Development of resistance by fall armyworm (*Spodoptera frugiperda*) to Cry proteins in Bt maize

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Abstract

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) has become one of the most damaging agricultural pests since it was first recorded outside of its native area of the Americas in 2016. In the Americas, genetically modified Bt maize is used as a control strategy for control of this pest. Although FAW is not one of the target pests of Bt maize in South Africa, it has suppressive effects on pest populations in areas where it occurs. Large scale cultivation of Bt maize exerts strong selection pressure on FAW populations, which subsequently evolve resistance to Bt toxins. The development of resistance to Bt proteins by FAW populations could lead to the unsuccessful implementation of Bt technologies, yielding them unreliant and unsustainable. In an attempt to mitigate this threat, single-toxin Bt cultivars were replaced by multi-toxin (pyramid) maize cultivars, and the deployment of specific insect resistance management (IRM) strategies. The most commonly used IRM strategy is the high-dose and refuge (HDR) strategy which is based on certain assumptions regarding the biology and lifecycle of target pests. In order for this HDR strategy to be successful, assumptions regarding the biology of target pests as well as insect-host plant interactions must be met. These assumptions require transgenic crops to express high doses of Bt toxins, which will cause the death of all susceptible (SS) and heterozygous resistant (RS) larvae, permitting only homozygous resistant (RR) individuals to complete lifecycles on Bt maize. Furthermore, this IRM strategy also requires the planting of a designated refuge area consisting of non-Bt maize to provide areas where populations of susceptible pests will be able to survive and reproduce. It is then assumed that Bt-resistant moths emerging from Bt maize will have the ability to fly and mate with susceptible moths emerging from refuge areas, diluting the resistance alleles and producing heterozygous resistant offspring, which will once again succumb to the high-dose produced by Bt maize. The aim of this study was to determine if changes occur in the life history parameters of *S. frugiperda* after exposure to Bt maize as host plant over different generations. The specific objectives of this research were to rear a field-collected FAW population on non-Bt maize, as well as MON810 and MON89034 maize cultivars for nine generations, and to determine if changes in life history parameters, larval preference and moth flight ability occur with continuous exposure over generations. Field collected FAW larvae were reared on three maize varieties (feeding groups), for eight or nine generations. These varieties were: a single-gene Bt maize hybrid (MON810) expressing Cry1Ab protein, a pyramid-gene Bt maize hybrid (MON89034) expressing Cry1A.105 + Cry2Ab2 proteins, and a non-Bt iso-hybrid. Results showed notable changes in life history parameters of larvae, pupae and moths reared on Bt plant tissue. Larval survival on Bt maize was significantly lower than on non-Bt maize and the duration of development period of larvae was significantly extended in the Bt maize feeding groups. Pupal mass was lower and the duration of the pupal period was longer in groups that were exposed to Bt maize. These extended development periods will result in poor synchronization of moth emergence patterns between Bt fields and refuge plantings, which will contribute to increased selection for resistance. Moth flight ability was adversely affected in the feeding groups reared on Bt maize. Adverse effects on moth flight ability could imply that resistant moths have limited capacity to fly and may be more likely to mate with other resistant moths that emerge from the same Bt maize field, increasing the selection for resistance evolution.

Key words: *Spodoptera frugiperda*, Bt maize, resistance development, MON810, MON89034, fitness, flight ability

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Chapter 1

Literature review

1.1 Introduction

Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae) is a polyphagous insect pest, more commonly known as the Fall armyworm (FAW) (Goergen *et al*., 2016). FAW is reported to feed and survive on 353 host plants, making them one of the most damaging agricultural pests in the world (Montezano *et al*., 2018). Although this insect is native to the tropical and sub-tropical regions of the Americas, where it is one of the most important pest species (Sparks, 1979), its migratory nature (which is due to its wide host range and ability to survive in warmer climates) has allowed it to spread to Asia, Africa and parts of Australia in only the past few years (Du Plessis *et al*., 2020; Montezano *et al*., 2018; EPPO, 2020, Piggott *et al*., 2021). The ability of FAW to rapidly adapt to its environment is not only due to the polyphagous nature of these larvae, but can also be attributed to the ability of larvae to evolve resistance to chemical pesticides as well as Bt (*Bacillus thuringiensis*) toxins expressed in genetically modified crop varieties (Zhang *et al*., 2020). This agricultural pest can however not withstand colder climates as its lifecycle does not include a diapause phase (Sparks, 1979).

The process of domestication in crop plants, which happens over hundreds (even thousands) of years, leads to the reduction of plant defense mechanisms, making them more accessible and vulnerable to herbivorous insects (Guera *et al*., 2020). As in the case of the FAW, this pest took advantage of the domestication process, and have become a key pest to many crop species, including maize (Guera *et al*., 2020). Whilst most lepidopteran herbivores are specialists, the polyphagous nature of the FAW makes it one of the most important noctuid pests worldwide (Gouin *et al.,* 2017).

The selection of host plants is critical for herbivorous insects as this ultimately determines the fate of offspring (Zalucki *et al*., 2002). Host plant preferences are dependent on plant characteristics such as nutrient value (chemical characteristics) and the presence of trichomes (physical characteristics) (Zalucki *et al*., 2002;

Knolhoff & Heckel, 2014; Visser *et al*., 2020). The recognition processes, such as host-plant detection and sexual communication, in insects is due to the presence of chemosensory organs that can detect chemical and physical cues. Non-volatile molecules are detected on taste sensilla, ovipositors and other mouthparts, due to the presence of gustatory receptors (GR). It was found by Gouin (2017) that the number of GR genes present in the genome of *S. frugiperda* was almost four times higher than that of non-polyphagous lepidopteran species. Pashley and Martin (1987) reported two distinctive *S. frugiperda* genotypes, referred to as 'strains' or 'sister species'. The one strain is referred to as the maize strain, which preferably feeds on maize and sorghum. The other sister species is the rice strain which prefers feeding on rice and forage grasses. These two sympatric host-plant strains are indistinguishable morphologically but differ in terms of fitness on different host-plant species (Gouin *et al.,* 2017) as well as sex pheromone composition (Zhang *et al*., 2020).

When present in large numbers in crop fields, substantial damage can be caused to crops such as maize, rice and sugarcane (Overton *et al*., 2021), which in turn results in yield losses, and threatens food security especially in developing countries (Bortolotto *et al*., 2016). An initial attempt to control FAW populations in crop fields saw farmers spraying large amounts of pesticides, after the introduction of organosynthetic pesticides in the 1940's. This was however only successful for a short period, as in the late 1950's concerns were being raised about the prevalence of pesticides in agricultural fields. Overreliance on the spraying of insecticides has proven to be ineffective and unreliable as it has increased the occurrence of pesticide resistance amongst FAW populations worldwide (Gutiérrez-Moreno *et al*. 2016).

1.2 Distribution of *Spodoptera frugiperda*

The FAW originates from the Western hemisphere, where it thrives in tropical and sub-tropical climates, ranging from parts of the Unites States of America to Argentina (Liu *et al*., 2020). The ability of FAW ability to survive in warmer climates, wide range of host plants as well as the ability of FAW pest populations being able to re-distribute itself annually (Luginbill, 1928) has allowed this insect pest to migrate across continental borders and thrive (Montezano *et al*., 2018). Outbreaks in

Western and Central Africa were reported in early 2016, where the FAW became an invasive species and the latest threat to grain production on the African continent (Goergen *et al*., 2016; Du Plessis *et al*., 2018). Entry to Africa was likely through flight of adult moths, or egg masses transported via direct flights between America and West Africa (Early *et al*., 2018), whilst dispersal through Africa took place by means of adult flight (Cock *et al*., 2017). The theory that there were multiple introductions of FAW into the African continent is strongly supported by two very distinct haplotypes that occur in samples collected from different locations in Western and Central Africa (Goergen *et al*., 2016; Nagoshi *et al*., 2017, 2018, 2021). The first detection of FAW on the African continent occurred in April 2016, on the island nations of São Tomé and Príncipe. Outbreaks in Western African countries (Benin, Nigeria, Ghana and Togo) occurred during June 2016. By October 2016, FAW populations had migrated throughout sub-Saharan Africa (Nagoshi *et al*., 2018). According to Liu *et al*. (2020), by 2018, reports confirmed the presence of this pest in South Asia (India). During 2018, FAW also spread to Thailand (FAO, 2019a), Myanmar (FAO, 2019b), China (FAO, 2019c), Republic of Korea (FAO, 2019d), Japan (FAO, 2019e), Philippines (FAO, 2019f), Indonesia (FAO, 2019g) and Australia (Piggott *et al*., 2021 & EPPO, 2020).

1.3 Lifecycle

Temperature plays a significant role in the survival and development of FAW populations. The optimal temperature range for development is between 26 and 30°C (Du Plessis *et al*., 2020). When temperatures are optimum, a single FAW generation can be completed in four weeks, however, in colder regions, a single FAW generation could take between 80 and 90 days to complete (Sparks, 1979). In tropical regions, FAW populations can complete up to eight generations per year (Du Plessis *et al*., 2018).

Eggs

Females lay eggs in batches, and there may be up to 200 eggs in each batch. A dense layer of scales protects the eggs from desiccation as well as environmental conditions (Sparks, 1979). Once the eggs have been laid, it appears an opaque white/green in colour, as it matures, it becomes more of a darker brown colour (Du

Plessis *et al*., 2018). The eggs hatch within two to four days from the day of oviposition, provided that environmental and temperature conditions are optimum (Sparks, 1979). Upon hatching, the neonate larvae devour this protective covering and then initiate feeding on host plants (Sparks, 1979).

Larvae and pupae

Six larval instars occur and developing larvae are able to feed and survive on a wide range of host plants. Larvae may feed on different plant parts, of which the suitability depends on the host plant species, the stage of host plant development, the stage of larval development (Sparks, 1979). Once larvae hatch, migration takes place by crawling or ballooning, when larvae spin a silk thread which allows them to drop from plants (Visser *et al.,* 2020). Instars 1 to 3 only consume about 2% of the total amount of leaf foliage consumed during the entire larval development stage (Sparks, 1979). When larger FAW larvae co-occur with smaller larvae, high levels of cannibalistic behaviour taks place where larger larvae often consume smaller larvae (Luginbill, 1928). Research done by Chapman (2000) reported that cannibalism accounts for up to 40 % of all FAW mortalities in infested plants. Once the larvae reach the 6th instar, preparation for pupation begins and larvae move from plants to the soil. Depending on the soil firmness, pupae can burrow up to 7 cm into soil or pupate amongst plant debris. Some larvae may also pupate in maize ears (Luginbill, 1928).

Moths

Moth emergence occur within one to five weeks after pupation, depending on soil temperature, texture and moisture (Sparks, 1979). Upon emergence from the pupal casing, moths burrow out of the soil and seek refuge on plants or plant debris. Sparks (1979) noted that newly emerged moths do not immediately engage in mating, and it is suspected that the virgin moths will instead spend the first night feeding. FAW moths are nocturnal, and movement regarding feeding, oviposition and mating mostly takes place during dusk or early hours of the morning (Sparks, 1979). As the sun sets, feeding takes place for around 2 hours, after which females start emitting a pheromone to attract males. Males can detect these pheromones from 9 to 12 m away. It is important to note that wind velocity and temperature play important roles in determining the distance from which males are able to detect females (Sparks, 1979). FAW moths are also attracted to lights that emit strong ultra-violet components (Abrahams *et al*., 2017). Once mating has taken place and a suitable host plant is located by the female moth, oviposition takes place in the early hours of the morning (Sparks, 1979). The average lifespan of FAW moths is two to three weeks. During this time, female moths mate multiple times and lay multiple egg batches. The potential fecundity of a female FAW moth is ±1000 eggs (Sparks, 1979).

1.4 Control of FAW

When pesticides are applied to crops, it is important to take the larval developmental stage into consideration. It is known that some larvae move to upper areas of the whorl at dusk/dawn when daily temperatures are relatively low. In order for pesticide application to be effective, it was recommended by Assefa & Ayalew (2019) that spaying times be adjusted to times when larvae are actively feeding and exposed. Additionally, the larval developmental stage is also important, as larger larvae penetrate and feed deeper into the whorl of maize plants, depositing frass (faecal matter) as it move downwards. This deposition of frass creates a blockage, which limits the movement of insecticides downwards into the whorl, making the spraying of pesticides ineffective (Assefa & Ayalew, 2019).

Synthetic pesticides are spayed to combat damage of maize by FAW larvae. The Insect Resistance Action Committee (IRAC), which focuses on the long-term efficacy of synthetic pest control products, has classified synthetically produced pesticides by their mode of action (MoA) (Sparks & Nauen, 2015). More than 25 different modes of action (MoA's) and 55 different chemical classes are covered by the MoA system developed by IRAC, which helps to establish appropriate insect resistant management (IRM) strategies. Although any of these pesticides may provide effective pest control, resistance may evolve, necessitating the need for IRM strategies such as the rotation of pesticide MoA's and crop rotation practices (Sparks & Nauen, 2015).

Integrated Pest Management

Alternative methods of control must be integrated into pest management strategies for FAW. Integrated Pest Management (IPM) is described by Kogan (1998) as "a pest management system that, in the context of the associated environment and the population dynamics of the pest species, utilizes all suitable techniques and methods in the most compatible manner and maintains the pest population at levels below those causing economic injury.” An IPM strategy is an approach to plant protection, with the biggest developments happening in the areas of genetic engineering of plants. Regarding the control of FAW populations, the use of genetically modified Bt crops makes a significant contribution to the reduction in yield losses in crops, not to mention the significant reduction in pesticide applications and associated environmental impact (Kogan, 1998; Brookes & Barfoot, 2018). According to Ehler (2006), IPM is ‘a decision-based process involving coordinated use of multiple tactics for optimizing the control of all classes of pests in an ecologically and economically sound matter.’ IPM strategies are developed in order to manage and keep the population numbers below an economic threshold, as well as to reduce the use of pesticides, which benefits both the environment as well as the economy (Kogan & Bajwa, 1998). The four pillars upon which IPM is based on are chemical control, host plant resistance (HPR)/genetics, biological control, and lastly cultural control. All these control methods are integrated to reduce pest populations whilst minimizing impact on the ecosystems.

Biological control

According to Stern (1959) biological control is a natural, permanent control method regarding the population density of pests. They further defined it as: “the action of parasites, predators, or pathogens on a host or prey population which produces a lower general equilibrium position than would prevail in the absence of these agents.” Pests that invade crops have naturally occurring enemies, biological control comes into play when natural ‘enemies’ or predators of pests supress or control the pest population. These natural predators are known as biological control agents, and after extensive research is done, these predators are released into areas where there are known, uncontrolled populations of pests (Kogan & Bajwa, 1999). Naturally occurring enemies of pests could be found in the immediate surrounding environment, or, if these kinds of populations are absent in the environment where pest populations occur, biological control agents can be introduced into an area (Stern *et al*., 1959).

A study done by Sisay *et al*. (2018) on the natural biological control agents of FAW, revealed four Hymenoptera species and one dipteran species that parasitize the eggs of FAW moths. No previous reports from Africa, North and South America associated these five species with the biological control of FAW. However parasitic wasps and tachinid flies are the most well-known biological control agents of FAW. Furthermore, studies done by Abang *et al*. (2021) found that *Telenomus remus* (Nixon) (Hymenoptera: Platygastridae) and *Trichogramma* spp. (Hymenoptera: Trichogrammatidae) showed relatively high levels of egg parasitism when compared to other endoparasitoids. Abang (2021) also stated that the use of parasitoids to biologically control pest populations of spodopterans is the most common method.

Cultural control

Cultural control aims to change the environment and make it unfavourable for the invading pest population, therefore making the survival, growth and reproduction of the pest unlikely (Harrison *et al*., 2019). This type of control relies on practices such as advancing or delaying planting dates, crop rotation and the changing of harvesting dates to name a few. The use of the above-mentioned practices interrupts the interactions between S*. frugiperda* populations and crops which may not be present at that time, resulting in the disruption of pest ecology (Harrison *et al*., 2019).

Chemical control

Chemical control makes use of the different synthetic, toxic and harmful compounds which can be administered in various ways, either onto the plant or its immediate surroundings. Chemical control usually provides immediate results, which results in only temporary relief or control of pest populations (Stern *et al*., 1959). When utilized as component of an IPM strategy, chemical control is only relied upon in situations where pest populations have exponentially increased due to environmental suppressions being inadequate (Stern *et al*., 1959). The use of these synthetic chemical compounds in ecosystems should be carefully planned to avoid or reduce effects on non-target populations of organisms (Jepson *et al*., 2020).

1.5 Evolution of resistance to insecticides

Resistance, as defined by Tabashnik (2014) is the “genetically based decrease in susceptibility to a pesticide" which differs from field-evolved/selected resistance, defined as "genetically based decrease in susceptibility to a pesticide in a population caused by exposure to the pesticide in the field" (Tabashnik *et al*., 2014).

Group 1B Organophosphates and 3A Pyrethroids-Pyrethrins are the cheapest and most commonly used pesticides in Africa. It is however unclear whether populations of FAW that migrated to Africa are resistant to these compounds, due to prior exposure in areas of origin (Abrahams *et al*., 2017). According to Tabashnik (2014), the evolution of resistance in pest populations is quickly becoming a global agricultural problem, and poses threats to human health (Tabashnik *et al*., 2014). A study done by Zhang (2020), in which an analysis of FAW genes relating to pesticide resistance was done, showed that the risk of FAW populations developing resistance to conventional pesticides is very high.

When pesticides with the same MoA are repeatedly applied over successive generations of FAW, it results in the evolution of resistance, not only to a specific pesticide but to all pesticides with the same MoA (Sparks & Nauen, 2015). In order to limit the evolution of resistant populations, several strategies may be employed. Firstly, instead of solely relying on pesticides, a combination of strategies should be applied. Pesticides with the same MoA should not be used repetitively, instead, a rotation of pesticides with a different MoA should be implemented. The application of pesticides should follow the close monitoring of crops and thresholds and should not be used as a preventative solution. Furthermore, recommended dosages should be followed closely (Sparks & Nauen, 2015).

1.6 Host plant resistance

Host plant resistance (HPR) is resistance that is naturally occurring within host plants and is also referred to as 'native' resistance (Prasanna *et al.,* 2018). HPR is one of the pillars of IPM which needs to be developed and implemented in order to carry out a successful strategy focused on controlling FAW populations. HPR can be successfully incorporated into control strategies for small holder farmers for which FAW control options are limited. In the Americas, HPR is a trait that has been integrated into conventional breeding programs, and, as a result, several maize breeding lines with resistance have been identified in different maize populations. In a 2020 field study in Kenya, no significant differences in plant damage occurred among six locally popular maize cultivars naturally or artificially infested by FAW (Morales *et al*., 2021). However, in laboratory experiments, FAW preference, acceptance, and growth did vary significantly among cultivars indicating the potential for identifying native host plant resistance traits (Morales *et al*., 2021).

Differences in levels of natural resistance of maize to FAW damage1992) can be assessed using the Davis Scale (Davis *et al*. 1992), in which the responses of the germplasm of maize plants is rated on a scale of 1 (highly resistant) to 9 (highly susceptible). Host plant resistance is however not limited to only naturally occurring genetic variation, as genetically modified (GM) Bt maize with insecticidal properties may also be considered a form of HPR.

It is however important to note that naturally occurring HPR is more durable because it is quantitative (meaning several genes code for the resistance traits), and also exerts less selection pressure on the pest population. This implies that FAW populations will evolve resistance to these traits at a much slower rate than what resistance would evolve to transgenes. This is because Bt maize plants usually contain a single gene that encodes for insecticidal proteins with a high success rate in controlling pest populations (Prasanna *et al*., 2018). FAW populations easily evolve resistance against traits that are based on only one or a few genes (Tabashnik, 1994). Breeding for HPR is therefore an ongoing process, which sees a never-ending race between the evolution of host plants and pest populations (Prasanna *et al*., 2018).

1.7 Genetically modified crops

Genetic modification, which is also referred to as genetic engineering, is seen as a modern-day extension of traditional cross breeding and selection methods to develop crop varieties with selected traits, using molecular technologies and recombinant DNA rDNA) to produce Genetically Modified Organism (GMOs) (Lemaux, 2009). Unlike traditional cross breeding methods where thousands of traits are combined when two plants are crossed, genetic modification allows for a more specified outcome, transferring only a few genes, selected for specific traits. GM crops are referred to as plants whose genetic make-up has been modified using molecular methods and techniques which alter the genome to produce specific phenotypic traits (Okigbo *et al*., 2011).

The altering of genomes makes use of recombinant DNA technology, and starts by the isolation of a desired gene, followed by the modification of the gene, and lastly the insertion of the gene into the genome of the desired organism (Okigbo *et al*., 2011). Genetic modification may also include the removal of specific genes. Genetic modification allows for plants and organisms to exhibit phenotypic or physiological properties that were not present before the genetic modification took place (Okigbo *et al*., 2011).

