Resistance to pyrethroids in the maize weevil, *Sitophilus zeamais*

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Abstract

The maize weevil is a pest of stored crop products, predominantly maize across humid areas of the world. The *Sitophilus zeamais* were controlled with pyrethroids until resistance emerged in areas across Brazil. This resistance was found to be conveyed by a super-kdr mutation in the voltage gated sodium channel (VGSC). A change from threonine to isoleucine at the 929 position in domain II, segment 5 of the VGSC was found to be the cause of resistance. This mutation also conveyed cross resistance to DDT. The project aimed to assess the level of resistance conveyed by the T929I mutation in lab populations of the maize weevil, to test for the presence of this mutation in the field, and finally to assess mutations at the T929 site using electrophysiology.

Bioassays showed resistant lab populations of the maize weevil to be 800-1500-fold less sensitive to deltamethrin and 80-105-fold less sensitive to DDT. The bioassays also allowed the calculation of a concentration of insecticide that could be used to select for resistance in populations of unknown resistance. 12 field populations of the maize weevil were selected for resistance using this discriminating dose before being tested for the T929I allele using a taqman diagnostic assay. No individuals were found to carry the T929I allele, therefore suggesting it is no longer a problem in the field and this assay would not be a useful field tool.

Two electrode voltage clamping (TEVC) on Xenopus oocytes expressing the T929V *para* VGSC mutation, found in the cat flea, showed that 1µM permethrin was unable to illicit a tail

current. Therefore this mutation conveys very high levels of resistance to pyrethroids, similar to that of the T929I mutation.

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Abbreviations

ATX-II	Anemonia sulcata toxin
cDNA	Complementary Deoxyribonucleic Acid
DDT	1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
DMSO	Dimethyl Sulphoxide
DSCI	Drosophila Sodium Channel I
FAM	5-car boxyfluoroscein
HRM	High resolution melt
JA	Jacarezhino
JF	Juiz de Fora
K^+	Potassium ion
Kdr	Knockdown resistance
LD	Lethal Dose
mRNA	Messenger ribonucleic acid
Na^+	Sodium ion
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase Chain Reaction- restriction fragment length polymorphism
RH	Relative Humidity
SL	Sete Lagoas
SNP	Single Nucleotide Polymorphism
STX	Saxitoxin
Super-kdr	Super knockdown resistance
TEVC	Two Electrode Voltage Clamp

TRP	Transient Receptor Potential
TTX	Tetrodotoxin
VGSC	Voltage Gated Sodium Channel
VIC	Applied biosystems proprietary dye

1. Introduction

1.1 The Voltage gated sodium channel

1.1.1 Introduction

The VGSC is part of a superfamily of ion channels that includes the voltage gated potassium channel, the voltage gated calcium channel, TRP-related channels and cyclic nucleotide gated channels (Yu and Catterall, 2003). The VGSC was the first of these to be cloned and fully sequenced (Catterall, 2007). Voltage gated sodium channels are responsible for the rising phase of action potentials in nerve, muscle and endocrine cells.

