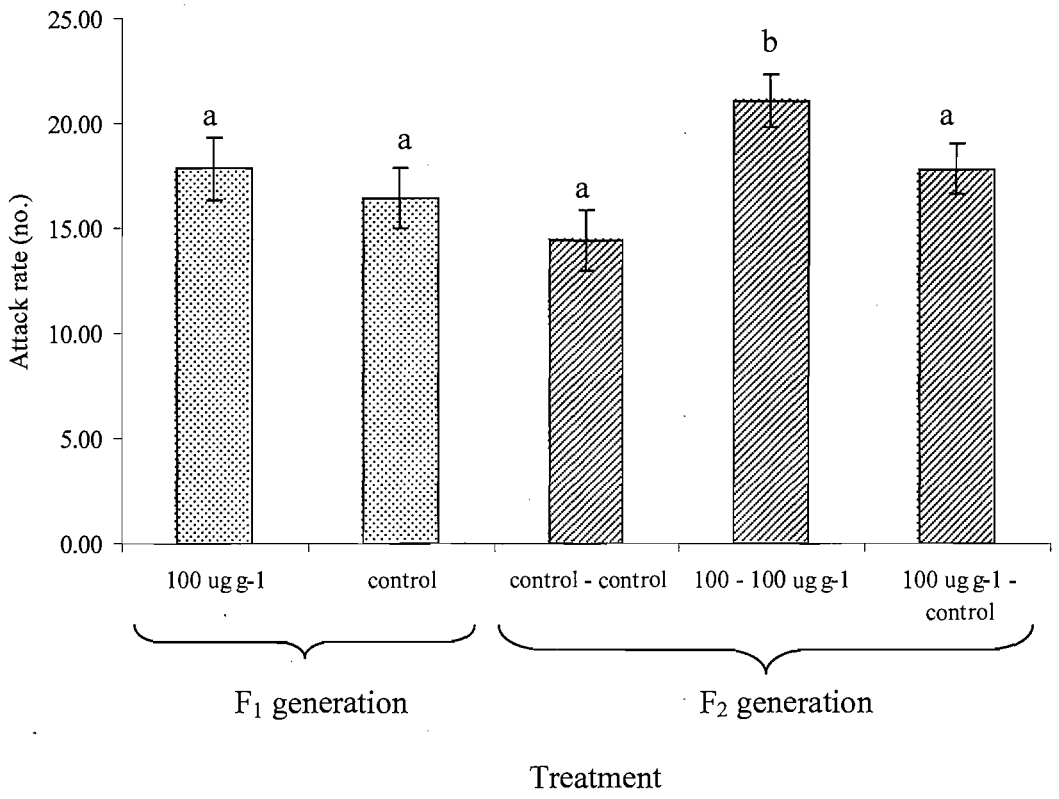


Figure 5.12 - Number of attacks on *S. littoralis* hosts by *C. marginiventris* females over a 10 minute period in the first and second generation exposure treatments (+S.E.). Columns with different letter represent treatment means that are significantly different at P<0.05.

5.3.2.vi Parasitism success

The lowest parasitism success when compared to the number of attacks was in the second generation adults raised in the F_1 generation on 100µg g^{-1} Cry1Ab and then on the control diet in the F_2 generation, parasitism success was 48%. The highest rate of parasitism success was in the F_1 generation control group at 65% (figure 5.3.14) however, parasitism success was not significantly greater than the F_2 generation raised on 100µg g^{-1} Cry1Ab then returned to the control diet ($F_{4,54}$=2.5, P=0.41). There was no significant difference in the parasitism success of the remaining groups with success ranging from 51% to 60% (figure 5.13).

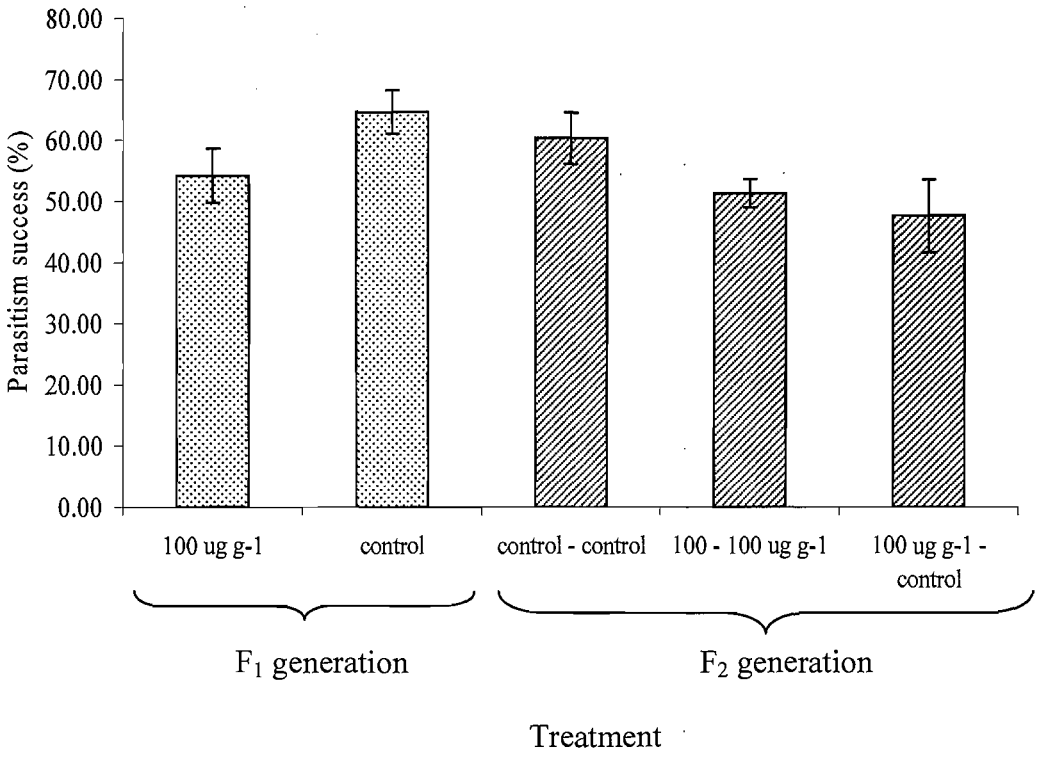


Figure 5.13 – Success of *C. marginiventris* attacks as a proportion of the number of cocoons formed for each attack. There was no significant different between the treatments at P>0.05.

5.3.2.vii Emergence rate

As with previous treatments emergence success remained consistently high and there was no significant difference between any of the treatments ($F_{4,53}$=0.259, P=0.903) with the least successful group being those parasitoids raised on 100µg g^{-1} Cry1Ab in the F_1

generation and control diet in the second generation with 76% emergence success. The highest emergence success was in the F_1 generation control group at 90% emergence. All remaining treatments ranged between 86-88% (figure 5.14).

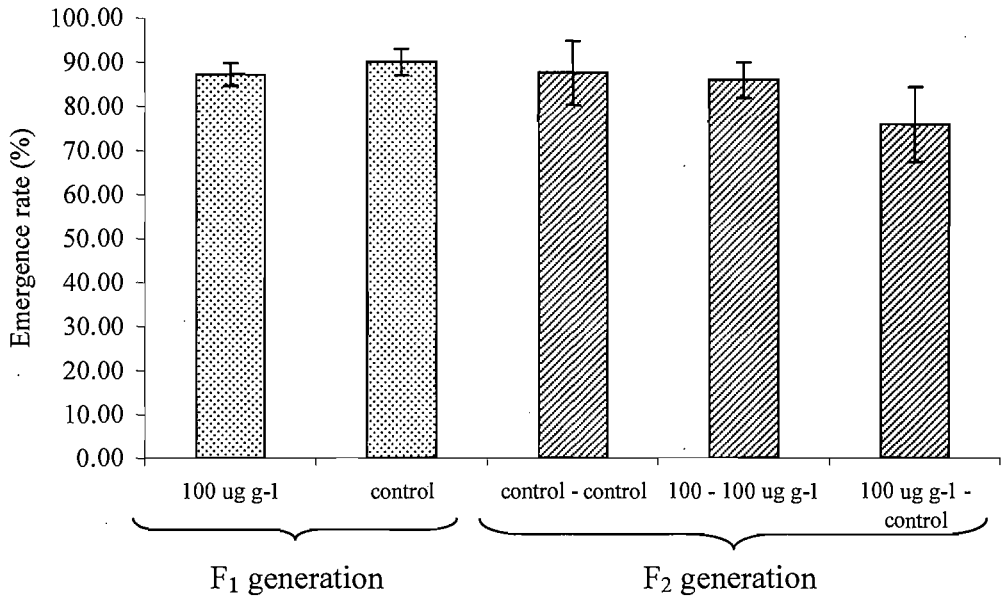


Figure 5.14 - Proportion of *C. marginiventris* adults emerging from spun cocoons (+S.E). No significant difference between the groups ($F_{4,53}$=0.259, P=0.903).

5.3.2.viii Sex ratio

The proportion of females in the F_2 generation was lower than in the F_1 generation with the proportion of females ranging from 18% to 29%. Whereas the highest proportion of females was in the F_1 generation control group at 57% with 41% female in the F_1 generation reared on host raised on 100 μg g^{-1} Cry1Ab (figure 5.15). The proportion of females in the F_1 control group and Cry1Ab group was not significantly different ($F_{4,54}$=6.231,P=0.421). There were significantly fewer females in all F_2 generation treatments when compared to the control (($F_{4,54}$ =6.231,P<0.001) figure 5.15).

