Response of selected non-target Lepidoptera, Coleoptera and Diptera species to *Cry1Ab* protein expressed by genetically modified maize

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ABSTRACT

The environmental impacts of genetically modified (GM) crop plants such as Bt (*Bacillus thuringiensis*) maize have not yet been fully assessed in South Africa. Bt maize designed to express Bt endotoxin for control of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) is planted on approximately 1.103 million hectares in South Africa. The monitoring of GM crops after release is important in order to assess and evaluate possible environmental effects. No risk assessment for Bt maize was done in South Africa before its release in 1998 and no targeted post-release monitoring of possible resistance development or impact on non-target species have been done. Awareness has risen in South Africa through research highlighting the possible effects GM crops may have. The aim of this study was to determine, through feeding experiments, the effects of Bt maize on selected non-target Lepidoptera, Coleoptera and Diptera species that occur in maize agro-ecosystems in South Africa. Results provide information for use in future risk assessment studies on Bt maize and indicate which species could possibly be of importance in post-release monitoring of Bt maize. Priority insect species were identified and laboratory- and semi-field experiments were conducted to evaluate the effect of Bt maize on these species. In the light of the reportedly lower toxicity of Bt maize to certain noctuid borers, the effect of Bt maize was evaluated on *Sesamia calamistis* (Hampson), *Agrotis segetum* (Denis & Schiffermüller), and *Helicoverpa armigera* (Hubner). Feeding studies were also conducted to determine the effect of Bt maize on non-target Coleoptera, i.e. *Heteronychus arator* Fabricius (Coleoptera: Scarabaeidae) and *Somaticus angulatus* (Fahraeus) (Coleoptera: Tenebrionidae). The effect of indirect exposure of the stem borer parasitoid *Sturmiopsis parasitica* (Curran) (Diptera: Tachinidae) to Bt toxin was evaluated to determine if there is any effect when it parasitizes Bt-resistant *B. fusca* larvae that have fed on Bt maize. Results from the study conducted with *S. calamistis* indicated that Bt maize of both events (Bt11 and MON810) were highly toxic to *S. calamistis*. The behavioural characteristic of *S. calamistis* to feed behind leaf sheaths and to enter stems directly did not result in escape of exposure to the toxin. Larval feeding on leaf sheaths therefore resulted in the ingestion of sufficient toxin to kill larvae before they

entered maize stems. Results showed that the effect of Cry1Ab toxin on the biology of *A. segetum* larvae and moths were largely insignificant. Whorl leaves were observed to be an unsuitable food source for *H. armigera* larvae and larval growth was poor. No larvae survived to the pupal stage on any of the Bt maize treatments. When feeding on maize ears *H. armigera* larval mass increased on non-Bt maize whereas no increase occurred on Bt maize. The feeding study conducted with Coleoptera showed that the effect of Bt maize on *H. arator* and *S. angulatus* was insignificant and no differences were observed in any of the parameters measured for the two species. Although not always significant, the percentage parasitism of Bt-consuming host larvae by *S. parasitica* was always higher compared to host larvae that fed on non-Bt maize. It could be that Bt toxin affects *B. fusca* fitness to such an extent that the immune systems of host larvae were less effective. The different parameters tested for *S. parasitica* indicated only one case where fly maggots originating from diapause host larvae feeding on non-Bt maize had a greater mass compared to host larvae that fed on Bt maize. The same applied to *S. parasitica* pupal length. For other parameters tested there were no significant differences. *Sesamia calamistis* is stenophagous and occurs in mixed populations with other borer species. It was therefore concluded that the ecological impact of local extinctions of *S. calamistis* caused by Bt maize is not expected to be great. Bt maize will most likely not have any significant effect on the control of *A. segetum* under field conditions. The feeding study conducted with *H. armigera* quantified the effects of Bt maize on this species and provided important information on the potential of Bt maize as protection against this polyphagous pest. However, the likelihood of *H. armigera* becoming an important secondary pest is high. It can be concluded that the Cry1Ab toxin targeting lepidopteran pests will not have adverse effects on *H. arator* or *S. angulatus*. Although some adverse effects were observed on *S. parasitica* mass and pupal length it is most likely that this will not contribute to adverse effects in the field, but that there rather be synergism between Bt maize and *S. parasitica*. An ecological approach was followed in which the potential effects of exposure of priority species to Bt toxin in maize was investigated. A series of selection matrixes were developed in which each of the above mentioned species was ranked for its maximum potential exposure to Bt toxin by assessing it occurrence**,** abundance**,** presence and linkage in the maize ecosystem. Through the use of

these selection matrixes, knowledge gaps were identified for future research and to guide the design of ecologically realistic experiments. This study contributes to knowledge regarding the possible effects of Bt maize on the most economically important non-target pests in South Africa. There is, however, a need to evaluate other non-target species in feeding studies, as well as in field studies. From this study it can be concluded that some species can be eliminated from further testing since Bt maize had no adverse effect while more research have to be conducted on other species.

Keywords: *Agrotis segetum*, Bt maize, ecological model, *Helicoverpa armigera*, *Heteronychus arator*, non-target species, risk assessment, *Sesamia calamistis*, *Somaticus angulatus*, *Sturmiopsis parasitica*.

OPSOMMING

Titel: Die reaksie van geselekteerde nie-teiken Lepidoptera, Coleoptera en Diptera spesies op Cry1Ab proteïen uitgedruk deur geneties-gemodifiseerde mielies

Die omgewingsimpak van geneties-gemodifiseerde (GG) gewasse soos Bt mielies is nog nie volledig in Suid-Afrika ondersoek nie. Bt mielies is ontwikkel om Bt-endotoksiene uit te druk vir die beheer van *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) en *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) en word op ongeveer 1.103 miljoen hektaar geplant in Suid-Afrika. Die monitering van GG gewasse na die kommersiële vrystelling daarvan is belangrik om sodoende moontlike omgewingseffekte waar te neem. Geen risiko-analise vir Bt mielies is in Suid-Afrika gedoen voor die vrystelling daarvan in 1998 nie en geen gerigte post-vrystelling monitering vir moontlike weerstandsontwikkeling of impak op nie-teiken spesies is gedoen nie. Bewustheid van moontlike effekte wat GG gewasse kan hê, het onlangs eers begin opvlam in Suid-Afrika. Die doel van die studie was om die effek van Bt mielies op geselekteerde nie-teiken Lepidoptera, Coleoptera en Diptera spesies vas te stel wat kan voorkom in die mielie-agro-ekosisteem, deur gebruik te maak van voedings-eksperimente. Die resultate voorsien inligting vir die gebruik in toekomstige risiko-analises op Bt mielies. Prioriteit insekspesies is geïdentifiseer en laboratorium- en semi-veldeksprimente is gedoen om die effek van Bt mielies op hierdie spesies te evalueer. In die lig van die gerapporteerde laer toksiese effek van Bt mielies teen sekere Noctuidae spesies, is die effek van Bt mielies op *Sesamia calamistis* (Hampson), *Agrotis segetum* (Denis & Schiffermüller) en *Helicoverpa armigera* (Hubner) geëvalueer. Voedingstudies is ook gedoen met *Heteronychus arator* Fabricius (Coleoptera: Scarabaeidae) en *Somaticus angulatus* (Fahraeus) (Coleoptera: Tenebrionidae) om die effek van Bt mielies op die nie-teiken Coleoptera-spesies te bepaal. Die parasitoïed *Sturmiopsis parasitica* (Curran) (Diptera: Tachinidae) is ook geëvalueer om die effek te bepaal wanneer dit Bt-weerstandbiedende *B. fusca* larwes, wat gevreet het op Bt mielies, parasiteer. Resultate van die studie met *S. calamistis* het getoon dat Bt mielies van altwee uitkomstes (Bt11 en MON810) uiters toksies is vir hierdie spesie. Die gedragseienskap van *S. calamistis* om agter die

blaarskede te voed en dan die stam direk te penetreer het nie gelei tot die ontsnapping van toksiene nie. Larwes wat op die blaarskede gevoed het neem dus genoeg toksiene in om gedood te word voordat die stam binnegedring word. Resultate wys dat die effek van Cry1Ab toksiene op die biologie van *A. segetum* larwes en motte grootliks nie-betekenisvol is. Dit is gevind dat kelkblare nie ‘n geskikte voedingsbron vir *H. armigera* larwale ontwikkeling is nie aangesien larwale ontwikkeling swak was. Geen larwes het tot die papiestadium oorleef op Bt mielies nie. Indien *H. armigera* larwes op mieliekoppe gevoed het, het hul massa toegeneem op die nie-Bt koppe, maar geen toename is waargeneem op Bt-koppe nie. Voedingstudies het getoon dat daar geen betekenisvolle effek van Bt mielies op die Coleoptera *H. arator* en *S. angulatus* was nie. Geen betekenisvolle verskil is waargeneem in enige van die parameters wat gemeet is vir die twee Coleoptera-spesies nie. Al was daar nie altyd ‘n betekenisvolle verskil nie, was die persentasie parasitisme van *S. parasitica* op die gasheerlarwes wat gevreet het op Bt mielies altyd hoër in vergelyking met gasheerlarwes wat gevoed het op nie-Bt mielies. Dit kan wees dat Bt-toksiene *B. fusca* larwes so beïnvloed dat die immuunstelsel van die gasheerlarwes minder effektief is. Die verskillende parameters wat vir *S. parasitica* ge-ëvalueer is toon slegs een geval waar vliegmaaiers afkomsig van diapouse-gasheerlarwes wat gevoed het op nie-Bt mielies ‘n groter massa het as dié afkomstig van gasheerlarwes wat gevoed het op Bt mielies. Dieselfde tendens is met *S. parasitica* papielengte waargeneem. Vir die ander parameters is geen betekenisvolle verskille waargeneem nie. *Sesamia calamistis* is ‘n stenofage spesie en kom in gemengde populasies met ander stamboorderspesies voor wat tot gevolg het dat die ekologiese impak van lokale uitwissing deur Bt mielies vermoedelik nie groot sal wees nie. Bt mielies sal waarskynlik nie ‘n betekenisvolle effek op die beheer van *A. segetum* onder veldtoestande hê nie. Die voedingstudies met *H. armigera* het die effek van Bt mielies op hierdie spesie gekwantifiseer en voorsien belangrike inligting oor die potensiaal wat Bt mielies bied teen vreetskade van hierdie plaag. Die moontikheid dat *H. armigera* ‘n belangrike sekondêre plaag kan word is egter groot. Die gevolgtrekking wat uit hierdie studie gemaak word is dat Cry1Ab proteïen wat Lepidoptera teiken nie ‘n negatiewe effek sal hê op *H. arator* of *S. angulatus* nie. Daar is sekere negatiewe effekte op *S. parasitica* massa en papielengte waargeneem, maar dit is hoogs onwaarskynlik dat dit sal bydra tot

negatiewe effekte in die veld. Daar mag dalk eerder ‘n sinergistiese effek tussen Bt mielies en *S. parasitica* wees. In hierdie studie is ‘n ekologiese benadering gevolg waarin die potensiële effek van blootstelling van prioriteit-spesies aan Bt-toksiene in mielies ondersoek is. ‘n Reeks seleksie-matrikse is ontwikkel waarin elkeen van die bogenoemde spesies gerangskik is volgens maksimum potensiële bloodstelling aan Bt- toksiene deur evaluering van verspreiding, volopheid, teenwoordigheid en skakeling in die mielie-ekosisteem. Deur die gebruik van die seleksie-matrikse, is leemtes geïdentifiseer vir verdere navorsing en om leiding te gee in die ontwikkeling van verdere ekologiese realistiese eksperimente. In hierdie studie is slegs enkele ekonomies-belangrike nie-teikenspesies ge-evalueer wat moontlik deur Bt mielies geaffekteer kan word. Daar is ‘n noodsaaklikheid om ander moontlike nie-teiken spesies te evalueer vir moontlike effekte. Uit hierdie studie kan die gevolgtrekking gemaak word dat sommige spesies uitgeskakel kan word van verdere evaluering, aangesien resultate uit voedingstudies toon dat Bt mielies nie ‘n negatiewe effek het nie. Die teenoorgestelde is egter ook moontlik waar sekere negatiewe effekte waargeneem word en waar verdere studies nodig is om tot ‘n gevolgtrekking te kan kom.

Sleutelwoorde: *Agrotis segetum*, Bt mielies, ekologiese model, *Helicoverpa armigera*, *Heteronychus arator*, nie-teiken spesies, risiko-analise, *Sesamia calamistis*, *Somaticus angulatus*, *Sturmiopsis parasitica*.

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CHAPTER 1: Introduction and literature review

1.1. Introduction

Genetically modified (GM) crops are here to stay. In 2008 the global area of transgenic crops reached 800 million hectares (James, 2008). The question is therefore not to grow or not to grow GM crops, but how to manage the use of transgenic crops. Scientists recognize the benefits of GM crops, but also note that releases into the environment could have adverse impacts under some circumstances and therefore urge continued science-based assessment of benefits and risks (Bhatia *et al*., 1999; Barton & Dracup, 2000; Sharma *et al*., 2000; Hill & Sendashonga, 2006). Although GM crops have many advantages, it also has like any other pest management technology some disadvantages. The most important advantage of GM crops is the reduction in the use of insecticides. This reduction in the number of insecticide applications result in economic benefits to farmers (Cannon, 2000; Meeusen & Warren, 1989; Nottingham, 2002) and is also beneficial to the environment. A GM crop that is more target specific can be an alternative for widespread application of broad-spectrum insecticides that result in high insect mortality (Musser & Shelton, 2003). Target pest resurgence is a phenomena often observed after insecticide applications, which also have substantial and deleterious impacts on the natural enemy complex (Armenta *et al*., 2003; Deedat, 1994; Eckert *et al*., 2006).

The first and most important disadvantage that a GM crop may have is the non-target effect on the environment. Transgenic crops are not inherently harmful; they only present problems where the new traits, or combinations of traits, made possible by modern gene technology producing unwanted effects in the environment. Different genetically engineered crops will present different problems depending on the new genes they contain, the characteristics of the parent crop and the region (environment) in which they are grown (Rissler & Mellon, 2000). If such problems arise it could open a whole new

dimension on the unexpected impacts of transgenic crops on non-target organisms that play key or sometimes unknown roles in the ecosystem (Altieri, 2004).

Ecological interactions are complex, and adverse environmental impacts may be experienced along food chains and throughout ecosystems (Nottingham, 2002). Because the number of crops and genes is so large and varied, identifying and categorizing potential risks of transgenic crops remains a challenge (Rissler & Mellon, 2000). The push for "monoculture crop" uniformity will not only destroy the diversity of genetic resources, but also disrupt the biological complexity that underlies the sustainability of indigenous farming systems, for example, on the Africa continent. There are many unanswered ecological questions regarding the impact of releasing transgenic plants and microorganisms into the environment (Altieri, 2004).

Another potential disadvantage is that biotechnology is being pursued to repair the problems caused by previous agrochemical technologies. Based on the fact that more than 500 species of pests have already evolved resistance to conventional insecticides, surely pests can also evolve resistance to Bt toxins in GM crops (Altieri, 2004). This was confirmed by the first report of field resistance by the stem borer, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) to Bt maize in the Christiana region of South Africa (Van Rensburg, 2007).

Ecological risk should be assessed before GM crops are released into the environment. To say whether there are risks, ecologists need to make comparisons with and without a GM crop. This comparison with the existing situation is particularly important in agricultural ecosystems, as modern farming methods have already had a large impact on biodiversity. Experiments of this type are scarce and mostly laboratory-based or small-scale field studies where no ecological data is collected. Nevertheless, a larger picture is starting to emerge, from which a framework for assessing risk can be developed. Although the risks in many cases are relatively small, there is potential for a wide range of direct and indirect ecological effects that could result from release of GM crops. Identifying ecological risks at an early stage is therefore important (Nottingham, 2002).

1.2. GM crops in Africa compared with global status of commercialized GM crops

During 2008, the global area of GM crops continued to grow strongly reaching 125 million hectares, up from 114.3 million hectares in 2007 (James, 2008). In 2008, the number of countries growing GM crops increased to 25, and comprised 15 developing countries and 10 industrial countries. These 25 countries growing GM crops in descending order of hectares are the USA, Argentina, Brazil, India, Canada, China, Paraguay, South Africa, Uruguay, Bolivia, Philippines, Australia, Mexico, Spain, Chile, Colombia, Honduras, Burkina Faso, Czech Republic, Romania, Portugal, Germany, Poland, Slovakia and Egypt (James, 2008). The growth rate between 1996 and 2008 was an unprecedented 74-fold increase making it the fastest adopted crop technology in recent history. Significant progress was made during 2008 in Africa, with an increase from one country in 2007 to three countries in 2008, with South Africa being joined by Burkina Faso and Egypt as the only countries on the continent that has approved release of GM crops. South Africa was ranked number eight in the world with a total of 1.8 million hectares grown to GM crops in 2008. Genetically modified maize, cotton and soybean are grown in South Africa and the cropping area continuously increased since the first plantings in 1998 (James, 2008). Accordingly, Burkina Faso grew 8 500 hectares of Bt cotton for seed multiplication before initial commercialization took place and Egypt grew 700 hectares of Bt maize for the first time in 2008. During December 2008, Kenya, a pivotal GM crop country in east Africa, enacted a Biosafety Law, which will facilitate the adoption of GM crops (James, 2008).

1.3. Event MON810 and Bt11 commercialized in South Africa

Events MON810 (Monsanto) and Bt 11 (Sygenta) are the only two Bt maize events that have been approved for release in South Africa. MON810 was the first event released and hybrids containing it was planted in 1998 (first Bt maize that was planted in South Africa) (Van Rensburg, 2007). Bt 11 was only approved for release and planted for the

first time during the 2006/07 growing season. Stacking of event MON810 with the Round-up-Ready gene for herbicide tolerance has also been approved and hybrids released in South Africa.

Before understanding the concept of different events one must know what an event is. Briefly, the transgene is constructed into a plasmid, which is absorbed onto micro-projectiles that are shot into plant cells, the delivered DNA elutes from the micro-projectiles and is integrated into the plant genome, creating a transgene locus. After transformation, plant cells are selected, usually aided by a selectable marker gene, and the transformed cells are regenerated into whole plants. Transformed plants are selected for the target trait, and then incorporated into plant breeding programmes, where commercial varieties can be produced. A transgenic lineage derived from a single transformed cell is referred to as a transformation "event" (Andow *et al*., 2004).

Bt 11 was commercialized by Syngenta. It has one copy of a truncated Cry1Ab gene with the cauliflower mosaic virus (CaMV) 35S promoter. This gene is not truncated down to the active Cry1Ab toxin, but is shortened from the original bacterial gene. The marker is a phosphinothricin herbicide resistance gene, which is regulated by the CaMV 35S promoter, and the event has an intron of the maize alcohol dehydrogenases 1S gene to facilitate expression in maize (Andow, 2002).

Event MON810 has not been adequately described in the public literature, lacking both detailed characterization of the toxin and a published linkage map (Andow, 2002). MON810 was commercialized by Monsanto and was formed from two different constructs. It contains at least one copy of a truncated Cry1Ab gene with the CaMV 35S promoter. This gene is not truncated down to the active Cry1Ab toxin, but is shortened from the original bacterial gene. Although the original gene is the same truncated gene that was used to produce Bt 11, it is further reduced in size in MON810. The number of gene inserts in MON810 is not specified, and the diversity of expression products may indicate that there is more than one. The markers are *nptII*, an antibiotic-resistance gene,

and a glyphosate herbicide-resistance gene, with unspecified promoters. Expressed products of *nptII* are not detected in maize plants (Andow, 2002).

The different events can result in phenotypic differences in expression of activated *Cry* toxin in different maize hybrids. Bt 11 and MON810 have similar, but not identical, levels of expression in the whole plant (Table 1.1) (Andow, 2002). This similarity was expected because these events share a similar truncated cry gene and use the same promoter, but the differences suggest real differences in the expression. Seed companies should recognize and publish the linkage maps and details of the structure of the toxins in their events (Andow, 2002).

Expression of Bt toxins in maize is often cited in the literature to be constitutive, meaning that expression occurs in all tissues at all times (Dutton *et al*., 2003). This is misleading since different promoters have been used for the various commercial maize hybrids and these different hybrids have been shown to express different amounts of toxin in different plant tissues (Table 1.2) (Dutton *et al*., 2003). It seems that the mortality level of target pests that could be expected depends on the toxicity of different Bt maize varieties. In laboratory assessments conducted by Van Rensburg (2001), in which *B. fusca* larvae were force fed on a hybrid containing MON810, the results obtained with stem tissue during the early vegetative stages indicated that the stems of Bt maize contained sufficient levels of protein to ensure effective control. In a review on risks and management of Bt maize in Kenya, Fitt *et al*. (2004) indicated that the toxicity of currently available Bt maize varieties in that country was considered to be low and that toxicity depended on the event used. The expression levels of Bt toxin in different varieties containing the same event therefore seem to vary, although the expression of that event was high in the mother line. These aspects need careful consideration in risk assessments and decisions pertaining to the release of Bt maize varieties.

Table 1.1. Comparison of *Cry* toxin expression in some transgenic Bt maize varieties (Andow & Hilbeck, 2004b).

Event and company	Promoter	Transgene	Molecular weight of transgene product expressed in plant (kilodaltons)
176 (Syngenta)	*PEPC* and *POL* (Pollen-specific promoter)	Cry1Ab (synthetic)	65*
Bt 11 (Syngenta)	CaMV35S (modulated by IVS6 intron)	Cry1Ab (truncated, synthetic)	Possibly 65**
MON810 (Monsanto)	CaMV35S (enhanced; modulated by HSP70 intron)	Cry1Ab (truncated, synthetic)	91
CBH 351 (Aventis)	CaMV35S	Cry9C (truncated, N-, C-terminal)	68 (can be partially degraded to a 55-kDa form)
DBT 418 (Dekalb)	CaMV35S (two copies octopine synthase enhancer and introns)	Cry1Ac	66

* Three immunoreactive proteins weighing approximately 60, 40, and 36 kilodaltons were also detected in leaves, but not in pollen.

** The Cry1Ab toxin extracted from maize leaf tissue displays characteristics and activities similar to those produced in *Escherichia coli* transformed to produce Cry1Ab. The purified tryptic core proteins from both plant and microbe were shown to be similar in molecular weight by SDS-Page.

Table 1.2. Expression of *Cry* toxin in different parts of Bt maize plants (mg/g) (Andow, 2002; Dutton *et al*., 2003).

Event	Grain	Leaf	Stem	Pollen	Pith	Root	Whole plant
Event 176	0.05	2.8-4.4	0.08	7.1		0.08	0.6
Bt 11*	1.4 (kernel)	3.3	Not detected	< 0.09 (pollen dry weight)		2.2-37.0 (protein)	6.3
MON810*	0.19-0.39 (grain)	10.34	Not detected	< 0.09 (pollen dry weight)		Not detected	4.65
CBH 351	18.6 (kernel)	44	2.8	0.24	2.8	25.9	250
DBT 418	43	1.2		Not detected			0.15-1.0

Note: All values are expressed per fresh tissue weight unless otherwise noted.

* Events commercialized in South Africa.

1.4. Models for assessing the risks of transgenic crops

Risk assessment is a process by which risks are identified and the seriousness of the risk is characterized so that decisions can be made on whether or how to proceed with the technology (Andow & Hilbeck, 2004b; Hillbeck *et al*., 2006). There are different opinions of how to assess the risk of transgenic crops, but one thing they all have in common is that possible effects must be identified. Three approaches are largely used for assessing risks of genetically modified plants. These are the ecotoxicology model, non-indigenous-species model, and the ecological model (Table 1.3.).

The *ecotoxicological model* aims to evaluate the potential non-target effects of chemicals released into the environment and has been suggested for use in evaluation of non-target species effects of GM crops (Andow & Hilbeck, 2004a). Universal indicator species are chosen because of their supposed sensitivity to chemical toxins, their wide availability, their ease of culture, and their genetic uniformity (Chapman, 2002). Eckert *et al*. (2006) suggested identifying indicator organisms and developing simple methods that combined suitability and cost effectiveness for ecological risk assessment under field conditions. Such species are supposed to provide information on the likely effects of the chemical on a wider range of species (Andow & Hilbeck, 2004a). The most serious problem with this approach is that it is not consistent with the need for case-by-case risk assessment that considers the relevant transgene, crop plant, and environment. In the ecotoxicology model, the primary end point is mortality or some other acute response from short-term exposure to the chemical. These responses, however, reveal little about other ecological impacts at the population, community or ecosystem level (Elmegaard & Jagers op Akkerhuis, 2000).

Private companies that develop GM crops usually test the effect of these crops on non-target species by identifying indicator species such as honey bees, green lacewing, parasitic Hymenoptera, ladybird beetles, *Daphnia*, earthworms and Collembola (AGBIOS, 2007). However, using earthworm for example, as an indicator species is not of much value because temperature and moisture seem to be the main inducing factors

(Reinecke & Ryke, 1972), where temperature and moisture are not sufficient earthworms will not be present. In South Africa it has been reported that earthworms are not too likely to be found in maize fields because the temperature and moisture is not suitable. This is the kind of mistake that can be made if the ecotoxicological model is used for assessing the risk of GM crops.

Table 1.3. Comparison of three models for assessing the risks of transgenic plants to non-target organisms (Andow & Hilbeck, 2004b).

	Ecotoxicology model	**Non-indigenous-species model**	**Ecological model**
Species selection criteria	Indicator species	Species at risk and other non-target species	Representatives of functional groups
End point	Acute toxicity	Invasiveness	Fitness
Clarity and measurability	Clear and measurable	Difficult to measure, estimated by expert opinion	Clear, but requires careful experimentation
Exposure within individuals	Short-term exposure	Long-term exposure	Long-term exposure
Exposure across generations	No cross-generation exposure	Considers long-term exposure across generations	Fitness can be extrapolated across generations
Test methodology	Single-chemical, dose-response assay	Synthesize expertise	Exposure to whole plant and single-chemical assay
Repeatability and consistency	Repeatable and consistent	Possibly repeatable and consistent	Repeatable and consistent
Relevance to risk	Not very relevant	Relevant	Relevant
Relation to decision making process	Linked, weak scientific justification	Often linked	Can be linked

Although acute toxicity testing of the transgene product in the laboratory should be part of initial testing of GM crops, it is insufficient to ensure accurate decision making in risk assessment. It will also be critical to abandon the use of universal indicator species and develop a species selection process that allows risk assessment to adapt on a case-by-case basis to the particularities of the transgene, crop plant, and environment in which the transgenic plant will be used (Andow & Hilbeck, 2004b).

The *non-indigenous-species model* has been repeatedly proposed as a useful model for understanding the environmental effects of transgenic crops, but little consideration has

been given to the applicability of the risk assessment methods (Andow & Hilbeck, 2004b). The risk assessment is initiated by identifying a commodity involved in international trade. The next step is identification of all non-indigenous species that are associated with the commodity and which may pose an environmental risk as potential pests in the country of importation. The only non-target species risks that are evaluated using this model are potential plant pest risks (Andow & Hilbeck, 2004b). When assessing the possible impact of transgenic crops, it will probably be insufficient to consider only potential plant pest risk.

Species selection based on an *ecological model* as suggested by Andow & Hilbeck (2004a, b), and Hilbeck *et al*. (2006) is case specific, depends on the transgenic crop and its cropping context, and prioritizes species that could be adversely affected by the transgenic crop. Species selection follows certain steps: (1) identification and screening for appropriate functional groups of biodiversity, (2) list and prioritize non-target species and processes for use in a selection matrix, (3) trophically mediated exposure path ways to transgenic plant and trans-gene products, (4) adverse effect scenarios for trophically mediated and other ecological effects, and (5) testing hypotheses and experimental designs to test for adverse effects (Hilbeck *et al*., 2006).

An appropriate experimental end point when using the ecological model is generational relative fitness which comprises the relative lifetime survival and reproduction of the non-target species. Survival experiments on the species that will be exposed to transgenic plants should last through one full generation, including all the immature stages (Andow & Hilbeck, 2004b). Generational relative fitness is a particularly useful end point, because it relates directly to risk. If the transgenic plant adversely affects a non-target species, its effects will come through some component of relative fitness. Two methodologies are needed to provide adequate information for non-target risk assessment. First, the methodology of the ecotoxicology model should be modified to use long-term exposure of the transgene product to the test species, mimicking potential exposure in the environment. The second methodology which follows on this is the “whole plant” method. This method evaluates the effects of the transgenic plant, which

may be greater than the isolated effect of the transgene product (Andow & Hilbeck, 2004b). The use of the ecological model is thus the most appropriate to asses the risk of transgenic crops. In a study conducted by Van Wyk *et al*. (2007) the ecological model was used to identify priority Lepidoptera species on maize in South Africa. In this ecological model priority non-target Lepidoptera species were identified for monitoring as well as further research, to determine the effect of Bt maize on these non-target species.

1.5. Concerns related to GM crops in South Africa

Concerns have been raised that environmental impacts have not yet been fully assessed for genetically engineered crop plants such as Bt maize. Bt maize designed to express Bt endotoxin for control of *B. fusca* and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) is planted on approximately 1.103 million hectares in South Africa (Gouse *et al*., 2009). The monitoring of GM crops after release is important in order to assess and evaluate possible environmental effects (Lang, 2004). Smale & De Groote (2003) suggested that diagnostic research of transgenic crops is important before rather than after release. The lack of a pre-release risk assessment of GM crops and post-release monitoring as suggested by Andow & Hilbeck (2004a) can become a future problem in South Africa. No risk assessment for Bt maize was done in South Africa before its release in 1998 and no post-release monitoring of possible resistance development or impact on non-target lepidopterans have been done. Recently awareness of biosafety issues increased in South Africa through highlighting the possible effects GM crops can have (Kruger *et al*., 2009; Van den Berg *et al*., 2007; Van Wyk *et al*., 2007; Van Wyk *et al*., 2008).

Pest management can have substantial impacts on non-target species both within and outside the units being managed (Dutton *et al*., 2003). Assessment of these impacts is hampered by the lack of even the most basic checklist of the species present in most systems (Losey *et al*., 2003). The first step towards a comprehensive insect management

program that would provide adequate pest suppression, maintenance of ecological services, and minimal impact on rare species is a detailed assessment of which insect species are likely to exist in the managed system. Unfortunately, this baseline accounting of insect species is lacking for almost every managed system (Losey *et al*., 2003). In South Africa, research conducted by Van Wyk (2006) started to address this issue.

Although there are few data on the ecological roles of most Lepidoptera in maize, it has been documented across several systems that many lepidopteran species contribute to the biological control of important weed species, and they provide alternate prey for the natural enemies of important pests (Losey *et al*., 2003).

