STUDIES OF THE POTENTIAL NON-TARGET EFFECTS OF *BACILLUS THURINGIENSIS* CRY1AB TOXIN ON TWO STEMBORER PARASITOIDS, *COTESIA FLAVIPES* CAMERON, *XANTHOPIMPLA STEMMATOR* THUNBERG, AND THE BUTTERFLY *ACRAEA EPONINA* CRAMER

By

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DECLARATION

I, Wanyama, Dennis Ochieno, hereby declare that this thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

This thesis is dedicated to the Ariada's family, Jack, Elizabeth, Angelina, Robert, Betty, Dennis, Anne, Patricia, Judy, Vincent, Irene, Victor, Albert, George, Maria, Andrew, Regina, Audrey. I thank them for their sacrifice and support which ensured my success.

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ABSTRACT

Studies of the potential non-target effects of *Bacillus thuringiensis* Cry1ab toxin on two stemborer parasitoids, *Cotesia flavipes* Cameron, *Xanthopimpla stemmator* Thunberg, and the butterfly *Acraea eponina* Cramer

A risk assessment was used for measuring the harmfulness caused by *Bacillus thuringiensis (Bt)* labelled MON810 maize to parasitoids that feed on maize stemborer pests in Kenya. The same factor was used for measuring the harm caused to butterfly caterpillars that accidentally ingest pollen deposited onto their host plants. Experiments were conducted using purified Cry1Ab toxin and two stemborer wasp parasitoids, *Cotesia flavipes* Cameron, (Braconidae) and *Xanthopimpla stemmator* Thunberg (Ichneumonidae). Larvae of the butterfly *Acraea eponina* Cramer (Acraeidae) were used in the studies on effects of *Bt* maize pollen spores on caterpillars.

In the experiments on the effects of host-ingested *Bt* toxins on parasitoids, neonate larvae of the stemborer *Chilo partellus* (Swinhoe) (Crambidae) were fed on artificial diet with purified Cry1Ab toxin at 0.005µg/ml and 0.01µg/ml. Controls included phosphate buffered saline (PBS) to check for its effect as a solvent for the toxin, and a normal diet. *C. partellus* larvae at the age of 25 days were brought into contact with mated females of *Co. flavipes* and parasitized. Three days old pupae were parasitized by *X. stemmator* in paper straws. Parameters like host weights, developmental time, number of progeny, immature mortalities, egg loads, oviposition rates, longevity and adult sizes were assessed. Experiments on effects of *Bt* maize pollen spores on butterfly larvae involved planting of non-*Bt* maize on plots, while *Waltheria indica* L., a host plant for *Acraea eponina,* were planted in plastic pots. The host plants were arranged at different distances in four directions of maize plots during anthesis. Densities of maize pollen

deposited on the host plant leaves were estimated using a 0.25cm^2 wire quadrat. The maximum density encountered was 492 grains / cm^2. These densities were converted to *Bt* toxin concentrations basing on Cry1Ab levels in MON810 variety. Two toxin concentrations that corresponded to 246 and 492 grains / cm^2 were prepared. The toxins were fed to *A. eponina* 2nd and 4th instar larvae on leaf discs and their mortalities were recorded daily.

The effects of *Bt* Cry1Ab toxin on *Co. flavipes* included reduction in host weights, production of few progeny, longer development time and higher immature mortality, oviposition of fewer eggs, smaller egg loads, short life span, and small adult size. For the parasitoid *X. stemmator,* the toxin caused reduction in weights of host pupae that suffered high mortality when parasitized by the wasp. The experiments on effects of *Bt* maize pollen spores on *A. eponina* larvae recorded high mortality of both 2nd and 4th instar larvae. The results indicate that introduction of *Bt* maize would harm parasitoids and butterfly larvae. An ecological imbalance may result as a consequence of these effects. More research should therefore be carried out using the actual transgenic plants prior to any introduction of *Bt* maize in Kenya.

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CHAPTER ONE

1.0 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

Precautionary risk assessments are necessary prior to the adoption of transgenic crops and other genetically modified organisms (GMOs). Through genetic engineering (GE), crops like *Bacillus thuringiensis* (*Bt*) maize that have varying desirable qualities are being produced. These maize varieties express *Bt* toxins (Cry-proteins) that are targeted to control cereal stemborer pests like *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae).

Despite their activity against the pests, the toxins could also be harmful on non-target organisms within the maize fields. Such organisms include natural enemies of pests, pollinators, symbionts and minor pests. All these organisms play specific roles in maintaining equilibrium in agroecosystems. Some may be of aesthetic value, while others could have important functions that are yet to be exploited. Adoption of these crops in new agroecosystems may therefore have unknown impacts on ecological balance and biodiversity.

Lack of clear information on potential risks on non-target organisms has raised mixed reactions on registration of GE-crops in many nations. Kenya is one of the countries that is considering the adoption of such *Bt* crops, although there is still no adequate data to clarify their effects on non-target fauna. It is therefore important to

identify endangered species and the situations under which they are exposed to the toxins. Experiments that predict or verify the risks must then be designed.

This work was part of a risk assessment programme on maize genetically modified with *Bt* (MON810 *Bt* maize variety) at the International Centre of Insect Physiology and Ecology (ICIPE). This maize variety expresses toxic substances labelled Cry1Ab toxins that target different stemborer species including *C. partellus*. The studies were simulatory and were carried out using purified Cry1Ab toxins. The studied non-target organisms were two hymenopterous parasitoids of stemborers (*Cotesia flavipes* Cameron and *Xanthopimpla stemmator* Thunberg), and a butterfly species *Acraea eponina* Cramer. The concern was whether Cry1Ab toxins that are ingested by the stemborer would harm the two parasitoids. On the other hand, *A. eponina* larvae could be harmed if they accidentally feed on certain densities of the toxic maize pollen, which is deposited on their host weed *Waltheria indica* L. Adverse effects of toxins would lead to extinction of the parasitoids and the elegant butterfly species. As a result, pest and populations would rise above economic threshold levels in the absence of natural control.

Effects of the toxins on the parasitoids were studied using tritrophic experiments. Data on host suitability and parasitoid fitness was recorded. Impact of *Bt* toxins on survivorship of the butterfly were studied by feeding the larvae on toxin concentrations, which were simulated from maize pollen densities that were encountered on the host plants. The methodologies and results of these studies will therefore be important in policy formation for the registration of *Bt* maize in Kenya.

1.2 Literature review

1.2.1 Risk assessment of *Bt* maize

Risk assessment is the method of evaluating hazards in GMOs. It is a scientific process with the objective of quantifying the probability that harm will occur and magnitude of resulting effects (Zakri, 2001). Regulatory processes for GM crops involve different criteria, which clarify uncertain risks before permitting the use of the crops (Levidow, 2003). Important issues include appropriate design of safety tests to yield meaningful results; cause effect models of non-target harm; and the acceptability of such harm (Levidow, 2003). Producers of GM foods would claim that their products are safe if they pass a series of tests under a risk assessment regime (Zakri, 2001). Different risk assessments on effects of MON810 and other *Bt* maize strains are being carried out in different countries (EcoStrat, 2000; CFIA, 2001; AGBIOS, 2001a, b; Monsanto, 2002; Hilbeck and Andow, 2004)

1.2.2 *Bt* maize benefits and potential risks

Bt maize hybrids were primarily targeted at controlling the European corn borer, *Ostrinia nubilalis* (Hb.) and the South-western corn borer, *Diatraea grandiosella* Dyar (Lep: Crambidae) (Storer *et al.*, 2001). Many biotechnologists considered these varieties and other crops that were genetically modified to produce insecticidal proteins derived from the bacterium *Bacillus thuringiensis* as the most important technological advancement in insect pest management since the development of synthetic pesticides (Obrycki *et al.*, 2001). The first genetically engineered crop plants containing the *Bt* gene

(corn, cotton, and potatoes) were commercially produced and harvested in the United States in 1996 (Hilbeck *et al.*, 1998a). Planting of *Bt* maize varieties has increased (Kota *et al.*, 1999).

Bt maize strains have been developed using different *cry*-genes (Crickmore *et al.*, 1998; Catangui and Berg, 2002). Expression of the toxins has also been found to vary in different plant tissues (Table 1.1). Both *Bt* 11 and Mon810 varieties express Cry1Ab toxin, and the 35S promoter from the cauliflower mosaic virus is involved in their transformation (Armstrong *et al.*, 1995). In Mon810, Cry1Ab toxin is produced at high concentrations throughout the growing season in leaves, pollen, silk and kernels (Verzola, 1999). Toxin production is also influenced by nitrogen levels in soil, and atmospheric concentrations of carbon dioxide (Coriella *et al.*, 2000).

Table 1.1 Expression of Cry1Ab toxin in different tissues of MON810 maize variety

Tissue	Concentration per tissue (fw)
Pollen	0.09µg / g
Leaves	7.93 to 10.34µg / g
Grain	0.19 to 0.39µg / g
Whole plant	3.65 to 4.65µg / g

(Sources: CFIA, 2001; Monsanto, 2002)

The benefits of planting *Bt* maize include increased savings in resources devoted to scouting for pests (Obrycki *et al.*, 2001) since there will always be toxins on the crops. The compatibility of *Bt* toxin with parasitoids has been considered in several integrated pest management (IPM) systems and has shown synergistic interactions (Chenot and Raffa, 1998; Soares *et al.*, 1993), which occur where there is toxin mediated partial resistance in the host (Johnson and Gould, 1992). In such situations, host mortality

caused by the combination of the toxin and parasitiods is greater than for parasitoids alone (Chilcutt and Tabashnik, 1997). The adverse effects of broad-spectrum pesticides will reduce, hence protecting non-target and beneficial insects like pollinators. Crop yields will increase and be protected due to season long control (Rice and Pilcher, 1998), since the germplasm gives exceptional control over the stemborers (Abel *et al.*, 2000). Stored maize will have adequate protection from lepidopterous storage pests (Giles *et al.*, 2000). There will also be lower carcinogenic mycotoxin levels in the maize due to reduction in saprophytic fungal pathogens like *Aspergillus flavus* L. and *Fusarium spp.*, which are associated with cob and stemborer feeding (Munkvold *et al.*, 1997; Storer *et al.*, 2001).

Potential risks of adopting *Bt* maize have raised reactions in many countries (Commandeur *et al.*, 1996; Hilbeck, 2001; Levidow and Carr, 2001; Dale *et al.*, 2002; Levidow, 1999, 2003). Much concern has been raised over the long-term yield and economic performance of *Bt* maize (Catangui and Berg, 2002); the unpredictability of stemborer infestations (Rice and Pilcher, 1998); and how the transgenic plants would interact with other non-target organisms at different trophic levels (Tappeser and Meyer, 1997; Hilbeck *et al.*, 1998a; Hilbeck *et al.*, 1998b; Obrycki *et al.*, 2001; Levidow, 2003). It is generally believed that large-scale uniform cultivation of *Bt* maize will exert high selection pressure on target pest species and accelerate the build up of resistance (Gould, 1998). Habitat modification, which could be a result of simplification with a monoculture, may differentially favour pests and dissociate them from their natural enemies, leading to more serious pest problems (Dent, 2000). Exchange of genetic

material between the transgenic crop and related species (Quist and Chapela, 2001; Luna *et al.*, 2001), could spread the insecticidal genes hence extending the risks.

1.2.3 Effects of *Bt* toxins on natural enemies

The quality of host plants influences the feeding, growth and development of phytophagous insects and can profoundly affect tritrophic interactions between plants, herbivores and their natural enemies (Price, 1986). A phenological change in the biochemistry of plant tissues, like the expression *Bt* toxins in transgenic crops affects growth and survival of herbivores (Murugan and George, 1992), which in turn influences their natural enemies (Bloem and Duffey, 1990; Werren *et al.*, 1992).

Chenot and Raffa (1998) exposed gypsy moth *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) larvae to sub-lethal concentrations of *Bt* toxins, and they observed higher emergence of the parasitoid *Cotesia melanoscela* (Ratzeburg) (Hymenoptera: Braconidae) due to increased parasitism in the field. Larval developmental time for the parasitoids was longer, and there was decrease in cocoon lengths. Tounou *et al.* (2005) observed that the wasp *Cotesia sesamiae* (Cameron) (Braconidae) produced larger clutch sizes and higher parasitism rates in three *Bt* fed lepidopterous hosts *Eldana saccharina* Walker (Pyralidae), *Busseola fusca* (Fuller) (Noctuidae) and *Sesamia calamistis* Hamps. (Noctuidae). The results were attributed to lowered immune response. Soares *et al.* (1993) reported that larvae of *Spodoptera exigua* (Hb.) and *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) that are intoxicated by sub-lethal doses of the *Bt* pesticide MVP®, could serve as suitable hosts for the attraction and reproduction of the hymenopterous parasitoid *Cotesia marginiventris* (Cresson)

(Braconidae). Blumberg *et al.* (1997) observed that pre-feeding of *Helicoverpa armigera* (Hübner) (Noctuidae) with lethal concentrations of *Bt* toxin did not prevent the parasitoid *Microplitis croceipes* (Cresson) (Braconidae) from ovipositing in the infected host larvae. They also found that long pre-feeding periods for hosts on the toxin prevented successful development and pupation of the parasitoids due to mortality of hosts.

Prütz and Dettner (2004) verified that *C. partellus* larvae that fed on *Bt* diet exhibited reduction in feeding, and suffered high mortality when parasitized; the rate of successful development among *Co. flavipes* that developed in these hosts was low, while their pupal and adult weights were reduced. However they did not detect any effect on parasitoid development time, sex ratios and cocoon mortality. Chilcutt and Tabashnik (1997) noticed that a resistant colony of *Plutellae xylostella* (L.) (Lepidoptera: Plutellidae) to *Bt* toxin had no effect on the performance of the parasitoid *Cotesia plutellae* (Kurdjumov) (Braconidae). In the *Bt* susceptible colonies, host mortality caused by the combination of *Bt* toxin and parasitoids was greater than for parasitoids alone. Artwood *et al.* (1997) found that high concentrations of *Bt* toxin in the artificial diet for the tobacco budworm larvae *Heliothis virescens* (Fabricius) (Noctuidae), caused low emergence rates of the parasitoid *Co. marginiventris*. They also observed that longer pre-exposure periods to the *Bt* diets for hosts that had been parasitized increased the rates of parasitoid emergence.

Ahmed *et al.* (1978) found that treatment of the gypsy moth *L. dispar* larvae with a combination of the parasitoid *Apanteles melanoscelus* Ratzeburg (Braconidae) and *Bt* toxin caused higher mortality than when each treatment was done alone. Salama and Zaki (1985) found no effect on longevity, productivity, or capacity to parasitize on adult

parasitoid *Trichogramma evanescens* Westwood (Hymenoptera: Trichogrammatidae) that had been fed on honey solution containing 500µg of *Bt* toxin. When eggs of the two hosts *Spodoptera littoralis* (Boisduvalle) (Noctuidae) and *Anagasta kuehniella* (Zeller) (Pyralidae) were sprayed with the same concentration, there was reduced parasitism, but no effect on development of immature stages or percentage emergence.