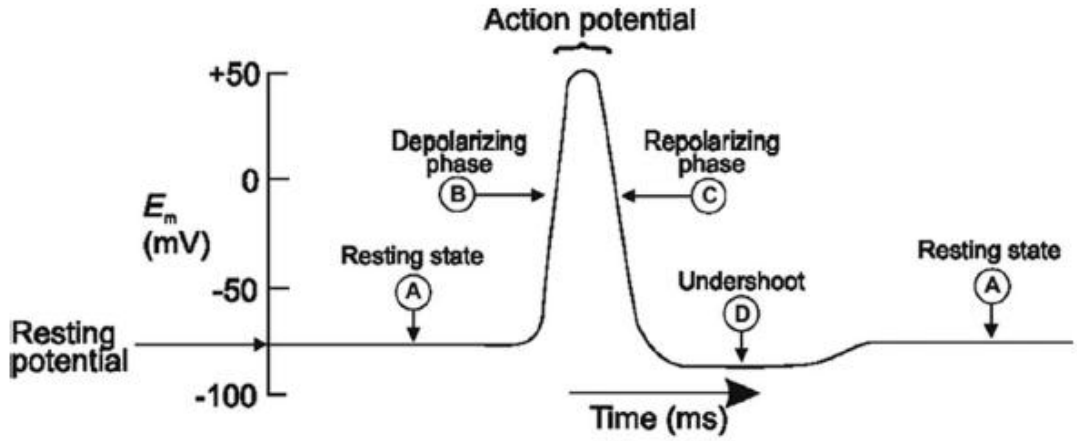

Fig1-1. Showing the phases of the action potential which represent the depolarization of the membrane and its repolarisation through the movement of ions across the membrane (Davies *et al*, 2007). See text for details.

"Voltage gated sodium channels conduct inward currents across cell membranes in electrically excitable tissues of the body" (French and Zamponi, 2005). **Fig1-1** shows the different stages of an action potential. When resting (**Fig1-1(A)**) the inside of the cell is negatively charged due to an uneven distribution of sodium and potassium ions across the

membrane, created by an ion pump in the cell membrane. When the voltage gated sodium channels are activated they allow an influx of sodium ions into the cell, producing the rising phase of an action potential (Benzanilla, 2005). The influx of sodium ions causes the inside of the cell to become more positive, this is depolarisation (**Fig1- 1(B)**) (Ganetzky and Wu, 1986).Once the sodium channels have become inactivated, the membrane is restored to its resting potential by movement of potassium ions through potassium channels from the inside to the outside of the cell (**Fig1-1(C)**). An action potential can move along an entire axon or membrane, as depolarization of one area causes the sodium channels to open in the adjoining region. This depolarization only spreads in one direction as the VGSC in the region the action potential has already spread through are inactivated and cannot re-open again until the membrane has become repolarised. Hodgkin and Huxley (1952) were the first to find that a current could be carried through a membrane by movement of sodium ions, their work was carried out on the squid giant axon using voltage clamping. More detail was discovered throughout the 1960's and 70's via voltage clamping of squid giant axons and vertebrate myelinated nerve fibres (Catterall, 2000).

1.1.2 Discovery

The sodium channel was the first voltage gated ion channel to be isolated from eel electroplax, which is rich in excitable cells (Benzanilla, 2005). A derivative of an α-scorpion toxin was first used to identify the α and β subunits of brain sodium channels in rats (Beneski and Catterall, 1980). Noda *et al* (1984) purified the channel from *Electrophorus electricus* electroplax using TTX (tetrodotoxin) and STX (saxitoxin). These bind to the extracellular part of the channel (Catterall, 1986). cDNA was isolated from expression libraries of

electroplax mRNA using oligonucleotides corresponding to partial sequences of electric eel electroplax sodium channel, and antibodies. The isolated protein was large and found to have 4 internally homologous domains with transmembrane α-helixes. These domains together form a pore of minimum 3Å x 5Å (Hille, 2001). Homologous mRNA for the sodium channel was later isolated from the rat brain (Noda *et al*, 1986; Loughney *et al*, 1989), where 3 different types were found. mRNA transcribed from VGSC cDNA from rat brain enabled the first voltage gated sodium channel to be successfully expressed in Xenopus oocytes (Suzuki *et al*, 1988), . The α subunits alone gave a functional channel but the β subunits were needed for normal kinetics and increased expression (Hille, 2001).

