

**Toxicity bioassays with insecticide
formulations used for control of
Spodoptera frugiperda (Lepidoptera:
Noctuidae)**

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“A dream does not become reality through magic: it takes sweat, determination and hard work” – Collin Powell

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Abstract

The fall armyworm, *Spodoptera frugiperda*, is an exotic pest of maize in Africa. It is polyphagous and has the ability to develop on a wide range of host plants with different nutritional indices. Rearing of insects is important for entomological research. After evaluation of several artificial diets, the most suitable artificial diet for rearing of *S. frugiperda* larvae was determined in this study. The following four diets were evaluated: *Busseola fusca* diet, *Anticarsia gemmatalis* diet, Stonefly *Heliothis* diet, *Chillo partellus* diet and maize leaves. The following fitness parameters were used to compare suitability of the different diets for *S. frugiperda* development: larval and pupal development, pupal mass, survival, adult eclosion, fecundity and fertility. The *B. fusca* artificial diet was determined as the most suitable for rearing of *S. frugiperda*. The nutritional composition of the respective diets differed as well as the water content. These factors affected the fitness parameters. The *B. fusca* diet is, however, not suitable for toxicological studies with insecticides incorporated into the diet since the temperature while preparing the diet is too high for incorporation of insecticides. The Stonefly *Heliothis* diet was therefore selected for rearing and use in toxicological studies. Monitoring of insecticide efficacy is used for proactive evidence-based resistance management. Baseline susceptibility of *S. frugiperda* should be determined to monitor its susceptibility to pesticides in future. Four bioassay methods viz. leaf dipping, topical application, insecticide overlay onto and incorporation in artificial diets were evaluated for use in toxicological studies with insecticides with different modes of entry and action. These insecticides were chlorantraniliprole (diamide), lufenuron (benzoxazole), pyridalyl (unknown) and methomyl (carbamate). Dose responses of third-instar *S. frugiperda* larvae were evaluated with PoloSuite, and statistical parameters were analysed to determine the most appropriate bioassay for the different insecticide groups. The insecticide incorporation into artificial diet bioassay was identified as the most suitable for susceptibility evaluation of *S. frugiperda* to chlorantraniliprole and methomyl. For lufenuron, the most suitable method was the insecticide overlay onto artificial diet. No suitable bioassay could be developed for the evaluation of susceptibility to pyridalyl, which may be explained by its unknown mode of entry and action. The difference in suitability of bioassays determined for each insecticide showed that the mode of entry into the insect has a profound effect on the effectivity of bioassays and also on the estimate of the median lethal dose.

Key words: artificial diets, bioassays, *Spodoptera frugiperda*, rearing, toxicological studies

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CHAPTER 1

Introduction and literature review

1.1 General introduction

Maize (*Zea mays* L.) (Poaceae) and sorghum (*Sorghum bicolor* L) (Poaceae) are important grain crops in South Africa. The total area planted to maize in South Africa during the 2016/17 production season was 2 628 600 ha with a production of 16 820 000 tons. The 42 350 ha of grain sorghum yielded 152 000 ton grains (DAFF, 2018). These grain crops are hosts to several native lepidopteran pests of economic importance in Africa, such as *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae), *Eldana saccharina* (Walker) (Lepidoptera: Pyralidae) (Kfir *et al.*, 2002; Ong'amo *et al.*, 2006) and *Mussidia nigrivenella* (Ragonot) (Lepidoptera: Pyralidae) (Goergen *et al.*, 2016). It also hosts exotic lepidopteran pests viz. *Chilo partellus* (Swinhoe) (Crambidae) and the Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Noctuidae) (Goergen *et al.*, 2016; Abrahams *et al.*, 2017). *Spodoptera frugiperda* was reported in Africa after its invasion from the Americas early in 2016 (Goergen *et al.*, 2016).

1.2 *Spodoptera frugiperda*



Figure 1: Distinctive characteristics of (A) a *Spodoptera frugiperda* larva and (B) a male moth.

Spodoptera frugiperda (Figure 1) is an important economic pest in Central America (Andrews, 1980) and is responsible for major yield losses in several crops (Murúa *et al.*, 2006). Two strains have been identified and although these strains are morphologically

identical, they can be differentiated based on their host plant preferences (Levy *et al.*, 2002; Virla *et al.*, 2008; Nagoshi *et al.*, 2015). The maize strain is known to feed on maize as well as cotton and sorghum while the rice strain feeds predominantly on rice, Bermuda grass (Levy *et al.*, 2002; Nagoshi and Meagher, 2008; Virla *et al.*, 2008; Jeger *et al.*, 2017) and Johnson grass (Velez *et al.*, 2013). There is a high level of genetic variation in as well as behavioural and biochemical differences between these two strains (Jeger *et al.*, 2017). The strains differ in allelic frequencies (Velez *et al.*, 2013), several glycolytic enzymes and in mitochondrial DNA sequences (Levy *et al.*, 2002). Other differences include differences in sex pheromone blends and mating that occurs at different times during the night. Levy *et al.* (2002) reported these aspects to thwart the ability for the two strains to interbreed. Each strain develops differently on their host plants due to different feeding preferences and the respective strains follow different migratory pathways (Levy *et al.*, 2002).

1.3 Distribution of *Spodoptera frugiperda*

This species originated from the tropical and subtropical regions of South America, (Andrews, 1980; Nagoshi *et al.*, 2015; Abrahams *et al.*, 2017; Jeger *et al.*, 2017). *Spodoptera frugiperda* populations annually migrate into the southern and northern temperate regions of America. The population numbers increase with time and become abundant in late summer and autumn (Jeger *et al.*, 2017).

Outbreaks of *S. frugiperda* in West and Central Africa were recorded for the first time in early 2016, with initial populations found in Benin, Nigeria, Sao Tome, Príncipe and Togo (Goergen *et al.*, 2016; Jeger *et al.*, 2017). The means of introduction of *S. frugiperda* into Africa is unknown, but it is speculated that it may have entered via agricultural trade or possibly with the weather systems associated with El Nino events during 2014-2016 (Goergen *et al.*, 2016; Jeger *et al.*, 2017). The pest has spread to several countries in West Africa and occurs throughout sub-Saharan Africa (Goergen *et al.*, 2016; Jeger *et al.*, 2017). As a result the presence of FAW was recently reported in India (Shylesha *et al.* 2018). The global distribution of FAW, without the recent invasion into India is shown in Figure 2.

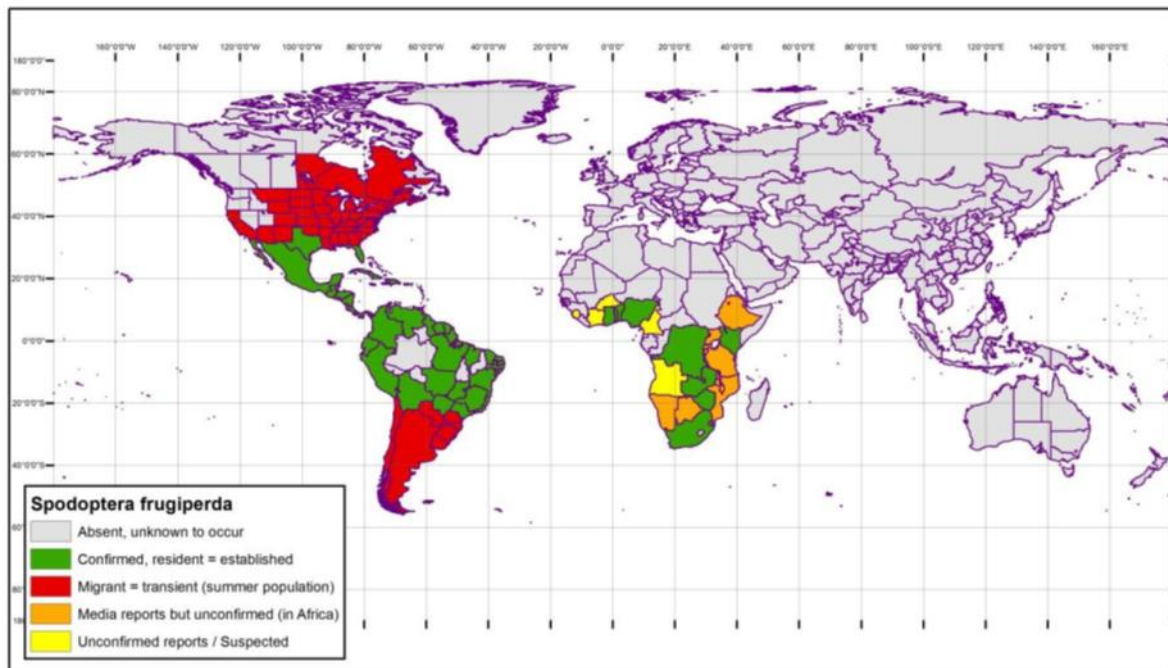


Figure 2: Global distribution of *Spodoptera frugiperda* documented during April 2017 (Jeger *et al.*, 2017).

To conduct research on *S. frugiperda* in South Africa, artificial diets should be evaluated to determine the most suitable diet for mass rearing as well as toxicological studies. Laboratory colonies of herbivorous insects are commonly reared on artificial diets to reduce the labour, time, space and costs associated with growing their host plants (Hervet *et al.*, 2016). These diets also simplify the synchronisation of insect development with the availability of food and can be optimised to increase insect fitness above that of insects reared on host plant tissue (McMorran, 1965).

1.4 Insect rearing

1.4.1 Development of artificial diets

Insects require nutritional substances for growth, tissue maintenance, reproduction and energy to maintain physiological functions (Chapman, 1998; Gullan and Cranston, 2008). Insects often have unusual or restricted diets, but the diet provides a complete range of the chemicals essential to the insect's metabolism (Gullan and Cranston, 2008). When insects have a restricted diet they utilize microorganisms to supplement the directly available nutrients (Gullan and Cranston, 2008). When artificial diets are developed it is therefore of great importance to understand the feeding and nutritional ecology of the species that needs

to be reared (Genc, 2006). Occasionally, a diet that has been designed for a specific species may be appropriate for another species with minor adaptations (Grenier, 2012).

1.4.2 Principals of insect's nutritional ecology

Nutritional balances are very important for insects as it affects the development, fertility and fecundity (Chapman, 1998; Genc, 2006). In terms of nutrition, optimal nutrition ensures progeny for the next generation. As insects are small they are adversely affected and are placed under physiological stress when nutrients are not sufficient (Genc, 2006). Insects react differently to nutritional imbalances, for example, they alter the total amount of food they ingest, larvae may move to another crop with a different nutritional balance or they regulate the effectiveness of the nutrients (Dadd, 1985; Chapman, 1998; Genc, 2006). The natural food of the species serves as indication which nutrients are required. Phytophagous insects such as lepidopteran larvae generally require equal amounts of proteins, amino acids and carbohydrates in their diets (Genc, 2006).

1.4.3 Different nutrient requirements

1.4.3.1 Protein and amino-acids

All arthropods utilise proteins to enable the synthesis of structural proteins, enzymes, receptors and storage structures. Thus proteins are vital for the development and growth of individuals (Chapman, 1998; Genc, 2006). Protein balance is therefore important for optimum growth and longevity (Haydak, 1953; Genc, 2006). The intake of protein is essential for the maturation of the ovaries and eggs since proteins enable the synthesis of hormones such as the juvenile hormone which is required for ovary and egg development. However, male insects do not require protein to mature their sperm (Genc, 2006). Optimal nutritional prerequisites alter with age, sex, and physiological stress (Chapman, 1998; Nation, 2001; Genc, 2006).

Amino acids are obtained when insects digest proteins. Insects need the same essential amino acids that other animals require from dietary sources (Chapman, 1998). These amino acids include arginine, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Genc, 2006; Thompson and Simpson, 2009). Other amino acids can be synthesized or derived from these essential amino acids. While all amino acids

are necessary for growth and development some participate in morphogenesis (Chapman, 1998). Tyrosine, for example, is critical for cuticle sclerotisation (Thompson and Simpson, 2009) while tryptophan is necessary for visual screening pigments. Other proteins such as γ -aminobutyric acid and glutamate are neurotransmitters, while proline serves as an important energy source in certain insects (Chapman, 1998). In some lepidopteran species such as *Helicoverpa zea* (Boddie) (Noctuidae) growth and development ceases in the absence of essential amino acids (Genc, 2006). Occasionally, nonessential amino acids stimulate growth and development, however this only happens when nutrients are balanced and the biochemical pathways involved in the synthesis of nonessential amino acids are organised (Genc, 2006).

1.4.3.2 Carbohydrates

Carbohydrates are primarily utilised for energy (Reinecke, 2013). Some insect species require specific carbohydrates in their diet (Chapman, 1998; Thompson and Simpson, 2009). However, they are not essential as carbohydrates can be synthesised from fats or amino acids (Chapman, 1998; Nation, 2001; Genc, 2006). Numerous carbohydrates, particularly sugars are potent feeding stimulants. Insects utilise carbohydrates differently; the utilisation depends on the ability of insects to hydrolyse polysaccharides (Chapman, 1998). Several insects have the ability to utilise a broad range of carbohydrates, this is more likely due to their ability to digest more complex structures (Chapman, 1998; Genc, 2006). Lepidopteran insects use both carbohydrates and lipids to supply energy for flight, but most insects can only utilise carbohydrates to support flight (Genc, 2006).

1.4.3.3 Lipids, polyunsaturated fatty acids and sterols

All cell membranes in biological organisms consist of fatty acids, phospholipids and sterols in addition to other specific functions (Chapman, 1998). Lipids mainly consists of free and bound fatty acids, long and short chain alcohols, steroids and their esters, phospholipids and other groups of compounds (Genc, 2006). Although carbohydrates are transformed into lipids, many insects have the ability to also synthesise lipids and accumulate them in fat body tissue (Nation, 2001; Genc, 2006). Since insects are able to synthesise various fatty acids and phospholipids they are not essential for dietary intake (Thompson and Simpson, 2009). Lepidopteran insects do, however, require dietary sources of fatty acids such as linoleic and linolenic acids (Fraenkel and Blewett, 1946; Chapman, 1998; Nation, 2001).

Fatty acids are present in both larval and adult stages (Genc and Nation, 2004). Polyunsaturated fatty acids and sterols are very important and are obtained through dietary nutrition as insects are incapable of synthesising these compounds (Chapman, 1998; Reinecke, 2013). When there is a deficiency of fatty acids several defects may occur, including wing malformation and the scales adhere to the pupal case when adults emerge (Vanderzandt, 1974). Genc and Nation (2004) reported larval survival of *Phyciodes phaon* (Edwards) (Lepidoptera: Nymphalidae) to be enhanced by the inclusion of linseed, olive and wheat germ oil in artificial diets.

The dietary intake of sterols is essential since most insect species are incapable of synthesising the sterol rings. However, if they are capable of synthesising sterols they may not be able to produce enough to meet their physiological prerequisites (Chapman, 1998). Sterols are essential in cellular membranes as well as for the synthesis of hormones (Thompson and Simpson, 2009; Reinecke, 2013). Sterol is the precursor for the synthesising of the ecdysteroid moulting hormone of insects (Chapman, 1998). Insufficient amounts of sterols in the diet will prevent successful moulting and this result in early-instar death (Nation, 2001; Genc, 2006). Most insects have the ability to utilise cholesterol which usually satisfies the sterol requirement. Even in minute amounts, lipids and sterols may stimulate growth and development (Chapman, 1998). Deficiency of sterols may manifest in any stage of the insect's life cycle and may also cause a reduction in fertility (Vanderzant, 1974; Nation, 2001).

1.4.3.4 Vitamins

Insects cannot synthesise vitamins and therefore obtain it through dietary intake (Chapman, 1998). Insects require different vitamins such as thiamine (B1), riboflavin (B2), nicotinic acid, pyridoxine, pantothenic acid, folic acid (B11), and biotin (Vitamin H) in small amounts (Chapman, 1998; Thompson and Simpson, 2009; Reinecke, 2013). Vitamins play important roles as cofactors of several enzymes that catalyse metabolic pathways (Chapman, 1998; Thompson and Simpson, 2009). Biotin contributes as cofactor to numerous enzymatic steps during the synthesis of fatty acids, and is a component of the enzyme pyruvate carboxylase (Tu and Hagedorn, 1992; Tuzz, 1992). Folic acid is essential for the biosynthesis of nucleic acid (Chapman, 1998). Deficiency of β -carotene may cause an abnormal green and yellow colour development and melanisation may also reduce (Chapman, 1998). When carotene is excluded from the artificial diet it may lead to delayed growth and moulting. In addition to

these phenomena, insects are generally smaller and less active than usual (Chapman, 1998). Carnitine is a main contributor in the channelling of fatty acids across mitochondrial membranes of insects (Genc, 2006). Vitamin E is a prerequisite for reproduction in some insects and it is also known to improve fecundity of some moths (McFarlen, 1992). Ascorbic acids are essential to maintain normal growth and development (Nation, 2001). Deficiency of ascorbic acid (Vitamin C) may cause abnormalities during ecdysis, this suggest that it may take part in cuticular sclerotisation (Chapman, 1998).

1.4.3.5 Minerals

Mineral requirements of insect are unknown and presumptions are made regarding their requirements (Chapman, 1998). Given the composition of insects and their physiology, it is rational to assume that sodium, potassium, calcium, magnesium, chloride and phosphate are essential minerals (Nation, 2001; Genc, 2006; Thompson and Simpson, 2009). Metal ions also serve as co-factors for enzymes. Molybdenum forms part of the xanthine dehydrogenase enzymes which is important in purine metabolism of insects (Genc, 2006). Insects require only trace amounts of iron and calcium. Salt mixtures have also been found to support the development of lepidopteran insects (Nation, 2001; Genc, 2006). Since the central element of cytochromes central element is iron, this element must be present in insect diets. Zinc and manganese play important roles in hardening of the cuticle and insect mandibles (Chapman, 1998)

1.4.3.6 Phagostimulants

Feeding on specific diets or crops is stimulated by certain chemicals which are also known as phagostimulants (Genc, 2006). Insects are attracted to or repelled from hosts or food due to the presence or absence of certain chemicals (Genc, 2006). Foods are identified and tasted with taste receptors on an insect's mouthparts, tarsi, antennae or other body parts. Potential phagostimulants may be nutritional components or non-nutritional allelochemicals (Nation, 2001; Genc, 2006).

1.5 Insecticides

1.5.1 Modes of action of insecticides

The mode of action explains what happens at a cellular level to an organism when it is exposed to particular chemical compounds (Yu, 2008; Sparks and Nauen, 2015). Insecticides kill their target species by interacting with a primary site of action within an arthropod whereby at least one basic physiological process is altered leading to death (Guedes *et al.*, 2016). These physiological alterations are the basis on which insecticides are developed (Guedes *et al.*, 2016).

