The indirect effect of Cry 1Ab protein expressed in Bt maize, on the biology of *Chrysoperla pudica* (Neuroptera: Chrysopidae)

JF Warren

21660557

Dissertation submitted in fulfilment of the requirements for the degree *Magister Scientiae* in Environmental Sciences at the Potchefstroom Campus of the North-West University

Supervisor: Prof J van den Berg

Co-supervisor: Prof H du Plessis

May 2014

Acknowledgements

I would like to thank my Heavenly Father who gave me this opportunity to study and appreciate His creation.

I would also like to thank my study leaders Prof. Johnnie van den Berg and Prof. Hannalene du Plessis for all their guidance, patience and advice during this year, without you this dissertation would not have been possible.

Then a very special thanks to my loving mother Tokkie Warren, father Stanley Warren, sister Nicolene de Klerk, friends Louis Lourens and Mabel Schoeman for assisting me with the time consuming field work, and *Chrysoperla pudica* feeding experiment, also all the emotional support and advice throughout the year. Love you all!

Mom and dad thank you so much for collecting the aphids; you were always just a phone call away.

Abstract

Genetically modified (GM) maize was developed mainly to control lepidopteran pests such as the maize stem borer (*Busseola fusca*) (Lepidoptera: Noctuidae). Since the first commercialization of GM crops with insecticidal properties, possible non-target effects such as the effect at the third trophic level on important predators for example lacewing species (*Chrysoperla* spp.) have been of concern. Contradicting results were reported in previous studies with regard to the effect of Cry 1Ab protein produced by Bt maize on the performance of lacewings. Some studies found that Bt proteins had no effect while others reported that *C. carnea* performed poorly if they consumed prey that consumed Cry 1Ab protein. In South Africa one of the most common chrysopid species in maize ecosystems is *Chrysoperla pudica* (Navás) (Neuroptera: Chrysopidae). Evolution of Bt resistant pests, such as *B. fusca* in South Africa facilitates a new pathway for exposure of predators to healthy prey that consumes Cry 1Ab proteins. The aims of this study was to determine the effect of the Cry 1Ab protein expressed in Bt maize on a non-target organism's (*C. pudica*) biology via indirect exposure, and to determine the concentration of Cry 1Ab protein in the plant, prey and predator. *Chrysoperla pudica* larvae were indirectly exposed to the Bt-toxin through healthy Bt-maize feeding prey (*B. fusca* larvae) in two feeding experiments and lacewing survival and life history parameters recorded. Bt had a limited effect on some parameters that were evaluated. The larval and pupal periods of *C. pudica* larvae that were exposed to the Bt-toxin had a significant difference from that of the control treatment. The Bt-toxin had a significant effect on fecundity, fertility and malformation after emergence of *C. pudica* adults of which larvae fed only on Bt resistant *B. fusca* larvae, but not on the mortality rate. Cry 1Ab concentration was the highest in the plant, followed by the prey and lacewing larvae. This study showed that the Cry 1Ab protein had a slight adverse effect only on certain life parameters of *C. pudica*, and that Cry 1Ab protein was hardly detectable in *C. pudica* larvae. However, since this study represented a worst-case scenario where diverse prey was not available, insignificant effects is expected under field conditions where prey is diverse.

Key words: Bt maize, *Busseola fusca*, *Chrysoperla pudica*, *lacewings*, natural enemies, risk assessment, third trophic level.

Uittreksel

Geneties-gemodifiseerde (GM) mielies is hoofsaaklik ontwikkel om Lepidopteraplae soos die mieliestamboorder (*Busseola fusca*) (Lepidoptera: Noctuidae) te beheer. Die moontlike effek van GM gewasse op voordelige nie-teiken organismes soos goudogies (*Chrysoperla* spp.) was van groot kommer vanaf die eerste kommersialisering van GM-gewasse met insekdodende eienskappe. Vorige studies het teenstrydige resultate opgelewer met betrekking tot die effek van Cry 1Ab proteïne op die lewensparameters van goudogies. Sommige studies toon dat Cry 1Ab proteïne geen effek het nie terwyl ander bevind dat dit wel ‘n effek op *C. carnea* het. In Suid-Afrika is een van die mees algemene chrysopid spesies in mielie-ekostelsels, *Chrysoperla pudica* (Neuroptera: Chrysopidae). Evolusie van Bt-weerstandbiedende plae soos *B. fusca* fasiliteer ‘n nuwe weg van blootstelling van voordelige insekte aan die Bt-toksien, wat mag lei tot nadelige gevolge op hierdie organismes. Die doel van hierdie studie was om die effek van Cry 1Ab proteïene, wat uitgedruk word in Bt-mielies, op *C. pudica* se biologie te bepaal, en om die konsentrasie van die Bt-toksien in die plant, prooi en predator te bepaal. *Chrysoperla pudica* larwes was indirek blootgestel aan die Bt-toksien deur middel van gesonde Bt-mielie-etende prooi (*B. fusca*) tydens twee voedingeksperimente waartydens die lewensiklusparameters en oorlewing van *C. pudica* bepaal is. Bt het ‘n geringe effek op enkele parameters getoon. Die *C. pudica* larwes wat aan die toksien blootgestel was, het betekenisvolle verskille op die larf- en pupaperiode gehad. Die Bt-toksien het ‘n betekenisvolle invloed gehad op die getal eiers, vrugbaarheid asook misformdheid van die volwassenes. Die Bt-toksien het egter nie die mortaliteit van *C. pudica* beïnvloed nie. Bt-konsentrasies was die hoogste in die plant, gevolg deur die prooi en goudogie-larwes. Resultate dui daarop dat Cry 1Ab proteïene slegs betekenisvolle effekte het op sekere lewensparameters van *C. pudica* en dat die proteïene skaars teenwoordig was in *C. pudica* larwes. Aangesien hierdie studie die ergste scenario verteenwoordig, waar geen diverse prooi beskikbaar was nie, word daar weglaatbaar-klein effekte onder veldtoestande verwag waar meer diverse prooi beskikbaar is.

Sleutelwoorde: Bt mielies, *Busseola fusca*, *Chrysoperla pudica*, derde trofiese vlak, goudogies, natuurlike vyande, risiko assessering.

Table of contents

Chapter 1

Introduction and literature overview

1.1 Bt maize in South Africa, history and impact

The most economically important crop in South-Africa is maize (Kfir *et al*., 2002). Sustainable production, taking into account all aspects of integrated pest management is therefore important (Kfir *et al*., 2002). *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) (Fig. 1.1), is one of the most important pests of maize and may cause between 10 and 100% yield loss, depending on planting date (Kfir *et al*., 2002).

Figure 1.1: *Busseola fusca* larvae inside a maize stem (Akol, 2011).

Genetically modified (GM) crops are modified through the use of genetic engineering whereby the DNA of the plant is manipulated and modified (Altieri, 2000). Some bacteria species can be used as gene-donors to donate a desirable trait to crop plants, for example a single insect-resistance gene from the bacterium *Bacillus thuringiensis* (Fig. 1.2) can be transferred to maize to enable the plant to produce Cry 1Ab proteins that kills lepidopteran species that feed on the crop (Tabashnik *et*

al., 2009). *Bacillus thuringiensis* is a gram-positive, aerobic, sporulating bacterium which synthesises insecticidal crystalline proteins during sporulation (Ranjekar *et al*., 2003). These proteins (toxins) are effective against different groups of insects and are not known to be toxic to mammals and other organisms, in its bacterial form as an insecticide spray, it is therefore accepted worldwide as an eco-friendly bio-pesticide (Ranjekar *et al*., 2003).

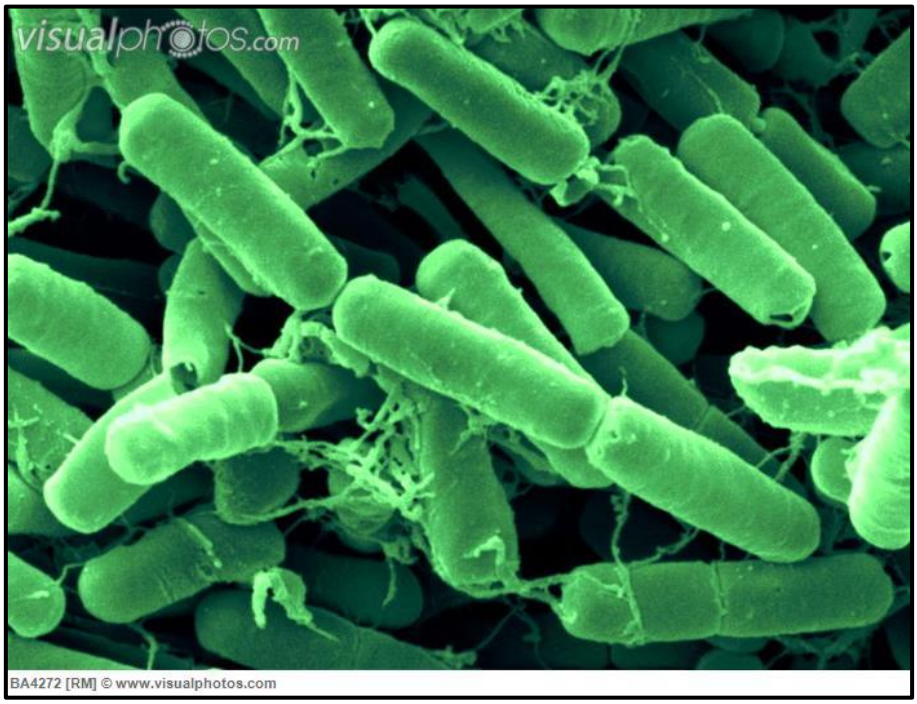

Figure 1.2: A scanning electron micrograph of the soil bacterium *Bacillus thuringiensis* (Anon, 2011).

GM crops that express novel traits aim to ensure a reduction in crop losses due to biotic and abiotic stresses such as drought, insect pests, weeds and pathogen infestations (Altieri, 2000). Because of the growing human population it is important to develop strategies to produce more food on limited agricultural land (Altieri, 2000). Novel crop genotypes that are modified through molecular biology and genetic engineering could ensure safe and sustainable agriculture for the demanding world population (Ranjekar *et al*., 2003). The main goals of GM crops are to directly benefit the producer by aiming to increase productivity per hectare, reduce production costs and chemical usage as well as to improve grower health (Anon, 2011).

Many farmers have turned to growing these modified crops because they are less expensive to maintain and easier to grow (Anon, 2011). Global GM crop planting has increased (Fig. 1.3) 100-fold in hectares from 1.7 million hectares in 1996 to 170 million hectares in 2012, grown by more than 17.3 million farmers in 29 countries (Tribe, 2012). South Africa is ranked 9th in the world with 2.3 million hectares consisting of three major crop species i.e. maize, cotton and soybeans (James, 2011).

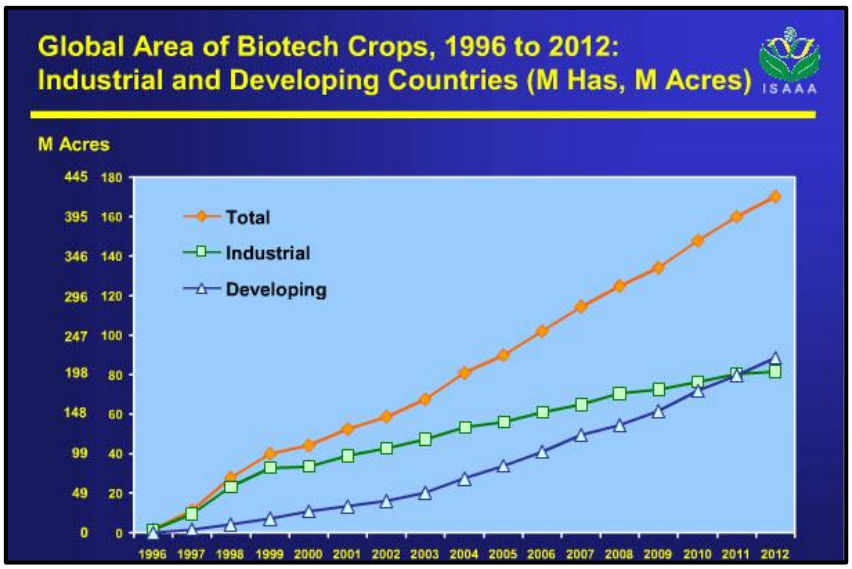

Figure 1.3: A graphical illustration of the global area planted with GM crops since the first commercialization thereof (Tribe, 2012).

Sixty percent of the world's population live within the 29 countries that adopted GM crops (James, 2011). South Africa, China, India, Brazil and Argentina are the five leading developing countries that collectively plant up to 52% of the total global hectares of GM crops, during 2012 (Tribe, 2012). Africa, a continent of developing countries, which includes South Africa, Burkina, Faso and Egypt account for 2.5 million hectares of the global area planted to GM crops (James, 2011).

Bt cotton was approved for the first time during 1997 in South Africa and Bt maize was approved during the following year (Gouse *et al*., 2005). Only 50 000 ha of Bt crops were planted during the 1999/2000 cropping season which accounted for only 3% of the total maize production area (Gouse *et al*., 2005; James, 2011). Between

2000 and 2011, the cropping area increased considerably, up to 2.3 million ha (Gouse *et al*., 2005; James, 2011).

1.2 GM crops and traits in South Africa

The three GM crops planted in South Africa are cotton, soybean and maize (James, 2011).

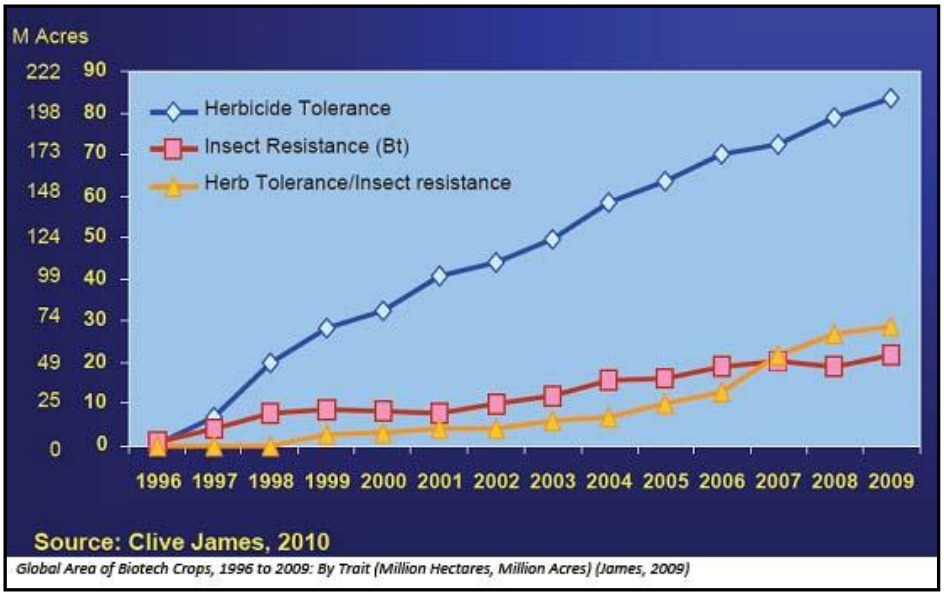

Figure 1.4: The number of hectares planted worldwide with genetically modified crops, with the three different traits since the first commercialization thereof (James, 2011).

There are two GM traits available in South Africa namely insecticidal resistance and herbicide tolerance (James, 2011). These two traits are also combined to form a stacked trait, with both herbicide tolerance and insecticidal properties. The cultivation of crops with stacked traits has increased sharply since 2006 (Fig. 1.4) (James, 2011).

1.3 Advantages and disadvantages of GM maize

The main advantage of GM crops with insecticidal traits is to reduce the use of insecticides that can have an impact on human health as well as the environment (Kumar *et al*., 2008). Reduced use of insecticides may lead to a more stable ecosystem because natural-enemies are not known to be affected negatively by GM

crops, which have been found to reduce target pest populations to below the economic injury level (EIL) (Kumar *et al*., 2008).

1.3.1 The advantages of Bt crops with insecticidal properties

Bt-protected crops, particularly maize and cotton have demonstrated significant benefits since their introduction into world agriculture during the 1995/1996 season (Betz *et al*., 2000). For example Bt-protected maize and cotton provide higher economic values and crop yields to producers (Betz *et al*., 2000).

The reduction in pest management costs, greater crop production flexibility, increased crop yield, highly effective pest control, reduced levels of fungal toxins, a reduction in the risk of chemical misuse and a reduction in ineffective timing of applications of chemicals (Betz *et al*., 2000; Kruger *et al*., 2009; Tabashnik *et al*., 2013). There is also a reduced risk in poisoning workers and it is less harmful to the environment (Betz *et al*., 2000). Bt crops do not require any special equipment and can therefore be effective on farms of all sizes (Meeusen and Warren, 1989). Lastly the control of pests is no longer affected by weather, the crop is protected continuously and scouting for pests may no longer be needed (Meeusen and Warren, 1989).

1.3.2 The disadvantages of Bt crops with insecticidal properties

The main threat to the continued success of Bt crops is the evolution of resistance by pests. Arthropods possess the remarkable ability to adapt to insecticides and other control tactics (Tabashnik *et al*., 2013). Resistance to Bt crops developed by target pests can unleash potential negative effects, affecting ecological processes and non-target organisms such as predators and parasitoids (Tabashnik *et al*., 2013). For example several lepidopteran species have been reported to develop resistance to the Bt toxin in both laboratory and field test studies (Tabashnik *et al*., 2013). The above mentioned suggests that major resistance problems are likely to develop in Bt crops, which, through the continuous expression of the toxin in plants creates strong selection pressure (Tabashnik *et al*., 2013).

Bt crops can also starve natural enemies as they need a small amount of prey to survive in the agro-ecosystem (Tabashnik *et al*., 1998). Some predators could theoretically thrive on dead or dying prey and therefore parasites and parasitoids would be most affected because they are more dependent on living hosts for survival and development (Tabashnik *et al*., 1998). Natural enemies could also be affected directly through inter-trophic level interactions (Tabashnik *et al*., 1998). Aphids for example, are capable of ingesting the toxin from Bt crops and, when preyed upon by coccinellids larvae or beetles, the latter are indirectly exposed to the toxin. This may affect the reproduction and longevity of these beneficial beetles (Birch *et al*., 1997). The potential of Bt toxins moving through food chains may pose serious implications for natural bio-control in agro-ecosystems (Hilbeck *et al*., 2012).

Since the first commercialization of GM crops with insecticidal properties, the possible effect on non-target organisms as well as resistance development of target organisms has been of great concern (Dutton *et al*., 2002).

When Bt was applied as a spray formulation (biopesticide), only one insect pest species developed resistance, *i.e.* the diamondback moth (*Plutella xylostella* L.) (Lepidoptera: Plutellidae) (Ferré and Rie, 2002). The first report of field resistance to Bt maize was made during 2006, in Christiana (Northern Cape province), South Africa when the African stem borer *B. fusca* was reported to survive on Bt maize under field conditions (Van Rensburg, 2007).

In 2007 only three cases of resistance had been detected in the world. These were *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) to Bt cotton producing Cry 1Ac, *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) to Bt maize producing Cry 1F and *B. fusca* to Bt maize producing Cry 1Ab (Fig. 1.5) (Tabashnik, 2008). *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) also became resistant in 2008 and *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae) developed resistance in 2011 (Fig. 1.5) (Tabashnik *et al*., 2013). Thus there are five lepidopteran species that have developed resistance against transgenic crops up and till 2011 (Fig. 1.5) (Tabashnik *et al*., 2013).