Molecular modelling of the sodium channel predicted a 2 dimensional folding pattern (**Fig1-2**) (Catterall, 2000). 6 α-helical transmembrane segments (S1-S6) in four homologous domains (I-IV) were predicted with a re-entrant loop that enters the transmembrane region between S5 and S6 and forms the outer pore. Large intracellular N and C terminal domains were also predicted. The sodium channel was thought to be voltage dependent when the S4 transmembrane segments were found to carry repeated motifs of positively charged amino acid residues followed by 2 hydrophobic residues. It is believed that the negative internal electric field exerts a strong force on these positive charges and holds the S4 segments in a 'cocked' position towards the inner face of the membrane (Catterall, 2000). S4 is the most conserved region between voltage gated channels (Zlotkin, 1999).

1.1.3 Mammalian VGSC

The mammalian voltage gated sodium channel α subunits which form the pore are encoded by 9 genes (Soderlund, 2010). A 10th gene was found to encode a similar protein but it is not yet known if this is a sodium channel, as functional expression has yet to be achieved. The channels are named $Na_v1.1$-$Na_v1.9$ and have >50% homology (Bosmans and Tytgat, 2007). They are co-expressed with β subunits that modulate the gating and kinetics of the channel, and also regulated expression (Catterall, 2000). There are 4 β subunits: β1-β4. They have a similar structure but different amino acid sequences. The β subunits consist of a large glycosylated extracellular domain with a single transmembrane segment and a small intracellular domain.

$Na_v1.9$ and $Na_v1.8$ are expressed in the peripheral nervous system, particularly in sensory neurons. $Na_v1.2$ is expressed in the central nervous system in the brain, myelinated and unmyelinated axons. $Na_v1.4$ is expressed in skeletal muscle and $Na_V1.5$ in cardiac muscle. Mammalian VGSC are 1000 times less sensitive to pyrethroids than insect types, independent of the temperature dependence of pyrethroids (Vais *et al*, 2001).

Mammalian sodium channels are less sensitive to pyrethroids and DDT than those of insects partly due to a difference at the 918 site in the linker between S4 and S5 of domain II which is M in insects and I in mammals (Vais *et al*, 2001) The 918 position in insects is equivalent to 874 in mammals. Peng *et al* (2009) studied the mammalian rat $Na_V1.2$ VGSC in Xenopus oocytes. The wild type I874 channel was found to be 100-fold less sensitive to deltamethrin than when this amino acid was mutated to M, as is found in insects. The M874 channel showed slower open-closed kinetics and slower kinetics of fast inactivation. This and other

factors, such as temperature difference and metabolism of the insecticide are thought to be why pyrethroids and DDT have low toxicity to mammals (Zlotkin, 1999)