In South Africa Bt maize events MON810 (Monsanto) and Bt 11 (Syngenta) are commercialized. Both these events express Cry1Ab in leaves and pollen (Dutton *et al*., 2003). Studies were conducted on target species of Bt maize (Van Rensburg, 2001), but no evaluation of the effect of Bt maize have been conducted on non-target species in South Africa (Van Wyk, 2006). Furthermore, no checklist of non-target insect species that might be affected by Bt maize through feeding on the plant or by ingesting Bt pollen have been compiled in South Africa. Dutton *et al*. (2003) suggested that laboratory, semi-field and field studies should be conducted on selected species, and, if these studies should show any effect, risk management must take place.

Studying the effect of Bt maize at the third trophic level is also of importance in the assessment of their possible ecological risks. Environmental risks are most easily assessed after damage has occurred, yet risk assessment is useful for decision making only when the risks are assessed before damage actually occurs (Andow & Hilbeck, 2004b).

As pointed out by McGeoch & Rhodes (2006), the protocols and guide lines for risk assessment of GM crops in South Africa has yet to be developed. Since Bt maize has already been released in South Africa this study and field research on Bt maize (Van Wyk *et al*., 2008) largely contributes to focusing post-release monitoring of potential

ecological impact, and possible risk assessment for future release of other Bt events in South Africa and the rest of Africa. Although Bt maize is considered as an environmentally friendly alternative to insecticides (Meeusen & Warren, 1989; Cannon, 2000), concerns have been raised that there may be adverse effects of Bt maize use on non-target lepidopterans (Meeusen & Warren, 1989; Wraight *et al*., 2000; Lang, 2004; Birch *et al*., 2004) and their consumers (Peacock *et al*., 1998; Dutton *et al*., 2003; Andow & Hilbeck, 2004a; Lövei & Arpaia, 2005).

1.5.1. Non-target insects feeding on Bt maize

The risks that transgenic crops pose to non-target organisms need to be addressed as part of the environmental risk assessment that precedes the commercialization of any novel transgenic crop (Romeis *et al*., 2006; Romeis *et al*., 2008). Like conventional agricultural pest control products, one of the risks associated with the growing of transgenic crops is their potential impact on non-target organisms including a range of arthropod species that fulfill important ecological functions (Romeis *et al*., 2006).

It has been estimated that there are over 250 different exposure pathways by which a transgene product or its metabolites could affect a secondary consumer, of which only a few are direct effects of the transgene product (Andow & Hilbeck, 2004a). Although this complexity can make testing and assessment difficult, uncertainty can be minimized by selecting appropriate species, and by conducting suitable tests to produce meaningful crop specific results (Dutton *et al*., 2003). Van Wyk *et al*. (2007) identified several non-target lepidopteran species as important and which is directly exposed to Bt maize through feeding on different plant parts. These species were suggested as high-priority species for use in risk assessment studies. These species can be classified in the functional group of non-target primary consumers, which constitutes herbivore species that are not the target of the transgene but feeds directly on the GM crop. The following lepidopteran species was recognized as important by Van Wyk *et al*. (2007): *Acantholeucania loreyi* (Noctuidae), *Agrotis segetum* (Noctuidae), *B. fusca* (Noctuidae), *C. partellus* (Crambidae), *Eublemma gayneri* (Noctuidae), *Helicoverpa armigera* (Noctuidae), *Sesamia calamistis* (Noctuidae), and *Spodoptera exigua* (Noctuidae).

1.5.2. Effect of Bt pollen on non-target Lepidoptera

Losey *et al*. (1999) demonstrated that exposure to Bt maize pollen can cause mortality in neonate monarch caterpillars, *Danaus plexippus* Linnaeus (Lepidoptera: Nymphalidae). Despite the fact that the authors cautioned that it would be inappropriate to draw any conclusion about the risk to monarch populations in the field based solely on their initial results, the study created a wide-spread perception of risk. Hansen-Jesse & Obrycki (2000) fed milkweed foliage, which was "naturally dusted" under field conditions with pollen from Bt maize, to monarch caterpillars in laboratory feeding trails. They reported significantly greater mortality of larvae that consumed foliage contaminated with Bt pollen, although no close-dependent effect of pollen concentration was observed. Wraight *et al*. (2000) reported that no mortality of black swallowtail caterpillars, *Papilio polyxenes* Fabricius (Lepidoptera: Papilionidae) could be directly attributable to exposure to MON810 maize pollen under field conditions. They suggested from their results that at least some potential non-target effects of the use of transgenic plants may be manageable. These results were confirmed in another study where pollen of Bt maize (MON810) failed to affect the black swallowtail in either the field or the laboratory (Zangerl *et al*., 2001).

Field experiments published to date have highlighted possible adverse effects of the Bt maize Event 176 on some butterfly larvae, while event MON810 seems to be much less toxic (Zangerl *et al*., 2001; Lang, 2004). Peacock *et al*. (1998) reported significant mortality for 27 of 42 lepidopteran species evaluated against Foray 48B (formulation of *B. thuringiensis*), and 8 of 14 species evaluated against Dipel 8AF (formulation of *B. thuringiensis*). Considering the wind dispersal of maize pollen, the possible deposition of pollen on host plants of non-target lepidopteran larvae near and in maize fields, and possible adverse effects of Bt maize pollen consumption on lepidopteran larvae, a survey of Lepidoptera occurring in field margins appears to be essential to determine the effect of commercial cultivation of transgenic Bt maize on Lepidoptera ecology.

1.5.3. Bt maize and tri-trophic interactions

There is also a concern over the potential for GM crops to affect natural enemies and to disrupt biological control (Hilbeck, 2002; Kennedy & Gould, 2007; Romeis *et al*., 2008; Wolfenbarger *et al*., 2008). Bt maize can also have an effect at the tri-trophic level. Recent studies have shown that transgenic insect resistant plants can have negative effects on non-target herbivores as well as on beneficial insects (Vojtech *et al*., 2005). Results of studies conducted on *Spodoptera littoralis* (Lepidoptera: Noctuidae) and the parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae) sustain that *C. marginiventris* survival, developmental times and cocoon weights were significantly negatively affected if their *S. littoralis* host larva had been fed Bt maize (Vojtech *et al*., 2005). Studies evaluating the induced-odour emission of Bt maize indicated that *C. marginiventris* and *Microplitis rufiventris* (Hymenoptera: Braconidae) could not distinguish between the transgenic and the isogenic line (Turlings *et al*., 2005). The same conclusion was drawn by Van den Berg & Van Wyk (2007) with *S. calamistis*. Because of these non-target natural enemies not distinguishing between Bt- and non-Bt maize there is a need for research to determine the effect of Bt maize on stem borer parasitoids in South Africa.

The tiered approach to assessing ecological risk of GM crops assumes that lower tier laboratory studies, which expose surrogate non-target organisms to high doses of insecticidal proteins, can detect harmful effects that might be manifested in the field. To test this assumption, Duan *et al*. (2009) performed meta-analyses comparing results for non-target invertebrates exposed to Bt toxin in laboratory studies with results derived from independent field studies examining effects on the abundance of non-target invertebrates. They concluded that laboratory studies incorporating tri-trophic interactions with Bt plants, herbivores and parasitoids were better correlated with the decreased field abundance of parasitoids than were direct exposure assays. For predators, laboratory tri-trophic studies predicted reduced abundances that were not realized in field studies and thus overestimated ecological risk (Duan *et al*., 2009). Therefore it is important to not only test risks in laboratory assays, but also to determine if there will be effects at field level.

1.6. Objectives

The aim of this study was to determine, through feeding experiments, the effects of Bt maize on selected non-target Lepidoptera, Coleoptera and Diptera species that occur in maize agro-ecosystems in South Africa. Results provide information for use in risk assessment studies on GM maize. Priority insect species were identified and laboratory- and semi-field experiments were conducted to evaluate the effect of Bt maize on these species.

The specific objectives of this study were addressed under the following topics:

- The effect of Bt maize expressing Cry1Ab toxin on the survival and fitness of priority non-target arthropod species. The following species were evaluated in feeding studies, *Sesamia calamistis* (Lepidoptera: Noctuidae), *Helicoverpa armigera* (Lepidoptera: Noctuidae), *Agrotis segetum* (Lepidoptera: Noctuidae), *Heteronychus arator* (Coleoptera: Scarabaeidae), and *Somaticus angulatus* (Coleoptera: Tenebrionidae).

- The effect of Bt maize at the third trophic level was evaluated using a natural enemy of a target stem borer *B. fusca* in experiments with the parasitic fly, *Sturmiopsis parasitica* (Diptera: Tachinidae).

- Ecological theory was used to improve environmental risk assessment and to tailor it to the specific maize field environment. Using an ecological model to identify priority species for non-target risk assessment, local species were classified functionally and prioritized using risk based ecological criteria to identify potential test species, assessments and end points.

To place all of the above mentioned points into perspective, this study also provides important information with respect to the successful deployment of Bt maize as a tool in integrated pest management.

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CHAPTER 2: Comparative efficacy of Bt maize events MON810 and Bt11 against *Sesamia calamistis* (Lepidoptera: Noctuidae) in South Africa

2.1. Abstract

Maize, expressing Cry1Ab insecticidal proteins produced by the bacterium *Bacillus thuringiensis* (Bt), was introduced for control of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) in South Africa in 1998. In the light of the reportedly lower toxicity of Bt maize to certain noctuid borers, the effect of Bt maize was evaluated on *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) in South Africa. The characteristic larval behaviour of *S. calamistis* may result in reduced exposure to Bt toxin and subsequent high levels of survival, since larvae do not feed on plant whorls like other borer species, but penetrate stems directly from behind leaf sheaths. Growth and survival of larvae were determined in a greenhouse bioassay with two Bt maize hybrids (Monsanto event MON810 and Syngenta event Bt11) and their non-Bt, iso-hybrids. Potted plants were artificially infested with first instar larvae. Percentage larval survival and mean larval mass were recorded over time. Bt maize of both events were shown to be highly toxic to *S. calamistis*. No larvae survived longer than nine days on plants of either of the Bt events. *Sesamia calamistis* is stenophagous and occurs in mixed populations with other borer species with which it shares several parasitoid species in Africa. The ecological impact of local extinction of *S. calamistis* caused by this highly effective transgenic event is therefore not expected to be great.

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2.2. Introduction

Bt maize was initially developed for the control of two stem borers in North America, i.e. *Diatraea grandiosella* (Dyar) (Lepidoptera: Crambidae) (Archer *et al*., 2001) and *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) (Ostlie *et al*., 1997) before it was introduced for control of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) in South Africa (Gouse, 2005). Currently there are two Bt maize events commercialized in South Africa namely MON810 and Bt11. Although the Bt maize that is used in South Africa effectively controls *B. fusca*, survival of this species on certain plant parts has been reported (Van Rensburg, 1998; 2001). The MON810 event is, however, reported to cause 100% mortality of *C. partellus* (Van Rensburg, 1998; Singh *et al*., 2005). The pink stem borer, *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae), which occurs widely throughout sub-Saharan Africa, was not initially intended as a target organism of Bt maize but a previous study showed that various plant parts of event MON810 was toxic to *S. calamistis* (Van den Berg & Van Wyk, 2007).

Sesamia calamistis is economically important in West Africa (Ajala *et al*., 2001) and does not often attain pest status in eastern and southern Africa in spite of its wide occurrence on several crops (Harris, 1962; Overholt & Maes, 2000). In South Africa *S. calamistis* was initially only recorded as a pest of maize in the coastal belt of the Western Cape region but its importance has increased since the 1990s in maize on the Highveld regions (< 1300 m above sea level), especially in irrigated maize (Van den Berg & Drinkwater, 2000).

Sesamia calamistis is probably the most widely distributed stem borer species in Africa. It occurs throughout sub-Saharan Africa below 2400 m above sea level (Polaszek & Khan, 1998; Muhammad & Underwood, 2004). It often occurs in mixed populations with other stem borers such as *B. fusca* and *Eldana saccharina* (Walker) (Lepidoptera: Pyralidae) which comprise the three indigenous economically important borer species on maize. Together with *C. partellus* these species form the complex of stem borers that is

targeted by Bt maize. Although *S. calamistis* is not considered a target organism of Bt maize in South Africa, it was listed as a target organism in Kenya (Hilbeck & Andow, 2004), where Bt maize is intended for release. Van Wyk *et al*. (2007) suggested that *S. calamistis* be included as a test species to determine the effect of Bt maize on non-target Lepidoptera species for risk assessment.

Although the general biology of *S. calamistis* is similar to that of other stem borers there is one major difference in larval behaviour. A unique characteristic of this behaviour is that neonate larvae do not migrate to plant whorls after hatching. Eggs are laid between leaf sheaths and the stem (similar to *B. fusca*) but neonate larvae feed on the leaf sheath for a short time before penetrating the stem directly (Fig. 2.1) (Shanower *et al*., 1993; Ajala *et al*., 2001). This aspect of its biology may affect the effectiveness of Bt against *S. calamistis* since larval feeding on whorl and leaf tissue, in which the expression levels of Bt toxin is high, is limited. The aim of this study was to determine the comparative efficacy of events MON810 and Bt11 against *S. calamistis*.

Figure 2.1. *Sesamia calamistis* larva penetrating maize stem directly.

2.3. Materials and methods

A greenhouse study was conducted to determine larval survival on Bt and non-Bt maize plants, grown in pots. Commercial hybrids of the events MON810 and Bt11 with their iso-hybrids were used. Hybrid DKC 78-15B (event MON810) with iso-hybrid CRN 3505 and hybrid NK Mayor B (event Bt11) with its iso-hybrid Brasco were used. An experiment was conducted using 100 potted plants of each hybrid. The experimental lay-out was a completely randomized design.

One hundred four-week old plants of each hybrid were inoculated with 10 neonate larvae. Larvae were obtained from a mass-rearing colony where moths were allowed to oviposit on non-Bt maize plants. The colony was initiated from field collected larvae that were maintained in a laboratory for two generations before use in this experiment. A camel-hair brush was used to transfer first instar larvae onto plants between the stem and the last unfolded leaf sheath (Fig. 2.2.) of the plant where larvae would normally hatch from eggs and commence feeding on the leaf sheath.

Figure 2.2. First instars transfered onto maize plant between stem and leaf sheath.

The number of surviving larvae and the mean mass of larvae per plant were determined at weekly intervals. Nine randomly selected plants of each maize hybrid were dissected at 3, 6, 9, 12, 20, 33 and 42 days after inoculation. Dissections of plants were terminated on day 42 due to the onset of the pupal stage. Repeated measures ANOVA were used to analyse percentage larval survival and mean larval mass over time (StatSoft, Inc., 2009).

2.4. Results

Larval survival decreased rapidly over the first nine days after inoculation (Fig. 2.3 and 2.4). Larval survival differed significantly over time between the Bt and non-Bt hybrids ($F_{(8,56)} = 4.507$; $P < 0.000003$). The level of survival also differed significantly between DKC 78-15B and iso-hybrid CRN 3505 ($F_{(4,28)} = 10.0719$; $P = 0.000003$) (Fig. 2.3). There was also a significant difference between variety NK Mayor B and iso-hybrid Brasco ($F_{(4,28)} = 4.919$; $P < 0.001019$) (Fig. 2.4). No surviving larvae were recorded on Bt maize plants from nine days after inoculation onwards on both Bt maize hybrids. Mean percentage of surviving larvae on DKC 78-15B (MON810) was 3.3% six days after inoculation, and 1.1% on NK Mayor B (Bt11). Mean percentage surviving larvae on the non-Bt plants was 23.3% on CRN 3505 and 14.4% on Brasco, 42 days after infestation. Larvae that were recovered from Bt plants were never larger than 5mm indicating that they did not develop beyond the second instar.

There was a significant difference between the mean larval mass of larvae feeding on DKC 78-15B (MON810) and CRN 3505 (non-Bt hybrid) ($F_{(4,28)} = 40.129$; $P < 0.000001$) (Fig. 2.5), and for larval mass on NK Mayor Bt (Bt11) versus Brasco (non-Bt iso-hybrid Bt11) ($F_{(4,28)} = 59.131$; $P < 0.000001$) (Fig. 2.6). Larval mass on Bt plants did not increase between three and nine days after commencement of the experiment, but increased on non-Bt plants. A decrease in mass was observed between 33 and 42 days on Brasco (non-Bt) when larvae started changing into pre-pupae on non-Bt stems (Fig. 2.6). A large difference in larval mass between Bt and non-Bt feeding larvae was observed

after three days of feeding (Fig. 2.6). Larval mass never increased on the Bt maize hybrids but increased rapidly on the non-Bt hybrids from day 12 onwards.

2.5. Discussion and conclusions

Expression of Bt toxins in maize is often cited in literature to be constitutive, meaning that expression occurs in all tissues at all times. Castro (2002) reported protein expression for MON810 and Bt11 in all plant tissue, season-long and high protein expression. This is misleading since different promoters have been used for the various commercial maize hybrids and these different hybrids have been shown to express different amounts of toxin in different plant tissues (Dutton *et al.*, 2003). For example, Cry1Ab protein expression in genetically modified maize varieties containing the cauliflower mosaic virus (CaMV) 35S promoter (MON810 and Bt11) expresses the toxin throughout the season in leaves, stem, roots, and kernels (EPA, 2000). Important behavioural implications may arise if differences in Bt toxin concentrations exist within the plant. For example, if larvae feed on silks and kernels with a lower toxin concentration, and only then penetrate the stems as third instars, they may be able to survive inside stems.

Results from a previous study where the "whole plant" method was also used Van den Berg *et al.* (2007) reported high susceptibility of *S. calamistis* to Bt maize (event MON810). Results from this study indicate that *S. calamistis* was just as highly susceptible to Bt11 than to event MON810. The behavioural characteristic of larvae to feed behind leaf sheaths and to enter stems directly did not result in escape of exposure to the toxin. Larval feeding on leaf sheaths therefore resulted in the ingestion of sufficient toxin to kill larvae before they entered maize stems. It was expected that *S. calamistis* larvae may survive on the Bt11 event to some extent, because of possible differential expression between Bt11 and MON810 (Letourneau & Burrows, 2001).

The high mortality level of a non-target Noctuidae species observed in this study is in contrast with another study which reported that Bt maize does not effectively control

other Noctuidae species such as corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) and fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) in the United States (Archer *et al*., 2001; Hilbeck & Andow, 2004). Other stem borer species against which MON810 is also highly effective are *B. fusca* (Van Rensburg, 2001), *C. partellus* (Van Rensburg, 1998; Singh *et al*., 2005) and *E. saccharina* (Keeping *et al*., 2007). It was, however, observed that the plant part on which *B. fusca* larvae feed significantly affected larval survival. Van Rensburg (2001) observed that protein expression was high enough during the vegetative stages of plant development when larvae feed only on leaf and stem tissue but *B. fusca* first instars survived when fed on maize silks. This could possibly contribute to survival of *H. zea* and *S. frugiperda* where they often feed on ears of Bt maize.

In a review on risks and management of Bt maize in Kenya, Fitt *et al*. (2004) indicated that the toxicity of currently available Bt maize varieties in that country was considered to be low and that toxicity depended on the event used. Mugo *et al*. (2005), in leaf disk bioassays, observed that not all events were equally efficient against different stem borers and that especially *B. fusca* was difficult to control. This aspect will need careful consideration in risk assessments and decisions on deployment of Bt maize varieties where *B. fusca* and other non-target Lepidopterans occur.

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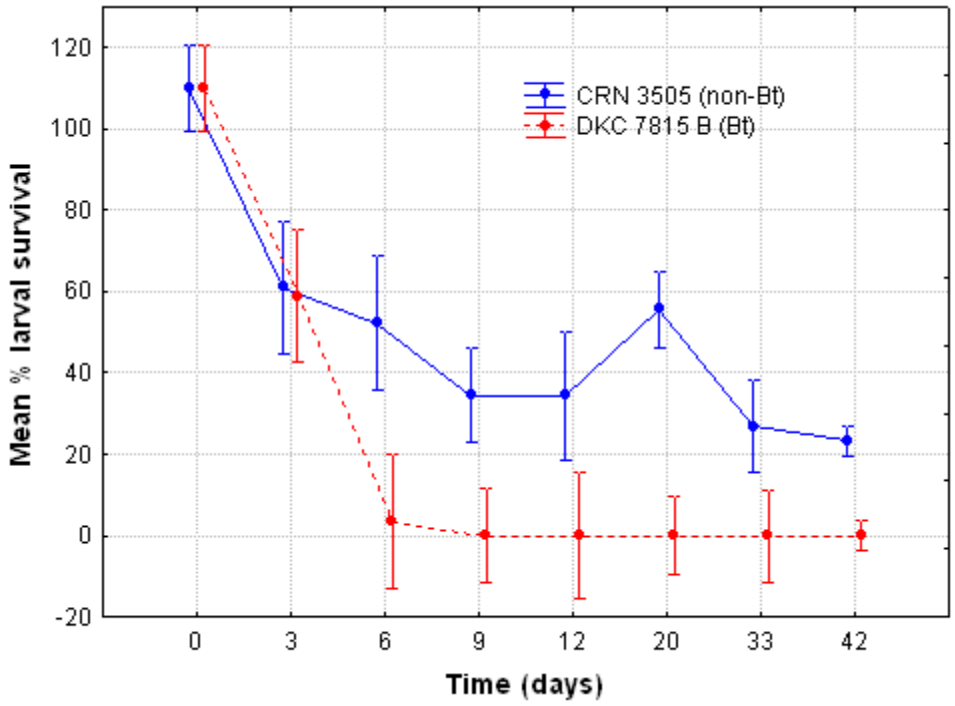

Figure 2.3. Mean percentage of *Sesamia calamistis* larval survival on event MON810 (hybrid DKC 78-15 Bt) and its iso-hybrid (CRN 3505) (Bars = standard error).

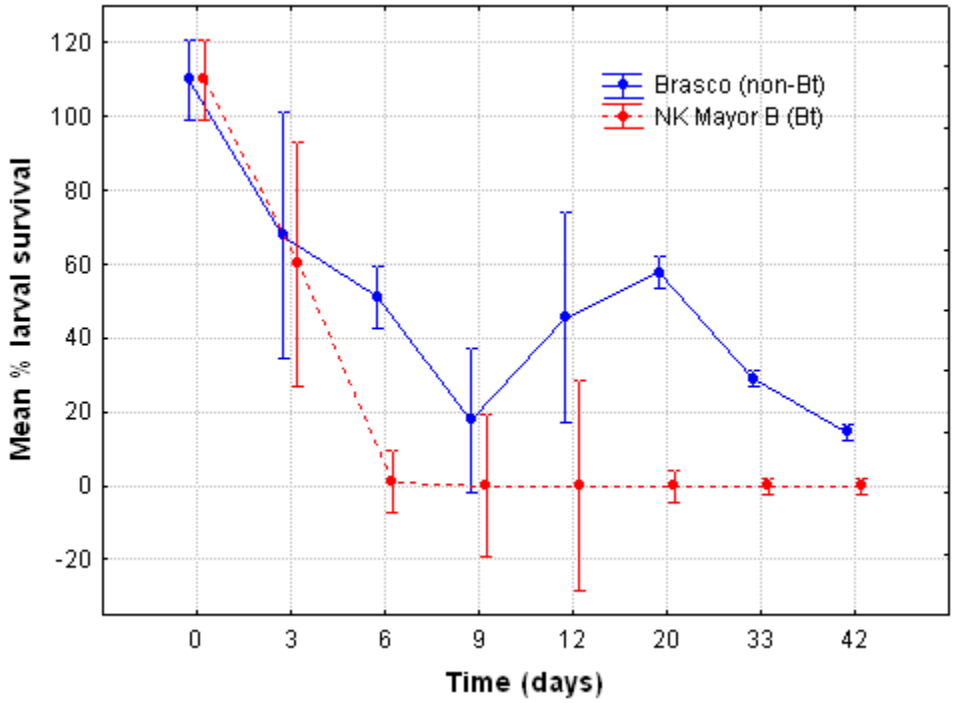

Figure 2.4. Mean percentage of *Sesamia calamistis* larval survival on event Bt11 (NK Mayor B) and its iso-hybrid (Brasco) (Bars = standard error).

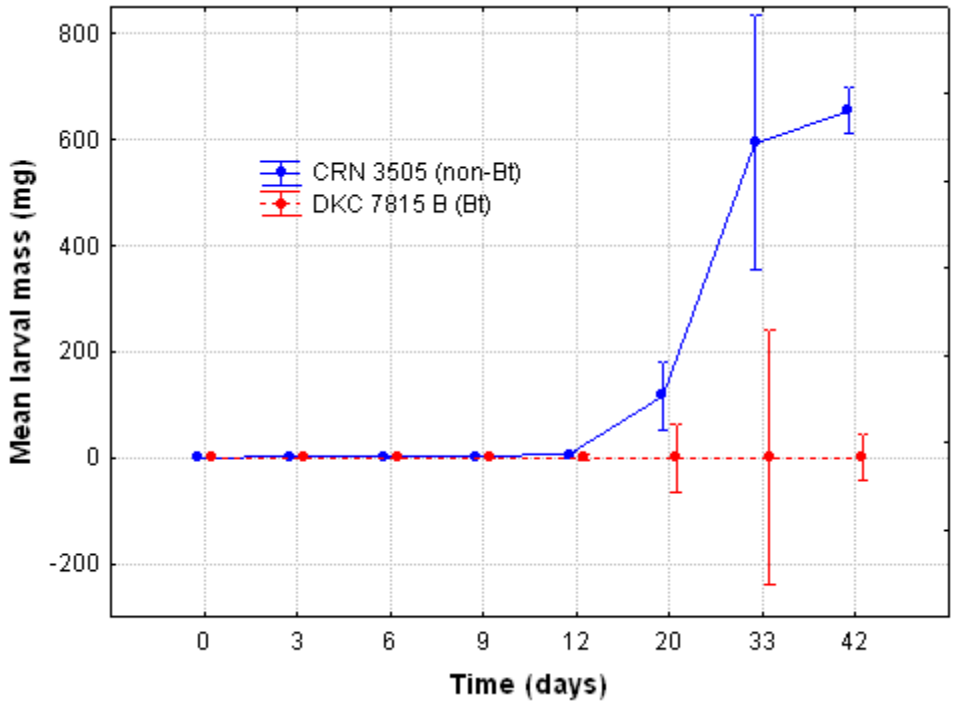

Figure 2.5. Mean larval mass of *Sesamia calamistis* on event MON810 (hybrid DKC 78-15 Bt) and its iso-hybrid (CRN 3505) (Bars = standard error).

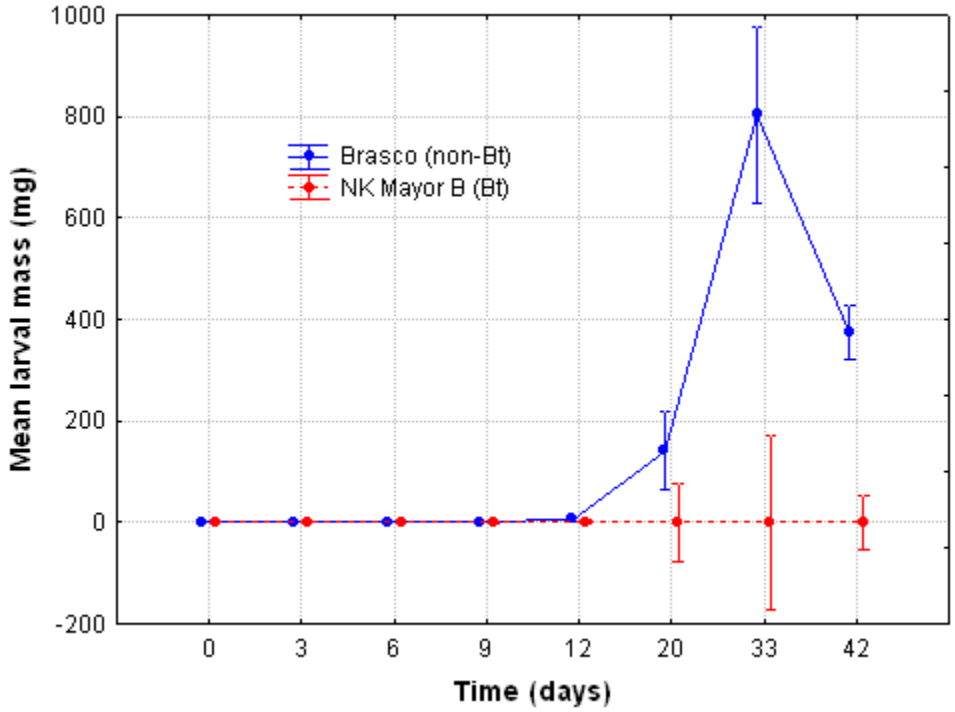

Figure 2.6. Mean larval mass of *Sesamia calamistis* on event Bt11 (hybrid NK Mayor B) and its iso-hybrid (Brasco) (Bars = standard error).

CHAPTER 3: Effects of Bt maize on the cutworm, *Agrotis segetum* (Lepidoptera: Noctuidae), a pest of maize seedlings

3.1. Abstract

The lepidopterous stemborers *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) are effectively controlled by Bt maize that express the Cry1Ab insecticidal protein. Another noctuid species, the cutworm *Agrotis segetum* (Denis and Schiffermüller) (Lepidoptera: Noctuidae), which is the most common and injurious pest of maize seedlings in South Africa, is exposed to Bt toxin for a part of its life cycle. The effect of this exposure to Bt maize has not been studied yet. The aims of this study were to determine the effects of Bt maize (events MON810 and Bt11) on larval mass, development time, survival, and fecundity of *A. segetum*. Laboratory studies were conducted with first- and fourth instar larvae, and moths. Results showed that the effect of Cry1Ab toxin on the biology of *A. segetum* larvae and moths were largely insignificant. The effects of the two Bt maize events on the different parameters measured in this study was not similar between the Bt events and their respective iso-hybrids. Compared with larvae that fed on conventional (non-Bt) maize, Bt maize did not affect survival of first instar larvae. However, mean mass of larvae that fed on Bt maize (Bt11) was significantly lower. Feeding on Bt maize did not have a significant effect on development period to pupa formation. Fewer eggs were laid by moths fed as larvae on maize event Bt11 compared with MON810. This study indicates that Bt maize will most likely not have any significant effect on the control of *A. segetum* under field conditions.

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3.2. Introduction

The target pests of Bt maize in South Africa are the lepidopterous stem borers *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae). These pests are effectively controlled by Cry1Ab toxin of events MON810 and Bt11 (Van Wyk *et al*., 2009). Various cutworm species (Lepidoptera: Noctuidae) occur in South Africa, namely the black cutworm (*Agrotis ipsilon*), grey cutworm (*A. subalba*), brown cutworm (*A. longidentifera*), spiny cutworm (*A. spinifera*) and the common cutworm (*A. segetum*). The latter is the only economically important species and recognized as the most common and injurious to maize seedlings (Du Plessis, 2000). Damage caused by *A. segetum* larvae (Fig. 3.1) can be severe. During the day, larvae occur beneath the soil surface from where they emerge to be active nocturnally. At night, larvae move from one seedling to another, cutting stems near ground level which cause seedlings to die (Annecke & Moran, 1982). Larvae consume part of the seedling stem and one larva can destroy several seedlings in one night (Drinkwater, 1980). Larvae attacking crop seedlings are usually in the fourth and later instars of development (Blair, 1975). Because moths lay their eggs on weeds and larvae are active throughout the winter it is generally accepted that an abundance of winter weeds may enhance cutworm infestations (Drinkwater, 1980).