The Insecticide Resistance Action Committee (IRAC) grouped chemical compounds with the same mode of action and primary site of action into main groups and sub-divided these into chemical sub-groups or examples of active ingredients (Sparks and Nauen, 2015). More than 25 different modes of action have been identified and grouped in the IRAC classification scheme (Sparks and Nauen, 2015). Of these groups, 85% act on the insect's nerve-muscle system (Casida and Durkin, 2013; Sparks and Nauen, 2015). Of the total insecticide sales, only 9% belong to insecticides that alter growth and development and 4% have a mode of action that disrupts energy production (respiration targets) (Sparks and Nauen, 2015). Any alterations in the nervous system are quickly amplified. The nervous system has been and remains the main target for development of insecticides.

Insecticides with modes of action which target the nervous system, either by inhibiting acetylcholinesterase or by affecting nerve cells directly cause acute effects after application (Yu, 2008; Sparks and Nauen, 2015). Other insecticidal compounds influence the developmental or metabolic processes of insects by mimicking or altering the action of hormones, or by altering the biochemistry of cuticle production (Yu, 2008; Sparks and Nauen, 2015).

Currently, organophosphates, carbamates, and pyrethroids dominate with 31% of the world market in insecticides. Among the nerve-muscle acting insecticides, neonicotinoids predominate with 27% of the world market (Simon-Delso *et al.*, 2014; Sparks and Nauen, 2015). Diamides that act on the ryanodine receptors are relatively new and account for 8% of total global insecticide sales but sales are steadily increasing (Sparks and Nauen, 2015).

Resistance towards insecticide modes of action correlates with the market share of the different insecticide products (Sparks and Nauen, 2015).

1.5.1.1 Insecticide affecting voltage gated sodium channels

Several classes of insecticides are axonic toxins, these insecticides interfere with axonal conduction. Insecticides such as pyrethroids bind to sodium channels (Khambay and Jewess, 2010), causing delays in the closing of the channel resulting in prolonged sodium inactivation (Yu, 2008; Soderlund, 2012; Meijer *et al.*, 2014). It causes a negative after potential, resulting in a belated recovery of its resting stage. Repetitive discharges of axonal action potentials occur in response to insecticide stimuli (Yu, 2008). This causes the axon to be easily excited again. These insecticides cause excessive neuro-excitation and hyperactivity, tremors, and rigid paralyses may occur (Yu, 2008).

Indoxacarb is a pro-insecticide that is easily metabolised by an esterase/amidase (Yu, 2008; Ghanim and Ishaaya, 2011) to its analogous N-decarbomethoxylated metabolite (DCJW) (Wing *et al.*, 2000; Lapied *et al.*, 2001; Silver *et al.*, 2010; Bird, 2015). This metabolite acts as an antagonist (Sánchez-Bayo, 2011) and is a very strong sodium channel blocker in insects (Pang *et al.*, 2012; Bird, 2015), causing flaccid paralysis and death (Lapied *et al.*, 2001). Both indoxacarb and pyrethroids bind to the sodium channel, but at different sites and exert different actions (Yu, 2008). Pyrethroids are responsible for lingering membrane depolarisation leading to repetitive nerve firings whereas DCJW suppresses spontaneous central nervous system action potentials (Yu, 2008). Thus, pyrethroids bind to sodium channels and keep them open whereas DCJW binds to certain types of sodium channels and prevent sodium ions from flowing into the axon (Yu, 2008).

1.5.1.2 Insecticides affecting calcium channels

Calcium channels are situated in nerve terminals and muscles. Insecticides such as flubendiamide affect the calcium channels and cause a gradual contraction of the insect's body (Yu, 2008). Flubendiamide induces the release of intracellular calcium, mediated by calcium channels such as the ryanodine receptor resulting in the contraction of an insect's muscle (Yu, 2008). Chlorantraniliprole is a selective ryanodine receptor agonist (Bird, 2015). Binding of this insecticide stimulates release of calcium from the sarcoplasmic reticulum resulting in impaired regulation of muscle contraction (Yu, 2008; Bird, 2015). Ingestion is the

primary route of exposure. The cardiac and skeletal muscles are rapidly affected after ingestion, causing instant feeding cessation and immobility (Yu, 2008).

1.5.1.3 Insecticides interfering with GABA-gated chloride channels

The GABA receptor chloride ionophore complex can be found in the central nervous system and also at peripheral neuromuscular junctions (Yu, 2008) and ganglia (Sánchez-Bayo, 2011). An ionophore is a substance which is able to transport particular ions across a lipid membrane in a cell (Lackie, 2007). Avermectins such as emamectin benzoate (Yu, 2008) bind to GABA receptors and act as a partial agonist (Sánchez-Bayo, 2011; Ghanim and Ishaaya, 2011). Avermectins mimic the chemical normally responsible for the regulation of the GABA receptor, and opens the chloride channel (Yu, 2008). This causes chloride ions to flow into the postsynaptic neuron (Yu, 2008; Das, 2013; Ghanim and Ishaaya, 2011). The effect is similar to that of GABA but it is irreversible (Yu, 2008). When avermectin-dependant conductance increased (Yu, 2008), sensitivity and paralysis results (Das, 2013; Bird, 2015). These insecticides may also affect an insect's glutamate-gate chloride channels resulting in paralysis (Yu, 2008).

1.5.1.4 Insecticides that bind to nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChR) are found in the insect's nervous system, situated on both pre- and postsynaptic nerve terminals (Sánchez-Bayo, 2011) on the cell bodies of interneurons, motor neurons and sensory neurons (Yu, 2008). Nicotinic acetylcholine receptors are so called because the bond is held more tightly due to nicotine (Yu, 2008). These insecticides are known to induce neuronal over-excitation by targeting nicotinic acetylcholine receptors (Matsuda *et al.*, 2009; Meijer *et al.*, 2014). Insecticides such as spinosyns mimic acetylcholine by acting as an agonist to activate the nicotinic acetylcholine receptor. The activation of the receptor causes an influx of sodium ions and creates action potentials (Yu, 2008). During normal physiological conditions, the synaptic action and binding is terminated by the enzyme acetylcholinesterase which hydrolyses the neurotransmitter. When insecticides bind they are not hydrolysed or destroyed by AChE (Yu, 2008). This persistent activation leads to an over-stimulation of cholinergic synapses (Yu, 2008; Salgado and Sparks, 2005), resulting in hyper-excitation (Sánchez-Bayo, 2011), convulsion, paralysis and finally death of the insect (Yu, 2008). Both spinosad and

spinetoram have a neurotoxic modes of action through contact or ingestion (Bacci *et al.*, 2016).

The nereistoxin analogs such as cartap hydrochloride are pro-insecticides that must be activated in vivo to become a nereistoxin (Yu, 2008). A nereistoxin acts as an antagonist of the acetylcholine receptor. Symptomatic effects are different with a nereistoxin as opposed to nicotine. Insects treated with nereistoxin are rapidly immobilized without convulsive symptoms which is ascribed to the fact that nereistoxin does not induce depolarisation (Yu, 2008).

1.5.1.5 Insecticides affecting biosynthesis

Benzoylphenylureas such as lufenuron are inhibitors of chitin biosynthesis (Yu, 2008; Sánchez-Bayo, 2011). These insecticides inhibit the formation of chitin (Ghanim and Ishaaya, 2011), thus the elasticity and firmness of the endocuticle is affected (Yu, 2008). Symptomology is observed during moulting (Das, 2013). The integrity of the cuticle is compromised and the cuticle is unable to support the insect and withstand the rigors of moulting, and this ultimately results in death of the insect (Yu, 2008).

1.5.1.6 Insecticides acting as ecdysone agonists

Diacylhydrazine insecticides such as methoxyfenozide bind to the ecdysone binding site of the ecdysone receptor-usp dimer, which activates the ecdysone responsive genes that are normally activated during moulting and metamorphosis (Yu, 2008; Zarate *et al.*, 2011). Feeding is then interrupted within 3 to 14 hours. Diacylhydrazine insecticides such as methoxyfenozide interact as a nonsteroidal ecdysone agonist (Yu, 2008). The insecticides bind to specific ecdysteroid receptor binding proteins, thereby accelerating the moulting process (Ghanim and Ishaaya, 2011) which disrupts the natural sequence of events and causes incomplete precocious moult (Sánchez-Bayo, 2011). This causes mortality of the larva (Yu, 2008; Zarate *et al.*, 2011).

1.5.1.7 Insecticides affecting ryanodine receptors

Modulators such as diamide insecticides activate ryanodine receptors, which are calcium-activated channels in the sarcoplasmic reticulum of muscle cells. Their function is to amplify

a small trigger calcium signal to produce a massive calcium release from intracellular stores that is needed for muscle contraction (Yu, 2008). Ryanodine receptors are also found in neurons of the central nervous system where it may be involved in calcium-signalling (Yu, 2008). Direct activation of ryanodine receptors by these insecticides causes sustained muscle contractions leading to rapid feeding cessation, regurgitation, lethargy and tetany (Yu, 2008). Chlorantraniliprole also known as an anthranilic diamide that binds selectively to ryanodine receptors in the muscles of insects (Bassi *et al.*, 2009; Hannig *et al.*, 2009; Sial *et al.*, 2011). This causes an uncontrolled release of calcium from internal stores in the endoplasmic reticulum. Impaired regulation of muscle contraction is observed resulting in feeding cessation, lethargy, paralysis and death (Sial *et al.*, 2011).

1.5.1.8 Insecticides inhibiting acetylcholinesterase

Acetylcholinesterase is responsible for the removal of the excitatory neurotransmitter acetylcholine from the cholinergic synapses (Yu, 2008). Organophosphates and carbamates inhibit and adhere to acetylcholinesterase while acetylcholine accumulates and causes prolonged stimulation (Yu, 2008; Das, 2013). This in turn causes desensitisation of the acetylcholine receptors and severe neurological disruption, and ultimately death (Yu, 2008).

Organophosphorous insecticides (OPs) are structurally similar to acetylcholine and compliments the AChE enzyme molecule (Singh, 2012). OPs are potent irreversible inhibitors of acetylcholinesterase (Meijer *et al.*, 2014). OPs alter the serine hydroxylgroup within the enzyme active site, phosphorylation of the hydroxyl group occur yielding a hydroxylated leaving group. This sequence inactivates the enzyme and impedes the degradation of the neurotransmitter acetylcholine (Singh, 2012). The synaptic concentration of acetylcholine increases, this results in hyper-excitation of the central nervous system. Phosphorylation of acetylcholine is persistent and reactivation of the enzyme may take hours or days (Singh, 2012). Chlorpyrifos is an example of an organophosphate.

Carbamates react with acetylcholinesterase in a similar manner as OPs. They bind to the enzyme cholinesterase forming a reversible complex. A carbamylation reaction of the serine hydroxyl group results (Singh, 2012). The complex degrades to a stable carbamylated enzyme and a hydroxyl leaving group. Sequentially the carbamylated enzyme is hydrolysed to regenerate the free enzyme and methylcarbanic acid (Singh, 2012). The differences between these two groups are that the phosphorylated enzyme hydrolyses at much slower

rate compared to the carbamylated enzyme. Thus recovery of carbamate poisoning occurs within hours after exposure (Singh, 2012). Methomyl is an example of a carbamate.

1.5.1.9 Microbial disruptors of insect mid-gut membranes

Bacillus thuringiensis (Bt), is a gram-positive, rod shaped bacterium, characterised by its ability to form crystal-like parasporal inclusions during sporulation (Whalon and Wingerd, 2003; Vachon *et al.*, 2012). Proteins also known as endotoxins are found within parasporal inclusions (Vachon *et al.*, 2012). A wide variety of crystal (Cry) proteins are known for their insecticidal activities (Whalon and Wingerd, 2003; Bravo *et al.*, 2010). Larvae ingest the Bt crystal inclusion, which is then solubilised into a protoxin. The protoxin is proteolytically processed to smaller protease-stable polypeptides which are the active toxins. These active toxins with high affinity to specific receptors bind at the surface of the midgut epithelial cells. This allows the irreversible insertion of the toxin into the membrane causing pores to form which are permeable to small molecules (Jenkins *et al.*, 2000; Whalon and Wingerd, 2003) such as inorganic ions, amino acids and sugars (Vachon *et al.*, 2012). The manifestation of these pores in the plasma membrane disrupts the cell physiology by eliminating the transmembrane ionic gradients and may lead to colloid-osmotic lysis of the cells due to substantial influx of solutes from the midgut lumen. Destruction of these cells results in extensive damage to the midgut epithelial tissue and death of the intoxicated larvae (Vachon *et al.*, 2012).

1.5.2 Modes of entry of insecticides into insects

Insecticides have different pathways of entering the body of an insect and can be differentiated as dermal/contact insecticides or stomach insecticides (Perry *et al.*, 1998; Yu, 2008; Sparks and Nauen, 2015). However, there are also several other insecticides with different modes of entry and a single insecticide may have more than one of these characteristics (Yu, 2008; Sparks and Nauen, 2015).

1.5.2.1 Contact and residual insecticides

These insecticides come into contact with the peripheral of the insect, penetrate the cuticle (Van Emden and Service, 2004; Singh and Merchant, 2017) and dissolve in the haemolymph (Yu, 2008). The haemolymph transports the insecticide to the target internal

organs (Yu, 2008). Ephemeral contact insecticides have short half-lives (Van Emden and Service, 2004) and should come into contact with the insect at the time of application to ensure efficacy (Singh and Merchant, 2017). Some insecticides remain active for a long time and form a residue on the surface it is applied to. A toxic dose is administered when an insect comes into contact with the residue (Singh and Merchant 2017). The time that the residual layer is effective vary greatly with insecticide, dose and environmental conditions (Van Emden and Service, 2004).

1.5.2.2 Stomach insecticides

Stomach insecticides are activated when certain stomach enzymes bind to the active ingredient (Van Emden and Service, 2004) and these insecticides therefore need to be ingested to be effective (Van Emden and Service, 2004; Singh and Merchant, 2017). Stomach insecticides have a significant advantage over contact insecticides since only insects which feed on the plant are affected and other beneficial insects are not harmed (Van Emden and Service, 2004). Stomach insecticides are ingested by feeding on the surface of plants where insecticides had been applied to as well as on plant material from plants sprayed with insecticides with a systemic or translaminar action in the plant (Van Emden and Service, 2004).

1.5.3 Insecticide resistance

Since arthropod pests are a major constraint to agricultural production (Abate *et al.*, 2000), insecticides play an important role in agriculture by protecting and improving productivity through pest management (Handford *et al.*, 2015). The application of insecticides has been practiced for more than two millennia in several countries such as China, India, Greece and Egypt, and for more than 150 years in Europe and North America (Isman, 2006). The significant increase in the use of insecticides over the past 50 years caused an increase in selection pressure for insecticide resistance (Head and Savinelli, 2007; Whalon *et al.*, 2008; Sparks and Nauen, 2015). Resistance evolution to insecticides is an urgent problem that threatens agriculture worldwide (Tabashnik *et al.*, 2014). Resistance is a micro-evolutionary process whereby arthropods are genetically adapted to selected insecticides resulting in populations of insects with unique and difficult management challenges (Whalon *et al.*, 2008; Tabashnik *et al.*, 2014). The majority (56.1%) of insecticide resistant insect species are

agricultural pests, while only 4.6% of resistant insects are beneficial. This proves that agrochemical practices cause evolution of resistance (Yu, 2008).

Exacerbated application of conventional insecticides led to many resistance problems over the years (Ghanim and Ishaaya, 2011). Insecticide resistance have been reported to all major insecticide classes, in recent years (Head and Savinelli, 2007; Ghanim and Ishaaya, 2011). Certain arthropod pests have developed insecticide resistance to numerous active ingredients from a variety of different classes while other pests have little or no history of resistance (Head and Savinelli, 2007).

Most of these arthropods belong to a relatively small number of families *viz.* Tetranychidae (mites), Culicidae (mosquitos), Noctuidae (moths) and Aphididae (aphids) (Pittendrigh *et al.*, 2007). These pests develop resistance faster due to frequent exposure to the same or similar insecticides on different crops (Head and Savinelli, 2007). The biology of pest species enables the development of resistance and once resistance is established, the frequency of resistance within the population may increase rapidly (Head and Savinelli, 2007). The abovementioned species are generally biochemically pre-adapted to develop resistance. Many herbivore species are polyphagous and they are therefore adapted to deal with a variety of plant defensive chemicals, which include alkaloids. These insects already possess mechanisms to detoxify or excrete novel toxins (Head and Savinelli, 2007; Dawkar *et al.*, 2013). Pests also have great dispersal capabilities, for example moths, while human activities can also contribute to their dispersal (Head and Savinelli, 2007). In addition, lepidopterans are highly adaptive to various stressors such as climate, the environment and food, which benefit them in evolving multiple survival mechanisms (Dawkar *et al.*, 2013).

1.5.3.1 General mechanisms for resistance

The development of new insecticides as well as resistance against different insecticide groups are on-going processes (Pittendrigh *et al.*, 2007; Yu, 2008; Dawkar *et al.*, 2013). Physiological responses toward a certain insecticide at individual level comprise not only the dose-response relationship but also nontoxic or protective responses (Guedes *et al.*, 2016). Studies conducted over several decades indicate that there are a limited number of mechanisms contributing to insecticide resistance (Pittendrigh *et al.*, 2007; Yu, 2008). Resistance mechanisms are often monogenic and it primarily resists the effect of insecticides by increasing the metabolic degradation (Ghanim and Ishaaya, 2011) of the

foreign substance, or the target site is changed in such a way that the effect of the insecticide is reduced or eliminated (Pittendrigh *et al.*, 2007; Yu, 2008; Dawkar *et al.*, 2013; Guedes *et al.*, 2016). The reduced rates of uptake (Ghanim and Ishaaya, 2011) and enhanced internal binding of the active ingredient to neutral molecules also aid in resistance development (Pittendrigh *et al.*, 2007; Yu, 2008; Dawkar *et al.*, 2013).

1.5.3.2. Reduced penetration

Reduced penetration (Heong *et al.*, 2011) is caused by a mechanism that prevents or reduces the entry or penetration of an insecticide into the insect's body (Nishida, 2002; Pittendrigh *et al.*, 2007; Yu, 2008). It has been hypothesised that limited penetration could delay the pesticide from reaching the target site, resulting in detoxifying enzymes having more time to metabolise the pesticide before it reaches its target (Nishida, 2002; Pittendrigh *et al.*, 2007; Yu, 2008).

1.5.3.3 Increased sequestration or excretion

Increased sequestration or excretion occurs when enzymes or proteins that are found in the body of an insect bind to the insecticide molecules and consequently transfer or transport these molecules away from the target site to several organelles such as fat bodies and haemolymph for safe storage (Pittendrigh *et al.*, 2007). This mechanism could have evolved early in the evolution of insects due to their interactions with flowering plants of which many may have contained toxic secondary compounds (Pittendrigh *et al.*, 2007; Yu, 2008; Dawkar *et al.*, 2013). Many resistant insects sequester insecticides and this process is frequently mediated by the esterase enzymes (Pittendrigh *et al.*, 2007; Yu, 2008; Dawkar *et al.*, 2013). Two different types of esterase-based resistance mechanisms exist. Firstly, increased levels of insecticide sequestration where insecticides swiftly attach to the esterase enzyme may take place. This action causes a broad spectrum of resistance. Secondly, point-mutations may occur which results in altered substance specificity, wherein a group of insecticides with a mutual ester bond are metabolised and reduced to less toxic forms, which causes narrow spectrum resistance (Pittendrigh *et al.*, 2007; Yu, 2008; Dawkar *et al.*, 2013).