Johnson *et al.* (1997) recorded low parasitism by *Campoletis sonorensis* (Cameron) (Hymenoptera: Ichneumonidae) on *H. virescens* larvae that fed on *Bt* tobacco than on non-*Bt* plants; and lower parasitism for non-adapted larvae than for the larvae that were adapted to the *Bt* plants. They attributed the differences in parasitism to decreased larval feeding on *Bt* plants, since the parasitoid locates host larvae using cues from damaged plant tissues. Kok and Acosta-Martinez (2001) found that adults of *Cotesia orobenae* Forbes (Braconidae), parasitoids of the cross-stripped cabbage worm, *Evergestis rimosalis* Guenee (Pyralidae), were not affected by *Bt* toxin when they came in contact with, or ingested the toxin in honey solution.

Ashouri *et al.* (2001) recorded reduced immature survival and adult size in the parasitoid *Aphidius nigripes* Ashmead (Hymenoptera: Aphidiidae), which developed in the potato aphid *Macrosiphum euphorbiae* (Thomas) (Homoptera: Aphididae) that fed on 'Superior-*BT*' potato expressing CryIIIA toxin. Erb *et al.* (2001) fed gypsy moth larvae on the *Bt* pathogen; they observed higher preference and higher super parasitism by *Compsilura concinnata* (Meigen) (Diptera: Tachinidae) on the non-infected hosts than on the *Bt* treated ones. Parasitoids in *Bt* treated, super parasitized hosts had shorter larval development times and smaller pupal masses than parasitoids in untreated larvae.

Hilbeck *et al.* (1998a, 1998b, 1999) observed increased developmental time and high rates of immature mortality in the predaceous larvae of *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) that were reared on two *Bt* fed lepidopterous hosts *S. littoralis* and *O. nubilalis*. They also recorded increased immature mortality when the predators were directly fed on artificial diet that contained Cry1Ab toxin (Hilbeck *et al.*, 1998b). Ponsard *et al.* (2002) reared four heteropterous predatory species on larvae of *S. exigua*, which had ingested tissues from transgenic cotton expressing Cry1Ac toxin. There was significant decrease in longevity for *Orius tristicolor* White (Anthocoridae) and *Geocoris punctipes* Say (Geocoridae). No significant effect on longevity was observed in *Nabis* sp. (Nabidae) and *Zelus renardii* Kolenati (Reduviidae).

Zwahlen *et al.* (2000) found no significant difference in mortality between predaceous *Orius majusculus* Reuter (Anthocoridae) nymphs that were reared on *Bt* fed and *Bt* unfed *Anaphothrips obscurus* (Müller) (Thysanoptera: Thripidae). Hafez *et al.* (1995) performed experiments on the effects of the commercial product Dipel 2X which is based on *Bt*, on *Orius albidepennis* (Anthocoridae), that predates on the eggs and larvae of the cutworm *Agrotis ypsilon* (Hufnagel) (Noctuidae). They found that cutworm neonates that had been pre fed on toxin diet, and eggs that were sprayed with toxins, caused low rate of feeding, increased nymphal duration, and reduced female reproductive capacity for the predator. Armer *et al.* (2000) found no significant difference in longevity for five species of phytophagous predators namely *Geocoris punctipes, G. pallens* Stäl, *O. tristicolor*, *Nabis* spp., and *Lygus hesperus* Knight (Heteroptera: Miridae); that were reared on transgenic *Bt*t and non-transgenic potato leaves.

Hough-Golstein and Keil (1991) offered *Bt* sprayed *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) eggs, nymphs, and adults to the predaceous *Perillus bioculatus* (F.) (Heteroptera: Pentatomidae). They observed very little mortality on the tested stages of the predator. Dogan *et al.* (1996) did not observe significant effect on survival, rate of food consumption, development or reproduction in the convergent lady beetle *Hippodamia convergens* Guérin-Ménenville (Coleoptera: Coccinellidae), which had been fed on green peach aphids *Myzus persicae* (Sulzer) (Aphididae) reared on *Bt* potatoes. Riddick and Barbosa (1998) found no observable effect on the predaceous *Coleomegilla maculata* (De Geer) (Coccinellidae) reared on *Bt* fed *L. decemlineata.* Lundgren and Wiedenmann (2002) did not detect any effects on fitness of the polyphagous predator *C. maculata* that were fed on mixtures of transgenic corn pollen expressing Cry3Bb toxin.

1.2.4 Pollen dispersal and effects of *Bt* toxins on butterfly larvae

The potential for non-target species to be affected by *Bt* maize pollen spores remains under study (USEPA, 2001). Insect larvae that feed on *Bt* toxins usually stop feeding and eventually die by septicaemia and starvation (Knowles and Ellar, 1987; Ceron *et al.*, 1995; Fiuza *et al.*, 1996; Kwa *et al.*, 1998). Losey *et al.* (1999) demonstrated that exposure to *Bt*11 maize pollen can interfere with feeding, retard growth and cause mortality in neonate Monarch caterpillars *Danaus plexippus* L. (Lepidoptera: Danaidae). Wraight *et al.* (2000) could not directly attribute mortality of *Papilio polyxenes* Fabricius (Lepidoptera: Papilionidae), the Black swallowtail to exposure to MON810 maize pollen

under field conditions. However, laboratory studies showed that the butterfly was sensitive to pollen from Norvatis event 176 that has higher toxin concentrations.

Zangerl *et al.* (2001) found that event-176 pollen caused reduction in growth rates and mortality under field conditions in both *D. plexippus* and *P. polyxenes*, although the survivorship of the latter butterfly was higher under similar conditions. They found mortality to occur at pollen counts of 100 grains / cm^2 and above; while the LD_{50} was estimated at 613 grains / cm^2.

Hellmich *et al.* (2001) performed bioassays of event-176 pollen fed to first instar monarch larvae on leaf discs and whole leaves of the common milkweed *Asclepias syriaca* L. (Asclepiadaceae); growth inhibition and mortality were recorded. Specifically, growth inhibition could be detected at 5-10 grains/cm^2 for event-176, but pollen from other varieties including MON810 and *Bt*11 did not demonstrate lethal and sub-lethal effects even at pollen counts above 1000 grains / cm^2. They also estimated 0.09µg of Cry1Ab toxin to occur at pollen counts of 366 grains in MON810 maize. According to efficacy assays for Cry1A proteins by USEPA (1995) on Monarch larvae, doses estimated to cause mortality (LD_{50}) were rated at 3.3ng of protein / ml diet, while for growth inhibition (EC_{50}) was 0.76 ng / ml.

1.2.5 *Cotesia flavipes* Cameron (Hymenoptera: Braconidae)

Cotesia flavipes (Plate 1.1) is a gregarious endoparasitoid that was introduced in Kenya in 1993 from Pakistan (Overholt *et al.*, 1994). This parasitoid wasp has now established stable populations and therefore it is a localized insect species in Kenya (Overholt, 1998). The wasp is pro-ovigenic with a fixed complement of around 150 eggs upon emergence (Potting *et al.*, 1997). They parasitize medium and large sized stemborer larvae (Overholt *et al.*, 1994) by the ingress-and-sting strategy (Smith and Wiedenmann, 1997), and lay 35 to 45 eggs per host (Potting *et al.*, 1997). The egg / larval period of this parasitoid lasts 9 to 13 days, after which the larvae exit the host and spin cocoons, adults emerge from the cocoons 5 to 6 days later (Kajita and Drake, 1969). The parameters of interest in host suitability tests for *Cotesia spp.* performed by Ngi-Song *et al.* (1995) were developmental time, the proportion of hosts that produced cocoons, the proportion of hosts that did not produce cocoons, and were still alive 20 days after parasitization, the total progeny produced per parasitized host, and the proportion of female progeny.

1.2.6 *Xanthopimpla stemmator* Thunberg (Hymenoptera: Ichneumonidae)

Xanthopimpla stemmator (Plate 1.2) is one of the pupal parasitoids that have shown potential for the control of paleotropical lepidoptera stemborers (Vinson, 1942). This wasp applies the drill-and-sting strategy of parasitization in which their sufficiently long ovipositors help them to oviposit in concealed host pupae (Nikam and Basarkar, 1981; Smith and Wiedenmann, 1997). All stages of pupae are acceptable to the ovipositing female, but pupae in the first half of the developmental period are most suitable (Smith *et al.*, 1993). Eggs hatch in one day and the larva moults 4 times in 5 to 7

days followed by pupation that lasts 11 to 12 days (Smith *et al.*, 1993). The average period of immature stage is 19 days in addition to a 1-day pre-oviposition period (Nikam and Basarkar, 1981), while adult longevity ranges between 22 to 56 days (Hailemichael *et al.*, 1994). Although some host pupae are superparasitized, early instar larval cannibalism or the host immune defences result in only one survivor (Smith *et al.*, 1993). Hailemichael *et al.* (1994) performed host suitability tests for *X. stemmator* by measuring development time in pupae of different stemborers that were offered to their parasitoids in paper straws.

1.2.7 *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae)

Chilo partellus (Plate 1.3) is a host to many species of parasitoids which include *Co. flavipes* and *X. stemmator* (Bonhof *et al.*, 1997). It is one of 21 economically important species of lepidopteran cereal stemborers, which attacks both cereal crops and wild grass species (Maes, 1997). Adults of this pest are nocturnal and live 2 to 3 days during which each female lays 200 to 600 scale-like eggs in batches of 10 to 80 overlapping eggs on the underside of leaves near the midrib (Harris, 1990). Hatching of larvae takes place after 4 to 5 days during which they feed in the leaf whorls tunnelling into stems (Harris, 1990). In a laboratory rearing experiment, Ampofo (1988) observed that there were six instars with durations of 2 to 3 days for instars I to IV; the fifth instar had the longest duration of 8 days while the VI instar took around 6 days to pupation. The pupal stage, which shows clear sexual differentiation on their ninth abdominal segments (Marston *et al.*, 1983; Ampofo, 1988), lasts for 5 to 12 days (Maes, 1998). Generally, it takes between 25 to 50 days for this insect to complete its life cycle (Maes, 1998).

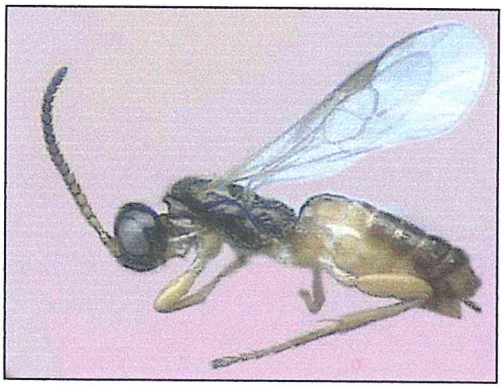


Plate 1.1 *Cotesia flavipes* Cameron (Braconidae), a larval parasitoid of cereal stemborer pests.

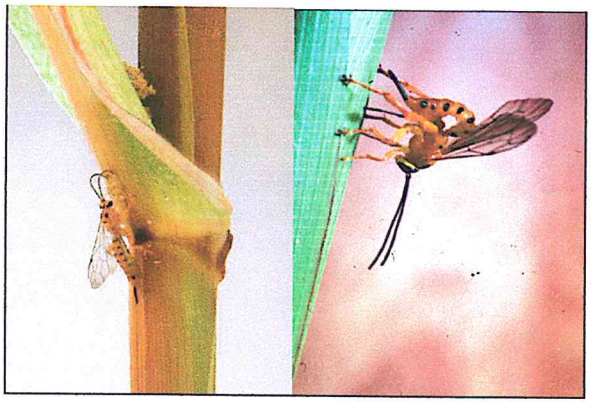
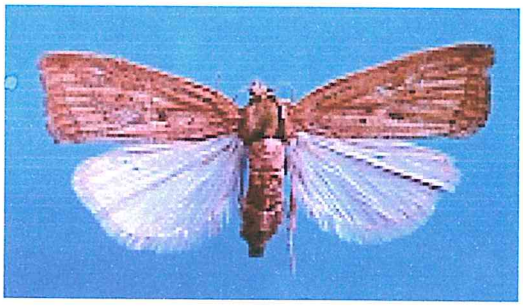


Plate 1.2 *Xanthopimpla stemmator* Thunberg (Ichneumonidae) probing for cryptic stemborer pupae in maize stems (left), and another one (right) drilling its ovipositor through a maize sheath to lay eggs in concealed host pupae.



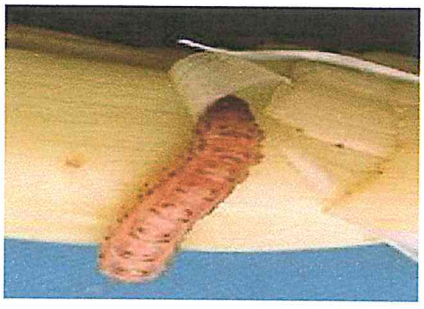


Plate 1.3 The stemborer moth *Chilo partellus* (left) and larva boring into maize stem (right)

1.2.8 *Acraea eponina* Cramer (Lepidoptera: Acraeidae)

Acraea eponina (Plate 1.4), which is also known as 'small orange Acraea' (Williams, 1969), is medium sized to large with relatively thin, narrow bodies, and a slow flapping flight (Boorman, 1991). The larvae have sharp, branched spines arranged in pairs along the sides of the body (Boorman, 1991). The species is found throughout many regions like the Ethiopian region, Madagascar and Tanzania; it is extremely common in woodland, forest margins, marshy areas and open habitats, from sea level to 2200m (Kielland, 1990). The larvae feed on a variety of low growing plants, which include *Triumfetta macrophyla*, *T. rhomboidea* (Tiliaceae); *Hermania sp.* (Sterculiaceae); *Hibiscus sp.* (Malvaceae); *Nicotiana tabacum* (Solanaceae) (Williams, 1969; Kielland, 1990; Larsen, 1996). During field surveys at the Kenyan coast, the species was found to extensively exploit *Waltheria indica* L. (Sterculiaceae), which grows on many farms (Own observation).