Cutworm moths can be identified by characteristic markings on the wing. The common cutworms have brown to grey fore wings, and pale whitish hind wings (Drinkwater, 1980). Moths lay eggs singly or in groups on the soil surface or lower plant parts. Hatching time of eggs and duration of subsequent stages are influenced by environmental conditions. Larvae moult five times and the last larval instar is followed by a pupal stage after which moths emerge. During the summer, the life cycle takes approximately 50 days to complete (Annecke & Moran, 1982; Du Plessis, 2000).

Neonate larvae hide under and feed on leaves of weeds or grain crops, on and near the soil surface. Larvae burrow into the soil after their second moult. From this stage onwards larvae only emerge at night (Du Plessis, 2000). When eggs are laid during the autumn and winter, various sizes of larvae overwinter in the soil until spring. During August and September, these larvae develop into pupae in pupal cells in the soil. First generation moths

for the new season will emerge from these pupae approximately two weeks later. These moths lay their eggs on leaves of weeds and volunteer plants in fields (Du Plessis, 2000).

A neat round hole is chewed into stems of older plants (four leaf stage and older). This damage is easily distinguished from that caused by black maize beetle (*Heteronychus arator* (Coleoptera: Scarabaeidae)) or false wire worms (*Somaticus angulatus* (Coleoptera: Tenebrionidae)), where the edges of feeding holes have a frayed appearance. Above ground symptoms of cutworm damage is similar to that of black maize beetle and false wire worm. Initial wilting of the central whorl leaf is followed by wilting of the entire plant (Drinkwater, 1980).

Figure 3.1. Common cutworm, *Agrotis segetum* larva.

Abundant autumn rains may also lead to increased cutworm populations during the following spring as a result of increased winter weed populations. A spring cultivation of fields at least 35 days prior to seeding is, therefore, generally recommended in order to reduce the resident cutworm populations by starvation (Drinkwater & Van Rensburg, 1992). In spite of this, crops grown on relatively weed-free fields have been observed to suffer severe cutworm damage in early summer, while weed-infested fields are not necessarily subject to cutworm infestations. The possibility, therefore, exists that an

abundance of cutworm larvae in a given field may be determined by the spectrum of weed species rather than by the number of weed plants per se (Drinkwater & Van Rensburg, 1992).

There are currently no transgenic maize hybrids in South Africa targeting *A. segetum* and the effect of Cry1Ab toxin on this species has not been reported. Lambert *et al*. (1996) did, however, reported that Cry9Ca was toxic to *A. ipsilon* and *A. segetum* in Belgium. The Cry1Ab toxin is very selective for Lepidoptera (Pons *et al*., 2005; Eizaguirre *et al*., 2006) and the Monsanto user guide for the production of YieldGard (MON810) maize states that MON810 has no effect on cutworm (Monsanto, 2007). In a field evaluation on Bt maize in Spain conducted by Eizaguirre *et al*. (2006), no effects by Cry1Ab (Event 176) on the percentage of plants killed by *A. segetum* were observed. Van Wyk *et al*. (2008) reported that the incidence of seedling damage caused by *A. segetum* in South Africa was significantly higher on a non-Bt than a Bt field. In the latter study, however, again only the incidence of cutworm damaged plants was recorded and the possible effect that exposure to Bt toxin could have had on larvae was not studied.

An assessment of the ecological effects of Bt maize on components of the maize biocenosis other than stem borers is essential (Pons *et al*., 2005). Crawley (1999) emphasized the need to study the effects of genetically modified crops on the demography of non-target species over their entire life cycle and several generations in the field. In selection of non-target Lepidoptera species for ecological risk assessment of Bt maize in South Africa, Van Wyk *et al*. (2007) identified species that can be regarded as priority species for testing. Five non-target Lepidoptera species including *A. segetum* were recommended for inclusion in post-release monitoring of Bt maize in South Africa. Based on their distribution and the fact that the bionomics of *A. segetum* is well known, it could also be recommended for inclusion in pre-release testing (Van Wyk *et al*., 2007).

The objective of this study was to determine the effect of Bt maize expressing Cry1Ab toxin (events MON810 and Bt11) on *A. segetum* larval survival and mass gain, as well as fecundity and fertility of moths.

3.3. Materials and methods

3.3.1. Larval survival studies

Two studies, one with neonate larvae and the other with fourth instars, were conducted to determine the effect of Bt maize on larval growth and survival. These studies involved laboratory bioassays in which maize seedlings were fed to larvae. The "whole plant method" suggested by Birch *et al*. (2004) was used to evaluate the effect of transgenic maize (not only the transgenic product), which in the case of cutworm is only the maize seedling.

The Bt maize events MON810 and Bt11 are the only registered Bt maize events with insecticidal properties in South Africa and both were evaluated in this study. The following four varieties were used: DKC 78-15B (genetically modified, MON810), CRN 3505 (non-Bt iso-hybrid for DKC 78-15B), NK Mayor B (genetically modified, Bt11), and Brasco (non-Bt iso-hybrid for NK Mayor B).

Experiment 1: Neonate larvae

Larvae for the use in this experiment originated from larvae that were reared on artificial medium for one generation after collecting larvae from maize fields. First instar survival and mass increase were evaluated under laboratory conditions. The experimental lay-out was a completely randomized design. Seven to ten day old seedlings were placed in test tubes (15 X 1.5 cm). One first instar larva was placed per test tube that was plugged with cotton wool. Test tubes were kept in an incubator at 25 ˚C and 65% relative humidity. Each maize hybrid was replicated 50 times. Seedlings were replaced with new seedlings and larvae were weighed at 3 - 4 day intervals. Larvae were weighed until the pupal stage was reached. Percentage pupation over time was also determined. Autoclaved soil was placed in the test tubes when larvae reached the second instar to prevent accumulation of unnecessary moisture.

Experiment 2: Fourth instars

This experiment was conducted using fourth instars, using the same methods described above. To obtain larger larvae of uniform age, first instar larvae were fed spinach until they reached the fourth instar before they were used in the experiment. Because the weed

species, *Amaranthus hybridus* (Amaranthaceae), was reported to support high levels of survival of A. *segetum* larvae (Mabuda, 2001), larvae used in this study were reared on spinach [(*Spinacia oleracea* (Amaranthaceae)], which belongs to the same plant family as *A. hybridus*. Each treatment was replicated 70 times. Larvae were starved for one day before the onset of the experiment. Larval survival and mean larval mass were recorded every 3 - 4 days until the onset of pupation. The duration of larval development from the onset of the experiment to pupation was recorded.

3.3.2. Oviposition experiment

The fecundity, fertility, and longevity of moths derived from larvae fed on Bt- and non-Bt maize from the fourth instar until pupation was determined. Two larval colonies were maintained on Bt and non-Bt maize seedlings. One male and one female moth were kept in a round plastic container (9 X 12cm) and were replicated 20 times. The container's opening was covered with gauze to serve as oviposition site. A zig-zag folded white paper (5 X 8cm) was placed inside each container to allow for a daytime hiding place for moths, as well as extra oviposition sites. A non-Bt maize seedling leaf was placed in each container to provide possible oviposition stimuli to moths. Drinking water was provided by means a sponge (1.5 X 1.5 cm) saturated with sugar water. Mortality of male and female moths was recorded at 24h intervals, and eggs collected until moths died. The number of eggs laid each night as well as the number of eggs that hatched per moth was recorded and expressed as a percentage.

3.4. Data analysis

Repeated-measures analysis of variance (ANOVA) were used to analyze larval mass, larval survival, and percentage pupation over time (StatSoft, Inc., 2009). Data on larval mass were log(x + 1) transformed before analyses. Untransformed data are, however, provided in the figures. Longevity, fertility and fecundity data were analyzed using one-way ANOVA (StatSoft, Inc., 2009).

3.5. Results

3.5.1. Larval survival studies

Experiment 1: Neonate larvae

A differential response of larvae to the different Bt maize events were observed with the mass of larvae feeding on Bt maize (NK Mayor B) being significantly lower than those that fed on the non-Bt hybrid (Brasco) ($F_{(1,98)}$ = 18.179; P = 0.00005) (Fig. 3.2). However, mean larval mass was not significantly different between larvae feeding on the other pair of hybrids (CRN 3505 and DKC 78-15) ($F_{(1,98)}$ = 2.4038; P = 0.1242) (Fig. 3.2.). Mass of larvae that fed on Brasco (non-Bt) seedlings increased more rapidly than larvae that fed on NK Mayor B (Bt). There was a significant difference in larval mass between the two non-Bt hybrids ($F_{(1,98)}$ = 4.419; P = 0.038) but not between the two Bt hybrids ($F_{(1,98)}$ = 0.144; P = 0.705).

Larval survival decreased slowly over time but did not differ significantly between Bt and non-Bt maize seedlings for treatments CRN 3505 (non-Bt) and DKC 78-15B (Bt) ($F_{(1,8)}$ = 1.7925; P = 0.217413) or Brasco (non-Bt) and NK Mayor (Bt), ($F_{(1,8)}$ = 4.886; P = 0.058) (Fig. 3.3.).

The percentage pupation of larvae fed on maize as first instars did not differ significantly between CRN 3505 (non-Bt) and DKC 78-15B (Bt) ($F_{(1,8)}$ = 0.021; P = 0.887) (Fig. 3.4.) or Brasco (non-Bt) and NK Mayor (Bt), ($F_{(1,8)}$ = 3.741; P = 0.089) (Fig. 3.4.).

This study showed that feeding on Bt maize did not have a significant effect on survival of first instar *A. segetum* larvae compared with feeding on conventional maize. Some effects were, however, observed with regard to mean larval mass. When first instar larvae fed on maize event Bt11 seedlings for their entire larval period, larvae were smaller compared with larvae feeding on non-Bt seedlings, larvae feeding on non-Bt seedlings reached the maximum mass sooner than larvae feeding on Bt seedlings, indicating that the former will reach physiological maturity faster. Larvae feeding on the non-Bt hybrid, Brasco, were significantly heavier than on any other hybrid indicating that this hybrid was more suitable as host for larval development.

Experiment 2: Fourth instars

There were no significant differences between the mass of fourth instars feeding on either CRN 3505 (non-Bt) and DKC 78-15B (Bt) ($F_{(1,142)}$ = 0.703; P = 0.403), or Brasco (non-Bt) and NK Mayor B (Bt) ($F_{(1,142)}$ = 0.086; P = 0.769) (Fig. 3.5). Similarly, no significant differences were observed in larval survival between Bt and non-Bt hybrids (Fig. 3.6.) [($F_{(1,12)}$ = 1.630; P = 0.226) for CRN 3505 (non-Bt) and DKC 78-15B (Bt); ($F_{(1,12)}$ = 0.412; P = 0.533) for Brasco (non-Bt) and NK Mayor B].

The pupal stage started on day 9 on the non-Bt hybrid CRN 3505 and day 13 for the other hybrids (Fig. 3.7.). The incidence of pupation of larvae over time was significantly higher on non-Bt maize (Brasco) than on Bt maize (NK Mayor B) ($F_{(1,12)}$ = 29.045; P = 0.00016). However, no significant differences were observed between treatments CRN 3505 (non-Bt) and DKC 78-15B (Bt) ($F_{(1,12)}$ = 2.605; P = 0.1325).

3.5.2. Oviposition experiment

Data on the effects of the consumption of Bt maize on fertility and fecundity of moths are provided in Table 3.1. In one of the treatments combinations, moths originating from larvae feeding on the non-Bt hybrid (Brasco) produced significantly more eggs than those from the Bt hybrid. Also, only in one of the treatments combinations was fertility significantly higher in moths originating from larvae feeding on Bt maize (DKC 7815B) (Table 3.1). The mean longevity of female or male moths did not differ significantly between any of the treatments (Table 3.1).

3.6. Discussion and conclusion

Results showed that the effect of Cry1Ab toxin on the biology of *A. segetum* larvae and moths were largely insignificant. Because small cutworm larvae do not feed on maize seedlings under field conditions, it is not realistic to extrapolate these results on first instars to field situations. This study was, however, conducted to determine the effects of Bt maize on cutworm larvae at the highest levels of exposure possible.

Cutworm moths lay their eggs on weeds (Drinkwater & Van Rensburg 1992) where larvae start feeding until the fourth instar. Only these larger larvae feed on maize seedlings. Blair (1975) reported that larvae attacking crop seedlings are usually fourth instars, which is why it was scientifically more realistic to rear larvae on another host plant until the fourth instar before using them in experiments.

Although there were no significant differences between survival and mass of fourth instar larvae in the different treatments, significant differences were observed in the percentage pupation over time. Larvae feeding on non-Bt seedlings of hybrid Brasco reached a higher percentage pupation over a shorter period of time compared to larvae feeding on event Bt11. Under field conditions, this can possibly influence the number of seedlings that larvae may feed on before pupation. A delay in the onset of pupation may therefore result in more seedlings being damaged in a Bt maize field. Dutton *et al*. (2002) also showed that another noctuid species, *Spodoptera littoralis* (Boisduval), a polyphagous lepidopteran pest, was partially affected by Cry1Ab. The survival rate and the time required to reach the second instar were affected significantly when larvae were reared on Bt maize compared with larvae reared on non-Bt maize.

No detrimental effects were observed with regard to moth longevity when fourth instars were fed Bt seedlings. Because pupal mass differed in one case, being lower in moths derived from Bt maize, it can be expected that these moths will lay fewer eggs, in accordance with observations by Moawad (1983) on *A. ipsilon*. Moawad (1983) reported a positive relationship between the numbers of eggs laid and the weight of the female pupa of *A. ipsilon*. It can thus be concluded that if the mass of pupae are reduced on Bt maize, fewer eggs will be laid in the new generation, compared to pupae derived from non-Bt maize. This was, however, only the case for the Bt 11 and not for MON810.

Weeds in maize fields cause an increase in cutworm numbers (Norris & Kogan, 2000). In South Africa, this leaves the farmers with a choice between early cultivation or the use of chemicals to control *A. segetum* (Du Plessis, 2000). It seems that Bt maize events MON810 and Bt11 will have no effect as control method for cutworm. Although some adverse effects, which were dependent on the Bt maize event, were observed on larval mass and number of eggs laid, it seems that the effect on cutworm under field conditions will be

negligent. Pilcher *et al*. (1997) and Koziel *et al*. (1993) reported that *A. ipsilon* was not affected by the *Bacillus thuringiensis* protein (Cry1Ab), even when high concentrations of Bt protein were present in the leaves. However, *A. ipsilon* showed susceptibility to a separate subspecies (Burges, 1981), so there is potential for using a *B. thuringiensis* crystal protein to control this pest.

The crystal protein, Cry9Ca1, from *Bacillus thuringiensis serovar tolworthi* has a fairly broad spectrum of activity against lepidopteran insects, including members of the families Pyralidae, Plutellidae, Sphingidae, and Noctuidae. It is also the first insecticidal crystal protein with activity against cutworms (Lambert *et al*., 1996). From the latter study Lambert *et al*. (1996) reported that the Cry9Ca1 toxin was highly toxic to members of the Noctuidae such as *Spodoptera exigua*, *S. littoralis*, *Mamestra brassicae* and *A. segetum*. The novel crystal protein Cry9Ca1 is not just another crystal protein with a highly toxic activity against lepidopteran larvae, but its discriminative spectrum of activity makes it one of the most appealing insecticidal crystal proteins for the control of agronomically important insect larvae either as sprays or through genetically engineered crop plants. Bioassay data indicated that this toxin was toxic to *A. segetum* and preliminary experiments also indicated activity against *Agrotis ipsilon* (Lambert *et al*., 1996). De Maagd *et al*. (2003) reported that Cry9Ca was the most toxic, followed by Cry1Aa and Cry1Fb to *A. ipsilon*. Overall, *A. ipsilon* appeared not to be susceptible to most available Cry1 toxins with known activity against lepidopterans (De Maagd *et al*., 2003).

It can be concluded that, although significant effects of genetically modified maize expressing Cry1Ab on *A. segetum* was observed in some instances under laboratory conditions, Bt maize events MON810 and Bt 11 will most likely not have any effect on this non-target pest under field conditions.

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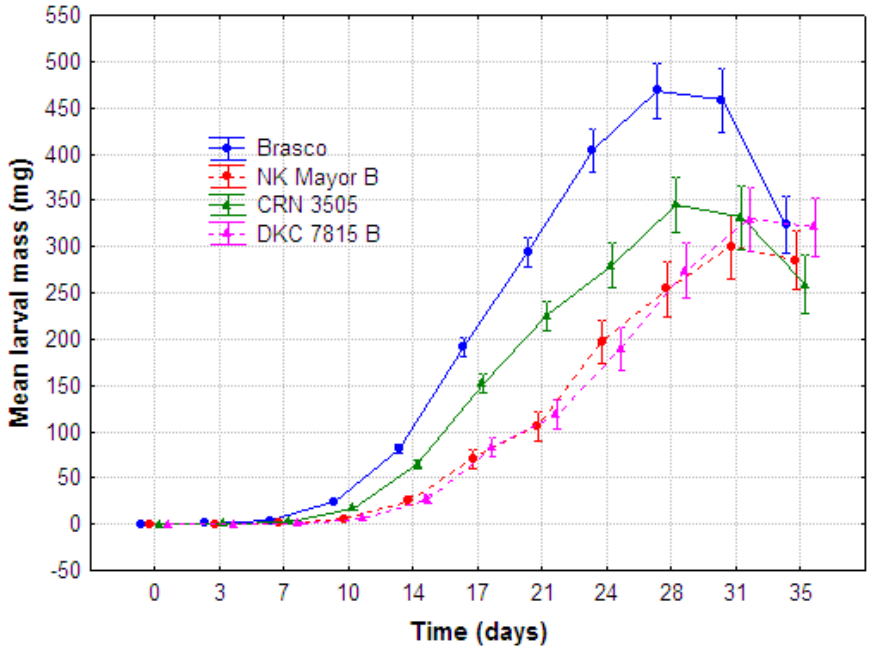

Figure 3.2. Mean mass of *Agrotis segetum* larvae feeding on maize seedlings from the 1st instar onwards. [Event MON810 (DKC 78-15B) and its non-Bt iso-hybrid (CRN 3505) and event Bt11 (NK Mayor B) and its non-Bt iso-hybrid (Brasco)]. (Bars indicate SE).

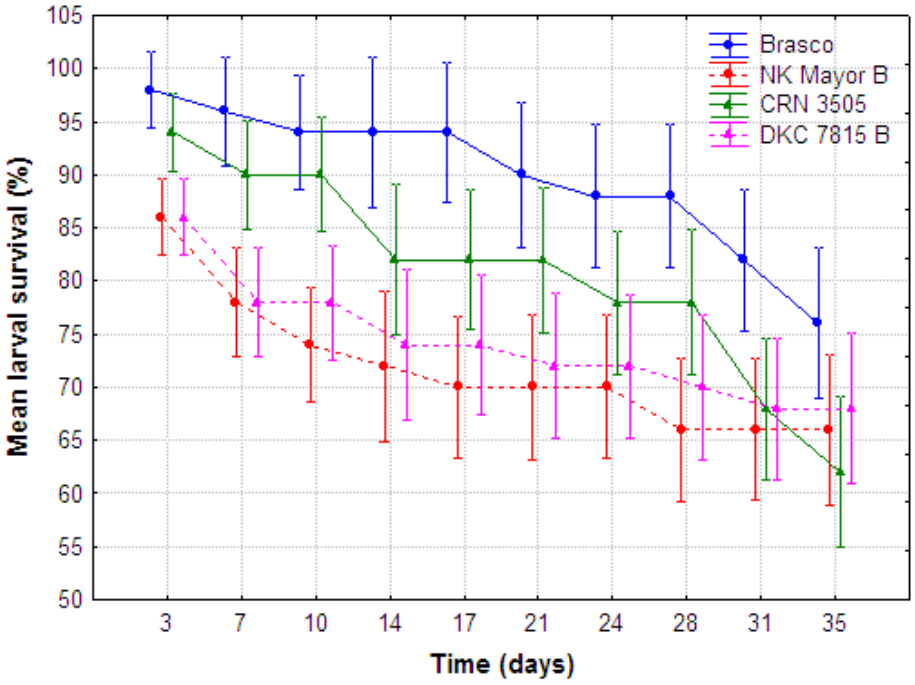

Figure 3.3. Mean percentage survival of *Agrotis segetum* larvae feeding on maize seedlings from 1st instar onwards. [Event MON810 (DKC 78-15B) and its non-Bt iso-hybrid (CRN 3505) and event Bt11 (NK Mayor B) and its non-Bt iso-hybrid (Brasco)]. (Bars indicate SE).

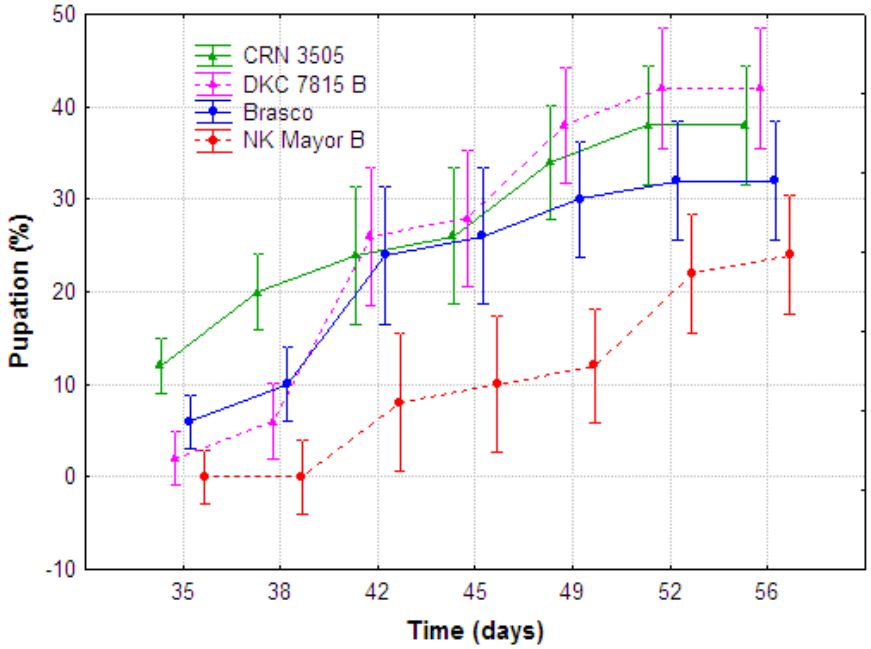

Figure 3.4. Mean percentage pupation of *Agrotis segetum* larvae feeding on maize seedlings from the 1st instar onwards [event MON810 (DKC 78-15B and non-Bt iso-hybrid CRN 3505) and event Bt11 (NK Mayor B and non-Bt iso-hybrid Brasco)]. (Bars indicate SE).

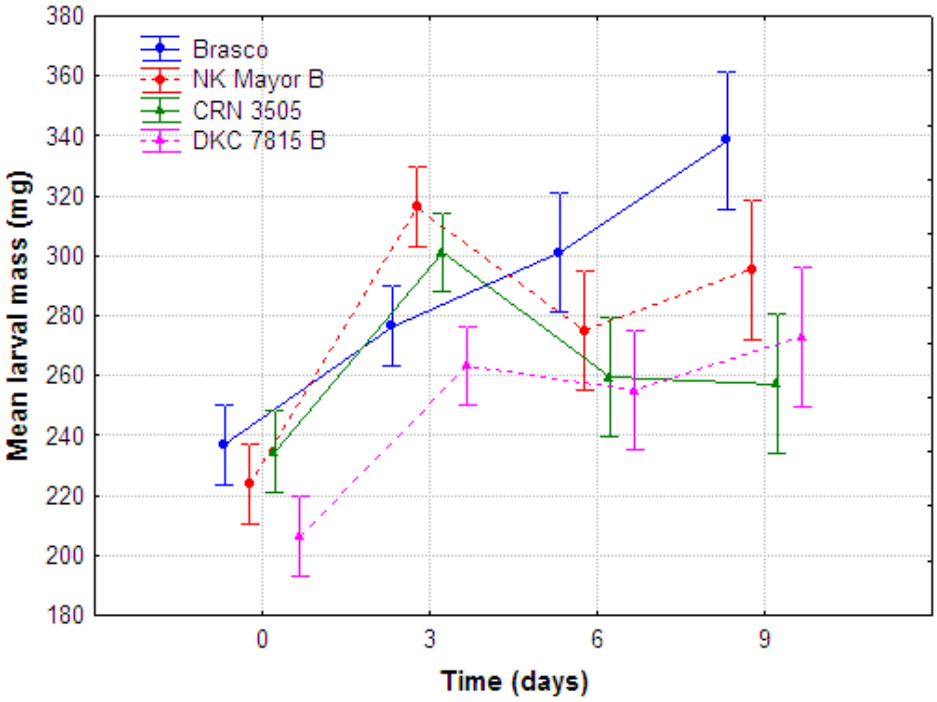

Figure 3.5. Mean mass of *Agrotis segetum* larvae feeding on maize seedlings as 4th instar larvae [event MON810 (DKC 78-15B and non-Bt iso-hybrid CRN 3505) and event Bt11 (NK Mayor B and non-Bt iso-hybrid Brasco)]. (Bars indicate SE).

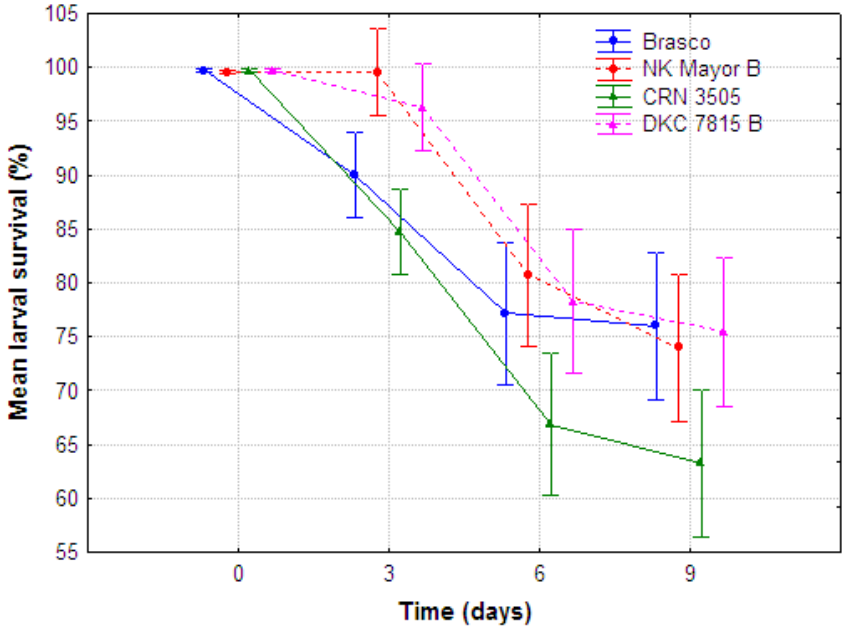

Figure 3.6. Mean percentage survival of *Agrotis segetum* larvae feeding on maize commencing seedlings as 4th instar larvae [event MON810 (DKC 78-15B and non-Bt iso-hybrid CRN 3505) and event Bt11 (NK Mayor B and non-Bt iso-hybrid Brasco)]. (Bars indicate SE).

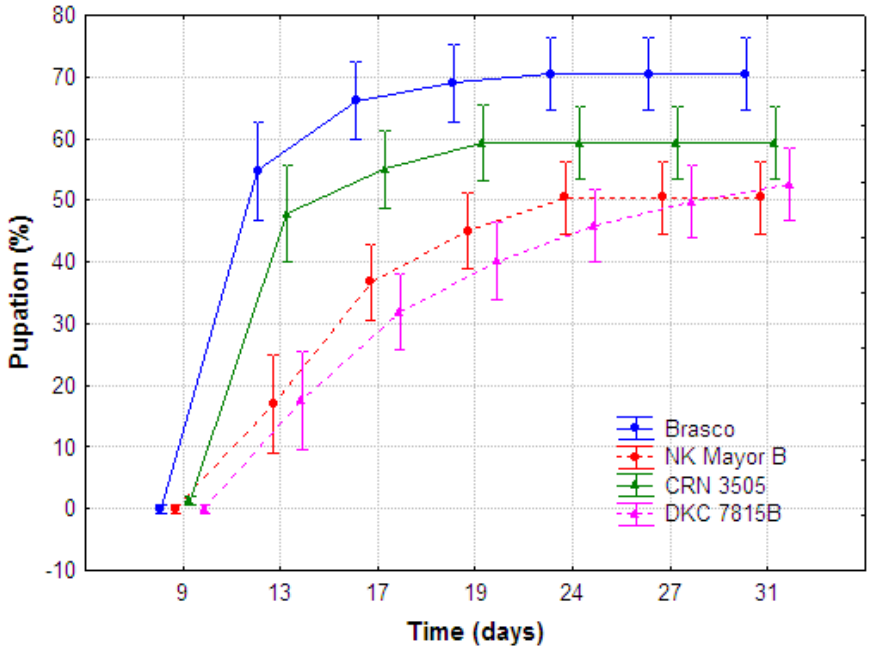

Figure 3.7. Mean percentage pupation of *Agrotis segetum* larvae feeding on maize seedlings from the 4th instar onwards [event MON810 (DKC 78-15B and non-Bt iso-hybrid CRN 3505) and event Bt11 (NK Mayor B and non-Bt iso-hybrid Brasco)]. (Bars indicate SE)

Table 3.1. Fecundity, fertility, female- and male longevity of *Agrotis segetum* moths originating from larvae fed on Bt and conventional maize seedlings from the 4th instar onwards [event MON810 (DKC 78-15B and non-Bt iso-hybrid CRN 3505) and event Bt11 (NK Mayor B and non-Bt iso-hybrid Brasco)].