1.5.3.4 Behavioural resistance

Behavioural changes in arthropods occur as a result of physiological alterations after interactions with the environment. Behavioural responses to pesticide exposures are a useful early warning of resistance evolution (Guedes *et al.*, 2016). Behavioural alterations resulting from the presence of insecticides are caused by the mode of action, innate responses toward the insecticide itself, or alterations in the environment. These responses can minimise or enhance the effect of the insecticide (Guedes *et al.*, 2016). Any behaviour such as avoidance (Heong *et al.*, 2011) or irritability after exposure is classified as behavioural resistance (Pittendrigh *et al.*, 2007; Yu, 2008). Behavioural changes may also occur during oviposition and feeding (Pittendrigh *et al.*, 2007; Yu, 2008). Behavioural responses to insecticides may be stimulus dependant. Alteration in behaviour occurs after insecticide detection and the response is enhanced by the stimuli. Responses may also be stimulus independent, due to an innate behavioural trait. Both stimulus dependant and independent responses may co-occur in an organism (Guedes *et al.*, 2016).

1.5.3.4 Metabolic resistance

Metabolic resistance to insecticides is a common resistance mechanism which occurs in many lepidopteran species (Scott and Wen, 2001; Enayati *et al.*, 2005; Pittendrigh *et al.*, 2007; Yu, 2008). Toxicity and persistence of an insecticide in the body of the insect depend on the ability of the insect to metabolise and excrete the insecticide (Singh, 2012). Insects often have the ability to increase their metabolism rate in response to a given insecticide (Pittendrigh *et al.*, 2007; Yu, 2008). The level of a specific enzyme is increased (Heong *et al.*, 2011) which degrades or alters the insecticide to a less toxic form, or an enzyme can be structurally changed which allows for the insecticide to be more easily processed (Pittendrigh *et al.*, 2007; Yu, 2008). Xenobiotics such as insecticides are mainly lipophilic in nature. This characteristic enables the insecticide to penetrate lipid cell membranes (Singh, 2012). As these molecules are insoluble in water, they are not easily excreted, unless the molecule is transformed to a polar compound (Singh, 2012). Therefore, the first step in xenobiotic metabolism is to alter the molecule into a more or less polar molecule. Sequentially there are two phases of reaction. During phase one, the xenobiotic compound is converted to a polar molecule (Singh, 2012). This alteration may be through oxidation, reduction or hydrolysis reactions. The product may serve as a substrate for the second phase of the two reactions (Singh, 2012). The xenobiotic compound or phase one product

conjugates with various endogenous molecules such as such as sugars, amino acids, glutathione, phosphate and sulphate. These products are generally more polar, less toxic and easily defecated (Singh, 2012). There are a few general metabolic resistance mechanisms which include the role of cytochrome P450s, glutathione S-transferases (GSTs), or esterase's (Enayati *et al.*, 2005; Pittendrigh *et al.*, 2007; Yu, 2008).

Specific enzyme classes are found in most organisms (Heong *et al.*, 2011). Metabolic resistance can be associated with over transcription of detoxification enzymes (Pittendrigh *et al.*, 2007; Yu, 2008). P450 enzymes, such as cytochrome P450 metabolise insecticides by N-, O-, and S-alkyl hydroxylation, aromatic hydroxylation, aliphatic hydroxylation and expoxidation, ester oxidation as well as thioether and nitrogen oxidation (Pittendrigh *et al.*, 2007; Yu, 2008).

Glutathione S-transferases are responsible for a variety of biological functions within the cell, including detoxification of xenobiotics such as insecticides (Heong *et al.*, 2011), carcinogens and drugs. GSTs are found in the cytosol and the membrane of all eukaryotic cells (Pittendrigh *et al.*, 2007; Yu, 2008). The expression levels of GSTs are in some cases directly related to the tolerance of the organism to the insecticides. GSTs are often responsible for resistance to certain insecticides of which organophosphates, organochlorides, DDT and pyrethroids are included (Pittendrigh *et al.*, 2007; Yu, 2008). Hydrophobic toxic compounds are converted to hydrophilic products by the action of the GST enzyme (Dawkar *et al.*, 2013).

An esterase such as acetylcholinesterase (AChE) is a hydrolase enzyme that splits the ester bonds in insecticides to yield an acid and an alcohol (Enayati *et al.*, 2005; Pittendrigh *et al.*, 2007; Yu, 2008). There is a variety of esterases that differ in their substance specificity, protein structure as well as their biological function. Insects attain resistance to organophosphates, carbamates and pyrethroids through esterases (Enayati *et al.*, 2005; Pittendrigh *et al.*, 2007; Yu, 2008).

1.5.3.5 Target site insensitivity

This mechanism refers to a change in the target site (Heong *et al.*, 2011). Mutations occur at the enzymatic target site and may occur with one or more amino acids that change (Heong *et al.*, 2011). Modified enzymes display a various degrees of insensitivity (Heong *et al.*,

2011). The target molecules which directly act with the pesticide, are altered and result in decreased toxicity of the insecticide (Pittendrigh *et al.*, 2007; Yu, 2008).

1.6 Dose-response bioassays

Determining the appropriate dose is a crucial endeavour (Duke, 2017). Insecticide dosage rates are grounded on the dose that effectively kills the most tolerant insect (Duke, 2017). Recommended rates are often much higher than needed for effective management of susceptible target species (Duke, 2017). High dosages as well as low dosages (Duke, 2017) could cause selection pressure for resistance in insect populations (Helps *et al.*, 2017).

1.6.1 Toxicology and dose-response

Insecticides should be studied to determine their adverse effects on a certain species (IPCS, 2009; Singh, 2012). Hence, toxicology is the science of characterising and quantifying the toxic or adverse effects of a chemical agent on a living organism (IPCS, 2009; Heong *et al.*, 2011; Singh, 2012; Roberts *et al.*, 2015). Insect toxicology focuses on the effects of chemicals that cause death or delay insect development, growth, and metamorphosis and/or reproduction (Heong *et al.*, 2011).

Toxic interactions of an insecticide with an insect's biological system are dose dependant (Paramasivam and Selvi, 2017). Toxicological studies are therefore all based on dose-response principals. Acute toxicity studies also known as LD₅₀ are defined as the dose that causes 50% mortality of test subjects after oral or dermal exposure to a selected insecticide (Heong *et al.*, 2011; Singh, 2012; Arome and Chinedu, 2014; Roberts *et al.*, 2015; Muntz *et al.* 2016; Paramasivam and Selvi, 2017).

Quantal dose response relationships show the variation in response to escalated dosage rates as a representation of the effects that may occur within a population (Guedes *et al.*, 2016). There are two indirect assaying methods, these methods are performed by exposing groups of individuals to standard doses and recording the responses, which may be death, knockdown, deformity or discoloration (Heong *et al.*, 2011). It is critical to understand what is meant by “dose” in dose-response analyses (IPCS, 2009). There are three basic types of “dose”, *viz.* the administered or external dose, the internal (absorbed) dose, and the target or tissue dose. External dose refers to the amount of an insecticide that is administered to the insect in a controlled experimental setting by a specific route at a specific frequency; the

dietary exposure of insecticides also refers to external dose (IPCS, 2009). The systemic availability of a toxicant denotes to the internal dose, which is available and resulting from absorption, distribution, metabolism, and excretion of the toxicant. The tissue dose refers to the quantity of toxicant that is distributed to and present in a specific tissue (IPCS, 2009). There are two parameters that influence dose-response experiments, viz. the dose frequency and duration of dosing. When evaluating resistance of insects to a specific insecticide the dosage is acute and the experiment does not last longer than seven days. After administration the response of the insect to the specific insecticides is observed and evaluated (IPCS, 2009; Roberts *et al.*, 2015).

The response to a specific insecticide concentration may differ considerably between individuals within a population since random variation may be found within a selected population (IPCS, 2009). Thus it is possible to conduct median lethal dose testing within a selected population because each individual has the possibility to respond differently to a selected dose (IPCS, 2009).

1.6.2 Variability in dose-response bioassays

The source of variability in dose-response may be due to differences in age, sex, rearing temperature, food supply, heterogeneity and illumination (Yu, 2008; Heong *et al.*, 2011).

1.6.2.1 Age

Active stages (larva, adult) of an insect are more susceptible than inactive stages (egg, pupa) due to the anatomical reorganisation and associated changes in metabolism (Kranthi, 2005; Yu, 2008). Resistance mechanisms may not manifest in younger larvae (Kranthi, 2005). This was reported by Yu (2008) with susceptibility results of *S. frugiperda* to selected insecticides (methomyl, diazinon and permethrin). A decrease in susceptibility to these insecticides was observed with later larval instars (Cook *et al.*, 2004; Yu, 2008). The LD₅₀ of sixth instar larvae were 135, 154, and 236 times higher for the abovementioned insecticides, but third instars had a tolerance of only 3.1, 3.2, and 5.6 fold respectively, on a body weight basis. This phenomenon has also been observed in the corn earworm (Yu, 2008).

1.6.2.2 Sex

In general, compared to males, female insects have been found to be more tolerant to insecticides. This is especially true for adult stages as the female is often larger than the male (Yu, 2008).

1.6.2.3 Rearing temperature

Differences in tolerance have been found in insects reared at different temperatures prior to treatment (Yu, 2008). Tolerance of DDT was greatest in American cockroaches that were acclimatised to lower temperatures. Yu (2008) ascribed this to insects reared at lower temperature having more unsaturated lipids. This causes greater solubility of an insecticide, which is then stored in inert fatty tissues rendering them unable to bind to the target site (Yu, 2008).

1.6.2.4 Food supply

Insect size and survival capacity are directly influenced by the quality and quantity of their diet (Yu, 2008). *Spodoptera frugiperda* larvae fed with maize leaves were less susceptible to methomyl, acephate, methamidophos, diazinon, trichlorfon, monocrotophos, permethrin and cypermethrin compared to larvae that fed on soybean leaves (Yu, 2008). Although nutrition may play a role, insecticide tolerance resulting from larval feeding on certain host plants is mainly due to plant allelochemicals, which induce detoxification enzymes in the insects (Yu, 2008).

1.6.2.5 Heterogeneity

Genetic differences occur between individuals within a population. A certain percentage of the population may therefore be more resistant towards a selected insecticide (Yu, 2008).

1.6.2.6 Illumination and environment

The intensity of illumination affects the activity of numerous insect species. It may therefore influence tolerance toward a specific insecticide due to differences in rates of metabolism

and uptake (Yu, 2008). Sub-optimal temperatures and varying relative humidity may cause variability in dose response bioassays (Kranthi, 2005).

1.6.3 Importance of bioassays

The main aim with the use of bioassays is to determine the most appropriate dose that effects selected insect pests (Kranthi, 2005). Bioassays refer to several methods in which a certain characteristic of a substance is measured in terms of response (Dewey, 1958). Insecticide bioassays conducted with a specific insect species enable the evaluation of its susceptibility towards a certain insecticide or mode of action group. It also enables scientists to determine the levels of resistance to a particular chemical compound within a given population (Paramasivam and Selvi, 2017). Since insecticides have different modes of action and modes of entry into an insect's body, numerous techniques are used to ensure that the insecticide reaches the target site (Paramasivam and Selvi, 2017). Recent advances in research and technology renewed interest in resistance risk assessment and the development of different bioassay methods (Durmusoglu *et al.*, 2015; Paramasivam and Selvi, 2017). Evaluation and development of new bioassay methods enables scientists to evaluate toxicity of insecticides with different modes of action towards the same species under the same test conditions (Paramasivam and Selvi, 2017).

Insecticide resistance monitoring is one of the first steps in the development of an insecticide resistance management (IRM) program (Sparks and Nauen, 2015; Zhu *et al.*, 2016; Pittendrigh *et al.*, 2007). Reliable, quick and effective bioassay techniques are needed to obtain effective resistance management (Gunning, 1993). Bioassay methods should closely resemble field conditions to ensure predictability of susceptibility of a population in the field from data obtained through laboratory measured resistance (Kranthi, 2005). Bioassays should be designed in such a manner to ensure reliability, replicability and consistency. It should be robust enough not to be influenced by variations in operator skills, materials, extraneous factors and handling procedures (Kranthi, 2005).

Conducting resistance studies also enables suggestions for more effective and safer insecticides to delay resistance development (Roush and Tabashnik, 1990). To report and compare results from susceptibility studies, a standard method should be used to ensure accurate analysis. IRAC developed several test methods in accordance with the technique used for insecticide application (IRAC, 2018). These methods can be grouped into four

categories viz. dipping, diet overlay, topical and feeding (Ffrench-Constant and Roush, 1990).

1.6.4 Commonly used bioassay methods

1.6.4.1 Topical application

This method is very effective for the evaluation of the efficacy of contact insecticides. Conventional techniques such as Potter's tower and Burkhard's micro-applicator were replaced by the hand held Hamilton repeating dispenser (Kranthi, 2005). This technique is one of the most convenient methods of dispensing known amounts of insecticides accurately onto insects (Singh, 2012). Insecticides are dissolved in a relatively nontoxic and volatile solvent such as acetone and a pre-calibrated one microliter solution is dispensed on the dorsal surface of the prothoracic region of the larvae (Kranthi, 2005; Yu, 2008; Singh, 2012). This method has numerous advantages. A high degree of precision and reproducibility can be attained. Large quantities of specimens can be tested in a relatively short time while simple and inexpensive equipment are used to perform these experiments (Singh, 2012).

1.6.4.2 Insecticide surface coating (dipping)

These bioassays are commonly referred to as residual tests (Perry *et al.*, 1998; Kranthi, 2005). When using this technique leaves, paper or plastic surfaces are coated with a thin film of diluted insecticide solution (Kranthi, 2005; Singh, 2012). The leaf residue assays closely simulate field exposures and have been used to monitor resistance levels in several insect species. However, this method tends to show variable results because of variation in the age of the leaf, stage of the plant, variety, environmental stress to plants and poor feeding capability of larvae, in addition to the risk of avoidance of the treated surface (Kranthi, 2005). Surface coating tests are primarily effective for the evaluation of oral insecticide efficacy (Perry *et al.*, 1998; Kranthi, 2005; Singh, 2012).

1.6.4.3 Diet incorporation

Diet incorporation, is also effective for the evaluation of oral insecticides (Perry *et al.*, 1998; Kranthi, 2005; Singh, 2012). These bioassays are fairly simple but depend on several factors

that include the availability of large amounts of toxins, thermal stability, consistent bioactivity under bioassay conditions and the suitability of the diet used in the bioassay (Kranthi, 2005).

1.7 *Spodoptera frugiperda* insecticide resistance`

Severe *S. frugiperda* infestations and the resultant economic losses caused reliance on rigorous applications of chemical insecticides (Yu *et al.*, 2003; Carvalho *et al.*, 2013; Perez-Zubiri *et al.*, 2016; Abrahams *et al.*, 2017). These insecticides are often not successful in controlling the pest when not applied during the susceptible stages of the insect's life cycle (Yu *et al.*, 2003). When insecticides are not applied effectively, resistance to these chemicals occur within a population. The indiscriminate use of insecticides for control of *S. frugiperda* where the modes of action are not rotated or the concentration of the insecticides is not applied according to regulations also lead to resistance development (Capinera, 1999; Hardke *et al.*, 2011; Valladares-Cisneros *et al.*, 2014). Larvae of this pest species are resistant to many insecticides in America (Carvalho *et al.*, 2013). Several cases of *S. frugiperda* resistance against many insecticides with different modes of action have been documented over years at different areas in South and Central America (Table 1). It causes great concern for the management of *S. frugiperda* in Africa (Goergen *et al.*, 2016; Jeger *et al.*, 2017). Several characteristics of *S. frugiperda* contribute to resistance development such as the ability to disperse rapidly over vast geographical areas where farmers have primarily relied on intensive application of chemical insecticides to control the pest (Carvalho *et al.*, 2013), the short life cycle, high reproductive capacity and their ability to feed on a wide variety of host plants (Bernardi *et al.*, 2015). Larvae feed in the funnel of maize plants where insecticides cannot reach them easily or they are exposed to small amounts contributing to resistance development (Abrahams *et al.*, 2017).

Since farming practices and social standards vary in different areas (Pittendrigh *et al.*, 2007), the IRM strategies for *S. frugiperda* are complicated. The greatest difficulties are in areas such as Africa and Central America where people have poor knowledge on basic biology and ecology of insect pests (Pittendrigh *et al.*, 2007).

Table 1: The mode of action, chemical group and active ingredients which *Spodoptera frugiperda* developed resistance at various localities.

Mode of action	Chemical group	Active ingredient	Locality
Acetylcholine esterase inhibitors	Organophosphates	Acephate	Puerto Rico (Zhu <i>et al.</i> , 2014)
		Chlorpyrifos	Brazil (Carvalho <i>et al.</i> , 2013), USA, Florida (Yu, 1991, 1992)
		Diazinon	USA, Florida (Yu, 1991, 1992)
		Dichlorvos	USA, Florida (Yu, 1991)
		Malathion	USA, Florida (Yu, 1992, 1991)
		Sulprofos	USA, Florida (Yu, 1991)
		Trichlorfon	USA, Louisiana (Wood <i>et al.</i> , 1981)
	Carbamates	Thiodicarb	USA, Florida (Yu, 1992)
		Methomyl	Mexico (Leon-Garcia <i>et al.</i> , 2012), Venezuela (Morillo and Notz, 2001), Florida (Yu, 1992, 1991)
		Carbaryl	USA, Florida (Yu, 1992, 1991; Yu <i>et al.</i> , 2003), USA, Louisiana (Wood <i>et al.</i> , 1981), USA, Georgia (Young and McMillan, 1979), Mexico (Gastelum, 1985)

Mode of action	Chemical group	Active ingredient	Locality
Sodium channel modulators	Pyrethroids, Pyrethrins and DDT	Bifenthrin	USA, Florida Gainesville (Yu, 1991)
		Cyfluthrin	Mexico, (Leon-Garcia <i>et al.</i> , 2012)
		Cyhalothrin	USA, Florida (Yu, 1991)
		Lambda-cyhalothrin	Colombia (Rios-Diez and Saldamando-Benjumea, 2011), Mexico (Leon-Garcia <i>et al.</i> , 2012), Brazil (Diez-Rodriguez and Omoto, 2001; Carvalho <i>et al.</i> , 2013) Venezuela (Morillo and Notz, 2001)
		Cypermethrin	USA, Florida (Yu, 1992; AL-Sarar <i>et al.</i> , 2006)
		Deltamethrin	Mexico (Leon-Garcia <i>et al.</i> , 2012)
		Fluvalinate	USA, Florida (Yu, 1991)
		Fenvalerate	USA, Florida (Yu, 1992)
		Deltamethrin	Mexico (Leon-Garcia <i>et al.</i> , 2012)
		Cypermethrin	USA (Yu,1992; Al-Sarar <i>et al.</i> , 2006)
		Tralomethrin	USA, Florida (Yu, 1991)
		Tau-fluvalinate	USA, Florida (Yu, 1992)
		Permethrin	USA, Florida (Yu, 1991, 1992), USA, Louisiana (Wood <i>et al.</i> , 1981)

Mode of action	Chemical group	Active ingredient	Locality
Inhibitors of chitin biosynthesis, type 0	Benzolureas	Lufenuron	Brazil (Nascimento <i>et al.</i> , 2015)
GABA-gated chloride channel antagonists	Cyclodiene organochlorides	Aldrin	Bolivia (APRD, 2018)

1.8 Problem statement

Spodoptera frugiperda invaded South Africa recently. The origin of this population is not known, but it is known that *S. frugiperda* is resistant to various insecticides with different modes of action in South and Central America. It is therefore not known if the population that invaded South Africa, carries any of these insecticide resistance genes, and if so to which insecticide group(s). The susceptibility of *S. frugiperda* to registered insecticides used for control of the pest in South Africa needs to be determined. The most appropriate bioassay for determining the susceptibility status of each insecticide group should first be determined. Rapid susceptibility testing of insecticides will enable timely detection of resistance evolution by *S. frugiperda* towards a given insecticide or group. This will allow for better management to prevent the development of insecticide resistance.