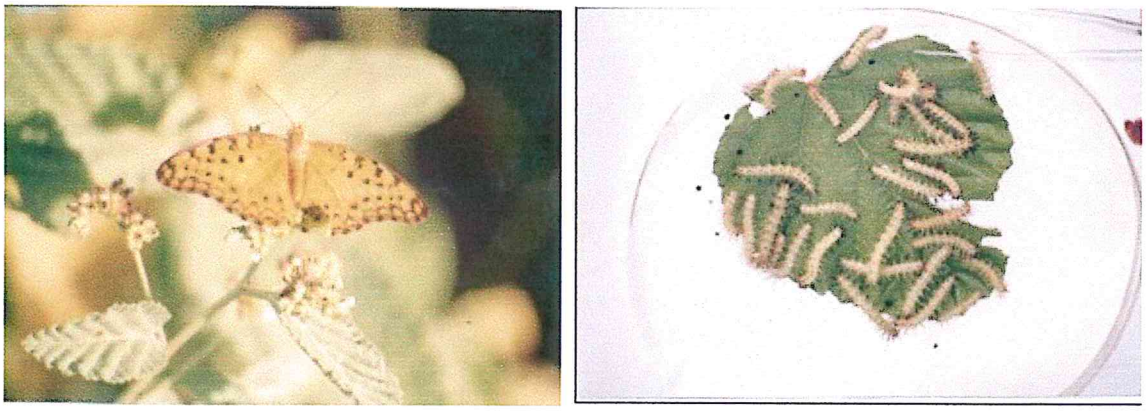


Plate 1.4 The butterfly *Acraea eponina* on flowers of the host plant *Waltheria indica* L. (left), and its gregarious larvae (right)

1.3 JUSTIFICATION

- Kenya is a signatory to the Cartagena protocol on biosafety that requires risk assessments as a precautionary measure in the transportation and use of living modified organisms. The studies need to be carried out in a scientifically sound manner, and taking into account recognized risk assessment techniques and other available scientific evidence, in order to identify and evaluate the possible adverse effects of living modified organisms on the conservation and sustainable use of biological diversity. This study would therefore serve as a basis in the registration of the *Bt* maize by the Kenyan government.
- Small-scale farmers who basically rely on natural enemies in stemborer management produce most of the maize in Kenya. More pest problems may arise in case the fitness of the biological control agents is lowered as the stemborers develop resistance against the *Bt* toxins. More weed problems will arise if the herbivorous butterfly larvae that help in suppressing populations of some plants are intoxicated by the *Bt* maize pollen spores. Also, in this period when conservation farming is emphasized, butterflies and other pollinators are required in the propagation of plants that cover soil and prevent it from drying and leaching of nutrients. Destruction of the parasitoid and butterfly larvae would therefore lead to decline in populations of such plants resulting in drying of soil and loss of nutrients, and hence poor crop yields.
- Impact of *Bt* crops on non-target organisms has been a contentious issue between scientists in the fields of biotechnology and ecology. Lack of scientific data on

such controversies would lead to delays in decision-making on the registration process of such crops in Kenya. These findings will serve as a basis in enlightening scientists on the impacts of *Bt* maize on non-target organisms. The data would facilitate in decision-making on registration of *Bt* maize.

- The pending adoption of *Bt* maize is need driven, as there are usually acute shortages in this cereal crop each year due to stemborer damage among other factors. The high demand for such crops may therefore tempt the seed companies and related industries to produce more *Bt* germplasm regardless of the potential detrimental effects. This study would therefore clarify such effects so that alternatives and remedial measures are formulated.
- Majority of Kenyans who are the actual consumers of maize are still ignorant of the actual issues that surround the adoption of *Bt* maize. The findings in study are therefore important in informing the public on the importance of different fauna in maize fields and the actual issues that need to be clarified regarding their survival under *Bt* maize regimes.

1.4 Hypothesis

Cry1Ab toxins produced by *Bt* maize have no effect on biological control and pollination processes, through tritrophic interactions with parasitoids of stemborers, or when butterfly larvae ingest dispersed *Bt* maize pollen spores.

1.5 General objective

The main objective of these studies was to elucidate the potential risks of adopting *Bt* maize on parasitoids of the stemborer *Chilo partellus* and larvae of the butterfly *Acraea eponina*.

1.6 Specific objectives

1) To evaluate the effects of Cry1Ab toxins on the fitness of the parasitoid wasp *Cotesia flavipes* in the control of *Chilo partellus* larvae.

2) To evaluate the effects of Cry1Ab toxins on the fitness of the parasitoid wasp *Xanthopimpla stemmator* in the control of *Chilo partellus* pupae.

(3) To evaluate the effects of Cry1Ab toxins in *Bt* maize pollen spores on the survivorship of larvae of the butterfly *Acraea eponina*.

CHAPTER TWO

2.0 EFFECTS OF CRY1AB TOXINS THAT ARE INGESTED BY *CHILO PARTELLUS* LARVAE ON THE FITNESS OF THE PARASITOID WASP *COTESIA FLAVIPES*

2.1 Introduction

The purpose of conducting this study was to investigate the effects of host-ingested Cry1Ab toxins on the parasitoid *Co. flavipes*, which develops in actively feeding *C. partellus* larvae. The physiological status of stemborer larvae that ingest *Bt* toxin is usually altered; there are also chances that the toxins would migrate into the haemocoel through pores that are usually formed in the gut walls due to intoxication. Immature parasitoids could be affected through alteration of the host's physiological status or by direct contact with toxins in the haemocoel. In such cases the suitability of host larvae for parasitoid development is lowered. Parasitoids that develop in the *Bt*-intoxicated hosts could be less fit and hence produce defective progeny. The first part of this experiment was conducted to investigate the suitability of *C. partellus* larvae that ingest *Bt*-toxins for the development of *Co. flavipes*, while the second part analyzed the quality of their progeny. Parameters that were considered included the host sizes, adult parasitoid sizes, longevity, reproductive capacities and the quality of the progeny that they produced.

2.2 Materials and Methods

Experimental insects

The wasps of the species *Co. flavipes* which parasitize larval moths were maintained at 25°C, R.H. 65-75 % and a 12L: 12D hr light-dark cycle on laboratory reared *C. partellus* larvae using the methods described by Ochieng *et al.* (1985) and Overholt *et al.* (1994). Artificial diet obtained from Animal Rearing and Containment Unit (ARCU) of ICIPE was used in rearing of *C. partellus* larvae (Odindo and Onyango, 1998). Parasitoid cocoons (F_0) were collected in glass vials and kept in a clean perspex cage until emergence. After emergence, adult parasitoids were provided a 20 % honey / water solution as diet. The colony of *Co. flavipes* had reached the 162nd generation when experiments were conducted.

Preparation of artificial diet with Cry1Ab toxin

Cry1Ab toxin was obtained and purified from a culture of a recombinant *Escherichia coli* strain XLI Blue (Ruud de Maagd of Wageningen University and Research centre, Netherlands), and evaluated for toxicity on different stemborer species (Dr. Ellie Osir and Dr. Matilda Oketch, ICIPE). Biuret technique was used in determining the concentration of the toxin that was dissolved in Phosphate Buffered Saline (PBS) (8.5g NaCl, 6.0g K_2HPO_4 and 3.0g KH_2PO_4 per litre, pH 7.0) (Navon, 2000), which was found to be 7.86 µg/µl. The toxin was rectified to 1µg/µl by diluting it with 6.86µl of PBS. Efficacy of the toxin on *C. partellus* neonates was tested and the two concentrations 0.005 µg/ml and 0.01µg / ml were selected as the suitable sub-lethal doses. Larvae in these concentrations were ready for parasitization after 25-days.

Serial dilutions were involved in preparing toxin concentrations for the assays (Seal and Leibee, 2003). The rectified toxin above was diluted with 99µl of PBS to obtain a toxin concentration of 0.01µg/µl. The other concentration of 0.005µg/µl was obtained by diluting volumes of the previous toxin solution with equivalent volumes of PBS. Artificial stemborer diet was prepared at the ICIPE'S ARCU by the method described in Odindo and Onyango (1998). The diet was maintained in fluid state at 50°C by placing the containers in water baths. Fifty microlitres of each toxin concentration were transferred using a micropipette into a beaker containing 50ml of artificial diet and stirred up. This resulted in two diets with *Bt* toxin at concentrations of 0.005µg/ml and 0.01µg/ml respectively. Controls to this experiment included a diet with PBS to check for the effect of the buffer, and a diet with no toxin or buffer. Approximately 10ml of each diet was poured into 7.5 × 2.5 cm glass vials and allowed to solidify at room temperature.

Rearing of *Chilo partellus* larvae in *Bt* diet

Neonates of *C. partellus* were placed singly into four sets glass vials measuring 7.5 × 2.5 cm, that contained the four diets prepared as indicated above. A single hair of a fine camel hairbrush was carefully brought into contact with the head of the neonate that adhered to it by a silk thread; the suspended neonates were then lowered onto the surface of the diet in the vials, and detached from the brush by gently dragging them over the diet. The vials were incubated at 26 ± 1°C, 50-70% relative humidity, and 12L:12D hr photoperiod. The larvae were reared for 25 days prior to parasitization as all the four groups were ready for the experiment at this age.

Host suitability

Cotesia flavipes cocoons were collected and kept in Perspex cages awaiting adult emergence. Upon emergence, adult F_0 parasitoids were allowed to mate for 24 hours under bright light that stimulates mating (Smith *et al.*, 1993), in sleeve cages measuring 20 × 20 × 20cm. A mating ratio of 1 male to 2 females was conserved in each cage so as to control the mating chances and hence the number of expected female progeny. The wasps were provided with diet consisting of 20% honey/water solution soaked in cotton.

Host larvae were removed from their respective diets and weighed using a Mettler AM100 analytical balance (Mettler-Toledo International Inc., USA). They were smeared with fresh maize frass to enhance acceptance by the parasitoids. Each larva was offered to a 1-day old mated female parasitoid for oviposition by the hand stinging method, in which a mature female parasitoid readily attacks a host that is brought close to it (Smith *et al.*, 1993). After oviposition, stung larvae were returned to their respective diets and reared as indicated above; the parasitoids used were kept in separate labelled eppendorf tubes, killed by freezing and stored at 4°C.

Parasitized host larvae were monitored daily for emergence of F_1 parasitoids or host death. Host larvae that yielded parasitoid cocoons were considered successfully parasitized. After parasitoid emergence, each cocoon mass was transferred into a clean vial and incubated in the same environmental conditions as above awaiting the emergence of adult parasitoids. The number of adult progeny from each cocoon mass and the female ratios (number of females per brood) were recorded. Other parameters recorded included the development time of the parasitoids i.e. egg-to-cocoon formation (i.e. oviposition to pupation), cocoon-to-adult and egg-to-adult. The number of immature

parasitoids that were found dead on the cocoons and in the diet was recorded. Twenty days after parasitization and when cocoon formation had ceased, all larvae were dissected in 0.85% saline solution and the number of parasitoid larvae that were found inside their host was counted. Cocoons containing dead parasitoids were counted four days after adult parasitoid emergence had stopped. Immature mortalities per host were expressed as ratios of the sum of adult progeny and the total number of dead immature wasps.

The post-oviposition egg loads for parasitoids that had stung, which were preserved in eppendorf tubes were estimated by dissection within three days. Each parasitoid was placed on a glass slide and dissected to expose the gravid ovaries, in a drop of 0.85% saline using insect pins and very fine forceps, at 40 × magnifications. The eggs that remained were released by gently macerating the ovaries, and counted with the aid of a tally counter. The hind tibial lengths of dissected wasps were also recorded as estimates of adult female wasp body sizes. The post-oviposition egg load was considered as an inverse function of the number of eggs that were oviposited.

Fitness of adult F_1 progeny

The longevity of adult wasps that had emerged from the four sets of hosts was studied by selecting fifty unmated male and female parasitoids from each diet. The wasps were placed in sleeve cages that measured 20 × 20 × 20cm. They were reared at a temperature of 26°C, a relative humidity of 70% and a 12L:12D photoperiod. A diet consisting of 20% honey / water solution, soaked in cotton wool was provided after every 12 hours. The number of dead parasitoids was recorded at 12-hr intervals. The dead parasitoids were stored at 4^0C, and their hind tibial lengths measured using an ocular

micrometer at 40× magnifications to check whether there was any correlation with their longevities.

The F_1 wasps that had emerged from the *C. partellus* larvae in the four diets were allowed to produce F_2 progeny in four groups of third instar larvae that had not been exposed to the *Bt* toxin; their post-oviposition egg loads were recorded when they had stung. Another group of naïve (unmated, not oviposited) F_1 parasitoids were selected for estimation of pre-oviposition egg loads. Data on parasitism, brood size, female ratio, developmental time, pre-oviposition egg loads, longevity and size for the emerging F_2 wasps was recorded as previously indicated.

Data analysis

Data were analyzed using the Statistical Analysis System (SAS) (SAS Institute 2000). Analysis of variance (ANOVA) that is sensitive in detecting differences between classified numerical data was used to compare variables like host weights, developmental time, progeny and immature mortalities among diet treatments. Means were separated using Student-Newman-Keuls test (SNK) when ANOVA was significant. Correlations between variables like adult size and egg loads were done using PROC CORR, while PROC FREQ analyzed data on frequencies e.g. successful parasitism. Prior to analysis, data were checked for normality and transformed where necessary. Data on counts e.g. egg counts and number of progeny and measurements e.g. development time and tibial lengths were log transformed, while arcsine transformation was carried out on ratios e.g. female ratios where necessary. Data were analyzed separately whenever there were sex specific differences e.g. males are significantly larger than females in adult *Co. flavipes*.

2.3 Results

Effects of *Bt* toxins on the suitability of *Chilo partellus* larvae for *Cotesia flavipes* development

The weights of *C. partellus* larvae that fed on the diet with on 0.01 µg/ml *Bt* diet were significantly lower than those that fed on the other diets *(P<0.05)* (Table 2.1a). This was attributed to the mode of action of the *Bt* toxin, which interferes with digestion and causes reduction in feeding rates (Prütz & Dettner, 2004). The parasitoids that developed in these hosts may have encountered nutrient shortages, which was detrimental to their growth and development.

The post oviposition eggloads in the F_0 parasitoids that oviposited in larvae that fed on the 0.01 µg/ml *Bt*-diet were slightly larger than those fed on the other three diets *(P<0.05)* (Table 2.2); which indicates that fewer eggs were laid. This was a reproductive response to smaller hosts, since parasitoids usually allocate fewer eggs to poor quality hosts (Bernal *et al.*, 2001). Since *Co. flavipes* is proovigenic, females that oviposited few eggs in the smaller host that grew in *Bt* toxin diet remained with more eggs in their ovaries. This was evident in the progeny whereby there were signs of decreasing numbers in the diet with the higher *Bt* toxin concentration (Table 2.1a).

Similar levels of successful parasitism were observed among the four groups of hosts *(P>0.05)*. The hosts might have developed tolerance to the sub-lethal concentrations of *Bt* toxins after the long period exposure (Rahman *et al.*, 2004). Therefore they were capable of exerting equivalent resistance that made the *Bt* toxin fed-hosts to attain similar parasitism levels as the controls.

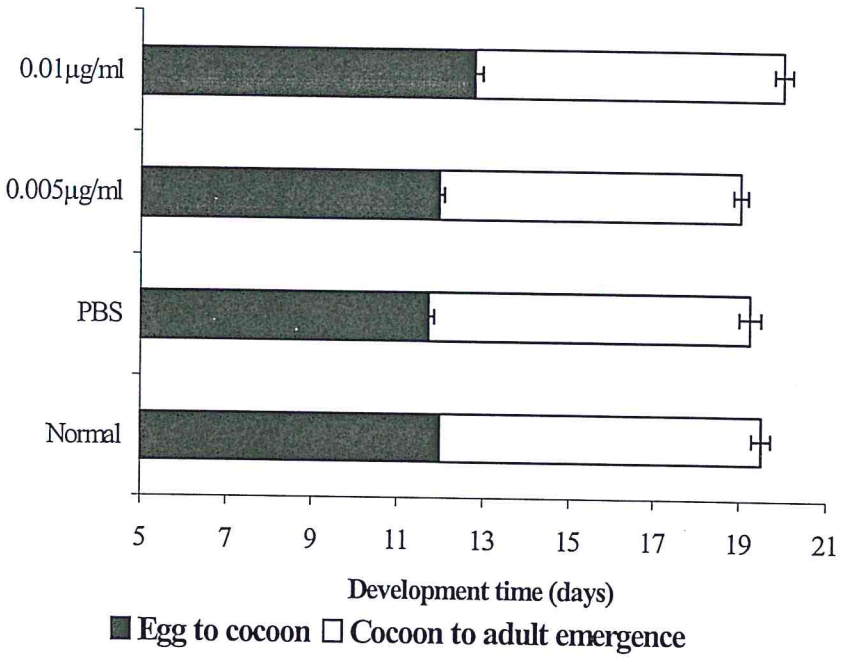


Figure 2.1 Development times for *Cotesia flavipes* that grew in hosts that were reared in normal diet, diet with PBS, a diet with 0.005 and 0.01µg/ml of *Bt* toxin. A significant increase in time was exhibited between egg to cocoon development in the 0.01µg/ml diet *(P<0.0001, F=12.04, Df=3, 113)*; there was no difference cocoon to adult time *(P>0.05)*.