1.8 Introduction to Bt maize

The introduction of biotech crops has revolutionized agricultural pest management practices and became a tool for the integration of various control methods to combat specific insect pest species (Yang *et al*., 2018). Genetic manipulation has allowed for the design of transgenic plants that have specific insecticidal traits. *Bacillus thuringiensis* (Bt), is a bacterium that occurs naturally in soils. Genes from *B. thuringiensis* are commonly used in genetic manipulation of crops, largely due to the naturally produced Cry proteins which have insecticidal properties against certain groups of insects. These Cry proteins are d referred to as Bt toxins and occur as crystals in the bacterium itself (Lemaux, 2009). Genes that encode for the production of these Cry proteins are important components used in genetic modification of maize. The insertion of these genes into maize genomes gives maize plants the ability to naturally produce crystal proteins within the plant tissues (Lemaux, 2009).

According to Yang *et al.* (2018), there are different classes of Bt proteins that are used for the control of lepidopteran pests, namely Cry1, Cry2 and Vip3A. These classes differ from one another, as the Cry proteins are produced during the reproductive growth phase of *B. thuringiensis*, whilst Vip proteins are produced during the vegetative growth phase, and are then excreted (Tabashnik & Carierre, 2017). It is also important to note that the Vip3A and Cry proteins do not share any binding sites or sequence homologies (Yang *et al*., 2018). When Cry toxins are present in full length, they are inactive, and need to be ingested by target pests to cause mortality (Abbas, 2018). Proteolytic enzymes present in the gut of larvae activate the Cry toxin, which is followed by the binding of the active form of Cry proteins to receptors on the membrane of the midgut, this process is essential for toxicity. The toxins result in the swelling of surrounding cells, ultimately resulting in cell lysis. This cell lysis causes the separation of the basement membrane from the midgut epithelium, allowing the alkaline gut fluid to leak into the hemocoel. The resultant rise in haemolymph pH leads to the paralysis and eventual death of the larvae (Abbas, 2018, Roh *et al*., 2007). The high degree of target pest specificity is largely due to the proteins' mode of action within insect larvae (Roh *et al*., 2007).

The adoption of Bt crops provide several benefits to farmers. These include decreased yield losses due to pest damage, reduced use of synthetic pesticides and simplified management (Carrière *et al*., 2020). According to Brookes (2007) benefits of Bt maize cultivation by various states in the EU included increased yield benefits of more than 10% (when compared to non-GM maize), increased income levels of farmers, as well as reductions of mycotoxins present in grain, therefore improving the quality of grains. Based on the value for commercial farmers, the estimated cumulative benefit of Bt maize to farmer income was $2.2 billion from 1998 to 2016 (Brookes and Barfoot, 2018).

In 1997, South Africa became the first African country to commercialize the production of Bt crops (Van den Berg *et al*., 2013). It was reported by Botha *et al.* (2019) that MON810 maize, which expresses Cry1Ab protein and MON89034 maize, which expresses Cry1A.105 + Cry2Ab2 proteins, are the Bt maize events that are currently cultivated in South Africa. The target pests of Bt maize in South Africa are *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) and *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) (Van Rensburg, 1999; Van Wyk *et al*., 2009). Although Bt maize can provide effective control of FAW (Botha *et al*. 2019), no Bt maize event is registered in South Africa for control of this pest. Bt maize does, however, have a significant effect on this pest and exerts selection pressure for evolution of resistance to Cry proteins expressed in local maize varieties.

1.9 Resistance evolution to Bt maize

Any type of control method used to reduce pest populations introduces a selection pressure (Hackett & Bonsall, 2016). Although the development of insect resistance is inexorable, when high levels of selections pressure are present, the rate of resistance is more rapid. It is essential to consider practices which increase the rate of resistance evolution in pest populations (Prasanna *et al*., 2018). The lack of crop rotation practices, and resultant recurrent planting of Bt maize events that express the same insecticidal toxin increases selection pressure, consequently increasing of development of resistance in target pest populations (Van den Berg *et al*., 2013). Non-compliance by farmers to the refuge requirements also contributes to increasing selection pressure on pest populations (Carrière *et al*., 2020). Additionally, when Bt toxin concentrations in plants are low, it results in the survival of heterozygous (RS) individuals. Pest behaviour may also contribute to an increased rate of resistance evolution (Tabashnik *et al.,* 2013). Studies done by Visser *et al.* (2020), showed avoidance behaviour of neonate larvae for plant tissues containing Bt proteins.

There have been several cases of resistance evolution of FAW to different Cry proteins. Field resistance to Cry1F proteins in Bt maize has been reported in *S. frugiperda* populations in Puerto Rico, Brazil, North America (Yang *et al*., 2018) and Argentina (Zhu *et al*., 2019). Resistance alleles against a relatively new Bt protein, Vip3A, were also detected in field populations collected in USA and Brazil (Zhu *et al.,* 2019). In 2010, new pyramided maize cultivars containing multiple *cry* genes were introduced as a more durable strategy to delay of resistance evolution in target pest populations (Santos-Amaya *et al*., 2015). These cultivars contained pyramided genes of two or more unrelated Bt proteins with different MoA's for the same target pest (Zhu *et al*., 2019), since multiple gene traits are more effective and durable than single gene traits (Prasanna *et al*., 2018). One concern regarding the pyramiding of Bt genes in transgenic crops is that previous exposure (and resultant resistance evolution) to single-toxin Bt events that contain one of the toxins present in pyramided cultivars, may accelerate the rate of resistance evolution to the two-toxin Bt events (Zhao *et al*., 2005). Pyramids are expected to be especially effective for delaying pest resistance when each toxin in the pyramid is highly effective on its own, resistance to each toxin in the pyramid does not confer cross-resistance to any other toxin in the pyramid, and pyramids are not grown together with single-toxin plants producing one of the toxins in the pyramid (Carrière *et al*., 2016). However, according to Santos-Amaya *et al*. (2015), resistance to pyramided maize cultivars was found to be 'recessive, incomplete and autosomal', showing no indications of cross resistance to the Vip3A gene, and no maternal effects. It is however important to note that the selection for resistance to Cry1A.105 + Cry2Ab reduced the effectiveness of Cry1Fa, indicating cross resistance (Santos-Amaya *et al*., 2015).

1.10 Insect resistance and Insect Resistance Management

Evolution of resistance in pest populations against pesticides and transgenic crops directly challenges sustainable agriculture and threatens food security (Hackett & Bonsall, 2016). The sustainable use of Bt crop technology could be compromised by field-evolved resistance to Bt proteins. The accelerated rate of resistance evolution in pest populations is ascribed to the worldwide large-scale adoption of Bt maize cultivars (Zhu *et al*., 2019). This emphasizes the need for the implementation of insect resistance management (IRM) strategies and highlights the need for grower compliance if the sustainability of Bt crops is to be maintained. Tabashnik (2014) defined field-evolved resistance as the 'genetically based decrease in susceptibility to a pesticide in a population caused by exposure to the pesticide in the field'. According to Mendelsohn and Kough (2003), the development of a field-resistant target pest species is highly likely when combining the unrestricted use of Bt maize with a lack of measures put in place to delay the evolution of resistance in target pest species. Additionally, the emergence of resistance would negatively affect the benefits of cultivating Bt crops and could have detrimental effects of the efficacy of Cry proteins on the target pest species. The benefits that the cultivation of Bt crops provides, could result in the overuse of Bt crops, which in turn could speed up the rate of resistance evolution in pest populations (Carrière *et al*., 2020). It is therefore required by the Environmental Protection Agency (EPA) in the USA that farmers who plant Bt crops implement an IRM program to limit the evolution of resistance by target pests (Mendelsohn & Kough *et al.,* 2003). Countries that allow the cultivation of Bt crops are expected to provide governance systems that will aid in the preservation of the benefits that Bt crops provide to the public (Carrière *et al.,* 2020). Strict adherence to a proper IRM strategy is of benefit to all, from producers to the public (Van den Berg *et al*., 2013), and usually includes frequent monitoring and inspection to ensure that insect resistance present in a field does not exceed the baseline level, a high dose expression of insecticidal proteins by crop plants, and a sufficient refuge which will be able to support a population of susceptible insect pests is present (Van den Berg *et al*., 2013).

1.11 High dose and refuge strategy

The high dose/refuge strategy was introduced as an IRM strategy to delay evolution of resistance in target pest species that feed on Bt crops as well as crops sprayed with chemical pesticides (Tabashnik *et al*., 2004).

For the implementation of the high dose/refuge strategy, farmers who plant Bt cultivars are required to designate a specified portion of their field to planting non-Bt maize plants. These areas serve as refuge for susceptible pests and provide a sustainable habitat for pest development, thereby decreasing the selection pressure on pest populations (Tabashnik *et al*., 2013). Current refuge requirements in South Africa are a 20% non-Bt refuge area that may be sprayed with pesticides, or a 5% refuge area that may not be sprayed with pesticides (Van den Berg *et al*., 2013). Furthermore, the HDR strategy is based upon assumptions regarding the insect-host plant interactions (Tabashnik *et al*., 2013). Assumptions supporting this strategy are, firstly, that alleles that confer resistance are rare, recessive and conferred by a single locus. Secondly, Bt plants are expected to express high doses of the toxin, and lastly, that most surviving resistant adults will randomly mate with susceptible adults that emerge from refuge areas (individuals emerging from refuge areas are expected to be homozygous (SS)) (Tabashnik *et al.,* 2004). In regard to a high-dose, the toxin concentration within maize plants should be high enough to kill 95% of heterozygous (RS) individuals (with only one resistance allele), and is estimated to be about 25 times the amount needed to kill susceptible (SS) individuals (Van den Berg *et al.,* 2013).

In order to expand the knowledge on FAW and enhance the effectiveness of IRM strategies, it is important track the dispersal of individuals and assess its fitness, e.g. flight ability (Vilarinho *et al*., 2011). Data were collected by Vilarinho (2011) by means of mark-release-recapture techniques in order to determine the dispersal capacity of FAW adults. Results indicated that the furthest recapture distance for males was 806 m, and for females 608 m. It is important to keep these values in mind when setting up strategies for the establishment of refuge sites (Vilarinho *et al*., 2011). It is therefore important to study the behaviour and fitness parameters of FAW larvae that developed on Bt maize (Visser *et al*., 2020). For example, reduced flight ability of moths from Bt maize fields will compromise the IRM strategy if mots remain local and do not fly off to mate with susceptible moths emerging from refuge areas.

1.12 Fitness costs associated with resistance

According to Hackett and Bonsall (2016), the fitness costs that are specifically associated with resistance to Bt crops are poorly understood, and variable results have been obtained regarding fitness costs related to field evolved resistance. When there is strong selection pressure on populations, the resultant surviving individuals may show fitness costs in the absence of selection pressure, compared to individuals who do not express the same resistant genotypes (Sosa-Gómez & Miranda, 2012). A diverse range of field-related fitness costs have been reported, however, they have been generalized as overall population survival and viability, and do not take ecological conditions into account (Hackett & Bonsall, 2016). According to Jakka (2014), the rate of resistance evolution can be impacted by fitness costs. By comparing the biology and lifecycles of susceptible and Bt-resistant populations of FAW, the existence of fitness costs, and the impacts on resistance evolution can be evaluated. Larval developmental time, pupal weight and duration, pupal emergence, moth flight ability, fecundity and fertility are of the most common traits compared between susceptible and Bt-resistant populations (Jakka *et al.,* 2014). The most evident difference in the comparison of the biological performance of susceptible vs resistant FAW populations, was an increase in larval developmental time for resistant populations that fed on Bt maize (Jakka *et al.,* 2014).

Knowledge regarding fitness costs (which are independent regardless of environmental conditions) may be a great aid in resistance management and could contribute to developing strategies that improve the effect that is served by the refuge (Hackett & Bonsall, 2016). According to Gassman *et al*. (2009), individuals that possess alleles that confer resistance to Bt toxins, find themselves in a predicament when in the absence of plant material containing Bt toxins, compared to individuals who do not possess such alleles. This compromise is referred to as a ‘fitness cost’. In order to evaluate the existence of fitness costs, comparisons of the biology, lifecycles, and performance of Bt-resistant and susceptible pest populations.

In a study conducted by Boersma and Carpenter (2016), flight ability of moths was used as a parameter to determine the fitness costs of rapidly chilling moths. Rapid chilling is a standard procedure used in the sterile insect technique (SIT), to immobilize moths. Results from the latter study showed that moths treated with gamma rays and subjected to cold temperatures were much less likely to be captured in pheromone traps around the orchardswhere reeasing took place. The fitness cost of these treatments were reduced flight ability which directly affected the competitiveness and dispersal of moths (Boersma & Carpenter, 2016).

A study on the fitness costs of Bt-resistance in populations of velvetbean caterpillar (*Anticarsia gemmatalis* Hübner) (Lepidoptera: Noctuidae), showed a significant increase in larval developmental time and pupal weight of individuals of Bt-resistant populations. Additionally, there was also a decrease in the survival and number of instars completed by larvae. There was no significant difference in parameters such as pupal duration, longevity of adults, number of eggs laid per female and egg fertility (Sosa-Gómez & Miranda, 2012). Research conducted by Barros *et al*. (2020) on the effects of insecticides on the development and flight performance of *Chloridea virescens* (Fabricius) (Lepidoptera: Noctuidae) (tobacco budworm), found that not only was there an increase in the average larval and pupal duration times, but also a decrease in overall flight performance of moths. The developmental and transgenerational effects of chlorantraniliprole in *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Diamondback moth) populations was studied by Gou *et al.* (2013), and results showed an increase in the developmental time of larvae, a decrease in pupal weight, decreased fecundity in adults as well as a decrease in the percentage of eggs that hatch.

1.13 Cross-resistance between different Cry proteins in FAW

Cross resistance occurs when populations of a target pest that are resistant to a Bt protein in turn have a genetically based resistance to other Bt proteins. The development of cross resistance to proteins used in Bt crops could drastically affect the sustainability and durability of gene-pyramided corn (Yang *et al*., 2017). According to Zhu (2019), populations of *S. frugiperda* that are resistant to Cry1F proteins show cross resistance to other Cry1 proteins, including Cry1Ab, Cry1Ac and Cry1A.105 (Zhu *et al*., 2019). However, studies done by Yang (2017) which aimed at evaluating the possible presence of cross resistance between Cry2Ab2 and multiple other Bt proteins found no cross resistance between Cry2Ab2-maize-selected FAW populations and Cry1F, Cry1A.105 and the Vip3A proteins, as well as maize plants expressing any of these proteins. According to Yang *et al.* (2018), Bt crops containing the Vip3A gene are effective against *S. frugiperda* populations that developed resistance against Cry proteins.

1.14 Resistance of FAW under laboratory conditions

More than a dozen pest species have been reared in laboratories where selection for Bt resistance took place (Tabashnik *et al*., 2002). Studies conducted by Tabashnik *et al*. (2002) on inheritance of resistance to Cry1Ac Bt toxins in a population of *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) (pink bollworm) found that resistance was recessive when the toxin concentrations were high. On the contrary, it was found that as the toxin concentration decreased, the dominance of resistance increased. Additionally, results reflected that there was a high likelihood that resistance is inherited and controlled by one or a few major loci, showing that after one round of selection, the resistant strain had a 300-fold resistance compared to the susceptible strain.

1.15 Comparative behavioural responses of target pests to Bt and non-Bt crops

There is a very high possibility that in the process of selecting a host plant, foraging larvae are able to detect the presence of Bt toxins within plant tissues, which could influence its choice when selecting a suitable host plant (Visser *et al*., 2020). Comparative studies were done by Visser *et al*. (2020) in populations of *B. fusca* larvae, to investigate whether the presence of Bt toxins in maize plants affected ovipositional preferences, larval feeding behaviour and host plant selection. The behaviour of susceptible larvae were then compared to the behaviour of larvae from populations that have already developed resistance by being exposed to Bt toxins.

The outcomes revealed that neonate larvae of *B. fusca*, from both susceptible as well as resistant populations, showed complete feeding avoidance behaviour coupled with higher rates of plant abandonment when exposed to maize tissue containing Bt toxins (Visser *et al*., 2020). There were however no distinct ovipositional preferences by female moths.

This study tested the hypothesis that the utilization of Bt maize as a host plant over different generations affected life history parameters of *Spodoptera frugiperda* populations. Life history parameters recorded were larval duration, larval survival, pupal mass, pupal duration and moth emergence. The specific objectives of this research were:

1. To rear a field-collected FAW population on non-Bt maize, as well as MON810 and MON89034 maize cultivars for 9 generations.
2. To determine if changes in life history parameters, larval preference and moth flight ability occur with continuous exposure over generations.

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Chapter 2

Changes in life history parameters of *Spodoptera frugiperda* following generational exposure to Bt and non-Bt maize.

Abstract

This study aimed to determine the changes in life history parameters of *Spodoptera frugiperda* populations which were reared on maize leaf material of different maize varieties for eight or nine generations. The maize varieties used were a non-Bt maize variety and two Bt maize varieties that express either Cry1Ab protein or both Cry1A.105 + Cry2Ab2 proteins. Comparisons of larval survival, duration of the larval and pupal stages as well as pupal mass were determined after each generation between the three feeding groups in order to identify anychanges within or across feeding groups. Results indicated delays several life history parameters over generations, for larvae and pupae reared on the two Bt maize varieties.Extended duration of larval and pupal periods were the life history parameters most affected by Bt toxins. Furthermore, reduced larval survival and moth emergence was also observed in Bt feeding groups.

Key words: Cry proteins, life history, pest management, *Spodoptera frugiperda*, resistance

2.1 Introduction

Pest control methods such as the use of insecticides or genetically modified crops with insecticidal traits exert selection pressure on target pest populations to evolve resistance (Hackett & Bonsall, 2016). Crystalline (Cry) proteins expressed within the tissues of genetially modifed Bt maize plants for example, exert a stong selection pressure on target pest populations, favouring the evolution of resistance within these populations (Tabashnik, 1994).

Bt maize varieties that express Cry1F, Cry1Ab, Cry1A.105 + Cry2Ab2 proteins that kill lepidopteran pests is cultivated widely in the world (Tabashnik *et al*., 2013), including South Africa (Visser & Van den Berg 2020). This wide-spread use of Bt maize has led to the development of resistance in Fall armyworm (FAW) (*Spodoptera frugiperda)* (J.E. Smith) (Lepidoptera: Noctuidae) populations to Cry1F, Cry1Ab and Vip3A (Huang, 2020, Botha *et al.,* 2019; Zhu *et al*., 2019), and Cry1Ab in *B. fusca* (Kruger *et al*., 2014; Van den Berg *et al*., 2013; Strydom *et al*., 2019). The rapid development of resistance, which is the predominant threat to the successful implementation and longevity of Bt crop technology (Van den Berg *et al.,* 2013), is due to the continuously high selection pressure exerted on FAW populations by Bt toxins (Tabashnik 1994; Visser & Van den Berg, 2020).

Studies on the life history of organisms often aim to determine how evolution takes place in order to adapt to favourable or unfavourable conditions in which the organisms are found (Stearns, 2000). In order to study the changes in life history parameters of populations, any changes or variations in response to beneficial or stress conditions within these major life events must be investigated (Chapin, 2017). When there are strong selection pressures on populations to adapt to certain conditions, the resultant surviving individuals may exhibit changes in fitness which may affect its ability to pass on the resistance to future generations (Sosa-Gómez & Miranda, 2012).

Monitoring of life history parameters over generations may provide indicators of adaptions to different selection pressures. For example, Liu *et al*. (1999) found that larvae of a Bt-resistant strain of pink bollworm (*Pectinophora gossypiella)* (Saunders) (Lepidoptera: Gelechiidae) that fed on Bt-cotton had significantly longer developmental times than susceptible larvae feeding on non-Bt cotton, with resistant larvae taking approximately five days longer to develop to pupae. Results obtained in a study conducted by Groeters *et al*. (1994), showed a difference in fitness components when life history parameters of a Bt-resistant population of diamondback moth (*Plutella xylostella*) (L.) (Lepidoptera: Plutellidae) were compared to those of a susceptible population. Within the resistant population, the larval survival was reduced by 25% compared to the susceptible population. Furthermore, the fecundity and fertility within the resistant population were reduced by 10% and significant differences in developmental times and pupal weights were also observed. However, Kruger *et al*. (2014) found no changes in fitness when comparing life history parameters of Bt-resistant and susceptible populations of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) on Bt maize.