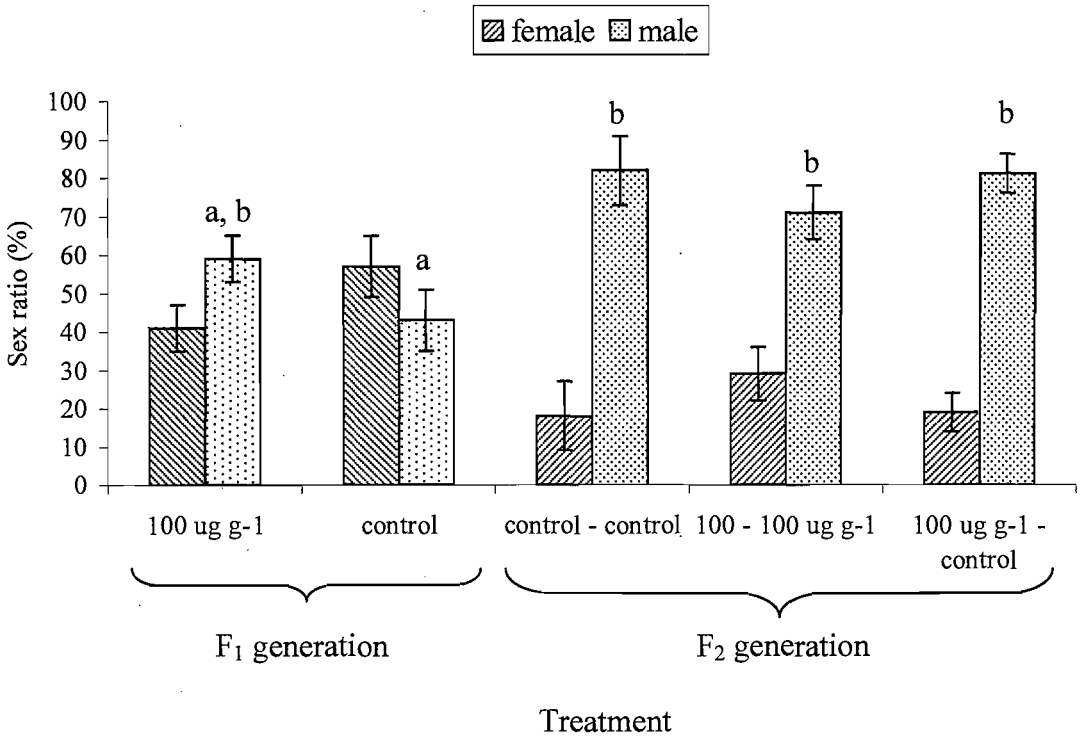


Figure 5.15 – Sex ratio of adult *C. marginiventris* adults (+S.E). Columns with different letter represent treatment means that are significantly different at $P<0.05$.

5.4.1 Cry1Ab effects on a single generation – host mass and parasitoid choice

This study showed that there were significant differences between host mass when host larvae were reared on Cry1Ab treatments or Bt176 maize when compared to controls. However this difference in host mass did not affect parasitoid attack rate despite a trend towards parasitoids carrying out more attacks on smaller hosts between 50-100mg. There was also no significant difference between either the attack rate, proportion of attacks resulting in cocoon formation or an emerging adult.

However, adult parasitoids had significantly shorter hind tibias when reared on host consuming diets containing the Cry1Ab toxin (i.e. smaller hosts). This supports the findings of other studies that suggest larger hosts provide a preferential nutritional environment than smaller hosts as they contain more resources for development (Godfray, 1994) and that ovipositing females allocate daughters to larger hosts as they realize a greater reproductive potential (Ode and Heinz, 2002). This is also confirmed by the work of this study (chapter 4) that parasitoids reared in smaller hosts (those reared on hosts consuming Cry1Ab) had shorter tibia lengths and longer pupation time. This is an indication of the stress to the parasitoid which may be in part due to reduced resources (chapter 4 section 3.2 Host mediated effects). As these findings of reduced fitness are following a single generation it is important to consider any effects following more than one generation of exposure to ensure these effects are not cumulative (see section 5.4.2).

The finding that there is no significant difference in attack rate suggests that whilst these smaller hosts produce less fit adult parasitoids given no choice, female parasitoids will parasitize the available hosts irrespective of size. The host larvae of between 50-100mg may represent the upper limit of parasitoid preference as *S. littoralis* hosts are aggressive towards parasitoid attack as they increase in size and able to defend themselves from oviposition attempts. This also supports Ode and Heinz (2002) that a relative rather than absolute host size is important for oviposition attack and success. Further when host mass was controlled for adult parasitoids showed no significant preference for hosts raised on either transgenic Bt176 or its near isoline confirming that parasitoids did not preferentially attack non-Cry1Ab containing hosts.

Whilst there was no significant difference in either the attack rate, proportion of attacks resulting in cocoon formation or an emerging adult the artificial diet control groups consistently had the highest success in these measures than the other treatments. This was reflected in the significant difference in adult tibia length in both the near isoline and control treatment when compared to the Cry1Ab and Bt176 maize treatments. The tibia length of adults reared on host consuming Bt176 maize being 6% shorter than the near isoline maize treatment. The trend in reduced oviposition and emergence success is also reflected when the actual numbers of emerging adults in each treatment is considered. For example only 12 adults in total emerged when combining all 10 replicates of the Bt176 treatment whereas there were 38 adults in the control treatment. Therefore, whilst individually the measures of parasitoid oviposition success may not appear significant, taking a holistic approach there were over 3 times as many adults in the non-Cry1Ab treatment and this could have a substantial impact on the population as a whole particularly when adult parasitoids show no difference in their preference for either size of host or host diet.

The trend seen in these results with no statistically significant difference in attack rate, oviposition rate and emergence rate yet large differences in actual numbers of adults and reduced tibia length may indicate a biologically relevant finding that is possibly masked statistically by biological noise (see chapter 2). A small sample size may be one explanation for the lack of statistical significance or it may simply be due to biological variation in measurement endpoints. If the noise in the system is due to biological variation then irrespective of the increase in sample size and number of replicates a statistically significant result is unlikely and the biological noise will remain.

As experimentation moves towards ecological realism and away from laboratory conditions this may lead to a reduction in statistical confidence but an increase in biological relevance. The appearance of a negative trend as seen in this study may be utilised in the risk assessment process to indicate a particular need for post market monitoring to ensure over the long-term this trend does not lead to a significantly reduced population size.

The noise in the biological system (chapter 2) leading to over 60% fewer adults in the Cry1Ab treatment is therefore an important consideration when assessing any risk due to the introduction of GM crops. This apparent large difference in actual offspring numbers but no statistically significant difference in specific measurement endpoints needs to be considered in light of natural variation in non-GM populations for regulatory decision making. Therefore, it is important that confidence limits and clear hypotheses are laid down prior to testing (Johnson et al., 2007) (Romeis et al., 2006a). The use of, for example, power analysis can give a clear indication of the confidence in the result and avoid type I and type II errors (Andow, 2003) (EFSA, 2007).

The use of the concept of substantial equivalence can also help inform decision making particularly if there is "biological noise" in the system. The concept of substantial equivalence is widely used and was first described by the OECD (Organization for Economic Cooperation and Development) in 1993 prior to the introduction of GM foods (Kearns and Mayers, 1999) (Burke, 1999). This tool can help ensure that any biological variation seen in measurement endpoints and subsequent population effects do not differ to those seen in comparative conventionally bred varieties.

5.4.2 Cry1Ab effects on F_1 and F_2 generations – oviposition success and parasitoid fitness

Whilst the size of hosts as they developed varied with exposure to the Cry1Ab toxin this did not affect the emergence rate of adult parasitoids following oviposition or sex ratio when compared to controls. However, parasitism success was highest in the F_1 generation reared from hosts raised on control diet and lowest in the F_2 generation initially reared on hosts consuming 100μg g^{-1} Cry1Ab and then switched in the F_2 generation to control diet. This change was apparent even though hosts at the time of parasitism were of the same size as this was controlled for and were not exposed to the toxin until immediately after parasitism.

The trend towards reduced fitness of adult parasitoids following exposure to the Cry1Ab toxin in the F_1 generation is not reflected in a significantly reduced tibia length of the adults following 2 generations of exposure, this confirms that the effects of continued exposure to Cry1Ab does not have a cumulative effect. Therefore, there appears to be no direct effect of host diet and size on adult

parasitoid size as has been seen in other studies (Greenblatt and Barbosa, 1981) smaller hosts are not in this instance producing smaller parasitoids over successive generations at this level of exposure. As variation in adult size can influence reproductive success by increasing longevity, fecundity and search efficiency (Harvey et al., 1994 and references therein) these fitness indicators are less likely to be affected at the population level.

However, pupation time of adult parasitoids was significantly longer when pupae had been exposed to the Cry1Ab toxin in the F_1 generation and those exposed to the toxin over 2 generations having the longest pupation time however differences in the second generation were not significant when compared to controls and this reaffirms that any negative effects seen in the first generation are not cumulative into the second generation. Pupation time has been shown to be a consistently sensitive measure of parasitoid stress (chapter 4). As with sex ratio (discussed later) the extension in pupation time across all treatments in the F_2 generation may reflect some experimental stress due to small population size and lack of outbreeding.

The lack of accumulation of the negative effects on measurement endpoints over 2 generations such as increased pupation time and reduced tibia length is an important element of assessing the potential risk of GM crops. This is particularly the case when some potential direct toxicity has been shown (chapter 4). The lack of accumulation of any negative effects on fitness and no bioaccumulation of the toxin itself through the food chain (chapter 4) allow the potential to make an informed risk decision whilst potentially avoiding costly field trials (chapter 6). However, given the negative trend seen in the number of emerging adults (section 5.4.1) and potential direct toxicity (chapter 4) careful post market monitoring would be required if field trials were not undertaken.

Given that previous innovations in the first green revolution with the widespread introduction of organopesticides such as DDT to agriculture after WWII lead to the loss of bird species due to bioaccumulation (Ach, 2000) (Portmann, 1975) (Turusov et al., 2002) also see Rachel Carson, Silent Spring) it is vital that any new GM crops do not have the potential to bioaccumulate or behave in this manner.