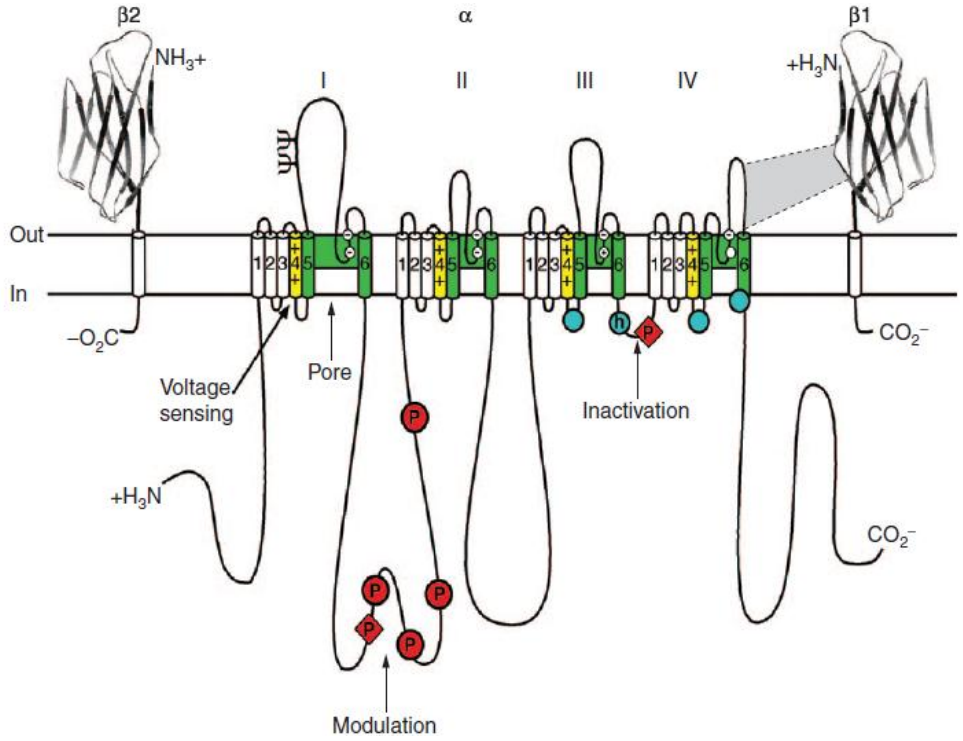

Fig1-2. Schematic representation of the voltage gated sodium channel, including the mammalian β1 and β2 subunits. The voltage sensing 4th segments are highlighted in yellow, and the pore linging segments 5 and 6 are highlighted green. The inactivation gate and its receptor are shown in blue (h, inactivation gate) and P represents sites of phosphorylation (Yu and Catterall, 2003).

1.1.4 Insect VGSC

First cloned from fruit fly, *Drosophila malanogaster*, the gene encoding the insect VGSC was found in the *para* locus on the X chromosome (Loughney *et al*, 1989). It was discovered via mutation experiments that caused dysfunction in the sodium channel. The first insect genes proposed to express the insect sodium channel were DSCI in Drosophila, found on the right arm of the second chromosome and genes at the *para* locus (Zlotkin, 1999). The mutations at the *para* locus caused temperature sensitive paralysis, hence their name. The

first of these was para^{ts} which caused complete paralysis at 29°C. The flies then recovered when returned to normal temperatures (below 25°C) (Suzuki *et al*, 1971). Mutation experiments by Ganetzky and Wu (1986) showed mutations at the *para* locus caused temperature sensitive paralysis to both larval and adult stages, it was therefore concluded that this was the primary gene for insect sodium channels. Other mutations were found to affect sodium channel function, including nap^{ts}, and sae^{ts}.

The main protein of the insect sodium channel was found to be homologous to the mammalian α subunit and is encoded on a single para gene in *D. melanogaster*. Alternative splicing of the *para* mRNA at 5 sites gives 48 splice variants and so different sodium channel isoforms with different functions and expression patterns of the channels (Thackeray and Ganetzky, 1994). The insect channel is only expressed well in Xenopus oocytes when co-expressed with tipE synthetic RNA of *D.malanogaster* (Soderlund, 2010; Warmke *et al*, 1997). TipE is an acidic membrane protein of 50kDa with 2 transmembrane regions (unlike the mammalian β-subunit which has only one) (Warmke *et al*, 1997) and is needed for good expression of the sodium channel (Feng *et al*, 1995). Although different in structure to the β-subunit in mammalian channels, TipE plays a similar role in modifying the channel kinetics, expression and stabilization (Lee *et al*, 2000; Zlotkin, 1999). Equivalents to TipE have been found in the housefly (Vsscβ) (Ingles, 1996) and TipE has been found to help expression of the sodium channel of *Blattella germanica* (Hall *et al*, 1999).

Since the discovery of the insect sodium channel, homologous channels from many insects have been isolated and expressed in oocytes. Housefly Vssc1(Soderlund and Knipple, 2003), cockcroach Para^{CSMA} (Tan *et al*, 2005) and cockroach BgNa$_V$1.1 (Bosmn and Tytgat, 2007). Others have been isolated but not yet functionally expressed: arachnid genes from the

Varroas mite (VmNa) (Wang *et al*, 2003), scorpion Buthus mortensii ($BmNa_V1$), spider Ornithoctonus huwena ($OhNa_V1$) (Zuo *et al*, 2006).