The effect of Bt toxins on the fitness of individuals within a FAW population could be better outlined and understood by the comparison of biology and lifecycles of Bt-resistant and susceptible populations (Jakka *et al*., 2014). Comparison of the larval developmental time, pupal weight and duration, pupal emergence success, moth flight ability, fecundity and fertility of susceptible and Bt-resistant populations, shows that changes in fitness of individuals play a major role in the evolution of resistance (Jakka *et al*., 2014). Results from a study done by Jakka *et al.* (2014) showed that Bt-resistant moths took an average of three days longer to emerge from pupae than susceptible moths, which in the case of field-developed resistance would result in asynchronicity in emergence of resistant and susceptible moths. The latter lack of synchronization between moth emergence patterns from Bt and non-Bt maize has a direct negative effect on insect resistance management strategy, since mating of susceptible and resistant moths will not be possible, therefore failing to dilute the frequency of resistance alleles in the pest population (Van den Berg *et al.,* 2014; Gould, 1998). Furthermore, according to Simmons and Marti (1992), *S. frugiperda* adults have a mating period of three days after emergence from pupae, with optimum fertility and fecundity occurring only for two days after emergence. Jakka *et al.* (2014) therefore suggested that in the case of field-evolved resistance, relating to the high dose/refuge (HDR) resistance management strategy, mating between susceptible and resistant FAW adults would mostly be unsuccessful.

The most evident difference in biological performance of susceptible and resistant *S. frugiperda* populations has been reported to be an increase in larval developmental time for resistant populations (Jakka *et al*., 2014). In Brazil, studies regarding the changes in fitness associated with the resistance of *S. frugiperda* populations to Spinosad indicated a 41% reduction in larval survival, and a 45% decrease in reproductive rates of Spinosad-resistant populations (Okuma *et al.,* 2018).

This study tested the hypothesis that the utilization of Bt maize as a host plant over different generations affected life history parameters of *Spodoptera frugiperda* populations. Life history parameters recorded were larval duration, pupal mass and pupal duration.

2.2 Materials and methods

2.2.1 Maize

To provide maize leaf tissue for continuous rearing of larvae, seeds of three different varieties were planted at two-week intervals to ensure constant availability of plants. Whorl leaves of these plants were provided as food for larvae.

The three maize varieties were a single-gene Bt maize hybrid (MON810; DKC78-15B), a pyramid-gene Bt maize hybrid (MON89034; DKC78-45 BRGEN), and a non-Bt iso-hybrid (CRN3505). The MON810 hybrid expresses the Cry1Ab protein while the MON89034 hybrid expresses the Cry1A.105 + Cry2Ab2 proteins. All maize plants used in the study were grown in 5-l plastic pots (22.5 x 18 cm) in a horticultural tunnel. The plants were watered regularly and received a single application of Nutrifeed solution (Starke Ayres, Johannesburg, South Africa).

2.2.2 Insects

FAW larvae were collected in non-Bt maize fields at two localities were natural infestations occurred. These fields were in Malelane (-25.5645° S, 31.6573° E) and at the Makhatini Flats (27.3953° S, 32.1764° E). Larvae of these parental generations were transported to the insect rearing facilities at North-West University in Potchefstroom where rearing to the pupal stage took place on non-Bt maize plant leaves before the continuous rearing on different maize varieties commenced.

Rearing was done in a rearing chamber maintained at a 28 ± 1° C, relative humidity (RH) of 65 ± 5% and 14L:10D photoperiod.

After pupation of larvae of the field-collected populations, pupae were kept in small plastic containers with a height of 5 cm and a diameter of 3 cm. Upon emergence, male and female moths were paired and placed into 2 L plastic containers to lay eggs. Each container contained small wet crusher stones that helped to keep maize stem cuttings upright and maintain moisture. A 10% sugar-water solution and a maize stem cutting was provided in each container. Eggs that were laid were collected and kept in small plastic tubes which were then placed in a desiccator. The RH inside the desiccator was 70 ± 5%, which was kept constant by placing a potassium hydroxide solution in the bottom compartment of the desiccator according to the method of Solomon (1951). The desiccator was kept in a rearing chamber at 28 ± 1° C, relative humidity (RH) of 65 ± 5% & 14L:10D photoperiod until the neonates hatched.

The parental (field-collected) populations of each of the two different populations were kept separately according to the two localities at which collections took place. F1 neonate-larvae of each population were separated into three respective treatment groups (feeding groups), namely: non-Bt, MON810 and MON89034. Each of the two separate populations were reared separately four generations (F1 to F4), after which the moths of the F4-generation of the two different geographic populations were combined into a single population according to the feeding group treatment on which rearing took place. Moths that emerged from each generation were mated with moths that fed on the same maize variety as the larvae. Therefore, there was no genetic exchange between the different feeding groups. This process is illustrated in figure 2.1.

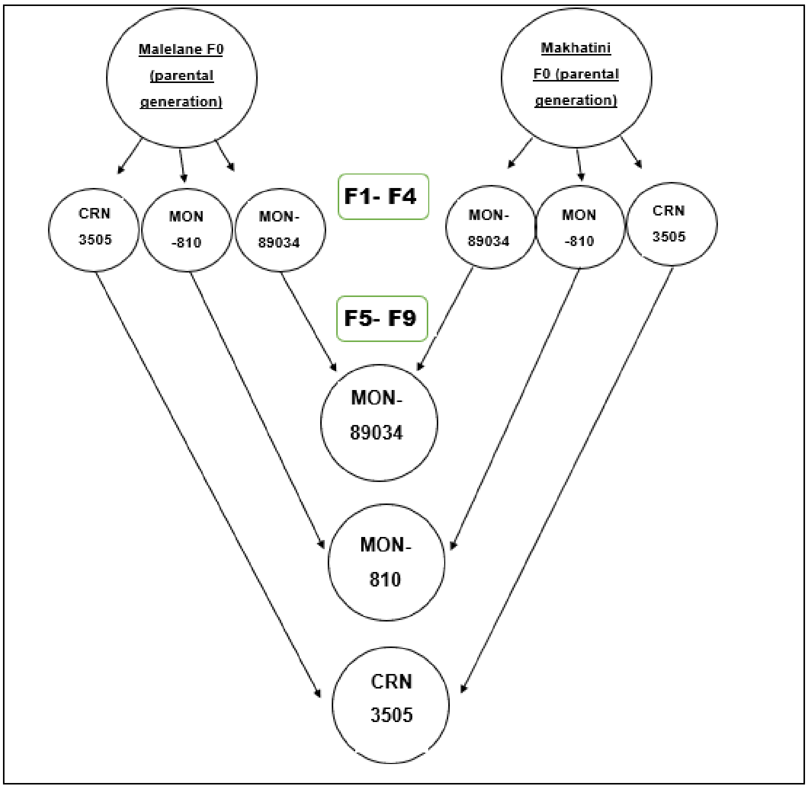

Figure 2.1. Diagram illustrating the process of how the two field-collected parental generations of *Spodoptera frugiperda* were divided into three separate feeding groups on different maize varieties and combined into a single population from the F5-generation onwards. CRN3505= non-Bt maize; MON810 and MON89034 are the Bt maize varieties.

Fourth-generation moths from both geographical areas which fed on the same maize cultivar were then put into a large cage where mating took place, proceeding mating, eggs were laid eggs. This was done to ensure mixing of the two geographical populations and genetic diversity of the populations that would be reared up to the 9th generation. Within each of the respective feeding groups, two hundred neonate-larvae were individually placed in plastic test tubes with aerated lids (height: 55 mm, diameter: 30 mm) and provided with fresh maize leaf tissue of the respective varieties every two days. This process was repeated until nine generations of rearing were completed.

Data collection

The number of surviving larvae were recorded at each feeding interval until pupation and the duration of the larval period calculated. Pupal mass was determined for each pupa separately, one day after pupation. The number of days from commencing of pupation to moth emergence was also recorded to determine the duration of the pupal period. The number of moths that successfully emerged from pupae was also recorded.

2.2.3 Statistical analysis

Data on larval duration, pupal mass and duration for the non-Bt, MON810 and MON89034 feeding groups and respective generations were tested for homogeneity of variance (Levene's test) and normality (Shapiro-Wilk test), but did not meet these assumptions. Data were therefore analysed by means of the non-parametric Kruskal-Wallis test followed by Duncan's multiple comparison post hoc test. Student t-tests were conducted where life history parameters were compared between only two of the feeding groups. The analyses were performed with TIBCO Statistica™ 13.3 (TIBCO Software, Inc., 2017).

2.3 Results

Rearing was successfully done for nine generations on non-Bt and MON89034 maize but the population reared on MON810 maize did not complete its development in sufficient numbers to continue with the F9-generation.

Results that compare the larval and pupal development periods at the end of the eight or nine generations of rearing are provided in table 2.1. The mean duration of the larval period after the 8th or 9th generations on non-Bt, MON810 and MON89034 maize leaf tissue differed significantly between feeding groups (H (2, N=3300) =1129.220; $p < 0.001$) (Table 2.1). The mean larval period of those that fed on MON89034 for nine generations was significantly longer compared to those that fed on non-Bt (5 days) and MON810 (2 days) maize. The mean larval period for those that fed on MON810 maize was significantly longer (4 days) than those fed on non-Bt. The shortest larval duration recorded was on non-Bt maize.

The mean pupal duration after eight or nine generations of populations that fed on non-Bt, MON810 and MON89034 maize differed significantly (H (2, N=2602)

=710.8333; p<0.001) (Table 2.1). The longest mean pupal duration was recorded on MON89034, which was significantly longer (≈ 2 days) than for the larvae that fed on non-Bt and MON810 maize. The shortest pupal period was recorded for larvae that were reared on leaf material of non-Bt maize.

The mean pupal mass of larvae that fed on the non-Bt, MON810 and MON89034 maize for eight or nine generations differed significantly (H (2, N=3299) =871.5186; p< 0.001) (Table 2.1). The lowest mean pupal mass was recorded for larvae that fed on MON89034, which was significantly lower than the pupal mass of larvae that fed on non-Bt or MON810 maize. The highest mean pupal mass was recorded for larvae that were reared on non-Bt maize. The variability in pupa size can be observed in figure 2.2.

Table 2.1. Mean (± SE) larval and pupal duration periods and pupal mass of the respective feeding groups **after** 9 generations of rearing on non-Bt maize and two Bt maize varieties. SE = Standard Error.

Maize variety	**Mean larval duration (days ± SE)**	**Mean pupal duration (days ± SE)**	**Mean pupal mass (g ± SE)**
non-Bt maize	15.63 ± 0.08a*	7.98 ± 0.04a	0.19 ± 0.001a
MON810	19.99 ± 0.10b	9.58 ± 0.05b	0.15 ± 0.001b
MON89034	20.14 ± 0.12b	9.72 ± 006b	0.13 ± 0.002c

*Means within columns followed by the same letter do not differ significantly.

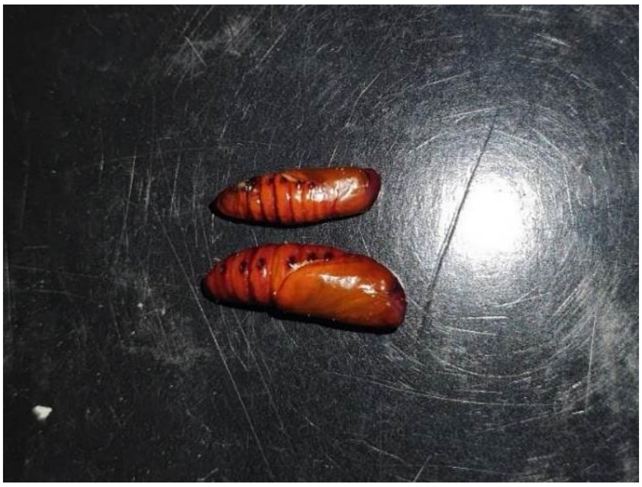

Figure 2.2. Size difference in pupae from the non-Bt maize (bottom) and MON89034 maize (top) feeding groups.

Larval survival

The percentage larval survival of the non-Bt maize feeding group remained high and relatively similar between generations (between 88.7 and 100%) over the duration of the study (Table 2.2). Larval survival on MON810 ranged between 16.4 and 95.9% over the duration of the study. The highest larval survival was recorded for the F7-generation (95.9%), whilst the lowest was recorded in the F8-generation (16.4%). Larval survival on MON89034 was generally lower than those of the non-Bt and MON810 feeding groups with the lowest in the F1-generation (19%), and the highest in the F7-generation (82.5%).

Larval development period

The mean duration of the larval period of F1-larvae differed significantly between feeding groups, with larvae taking approximately nine to ten days longer to develop to pupae on the two Bt maize varieties (Table 2.2). Comparison of larval development periods of different generations between feeding groups showed that larvae feeding on non-Bt maize completed life cycles in significantly shorter time periods than those on the Bt maize varieties. Variation in larval development rates over generations on non-Bt maize ranged between only 13.8 – 16.7 days compared to those on MON810 and MON89034 which ranged between 14.6 – 25.6 and 16.8 – 26.9 days respectively.

Comparisons of the mean larval development periods between generations within the non-Bt maize feeding group showed that it differed significantly (H (8, N=1515) = 439.717; p < 0,001) and that the developmental period was approximately two days shorter for the F7 and F8 generations compared to the F1-generation (Table 2.2). The F5-generation had the longest mean larval duration period (16.79 days), whilst the F7-generation had the shortest larval period (13.88 days). The duration of the larval development periods on MON810 differed significantly (H (8, N=1023) = 664.4635 p < 0,001) between generations, with the F1-generation having the longest mean larval duration period (24.64 days). Larval development periods of the F4 to F8 generations on MON810 were significantly shorter than those of the first two generations on MON810, with the development periods of the last two generations being the shortest (14.6 – 15.6 days) compared to the 25.6 days of the F1-generation. The mean duration of the larval period differed significantly (H (8, N=762) = 357.5116; p< 0,001) between generations for larvae that fed on MON89034. Within this feeding group, the F5 to F9 generation larvae (except F7) had significantly shorter development periods (16.8 – 19.8 days) than those of the first three generations (22.1 – 26.9 days).

Pupal mass

Mean pupal mass differed significantly between feeding groups and ranged between 0.16 and 0.23 mg for the non-Bt feeding group, and between 0.12 and 0.22 mg and 0.09 and 0.16 mg for the MON810 and MON89034 feeding groups respectively (Table 2.3). Pupal mass was significantly higher in the non-Bt maize feeding group compared to the two Bt maize varieties with that of the MON89034 also being significantly lower for nearly all the generations. The mean pupal mass of larvae that were reared on non-Bt maize differed significantly (H (8, N= 1514) = 319.3877; p< 0.001) between generations but were never lower for any generation except the F4 and F9, than that of the F1 or F2 generations (Table 2.2). In the MON810 feeding group, pupal mass of most of the generations from F4 to F7 were significantly higher (H (7, N= 1023) = 308.5312; p< 0.001) than those of the first two generations. None of the generations from F2 to F8 showed lower pupal mass than that of the F1-generation. From the F4-generation onwards the mean pupal mass for larvae that fed on MON89034 was significantly (H (7, N= 762) = 173.0119; p< 0.001) higher than those of the first three generations.

Table 2.2. Mean (± SE) larval duration and larval survival of *Spodoptera frugipera* over nine generations of rearing on three different maize varieties (feeding groups). SE = Standard Error.

	Larval duration (days ± SE)			Larval survival (%)		
Generation	**non-Bt**	**MON810**	**MON89034**	**non-Bt**	**MON810**	**MON89034**
F1	16.27 ± 0.13ab*A**	25.64 ± 0.23aB	26.95 ± 0.38aB	88.7 aA	73.8 aB	19.0 aC
F2	16.25 ± 0.12abA	23.07 ± 0.22bB	23.45 ± 0.34abB	93.3 aA	73.0 aB	24.5 aC
F3	16.09 ± 0.13abA	22.05 ± 0.25bB	22.11 ± 0.30bdB	91.7 aA	72.4 aB	30.1 aC
F4	16.15 ± 0.14abA	19.34 ± 0.18cB	20.42 ± 0.21cdC	100 bA	75.7 bB	42.3 abC
F5	16.79 ± 0.14aA	18.31 ± 0.24cB	18.97 ± 0.20efB	96.6 aA	44.0 bB	45.3 abBC
F6	15.96 ± 0.15bA	19.68 ± 0.37cB	19.84 ± 0.28ceB	93.3 aA	17.7 bB	23.6 aBC
F7	13.88 ± 0.11cA	14.61 ± 0.21dB	21.20 ± 0.24bdC	98.0 bA	95.9 bB	82.5 abC
F8	14.10 ± 0.15cA	15.63 ± 0.44dB	16.86 ± 0.24gC	94.7 aA	16.4 bB	31.6 aC
F9	15.93 ± 0.15ab***A******	-	17.73 ± 0.26fg***B***	95.3 aA	-	45.8 abB

*Means within columns followed by the same lower case letters do not differ significantly.
**Means within rows followed by the same higher-case letter do not differ significantly (student's t-test = -6.858; P < 0.00001).
***Means within the row followed by a different higher-case letter in italics for larval duration, do not differ significantly (student's t-test = 3.163; P = 0.001).

Table 2.3. Mean (± SE) pupal duration, pupal mass and moth emergence (%) for each respective feeding group over 9 generations. SE = Standard Error.

	Pupal mass (g ± SE)			Pupal duration (days ± SE)			Moth emergence (%)		
Generation	**Non-Bt**	**MON810**	**MON89034**	**non-Bt**	**MON810**	**MON89034**	**Non-Bt**	**MON810**	**MON89034**
F1	0.18 ± 0.003ab*A**	0.12 ± 0.003aB	0.09 ± 0.005aC	7.76 ± 0.08aA	10.75 ± 0.15aB	11.43 ± 0.22abB	83.6 A	69.6 B	57.5 B
F2	0.16 ± 0.003dA	0.12 ± 0.003aB	0.09 ± 0.005aC	7.59 ± 0.07aA	11.12 ± 0.15aB	9.79 ± 0.20cdB	92.3 A	69.1 B	61.2 B
F3	0.18 ± 0.003bA	0.13 ± 0.003aB	0.11 ± 0.004abC	7.84 ± 0.08abA	10.02 ± 0.16aB	11.29 ± 0.17aB	89.1 A	69.5 B	62.3 B
F4	0.23 ± 0.003eA	0.14 ± 0.002bB	0.12 ± 0.003bcC	7.82 ± 0.08abA	8.63 ± 0.11bB	10.00 ± 0.11bcC	95.3 A	75.8 B	70.9 B
F5	0.20 ± 0.003acA	0.14 ± 0.003bB	0.13 ± 0.003cdC	8.19 ± 0.08bcA	9.94 ±0.14aB	10.29 ± 0.10acB	95.8 A	84.2 B	77.9 B
F6	0.21 ± 0.003cA	0.14 ± 0.005abB	0.12 ± 0.004bcdB	8.45 ± 0.08cA	8.20 ± 0.30bA	9.44 ± 0.25ceB	97.1 A	47.2 B	25.3 C
F7	0.19 ± 0.003acA	0.22 ± 0.003cB	0.16 ± 0.003eC	8.51 ± 0.06cA	8.65 ± 0.12bAB	8.88 ± 0.17defB	96.3 A	83.5 BC	41.4 BC
F8	0.18 ± 0.003abA	0.13 ± 0.005abB	0.13 ± 0.003cdB	7.68 ± 0.08aA	8.76 ± 0.32bB	8.45 ± 0.15efB	100 A	45.6 B	54.1 B
F9	0.16 ± 0.003bd***A******	-	0.15 ± 0.004de***A***	7.82 ± 0.09a***A*******	-	8.08 ± 0.15f***A***	85.3 A	-	61.7 B

*Means within columns followed by the same lower-case letter do not differ significantly.
**Means within rows for each life history parameter followed by the same higher-case letter do not differ significantly.
***Means within the row followed by the same higher-case letter in italics do not differ significantly (Student t-test = 3.163; P = 0.001).
****Means within the row followed by the same higher-case letter in italics for separate life history parameters do not differ significantly (Student t-test = -1.630; P = 0.104).

Pupal duration

The mean duration of the pupal period differed significantly between feeding groups and ranged between 7.59 and 8.19 days for the non-Bt feeding group, and between 8.2 and 11.1 days 9.0 and 11.4 days for the MON810 and MON89034 feeding groups respectively (Table 2.3). The pupal periods of larvae reared on the Bt maize varieties were significantly longer than those on the non-Bt maize variety. There were however no significant differences between the pupal periods recorded between generations within the two Bt maize feeding groups for nearly all the generations. The mean pupal periods of larvae that were reared on non-Bt maize differed significantly (H (8, N=1404) = 159.2482, p< 0.001) between generations and mean pupal periods of all successive generations were either equal to or longer than that of the F1-generation (Table 2.3). In the MON810 feeding group, the pupal period became significantly (H (7, N=750) = 226.4479; p< 0.001) shorter towards later generations, with those of the last three generations being significantly shorter than that of the F1 to F3-generations. The mean pupal period of the MON89034 feeding group also became progressively shorter over generations with significantly (H (8, N= 448) = 218.1082; p< 0.001) shorter periods recorded for the last four generations.