This study helps illustrate how moving through a tiered system of risk assessment by first identifying direct and host mediated effects (chapter 4) and then effects over successive generations any potential harm can be identified without using field scale studies (see chapter 6 for a discussion of caveats to this).

The changes in sex ratio in this study in the second generation group suggest that the experimental set up may have caused some stress in to the adult parasitoids. This stress may be linked to reduced outbreeding in the F_2 generation not seen in the F_1 generation. Several studies report female biased sex ratios in field populations (Tagawa, 2000) particularly as the reduction in the proportion of females in the F_2 generation is across all treatment groups. A increase in the proportion of males with female age can occur (Tagawa, 2000) however this is unlikely to be the case in this study as females were used between 2-7 days post emergence.

A question that remains unanswered however is the egg load of individual parasitoids when exposed or not exposed to the Cry1Ab toxin. It is not clear if despite no differences in emergence rates, survivorship or attack rate if parasitoids with a high egg load would die before becoming egg limited or if parasitoids with a low egg load would become egg limited before they die (Collier et al., 1994) if given access to unlimited hosts. Rivero-Lynch (1997) found that in synovigenic parasitoids egg load remained constant despite their experience of host abundance - the effect of this in pro-ovigenic parasitoids is unclear.

Any trend towards smaller adults due to an environmental stress such as GM crops is an important consideration as any variation in the female size can affect egg production and therefore have a cumulative effect on subsequent generations (Fox, 1996). Whilst tibia length did not continue to reduce over the generations in this study the significantly increased pupation time in adults exposed to the Cry1Ab toxin in the first and second generation may indicate stress due to exposure to the toxin across more than one generation.

As with previous findings (section 5.4.1) were a trend towards reduced numbers of adults was identified an increase in the number of generations and individuals exposed to the Cry1Ab toxin may produce a statistically significant result and

reduce biological noise. The continued increase in pupation time over 2 generations but no continued decrease in tibia length (indicator of fitness) could also be used as a trigger to indicate the need for careful post market monitoring.

If effects are transmitted across several generations then clearly this may have evolutionary implications for the fitness, timing of mating and the emergence for populations as a whole. To elucidate this fully would require studies of multiple generations and a larger sample size to ensure that any effects were not the result of inbreeding pressure. It also reinforces the case for freely interbreeding populations not exposed to GM crops to ensure sympatric speciation due changes in pupation time for example do not occur i.e. the use of refuges.

Refuges are used widely for resistance management and are areas of planting of untransformed crops and can be both spatial and temporal (Hunter, 2000). The concept of refuges can help restrict non-assortative (i.e. non-random) mating by allowing both susceptible and resistant offspring to mate (Hunter, 2000). This strategy is currently used for the control of the build up of insect resistance which has occurred in *Plutella xylostella* in field sprayed populations (Raymond et al., 2007) (Hunter, 2000). Therefore, whilst this strategy is currently deployed to avoid resistance build up and is mandatory in the United States (Hunter, 2000) it may also have benefits in avoiding speciation due to changes in other phenotypic traits. However, without long-term, clearly defined monitoring of the impact of refuges their success is impossible to evaluate.

Interestingly, the highest attack rate of adult parasitoids were those having 2 generations exposure to the toxin however this increased attack rate did not translate in to a greater numbers of offspring. The lack of greater numbers of offspring despite higher rates of parasitism in smaller host reared on Cry1Ab over two generations is explained by greater variation in the control group and slightly higher yet not significantly different levels of parasitism success in this group.

This study gives an insight into the possible changes and stresses that parasitoids encounter over two populations of exposure to the Cry1Ab endotoxin. However, the response of the parasitoids is not entirely clear (e.g. changes in sex ratio) and a larger

scale study reducing experimental stress and increasing population size in a glasshouse environment would be extremely beneficial. In terms of risk decision making this study illustrates that laboratory studies of worse case scenarios using only a single generation may not always be sufficient and at least limited population studies may be necessary and desirable. It is important to remember however, that exposure levels in this study were 5 times those seen in the field (chapter 2) and that any reduced parasitoid fitness was extremely limited.

This study also identifies that trends which may be biologically relevant, but not statistical significant, may be apparent and these findings could provide a trigger for either larger scale (semi-field) studies to clarify potential risk and/or alternatively provide a trigger for post market monitoring.

Chapter 6

General Discussion and Conclusions.

6. General Discussion

The aim of this study was to place host mediated and direct exposure experiments within an ecological risk assessment (ERA) framework to enhance and inform the decision making process already in place and avoid where appropriate unnecessary, costly and time-consuming testing at a higher tier. Avoiding higher tier testing when appropriate may be prudent as with increasing complexity comes increased cost and with increasing realism comes decreasing statistical power (Romeis et al., 2006a).

Therefore, this work was conducted within an ecological risk assessment (ERA) framework with experiments at the worst case scenario first tier level and at a small population level incorporating increased realism. The main objective of the project was to establish the effect of expression of Cry1Ab toxin incorporated by Bt176 transgenic maize on the fitness of the parasitoid *Cotesia marginiventris*. Research on non-target parasitoids is a vital component of assessing any risk of novel genetically modified plants (GMPs) as they are a beneficial insect that can help regulate pest populations.

This main objective was further divided into experimental objectives within the ERA framework they were:

1. to establish the amount of natural variation in the system,
   Chapter 2 – Baseline data.
2. to establish if any effects seen were due to direct or indirect (host mediated) toxicity,
   Chapter 4 - Direct and Host mediated toxicity (worst case scenario).
3. to establish the dose at which (comparative to plant expression levels) these effects (if any) were evident,
   Chapters 3 and 4.
4. to establish if the parasitoid was able to recover from the effects of the toxin after more than one generation of exposure,
   Chapter 5 – Population and multigenerational effects (second tier).

This chapter discusses the main findings of this study and how this could inform future work. However, it does not set out the answer the question of how much risk is acceptable, this is still and rightly a question for the public, scientists, government and other stakeholders.

6.1 Summary of findings

6.1.i Natural variation experiments showed the considerable variation in host larvae size assessed via measures of larval mass. Larvae showed considerable variation in size both between diet treatments (artificial diet, isoline maize and Bt176) and between cohorts. These findings indicated the suitability of artificial diet as an optimal diet and the amenability of study using this diet source. However, the use of artificial diet did not completely negate the biological variation (see chapter 2) however this was reduced in subsequent studies in part due to changes in experimental technique and also due to the reduced lifespan and growth of the larvae due to parasitism (see chapter 4).

6.1.ii Cry1Ab expression levels in Bt176 maize plants was established using ELISA analysis. Expression levels ranged from 15.3 to 8.8 $\mu g\ g^{-1}$ fresh weight dependent on leaf stage and plant age (see chapter 2) with a mean expression level of $11 \mu g\ g^{-1}$ fresh weight. Levels of purified Cry1Ab incorporated into artificial diet were set at 1, 2, 10, 20 and $100 \mu g\ g^{-1}$ (see chapter 4). This range accounted for the levels of natural variation in plant expression levels i.e. between $10\text{-}20 \mu g\ g^{-1}$ and also extended the range outside the natural variation of plant expression.

The aim of this complete range of Cry1Ab levels was to establish the point at which no observable effect (NOEC) occurred and at what concentration any effect occurred relative to plant expression levels. The highest dose of $100 \mu g\ g^{-1}$ is the point at which *S. littoralis* are still amendable to experimentation following ingestion of Cry1Ab, at higher doses *S. littoralis* larvae suffer very high mortality and growth retardation ((Ramirez-Romero et al., 2007) due to only partial resistance to the protein (Keller et al., 1996).

6.1.iii The effect of direct exposure of juvenile *Cotesia marginiventris* to the Cry1Ab toxin was assessed using a subcutaneous injection of toxin into the parasitized host larvae. Cry1Ab mode of action is via the host gut where it binds to receptors on the

brush border membrane, following binding pores are opened in the membrane disrupting the osmotic balance and ultimately leading to larval death (de Maagd et al., 2001; Hilder and Boulter, 1999; Sanchis et al., 1994). Whilst *S. littoralis* are partially resistant to Cry1Ab (Keller et al., 1996) larvae still suffer some retardation in growth when exposed orally to the toxin (Vojtech et al., 2005). The direct injection of host larvae avoided the host gut and the detrimental effects on host growth seen when host larvae are exposed to the toxin via artificial diet. However, the injection technique produced significantly higher mortality in injected groups when compared to control groups (chapter 4).

High mortality was not reflected in significant differences between control injection and uninjected controls with respect to tibia length or sex ratio. Pupation time was the only measurement endpoint that was significantly extended when comparing exposure between the Cry1Ab and distilled water control injection (chapter 4).

6.1.iv Host mediated effects of the toxin on *C. marginiventris* were generally more pronounced and effected a range of measurement endpoints when compared to direct toxicity experiments. Significantly longer pupation time and significantly shorter tibia length were both evident when compared to controls. These differences in host mediated exposure were only seen at five times the plant expression levels (chapter 4).

6.1.v Cry1Ab movement through the food chain (host mediated exposure) was monitored via ELISA analysis. As would be expected levels of detectable Cry1Ab in larvae, larval frass and parasitoid cocoons increased with increasing exposure dose. No Cry1Ab was detected in parasitoid adults whilst it was detectable in parasitoid cocoons, this suggests that the parasitoids do take up the Cry1Ab as juveniles but that it is subsequently excreted prior to adult emergence. The detection of Cry1Ab in parasitoid cocoons but the lack of accumulation of Cry1Ab in adult parasitoids has also been reported in previous studies (Vojtech et al., 2005) and in other species of parasitoid that consume the entire host including the gut (Sanders et al., 2007). No Cry1Ab was detected in direct exposure trials and this reflects the small quantity and single dose method of exposure (chapter 4).