The development times of parasitoids reared on host larvae that were fed on the 0.01 µg/ml *Bt*-diet were significantly longer from egg to cocoon formation, and the total development time *(P<0.05)*; however, there was no significant difference in duration between cocoon formation and adult emergence among the four groups *(P>0.05)* (Figure 2.1). The development time from egg to cocoon formation was negatively correlated with the host weights ($P < 0.0001$, $Rh_o = -0.48064$, $n = 99$). The slow growth of the parasitoids may have been an effect of small weights of the *Bt* toxin-fed hosts. Scramble competition among immature parasitoids is serious in smaller a host, that affects their development (Ngi-Song *et al.*, 1995; Elzinga *et al.*, 2003). Increased development times for parasitoids that grow in *Bt* intoxicated hosts has also been observed in *Co. melanoscela* (Ratzeburg) (Braconidae) (Chenot & Raffa, 1998), *Co. marginiventris* (Cresson) (Baur & Boethel, 2002), and in *P. pyralophagus* (Marsh) (Braconidae) (Bernal *et al.*, 2002). However, Prütz & Dettner (2004) did not detect any effects in development time of *Co. flavipes* in *Bt*-intoxicated *C. partellus*.

The parasitoid yielded similar numbers of progeny in the four groups of hosts *(P>0.05)* (Table 2.1a). This was attributed to the attainment of a critical minimum viable size for the completion of parasitoid development (Elzinga *et al.*, 2003), and for the sustenance equivalent progeny as controls.

Cotesia flavipes developing on *Bt* and non-*Bt* fed hosts produced progeny with similar sex ratios *(P>0.05)* (Table 2.1a). This absence of difference indicates that reduction in host size due to ingestion of *Bt* toxin, does not influence sex ratios in *Co. flavipes*. Host size has been reported to have no influence on the sex ratios of *Co. flavipes*

(Ngi-Song *et al.*, 1995). Other observations on the absence of effect of *Bt* toxin on parasitoid sex ratios were reported by Bernal *et al.* (2002) and Prütz & Dettner (2004).

There was no effect of *Bt* toxin on mortality of immature wasps in the three different sites (in diet, cocoons, and inside the hosts) *(P>0.05)* (Table 2.1a). This absence of mortality was attributed to the large sizes of the hosts in the four diets, which could sustain the equivalent parasitoid progeny. In 22-days old hosts however, more dead parasitoids were found in the diet with *Bt* toxin than in the other treatments: normal diet 1.82 ± 0.06b, PBS diet 3.80 ± 1.12ab, and 0.005µg/ml *Bt* diet 5.12 ± 0.11a *(P=0.0046, F=5.98, Df=2, 52)*; mortality was not detected inside the hosts and inside cocoons between the treatments *(P>0.05)*. Since mortality in the diet did not occur in the *Bt* treatments among the 25 days old hosts, it implies that *Bt* toxins cause immature mortalities of *Co. flavipes* through reduction of host sizes (Ngi-Song *et al.*, 1995). The eggressing immature wasps cannot develop into cocoons, as they are weakened by the competition for the limited host resources (Elzinga *et al.*, 2003). Parasitoids that attain the critical size for pupation (Davidowitz *et al.*, 2003) are therefore capable of spinning cocoons and successfully developing. This result is consistent with Prütz & Dettner (2004) who verified no effect of on mortality of *Co. flavipes* inside cocoons.

Fitness of adult *Co. flavipes* F_1 progeny

The adult sizes of female and male wasps in the toxin diets were significantly reduced *(P<0.05)* (Figure 2.2 a & b). This reduction in size was attributed to competition for limited nutrients and space in their small hosts, which limited their growth and caused reduction their adult sizes (Allen & Hunt, 2001). Reduction in size of *Co. flavipes*

cocoons from *Bt* toxin-fed hosts has been reported by Prütz & Dettner (2004). However, decrease in adult size due to host-ingested Cry-proteins did not exist in the idiobiont parasitoid *P. pyralophagus* (Bernal *et al.*, 2002), suggesting that host internal space is the limiting factor on the sizes of progeny in koinobiont wasps like *Co. flavipes*.

The toxin caused a significant reduction in pre-oviposition eggloads of wasps that emerged from 0.01µg/ml *Bt*-fed hosts *($P<0.05$)* (Table 2.1b); these eggloads were positively correlated with female size *($P=0.0439$, $Rh_o=0.14267$, $n = 200$)*. The reduction in pre-oviposition eggloads for the *Bt*-fed wasps was an effect of poor nutrient supply for oogenesis. The fact that the parasitoids were small in size indicates that they were malnourished, as host-derived nutrients are exclusively necessary for egg formation in proovigenic parasitoids like *Co. flavipes*. The small weights of the *Bt* toxin-fed larvae therefore resulted in production of small wasps, whose eggloads were relatively reduced. Reduction in pre-oviposition eggloads for wasps that develop in *Bt*-fed hosts was also observed by Baur & Boethel (2002) in the parasitoid *Co. marginiventris*. However, this effect did not occur in *P. pyralophagus* (Bernal *et al.*, 2002) as it is a koinobiont wasp.

The longevities of both female and male wasps were significantly reduced in the *Bt* toxin diets *($P<0.05$)* (Figure 2.3 a & b). For both sexes, longevity and tibial lengths were positively correlated *(*female: *$P<0.0001$, $Rh_o=0.32852$, $n=404$;* male: *$P<0.0001$, $Rh_o=0.28916$, $n=325$)*. This reduction in longevity was therefore a consequence of their small body sizes, which were indications of decrease in their fat bodies that act as energy reserves (Strohm, 2000). The food reserves of such small wasps get depleted faster, resulting in short life spans. Reduction in longevity due to host-ingested *Bt* toxin was also reported by Bernal *et al.* (2002) and Baur & Boethel (2002).

Quality of progeny produced by the F_1 *Co. flavipes* in normal host larvae

The third instar *C. partellus* larvae that were offered to the parasitoids from the 0.001µg/ml *Bt* diet were smaller than the other three groups *(P<0.05)* (Table 2.1b). This was attributed to bias towards selection of large larvae for parasitization, as the parasitoids from the 0.001µg/ml *Bt* diet were offered hosts after the other three groups. In such a case the experimenter selected the best larvae, which increased the proportion of smaller ones. Therefore, the number of lighter hosts was high by the time the wasps from the 0.001µg/ml *Bt* diet were stinging.

The post-oviposition eggloads of the four groups of parasitoids were not significantly different *(P>0.05)* (Table 2.1b). The hosts that were offered to the four parasitoid groups were slightly different in size, while the pre-oviposition eggloads of the four groups of wasps (F_1) were also equivalent. For this reason there were no host size related effects that could have influenced oviposition (Bernal *et al.*, 2001). This resulted in allocation of equivalent eggs, and hence similar post-oviposition loads.

All four groups of *Co. flavipes* exerted similar levels of successful parasitism on their hosts *(χ^2=3.0332, Df=3, P=0.3865)*. This result suggests that parasitoids developing in *Bt* toxin-fed host larvae can bear normal-developing progeny when offered suitable hosts. Parasitoids like *Cotesia* spp. rely on a polydnavirus (PDV) as a host immune suppressant during oviposition (Mochaiah *et al.*, 2002), therefore the results could also imply that the production of such factors may not have been affected when the F_1 wasps developed in *Bt* toxin-fed hosts.

The progeny of parasitoids that developed in the 0.01µg/ml *Bt* diet suffered high rates of immature mortality inside the hosts and in the diet *(P<0.05)*, and both mortalities

were negatively correlated with the host weights (Table 2.1b). The results indicate that the mortalities occurred because larvae that were parasitized by the F_1 wasps from the 0.01µg/ml *Bt*-fed hosts were smaller in size. These small hosts were relatively inadequate in nutrients and space. The immature wasps larvae that grew in the small hosts could have experienced developmental failures due to malnutrition. Mortality of immature *Co. flavipes* that is induced by the small size of *C. partellus* was also observed by Ngi-Song *et al.* (1995). However, the absence of difference in cocoon mortalities indicates that the survival of this stage is less dependent on host quality. Therefore, development of the F_1 wasps in *Bt* toxin-fed larvae did not influence immature mortalities of their progeny.

Female wasps that emerged from the *Bt* toxin diet produced fewer progeny *(P<0.05)*, which did not vary in female ratios *(P>0.05)* (Table 2.1b). The production of few F_2 progeny was attributed to the higher rate of immature mortality. Uniform female ratios were realized indicating that, development of *Co. flavipes* in *Bt*-fed hosts does not affect their mating capacities influences sex ratios in their progeny.

There was no significant difference in development time from egg to cocoon formation between the parasitoids *(P>0.05)*. The duration between cocoon formation and adult emergence significantly varied, although there were no toxin related trends (Normal $\geq$ 0.01 µg/ml $\geq$ PBS $\geq$ 0.005 µg/ml: *P=0.0086, F=4.01, Df=3, 183*). The hosts were in the same stage of development, which allowed the growth of the pre-cocoon stages to be synchronized with that of their hosts (Beckage & Riddiford, 1983). The variations in durations for adult emergence could not be linked to any factor.

A significant decrease in size was detected in the female wasps *(P<0.05)*, while the males were not different in size *(P>0.05)* (Figure 2.2). However, these small female

wasps carried normal pre-oviposition eggloads *(P>0.05)* (Table 2.1b). This observation indicates that the females were maximizing oocyte development at the expense of somatic growth (Stevens *et al.*, 2000). The fact that wasps that were reared on *Bt* toxin fed hosts had reduced eggloads, indicates that their eggs could also have been poor in nutrients. The embryonic stages that developed in such poor eggs may have gained little mass by the time of eclosion. During their growth in the hosts, the young female parasitoids (Harvey & Gols, 1998) had to preferentially allocate the host-derived limited resources between somatic and reproductive development (Stevens *et al.*, 2000). Therefore, investment of more food in reproduction may have caused reduction in female body size (Stevens *et al.*, 2000). However, the male wasps attained normal size since reproductive development is less important in this sex (Harvey and Gols, 1998).

There were irregular trends in longevities of the four groups of parasitoids in both sexes which did not give any indication of *Bt* toxin influence.

Table 2.1 Host weights (g), post-oviposition egg loads, immature mortality, progeny and sex ratios for F_1 *Cotesia flavipes* that developed in hosts that were reared under four diet treatments (a) and for the F_2 progeny that developed in non-intoxicated hosts (n, mean ± SE).

	Diet	Host weights		Post-oviposition eggs		immature mortality (%)		Progeny		Sex ratio
a)	Normal	30	0.11 ± 0.06a	30	113.7 ± 4.9ab	24	10.0 ± 0.03	24	25.2 ± 0.08	0.45 ± 0.07
	PBS	29	0.10 ± 0.05a	28	107.7 ± 11.2b	22	28.0 ± 0.07	22	21.2 ± 0.14	0.43 ± 0.09
	0.005µg/ml	30	0.09 ± 0.10b	29	107.1 ± 7.0ab	39	17.0 ± 0.05	39	23.2 ± 0.09	0.41 ± 0.07
	0.01µg/ml	35	0.04 ± 0.07c	36	136.8 ± 5.2a	26	18.0 ± 0.05	26	19.2 ± 0.09	0.45 ± 0.07
b)	Normal	50	0.07 ± 0.03a	49	108.6 ± 5.8	48	13.0 ± 0.02ab	48	41.4 ± 2.47ab	0.75 ± 0.04
	PBS	50	0.07 ± 0.03a	48	108.4 ± 5.0	49	10.0 ± 0.02b	49	42.7 ± 2.06a	0.81 ± 0.03
	0.005µg/ml	50	0.07 ± 0.06a	50	118.2 ± 5.4	47	16.0 ± 0.02ab	47	36.1 ± 2.01ab	0.77 ± 0.44
	0.01µg/ml	50	0.06 ± 0.03b	49	115.5 ± 6.2	43	20.0 ± 0.03	43	33.8 ± 1.94b	0.80 ± 0.03

Host weights (a) F=41.07, d.f. 3, 120, P<0.0001 (b) F=3.58, d.f. 3, 196, P=0.015; **Post-oviposition eggs** (a) F=2.09, d.f. 3, 113, P=0.1053 (b) F=0.73, d.f. 3, 192, P=0.5372; **Immature mortality** (a) F=1.48, d.f. 3, 107, P=0.2248 (b) F=3.52, d.f. 3, 183, P=0.0163; **Progeny** (a) F=1.15, d.f. 3, 107, P=0.3327 (b) F=2.99, d.f. 3, 183, P=0.0323; **Sex ratio** (a) F=0.32, d.f. 3, 107, P=0.8130 (b) F=1.03, d.f. 3, 183, P=0.3792. Within a column, means ± SE followed by the same letter are not significantly different (P>0.05; SNK test)