Moth emergence was significantly higher on non-Bt maize (83.6 – 100%) than on MON810 (45.6 – 84.2%) and MON89034 (25.3 – 77.9%) (Table 2.3). The percentage moth emergence for the population reared on non-Bt maize remained relatively similar over the duration of the study. Percentage moth emergence for the Bt maize feeding groups did not differ significantly between groups for most of the generations and ranged between 45.6 and 84.2% for the MON810 and between 41.4 and 77.9% for MON89034.

2.4 Discussion

By exposing successive generations of FAW to Bt-maize expressing Cry proteins, and by comparing the life history parameters of different populations feeding on non-Bt and Bt-maize cultivars, changes in life history parameters and possible costs in terms of fitness were identified.

Larval survival

Results indicated that the fitness of the population maintained on non-Bt maize was not affected during the rearing process over nine generations. Larval survival on non-Bt maize was high (88-100%) over all generations, and fitness of this feeding group after nine generations of rearing was similar to that of the 1st generation. The results observed on the effects exposure to two Bt maize varieties on various life history parameters of FAW populations can therefore be ascribed to susceptibility of *S. frugiperda* to the Cry proteins expressed in these plants.

Larvae exposed to MON810 maize showed lower survival rates (16.4 – 95.9%) than larvae reared on non-Bt maize while survival on MON89034 was the lowest (19.0 – 82.5%). These levels of survival on Bt maize are higher than those reported by Botha *et al*. (2019) who used similar methods and rearing conditions to those used in this study.

Sousa *et al*. (2016) reported survival of between 20-80% on Cry1Ab maize, whilst Botha *et al.* (2019) reported moderate survival of between 20 and 86% after ten days on MON810 maize for nine *S. frugiperda* populations from South Africa. In this study, the high levels of larval surival on MON810 maize were to be expected, as Omoto *et al*. (2016), Roush *et al*. (1997) and Onstad *et al*. (2013) reported that MON810 maize cultivars do not meet the requirements of a high-dose. Additionally, Huang (2020) and Sousa *et al.* (2016) previously also reported that MON810 maize varieties do not produce a dose high enough to cause the death of RS individuals of *S. frugiperda*. Furthermore, the partial efficacy of Cry1Ab maize against FAW in South Africa is similar to results reported by Niu *et al.* (2016), where high rates of survival and resistance against Cry1F toxins were recorded in *S. frugiperda* populations collected in Puerto Rico

Botha *et al*. (2019) reported minimal survival (less than 1%) on MON89034 maize leaf tissue after ten days, with larvae not being viable and not completing development to the pupal stage. Similarly, Bernardi *et al*. (2016) also reported a 100 % mortality for larvae reared on Cry1A.105 + Cry2Ab2 maize. The high mortality of larvae on MON89034 is ascribed to the presence of two Cry proteins (Cry1A.105 + Cry2Ab2) in this maize variety, therefore killing all SS and most RS individuals (Huang, 2020). Recent research conducted by crossing a known single-gene resistant population (Cry1A.105) with another known single-gene resistant population (Cry2Ab2), yielded a *S. frugiperda* population that was resistant to dual-gene Bt maize cultivar (Cry1A.105/Cry2Ab2) (Niu *et al*., 2019; Zhu *et al*., 2019).

Larval survival rates of both feeding groups on Bt maize varied between generations. However, a sharp increase in survival in the F7-generations indicated an increase in resistance within the population. This was followed by a decrease in F8-generation. The explanation for this decrease is unclear. It was observed that a large number of the F8-larvae that were offspring of F7-moths had unusually extended larval duration periods of up to 27 days and where after larvae ceased feeding on plant material. Subsequently, larvae would attempt to form pre-pupae, where-after pre-pupae turned black in colour and died (Figure 2.3a). As the F7-generations of Bt-reared larvae had high survival rates, it is possible that the frequency of resistance alleles within the F7-population was high, resulting in the transfer of resistance to offspring in the F8-generation. However, the low incidence of successful pupa formation observed in the succeeding F8-generations could indicate a possible decline in fitness associated with resistance evolution.

The high levels of larval survival in the MON810 and MON89034 feeding groups, and successful reproduction of follow-up generations illustrated that a high frequency of the individuals within these feeding groups were resistant to the Cry proteins produced by the respective maize varieties. Furthermore, this indicates that alleles with resistance to Cry1A.105 + Cry2Ab2 proteins were possibly present in the *S. frugiperda* population that invaded Africa, and that the Cry1Ab event does not meet the required high-dose criteria (Botha *et al.,* 2019). Gould (1998) defined a high-dose event, in the context of Bt crops, to be a dose that kills 99.99% of susceptible individuals in the field.

Larval duration

Duration of the larval period was significantly delayed for larvae reared on both MON810 and MON89034 maize cultivars for all generations, compared to those reared on non-Bt maize. These results are supported by the findings of Botha *et al*. (2019) who reported delays of up to 10 days in larval periods for populations feeding on MON810 plant material. Botha *et al*. (2019) reported larval developmental periods of between 18.3 and 23.6 days for nine different populations, which is similar to the range reported in this study (14.6 to 25.6 days). Sousa *et al*. (2016) also found that FAW larvae that fed on maize tissue containing Cry1Ab toxins exhibited a seven-day delay in developmental time when compared to larvae that fed on non-Bt maize tissue. Similar observations were made by Buntin *et al*. (2001) and Omoto *et al.* (2016), who reported extended periods of larval development for larvae feeding on Cry1Ab maize leaf tissue. Larval duration within the MON810 feeding group for the 1st generation had a mean of 25.64 days, which is high compared to the 1st generation of the non-Bt feeding group (16.27 days). For the population feeding on MON810, the larval duration of the 1st generation was approximately 24 days, whereas the larval duration of the 7th generation was significantly shorter (approximately 14 days). Results therefore indicate increased levels of resistance to the Cry1Ab protein over successive generations since the larval duration period became shorter with each generation. We can therefore assume that the response of FAW populations to Bt toxin exposure is to select for resistance within the population.

Lengthy larval duration periods were exhibited in the MON89034 feeding group, with some larval development periods being as long as 30 days. Similar research conducted by Botha *et al.* (2019) showed the survival of only one larvae on MON89034 leaf tissue, with an extended larval period of 29 days. This is similar to results of Hardke *et al*. (2011) who reported only 25-76% of larvae that fed on maize containing Cry1Ab proteins successfully pupated. Transgenerational effects were also reported by Sousa *et al*. (2016) who showed that prolonged developmental times were one of the main observations when continuously exposing larvae to Bt toxins, regardless of previous exposure to Cry proteins. Shorter developmental delays for larvae exposed to Cry1Ab toxins over generatons were recorded in larvae that were previously exposed to Cry1Ab maize under field conditions (Sousa *et al*., 2016). Furthermore, Sousa *et al*. (2016) reported that exposure of immature stages of FAW over generations drastically effected the rates of larval survival, growth, development, as well as larval and pupal weights. Sousa *et al.* (2016) reported that larvae reared in on non-Bt had an average larval developmental time of 21.3 days, whilst larvae feeding on Cry1Ab maize foliage had an average larval development time of 28.5 days, resulting in a 7-day delay. The longer developmental times recorded by Sousa *et al*. (2016) could however also be attributed to larvae being reared at lower temperatures (25 ± 2 °C) in comparison to this study. Studies done on the sub-lethal exposure of *H. armigera* neonates to Bt cotton showed that the duration of various life stages was significantly affected (Sedaratian *et al*., 2012)

Pupal mass

The mean mass of pupae from the two Bt maize feeding groups were significantly lower than those of all the generations of the non-Bt maize feeding group. The pupal mass of the MON89034 group was significantly lower than that of the MON810 group for six of the eight generations, which shows the higher level of susceptibility of FAW to MON89034 maize. Botha *et al*. (2019) reported moderate survival of FAW populations feeding on Cry1Ab maize, and that larvae reared on Bt maize could weigh as much as 82% less than larvae reared on non-Bt maize. Lower incidences of pupae formation were reported for larvae feeding on Cry1Ab maize (Botha *et al.,* 2019), and Hardke *et al.* (2011) reported pupation rates of 25-73% of larvae reared on Cry1Ab maize. Mean pupal mass of the F1-generation of the MON810 feeding group was significantly lower than that of any generation within the control population, also indicating adaptation to Cry1Ab protein over generations. Initially, very long pupal periods were observed and moths from the MON810 feeding group took at least three to four days longer to emerge than those of the non-Bt maize feeding group. A decrease in pupal period was observed over generations, conversely by the 8th generation, moths from the MON810 feeding group still took longer to emerge from pupae than moths from the non-Bt feeding group. The delay in development of this feeding group could be ascribed to extended larval stages, which is similar to findings by Omoto *et al*. (2016).

Omoto *et al*. (2016) ascribed the lower mass of larvae feeding on MON810 to growth inhibition, due to the susceptibility of larvae to Cry1Ab toxins. Similarly, Sousa *et al*. (2016) observed a reduction in weight gain of *S. frugiperda* larvae that fed on Cry1Ab maize foliage when compared to weight gain of larvae that fed on non-Bt maize foliage. Sousa *et al*. (2016) reported that larvae feeding on non-Bt maize foliage weighed 0.257 g while those reared on Cry1Ab maize weighed 0.048 g after 14 days. However, compensation by FAW populations for the sublethal effects of Bt toxins on growth and development was recorded by Sousa *et al*. (2016). The latter study also reported that the relative difference in reduction of pupal mass between larvae feeding on Cry1Ab and non-Bt maize foliage was much smaller than the differences in larval mass between the two feeding groups (Sousa *et al.,* 2016; Botha *et al*., 2019).

Pupal duration

Mean pupal duration of the MON810 and MON89034 feeding groups was longer than that of the non-Bt feeding group for almost all generations. This reduction in fitness is also ascribed to the delaying effect of Bt toxins on larval development (Jakka *et al.,* 2014). Yet, similar studies done by Sosa-Gomez and Miranda (2012) to determine costs associated with resistance, found that no or very little costs in terms of pupal stages, adult longevity and survival, as well as fecundity and fertility, were associated with resistance to Cry1Ac maize. Botha *et al*. (2019) reported that all of the F1-generations of nine populations of *S. frugiperda* exposed to Bt toxins exhibited low pupation rates preceeded by extended larval periods.

Moth emergence

The initially high rates of moth emergence were also followed by varying emergence rates. Pupae from MON810 showed signs of deformation and many died (Figure 2.3 b). This observation was similar to that by Botha *et al.* (2019) where approximately 86% of larvae feeding on MON810 survived up to the 10th day, but only 54% of larvae were able to successfully form pupae. Moth emergence in the MON89034 feeding group showed a steady incline up to the F5-generation followed by a decline. From the onset of pupation, damage was observed in many of the pupae, and pupae that appeared undamaged yielded moths that had considerable damage to wings and other body parts, inhibiting them from flying and reproducing (Figure 2.3 c and d). Botha *et al*. (2019) also described lower incidences of pupa formation in populations that fed on maize tissue containing Bt toxins.

Another observation made in the MON89034 feeding group was that moths that emerged and reproduced did not have a very long life span (data not shown). These moths were only able to lay small clusters of eggs that had a luminescent green glow, which were prone to drying out and only a small number of neonate larvae emerged from these egg batches. (Figure 2.3 e, f, g and h)). The number of eggs within batches could not be quantified due to the multi-layering of batches and the necessity of leaving eggs unhindered.

Other studies did not provide any sufficient evidence to support the finding of the green luminescence of eggs produced by MON89034-resistant moths.

This study showed that *S. frugiperda* fitness was initially very low on the Bt maize varieties but that fitness in terms pupal mass and the duration of larval and pupal periods improved slightly over generations. The tendency that larval survival increased over generations indicate that there was successful adaptation to selection pressure and that survival was actually very high, viewed against the back ground of the expected high-dose response to MON89034 maize. A tendency of increased pupal mass and shorter larval and pupal periods was also observed over generations on both the Bt maize varieties. This indicates successful selection for resistance alleles in the two Bt maize feeding groups over successive generations. It was reported by Honěk (1993), that shorter developmental periods, increased larval and pupal mass and moth fecundity and fertility are associated with higher suitability of a food source for a particular insect. The increase in fitness, based on the life history parameters evaluated in this study may therefore be indicative of the adaptation of the two *S. frugiperda* feeding groups to the Cry proteins expressed by these maize varieties, and the selection of resistant individuals over time.

The parental generations of FAW used in this study were most likely exposed to Bt maize at the Malelane site where collections took place. This was not the case at the site on the Makhatini Flats. In this study, these two populations were however mixed from the 5th generation onwards. The genetic background plays a pivital role in determining the response of *S. frugiperda* populations when exposed to Cry proteins, and variable responses were recorded for populations collected from different geograhical areas in South America (Monnerat *et al.,* 2006). A study done on *S. frugiperda* populations that were previously exposed to Cry1Ab maize hybrids under field conditions (Sousa *et al*., 2016), showed high levels of survival (22.8-73.6%) and reproduction of RS individuals previously exposed to Cry1Ab under laboratory conditions. These larval survival rates reported by Sousa *et al*. (2016) is similar to the ranges of survival observed in the MON810 feeding group during this study. Results from Sousa *et al.* (2016) also reported increased fitness of larvae of some populations over generations on MON810 maize. Three out of five populations previously exposed to Cry1Ab maize under field conditions, showed no reduction in terms of fitness when feeding on MON810 maize varieties, compared to larvae of populations that were collected from non-Bt maize fields (Sousa *et al.*, 2016). The repsonses of FAW to Cry toxins prodcued in Bt maize cultivars will therefore yield variable results dependant on previous exposure as well as the presence of resistance allels within populations (Sousa *et al*., 2016).

*Spodoptera frugiperd*a is the only pest that has developed practical resistance, accompanied by field control failures of Bt maize in multiple countries across the world (Storer *et al*., 2010; Farias *et al*., 2014; Huang *et al*., 2014, 2020; Omoto *et al*., 2016; Chandrasena *et al*., 2018). Luong *et al*. (2016) evaluated survival two different *Helicoverpa amigera* (Hübner) (Lepidoptera: Noctuidae) strains (susceptible and resistant) on non-Bt and Bt-cotton (Bollgard II®) and observed a significant difference between the mortality of susceptible larvae on Bt-cotton vs non-Bt cotton. An interesting find by Arshad *et al*. (2009) was that susceptible larvae were more likely to feed on flower structures of Bt-plants, which was revealed to contain low concentrations of Bt toxins. In doing so, these larvae observed by Luong *et al*. (2016) increased chances of survival by ingesting tissue that exposes them to relatively low-doses of Bt toxins. The latter results therefore showed no significant differences between the survival rates of neonates feeding on the flowers of Bt and non-Bt plants

As confirmed by findings of Sousa *et al*. (2018), the generational and continuous exposure of *S. frugiperda* to Cry proteins affected and changed duration of larval developmental periods, as well as larval survival. Similarly, a decrease in pupal weight was accompanied by a decrease in moth emergence. This therefore supports the theory that high selection pressure exerted on populations by exposure to Bt toxins causes changes in life history parameters of populations (Tabashnik *et al.,* 1994). Comparison of the duration of the larval development period between different feeding groups show that larval duration on MON89034 was significantly longer than those of the other feeding groups. According to Sousa *et al*. (2016), the reduction in fitness of resistant individuals within populations allows for natural selection against resistance development.

The high-dose and refuge strategy is the most recognized and accepted method for delaying the evolution of resistance in pest populations (Tabashnik, 1994). This strategy ensures the death of RS individuals due to the high dose of Cry proteins in Bt maize, sustaining of SS individuals becomes possible by planting non-Bt refuge areas. The non-Bt maize refuge provides SS individuals with a chance at survival in predominantly Bt maize fields, and provides suitable mates for surviving RR individuals from designated Bt areas. The mating of SS and RR individuals produces RS individuals, which will then in turn not survive when feeding on Bt maize that has a high concentration of Cry proteins, ensuring no build-up of resistance alleles within populations (Jakka *et al*., 2014). The HDR strategy relies on certain assumptions that have to be met in order to be successful in the delay of resistance evolution. If these assumptions are not met, the rate of resistance evolution is rapid (Ives and Andow, 2002). Findings by Santos-Amaya *et al*. (2015) showed that the assumptions of the HDR strategy did not fit the genetic basis of resistance, which would lead to the rapid evolution of resistance within pest populations that are exposed to Bt toxins under such circumstances.

Whilst recognition has been given to the importance of sub-lethal effects of Bt toxins on target pests of genetically modified crops, studies on the lethal and sub-lethal effects of Bt toxins on the life history parameters of *S. frugiperda* and other target pest species are rare (Sousa *et al*., 2016).

2.5 Conclusion

This study describes the changes in fitness that occur after generational exposure of susceptible populations of *S. frugiperda* to maize tissues containing Bt toxins. The onset of resistance development within FAW populations goes along with changes in various life history parameters. The life history parameters that were affected the most in *S. frugiperda* populations exposed to Bt maize were decreases in percentage larval survival, as well as the extension of larval and pupal development periods. The significance of these findings indicate detrimental effects with regard to efforts such as the HDR strategy, which aim to delay resistance evolution within target pest species. Results indicate that assumptions necessary to be met, in order for the successful implementation of the HDR strategy, will not be met and will ultimately result in failure of efforts made to reduce the resistance alleles within populations.

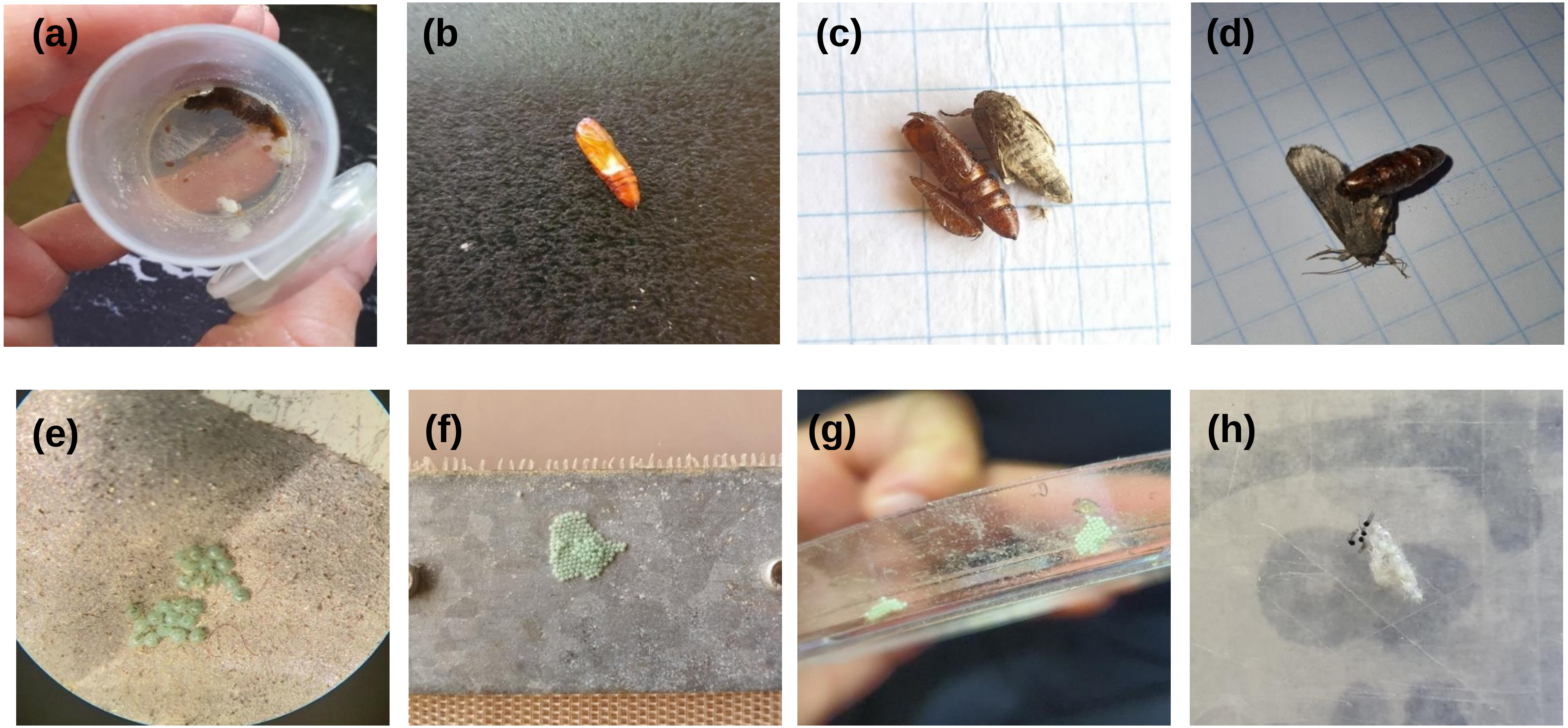

Figure 2.3. (a) Pre-pupae from MON89034 after failure to pupate, (b) one-day old pupa on MON810 showing signs of damage, (c) due to malformation observed in many pupae, MON89034 moths struggled to emerge from pupal casing and damaged wings, (d) moth from MON810, with pupal casing stuck to damaged wing, (e) microscopic view of fluorescent green egg batch laid by moth reared on MON89034, (f) egg batch of moth from MON89034, (g) small size of egg batch of moth from MON89034, (h) only four neonate larvae emerge from relatively dry MON810 egg batch.