1.1.5 Activation and gating

1.1.5.1 Structure

The voltage gated sodium channel consists of 4 internally homologous domains (I-IV) connected by linkers. Each domain contains 6 membrane spanning regions (S1-S6). S5 and S6 with the pore loop make up the ion conducting pore (Yu and Catterall, 2003) and S1-S4 gives the channel its voltage sensitivity. The ion selectivity filter is at the extracellular side of the channel and formed from the P-loops between S5 and S6. This was found using the channel blockers tetrodotoxin and saxitoxin. In the VGSC, the selectivity filter is made up of the amino acid sequence: DEKA. Each of these amino acids is found at corresponding locations on each of the P-loops between S5 and S6 on each of the four domains. If DEKA is changed to EEEE, the selectivity of the channel is switched to calcium ions (Catterall, 2000). The intracellular pore is made up from the S6 segments and small extracellular loops that connect the transmembrane segments, with the largest connecting S5 or S6 with the P-loop. The domains are linked to each other via larger intracellular loops (Yu and Catterall, 2003). Cryo-electron microscopy and single particle image analysis was used by Sato *et al* (2001) to study the 3D structure of the channel from *Electrophorus electricus*. As can be seen in **Fig1-3** the channel is bell shaped and consists of several inner cavities which are connected to four small holes and eight orifices close to the cytoplasmic and extracellular surfaces of the

membrane when the channel is in its closed conformation. It is thought that when the channel activates, the central mass pushes out into the 4 cavities. The channel contains 2 gates: the activation gate known as the 'm gate' and the inactivation gate, known as the 'h gate' (Davies *et al*, 2007). When the membrane is depolarized a conformational change causes influx of Na^+ ions into the cell through the channel. This conformational change is brought about by movement of the S4 segments. This movement was detected via fluorescent labelling experiments (Banzanilla, 2000).

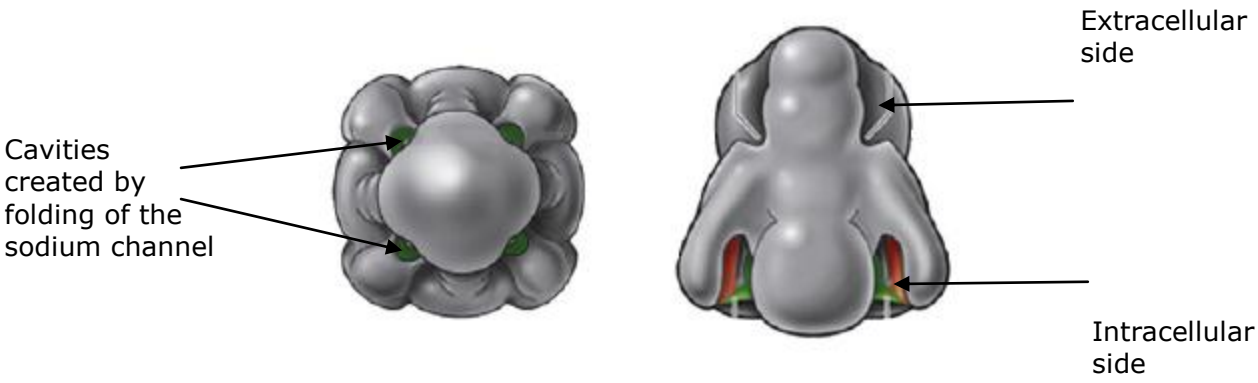

Fig1- 3. 3D image of the VGSC showing the cavities, indicated by the black arrows. The image on the left is from the extracellular side as though looking down on the channel and the image on the right shows the channel as it would sit in the membrane (Adapted from Catterall, 2010).