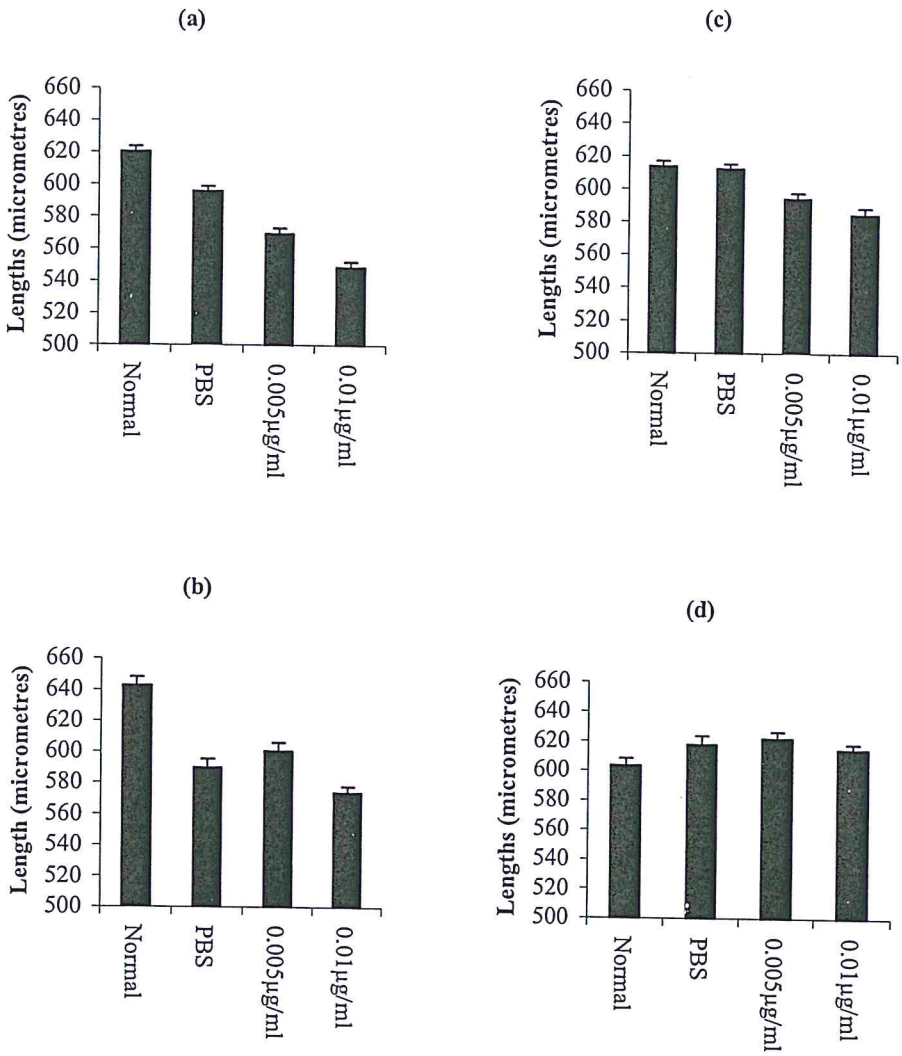
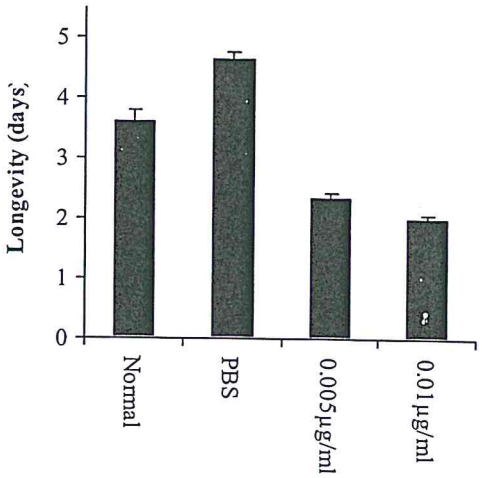


Figure 2.2 Left hind tibial lengths of F_1 and F_2 *Cotesia flavipes* that grew in hosts that were reared in normal diet, diet with PBS, a diet with 0.005 and 0.01µg/ml of *Bt* toxin. Both generations of adult female wasps, and the F_1 male generation emerging from *Bt*-fed hosts were small in size. ***Female F1*** (fig a) *($P < 0.0001$, $F = 87.79$, $Df = 3, 600$)*, ***F2*** (fig b) *($P < 0.0001$, $F = 16.6$, $Df = 3, 746$)*; ***Male F1*** (fig c) *($P < 0.0001$, $F = 36.73$, $Df = 3, 321$)*, ***F2*** (fig d) *($P = 0.0913$, $F = 2.17$, $Df = 3, 381$)*.

(a)



(b)

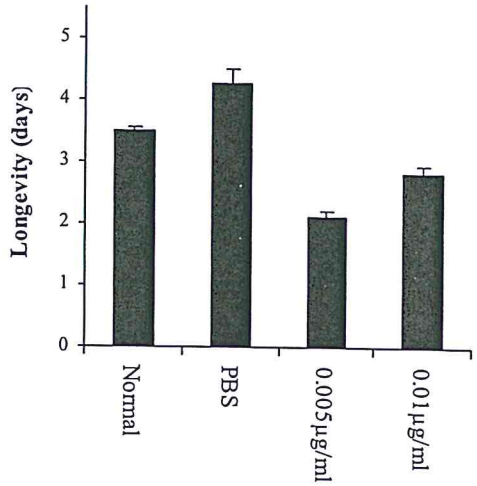


Figure 2.3 Longevity (days) of F_1 female (a) and male (b) *Cotesia flavipes* that grew in hosts that were reared in normal diet, diet with PBS, a diet with 0.005 and 0.01µg/ml of *Bt* toxin. Both sexes of wasps from the two toxin diets had shortened longevities: females *($P < 0.0001$, $F = 82.61$, $Df = 3, 400$)*; males *($P < 0.0001$, $F = 59.16$, $Df = 3, 321$)*.

2.4 Discussion

Bacillus thuringiensis toxins could be detrimental to biological control since they lowered the suitability of host *C. partellus* larvae and hence the fitness of *Co. flavipes* in the laboratory studies. Important qualities for biological control that were negatively affected included host sizes, parasitoid oviposition rates, developmental time, number of progeny, egg loads, longevity, and adult sizes.

The reduction in size of the *Bt*-fed *C. partellus* larvae would lead to shortages in host resource for parasitoids like *C. flavipes*. This would lead to reduction in adult sizes and poor development of parasitoid progeny (Hirose, 1994), while fewer numbers of these natural enemies would be produced per host (Salt, 1941). The richness of parasitoid assemblages in agroecosystems would therefore be poorer as a consequence of reduction in host sizes (Hirose, 1994).

Egg load and oviposition rates in parasitoids determine the number of progeny produced by parasitoids. Production of large quantities of eggs per oviposition in each host enables parasitoids to attain a large number of progeny, which ensures higher chances of survival (Potting *et al.*, 1997). Ecological studies have verified that in a random parasitoid population, the number of hosts encountered is a linear function of host density, parasitoid density, and the area searched by the parasitoid (Nicholson and Bailey, 1935). The small number of parasitoid progeny that was exhibited in the *Bt*-fed hosts would lower the wasp populations leading to decreased chances of encounter with hosts (Knipling, 1992), and hence inadequate pest control.

Developmental time is an important aspect of parasitoid fitness for biological control. This is because the relative efficiency of natural enemies as pest control agents is

negatively correlated with their generation time relative to that of their prey (Kindlmann and Dixon, 1999). Changes in numbers of individuals tend to be large if generation time of the parasitoid species is short, as short living natural enemies complete more generations per unit time and can deplete the pests faster than longer living parasitoid species (Kindlmann and Dixon, 1999). The impaired growth rates among *Co. flavipes* developing in *Bt*-fed hosts can prolong generation time, which dramatically reduces a population's intrinsic rate of increase (Cole, 1954). Prolonged development time also increases the risk of attack by enemies during vulnerable immature stages (Feeny, 1976). For effective pest suppression, synchrony between the host and parasitoid development must occur, and the suitable stage of host should overlap with the parasitizing stage of the parasitoid (Weseloh, 1976; Kindlmann and Dixon, 1999).

Longevity of a parasitoid has a direct relationship with its efficiency for the suppression of pests. Short life spans that were found in *Co. flavipes* that developed in *Bt*-fed hosts would result in low numbers of expected host encounters (Potting *et al.*, 1997).

Body size in parasitoids is a direct function to their reproductive capacity (Van Den Assem, 1985; Bernal *et al.*, 2001). Smaller size results in lower fecundity that lowers the rate of population increase (Price *et al.*, 1980). Specifically, size is positively related with semen quantity in males (Van Den Assem, 1985) and egg production for lifetime reproductive success in females (Jervis and Copland, 1996).

Toxins that are expressed in *Bt* maize therefore lower the suitability of stemborer larvae and hence the fitness of larval parasitoid wasps like *Co. flavipes*.

CHAPTER THREE

3.0 EFFECTS OF CRY1AB INTOXICATED PUPAE OF THE MOTH *CHILO PARTELLUS* ON THE FITNESS OF THE PARASITOID WASP *XANTHOPIMPLA STEMMATOR*

3.1 Introduction

In the previous studies, the effects of host-ingested *Bt* toxins on a gregarious larval endoparasitoid *Co. flavipes* were observed. Such wasps could be more vulnerable to the toxins, since they develop in the haemolymph of actively feeding and growing host larvae. However, different effects could be observed in solitary parasitoids that develop in the quiescent pupal stage of their hosts. This study investigated the effects of *Bt* toxins that are ingested by *C. partellus* larvae on the suitability of their pupae for the development of parasitoid wasp *Xanthopimpla stemmator*.

3.2 Materials and Methods

Chilo partellus neonates were reared in the four diets (Normal diet, PBS diet, and the two toxin diets) as indicated in section 2.2. Four days after pupation, they were sexed by examining their external genitalia on the ninth abdominal segment at 20× magnifications as described by Ampofo (1988). Their weights were recorded using a Mettler AM100 analytical balance (Mettler-Toledo International Inc., USA).

Five pupae from the same diet were then inserted into a paper straw measuring 6 cm with a diameter of 0.5 cm. This was based on preliminary work, which indicated that high parasitism occurred with decrease in number of pupae, or increase in time, and 5 pupae were selected since they could be adequately parasitized within 6 hours. The straws were smeared with fresh frass to boost acceptance by the parasitoid. The identity number of each pupa was indicated on the straws. Active mated female parasitoids were placed singly in glass vials. They were provided with a diet that consisted of 20 % honey / water solution. Each parasitoid was then offered one paper straw that contained five pupae for parasitization within a period of six hours. Only female pupae were used for acceptance tests since they were adequate in number. The paper straws were placed vertically in the vials on a clay substrate during exposures.

The pupae were removed from the straws after they had been parasitized and placed individually in glass vials for 12 hours for the wounds that were inflicted by the ovipositor to undergo necrosis and darken, hence increasing their visibility. Pupae were observed at 25× magnifications, the presence of ovipositor probe wounds was recorded as acceptance. The wounds were also counted as estimates of acceptance level. Each member of the set of parasitized pupae was kept separately in a vial that was labelled. Data on their sex and weight was also recorded. The vials were lined with paper towel and maintained at 26 ± 1 °C, 50-70 % RH and 12:12 (LD) hr photoperiod in an incubator.

Parasitoid emergence was checked everyday up to the twenty-five days after oviposition. The developmental period in days for each parasitoid was recorded. The number of pupae in which oviposition was successful that yielded parasitoids was recorded as successful parasitism. Pupae that emerged into moths and those that did not

yield parasitoids or moths were recorded as unsuccessfully parasitized. Twenty five days after oviposition, pupae that were accepted but did not produce moths or parasitoids were dissected to check for mortality of immature wasps, unemerged moths, or dead pupae.

At emergence the sexes of the wasps were recorded. Unmated male and female parasitoids were transferred to clean vials in which they were reared singly under the same environmental conditions. They were fed with the honey solution daily until they died. The number of days the wasps lived from emergence to death occurred was recorded as longevity. The size of each dead parasitoid was estimated by measuring the left tibia and wing spans as an index of body size. Data were analysed in a similar way as indicated in section 2.2 for variations in host weights, development time, successful parasitism, adult sizes and longevity.

3.3 Results

The toxin caused significant reduction in weights of both sexes of host pupa *(P>0.05)* (Figure 3.1). This observation is consistent with the fact that insects growing in poor diets usually attain lower critical weights for pupation (Davidowitz *et al.*, 2003). Therefore, the nutritional value of the small pupae, which is necessary for parasitoid development was relatively lower.

There were no significant variations in development times of both female and male parasitoids *(P>0.05)* (Table 3.1). This was an indication that the immature wasps were not affected by ingestion of toxins that could have been bound onto host tissues (Karim & Dean, 2000). Also, unlike larval parasitoids whose development is usually dependent on the host's growth rate (Beckage & Riddiford, 1983); pupal parasitoids like

X. stemmator do not require synchronization. This is because their host pupae are quiescent with minimal growth, and are usually completely devoured. Therefore, despite the slow growth rate of *C. partellus* after ingesting the *Bt* toxins, the host pupae had no influence on the developmental rates of *X. stemmator*.

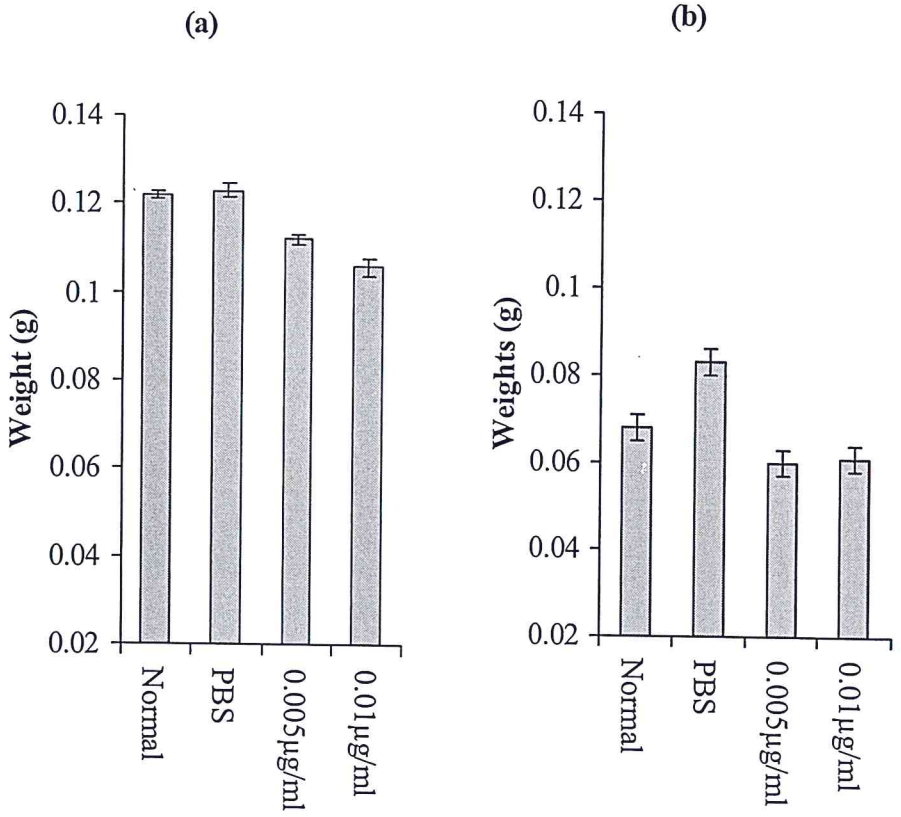


Figure 3.1 Weight reduction in female(a) and male(b) *Chilo partellus* pupae that were reared in diets with 0.005 and 0.01µg/ml of *Bt* toxin as compared with controls (a) $P<0.0001$, $F=8.06$, d.f.=3, 270 (b) $P=0.0004$, $F=6.24$, d.f=3, 295.

There was no significant effect of the *Bt* toxin on host acceptance among the four groups of pupae *(P>0.05)* (Table 3.2). This parasitoid species relies on vibrational sounding strategy for detecting cryptic pupae (Fischer *et al.*, 2003). This method could be of low fidelity in the discrimination of finer cues that arise from concealed pupae. Therefore, poor perception could have been the reason behind these unbiased levels of host acceptance among the four groups of host pupae.

The female ratios of parasitoids did not significantly vary among the four groups of hosts *(P>0.05)* (Table 3.2). This is not consistent with the expectation that parasitoids would preferentially allocate different sexes of progeny basing on host size (Bernal *et al.*, 2001). The vibrational sounding strategy could also have been ineffective in detecting the slight variations in sizes of pupae in paper straws, and could not lead to preferential allocation of eggs by sexes. For this reason similar female ratios were produced.