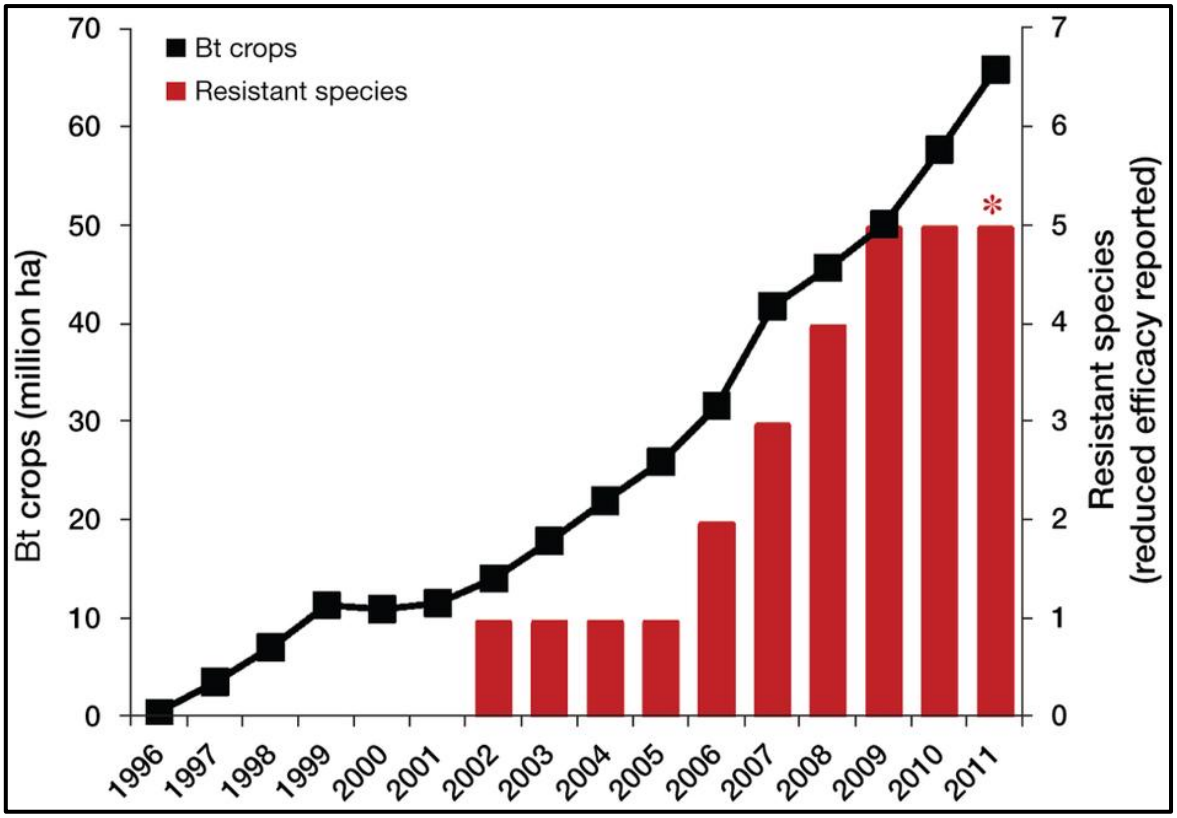

Figure 1.5: Planting of Bt crops globally each year and cumulative number of insect species with field-evolved resistance and reduced efficacy reported (Tabashnik *et al*., 2013).

Initially *Bt kurstaki* Bt-strains were effective only against lepidopteran insect pests but with the discovery of new Bt-strains such as *Bt tenebrionis*, various other pests such as coleopterans can also be controlled (Ranjekar *et al*., 2003). Other insecticidal agents that have been developed to control pest species include proteins known as vegetative insecticidal proteins (VIPs), proteinase inhibitors (PIs), plant lectins, α-amylase inhibitors (α-AIs), chitin and insecticidal viruses (Ranjekar *et al*., 2003).

1.4 Different pathways of exposure of organisms to Bt protein

There are various exposure pathways through which target and beneficial organisms may be exposed to Bt proteins. The specific exposure pathway affects the non-target organisms' level of exposure to these insecticidal proteins and affects the potential effects that it may have on non-target organisms (Schuler, 2004).

Four different pathways will be explained below:

Exposure pathway 1: Direct exposure happens through the ingestion of the insecticidal protein, for example when a *B. fusca* larva directly feeds on a Bt maize plant (Fig. 1.6). This specific pathway may be affected by the time of protein expression in the plant as well as how the herbivore ingests the plant tissue (Dutton *et al*., 2002). Herbivores that are phloem-sap feeders such as aphids will not ingest the insecticidal protein, since the protein does not occur in the phloem (Raps *et al*., 2001).

Figure 1.6: *Busseola fusca* larva feeding directly on a maize stem (Khan, 2007).

Exposure pathway 2: Exposure to insecticidal proteins may also be indirect through consumption of plant tissues that are transported by wind and/or water (Losey *et al*., 1999). Maize pollen that is transported by wind can expose other arthropods to insecticidal proteins within or beyond the crop border (Losey *et al*., 1999). For example the monarch butterfly caterpillar, *Danaus plexippus* (Linnaeus) (Lepidoptera: Danaidae) can be affected by Bt maize pollen which can drift onto its host plant the tropical milkweed (*Asclepias syriaca*) (Fig. 1.7) (Losey *et al*., 1999). This will be discussed in more detail later on (1.6.1).

Figure 1.7: A monarch caterpillar feeding on a tropical milkweed plant dusted with Bt maize pollen (Losey *et al*., 1999).

Exposure pathway 3: Certain plants that express lectins transport the insecticidal proteins inside the phloem, the opposite of what happens in Bt crops (Shi *et al*., 1994). These insecticidal proteins will therefore be present in the honeydew of sap-feeding Hemiptera (Fig. 1.8) (Kanrar *et al*., 2002). These herbivores do then contain the insecticidal proteins which could be detrimental to their predators if they were susceptible to the particular proteins (Kanrar *et al*., 2002).

Figure 1.8: Aphids feeding on cabbage leaves (Glen, 2012).

Exposure pathway 4: This is the most important pathway of exposure for entomophagous arthropods. The prey or host is usually an herbivore that feeds on the GM plant. For example, *B. fusca* larva feeds on a Bt maize plant after which it may be consumed by a *Chrysoperla pudica* (Neuroptera: Chrysopidae) larva, similar to an aphid being consumed by a chrysopid larvae (Fig. 1.9) or other entomophagous species. In either case the level of exposure is highly variable and difficult to predict. This is also known as third trophic level exposure. The study presented in this dissertation addresses the exposure of *C. pudica* to Cry 1Ab proteins through this pathway.

Figure 1.9: *Chrysoperla pudica* larva eating an aphid.

Natural enemies such as predatory arthropods can therefore ingest the Cry 1Ab protein either through feeding on herbivore species that have fed on Bt plant tissue (inter-trophically), feeding directly on the plant parts that contains the protein or via the environment (De la Poza *et al*., 2005).

1.5 Effects of GM crops on non-target species

The potential impact of GM crops on biodiversity is a topic of great interest (Carpenter, 2011). In both natural and agricultural environments, GM crops and their transgene products may come into contact with hundreds of non-target species with

important ecosystem functions (Carpenter, 2011). These non-target organisms may be affected in a negative or positive manner while others may be unaffected.

If a target or non-target organism is eliminated in a specific guild or functional group, it may change the guild structure which can then lead to the development of secondary pests (Van Wyk *et al*., 2007). It is therefore important to assess the potential risks that GM crops may hold to non-target organisms (Van Wyk *et al*., 2007). Target pests may be affected directly if they are susceptible to the transgenic Bt crop, whereby natural enemies of the target pest may be indirectly affected by the modified crop when they feed on these intoxicated host species that fed directly on the modified crop plant (Schuler, 2004). The degree of the indirect effect can be influenced by prey quality, prey behaviour, prey availability and crop management practices (Schuler, 2004).

The Cry 1Ab proteins are produced in Bt plants throughout the entire growing season and therefore target and non-target arthropods have the opportunity to encounter Cry 1Ab proteins on a continuous basis in high concentrations (Sisterson *et al*., 2004). For example natural enemies such as lacewings (*Chrysoperla* spp.) can directly be affected through intertrophic level effects of the Bt toxin, through pathway 4 (Altieri, 2000). Another possible direct effect of Bt toxins on natural enemies could be in an indirect form for example, if the natural enemy is species-specific on the target organism they can starve if the target pest is wiped-out as a result of the Bt toxin (Altieri, 2000). However this has not proven to be the case under field or laboratory conditions.

A meta-analysis conducted by Wolfenbarger *et al*. (2008) showed no uniform negative or positive effects, when comparing Bt plants to their non-transgenic counterparts on different ecological functional groups (Wolfenbarger *et al*., 2008). The only functional guild that was slightly lower in abundance in Bt cotton was the predators, of which an overall moderate reduction in two predaceous families (Nabidae and Coccinellidae) was detected (Wolfenbarger *et al*., 2008). Aphids (a common prey for coccinellids) showed no change in abundance, therefore a reduction in common prey probably does not explain the decrease of these predators (Wolfenbarger *et al*., 2008). Reductions in target prey could be a contributing factor

as well as sub-lethal effects of feeding on Bt pollen or other prey abundance or quality in Bt crops (Naranjo, 2005; Wolfenbarger *et al*., 2008). No changes in the abundance of *Chrysoperla* spp. could be detected in Bt cotton or Bt maize fields (Wolfenbarger *et al*., 2008). In other words Bt crops favoured the abundance of non-target arthropods relative to insecticide-treated controls, especially within the predator, mixed, and herbivore functional guilds (Wolfenbarger *et al*., 2008).

Many studies involving the effect of the Cry 1Ab proteins have been done on different organisms, including arthropods, birds, fish and even mammals. In the above mentioned studies the test subjects were exposed directly or indirectly to the Bt protein (Clark *et al*., 2005). There is not much reason to expect non-target toxicity in these organisms and it has been used in direct testing with no adverse effects (Clark *et al*., 2005).

1.5.1 Effect of the Bt toxin on the monarch butterfly

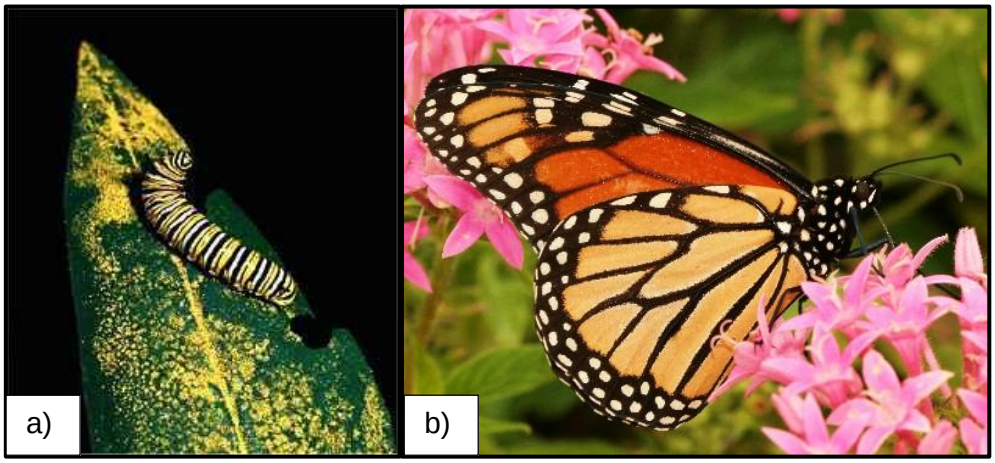

Figure 1.10 a) A monarch caterpillar feeding on milkweed dusted with Bt maize pollen (Losey *et al*., 1999), b) An adult monarch butterfly (Anon, 2010).

The monarch butterfly (*D. plexippus*) (Fig. 1.10b) is another example of a non-target organism that could be affected by Bt crops (Losey *et al.* 1999). Under laboratory conditions, when the monarch larvae is fed milkweed (*Asclepias curassavica*), its primary host, that is dusted with Bt-pollen, it can suffer adverse effects such as high mortality, impaired feeding and impaired growth (Losey *et al.* 1999). This resulted in huge public concern over the butterfly as well as other negative effects of GM crops

(Losey *et al.,* 1999). Research did, however, further show that the monarch butterfly has to be exposed to Bt pollen levels greater than 1000 grains/cm^2 to have a toxic effect (Sears *et al*., 2001). Monarch butterfly caterpillars (Fig. 1.10a) were present on milkweed leaves during pollen shed of maize but on an average, the pollen load on leaves were very low with only ±170 pollen grains/cm^2 (Sears *et al*., 2001).

1.5.2 Non-target effects and fate of the Bt protein in the soil

Plants have a major influence on communities of micro- and other organisms in the soil, which are fundamental to many functions of soil systems such as decomposition of waste, mobilization of nutrients and nitrogen cycling (Carpenter, 2011). It has been reported that these toxins may cause suppression in fungal populations, alter soil enzymatic activity, increase carbon turnover, reduce protozoan populations and cause displacement of indigenous soil populations (Naseby and Lynch, 1998). Bt toxins can be present in the soil for 2-3 months (Palm *et al*., 1996). The above mentioned can happen when a Bt plant decomposes and the Bt toxins incorporate into the soil (Palm *et al*., 1996). In the soil, Bt toxins can resist degradation by binding to the humic acids, organomineral complexes and clay particles, while their toxic activity is maintained (Donnegan *et al*., 1995).

Roots of Bt crops can also exude active Bt toxins into the soil which can also filter into the soil water thus enhancing the Bt toxin concentration in aquatic ecosystems (O'Callaghan *et al.,* 2005). Earthworms, bacteria, fungi, protozoans and nematodes were however not significantly affected by the above mentioned Bt-toxin that exudes from the roots of Bt crops (O'Callaghan *et al.,* 2005). In soil the Bt toxin is more readily degraded than in aquatic ecosystems (Douville *et al.,* 2008). Soil that contains Bt toxin has an effect on the microbial community composition, whereby there is an enhancement of soil respiration observed in the first 72 hours (Mulder *et al.,* 2006). Only short Bt-induced ecological shifts occur in the microbial communities of soils containing Bt toxins (Mulder *et al.,* 2006). The introduction of transgenic maize influences diversity, abundance, and ecosystem functioning of the bulk soil bacteria (Mulder *et al.,* 2006). Bt-induced adaptive radiation may occur rapidly in the microbial communities below maize fields (Mulder *et al.,* 2006).

1.5.3 Non-target effects of Bt proteins in aquatic ecosystems

Aquatic ecosystems can obtain the Bt toxin either through the decomposition of Bt crop residues that end up in water and proteins that leach into ground water or through the dispersion of Bt pollen by means of wind (Rosi-Marshall *et al.,* 2007). The insecticidal protein can then be dispersed through water currents and can be ingested by aquatic organisms (Rosi-Marshall *et al.,* 2007). Laboratory trials suggested that the consumption of Bt maize detritus may affect stream-dwelling invertebrates (Rosi-Marshall *et al.,* 2007). Another way for overland transport of maize detritus to stream channels via wind and entrainment in surface runoff from heavy precipitation, is through the common agricultural practice of leaving crop residues on fields postharvest (i.e., conservation tillage) (Tank *et al*., 2010). Large accumulations of maize detritus along the riparian zone and within stream channels in numerous streams several months after harvest suggests the high potential for Cry1Ab protein to occur in entrained detritus and to be dissolved in stream water (Tank *et al*., 2010).

Figure 1.11: *Daphnia magna* adult (Clare, 2002).

The water flea, *Daphnia magna* (Straus) (Cladocera: Daphniidae) (Fig. 1.11) is an indicator species that is often used in toxicological- and ecotoxicological studies (Bøhn *et al.,* 2008). Because of *D. magna*'s asexual reproductive strategy, minimal genetic variability and rapid lifecycle it is an ideal organism to test the effects of Bt toxins within the aquatic ecosystem (Bøhn *et al.,* 2008). When *D. magna* was fed on

dried Bt maize kernels that were grinded, it showed significant long-term effects such as delayed maturity, reduced fecundity, reached sexual maturity earlier, higher mortality rate and reduced body weight (Bøhn *et al.,* 2008).

1.5.4 Effect of the Bt toxin non-target organisms

Previous studies showed contradicting results with regard to the effect of the Bt toxin on the biology of the predatory lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae). Some studies suggest that *C. carnea* is not affected when it indirectly or directly ingested Cry 1Ab protein (Dutton *et al*., 2003). Other studies show that when *C. carnea* fed on larvae that were reared on Cry 1Ab expressing Bt maize, they suffered delayed development and had a reduced survival rate. It was however reported that during a choice test *C. carnea* preferred to eat *Rhopalosiphum padi* (Koch) (Homoptera: Aphidae) that had not consumed Bt (Meier and Hilbeck, 2001; Dutton *et al*., 2002). Another choice test was done between *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and *R. padi* and *C. carnea* preferred the aphids (*R. padi*) whether it had consumed Bt or not (Meier and Hilbeck, 2001). This will be discussed in more detail later on (1.6.2). However, this study was only a bitrophic feeding trial, which is not realistic in the field.

1.6 Previous research on the indirect effects of the Bt toxin on non-target organisms

Certain Cry proteins are specific for certain pests, for example Cry 1Ab protein is specific for lepidopteran pests (Tabashnik *et al*., 2009). Cry 1F and Cry 1Ac was also developed to control lepidopteran pests (Tabashnik *et al*., 2009). Cry 3Bb1 and Cry 34Ab1 +Cry 35Ab1 was developed to control coleopteran pests (Tabashnik *et al*., 2009).

Cry 1Ab proteins could have adverse effects on beetles and lacewings even if it is specific to lepidopterans, and can therefore affect the third trophic level via the food chain (Dutton *et al*., 2002). Previous research on the indirect effect of the Bt toxin on beneficial insects such as the Two spotted ladybeetle (*Adalia bipunctata*) (Linnaeus)

(Coleoptera: Coccinellidae) (Fig. 1.12) and predatory lacewing (*C. carnea*) show contradicting results. These studies are reviewed below (1.6.1 and 1.6.2).

1.6.1 Two spotted ladybeetle (Adalia bipunctata)

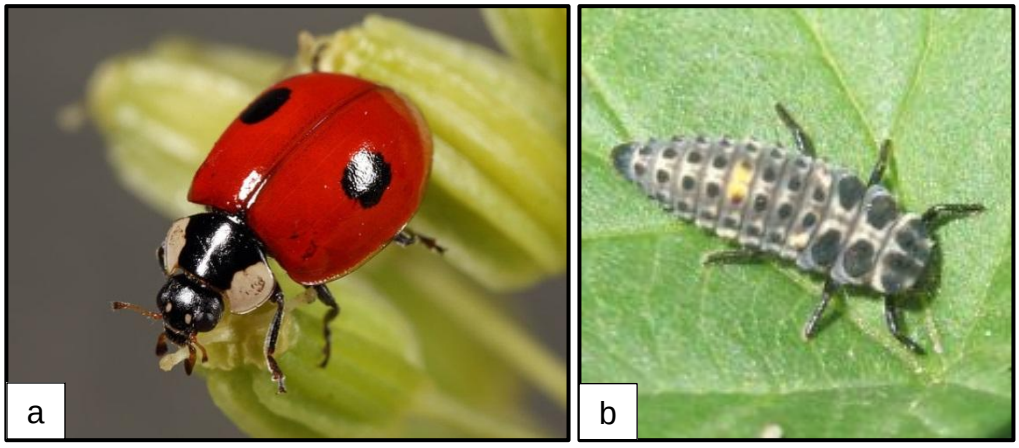

Figure 1.12: *Adalia bipunctata* a) adult (De Wilder, 2001) b) larva (Edkins, 2002).

Research showing adverse effects of Cry 1Ab protein

The microbial Bt toxin, Cry 1Ab, had lethal effects on the two-spotted lady beetle *A. bipunctata* (Fig. 1.12) (Schmidt *et al*., 2009). *Adalia bipunctata* died at a significantly higher rate, when reared on meal moth eggs (*Ephestia kuehniella*) (Zeller) (Lepidoptera: Pyralidae) coated with a solution containing purified Bt toxins (Schmidt *et al*., 2009; Hilbeck *et al*., 2012). However, this study was only a bitrophic feeding trial, which is not realistic in the field.