6.1.vi Parasitoid attack preference was not affected by the diet treatments on which the hosts were raised or the size of the hosts. There was a trend towards preferential selection for attacking smaller hosts (50-100mg) compared to larger hosts (<150mg), but this was not significant. Ovipositing adult parasitoids did not avoid hosts raised on GM maize or artificial diet containing the Cry1Ab toxin.

Host mediated effects over successive generations showed some significant differences in the first generation of exposure however these differences did not relate to continued poor fitness when the next generation of parasitoids were exposed to the toxin i.e. there were no transgenerational effects (Chapter 5).

6.2 Main Conclusions

6.2.i Measurement and Assessment endpoints

- Host larvae experience considerable biological variation in host mass.
- Direct toxicity may be evident due to extended pupation time.
- Host mediated effects were only evident at 5 times plant expression levels.
- Cry1Ab was not accumulated into adult parasitoids.
- Parasitoids did not avoid attacking host of larvae reared on Cry1Ab containing diet.
- Negative impacts of exposure to Cry1Ab did not accumulate over two generations.
- Pupation time is a highly sensitive measure and indicator of parasitoid stress.

6.2.ii Ecological Risk Assessment

The use of the tiered approach allowed the following conclusions to be made:

- Tiered approach allows for recognition of the type of toxicity i.e. direct or host mediated (chapter 4).
- The implications of toxicity at lower tiers can be confirmed at a higher level whilst avoiding field trials i.e. transgenerational effects (chapter 5).
- Level of exposure relative to field exposure levels must be confirmed to inform risk decision making process (chapters 4, 5 and 6).

6.3 General Discussion.

"No instrument has yet been designed that can measure toxicity. Chemical concentration can be measured with an instrument but only living things can be used to measure toxicity." (Cairns and Mount, 1990).

As stated at the start of this chapter the aim of this study was to place host mediated and direct exposure experiments within an ecological risk assessment framework (figure 6.1) to enhance the informed decision making process and avoid where appropriate unnecessary testing higher tier testing. With this comes two important questions *1 – If there is no effect at the tier 1 worst case scenario test does this negate the need for higher tier testing (tiered approach)* and therefore *2 – Are full or small scale field trials necessary for each new plant incorporated protectant alongside laboratory based worst case scenario testing* ?

It is important to note that the considerations here focus on the affect to non-target organisms and specifically parasitoids. Whilst the discussion may be relevant to other areas it does not specially consider for example the affects of out-crossing, weediness and human allergenicity. Other considerations following the introduction of a GM crop may also include the affects of changes in farm management practices and consequently have implications for biodiversity in general.

This study looks at a laboratory approach of worst case scenario testing using the parasitoid *C. marginiventris* as a surrogate species representing beneficial parasitoids not consuming the host gut (see chapter 1 section 1.3 regarding Cry1Ab mode of action). Similarly the *S. littoralis* host represents non-target lepidopteran herbivores. However, this study recognises that a case by case approach using specific non-surrogate species that may be key species in a farming environment or a field approach may be necessary. If a case by case approach is required it can still follow the approach discussed here if the species is amendable to this type of bioassay technique.

A case by case approach can either be laboratory or field based, or both where necessity and pragmatism dictate. A field approach may be necessary when species are not amenable to laboratory culturing or when they are key species within an ecosystem or both. Some experts argue that there is simply an insufficient data set to ensure that no

further testing is required if no effect is seen at the worst case scenario level (EFSA, 2007) and whilst the need the need for extra testing should not be a means for restricting the use of novel GM crops or existing varieties it would seem prudent to ensure sensitive species or ecosystems are not put at risk by this technology. Worst case scenario testing within the laboratory simply does not account for the effect on local biodiversity that can be compounded by the stress of the intensification of farming following the introduction of this new technology (Andow et al., 2001) page 199). Therefore, this may be a compelling reason to carryout both worst case scenario and case-by-case field scale trials. Field trials should not be excluded by an inflexible and rigid approach to risk assessment if the receiving environment is unique or long-term effects difficult to predict.

Whilst, issues exist regarding the comparability of laboratory trials with a field situation, laboratory trials are easier to standardize and considerably less costly than field trials. The regional, national and geographical differences may simply make field trials prohibitively costly and difficult to compare even within a single county. However, trials comparing laboratory, glasshouse and field scale experiments found no contradiction between laboratory results and field results and any changes were either transient or no greater than changes due to impacts of crop type, tillage or pesticide use (Birch et al., 2007).

The economic constraints of field trials are a major consideration when planning such experiments, this is reflected in the cost of the UK farm scale evaluations (FSE) of genetically modified herbicide-tolerant (GMHT) crops (Firbank, 2003) (Firbank et al., 1999). The UK FSE were the largest of its kind carried out in the world costing approximately £5 million and give an indication of the expense and problems associated with this type of testing (Williams, 2003).

"The FSE were are an ecological experiment designed to examine the effects of GMHT crops on the biodiversity and functioning of arable fields in Great Britain" (Squire et al., 2003). Therefore they considered the effect of different choices of weed management on the survival of wildlife dependant on weeds for development and growth (Amman, 2003). The data collected was then used to compare the differences between GMHT and conventional management practices (Squire et al., 2003). The experimental design

also allowed sufficiently stringent control of variation to get statistically significant results and allow up-scaling to predict the effect on countrywide biodiversity should widespread use of GMHT crops be introduced (Squire et al., 2003). However, whilst these criteria set the FSE within a conceptual framework and allowed the formulation of testable hypotheses, they did not necessarily ask the correct question to make risk analysis and risk decision making objective and linked to science based findings. Therefore, to ensure that decision making based on the risk posed by GM crops is objective and based on fact and not subjective and based on how stakeholders and regulators feel about the technology requires a clear link between the question asked by the risk assessment and the regulatory decision required.

As the risk decision of what risk is acceptable is linked to what change in biodiversity compared to conventional agriculture is acceptable, a complete conceptual framework including risk decision making is critical to make the risk decision making process less subjective. This is particularly important with the type of investment on the scale of the FSE as it should ensure that the scientific data and analysis are linked to the decision making process.

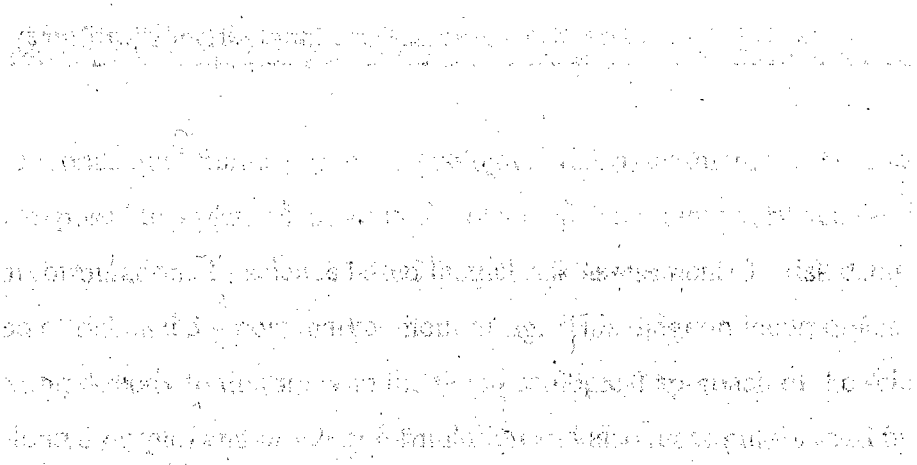
6.3.i Hypothesis testing and conceptual frameworks.

In order to carryout meaningful and cost effective risk assessment a clear conceptual framework is required along with clear and testable hypothesis (Johnson et al., 2007). The FSE are a good example of the results being easily misinterpreted by all sides and being a baseline for farming practices (Nature, 2003) news in brief)). Therefore whilst studies of this type are valuable and highly desirable it is vial that clear hypotheses and conceptual frameworks are laid down prior to the start of any ERA to avoid later confusion and ease decision making. Sanvido (2005) lays down a clear and effective conceptual model for ERA which contains not only laboratory and field trial assessments but also post market monitoring. Whilst the conceptual framework devised by Sanvido (2005) is a useful model for the science base function of ecological risk assessment this needs to link with the model for example like that described by Johnson (2007) which also encompasses risk decision, key issue identification and risk communication, these are all vital components of a robust ERA conceptual model. When conceptual models are linked with clear and measurable assessment and

measurement endpoint (section 6.3.iii) this can help ensure more readily assessable data on which decisions about risk can be based.

The advantage of clear hypothesis testing and conceptual frameworks are also in the area of public and risk communication as it is easier to communicate what has been tested, how and why a vital component of communicating risk to a lay audience. A clear initial definition of what the ERA can and can't achieve also ensures that expectations of the process are not unrealistic and all stakeholders are clear what it can and importantly can't provide.

Figure 6.1 lays out a conceptual framework in which the techniques discussed in this project utilise the risk assessment process and the decision making through each tier. This tiered approach follows conceptual frameworks reported and widely used within the risk assessment arena (Poppy and Sutherland, 2004b) (Johnson et al., 2007) (Sanvido, 2005) (Romeis et al., 2006a) and as discussed above may require incorporation into existing schemes. The post market monitoring (PMM) function of risk assessment would require constant review to ensure any new data complies with stakeholder agreement of acceptable risk.