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Chapter 3

The effect of transgenerational exposure of *Spodoptera frugiperda* larvae to Bt maize on larval preferences and moth flight ability

Abstract

The success and sustainability of Bt crop varieties to control target pest populations is threatened by the evolution of resistance against Bt toxins by target pests. *Spodoptera frugiperda*, which is one of the most damaging maize pests worldwide has evolved resistance to Cry proteins expressed in Bt maize. In an attempt to delay the evolution of resistance, insect resistance management (IRM) strategies must be implemented in fields where Bt crops are planted. This study evaluated the effects of generational exposure to Bt toxins on food preferences of neonate larvae, as well as the effect on flight ability of *S. frugiperda* adults. Results of no-choice tests showed that larvae that were exposed to Bt toxins over generations showed a reduced preference for leaf tissue of these maize varieties. In two-choice tests with non-Bt maize and Bt maize leaf tissue, there was a significant preference for non-Bt maize tissue. In multiple-choice tests, non-Bt maize was also the predominant choice of most larvae, regardless of feeding group. This could indicate that FAW larvae have the ability to detect the presence of Bt toxin within maize tissues. Results obtained from moth flight ability tests revealed lower flight ability of moths that were generationally exposed to Bt toxins, compared to the flight ability of the population not exposed to Bt toxins. Findings from larval choice tests as well as moth flight ability tests can result in the decrease in effectiveness of the HDR strategy as: larvae showing feeding avoidance behaviour will not feed on plant material containing Bt toxins, therefore permitting the survival and mating of all heterozygous individuals, eventually leading to the exponential increase of resistance alleles within populations. Additionally, the poor flight ability of moths exposed to Bt toxins will not result in optimum mating behaviours, as resistant moths will not be able to fly to refuge areas where homozygous susceptible moths reside.

Key words: Bt maize, Cry proteins, flight ability, pest management, *Spodoptera frugiperda*, resistance

3.1 Introduction

Pest biology, feeding behaviour and movement between host plants plays a pivotal role in determining the level of exposure of target pests on Bt maize to Cry toxins expressed by these genetically modified plants (Visser & Van den Berg 2020). These aspects should therefore be taken into consideration when developing and implementing insect resistance management (IRM) strategies. The longevity and sustainability of Bt maize that expresses insecticidal Bt proteins is threatened by the evolution of resistance in target pest species (Tabashnik *et al*., 2013). Over the past decade, several lepidopteran pest species have evolved resistance to Cry proteins (Tabashnik *et al.,* 2013). Field resistance to Cry 1F proteins in Bt maize varieties has been reported in *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) populations in Puerto Rico, Brazil, North America (Yang *et al*., 2018) and Argentina (Zhu *et al*., 2019). Resistance alleles against the relatively new Bt protein, Vip3A, were also detected in field populations collected in the USA and Brazil (Zhu *et al.,* 2019). Several IRM strategies can be deployed to assist in the management of resistance evolution. Seed mixtures is an IRM approach which is implemented against lepidopteran pests and is often referred to as 'refuge in a bag' strategy. Seed mixtures imply the mixing of Bt and non-Bt seeds at pre-determined ratios, which allow for the simplification of refuge requirements (Erasmus *et al*., 2016; Towles *et al*., 2021). The High Dose and Refuge (HDR) strategy is however the most commonly used IRM strategy and is based on a number of assumptions regarding the ecology and biology of target pest species as well as interactions with host plants (Tabashnik *et al.,* 2013).

The HDR strategy is based on the following assumptions: 1) there is an initially low frequency of resistance alleles within populations, 2) the resistance allele is functionally recessive, and 3) random mating will occur between resistant moths who successfully completed lifecycles on Bt maize cultivars, and susceptible moths produced from areas of refuge (Tabashnik & Carrière, 2001). Furthermore, any IRM strategy is more effective if the feeding stage behaviour of the pest is indicates limited movement between host pants (Panuti *et al*., 2016). Movement off, as well as between host plants could result in larvae being exposed to sub-lethal doses of Bt toxins, which would in turn increase selection for RS individuals and lead to an increase in the rate of resistance evolution (Razze & Mason, 2012; Davis & Onstad, 2000, Heuberger *et al*., 2011; Ives *et al*., 2011). It is also a requirement that moths that emerge from refuges should be of high quality to ensure normal flight behaviour (Head, 2004). Decreased fitness of moths that survive on Bt maize may also influence movement and mating incidence with susceptible moths. Larval preference and subsequent migration behaviour as well as moth fitness are therefore relevant to refuge design.

In a cropping system where the HDR strategy is implemented, the goal would be for larval food plants to consist either of maize containing no Bt toxins (refuge planting), or maize expressing a lethal dose of Bt toxins (Gould, 1998). According to Juma *et al*. (2008) and Zaluki *et al*. (2002) not only gravid females, but also neonate larvae demonstrate preferences when it comes to host plant selection. Physical and chemical characteristics play major roles in the process of host selection by herbivores, and there is a need for practical methods to evaluate the effect of exposure to Bt traits on the altering of physical and chemical cues released by host plants (Turlings *et al*., 2005; Han *et al*., 2016). Han *et al*. (2016) and Turlings *et al*. (2005) showed that molecular and physical characteristics of plants that play a role in host plant selection may be altered once crops have been genetically modified. According to Nascimento *et al*. (2020), the selection of appropriate host plants is a critical in determining the success and survival of neonate larvae, as neonate larvae are especially vulnerable to defence mechanisms exhibited by plants. Nascimento *et al*. (2020) reported that initial dispersion of neonate larvae from hatching site is inevitable, as FAW adults are known to produce large egg batches. Upon hatching, neonate larvae disperse in order to reduce intraspecific competition, reduce the effects of cannibalism and to prevent depletion of food resources (Singer *et al.*, 2002). The final stages of host plant acceptance therefore include the occupation and feeding of larvae on host plants. Any factor that could alter pest interactions with Bt plants could have an impact on the success of HDR strategy and sustainability of Bt crops (Visser *et al*., 2019). Plant abandonment by neonate larvae in Bt maize fields could result in larvae consuming varying proportions of toxic and non-toxic maize tissue, therefore influencing toxicity levels and putting the HDR strategy at risk (Gould, 1998).

It is presumed that FAW larvae do not migrate over long distances and are limited in terms of mobility (Rojas *et al.*, 2018). Neonate larvae are able to disperse randomly through ballooning, which occurs when wind sweeps larvae up by catching onto silk threads produced by silk glands. In the absence of wind currents, larvae use this silk thread to balloon off plant structures until contact is made with an alternative host plant or the ground (Rojas *et al.,* 2018). Larvae generally disperse from the original oviposition/hatching site in order to occupy suitable host plants within a limited area (Zalucki *et al.,* 2002), a behavioural trait that will be accentuated if larvae exhibit non-preference for host plants such as Bt maize.

Nascimento *et al*. (2020) recommended urgent evaluation of the effects of transgenic crops on the foraging behaviour of insects. By using maize hybrids that consist of identical morphological compositions, with the only difference being genetic modification for production of Bt toxins, it was found by Nascimento *et al*. (2020) that FAW larvae avoided leaf-tissues containing Bt toxins. This indicates that larvae have the ability to detect the presence of toxins within leaf tissues (Nascimento *et al*., 2020). Nascimento (2020) and found that no link existed between ovipositional preferences of FAW moths and preferences of FAW larvae and reported that moths preferred to lay eggs on transgenic hybrids whilst non-Bt hybrids were the preferred food choice of larvae. Goncalves *et al*. (2020) found that the oviposition preference of gravid female *S. frugiperda* moths on maize plants was completely random, though a slight preference was shown for plants which had minimal prior herbivory and favourable physical plant traits, which could be the reason why female moths seem to prefer Bt plants as there is little herbivory exhibited.

Fall armyworm adults are nocturnal and are considered to have great flight abilities (Johnson, 1987). In order to maximize the effectiveness of IRM strategies, the fitness of individuals should be assessed by tracking the dispersal of individuals, e.g., flight ability (Vilarinho *et al*., 2011). Vilharinho *et al*. (2016) used mark-release-recapture techniques to record dispersal capacity of *S. frugiperda* adults, results determined showed that the furthest recapture distance for males was 806 m and for females 608 m. Van Handel (1974) tracked and reported 16-30 hrs of tethered flight by FAW males. A study conducted by Liang *et al.* (2007) showed that flight ability can be influenced by the presence of Cry proteins in the larval host plant. Liang *et al.* (2007)

compared the flight ability of a Cry1Ac-resistant (BtR) population of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) to that of a susceptible population, and reported that the flight distance of Bt-resistant individuals which were reared on diet free of Bt toxins was 56.2 km, almost double the distance of that of individuals feeding on plant material containing Bt toxins, which was 26.2 km. It was noted by Liang *et al.* (2007) that the resistant strain of larvae that were able to complete its lifecycle whilst feeding on Bt plant material yielded moths that exhibited a weaker flight ability overall, compared to months that were yielded from susceptible larvae fed on non-Bt diets. It should be highlighted that the effect of Bt toxins on the flight ability of FAW adults has not yet been extensively researched, although flight is a major component of the HDR strategy when it comes to mating of moths from separate Bt and refuge areas. In addition, the spatial scale of Bt and refuge maize relative to the movement of adult moths must be taken into consideration. If homozygous resistant (RR) moths are unable to fly sufficient distances in order to find homozygous susceptible (SS) mates, or vice versa, this could compromise the success of HDR strategies (Gould, 1998). The ballooning behaviour (Rojas *et al*., 2018) and preference of larvae of the target pest species for Bt or non-Bt maize plants (Visser *et al*. 2019), as well as the fitness of moths to fly, mate and select suitable host plants for oviposition (Rojas *et al*., 2018) therefore directly influences the efficacy of an IRM strategy.

This study tested the hypothesis that the utilization of Bt maize as a host plant over different generations affected the preferences of larvae for Bt maize tissue, and caused a decline the flight ability of adults.

3.2 Materials and methods

The *S. frugiperda* larvae and moths used in these bioassays were reared for nine generations on either non-Bt maize, or one of two Bt maize varieties (feeding groups). Larval preferences for Bt and non-Bt maize as well as flight ability of moths of which larvae were reared on these maize varieties were determined after completion of each of the generations.

The following three maize varieties were used: 1) a single-gene Bt maize hybrid (MON810; DKC78-15B), 2) a pyramid-gene Bt maize hybrid (MON89034; DKC78-45

BRGEN), and 3) a non-Bt iso-hybrid (CRN3505). The MON810 hybrid expresses the Cry1Ab protein while the MON89034 hybrid expresses the Cry1A.105 + Cry2Ab2 proteins. All maize plants were grown in 5-l plastic pots (22.5 x 18 cm) in a horticultural tunnel. The insect rearing procedures as well as the plant growth conditions are described in Chapter 2.

3.2.1 Larval preference bioassays

Bioassays were conducted to determine if changes in neonate larval preference occurred over generations if *S. frugiperda* was continuously reared on either Bt or non-Bt maize over generations. Larval preferences were also compared between each of the feeding groups over generations.

The feeding preferences of neonate larvae were recorded by administering no-choice, two-choice and multiple-choice tests. These assays were conducted in petri dishes (diameter: 12 cm) that were fitted with a sheet of moist filter paper to retain moisture and prevent desiccation of plant material and neonate larvae. Leaf tissue was cut from the inner whorls of maize plants from the three different varieties, and a standardized size of maize leaf material (3 x 3 cm) was used in preference tests throughout the study. Each of the three choice-tests were replicated four times. Each repetition consisted of five petri dishes, each containing one larva, resulting in twenty larvae per maize variety (no-choice) as well as for each of the maize variety combinations (two-choice and multiple-choice).

The no-choice tests were conducted by placing the maize leaf tissue in the centre of the petri dish and placing the neonate larvae on the inside perimeter of the petri dish, 3 cm from maize tissue (Figure 3.1 (a)). For the two-choice tests, two pieces of maize leaf tissue from different cultivars were placed on the outside perimeter of a petri dish and the neonate larvae were placed in the centre of the petri dish (Figure 3.1 (b)). In the multi-choice tests, similarly to the two-choice tests, the maize leaf tissue was placed on the outside perimeter of the petri dish and one neonate larvae was placed in the centre (Figure 3.1 (c)). Following the insertion of the larva, all petri dishes were closed and sealed by means parafilm. Petri dishes were then placed in a temperature-controlled room for the duration of the study. The position of each larvae was recorded after 24. Wandering larvae that were not present on leaf tissue were also noted as no choice. Petri dishes were kept in a rearing chamber at a temperature of 28 ± 1 °C, RH of 65 ± 5% and a 14L: 10D photoperiod.

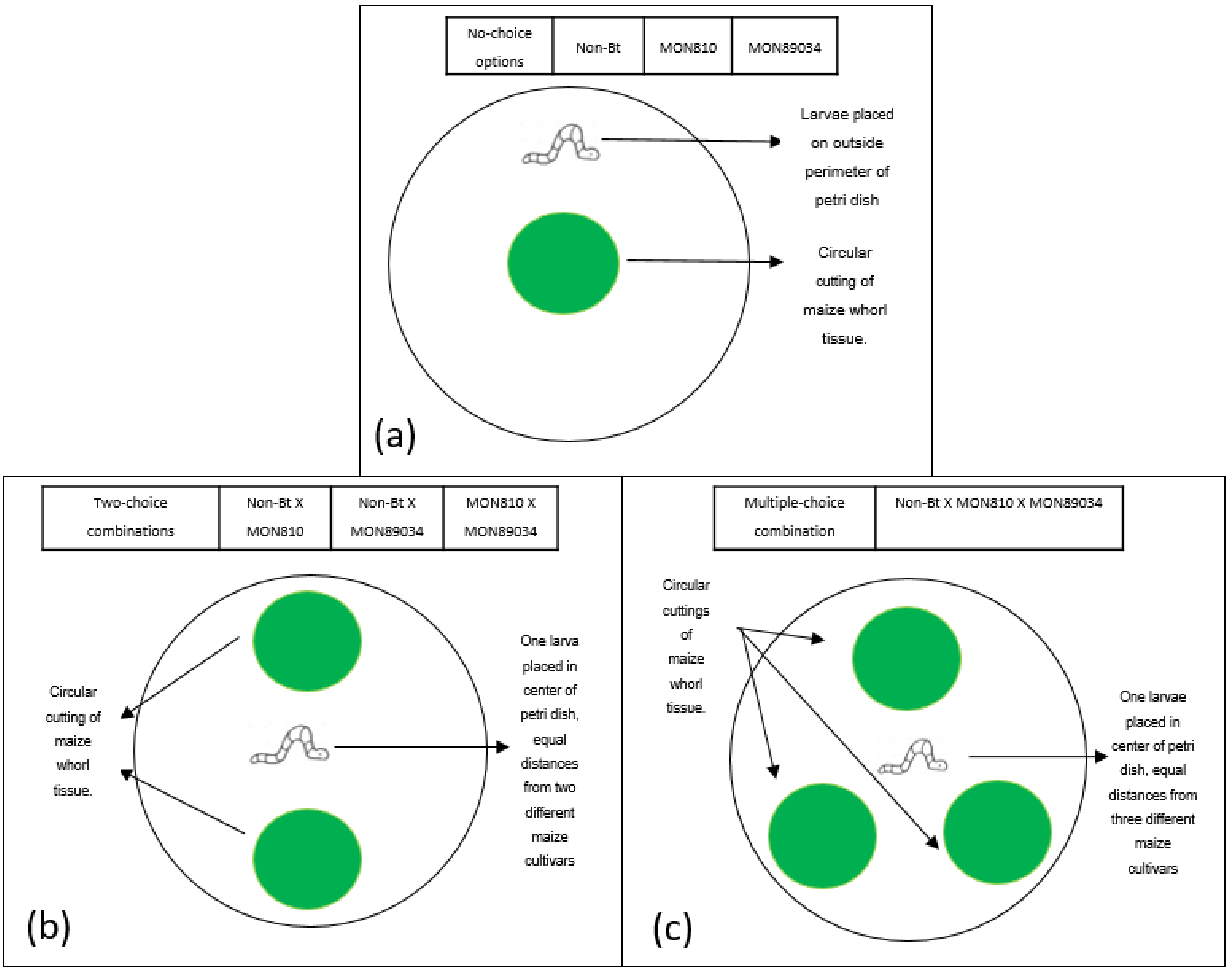


Figure 3.1. Diagrams illustrating the experimental set-up of (a) no-choice tests, (b) two-choice tests and (c) multiple-choice tests.

3.2.2 Flight ability tests

All female moths that emerged from each of the F1 to F9 generations of the respective treatment groups were used to determine flight ability after larvae were reared on the respective maize varieties. White plastic cylinders of different heights were used to evaluate the ability of moths to fly out of these cylinders, similar to methods used for evaluation of flight ability of mass reared *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) moths for use in sterile insect technique strategies (Boersma & Carpenter, 2016). The heights of the cylinders were: 15 cm, 25 cm and 40 cm high, and the diameter was 10 cm. The three cylinders were placed adjacent to each other (Figure 3.2) on a flat surface in the middle of a laboratory. A mosquito net was suspended from the roof of the laboratory, over the cylinders to prevent moths from escaping the experimental arena (Figure 3.3). A light source was fitted at the top of the mosquito net in order to provide stimulation for moths to fly upwards. Each replicate consisted of no more than six female moths per cylinder, until all emerged female moths were tested in each feeding group. Within each replicate, six newly emerged female moths were placed in the bottom of one of the cylinders and observed for 30 minutes, after which the number that succeeded to fly out of the cylinder were recorded. The six moths were first placed in tallest cylinder, followed by the 250 mm and 150 mm cylinders. Flight ability tests were conducted in a laboratory between 07:00 and 09:00 am.

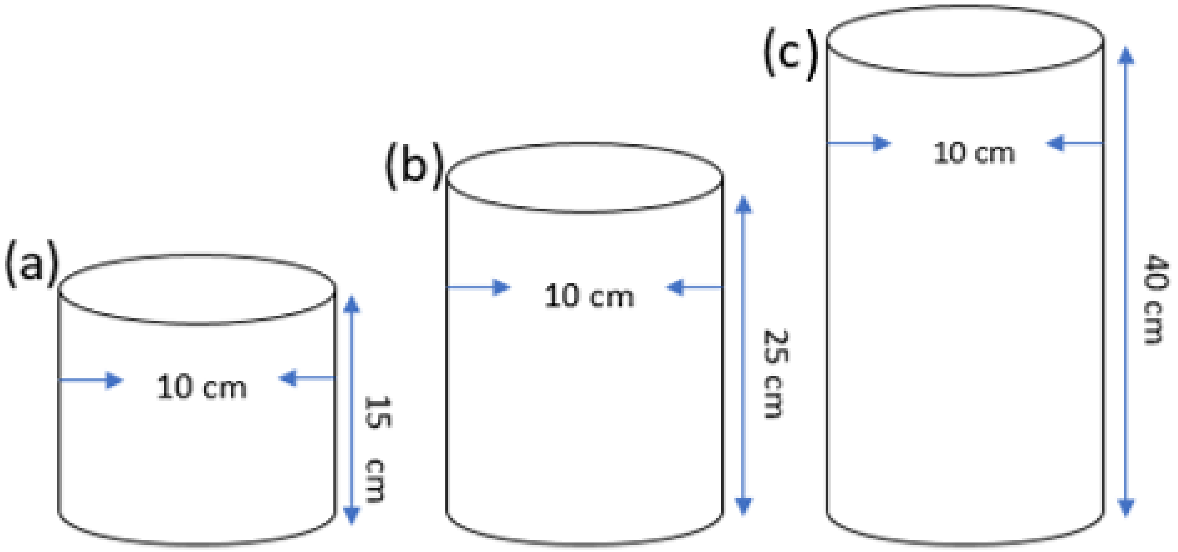


Figure 3.2. Diagram illustrating dimensions of pipes used to assess moth flight ability.

Figure 3.3. Mosquito net placed over cylinders to prevent escape of moths.