Research showing no adverse effects of Cry 1Ab protein

A laboratory study on the toxicity of Cry 1Ab protein to *A. bipunctata* (Fig. 1.12), which also focussed on the importance of the study design, found no adverse effects (Álvarez-Alfageme *et al*., 2011). A tritrophic study was conducted using spider mites, that fed on Bt maize, as a food source for *A. bipunctata* to make the exposure levels more realistic (Álvarez-Alfageme *et al*., 2011; Romeis *et al*., 2012). Spider mites (*Tetranychus urticae*) (Koch) (Trombidiformes: Tetranychidae) was used as a food source since they are not affected when feeding on Cry 1Ab expressing maize, thus they are healthy prey (Álvarez-Alfageme *et al*., 2011). The Cry 1Ab protein had no adverse effect on *A. bipunctata* development time, larval mortality or weight

(Álvarez-Alfageme *et al*., 2011). These results were confirmed when *A. bipunctata* was fed directly with a sucrose solution that contained a higher dose of the protein that could be expressed in maize (Álvarez-Alfageme *et al*., 2011). The risk of Bt maize to this predator is therefore considered to be insignificant, as it will be exposed to Cry 1Ab proteins in very small concentrations in the field (Romeis *et al*., 2012).

1.6.2 Predatory lacewing (*Chrysoperla carnea*)

Research showing adverse effects of Cry 1Ab protein

Research showed that indirect consumption of Cry 1Ab proteins had some adverse effects on *C. carnea,* exposed to *Ostrinia nubilalis* (Hübner) (Lepidoptera: Noctuidae) and *S. littoralis* larvae as prey (Hilbeck *et al*., 1998a). They found that *C. carnea* had a prolonged development time as well as a higher mortality rate because of the Cry 1Ab proteins, but concluded that it could have been due to poor food quality (Hilbeck *et al*., 1998a). In a follow-up study, a bioassay technique was used to incorporate the Cry 1Ab protein into a liquid diet that was encapsulated within small paraffin spheres (Hilbeck *et al*., 1998b). This study showed that the Cry 1Ab protein was toxic to chrysopid larvae because higher mortality rates as well as a longer larval stage was recorded when the larvae consumed the Cry 1Ab protein (Hilbeck *et al*., 1998b).

Predator development and survival was also shown to be adversely affected when feeding on lepidopteran larvae (*S. littoralis*) that consumed only a low concentration of the Cry 1Ab proteins (Dutton *et al*., 2002). Poor food quality (sick/affected prey) in combination with Cry 1Ab proteins could be the reason why negative effects were observed (Dutton *et al*., 2002).

Research showing no adverse effects of Cry 1Ab protein

Cry 1Ab proteins had an effect on the mortality rate of *C. carnea*, but this could have been due to poor food quality as the prey could have become sick/affected due to the Cry 1Ab protein, which could then have had an adverse effect on *C. carnea* (Dutton *et al*., 2002). Further investigation was therefore needed.

In a study by Romeis *et al*. (2004) *C. carnea* was fed an artificial diet with higher a concentration of Cry 1Ab protein than would be present under field conditions (in maize) or through a more realistic tritrophic feeding experiment. The weight and longevity of the first instar was recorded and this study showed that the Bt-toxin had no effect on *C. carnea* (Romeis *et al*., 2004).

Another study was conducted with a tritrophic feeding experiment (Obrist *et al*., 2006). *Chrysoperla carnea* was exposed to Cry 1Ab proteins through *T. urticae* that fed on Bt maize and there was no adverse effect on the biology of the non-target organism (Obrist *et al*., 2006).

In another study by Lawo and Romeis (2007) it was reported that Cry 1Ab proteins did not cause negative effects on *C. carnea* when they ingested a sucrose solution containing the protein. They tested the hazard potential of a transgenic protein using high-dose toxicity tests by mixing purified proteins into an artificial diet, and concluded that Cry 1Ab protein had no significant risk to the predator (Lawo and Romeis, 2007). Lawo and Romeis, (2007) concluded that Cry 1Ab protein did not have an adverse effect on the development of *C. carnea*.

Chrysoperla carnea is known to be prevalent pollen-consumers in maize fields (Yunhe *et al*., 2008). At the peak of pollen shedding, field collected *C. carnea* females was reported to contain an average of approximately 5000 maize pollen grains in their gut (Yunhe *et al*., 2010). A study was conducted using Bt maize pollen and non-Bt maize pollen, to determine the effect on survival, weight and pre-oviposition period and the results showed that Bt had no effect (Yunhe *et al*., 2008). In another experiment which was conducted using artificial diets that expressed about 10 times the concentration of the Cry 1Ab protein in maize pollen, an adverse effect was detected with the pre-oviposition period, fecundity and dry weight being significantly negatively affected (Yunhe *et al*., 2008). The uptake of Cry proteins in significant levels by *C. carnea* was therefore also confirmed (Yunhe *et al*., 2008).

When *C. carnea* fed on susceptible *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) larvae that fed on Bt cotton there was a significant decrease in predator survival and an increase in development time (Lawo *et al*., 2010). Another

experiment in which *C. carnea* fed on resistant *H. armigera* larvae that fed on Bt cotton showed no significant decrease in predator survival or difference in development time (Lawo *et al*., 2010). The quality of the prey is therefore very important as the susceptible prey will be sick/affected and can cause adverse effects due to reduced prey-quality (Lawo *et al*., 2010).

The different studies reported above were done using different methods of exposure. The method of exposure could have an effect on the results that were found. For example, some might argue that in some cases there was no exposure, while in other studies the prey was sick/affected, which may have resulted in the poor performance of the non-target species that were tested.

1.7 Importance of *Chrysoperla* spp. as biocontrol agents

1.7.1 Biology of Chrysoperla pudica

In South Africa one of the most common chrysopid species in maize ecosystems is *C. pudica*. These little green insects, with eyes of yellow, metallic lustre is commonly known as green lacewings or golden eyes (Skaife 1979). *Chrysoperla pudica* adults (Fig. 1.13) will feed on pollen, honeydew and nectar (Canard and Volkovich, 2007).

Figure 1.13: An adult *Chrysoperla pudica* (Anon, 2012).

The eggs of the *Chrysoperla* spp. are unique to its family as they are laid on stalks (Fig. 1.14a) (Skaife, 1979). The advantage of being laid on a stalk is that it minimizes the chance of being found by other crawling insects as well as their own larvae, as they are fiercely cannibalistic (Skaife, 1979). "Imagine an ant walking on the surface of a leaf, the stalk of the egg will just seem like another hair on the leaf and not potential food, thus being laid on a stalk maximizes the chance of survival" (Skaife, 1979).

These stalks (Fig. 1.14a), on which the eggs are deposited are formed when the female touches the leaf surface with the tip of her abdomen and then ejects a drop of sticky fluid from glands that are associated with her ovaries (Skaife, 1979). The female then lifts her abdomen, causing the sticky drop to form a thread which rapidly hardens in the air, forming the stalk (Skaife, 1979). At the top end of this stalk the female will deposit an egg. Eggs can also be deposited in different patterns: as a single egg (Fig 1.14a), in a batch (Fig. 1.14b) or in a cluster (Fig. 1.14c) (Skaife, 1979).

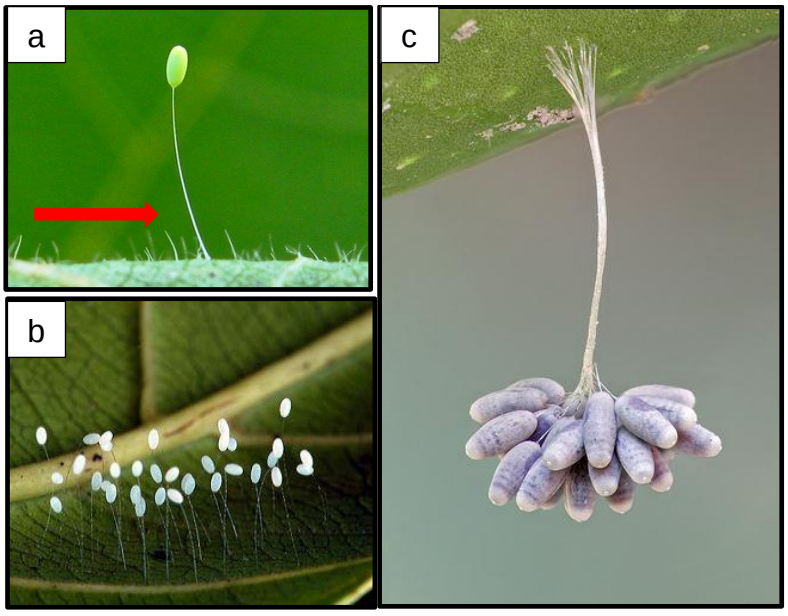

Figure 1.14: Different patterns of chrysopid eggs a) A single chrysopid egg laid on a stalk indicated by the red arrow (Anon, 2008), b) Chrysopid eggs that were laid in a batch (Sikes, 2012) and c) Chrysopid eggs that were laid in a cluster (Baliga, 2012a).

Figure 1.15: A photo taken of two *Chrysoperla pudica* eggs with the larvae visible on the inside.

As the egg develops it changes colour, from green to light purple just before it hatches (Fig.1.15). After the larvae hatches it will sit on the empty egg shell (Fig. 1.16) for a few hours (Canard and Volkovich, 2007). In this resting period the final embryonic stage (closing of the mouth) will be completed (Fig. 1.17a and 1.17b) (Canard and Volkovich, 2007). During this period the larva are defenceless, and it is therefore an advantage that it stayed on the egg (Canard and Volkovich, 2007). The mouthparts of the larvae can be under-developed if there is any disturbance during the final embryonic stage which will result in insufficient future food uptake (Canard and Volkovich, 2007).

Figure 1.16: First instar larvae sitting on empty egg shells (Baliga, 2012b).

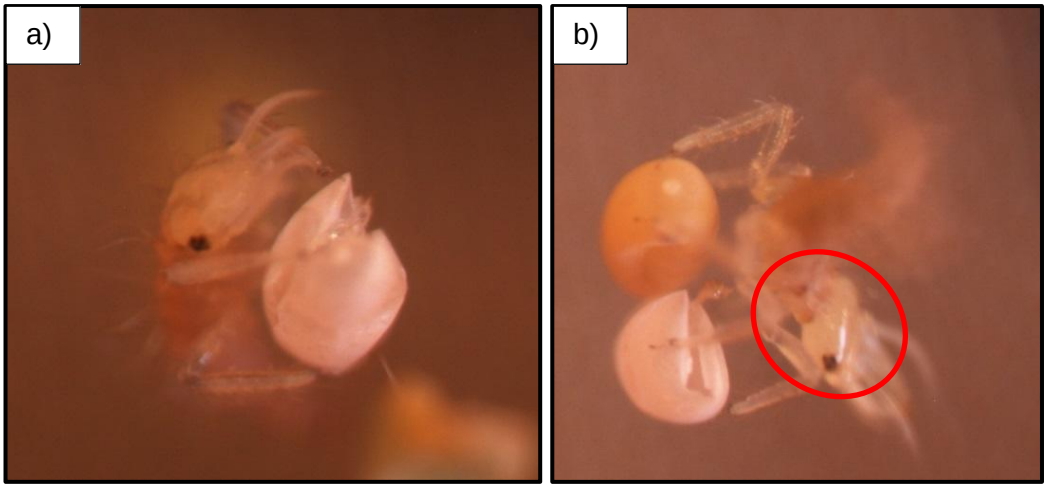

Figure 1.17: a) The 1st instar nymph sitting on the empty egg shell, b) the red circle indicates that the mouthparts haven't fully developed yet as it still has a hard shell on the head.

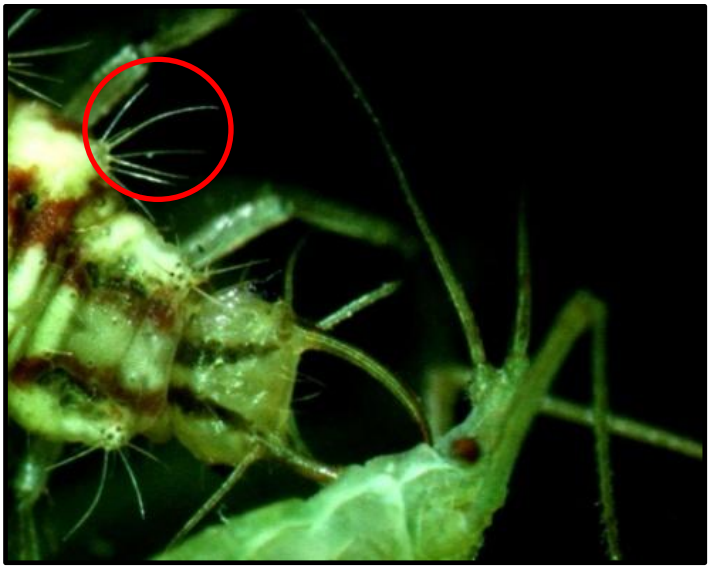

Figure 1.18: Hair on the dorsal side of *Chrysoperla pudica* larvae, indicated by red circle.

There are three larval instars and they can be smooth or have hair on the dorsal side (Fig. 1.18), depending on the species (Scholtz and Holm, 1986). The food consumption of the larvae increases as they grow (Barnes, 1975). The 3rd instar *C. pudica* larvae will consume the most food and the 1st instar larvae the least.

After the 3rd instar larvae have grown to maturity it usually spin the cocoons (Fig. 1.19) (Canard and Volkovich, 2007). The cocoon spinning process is a complicated and long process which can last between 24 and 48 hours (Canard and Volkovich, 2007).

Figure 1.19: A *Chrysoperla pudica* cocoon.

The cocoon also changes colour, as the pupa inside develops, it is white at first and then near the time of adult emergence it turns to a light green (Fig. 1.20, red arrow) (Barnes, 1975). The eyes of the adult *C. pudica* also become visible from the outside of the cocoon (Fig. 1.20, blue arrow), when the adult is near emergence. The adult emerges from the cocoon by putting pressure at one side of the cocoon, this side tears and forms a lid where the adult crawls out (Barnes, 1975).

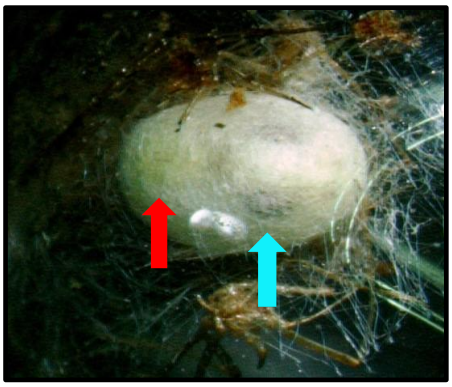

Figure 1.20: The adult of *Chrysoperla pudica* is near emergence, the red arrow indicates the cocoon turning light green and the blue arrow show the eyes of the adult becoming visible.

After emergence from the cocoon, the adult moults (Fig. 1.21 and Fig. 1.22) outside the cocoon and within an hour the adult will expand its wings (Canard and Volkovich, 2007). Sometimes the adults do not go through the final moulting stage (Fig. 1.23) and then they die or sometimes they do not complete the final moulting stage entirely and then they are malformed individuals with crooked wings (Canard and Volkovich, 2007).

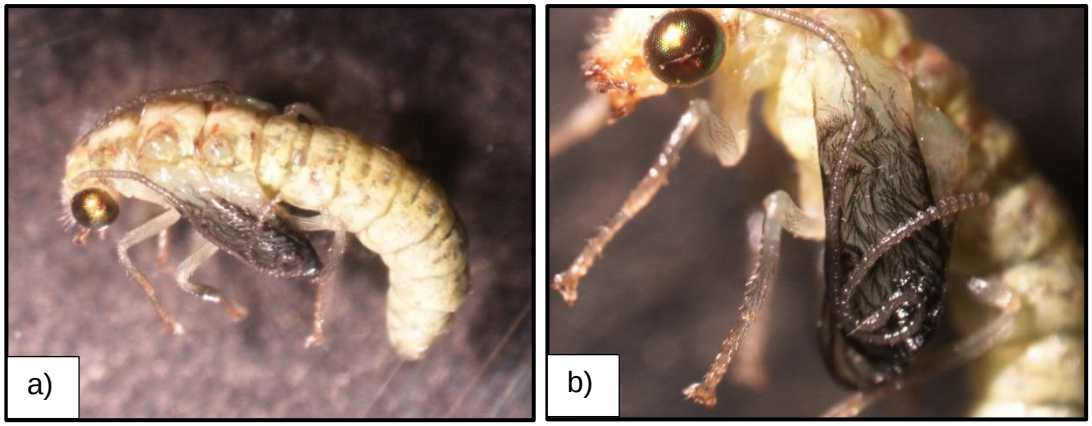

Figure 1.23: a) *Chrysoperla pudica* adult after emergence still busy with the final moulting stage outside the cocoon b) a close-up on the wing of the adult.

Figure 1.22: The final moult of a *Chrysoperla pudica* adult outside the cocoon.

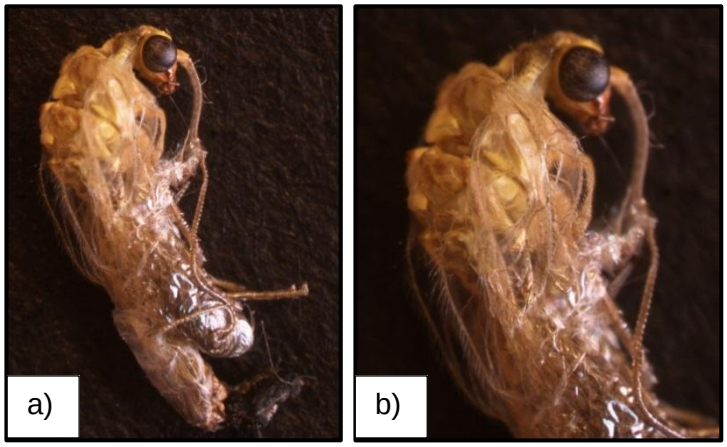

Figure 1.23: a) An adult *Chrysoperla pudica* that was unable to go through the final moulting stage outside the cocoon and spread its wings, also known as malformed adults b) a close-up of the malformed adult.

Shortly after emergence the adults still cannot mate as they still have immature gonads. This stage is known as the pre-mating period (Canard and Volkovich, 2007). It is during this period that they find partners and during which the gonads mature (Canard and Volkovich, 2007).

1.7.2 The importance of lacewings

Chrysoperla larvae are voracious predators with a very high consumption rate and effective searching capacity, thus they are an effective natural enemy and active predator (Senior and McEwen, 2007). Lacewings are important because their larvae can be used to control several arthropod pests. *Chrysoperla zastrowi* (Esben-Petersen) (Neuroptera: Chrysopidae) can consume 488 aphids or 906 potato tuber moth eggs during their larval stage (Barnes, 1975). By the third instar the lacewing larvae can eat up to 84 aphids or 200 potato tuber moth eggs a day (Barnes, 1975). Lacewing larvae consume large or small soft-bodied arthropod pests, some of which are of economic importance such as whiteflies, mealy bugs, aphids and mites (Senior and McEwen, 2007).

Chrysoperla species are effective biological control agents (Senior and McEwen, 2007). *Chrysoperla carnea* are also frequently used as test species in insecticide non-target effect studies as well as studies with Bt proteins (Hilbeck *et al*., 1998a; Hilbeck *et al*., 1998b; Lawo and Romeis, 2007; Li *et al*., 2010; Romeis *et al*., 2004). As early as 1949 *Chrysoperla* played an important role in successful integrated pest management (IPM) programmes (Senior and McEwen, 2007).

There are three strategies where *Chrysoperla* spp. can be used in biological control namely: classical -, augmentative - and conservation biological control (Senior and McEwen, 2007).

1.7.2.1 Classical biological control

This method is used to control pest species that were introduced into the crop environment from foreign areas (Senior and McEwen, 2007). Natural enemies of this new pest (from its native area) are mass reared and released to control this pest.

It is important that the newly introduced natural enemy does not cause more harm than the pest that it controls (Senior and McEwen, 2007). At least one of the *Chrysoperla* spp. is usually already present in the crop (native area) thus *Chrysoperla* spp. is not commonly used in classical biological control (Senior and McEwen, 2007).

1.7.2.2 Augmentative biological control

This can be done through the inoculative (small number of natural enemies are introduced early into the crop cycle in the hopes that they will reproduce) or indundative (the mass production and release of natural enemies where immediate control is needed) releases of natural enemies (Senior and McEwen, 2007). Augmentation increases the already existing natural enemy population densities to effectively control insect pests (Senior and McEwen, 2007).