Table 3.1 Development times in days (mean ± se) for *Xanthopimpla stemmator* that developed in pupae that were reared in different diets

Diets	Female	n	Male	n
Normal	18.13 ± 0.26a	16	16.67 ± 0.14a	18
PBS	18.04 ± 0.16a	24	17.12 ± 0.12a	17
0.005 µg/ml	17.76 ± 0.19a	34	17.04 ± 0.22a	28
0.01 µg/ml	17.64 ± 0.28a	11	17.62 ± 0.24a	13

Female: F=1.01, d.f. 3, 81, P=0.3939; Male: F=2.5, d.f. 3, 72, P=0.0664
Means followed by the same letter(s) in the same column are not significantly different (SNK test, P>0.05).

The ratios of dissected pupae that contained dead parasitoids were significantly low in the two toxin diets, while mortality of parasitized pupae was significantly high in those groups *(P>0.05)* (Figure 3.2). This was attributed to the high mortality of the parasitized pupae from the *Bt* toxin-diet. Mortality of pests that ingest the toxin is higher when they get parasitized (Chilcutt & Tabashnik, 1997), which prevents development of the parasitoids (Blumberg *et al.*, 1997).

The four groups of pupae did not exhibit any significant difference in percentages of successful parasitism *(P>0.05)* (Table 3.2). The eggs of *X. stemmator* do not hatch in dead pupae; this was confirmed in an experiment in which freeze-killed pupae were stung by the wasps but did not yield adult parasitoids (Personal Observation). However inside vivid hosts, the eggs hatch into actively feeding larvae within 1 day (Smith *et al.*, 1993). In the absence of toxins, the major limiting factor for survivorship of the voracious larvae is food quantity. The results therefore imply that the living pupae from the *Bt* diets favoured the hatching of wasp eggs, and they were considerably of adequate size for successful development of the parasitoid. However, the *Bt* toxin lowered the capacity of host pupae to survive after stinging.

The longevities of adult wasps did not significantly vary among the four groups *(P>0.05)* (Table 4.3). Also tibia lengths were not significantly different among adult female and male parasitoids from the four diets *(P>0.05)* (Table 3.3). Both adult size and longevity in wasps are directly dependent on host size (Elzinga *et al.*, 2003). Despite the smaller weights of the *Bt* toxin-fed hosts, the variations in size could have been very slight to cause reduction in parasitoid longevity and body size.

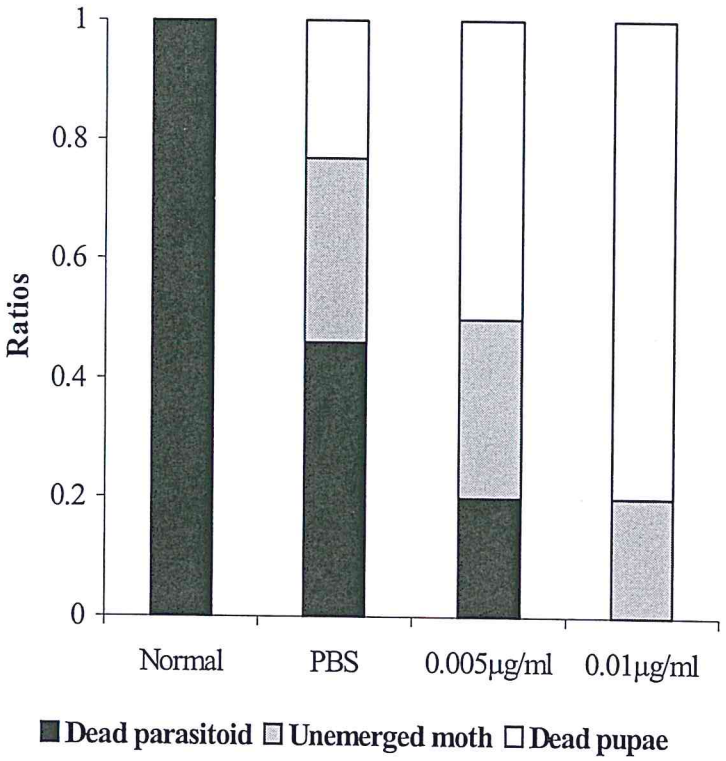


Figure 3.2 Dead parasitoids, unemerged moths, and dead pupae that were recorded after the dissection of pupae that did not produce adult wasps and moths. A significantly low number of pupae from the controls contained dead parasitoids; while the number of dead pupae was significantly high in the 0.005 and 0.01μg/ml *Bt* diet *(Df=3, $\chi 2$=15.8960, P=0.0143).*

Table 3.2 Host acceptance, Successful parasitism, and female ratios for *Xanthopimpla stemmator* that developed in pupae that were reared in different diets

Diets	Acceptance		Parasitism		Female ratio	
	%	n	%	n	%	n
Normal	82.21	19	43.75	16	42.50	40
PBS	69.70	33	28.00	25	58.54	41
0.005 µg/ml	61.22	49	50.00	30	54.84	62
0.01 µg/ml	67.35	49	26.47	34	45.83	24

Acceptance: χ^2=3.3815, d.f.=3, P=0.3365; Parasitism: χ^2=4.9906, d.f.=3, P=0.1725; Female ratio: χ^2=2.6951, d.f.=3, P=0.4411

Table 3.3 Longevities and adult sizes (tibia lengths) for *Xanthopimpla stemmator* that developed in pupae that were reared in different diets

Diets	Longevity in days (mean ± se)		Hind tibia lengths micrometers (mean ± se)			
			Female	n	Male	n
Normal	33	37.91 ± 2.25a	2254.62 ± 66.03a	13	2135.46 ± 88.15a	11
PBS	33	41.49 ± 2.39a	2375.22 ± 34.19a	23	2148.75 ± 35.38a	8
0.005 µg/ml	46	40.85 ± 2.53a	2292.69 ± 32.45a	26	2126.47 ± 51.83a	17
0.01 µg/ml	15	43.20 ± 5.32a	2355.00 ± 54.97a	8	2283.00 ± 78.92a	10

Longevity: F=0.28, d.f. 3, 123, P=0.8381; Female tibial lengths: F=1.58, d.f. 3, 66, P=0.2015; Male tibial lengths: F=1.02, d.f. 3, 42, P=0.3943. Longevities for the two sexes were pooled as they did not vary significantly. Female tibial lengths were significantly longer than for males, therefore data were analyzed separately. Means followed by the same letter(s) in the same column are not significantly different (SNK test, P > 0.05)

3.4 Discussion

Bacillus thuringiensis Cry1Ab toxins did not cause serious negative effects on the suitability of host *C. partellus* pupae for the wasp *X. stemmator,* and hence the fitness of the parasitoid. An important quality for biological control that was slightly affected was host pupal size and its post-parasitism survivorship. However, no adverse effects on fitness were realized on the emerging adult wasps.

The stemborer *C. partellus* could grow and pupate at the very low concentrations of the Cry1Ab toxin. A slightly lower pupation weight was attained that signified a decrease in nutrient resource for a parasitoid development (Hirose, 1994). The larvae of *X. stemmator* that developed in those pupae may have encountered nutrient shortages. However, the nutrient deficit was very small to cause any adverse effects on the fitness of the parasitoid wasp. Therefore, *C. partellus* larvae that feed on *Bt* toxins and pupate can serve as suitable hosts for the parasitoid wasp *X. stemmator.*

Specificity of parasitoids like *X. stemmator* to the pupal stage of their hosts is very precise, suggesting that the developmental status of the host is critical in determining host suitability (Vinson and Inwantsch, 1980). Inability to pupate at higher toxin levels, longer development times for pupation and mortality of parasitized hosts among *C. partellus* larvae that ingest *Bt* toxins would lead to scarcity of host pupae. This can cause a decrease in the rate of growth of *X. stemmator* populations as there would be scarcity of resources for oviposition.

The results indicate that the *Bt* toxin does not affect the parasitoid *X. stemmator* by reducing the host size, or through direct contact with host-ingested toxin. However, the use of *Bt* toxin could lead to mortality of parasitized hosts, which would reduce the

wasp population. However, this could also be considered an advantage in *C. partellus* management, as the use of the toxins in *Bt* maize would act synergistically with the wasp to increase mortality of the stemborer (Soares et al., 1993; Chenot and Raffa, 1998). In addition, *X. stemmator* is highly polyphagous and would establish populations on the stemborers like *Busseola fusca* (Fuller) (Noctuidae) that are not susceptible to the *Bt* toxins (Saxena and Stotzky, 2005).

Effects of host-ingested Cry-proteins are therefore not serious on pupal parasitoids like *X. stemmator* that develop in quiescent host pupae. Since there are no signs of direct intoxication through ingestion of tissue-bound *Bt* toxin, indirect mechanisms are the most likely way that the parasitoids are affected. This is by interfering with the host's food intake and growth, which can only occur among larval endoparasitoids like *Co. flavipes*. However, experimentation with other pupal parasitoids such as *Dentichasmias busseolae* Heinrich (Ichneumonidae), and the gregarious wasps like *Pediobius furvus* (Gahan) (Hymenoptera: Eulophidae) is necessary to confirm this view. Therefore, Cry1Ab toxins have no direct effects on the pupal parasitoid *X. stemmator*, which may act synergistically with the toxin in *Bt* maize. This polyphagous wasp can therefore help control pupae of less susceptible stemborers in *Bt* maize.

CHAPTER FOUR

4.0 EFFECTS OF POLLEN DENSITIES AND *BT* TOXINS ON SURVIVORSHIP OF THE BUTTERFLY *ACRAEA EPONINA*

4.1 Introduction

The current study investigated bitrophic effects of Cry1Ab toxin in *Bt* maize pollen spores on herbivorous like butterfly larvae through accidental intoxication. This differs from the two previous studies whereby tritrophic interactions were investigated. Butterflies could be harmed by *Bt* maize when their larvae accidentally ingest pollen deposited onto their host plants. Risk assessments for *Bt* maize on butterfly larvae have been conducted by estimation of lethal densities of *Bt* pollen spores, or through toxicological assays with purified toxins. However, most methods that are used in the estimation of pollen densities on leaves are labour intensive. There has also been lack of substantial methods of relating the encountered pollen densities to concentrations of purified toxins. The current study aimed at developing an alternative risk assessment method for *Bt* pollen spores on non-target butterfly species like *A. eponina* that inhabit many farms at the Kenyan coast. The first part of this work estimated densities of maize pollen spores that are deposited on host plants for butterfly larvae. The pollen densities were then converted to Cry1Ab concentrations basing on toxin expression in the pollen of MON810 maize variety; the determined *Bt* concentrations were then tested for toxicity on butterfly larvae.

4.2 Materials and Methods

Estimation of pollen densities

Maize pollen spores are usually dispersed by wind to distances of up to 200 m, although very low densities have been found deposited on weeds growing 2 m from maize fields (Pleasants *et al.*, 2001). An experimental design that would take into account the direction of prevailing winds, and dispersal distances was adopted. This comprised of a standard experimental plot for maize (6m × 6m) (NARL, 1998), with an east-west orientation. Potted host plants for the butterfly species would be arranged in four compass directions at 1 m intervals from the middle to a distance of 4 m from the edges (Figure 4.1). The plot would carry 29 potted host plants per sampling session.

A leading plot was prepared at ICIPE's Muhaka field station and Pwani hybrid-4 maize (PHO4) variety (www.kenyaseed.com/pwanihybrid4.htm) was planted at a spacing of 75 cm × 30 cm (NARL, 1998). This leading plot was important in prediction of the anthesis for the experimental maize plots that would be planted. The actual experimental maize was planted three weeks later on four identical plots, which were separated at distances of 200 m so as to avoid pollen drifts between neighbouring plots (Pleasants *et al.*, 2001). Seedlings of *Waltheria indica* L. (Sterculiaceae), a host plant for *A. eponina* were collected and sown in plastic pots.

When the proportion of maize plants that had tasselled on the plots was around 70% (Stanley-Horn *et al.*, 2001), the potted host plants were arranged on each plot so as to trap the dispersed pollen grains (figure 4.1). Sampling for pollen was done after a 24 hr

period, and was repeated after two days within the anthesis period that occurs within one week (Kling, 1997). During this process, changes in wind direction were also monitored.

Two upper leaves were plucked from the potted plants and placed horizontally in labelled Petri dishes to avoid loss of pollen from their surfaces. The number of maize pollen grains on the leaves was then estimated at ×25 magnifications using an improvised thin-wire quadrat (5 mm × 5 mm), to match the field of view of a 0.25cm^2 ocular grid used in pollen density estimation (Stanley-Horn *et al.*, 2001) (Own instructions to ICIPE workshop). Pollen was counted around the base, apex, middle and two sides of each leaf, since densities of deposited pollen have been reported to have a biased distribution to some sections of the leaf blades (Pleasants *et al.*, 2001).

Effect of *Bt* toxin on survivorship of *Acraea eponina* larvae

The maximum pollen density that was obtained (492 grains / cm^2) (Section 4.4) was used in preparation of toxin concentrations. The concentration of 0.09 µg/g toxin in Mon810 pollen (CFIA, 2001) that occurs at 366 grains (Hellmich *et al.*, 2001) was used in the transformation of pollen densities into toxin concentrations. Basing on this data, a quantity of 492 grains would contain 0.12 µg of *Bt* toxin. This amount of toxin (0.12 µg) was to be carried on a 1cm^2 leaf surfaces to simulate the pollen count of 492 grains / cm^2; and a 2.54 cm^2 leaf disc cut out by a cork borer would therefore carry an equivalent of 0.31µg of toxin. Twenty microlitres of toxin were found to adequately spread over a 2.54 cm^2 leaf disc. A toxin stock solution with a predetermined concentration of 800 µg / ml was rectified to 15.5 µg / ml for use in the bioassay; this ensured that 0.31µg of toxin are

carried in a 20 µl drop. The same process was used in preparing another rectified toxin solution with half the concentration i.e. 0.15 µg of toxin per 20 µl.

Eggs of *A. eponina* were collected from the field and allowed to hatch at room temperature. Emerging larvae were fed on *W. indica* leaves until they attained an adequate size for experiments. Caterpillars in the second and fourth instars were preferred for bioassays; this was based on microscopic observations whereby their head capsules were larger than a pollen spore, and were therefore considered capable of accidentally ingesting the grains. They were placed separately in sterile Petri dishes and grouped into three sets of 40 to 50 insects. Two of the groups were offered leaf discs with the two toxin concentration, while the third group was a control.

Leaf discs of *W. indica* (2.54 cm^2) were cut out using a cork borer and sterilized in 0.5 % hypochlorite solution. Twenty microlitre volumes of the two rectified solutions estimated to contain 0.31µg and 0.15µg of toxin were spread over the surface of two groups of the discs using a micropipette to give assumed resultant toxin concentrations of 0.12 µg / cm^2 and 0.06 µg / cm^2 respectively. Fresh leaf discs were prepared daily and offered to the insects daily. Observations were made daily for survivorship of larvae. Longevity of larvae was recorded up to the day when all treated larvae had died. The percentage of larvae that were alive out of the initial population was recorded daily.