	Event: Bt 11 with iso-hybrid		**Event: MON810 with iso-hybrid**	
	Brasco	**NK Mayor B**	**CRN 3505**	**DKC 7815 B**
Mean number of eggs laid (±SE)	292.5 (±40.67)	134.4 (±35.06)	209.1 (±36.76)	201.0 (±30.51)
F – value	$F_{(1,36)} = 0.42$		$F_{(1,49)} = 0.02$	
P – value	0.0077		0.8653	
% Hatched (±SE)	64 (±7.82)	70 (±11.79)	40 (±7.26)	81 (±6.97)
F – value	$F_{(1,34)} = 0.18$		$F_{(1,48)} = 16.35$	
P – value	0.6726		0.2462	
Mean female moth longevity (days) (±SE)	8.7 (±0.517)	8.1 (±0.811)	9.3 (±0.812)	7.7 (±0.550)
F – value	$F_{(1,36)} = 0.42$		$F_{(1,41)} = 3.05$	
P – value	0.5206		0.0878	
Mean male moth longevity (days) (±SE)	7.0 (±0.716)	7.5 (±0.609)	5.4 (±0.658)	6.5 (±0.614)
F – value	$F_{(1,36)} = 0.16$		$F_{(1,41)} = 1.38$	
P – value	0.6871		0.2462	

CHAPTER 4: Response of the African bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae) to Bt maize in South Africa

4.1. Abstract

Due to its sporadic occurrence and low levels of damage, *Helicoverpa armigera* (Lepidoptera: Noctuidae) is considered of minor importance and a secondary pest of maize. Damage to maize by *H. armigera* is usually only limited to the ears. In severe cases the silks can be damaged to such an extent that poor pollination occurs. *Helicoverpa armigera* forms part of the ear-feeding guild of maize pests and is directly exposed to Cry1Ab, Bt toxin produced by the plant to control noctuid and crambid stem borers. The effect of Bt maize on *H. armigera* has not yet been studied in South Africa. The objective of this study was to determine the effect of Bt maize on *H. armigera* growth and survival. A laboratory (hybrids used events MON810 and Bt11, with iso-hybids) and greenhouse study (hybrid used MON810 with iso-hybrid) was conducted with 1st instar larvae feeding on whorl leaves and ears respectively. Whorl leaves was observed not to be a suitable food source for *H. armigera* larvae and larval growth was poor. No 1st instar larvae survived to the pupal stage on any of the Bt maize treatments. When feeding on ears larval mass increased on non-Bt maize whereas no increase occurred on Bt maize (significant at P = 0.05). The concomitant data on larval survival provided a similar result. In conclusion, this study has quantified the effects of Bt maize on *H. armigera* and provides important information on the potential for Bt maize to protect maize from feeding damage. However, the likelihood of *H. armigera* becoming an important secondary pest is high.

4.2. Introduction

Busseola fusca (Fuller) (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) are the target pests of Bt maize in South Africa. There are also

several other economically important maize pests that occasionally attack the crop such as *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae). *Helicoverpa armigera* is considered an economically important pest of maize wherever maize is grown in South Africa (Matthee, 1974). Although this pest is commonly associated with maize, it is regarded as a minor or sporadic pest. However, when all crops are considered Moran (1983) ranked *H. armigera* as the most important pest species in South Africa. It is also a significant pest of many other crops and vegetables in the world (Fitt *et al*., 2004). The effects of Bt maize on *H. armigera* as a secondary pest has not been reported previously, but could be relevant in integrated pest management programs in maize. The importance of secondary pests are sometimes overlooked, for example, a study conducted by Ebora *et al*. (1994) underlined the importance of the target species, *Phthorimea operculella* (Zeller) (Lepidoptera: Gelechiidae), but warned that *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) can also significantly affect Bt potato (Cry1Ac) as a secondary pest.

Damage to maize caused by *H. armigera* is usually only limited to the ears. Larvae initially feed on the silks and later penetrate the tips of ears (Fig. 4.1). Due to cannibalism usually only one fully grown larva is found on each ear (Nye, 1960; Matthee, 1974). When ears are still young and bollworm infestations are severe, the silks may be damaged to an extent where poor pollination occurs. Husk leaves covering young ears can also be damaged and should this occur during rainy periods, water can enter the ears. This may lead to fungal growth that will cause kernels to become discoloured and prone to ear rot infection. However, in most cases only the tips of ears are damaged and yield losses are slight. Kernels at the tips of ears are of inferior quality and usually discarded during the harvesting process (Du Plessis & Van den Berg, 1999).

Helicoverpa armigera moths lay yellow-white eggs singly on or near maize ears. One female can lay more than a 1 000 eggs during her life span. Prior to mating and laying eggs, moths feed on nectar and other sources of sugar. Eggs hatch within three to five days (Du Plessis & Van den Berg, 1999). Larvae moult five to six times during which time larvae change colour. When fully grown, larvae leave the plants and burrow 60 to

100 mm deep into the soil where pupation takes place. The pupal stage lasts about two weeks, but may be extended during winter when pupae enter a dormant stage. In the absence of dormancy, the duration of the life cycle is about 50 days (Hill, 1987).

Figure 4.1. *Helicoverpa armigera* larva on maize ear.

Helicoverpa armigera can be controlled by means of natural enemies, cultural control measures and chemical control. The only transgenic crop commercialized in South Africa for the control of bollworms is Bt cotton encoding for the Cry1Ac protein. *Helicoverpa armigera* is susceptible to both Cry1Ab (Bt maize) and Cry1Ac (Bt cotton) proteins, though considerably less so than other Heliothine species such as *Heliothis virescens* (primary target for Bt cotton in USA) (Fitt *et al*., 2004). Although *H. armigera* is considered a non-target species for Bt maize in South Africa, Van Wyk *et al*. (2008) observed that at field level, the incidence of damage caused by *H. armigera* was always significantly lower on Bt maize than on non-Bt maize. This pest is one of the main non-

target pests of concern in risk assessments for release of maize expressing *Cry* proteins with insecticidal properties (Van Wyk *et al*., 2007).

The objectives of this feeding study were to determine the effect of feeding on Bt maize whorl leaves and ears on larval survival and development of *H. armigera*.

4.3. Materials and methods

4.3.1. Larval survival and mass gain

Larvae were collected from ears of non-Bt maize in the Potchefstroom area (46° 43` S, 27° 06` E) of the North West Province, South Africa. Larvae were reared on artificial diet (chickpea based agar diet, developed for *C. partellus*) until pupation. Moths derived from these pupae were allowed to lay eggs on nylon gauze. The first instars were used in various bioassays. Larval survival and mass gain on Bt and non-Bt maize leaves and ears were evaluated in a laboratory bioassay and a greenhouse trail respectively. In the laboratory first instar larvae were allowed to feed on whorl leaves, whereas ears were used under greenhouse conditions. In this study the "whole plant method" approach was used to expose the insect to the actual plants parts that it would consume under natural conditions, as suggested by Birch *et al*. (2004). Both experimental lay-outs were completely randomized designs.

Experiment 1: First instars on maize whorl leaves

One first instar larva was placed in a glass test tube with a 15 cm long piece of maize leaf cut from the central whorl leaves of three to four week old maize plants of the various hybrids. The hybrids were DKC 78-15B (event MON810) with iso-hybrid CRN 3505 and NK Mayor B (event Bt11) with its iso-hybrid Brasco. In this experiment each treatment was replicated 50 times. Test tubes were kept under natural day/night conditions in a laboratory where temperatures fluctuated between 20 and 25 ˚C. Larval mass was determined every fourth day and leaf material was replaced with each assessment. Larval survival as well as mean larval mass was determined.

Experiment 2: First instars on maize ears

Non-Bt hybrid CRN 3505 and Bt hybrid DKC 7815B were used in this study. Ears in the soft dough stage were infested with 10 first instars per ear without removing ears from the plants. Larvae were placed on the tips of ears between the silks by means of a camel-hair brush. Seventy ears of each hybrid were infested. Each infested ear was covered with a white fine organza (see-through material) bag. Ten ears were removed from maize plants of each hybrid twelve days after infestation and dissected to determine larval mass and survival. Seven further samplings were done at three day intervals until larvae reached the pre-pupal stage.

4.4. Data analysis

Repeated-measures analyses of variance (ANOVA) were used to analyze larval mass, and larval survival (StatSoft, Inc., 2009). Data on larval mass were log(x + 1) transformed before analyses. Untransformed data are, however, provided in the figures.

4.5. Results

Experiment 1: First instars on maize whorl leaves

Mass of larvae was significantly lower when feeding on Bt, as compared to non-Bt maize whorl leaves. Differences were significant between CRN 3505 and DKC 78-15B ($F_{(1,98)}$ = 28.16; $P < 0.00001$) as well as Brasco and NK Mayor B ($F_{(1,98)}$ = 79.78; $P < 0.00001$) (Fig. 4.3). Larval mass also differed significantly between the two non-Bt hybrids ($F_{(1,98)}$ = 9.00; $P = 0.003$). The mass of larvae feeding on Brasco increased rapidly over the first 12 days followed by a rapid decrease until day 17. A slow but consistent increase in mass was observed on the non-Bt hybrid CRN 3505, whereas for the two Bt hybrids, larval mass never increased in mass over the trail period (Fig. 4.3).

Larval survival decreased rapidly on both Bt hybrids over the first four days when compared to the non-Bt hybrids. Larval survival was significantly lower on DKC 78-15B compared to CRN 3505 ($F_{(1,98)}$ = 69.74 ; P = 0.00003) as well as on NK Mayor B compared to Brasco ($F_{(1,8)}$ = 200.30; P < 0.00001) (Fig. 4.4).

Experiment 2: First instars on maize ears

Mass of larvae feeding on Bt maize (DKC 78-15B) was significantly lower than those feeding on the non-Bt iso-hybrid (CRN 3505) ($F_{(1,18)}$ = 161.373; P < 0.00001) (Fig. 4.2) (Fig. 4.5). Mass of larvae feeding on the Bt hybrid did not increase over time but a consistent increase was observed when feeding on the non-Bt hybrid. A decrease in mass observed from day 30 onwards of larvae feeding on non-Bt ears was due to the onset of the pre-pupal stage.

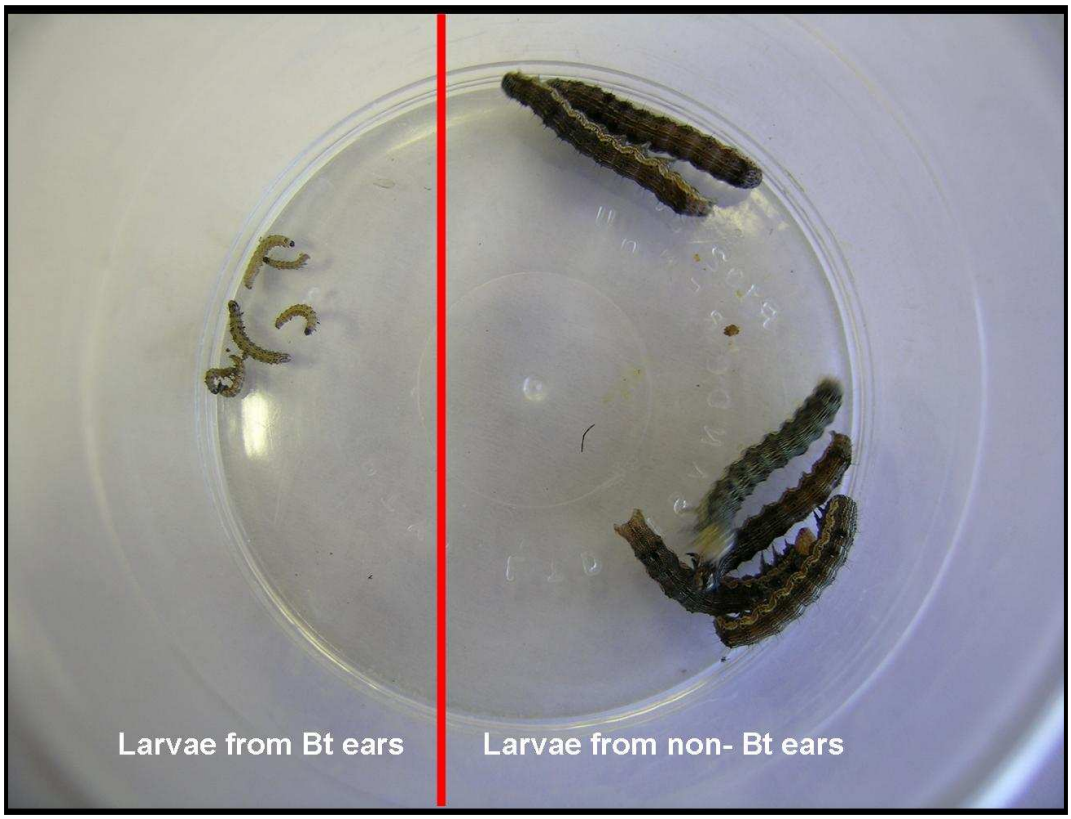

Figure 4.2. A significant difference in *Helicoverpa armigera* larval mass when feeding on Bt (left) and non-Bt (right) ears.

Larval survival decreased slowly over time (Fig. 4.6) and differed significantly between Bt and non-Bt ears for treatments DKC 78-15B and CRN 3505 ($F_{(1,18)}$ = 523.986; P < 0.00001). The more rapid decline in larval survival on CRN 3505 between days 21 and 24 is ascribed to cannibalism, since only one fully grown larva is usually found per ear (Matthee, 1974; Nye, 1960).

4.6. Discussion

These results show that maize leaf tissue is not a very suitable food source for *H. armigera* first instars. Similar observations were made by Wu *et al*. (2002) who reported that *H. armigera* first instar larvae feeding on maize whorls generally did not perform well due to their preference for ears. However, in this study significant differences were observed in both larval mass and larval survival when comparing Bt and non-Bt hybrids. Mass of larvae feeding on leaves of the non-Bt hybrid, Brasco was greater than for any other hybrid indicating that this hybrid was a more suitable host for larval development. Larval mass when feeding on leaves of Bt hybrids, never increased and the survival rate decreased rapidly over the first four days. For larvae feeding on leaves of non-Bt hybrids, mass increased and survival decreased but not as rapidly as when feeding on Bt hybrids. A study conducted by Van Wyk *et al*. (2008) indicated that *H. armigera* larvae do survive on Bt maize ears under field conditions but their numbers were always significantly lower in Bt maize fields compared to the non-Bt fields. The latter result is confirmed in this study conducted under greenhouse conditions.

Pilcher *et al*. (1997) reported that *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larvae, which are secondary pests in the maize ecosystems where *Ostrinis nubilalis* (Lepidoptera: Crambidae) is the main pest, also do not survive when feeding on maize leaf tissue but that larvae do survive in much higher numbers in maize ears than the target pest, *O. nubilalis*. The differences in numbers of surviving larvae between different plant parts (leaf and ear) probably relate to the levels of Bt protein expressed in different tissues (Pilcher *et al*., 1997). Pilcher *et al*. (1997) suggested that a higher dose of

Cry1Ab protein will be required to affect *H. zea* to the same extent as *O. nubilalis*, the target species in Georgia, U.S.A. MON810 maize expresses the *Cry* protein in silk tissue and kernels as well as leaves, tassels, and stalk, thus it is also biologically active against *H. zea* (Horner *et al*., 2003). Although many *H. zea* larvae are able to survive and complete development in Bt maize ears, many suffer negative fitness effects (weight loss and development delay) because of sub-lethal exposure (Horner *et al*., 2003). Padidam (1992) showed that Cry1Ac was about 12 times more toxic to *H. armigera* than Cry1Ab. Consequently, only two insecticidal protein options for sustainable control of *H. armigera* have been identified, Cry1A and Cry2A. Cry1Ab and Cry2Ab were less toxic but potentially useful (Liao *et al*., 2002). Studies conducted by Chakrabarti *et al*. (1998) showed that Cry1Ac protein was the most potent toxin tested followed by Cry1Aa, Cry2Aa and Cry1Ab.

From this study it is concluded that Bt maize will suppress *H. armigera* infestations but not to levels approaching 100%. In a two year study by Burkness *et al*. (2001) control of *H. zea* in Bt hybrids was reported to range between 85 and 88% when compared with the appropriate non-Bt hybrids, suggesting that Bt hybrids provide high levels of larval control. Archer *et al*. (2001) also studied ear damage caused by *H. zea* to four events of Bt maize (MON810, Bt11, Bt176 and CBH354) and reported that no Bt maize hybrid provided consistent control of *H. zea* larvae feeding on kernels. A study conducted by Dowd (2001) indicated that although *H. zea* feeding was slowed down on Bt maize expressing high levels of the protein in the kernels, incidence of infestation was often not affected, and caterpillars remained alive and can eventually damage an equivalent number of kernels. Wu *et al*. (1999) found 100-fold differences in the susceptibility to Cry1Ac of different *H. armigera* populations in China. It is therefore unrealistic to compare the susceptibility of different species unless many independent populations have been tested, preferably with the same protocols (Liao *et al*., 2002).

In this study larvae feeding on Bt ears were always smaller than larvae feeding on non-Bt hybrids, which contributed to a delay in development. Larval establishment did occur on a few ears of Bt maize plants, but once established in ears, larvae developed more slowly.

Because of this delay in development much less ear damage can be expected to occur compared to non-Bt plants. Continued mortality during the extended development of larvae on Bt ears explains the lower incidence of pre-pupa formation. Similarly, Buntin *et al*. (2001) reported that Bt maize with events Bt11 and MON810, reduced whorl infestation and damage of both *S. frugiperda* and *H. zea* in maize in the USA. Bt maize of the events MON810 and Bt11 was observed to cause a steady mortality of *H. zea* larvae during development, but permitted 15 - 40% survival to the prepupal stage compared with non-Bt maize (Storer *et al*., 2001). A delay in development was also observed by Storer *et al*. (2001) who reported that larvae of *H. zea* that did survive developed more slowly on Bt than on non-Bt maize, and that pupation and adult eclosion were delayed by 6 – 10 days when feeding on Bt maize ears.

Because of reduced numbers of *H. armigera* on Bt ears, indirect effects can be expected to occur that can influence parasitoids and predators. Larvae and adults of sap beetles, *Carpophilus* spp. (Coleoptera: Nitidulidae), and larvae of the otitid fly, *Euxesta stigmatis* Loew (Diptera: Otitidae) were observed to be less abundant on Bt than non-Bt maize ears in the USA, mostly because kernel damage caused by *H. zea* was less in Bt maize, which presumably made Bt maize ears less attractive to these insects (Daly & Buntin, 2005). Significant reductions in numbers of larvae of non-target lepidopterans following the use of Bt can indirectly affect other species that rely on lepidopterous larvae as a primary source of food (Peacock *et al*., 1998).

Helicoverpa armigera has a history of demonstrated potential in developing resistance to virtually all the insecticide molecules used against it (Kranthi *et al*., 2005). With constitutive expression of Bt toxins throughout the plant and for the entire growing season, Bt crops have the potential to place the highest selection pressure for such resistance of any insecticide deployed to date (Storer *et al*., 2003). Because the insecticidal activity of transgenic plants also declines significantly as the plants mature (Fitt & Wilson, 2000; Fitt *et al*., 2004), some *H. armigera* larvae are able to complete their development later in the season (Van Wyk *et al*., 2008). This survival poses a

serious risk to sustainability of the technology because it will facilitate resistance development in the pest.

In conclusion, this study has quantified the effects of Bt transgenic maize hybrids on bollworm, *H. armigera* feeding on maize ears and it provides important information on the potential for Bt maize to protect maize from *H. armigera* ear feeding damage. However, based on it ability to develop resistance, the likelihood of *H. armigera* becoming an important secondary pest is high. Although this pest is currently suppressed by Bt maize it could develop resistance, which, in that case, would make it the only ear feeding lepidopteran of importance, with the opportunity of invading the vacant niche usually occupied by other ear feeding lepidopterans (target pests). If this was to happen, chemical control measures, similar to those applied against stem borers before the advent of Bt maize, would again be required.

4.7. Resistance development and monitoring

Helicoverpa armigera has a history of demonstrated potential in developing resistance to virtually all the insecticide molecules used against it (Kranthi *et al*., 2005). Synthetic pyrethroid resistance in field strains of *H. armigera* in South Africa was reported by Van Jaarsveld *et al*. (1998). The Nelspruit populations appeared to be the most resistant to pyrethroids, followed by Pongola, Tala Valley and Brits, Bela-Bela and Vaalharts (Van Jaarsveld *et al*., 1998). Pyrethroid resistance was also found in 54 field strains of *H. armigera* collected between 1995 and 1999 from 23 districts in seven states of India (Kranthi *et al*., 2001). Chakrabarti *et al*. (1998) also reported resistance of *H. armigera* to many conventional organic and synthetic insecticides in many parts of India. Because *H. armigera* have evolved resistance to some insecticides, it also has the potential to become resistant to genetically modified crops with insecticidal properties. The high dose-refuge strategy is the currently recommended world-wide as resistance management strategy for Bt crops (Gujar *et al*., 2007).

With constitutive expression of Bt toxins throughout the plant and for the entire growing season, Bt crops have the potential to place higher selection pressure for resistance development than any insecticide deployed to date (Storer *et al*., 2003). Because the insecticidal activity of transgenic plants also declines significantly as plants mature (Fitt & Wilson, 2000; Fitt *et al*., 2004), some *H. armigera* larvae are able to complete their development later in the season (Van Wyk *et al*., 2008). This survival poses a serious risk to sustainability of the technology because it will facilitate resistance development in the pest.

The toxins expressed in currently available Bt varieties of maize (Cry1Ab) and cotton (Cry1Ac) are very similar in structure and mode of action. Cross-resistance to these toxins has been reported in populations of *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) (Tabashnik *et al*., 1997) and *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) (Gould *et al*., 1992). One implication is that a polyphagous pest, such as *H. zea*, attacking transgenic cotton that produces Cry1Ac might be selected for cross-resistance to transgenic maize that produces Cry1Ab (Tabashnik *et al*., 1997). The same issue was highlighted by Fitt *et al*. (2004) with *H. armigera*, who indicated that if this species is exposed to Cry1Ab in maize ears of Bt maize it would add to selection pressure in a Bt cotton system that would likely express Cry1Ac in a cotton producing region. Although one gene can confer resistance to at least four toxins, genes that confer resistance to fewer toxins also occur in insect populations. For example, resistance to Cry1Ab, but not to Cry1Aa and Cry1Ac, was found in a field-selected strain of diamondback moth, *P. xylostella* in the Philipines (Ballester *et al*., 1994) and in a laboratory-selected strain of the cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) (Estada & Ferré, 1994).

The relatively low susceptibility of *Helicoverpa armigera* to Cry1Ac, its history of resistance development to chemical insecticides and the seasonal decline in expression of Cry1Ac in transgenic cotton necessitated the development of cotton expressing two insecticidal proteins to provide sustainable control of this pest (Liao *et al*., 2005). For an effective insect resistance management strategy for *H. armigera* it was essential that the

second insecticidal protein has a significantly different mode of action to Cry1Ac. Liao *et al*. (2005) conducted a study to determine binding sites for some *Cry* proteins in the brush border membrane vesicles of *H. armigera* and *H. punctigera* (Wallengren) (Lepidoptera: Noctuidae). They found that the binding affinity for Cry1Ac was higher than for Cry1Ab, matching their relative toxicities, and Cry1Ac and Cry1Ab were found to share at least one binding site in both *H. armigera* and *H. punctigera*. However, Cry2Aa did not compete with Cry1Ac for binding and so could be used in transgenic cotton in combination with Cry1Ac to control *H. armigera* and manage resistance (Liao *et al*., 2005). Two strains of pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) selected in the laboratory for resistance to *Bacillus thuringiensis* toxin Cry1Ac had substantial cross-resistance to Cry1Aa and Cry1Ab. The narrow spectrum of resistance and the cross-resistance to activated toxin Cry1Ab suggest that reduced binding of toxin to midgut target sites could be an important mechanism of resistance (Tabashnik *et al*., 2000). Akhurst *et al*. (2003) reported that a composite strain of first instar *H. armigera* from generation 25 were able to complete their larval development on transgenic cotton expressing Cry1Ac and produce fertile adults. The strain was also resistant to Cry1Ab.

Development delays of *H. zea* could also increase the rate of resistance development to Cry1Ab (Peck *et al*., 1999). Emergence of moths from Bt maize in late summer or those emerging the following spring may not be synchronous with non-Bt moths emerging from maize. This asynchrony could result in mating and oviposition by individuals from refuges before adults from the Bt crop have emerged (Gould, 1998; Storer *et al*., 2001; Wu *et al*., 2002). This situation would lead to reproductive isolation of Bt-selected adults and thus partially increase the rate of resistance development. On cotton, for example, Bird & Akhurst (2004) reported that life history parameters of *H. armigera* larvae feeding on young cotton plants showed a significant developmental delay of up to seven days for the resistant strain compared with the susceptible strain on non-Bt cotton. Delays in larval development, such as those experienced by *H. zea* larvae exposed to moderate doses of Cry1Ab in MON810 Bt maize (Storer *et al*., 2001), may delay pupation late enough for environmental conditions to trigger diapause. This would result in a greater proportion of

pupae remaining in the soil, thus not contributing to the fall moth population (Horner *et al*., 2003).

All these studies mentioned above highlight the importance of resistance monitoring. Fitness costs may help to delay or prevent the spread of alleles conferring resistance to Bt crops when refuges, but also the most appropriate refuges, of non-Bt host plants are present (Carrière *et al*., 2004; Wu *et al*., 2002). Implications such as *H. armigera* history of insecticide resistance, decline of Bt toxicity through the growing season, some survival of *H. armigera* on Bt ears, and delay in development create a complexity of problems to overcome. The following factors must also be kept in mind when considering the rate of resistance evolution in an insect population to a Bt crop: pest bionomics, initial frequency of resistance alleles in the pest population, genetic mode and stability of resistance, fitness of resistant individuals, temporal and spatial distribution of the insect pest on different host plants, and gene flow among different geographical populations (Wu *et al*., 2002). Although no evidence of development of field-level resistance in *H. armigera* has been reported, Gujar *et al*. (2007) highlighted the importance of the regular monitoring of insect susceptibility to Bt toxin and request that this becomes an essential pre-requisite of Bt resistance management for detecting and quantifying resistance development in the target insect pests. It is important to detect resistance in its early developmental phase, so that proper management measures can be initiated in time (Kranthi *et al*., 2005).

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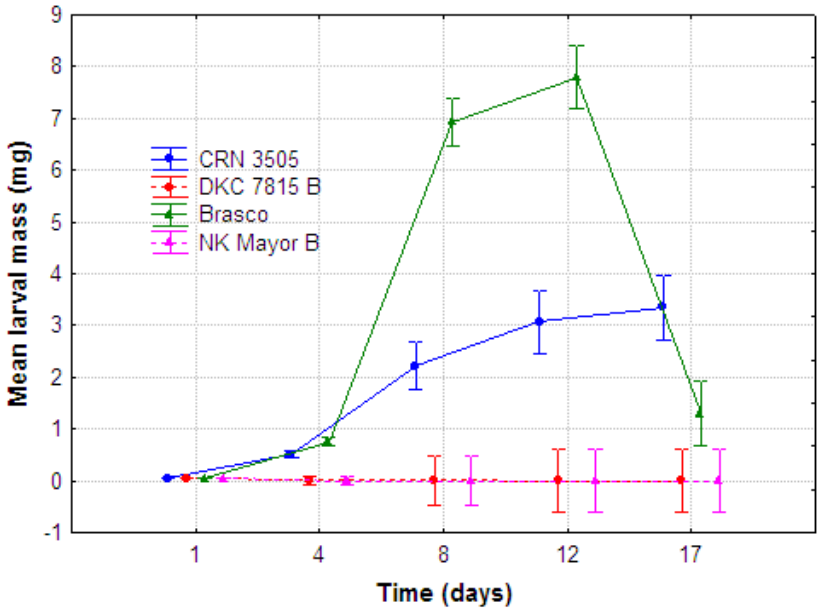

Figure 4.3. Mean mass of *Helicoverpa armigera* larvae feeding on maize whorl leaves from the 1st instars onwards (Event MON810 hybrid, DKC 78-15 B with non-Bt iso-hybrid, CRN 3505 and event Bt11 hybrid, NK Mayor B with non-Bt iso-hybrid, Brasco) (Bars indicate SE).

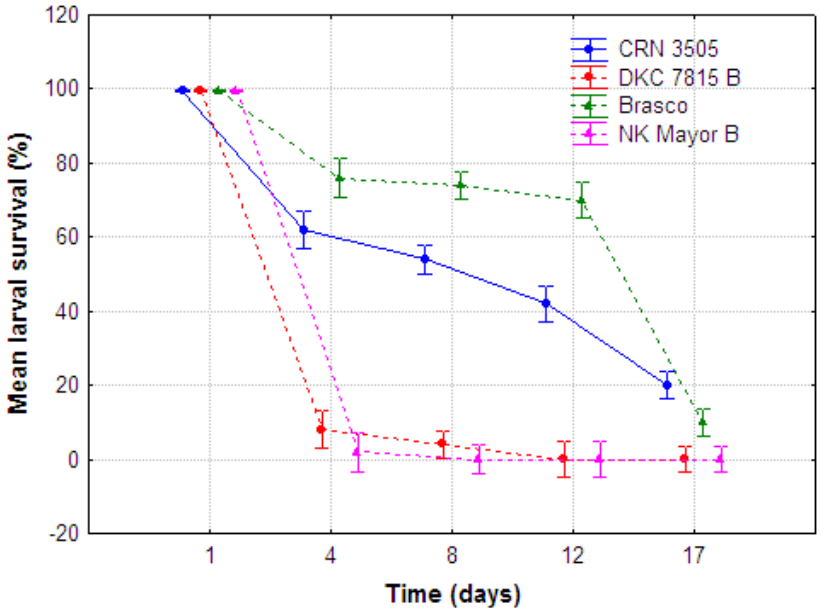

Figure 4.4. Mean percentage survival of *Helicoverpa armigera* larvae feeding on maize whorl leaves from 1st instars onwards (Event MON810 hybrid, DKC 78-15 B with non-Bt iso-hybrid, CRN 3505 and event Bt11 hybrid, NK Mayor B with non-Bt iso-hybrid, Brasco) (Bars indicate SE).

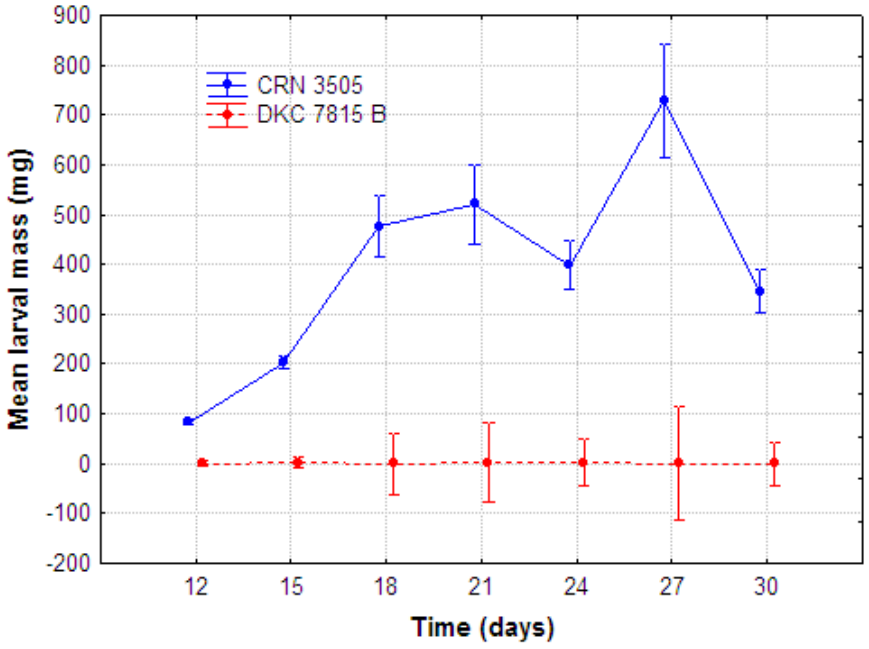

Figure 4.5. Mean mass of *Helicoverpa armigera* larvae feeding on maize ears from 1st instars onwards (Event MON810 hybrid, DKC 78-15 B with non-Bt iso-hybrid, CRN 3505) (Bars indicate SE).