1.9 General objective

The general objective of this study was to evaluate and compare four toxicity bioassays to determine the most appropriate bioassay for baseline dose-response studies with insecticides with different modes of action against *S. frugiperda*.

1.9.1 Specific objectives

The specific objectives were to:

- Determine which artificial diet is the most suitable for rearing of *S. frugiperda* larvae and for use in toxicity bioassays.

- Compare four toxicity bioassays, viz. leaf dipping, topical application, artificial diet overlay and artificial diet incorporation for susceptibility testing to insecticides with different modes of action.
- Determine susceptibility status of third instar *S. frugiperda* larvae originating from one population to insecticides with different modes of action.

The results of this study are presented in the form of chapters with the following titles:

- Chapter 2: Suitability of four artificial diets for rearing of *Spodoptera frugiperda* larvae.
- Chapter 3: Comparison of four toxicity bioassays for susceptibility testing of *Spodoptera frugiperda* to insecticides with different modes of entry and action.
- Chapter 4: Conclusions and recommendations.

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Chapter 2

Suitability of four artificial diets for rearing of *Spodoptera frugiperda* larvae

2.1 Abstract

The fall armyworm, *Spodoptera frugiperda*, is a polyphagous pest with the ability to develop on a wide variety of host plants with different nutritional indices. There are 353 reported host plants, but maize is the most preferred. The suitability of four artificial diets, viz. *Busseola fusca*, *Anticarsia gemmatalis*, Stonefly *Heliothis* and *Chillo partellus* diets as well as maize leaves were studied for mass rearing of *S. frugiperda*. Suitability of the diets was evaluated according to the fitness parameters, larval and pupal development, pupal mass, survival, adult eclosion, fecundity and fertility. Fitness parameters of *S. frugiperda* was the most favourable on the *B. fusca* artificial diet. This diet promoted larval and pupal development, pupal mass, percentage oviposition, fecundity and fertility. Maize was relatively suitable considering the short larval and pupal periods, and high fertility. However, pupal mass was significantly lower than those of larvae feeding on artificial diets. Larval feeding on artificial diets resulted in reduced fertility of moths compared to larval feeding on maize leaf tissue. *S. frugiperda* performed the poorest on the *C. partellus* diet. Larvae and pupae had prolonged developmental periods, low survival, low pupal mass and moths did not lay any eggs. Diets had different nutritional compositions as well as water content. These factors influenced all fitness parameters.

Key words: artificial diets, fitness parameters, *Spodoptera frugiperda*

2.2 Introduction

The fall armyworm, *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae), is native to the Americas (Prasanna *et al.*, 2018), but it was reported on the African continent for the first time in 2016 (Goergen *et al.*, 2016). Populations of *S. frugiperda* established in sub-Saharan Africa (Prasanna *et al.*, 2018) and caused losses to maize, sorghum, rice and sugarcane estimated at approximately \$13 383000 (Abrahams *et al.*, 2017). The fall armyworm completes its life cycle within 30-40 days depending on

the temperature, humidity and the nutritional value of its food sources (Andrews, 1980, 1988; Jeger *et al.*, 2017). The life cycle consists of a pupal stage from which adults emerge as moths (Andrews, 1980, 1988; Jeger *et al.*, 2017). Mated female moths oviposit 100 to 1500 eggs on maize leaves or any other host (Andrews, 1980, 1988; Jeger *et al.*, 2017). Larvae emerge from the eggs and moult five times before pupation (Jeger *et al.*, 2017).

This pest is polyphagous and have the ability to develop on several host plants with different nutritional indices (Silva *et al.*, 2017). Although *S. frugiperda* can develop on different crops (Silva *et al.*, 2017), nutrient levels should be balanced for optimal growth (Chapman, 1998). The nutritional requirements of insects are primarily derived from knowledge of the chemical composition of their natural diet (Thompson and Simpson, 2009).

Herbivorous insects are commonly reared on artificial diets as it is less labour intensive, it reduces time, space and associated costs of growing specific host plants (Hervet *et al.*, 2016). Artificial diets enable synchronisation of the insect's development for entomological experiments. These diets may increase the fitness of an insect opposed to insects being reared on host plants (Hervet *et al.*, 2016). The application of artificial diets is wide and it is utilised in numerous different studies (Reinecke, 2013). Reliability of entomological studies does, however, depend on the health of the insects, which depends on the quality and the nutritional value of the artificial diets on which the insects are reared (Cohen, 2001). Insect nutrition affects the physiology, ecology and evolution of insects (Santos *et al.*, 2003). The nutritional quantity and quality during larval stages affects the growth rate, development time, body weight and survival, as well as the adult fecundity and longevity (Santos *et al.*, 2003). For any artificial diet to be successful, it should fulfil the sensory requirements, be nutritious, reasonably stable and economically affordable (Cohen, 2001). Artificial diets contain several nutritional compounds and consist of a well-balanced amount of protein (nitrogen), carbohydrates, vitamins, sterols, fatty acids and minerals (Thompson and Simson, 2009). Each of these ingredients perform a specific function during larval development and other ingredients are added to preserve the artificial diet to obtain a long shelf life (Prasanna *et al.*, 2018).

Spodoptera frugiperda has been reared successfully on a meredec artificial diet in the United States (Perkins, 1979). A meredec artificial diet is semi-chemically defined (Piper, 2017) and since *S. frugiperda* is polyphagous a number of diets can be used to rear the pest successfully (Prasanna *et al.*, 2018). Many of the diets used for rearing of *S. frugiperda* were developed for rearing of other insects (Prasanna *et al.*, 2018). Institutions in Africa have optimised different synthetic diets for this purpose. Both the International Maize and Wheat Improvement Centre (CIMMYT) in Zimbabwe and the International Centre of Insects Physiology and Ecology (ICIPE) in Kenya use artificial diets that are similar to the maize stem borer (*Busseola fusca*) (Fuller) (Lepidoptera: Noctuidae) diet (Prasanna *et al.*, 2018). The Agricultural Research Council (ARC) in South Africa use a diet similar to the diet used to rear the spotted stem borer, *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) (Prasanna *et al.* 2018).

The aim of this chapter was to compare the suitability of four artificial diets, viz. *Busseola fusca*, *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Noctuidae), *Chilo partellus* and the Stonefly *Heliothis* diet for rearing of *S. frugiperda*.

2.3 Materials and methods

2.3.1 Rearing of *Spodoptera frugiperda*

Spodoptera frugiperda larvae were collected from maize near Groblersdal (25°12'24.5"S; 29°13'22.1"E), Mpumalanga province, South Africa. The larvae were reared individually in small plastic containers (52 mm high and 30 mm in diameter) with aerated lids. The first instar larvae were fed with leaves from maize whorls, but later instars were fed with older leaves. The larvae and pupae were kept in a rearing room at 26±2 °C, 60-65% RH and 14L:10D photoperiod. Pupae were observed daily until the moths emerged. Single male-female pairs were confined to oviposition chambers in the same rearing room. The chambers and method used are according to that described by Kruger *et al.* (2012). A plastic bottle (22 cm in height and 10 cm in diameter) was cut open at the top and filled with small crusher stones up to a height of 5 cm. A maize stem (25 - 30 mm diameter) with the whorl intact and 18 cm in length were placed in an upright position in the bottle. The stem was inserted 3 - 4 cm into

the crusher stones to keep it upright. Water was added up to a level three-quarter of the height of the stones to provide humidity and to keep the plant parts fresh. A 10% sugar solution was provided as food for moths. The containers were covered with a fine gauze mesh to prevent the moths from escaping. The containers were observed and egg batches found were removed daily. Egg batches were placed individually into small plastic containers (52 mm high and 30 mm in diameter) with a steel mesh infused lid. These plastic containers were kept in a glass desiccator (150 mm diameter) in which RH was maintained at $70 \pm 2\%$ using a potassium hydroxide solution according to the method of Solomon (1951). The desiccators were also kept in the same rearing room as described above. Larvae that hatched from these eggs were used in the assays to determine the most suitable diet for rearing of *S. frugiperda*.

2.3.2 Rearing of *Spodoptera frugiperda* larvae on respective diets

The leaves as well as the soft whorl tissue of the maize were cut into pieces. These whorl and leaf pieces (served a standard treatment) as well as 15 ml of each of the four artificial diets, viz. *B. fusca* diet, *C. partellus* diet, *A. gemmatilis* diet (Greene *et al.*, 1976) and Stonefly *Heliothis* diet [Ward's Natural Science Establishment, LLC (www.wardsci.com)] were kept separately in small plastic rearing containers (52 mm high and 30 mm in diameter) with mesh infused lids. Ingredients of the respective artificial diets are provided in Table 2.1.

Four neonate larvae were inoculated onto the food in each container using a soft camel hair brush. There were 170 containers per diet and therefore 680 neonate larvae were inoculated onto each diet and also onto maize whorl tissue. Larvae from at least five egg batches of different females were inoculated per rearing diet. The containers were kept in a rearing room at 25 ± 2 °C, 65 ± 5 % humidity and a 14L:10D photoperiod. Once the larvae became pre-pupae, they were removed from the diet and the larval development time for each larva was calculated. Each pre-pupa was placed in a separate container to complete pupation. The mass and sex of pupae were determined two days after pupation. Single male-female pairs were transferred to oviposition chambers as described in paragraph 2.3.1. The moths were kept in a rearing room under conditions described above. There were 40 male-female pairs per diet. Egg batches were collected daily, placed in a desiccator and kept in a rearing

room with the moths. Fecundity and fertility was calculated by counting the neonate larvae as well as the eggs that failed to hatch.

2.3.3 Preparation of diets

The quantities of the ingredients used for the *B. fusca*, *C. partellus* and *A. gemmatalis* diets are provided in Table 2.1.

2.3.3.1 *Busseola fusca* diet

Dried maize leaves were milled to a course powder, autoclaved and stored in a refrigerator. Sorbic acid was added to and dissolved in 2 000 ml boiling water. Agar-agar was added to 2000 ml water and mixed thoroughly. The Agar-agar solution was added to the sorbic acid solution and boiled for a further 15 minutes left to cool down to 60 °C. Dry ingredients, viz. maize powder, chick pea, brewer's yeast, ascorbic acid and Vitamin E were mixed with 3 500 ml water to a paste. Formaldehyde was added to the paste and mixed thoroughly with a blender. Methyl-4-hydroxybensoate and ether was mixed together and also added to the paste. This paste was added to the agar mixture when the latter cooled down to a temperature of 60 °C and mixed thoroughly. From this mixture (artificial diet), 15 ml was poured into small plastic containers (52 mm high and 30 mm in diameter). These containers were kept at room temperature (± 24 °C) for at least two hours to cool down before neonates were inoculated onto the diet.

2.3.3.2 *Chilo partellus* diet

Sorbic acid was added to and dissolved in 2000 ml boiling water. Agar-agar was added to 2000 ml water and mixed thoroughly. The Agar-agar solution was added to the sorbic acid solution and boiled for a further 15 minutes left to cool down to 60 °C. Dry ingredients, viz. chick pea, wheat germ, sugarcane, milk powder, brewer's yeast, ascorbic acid and Vitamin E were mixed with 3 500 ml water to a paste. Formaldehyde was added to the paste and mixed thoroughly with a blender. Nipagin, cholesterol and ether were mixed and added to the above mentioned paste. This paste was added to the agar mixture after the latter was cooled down to 60 °C and mixed thoroughly. This

mixture (artificial diet) (15 ml) was poured into small plastic containers (described above, refer to subheading 2.3.2.1). These containers were kept at room temperature ($\pm 24^{\circ}\text{C}$) for at least two hours to cool down before neonates were inoculated onto the diet.

2.3.3.3 *Anticarsia gemmatalis* diet

Small white canning beans (cultivar: Teebus-RR1) were milled to a coarse powder, autoclaved and stored in a refrigerator. Agar-agar was added to 1 600 ml water and boiled for 10 minutes. Dry ingredients, viz. wheat germ, milk powder, brewer's yeast and white bean powder were mixed with 2 400 ml water to a paste. In a separate bowl, ascorbic acid, sorbic acid, nipagin, antibiotics (Doxycycline), vitamin B complex, and 10% formaldehyde were mixed together. The three mixtures were then added and mixed together. Fifteen millilitres of this mixture (artificial diet) were poured into small plastic containers (described above, refer to subheading 2.3.2.1). These containers were kept at room temperature (24°C) for at least two hours to cool down before neonates were inoculated onto the diet.

2.3.3.4 Stonefly *Heliopsis* diet

The Ward's science Stonefly *Heliopsis* diet is a premixed formulation. To prepare this diet, 2 720 ml water was mixed thoroughly with 680 g of premix powder. Diet (15 ml) was added with a large syringe to each small plastic container (described above, refer to subheading 2.3.2.1). The diet was replaced weekly to prevent desiccation.

Table 2.1: Ingredients of artificial diets used for rearing of *Spodoptera frugiperda*.

Ingredients	<i>B. fusca</i>	<i>C. partellus</i>	<i>A. gemmatalis</i>
Fraction A			
Agar	72 g	80 g	80 g
Distilled water	4000 ml	4000 ml	1200 ml
Sorbic acid	8 g	15 g	7.92 g
Fraction B			
Wheat germ	-	450 g	240 g
Maize powder	320 g	-	-
Chick pea	900 g	500 g	-
Sugarcane	-	100 g	-
Brewer's yeast	64 g	90 g	150 g
Asorbic acid	21 g	30 g	15.84 g
Vitamin E	9 g	8 g	-
White bean	-	-	300 ml
Milkpowder	-	90 g	240 g
Formaldehyde 10%	6 ml	2 ml	6 ml
Distilled water	3500 ml	3000 ml	2400 ml
Fraction C			
Nipagin	13 g	6 g	13.2 g
Doxycycline (antibiotic)	-	-	0.496 g
Ether	150 ml	150 ml	-
Vitamin complex (B1 & B6)	-	-	1.98 g:1.98 g:19.8 ml Distilled water
Cholesterol	-	2.4g	-

Busseola fusca and *C. partellus* diets adapted from Prasanna *et al.* (2018). *Anticarsia gemmatalis* – adapted from Greene *et al.* (1976). g = grams and ml =millilitres.

Table 2.2: Ingredients of Nespray full cream instant milk powder (Nestle South Africa).

Ingredients	Per 100 g powder
Protein	23.6 g
Glycaemic carbohydrates of which total sugars	39.9 g
Saturated fat	17.7 g
Dietary fibre	0 g
Total sodium	335 mg
Vitamin A	540 µg RE
Vitamin B2	1.4 mg
Vitamin B12	1.8 µg
Vitamin C	50 mg
Biotin	50 µg
Folic acid	200 µg
Calcium	860 mg
Phosphorus	700 mg
Iron	10 mg
Zinc	4.5 mg

g = gram, mg = milligram, µg = microgram

2.3.4 Fitness measurements

Eight fitness parameters were recorded viz. larval development, larval survival, pupal duration, pupal mass, adult emergence, adult oviposition, fecundity and fertility. Fitness data for each larva and pupa were recorded individually.

2.4 Statistical analyses

Percentage *S. frugiperda* larval survival, pupation, adult eclosion, fecundity and fertility per female, percentage egg hatch, as well as the percentage females that laid eggs from larvae reared on the respective diets, were analysed by means of a binomial distribution test. Bonferroni correction was used to adjust for multi means comparisons of the multi-choice test. Pairwise comparisons of the development time of male and female pupae was compared by means of Mann-Whitney U-tests. Data on the mean larval and pupal development times, pupal mass, mean fecundity and fertility of females for larvae reared from the respective diets, were tested for homogeneity of variance (Levene's test) and normality (Shapiro-Wilk test), but the assumptions were

not met. The respective data sets were therefore analysed by means of Kruskal-Wallis tests and pair-wise comparison of means. All analyses were performed with TIBCO Statistica™ 13.3 (TIBCO Software, Inc., 2017)

2.5 Results

Significantly more *S. frugiperda* larvae reared on the *B. fusca* diet survived compared to those reared on all the other diets as well as maize leaves (Table 2.3). The percentage survival of larvae reared on the *A. gemmatalis* diet, Stonefly *Heliothis* diet and maize leaf tissue did not differ significantly. The percentage survival of larvae that were reared on the *C. partellus* diet was significantly lower than on any of the other diets (Table 2.3).

Table 2.3: Percentage survival, pupation and percentage adult emergence of *Spodoptera frugiperda* reared on different artificial diets and maize.

Artificial diet	N (neonates)	% Survival	N (pupae)	% Adult eclosion
<i>Busseola fusca</i>	680	76.3a	519	47.0a
<i>Anticarsia gemmatalis</i>	680	49.3b	335	69.6b
Stonefly <i>Heliothis</i>	680	47.4b	322	64.6b
<i>Chilo partellus</i>	680	17.9c	122	18.9c
Maize	560	40.2b	225	64.9b

Means within a column followed by the same letter are not significantly different at $P < 0.05$ (binomial distribution test with Bonferroni correction to adjust for multi means comparisons)

The percentage FAW larvae which completed their development (adult eclosion) were significantly higher on the *A. gemmatalis* and Stonefly *Heliothis* diet and maize compared to the *B. fusca* and *C. partellus* diet. The percentage adult emergence from FAW larvae reared on the *C. partellus* diet was significantly lower than from larvae reared on all the other diets (Table 2.3).