3.2.3 Statistical analysis

Larval choice tests

The positions of larvae within the petri dishes were recorded at 24 hours. Larvae were either noted as on/at the leaf sample(s), or as a no-choice (NC) for wandering or missing larvae. Binomial distribution tests were conducted in order to determine if a significant difference existed between the proportions of larvae present or absent on leaf samples. In cases where two opposing outcomes were possible, binomial tests were used to determine if there was a significant difference between the two proportions. Wandering or missing larvae that qualified as 'NC' were excluded from analysis but were indicated in figures/tables as NC. Therefore, the proportion of larvae found on/at leaf material were used in binomial distribution tests. Bonferroni correction was used to adjust for multi means comparisons in the no-choice tests, while choices made in the two-choice tests were tested against a 50% preference ratio.

Moth flight ability

All female moths produced in every generation from each of the three feeding groups were subjected to flight ability tests. The number of moths that were able to fly out of each of the cylinders were recorded and the relative proportions were determined. Data for each test were analysed by means of binomial distribution tests. Bonferroni correction was used to adjust for multi means comparisons. Data collected for moth flight ability were log transformed data and tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) but did not meet these assumptions. The data were therefore analysed by means of the non-parametric Kruskall-Wallis test, followed by Dunn's multiple comparison post hoc test. The analyses were performed with TIBCO Statistica™ 13.3 (TIBCO Software, Inc., 2017).

3.3 Results

Larval choice as well as moth flight ability tests were conducted for each of the nine generations reared on non-Bt maize and MON89034. Due to a too small population of the feeding group reared on MON810 maize after generation eight, it was not included in analyses and comparisons of larval preference and moth flight ability of the F9-generation.

No-choice tests

F1-generation larvae of all three feeding groups exhibited very high preference (75-100%) for all three maize varieties (Figure 3.4). The proportion of F1-larvae that occurred on non-Bt maize and MON810 maize after 24 hr did not differ significantly (Table 3.1).

The no-choice tests demonstrated that the probability of larvae settling on Bt-maize leaf tissue was significantly lower than that of non-Bt maize. Larvae that were reared on non-Bt maize continuously settled in very high numbers (< 95%) on non-Bt maize leaf tissue over generations and there were no significant differences between the proportions that settled on this leaf tissue over generations (Table 3.1). Within the first generation of the MON810 feeding group, 100% of larvae were settled on MON810 maize leaves after 24 hr. The percentage of larvae that settled on MON810 leaf tissue declined from 95% in the F2-generation to 65% in the F8-generation.

Within the MON89034 feeding group, an initially high number of larvae (75%) chose to settle on the MON89034 leaf tissue in the F1-generation but preference in the subsequent generations were low (between 30 – 50%) (Figure 3.4).

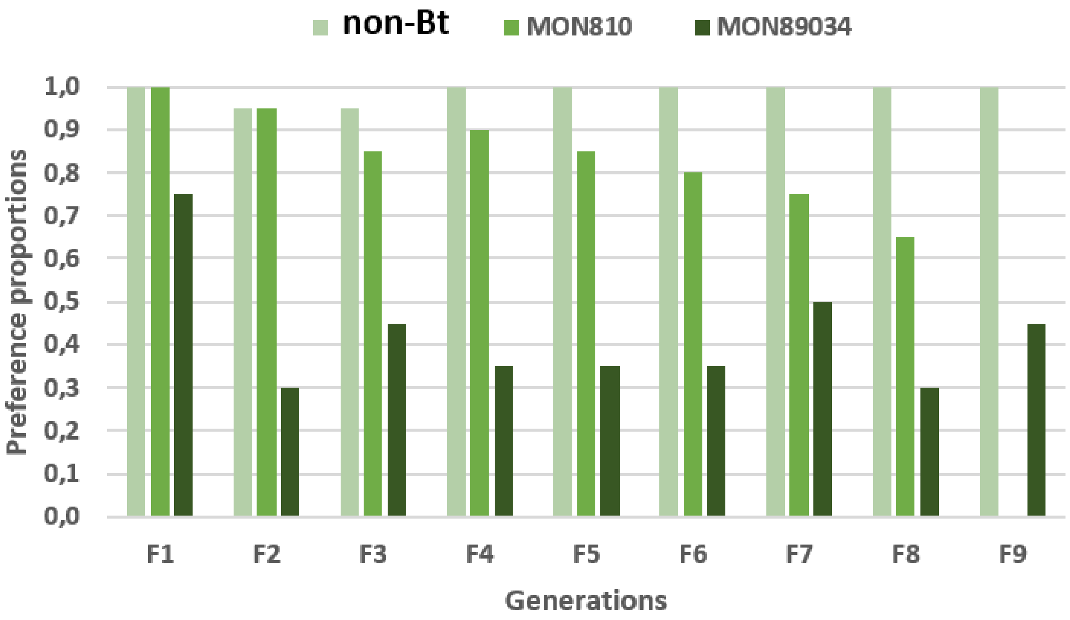


Figure 3.4. The preference responses of neonate *Spodoptera frugiperda* larvae after 24 hr in no-choice tests within feeding groups, non-Bt maize, MON810 and MON89034.

Table 3.1: Results of the binomial distribution tests conducted with data of **no-choice** tests with neonate larvae of *Spodoptera frugiperda* of the non-Bt, MON810 and MON89034 feeding groups after 24 hours

Generation	Non-Bt maize feeding group	MON810 maize feeding group		MON89034 maize feeding group	
F1 x F2	NS	F1 x F2	NS	F1 x F2	**
F1 x F3	NS	F1 x F3	NS	F1 x F3	NS
F1 x F4	NS	F1 x F4	NS	F1 x F4	*
F1 x F5	NS	F1 x F5	NS	F1 x F5	*
F1 x F6	NS	F1 x F6	NS	F1 x F6	*
F1 x F7	NS	F1 x F7	NS	F1 x F7	NS
F1 x F8	NS	F1 x F8	NS	F1 x F8	**
F1 x F9	NS	F2 x F3	NS	F1 x F9	NS
F2 x F3	NS	F2 x F4	NS	F2 x F3	NS
F2 x F4	NS	F2 x F5	NS	F2 x F4	NS
F2 x F5	NS	F2 x F6	NS	F2 x F5	NS
F2 x F6	NS	F2 x F7	NS	F2 x F6	NS
F2 x F7	NS	F2 x F8	NS	F2 x F7	NS
F2 x F8	NS	F3 x F4	NS	F2 x F8	NS
F2 x F9	NS	F3 x F5	NS	F2 x F9	NS
F3 x F4	NS	F3 x F6	NS	F3 x F4	NS
F3 x F5	NS	F3 x F7	NS	F3 x F5	NS
F3 x F6	NS	F3 x F8	NS	F3 x F6	NS
F3 x F7	NS	F4 x F5	NS	F3 x F7	NS
F3 x F8	NS	F4 x F6	NS	F3 x F8	NS
F3 x F9	NS	F4 x F7	NS	F3 x F9	NS
F4 x F5	NS	F4 x F8	NS	F4 x F5	NS
F4 x F6	NS	F5 x F6	NS	F4 x F6	NS
F4 x F7	NS	F5 x F7	NS	F4 x F7	NS
F4 x F8	NS	F5 x F8	NS	F4 x F8	NS
F4 x F9	NS	F6 x F7	NS	F4 x F9	NS
F5 x F6	NS	F6 x F8	NS	F5 x F6	NS
F5 x F7	NS	F7 x F8	NS	F5 x F7	NS
F5 x F8	NS			F5 x F8	NS
F5 x F9	NS			F5 x F9	NS
F6 x F7	NS			F6 x F7	NS
F6 x F8	NS			F6 x F8	NS
F6 x F9	NS			F6 x F9	NS
F7 x F8	NS			F7 x F8	NS
F7 x F9	NS			F7 x F9	NS
F8 x F9	NS			F8 x F9	NS

Significance indicated by *P<0.05; **P<0.01

Two-choice tests

Results on preference of larvae from the non-Bt maize feeding group for MON810 (Figure 3.5a) and MON89034 (Figure 3.5b) maize leaf tissue. The proportion of larvae that settled on non-Bt maize leaf tissue was significantly greater than the proportion that settled on leaf tissue of the Bt maize varieties.

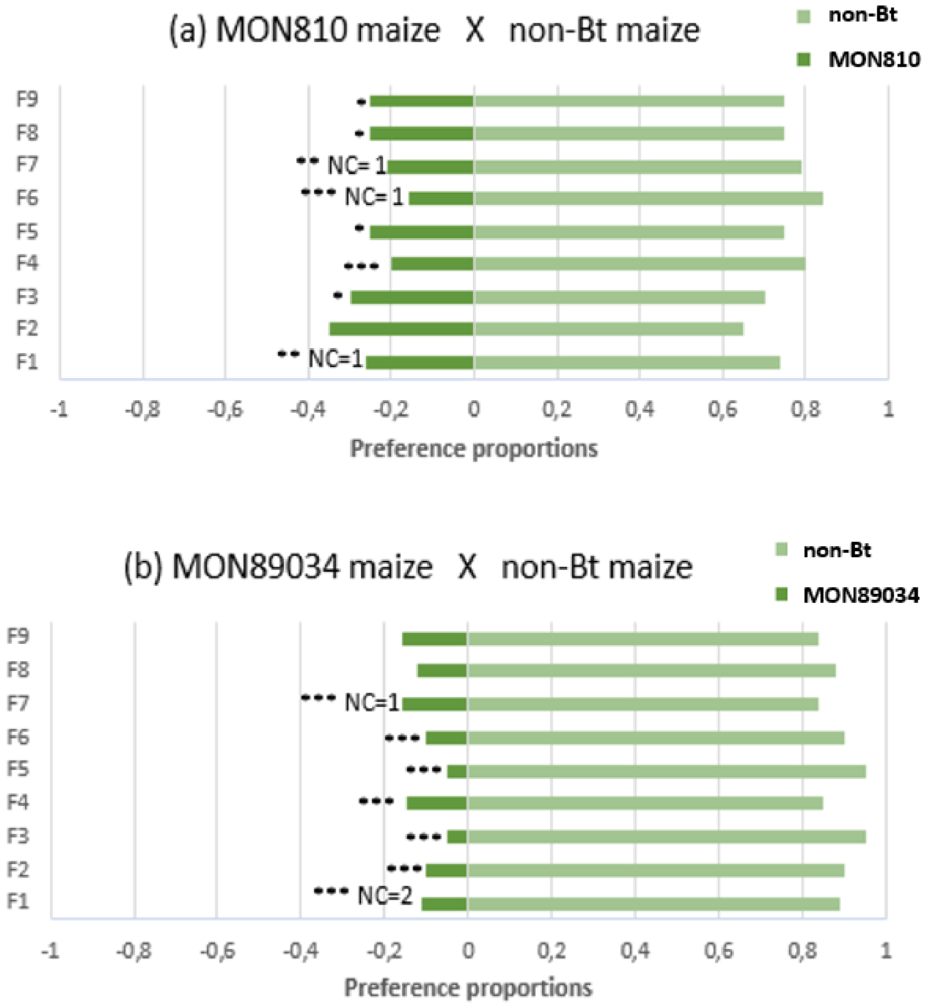


Figure 3.5. The preference responses of neonate *Spodoptera frugiperda* larvae of the **non-Bt maize feeding group** in two-choice tests with the following combinations of choices: (a) non-Bt maize and MON810, and (b) non-Bt maize and MON89034 after 24 hours. Significance indicated by *P<0.05; **P<0.01 and P<0.001. NC' indicates the number of larvae (out of 20 per combination) that did not make a choice.

The proportion of larvae from the MON810 feeing group that settled on the non-Bt maize leaf tissue was significantly greater than on the MON810 leaf tissue (Figure 3.6a). When larvae of the MON810 feeing group were provided with a choice between leaf tissue of MON810 and MON89034, there were no difference in preferences (Figure 3.6b).

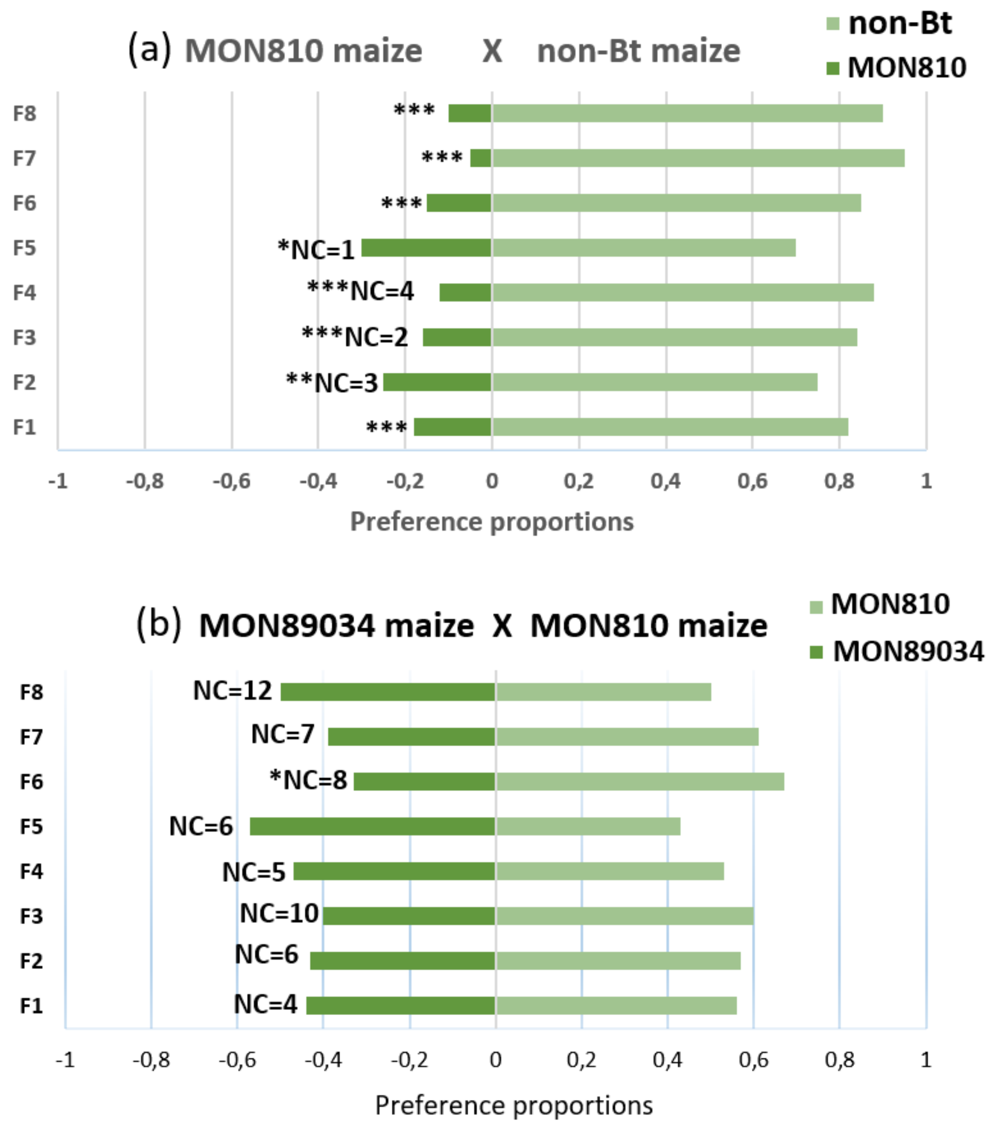
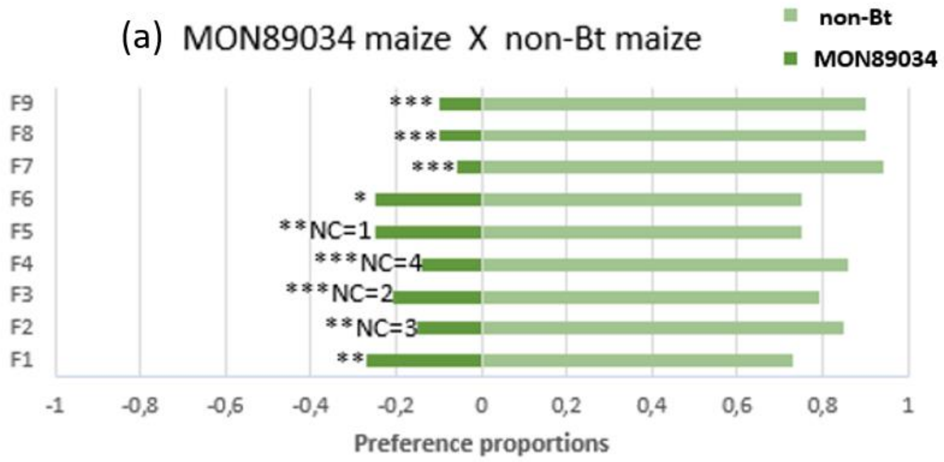


Figure 3.6. The preference of neonate *Spodoptera frugiperda* larvae of the MON810 feeding group in two-choice tests with the following combinations of choices: (a) MON810 and non-Bt maize and (b) MON810 and MON89034 after 24 hours. Significance indicated by *P<0.05; **P<0.01 and ***P<0.001. 'NC' indicates the number of larvae (out of 20 per combination) that did not make a choice.

A significantly higher proportion of larvae of the MON89034 feeing group settled on non-Bt maize leaf tissue than on MON89034 leaf tissue (Figure 3.7a). When neonate larvae of the MON89034 feeding group was provided with a choice between the MON89034 and MON810 leaf tissue, significant preference was exhibited for MON810 maize tissue (Figure 3.7b). There were also higher occurrences of 'no-choices' in preference tests with maize tissues containing Bt toxins.



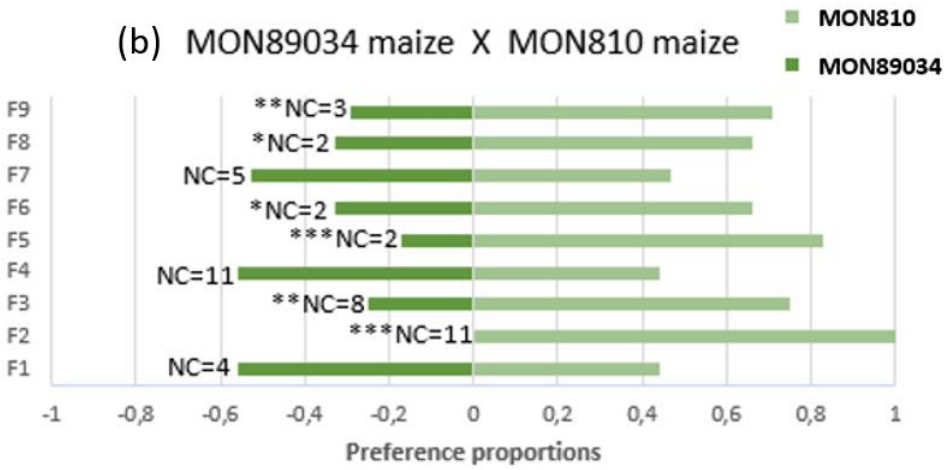


Figure 3.7. The preference of *Spodoptera frugiperda* neonate larvae of the MON89034 feeding group in two-choice tests with (a) MON89034 and non-Bt maize and (b) MON89034 and MON810 after 24 hours. Significance indicated by *P<0.05; **P<0.01 and ***P<0.001. 'NC' indicates the number of larvae (out of 20 per combination) that did not make a choice.

Multiple-choice tests

A significantly higher proportion of larvae of the non-Bt maize feeding group settled on leaf tissue of non-Bt maize compared to those that settled on leaf tissue of any of the two Bt maize varieties (Figure 3.8). There were however no significant differences between the proportion of larvae that exhibited preference for MON810 and MON89034 leaf tissue (Table 3.2).

Comparison of preferences of larvae of the MON810 feeding group in multiple-choice tests showed a significant difference between proportions that settled on non-Bt maize leaf tissue and MON810/MON89034 leaf tissues. There were no significant differences between proportions of larvae between MON810 and MON89034 leaf tissues. No significant differences were noted for 1st and 5th generations (Table 3.2).

Comparison of the proportions of larvae of the MON89034 feeding group showed that significantly higher numbers settled on non-Bt maize leaf tissue and MON810/MON89034 leaf tissues (Figure 3.8). There were no significant differences between proportions of larvae that settled on MON810 and MON89034 leaf tissues in any of the multiple-choice tests (Table 3.2).