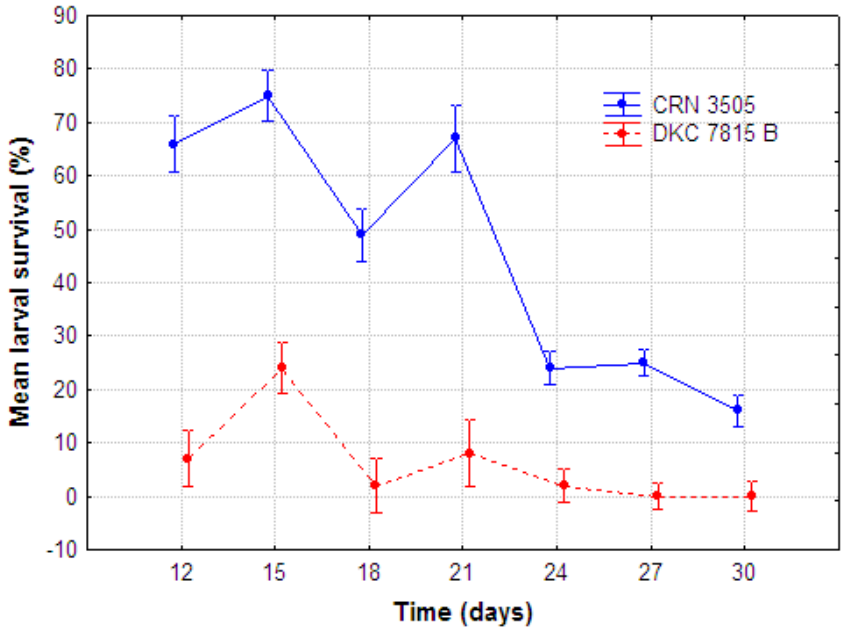

Figure 4.6. Mean percentage survival of *Helicoverpa armigera* larvae feeding on maize ears from 1st instars onwards (Event MON810 hybrid, DKC 78-15 B with non-Bt iso-hybrid, CRN 3505) (Bars indicate SE).

CHAPTER 5: Effect of Bt maize expressing Cry1Ab toxin on non-target coleopteran insect pests, *Heteronychus arator* (Scarabaeidae) and *Somaticus angulatus* (Tenebrionidae)

5.1. Abstract

Many studies have been done on the controlling effect of Bt maize on the target pests of maize, but literature dealing with the effect of Bt maize on non-target pests of maize is scarce. *Heteronychus arator* Fabricius (Coleoptera: Scarabaeidae) and *Somaticus angulatus* (Fahraeus) (Coleoptera: Tenebrionidae) are regarded as sporadic but serious pests of maize in South Africa. Little information is available on the effect of Bt maize expressing Cry1Ab on other agronomically important pests. The objective of this study was to determine the effect of Bt maize expressing Cry1Ab on these two non-target coleopteran pests although Cry1Ab is only targeting lepidopteran stemborers. Direct effects of Bt maize on non-target pests can easily be measured in laboratory experiments that stretch over the entire lifecycle of the pests. Feeding studies were conducted to determine the effect of Bt maize on mortality, growth and fertility of *H. arator*. Larval survival and mass gain as well as beetle fertility were determined for *S. angulatus*. The study showed that the effect of Cry1Ab toxin on the biology of *H. arator* and *S. angulatus* was insignificant. No significant differences were observed in any of the parameters measured in this study. It can be concluded that the Cry1Ab toxin targeting lepidopteran pests will not have an adverse effect on either *H. arator* or *S. angulatus*.

5.2. Introduction

The family Scarabaeidae is reported as one of the 25 most important families of insects and mites on cultivated plants in South Africa (Moran, 1983). The Scarabaeidae is the second most important family in the Coleoptera order with a pest status of 229, while 33 of these species are recognized pests (Moran, 1983). *Heteronychus arator* Fabricius

(Coleoptera: Scarabaeidae) is ranked with a pest status of 62 among the 101 most important plant-feeding pests. Larvae of soil-inhabiting Tenebrionidae (false wireworms) are recognized worldwide as pests of maize (Allsopp, 1980). The family Tenebrionidae is not reported under the 25 most important families of insects and mites on cultivated plants in South Africa by Moran (1983). However, it ranks 12th in importance in the Coleoptera order, with a pest status of 6, while only one species is recognized as a pest (Moran, 1983).

The black maize beetle, *H. arator* (Fig. 5.1), is indigenous to Africa and is a sporadic but serious pest of maize in South Africa (Du Toit, 1998). It has only one generation per year (Fig. 5.2) (Du Toit, 1998). Damage to maize is caused by adult beetles that feed on the subterranean part of maize seedlings. Larvae (Fig. 5.3) do not cause damage when feeding on decomposing organic matter in the soil. Damage to maize seedlings during the planting season is done by "old" beetles that are already present in the maize field. Some damage may occur in late summer when the next generation of adults emerges. *Heteronychus arator* causes damage to maize during the early growth stages up to seven weeks after planting, with the peak period of damage occurring three to five weeks after planting. In some cases the extent of damage to maize may be so severe that replanting is justified (Du Toit, 1998).

Figure 5.1. Black maize beetles damaging maize stem.

Figure 5.2. Black maize beetle eggs.

Figure 5.3. White grub, larva of *H. arator*.

As a result of feeding the damaged underground stem has a ragged and frayed appearance, which distinguishes *H. arator* damage from that of the common cutworm, *Agrotis segetum* (Denis & Schiffermüller) (Lepidoptera: Noctuidae) (Du Toit, 1998) that cut through the stem. Two above-ground damage symptoms to maize seedlings can be distinguished. Feeding damage results in death of the growth point called “dead-heart” and the appearance of longitudinal yellow stripes on the leaves. Older plants normally survive attack but may remain weakened and are prone to lodging due to large numbers of beetles feeding at the bases of maize plants (Du Toit, 1998).

In a survey of *Somaticus* species in the main maize growing area of South Africa (the Maize Triangle), 15 species were recorded. *Somaticus terricola* (Fahraeus) (Coleoptera: Tenebrionidae) was the most widely distributed in this area, while the most injurious species to maize, *S. angulatus* (false wireworm), had the widest distribution of those species that occurred mainly in the western part of the growing area (Drinkwater, 1990). Subterranean damage to maize seedlings by *S. angulatus* can result in plant population reductions of up to 60% in individual fields (Drinkwater, 1987). In a study conducted by Drinkwater (1989), all cases where damage to maize seedlings by tenebrionid larvae was

investigated, only *Somaticus* species were found to be the cause. Although more than 25% of the sampled maize fields were infested by *S. angulatus* and *S. terricola*, the former was responsible for damage in about 75% and the latter in about 6% of the cases. This can possibly be ascribed to differences in the geographical distribution of the two species. Unlike *S. terricola*, *S. angulatus* was found in the drier western part of the maize production area where sporadic drought conditions often lead to less vigorous growth, rendering the seedlings more vulnerable to attack (Drinkwater, 1989). *Somaticus angulatus* larvae (Fig. 5.4) damage seedlings by chewing holes into the subterranean stems. The adult beetles (Fig. 5.6) do not cause any damage (Drinkwater *et al*., 2002).

Figure 5.4. *Somaticus angulatus* larvae.

Figure 5.5. *Somaticus angulatus* eggs.

Figure 5.6. *Somaticus angulatus* beetle.

Van Wyk *et al*. (2007) used an ecological model to identify and prioritize Lepidoptera species that are primary consumers but not target pests of Bt maize. Except for studies

on cutworm (*Agrotis segetum*) (Lepidoptera: Noctuidae) (Erasmus *et al*., 2010), no evaluation of the effect of Bt maize on other non-target primary consumers of Bt maize has yet been done in South Africa. The effect of exposure of non-target primary consumers to Bt maize would not necessarily result in death but could be some form of individual reduced fitness (Van Wyk *et al*., 2007).

Only chemical control is used in South Africa to control *H. arator* and *S. angulatus*. Various seed dressings and granular insecticides, two emulsifiable concentrates and one bait formulation are registered for control of *H. arator* and *S. angulatus* (MIG, 2008). There is currently no genetically modified maize registered in South Africa for control of Coleoptera species. The Bt toxin, Cry1Ab (commercialized in South Africa) is selective for Lepidoptera and therefore the impact of Bt maize on non-Lepidopteran pests is expected to be minimal (Eizaguirre *et al*., 2006; Pons *et al*., 2005). The efficacy of granwar insecticide treatments depends on factors such as placement of the chemical and planting depth. In the traditional pest distribution area, preventative insecticide treatments are recommended. Seed dressings and granular insecticides are registered for preventative treatment applied during planting. Corrective spray applications are advisable in those areas beyond the traditional distribution area, and then only in maize fields which are notably infested (Du Toit, 1998).

Little quantitative data are available on the insecticidal spectrum of single purified *Bacillus thuringiensis* proteins against a wide range of agronomically important pests (MacIntosh *et al*., 1990). Many studies have been done on the controlling effect of Bt maize on the target pests of maize but literature dealing with the effect of Bt maize on non-target pests of maize is scarce. Bt maize can potentially negatively affect population densities of non-target phytophagous insects due to the toxin. Direct effects of Bt maize on the non-target pests can easily be measured in laboratory experiments that stretch over their entire lifecycle (Pons *et al*., 2005). Consequently, non-target pests may ingest Bt-toxin as noted by Dutton *et al*. (2004). Although stemborers are the most harmful pests of maize in South Africa, other herbivore pests such as *H. arator* and *S. angulatus* may also affect crop yield. However, differential expression of toxin in Bt maize during the life

cycle of the genetically modified plant make it necessary to asses its impact fully (Eizaguirre *et al*., 2006).

The objective of this study was to determine the effect of Bt maize expressing Cry1Ab on the non-Lepidopteran pests of maize, i.e. maize beetle, *H. arator* and *S. angulatus*, the false wireworm.

5.3. Materials and methods

5.3.1. *Heteronychus arator* adult mortality, mass and oviposition

Overwintered sexually immature beetles that are active from late January until late April were collected from the field in the eastern region of the maize production area in South Africa. Beetles were collected by using light traps at the end of February. Two laboratory experiments were conducted to compare beetle mass, mortality, and fertility when feeding on two to four week old maize stems of Bt and non-Bt maize. The experimental lay-out was a completely randomized design.

Experiment 1a: Comparison of male and female mortality and mass gain

Beetles were fed on 7 cm long pieces of stem of maize hybrids DKC 78-15B (Bt – MON810) and CRN 3505 (non-Bt). Thirty male and 30 females were evaluated per hybrid. Beetles were kept separately in 10 cm long glass vials provided with 20 ml washed sand. Beetle mass was recorded at one week intervals from capture until day 47 and then at fortnightly intervals until day 119 when beetles started to die off. Beetle mortality was also monitored until the end of the study.

Experiment 1b: Comparative mortality and oviposition

Thirty female and 20 male beetles were placed per (40 × 40 × 22 cm) plastic container and replicated three times for each hybrid. The two hybrids used in the experiment were DKC 78-15B (Bt – MON810) and CRN 3505 (non-Bt iso-hybrid). The containers were provided with a 10 cm layer of washed sand. Drinking water was provided using water-

filled test-tubes (7 × 1 cm) topped with cotton wool plugs. The beetles were fed four-week old maize stems that were replaced once a week. Each hybrid was replicated three times. Beetles were monitored over a 320 day period. Therefore, beetle mortality was monitored at weekly intervals but during winter months this was done every second week. The sand was sifted once a week and later in the season every second week to collect eggs. The total number of eggs laid per 30 females was determined. The eggs were kept in glass vials on a mixture of moist peat and sandy soil to determine the number of viable eggs per 30 females.

5.3.2. *Somaticus angulatus* larval survival, mass gain and oviposition

Adults were collected by hand from fields in the Hoopstad area (Free State Province, South Africa) during March. Beetles were kept in plastic containers (see above) with the bottoms covered with a 5 cm layer of sifted, washed sand. Beetles were fed apples and green maize leaves which also provided shelter. Drinking water was provided as described above. First instars were kept at 25˚C and 65% humidity since larvae do not feed until the second instar (Drinkwater, 1987).

Two studies were done to compare mean larval mass and mean larval survival on Bt- and non-Bt maize seedlings. These studies involved laboratory bioassays where maize seedling stems were fed to larvae. The "whole plant method" suggested by Birch *et al.* (2004) was used to evaluate the effect of the transgenic plant and not only the transgene product. The following four hybrids were used: DKC 78-15B (Bt: MON810), CRN 3505 (non-Bt iso-hybrid for 78-15B), NK Mayor B (Bt: Bt11), and Brasco (non-Bt iso-hybrid for NK Mayor B).

Experiment 2a: Second instar larvae

Survival of second instar larvae were evaluated on maize seedlings in the laboratory. Cuttings (1.5 cm long) of seedling stems of 1-2 week old maize plants were placed in test tubes (75 mm long, 10 mm diameter). One second instar larva was placed per test tube. Test tubes were held in the incubator at 25 ˚C and 65% humidity. Each hybrid was replicated 50 times. The seedlings were replaced with new seedling stems every 3-4 days

when larval mass was determined. Test tubes contained autoclaved soil in sufficient quantity to cover stem cuttings completely. During the first evaluation using hybrids CRN 3505 (non-Bt iso-hybrid) and DKC 78-15B it was noticed that larvae were reluctant to start feeding. In the subsequent evaluation of hybrids Brasco (non-Bt iso-hybrid) and NK Mayor B (Bt), dry Pronutro cereal was added to the soil to serve as a feeding stimulus. The cereal was removed from the soil between day 36-49, when stem cuttings started to show visible feeding symptoms. Mass of larvae was determined until the pre-pupal stage was reached.

Experiment 2b: Fourth instar larvae

To obtain larger larvae of uniform age, second instar larvae were reared as described above, on Pronutro, until they reached the fourth instar before they were used in the experiment. The same maize varieties as above were used to compare larval survival and mean mass of fourth instar larvae when feeding on Bt- and non-Bt maize seedling stems. Each hybrid was replicated 50 times. One larva and a 1.5 cm piece of seedling stem cutting were placed per test tube (125 mm long, 13.5 mm diameter) and were covered with Pronutro soil mixture. The mixture was used for the first week after which only soil was used. Survival and mass were recorded every 3-4 days until pre-pupae started to form.

Experiment 2c: Oviposition

Beetles were collected as describe above before the onset of the experiment to evaluate the possible effect that feeding on Bt maize could have on fertility and fecundity. Beetles were sexed by size since female beetles are larger than males. Ten male and 10 female beetles were placed in each of 22 plastic containers (17 × 12 × 8 cm), using 11 containers for each of the two maize hybrids. These were DKC 78-15B (Bt: MON810) and CRN 3505 (non-Bt iso-hybrid for DKC 78-15B). The containers were provided with a 3 cm layer of sand. Green maize leaves of each hybrid were provided as food and replaced daily. Sand was sifted every third day to collect eggs. The number of eggs laid per container and egg mass was determined. Eggs were kept in an incubator at 25°C and 65% humidity until hatching, when larval numbers were recorded.

5.4. Data analysis

Repeated measures ANOVA were used to analyze beetle mortality, beetle mass, larval survival, larval mass, fertility and fecundity over time (StatSoft, Inc., 2009).

5.5. Results

5.5.1. *Heteronychus arator* mortality, mass gain and oviposition

Experiment 1a: Comparison of male and femal mortality and mass gain

The percentage mortality of male and female beetles increased over time but did not differ significantly over a 119 day period between the Bt and non-Bt iso-hybrid. No differences were observed between mortality of male beetles feeding on DKC 78-15B and CRN 3505 ($F_{(1,4)} = 0.062$; $P = 0.816$) as well as for female beetles ($F_{(1,4)} = 4.252$; $P = 0.108$) (Fig. 5.7). Beetle mass decreased slowly over time with no differences between the hybrids for male ($F_{(1,58)} = 0.288$; $P = 0.593$) and female beetles ($F_{(1,58)} = 1.472$; $P = 0.229$) (Fig. 5.8).

Experiment 1b: Comparative mortality and oviposition

The mortality of male ($F_{(1,4)} = 0.919$; $P = 0.392$) and female ($F_{(1,4)} = 0.161$; $P = 0.705$) beetles feeding on DKC 78-15B and CRN 3505 were not significantly different over a 173 day period, although mortality increased slowly over time (Fig. 5.9). Fertility of female beetles peaked between 182 and 265 days for both hybrids with no significant differences observed between the total number of eggs laid per 30 females ($F_{(1,4)} = 0.002$; $P = 0.969$) (Fig. 5.10). The number of eggs that hatched per 30 females also did not differ significantly between hybrids ($F_{(1,4)} = 0.063$; $P = 0.814$) (Fig. 5.11).

5.5.2. *Somaticus angulatus* larval mortality and oviposition

Experiment 2a: Second instar larvae

Mean survival of larvae feeding on CRN 3505 and DCK 78-15B decreased rapidly with no significant differences observed between hybrids ($F_{(1,18)}$ = 0.169; P = 0.686) (Fig. 5.12). A slower decrease was observed for Brasco and NK Mayor B ($F_{(1,18)}$ = 1.195; P = 0.289) (Fig. 5.12) which was not significantly different between the two. The results were due to the absence of Pronutro cereal in the experiment with CRN 3505 and DKC 78-15B. From these data it can be concluded that Pronutro can serve as a valuable stimulus to the onset of larval feeding under laboratory conditions.

There were no significant differences in mean larval mass when feeding on CRN 3505 and DKC 78-15B ($F_{(1,198)}$ = 0.497; P = 0.481) with a slow increase in mass observed over time. A more rapid increase was observed on Brasco and NK Mayor B but also without significant difference ($F_{(1,198)}$ = 2.794; P = 0.096) (Fig. 5.13). There were, however, significant differences between CRN 3505 and Brasco ($F_{(1,198)}$ = 51.729; P < 0.0001), and between DKC 78-15B and NK Mayor B ($F_{(1,198)}$ = 21.322; P < 0.0001) (Fig. 5.13).

Experiment 2b: Fourth instar larvae

A low initial incidence of larval mortality persisted until day 50 followed by a rapid decrease in survival onwards. Larval survival did not differ significantly between CRN 3505 and DKC 78-15B ($F_{(1,8)}$ = 1.941; P = 0.201), as well as between Brasco and NK Mayor B ($F_{(1,8)}$ = 2.149; P = 0.181) (Fig. 5.14). No significant differences were observed between mean larval mass for CRN 3505 and DKC 78-15B ($F_{(1,48)}$ = 0.888; P = 0.351), and for Brasco and NK Mayor B ($F_{(1,48)}$ = 0.244; P = 0.624) (Fig. 5.15). The highest larval mass was observed around 63 days after commencement of the experiment after which there was a general decrease in mean larval mass on all the different hybrids. This decrease in larval mass can be due to an insufficient diet or the onset of pupuation.

Experiment 2c: Oviposition

There were no significant differences between the mean number of eggs laid per 10 female beetles when feeding over a 29 day period on CRN 3505 and DKC 78-15B ($F_{(1,20)}$ = 1.728; P = 0.204). There were also no differences with regard to the number of eggs that hatched ($F_{(1,20)}$ = 4.138; P = 0.055) over the same period (Fig. 5.16). The mean mass of eggs also did not differ significantly ($F_{(1,20)}$ = 1.429; P = 0.246) (Fig 5.17).

5.6. Discussion and conclusion

Little information is available on the effects of the Bt toxins on non-target insects (Deml *et al*., 1999). Most of the previous studies in South Africa were performed with target insect species of which the results were partially predictable (Van Rensburg, 1998; Van Rensburg, 2001, Van den Berg & Van Wyk, 2007), since it is known that Cry1Ab targets lepidopteran stemborers. It is most recently that focus has fallen on non-target species in South Africa (Erasmus *et al*., 2010). Considering this situation, this study was set up to investigate the influence of Bt toxin (Cry1Ab) on the growth and survival of insect pests from other taxa. Because of this we deemed it necessary to determine the effect of Bt maize (Cry1Ab) on the two most economically important coleopteran pests of maize in South Africa. Deml *et al*. (1999) found that CryIIIA was effective against Lepidoptera, which was quite unexpected because according to literature this endo-toxin should be harmful only to certain Coleoptera (Knowles, 1994). The opposite can be true, Cry1Ab could have an effect on coleopterans, especially *Heteronychus arator* and *Somaticus angulatus* which have never been evaluated on Bt maize before this study. The reason for using *H. arator* and *S. angulatus* is that these two species differ in the life stage that damages maize and that is therefore exposed to Bt toxin. *Heteronychus arator* beetles cause damage, whereas for *S. angulatus* the larval stage is the pest. In such studies of plants producing Bt toxins, Deml *et al*. (1999) suggested that tests at the species-level should be mandatory, and that target and non-target insects should be used which are really relevant to the plants in the field.

However, this study indicated that there were no significant effects on *H. arator* mortality, mass, fertility or fecundity when feeding on Bt maize. The same results were observed for *S. angulatus*, with no effect on survival of second and fourth instars. Also no significant effect was observed on larval mass, number of eggs laid and hatched. Therefore, it did not matter if a coleopteran beetle or larvae fed on Bt maize, there were no adverse effects.

Romeis *et al*. (2008) proposed that the process of testing surrogate species is intended to be efficient and rigorous, focusing the resources to address potential risks or uncertainties and eliminating from further consideration the risks that are negligible. *Heteronychus arator* and *S. angulatus* can be seen as surrogate species in this study and, therefore, no future testing will be necessary on coleopteran species in South Africa to determine the effect of Bt maize expressing Cry1Ab, because the different parameters measured in this study indicated that there was no adverse effect of Bt maize on Coleoptera.

Similar results were reported for non-target species occurring elsewhere. Dowd (2000) reported that control of non-caterpillar pests such as sap beetles by Bt maize expressing Cry1Ab is expected to be low because of the lack of efficacy of the protein against Coleoptera, but may occur indirectly because caterpillar damage, which attracts sap beetles, is reduced. The two coleopteran species used in this study can be considered to be polyphagous, especially the *Somaticus* beetles, which are adapted to live in semi-arid conditions where it feeds on detritus. The level of feeding that beetles and larvae were subjected to on Bt maize in this study was unrealistically high since under field conditions these beetles also feed on other plants species.

Defining potential exposure of insects to selection by Bt toxin is an important aspect of resistance risk assessment (Fitt *et al*., 2004). The maximum potential exposure of a non-target species to a transgenic crop is based on geographic range, habitat specificity, local abundance, prevalence and temporal association with the crop (Andow & Hilbeck, 2004). The following criteria are used to rank species for maximum potential exposure to Bt-toxin: occurrence**,** abundance**,** presence and linkage in the maize ecosystem as well as

potential adverse effects that exposure may have on the non-target species (Andow & Hilbeck, 2004). In this context "occurrence" refers to the presence of a non-target species in the agroecosystem, its geographic range and prevalence. "Abundance" refers to local abundance and prevalence while "presence" involves temporal association with the crop. "Linkage" refers to habitat specificity and the degree of specialization of the non-target species on maize. Linkage might also be called feeding specialization and focuses on trophic relationships. The linkage of both *H. arator* and *S. angulatus* to the maize ecosystem is very low and potential exposure to Bt maize expressing the Cry1Ab protein is therefore low.

Pons *et al*. (2005) evaluated the impact of Bt maize, expressing Cry1Ab protein, on wireworms at farm-scale by comparing their abundance on Bt- and non-Bt plots in Spain. The Bt maize did not affect the incidence of the wireworm, *Agriotes lineatus* (Linnaeus) (Coleoptera: Elateridae) that attack maize seed and seedlings (Pons *et al*., 2005). Eizaguirre *et al*. (2006) also reported during a six year study in Spain that Bt maize did not have a negative impact on *A. lineatus*. Results by Li *et al*. (2007) in China showed no significant differences in arthropod community-specific parameters between Bt and non-Bt rice. Based on their findings they concluded that Bt rice generally exerts no marked negative effects on the arthropod community in paddy fields. Daly & Buntin (2005) also found no consistent effect of Bt maize event MON810 on phytophagous coleopterans.

Bt maize expressing Cry3Bb1 is planted to control *Diabrotica virigifera virgifera* LeConte (Coleoptera: Chrysomelidae) (Al-Dee & Wilde, 2003) in the USA. The impact of this toxin was evaluated by Al-Dee & Wilde (2003) who concluded that direct impact will occur only on chrysomelids and possibly related taxa. They reported no significant difference between Bt maize expressing Cry3Bb1 and non-Bt maize in the number of beneficial insects visually observed in fields.

From this study and examples listed above, it appears that Bt maize events expressing Cry1Ab toxin targeting lepidopteran pests will not have an adverse effect on Coleoptera species. Because Bt maize showed no effect on *H. arator* and *S. angulatus* these pests

may still be important pests in Bt maize fields and applications of insecticides may still be required if the population exceeds the economic threshold in South Africa.

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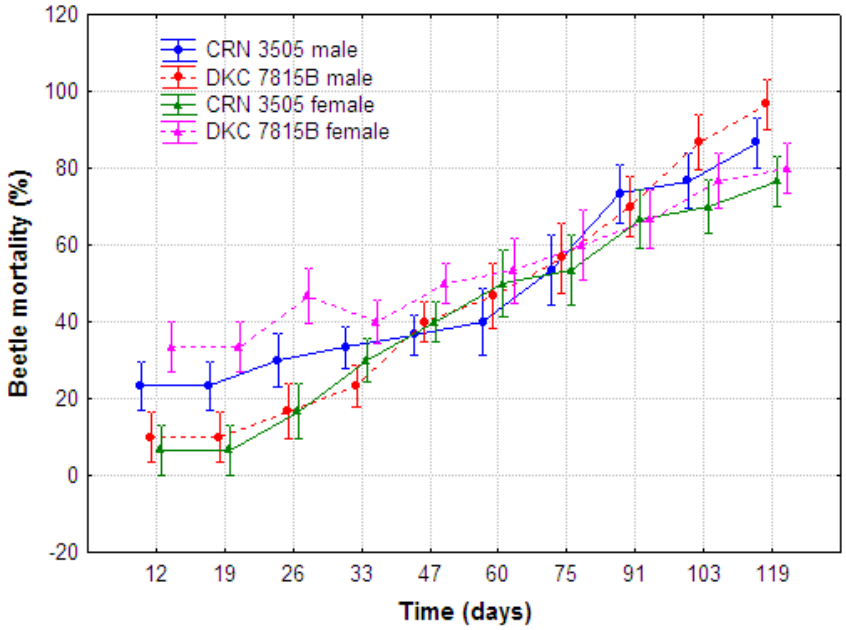

Figure 5.7. Mean percentage mortality of male and female *Heteronychus arator* beetles feeding on Bt (DKC 78-15B) and non-Bt maize (CRN 3505) in glass vials. (Bars indicate SE).

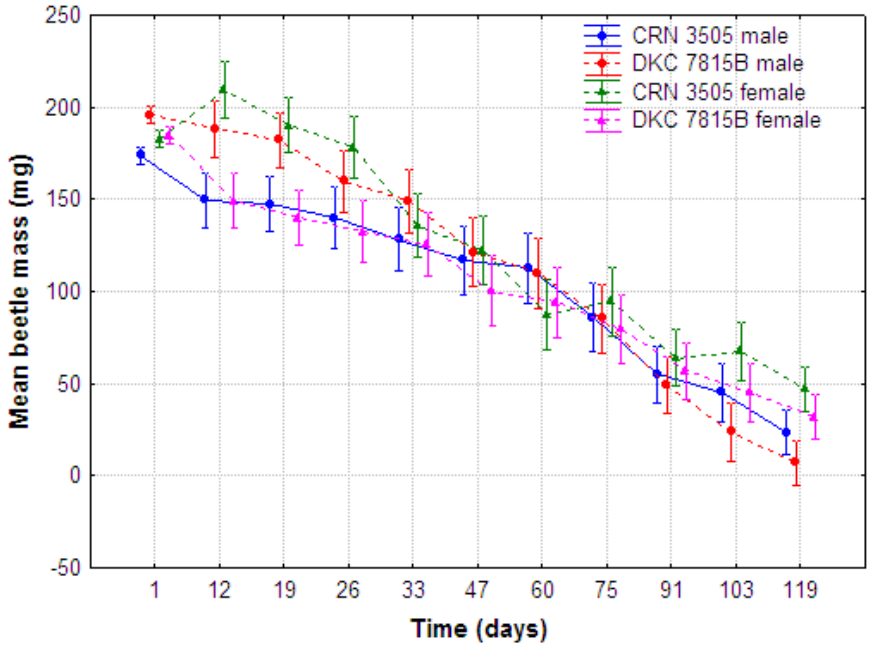

Figure 5.8. Mean mass of male and female *Heteronychus arator* beetles feeding on Bt (DKC 78-15B) and non-Bt maize (CRN 3505) in glass vials. (Bars indicate SE).