There were significant differences in larval development time of larvae reared on the respective diets and maize ($\chi^2 = 85.15$, $df = 9$, $P < 0.001$). Larvae that were reared on *B. fusca* artificial diet and maize developed significantly faster compared to larvae reared

on the *A. gemmatalis*, Stonefly *Heliothis* and *C. partellus* diets (Table 2.4). There was no significant difference in development time of larvae reared on the *B. fusca* artificial diet and maize. Development time on the *A. gemmatalis* and Stonefly *Heliothis* diets was similar. Larvae that were reared on the *C. partellus* diet developed significantly slower compared to larval development time on all the other diets and maize leaf tissue (Table 2.4).

Table 2.4: Larval development time of *Spodoptera frugiperda* reared on different artificial diets and maize.

Larval development (Days)				
Diet	Sex	N	Mean $\pm$ SE	Median
<i>Busseola fusca</i>	♀	119	17.7 $\pm$ 0.1 a	17
	♂	125	17.7 $\pm$ 0.1 a	17
<i>Anticarsia gemmatalis</i>	♀	137	18.4 $\pm$ 0.1 b	18
	♂	96	18.6 $\pm$ 0.1 b	18
Stonefly <i>Heliothis</i>	♀	88	18.8 $\pm$ 0.1 b	18
	♂	120	18.6 $\pm$ 0.1 b	18
<i>Chilo partellus</i>	♀	12	22.1 $\pm$ 0.4 c	22
	♂	11	22.2 $\pm$ 0.4 c	22
Maize	♀	72	16.9 $\pm$ 0.1 a	17
	♂	74	17.7 $\pm$ 0.1 a	17

Means within the column followed by the same letter are not significantly different at $P < 0.05$ (Kruskal-Wallis tests with pair-wise comparison of means)

Pairwise comparisons of the development time of male and female pupae from larvae reared for each of the respective diets did not differ between diets. Pooled data on the pupal duration for male and female pupae of *S. frugiperda* larvae reared per diet, did, however, differ significantly between the diets ($\chi^2 = 97.88$, $df = 4$, $P < 0.001$). Pupal development time from larvae reared on the *B. fusca*, *A. gemmatalis* and *C. partellus* diets did not differ, but was significantly shorter than that of larvae reared on the Stonefly *Heliothis* diet (Table 2.5). Pupal development time of larvae reared on the Stonefly *Heliothis* diet was significantly longer than on any of the other diets and maize (Table 2.5).

Table 2.5: Mean development time of male and female *Spodoptera frugiperda* pupae from larvae reared on four artificial diets and maize leaves

Pupal development period (Days)				
Diet	Sex	N	*Mean no of days $\pm$ SE	#Mean no of days $\pm$ SE
<i>Busseola fusca</i>	♀	119	10.2 $\pm$ 0.1	10.9 $\pm$ 0.1a
	♂	125	11.6 $\pm$ 0.1	
<i>Anticarsia gemmatalis</i>	♀	137	10.5 $\pm$ 0.1	10.7 $\pm$ 0.1a
	♂	96	11.1 $\pm$ 0.2	
Stonefly <i>Heliothis</i>	♀	88	11.5 $\pm$ 0.2	12.0 $\pm$ 0.1c
	♂	120	12.5 $\pm$ 0.2	
<i>Chilo partellus</i>	♀	12	10.2 $\pm$ 0.4	10.5 $\pm$ 0.2ab
	♂	11	10.8 $\pm$ 0.5	
Maize	♀	72	9.5 $\pm$ 0.4	10.3 $\pm$ 0.2b
	♂	74	11.0 $\pm$ 0.5	

*There was no difference in male and female pupal development time of larvae reared on each of the respective diets (Mann-Whitney U test).

#Means within the column followed by the same letter are not significantly different at $P < 0.05$ (Kruskal-Wallis tests with pair-wise comparison of means)

Pupal mass from larvae reared on the respective diets and maize leaves differed significantly ($\chi^2 = 480.99$, $df = 9$, $P < 0.001$). The pupal mass of males and females that were reared on *B. fusca* diet and *A. gemmatalis* diets did not differ (Table 2.6). These pupae were significantly heavier than pupae from larvae reared on any of the other diets and maize leaves (Table 2.6). The pupal mass of male and female larvae that were reared on Stonefly *Heliothis* diet as well as *C. partellus* diet were similar. Pupae from larvae reared on maize leaves weighed less than pupae from larvae reared on all the other diets except for female pupae from larvae reared on the *C. partellus* diet (Table 2.6).

Table 2.6: Mass of *Spodoptera frugiperda* pupae that developed from larvae reared on different artificial diets and maize.

Pupal mass (mg)				
Diet	Sex	N	Mean±SE	Median
<i>Busseola fusca</i>	♀	249	228.4 ± 2.2 a	228.1
	♂	270	230.5 ± 2.3 a	233.0
<i>Anticarsia gemmatalis</i>	♀	175	230.4 ± 2.6 a	235.8
	♂	160	232.2 ± 3.0 a	240.8
Stonefly <i>Heliiothis</i>	♀	144	187.8 ± 2.9 b	188.4
	♂	178	184.7 ± 2.8 b	187.5
<i>Chilo partellus</i>	♀	52	173.5 ± 4.8 bc	177.8
	♂	70	182.5 ± 4.5 b	184.4
Maize	♀	113	149.5 ± 3.5 c	145.4
	♂	112	166.0 ± 3.5 c	162.8

Means within the column followed by the same letter are not significantly different at $P < 0.05$ (Kruskal-Wallis tests with pair-wise comparison of means)

The highest percentage of *S. frugiperda* females that oviposited were from larvae that were reared on the *B. fusca* diet and maize leaves (Table 2.7). There was, however not a significant difference between the percentage of females from larvae that were reared on the *A. gemmatalis* diet and those from larvae that were reared on maize leaves (Table 2.7). The lowest percentage females that laid eggs were from larvae that were fed with the Stonefly *Heliiothis* diet that also did not differ from the percentage females reared from larvae fed with the *A. gemmatalis* diet (Table 2.7). Only 23 of the 680 neonate larvae that were initially inoculated onto the *C. partellus* diet developed into pupae. None of the moths that eclosed from these pupae oviposited (Table 2.7).

The mean fecundity per female reared on the respective diets and maize leaves differed significantly ($\chi^2 = 20.88$, $df = 9$, $P < 0.0001$). The mean fecundity of females from larvae reared on the *B. fusca* diet was significantly higher than from females reared from larvae fed on the *A. gemmatalis* diet and maize leaves (Table 2.7). The mean fecundity of females from larvae reared on the *B. fusca* and Stonefly *Heliiothis* diets did, however, not differ significantly. There was also no significant difference in the

number of egg batches laid by females from larvae reared on the *B. fusca* and Stonefly *Heliothis* diets as well as on maize leaves (Table 2.7).

There were significant differences in the fertility per female ($\chi^2=12.83$, $df = 3$, $P < 0.01$). There was no significant difference in fertility of *S. frugiperda* eggs originating from larvae reared on *B. fusca*, Stonefly *Heliothis* diets and maize leaves. Fertility from eggs of larvae that were reared on the *A. gemmatalis* diet was, however significantly lower than from larvae reared on the *B. fusca* and Stonefly *Heliothis* diets (Table 2.7). The percentage of eggs that hatched from larvae that were reared on the Stonefly *Heliothis* diet and maize was significantly higher than the percentage of eggs that hatched from larvae reared on the *B. fusca* and *A. gemmatalis* diets. Hatching success from the latter two diets did however not differ significantly (Table 2.7).

Although females reared on the *B. fusca* diet had the highest fecundity, higher fertility was recorded on maize. Although the fecundity was not high from females of larvae that were reared on Stonefly *Heliothis* diet, the fertility of the eggs was high. Fertility of eggs was the lowest from larvae that were reared on the *A. gemmatalis* diet (Table 2.7).

Table 2.7: Fecundity and fertility of *Spodoptera frugiperda* female moths from larvae reared on different artificial diets and maize.

Artificial diet	*Females (oviposited %)	#Mean fecundity/female ±SE	#Mean fertility/female ±SE	*Eggs hatched (%)
<i>Busseola fusca</i>	90a	1028.9 ± 62.0a	663.2 ± 55.2a	64.5a
<i>Anticarsia gemmatalis</i>	65bc	521.2 ± 72.9b	332.3 ± 65.0b	63.8a
Stonefly <i>Heliothis</i>	48c	663.7 ± 85.3ab	608.6 ± 76.0a	91.6b
Maize	78ab	500.2 ± 66.8b	479.45 ± 59.5ab	95.9b

*Means within a column followed by the same letter are not significantly different at $P < 0.05$ (binomial distribution test with Bonferroni correction to adjust for multi means comparisons)

#Means within a column followed by the same letter are not significantly different at $P < 0.05$ (Kruskal-Wallis tests with pair-wise comparison of means)

2.6 Discussion

Considering all fitness parameters evaluated during this study it is evident that the *B. fusca* diet was the most suitable for development of *S. frugiperda* larvae. Larval and pupal development time was shortest and pupal mass was the highest on this diet. Fecundity of moths from larvae that fed on the *B. fusca* diet was also high although only 65% of the eggs hatched. The quality as well as quantity of nutritional compositions may have adverse effects on all insect developmental stages. Nutrient imbalances may cause the insect to ingest and process excessive amounts of food to acquire enough of a specific compound that is present in low concentrations or the insect has to interconvert one compound to the other. These processes are metabolically costly and the rates of production may be limited (Chapman, 1998). The major nutritional constituents such as amino acid/protein and carbohydrate content of diets are generally adapted to levels similar to those in the natural host plant that the insect consumes (Chapman, 1998). Plant-feeding lepidopteran insects require equal amounts of amino acids and carbohydrates in their diet (Chapman, 1998). Alterations in nutritional requirements and capabilities are general phenomena during lepidopteran larval development. Larvae undergo extensive changes in body size and physiology across larval instars (Chapman, 1998). Nutritional requirements change from early larval instars which need nutrients essential for growth to later instars, requiring nutrients needed for metamorphosis and adulthood (Ojeda-Avila *et al.*, 2003). The differences in developmental times, pupal mass, percentage survival and eclosion as well as fecundity and fertility may therefore be related to the differences in nutritional compositions between the diets the larvae were reared on.

Larvae that were reared on maize and *B. fusca* artificial diet had the shortest larval development time. Nutritional balance and growth is inversely correlated (Chapman, 1998). When nutritional composition is optimal and environmental factors are favourable the insects will complete their life cycle in a shorter period of time (Chapman, 1998). The longer larval development time of larvae that were reared on the *A. gemmatilis* and Stonefly *Heliothis* diets may indicate that certain nutritional constituents were present in inadequate quantities compared to the *B. fusca* diet and maize. The Stonefly *Heliothis* diet is a premixed diet to which only water was added. The moisture to dry ingredients ratio was less than in the other artificial diets. While

the neonates were inoculated only once on the other artificial diets, the Stonefly *Heliothis* diet had to be replaced regularly due to desiccation. A study conducted on *Manduca sexta* (L.) (Lepidoptera: Sphingidae) indicated that larvae grew slower on diets with a low-water content (Ojeda-Avila *et al.*, 2003). However, water content becomes less growth limiting with larval age and size (Ojeda-Avila *et al.*, 2003). Not only nutritional value of a diet is therefore important, but also water content and a combination of these requirements could have been limiting and might have resulted in prolonged larval development. Since the *A. gemmatalis* diet did not desiccate during this study, the prolonged larval development can be attributed to nutritional imbalances of the artificial diet for rearing of *S. frugiperda* larvae. The long development time of *S. frugiperda* larvae on the *C. partellus* diet showed that this diet was inferior compared to all the other diets. When nutritional targets are not achieved larvae prolong their development time or increase the number of larval instars (Chapman, 1998). Longer development time and reduced growth rate is considered a disadvantage since the chance of mortality increases. This phenomenon is also known as the slow-growth-high-mortality hypothesis (Frago and Bauce, 2014). Larvae reared on *C. partellus* diet also had the lowest larval survival confirming the abovementioned hypothesis.

Prolonged pupal duration observed for *S. frugiperda* larvae reared on the Stonefly *Heliothis* diet may result from nutritional imbalances. The pupal mass of larvae reared on the *B. fusca* and *A. gemmatalis* diets was higher than for pupae from larvae reared on Stonefly *Heliothis* diet, *C. partellus* diet and maize. Amongst all these diets provided to *S. frugiperda* larvae, pupal mass of larvae reared on maize, was the lowest. The larvae of *S. frugiperda* feed mainly inside the whorls of maize plants (Abrahams *et al.*, 2017). These larvae are cannibalistic and they were therefore kept individually in rearing containers. Since it was not practically possible to provide each larva with a fresh maize whorl on a daily basis for the duration of its developmental period in mass rearing, the larvae in this experiment were provided with older leaf material. Differences in pupal mass may therefore be attributed to the nutritional composition of the respective diets as well as availability of moisture in the diets during the larval development period. Fat accumulation during larval development is crucial for the success of later stages. Fat content increases with larval instar and it is also a general characteristic of insect development (Slansky and Scriber, 1985). Fat is commonly stored in the fat bodies of insects as triacylglycerol's. The fat body in insects

are analogous to adipose tissue and liver in vertebrates (Ojeda-Avila *et al.*, 2003). The fat bodies serve as a storage place and also play a central role in mobilizing fat into circulation during muscle efforts. Triacylglycerols are a major source of metabolic energy during non-feeding stages of development (Ojeda-Avila *et al.*, 2003). Larval diet has a significant effect on the fat content in the body. Larvae are capable of dietary transformation of carbohydrates into storage forms of fat. Fat content increases with larval instars to the mid-pupal stage and further increases occur on the first day after adult eclosion. Dietary compositions of the different artificial diets therefore had an effect on the pupal mass and specifically fat deposition. All the diets used in this study had different ratios and carbohydrate sources. The *B. fusca* diet contained higher quantities of chick pea flour while maize leaf powder was also included. The maize leaf powder was, however, autoclaved and could have lost its nutritional value in the process. The *C. partellus* diet contained a combination of chick pea flour as well as wheat germ. The quantity of wheat germ was the highest in the *C. partellus* diet and sugar cane was also added. The *A. gemmatalis* diet contained a combination of wheat germ and white bean flour, with white bean flour being added in larger quantities as a carbohydrate source. The composition of Stonefly *Heliothis* diet is unknown. The composition of carbohydrates in *B. fusca* and *A. gemmatalis* diets was adequate, but less adequate in the Stonefly *Heliothis* and *C. partellus* diets for *S. frugiperda* development. The addition of sugarcane powder in the *C. partellus* diet increased the sucrose levels of the diet and sugar was therefore present in a higher ratio than the protein sources. This may have caused excess carbohydrates that are difficult to process or utilise. The low survival rate of larvae reared on the *C. partellus* diet was also accompanied with prolonged instar development. Most of the larvae did not reach the pre-pupa stage and died, indicating that this diet was not suitable for rearing *S. frugiperda* larvae.

The nutritional balance of all the ingredients in the respective diets is, however, important and changes in the ratios may improve the suitability of the diets for rearing of *S. frugiperda* larvae. Pupal mass of larvae reared on maize was the lowest and could be explained by the age of the leaves the larvae were reared on. The older leaves were less nutritional and also contains less moisture than leaf material deep inside the whorls where the larvae usually feed. This also explains the difference in results obtained by Silva *et al.* (2017) who reported optimal larval growth and

development on maize. Larval survival on maize reported by Silva *et al.* (2017) was also higher compared to this study.

The disadvantages of rearing *S. frugiperda* on maize is that it is labour intensive, time consuming and maize plants should be available in mass since ideally only whorl tissue should be provided. Larvae were also more exposed to stress due to regular handling during food replacement which could also have hampered their development and survival.

Larval survival was highest for larvae reared on *B. fusca* diet, but percentage eclosion of moths from pupae was low. It may be attributed to handling during weighing and sexing of the pupae. It did not seem to be due to a lack of certain nutritional components in the diet, since a very high percentage of the moths were able to oviposit and also laid the most eggs compared to moths from larvae reared on the other diets. Larval survival on *A. gemmatilis*, Stonefly *Heliothis* diet and maize were all relatively low and the percentage pupae eclosed was also similar. It may be attributed to the nutritional composition, and for larvae fed with the Stonefly *Heliothis* diet and maize, to mechanical injury caused by handling during food replacement.

Oviposition of *S. frugiperda* females that originated from the *A. gemmatilis*, Stonefly *Heliothis* diets and maize was lower compared to those from larvae reared on the *B. fusca* diet. No females reared on the *C. partellus* diet, oviposited. The adequacy of immature food sources is a reflection of the quantity and quality of nutrients stored for subsequent egg production (Muller *et al.*, 2015). The quality and/or quantity of the nutrients in the *A. gemmatilis*, Stonefly *Heliothis* and *C. partellus* diets were therefore not adequate to promote optimal fecundity. In contrast to fecundity, the fertility of the eggs is determined by both the females and the males. A study conducted on *Lobesia botrana* (Denise and Schiffermüller) (Lepidoptera: Tortricidae) indicated that larval nutrition adversely effects the volume of spermatophores that is produced as well as the number of sperm cells that is transferred by males (Muller *et al.*, 2015). Quantity and quality of spermatophores influences female reproductive outputs (Muller *et al.*, 2015). Male pupae that were heavier produce adults which are more energetic with more metabolic reserves which results in males that produce bigger spermatophores (Muller *et al.*, 2015). Large ejaculations of male moths cause females to oviposit more

eggs (Muller *et al.*, 2015). However, the volume of spermatophores are not the only factor that influences fecundity and fertility of females. Quality of spermatophores affects the number of eggs that are laid as well as the fertility of the eggs (Muller *et al.*, 2015). Therefore, low fertility and fecundity may be attributed to nutritional imbalance causing low quantity and quality of spermatophores. In nature females may possibly be able to modulate their behaviour to only mate with males with adequate spermatogenic abilities (Muller *et al.*, 2015). During this study, adults were paired and not allowed to choose a male or female, which could have influenced results on fecundity and fertility observed in this study.

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Chapter 3

Comparison of four toxicity bioassays for susceptibility testing of *Spodoptera frugiperda* to insecticides with different modes of entry and action.