Data analysis

Both data on pollen densities and survivorship of larvae were analysed by the SAS programme (SAS Institute, 2000). Pollen densities were converted into grains per cm^2 by multiplying by a factor of 4 before analysis. PROC GLM (SAS) was used in

analysing variations in pollen densities and the effect of *Bt* toxin on larval survival. Mean separation was done by SNK-test when there were significant variations.

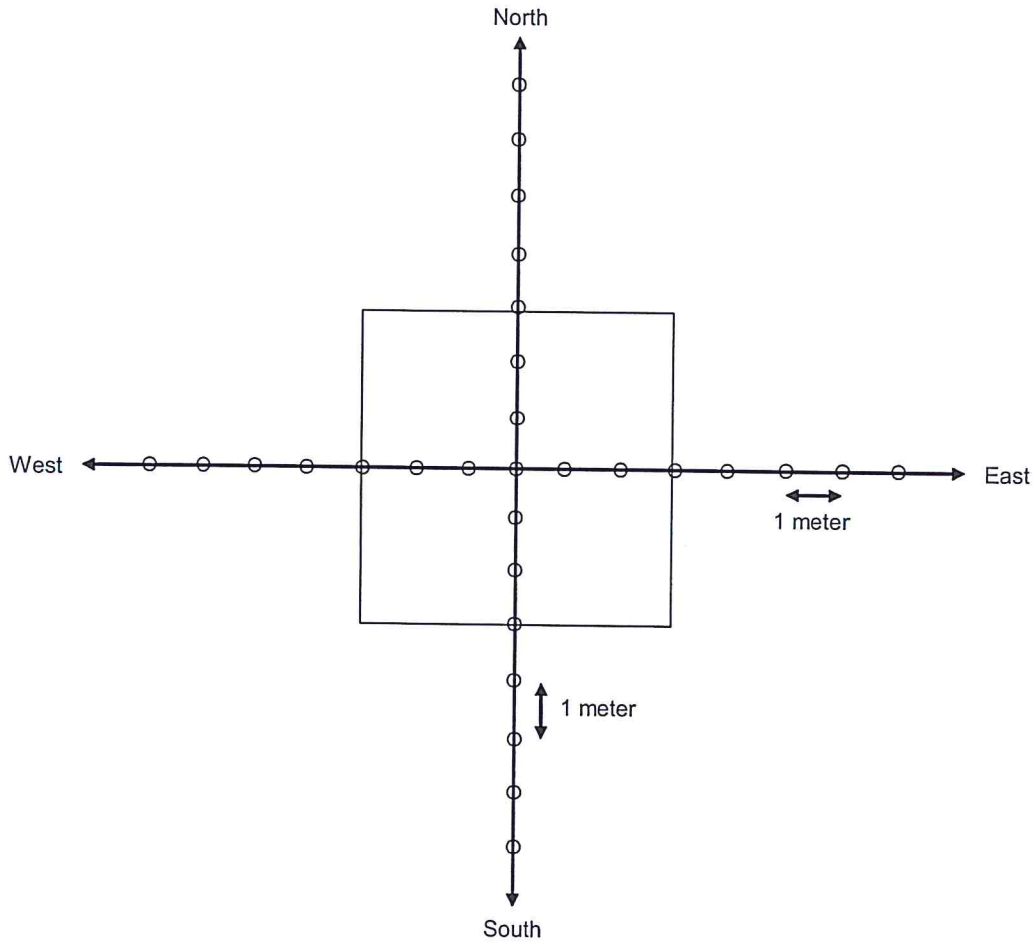


Figure 4.1 Arrangement of potted host plants on the 6 × 6 m plots. The plot is represented by the square section; while the pots are illustrated by small circles aligned in four compass directions at 1m intervals. One pot was in the middle while four of them at the edge.

4.3 Results

Both Cry1Ab toxin concentrations that corresponded to 246 and 492 pollen grains / cm^2 respectively caused significant reduction in longevity for both second and fourth instar larvae of *A. eponina* (Table 4.1). Larvae from both *Bt* toxin concentrations died within 10 days of treatment while most of the controls were still alive (Figure 4.3). These results indicate that pollen from Mon810 maize can cause mortality of this larval species at densities that are low as 246 grains per cm^2. However, Hellmich *et al.* (2001) recorded no effect of Mon810 pollen on *D. plexippus* larvae at densities above 1000 grains per cm^2. This difference is attributed to the conversion factor for pollen into toxin concentrations. The concentration of Cry1Ab toxin in pollen of Mon810 variety is 0.09µg/g (Hellmich *et al.*, 2001). However, 1g of maize pollen constitutes pollen counts that are far above 366 grains. A single maize pollen grain weighs about 247×10^{-9} g (Emberlin *et al.*, 1999); 1g of these gametes would therefore comprise of over 40, 000 grains and not 366 grains. Therefore, 0.09µg of *Bt* toxin occur in 4 million grains. Basing on this conservative concentration, larvae that encounter pollen densities of 246 and 492 grains/cm^2 would encounter 5.54×10^{-6} and 11.07×10^{-6} µg of toxin / cm^2 respectively. These concentrations may be very low to cause mortality in the larvae, considering that they are below the equivalent of 1000 grains/cm^2 that showed no adverse effects on monarch larvae (Hellmich et al., 2001).

Pollen densities on leaves of *W. indica* ranged between 0 to 492 grains per cm^2, with a mean of 32.14 ± 1.12 for the 3200 samples. The densities were significantly high on the second sampling day (34.94 ± 1.61) than in the first day (29.33 ± 1.56) *($P<0.0001$,*

F=17.23, Df = 1, 3198). This was a sign of increase in the quantity of pollen that is shed by maize plants with advancement in anthesis (Uribelarrea *et al.*, 2002).

Significantly high pollen densities were recorded on host plants in the northern direction *(P<0.05)* (Fig 4.4). During the sampling period, winds were blowing towards the north. Since maize pollen usually drifts in wind currents (Luna *et al.*, 2001), the plants on the windward side received more pollen grains.

Table 4.1 Longevities in days (mean ± se) for *Acraea eponina* larvae that fed on purified Cry1Ab toxin

	Second Instars		Fourth Instars	
Treatment	**n**	**mean ± se**	**n**	**mean ± se**
Controls	50	5.84 ± 0.11a	39	8.54 ± 0.51a
Bt toxin (0.06µg/cm^2)	48	2.67 ± 0.15b	40	4.53 ± 0.29b
Bt toxin (0.12µg/cm^2)	50	2.22 ± 0.13c	38	3.58 ± 0.27c

Second Instars: F=98.67, d.f. 2, *145*, P<0.0001; Fourth Instars: F=18.84, d.f. 2, 114, P<0.0001. Means followed by the same letter(s) in the same column are not significantly different (SNK test, P>0.05)

Plants that were in close proximity to the maize plots exhibited significantly high pollen densities than the distant ones *(P<0.05)* (Figure 4.5). This reduction in pollen densities on distant host plants is attributed to the relatively high density of the grains, which influences their range of deposition (Pleasants *et al.*, 2001). The distribution of pollen on leaf surfaces was significantly high on the middle and the base than on the other parts *(P<0.05)* (Figure 4.2). The leaves of *W. indica* tend to be inwardly curved with prominent veins in the middle and basal sections. This structure favours the deposition of pollen along these areas than on the apex and sides (Pleasants *et al.*, 2001).

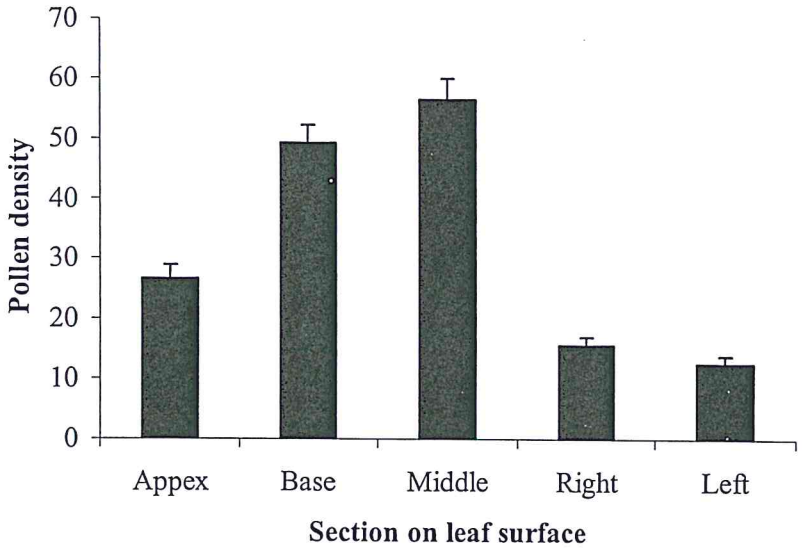


Figure 4.2 Mean comparison for pollen densities on different sections of leaves. High densities were recorded in the middle and at the bases *(Df=4, 3195; F=75.32; P<0.0001)*.

4.4 Discussion

The study has shown that larvae of the butterfly *A. eponina* are susceptible to *Bt* Cry1Ab toxins, and that pollen spores from *Bt* maize deposited onto the leaves of *W. indica*, a host plant for the butterfly *A. eponina* are harmful to their larvae. The same effects may arise on other butterfly species that feed on various weeds around maize farms in Kenya. This is due to the fact that different butterfly species like *D. plexippus*, *P. polyxenes*, and *Pieris* spp. (Lepidoptera: Pieridae) are highly susceptible to the *Bt* toxins (Zangerl *et al.*, 2001; Hellmich *et al.*, 2001; Felke *et al.*, 2002).

The loss of butterflies through *Bt* maize pollen spore intoxication would disrupt the ecological balance in agroecosystems in several ways. Adults of many butterflies are

pollinators of some plants (Kerr, 2001). Extinction of these insects would result in interference with pollination and hence propagation of some plants. This would result in inadequate plant cover in non-cropping seasons, which leads to land degradation.

Elimination of the butterfly larvae amounts to disruption natural control, as these insects suppress some host plants below weed levels (Guretzky and Louda, 1997). Weed infested fields require more inputs in terms of tillage, pesticide and fertilizer application. In such cases, the cost of production increases while yields are reduced leading to food shortage. Mortality of some butterflies that feed on carrion and dung like *Charaxes* spp. (Larsen, 1996) would retard the processes of biodegradation and recycling of nutrients.

Decline in populations of butterfly larvae would cause food shortages for other organisms like the insectivorous birds that feed on *D. plexippus* (Alonso-Mejia, 1998). Such organisms may resort to feeding on crops and other organisms that serve other important ecological functions like the predaceous mantids.

Therefore *Bt* maize pollen densities that are deposited on host plants of butterfly larvae that feed on the windward direction, closer to *Bt* maize that is at the pick of anthesis are likely to be exposed to higher densities of the toxic pollen. The effects of the toxins could be serious if the larvae feed on the mid and basal sections of host leaves.

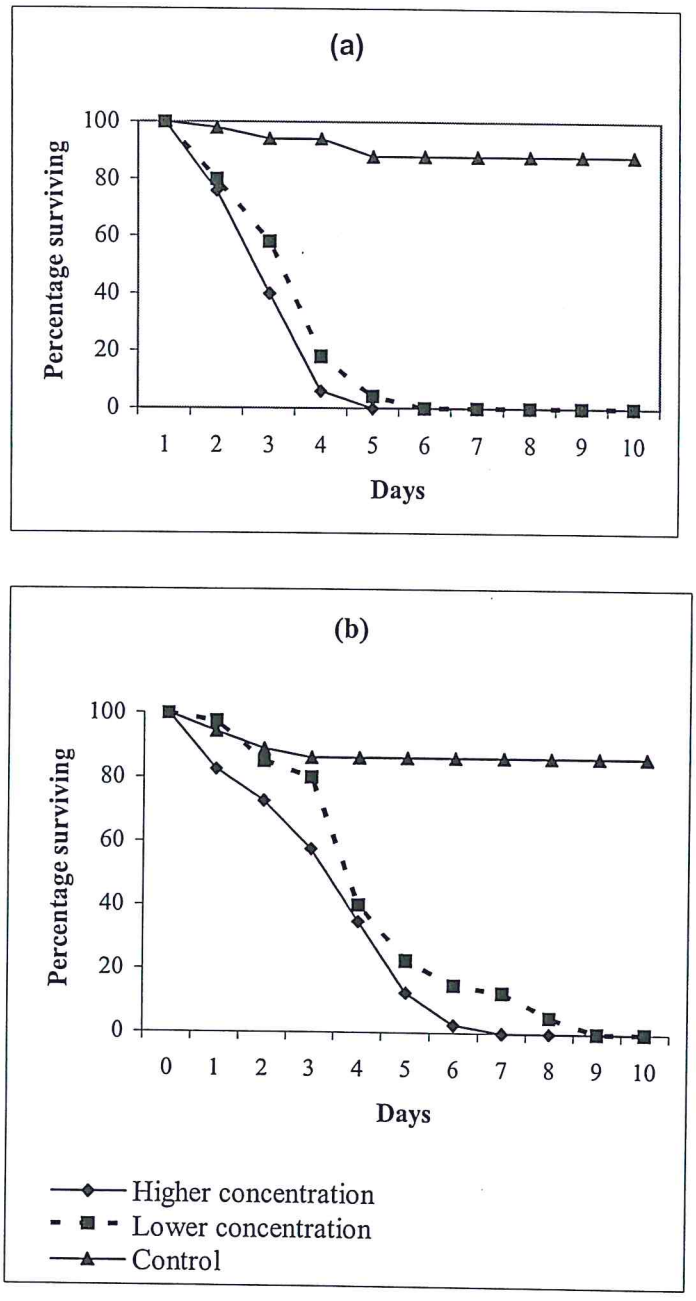


Figure 4.3 Daily mortalities of second (a) and fourth (b) instar larvae of *Acraea eponina* that fed on different *Bt* toxin concentrations. Caterpillars in both toxin concentrations had died by the 6^{th} and 10^{th} day respectively.