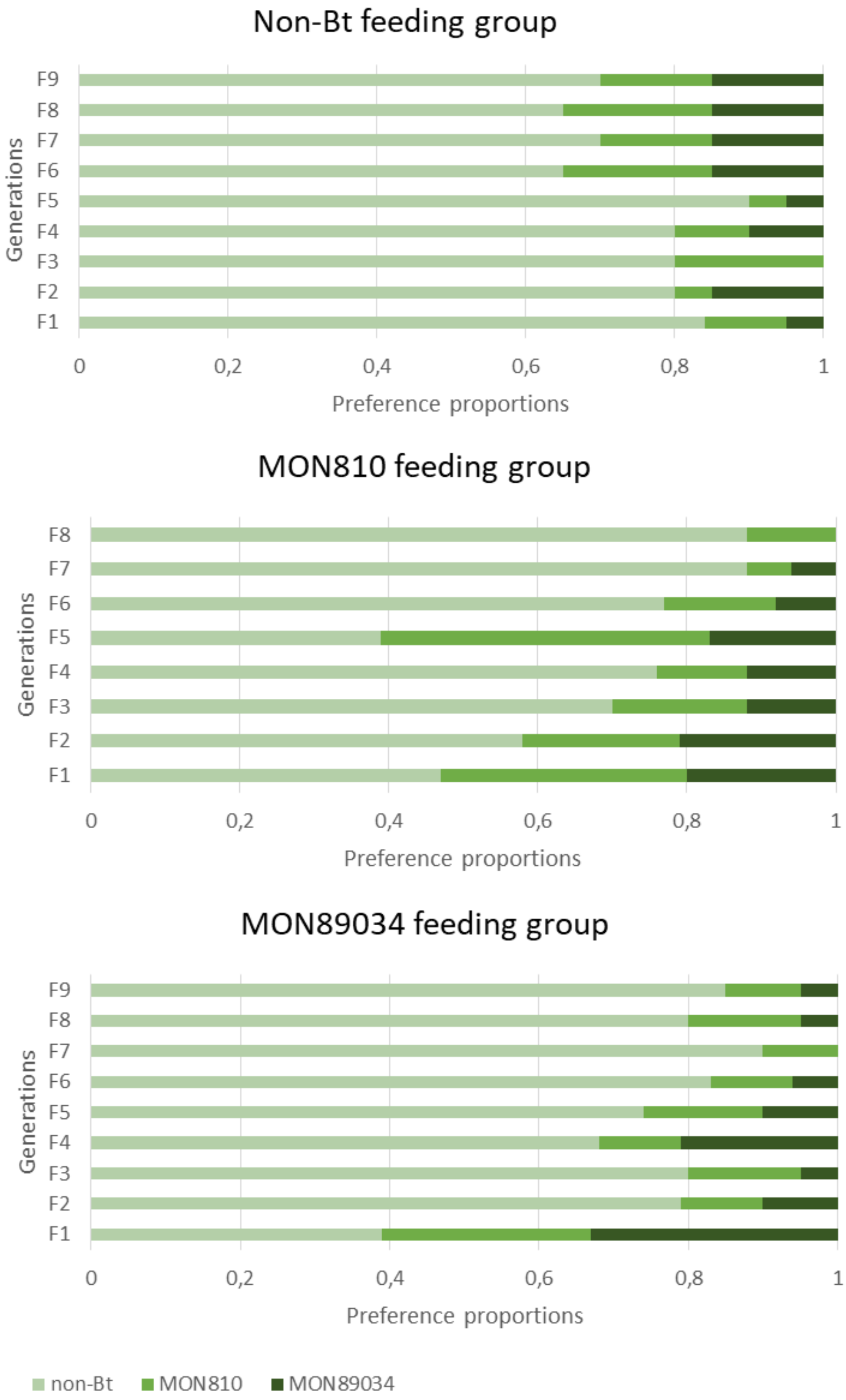


Figure 3.8. The preference of neonate *Spodoptera frugiperda* larvae of the non-Bt, MON810 and MON89034 feeding groups in multiple-choice tests with (a) non-Bt, (b) MON810 and (c) MON89034 maize leaf tissue after 24 hours. Significant differences in preferences are provided in table 3.2. ‘NC’ indicates the number of larvae (out of 20 per combination) that did not make a choice.

Table 3.2. Results of the binomial distribution test conducted on data of multiple-choice preference tests with neonate *Spodoptera frugiperda* larvae reared for multiple generations on either non-Bt, MON810 and MON89034.

		Non-Bt feeding group		**MON810 feeding group**		**MON89034 feeding group**	
Generation	**Combinations**	**P-value**	**Bonf.**	**P-value**	**Bonf.**	**P-value**	**Bonf.**
F1	non-Bt vs MON810	***	***	NS	NS	NS	NS
	non-Bt vs MON89034	***	***	NS	NS	NS	NS
	MON810 vs MON89034	NS	NS	NS	NS	NS	NS
F2	non-Bt vs MON810	***	***	*	NS	***	***
	non-Bt vs MON89034	***	***	*	NS	***	***
	MON810 vs MON89034	NS	NS	NS	NS	NS	NS
F3	non-Bt vs MON810	***	***	***	**	***	***
	non-Bt vs MON89034	***	***	****	**	***	***
	MON810 vs MON89034	NS	NS	NS	NS	NS	NS
F4	non-Bt vs MON810	***	***	***	***	***	***
	non-Bt vs MON89034	***	***	***	***	***	**
	MON810 vs MON89034	NS	NS	NS	NS	NS	NS
F5	non-Bt vs MON810	***	***	NS	NS	***	***
	non-Bt vs MON89034	***	***	NS	NS	***	***
	MON810 vs MON89034	NS	NS	NS	NS	NS	NS
F6	non-Bt vs MON810	**	*	***	***	***	***
	non-Bt vs MON89034	**	**	***	***	***	***
	MON810 vs MON89034	NS	NS	NS	NS	NS	NS
F7	non-Bt vs MON810	***	**	***	***	***	***
	non-Bt vs MON89034	***	**	***	***	***	***
	MON810 vs MON89034	NS	NS	NS	NS	NS	NS
F8	non-Bt vs MON810	***	**	***	***	***	***
	non-Bt vs MON89034	**	**	***	***	***	***
	MON810 vs MON89034	NS	NS	NS	NS	NS	NS
F9	non-Bt vs MON810	***	**	-	-	***	***
	non-Bt vs MON89034	***	**	-	-	***	***
	MON810 vs MON89034	NS	NS	-	-	NS	NS

Significance indicated by *P<0.05; **P<0.01 and ***P<0.001, NS = not significant.

Flight ability tests

Results showed that there were no changes in flight ability of moths within the non-Bt feeding group, over generations (Table 3.3, Fig. 3.9). High proportions (<86%) of moths were able to fly out of the lowest and medium height cylinders while the success rate was between 80-89% in the case of the highest cylinder. When comparing the general flight ability of moths of the MON810 feeding group, it was evidently lower than that of the non-Bt group and only 84%, 74% and 58% of the moths from the F1-generation successfully flew out from the 15, 25 and 40 cm high cylinders respectively (Fig. 3.9, Table 3.4). The general flight ability of moths of the MON89034 feeding group was lower than that of the other groups and only 50%, 38% and 13% of the F1-generation moths successfully escaped from the 15, 25 and 40 cm high cylinders respectively (Table 3.4). Significant differences in the flight ability of moths from different generations of the MON810 feeding group were observed (Table 3.3) and flight ability of the 8th generation was similar to that of the 1st generation. No significant changes in flight ability of moths from the MON89034 feeding group were observed over 9 generations (Table 3.3).

However, when comparisons were done between feeding groups for each generation, significant changes in flight ability were observed (Table 3.5). The proportions of moths of each generation that successfully flew out of the cylinders are presented in Figure 3.9, which shows that from non-Bt feeding group exhibited overall better flight performance than the two Bt feeding groups. Flight ability of moths of the Bt feeding groups was lower than that of the non-Bt group, from the F1-generation onwards (Table 3.5, Fig. 3.9). The lowest fitness of moths to fly out of cylinders was observed for the MON89034 feeding group at 40 cm where only between 13 and 45% succeeded (Table 3.5).

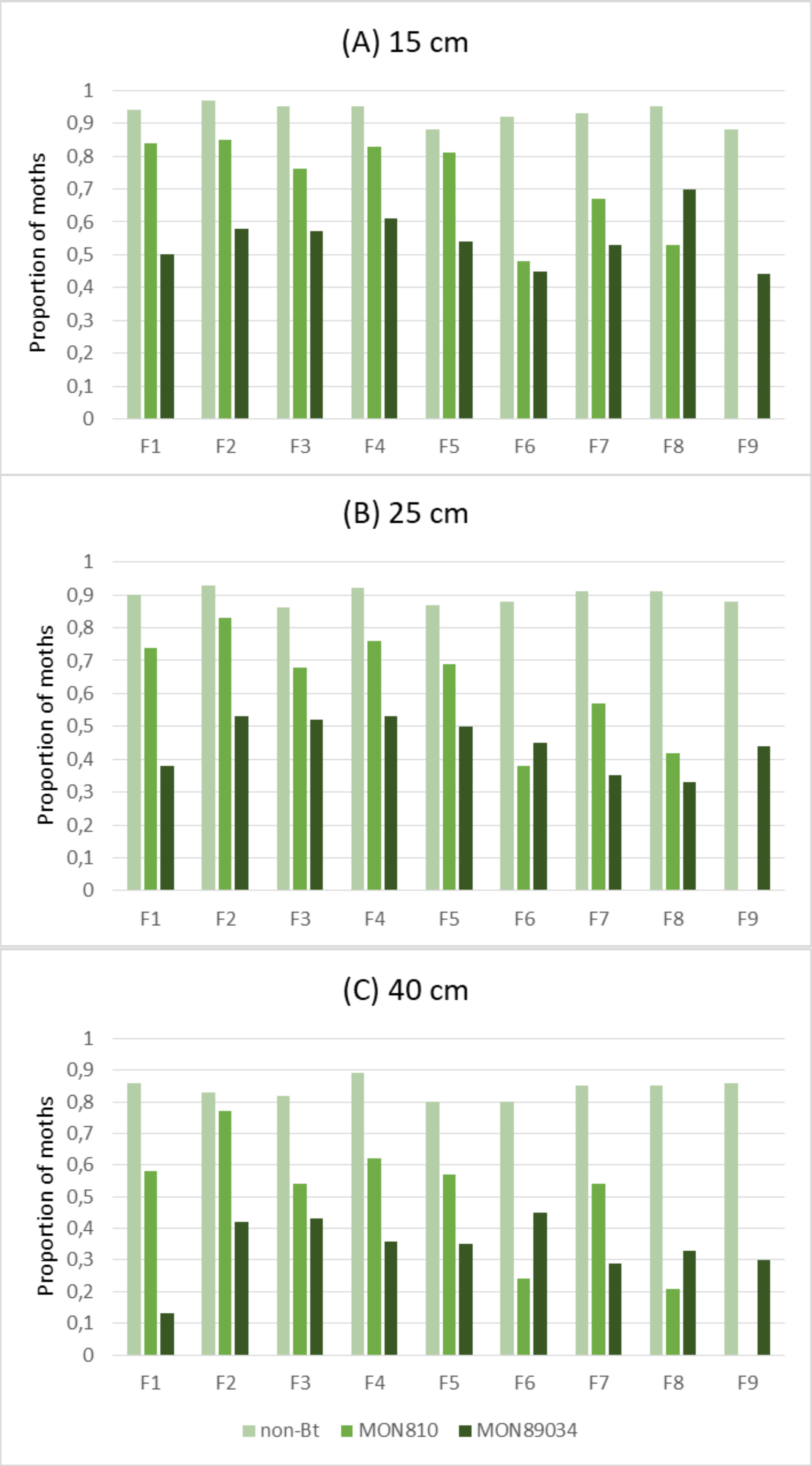


Figure 3.9. Proportion of moths that succeeded to fly out of cylinders of different heights after being reared on non-Bt, MON810 and MON89034 maize for eight or nine consecutive generations. A) 15 cm, B) 25 cm, C) 40 cm.

Table 3.3: Results of the binomial distribution test data on *Spodoptera frugiperda* moth flight ability within different feeding groups over generations.

	Non-Bt feeding group			MON810 feeding group			MON89034 feeding group		
	15 cm	**25 cm**	**40 cm**	**15 cm**	**25 cm**	**40 cm**	**15 cm**	**25 cm**	**40 cm**
F1 x F2	NS	NS	NS	NS	NS	NS	NS	NS	NS
F1 x F3	NS	NS	NS	NS	NS	NS	NS	NS	NS
F1 x F4	NS	NS	NS	NS	NS	NS	NS	NS	NS
F1 x F5	NS	NS	NS	NS	NS	NS	NS	NS	NS
F1 x F6	NS	NS	NS	NS	NS	NS	NS	NS	NS
F1 x F7	NS	NS	NS	NS	NS	NS	NS	NS	NS
F1 x F8	NS	NS	NS	NS	NS	NS	NS	NS	NS
F1 x F9	NS	NS	NS	X	X	X	NS	NS	NS
F2 x F3	NS	NS	NS	NS	NS	NS	NS	NS	NS
F2 x F4	NS	NS	NS	NS	NS	NS	NS	NS	NS
F2 x F5	NS	NS	NS	NS	NS	NS	NS	NS	NS
F2 x F6	NS	NS	NS	*	**	not	NS	NS	NS
F2 x F7	NS	NS	NS	NS	*	NS	NS	NS	NS
F2 x F8	NS	NS	NS	NS	*	**	NS	NS	NS
F2 x F9	NS	NS	NS	X	X	X	NS	NS	NS
F3 x F4	NS	NS	NS	NS	NS	NS	NS	NS	NS
F3 x F5	NS	NS	NS	NS	NS	NS	NS	NS	NS
F3 x F6	NS	NS	NS	NS	NS	NS	NS	NS	NS
F3 x F7	NS	NS	NS	NS	NS	NS	NS	NS	NS
F3 x F8	NS	NS	NS	NS	NS	NS	NS	NS	NS
F3 x F9	NS	NS	NS	X	X	X	NS	NS	NS
F4 x F5	NS	NS	NS	NS	NS	NS	NS	NS	NS
F4 x F6	NS	NS	NS	*	*	*	NS	NS	NS
F4 x F7	NS	NS	NS	NS	NS	NS	NS	NS	NS
F4 x F8	NS	NS	NS	NS	NS	*	NS	NS	NS
F4 x F9	NS	NS	NS	X	X	X	NS	NS	NS
F5 x F6	NS	NS	NS	NS	NS	NS	NS	NS	NS
F5 x F7	NS	NS	NS	NS	NS	NS	NS	NS	NS
F5 x F8	NS	NS	NS	NS	NS	NS	NS	NS	NS
F5 x F9	NS	NS	NS	X	X	X	NS	NS	NS
F6 x F7	NS	NS	NS	NS	NS	NS	NS	NS	NS
F6 x F8	NS	NS	NS	NS	NS	NS	NS	NS	NS
F6 x F9	NS	NS	NS	X	X	X	NS	NS	NS
F7 x F8	NS	NS	NS	NS	NS	NS	NS	NS	NS
F7 x F9	NS	NS	NS	X	X	X	NS	NS	NS
F8 x F9	NS	NS	NS	X	X	X	NS	NS	NS

Significance indicated by *P<0.05; **P<0.01 and ***P<0.001,

NS = not significant, X = not determined

Table 3.4: Percentage (%) of *Spodoptera frugiperda* moths of different generations able to successfully fly out of cylinders of different heights after being reared on non-Bt, MON810 and MON89034 maize for nine generations.

Gene-ration	Non-Bt feeding group			MON810 feeding group			MON89034 feeding group		
	15 cm	25 cm	40 cm	15 cm	25 cm	40 cm	15 cm	25 cm	40 cm
F1	94	90	86	84	74	58	50	38	13
F2	97	93	83	85	83	77	58	53	42
F3	95	86	82	76	68	54	57	52	43
F4	95	92	89	83	76	62	61	53	36
F5	88	87	80	81	69	57	54	50	35
F6	92	88	80	48	38	24	45	45	45
F7	93	91	85	67	57	54	53	35	29
F8	95	91	85	53	42	21	70	33	33
F9	88	88	86	X	X	X	44	44	30

X = not determined

Table 3.5: Results of the binomial distribution of data on the comparison of *Spodoptera frugiperda* moth flight ability between the non-Bt, MON810 and MON89034 feeding groups of similar generations.

	Non-Bt X MON810			**Non-Bt X MON89034**			**MON810 X MON89034**		
Generation	**15 cm**	**25 cm**	**40 cm**	**15 cm**	**25 cm**	**40 cm**	**15 cm**	**25 cm**	**40 cm**
F1 x F1	NS	**	***	***	***	***	**	**	**
F2 x F2	**	*	NS	***	***	***	**	**	**
F3 X F3	**	*	**	***	***	***	NS	NS	NS
F4 X F4	*	**	***	***	***	***	**	**	**
F5 X F5	NS	**	**	***	***	***	**	*	*
F6 X F6	***	***	***	***	***	***	NS	NS	NS
F7 X F7	***	***	***	***	***	***	NS	NS	*
F8 X F8	***	***	***	***	***	***	NS	NS	NS
F9 X F9	X	X	X	***	***	***	X	X	X

Comparisons within row for each feeding group; Significance indicated by *P<0.05; **P<0.01 and ***P<0.001,

NS = not significant, X = not determined

3.4 Discussion

No changes in larval preference or moth flight ability was observed over generations for larvae and moths of the feeding group reared on non-Bt maize. This indicated that the rearing under artificial conditions did not influence the fitness of larvae in terms of preference and moths in terms of flight ability.

No-choice larval preference tests allowed us to observe the initial responses of larvae to the different maize cultivars, and through comparison with larval preference responses of individuals reared on non-Bt and Bt maize, transgenerational changes were observed. No-choice tests with larvae of the MON810 and MON89034 feeding groups showed variable results.

Within the MON810 feeding group, a large proportion of larvae from the 1st generation preferred to settle on MON810 and MON89034 leaf tissue within 24 hours. While the preferences of the MON810 feeding group remained largely the same over generations, a slight decline was observed toward later generations. Larvae of the MON89034 feeding group did however show lower proportions of initial preference for MON89034 leaf tissue. This could indicate that the presence of Bt toxins within the maize did not initially deter larvae who have not previously been exposed to Bt toxins, and that in the absence of other host plants, larvae would probably colonize MON810 maize cultivars. However, neonate larvae from successive generations showed continuously lower initial preferences for maize tissue containing Bt toxins over generations. Another reason for high initial preference of 1st-generation MON810 fed larvae for MON810 maize cultivars, could be that the high susceptibility of these larvae to Bt toxins in MON810 left them immobile. Berdegué *et al.* (1996) reported that the mobility of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) larvae was adversely affected after ingestion of Bt toxins.

Although the proportions of larvae of the different feeding groups exhibited varying preferences over generations, the high proportion of larvae that settled on MON89034 maize leaf tissue was significantly lower in all successive generations. Lower preferences for Bt maize tissues and higher proportions of larvae of the Bt maize feeding groups found wondering in the testing arena may be ascribed to a higher inclination of resistant larvae to migrate (Visser *et al*., 2019). Studies done by Visser *et al*. (2020) compared plant abandonment rates of B. fusca larvae exposed to different Bt maize cultivars. Results indicated that neonate *B. fusca* larvae showed higher rates of plant abandonment off Bt maize plants than non-Bt maize plants. Furthermore, Singer *et al*. (2002) stated that host preference choices by *S. frugiperda* is influenced by its ability to recognize the presence of Bt toxins in food sources. Calatayud *et al.* (2015) also noted that *B. fusca* larvae are able to survive for at least 49 hours without ingesting plant tissue, leaving ample time for larvae to search for alternative food sources after disapproving of host plants. It has been observed that *B. fusca* larvae use sensory structures that are located on mouthparts to differentiate between Bt and non-Bt plants, according to chemical composition (Schoonhoven & van Loon, 2002; Juma *et al*., 2008). Clarke *et al*. (2006) also found that feeding avoidance behaviour was induced by exposing *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae) to Bt maize, indicating that the detection of Bt toxins within plant tissues is not limited to only lepidopterans.

Two-choice tests allowed us to monitor the change in larval preferences between maize varieties proceeding generational exposure to Bt maize. Within the non-Bt maize feeding group, changes in generational preferences for non-Bt and Bt maize cultivars were observed. Although a small proportion of larvae of the non-Bt maize feeding group chose to settle on MON810 and MON89034 maize tissue, significant preference for non-Bt maize was observed in almost all of the two-choice and multiple-choice tests conducted with this feeding group. Most larvae of the non-Bt maize feeding group preferred to settle on leaf tissue of this variety on which rearing took place. Visser *et al*. (2019) found that *B. fusca* larvae showed a higher preference for plants that expressed no Bt toxins and that migration took place at higher rates from plant tissues containing Bt toxins.

Neonate larvae from MON810 feeding group showed relatively equal preference proportions in two-choice tests with MON810 X MON89034 maize. However, in two-choice tests with MON810 X non-Bt maize significant differences were observed, and a larger proportion of larvae settled on non-Bt maize leaf tissue than MON810 leaf tissue. Dos Santos *et al.* (2017) tested preferences of neonate larvae of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) for non-Bt soybean and Bt-soybean containing Cry1Ac toxins and observed that the presence of Cry1Ac toxins did not deter larvae and had no effect on preference.