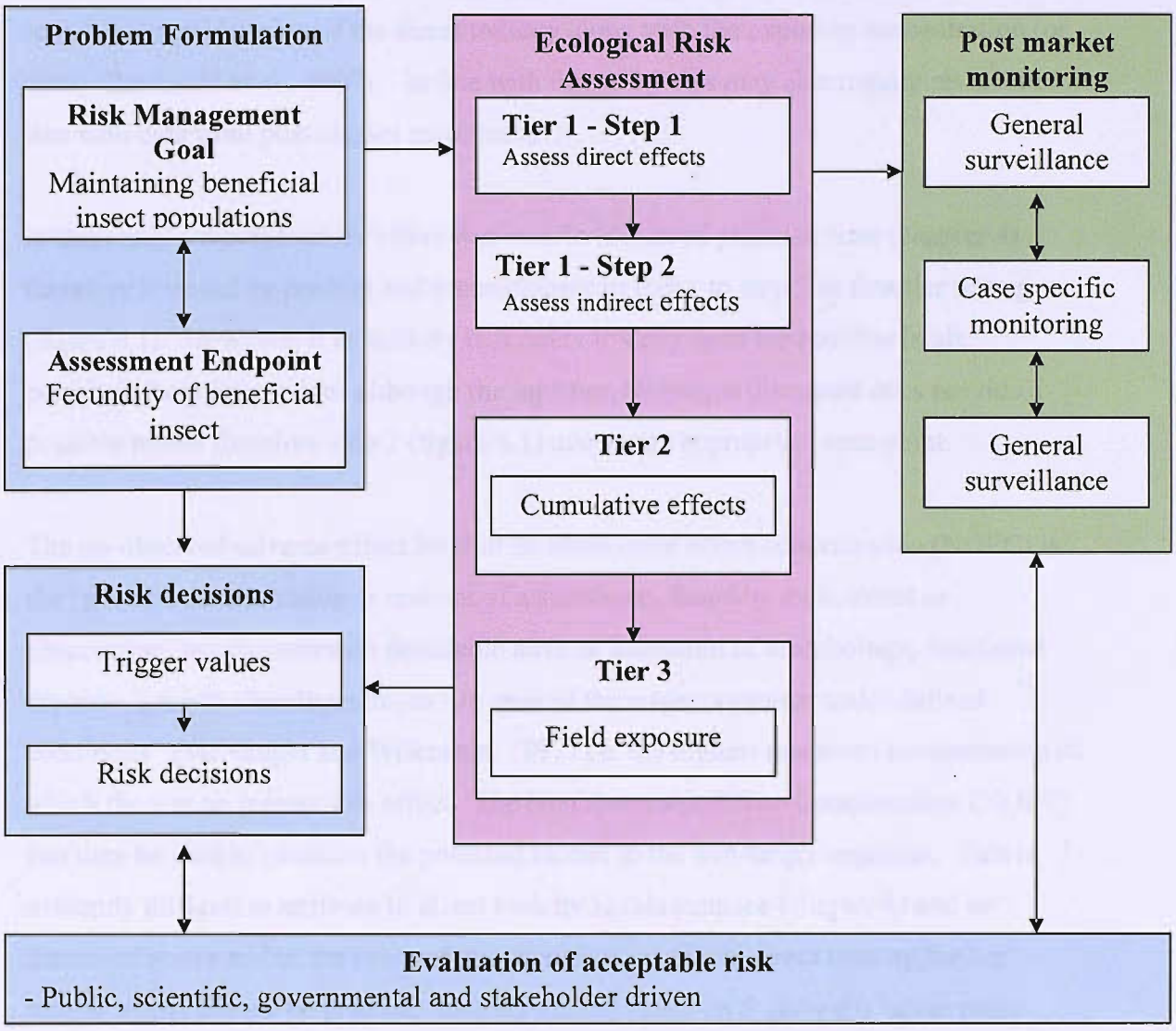


Figure 6.1 Conceptual framework of an ecological risk assessment for a non-target organism exposed to a plant incorporated protectant. The framework depicts 5 stages; 1 - problem formulation, 2 - science based factual risk assessment, 3 - risk decision, 4 – evaluation of risk and 5 – post market monitoring. This diagram incorporates specific ideas relating directly to this study in the tiered ecological approach of the science based ERA (coloured purple) and problem formulation and also ideas put forward by Johnson (2007) and Sanvido (2005).

6.3.ii Trigger values and assessing hazard.

Assessing hazard i.e. the potential of this new technology to cause harm and making informed and financially costly decisions about when to move to the next tier of testing requires a consideration of the direct toxicity along with the exposure concentration (or dose) (Raybould et al., 2007). In line with figure 6.1 this may also require review in line with data from post market monitoring.

In this study a direct toxicity effect was seen in increased pupation time (chapter 4) therefore it would be prudent and precautionary to move to step 2 of first tier testing (figure 6.1). However, it is unlikely that direct toxicity tests are possible in all parasitoid/host interactions although the injection technique discussed does provide a possible model therefore step 2 (figure 6.1) may be an appropriate start point.

The no-observed-adverse-effect level or no observable effect concentration (NOEC) is the "greatest concentration or amount of a substance, found by experiment or observation, which causes no detectable adverse alteration of morphology, functional capacity, growth, development, or life span of the target organism under defined conditions" (McNaught and Wilkinson, 1997) i.e. the highest measured concentration at which there is no measurable effect. The No Observable Effect Concentration (NOEC) can then be used to establish the potential hazard to the non-target organism. This is evidently difficult to attribute to direct toxicity in this instance (chapter 4) and as discussed above and in the event of the identification of any direct toxicity further testing would always be prudent. Indirect toxicity trials on *S. littoralis* larvae mass show a NOEC of 2μg g^{-1} and importantly a NOEC in *C. marginiventris* at 20μg g^{-1}.

Having established the NOEC this can then be used to inform whether testing at the 2nd tier 3rd tier is prudent. The hazard quotient (HQ) for the substance can then be calculated. The hazard quotient is simply the ratio of the exposure dose divided by minimum dose above which adverse effects can be seen ((Raybould et al., 2007) also see http://www.epa.gov/R5Super/ecology/html/glossary.html#hazard last updated 01/06/07) in practical terms the NOEC.

Raybould et al. (2007) suggest that at a HQ below 1 there is minimal risk to the non-target and therefore potentially no need for further testing. This is the point at which

field exposure is equal to or less than the NOEC. In this study of indirect testing the conservative estimate of the HQ is 1 i.e. at 20μg g^{-1} Cry1Ab (the highest dose seen in plant expression levels, mean 11μg g^{-1}) there is no observable effect and any adverse effects are only seen at 100μg g^{-1} Cry1Ab. At this point therefore no further testing may be deemed necessary however if following the precautionary principle (i.e. reducing the likelihood of type II errors (Batie, 2003)) further testing could be conducted to ensure small changes at the individual level are not compounded with each generation of continued exposure (chapter 5). Then, if no effects are seen at this point it may be safe to conclude testing particularly if previous studies with a range of species and substances show similar results. The only caveat here is as discussed earlier if there are likely to be significant changes in farm management practice, the area is a sensitive area for biodiversity and/or species of key ecological importance are resident in the area.

A further final consideration for triggering higher tier testing is that whilst it is vital to have accurate reporting of statistical power and sufficient replication any calculation must have sufficient power to avoid unrealistically large sample sizes (Andow, 2003) as this is unlikely to be practical or economic.

It is also important to understand both the direct and indirect effects of any GM plants incorporating insecticidal proteins as this technology has the ability to disrupt current biological controls (Poppy and Sutherland, 2004b). The difference between direct and indirect (host mediated) effects is particularly pertinent to the field of parasitoids an important beneficial insect in many agricultural settings. Many studies have shown detrimental effects to parasitoids (Meissle et al., 2005; Sanders et al., 2007) however what is less clear is if these effects are of the direct toxicity of the compound to the parasitoid or an effect of the host showing reduced nutritional quality (sub-optimal host syndrome) i.e. indirect host mediated effects. This is an important distinction as if the incorporated compound is directly toxic this is potentially much more damaging to the parasitoid than if the effects seen in the parasitoid or due to a poor quality host. In a field situation a parasitoid is potentially less likely to oviposit in a poor quality host but as shown in this study (chapter 5) does not avoid a host of the same size containing the GM protein. Therefore, if the compound showed direct toxicity this could negatively impact the parasitoid population very rapidly.

Generational studies are also vital as they have the ability to characterize any potential evolutionary pressures which may have implications at the population level e.g. changes in sex ratio. The incorporation of longitudinal studies ensures that any longer term effects of a GM protein can be identified.

6.3.iii Measurement endpoints

The work detailed in this study has expressly laid out specific and measurable endpoints, these are specific ecological attributes that are readily measurable (USEPA., 1994) for example pupation time, tibia length, sex ratio and mortality. The use of endpoints which indicate adverse affects on fitness have clearly been shown to indicate very subtle changes in fitness e.g. changes in parasitoid pupation time which may not have been evident if endpoints were restricted solely to lethality. Therefore, a consideration of a range of fitness (sub-lethal) endpoints is important rather than measuring mortality as a large proportion of the population are likely to be lost to natural mortality and it is those that remain and their ability to undertake a biological control function that is important. Clearly, these endpoints much remain unambiguous and susceptible to the stressor (Poppy, 2003).

This use of both lethal and sub-lethal measurement endpoints also takes account of the exposure pathway of the non-target organism from stressor e.g. GM crop to receiver e.g. parasitoid. "A complete exposure pathway is how a chemical can be traced, or expected to travel, from a source to a plant or animal that can be affected by that chemical", http://www.epa.gov/R5Super/ecology/html/glossary.html#complexposure updated 01/06/07). The use of lethal and sub-lethal endpoints account for both direct toxicity (direct contact with the stressor) or indirect toxicity that can be host mediated as in the case of parasitoid insects.