If there is not enough prey in the crop to sustain the natural enemy, early in the cropping season, with an inoculative release, the adults will not stay in the crop environment (Larock and Ellington, 1996). The indundative control method is usually used where immediate control is necessary on short term crops (Senior and McEwen, 2007).

1.7.2.3 Conservation biological control

The aim of this strategy is to enhance agricultural fields as habitats for natural enemies by providing hibernation shelters and food supplements, so that population numbers can increase (Senior and McEwen, 2007). This strategy therefore identifies and rectifies factors that adversely affect, and suppress natural enemy reproduction (Senior and McEwen, 2007). Conservation biological control is more affordable because it doesn't depend on the mass production of natural enemies (Senior and McEwen, 2007).

1.8 Aim of this study

The aims of this study were to:

- evaluate the effect of the *Cry* 1Ab protein on the biology of *Chrysoperla pudica* through indirect exposure, via healthy Bt-maize feeding prey (*Busseola fusca*) and
- to determine the concentration of *Cry* 1Ab protein present at different trophic levels (the plant, prey and predator), through the use of Enzyme-Linked ImmunoSorbent Assay (ELISA) tests.

1.9 References

Akol, A.M., Chidege, M.Y., Talwana, H.A.L. and Mauremootoo, J.R. 2011. Busseola fusca (Fuller, 1901) – African Maize Stalkborer. [Web:] http://www.keys.lucid-central.org/keys/v3/eafrinet/maize_pests/Media/Html/ Busseola_fusca_(Fuller_-1901)_-_African_Maize_Stalkborer.htm. Date of access 06 September 2013.

Altieri, M.A. 2000. The ecological impacts of transgenic crops on agroecosystem health. *Ecosystem Health* 6(1): 13-23.

Álvarez-Alfageme, F., Bigler, F. and Romeis, J. 2011. Laboratory toxicity studies demonstrate no adverse effects of Cry 1Ab and Cry 3Bb1 to larvae of *Adalia bipunctata* (Coleoptera: Coccinellidae): the importance of study design. *Transgenic Research* 20:467-479.

Anon. 2008. Flickr from yahoo. Chrysoperla's egg [Web:] http://farm4.static-flickr.com/3455/3228345745_2acc79a139_m.jpg. Date of access 25 October 2012.

Anon. 2010. Bt corn and Monarch butterflies. [Web:] http://www.ars.usda.gov/is/br/-btcorn/-index.html?pf=1. Date of access 20 September 2012.

Anon. 2011. *Bacillus thuringiensis*. SciMat. [Web:] http://www.visualphotos.com/-image/1x3740102/bacillus_thuringiensis_bacteria_scanning_electron. Date of use 06 September 2013.

Anon, 2012. Green lacewing insect. [Web:] http://commons.wikimedia.org/wiki-/File:Green_Lacewing_Insect.jpg. Date of access 30 October 2013.

Baliga, V. 2012a. Flickr from yahoo. Crysopid egg cluster. [Web:] http://farm5.static.-flickr.com/4145/4987873392_6f894a8c0c.jpg. Date of access 25 October 2012.

Baliga, V. 2012b. Flickr from yahoo. Green Lacewing eggs after 112 hrs. [Web:] http://www.flickr.com/photos/10567324@N03/6672448535. Date of access 25 October 2012.

Barnes, B.N. 1975. The life history of *Chrysoperla zastrowi* ESB.-Pet. (Neuroptera: Chrysopidae). *Journal of the Entomological Society of southern Africa* 38: 47-53.

Betz, F.S., Hammond, B.G. and Fushs, R.L. 2000. Safety and advantages of *Bacillus thuringiensis* - Protected plants to control insect pests. *Regulatory Toxicology and Pharmacology* 32:156–173.

Birch, A.N.E. et al. 1997. Interaction between plant resistance genes, pest aphid populations and beneficial aphid predators. Scottish Crops Research Institute (SCRI). *Annual Report 1996-1997*, pp. 70-72.

Bøhn, T., Primicerio, R., Hessen, D.O. and Traavik, T. 2008. Reduced fitness of *Daphnia magna* fed a Bt-trangenic maize variety. *Archives of Environmental Contamination and Toxicology* 55: 584-592.

Canard, M. and Volkovich, T.A. 2007. Outlines of lacewing development. *In* McEwen, P.K., New, T.R. and Whittington, A.E. *ed.* Lacewings in the crop environment. Cambridge University Press. Pp. 130-153.

Carpenter, J.E. 2011. Impact of GM crops on biodiversity. *Landes Bioscience*. 2(1): 7-23.

Clark, B.W., Phillips, T.A. and Coata, J.R. 2005. Environmental fate and effect of *Bacillus thuringiensis* (Bt) proteins from transgenic crops: a review. *Journal of Agriculture and Food Chemistry* 53: 4643-4653.

Clare, J. 2002. Daphnia. *An Aquarist's Guide*. [Web:] http://www.caudata.org/-daphnia/. Date of access 25 July 2013.

De la Poza, M., Pons, X., Farinós, G.P., López, C., Ortego, F., Eizaguirre, M., Castañera, P. and Albajes, R. 2005. Impact of farm-scale Bt maize on abundance of predatory arthropods in Spain. *Crop Protection* 24: 677-684.

De Wilder, A. 2001. Tweestippelig lieveheersbeestje (*Adalia bipunctata*). [Web:] http://www.ahw.me/img/adalia_bipunctata007b.html. Date of access 06 September 2013.

Donnegan, K.K., Palm, C.J., Fieland, V.J., Porteous, L.A., Ganis, L.M., Scheller, D.L. and Seidler, R.J. 1995. Changes in levels, species, and DNA fingerprints in soil microorganisms associated with cotton expressing the *Bacillus thuringiensis* var *kurstaki* edotoxin. *Applied Soil Ecology* 2: 111-124.

Douville, M., Gagné, F., André, C. and Blaise, C. 2008. Occurrence of the transgenic corn *cry1Ab* gene in freshwater mussels (*Elliptio complanata*) near corn fields: Evidence of exposure by bacterial ingestion. *Ecotoxicology and Environmental Safety* 72: 17-25.

Dutton, A., Klein, H., Romeis, F. and Bigler, F. 2002. Uptake of Bt-toxin by herbivores feeding on transgenic maize and consequences for the predator *Chrysoperla carnea*. *Ecological Entomology* 27: 441-447.

Dutton, A., Klein, H., Romeis, F. and Bigler, F. 2003. Assessing the risks of insect resistant transgenic plants on entomophagous arthropods: Bt-maize expressing Cry 1Ab as a case study. *BioControl* 48: 611-636.

Edkins, K. 2002. Photos of insects, coccinellidae larvae. [Web:] http://www.gwydir.-demon.co.uk/insects/coccinellidae.htm. Date of access 06 September 2013.

Ferré, J. and Rie, J. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annual Review of Entomology* 47: 501-533.

Glen, C. 2012. How to control aphids in vegetables crops. [Web:] http://pender.ces.-ncsu.edu/2012/10/how-to-control-aphids-on-vegetable-crops/. Date of access 24 July 2013.

Gouse, M., Pray, C.E., Kirsten, J. and Schimmelpfennig, D. 2005. A GM subsistence crop in Africa: The case of Bt white maize in South Africa. *International Journal of Biotechnology* 7: 84-94.

Hilbeck, A., Baumgartner, M., Fried, P.M. and Bigler, F. 1998a. Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environmental Entomology* 27: 480-487.

Hilbeck, A., McMillan, J.M., Meier, M., Humbel, A., Schlaepfer-Miller, J. and Trtikova, M. 2012. A controversy re-visited: Is the coccinellid Adalia bipunctata adversely affected by Bt toxins? *Environmental Sciences Europe* 24: 10.

Hilbeck, A., Moar, W.J., Pusztai-Carey, M., Filippini, A. and Bigler, F. 1998b. Toxicity of *Bacillus thuringiensis* Cry 1Ab Toxin to the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environmental Entomology* 27: 1255-1263.

James, C. 2011. Executive summary: Global status of commercialised biotech/GM crops: 2011. [Web:] http://www.isaaa.org/resources/publications/briefs/43. Date of access 10 August 2012.

Kanrar, S., Venkateswari, J., Kirti, P.B. and Chopra, VL. 2002. Transgenic Indian mustard (*Brassica juncea*) with resistance to the mustard aphid (*Lipaphis erysimi* Kalt). *Plant Cell Reports* 20: 976-981.

Kfir, R., Overholt, W.A., Khan, Z.R. and Polaszek, A. 2002. Biology and management of economically important Lepidopteran cereal stem borers in Africa. *Annual Review of Entomology* 47: 701-731.

Khan, Z.R. 2007. 'Push-pull' technology for the control of stemborers in Striga weeds. [Web:] http://www.push-pull.net/striga/stemborer.html. Date of access 12 July 2013.

Kruger, M., Van Rensburg, J.B.J. and Van den Berg, J. 2009. Perspective on the development of stem borer resistance to Bt maize and refuge compliance at the Vaalharts irrigation scheme in South Africa. *Crop Protection* 28(8): 684-689.

Kumar, S., Chandra, A and Pandey, K.C. 2008. *Bacillus thuringiensis* (Bt) transgenic crop: An environment friendly insect-pest management strategy. *Journal of Environmental Biology* 29(5): 641-653.

Larock, D.R. and Elligton, J.J. 1996. An integrated pest management approach, emphasizing biological control for pecan aphids. *Southwestern Entomologist* 21: 153-166.

Lawo, N.C. and Romeis, J. 2007. Assessing the utilization of carbohydrate food source and the impact of insecticidal proteins on larvae of the green lacewing, *Chrysoperla carnea*. *Biological Control* 44: 389-398.

Lawo, N.C., Wäckers, F.L. and Romeis, J. 2010. Characterizing indirect prey-quality mediated effects of a *Bt* crop on predatory larvae op the green lacewing, *Chrysoperla carnea*. *Journal of Insect Physiology* 56: 1702-1710.

Li, Y., Meissle, M., and Romeis, J. 2010. Use of maize pollen by adult *Chrysoperla carnea* (Neuroptera: Chrysopidae) and fate of Cry proteins in Bt-transgenic varieties. *Journal of Insect Physiology* 56: 157-164.

Losey, J.E., Raynor, L.S. and Carter, M.E. 1999. Transgenic pollen harms monarch larvae. *Nature* 399: 214.

Meeusen, R.L. and Warren, G. 1989. Insect control with genetically engineered crops. *Annual Review of Entomology* 35: 373-381.

Meier, M.S. and Hilbeck, A. 2001. Influence of transgenic *Bacillus thuringiensis* corn-fed prey on prey preference of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Basic Applied Ecology* 2: 35-44.

Mulder, C., Wouterse, M., Raubuch, M., Roelofs, W. and Rutgers, M. 2006. Can transgenic maize affect soil microbial communities? *PLoS Computational Biology* 2(9)e129: 1165-1172.

Naranjo, S.E., 2005. Long-term assessment of the effects of transgenic Bt Cotton on the function of the natural enemy community. *Environmental Entomology* 34:1211-1223.

Naseby, D.C. and Lynch, J.M. 1998. Impacts of wild type and genetically modified *Pseudomonas fluorescens* on soil enzyme activities and microbial population structure in the rhizosphere of sea. *Molecular Ecology* 7: 617-625.

O'Callagan, M., Glare, T.R., Burgess, E.P.J. and Malone, L.A. 2005. Effects of plants genetically modified for insect resistance on non-target organisms. *Annual Review of Entomology* 50: 271-292.

Obrist, L.B., Dutton, A., Romeis, J. and Bigler, F. 2006. Biological activity of Cry 1Ab toxin expressed by Bt maize following ingestion by herbivorous arthropods and exposure of the predator *Chrysoperla carnea*. *BioControl* 51: 31-48.

Palm, C.J., Schaller, D.L., Donegan, K.K.and Seidler, R.J. 1996. Persistence in soil of transgenic plant produced *Bacillus thuringiensis* var. k*urstaki* δ-endotoxin. *Canadian Journal of Microbiology* 42: 665-671.

Ranjekar, P.K., Patankar, A., Gupta, V., Bhatnagar, R., Bentur, J. and Kumar, P.A. 2003. Genetic engineering of crop plants for insect resistance. *Current Science* 84: 321-329.

Raps, A., Kehr, J., Gugerli, P. Moar, W.J., Bigler, F. and Hilbeck, A. 2001. Immunological analysis of phloem sap of *Bacillus thuringiensis* corn and of the non-target herbivore *Rhopalosiphum padi* (Homoptera: Aphididae) for the presence of Cry 1Ab. *Molecular Ecology* 10: 525-533.

Rosi-Marshall, E.J., Tank, J.L., Royer, T.V., Whiles, M.R., Evans-White, M., Chambers, C., Griffiths, N.A., Pokelsek, J. and Stephen, M.L. 2007. Toxins in transgenic crop byproducts may affect headwater stream ecosystems. *Proceedings of the National Academy of Science* 104(41): 16204-16208.

Romeis, J., Álvarez-Alageme, F. and Bigler, F. 2012. Putative effects of Cry 1Ab to larvae of *Adalia bipunctata* – reply to Hilbeck et al. (2010). *Environmental Sciences Europe* 24(18): 1-5.

Romeis, J., Dutton, A. and Bigler, F. 2004. *Bacillus thuringiensis* toxin (Cry 1Ab) has no direct effect on larvae of the green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae). *Journal of Insect Physiology* 50: 175-183.

Scholtz, C.H. and Holm, E. 1986. Insects of Southern Africa. Butterworths. pp. 185.

Schuler, T.H. 2004. GM Crops: Good or bad for natural enemies. *In* Van Emden, H.F. and Gray, A.J. *ed.* GM Crops-Ecological dimensions. *Aspects of Applied Biology* 74: 81-90.

Schmidt, J.E.U., Braun, C.U., Whitehouse, L.P. and Hilbeck, A. 2009. Effects of activated Bt transgene products (Cry 1Ab, Cry 3Bb) on immature stages of the ladybird *Adalia bipunctata* in laboratory ecotoxicity testing. *Archives Environmental Contamination Toxicology* 20: 467-479.

Sears, M.K., Hellmich, R.L., Stanley-Horn, D.E., Oberhauser, K.S., Pleasants, J.M., Matilla, H.R., Siegfried, B.D. and Dively, G.P. 2001. Impact of Bt corn pollen on monarch butterfly populations: A risk assessment. *National Academy of Science* 98(21): 11937-11942.

Senior, L.J. and McEwen, P.K. 2007. The use of lacewings in biological control. In McEwen, P.K., New, T.R. and Whittington, A.E. ed. Lacewings in the crop environment. Cambridge University Press. pp. 296-299.

Shi, Y., Wang, M.B., Powell, K.S., Van Damme, E., Hilder, V.A., Gatehouse, A.M.R., Boutler, D. and Gatehouse, J.A. 1994. Uses of the rice sucrose synthase-1 promotor to direct phloem-specific expression of β-glucuronidase and snowdrop lectin genes in transgenic tobacco plants. *Journal of Experimental Botany* 45: 623-632.

Sikes, D.S.S. 2012. Eggs on stalks of a green lacewing. [Web:] http://www.users.iab.uaf.edu/~ derek_sikes/INDO/images/05-05-14-0086a.jpg. Date of access 26 October 2012.).

Sisterson, M.S., Biggs, R.W., Olson, C., Carrière, Y., Dennehy, T.J. and Tabashnik, B.E. 2004. Arthropod abundance and diversity in Bt and non-Bt cotton fields. *Environmental Entomology* 33: 921-929.

Skaife, S.H. 1979. African insect life. Cape Town: Struik publishers. pp. 106.

Tabashnik, B.E. 2008. Delaying insect resistance to transgenic crops. *National Academy of Science* 105(49): 19029-19030.

Tabashnik, B.E., Brévault, T. and Carrière, Y. 2013. Insect resistance to Bt crops: lessons from the first billion acres. *Nature Biotechnology*. 31(6): 510-520.

Tabashnik, B.E., Liu, Y., Malvar, T., Heckel, D.G., Masson, L. and Ferré, J. 1998. Insect resistance to *Bacillus thuringiensis*: uniform or diverse? *The Royal Society* 353: 1751-1756.

Tabashnik, B.E., Van Rensburg, J.B.J. and Carrière, Y. 2009. Field-evolved insect resistance to *Bt* crops: definition, theory and data. *Journal of Economic Entomology* 102(6): 2011-2025.

Tank, J.L., Rosi-Marshall, E.J., Royer, T.V., Whiles, M.R., Griffiths, N.A., Frauendorf, T.C. and Treering, D.J. 2010. Occurrence of maize detritus and a transgenic insecticidal protein (Cry1Ab) within the stream network of an agricultural landscape. *PNAS Early Edition* 1-6.

Tribe, D. 2012. Global Status of Commercialized Biotech/GM Crops: 2012 - ISAAA Brief 44-2012 | ISAAA.org. [Web:] http://gmopundit.blogspot.com/2013/02/global-status-of-commercialized.html. Date of access 12 July 2013.

Van Rensburg, J.B.J. 2007. First report of field resistance by the stem borer, *Busseola fusca* to Bt-transgenic maize. *South African Journal of Plant and Soil* 24: 147-151.

Van Wyk, A., Van den Berg, J. and Van Hamburg, H. 2007. Selection of non-target Lepidoptera species for ecological risk assessment of Bt maize in South Africa. *African Entomology* 15: 356-366.

Wolfenbarger, L.L., Naranjo, S.E., Lundgren, J.G., Bitzer, R.J. and Watrud, L.S. 2008. Bt Crop Effects on Functional Guilds of Non-Target Arthropods: A Meta-Analysis. *PLoS ONE* 3(5): e2118: 1-11.

Yunhe, L., Meissle, M. and Romeis, J. 2008. Consumption of *Bt* maize pollen expressing Cry 1Ab or Cry 3Bb1 does not harm adult green lacewings, *Chrysoperla carnea* (Neuroptera: Chrysopidae). *PLoS ONE* 3(8): 1-8.

Yunhe, L., Meissle, M. and Romeis, J. 2010. Use of maize pollen by adult *Chrysoperla carnea* (Neuroptera: Chrysopidae) and fate of Cry proteins in *Bt*-transgenic varieties. *Journal of Insect Physiology* 56: 157-164.

Chapter 2

Indirect effect of Cry 1Ab proteins expressed in Bt maize, on the biology of *Chrysoperla pudica* (Neuroptera: Chrysopidae)

2.1 Abstract

Genetically modified Bt maize was commercialised in SA to control lepidopteran insect pests such as the maize stem borer (*Busseola fusca*) (Lepidoptera: Noctuidae). Possible non-target effects of Bt maize at the third trophic level on natural enemies such as lacewings (*Chrysoperla* spp.), have been of concern since its development. Contradicting results were reported in previous studies with regard to the performance of lacewings exposed to Cry 1Ab protein. The aim of this study was to evaluate the effect of Cry 1Ab protein on the biology of *Chrysoperla pudica* (Neuroptera: Chrysopidae), and to determine the concentration of Cry 1Ab proteins in the plant, prey and predator. Two experiments were conducted in which *C. pudica* larvae were indirectly exposed to the Bt-toxin through healthy Bt-maize feeding *B. fusca*. These lacewing larvae were maintained in glass test tubes in temperature controlled incubators. *Chrysoperla pudica* larval- and pupal development time, overall mortality, incidence of malformed adults, fecundity and fertility was determined. Results indicated that the larval and pupal periods of *C. pudica* larvae that were exposed to the Bt-toxin had a significant difference from that of the control treatment. Exposure also had an effect on fecundity and fertility as well as the percentage of malformed adults, but not on mortality rate. This study showed that the Cry 1Ab protein had an adverse effect only on certain life parameters of *C. pudica*, and that the Cry 1Ab protein was hardly detectable in *C. pudica* larvae that fed on B. fusca larvae for a period of 8 days. However, since this study represented a worst-case scenario where diverse prey was not available, insignificant effects is expected under field conditions where more diverse prey is consumed by lacewing larvae.

Key words: Bt maize, *Busseola fusca*, *Chrysoperla pudica*, ELISA-test, lacewings, natural enemies, risk assessment, third trophic level.