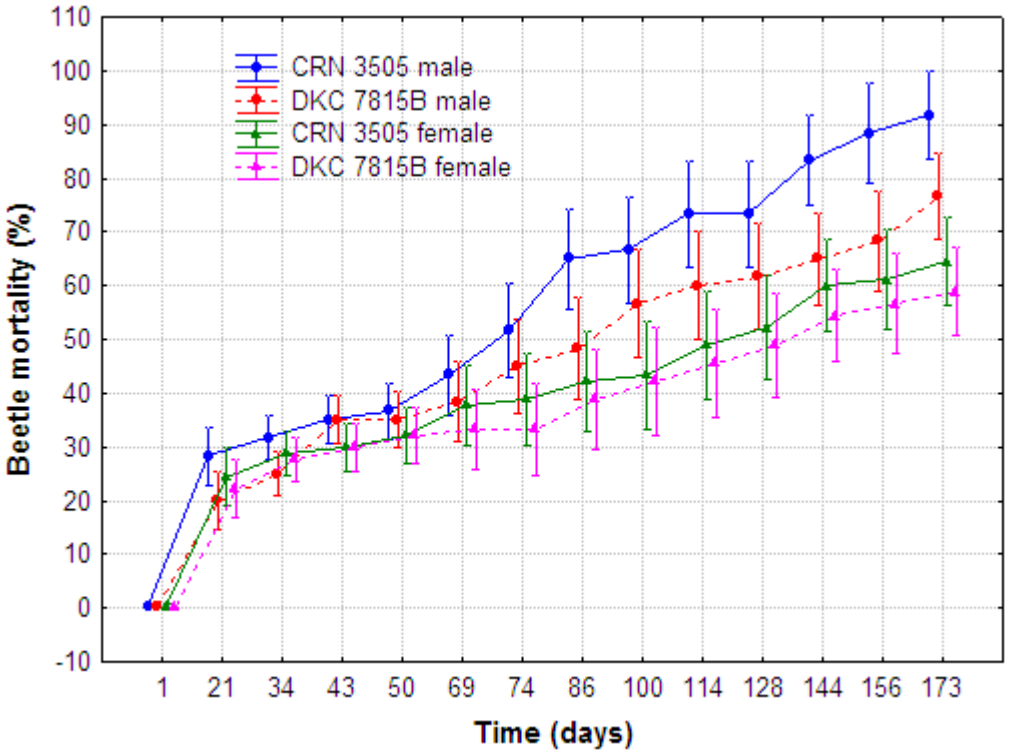

Figure 5.9. Mean percentage mortality of male and female *Heteronychus arator* beetles feeding on Bt (DKC 78-15B) and non-Bt maize (CRN 3505) in plastic containers. (Bars indicate SE).

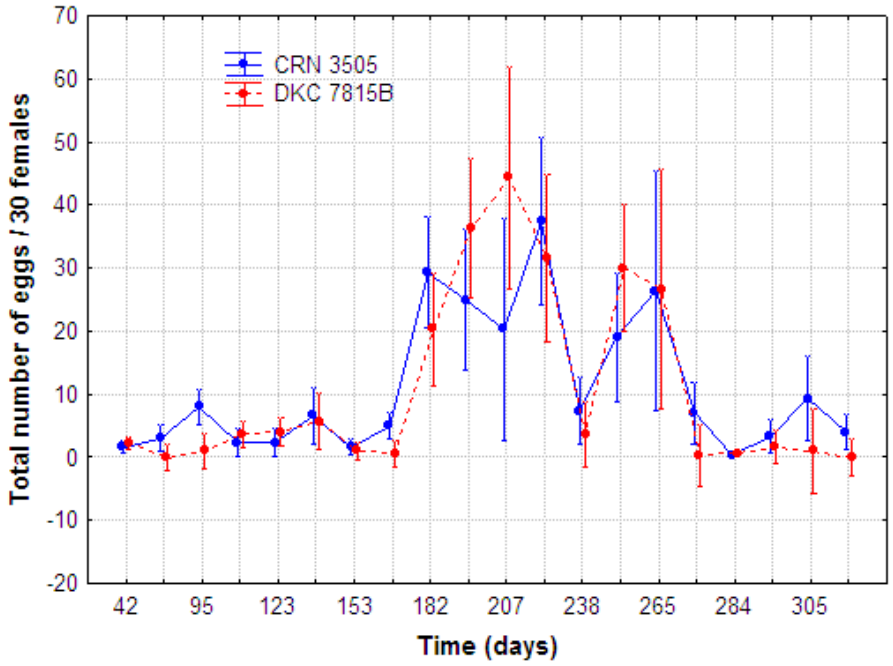

Figure 5.10. Total number of eggs laid per 30 female *Heteronychus arator* beetles feeding on Bt (DKC 78-15B) and non-Bt maize (CRN 3505) in plastic containers. (Bars indicate SE).

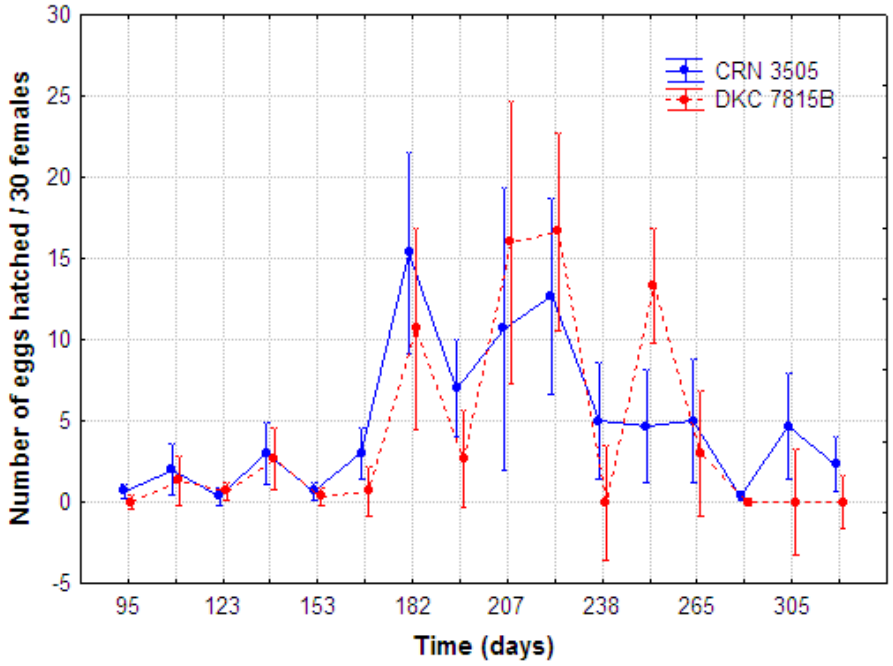

Figure 5.11. Total number of eggs hatched per 30 female *Heteronychus arator* beetles feeding on Bt (DKC 78-15B) and non-Bt maize (CRN 3505) in plastic container. (Bars indicate SE).

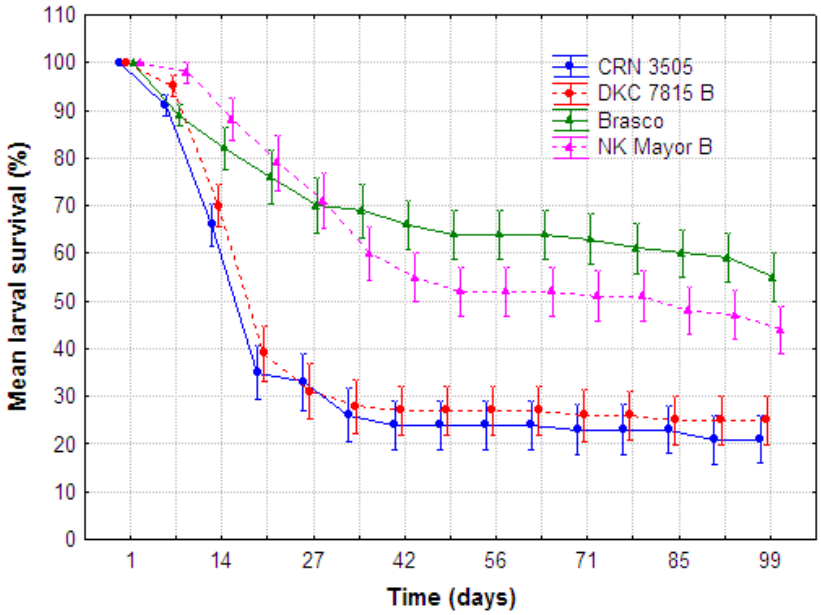

Figure 5.12. Mean percentage survival of *Somaticus angulatus* that commenced feeding on maize seedlings as 2nd instar larvae. [Event MON810 (DKC 78-15B) and its non-Bt iso-hybrid (CRN 3505) and event Bt11 (NK Mayor B) and its non-Bt iso-hybrid (Brasco)]. (Bars indicate SE).

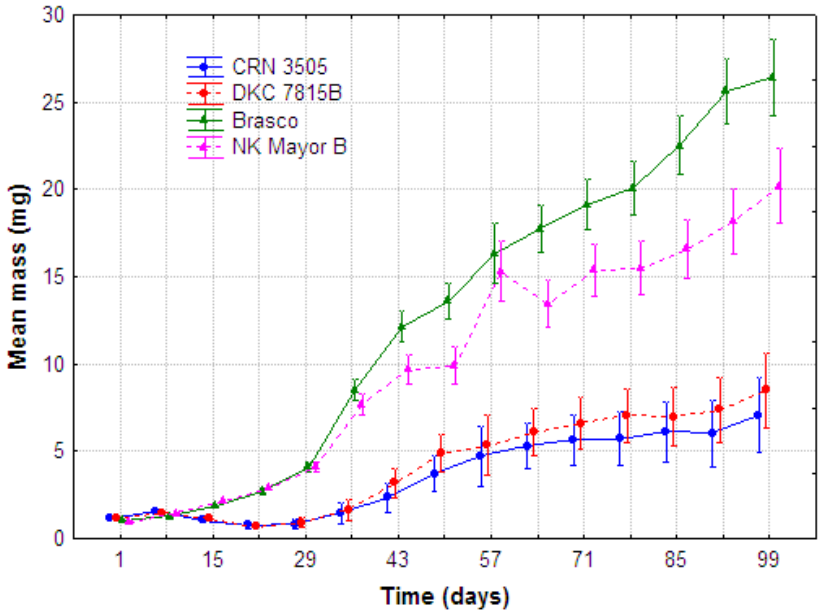

Figure 5.13. Mean mass of *Somaticus angulatus* larvae that commenced feeding on maize seedlings as 2nd instar larvae. [Event MON810 (DKC 78-15B) and its non-Bt iso-hybrid (CRN 3505) and event Bt11 (NK Mayor B) and its non-Bt iso-hybrid (Brasco)]. (Bars indicate SE).

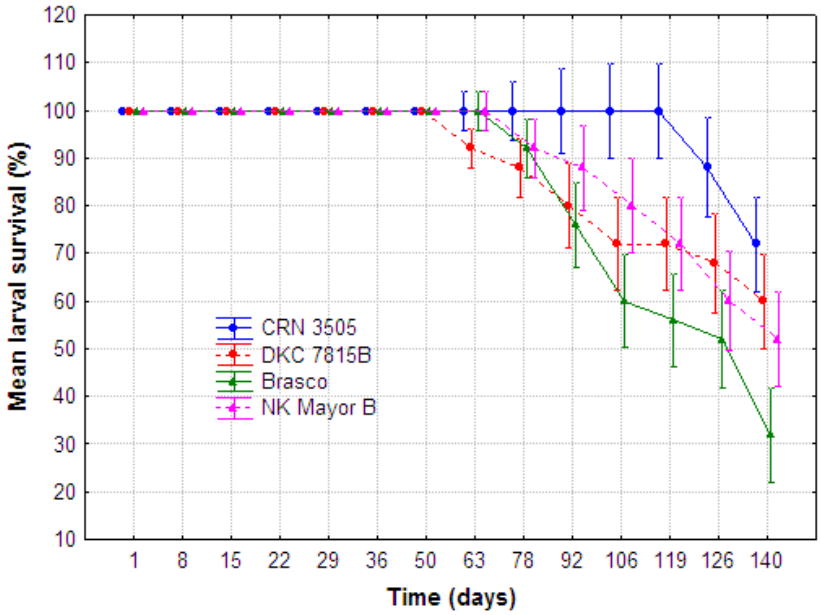

Figure 5.14. Mean percentage survival of *Somaticus angulatus* that commenced feeding on maize seedlings as 4th instar larvae. [Event MON810 (DKC 78-15B) and its non-Bt iso-hybrid (CRN 3505) and event Bt11 (NK Mayor B) and its non-Bt iso-hybrid (Brasco)]. (Bars indicate SE).

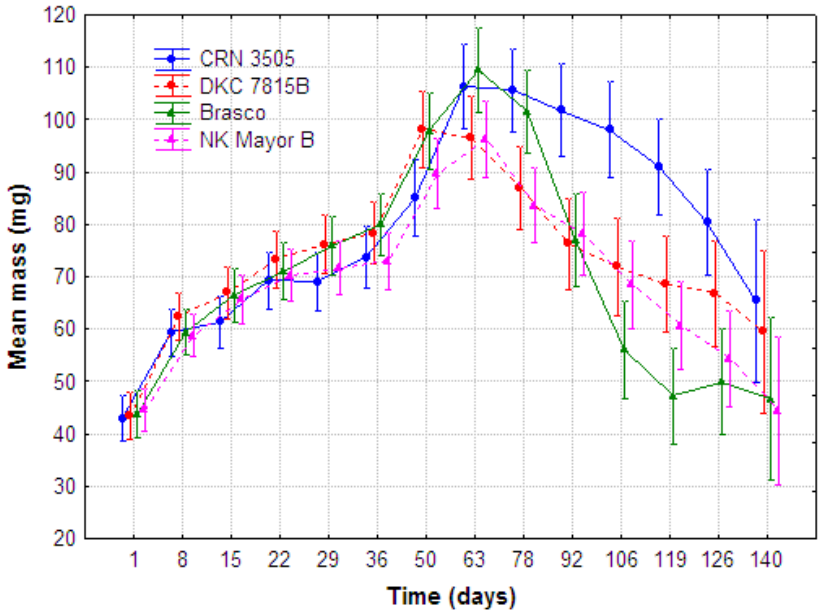

Figure 5.15. Mean mass of *Somaticus angulatus* larvae that commenced feeding on maize seedlings as 4th instar larvae. [Event MON810 (DKC 78-15B) and its non-Bt iso-hybrid (CRN 3505) and event Bt11 (NK Mayor B) and its non-Bt iso-hybrid (Brasco)]. (Bars indicate SE).

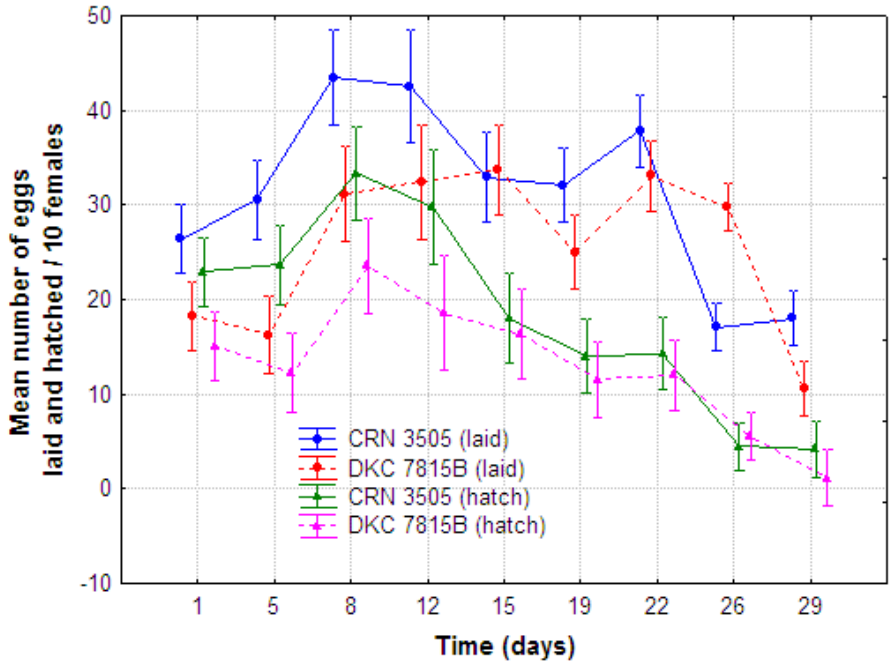

Figure 5.16. Mean number of eggs laid and hatched per 10 *Somaticus angulatus* female beetles feeding on maize leaves. [Event MON810 (DKC 78-15B) and its non-Bt iso-hybrid (CRN 3505)]. (Bars indicate SE).

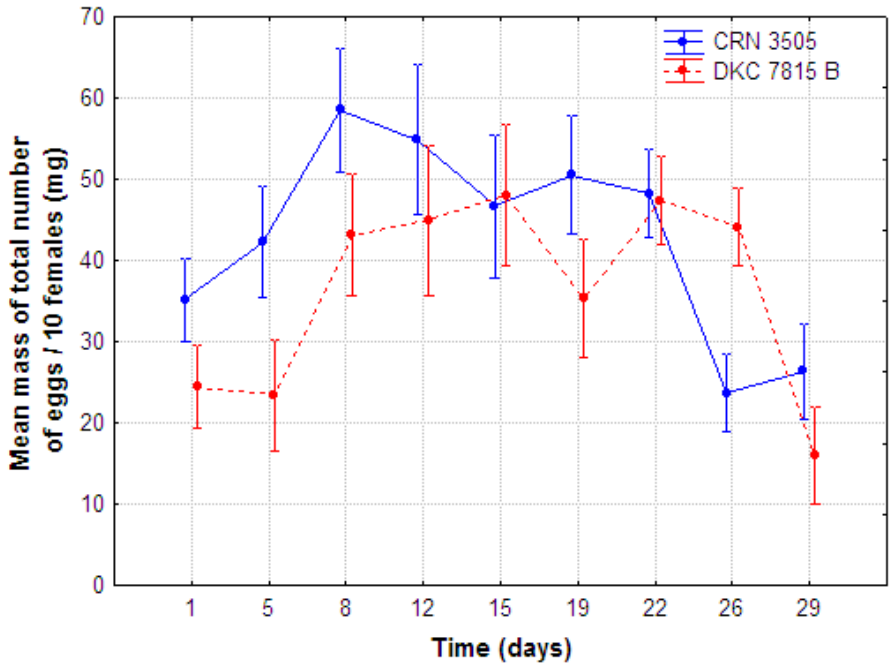

Figure 5.17. Mean mass of total number of eggs per 10 *Somaticus angulatus* female beetles feeding on maize leaves. [Event MON810 (DKC 78-15B) and its non-Bt iso-hybrid (CRN 3505)]. (Bars indicate SE).

CHAPTER 6: Survival of the parasitic fly, *Sturmiopsis parasitica* (Diptera: Tachinidae) on larvae of *Busseola fusca* (Lepidoptera: Noctuidae), feeding on Bt maize

6.1. Abstract

One of the most important components of integrated pest management is biological control and the preservation of natural enemies of pest arthropods. *Sturmiopsis parasitica* (Curran) (Diptera: Tachinidae), is an important larval parasitoid of gramineous stem borers in Africa. The large-scale cultivation of transgenic crops may carry potential ecological risks to natural enemies. To date no tri-trophic study was conducted in South Africa to determine if there is any effect of Bt maize on parasitoids. The objective of this study was to determine if there is any effect on *S. parasitica* when parasitizing Bt-resistant *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) diapause or fourth instar larvae that have fed on Bt maize. Bt-susceptibe and Bt-resistant *Busseola fusca* larvae, originating from different rearing populations were parasitized (inoculated) with two to four *S. parasitica* maggots each. Host larvae were screened daily until parasitoids emerged. Parameters measured for parasitoids were duration of maggot stage in host larvae, duration of the parasitoid pupal stage, as well as pupal mass and pupal size. Although not always significant, the percentage parasitism of Bt-consuming host larvae was always higher compared to host larvae that fed on non-Bt maize. It could be that Bt toxin affected the *B. fusca* larval fitness to such an extent that the immune systems were weakened, but that the larvae were still suitable for parasitization. The different parameters tested indicated only one case where maggots originating from diapause host larvae feeding on non-Bt maize had a greater mass compared to maggots from host larvae that fed on Bt maize. The same applied to *S. parasitica* pupal length. For the rest of the parameters tested there were no significant differences. Although some adverse effects were observed on *S. parasitica* mass and pupal length it is most likely that this will not contribute to adverse effects in the field, but that there may rather be synergism between Bt maize and *S. parasitica*.

6.2. Introduction

The tachinid fly, *Sturmiopsis parasitica* (Curran) (Diptera: Tachinidae), is an important larval parasitoid of gramineous stem borers in Africa (Chinwada *et al*., 2004). This parasitoid have been recorded on various lepidopteran stem borer species, including *Busseola fusca* (Fuller) (Noctuidae), *Chilo partellus* (Swinhoe) (Crambidae), *Chilo orichalcociellus* (Strand) (Crambidae) (Bonhof *et al*., 1997), *Coniesta ignefusalis* (Hampson) (Crambidae), *Eldana saccharina* (Walker) (Pyralidae), *Sesamia calamistis* Hampson (Noctuidae), *Sesamia nonagrioides* Tams & Bowden (Noctuidae) (Polaszek, 1998) and *Acigona ignefusalis* Hampson (Pyralidae) (Nagarkatti & Rao, 1975). Since the hosts of *S. parasitica* occur in sugar-cane, maize and sorghum, its value as a biocontrol agent is high (Nagarakatti & Rao, 1975).

Female *S. parasitica* individuals are easily recognized by the whitish frons (smoky grey in males) and the two long downwardly directed fronto-orbital bristles on either side (absent in males) (Nagarkatti & Rao, 1975). Females of average to large size can produce 500-900 maggots each (Nagarkatti & Rao, 1975). Being highly fecund and with an egg maturation period extending over only a few days, a single female can distribute its maggots over several borer tunnel entrances (Chinwada *et al*., 2004). In the case of a parasitoid like *S. prasitica*, the larvae consume the whole host larvae (Fig. 6.1) (Dent, 2000). The larval period inside the host is generally between 12-14 days at 26°C, occasionally lasting up to 35 days. The fully grown maggot then emerges from the host larva. The prepupal period is about 12h and the pupal period 12-19 days. The newly formed puparia (Fig. 6.2) are initially reddish brown, turning dark as development proceeds (Nagarakatti & Rao, 1975).

Figure 6.1. *Sturmiopsis parasitica* maggot emerging from *Busseola fusca* larva.

Figure 6.2. Newly formed pupa of *Sturmiopsis parasitica* next to the remains of the host larvae, *Busseola fusca*.

The exotic parasitoid *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae) has been introduced into many areas of the world as a biological control agent against several species of stemborers with varying degrees of success (Ngi-Song *et al*., 1995). There is a lack of understanding of the reasons underlying failures to establish *C. flavipes* in Africa (Ngi-Song *et al*., 1995). Parasitoids are not only unpredictable, but there is also uncertainty of how important they really are in controlling pests. Parasitoid effectiveness in keeping stem borer populations below acceptable thresholds has been doubted by several authors (Bonhoff *et al*., 1997; Kfir, 1997; Overholt *et al*., 1994). However, no studies are yet available that describes the effect of the absence of parasitoids on stem borer populations. Kfir (2002), however, concluded that the higher infestation level of stem borers in sprayed sorghum plots was due to the partial elimination of parasitoids and possibly other natural enemies by the pesticide. Other investigations into the effects of removal or partial removal of parasitoids from stem borer-infested crops by applying insecticides showed that borer populations could double (Kfir *et al*., 2002).

Concerns have been raised that large-scale production of transgenic crops may carry potential ecological risks to natural enemies (Hilbeck, 2002; Kennedy & Gould, 2007; Letourneau *et al*., 2002). Studies on tri-trophic interactions have indicated that natural enemy interactions with transgenic cultivars vary from synergism to antagonism (Bourguet *et al*., 2002; Schuler *et al*., 2004; Tounou *et al*., 2005; Romeis *et al*., 2006; Wei *et al*., 2008) Potential negative effects include (1) significant reductions of populations of the target pest, resulting in a lack of host availability to the natural enemy; (2) direct effects of transgenic plant-produced toxins on natural enemies; and (3) negative effects that are mediated through insect herbivore hosts (Pilcher *et al*., 2005). Conversely, potential benefits of large-scale transgenic product use could include (1) reduction in insecticide use, which leads to decreased natural enemy mortality; (2) increased secondary insect pest prey/host availability; and (3) indirect behavior or physiological impacts that could increase a herbivore's vulnerability to natural enemies. These benefits and / or negative effects would variably apply depending on whether the natural enemy is a generalist or specialist (Pilcher *et al*., 2005).

To date no study has been conducted in South Africa to determine the effect of Bt maize on any parasitoid or predator. Hilbeck (2002) highlighted the importance of unintended targets which include higher-trophic-level organisms, such as insect natural enemies of both the non-target herbivores and the original target species. For herbivores, valuable information can be gained from direct feeding studies. Such feeding studies are essential and form part of the toxicity screening at higher-trophic-level species. This is because organisms at higher-trophic-levels are exposed to the toxin in an altered form due to processing by the herbivores (Hilbeck, 2002). If no tri-trophic experiments are conducted, the effect of toxin processing in the herbivore gut is ignored entirely and, thereby, important ecological interactions among plants, herbivores, and natural enemies may be missed (Hilbeck, 2002). The presence of Bt-resistant populations of the target pest, *B. fusca*, in South Africa (Van Rensburg, 2007), provides the ideal opportunity to evaluate the possible indirect effects of the Cry 1Ab protein on the 3rd trophic level. Since larvae of *B. fusca* can be reared on Bt maize without a negative effect on host larval quality, the possible confounding factor of host quality can be excluded as an influencing factor.

The objective of this study was to determine the effect on *S. parasitica* when parasitizing *B. fusca* larvae consuming Bt maize.

6.3. Materials and methods

6.3.1. Comparison of parasitism on four *Busseola fusca* diapause populations

Busseola fusca diapause larvae were used in laboratory experiments. Diapause larvae were collected from maize fields in the winter from three different localities, following the methodology described by Van Rensburg & Van Rensburg (1993). Four populations of *B. fusca* diapause larvae were collected, two from Warden (Free State, South Africa) (one Bt and one non-Bt population used as control), one from Vaalharts (Northern Cape, South Africa) (one Bt population) and one from Ventersdorp (North West, South Africa)

(non-Bt population used as control for the Vaalharts Bt-resistant population). The diapause larvae were collected from dry maize stalks during the winter month of August, approximately four to five months after they commenced feeding on the then green maize plants. At the time of the experiment, larvae were in diapause and had ceased feeding on green maize approximately three to four months earlier. The South African Sugarcane Research Institute, Mount Edgecombe, provided *S. parasitica* flies from a mass rearing colony.

Fourteen days after mating, gravid female flies were dissected and their uteri ruptured in distilled water to release the maggots (Fig. 6.1). Using a fine camel-hair brush, active maggots were transferred to the ventral surface of the abdomen of a host *B. fusca* larva that had been dipped in a 1% NaOH-water solution and twice in distilled water before the time of innoculation. Host larvae were inoculated using two to four maggots and for each colony 150 larvae were used. Diapause larvae were provided with a dry mature maize stem because no feeding takes place during this stage. Larvae were kept in a round plastic container (5.5 cm long, 5 cm diameter) at 25°C and 60-65% RH. Host larvae were checked daily until parasitoid pupae formed. Parasitoid pupae were weighed, measured and held individually inside multi-cellular insect rearing trays until adult emergence. Duration (days) of the maggot stage in host larvae and duration (days) of parasitoid pupal stage until fly emergence were also determined.

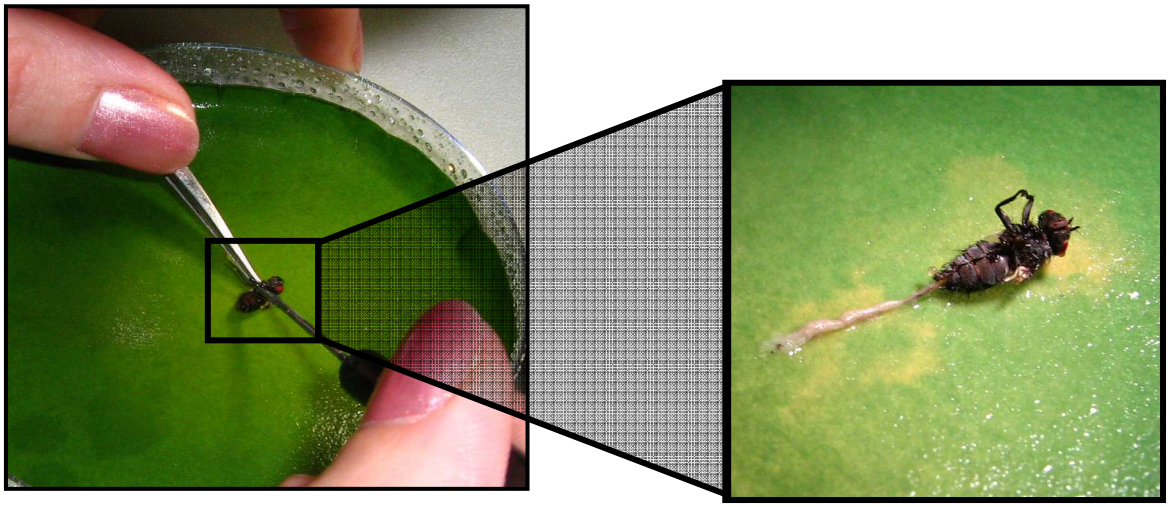

Figure 6.1. Gravid female fly being dissected.

6.3.2. Parasitism of *Busseola fusca* diapause larvae and 4th instars originating from diapause populations

Two diapause populations were collected from maize fields. One population was collected from a Bt maize field in Hartswater (North-Cape, South Africa) and the other from a non-Bt maize field (used as control) at Nampo Park, Bothaville (Free State, South Africa). Fourth instars used in the experiment originated from both these populations after moths from diapause larvae were allowed to mate and oviposit. First instars were reared on either Bt or non-Bt maize stems, corresponding with the plants they were originally collected from, until they reached the 4th instar for use in the experiment. The diapause and 4th instar larvae were inoculated with two to four maggots as described above.

Diapause and 4th instar larvae were kept under the same conditions as above. Fourth instars were provided with either Bt and non-Bt maize stems to feed on. Larvae were observed daily until parasitoid pupae formed. Parasitoid pupae were weighed, measured and then kept individually inside multi-cellular insect rearing trays until adult emergence. Duration (days) of the maggot stage in host larvae and duration (days) of the parasitoid pupal stage until fly emergence were also determined.

6.4. Data analysis

Statistical analysis was done with Statistica software (StatSoft, Inc., 2009). Two-by-two tables were used to determine significance of percentage parasitism by using Chi-square analysis. The 95% confidence interval, of the percentage parasitism was determined by using the odds-ratio. Parasitoid pupal mass, pupal dimensions, duration of maggots in host larvae, and duration of the parasitoid pupal stage were analyzed using a one-way ANOVA.

6.5. Results

6.5.1. Comparison of parasitism on four *Busseola fusca* diapause populations

The percentage parasitism of *B. fusca* diapause larvae by *S. parasitica* are summarized in Table 6.1. Parasitism of the stem borer population from Vaalharts (feeding on Bt maize during the previous growing season) did not differ significantly from that of Ventersdorp (feeding on non-Bt maize). The two Warden populations differed significantly (P = 0.038) (Table 6.1) with a higher percentage parasitism on the Bt population.

The mean mass and dimensions of *S. parasitica* pupae are provided in Table 6.2. Mean mass of pupae emerging from the Warden non-Bt stem borer population was significantly higher than that of the Warden Bt population but no significant difference was observed in the percentage pupal parasitism between the Vaalharts (Bt) and Ventersdorp (non-Bt) populations. A significant difference between *S. parasitica* pupal length was observed for the Vaalharts (Bt) and Ventersdorp (non-Bt) borer populations. Length of pupae emerging from the Ventersdorp non-Bt population was significantly greater. No significant differences were observed between the mean length of pupae emerging from the two Warden populations or between pupal mean width emerging from any of the other populations.