6.4 Concluding Remarks

Clearly whilst it is important to consider a case by case approach it is also important to reduce redundancy (Cairns and Mount, 1990). However, redundancy can only be reliably avoided if a database of all the affects is accessible to all stakeholders and there is a general consensus on a affective approach to accessing the effects of GM plants, importantly this needs to include work showing no effect which is often left unpublished and therefore unreviewed. Databases like BioOK (Mikschofsky et al., 2007) may be a step towards this with the criteria that they aim to be a neutral and independent interdisciplinary service (http://www.bio-ok.com/index.htm updated 22/10/2007). However, a wide ranging database does not guarantee that a decision on the acceptable risk will be made in the same way by agencies and government bodies and the same data set can give rise to different risk management decisions and these difference can originate from factors that are social, economic and political (Kamrin, 1990).

The use of power analysis can give a clear indication of the rigour of a study into the effects of GM crops and the likelihood of avoiding type I and type II errors. Ideally power analysis would be conducted prospectively rather than retrospectively however for the non-statistician this can prove complex but in a controversial arena such as the commercial use of GM crops which requires reporting of non-significant results a Type II error can be hazard (Andow, 2003) and lead to a lack of confidence in the risk assessment process. However, as the database increases so should the confidence in any decision regarding the safety of the substance (Cairns and Mount, 1990).

Ultimately, the simple worst case scenario tests described at tier 1 may not answer or predict outcomes to site-specific assessments and therefore may perform more of a screening role in some situations or for some new products. However the difficulty of high cost, standardisation and establishing causal relationships in a field setting remains (EFSA, 2007). It is important for the development of this technology into new and potentially lifesaving areas to not allow the issue of cost and comparability to be used as the reason to slow or stop new development. Which ever approach is adopted this needs to be supported by appropriate long-term monitoring (EFSA, 2007) however who is responsible for funding this post market monitoring would no doubt attract much discussion.

Clearly conceptual models and clear hypotheses are a vital component of meaningful and effective risk assessment to ensure results and risk can be communicated clearly and effectively. These are required along with clear, unambiguous and measurable measurement endpoints (Poppy, 2003)

The most pragmatic way forward for ERA be that a field or laboratory based case by case approach or a tiered system may be a joint framework taking the best codes of practice from each approach given a variety of environmental and geographical settings.

6.5 Future work - What next?

There are potentially two areas of the ecological risk assessment process related to this study that require continued research and development to move this field forward. These are areas of research related to:

(1) the direct scientific laboratory or field study of the effects of new genetically modified plant (GMP) varieties particularly those containing multiple traits or those not currently widely used in conventional insect control.

(2) those linked to desk based research formulating clear and robust conceptual models, assessment and measurement endpoints.

Clearly these are not mutually exclusive areas of research and particularly in the area of assessment and measurement endpoint formulation have significant overlap. Robust and well developed assessment and measurement endpoints ensure when data is collected and analysed it is able to effectively inform the decision making process.

6.5.i Laboratory and field based research

Within the scope of this project establishing the absolute NOEC for this substance and organism would be of considerable scientific and intellectual interest, however, this would not necessarily be money well spent or enhance the field rather add further data to the growing number of studies.

Whilst further testing of the same or similar species may not be economic, a robust and marketable approach to post market monitoring (PMM) is invaluable to ensure there are no unintentional effects or impacts on the environment following release. The PMM of potential long-term effects is laid down by EFSA ((2007) and references therein) as a

"characteristic time scale in the order of 10 to 100 generations. Thus for most organisms long-term effects should emerge after a minimum of 10 to 20 years". The formulation of a simple approach to PMM would also help inform the existing database and allow the modelling of any environmental impacts whilst reinforcing the credibility of the ERA process and therefore is a process that should continue to be a fundamental part of future risk assessment work. In a report for UK DEFRA (2006) post market monitoring of Bt toxins in the soil is specifically recommended to address the issue of persistence (Schuler, 2006) and therefore constitutes an area of active and continued research in this field.

Greater work on establishing variation and interpretation of what constitutes a robust measurement endpoint and hazard quotient would be valuable along with greater understanding of how any changes seen at tiers 1 and 2 impact when translated to the field. This work may involve mathematical modelling to compare worst case scenario testing and field trial results. Therefore, requiring an interdisciplinary approach between ecologists, statisticians and mathematicians.

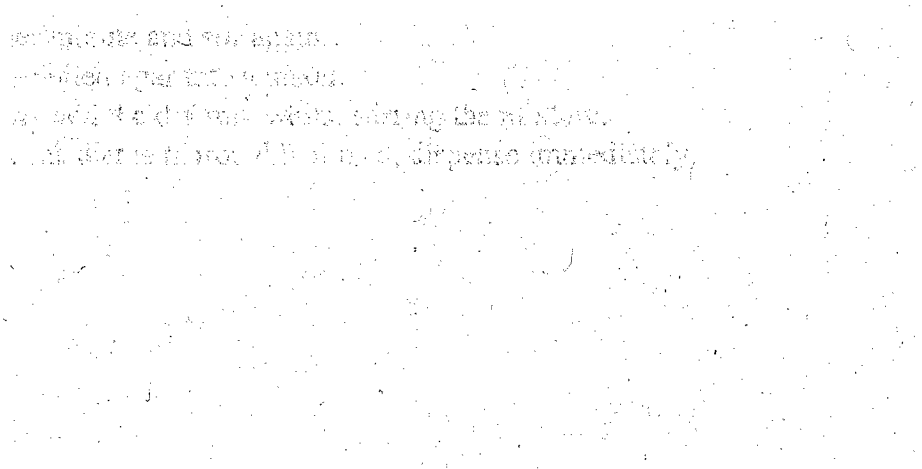
6.5.ii Conceptual models and decision making

With an increasing database of studies and assessment of existing G.M.P.s particularly in the field of non-target parasitoids it would be informative to use this information for the identification of trigger values (section 6.3.ii) that precipitate the move from tier 1 to tier 2 testing. This work would also necessarily tie-in to work using modelling to assess how changes at the worse case scenario level are manifested in the field. A clear and rigorous system for decision making about when to move to higher tier testing and when a product presents a sufficiently low enough risk to negate the need for further testing is a vital component of the risk assessment process (see caveats in section 6.3).

The formulation of clear and useable conceptual models is vital for working towards a system of incorporating both case by case and tiered testing and linking those individually to specific biota. This work would need to incorporate the concepts already laid down in this study including risk management goals (Chapter 1 and 6), risk assessment (chapter 1, 4, 5 and 6) and risk decision making (chapter 5 and 6) whilst also incorporating post market monitoring and risk communication.

Post market monitoring is an area that will become increasingly important over the next 10-20 years as the results of long-term effects (EFSA, 2007) help inform the risk assessment process. Therefore in terms of future work it is essential that this new data is formulated in a rigorous manner i.e. clear hypotheses and useable practical conceptual models rather than untestable data gathering. The formulation of updated conceptual models also needs to ensure that the decision making process is closely linked to the data gathered and data analysis to ensure risk decision making is objective rather than subjective and clearly informed by the scientific process.

This process of clear conceptual models and hypothesis testing will ensure that applications for approval for cultivation of GM crops become more standardized across the EU and ensure that indicator species, method of exposure and scale and scope of the study are more uniform. Whilst this study looks specifically at worst case scenario testing, formulation of trigger values (i.e. method of exposure, scope and scale of study) and a conceptual model to help inform the decision making process it does not specifically address species selection and post market monitoring.



Appendix 1

Diet for Beet Armyworm (*Spodoptera exigua*) and *Plutella xylostella* (Diamond back moth)

Ingredients

For 1 litre:

19.8g agar
161.6g dry mix (Beet Armyworm Diet, Bioserv F9219B) (store in fridge) containing:
casein
wheat germ (stabilized)
ascorbic acid
inisitol
locust bean gum
sodium proprionate
sorbic acid
methyl paraben
aureomycin
vitamin mix, lepidopteran #722
choline chloride
salt mix, Wesson
fructose
linseed oil (raw)
safflower oil

Method:

- Weigh agar and place in a 2 litre plastic jug. Add 820ml of cold water and stir.
- Place jug in the microwave and heat on full power until agar has boiled (approximately 6 minutes). Remove jug and stir for 15 seconds. Microwave for a further minute and stir again.
- Place boiled agar into a mixer.
- Slowly add the dry mix whilst stirring the mixture.
- Once the diet is thoroughly mixed, dispense immediately.

Levels of significance between larval mass of *Spodoptera littoralis* juveniles at day 7,10 and 14 development in trials 1, 2 and 3 using Tukey post hoc analysis following oneway ANOVA.

Trial 1 larval mass day 7
Oneway ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.812	2	2.406	25.903	.000
Within Groups	3.622	39	.093		
Total	8.434	41			

Post Hoc Tests - Multiple Comparisons
Tukey HSD

(I) condition trial 1	(J) condition trial 1	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
artificial diet	Bt176	.11623000	.000	-1.0547699	-.4884258
	Isoline	.11623000	.000	-.8313752	-.2650311
Bt176	artificial diet	.11623000	.000	.4884258	1.0547699
	Isoline	.13629175	.242	-.1086539	.5554434
Isoline	artificial diet	.11623000	.000	.2650311	.8313752
	Bt176	.13629175	.242	-.5554434	.1086539

* The mean difference is significant at the .05 level.

Trial 2 larval mass day 7
Oneway ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	47.502	2	23.751	339.432	.000
Within Groups	4.898	70	.070		
Total	52.400	72			

Post Hoc Tests - Multiple Comparisons

Tukey HSD

(I) conditon trial 2	(J) conditon trial 2	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
artificial diet	Bt176	.07526285	.000	-1.7215757	-1.3611327
	isoline	.08070103	.073	-.0131047	.3733825
Bt176	artificial diet	.07526285	.000	1.3611327	1.7215757
	isoline	.07424984	.000	1.5436973	1.8992889
isoline	artificial diet	.08070103	.073	-.3733825	.0131047
	Bt176	.07424984	.000	-1.8992889	-1.5436973

* The mean difference is significant at the .05 level.