2.2 Introduction

The main goal with GM crops was to directly benefit the producer through increasing productivity per hectare, reducing production costs and chemical usage as well as improving grower health (Kumar *et al*., 2008; Anon, 2011). The most widely planted crop with insecticidal properties is Bt maize (Ranjekar *et al*., 2003). Bt maize produces Cry proteins (toxins) that are effective against different groups of insects and is not known to be toxic to mammals and other organisms, therefore it is accepted worldwide as an eco-friendly bio-pesticide (Ranjekar *et al*., 2003). Four Bt delta-endotoxin genes (Cry1Ab, Cry1Ac, Cry2Ab, and Cry9C) are currently used commercially in cotton and maize to protect these crops against lepidopteran pests (Shelton *et al*., 2002). These toxins are produced in Bt plants throughout the entire growing season. Target and non-target arthropods may therefore encounter Bt toxins on a continuous basis and in high concentrations (Sisterson *et al*., 2004). The potential impact of GM crops on biodiversity is a topic of great interest (Carpenter, 2011). In the natural and agricultural environments, GM crops and their transgene products may come into contact with hundreds of non-target species with important ecological functions (Carpenter, 2011). If a target or non-target organism is eliminated from a specific guild, it may change the guild structure which may then lead to development of secondary pests. It is therefore important to assess the potential risks associated with GM crops with insecticidal properties (Van Wyk *et al*., 2007).

Target pests may be affected directly if they are susceptible to the specific Cry proteins produced by the transgenic crop, whereby natural enemies of the target pest may be indirectly affected, when feeding on herbivores exposed to Bt plants (Schuler, 2004). Pathways of indirect exposure of natural enemies to Cry proteins can be affected by prey quality, protein expression levels in plants, prey behaviour, prey availability and crop management practices (Schuler, 2004). For example natural enemies such as lacewings (*Chrysoperla* spp.) can directly be affected through the inter-trophic level effects of the Bt toxin (Altieri, 2000). Another direct effect could be if the natural enemy is species-specific for the target organism, whereby they can starve if the target pest is eradicated as a result of the Bt toxin (Altieri, 2000).

When non-target effects evaluation of a product such as Bt maize is done, it is important to use species that occur naturally in the receiving environment (Van Wyk *et al*., 2007). For example in South Africa one of the most common chrysopid species in maize ecosystems is *C. pudica* (Navás). Under natural conditions this species largely feeds on aphids (Homoptera: Aphidae) but may also be exposed to the Bt protein consumed by herbivorous larvae, such as *B. fusca*, which is resistant to the Bt protein. Lacewing larvae will therefore be indirectly exposed to Bt protein through consumption of healthy prey containing the Bt protein (Van Wyk *et al*., 2007).

Chrysoperla larvae are voracious predators with a very high consumption rate and effective searching capacity, making them effective natural enemies and active predators (Senior and McEwen, 2007). Lacewings are important because their larvae can be used to control several arthropod pests. For example, *Chrysoperla zastrowi* (Esben-Petersen) (Neuroptera: Chrysopidae) can consume 488 aphids or 906 potato tuber moth eggs during their larval stage (Barnes, 1975). By the third instar the lacewing larvae can eat up to 84 aphids or 200 potato tuber moth eggs a day (Barnes, 1975). Lacewing larvae consume large or small soft-bodied arthropods, some of which are of economic importance such as whiteflies, mealy bugs, aphids and mites (Senior and McEwen, 2007).

Previous studies showed contradicting results with regard to the effect of the Bt toxin on the biology of *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), a common species in Europe. Some reports indicate that *Chrysoperla pudica* (Stephens) (Neuroptera: Chrysopidae), was not adversely affected when it indirectly or directly ingested the Cry 1Ab toxin (Dutton *et al*., 2003). Other studies showed that when *C. carnea* fed on Lepidoptera larvae exposed to Cry proteins, they suffered delayed development and had a reduced survival rate (Hilbeck *et al*., 1998). The latter authors did, however, indicate that it could have been as a result of poor prey quality (sick/affected prey), as the prey they used were not resistant to Cry proteins, and not because of the Cry protein itself (Hilbeck *et al*., 1998). On the other hand it was also found that *C. carnea* preferred to eat the aphids

(*Rhopalosiphum padi*) (Koch) (Hemiptera: Aphidae) individuals that have not been exposed to Cry proteins (O'Callaghan *et al*., 2005).

The maize stemborer (*Busseola fusca*) (Fuller) (Lepidoptera: Noctuidae) developed resistance to Bt maize in South Africa (Van Rensburg, 2007). This evolution of resistance facilitated the rearing of *B. fusca* on Cry 1Ab expressing Bt maize with no observable negative effects. *Busseola fusca* reared on Bt maize plants are therefore considered to be of high food quality (healthy prey). These larvae provide a unique opportunity to study the effect of Cry 1Ab proteins on the biology of non-target organisms, such as *C. pudica* through exposure to healthy, Bt maize consuming prey.

The aim of this study was to evaluate the effect of the *Cry* 1Ab protein on the biology of *C. pudica* through indirect exposure, via healthy Bt-maize feeding prey.

2.3 Material and methods

Busseola fusca larvae and aphids were used in laboratory bioassays to study the effect of the Bt toxin (Cry 1Ab protein) on the biology of *C. pudica*. Two separate but identical experiments were conducted. The first experiment commenced during early February 2013, and the second, during early March 2013.

2.3.1 Insect rearing

Chrysoperla pudica as well as one of its food sources used in this study, *i.e. B. fusca* larvae, were reared at the North-West University, Potchefstroom, South Africa. The other food source used in this study, Bluegreen aphid (*Acyrthosiphon kondoi*) (Hemiptera: Aphididae), was collected daily from a lucerne field at Farm Leinster, Vredefordt, South Africa (26°56'26.11"S, 27° 3'45.16"E) using a sweep net (Fig. 2.3).

2.3.1.1 Busseola fusca rearing

Three pairs of male and female *B. fusca* adults were placed in plastic containers (25 x 15 cm) covered with a fine material to prevent the adults from escaping. Each

plastic container held a maize stem which served as an oviposition substrate. After eggs were laid they were removed and placed in plastic containers (22x16x8cm), provided with either Bt maize whorls or non-Bt maize whorls as a food source for the larvae. Neonate stem borer larvae fed on the whorl leaves for a period of 4 – 10 days until they reached second to third instars. These instars were then used as prey for *C. pudica* larvae. Rearing was done in an incubator at 26 ± 1 ºC, with a relative humidity of 60-70%. Detailed information on *B. fusca* rearing is provided in Appendix I.

2.3.1.2 Chrysoperla pudica rearing

Chrysoperla pudica adults were collected from a lucerne field in the Vredefordt district (26°56'26.11"S, 27° 3'45.16"E). This study was conducted using the F1-generation obtained from these adults. The adults were kept in plastic containers (honey jars) (10x10x15cm), at 26 ± 1 ºC with natural a day/night photoperiod. Each container was provided with a sugar water solution on cotton wool that served as food source for the adults. The plastic containers had a very fine sieve-like material in the cap that provided aeration and prevented the adults from escaping. Eggs were laid inside the bottles and the larvae that hatched were used in the feeding experiment. Detailed information on *C. pudica* rearing is provided in Appendix II.

2.3.2 Feeding experiment

Chrysoperla pudica eggs were removed from the plastic containers by carefully cutting the stalk of each individual egg (Fig. 2.1).

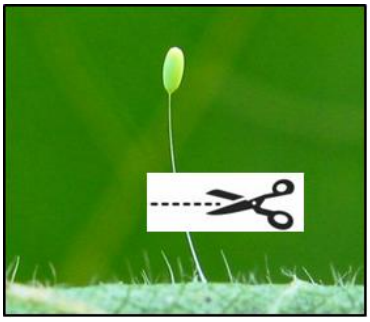

Figure 2.1: An illustration to indicate how the *Chrysoperla pudica* eggs were removed from the containers.

Each egg was placed individually into a glass test tube (75 x 10 mm) (Fig. 2.2a) that was covered with a very fine material to prevent the larvae from escaping (Fig. 2.2b), in the rare occasion that larvae did escape, the individual was removed from the experiment. The test tubes were kept at 26 ± 1 ºC with natural day/night photoperiod.

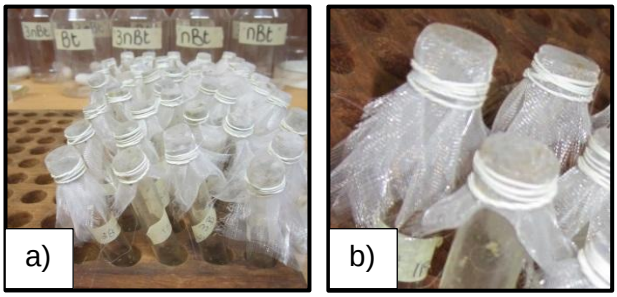

Figure 2.2: a) Test tubes where hatching and feeding of *Chrysoperla pudica* larvae took place, b) the test tubes were covered with a very fine material to prevent the larvae from escaping.

A description of the feeding treatments used in the study is provided in Table 1. This entire experiment with the five different treatments was repeated to ensure that the results that were found were correct and trustworthy.

Table 1: The five different diet treatments that *Chrysoperla pudica* larvae were exposed to during the study.

Treatments			
Diet group (Treatment)	**Bt exposure**	**Diet for 2nd to 3rd instar larva**	**Number/day**
1	+	Bt-resistant *B. fusca* reared on Bt maize	4-10 larvae
2	-	*B. fusca* reared on non-Bt maize	4-10 larvae
3	+	Bt-resistant *B. fusca* reared on Bt maize (3 days) followed by aphids (3 days)	4-10 larvae/ 10-20 aphids
4	-	*B. fusca* reared on non-Bt maize (3 days) followed by aphids (3 days)	4-10 larvae/ 10-20 aphids
5	-	Aphids	10-20 aphids

The day that the *C. pudica* eggs hatched was recorded as day one. During the first three days, all *C. pudica* larvae received 10-15 aphids (*A. kondoi*) per day inside the

test tube. On day three, individuals were randomly divided into five different diet groups (treatments) (Table 1). Each diet group was replicated ten times and each replicate consisted of five, 3-day old individuals.

All larvae were provided with food as indicated in Table 1, until they died or formed pupae. Diet group 1 received only Bt-resistant *B. fusca* larvae that was reared only on Bt maize, and therefore, presumably, the highest concentration of the Bt protein. *Chrysoperla pudica* individuals in diet group 2 served as a control for group 1 since they did not consume any Bt proteins, their prey consisted only of *B. fusca* larvae reared on non-Bt maize. Group 3 was less exposed to the Bt protein than group 1, the *C. pudica* larvae fed on Bt-resistant *B. fusca* reared on Bt maize for three days, followed by aphids (*A. kondoi*) for the following three days and so on. The aphids were collected from a lucerne field, to ensure that they had no traces of Bt proteins in their system. There has been reports that trace amounts of Bt Cry proteins could occur in low concentration in aphids, but it is believed that Cry proteins are not found in aphids at all (Romeis and Meissle, 2011). Group 4 served as a control for group 3 since the *C. pudica* larvae did not consume any Bt proteins but also received their food in three-day cycles. Group 5 only received 10-20 aphids (*A. kondoi*) daily and served as the overall control group.

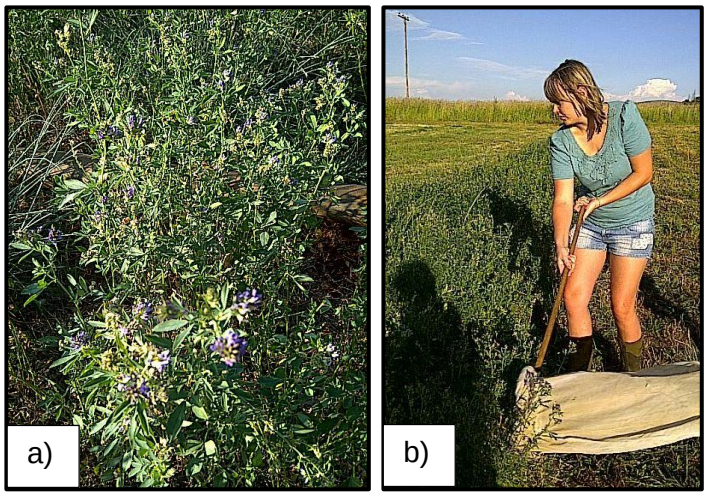

Figure 2.3: The collecting of aphids (*Acyrthosiphon kondoi*) in the a) lucerne field with a b) sweep net.

Chrysoperla pudica larvae received second- to third-instar *B. fusca* larvae (4-10 per day), depending on the size of the *C. pudica* larva. Chrysopid larvae that were ten

days and older only received third instar *B. fusca* larvae as food. Debris was removed from the test tubes daily, before fresh food was provided.

2.3.3 Fecundity and fertility experiment

After the feeding experiment (explained above) the *C. pudica* adults that emerged and successfully spread their wings were placed into plastic containers. For each diet group there were four plastic containers with sugar water as food source. Each plastic container contained ten adults (Fig. 2.4) thus giving 40 adults in total for each diet group. These adults were randomly selected from those that emerged from the pupae and no distinction was made between males and females.

Figure 2.4: Plastic containers with ten adults in each to determine the fecundity of the *Chrysoperla pudica* adults reared on the different diets.

The adults laid their eggs inside the plastic containers. The eggs were collected from the containers every third day, after moving the adults to new plastic containers. This was done until no more eggs were laid and all adults were dead. Fecundity (number of eggs laid) and fertility (number of fertile eggs) was recorded for the F_1 generation when the *C. pudica* adults emerged from the cocoons. Each egg was carefully cut off at its stalk and placed individually into a test tube (70x10mm). The number of eggs in each plastic container was then determined to provide an indication of the fecundity of adults after they have been exposed to the different feeding treatments. The test tubes were checked daily to count the number of eggs

that hatched for each treatment and to determine the fertility. Test tubes were kept in an incubator at 26 ± 1 ºC with a natural day/night photoperiod and a relative humidity of 60-70%. Each test tube was covered with Parafilm (Fig. 2.5), to prevent the larvae from escaping.

Figure 2.5: Test tubes covered with parafilm where *Chrysoperla pudica* eggs were kept to determine fertility.

2.3.4 Determining Cry 1Ab concentrations at different trophic levels.

A separate feeding trail was conducted using only the treatments in which Bt maize was involved, as explained in Table 1. The reason for this was to obtain enough individuals to use in the ELISA-tests to determine the concentration of Cry 1Ab protein present at the three different trophic levels

- 1st level – Bt/non-Bt maize (plant)
- 2nd level – *Busseola fusca* (prey)
- 3rd level – *Chrysoperla pudica* (predator)

Twenty 2nd-instar *C*. *pudica* larvae that was exposed to feeding treatment 1 (Table 1) and 20 2nd-instar *C. pudica* larvae from group three were frozen at -66 ºC. Thirty *B. fusca* second-instar larvae that fed only on Bt maize whorls and leaves of three Bt maize whorls were also frozen at -66 ºC. The frozen material was then transported in a liquid nitrogen container to the GMO testing laboratory at the University of the

Free State, where ELISA-tests were done according to the EnviroLogix QualiPlate Kit for Cry1Ab (Appendix III).

2.4 Data collection

The following data were collected: survival and mortality of larvae, the number of days until pupation, the number of days in the pupal period, whether the adults successfully spread its wings, as well as fecundity and fertility.

2.5 Data analysis

The data was converted into percentages to determine the overall effect of Bt on the 3rd trophic level. Analysis of variance (ANOVA) was conducted followed by a post-hoc Tukey-test to determine if there were any significant differences in the effects of exposure on life history parameters. ELISA-tests were used to determine the concentration of the Cry 1Ab protein present at each of the different trophic levels.

2.6 Results and discussion

Since the aim of this study was to evaluate the effect of indirect exposure to Cry 1Ab protein on the biology of *C. pudica*, comparisons were made between treatments 1 (Bt-feeding *B. fusca* larvae) and 2 (non-Bt feeding *B. fusca* larvae) and between 3 (aphids and Bt-feeding *B. fusca* larvae) and 4 (aphids and non-Bt feeding *B. fusca* larvae). Treatment 5 was not compared to other treatments and only served as control for the experiment since there were no Cry 1Ab proteins present in the diet (Table 1).

The larval and pupal periods were the shortest for the chrysopid larvae that fed only on aphids (Tables 4 and 5). The larval and pupal periods of individuals that received Cry 1Ab proteins were shorter than that of those that did not receive any Cry 1Ab proteins. The larval period of individuals that received only Bt-feeding larvae were 1.1 x days shorted than those that fed on non-Bt-feeding larvae. Thus the larval and pupal period of diet group 5 was the shortest, group 1 was shorter than group 2 and group 3 was shorter than group 4. The overall mortality was very low for all the

treatments (Table 6). The percentage of malformed adults after emergence was the lowest for the treatment that only received aphids, and the highest in both treatments that received Bt resistant *B. fusca* larvae that fed on Bt maize (Table 7). The *C. pudica* adults that only received aphids in their diet laid the least number of eggs but this treatment had the highest percentage of fertile eggs (Table 8). The two treatments of *C. pudica* adults that received only Bt resistant *B. fusca* larvae in their diet laid the most eggs but had the lowest percentage of fertile eggs as well (Table 8). These differences in development times between different diet groups were expected since the aphid diet is an optimum diet for rearing *Chrysoperla* species. Chrysopid food selection was highlighted in choice-tests, which showed that *C. carnea* larvae preferred aphids to Lepidoptera larvae (Meier and Hilbeck, 2001).

2.6.1 Larval period

Significant differences were observed in the duration of the larval period between the treatments for both experiments (Table 4; Fig. 2.6a and b).

Table 4: The mean duration of the larval period (mean number of days to pupation) of *Chrysoperla pudica*, after exposure to different treatments, for experiment 1 and experiment 2.

	Experiment 1	**Experiment 2**
Treatment	**Mean number of days in larval period**	
Aphids	13.4 a	13.1 a
Aphids + Bt *B. fusca* larvae	14.2 b	14.7 b
Aphids + non-Bt *B. fusca* larvae	15.1 c	15.4 c
Bt *B. fusca* larvae	15.0 bc	16.0 cd
non-Bt *B. fusca* larvae	15.1 c	16.3 d
F-value	$F_{4, 245} = 15.31$	$F_{4, 245} = 67.48$
P-value	0.00001	0.0000

Means within columns followed by the same letter do not differ significantly at P = 0.05.

The duration of the larval period of individuals that fed only on aphids (control) was significantly shorter than all of the other treatments (Table 4; Fig. 2.6a and b). Individuals that received Bt-resistant *B. fusca* larvae followed by aphids on a 3-day cycle, developed significantly quicker than those that received non-Bt-feeding *B. fusca* larvae and aphids (Table 4; Fig. 2.6a and b). There was no significant difference between the two treatments that only received *B. fusca* larvae (Table 4; Fig. 2.6a and b).