There were no significant differences in the developmental periods of the maggot or pupal stages in host larvae between any of the populations (Table 6.3).

6.5.2. Parasitism of *Busseola fusca* diapause larvae and 4th instars originating from diapause populations

There were no significant differences (P> 0.05) (Table 6.4 – 6.6) between any of the parameters tested between the two diapause populations collected from Bt- and non-Bt maize fields, and the two 4th instar populations reared from the same diapause larvae when they were parasitized by *S. parasitica*.

6.6. Discussion and conclusion

One of the most important components of integrated pest management is the preservation of natural enemies of pest arthropods (Daly & Buntin, 2005). Unfortunately, plant breeders have continued to attempt to breed for total resistance, and bio-control specialists have ignored the role of the plant in ensuring successful foraging behavior by insect natural enemies (Poppy & Sutherland, 2004). To date, studies have focused more upon the effects of Bt toxins on specific herbivores, without consideration of their persistence within arthropod food webs (Harwood *et al*., 2005). The movement of Bt toxin through trophic levels has received little attention (Wei *et al*., 2007). Harwood *et al*. (2005) reported significant quantities of detectable Cry1Ab endo-toxin within non-target herbivores and predators which indicate that long-term exposure to insecticidal toxins could occur in the field. There is limited understanding of the impact of the *Cry* toxins expressed in the transgenic plants on the growth, development, and distribution of natural enemies. To ensure that transgenic crops are environmentally sustainable, long-term evaluations of the possible effects of this technology on naturally occurring beneficial arthropods are necessary (Day & Buntin, 2005).

In the current study, laboratory studies were conducted which is the fist tier to determine if there is any effect of Bt maize on the selected species as suggested by Dutton *et al*. (2003). Although not always significant, the efficacy of parasitism on Bt-fed host larvae was always higher compared to host larvae that fed on non-Bt maize. A possible explanation for this can be that the non-Bt host larvae were more fit than host larvae that fed on Bt maize and therefore better equipped to defend themselves against parasitism. It has been reported with the stemborer parasitoid, *C. flavipes* is sometimes killed in the maize stem when foraging for *C. partellus*, probably by biting or spitting of the host larvae (Takasu & Overholt, 1997). In addition, a parasitoid might be able to insert the ovipositor into the host, but not have enough time to inject eggs before being attacked by the borer larvae. Tounou *et al*. (2005) also reported that the paralytic effects of the Bt toxin on *S. calamistis* larvae could make it easier for *Cotesia sesamiae* (Cameron) (Hymenoptera: Braconidae) to successfully attack and oviposit inside the host. Therefore,

further studies on the effect of *Cry* toxin on the fitness of *B. fusca* are required to determine whether there is synergism between Bt and *S. parasitica*.

The effect of Cry1Ac toxin on *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae), a parasitoid of the cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) was evaluated by Liu *et al*. (2005). They found that when female parasitoids parasitized host larvae that had been fed on a diet containing Cry1Ac toxin, their offspring's larval development were significantly delayed. Their pupal weight, adult weight, and adult longevity were also significantly less than those of the control treatment (Liu *et al*., 2005). Parasitoid larvae of *Campoletis sonorensis* (Cameron) (Hymenoptera: Ichneumonidae) was observed to develop significantly slower in host larvae, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), that fed on Bt maize (MON810) (Meissle *et al*., 2004). However, cocoon weight, time from pupation to emergence, sex ratio and total survival were not significantly different from the control treatment (Meissle *et al*., 2004). *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) survival, developmental times and cocoon weights were significantly negatively affected if their *S. littoralis* host larva had been fed Bt maize (Vojteck *et al*., 2005). Prütz and Dettner (2004) reported that the parasitoid *C. flavipes* (Cameron) completed its larval development in only 25% of all *C. partellus* hosts in the Bt group, whereas 83% of all hosts allowed completion of parasitoid larval development in the control. The parasitoid pupal weight and adult weight were also reduced in the Bt group compared to the control (Prütz & Dettner, 2004). Results from a study conducted by Bernal *et al*. (2002) showed that ingestion of Bt maize tissue by *Eoreuma loftini* Dyar (Lepidoptera: Pyralidae), a subtropical stemborer, negatively affected some fitness components in *Parallorhogas pyralophagus* (Marsh) (Hymenoptera: Braconidae), a gregarious, external idiobiont parasitoid, whereas other components were not affected.

In contrast to the above there are also many examples in literature which indicate that there are no adverse effects of Bt maize on other parasitoid species (Schuler *et al*., 2004; Romeis *et al*., 2006; Sharma *et al*., 2008; Wei *et al*., 2008). It is also evident from the examples above that different parameters can be used to identify possible effects of Bt

maize on parasitoids. In this study different parameters were used to identify possible adverse effects of Bt on *S. parasitica* when parasitizing *B. fusca*. In the case of *S. parasitica* only once did results indicate that maggots originating from diapause host larvae feeding on non-Bt maize had a greater mass compared to counterparts on host larvae that fed on Bt maize. This also applied to pupal length. For the other parameters tested there were no significant differences. Development period of maggots inside host larvae and parasitoid pupae were not influenced by host larvae that fed on Bt maize. These results highlight the importance of tri-trophic studies to determine if there are any adverse effects of Bt maize on parasitoids. The presence of any adverse effect might require additional research to determine if these effects will also be observed under field conditions.

Decreasing the target pest populations to minimal numbers can drastically change existing multi-trophic interactions in the field (Pilcher *et al*., 2005). The impacts of this elimination of parasitoid host larvae that are totally controlled by the Bt plant are not yet clarified. One of the most obvious ways in which transgenic maize may affect the level of tachinid parasitism is by decreasing density of the host larvae, as was indicated for *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Bourguet *et al*., 2002). However, *S. parasitica* parasitize more than one lepidopteran species and all of these host larvae attack various crops and wild grasses (Bonhoff *et al*., 1997; Polaszek, 1998; Kfir, 2000). Therefore, even if the number of tachinid parasitoids decline due to stem borer depletion in Bt maize fields, their persistence in the environment is probably not threatened.

6.7. References

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Table 6.1. Percentage parasitism by *Sturmiopsis parasitica* of four diapause *Busseola fusca* populations, comparing data when fed on Bt and non-Bt maize.

Colony	% Parasitized (N)	Chi-square	P-value	95% confidence interval
Vaalharts (Bt)	28.67 (150)	0.07	0.796	(0.666; 1.833)
Ventersdorp (non-Bt)	26.67 (150)			
Warden (Bt)	54.67 (150)	4.32	**0.038***	(0.979; 2.433)
Warden (non-Bt)	42.00 (150)			

Statistical significance is indicated as * P<0.05

Table 6.2. Mean mass, length and width of *Sturmiopsis parasitica* pupae originating from *Busseola fusca* diapause larvae that had fed on Bt and non-Bt maize.

Colony	Mean mass (SE) (N)	F-value	P-value	Mean length (SE) (N)	F-value	P- value	Mean width (SE) (N)	F-value	P-value
Vaalharts (Bt)	58.75 (±2.15) (43)	$F_{(1,81)}$= 1.17	0.28	7.80 (±0.12) (43)	$F_{(1,81)}$= 17.62	**< 0.01***	3.15 (±0.07) (43)	$F_{(1,81)}$= 3.16	0.08
Ventersdorp (non-Bt)	62.10 (±2.23) (40)			8.45 (±0.11) (40)			3.34 (±0.08) (40)		
Warden (Bt)	53.31 (±1.31) (82)	$F_{(1,143)}$= 5.41	**0.02***	7.90 (±0.14) (80)	$F_{(1,141)}$= 0.28	0.60	3.05 (±0.06) (80)	$F_{(1,141)}$=0.51	0.48
Warden (non-Bt)	57.92 (±1.49) (63)			7.79 (±0.16) (63)			3.12 (±0.07) (63)		

Statistical significance is indicated as * P<0.05

Table 6.3. Mean development period of *Sturmiopsis parasitica* maggots in host larvae and pupal period before fly emergence.

Colony	Mean days in host larvae (SE) (N)	F - value	P - value	Mean days as pupae before fly emergence (SE) (N)	F - value	P - value
Vaalharts (Bt)	14.69 (±0.50) (42)	$F_{(1,79)}$= 2.96	0.09	14.40 (±0.69) (5)	$F_{(1,12)}$= 0.04	0.84
Ventersdorp (non-Bt)	15.92 (±0.51) (39)			14.20 (±0.52) (9)		
Warden (Bt)	15.71 (±0.41) (77)	$F_{(1,138)}$= 0.71	0.40	13.00 (±0.55) (16)	$F_{(1,30)}$= 0.01	0.93
Warden (non-Bt)	14.71 (±0.46) (63)			13.06 (±0.55) (16)		

Statistical significance is indicated as * P<0.05

Table 6.4. Percentage parasitism by *Sturmiopsis parasitica* of different *Busseola fusca* populations that consumed either Bt or non-Bt maize.

Colony	% Parasitized (N)	Chi-square	P-value	95% confidence interval
Diapause (Bt)	13.80 (80)	0.03	0.867	(0.420; 2.535)
Diapause (non-Bt)	13.40 (82)			
4th instars (Bt)	27.10 (92)	2.24	0.135	(0.892; 4.007)
4th instars (non-Bt)	16.50 (79)			

Statistical significance is indicated as * P<0.05

Table 6.5. Mean mass, length and width of *Sturmiopsis parasitica* pupae originating from different *Busseola fusca* populations that consumed either Bt or non-Bt maize.

Colony	Mean mass (SE) (N)	F-value	P-value	Mean length (SE) (N)	F-value	P-value	Mean width (SE) (N)	F-value	P-value
Diapause (Bt)	65.70 (±2.98) (11)	$F_{(1,20)}$= 0.09	0.77	8.41 (±0.14) (11)	$F_{(1,20)}$= 0.21	0.65	3.41 (±0.12) (11)	$F_{(1,20)}$= 0.08	0.78
Diapause (non-Bt)	66.97 (±2.98) (11)			8.50 (±0.14) (11)			3.36 (±0.12) (11)		
4th instars (Bt)	59.48 (±2.73) (25)	$F_{(1,36)}$= 1.67	0.20	8.26 (±0.12) (25)	$F_{(1,36)}$= 0.06	0.82	3.20 (±0.08) (25)	$F_{(1,36)}$= 1.88	0.18
4th instars (non-Bt)	65.52 (±3.79) (13)			8.31 (±0.16) (13)			3.40 (±0.11) (13)		

Statistical significance is indicated as * P<0.05

Table 6.6. Mean development period of *Sturmiopsis parasitica* maggots in host larvae feeding on Bt or non-Bt maize and pupal period before fly emergence.

Colony	Mean days in host larvae (SE) (N)	F - value	P - value	Mean days pupae before fly emerge (SE) (N)	F - value	P - value
Diapause (Bt)	15.64 (±0.37) (11)	$F_{(1,20)}$= 2.44	0.13	15.40 (±0.23) (10)	$F_{(1,17)}$= 0.23	0.64
Diapause (non-Bt)	14.82 (±0.37) (11)			15.56 (±0.24) (9)		
4th instars (Bt)	16.48 (±0.43) (25)	$F_{(1,36)}$= 3.22	0.08	14.61 (±0.57) (23)	$F_{(1,34)}$= 0.17	0.68
4th instars (non-Bt)	15.15 (±0.60) (13)			15.00 (±0.75) (13)		

Statistical significance is indicated as * P<0.05

CHAPTER 7: Selection of non-target insect species for risk assessment by using feeding studies as endpoint to determine possible effects of genetically modified maize

7.1. Abstract

It is essential to assess the environmental risk that Bt maize may hold and to study its effect on species assemblages that fulfil a variety of different ecosystem functions. Ecological theory can be used to improve environmental risk assessment and, by applying it to specific environments, local species can be classified functionally and prioritized to identify potential test species, assessments and endpoints. Although the stem borers *Busseola fusca* and *Chilo partellus* are the target species of Bt maize in South Africa, various other Lepidoptera, Coleoptera and Diptera species are directly and indirectly exposed to Bt toxin. In this study, an ecological approach was followed in which the potential effects of exposure of priority species to Bt toxin in maize (event MON810) was investigated. Non-target Lepidoptera and Coleoptera that are primary consumers of maize and which could therefore be directly exposed to Bt-toxin were identified, and the possible effects that Bt maize may have on these species assessed. A natural enemy, *Sturmiopsis parasitica* that parasitizes *B. fusca* was also evaluated. A series of selection matrices were developed in which each species was ranked for its maximum potential exposure to Bt toxin by assessing its occurrence**,** abundance**,** presence and linkage in the maize ecosystem. Through the use of this selection matrix, knowledge gaps were identified for future research and to guide the design of ecologically realistic experiments. The following non-target species were identified in the matrix and were evaluated in feeding and tri-trophic experiments: *Sesamia calamistis*, *Helicoverpa armigera*, *Agrotis segetum*, *Heteronychus arator*, *Somaticus angulatus* and *Sturmiopsis parasitica*. During this study only a few non-target species were evaluated that may be affected by Bt maize. This study indicated that some species can be eliminated for further testing, since Bt maize had no adverse effect at all, whereas for others continued studies need to be considered before a conclusion can be drawn. These

possible effects need to be confirmed in the actual environment before any particular hypothesis can be sufficiently supported or refuted.

7.2. Introduction

Food webs are among nature's most complex creations (Eveleigh *et al*., 2007). With the increased awareness of the complexity and importance of food webs in agricultural systems conservation of these ecosystems have been highlighted. Artificial food webs are created in agricultural systems and the interactions between plants, herbivores and natural enemies in these systems may change from simple tri-trophic interactions to more complex food web interactions (Janssen *et al*., 1985). Crop plants and insect pests are part of a complex agricultural ecosystem that involves interactions between many trophic levels, often referred to as multi-trophic interactions (Poppy & Sutherland, 2004). Furthermore, it has been realized that life on earth depends on the proper functioning of several large-scale ecological processes, many of which provide humanity with irreplaceable benefits, termed "ecosystem services" (Daily *et al*., 1996).

Agriculture is an important environmental quality driver (Hails, 2002), and its effect is not likely to diminish in the future (Tilman *et al*., 2001). There are many agricultural practices and designs that have the potential of enhancing insect biodiversity, whereas others may have adverse effects. The management of pests can have substantial impacts on non-target arthropod species both within and outside the units that are being managed. The goal of insect pest management is to maintain, through directed strategies, insect pest numbers below threshold densities. These management practices interfere with the ability of insects to survive, reproduce or exploit resources, and the impacts of these tactics are very rarely confined to the target pest species (Losey *et al*., 2003). The idea is, therefore, to develop management strategies that enhance or regenerate levels of biodiversity that support sustainable agro-ecosystems by providing ecological services such as biological control (Panizzi, 2007).

The biodiversity of an agro-ecosystem is not only important for its intrinsic value, but also because it influences ecological functions that are vital for crop production in sustainable agricultural systems, as well as for wildlife and the surrounding environment. Changes in biodiversity could possibly alter these functions and harm the agro-ecosystem as well as surrounding natural ecosystems (Dutton *et al*., 2003; Birch *et al*., 2004; Hilbeck *et al*., 2006). For this reason it is essential to assess the environmental risk that the release of a genetically modified (GM) crop may hold and to study its effect on species assemblages.

Risk assessment is a process by which risks are identified and the seriousness of the risks are characterized so that decisions can be made on whether or how to proceed with the technology (Andow & Hilbeck, 2004a). Schmitz *et al*. (2003) stated that, considering the high diversity of herbivores, many species of which inhabit agricultural landscapes, and the high complexity of interactions even in agricultural biocoenosis, more biosafety research on and monitoring of the effect of Bt maize on the environment is needed. The adequate protection of herbivores, particularly Lepidoptera, in the agricultural landscape is important for general environmental protection efforts. In addition, integrated pest management strategies rely on sufficient non-target species that serve as alternative hosts for parasitoids of economic relevance (Schmitz *et al*., 2003).

The fist step towards a comprehensive insect management program that would provide adequate pest suppression, maintenance of ecological services, and minimal impact on rare species is a detailed assessment of which insect species are likely to exist in the managed system. Unfortunately, this baseline accounting of insect species is lacking for almost every managed system (Losey *et al*., 2003). To assess the risks of any insect resistant GM plant on non-target arthropods, as a first step, it is necessary to identify arthropods that occur inside cropping systems in specific regions into which GM crops will be introduced (Dutton *et al*., 2003).

Ecological theory can be used to improve environmental risk assessment and to tailor it to specific environments. In an ecological model for non-target risk assessment, local

species can be classified functionally and prioritized using risk based ecological criteria to identify potential test species, assessments and endpoints (Andow & Hilbeck, 2004b). The environmental risk assessment process described for Bt maize by Birch *et al*. (2004) assessed the possible risks of transgenic crops on biodiversity. In that model it is recommended that species be selected from assemblages, that the potential for risk be identified and that research protocols be developed to assess these risks.

In this study an "ecological model" approach was used to select species for research and to evaluate species for their susceptibility to Bt maize. A case-specific approach suggested by Birch *et al*. (2004), Andow & Hilbeck (2004a) and Hillbeck *et al*. (2006)) was followed during which selection of non-target species and the potential effects of direct exposure to Bt maize was investigated. No risk assessment for Bt maize was done in South Africa before its release in 1998. As pointed out by McGeoch and Rhodes (2006), the protocols and guide lines for risk assessment of GM crops in South Africa has yet to be developed. In this chapter we rely strongly on methods and examples used by Andow and Hilbeck (2004a, b) and, Hilbeck, Andow and Fontes (2006) in their case studies on Bt maize in Kenya and Bt cotton in Brazil respectively.

7.3. An ecological model for non-target risk assessment

Species selection in the ecological model is case specific, depending on the GM crop and its cropping context, and prioritizes species that could be adversely affected by the GM crop (Andow & Hilbeck, 2004a, b). The fist step begins by identifying and screening appropriate functional groups. Functional groups are established according to their ecological role or function in the ecosystem. The next step is to compile a list of species in these functional groups followed by prioritization of species found in the relevant environment. These species are prioritized on the basis of ecological principles. Then these species' trophically mediated exposure to the GM crop and the transgene products are analysed. As a fourth step, potential hazards are identified and hypotheses developed. The final step is the experimental endpoint where the identified parameters are measured

(Andow & Hilbeck, 2004b; Birch *et al*., 2004; Hilbeck *et al*., 2006). The above mentioned steps are discussed below with reference to Bt maize in South Africa.

7.3.1. Identification of functional groups

Qualitative field expertise and available data on biodiversity is crucial for determining the list of possible non-target species, their trophic relationships and relevant functions (Birch *et al*., 2004). This step involves the identification of important functions that could be considered in risk assessment for a given case. This list will be specific to the crop and its cropping context and agro-ecosystem (Hilbeck *et al*., 2006). Using ecological function allows focus on ecological processes and limits the number of species and functions that need to be tested (Birch *et al*., 2004).

Based on ecological function Birch *et al*. (2004) identified the following functional groups that needs to be considered in pre-release testing of GM plants: secondary pests, natural enemies, species of conservation concern, species that generate income, species of social or cultural value, competitors, non-target primary consumers, secondary consumers, pollinators, decomposers, nutrient recyclers, seed dispersers and species of unknown function. Hilbeck *et al*. (2006) identified the following functional groups for use in Bt cotton risk assessment in Brazil: non-target pest herbivores, pollinators and species of conservation concern, predators, parasitoids, weeds and soil ecosystem functions. These functional groups are not mutually exclusive. For example many species are both secondary pests and non-target primary consumers (Andow & Hilbeck, 2004b).

In this study on maize in South Africa the functional groups of non-target primary consumers and natural enemies of target primary consumers was used. Using the guild concept, the functional group of non-target primary consumers were further sub-divided, namely non-target primary consumers feeding on maize (1) stem, (2) ears, and (3) seedlings.

7.3.2. Prioritization of non-target species (selection matrix)

The second step in species selection is classification of the non-target species that occur in association with the crop in the region where the GM crop is intended to be released into functional groups, using available information and expertise (Hilbeck *et al*., 2006). Inclusion of species that actually occur in the region generates a case-specific set of potential non-target species (Andow & Hilbeck, 2004b).

Table 7.1. Insect species commonly found in maize fields in South Africa.

Scientific name	Feeding guild	Family	Reference
Lepidoptera			
Acantholeucania loreyi	Ear	Noctuidae	Van Wyk *et al*., 2007
Agrotis ipsilon	Seedling	Noctuidae	Annecke & Moran, 1982
Agrotis longidentifera	Seedling	Noctuidae	Annecke & Moran, 1982
Agrotis segetum	Seedling	Noctuidae	Van Wyk *et al*., 2007
Agrotis subalba	Seedling	Noctuidae	Annecke & Moran, 1982
*Busseola fusca**	Stem	Noctuidae	Annecke & Moran, 1982
Chilo orichalcociliellus	Stem	Crambidae	Kroon, 1999
*Chilo partellus**	Stem	Crambidae	Annecke & Moran, 1982
Eublemma gayneri	Ear	Noctuidae	Van Wyk *et al*., 2007
Helicoverpa armigera	Ear	Noctuidae	Van Wyk *et al*., 2007
Sesamia calamistis	Stem, ear	Noctuidae	Van Wyk *et al*., 2007
Spodoptera exempta	Seedling	Noctuidae	Annecke & Moran, 1982
Spodoptera exigua	Seedling	Noctuidae	Van Wyk *et al*., 2007
Spodoptera littoralis	Seedling	Noctuidae	Kroon, 1999
Coleoptera			
Astylus atromaculatus	Seedling, ear	Melyridae	Drinkwater *et al*., 2002
Heteronychus arator	Seedling	Scarabaeidae	Drinkwater *et al*., 2002
Heteronychus licas	Seedling	Scarabaeidae	Hill, 1987
Megalognatha rufiventris	Ear	Chrysomelidae	Hill, 1987
Nematocerus spp.	Seedling	Curculionidae	Hill, 1987
Protostrophus spp.	Seedling	Curclionidae	Drinkwater *et al*., 2002
Somaticus spp.	Seedling	Tenebrionidae	Drinkwater *et al*., 2002
Diptera			
Sturmiopsis parasitica	Natural enemy	Tachinidae	Bonhoff *et al*., 1997
Hymenoptera			
Cotesia flavipes	Natural enemy	Branconidae	Bonhoff *et al*., 1997
Cotesia sesamiae	Natural enemy	Branconidae	Bonhoff *et al*., 1997

***** Target species of Bt maize in South Africa.

Species selection for this study was only conducted by means of compiling a list using scientific literature (Table 7.1) to identify non-target primary consumers of maize and some potential natural enemies and is therefore not exhaustive. This list of non-target primary consumers and natural enemies are specific to maize and its cropping context in the agro-ecosystem. For the non-target primary consumers only species belonging to the

stem, ear, and seedling feeding guild were listed. Twelve lepidopteran species, excluding the two target species (*B. fusca* and *C. partellus*), seven coleopteran species and three natural enemies were listed.

Several criteria can be used to prioritize non-target species, including maximum possible exposure and potential adverse effects. Any non-target organism feeding on the GM plant or part of the plant would come in contact with the transgene and its product (Andow & Hilbeck, 2004b).

The maximum potential exposure of a non-target species to a GM crop is based on geographic range, habitat specificity, local abundance, prevalence and temporal association with the crop (Andow & Hilbeck, 2004b). Defining potential exposure of insects to Bt toxin is an important aspect of resistance risk assessment (Fitt *et al*., 2004). In order to provide a rational and transparent approach to support the selection of species for use in risk assessment analyses, Birch *et al*. (2004) developed a series of selection matrices. In this system each species is ranked for its maximum potential exposure to Bt-toxin by assessing it occurrence, abundance, presence and linkage in the maize ecosystem as well as for potential adverse effects that exposure may have on the non-target species (Birch *et al*., 2004) (Table 7.2). In this context "occurrence" refers to the presence of a non-target species in the agroecosystem, its geographic range and prevalence. "Abundance" refers to local abundance and prevalence while "presence" involves temporal association with the crop. "Linkage" refers to habitat specificity and the degree of specialization of the non-target species on maize. Linkage might also be called feeding specialization and focuses on trophic relationships with other host plant species of organisms in a particular functional group (Van Wyk *et al*., 2007).

Table 7.2. Selection matrix for prioritizing non-target species associated with maize in South Africa.*

		Maximum potential exposure				Possible adverse effect		
Guild	**Species**	**Occurrence**	**Abundance**	**Presence**	**Linkage*** **	**Significance**	**Damage**	**Rank****
Stem	***Sesamia calamistis***	Occasional	Low - medium	Anytime	Strong (oligophagous)	Low (sporadic pest)	High	1
Ear	***Helicoverpa armigera***	Certain	Abundant	Post-flowering	Weak (polyphagous)	Low (always present)	Low	1
Ear	***Acantholeucania loreyi***	Occasional	Medium - abundant	Pre- and post-flowering, also on tillers	Strong (monophagous)	Potential pest (sporadic presence)	Low	1
Ear	***Eublemma gayneri***	Sporadic	Low	Post-flowering	Doubtful	-	Very low	2
Seedling	***Agrotis segetum***	Occasional	Medium	Seedling	Weak (polyphagous)	High (important pest)	High	2
Seedling	***Spodoptera exigua***	Occasional	Low	Seedling	Weak (polyphagous)	Potential pest (occasional presence)	Sometimes	2
Seedling	***Heteronychus arator***	Occasional	High – low	Seedling	Weak (polyphagous)	Potential pest (occasional presence)	High	3
Seedling	***Somaticus angulatus***	Occasional	High – low	Seedling	Weak (polyphagous)	Potential pest (occasional presence)	High	3
Natural enemy	***Cotesia* spp.**	Certain	High - medium	Anytime	Strong	(Natural enemy)	None	1
Natural enemy	***Sturmiopsis parasitica***	Occasional (certain in Zimbabwe)	Low	Anytime	Strong	(Natural enemy)	None	1

*Based on a selection matrix developed by Andow & Hilbeck (2004a).

**Species that were considered most important were ranked 1.

***The degree of feeding specialization and association with the crop (host plant range); Weak = species of polyphagous nature, the species is not dependent on the crop for survival; Strong = narrow host range and the crop may be important in species ecology; Doubtful = not enough information available.

Although many species have an unknown ecological function, this does not imply that their ecological function is insignificant. Of the species with unknown ecological function, Andow & Hilbeck (2004a) suggested that those with a high standing biomass or those that are found in frequent association with the GM crop habitat should also be selected for testing. By explicitly considering such species for initial non-target testing, a scientifically justified precautionary approach is introduced into risk assessment.

Data collected, field observations and expert opinion was used to develop selection matrixes in which species were selected on the bases of their occurrence, abundance, presence and linkage in the maize ecosystem (Table 7.2). The aims with development of these matrixes were to establish options and identify knowledge gaps for future research and select possible species for post-release impact monitoring studies. The most common species were *S. calamistis, H. armigera, A. segetum, H. arator, S. angulatus,* and *St. parasitica*. The first five species are directly exposed to Bt maize but *St. parasitica* only indirectly through parasitization of host larvae that directly consumed Bt maize. The most important non-target species according to Table 7.2 was *S. calamistis, H. armigera, A. loreyi, Cotesia* spp. and *St. parasitica*.

7.3.3. Trophically mediated exposure to GM plant and transgene products

This step analyses possible causal pathways of exposure to the GM plant and toxin, and potential impacts of the GM plant for high priority species identified during the previous step (Birch *et al*., 2004). The purpose of this evaluation is to differentiate candidate test species likely and unlikely to be exposed to the Bt toxin, and for the former, to guide the design of the exposure system in the test protocols (Birch *et al*., 2004). Potential likely exposure can occur through many pathways. Any non-target organism feeding on the GM plant or parts of the plant may come in contact with the transgene and its product. The number of possible pathways is immense. It has been estimated that there are over 250 different exposure pathways by which a transgene product or its metabolites could affect a secondary consumer, of which only a few are direct effects of the transgene product (Andow & Hilbeck, 2004a).

Because information on the expression levels of Bt proteins in maize in general, as well as in different plant parts differ and in some instances are doubtful, all herbivores feeding on any Bt maize tissue should be expected to ingest Bt toxin (Birch *et al*., 2004). Except for the Diptera and Hymenoptera, all the non-target herbivores listed in Table 7.1 ingest Bt toxin and are considered primary consumers of Bt maize plants. Although these species feed directly on the plant, they feed on different plant parts, depending on guild, which can lead to ingestion of different amounts of Bt toxin on account of differential expression levels (Andow, 2002; Dutton *et al*., 2003).

Indirect exposure of natural enemies, such as *Cotesia* spp. and *St*. *parasitica* happens indirectly when stem borer or other host larvae feed on Bt maize and ingest Bt toxin. Parasitoid larvae are very likely to be tritrophically exposed to Bt toxin and/or metobolites if host larvae survive on Bt maize. It is also known that some parasitoids feed on pollen and could ingest Bt toxin, but when feeding on nectar-like guttation fluid it is less likely to ingest Bt toxin than when feeding on pollen (Birch *et al*., 2004).

Table 7.3. Ecological and behavioural attributes of Lepidoptera, Coleoptera and Diptera species, and the efficacy of Bt maize against each species. Information used to compile preliminary risk assessment. *

Attribute	***Busseola fusca***	***Chilo partellus***	***Sesamia calamistis***	***Helicoverpa armigera***	***Agrotis segetum***	***Heteronychus arator***	***Somaticus angulatus***	***Sturmiopsis parasitica***
Toxicity of current Bt plant ****	High	High	High	High to low	Low	None	None	None
Other Bt crops	None	None	None	Yes	?	None	None	Parasitoid, does not need to be controlled
Diapause	Yes, 3-6 months during winter	No, larvae becomes quiescent	No	Yes, in pupal stage	No	No	No	No
Larval dispersal	Larvae balloon one to several meters	Larvae balloon one to several meters	Larvae balloon one to several meters	Large larvae are cannibalistic	Larvae crawl several meters	Larvae crawl several meters	Larvae crawl several meters	?
Adult dispersal	?	?	?	?	?	?	?	?
Generations per year **	3	5	5	5 – 6	?	2	1	?
Generations in maize **	3?	5?	5?	?	?	1?	1?	?
Duration of life cycle (days) **	40 – 65	25 – 50	40 - 70	40 – 50	42 – 175	266 - 300	200 - 300	35 - 40
Abundance in wild hosts	Rare	Rare	Common	Common	?	?	Common	?
Fecundity **	1000 eggs	500 eggs	1000 eggs	1600 eggs	1000 eggs	20 – 80 eggs	15 – 40 eggs	500 – 900 maggots
Egg batch size **	10 – 80	50 – 100	20 - 100	Single eggs	Singly or in groups	Single eggs	Single eggs	Distribute maggots singly

(Compiled on the basis of experts opinions of: J.B.J. van Rensburg, T.W. Drinkwater, H. du Plessis, D. Conlong and J. van den Berg)

* Based on Fitt *et al*. (2004); ** May vary according to environmental conditions; *** Grist, 1975; **** Based on feeding studies conducted

7.3.4. Hazard identification and hypothesis development (Adverse-effect scenarios)

Those species that are given the highest priority should be the candidates for testing. This final selection process is not a purely scientific one, but it should be transparent (Andow & Hilbeck, 2004b). Scientific literature was used to compile Table 7.3 and in this table knowledge gaps can be highlighted for these identified priority species. These knowledge gaps can be considered in future research. An illustration of a possible foodweb (Fig. 7.1) was made to assist in development of hypotheses for further study. In this diagram, which represents only a few herbivorous species, the complexity of the food web in a maize ecosystem is realized and it highlights the complexity if the system has yet to be described. An estimated minimum number of 300 species of arthropds occur in maize fields in South Africa and attempts to describe this biodiversity are under way (Personal communication, J. van den Berg, North-West University).