1.1.5.2 Gating

When the channel is resting, the pore is blocked by the 'm gate'. Depolarization causes movement of the S4 regions in domains I and II, the S6 segments (forming the pore) rotate laterally causing the channel to open for a few milliseconds. This is known as the 'sliding

helix' model (Catterall, 1986) or 'helical screw' model (Guy and Seetharamulu, 1986), this model is represented in **Fig1-4**.

S4 is helical with 3.6 residues per turn of the helix. The S4 regions are positively charged and in the resting state are stabilized by the positive charges forming bonds with negatively charged amino acids in adjacent S1, S2 and/or S3 transmembrane segments. In the resting state the S4 segments are drawn inwards due to the negatively charged internal resting membrane potential. Depolarization releases this electrostatic force as the internal potential becomes positive, and the S4 segments are able to move outwards in a spiral pathway where each positive residue slides onto the next negative one (Catterall, 2010). Mutating the positive charges of the S4 segments has been found to alter voltage sensing in the VGSC (Benzanilla, 2005).

The movement of S4 then causes movement of the linker between S4 and S5 which exerts a force on S6 segments causing the pore to open (Yarov-Yaravoy, 2006). However, pulling on the S4-S5 linker alone was not thought to be enough to cause opening of the gate, it is predicted that this is supported by disulphide cross-linking between the extracellular ends of S4 and S5 and by a highly conserved interaction between an extracellular site at S1 and S5 which help cause opening of the channel by acting as a fulcrum for the voltage sensor to pull on the S4-S5 linker. Activation is followed by fast inactivation via closing of the 'h gate' which happens in 1-2 milliseconds.

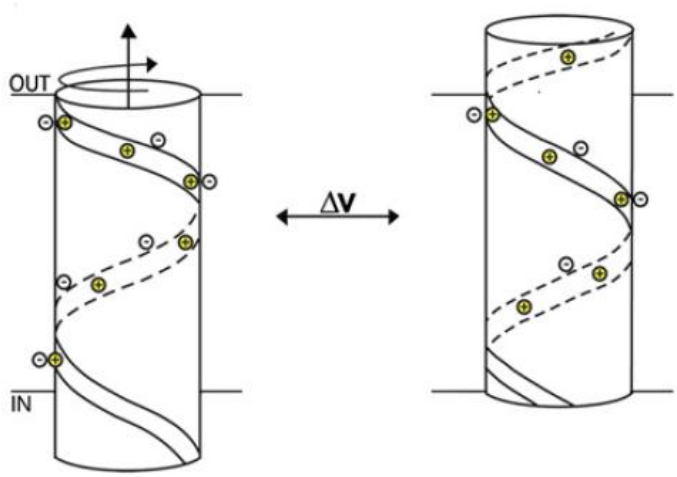

Fig1-4. The S4 segments are represented as cylinders where the positive charge resulting from arginine gating charges is represented by the ribbon. When the membrane is depolarized, S4 moves out in a spiral pathway and the positive charges slide onto the next negative charge in adjacent segments.(Catterall, 2010)

The 'h gate' consists of a hydrophobic triad of amino acids, which in the *para* sodium channel is MFM but in vertebrates is IFM. These amino acids are found on the intracellular loop between domains III and IV and latch the loop to a receptor site in the intracellular pore (Davies *et al*, 2007; Benzanilla, 2005). The IFM motif is flanked by glycine residues which are thought to form the hinge of the gate. It has been found that if the loop is cut then inactivation is noticeably slowed (Stuhmer *et al*, 1989). Inactivation is linked to activation. When the membrane becomes repolarised the m-gate returns to its starting position, closing the pore from the extracellular side, this is deactivation. This is followed by opening of the 'h gate' within 2-5 milliseconds and the channel is back at its resting state. Activation to deactivation does not have to follow this pattern, it can even bypass activation all together and go straight from the deactivated, resting state to closed state inactivation. The channel can also re-open without first inactivating.