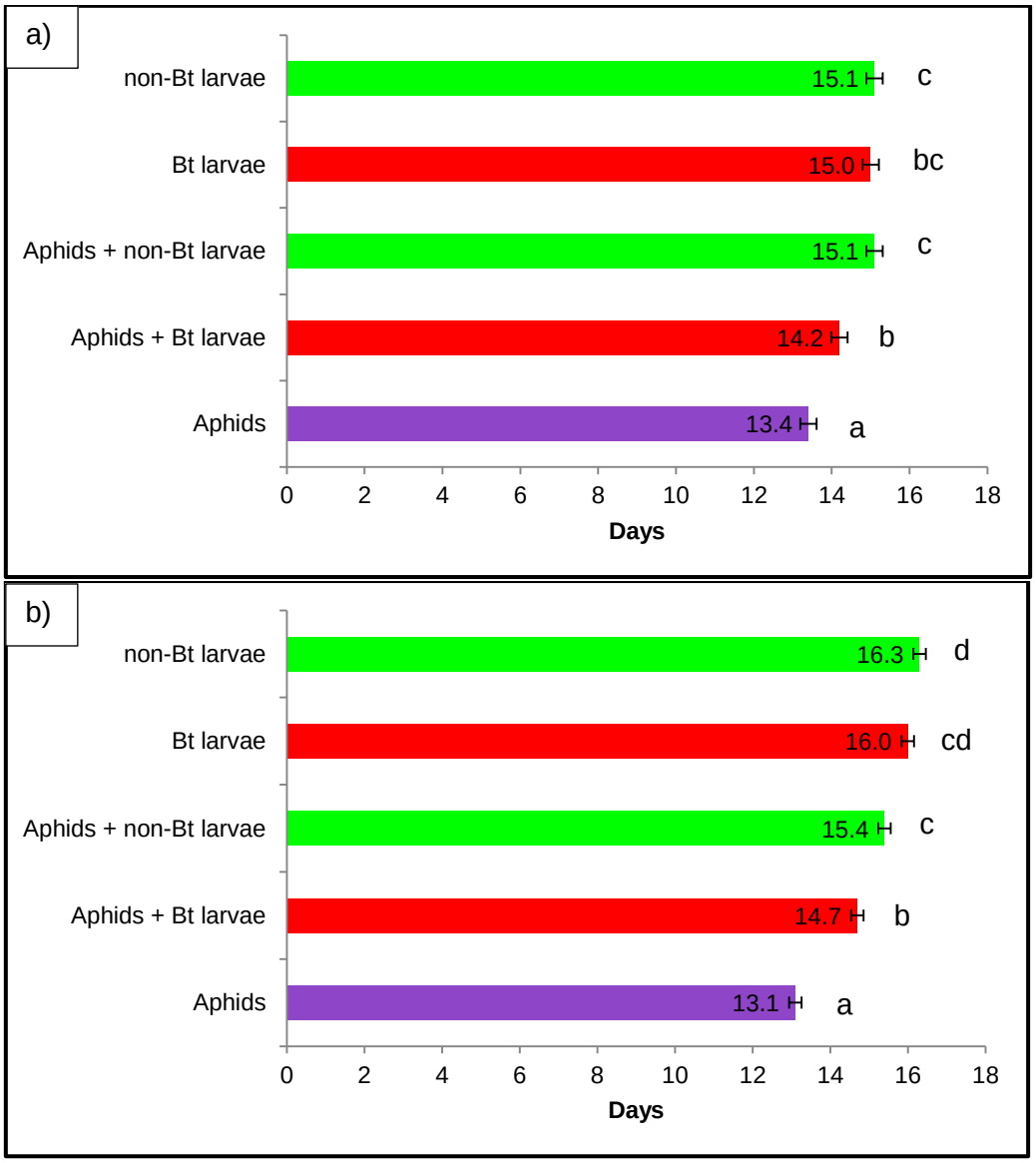

Figure 2.6: The mean number of days spent by *Chrysoperla pudica* in the larval period (mean number of days to pupation), after the exposure to different treatments, for a) experiment 1, and b) experiment 2. Bars followed by the same letter do not differ significantly at P = 0.05.

An interesting result was that the development period of *C. pudica* larvae that were in diet group 1 (Bt resistant *B. fusca* reared on Bt maize) and 2 (*B. fusca* reared on non-Bt maize) was longer than that of all the other treatments, indicating that a diet of stemborer larvae only resulted in delayed onset of the pupal stage (Table 4, Fig.2.6). The larval period of individuals subjected to treatment 1 (Bt resistant *B. fusca* reared on Bt maize) was shorter than treatment 2 (*B. fusca* reared on non-Bt maize), and treatment 3 (Bt resistant *B. fusca* reared on Bt maize and aphids) was shorter than treatment 4 (*B. fusca* reared on non-Bt maize and aphids), indicating that the larvae that had Bt protein in their diets had shorter larval periods (Table 4; Fig. 2.6). *Chrysoperla larvae* that received Bt in the diet (treatment 1 and 3) developed quicker than that of the control groups (treatment 2 and 4), it is as if the *C. pudica* larvae realises that something is wrong and that development must take place faster in order to reach maturity (Table 4; Fig. 2.6). The shorter duration of the larval stage of treatment 5 (aphids) is therefore ascribed to the preference of aphids as food (Table 4; Fig. 2.6).

The cumulative percentage of pupa that formed over time for each treatment is indicated in Fig. 2.7. The control group of *C. pudica* larvae that only had aphids in their diet developed quicker than all the other treatments (Fig. 2.7). The entire *C. pudica* larvae in the control group developed into pupae on day 17 (Fig. 2.7). The *C. pudica* larvae that consumed the diet that only consisted of *B. fusca* larvae took the longest for the onset of pupae (Fig. 2.7). *Chrysoperla pudica* larvae that received aphids and *B. fusca* took longer to develop than the control group and developed quicker than the two treatments that received *B. fusca* (Fig. 2.7).

The entire group of *C. pudica* larvae in the control diet group (treatment 5) formed pupae in only seven days (Fig. 2.7). *Chrysoperla pudica* larvae in treatment 2 (*B. fusca* reared on non-Bt maize), treatment 1 (Bt resistant *B. fusca* reared on Bt maize) and treatment 3 (Bt resistant *B. fusca* reared on Bt maize and aphids) formed into pupae in ten days (Fig. 2.7). The *C. pudica* larvae in treatment 4 (*B. fusca* reared on non-Bt maize and aphids) took the longest to develop into pupae, it took 15 days (Fig. 2.7).

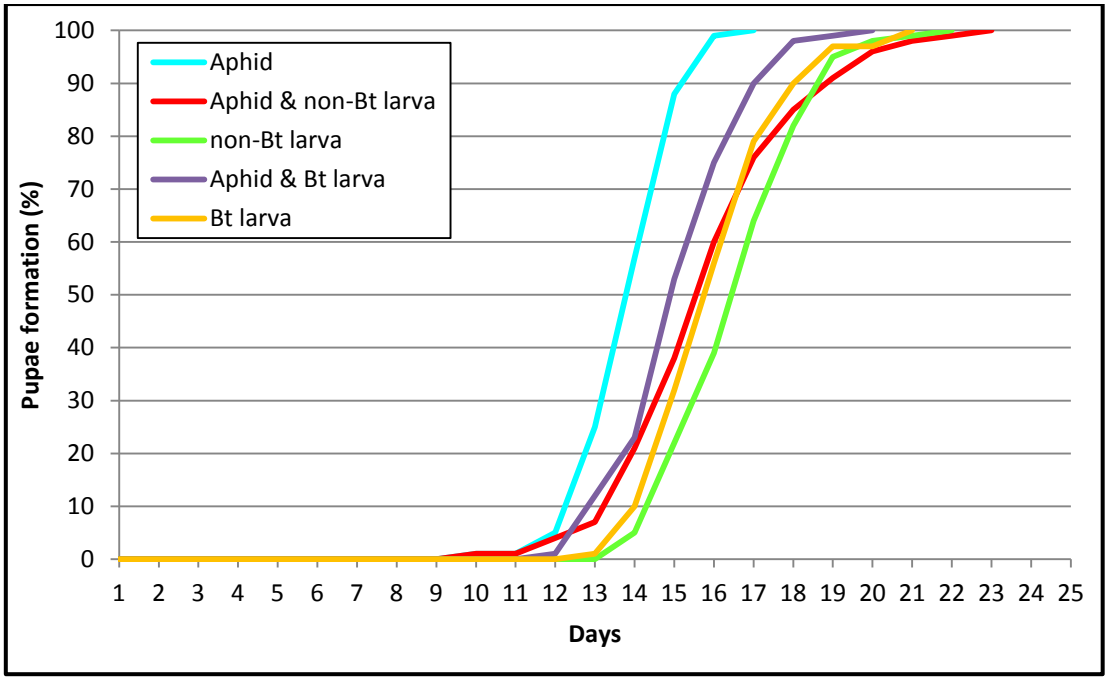

Figure 2.7: The cumulative percentage of pupa formation over time.

The two *C. pudica* groups that received Bt in the diet (treatment 1 and 3) developed quicker than that of the control groups (treatment 2 and 4), again as if the larvae knew something was wrong and that they needed to developed faster (Fig. 2.7).

2.6.2 Pupal period

There were significant differences observed in the duration of the pupal period between the different treatments (Table 5; Fig. 2.8a and b). The development of the *C. pudica* larvae group that received Bt-resistant *B. fusca* larvae and aphids was significantly shorter than the *C. pudica* larvae that received non-Bt feeding *B. fusca* larvae and aphids, for both experiments (Table 5; Fig. 2.8a and b). The *C. pudica* larvae that received only Bt-resistant *B. fusca* larvae developed significantly quicker than the *C. pudica* larvae that received non-Bt *B. fusca* larvae for both experiments (Table 5; Fig. 2.8a and b). The control group of *C. pudica* larvae that were only provided with aphids had the second shortest pupal development time in both experiments (Table 5; Fig. 2.8a and b), and this was significant. The *C. pudica* larvae that consumed Bt resistant *B. fusca* larvae had the quickest pupal development time (Table 5; Fig. 2.8a and b).

The *C. pudica* larvae that consumed the Bt toxin developed (treatment 1 and 3) quicker as if they sensed something was wrong (Table 5; Fig. 2.8). Both diets that only consisted of *B. fusca* larvae (treatment 1 and 2) had the longest pupal period (Table 5; Fig. 2.8), making it clear that *B. fusca* larvae is not *C. pudica* larvae preferred diet. The duration of the pupal stage was the shortest for individuals reared on Bt-resistant *B. fusca* larvae reared on Bt maize and aphids, indicating that the exposure to Bt protein did not have an adverse effect on pupal development, unless the more rapid development was in response to stress caused by exposure to the Bt protein (Table 5; Fig. 2.8). The longest pupal periods were observed for individuals reared on diets containing *B. fusca* larvae, irrespective of whether they consumed Bt or non-Bt maize (Table 5; Fig. 2.8).

Table 5: The mean number of days spent by *Chrysoperla pudica* in the pupal period (mean number of days until adult emergence), after the exposure to different treatments, for experiment 1 and experiment 2.

	Experiment 1	**Experiment 2**
Treatment	**Mean number of days in pupal period**	
Aphids	12.7 a	12.1 a
Aphids + Bt *B. fusca* larvae	11.1 b	11.8 a
Aphids + non-Bt *B. fusca* larvae	13.0 a	14.0 b
Bt *B. fusca* larvae	16.1 c	16.4 c
non-Bt *B. fusca* larvae	17.1 d	17.2 d
F-value	$F_{4, 245} = 109.51$	$F_{4, 245} = 135.19$
P-value	0.00001	0.00001

Means within columns followed by the same letter do not differ significantly at P = 0.05.

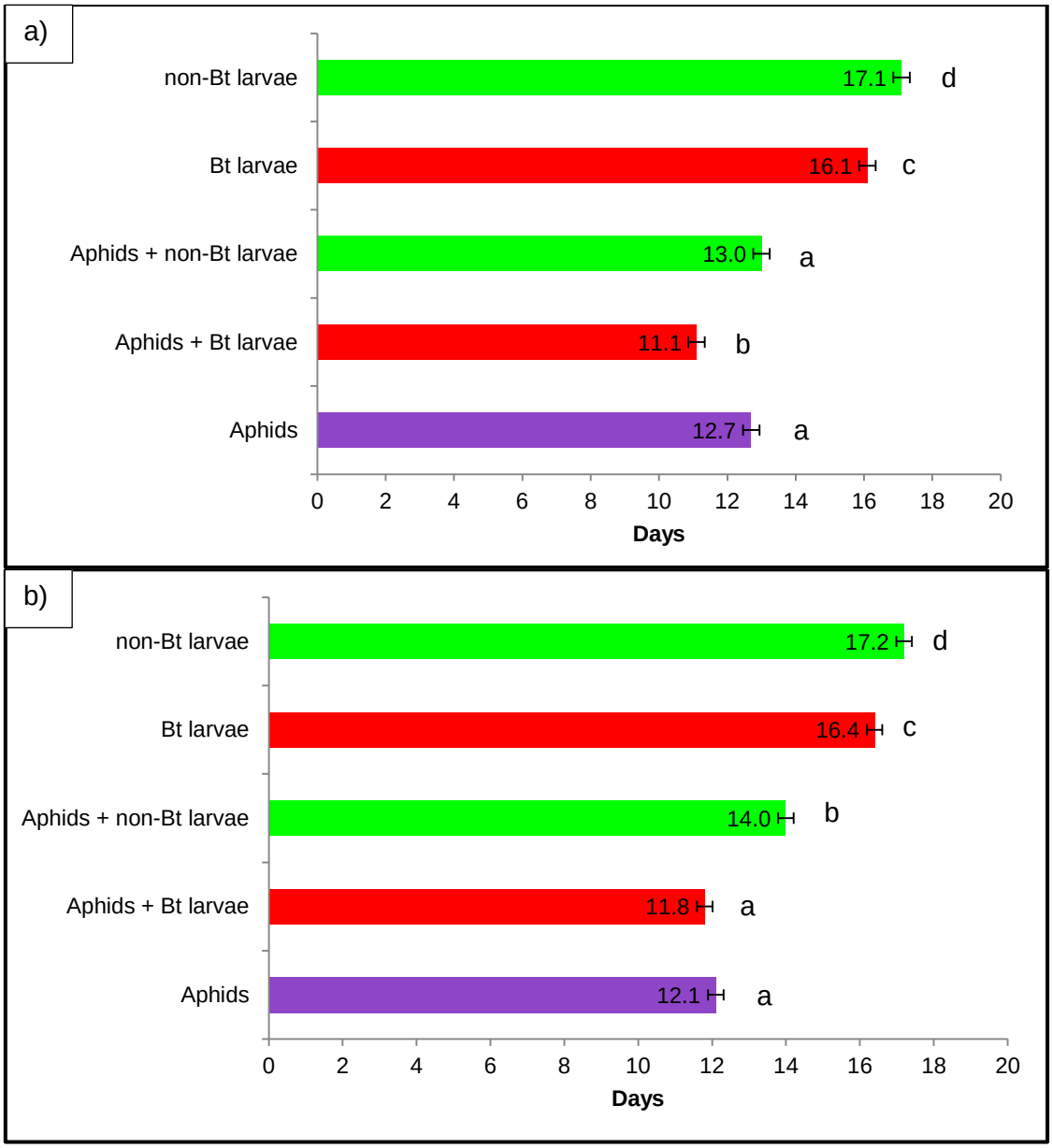

Figure 2.8: The mean number of days spent by *Chrysoperla pudica* in the pupal period (mean number of days until adult emergence), after the exposure to different treatments, for a) experiment 1 and b) experiment 2. Bars followed by the same letter do not differ significantly at P = 0.05.

2.6.3 Overall mortality

Overall mortality of the *C. pudica* was low for all the treatments (Table 6). The Cry 1Ab protein did not have an effect on the mortality rate of *C. pudica* (Table 6). There were no significant differences between any of the treatments in both experiments. Percentage mortality of *C. pudica* ranged between 0 – 4% for all the different

treatments, both experiments (Table 6). Therefore it can be seen from these results that Bt proteins does not have an effect on the mortality rate (Table 6).

Table 6: The overall mortality of *Chrysoperla pudica,* after exposure to different treatments, for experiment 1 and experiment 2.

	Experiment 1	**Experiment 2**
Treatment	**Percentage of overall mortality**	
Aphids	2.0 a	2.0 a
Aphids + Bt *B. fusca* larvae	2.0 a	4.0 a
Aphids + non-Bt *B. fusca* larvae	4.0 a	0.0 a
Bt *B. fusca* larvae	4.0 a	0.0 a
non-Bt *B. fusca* larvae	0.0 a	2.0 a
F-value	$F_{4, 245} = 0.591$	$F_{4, 245} = 0.884$
P-value	0.669	0.474

Means within columns followed by the same letter do not differ significantly at P = 0.05.

2.6.4 Malformed adults

There were significant differences between the percentages of malformed adults observed after emergence from cocoons between some treatments. This result was similar for both experiments (Table 7). The control treatment in which *C. pudica* larvae only received aphids had the lowest percentage of malformed adults (Table 7). Only 2% of *C. pudica* adults in the control group (treatment 5) were malformed (Table 7). The *C. pudica* adults that received Bt-feeding *B. fusca* larvae had the highest percentage of malformed adults after emergence (Table 7).

The only significant difference of malformed *C. pudica* adults after emergence was between the control group (treatment 5) and the two groups that consumed Bt (treatment 1 and 3) (Table 7). *Chrysoperla pudica* adults in treatment 1 and 3 were significantly more malformed (26% - 30%) than in treatment 5 (2%).

Table 7: The percentage of malformed *Chrysoperla pudica* adults, after exposure to the different treatments, for experiment 1 and experiment 2.

	Experiment 1	**Experiment 2**
Treatment	**Percentage of malformed adults**	
Aphids	2.0 a	2.0 a
Aphids + Bt *B. fusca* larvae	30.0 b	26.0 bc
Aphids + non-Bt *B. fusca* larvae	10.0 a	8.0 ab
Bt *B. fusca* larvae	30.0 b	28.0 c
non-Bt *B. fusca* larvae	14.0 ab	10.0 abc
F-value	$F_{4, 245} = 5.849$	$F_{4, 245} = 5.654$
P-value	0.0002	0.0002

Means within columns followed by the same letter do not differ significantly at P = 0.05.

2.6.5 Fecundity and fertility

There were some significant differences between the fecundity as well as fertility of *C. pudica* individuals that received Bt and those that did not have any Bt in their diet (Table 8). The *C. pudica* adults that only received aphids in their diet laid the least number of eggs (70) but this treatment also had the highest percentage of fertile eggs (70%) (Table 8). The only significant difference is between the *C. pudica* adults in the control group and in treatment 1 (Bt-resistant *B. fusca* reared on Bt maize) (Table 8). *Chrysoperla pudica* adults from treatment 5 (aphids) had a significantly lower fecundity (only 70 eggs) than *C. pudica* adults from treatment 1 (Bt-resistant *B. fusca* reared on Bt maize) (161 eggs) (Table 8). *Chrysoperla pudica* adults from treatment 5 (aphids) had a significantly higher fertility (70%) than *C. pudica* adults from treatment 1 (Bt-resistant *B. fusca* reared on Bt maize) (45%) (Table 8). *Chrysoperla pudica* adults from treatment 1 (Bt-resistant *B. fusca* reared on Bt maize) laid the most eggs but had the lowest percentage of fertile eggs (Table 8) this can be because the *C. pudica* adults sensed something was wrong and had to produce more eggs because a lower number would be fertile.

Table 8: The fecundity (number of eggs laid), fertility (number of eggs that hatched) and percentage fertility (% of eggs that hatched) of *Chrysoperla pudica* that fed on different diets.

Treatment	**Mean number of eggs / container**	**Mean percentage fertility**
Aphids	70 a	70.41 a
Aphids + Bt *B. fusca* larvae	144 ab	57.81 ab
Aphids + non-Bt *B. fusca* larvae	128 ab	60.98 a
Bt *B. fusca* larvae	161 b	45.47 b
non-Bt *B. fusca* larvae	96 ab	57.74 ab
F-value	$F_{4, 15} = 3.482$	$F_{4, 15} = 8.350$
P-value	0.033	0.0009

Means within columns followed by the same letter do not differ significantly at P = 0.05.

2.6.6 Cry 1Ab concentration at the different trophic levels

Through the use of *ELISA*-tests the concentration of the toxin could be identified in all three trophic levels. Cry 1Ab concentration was the highest in the Bt maize whorl tissue and lowest in the larvae from the treatment that received Bt resistant *B. fusca* larvae followed by aphids (Table 9).

Table 9: The concentration Cry 1Ab protein present in different samples (Appendix III).

Samples	**Mean toxin concentration**
Bt maize whorl leaf tissue	1.620 ug toxin/g tissue
Bt-resistant *Busseola fusca* larva reared on Bt maize	0.423 ug toxin/g tissue
Chrysoperla pudica larvae reared on Bt-resistant *Busseola fusca* larva that fed on Bt maize only	<1 ng/ml toxin
Chrysoperla pudica larva reared on Bt-resistant *Busseola fusca* larva reared on Bt maize and aphids	<1 ng/ml toxin

This study represents a worst-case scenario where diverse prey was not available to *C. pudica* larvae even under these worst-case scenario conditions only the pupal

development period *C. pudica* was significantly affected in individuals that were indirectly exposed to the Cry 1Ab protein. At field level, where prey is more diverse, this phenomenon will most likely not be observed. The cryptic behaviour of stem borer larvae which nearly exclusively feed deep inside plant whorls also makes them less likely to be exploited as food by chrysopid larvae. *Chrysoperla carnea* numbers were reported not to be reduced inside Bt maize fields, when compared numbers inside non-Bt maize fields, during a 2-year field study in France (Bourguet *et al*., 2002).