Trial 3 Larval mass day 7

Oneway ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	63.445	2	31.722	531.446	.000
Within Groups	4.536	76	.060		
Total	67.981	78			

Post Hoc Tests - Multiple Comparisons

Tukey HSD

(I) condition trial 3	(J) condition trial 3	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
aritificial diet	Bt176	.07101297	.000	-2.4732978	-2.1337877
	Isoline	.06362386	.000	-.9075067	-.6033236
Bt176	aritificial diet	.07101297	.000	2.1337877	2.4732978
	Isoline	.07052824	.000	1.3795313	1.7167239
Isoline	aritificial diet	.06362386	.000	.6033236	.9075067
	Bt176	.07052824	.000	-1.7167239	-1.3795313

* The mean difference is significant at the .05 level.

Trial 1 larval mass day 10- Artificial diet, Bt176 & Isoline

Oneway ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.370	2	3.685	33.830	.000
Within Groups	4.248	39	.109		
Total	11.619	41			

Post Hoc Tests - Multiple Comparisons

Tukey HSD

(I) condition	(J) condition	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Artificial diet	Bt176	.12587609	.000	-1.3384035	-.7250577
	Isoline	.12587609	.151	-.5463422	.0670036
Bt176	Artificial diet	.12587609	.000	.7250577	1.3384035
	Isoline	.14760280	.000	.4324554	1.1516672
Isoline	Artificial diet	.12587609	.151	-.0670036	.5463422
	Bt176	.14760280	.000	-1.1516672	-.4324554

* The mean difference is significant at the .05 level.

Trial 2 Larval mass day 10

Oneway ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.122	2	6.561	72.078	.000
Within Groups	6.827	75	.091		
Total	19.949	77			

Post Hoc Tests

Tukey HSD

(I) condition trial 2 day 10	(J) condition trial 2 day 10	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
artificial	Bt176	.08644959	.000	-1.1181689	-.7047473
	Isoline	.08709555	.360	-.3278285	.0886823
Bt176	artificial	.08644959	.000	.7047473	1.1181689
	Isoline	.07993666	.000	.6007474	.9830226
Isoline	artificial	.08709555	.360	-.0886823	.3278285
	Bt176	.07993666	.000	-.9830226	-.6007474

* The mean difference is significant at the .05 level.

Trial 3 Day 10 larval mass

Oneway ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	93.264	2	46.632	283.220	.000
Within Groups	14.160	86	.165		
Total	107.424	88			

Post Hoc Tests - Multiple Comparisons

Tukey HSD

(I) condition	(J) condition	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
artifical diet	Bt176	.10566893	.000	-2.7244056	-2.2203731
	isoline	.10566893	.000	-1.1125952	-.6085628
Bt176	artifical diet	.10566893	.000	2.2203731	2.7244056
	isoline	.10476960	.000	1.3619390	1.8616817
isoline	artifical diet	.10566893	.000	.6085628	1.1125952
	Bt176	.10476960	.000	-1.8616817	-1.3619390

* The mean difference is significant at the .05 level.

Trial 1 larval mass day 14

Oneway ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	28.473	2	14.237	347.944	.000
Within Groups	2.169	53	.041		
Total	30.642	55			

Post Hoc Tests - Multiple Comparisons

Tukey HSD

(I) condition trial 1 day 14	(J) condition trial 1 day 14	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
artificial diet	Bt176	.07518047	.000	-1.8123289	-1.4497690
	Isoline	.07209353	.000	-1.5521064	-1.2044332
Bt176	artificial diet	.07518047	.000	1.4497690	1.8123289
	Isoline	.09294024	.024	.0286757	.4768826
Isoline	artificial diet	.07209353	.000	1.2044332	1.5521064
	Bt176	.09294024	.024	-.4768826	-.0286757

* The mean difference is significant at the .05 level.

Trial 2 larval mass day 14

Oneway ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	47.020	2	23.510	363.180	.000
Within Groups	4.855	75	.065		
Total	51.875	77			

Post Hoc Tests - Multiple Comparisons

Tukey HSD

(I) condition trial 2 day 14	(J) condition trial 2 day 14	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Artificial diet	Bt176	.07290245	.000	-2.0381374	-1.6895013
	Isoline	.07344718	.000	-1.7688418	-1.4176007
Bt176	Artificial diet	.07290245	.000	1.6895013	2.0381374
	Isoline	.06741013	.000	.1094128	.4317834
Isoline	Artificial diet	.07344718	.000	1.4176007	1.7688418
	Bt176	.06741013	.000	-.4317834	-.1094128

* The mean difference is significant at the .05 level.

Trial 3 larval mass day 14

Oneway ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	88.391	2	44.195	240.551	.000
Within Groups	13.596	74	.184		
Total	101.987	76			

Post Hoc Tests - Multiple Comparisons

Tukey HSD

(I) condition trial 3 day 14	(J) condition trial 3 day 14	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Artificial diet	Bt176	.12779338	.000	-3.0879815	-2.4766779
	Isoline	.12861698	.000	-2.3147949	-1.6995516
Bt176	Artificial diet	.12779338	.000	2.4766779	3.0879815
	Isoline	.11162231	.000	.5081821	1.0421308
Isoline	Artificial diet	.12861698	.000	1.6995516	2.3147949
	Bt176	.11162231	.000	-1.0421308	-.5081821

- The mean difference is significant at the .05 level.

Appendix 3

Recipe - TES buffer – Crystal production from *E. coli*

50mM Tris	1.5g
50mM EDTA	4.6g
15% Sucrose	37.5g
Water	to 250ml

Adjust pH to 8.0 with a few 100µl of 10M NaOH

Appendix 4

Amino acid sequence similarity between Cry1Ab1 (Query) and Cry1Ab7 (subject). N-terminal sequence regions highlighted in red at line 1, amino acid sequences also highlighted in red.

Source - http://www.ncbi.nlm.nih.gov/

>gi|40278|emb|CAA31620.1| unnamed protein product [Bacillus thuringiensis]
Length=1155

Score = 2328 bits (6034), Expect = 0.0
Identities = 1149/1155 (99%), Positives = 1149/1155 (99%), Gaps = 0/1155 (0%)

```
Query  1    MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL  60
            MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL
Sbjct  1    MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL  60

Query  61   VDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEAD  120
            VDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEAD
Sbjct  61   VDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEAD  120

Query  121  PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ  180
            PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ
Sbjct  121  PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ  180

Query  181  RWGFDAATINSRYNDLTRLIGNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTV  240
            RWGFDAATINSRYNDLTRLIGNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTV
Sbjct  181  RWGFDAATINSRYNDLTRLIGNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTV  240

Query  241  LDIVSLFPNYDSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHLMDIL  300
            LDIVSLFPNYDSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHLMDIL
Sbjct  241  LDIVSLFPNYDSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHLMDIL  300

Query  301  NSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQGVYR  360
            NSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQGVYR
Sbjct  301  NSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQGVYR  360

Query  361  TLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEIPPQNNNV  420
            TLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEIPPQNNNV
Sbjct  361  TLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEIPPQNNNV  420

Query  421  PPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIPSSQITQIPLTKST  480
            PPRQGFSHRLSHVSMFRSGFSNSSVSIIR PMFSWIHRSAEFNNIIPSSQITQIPLTKST
Sbjct  421  PPRQGFSHRLSHVSMFRSGFSNSSVSIIRPPMFSWIHRSAEFNNIIPSSQITQIPLTKST  480

Query  481  NLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTS  540
            NLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQ HTS
Sbjct  481  NLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQLHTS  540

Query  541  IDGRPINQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYID  600
            IDGR INQGNFSATMSSGSNLQSGSFR VGFTTPFNFSNGSSVFTLSAHVFNSGNEVYID
Sbjct  541  IDGRIINQGNFSATMSSGSNLQSGSFR I VGFTTPFNFSNGSSVFTLSAHVFNSGNEVYID  600

Query  601  RIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHIDQVSNLVECLSDEFC  660
            RIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHIDQVSNLVECLSDEFC
Sbjct  601  RIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHIDQVSNLVECLSDEFC  660

Query  661  LDEKKELSEKVKHAKRLSDERNLLQDPNFRGINRQLDRGWRGSTDITIQGGDDVFKENYV  720
            LDEKKELSEKVKHAKRLSDERNLLQDPNFRGINRQLDRGWRGSTDITIQGGDDVFKENYV
Sbjct  661  LDEKKELSEKVKHAKRLSDERNLLQDPNFRGINRQLDRGWRGSTDITIQGGDDVFKENYV  720

Query  721  TLLGTFDECYPTYLYQKIDESKLKAYTRYQLRGYIEDSQDLEIYLIRYNAKHETVNVPGT  780
            TLLGTFDECY TYLYQKIDESKLKAYTRYQLRGYIEDSQDLEIYLIRYNAKHETVNVPGT
```

```
Sbjct 721  TLLGTFDECYLTYLYQKIDESKLKAYTRYQLRGYIEDSQDLEIYLIRYNAKHETVNVPGT 780

Query 781  GSLWPLSAPSPIGKCAHHSHHFSLDIDVGCTDLNEDLGVWVIFKIKTQDGHARLGNLEFL 840
            GSLW LSAPSPIGKCAHHSHHFSLDIDVGCTDLNEDLGVWVIFKIKTQDGHARLGNLEFL
Sbjct 781  GSLWRLSAPSPIGKCAHHSHHFSLDIDVGCTDLNEDLGVWVIFKIKTQDGHARLGNLEFL 840

Query 841  EEKPLVGEALARVKRAEKKWRDKREKLEWETNIVYKEAKESVDALFVNSQYDRLQADTNI 900
            EEKPLVGEALARVKRAEKKWRDKREKLEWETNIVYKEAKESVDALFVNSQYDRLQADTNI
Sbjct 841  EEKPLVGEALARVKRAEKKWRDKREKLEWETNIVYKEAKESVDALFVNSQYDRLQADTNI 900

Query 901  AMIHAADKRVHSIREAYLPELSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNG 960
            AMIHAADKRVHSIREAYLPELSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNG
Sbjct 901  AMIHAADKRVHSIREAYLPELSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNG 960

Query 961  LSCWNVKGHVDVEEQNNHRSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVT 1020
            LSCWNVKGHVDVEEQNNHRSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVT
Sbjct 961  LSCWNVKGHVDVEEQNNHRSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVT 1020

Query 1021 IHEIENNTDELKFSNCVEEEVYPNNTVTCNDYTATQEEYEGTYTSRNRGYDGAYESNSSV 1080
            IHEIENNTDELKFSNCVEEEVYPNNTVTCNDYTATQEEYEGTYTSRNRGYDGAYESNSSV
Sbjct 1021 IHEIENNTDELKFSNCVEEEVYPNNTVTCNDYTATQEEYEGTYTSRNRGYDGAYESNSSV 1080

Query 1081 PADYASAYEEKAYTDGRRDNPCESNRGYGDYTPLPAGYVTKELEYFPETDKVWIEIGETE 1140
            PADYASAYEEKAYTDGRRDNPCESNRGYGDYTPLPAGYVTKELEYFPETDKVWIEIGETE
Sbjct 1081 PADYASAYEEKAYTDGRRDNPCESNRGYGDYTPLPAGYVTKELEYFPETDKVWIEIGETE 1140

Query 1141 GTFIVDSVELLLMEE 1155
            GTFIVDSVELLLMEE
Sbjct 1141 GTFIVDSVELLLMEE 1155
```