Significant preference differences were noted for larvae from the MON89034 feeding group for non-Bt maize in two-choice tests. In two-choice tests with MON89034 X MON810 high numbers of larvae were recorded as ‘NC’, indicating that in the absence of non-Bt maize tissue, larvae were more hesitant to settle on Bt leaf tissues with large numbers of larvae found wandering in the arena. Nascimento *et al*. (2020) suggested that FAW larvae had the ability to detect the presence of Bt toxins within plant tissues, since most larvae selected non-Bt hybrids for feeding. Similarly, Garcia *et al.* (2015) found that in no-choice tests, *Mythimna unipuncta* (Haworth) (Lepidoptera: Noctuidae) larvae were less likely to settle on Bt maize tissue, compared to choice tests where non-Bt maize tissue was also available.

Larvae in multiple-choice tests within the MON810 feeding group showed initially high preferences for all three maize varieties. In the 5th generation, the proportion of larvae that settled on non-Bt maize was similar to that on MON810. However, preceeding the 5th generation, the proportion of larvae that preferred to settle on MON810 was noticeably decreased. Proportions of larvae that preferred MON89034 decreased with generations until a very low percentage (<6%) preferred leaf tissue of this variety.

Larvae of the 1st generation MON89034 feeding group showed relatively high preference for the other two varieties in multiple-choice tests. This could be as a result of these larvae the *S. frugiperda* population used in this study not having been exposed to Bt toxins previously. In subsequent generations the majority of larvae preferred to settle on non-Bt maize cultivars.

Another point to be made is that the number of larvae that were missing or found wandering, recorded as ‘NC’ in all figures, was minimal for all choice tests conducted with larvae from non-Bt maize feeding group. The number of larvae recorded as ‘NC” for choice tests carried out with larvae from MON810 and MON89034 feeding groups showed noticeable increases in this regard. According to Han *et al.* (2016) feeding avoidance behaviour on Bt plant tissue is a pervasive trait of the Lepidoptera. For example, it was found that *B. fusca* larvae were able to display feeding avoidance behaviour for up to 49 h as larvae searched for alternate and appropriate food sources (Calatayud *et al.*, 2015), whilst Visser *et al*. (2019) revealed that *B. fusca* larvae tended to remain sheltered in Bt leaf folds, even though no feeding was observed, possibly as a result of Bt toxin detection by larvae. Studies on plant abandonment (Visser *et al*., 2019), indicated that when Bt toxins were present in maize plant tissue, neonate *B. fusca* larvae showed higher rates of abandonment. Plant abandonment by neonate larvae in Bt maize fields could result in larvae consuming varying proportions of toxic and non-toxic maize tissue, therefore influencing toxicity levels and putting the HDR strategy at risk (Gould, 1998).

Choice-tests also indicated that Cry1Ab-resistant and susceptible larvae refrained from feeding on MON810 and MON89034 plant tissue (Visser *et al*., 2019). Results of field trials showed that *B. fusca* larvae migrated more successfully and to a greater extent in plots of non-Bt plants than in plots of MON89034 plants (Visser *et al*., 2019), which could be ascribed to the inhibition of larval mobility due to susceptibility to Bt toxins (Berdegué *et al.,* 1996).

Most transgenic maize events do not meet the high-dose requirements when it comes to the expression of Cry toxins, exposing target pests to sub-lethal levels of toxins (Sousa *et al*., 2016). According to Omoto *et al.* (2016), MON810 maize is wel known to express low doses of Cry1Ab toxins. Concerns have been raised regarding the field-level effects of low efficacy of maize hybrids that express Cry1Ab toxins (Garcia *et al*., 2015). As a result of this, heterozygous resistant (RS) pests are able to survive the sub-lethal effects of toxins, and are therefore able to mate with homozygous resistant (RR) individuals, speeding up the evolution of resistance against Bt toxins (Sousa *et al*., 2016).

No significant changes were observed in the flight ability of moths of the non-Bt feeding group over nine generations. Constant high rates of success in moths flying out from different heights was observed in the non-Bt feeding group, indicating that the fitness of moths was not affected. This is in accordance with the opinion of Johnson (1987), who stated that FAW adults are well known for great flight ability skills, within a single night FAW adults are able to fly up to 100 km.

Overall, there was no improvement or deterioration in the flight ability of moths from the MON810 and MON89034 feeding groups in consecutive generations. However, the flight ability of moths of the MON89034 feeding groups was lower than that of the other groups, from the F2-generation onwards. Slight variations in flight ability of moths of the MON810 feeding group were observed, however the similar flight ability of the first and last generation shows that flight ability did not change over generations. However, comparisons of flight ability between feeding groups showed significant differences. A study conducted by Liang *et al.* (2007) showed that flight ability can be influenced by the presence of Cry proteins in the larval host plant. Similar results were recorded in this study which showed reduced flight ability from the 2nd generatyion owards for the two Bt maize feeding groups. Results from this study are in accordance with results recorded by Liang *et al*. (2007), who showed decreased flight ability for *H. armigera* that ingested Bt toxins during larval stages. The mean flight duration of BtR individuals was 11.6 hours after feeding on normal diet, but only 7.3 hours after feeding on a Bt diet (Liang *et al*., 2007).

According to Njuguna *et al*. (2021), the high mobility of FAW adults should ease concerns on reproductive isolation of moths emerging from Bt areas and moths emerging from refuge areas. However, results from this study indicate that exposure to Bt toxins drastically affect the flight ability of moths. The success of the HDR strategy, which relies on mobility and flight of moths between non-Bt refuge areas and Bt fields, may be adversely affected if poor moth fitness result in assortative mating.

3.5 Conclusion

Much reliance is put on the HDR strategy to delay resistance evolution within FAW populations. Underlying assumptions of this strategy are that larvae do largely not move off host plants and that moths are healthy and mobile to promote random mating. Results from this study indicated that larvae show feeding avoidance behaviour on plant material containing Bt toxins, this could lead to the survival and mating of all heterozygous individuals, eventually resulting in the exponential increase of resistance alleles within field populations. Additionally, the poor flight ability of moths exposed to Bt toxins will not result in optimum mating behaviour, as resistant moths will not be able to fly to refuge areas where homozygous susceptible moths reside. Results from this study indicate that larval behaviour and moth flight ability are affected when FAW population are exposed to Bt maize, which may limit the success of the HDR strategy.

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Chapter 4

Conclusions

4.1 Introduction

The cultivation of maize in Sub-Saharan Africa (SSA) by subsistence as well as commercial farmers is essential in order to ensure adequate food supplies on the continent. The invasion of the African continent by one of the most damaging agricultural pests worldwide, *Spodoptera frugiperda* (FAW) (J.E. Smith) (Lepidoptera: Noctuidae), has left major concerns regarding food security in the years to come (Van den Berg *et al.,* 2021). In order to combat yield losses, it has been suggested to introduce the cultivation of Bt maize cultivars (Van den Berg *et al*. 2021), containing transgenes from *Bacillus thuringiensis,* for the control of FAW populations (Prasanna *et al*., 2018). Genetic manipulation of maize plants has allowed plants to have specific insecticidal traits, and by inserting genes from *B. thuringiensis* into the maize genome, plants are able to produce Cry proteins within tissues from the onset of germination (Prasanna *et al*., 2018). According to Lemaux (2009) certain groups of insect pests are very susceptible to Cry proteins, including FAW.

Thompson *et al.* (2019) confirmed that South African farmers have planted Bt maize since it was approved in 1998, and is currently the only country in SSA which has approved the cultivation of Bt maize. However, the adoption, success and sustainability of Bt maize technologies is threatened by the development of resistance to Bt toxins by target pest populations (Tabashnik, 1994). Due to an unusually high selection pressure exerted by Bt toxins on pest populations, several lepidopteran pest species have evolved resistance to Cry proteins (Tabashnik *et al.,* 2013; Zhu *et al*., 2019). Field resistance to Cry 1F proteins in Bt maize varieties has been reported in *S. frugiperda* populations in the Americas (Yang *et al*., 2018) where resistance alleles against Vip3A protein were also detected (Zhu *et al.,* 2019). In South Africa, Botha *et al*. (2019) reported high levels of survival of *S. frugiperd*a on Bt maize cultivars that express Cry1Ab but very low survival on cultivars that express Cry1A.105 + Cry2Ab2 proteins, indicating that the former is not effective against this pest. Maize cultivars that express Cry1A.105 + Cry2Ab2 proteins may therefore provide effective control of *S. frugiperda*, provided that it does not evolve resistance to these proteins over a short period of time.

The short life cycle of FAW, paired with previously reported survival on Bt maize, which exerts high selection pressure on exposed pest populations, favours resistance evolution in areas which are suitable for pest survival throughout the year. The duration of the FAW lifecycle is temperature dependant, and according to du Plessis *et al.* (2018), developmental times vary according to the climate of geographical region in which the population occurs. A single lifecycle could take up to 30 days to complete in summer, but can be extended up to 90 days during winter seasons (Capinera, 2002). FAW populations lack a diapause phase (Johnson, 1987) and can produce up to eight generations in one year in tropical areas with optimum temperatures (Busato *et al.,* 2005). Such rapid reproduction increases selection pressure and necessities management of insect resistance evolution which requires synchronised emergence of resistant and susceptible moths to allow for mating and to ensure dilution of resistance alleles in the pest population.

This study aimed to evaluate the response of FAW, if subjected to high selection pressure for resistance evolution, and to determine possible changes in life history parameters of larvae and pupae as well as moth fitness during this process.

The three maize varieties used in this study were a single-gene Bt maize hybrid (MON810; DKC78-15B), a pyramid-gene Bt maize hybrid (MON89034; DKC78-45 BRGEN), and a non-Bt iso-hybrid (CRN3505). The MON810 hybrid expresses the Cry1Ab protein while the MON89034 hybrid expresses the Cry1A.105 + Cry2Ab2 proteins.

Results from this study indicated high levels of resistance to MON810, and moderate levels of resistance to MON89034. For populations that were reared on these two Bt maize varieties, several differences and changes in life history parameters over generations were observed. These observed changes in larval preferences, duration of larval and pupal periods as well reduced flight ability of populations that were exposed to Bt toxins, may all contribute to rapid evolution of resistance.

The effect of exposure was most evident during the larval phase of development, as extended larval periods were recorded. Extensions of pupal period and poor moth emergence were also recorded for populations reared on Bt maize tissue. High larval survival was observed in the MON810 feeding group whilst relatively lower survival percentages were evident in the MON89034 feeding group, confirming that the use of MON89034 maize cultivars to control field populations of FAW would prove to be more effective than that of MON810.

Results of no-choice tests showed that larvae exposed to Bt toxins over generations developed a reduced preference for leaf tissue of these maize varieties. In two-choice tests with non-Bt maize and Bt maize leaf tissue, there was a significant preference for non-Bt maize tissue indicating that larvae could detect the presence of Bt toxins within maize tissues. Flight ability tests indicated that the ingestion of Bt toxins during larval stages reduced the flight ability of moths and, additionally, deformation of wings was observed in many of the moths reared on Bt maize. The consumption of Cry proteins by larvae has been reported by Liang *et al*. (2007) to affect the flight abilities of FAW moths, with moths of larvae that were reared on non-Bt diet flying almost double the distance of moths whose larvae fed on Bt-diet.

4.2 IRM

According to Hackett and Bonsall (2016), pest populations that develop resistance against pesticides and transgenic crops are not only major threats to sustainable agriculture, but also threaten food security. Rates of resistance evolution against Bt technologies have increased rapidly due to the worldwide adoption (Zhu *et al*., 2019). For this reason, according to Bates *et al.* (2005), the deployment of an IRM strategy is obligatory when Bt crops are cultivated. Whilst there are many IRM strategies used worldwide to delay the evolution of insect resistance, the most commonly used and widely accepted IRM strategy is the High-dose and Refuge (HDR) strategy. This strategy is based on assumptions regarding the biology of target pests and insect-plant interactions (Tabashnik *et al*., 2009), and requires transgenic crops to express high doses of Bt toxins. Another component of this IRM strategy is a designated refuge area where only non-Bt crops are planted in order to provide areas where populations of susceptible pests will be able to survive (USEPA, 1998). Hypothetically, high-doses of toxins in the Bt maize areas of fields will cause the death of all susceptible (SS) and heterozygous resistant (RS) larvae, permitting only homozygous resistant (RR) individuals to complete lifecycles on Bt maize. It is then assumed that resistant moths emerging from Bt maize will have sufficient capabilities to fly and mate with susceptible moths produced from refuge areas, diluting the resistance alleles and producing heterozygous resistant offspring, which will once again succumb to the high-dose produced by Bt maize (Tabashnik & Carrière, 2017). Major consequences have been seen as a result of farmers not adhering to refuge requirements and non-compliance to refuge plantings, resulting in *Busseola fusca* (Lepidoptera: Noctuidae) populations developing practical resistance to Cry1Ab toxins within 8 years in South Africa (van den Berg, 2017).

Various studies reported severe delays in larval and pupal developmental times for lepidopteran larvae reared on plant tissues containing Bt toxins (Botha *et al.*, 2019; Jakka *et al*., 2014; Liu & Tabashnik, 1999; Sousa *et al*., 2016). Gould (1998) suggested that delays in developmental times would have detrimental effects on resistance management strategies because the HDR strategy relies on the mating of susceptible moths from refuge areas with resistant moths from Bt fields. Jakka (2014) found that moths fed on Bt tissues took an average of three days longer to emerge than moths that fed on non-Bt maize during larval stages. According to Simmons and Marti (1992), after emerging from pupae, FAW adults have a mating period of about three days with optimum fertility and fecundity occurring only for two days after emergence. This raises concerns that the delays in development of populations feeding on Bt plant tissues would cause asynchronization between moths emerging from non-Bt and Bt areas (Jakka *et al.,* 2014), compromising the HDR strategy by not having the desired effect of dilution on resistance alleles within populations. It is therefore likely that mating between resistant and susceptible moths would mostly not occur. The required mating between resistant and susceptible moths would further be adversely affected if moths that emerge in Bt maize fields exhibit poor flight ability. It would also be important to consider long term effects of concurrently exposing field populations of FAW to Bt maize, as susceptible populations will follow normal cycles, and resistant populations will fall further behind with every generation in terms of developmental times

In South Africa, refuge areas are implemented in the form of structured refuge blocks, mandatory refuge areas have to make up 5% to 20% of all hectares planted (Van den Berg, 2017). Current refuge requirements in South Africa are a 20% non-Bt refuge area that may be sprayed with pesticides, or a 5% refuge area that may not be sprayed with pesticides (Van den Berg *et al*., 2013). According to Njuguna (2021), Kenya is set to be the next country in SSA to approve the cultivation of Cry1Ab maize cultivars, and according to Van den Berg *et al.* (2021), despite imperfections of the South African approach to resistance management, the refuge requirements for Kenyan Bt maize fields is set to be 5%. Additionally, the strategy is to initially introduce Cry1Ab maize, where after Cry1A.105 + Cry2Ab maize will be introduced. These refuge requirements are weaker than that of other countries in which FAW populations have quickly developed resistance against Bt crops (Tabashnik and Carrière, 2019). According to Van den Berg *et al.* (2021), the current strategies planned by African authorities for IRM will not suffice to delay resistant in the case of FAW.

4.3 Discussion of issues regarding low dose events

In order to delay the evolution of resistance within pest populations, insect resistance management (IRM) strategies may be implemented, and according to Tabashnik *et al.* (2013) the HDR strategy is established according to assumptions made about the biology and life cycle of target pest species, as well as interactions with host plants.

In most cases, the high-dose requirements of the HDR strategy are not met by transgenic plants, exposing target pets to sub-lethal doses of Bt toxins (Sousa *et al*., 2016). Transgenic maize expressing Cry1Ab toxins, according to Omoto *et al.* (2016), are known to produce low doses of Bt toxins, while Garcia *et al.* (2015) confirmed that concerns regarding the effects of low-efficacy maize expressing Cry1Ab toxins on the establishment of lepidopteran populations in maize fields. Sub-lethal effects of toxins allow for the survival of heterozygous individuals, if heterozygous resistant individuals mate with homozygous resistant individuals, the rates of resistance evolution within populations will inevitably be amplified (Sousa *et al*., 2016).

This study revealed the low-dosage of Bt toxins present in MON810 maize tissue as substantial numbers of larvae were able to survive and reproduce. This supports claims made by various studies that MON810 does not qualify as a maize event producing high doses of Cry toxins (Huang, 2020; Omoto *et al*., 2016; Onstad *et al.,* 2013; Sousa *et al*., 2016; Roush *et al.,* 1997). According to Botha *et al*. (2019), results from their studies showed the complete susceptibility (100% mortality) of FAW to a maize event expressing Cry1A.105 +Cry2Ab2. This study, however, showed varying percentages of larval survival and reproduction success within the MON89034 feeding group, indicating that the FAW populations were not highly susceptible to this maize variety, and confirmed the presence of resistant alleles against MON89034 in the populations.

4.4 Cross resistance

In order to combat the evolution of resistance in target pest populations, farmers worldwide have made a shift from planting single-toxin Bt cultivars to planting multi-toxin (pyramided) Bt cultivars (Carrière *et al.,* 2015). According to Zhao *et al.* (2005) pyramided maize cultivars produce two or more different Bt toxins, e.g. Cry1A.105 + Cry2Ab. Pyramid maize cultivars are expected to be highly effective at controlling pest populations, as well as delaying the onset of insect resistance development if a few conditions are met. Firstly, singular toxins included in the pyramid should be potent enough to cause the death of pests on their own, no-cross resistance is conferred by the pyramid to any toxins included in the pyramid, and lastly, no cultivars expressing any of the singular toxins included in the pyramid is grown in close proximity (Carrière *et al.,* 2015, Carrière *et al.,* 2016).

The presence of cross resistance within populations of target pests could have detrimental effects on the sustainability of pyramided Bt technologies. Yang *et al.* (2018) defined cross-resistance as occurrences where populations of target pests who are resistant to a specific Bt protein in turn show a genetic-based resistance to other Bt proteins. For example, cross resistance has been observed in populations of FAW that are resistant to Cry1F toxins, with these populations also showing resistance against other Cry1 proteins such as Cry1Ab, Cry1Ac and Cry1A.105 (Zhu *et al*., 2019). However, Yang *et al.* (2018) stated that populations of FAW that have developed resistance against Cry1 proteins are still susceptible against Vip3 Bt technologies.

4.5 Concurrent planting

The great potential of pyramided Bt maize cultivars to not only control FAW, but also delay the evolution of resistance to Bt toxins is put at risk by concurrently planting single-toxin cultivars, which are included in the pyramided cultivar, in immediate surroundings where pyramids are planted (Zhao *et al*., 2005). This is especially important in the case of FAW since this study showed reduced flight ability of moths after several generations of exposure to Bt maize. This could imply that resistant moths may have limited capacity to fly and might be more likely to mate with other resistant moths that emerge from the same Bt maize field, increasing the frequency of the resistance R allele. Long term concurrent planting of single and multi-toxin Bt plants will result in the failure of both cultivars to control target pest populations. Zhao (2005) found that selection for resistance against pyramided two-gene Bt crops will occur more rapidly when concurrently planting single-gene and multi-gene plants together, than only planting multi-gene Bt plants.

Larval survival on MON89034 indicated that alleles with resistance to Cry1A.105 + Cry2Ab2 proteins were possibly already present in the *S. frugiperda* population that invaded Africa, and that the Cry1Ab event does not meet the required high-dose criteria (Botha *et al.,* 2019). Omoto *et al*. (2016), Roush *et al*. (1997) and Onstad *et al*. (2013) reported that MON810 maize cultivars do not meet the requirements of a high-dose, which would lead to high survival rates in target pest populations. Huang (2020) and Sousa *et al.* (2016) previously also reported that MON810 maize varieties do not produce a dose high enough to cause the death of RS individuals of *S. frugiperda*. Furthermore, the partial efficacy of Cry1Ab maize against FAW in South Africa is similar to results reported by Niu *et al.* (2016), where high rates of survival and resistance against Cry1F toxins were recorded in *S. frugiperda* populations collected in Puerto Rico. Huang (2020) ascribed high rates of survival and resistance within *S. frugiperda* populations to an inherently low susceptibility of FAW to Cry1Ab protein.

4.6 Future recommendations

A recommendation for any future studies would be to also determine the fitness costs of resistant populations of FAW. This implies that the life history parameters of resistant FAW populations should also be determined when larvae are again exposed to non-Bt maize, to determine if there are fitness costs associated with resistance.

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APPENDIX A: DECLARATION OF LANGUAGE EDITING

Language editing statement

To whom this may concern,

I, Prof. Johnnie Van den Berg, hereby declare that the thesis titled: “Development of resistance by Fall armyworm *(Spodoptera frugiperda)* to Cry proteins in Bt maize” by Andriani Papa has been edited for language correctness and spelling. No changes were made to the academic content or structure of this work.

J van den Berg

Prof. Johnnie Van den Berg   Date: 22 November 2021