Obrist *et al*. (2006) reported the movement of Cry 1Ab proteins through the food chain, since its presence was recorded in chrysopid larvae that fed on *S. littoralis* larvae that consumed Bt maize. The above mentioned reason is why the ELISA-tests were conducted to show that Cry 1Ab proteins do move through the trophic levels. It is important to establish whether the Bt protein affects the prey itself (Dutton *et al*., 2002; Hilbeck *et al*., 1998). If the prey quality is affected by Bt proteins, it will in turn affect the predator, which could have resulted in the adverse effect of Bt maize observed on C. carnea Dutton *et al*. (2002) and Hilbeck *et al*. (1998).

The result of this study, in which the effect of food quality was excluded, showed that Cry 1Ab proteins did in fact reach the third trophic level, although at very low levels. It also showed that Cry 1Ab proteins only affected certain fitness components during the life cycle of *C. pudica*. However, since this study represented a worst-case scenario where diverse prey was not available to *C. pudica*, insignificant effects are expected under field conditions where prey is more diverse.

2.7 References

Altieri, M.A. 2000. The ecological impacts of transgenic crops on agroecosystem health. *Ecosystem Health* 6(1): 13-23.

Anon. 2011. *Bacillus thuringiensis*. SciMat. [Web:] http://www.magma.ca/~scimat-/B_thurin.htm. Date of use 12 June 2013.

Barnes, B.N. 1975. The life history of *Chrysoperla zastrowi* ESB.-Pet. (Neuroptera: Chrysopidae). *Journal of the Entomological Society of southern Africa* 38: 47-53.

Bourguet, D., Chaufaux, J., Micoud, A., Delos, M., Naibo, B., Bombarde, F., Marque, G., Eychenne, N. and Pagliari, C. 2002. *Ostrinia nubilalis* parasitism and the field abundance of non-target insects in transgenic *Bacillus thuringiensis* corn (*Zea mays*). *Environmental Biosafety* 1: 49-60.

Carpenter, J.E. 2011. Impact of GM crops on biodiversity. *Landes bioscience*. 2(1): 7-23.

Dutton, A., Klein, H., Romeis, F. and Bigler, F. 2002. Uptake of Bt-toxin by herbivores feeding on transgenic maize and consequences for the predator *Chrysoperla carnea*. *Ecological Entomology* 27: 441-447.

Dutton, A., Klein, H., Romeis, F. and Bigler, F. 2003. Assessing the risks of insect resistant transgenic plants on entomophagous arthropods: Bt-maize expressing Cry 1Ab as a case study. *BioControl* 48: 611-636.

Hilbeck, A., Baumgartner, M., Fried, P.M. and Bigler, F. 1998. Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environmental Entomology* 27: 480-487.

Kumar, S., Chandra, A and Pandey, K.C. 2008. *Bacillus thuringiensis* (Bt) transgenic crop: An environment friendly insect-pest management strategy. *Journal of Environmental Biology* 29: 641-653.

Meier, M.S. and Hilbeck, A. 2001. Influence of transgenic *Bacillus thuringiensis* corn-fed prey on prey preference of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Basic Applied Ecology* 2: 35-44.

Obrist, L.B., Dutton, A., Romeis, J. and Bigler, F. 2006. Biological activity of Cry 1Ab toxin expressed by Bt maize following ingestion by herbivorous arthropods and exposure of the predator *Chrysoperla carnea*. *BioControl* 51: 31-48.

O'Callagan, M., Glare, T.R., Burgess, E.P.J. and Malone, L.A. 2005. Effects of plants genetically modified for insect resistance on non-target organisms. *Annual Review of Entomology* 50: 271-292.

Ranjekar, P.K., Patankar, A., Gupta, V., Bhatnagar, R., Bentur, J. and Kumar, P.A. 2003. Genetic engineering of crop plants for insect resistance. *Current Science* 84: 321-329.

Romeis, J. and Meissle, M. 2011. Non-target risk assessment of *Bt* crops – Cry protein uptake by aphids. *Journal of Applied Entomology* 135: 1-6.

Schuler, T.H. 2004. GM Crops: Good or bad for natural enemies. *In* Van Emden, H.F. and Gray, A.J. *ed.* GM Crops-Ecological dimensions. The association of applied biologists. *Aspects of Applied Biology* 74: 81-90.

Senior, L.J. and McEwen, P.K. 2007. The use of lacewings in biological control. In McEwen, P.K., New, T.R. and Whittington, A.E. ed. Lacewings in the crop environment. Cambridge University Press. pp. 296-299.

Shelton, A.M., Zhao, J.Z. and Roush, R.T. 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. *Annual Review of Entomology* 47: 845-881.

Sisterson, M.S., Biggs, R.W., Olson, C., Carrière, Y., Dennehy, T.J. and Tabashnik, B.E. 2004. Arthropod abundance and diversity in Bt and non-Bt cotton fields. *Environmental Entomology* 33: 921-929.

Van Rensburg, J.B.J. 2007. First report of field resistance by the stem borer, *Busseola fusca* to Bt-transgenic maize. *South African Journal of Plant and Soil* 24: 147-151.

Van Wyk, A., Van den Berg, J. and Van Hamburg, H. 2007. Selection of non-target Lepidoptera species for ecological risk assessment of Bt maize in South Africa. *African Entomology* 15: 356-366.

Chapter 3

Conclusion

Genetically modified maize which produces the insecticidal Cry 1Ab protein is planted in South Africa to control *Busseola fusca*, a lepidopteran stem borer (Van Wyk *et al*., 2007). Since the first commercialization of GM crops with insecticidal properties, the possible effect on non-target organisms as well as resistance development of target organisms has been of great concern (Dutton *et al*., 2002). However, in South Africa it is required by law that post-release monitoring of genetically modified crops with insecticidal proteins has to be done, to determine the possible environmental impact as well as the effect on non-target organisms (Anon, 2004).

Natural enemies play an important role in the agro-ecosystem and are considered to be important natural resources (Dutton *et al*., 2003). Thus if natural enemies such as *Chrysoperla pudica* is affected by Cry 1Ab proteins, it could disrupt the beneficial interactions in agro-ecosystems (Dutton *et al*., 2003).

Lacewings have a high prey consumption rate, they must be able to migrate freely between different vegetation in order to survive and to follow their prey as the season and crop change. Field crops are only temporary habitats. For example some times of a year crops do not host prey for chrysopids and the environment is therefore unsuitable for chrysopids (Duelli, 2007).

Chrysoperla pudica larvae are known to feed on young lepidopteran prey (*B. fusca*) in the maize fields, however the *B. fusca* larvae are not preferred (Meier and Hilbeck, 2001). In some choice studies it becomes clear that *C. pudica* prefer prey such as aphids, spider mites or lepidopteran eggs all of which have no or only trace amounts of Cry 1Ab proteins (Principi and Canard, 1984; Bay *et al*., 1993).

Previous studies on the effects of Cry 1Ab protein on *Chrysoperla carnea* showed contradicting results. Some studies indicated no effect, while others ascribed the

poor performance of *C. carnea* to poor food quality (sick prey). In this study, however, the effect of food quality was eliminated, since Bt-resistant *B. fusca* was used as a food source.

In this study *C. pudica* larvae were separated into five different diet groups to determine the effect of Cry 1Ab protein on their biology. Each diet group was replicated ten times and each of these replicates consisted of five, 3-day old individuals. This whole experiment was repeated twice to ensure that the results that were found were not by accident. Larval- and pupal development time, the survival and mortality of the individual larvae, whether the adults spread its wings, adult fecundity and adult fertility. Lastly the concentration of the Bt toxin was determined in all three trophic levels.

Results showed that Cry 1Ab protein reached the 3rd trophic level and that the concentration of the protein was reduced in higher trophic levels. Cry 1Ab protein had a significant effect on the larval- and pupal period, although there were differences between the percentage of malformed adults as well as the fecundity and fertility. The summary of this study (Table 10) showed that Cry 1Ab protein only had adverse effects on certain fitness components. In addition, exposure to Cry 1Ab proteins in the field would generally be low for *C. pudica* which use aphids as their major food source. Furthermore, aphids ingest no or only trace amounts of Cry proteins when feeding on Bt maize (Álvarez-Alfageme *et al*., 2011). This study is therefore seen as a worst-case scenario where *C. pudica* did not have as many choices of prey as it would have under field conditions.

When the treatments are compared to the control groups (treatment 2 and 4) only and not to the overall control group (treatment 5), only minimal effects become clear. Treatment 1 (Bt-feeding *B. fusca* larvae) and treatment 2 (non-Bt feeding *B. fusca* larvae) are compared, as treatment 2 is the control for treatment 1, the only significant difference is that the pupal development stage of *C. pudica* is quicker than its control (Table 10). Treatment 3 (aphids and Bt-feeding *B. fusca* larvae) and 4 (aphids and non-Bt feeding *B. fusca* larvae) are compared, as treatment 4 is the control for treatment 3, there was significant differences in the larval- and pupal period of *C. pudica* as well as the percentage of malformed *C. pudica* adults (Table

10). The reason why the significant differences is less between treatment 1 and treatment 2 can be ascribed to the diet only consisting of lepidopteran larvae and this is not the preferred food source for *C. pudica* larvae. There are more significant differences between treatment 3 and 4 and this can be ascribed to the fact that the aphids form part of the diet. This is the case when it is only compared to its specific control, when it is compared to the overall control (treatment 5) more life parameters that are evaluated had significant differences.

Table 10: A summary of the effects of Cry 1Ab protein on *Chrysoperla pudica* exposed to different diets.

	Does Bt have an effect?			
	Aphids & Bt larvae	**Aphids & non-Bt larvae**	**Bt larvae**	**Non-Bt larvae**
Larval period	✓	✗	✗	✗
Pupal period	✓	✗	✓	✗
Fecundity	✗	✗	✗	✗
Fertility	✗	✗	✗	✗
% Mortality	✗	✗	✗	✗
% Malformed individuals	✓	✗	✗	✗

When the overall control treatment (treatment 5 - aphids) is compared to treatment 1 (Bt-feeding *B. fusca* larvae) and 3 (Bt-feeding *B. fusca* larvae and aphids), all life parameters evaluated, except mortality differed significantly.

Lacewings have long been appreciated as natural enemies of pests. As an indicator species, it is important to study the possible effects that long-term exposure to Bt protein may have on the populations, as well as their biology and ecology.

3.1 References

Álvarez-Alfageme, F., Bigler, F. and Romeis, J. 2011. Laboratory toxicity studies demonstrate no adverse effects of Cry 1Ab and Cry 3Bb1 to larvae of *Adalia bipunctata* (Coleoptera: Coccinellidae): the importance of study design. *Transgenic Research* 20: 467-479.

Anon. 2004. National Environmental Management: Biodiversity Act 10 of 2004. Pretoria: Government printer, South Africa.

Bay, T., Hommes, M. and Plate, H.P. 1993. Die florfiege *Chrysoperla carnea* (Stephens). *Federal Biological Research Centre for Agriculture and Forestry* 288: 3-175.

Dutton, A., Klein, H., Romeis, F. and Bigler, F. 2002. Uptake of Bt-toxin by herbivores feeding on transgenic maize and consequences for the predator *Chrysoperla carnea*. *Ecological Entomology* 27: 441-447.

Dutton, A., Klein, H., Romeis, F. and Bigler, F. 2003. Assessing the risks of insect resistant transgenic plants on entomophagous arthropods: Bt-maize expressing Cry 1Ab as a case study. *BioControl* 48: 611-636.

Duelli, P. 2007. Dispersal and oviposition strategies in *Chrysoperla carnea*. *In* Gepp, J., Aspock, H. and Hölzel, H. *ed*. Progress in world's Neuropterology, pp. 133-145.

Meier, M.S. and Hilbeck, A. 2001. Influence of transgenic *Bacillus thuringiensis* corn-fed prey on prey preference of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Basic Applied Ecology* 2: 35-44.

Principi, M.M. and Canard, M. 1984. Feeding habits. *In* Biology of Chrysopidae, *ed.* Canard, M., Séméria, Y. and New, T.R., pp. 76-92.

Van Wyk, A., Van den Berg, J. and Van Hamburg, H. 2007. Selection of non-target Lepidoptera species for ecological risk assessment of Bt maize in South Africa. *African Entomology* 15: 356-366.

Appendix I:

Busseola fusca rearing

A rearing colony was started by collecting *B. fusca* larvae from a maize field at the Vaalharts irrigation scheme in the Northern Cape, South Africa. The maize stems were cut open with a knife (Fig. 4.1) as the larvae occur inside the maize stem (Fig. 4.2). The larvae were then placed in a plastic container with maize leaves and cobs (Fig. 4.3) to keep them cool for transportation.

Figure 4.1: The maize stem being cut open to find the *Busseola fusca* larva inside the stem.

Figure 4.2: *Busseola fusca* larvae, indicated by the red oval, inside the maize stem.

Figure 4.3: The plastic container with maize leaves were *Busseola fusca* larvae were placed to remain cool during transportation.

As soon as the pupae developed they were sexed and males and females were placed in separate petri dishes (Fig. 4.5). After the adults (Fig. 4.6) emerged from the pupae they were placed (3 males and 3 females) in plastic bottles. The plastic bottles were used as oviposition chambers and were 30cm high and 15 cm in diameter and covered with a very fine material to prevent the adults from escaping (Fig. 4.7). A 20 cm maize stem with the bases of the leaves intact was place in an upright position inside the oviposition chamber, to serve as an oviposition substrate (Fig. 4.7). The maize stems were kept upright with the help of crusher stone, which filled the container with approximately 5cm (Fig. 4.7). Water was added to fill the container to just under the level of the crusher stone to provide humidity to the adults and to keep the stems fresh for as long as possible. Each container also had a small bottle with sugar water to give the adults more energy, prolong their lives and enhance their fecundity (Fig. 4.7).

Figure 4.5: *Busseola fusca* pupae in a Petri dish.

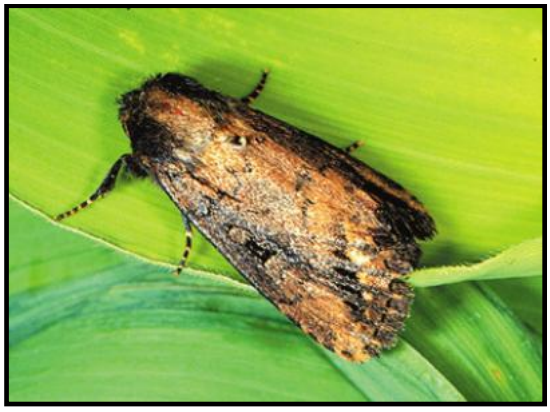

Figure 4.6: *Busseola fusca* adult on a maize leaf (Akol *et al*., 2011).

Figure 4.7: One of the plastic bottles, oviposition chambers, where the adult *Busseola fusca* laid their eggs.

The adults laid their eggs in batches on the leaf sheaths of the maize plants in batches (Fig. 4.8). The maize stems were switched with fresh stems as soon as it seemed as if there was no space left to lay new eggs, or every 3 days, whichever came first.

Figure 4.8: a *Busseola fusca* egg batch on a maize leaf (Akol *et al*., 2011).

The egg batches were carefully removed from each stem in three day intervals, by cutting off a small piece of leaf with the egg batch attached to it. After removal the egg batches was divided and placed in plastic containers with mesh in the lid (Fig. 4.9) with either Bt maize (MON 810) whorls or non-Bt maize whorls that served as food for the larvae that hatched. These plastic containers were kept in an incubator at 26 ± 1 ºC with a humidity of 60-70% until they hatched. The containers were cleaned and fresh food was provided in weekly intervals.

Figure 4.9: One of the plastic containers where rearing of *Busseola fusca* larvae took place.

References

Akol, A.M., Chidege, M.Y., Talwana, H.A.L. and Mauremootoo, J.R. 2011. Busseola fusca (Fuller, 1901) – African Maize Stalkborer. [Web:] http://www.keys.lucid-central.org/keys/v3/eafrinet/maize_pests/Media/Html/ Busseola_fusca_(Fuller_-1901)_-_African_Maize_Stalkborer.htm. Date of access 06 September 2013.

Appendix II:

Chrysoperla pudica rearing

Chrysoperla pudica adults were collected from a lucerne field (Fig. 4.10) in the Vredefordt district 26°56'26.11"S, 27° 3'45.16"E. The adults were kept in plastic containers, also known as honey jars (10x10x15cm), at 26ºC temperature with natural day/night photoperiod with sugar water (Fig. 4.11) that served as a food source. The plastic containers had a very fine sieve-like material in the cap that prevented the adults from escaping and to let oxygen through (Fig. 4.12). The adults laid their eggs inside the bottles.

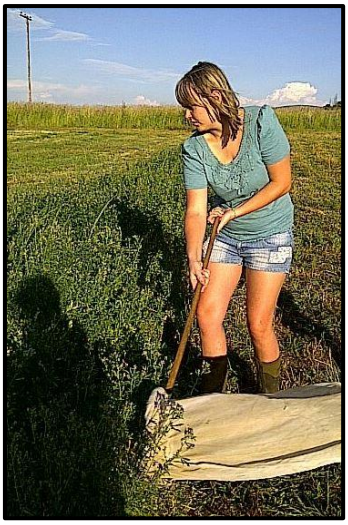

Figure 4.10: The lucerne field were the *Chrysoperla pudica* adults were collected.

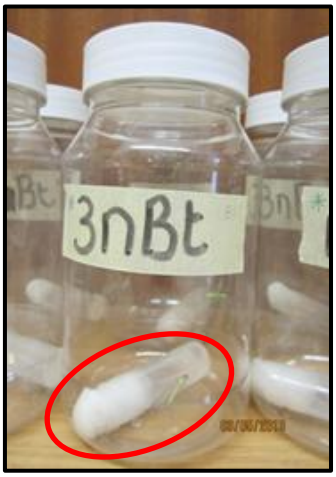

Figure 4.11: Plastic container used in the rearing of *Chrysoperla pudica* adults, with sugar water (indicated with the red circle).

Figure 4.12: The fine sieve-like material in the cap of the plastic containers where *Chrysoperla pudica* adults were kept, to prevent the adults from escaping.

The eggs that were laid were collected every third day, by moving the adults to a new plastic container, using a pooter (Fig. 4.13). The eggs were then carefully removed by cutting the egg (Fig. 4.14) of at its stalk. Each egg will then be placed individually into a test tube (70x10mm) (Fig. 4.15a) covered with a very fine material to prevent the larvae from escaping (Fig. 4.15b), where hatching took place. Test tubes were kept at 26 ± 1 ºC with natural day/night photoperiod and a humidity of 60-70% where they hatched.

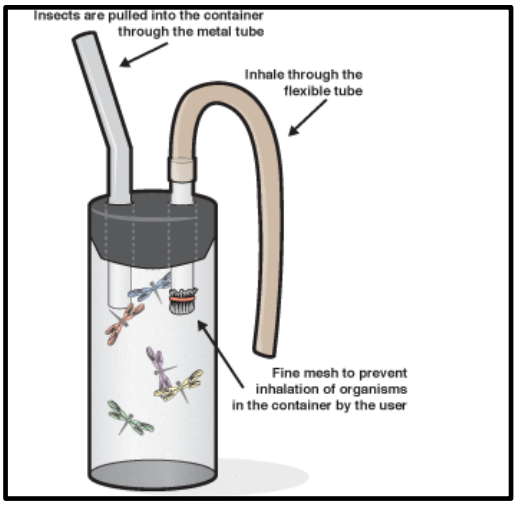

Figure 4.13: A schematically representation of a pooter, used to move *Chrysoperla pudica* adults between plastic containers (Ento, 2009).