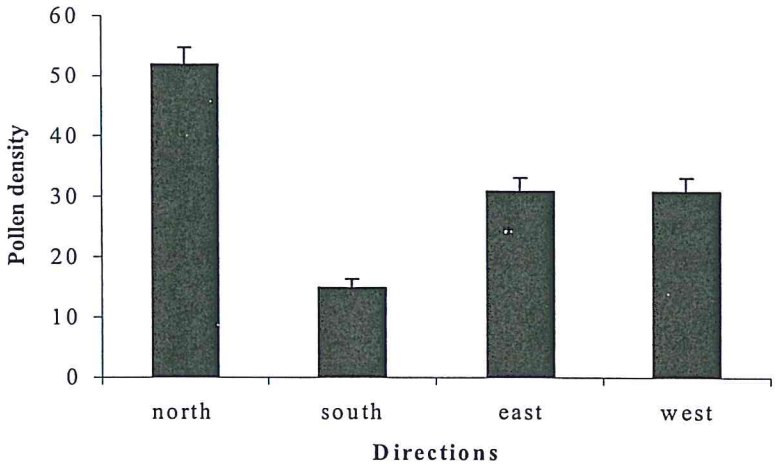


Figure 4.4 Mean comparison of pollen densities on leaves of *Waltheria indica* in four different directions. Leaves on the windward northern direction received more pollen than those on the other three directions *(Df=3, 3196; F=104.03; P<0.0001).*

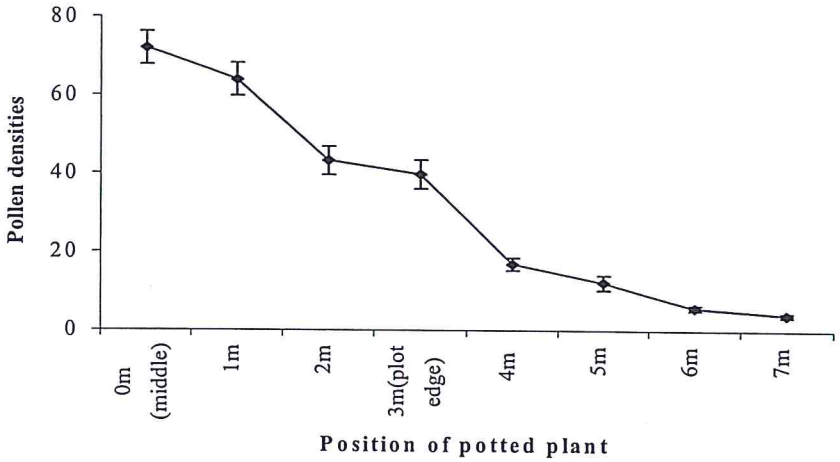


Figure 4.5 Pollen densities at different distances on maize plots. The figure shows decrease in mean pollen densities with distance from the centre of the plots on leaves of *Waltheria indica* (F=184.07, d.f. 7, 3192; P<0.0001).

CHAPTER FIVE

5.0 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

Difficulties in management of stemborers have led to the production of *Bt* maize as a new option (IRMA, 2002). These maize varieties also have the ability of indirectly controlling fungal pathogens that produce mycotoxin. This is by controlling stemborer feeding that encourages their infestation (Duvick, 2001). These transgenic maize varieties were first commercialized in the USA and Canada in 1996, and two years later in Argentina, South Africa and Spain; yield increases of up to 10 % due to *Bt* maize have been recorded especially in tropical areas that have more pest problems (Demont and Tollens, 2004). Kenya is among the developing countries that are in the process of registering these maize varieties for commercial use against the stemborers especially *C. partellus* and *B. fusca* (IRMA, 2002). Although *Bt* maize varieties are highly resistant to insects, there are potential non-target hazards associated with the cultivation of these plants (EcoStrat, 2000). Being one of the signatories to the Cartagena protocol on biosafety; the government of Kenya is required to carry out risk assessments in order to regulate the use of genetically modified crops such as the *Bt* maize. However, studies on the risks associated with *Bt* maize have not yet been implemented.

The main reason for the introduction of *Bt* maize in Kenya has been the need to control the cereal stemborer pests. Most of maize in Kenya is produced in the western high altitude zone, especially in Trans-Nzoia district where *C. partellus* is a minor pest, but colonizes the lower coastal region (Ngi-Song *et al.*, 1995). The major pest in highland

regions like Trans-Nzoia is *B. fusca* (Ngi-Song et al., 1998). This pest is not susceptible to Cry-toxins expressed in the currently produced *Bt* maize (IRMA, 2001a; Saxena and Stotzky, 2005). Besides insusceptibility to *Bt* toxins, *B. fusca* cannot be suppressed through biological control using *Co. flavipes* due to its strong immune system against the parasitoid (Ngi-Song *et al.*, 1995). Apparently, Kenyan scientists did not have a clear prior knowledge on the actual target stemborer species; this was clearly stated in America and Europe that *O. nubilalis* was the main problem (Chaufaux *et al.*, 2001), which justified the use of the *Bt* maize. This means that the introduction of the transgenic maize will have little impact on Kenya's annual maize production because the major stemborer, *B. fusca,* in the main maize growing areas is not susceptible to the *Bt* toxins. Therefore, it appears that Kenyans are simply embracing the technology irrespective of its capacity to control stemborers. This gives an impression that the only good thing with the *Bt* maize is the technology involved and the ability to kill *C. partellus*.

The toxins ingested by stemborers are harmful to non-target beneficial organisms by lowering parasitoid fitness, especially wasps like *Co. flavipes* that parasitize stemborer larvae (Chapter 2). This is because their parasitic developmental stages are dependent on actively feeding and developing stemborer larvae. However, parasitoids like *X. stemmator* that develop in quiescent host pupae are less affected by the *Bt* toxins (Chapter 3). The effect of Cry-proteins on parasitoids is therefore indirect by altering the growth of the stemborer host. There is still no clear evidence of direct intoxication of developing parasitoids by the host-ingested *Bt* toxins (EcoStrat, 2000). The biological control program for the management of cereal stemborers in sub Saharan Africa has spent large sums of money in capacity building and project implementation. This program was

running when there was no *Bt* maize, therefore introduction of *Bt* maize that expresses insecticidal factors would interfere with the already established populations of the parasitoid *Co. flavipes* (Overholt, 1998). New biological control programs would have to be initiated especially if the populations of *Bt*-resistant pests will emerge.

Kenya has been realizing a decline in crop harvests in the recent past (WFP, 2005). However, due to lack of clear government policy to protect the yields and motivate farmers through purchase of the produce, further losses have been common due to harsh weather and post harvest pests. Currently the larger grain borer *Prostephanus truncatus* Horn (Coleoptera: Bostrichidae) (Hill *et al.*, 2003), and the maize weevil *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) (IRMA, 2001b) are viewed by most farmers as the biggest threat to maize produce. Poor cereal storage facilities have also led to infestation by the fungus *Aspergillus flavus* (Muriuki and Siboe, 1995), which is responsible for production of afflatoxin that has recently caused human mortality in some Kenyan districts.

Bacillus thuringiensis toxins are also highly lethal to many lepidopterous insects (Fiuza *et al.*, 1996; Kwa *et al.*, 1998). Caterpillars that accidentally ingest toxic maize pollen that is deposited on host plants may therefore be harmed (Chapter 4). The reason is that densities of *Bt* maize pollen that are deposited on host plants for butterflies larvae like *A. eponina* are of lethal levels. The negative impacts of *Bt* maize pollen on butterfly larvae would lead to a collapse of income generating butterfly farming in Kenya. Just like other Afro-tropical counties Kenya is rich in diversity of elegant butterfly species (D'Abrera, 1997). The Kipepeo project at the Kenyan coast runs an income generating butterfly farming programme for local farmers (www.kipepeo.org). Pupae are bought by

the project and sold to live butterfly displays in Europe and North America. However, there have been concerns regarding the exportation of Kenyan butterflies. Some people think that it is an indirect way of re-establishing butterfly populations in countries where there are claims of mortality of butterfly larvae due to intoxication by pollen from *Bt* maize (Losey *et al.*, 1999).

Most people in the developing world are oblivious of what *Bt* maize is all about or the nature of technology involved in its production. This is in spite of their right to know the kinds of food they are consuming. This has raised a lot of suspicion among consumers since the technology is being exploited by very few researchers to develop crop varieties with genetic factors only understood by their collaborators. This has led to sharp decreases in *Bt* maize imports by the European Union from the USA (Greenpeace, 2003). Similar sentiments led to the rejection of tons of *Bt* maize as a relief food stuff from America by the Zimbabwean government in the year 2002. For instance, unregulated use of genetic engineering technology may lead to exploitation of genes from organisms that are morally unacceptable by some societies. A good example is the use of human genes (Inui and Ohkawa, 2005) or hereditary factors from 'unclean' mammals like pigs (www.fftc.agnet.org/library/abstract/rh2002009b.html) in the production of herbicide resistant rice. This may lead to rejection of GM food products and the technology as whole especially in sensitive societies like Muslim world.

A report by the World Health Organization (WHO) concluded that genetically modified foods can contribute to enhancing human health and development; but there is need for continued safety assessments on such foods before they are marketed, and to ensure they are evaluated from different social and cultural perspectives (WHO, 2005).

In this case, the effects of the genetically modified foods on human health were stressed. Among the factors in that report that needed investigation included Horizontal Gene Transfer (HGT), whereby viable undigested DNA fragments (Duggan, 2003), could get integrated into the genomes of intestinal microflora, and hence transforming them to dangerous organisms. Health complications could also arise if the genes are incorporated into cells that line the intestinal tracts of humans and animals through HGT (WHO 2005).

In Europe and America, *Bt* maize is basically planted as a fodder crop. In contrast, the crop is being introduced into Africa primarily for human consumption. Attempts to use GM crops for human consumption have been met by stiff resistance from consumers in the developed world. Some scientists involved in the development of the *Bt* maize are positive about the technology, yet they have strong reservations when it comes to consumption of such *Bt*-containing foodstuffs. Studies that investigate the effects of *Bt* toxins on humans and mammals have not been emphasized, while more funding has concentrated on ecological risk assessments basing on toxicity of the *Bt* toxins on invertebrate species. It is therefore important to note that successful adoption of these transgenic crop varieties will be greatly influenced by consumer confidence in the food and not the way the crop interacts with non-human organisms.

The technologies involved in the production of GM crops are usually protected by Intellectual Property Rights (IPR), which would encourage the monopolization of the seed industry (Léger, 2003). Introduction of *Bt* maize in Kenya is therefore viewed as a strategy by multinational seed companies to monopolize germplasm development. The emergence of monopolies that are not well regulated may lead to production of substandard crop varieties that are sold at elevated costs. This would ultimately kill out

the local seed companies that lack the capacity for mass production of GM seeds, which would lead to dependence on few companies for seeds and loss of jobs.

The exchange of genetic material between transgenic crops and the non-transgenic landraces has raised the concept of 'genetic pollution'. Most small scale maize producers do not buy planting materials from seed companies; maize seeds are usually obtained from successive harvests and shared among different farmers. Since maize is wind pollinated, there are chances of producing hybrids if pollen from transgenic maize fields fertilizes neighbouring non-transgenic plants (Chilcutt and Tabashnik, 2004). This may negatively alter the germplasm of conservative farmers who rely on their farm produce for seed production. Awareness of farmers about the negative impacts or gene flow from neighbouring transgenic crops may trigger legal disputes.

Wide spread planting of *Bt* maize may lead to selection of resistant populations of cereal stemborers to the Cry-toxins. This has been verified to occur in the European corn borer *O. nubilalis* under laboratory conditions (Huang *et al.*, 1999). The issue has necessitated research on resistance management through different strategies (Sharma and Ortiz, 2000). Some of the strategies include the use of more than one insecticidal gene in the same transgenic crop, and the creation of pockets of alternative non-*Bt* stemborer hosts known as refugia around *Bt* maize plantations (Sharma and Ortiz, 2000; Kanya *et al.*, 2004). In the first case, the presents of more than one insecticidal gene would disrupt the pest ability to develop resistance that would occur where one Cry-protein is expressed. In the latter, it is perceived that dominant *Bt*-susceptible stemborers in the non-*Bt* refugia would mate with recessive *Bt*-resistant populations to produce susceptible heterozygotes thereby reducing the abundance of resistant pests (Sharma and Ortiz,

2000). In South Africa, farmers are requested to plant 5-20 % non-*Bt* alternative hosts for stemborers for resistance management that may not be sustainable in maize growing areas in Kenya where there are many small holder farming systems due to land shortage (Mwangi and Ely, 2001). Another strategy for resistance management is the use of mixtures of *Bt* and non-*Bt* seeds (Davis and Onstad, 2000).

Maize production has always been directly limited by the declining soil fertility in different areas. Being a heavy consumer of macronutrients like Nitrogen and Phosphorus (Saleern *et al.*, 2003), its cultivation has degraded many virgin lands within few cropping seasons. This has led to high demand for nitrogenous fertilizers, which is becoming unsustainable for most farmers. The *Bt* maize varieties express Cry toxins that like other proteins require nitrogen for biosynthesis (Coviella *et al.*, 2000, 2002); this therefore makes the *Bt* maize have a higher demand for this macronutrient than the non-*Bt* crops. It has been verified that interaction between different levels of CO_2 and nitrogen may cause variations in toxin expression in some *Bt* crops (Coviella *et al.*, 2000, 2002). Improper soil fertilization due to unaffordable nitrogen fertilizers could therefore lead to production of sub-lethal *Bt* toxin levels by the transgenic maize varieties, which may encourage inherent resistance development among stemborer populations (Huang *et al.*, 1999).

Many institutions are training scientists in genetic engineering. However, most of such trainees lack prior exposure to ecology and other fields of biology, which would enable them to have a wider perspective of environmental issues. For example, people who have undergone training in biotechnology and biochemistry are preferred in genetic engineering-related tasks. The facts that there is early specialization in these courses, the students are deprived of biological subject matter. The same inadequate training also

affects scientists who are only trained in biological sciences, as they are less likely to be exposed to molecular techniques. The effect of such inadequate training was once experienced in the Kenyan media, whereby a leading plant breeding scientist dismissed the needs for risk assessment on *Bt* maize expressed by ecologists as mere academic argument. The world therefore faces a genetic crisis if people who are capable of manipulating organisms are not trained in biological sciences.

It is imprudent to think that a single factor like the *cry* gene in *Bt* maize would help in solving food shortages in Kenya, since crops are usually faced with more than one problem, which requires ecological strategies like integrated crop and pest management (Horowitz and Ishaaya, 2004). Reliance on GE to solve more crop problems would necessitate the introduction of more genes into a plant, which may lead to alterations of the genome structure yielding unsuitable crops for example, pleiotropic effects that are responsible for high levels of lignin in *Bt* maize varieties (Saxena and Stotzky, 2001).

Although the Cry toxins are lethal to some *Chilo* spp., there are other numerous pests that also cause substantial damage to maize. Good examples are the sap-sucking hemipterous insects like *Rhopalosiphum padi* (Aphididae), which are not affected by Cry toxin (Lumbierres *et al.*, 2004) that does not exist in the phloem vessels of *Bt* maize (Raps *et al.*, 2001; Dutton *et al.*, 2003). Also *Striga* spp. (Scrophulariaceae) or the 'witch weed' causes far much more damage to cereal crops (Gethia and Smith, 2004), especially on poorly nitrified soils in Africa (Cechin and Press, 1993; Reda *et al.*, 2005). In addition to these pests and weeds, maize crop is attacked by over fifty different diseases that are caused by pathogens like viruses, bacteria and fungi (CIMMYT, 2003).

To further complicate the challenges that are encountered in maize production, droughts have been the greatest factor that has caused most food shortages in the world. This has led to increased efforts by the International Maize and Wheat Improvement Centre (CIMMYT) to improve tropical maize for drought tolerance, since drought is an important factor that limits maize production in low-income countries (Edmeades et al. 1989). Therefore production of drought resistant crops and land irrigation should be given primary attention, as effects of pest damage are usually severe under dry conditions. *Bt* maize varieties should therefore not be adopted unless proper risk assessment studies are conducted with the actual *Bt* maize.

Recommendations

- Regular surveys should be conducted and databases of insect assemblages in maize growing zones maintained. The information would be important for monitoring biodiversity in case of *Bt* maize adoption.
- Precautionary risk assessments should be carried out on other transgenic cultivars, and on different species of non-target organisms. This would help in generating more reliable information since conclusions must not be based on isolated studies.
- Intervention and remedial strategies must be formulated as precautionary measures against the negative impacts of *Bt* expressing crops on non-target organisms.

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