3.1 Abstract

Application of insecticides is the main method of control for *Spodoptera frugiperda*. The pest has developed resistance towards various insecticide mode of action groups in South and Central America. It also causes great concern for control of *S. frugiperda* in Africa. It is not known if the population that invaded Africa, carried resistance alleles, and if so, to which insecticide groups. Baseline susceptibility of *S. frugiperda* should be known to monitor its susceptibility status in future. The most suitable bioassay method for each of the respective insecticide groups should therefore be determined. Four bioassay methods; viz. leaf dipping, topical application, insecticide overlay onto and incorporation in artificial diets were evaluated for use in toxicological studies with insecticides with different modes of entry and action. These insecticides and respective groups were: chlorantraniliprole (diamide), lufenuron (benzoxazinoids), pyridalyl (unknown) and methomyl (carbamate). Dose responses were evaluated with the PoloSuite computer program and statistical parameters were analysed to determine the most suitable bioassay for evaluation of the different insecticide groups. Insecticide incorporation into artificial diet was identified as the most suitable bioassay for the susceptibility evaluation of *S. frugiperda* to chlorantraniliprole and methomyl. For lufenuron, the most suitable method was the insecticide overlay onto artificial diet. No suitable bioassay could be determined for the evaluation of susceptibility to pyridalyl. The difference in suitability of bioassays for each insecticide showed that the mode of entry has a profound effect on the effectivity of bioassays and also on the estimate of the median lethal dose.

Keywords: bioassays, mode of actions, mode of entry, *Spodoptera frugiperda*

3.2 Introduction

Spodoptera frugiperda (Lepidoptera: Noctuidae) (Smith) cause economic losses to crops and the pest is primarily controlled with insecticides (Yu *et al.*, 2003; Carvalho *et al.*, 2013; Perez-Zubiri *et al.*, 2016; Abrahams *et al.*, 2017). The pest developed resistance towards different insecticide mode of action groups in South and Central America (Carvalho *et al.*, 2013). These groups include organophosphates, carbamates, pyrethroids, pyrethrins (Yu, 1991, 1992; Leon-Garcia *et al.*, 2012), benzolurease (Nascimento *et al.*, 2015) and cyclodiene organochlorides (APRD, 2018). It also causes great concern for the control of *S. frugiperda* in Africa (Goergen *et al.*, 2016; Jeger *et al.*, 2017).

The success of a resistance management strategy relies on the effective detection of insecticide resistance (Siegfried *et al.*, 2007). To implement management strategies, it is fundamental to development of appropriate bioassay methods and also to estimate the baseline susceptibility of an insect to an insecticide to be able to monitor changes in tolerance (Elghar *et al.*, 2005; Siegfried *et al.*, 2007). Successful resistance monitoring therefore depends on the availability of quick, reliable and effective bioassays (Gunning, 1993; Elghar *et al.*, 2005). Adaptation of bioassay techniques enables evaluation of resistance in lepidopterans towards insecticides with different modes of action and modes of entry (Elghar *et al.*, 2005).

Bioassays refers to the determination of the effectiveness of a physical, chemical or biological agent with the use of a biological indicator, for example larvae (Bliss, 1957). Bioassays represents a collection of methods where a characteristic of a treatment is measured in terms of response *viz.* lethal dosages, lethal time, contact and digestive mode of entries respectively (Dewey, 1958). The term “dose’ in a bioassay can therefore refer to lethal concentration (LC), lethal dose (LD), effective concentration (ED), inhibitory dose (ID) and lethal time (LT) (Robertson *et al.* 2007). Assessment and development of new bioassays enables scientists to evaluate the toxicity of insecticides with different characteristics towards the same pest under identical test conditions (Paramasivam and Selvi, 2017). Bioassays should be designed in such a manner that the bioassay closely resembles field conditions to ensure that the susceptibility of a population in the field can be predicted from data obtained from

laboratory experiments (Kranthi, 2005). To compare and report results from susceptibility studies, a standard method should be used to ensure accurate analysis (IRAC, 2018). The insecticide resistance action committee (IRAC) developed several test methods in accordance with the methods used for insecticide application (IRAC, 2018). These methods can be grouped into four categories viz. dipping, overlay, topical and feeding (Ffrench-Constant and Roush, 1990).

For a binary bioassay (measurement of dead or alive insects) to be valid, an appropriate linear response between the biological indicator and the dosage rates should exist (Bliss, 1957). Numerous methods have been developed to calculate lethal or effective doses of toxicants and their confidence limits. Probit analysis was initially developed by Bliss (1938) and improved by Finney (1952). Finney recommended fitting regression lines as precisely as possible and obtain statistical parameters. To evaluate bioassays various parameters should be taken into consideration. Confidence intervals may only be compared between groups with similar degrees of freedom (Bliss, 1957). The length of the confidence intervals of the regression line depends on the precision of the slope (Bliss, 1957). Slope values are also a measure of sensitivity of the dose response toward an insecticide. When a slope value is high it indicates a high degree of sensitivity and correlation between the concentration of the insecticide and the resultant mortality (Georghiou and Metcalf, 1961). It is important to preferably use doses at equal log-intervals for a median lethal dosage bioassay (Bliss, 1957). The number of responses also affects the confidence intervals, thus the larger the sample size the shorter the confidence intervals. Smaller standard deviation and shorter confidence intervals are good indications of precision (Bliss, 1957).

The aim of this study was to compare four toxicity bioassays viz. leaf dipping, topical application, insecticide overlay onto and incorporation into artificial diet for susceptibility testing to insecticides with different mode of actions.

3.3 Materials and methods

3.3.1 Rearing of *Spodoptera frugiperda*

Spodoptera frugiperda larvae were collected from maize near Groblersdal (25°12'24.5"S; 29°13'22.1"E), Mpumalanga province, South Africa. The larvae were reared individually in small plastic containers (52 mm high and 30 mm in diameter) with aerated lids. They were fed with leaves from maize whorls and kept in a rearing room at 26±2 °C, 60-65% RH and 14L:10D photoperiod until pupation. Pupae were kept in the same rearing room as the larvae and observed daily until the moths emerge. Once the moths emerge, single male-female pairs were confined to oviposition chambers in the same rearing room where the larvae were reared and the pupae were kept. The chambers and method used are according to that described by Kruger *et al.* (2012). A plastic bottle (22 cm in height and 10 cm in diameter) was cut open at the top and filled with small crusher stones up to a height of 5 cm. One maize stem (25 - 30 mm diameter) with the whorl intact and 18 cm in length were placed in an upright position in the bottle. The stem was inserted 3 - 4 cm into the crusher stones to keep it upright. Water was added up to a level three-quarter of the height of the stones to provide humidity and to keep the plant parts fresh. A 10% sugar solution was provided as food for the moths. The containers were covered with a fine gauze mesh to prevent the moths from escaping. The containers were observed and egg batches found were removed daily. One egg batch was placed per small plastic container (52 mm high and 30 mm in diameter) with a steel mesh infused lid. These plastic containers were kept in a glass desiccator (150 mm diameter) in which RH was maintained at 70 ± 2 % using a potassium hydroxide solution according to the method of Solomon (1951). The desiccators were also kept in the same rearing room as described above. Neonate larvae that hatched from these eggs were reared on maize and Ward's science Stonefly *Heliothis* premix diet [Distributors: Symbiolab and manufactured by Ward's Natural Science Establishment, LLC (www.wardsci.com)]. The premix diet was weighed and water was added in a ratio of 1:4. (water:diet) and mixed thoroughly to a smooth paste. Neonate larvae were inoculated onto the diet and kept in a rearing room (conditions described above) until the larvae reached the third instar. Third instar *S. frugiperda* larvae were used to evaluate the four bioassays viz.

leaf dipping, insecticide overlay onto artificial diet, topical application and insecticide incorporation into artificial diet.

3.3.2 Selected insecticides for bioassays

The most suitable bioassay to use in toxicological studies for insecticides with different modes of entry and action were evaluated. The following formulated insecticides were used: methomyl, (carbamate), lufenuron (benzoxazole), chlorantraniliprole (diamide), and pyridalyl (UN - unknown). The IRAC groups evaluated were 1B (carbamate), 28 (diamide), 15 (lufenuron) and UN (unknown). The mode of entry of methomyl, chlorantraniliprole and lufenuron are via contact and ingestion, while that of pyridalyl is unknown (Tomlin, 2009). The range of concentrations for the respective active ingredients used in the bioassays was predetermined in rangefinder assays. The predetermined range per active ingredient was used for all the bioassays done to be able to compare the four bioassays.

3.3.3 Preparation and dilution of stock solutions for bioassays

A stock solution for each of the respective insecticides (see 3.3.2) was prepared with deionised water and stirred to homogenise the solution. The concentration of the active ingredient in the formulation of each insecticide product used was taken into consideration for preparation of the respective stock solutions. A series of five dilutions (concentrations) was prepared from the stock solution and used as the insecticide treatments in the respective bioassays performed, viz. leaf dipping, insecticide overlay onto artificial diet, topical application and insecticide incorporation into artificial diet bioassays. For the leaf dipping bioassay, Triton X-100, a non-ionic surfactant, was added to the respective dilutions and stirred well with a magnetic stirrer to ensure that Triton X-100 was dissolved before use. The surfactant was added to obtain optimal leaf coverage. For the topical application bioassay, the stock solutions of each insecticide was prepared with a solution consisting of 5% deionised water and 95% acetone (Temple *et al.*, 2009). To ensure solubility of the insecticide deionised water was added and homogenised with the insecticide before acetone was added. The series of five dilutions (concentrations) prepared from this stock solution, were also prepared with a solution consisting of 5% deionised water and 95% acetone. A fresh

stock solution was prepared for each replicate of each bioassay and used immediately after preparation. The range of concentrations of each insecticide used in the respective bioassays were determined in preliminary susceptibility testing. The aim of this preliminary range finding was to ensure that the range include concentrations that will cause different degrees of mortality of third instar FAW larvae in all bioassays. For comparison of the bioassays per insecticide (representing different modes of entry and action), the same series of concentrations per insecticide were used in all four bioassays.

3.3.3 Insecticide incorporated artificial diet bioassay

The insecticide incorporated artificial diet bioassays was conducted according to the IRAC Susceptibility Test Methods Series no. 020 for lepidopteran larvae. The evaluation of each insecticide consisted of six treatments, viz. the five insecticide concentrations incorporated into the artificial diet and the control treatment (Figure 3.1, A). The control treatment consisted of diet prepared with deionised water only. There were three replicates for each insecticide evaluated. The insecticide solutions were stirred well before 20 ml of the insecticide solution with a known concentration was added to 5 g of Stonefly *Heliothis* artificial diet (Figure 3.1, B) and mixed thoroughly to a smooth paste. A measuring spoon was used to place 2 ml of the diet in each well of a bioassay tray (Frontier: scientific services). One, third instar *S. frugiperda* larva, reared on the Stonefly *Heliothis* diet was inoculated onto the insecticide incorporated and control diets in the respective wells using a fine, artist's brush. The bioassay trays were covered with a plastic lid and incubated at $26 \pm 2^{\circ}\text{C}$, 60-65% RH with a 16:8 L:D photoperiod. Ten larvae were used for each concentration as well as for the untreated control in each replicate.

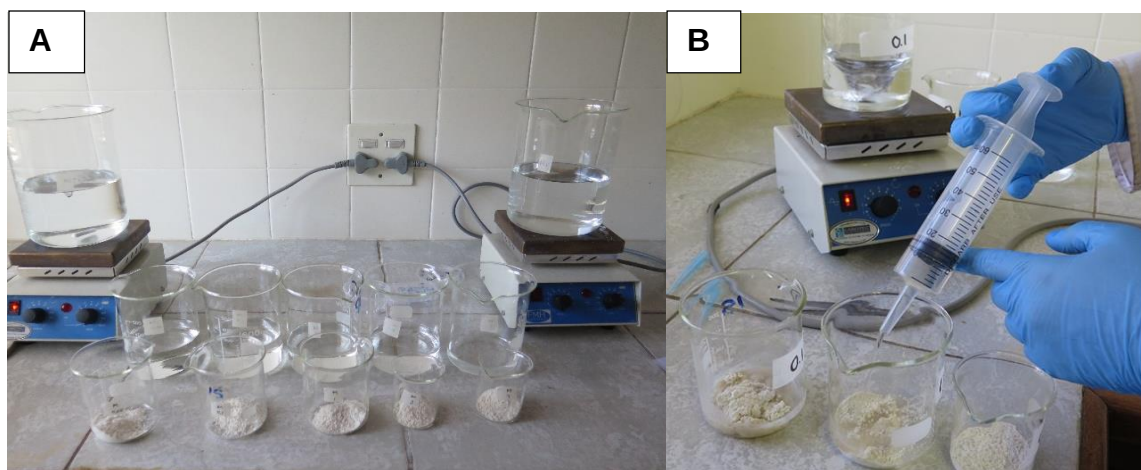


Figure 3.1: A: Preparation of insecticide incorporated Stonefly *Heliiothis* artificial diet. B: An insecticide solution added to the diet at predetermined concentrations.

3.3.4 Insecticide overlay onto artificial diet bioassay

This bioassay was conducted similar to IRAC method no. 020 (insecticide incorporated bioassay for lepidopteran larvae). The difference was that the five insecticide solutions (treatments) were not incorporated into the diet, but it was applied as overlays onto the artificial diet. The diet was prepared by mixing 5g Stonefly *Heliiothis* premix diet with 20 ml deionised water to a smooth paste. Two millilitre of the diet was dispensed with a sterile syringe into each well of the bioassay trays (Frontier: scientific services). For each insecticide evaluated, 130 μ l of insecticide solution was dispensed onto the diet (Figure 3.2, A), after application the diet absorbed the insecticide that was applied. For the control treatment no insecticide was applied onto the diet. There were three replicates per insecticide. One *S. frugiperda* larva (third instar), reared on the Stonefly *Heliiothis* diet, was inoculated onto the diet in each well using a fine artist's brush. Ten larvae were used for each insecticide concentration per replicate as well as for the untreated control in each replicate. The bioassay trays were covered with plastic lids and incubated at $26 \pm 2^{\circ}\text{C}$, 60-65% RH with a 16:8 L: D photoperiod (Figure 3.2, B).

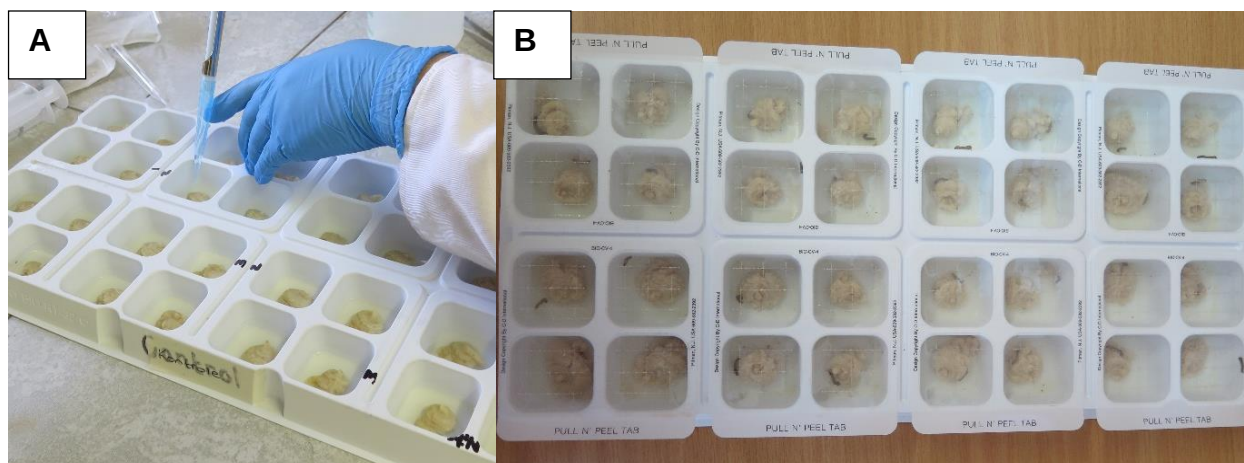


Figure 3.2: A: Application of 130 μ l of insecticide mixture onto artificial diet in each well. B: One third instar *Spodoptera frugiperda* larva was inoculated onto the diet in each well, which was sealed with a plastic lid.

3.3.5 Insecticide topical application bioassay

Spodoptera frugiperda larvae were reared on Stonefly *Heliopsis* diet. Third instar larvae were cooled at 4 °C for 4 minutes to ensure that they do not move during topical application of insecticides. A hand-operated micro-pipet was used to apply topically aliquots (1 μ l) of an insecticide solution onto the pro-thorax of these larvae (Figure 3.3). The insecticide solutions were prepared at five concentrations which represented the insecticide treatments and a control treatment (5% deionised water and 95% acetone only) as recommended by Kranthi (2005) and Yu (2008). The method was adapted according to Temple *et al.* (2009) to ensure solubility of insecticides. After application, a single larva was transferred with a fine artist's brush to each well of a polystyrene bioassay tray (Frontier: scientific services), containing artificial diet. Prior to placing the diet into the bioassay well, a 3 mm layer of 2% Agar-Agar was poured into each well and left to set. This was done to keep the diet from desiccating. There were three replicates per insecticide. Ten larvae were used for each insecticide concentration as well as for the untreated control in each replicate. The bioassay trays were covered with plastic lids and incubated at 26 $\pm$ 2°C, 60-65% RH with a 16:8 L: D photoperiod.



Figure 3.3: Topical application of an insecticide solution onto the prothorax of a third instar *Spodoptera frugiperda* larva.

3.3.6 Insecticide leaf dipping bioassay

The leaf dipping bioassay was conducted according to IRAC method no. 018 for *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae). Tender maize leaves were cut into small pieces of approximately 4 x 4 cm. The leaf pieces were dipped individually in the insecticide solution for 5 seconds with gentle agitation to ensure the entire surface is submerged equally (Figure 3.4). This procedure was done for all five the insecticide concentrations (treatments) evaluated as well as the control treatment which consisted of a deionised water and Triton X-100 solution only. Leaves were carefully drained of excess liquid and placed on fine mesh to air dry. Prior to transferring a single piece of treated leaf material to the wells, a 3 mm layer of 2% Agar-Agar was poured into each well of a polystyrene bioassay tray (Frontier: scientific services), and left to set. This was done to keep the leaf pieces from desiccating. One third instar *S. frugiperda* larva was placed onto the treated maize leaf tissue in each well, with a fine, soft brush. There were three replicates per insecticide. Ten larvae were used for each insecticide concentration as well as for the untreated control of each replicate. The bioassay trays were covered with plastic lids, each covered four wells and incubated at $26 \pm 2^{\circ}\text{C}$, 60-65% RH with a 16:8 L: D photoperiod.



Figure 3.4: Leaf pieces dipped individually in the insecticide solution for 5 seconds with gentle agitation.

3.3.7 Mortality assessment

Mortality was assessed according to the recommendations by IRAC for the respective insecticide groups, viz. unknown, benzolureas and diamides after four days and for the carbamate, assessment was done after three days. Larvae were considered dead when they did not respond to gentle prodding with an artist's fine brush.

3.4. Determining susceptibility

The most suitable bioassay determined for susceptibility testing of *S. frugiperda* larvae for the respective insecticide groups was used to determine the LC_{50} estimates. Therefore, susceptibility was determined for one population originating from Groblersdal F7.