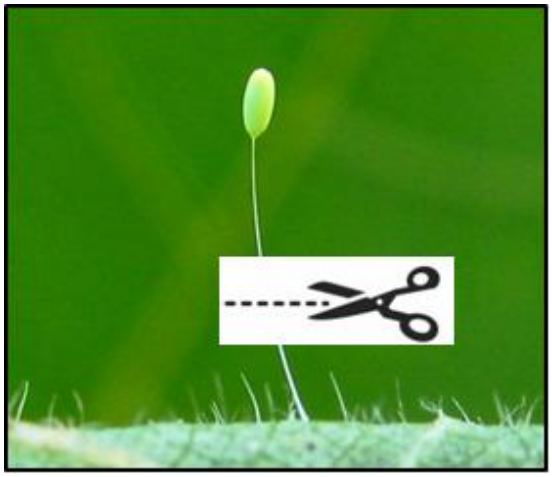

Figure 4.14: An illustration to indicate how the *Chrysoperla pudica* eggs were removed from the containers.

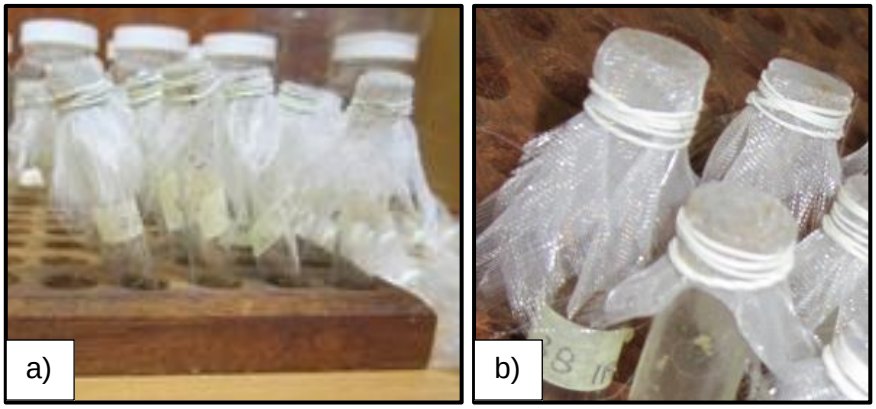

Figure 4.15: a) Test tubes were hatching and feeding of *Chrysoperla pudica* larvae took place, b) the test tubes were covered with a very fine material to prevent the larvae from escaping.

These individuals that hatched were either placed back into the rearing colony or used in the feeding experiments. Aphids were provided daily as food (Fig. 4.16) for the individuals that were placed back in the rearing colony. Debris was removed every day from the test tube before fresh food was provided. Pupation (Fig. 4.17) and adult emergence took place inside the test tubes. Newly emerged adults were moved to the plastic containers to uphold the rearing colony.

Figure 4.16: *Chrysoperla pudica* larva eating an aphid

Figure 4.17: Cocoon containing the pupae of *Chrysoperla pudica.*

References

Ento, M. 2009. Beetle and bug blog. [Web:] http://scene.asu.edu/habitat/equipment/illustrator/aspirator.gif. Date of access 21 October 2012.

Appendix III:

EnviroLogix QualiPlate Kit for Cry1Ab

Website: *www.envirologix.com*

Intended use:

The EnviroLogix QualiPlate Kit for Cry1Ab/Cry1Ac is designed for the non-quantitative laboratory detection of:

- Cry1Ab protein in Bt11, MON810 or Bt176 corn leaf tissue, or in Bt11 or MON810 corn seed samples or even insect samples containing Bt;

How the Test Works:

This EnviroLogix QualiPlate Kit is a "sandwich" Enzyme-Linked ImmunoSorbent Assay (ELISA). In the test, plant leaf, seed or insect sample extracts are added to test wells coated with antibodies raised against Cry1Ab toxin. Any residues present in the sample extract bind to the antibodies, and are then detected by addition of enzyme (horseradish peroxidase)-labeled Cry1Ab antibody.

After a simple wash step, the results of the assay are visualized with a colour development step; colour development is proportional to Cry1Ab concentration in the sample extract.

Lighter color = Lower concentration
Darker color = Higher concentration

Materials needed not provided by the Kit

- 1 N Hydrochloric acid (HCl) Stop Solution. Prepare by adding 83 mL of concentrated HCl (36.5-38.0%) to 917 mL of distilled or deionized water; work in a fume hood and use proper protective gear. This reagent may be stored at room temperature for 2 years.
- Disposable tip, adjustable air-displacement pipettes which will measure 50 and 100 microliters (µL), preferably of multi-channel style.
- Marking pen (indelible)

- Tape or Parafilm®
- Timer
- Microtiter plate reader
- Wash bottle, or microtiter plate or strip washer
- Orbital plate shaker (optional)
- Calibrators or Standards. This kit may be used in a quantitative fashion with user-supplied calibrators. For example, corn flour standards containing known percentages of Bt11- or MON810-expressing corn are available from the European Commission Joint Research Centre, Institute for Reference Materials and Measurements (Retieseweg, B-2440 Geel, Belgium), and can be used to calibrate this test for measurement of ground corn samples. In order for this to work, it is imperative that the samples be ground to the same consistency as the calibrators, and that both are extracted with the same extraction buffer, buffer-to-sample ratio, and extraction time. Alternatively, if the user can obtain pure Cry1Ab or Cry1Ac protein, the kit can be calibrated with these materials. In this instance, complete extraction of the protein from the sample is required to obtain the best estimate of the amount of Cry1 protein in the sample.

Preparation of Solutions

Wash Buffer: Add the contents of the packet of Buffer Salts (phosphate buffered saline, pH 7.4 - Tween 20) to 1 litre of distilled or de-ionized water, and stir to dissolve (Fig: 4.18). Store refrigerated when not in use; warm to room temperature prior to assay.

Figure 4.18: Prepare wash buffer and extraction solutions.

Sample Preparation

Sample Extraction:

Sample extraction protocols are to be designed and validated by the individual users of this kit. The following suggestions are guidelines, and define the manner in which the kit is performance tested by the manufacturer.

1. Green leaf samples: Extract green corn leaf samples (Fig: 4.19) that are 5-10 mm2 in size with 250 µL of Extraction Buffer. The extraction efficiency will vary proportionately with the amount of tissue disruption and mixing performed. Use extreme caution to prevent sample-to-sample cross-contamination with plant tissue or exudate.
2. Single seed samples: Crush corn seeds and extract each with 0.75 to 1 mL of Extraction Buffer. Mix thoroughly, and then allow solids to settle before transferring extract to the assay plate.

Figure 4.19: Punch leaf sample.

How to Run the Assay

- Read all of these instructions before running the kit.
- Allow all reagents to reach room temperature before beginning (at least 30 minutes with un-boxed plates and reagents at room temperature - do not remove plates from bag with desiccant until they have warmed up).
- Organize all reagents, sample extracts, and pipettes so that step 1 can be performed in 15 minutes or less. The use of a multichannel pipette is strongly recommended.

- If more than four strips are to be run at one time, the loading time will most likely exceed 15 minutes, and the use of a multi-channel pipette is recommended.
- If four or fewer strips are to be run, use a disposable-tip air-displacement pipette and a clean pipette tip to add each Calibrator and diluted sample extract to the wells. Conjugate, Substrate, and Stop Solution may be added in the same manner; alternatively, use a repeating pipette for these three reagents.
- If fewer than all twelve strips are used, reseal the unneeded strips and the desiccant in the foil pouch provided, and refrigerate (Fig: 4.20).

Figure 4.20: Remove unneeded strips.

- Use the well identification markings on the plate edge to guide you when adding the samples and reagents. It is recommended that at least two wells each of Blank (Extraction Buffer) and Cry 1Ab Positive Control be run on each plate. Additional quality control samples may be added at the discretion of the user. Sample extracts may be run in either single or duplicate wells. See example of typical assay setup, Figure 4.25.

1. Add 50 µL of Cry1Ab Enzyme Conjugate to each well of the plate. Immediately follow with 50 µL of Extraction Buffer Blank, 50 µL of Cry1Ab Positive Control, and 50 µL of each sample extract to their respective wells. Follow this same order of addition for all reagents (Fig. 4.21).

NOTE: It is strongly recommended that a multi-channel pipette be used in steps 1, 5, and 7.

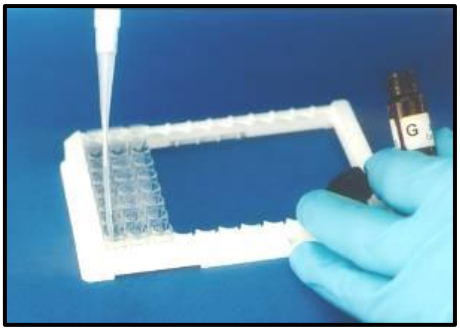

Figure 4.21: Add Conjugate, Control, and sample extract.

2. Thoroughly mix the contents of the wells by moving the plate in a rapid circular motion on the bench top for a full 20-30 seconds (Fig. 4.22). Be careful not to spill the contents!

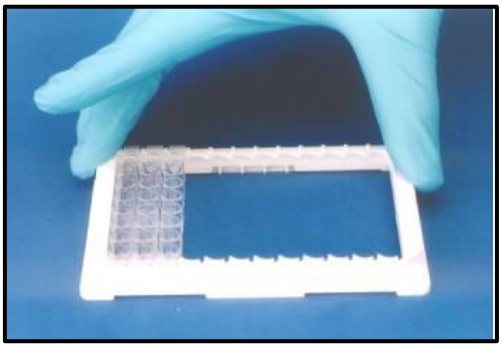

Figure 4.22: Mix plate.

3. Cover the wells with tape or Parafilm to prevent evaporation and incubate at ambient temperature for 1 to 2 hours. If an orbital plate shaker is available shake plate at 200 rpm.

NOTE: Users shall determine appropriate incubation times to give the best results with the tissue disruption/extraction methods in use.

4. After incubation, carefully remove the covering and vigorously shake the contents of the wells into a sink or other suitable container. Flood the wells completely with Wash Buffer, and then shake to empty (Fig. 4.23). Repeat this wash step three times. Alternatively, perform these four washes (300 µL/well) with a microtiter plate or strip washer. Slap the plate on a paper towel to remove as much water as possible.

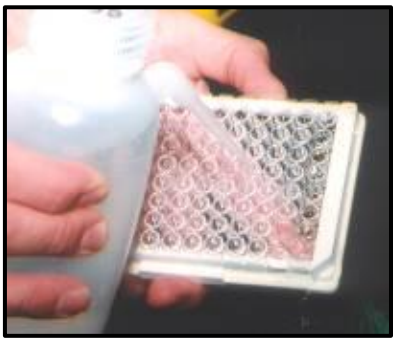

Figure 4.23: Bottle wash method.

5. Add 100 µL of Substrate to each well.

6. Thoroughly mix the contents of the wells, as in step 2. Cover the wells with new tape or Parafilm and incubate for 15 to 30 minutes at ambient temperature. Use orbital shaker if available.

NOTE: Users shall determine appropriate incubation times to give the best results with the tissue disruption/extraction methods in use.
Caution: Stop Solution is 1.0N Hydrochloric acid. Handle carefully.

7. Add 100 µL of Stop Solution to each well and mix thoroughly (Fig. 4.24). This will turn the well contents yellow.

NOTE: Read the plate within 30 minutes of the addition of Stop Solution.

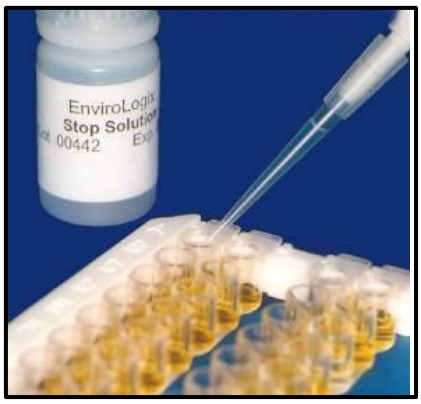

Figure 4.24: Complete protocol, and then add Stop Solution.

How to Interpret the Results

Spectrophotometric Measurement

1. Set the wavelength of your microtiter plate reader to 450 nanometers (nm). (If it has dual wavelength capability, use 600, 630 or 650 nm as the reference wavelength.)
2. Set the plate reader to blank on the Extraction Buffer Blank wells. If the reader cannot do this, measure and record the optical density (OD) of each well's contents, then subtract the average OD of the Blank wells from each of the readings.

Interpreting the Results

Compare the OD's of the sample extracts to those of the Positive Control to determine presence or absence of Cry1Ab endotoxin in your sample extract. Samples with absorbance's close to that of the Blank wells (and less than that of the Positive Control wells) are presumed to be free of Bt endotoxin. Samples with absorbance's significantly higher than those of the Blank wells are positive for Bt endotoxin content (Fig. 4.26).

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79	S87
B	PC	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80	S88
C	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73	S81	S89
D	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74	S82	S90
E	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75	S83	S91
F	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76	S84	S92
G	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77	S85	BL
H	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78	S86	PC

"BL" = Blank wells (Extraction Buffer)

"PC" = Cry1Ab Positive Control Wells

"S.." = sample extracts

Figure 4.25: Example of a typical assay setup

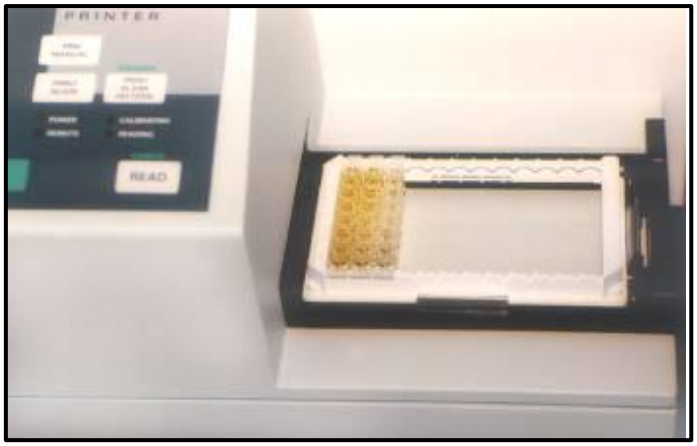

Figure 4.26: Read plates in a Plate Reader within 30 minutes of the addition of Stop Solution.

Precautions and Notes

- Store all Kit components at 4°C to 8°C (39°F to 46°F) when not in use.
- Do not expose Kit components to temperatures greater than 37°C (99°F) or less than 2°C (36°F).
- Allow all reagents to reach ambient temperature (18°C to 27°C or 64°F to 81°F) before use.
- Do not use kit components after the expiration date.
- Do not use reagents or test plates from one Kit with reagents or test plates from a different Kit.
- Do not expose Substrate to sunlight during pipetting or while incubating in the test wells.
- Do not dilute or adulterate test reagents or use samples not called for in the test procedure.
- Cry 1Ab Cry1Ac proteins can be degraded by heat and sunlight. Take samples from green, actively growing leaves. Leaf samples that cannot be extracted immediately may be stored frozen for up to 1 week prior to analysis. Seeds may be stored for at least 6 months under cool, dry conditions.
- As with all tests, it is recommended that results be confirmed by an alternate method when necessary.
- Observe any applicable regulations when disposing of samples and kit reagents.

Appendix IV:

ELISA-test plate readings

Table 10 shows that insect c (*B. fusca* larvae) optical density was very high, higher than 3.000 and did need to be diluted to get an accurate reading. The reading for the leaves first seem if it is going to work but as the analysis is done, it is clear that the sample falls outside of the standard curve and that the assay should be repeated at a lower dilution (Table 10 and 11).

The following will be the key throughout the entire *ELISA*-test:

Insect A *Chrysoperla pudica* larvae reared on Bt resistant *Busseola fusca* larvae that was reared on Bt maize and aphids.

Insect B *Chrysoperla pudica* larvae reared on Bt resistant *Busseola fusca* larvae that was reared on Bt maize.

Insect C Bt resistant *Busseola fusca* larvae reared on Bt maize.

Leaf 1 - 3 Bt maize whorls.

Table 10: The plate reading done from table 3 – optical density (OD) reading at 450 nm minus blank.

	1	2	3	4
A	0.29	0.007	?????	0.013
B	0.306	-0.007	?????	0.012
C	0.534	0.107	?????	0.006
D	0.575	0.099	0.019	0.009
E	1.536	0.105	0.02	
F	1.507	0.039	0.022	
G	2.389	0.052	0.014	
H	2.434	0.037	0.004	

Question marks indicate overflow ie reading is higher than OD of 3.000

Table 11: The results of the toxin present in each sample.

Since the standard curve ranges from 1 to 10 ng/ml values below 1 ng/ml are reported as <1 ng/ml and values above 10 ng/ml are reported as >10 ng/ml

Well ID	Well	OD (450 nm)	Duilution factor	Uncorrected toxin concentration (ng/ml)	Uncorrected toxin mean concentration (ng/ml)	SD	Volume used in assay (ul)	Extraction volume (ul)	Weight of sample (g)	Comments
Insect A	F5	0.107	Undiluted	<1	<1	NA	50	500	0.155	Sample contains <1 ng/ml toxin
	G5	0.099		<1						
	H5	0.105		<1						
Insect B	A6	0.039	Undiluted	<1	<1	NA	50	500	0.143	Sample contains <1 ng/ml toxin
	B6	0.052		<1						
	C6	0.037		<1						
Insect C	D6	?????	Undiluted	>10	>10	NA	50	500	0.043	Sample contains >10 ng/ml. Repeat analysis with dilution at 1:10, 1:100 and 1:1000
	E6	?????		>10						
	F6	?????		>10						
Leave - 1	G6	0.019	1:10000	<1	<1	NA	50	1000	0.101	Sample contains <1 ng/ml (outside standard curve). Repeat analysis with lower dilution at 1:1000
	H6	0.02		<1						
	A7	0.022		<1						
Leave - 2	B7	0.014	1:10000	<1	<1	NA	50	1000	0.105	Sample contains <1 ng/ml (outside standard curve). Repeat analysis with lower dilution at 1:1000
	C7	0.004		<1						
	D7	0.013		<1						
Leave - 3	E7	0.012	1:10000	<1	<1	NA	50	1000	0.1	Sample contains <1 ng/ml (outside standard curve). Repeat analysis with lower dilution at 1:1000
	F7	0.006		<1						
	G7	0.009		<1						

The second assay is conducted only on the samples that needed to be diluted because the Cry 1Ab toxin concentration was too high to determine correctly (Table 12 and 13).

Table 12: The plate reading done from table 4 – optical density (OD) reading at 450 nm minus blank.

	1	2	3	4
A	0.26	0.002	0.026	0.332
B	0.277	-0.003	0.006	0.346
C	0.473	0.958	0.004	0.351
D	0.484	0.897	0.547	0.343
E	1.28	0.968	0.54	
F	1.394	0.087	0.547	
G	2.095	0.109	0.296	
H	2.127	0.108	0.338	

Table 13: The result of the toxin present in each sample after the dilutions has been made.

Well ID	Well	OD (450 nm)	Duilution factor	Uncorrected toxin concentration (ng/ml)	Uncorrected mean toxin concentration (ng/ml)	SD	Volume used in assay (ul)	Extraction volume (ml)	Weight of sample (g)	Comments	Corrected mean toxin concentration for dilution (ug toxin/g tissue)
Insect C	C2	0.958	10	4.31	4.23	0.19	50	0.5	0.04		0.5
	D2	0.897		4.02							
	E2	0.968		4.36							
Insect C	F2	0.087	100	<1	<1	NA	50	0.5	0.04	Sample contains <1 ng/ml (outside standard curve)	NA
	G2	0.109		<1							
	H2	0.108		<1							
Insect C	A3	0.026	1000	<1	<1	NA	50	0.5	0.04	Sample contains <1 ng/ml (outside standard curve)	NA
	B3	0.006		<1							
	C3	0.004		<1							
Leave 1	D3	0.547	100	2.31	2.30	0.02	50	1	0.10		2.3
	E3	0.54		2.28							
	F3	0.547		2.31							
Leave 2	G3	0.296	100	1.09	1.22	0.11	50	1	0.11		1.2
	H3	0.338		1.29							
	A4	0.332		1.26							
Leave 3	B4	0.346	100	1.34	1.34	0.02	50	1	0.10		1.3
	C4	0.351		1.36							
	D4	0.343		1.32							