Knowledge on diversity, survival and infestation levels of non-target species on maize can be used to guide the hazard identification process and development of hypotheses relevant for risk assessment (Birch *et al*., 2004). From this study three non-target Lepidoptera species (*S. calamistis*, *H. armigera*, and *A. segetum*), two Coleoptera species (*H. arator* and *S. angulatus*), and one Diptera natural enemy species (*St. parasitica*) were considered in further testing the effect of Bt maize. Based on species distribution and the fact that some of these species are well studied, several species such as these could be recommended for inclusion in pre-release testing (Barton & Dracup, 2000; Birch *et al*., 2004) and impact assessments. The major hazards associated with these primary consumer species are that they might become significant secondary pests and may develop resistance because of their continuous exposure to Bt toxin.

In this study *S. calamistis* was the only Lepidoptera species closely related to the target stem borers evaluated in feeding studies, using maize varieties expressing Cry1Ab protein (event MON810 and Bt11) (Chapter 2). *Sesamia calamistis* was studied because it is a stemborer species that is closely associated with maize and wild host plants that occur around the maize cropping system. Although *S. calamistis* occurs at low infestation

levels it is ranked as a high priority species (Table 7.2) based on several aspects regarding its biology and ecology. *Sesamia calamistis* can occur during the whole cropping season and causes damage to the leaves, stems and ears of plants. The geographical distribution and prevalence of this species also changed during the past decade. This species used to be considered a pest in warmer coastal areas but has since 1995 been observed on sweet corn on the Highveld region as well as centre pivot irrigation systems in the North-West and Northern Provinces where it causes serious stand reductions (Van den Berg & Drinkwater, 2000). The fact that *S. calamistis* is noticed largely on irrigated maize adds to its linkage with Bt maize, since this is the preferred crop under centre pivot irrigation. The linkage of *S. calamistis* to maize may be weak in the more sub-tropical low-altitude areas where it has several wild host plants and also attacks other crops (Van den Berg *et al*., 2001). However, its linkage with maize in the Highveld region of South Africa and especially in semi-arid areas where maize is planted under irrigation may be strong since few or no wild hosts are present.

The ear-feeding Lepidoptera i.e., *H. armigera* and *A. loreyi* were also regarded as priority species and received high rankings (Table 7.2). The effects of Bt maize was evaluated for *H. armigera* (Chapter 4), but not for *A. loreyi*. These two species are abundant in many Bt and non-Bt maize fields during the post-flowering stage (Van Wyk *et al*., 2008). The presence and occurrence of *A. loreyi* on maize, as indicated in Table 7.2 was high since it attacked the crop from the seedling to the post flowering stages and was recovered in the majority of fields during previous surveys (Van Wyk *et al*., 2007). Feeding damage caused by *A. loreyi* to maize ears are the same as that of *H. armigera*, but this species is sometimes also a voracious feeder on maize leaves. The many uncertainties regarding the biology, distribution and host plant rage of *A. loreyi*, together with its capability to survive on Bt maize contributes to the importance of this non-target species. The linkage of *A. loreyi* with the maize ecosystem is uncertain and only a few wild host plants of this species are known (Van Wyk *et al*., 2007).

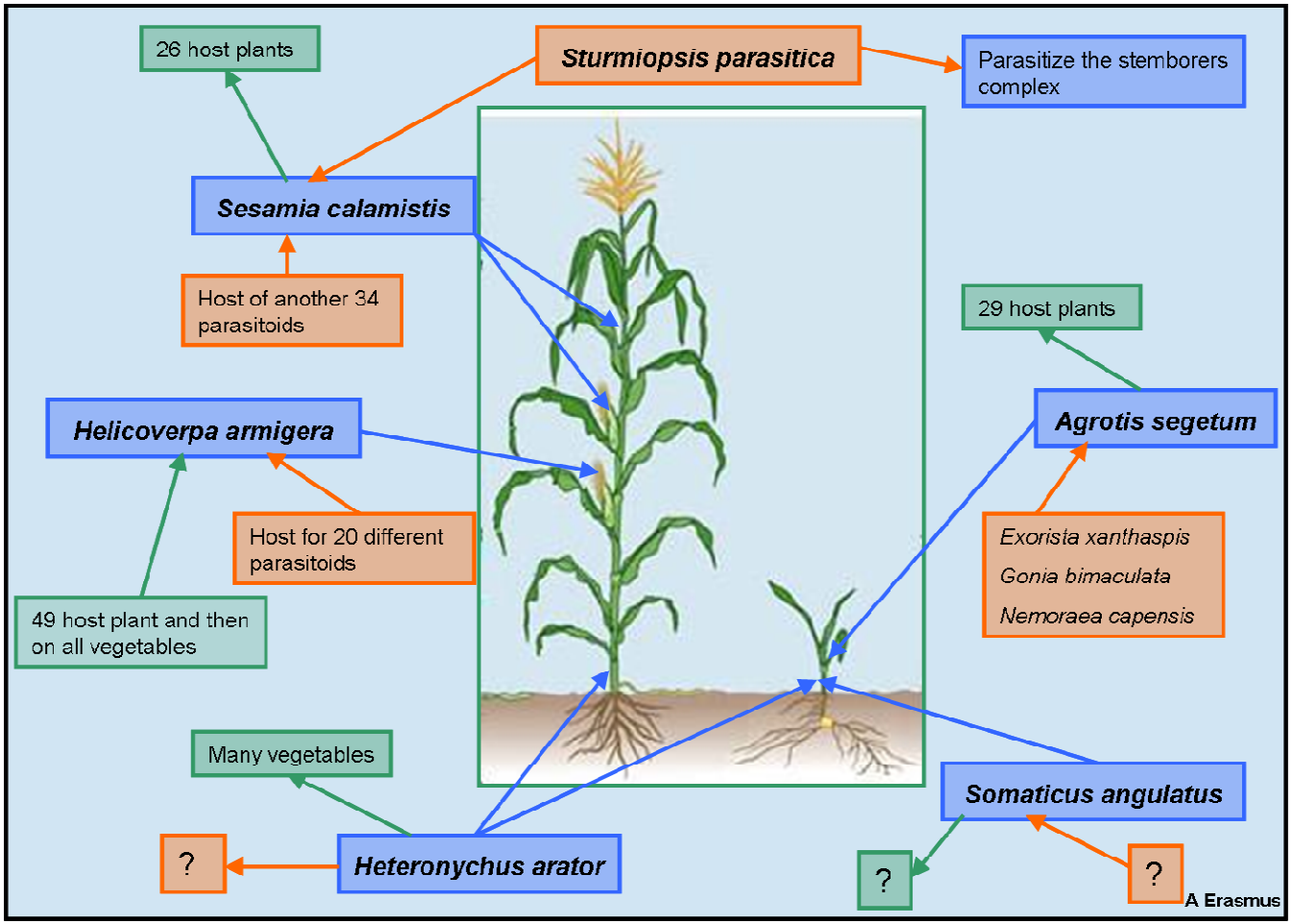

Figure 7.1. Illustration of the complexity of the maize ecosystem with only a few non-target species; what to consider when assessing the risk of a GM crop to non-target species and how to recognise knowledge gaps (all the statements made here are supported by scientific literature: Kfir, 1995; Polsazek & Khan, 1998; Van den Berg, 1993; Van den Berg et al., 2001; Visser, 2009).

Helicoverpa armigera has the potential to be exposed to Bt-toxin for prolonged periods, since its presence inside the maize crop can be from the seedling to soft dough stage. With its high abundance and common occurrence (Table 7.2) this species could become a significant secondary pest. A strong linkage between maize and *H. armigera* is evident when no other host plants are present, but linkage can also be weak when a wide host range occurs. The information matrix provided in Table 7.2, indicates that this species has the highest maximum potential exposure to Bt toxin. A study conducted by Van Wyk *et al*. (2008) indicated that *H. armigera* larvae do survive on Bt maize ears under field conditions, but their numbers were always significantly lower in Bt maize fields compared to non-Bt fields.

This study and others (Van Rensburg, 1998, 2001) showed that Bt maize is highly effective against the target stem borer species, *B. fusca* and *C. partellus* under laboratory and field conditions and that their numbers on plants are reduced to insignificant levels. It is important to keep in mind that once a niche such as that occupied by stem borers becomes vacant, the possibility exists that other Lepidoptera species could become secondary pests. It may, however, also be the case that the numbers of non-target pests decrease or are not affected at all (Peacock *et al*., 1998). Species that may possibly occupy such a vacant niche once *B. fusca* and *C. partellus* becomes locally extinct in an area could be *H. armigera* or *A. loreyi*.

Eublemma gayneri can be considered to be a "value unknown" species in the wild and it is not possible to speculate on the effects that may result should Bt maize have negative or positive effects on their numbers in the wild. It is ranked lower in importance than other species since little is known about it and its linkage with maize is probably not strong. The presence of this species on maize is uncertain and it has only been recovered from silk of plants during the post-flowering stage. Using the criteria of Andow & Hilbeck (2004b) *E. gayneri* is such a value-unknown species that could be considered for further testing.

Agrotis segetum and *S. exigua* were considered of lesser importance than the above mentioned non-target species (Table 7.2). These species could, however, also be considered in future pre-release impact assessments in countries where they occur and post-release monitoring of pest status and resistance development in South Africa. *Agrotis segetum* is an important maize pest, but it only damages maize seedlings. Its presence in the maize system is therefore short and its linkage weak since it is only directly exposed to maize during the seedling stage and because it prefers weeds to maize (Van Rensburg, 1994; Drinkwater & Van Rensburg, 1992).

Spodoptera exigua is considered a species of lesser importance because of its occasional occurrence and low abundance in maize in South Africa. Although the abundance of *S. exigua* is low in the majority of seasons, solitary larvae often feed on maize seedlings and cause damage resembling that of *C. partellus* (Van Rensburg, 1999). Outbreaks do occur from time to time, from there the common name, lesser army worm (Van Rensburg, 1999).

The two Coleopteran species, *H. arator* and *S. angulatus* were included in the ecological model because they can be regarded as two of the most abundant beetle crop pest species in South Africa (Drinkwater, 1990; Du Toit, 1998). Both these species received the lowest ranking (three) in table 7.1. These beetles can be considered of lesser importance because they belong to the Coleoptera and are known as sporadic pests. Although of lesser importance these species were considered in testing because no other coleopteran species were previously evaluated in South Africa to determine the effect of Bt maize.

Cotesia sesamiae Cameron (Hymenoptera: Braconidae) and *Sturmiopsis parasitica* (Curran) (Diptera: Tachinidae) are the most widespread and abundant indigenous larval parasitoids of stem borers (Bonhof *et al*., 1997). *Sturmiopsis parasitica* is an important larval parasitoid of gramineous stem borers in Africa (Chinwada *et al*., 2004). This parasitoid have been recorded on various lepidopteran stem borer species, including *B. fusca*, *C. partellus*, *Chilo orichalcociellus* (Strand) (Crambidae) (Bonhof *et al*., 1997),

Coniesta ignefusalis (Hampson) (Crambidae), *Eldana saccharina* (Walker) (Pyralidae), *S. calamistis*, *Sesamia nonagrioides* Tams & Bowden (Noctuidae) (Polaszek, 1998) and *Acigona ignefusalis* Hampson (Pyralidae) (Nagarkatti & Rao, 1975). *Cotesia sesamiae* have been recorded on *B. fusca*, *C. orichalcociellus*, *C. partellus*, *E. saccharina*, and *S. calamistis*.

Both these parasitoid species received a ranking of one because they constitute a very important component of the natural enemy complex of the target pests of Bt maize. It is difficult to speculate about the effect of Bt maize on natural enemies because this may be affected by the exposure pathway. In the case of *S. parasitica* and *Cotesia* spp. the effect is direct because parasitic larvae consume the whole host larvae (Dent, 2000). Kfir (2002) reported that a partial removal of natural enemies from a cereal crop field could bring about a substantial increase in stem borer numbers. This indicates that in South Africa indigenous natural enemies have the ability to suppress stemborer populations and reduce pest numbers. This is important for their conservation as resident natural enemies for the control of stem borers. Thus, if parasitoid larvae are negatively effected by feeding on stem borers that survive on Bt maize, stem borer numbers may increase.

7.3.5. Experimental endpoint for the ecological model

An appropriate experimental end point for initial testing is the generational relative fitness or some component of relative fitness (Andow & Hilbeck, 2004b). Generational relative fitness is the relative lifetime survival and reproduction of the non-target species. Thus, survival experiments should last at least through one full generation, including all the immature stages of the non-target species (Andow & Hilbeck, 2004a, b). The duration of the test should correspond to the time the non-target species would be exposed to the GM plant or plant parts. If the GM plant were to adversely affect non-target species in the environment, its effects would come through some component of relative fitness. The result from such an initial testing would guide the design of further ecologically realistic experiments (Birch *et al*., 2004), as was done with *S. calamistis* (Chapter 2), *A. segetum* (Chapter 3), *H. armigera* (Chapter 4), *H. arator* (Chapter 5), *S. angulatus* (Chapter 5),

and *St. parasitica* (Chapter 6). Feeding- and tri-trophic studies were conducted with these species which were selected from the ecological model.

Feeding studies conducted with *S. calamistis* showed that this stem borer species is highly susceptible to Bt maize events expressing Cry1Ab and that no survival occurred in the laboratory (Chapter 2) (Van Wyk *et al*., 2009) and greenhouse experiments (Van den Berg & Van Wyk, 2007). Because *S. calamistis* also belongs to the stem borer complex on maize in South Africa, this species can now also be regarded as a target species that is being controlled by Bt maize. *Sesamia calamistis* is stenophagous and occurs in mixed populations with other borer species with which it shares several parasitoid species in Africa, thus the ecological impact of local extinction of *S. calamistis* caused by this highly effective transgenic event is therefore not expected to be great.

Results of the feeding study conducted with *A. segetum* (Chapter 3) showed that the effect of Cry1Ab toxin on the biology of larvae and moths were largely insignificant. Although there were no significant differences between survival and mass of larvae feeding on Bt and non-Bt seedlings for a period of approximately two weeks, significant differences were observed in the percentage pupation over time. Larvae feeding on non-Bt seedlings of hybrid Brasco reached a higher percentage pupation over a shorter period of time compared to larvae feeding on event Bt11. Under field conditions, this can possibly influence the number of seedlings that larvae may feed on before pupation. It can be concluded that, although significant effects of Bt maize expressing Cry1Ab on *A. segetum* was observed in some instances under laboratory conditions, Bt maize events MON810 and Bt 11 will most likely not have any effect on this non-target pest under field conditions.

Feeding studies conducted (Chapter 4) with *H. armigera* indicates that larvae feeding on Bt ears were always smaller than larvae feeding on non-Bt hybrids which contributed to a delay in development. Because of the lower level of survival on Bt ears much less ear damage occured on Bt ears compared to non-Bt plants. In conclusion, this study has quantified the effects of Bt maize hybrids on the ear-feeding maize bollworm, *H.*

armigera. It provides important information on the potential of Bt maize to protect maize from *H. armigera* feeding damage. However, the likelihood of *H. armigera* becoming an important secondary pest is high.

In Chapter 5 the feeding studies indicated that there were no significant effects on *H. arator* mortality, mass, fertility or fecundity when feeding on Bt maize. The same results were observed for *S. angulatus*, with no effect on survival of second and fourth instars. Also no significant effect was observed on larval mass or fecundity. Therefore, it did not matter if these beetles or their larvae fed on Bt maize, there were no adverse effects. This statement makes it easier for future research to not even consider one of these coleopteran species in further testing of the effect of Bt maize containing Cry1Ab.

The tri-trophic study conducted with *St. parasitica* (Chapter 6) indicated that although not always significant, the percentage parasitism of Bt-consuming host larvae was always higher compared to host larvae that fed on non-Bt maize. It could be that Bt toxin affects *B. fusca* fitness to such an extent that the immune systems of host larvae were less effective than host larvae that fed on non-Bt maize. The different parameters tested indicated only one case where maggots originating from diapause host larvae feeding on non-Bt maize had a greater mass compared to host larvae that fed on Bt maize. The same applied to *St. parasitica* pupal length. For the rest of the parameters tested there were no significant differences. Although some adverse effects were observed on *St. parasitica* mass and pupal length it is most likely that this will not contribute to adverse effects in the field, but rather that there is synergism between Bt maize and *St. parasitica*.

Studies by Van Rensburg (1998; 2001) and Singh *et al*. (2005) have shown that the generational fitness of the target pests *B. fusca* and *C. partellus* feeding on Bt maize (event MON810) is extremely low with no survival to the adult stage reported. Appropriate methodologies and protocols to assess risk should be developed for high priority species (Birch *et al*. 2004), as identified and conducted in this study. Conventional ecotoxicology methodologies are suggested by Birch *et al*. (2004) to assess effects of exposure to the transgene products. A “whole plant” methodology is also

required to evaluate the effects of the whole transgenic plant, not just the transgene product (Birch *et al*., 2004), such as that used for all the feeding studies conducted throughout this entire study.

7.4. Conclusion

The selection matrix (Table 7.2), together with the information on species mentioned above, can be used to decide which species to use as test species for the future and which can be eliminated. In the case of event MON810 and Bt11, it can now be concluded what the possible effects of these proteins will be on each of these species. However, this is not where it stops, since there is still many non-target species in other functional groups that need to be tested. This methodology should be considered for each GM crop or new event produced.

Hillbeck *et al*. (2006) have developed this scientific, case-specific, step-by-step methodology to support non-target environmental risk assessment that aims to evaluate the actual potential environmental risks of a GM plant rather than rely on indicator species. This methodology is a screening process that considers all possible non-target species and adverse effects and eliminates those that are less likely to result in an adverse effect on the environment. It starts by using specific information about the crop and geographical region to develop a list of non-target species and ecological functions that could be most at risk (Hillbeck *et al*., 2006).

In conclusion, this chapter described the use of the ecological model to identify priority spesies and non-target species which could be affected by Bt maize in South Africa. There is still a further need to evaluate possible non-target species and adverse effects on these species which were not tested in this study. From the study conducted here some species can be eliminated for further testing, while research on others has to continue before a conclusion can be drawn. While this study largely made use of results based on

feeding studies in the laboratory, possible effects also need to be monitored in the actual environment following release of GM crops.

7.5. References

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CHAPTER 8: Conclusion

Civilization began with agriculture (Anon, 1996). A stable agricultural industry ensures a country of food security and food security is considered to be one of the primary requirements of any nation (Gravlee, 2009). The Food and Agriculture Organization (FAO) defines food security as the situation that exists when all people, at all times, have access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life (FAO, 2009). Sustainable agriculture and food security are critical foundations that underpin human society (Altieri, 1995; Gravlee, 2009) and it also refers to the ability of a farm to produce crops indefinitely and profitably, without damaging the ecosystem (Altieri, 1995).

At present there are about 6.79 billion people in the world (Wikipedia, 2009) some 800 million of whom are not receiving adequate nourishment, which places continuous pressure on agriculture to provide adequate food security. By 2020 the world population will have grown to almost eight billion people (IPC, 1998), which continue to be a challenge for agriculture to provide food security. There is no unique solution to the problem of sustainable agriculture, but the development of improved plant varieties with enhanced performance and reduced environmental impact is one beneficial strategy. The potential of GM crops to make major contributions to food security and agricultural sustainability worldwide is indisputable (Christou & Capell, 2009). It is recognized that biotechnology is not a magic wand that can achieve sustainable agriculture and free the world from poverty, hunger and malnutrition, but the use of GM plants as one component of a wider strategy including conventional breeding and other forms of agricultural research can contribute substantially towards the achievement of these goals, both now and in the future (Christou & Capell, 2009).

Currently, an estimated 37% of all crop production is annually lost to pests (13% to insects, 12% to plant pathogens, and 12% to weeds) in spite of the use of pesticides and non-chemical controls (Pimentel *et al*., 1993). The share of crop yields lost to insects has nearly doubled during the last 40 years preceding 1993, despite more than a ten-fold

increase in both the amount and toxicity of synthetic insecticides used (Pimentel *et al*., 1993). The losses sustained in crops due to the stemborer, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) (target species of Bt maize in South Africa) have been estimated in South Africa and more northerly countries from 5% – 75% and even higher (Annecke & Moran, 1982). Perhaps more in an attempt for consistency than the result of accurate observation, it is almost generally accepted that stem borer impact reduces crop yield, on average, by 10% (Annecke & Moran, 1982).

These losses need to be controlled and by using Bt maize this is possible. In comparison, the average positive yield impacts from using Bt maize in other parts of the world have been in the range of +5% (US and Canada) to +24% (the Philippines) (Brookes, 2009). Insect pests are therefore an important target for GM technology (Christou & Capell, 2009).

Pimentel *et al*. (1993) reported that approximately 62 million kg of insecticides are applied to 5% of the total agricultural land in the US. The wide adoption of Bt maize have led to significant reductions in insecticide use (Brookes & Barfoot, 2006; Fitt, 2008; Hellmich *et al*., 2008; Kennedy, 2008). The technology has reduced pesticide spraying by 224 million kg (equivalent to about 40% of the annual volume of pesticide active ingredient applied to arable crops in the European Union) and as a result, decreased the environmental impact associated with pesticide use by more than 15% (Brookes & Barfoot, 2006). This reduced insecticide use, in conjunction with the selective activity of Bt toxin, results in a more favorable environment for beneficial insects (Kennedy, 2008), including natural enemies of pests (Hellmich *et al*., 2008).

It is clear that it is of great importance to increase food production for the growing human populations, but we have to keep in mind while doing so to protect the sensitive environment. We need to ask ourselves the question, does the need to provide adequate food not overshadow conservation of the environment. The golden rule to remember is that without the environment it will be impossible to produce food. Therefore, we need to manage our environment to be as sustainable as possible. Agriculture is an important

environmental quality driver (Hails, 2002), and its effect is not likely to diminish in the future (Tilman *et al*., 2001). Unfortunately, the need to balance profitability and environmental stewardship is a significant economic and scientific challenge, since agriculture by its very nature is one of the most expensive and environmentally harmful practices carried out by humans (Christou & Capell, 2009). The extent and methods of agriculture have demonstrably led to extensive and permanent loss of biodiversity in many localities (Devine & Furlong, 2007).

Like all other control tactics that have adverse effects on the environment, it is likely that GM crops will not have any adverse effect. It is only a question of how do the producers use this technology to cause minimal impact on the environment and that is why it is necessary to do sufficient risk assessment by monitoring possible effects. To do risk assessment there are different suggestions to follow (Dutton *et al*., 2003; Losey *et al*., 2003; Andow & Hilbeck, 2004a; Andow & Hilbeck, 2004b; Romeis *et al*., 2006; Kennedy, 2008; Romeis *et al*., 2008; Romeis *et al*., 2009). For the purpose of this study the ecological model as described by Birch *et al*., (2004) and Hilbeck *et al*., (2006a) was followed.

Hillbeck *et al*. (2006a) have developed this scientific, case-specific, step-by-step methodology to support non-target environmental risk assessment that aims to evaluate the actual potential environmental risks of a GM plant rather than rely on indicator species. This methodology is a screening process that considers all possible non-target species and adverse effects and eliminates those that are less likely to result in an adverse effect on the environment. It starts by using specific information about the crop and geographical region to develop a list of non-target species and ecological functions that could be most at risk (Hillbeck *et al*., 2006b). In the present study a list of species found on and in maize fields was compiled and species that were most abundant were evaluated in feeding or tri-trophic studies. These species were also identified as priority species in the selection matrix.

Results from feeding studies indicated that *Sesamia calamistis* (Lepidoptera: Noctuidae) was just as highly susceptible to Bt11 than event MON810. The behavioral characteristic of larvae to feed behind leaf sheaths and to enter stems directly did not result in escape of exposure to the toxin. Larval feeding on leaf sheaths therefore resulted in the ingestion of sufficient toxin to kill larvae before they entered maize stems. *Sesamia calamistis* is stenophagous and occurs in mixed populations with other borer species with which it shares several parasitoid species in Africa. The ecological impact of local extinction of *S. calamistis* caused by this highly effective transgenic event is therefore not expected to be severe.

Feeding studies conducted in the laboratory with *Agrotis segetum* (Lepidoptera: Noctuidae) indicated that the effect of Cry1Ab toxin on the species' biology were largely insignificant, however, some differences were observed. Comparing first instar larvae that fed on conventional (non-Bt) maize, Bt maize did not affect survival. There were no significant differences between survival and mass of fourth instar larvae for the different treatments, however, significant differences were observed in the percentage pupation over time. Larvae feeding on non-Bt seedlings of hybrid Brasco reached a higher percentage pupation over a shorter period of time compared to larvae feeding on event Bt11. Under field conditions, this can possibly influence the number of seedlings that larvae may feed on before pupation. Fewer eggs were laid by moths when fed as larvae on maize event Bt11 compared to MON810. It can be concluded that, although significant effects of genetically modified maize expressing Cry1Ab on *A. segetum* was observed in some instances under laboratory conditions, Bt maize events MON810 and Bt 11 will most likely not have any effect on this non-target pest under field conditions.

In laboratory and greenhouse studies conducted with *Helicoverpa armigera* (Lepidoptera: Noctuidae) 1st instar larvae feeding on whorl leaves and ears respectively, it was observed that whorl leaves were not a suitable food source and when feeding on ears larval mass increased on non-Bt maize whereas no increase occurred on Bt maize. In this study larvae feeding on Bt ears were always smaller than larvae feeding on non-Bt hybrids which contributed to a delay in development. Larval establishment did occur on a few ears of Bt

maize plants, but once established in ears, larvae developed more slowly. From this study it is concluded that Bt maize will suppress *H. armigera* infestations but not to levels approaching 100%. A study conducted by Van Wyk *et al*. (2008) confirmed the same results, indicating that *H. armigera* larvae do survive on Bt maize ears under field conditions but their numbers were always significantly lower in Bt maize fields compared to the non-Bt fields. In conclusion, this study has quantified important information on the potential for Bt maize to protect maize from *H. armigera* feeding damage. However, the likelihood of *H. armigera* becoming an important secondary pest is high. *Helicoverpa armigera* has a history of demonstrated potential in developing resistance to virtually all the insecticide molecules used against it (Kranthi *et al*., 2005), therefore it could also be the case with Bt maize. Although this pest is currently suppressed by Bt maize it could develop resistance, which, in that case, would make it the only ear feeding lepidopteran of importance, with the opportunity of invading the vacant niche usually occupied by other ear feeding lepidopterans (target pests). If this would happen, chemical control measures, similar to those applied against stem borers before the advent of Bt maize, would again become necessary.

All three the above mentioned lepidopteran species belong to the Noctuidae family, but the effect Bt maize had on these species differ from one another. *Sesamia calamistis* which also belongs to the stemborer complex, is controlled effectively by the Bt maize events also controlling the two target stemborer species, *Busseola fusca* (Lepidoptera: Noctuidae) and *Chilo partellus* (Lepidoptera: Crambidae). The question still remains if this now allows us to regard *S. calamistis* as a target species of Bt maize or not. *Helicoverpa armigera* is being suppressed by Bt maize although not controlled, but still may cause damage to maize ears. However, *H. armigera* will need to be monitored for resistance development. In contrast to the above mentioned species, Bt maize almost have no effect at all on *A. segetum*. Thus is it clear that the Bt toxin has different effects on different species even though they belong to the same family, therefore it is of great importance to monitor these effects on all possible species that comes into contact with Bt maize, directly or indirectly.

The feeding studies conducted to determine the effect of Bt maize on mortality, growth and fertility of *Heteronychus arator* (Coleoptera: Scarabaeidae) and *Somaticus angulatus* (Coleoptera: Tenebrionidae) showed that the effect of Cry1Ab on the biology of these two species was insignificant. Most of the previous studies in South Africa focused on the effect of Bt maize on the target and non-target Lepidoptera. Considering this situation, this study also investigated the influence of Bt toxin (Cry1Ab) on the growth and survival of insect pests from other orders. We found it necessary to determine the effect of Bt maize (Cry1Ab) on the two serious pest coleopteran species found in South Africa. Experiments showed that it did not matter if one of the beetle species or their larva fed on Bt maize, there were no adverse effects. From this knowledge it can be concluded that these two beetle species can be left out in the risk assessment to determine the effect of Bt maize expressing Cry1Ab. The feeding studies conducted here were sufficient and further field studies will not be necessary.

To date no tri-trophic study had been conducted in South Africa to determine if there is any effect of Bt maize on parasitoids. Tri-trophic studies to determine if there is any effect of Bt maize on *Sturmiopsis parasitica* (Diptera: Tachinidae) when parasitizing Bt-resistant *B. fusca* diapause or fourth instar larvae that have fed on Bt maize indicated only one case where maggots originating from diapause host larvae feeding on non-Bt maize had a greater mass compared to host larvae that fed on Bt maize. Although some adverse effects were observed on *St. parasitica* mass and pupal length it is most likely that this will not contribute to adverse effects in the field.

Why should we be concerned about the potential non-target effects of GM crops or any other pest management tactic? The answer lies in the ecological roles or “services” provided by these species. These non-target species play vital roles in agroecosystems. If GM crops or any other factor has a negative impact on this species, those ecological functions may be threatened (Losey *et al*., 2001). Of the above mentioned species some can be included in risk assessment, such as, *S. calamistis, A. segetum, H. armigera* and *St. parasitica*, whereas some can be excluded, such as *H. arator* and *S. angulatus*.. The feeding- and tri-trophic studies conducted with these species in the laboratory, concluded

which species need to be evaluated further in the field to determine if there will be any adverse effect in the environment. Finally, the importance of observing the non-Bt refuge strategy can not be over emphasized. The refuge area does not only play an important role in resistance monitoring but also creates the perfect environment for all the non-target species to survive. The remaining question thus is: Is the refuge big enough to support these vital ecological functions?

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