3.5 Statistical analyses

Mortality data from the dose-response bioassays were subjected to probit analyses using PoloSuite 1.8. (LeOra software) The parameters from these analyses were evaluated according to Robertson *et al.* (2017). This was done to:

1. determine whether a linear relationship existed between the probits and the dose i.e. whether the slopes of the respective regressions were significant (t-ratio). These t-ratios should exceed 1.96 to indicate that the regression parameters are significant.
2. test the goodness of fit of the dose-probit regression model (χ^2 should be significant). When χ^2 is not significant, and the heterogeneity factor (χ^2/df) is greater than 1, standardize residuals should be plotted against doses, to identify sources of poor fit to the probit model, which can be observed as outliers outside the bounds of -2 to 2.
3. estimate and compare median lethal concentrations (LC_{50}s) and their corresponding 95% CL (confidence limits), the shorter the CL the better the bioassay.
4. determine lethal dose ratios with 95% CL. If the 95% CLs of the ratio include 1.0, the lethal concentrations (LCs) as estimated by the two bioassays compared were not significantly different.
5. test the likelihood ratio (LR) of equality and parallelism.

The slopes of the concentration-mortality regressions of the bioassays were compared for testing of the hypothesis of parallelism (Robertson *et al.*, 2017). Two slopes were considered significantly different when the χ^2 was associated with a probability of $P < 0.05$.

After the most suitable bioassay was determined, the LC_{50} estimates of the respective insecticides were compared to recommended dosage rates to determine the susceptibility of the population.

3.6 Results

Relative homogeneity of *S. frugiperda* larvae used in the bioassays was assumed, since the same number of third instar *S. frugiperda* larvae were used in each replicate of all four bioassays, for all the insecticides evaluated. All the larvae also originated from the same insect population.

The slope of the regression line from the data fitted to the probit-dose for each of the bioassays and the goodness of fit of these regression lines (χ^2) were evaluated. (Table 3.1). When the t-ratio of the regression lines was less than 1.96, the regression for that bioassay was not significant and denied the existence of a dose-responsive line (Robertson *et al.*, 2017). These bioassays (datasets) were therefore not considered further. The parameters are, however, still shown in Table 3.1 and indicated in yellow. Due to the consistency in the number of replicates used and homogeneity of the *S. frugiperda* population, the length of the 95% confidence limits (CLs) was used for comparison between the respective bioassays.

3.6.1 Chlorantraniliprole

The slopes of the regression lines of all four bioassays were significant ($t > 1.96$) indicating that a linear relationship existed between the probits and dose (Table 3.1). The high slopes of these bioassays showed a high degree of sensitivity of the larvae towards the insecticide. The χ^2 of the insecticide incorporation bioassay was the lowest compared to the other three bioassays and this bioassay method therefore had the best fit to the probit model, followed by the topical application, leaf dipping and insecticide overlay bioassays (Table 3.1). Comparison across bioassays indicated that the LC_{50} value estimated by the insecticide incorporated artificial diet bioassay was the lowest, followed by the leaf dipping, insecticide overlay and topical application (Table 3.1). The CL of the insecticide incorporated artificial diet bioassay was also the shortest, followed by the leaf dipping, insecticide overlay and topical application. Heterogeneity is also an indication of how well the data fit the model. The heterogeneity factor for all the bioassays were lower than 1.0 confirming that all four bioassays fitted the model. According to the LR test of equality and parallelism for all four bioassays evaluated simultaneously, the test for parallelism was accepted but the

lines were not equal. The regression lines of the respective bioassays were then compared pair wise and only the slopes of the insecticide incorporated artificial diet and topical application bioassays were equal. These slopes differed from that of the insecticide overlay and leaf dipping bioassays (Figure 3.5). The slopes of the latter two bioassays were not exactly, but almost similar, which may explain the assumption of parallelism when all four lines were evaluated simultaneously. The slopes of the leaf dipping bioassay as well as the insecticide overlay onto artificial diet bioassay were steeper compared to the slopes of the artificial diet incorporation as well as topical application bioassays (Figure 3.5). The regression lines were significantly different ($t > 1.96$) and the lethal dose ratio could therefore be calculated and compared between the respective bioassays. The CL of the lethal dose ratios all excluded 1, except for the insecticide incorporated artificial diet x leaf dipping bioassays indicating that the LC_{50} estimates of these two bioassays did not significant differ from each other (Table 3.2).

3.6.2 Lufenuron

Only two of the bioassays evaluated provided a linear dose-responsive line, *viz.* the insecticide overlay onto artificial diet bioassay and leaf dipping bioassay (Table 3.1). The LC_{50} estimate of the leaf dipping bioassay was lower than that of the insecticide overlay onto artificial diet bioassay (Table 3.1), but the CLs of the insecticide overlay onto artificial diet bioassay was the shortest, compared to the CLs of the leaf dipping bioassay, indicating the insecticide overlay onto artificial diet bioassay to be the better bioassay for estimating the LC_{50} (Table 3.1). The heterogeneity factors of both bioassays was lower than 1.0 and data of both therefore fitted the dose-response regression model. The χ^2 of the insecticide overlay onto artificial diet was, however, lower than that of the leaf dipping bioassay confirming a better fit to the probit model (Table 3.1). The slopes of the regression lines of the two bioassays were neither parallel, nor equal (Figure 3.6). The bioassay method and exposure rate therefore differed qualitatively. The slope of the insecticide overlay onto artificial diet bioassay was steeper compared to the slope of the leaf dipping bioassay. (Figure 3.6). As only the insecticide overlay onto artificial diet and leaf dipping bioassay had significant regression lethal dose ratios only these two could be compared. According to the CL

of the lethal dose ratios, the LC₅₀ estimates for the two bioassays differed significantly from each other (Table 3.2).

3.6.3 Pyridalyl

Pyridalyl has an unknown mode of action and the slope of only one of the bioassays, viz. leaf dipping, was significant ($t > 1.96$) with a linear relationship between the probits and dose (Table 3.1). The slope of the regression for the leaf dipping bioassay was low (Figure 3.7). The χ^2 was, however, high and the heterogeneity factor >1 explaining the incorrect and very long CLs of the LC₅₀ (Table 3.1). None of the bioassays evaluated in this study was therefore suitable for use in toxicological bioassays for pyridalyl with third instar *S. frugiperda* larvae.

3.6.4 Methomyl

The slope of only one of the bioassays, viz. insecticide incorporated artificial diet bioassay was significant ($t > 1.96$) indicating that a linear relationship existed between the probits and dose (Table 3.1). The χ^2 of this bioassay was low and the heterogeneity factor <1 confirming that this bioassay fitted the model. The 95% CL of the LC₅₀ was short indicating a good bioassay (Table 3.1). The slope of the regression line for the insecticide incorporation bioassay was very steep (Figure 3.8) Therefore, the only bioassay suitable to be used for toxicological bioassays for methomyl with third instar *S. frugiperda* larvae is the insecticide incorporated artificial diet bioassay.

3.6.5 Statistical criteria for the four bioassays

The suitability of the respective bioassays for use in toxicological studies with third instar *S. frugiperda* larvae for the four insecticides used in this study, was determined according to the statistical parameters provided in Table 3.1. By using these parameters, the bioassays were ranked and summarised in Table 3.3. The bioassay that performed best for each insecticide are indicated in orange. For chlorantraniliprole and methomyl, the insecticide incorporation bioassay was found to be the most suitable bioassay and the insecticide overlay onto artificial diet bioassay for lufenuron (Table 3.3). None of the bioassays evaluated was found to be suitable for toxicological

studies with pyridalyl, but some of the parameters needed for a suitable bioassay was satisfied by the leaf dipping bioassay (indicated in blue) (Table 3.3). Further evaluations of bioassays for toxicological studies with pyridalyl are therefore needed.

3.6.6 Determination of susceptibility

Spodoptera frugiperda larvae that originated from Groblersdal were very susceptible to the respective insecticides; chlorantraniliprole, lufenuron, pyridalyl and methomyl. LC₅₀ estimates determined in this study according to the most suitable bioassay identified were low. These were: chlorantraniliprole (0.06 ppm), lufenuron (0.91 ppm) and methomyl (40.13 ppm) (Table 3.1).

3.7 Discussion

The experimental design of toxicity bioassays with insects should minimize factors that may have an additional effect on the outcomes of bioassays. For example, it is important that insects should originate from the same population and generation, since their responses to an insecticide are more likely to be similar (Robertson *et al.*, 2007). When dose regression lines are not parallel, it may be attributed to differences in mode of action, mode of entry, exposure rate as well as different bioassays (Lei and Sun, 2018).

When all the statistical parameters estimated in this study were taken into account, the best bioassay for use in toxicological bioassays with *S. frugiperda* larvae to the diamide, chlorantraniliprole was determined as the insecticide incorporated artificial diet, but leaf dipping was also found to be suitable for toxicological studies. Leaf dipping may be used as an alternative to the insecticide incorporated artificial diet, since there was no difference in LC₅₀ estimates of the two bioassays and the leaf dipping bioassay may resemble field applications of insecticides more accurately. The insecticide incorporated artificial diet bioassay is, however, a good indicator of the degree of susceptibility. The standard method of insecticide incorporated artificial diet as recommended by IRAC (IRAC method no 020) for diamides against lepidopteran larvae, was therefore confirmed in this study. The poor performance of the topical

application bioassay may be explained by the mode of entry of chlorantraniliprole. The primary route of exposure of chlorantraniliprole is through ingestion, and secondary by contact (Tomlin, 2009). When chlorantraniliprole was applied topically the only mode of entry was through contact of the insecticides with the epidermis of the larvae. The mode of entry of an insecticide therefore has a profound effect on the effectivity of a bioassay and also on the estimate of the median lethal dose by the bioassay.

The best bioassay for susceptibility evaluation of *S. frugiperda* larvae to lufenuron, the insect growth regulator, was found to be the insecticide overlay onto artificial diet bioassay. The leaf dipping bioassay was less sensitive although recommended by IRAC (method no 007) for leaf-eating lepidopteran larvae for growth regulators. The leaf dipping bioassay may, however, also be used. All parameters of the leaf dipping bioassay were within acceptable bounds, but the dose response line of this bioassay did not fit the model as well as the insecticide overlay bioassay.

There is currently no IRAC method recommended for paridaly. The mode of entry of this insecticide is unknown. The biochemical mode of action is also not identified, but insects lose their vigour and die later on (Tomlin, 2009). No bioassay, other than leaf dipping provided a linear dose-responsive line for paridaly, but the dose responsive line showed a lack of fit. It may be ascribed to low sensitivity of the bioassay since the slope of the regression line changed gradually. The most suitable bioassay to use for paridaly could also not be determined in this study.

IRAC (method no 007) (leaf dipping) is recommended for susceptibility testing of leaf-eating lepidopteran larvae to carbamates. The mode of action of methomyl is contact and stomach (Tomlin, 2009). However, in this study the leaf dipping bioassay did not provide a linear probit-dose regression line while the insecticide incorporated artificial diet bioassay was suitable for susceptibility testing. It therefore, differs from the method recommended by IRAC for carbamates. It may be explained by the bigger cuticle surface of larvae that were constantly exposed to the toxin while feeding into diet with the insecticide incorporated diet bioassay, compared to the area exposed by larvae moving over and feeding on dipped leaf material.

The interpretation of toxicological bioassays with different modes of action are complex. Bioassays are influenced by a number of factors. The leaf dipping bioassay differed in feeding substrate since the larvae used in the other three bioassays were reared on and inoculated onto artificial diet during the bioassay. The degree of exposure to the insecticides in the respective bioassays was different although the dosage concentration was kept the same. Topical application was the least successful bioassay. A possible explanation may be that ingestion can be regarded as the more effective route of exposure compared to contact exposure. The fit of bioassay methods to a linear dose-responsive line may be improved by increasing the number of dosage rates evaluated as well as the number of larvae used per dosage rate.

The most appropriate bioassay for selected mode of action groups for toxicological studies with *S. frugiperda* larvae was determined in this study. The recommended dosages for the respective insecticides in South Africa, viz. chlorantraniliprole (67 ppm), lufenuron (167 ppm) and methomyl (600 ppm) is much higher than the LC₅₀ estimates determined in this study. LC₅₀ estimates that were determined with the most suitable bioassay for the respective bioassays were 0.06 ppm for chlorantraniliprole, 0.91 ppm for lufenuron and 40.13 ppm for methomyl. The bioassays determined to be the most suitable for susceptibility evaluation of *S. frugiperda* in South Africa, will be used in future studies.

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Table 3.1: Comparison of statistical parameters determined in toxicological bioassays with different active ingredients used for control of third-instar *Spodoptera frugiperda* larvae.

AI	Bioassay	N	LC ₅₀ (ppm) and CLs	Slope ±SE	Intercept ±SE	t-ratio	df	χ ²	h
Chlorantraniliprole	Overlay	180	0.91 (0.09 - 8.19)	1.21 ± 0.3640	0.05 ± 0.20	3.37	3	2.57	0.86
	Incorporated	180	0.06 (0.01 - 0.16)	1.01 ± 0.420	1.22 ± 0.30	2.42	3	0.50	0.17
	Leaf dipping	180	0.26 (0.00 - 0.74)	1.25 ± 0.38	0.72 ± 0.22	3.24	3	2.10	0.70
	Topical application	180	4.34 (1.67 - 311.32)	1.01 ± 0.38	-0.64 ± 0.21	2.66	3	1.20	0.41
Lufenuron	Overlay	180	0.91 (0.64 - 1.29)	1.97 ± 0.75	0.08 ± 0.24	2.63	3	0.38	0.13
	Incorporated	180	NA	NA	NA	NA	NA	NA	NA
	Leaf dipping	180	0.12 (0.00 - 0.82)	0.83 ± 0.21	0.76 ± 0.25	3.92	3	2.43	0.81
	Topical application	180	602.98 (0.00 - 1.36)	0.26 ± 0.20	-0.73 ± 0.24	1.33	3	2.20	0.73
Pyridalyl	Overlay	180	4485916.83 (0.00 - 982.18)	0.16 ± 0.21	-1.04 ± 0.23	0.76	3	0.36	0.12
	Incorporated	180	13.36 (6.40 - 356.16)	1.37 ± 0.74	-1.54 ± 0.48	1.84	3	0.88	0.29
	Leaf dipping	180	2.27 (0.25 - 40673.24)	0.61 ± 0.20	-0.22 ± 0.20	3.00	3	3.79	1.26
	Topical application	180	71.0 (18.22 – 15415.29)	0.83 ± 0.59	-1.54 ± 0.39	1.40	3	0.40	0.13
Methomyl	Overlay	180	75.29 (52.56 - 16.29)	6.11 ± 4.93	-11.46 ± 8.09	1.24	3	1.07	0.36
	Incorporated	180	40.13 (37.95 - 42.56)	7.41 ± 2.52	-11.89 ± 4.03	2.94	3	0.26	0.09
	Leaf dipping	180	50.25 (43.94 - 84.30)	4.10 ± 2.44	6.97 ± 3.92	1.68	3	0.38	0.13
	Topical application	180	62.98 (17.22 - 52.12)	11.14 ± 9.20	-20.04 ± 15.43	1.20	3	0.73	0.24

AI = Active Ingredient, N = Number of larvae, h = Heterogeneity, χ² = goodness of fit test was significant at α= 0.05, LC₅₀ = Concentration that confers 50% mortality (95% Confidence limits), t-ratio = regression significant at t > 1.96, df = degrees of freedom, Parameters in Yellow did not adhere to the models specifications.

Table 3.2: Lethal dose ratios between different bioassays for the respective active ingredients fitted to the probit-dose regression model.

AI	Comparison of bioassays	Lethal dose ratio of LC ₅₀ (95% CL)	Significance
Chlorantraniliprole	Incorporated x Leaf dipping	0.239 (0.027, 2.122)	NS
	Incorporated x Overlay	0.069 (0.008, 0.573)	*
	Incorporated x Topical application	0.014 (0.001, 0.154)	*
	Overlay x Topical application	0.069 (0.008, 0.573)	*
	Overlay x Leaf dipping	0.289 (0.089, 0.935)	NS
	Leaf dipping x Topical application	0.061 (0.012, 0.294)	*
Lufenuron	Leaf dipping x Overlay	0.134 (0.036, 0.494)	*

AI = Active Ingredient, NS = LC₅₀ not significantly different, * = LC₅₀ significantly different, CL = confidence limits.

Table 3.3: Summary of the statistical requirements used to validate the respective toxicological bioassays used for insecticides with different active ingredients, for control of *Spodoptera frugiperda* larvae.

AI	Statistical criteria	Bioassays			
		Overlay	Incorporated	Leaf dipping	Topical application
Chlorantraniliprole	t-ratio > 1.96 (Linearity)	√	√	√	√
	Residuals (-2;2)	√	√	√	√
	CL length of LC ₅₀ estimate	3	1	2	4
	χ^2	4	1	3	2
	Heterogeneity	4	1	3	2
Lufenuron	t-ratio > 1.96 (Linearity)	√	NA	√	≠
	Residuals (-2;2)	√	NA	√	≠
	CL length of LC ₅₀ estimate	1	NA	2	≠
	χ^2	1	NA	2	≠
	Heterogeneity	1	NA	2	≠
Pyridalyl	t-ratio > 1.96 (Linearity)	≠	≠	√	≠
	Residuals (-2;2)	≠	≠	√	≠
	CL length of LC ₅₀ estimate	≠	≠	1	≠
	χ^2	≠	≠	1	≠
	Heterogeneity	≠	≠	≠	≠
Methomyl	t-ratio > 1.96 (Linearity)	≠	√	≠	≠
	Residuals (-2;2)	≠	√	≠	≠
	CL length of LC ₅₀ estimate	≠	1	≠	≠
	χ^2	≠	1	≠	≠
	Heterogeneity	≠	1	≠	≠

√ = Requirement achieved, NA = Not applicable, ≠ statistical parameter did not satisfy minimal requirement, Numbers indicate order of suitability of bioassay, 1 - 4 = most to least suitable, CL= confidence limits.