Appendix 5

Levels of significance between larval mass of *Ostrinia nubalis* juveniles at 5 days development using Tukey post hoc analysis following oneway ANOVA.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	6	.000	77.176	.000
Within Groups	.000	141	.000		
Total	.000	147			

	(I) variable	(J) variable	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Tukey HSD	control	full 100	.00012810	.000	.0014370	.0022034
		full 50	.00012968	.000	.0014390	.0022148
		full 10	.00013327	.002	.0001360	.0009333
		trunc 100	.00012810	.000	.0016979	.0024643
		trunc 50	.00013140	.000	.0016053	.0023914
		trunc 10	.00013140	.000	.0012168	.0020029
	full 100	control	.00012810	.000	-.0022034	-.0014370
		full 50	.00013241	1.000	-.0003893	.0004028
		full 10	.00013592	.000	-.0016921	-.0008789
		trunc 100	.00013086	.423	-.0001305	.0006524
		trunc 50	.00013409	.837	-.0002229	.0005793
		trunc 10	.00013409	.703	-.0006114	.0001908
	full 50	control	.00012968	.000	-.0022148	-.0014390
		full 100	.00013241	1.000	-.0004028	.0003893
		full 10	.00013742	.000	-.0017033	-.0008812
		trunc 100	.00013241	.471	-.0001419	.0006502
		trunc 50	.00013560	.867	-.0002342	.0005771
		trunc 10	.00013560	.682	-.0006227	.0001886
	full 10	control	.00013327	.002	-.0009333	-.0001360
		full 100	.00013592	.000	.0008789	.0016921
		full 50	.00013742	.000	.0008812	.0017033
		trunc 100	.00013592	.000	.0011398	.0019530
		trunc 50	.00013904	.000	.0010478	.0018796
		trunc 10	.00013904	.000	.0006593	.0014911
	trunc 100	control	.00012810	.000	-.0024643	-.0016979
		full 100	.00013086	.423	-.0006524	.0001305
		full 50	.00013241	.471	-.0006502	.0001419
		full 10	.00013592	.000	-.0019530	-.0011398
		trunc 50	.00013409	.996	-.0004838	.0003184
		trunc 10	.00013409	.010	-.0008723	-.0000701
	trunc 50	control	.00013140	.000	-.0023914	-.0016053
		full 100	.00013409	.837	-.0005793	.0002229
		full 50	.00013560	.867	-.0005771	.0002342
		full 10	.00013904	.000	-.0018796	-.0010478

		trunc 100	.00013409	.996	-.0003184	.0004838
		trunc 10	.00013724	.077	-.0007990	.0000220
	trunc 10	control	.00013140	.000	-.0020029	-.0012168
		full 100	.00013409	.703	-.0001908	.0006114
		full 50	.00013560	.682	-.0001886	.0006227
		full 10	.00013904	.000	-.0014911	-.0006593
		trunc 100	.00013409	.010	.0000701	.0008723
		trunc 50	.00013724	.077	-.0000220	.0007990

Legend

full 100 – Full length uncleaved Cry1Ab toxin at a concentration of 100 ng g^{-1} when incorporated into artificial diet.

full 100 – Full length uncleaved Cry1Ab toxin at a concentration of 50 ng g^{-1} when incorporated into artificial diet.

full 100 – Full length uncleaved Cry1Ab toxin at a concentration of 10 ng g^{-1} when incorporated into artificial diet.

trunc 100 – Truncated cleaved Cry1Ab toxin at a concentration of 100 ng g^{-1} when incorporated into artificial diet.

trunc 50 – Truncated cleaved Cry1Ab toxin at a concentration of 50 ng g^{-1} when incorporated into artificial diet.

trunc 10 – Truncated cleaved Cry1Ab toxin at a concentration of 10 ng g^{-1} when incorporated into artificial diet.

control – Control diet containing no Cry1Ab toxin.

Appendix 6

Levels of significance between larval mass of *Ostrinia nubalis* juveniles at 12 days development using Tukey post hoc analysis following oneway ANOVA.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.061	4	.015	81.921	.000
Within Groups	.016	88	.000		
Total	.078	92			

Multiple Comparisons

Tukey HSD

(I) variable	(J) variable	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
control	100 ng uncleaved	.00478059	.000	.0567665	.0833957
	50 ng uncleaved	.00469839	.000	.0568046	.0829760
	10 ng uncleaved	.00434599	.000	.0196845	.0438929
	10 ng truncated	.00434599	.000	.0419599	.0661684
100 ng uncleaved	control	.00478059	.000	-.0833957	-.0567665
	50 ng uncleaved	.00491452	1.000	-.0138785	.0134968
	10 ng uncleaved	.00457878	.000	-.0510450	-.0255398
	10 ng truncated	.00457878	.006	-.0287695	-.0032644
50 ng uncleaved	control	.00469839	.000	-.0829760	-.0568046
	100 ng uncleaved	.00491452	1.000	-.0134968	.0138785
	10 ng uncleaved	.00449290	.000	-.0506150	-.0255882
	10 ng truncated	.00449290	.006	-.0283395	-.0033128
10 ng uncleaved	control	.00434599	.000	-.0438929	-.0196845
	100 ng uncleaved	.00457878	.000	.0255398	.0510450
	50 ng uncleaved	.00449290	.000	.0255882	.0506150
	10 ng truncated	.00412297	.000	.0107924	.0337585
10 ng truncated	control	.00434599	.000	-.0661684	-.0419599
	100 ng uncleaved	.00457878	.006	.0032644	.0287695
	50 ng uncleaved	.00449290	.006	.0033128	.0283395
	10 ng uncleaved	.00412297	.000	-.0337585	-.0107924

* The mean difference is significant at the .05 level.

Legend

control – Control diet containing no Cry1Ab toxin.

100ng uncleaved – Full length uncleaved Cry1Ab toxin at a concentration of 100 ng g^{-1} when incorporated into artificial diet.

50 ng uncleaved – Full length uncleaved Cry1Ab toxin at a concentration of 50 ng g^{-1} when incorporated into artificial diet.

10 ng uncleaved – Full length uncleaved Cry1Ab toxin at a concentration of 10 ng g^{-1} when incorporated into artificial diet.

10 ng truncated – Truncated cleaved Cry1Ab toxin at a concentration of 10 ng g^{-1} when incorporated into artificial diet.

Appendix 7

Results of one-way ANOVA following logarithmic transformation of *S. littoralis* larval mass prior to experimentation in *C. marginiventris* attack rate choice tests.

(a) comparison of larval mass of hosts raised on Bt176 transgenic maize and its isoline;

Replicate 1

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	1	.001	.210	.652
Within Groups	.086	18	.005		
Total	.087	19			

Replicate 2

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.002	1	.002	.959	.340
Within Groups	.042	18	.002		
Total	.044	19			

Replicate 3

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.004	1	.004	.706	.412
Within Groups	.103	18	.006		
Total	.107	19			

Replicate 4

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	1	.000	.000	.988
Within Groups	.055	18	.003		
Total	.055	19			

Replicate 5

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.003	1	.003	.554	.466
Within Groups	.101	18	.006		
Total	.104	19			

Replicate 6

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	1	.000	.001	.970
Within Groups	.082	18	.005		
Total	.082	19			

Replicate 7

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.002	1	.002	.443	.514
Within Groups	.091	18	.005		
Total	.093	19			

Replicate 8

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	1	.001	.134	.719
Within Groups	.084	18	.005		
Total	.085	19			

Replicate 9

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	1	.001	.748	.398
Within Groups	.031	18	.002		
Total	.032	19			

Replicate 10

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	1	.000	.032	.860
Within Groups	.061	18	.003		
Total	.061	19			

b) comparison of larval mass of *S. littoralis* host raised on control artificial marked for visual identification or unmarked;

Replicate 1

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.008	1	.008	2.477	.133
Within Groups	.062	18	.003		
Total	.070	19			

Replicate 2

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	1	.001	.087	.772
Within Groups	.122	18	.007		
Total	.123	19			

Replicate 3

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	1	.001	.330	.573
Within Groups	.063	18	.004		
Total	.064	19			

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