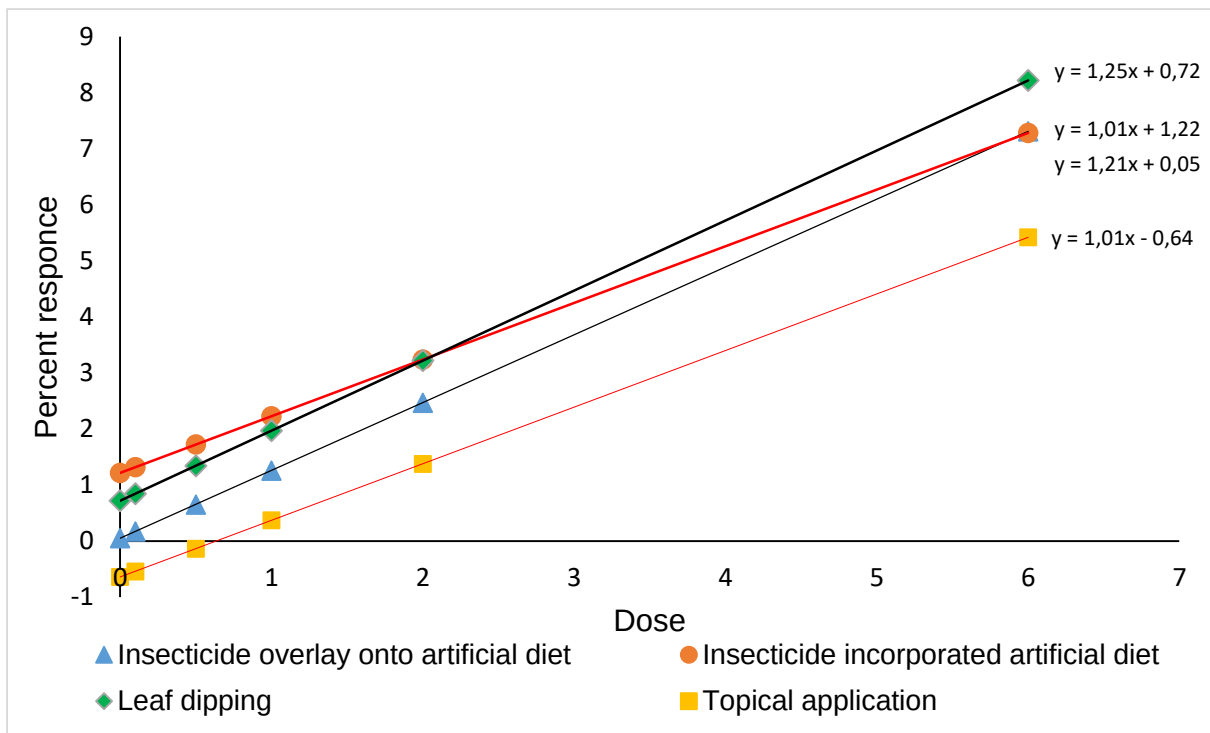


Figure 3.5: Dose-response relationships of third instar *Spodoptera frugiperda* larvae to chlorantraniliprole in four different bioassays.

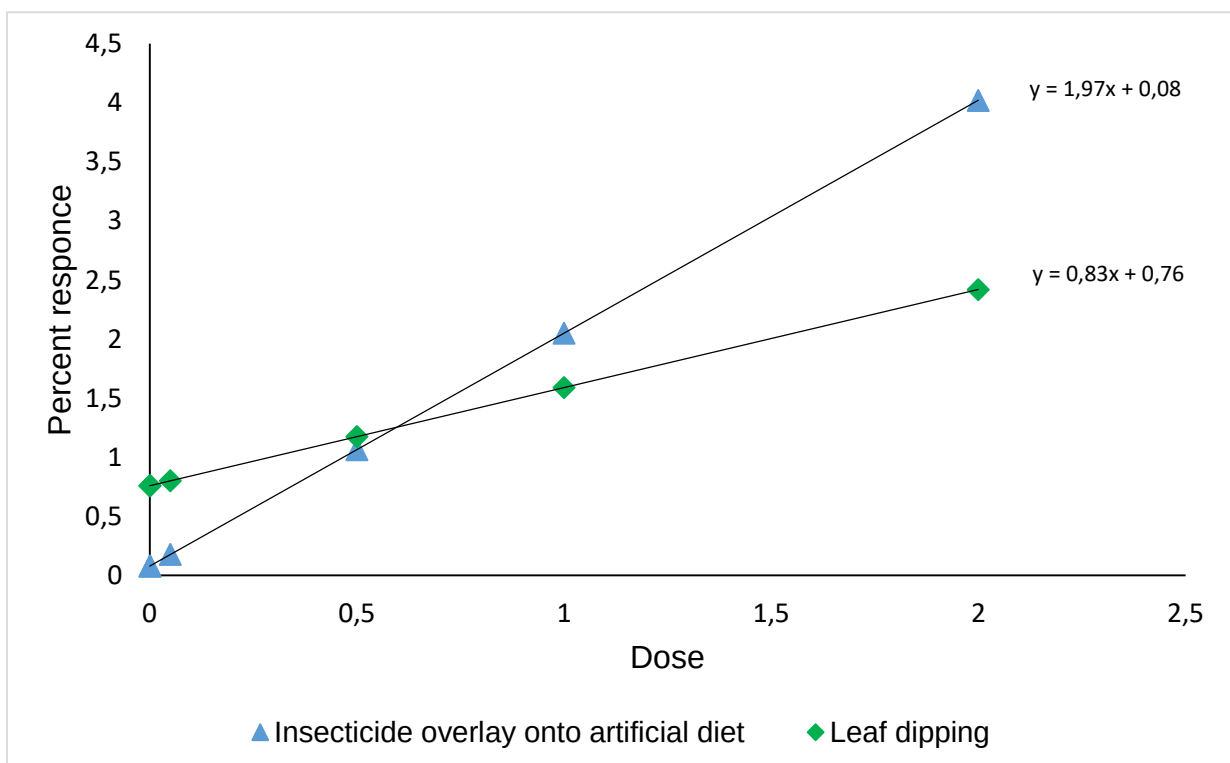


Figure 3.6: Dose-response relationships of third instar *Spodoptera frugiperda* larvae to lufenuron in a bioassay with insecticide overlay onto artificial diet and a leaf dipping bioassay.

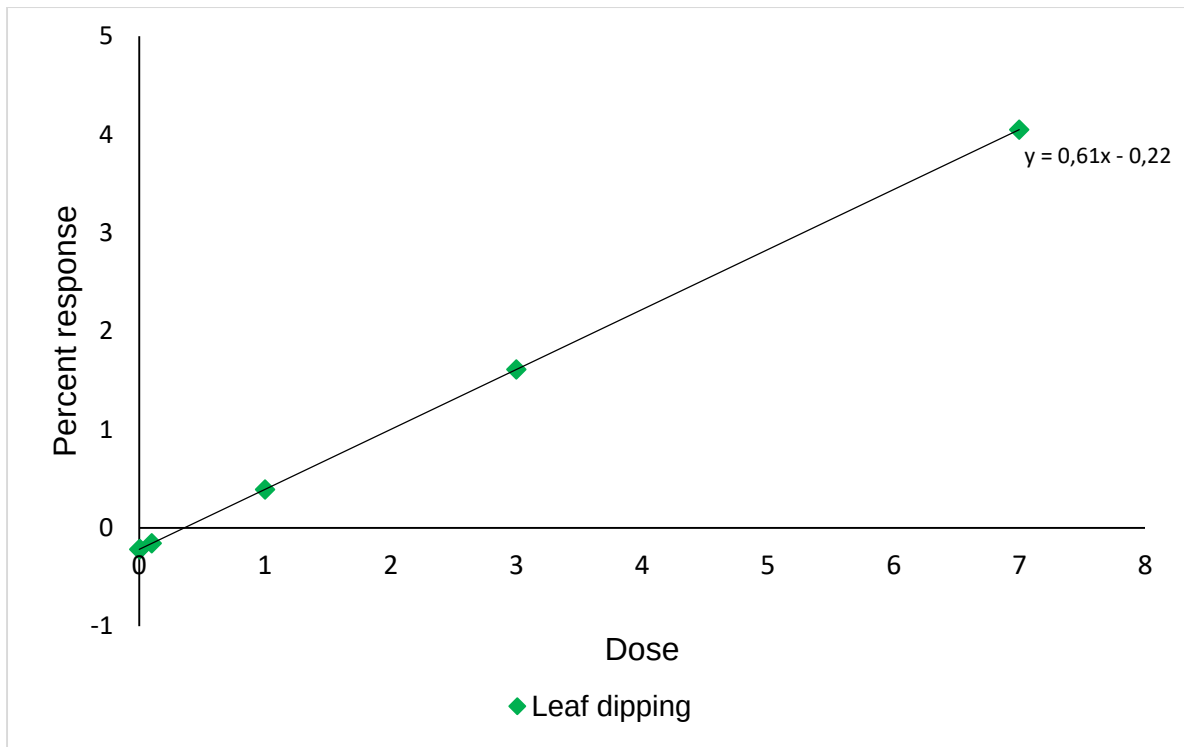


Figure 3.7: Dose-response relationships of third instar *Spodoptera frugiperda* larvae to pyridalyl in a leaf dipping bioassay.

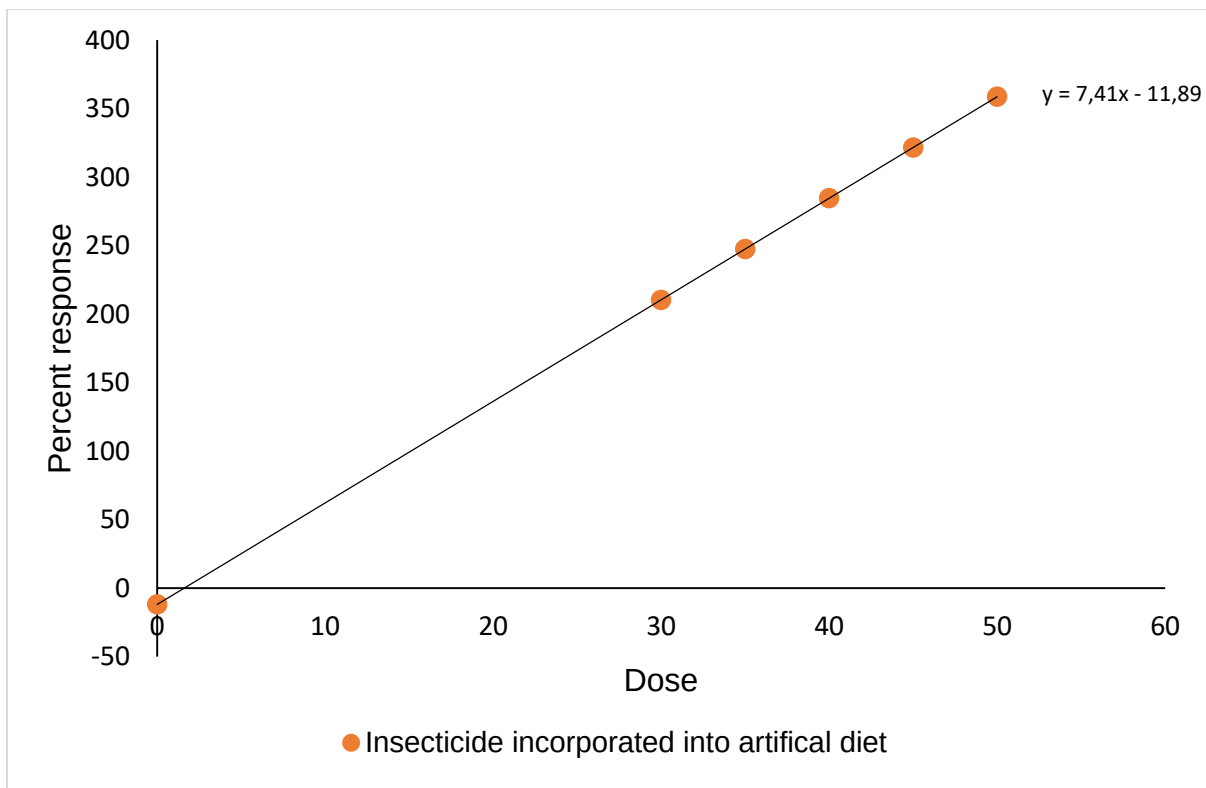


Figure 3.8: Dose-response relationships of third instar *Spodoptera frugiperda* larvae to methomyl in an insecticide incorporated artificial diet bioassay.

Chapter 4

Conclusion and recommendations

4.1 Discussion

The fall armyworm, *Spodoptera frugiperda*, is a polyphagous pest with the ability to develop on a wide variety of host plants with different nutritional indices. There are 353 *S. frugiperda* larval host plants reported in literature (Montezano *et al.*, 2018), but maize is the most preferred (Silva *et al.*, 2017). Rearing of insects is important for entomological research (Cohen, 2001). It is, however, not always possible to have enough maize plants available to rear *S. frugiperda* in masses throughout the year for research purposes. Herbivorous insects are commonly reared on artificial diets (Hervet *et al.*, 2016) which enables synchronisation the availability of insects for the use in entomological studies (Hervet *et al.*, 2016). Reliability of entomological studies does, however, depend on the health of the insects, which depends on the quality and the nutritional value of artificial diets (Cohen, 2001). The quantity and quality of food consumed during the larval stages affects the growth rate, development time, body weight and survival, as well as the adult fecundity and longevity of insects (Santos *et al.*, 2003).

The suitability of four artificial diets, viz. *Busseola fusca*, *Anticarsia gemmatilis*, Stonefly *Heliothis* and *Chilo partellus* diets and maize leaves were evaluated for mass rearing of *S. frugiperda*. Suitability of the diets was evaluated according to the following fitness parameters: development time of larvae and pupae, pupal mass, survival, adult eclosion, fecundity and fertility. Fitness parameters of *S. frugiperda* was the most favourable on the *B. fusca* artificial diet. This diet promoted larval and pupal development, pupal mass, percentage oviposition, fecundity and fertility. Although percentage fertility was lower compared to larvae reared on maize. Maize was relatively suitable considering low larval and pupal duration, and high fertility. However, pupal mass was significantly lower than all other artificial diets. *Spodoptera frugiperda* performed the poorest on the *C. partellus* diet. Larvae had prolonged larval and pupal duration, low survival, low pupal mass and no fecundity or fertility. Diets had

different nutritional compositions as well as water content. These factors influenced all fitness parameters. The *B. fusca* diet increased the fitness of *S. frugiperda*.

Compared to rearing of *S. frugiperda* larvae on maize plant tissue, rearing on the *B. fusca* diet is less labour intensive, it reduces rearing time as larvae develop faster and less maize plants were required. The maize leaves needed for the diet could be harvested, dried, milled, autoclaved and stored. This enables mass rearing outside the maize growing season and eliminates the problem of having a constant supply of fresh maize leaves and whorls needed for rearing of *S. frugiperda*.

Many of the diets published for rearing of *S. frugiperda* were developed for rearing of other insects (Prasanna *et al.*, 2018). Institutions in Africa have optimised different synthetic diets for this purpose. Both the International Maize and Wheat Improvement Centre (CIMMYT) in Zimbabwe and the International Centre of Insects Physiology and Ecology (ICIPE) in Kenya use artificial diets that are similar to the maize stem borer (*Busseola fusca*) diet (Prasanna *et al.*, 2018). The Agricultural Research Council (ARC) in South Africa recommends a diet used to rear the spotted stem borer, *Chilo partellus* (Lepidoptera: Crambidae) (Swinhoe) (Prasanna *et al.*, 2018) for rearing of *S. frugiperda*. The *B. fusca* diet was confirmed in this study to be suitable for rearing of *S. frugiperda*.

The artificial diet is cooked and when it cools down it sets. Adding of insecticide formulations at a high temperature may result in deactivation of the insecticide. Therefore, the Stonefly *Heliothis* diet were used in toxicological studies as recommended by IRAC (Insecticide resistance action committee) method 020. This diet was however not the most suitable for development of *S. frugiperda*.

With the introduction of *S. frugiperda* in Africa, chemical companies obtained emergency registrations for insecticides to control *S. frugiperda*. Nineteen active ingredients belonging to 11 mode of action groups were registered against *S. frugiperda* in South Africa (DAFF, 2017). *Spodoptera frugiperda* is known to have developed resistance to four of these mode of action groups in South and Central America. These groups are organophosphates, carbamates, pyrethroids, pyrethrins (Yu, 1991, 1992; Leon-Garcia *et al.*, 2012), and benzolurease (Nascimento *et al.*,

2015). This observation is cause for great concern for the control of *S. frugiperda* in Africa (Goergen *et al.*, 2016; Jeger *et al.*, 2017). It is not known if the population that invaded Africa, carried resistance alleles, and if so, to which insecticide groups. Monitoring of insecticide efficacy is the basic tool for proactive evidence-based resistance management (Roditakis *et al.*, 2017). Baseline susceptibility of *S. frugiperda* should therefore be determined and the most suitable bioassay method for each of the respective insecticide groups should be determined. Appropriate bioassays for various modes of entry and action enables susceptibility evaluation of *S. frugiperda*. Four bioassay methods; viz. leaf dipping, topical application, insecticide overlay onto and incorporation in artificial diets were evaluated for use in toxicological studies with insecticides with different modes of entry and action. These insecticides with the respective groups were: chlorantraniliprole (diamide), lufenuron (benzolureas), pyridalyl (unknown) and methomyl (carbamate). Dose responses were evaluated with PoloSuite computer program, and statistical parameters were analysed to determine the best bioassay for the insecticide groups evaluated. The insecticide incorporation into artificial diet bioassay was identified as the most suitable bioassay for the susceptibility evaluation of *S. frugiperda* to chlorantraniliprole and methomyl and should be standardised for susceptibility evaluation of *S. frugiperda* to chlorantraniliprole and methomyl. For lufenuron the most suitable method was the insecticide overlay onto artificial diet-bioassay which can be considered as a standard method for the susceptibility evaluation of lufenuron.

Standardisation of these methods will enable evaluation of susceptibility to insecticides from these groups over geographical areas, populations and time. Standard methods enable insecticide resistance monitoring and development of insecticide resistance management (IRM) programs (Sparks and Nauen, 2015; Zhu *et al.*, 2016). No suitable bioassay could, however, be determined for the evaluation of susceptibility to pyridalyl. An important factor to consider in bioassays is the mode of entry. The difference in suitability of bioassays determined for each insecticide in this study, showed that the mode of entry has a profound effect on the effectivity of bioassays and also on the estimate of the median lethal dose.

Previous studies comparing bioassay methods did not identify the most suitable bioassay for specific susceptibility evaluations, but only provide differences in LC₅₀

estimates determined in the respective bioassays (Prabhaker *et al.*, 1996; Perez *et al.*, 1997; Lowery and Smirle, 2003; Prabhaker *et al.*, 2006). It was also difficult to determine the most suitable bioassay for susceptibility evaluation of *S. frugiperda* to the respective insecticides. High mortality in a bioassay does not indicate better suitability of the bioassay. Therefore, an appropriate statistical model (Probit) was applied and analysed with PoloSuite software. Statistical comparisons using the parameters provided by these analyses were used for interpretations of dose mortalities from which appropriate conclusions were made. This study indicated that laboratory reared *S. frugiperda* larvae that originated from Groblersdal were highly susceptible to the following insecticides: chlorantraniliprole, lufenuron, and methomyl. LC₅₀ estimates were very low in comparison to recommended dosage rates of the respective insecticides.

4.2. Recommendations and future studies

A suitable bioassay for susceptibility evaluation of *S. frugiperda* to paridalyI should be determined. Since the moths of *S. frugiperda* can disperse over vast distances, baseline susceptibility of *S. frugiperda* populations in different regions in Africa should be determined to enable proactive evidence-based resistance management. These baseline susceptibility evaluations should also be conducted on either F1 or F2 insect populations to make accurate assumptions of susceptibility of *S. frugiperda* in the field.

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