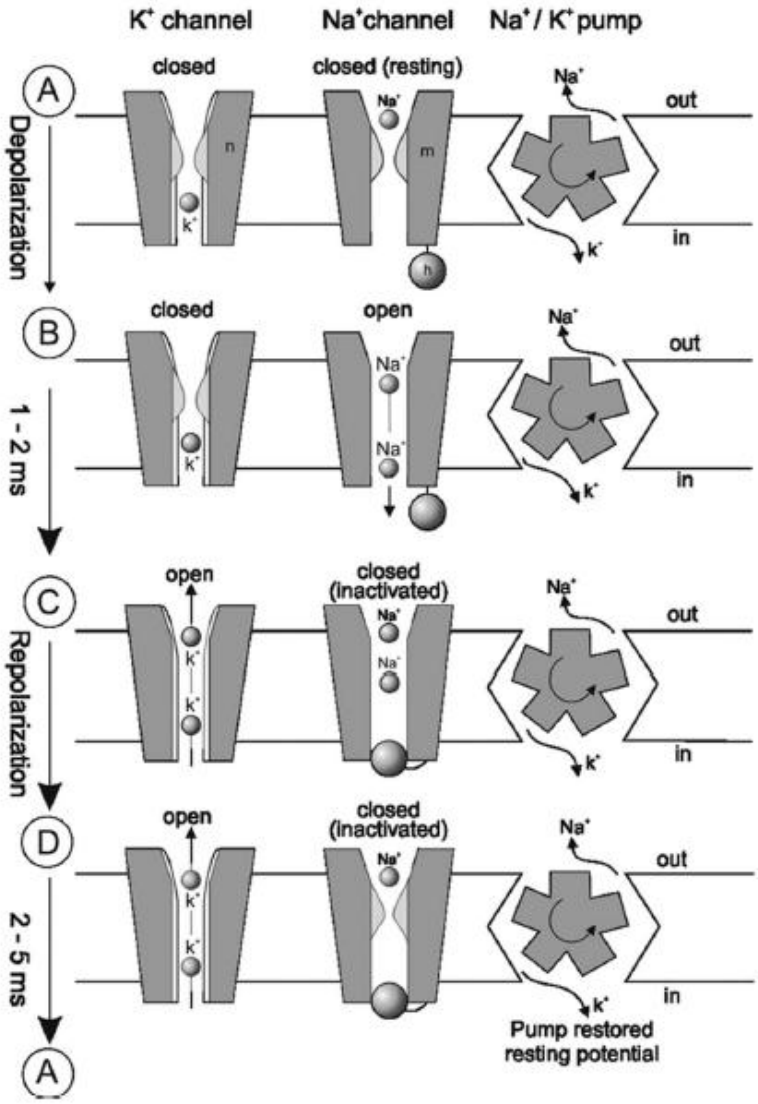

Fig1-5. (A) Shows the membrane in its resting state, the sodium-potassium pump maintain the resting potential of the membrane and the voltage gated sodium and potassium channels both remain closed. (B) represents depolarization, when the sodium channel opens allowing Na+ into the cell and so increasing the intracellular charge. After 1-2ms the potassium channel opens (C) allowing K+ out of the cell and the sodium channel becomes inactivated by blockage via the intracellular loop. This is repolarisation. The sodium channel m-gate then returns to its original position (D) and the h-gate will be removed. The NA+/K+ pump returns the membrane to its original resting potential. Adapted from Davies *et al* (2007).

1.2 Insecticides

1.2.1 History

Insecticides are natural or synthetic chemicals that have been used in some form for thousands of years to help protect plants from damage by some of the 10,000 insect species which feed on crops. There are many classes of insecticides with different target sites within the insect nervous system (Ware, 1999). The most notable targets are the ion channels that control the production of action potentials and inhibition of enzymes that degrade neurotransmitters. The focus of this study is on insecticides that target the insect voltage gated sodium channel VGSC, namely DDT and pyrethroids.

The VGSC is a large transmembrane protein which has many sites that can be targeted by neurotoxins (Catterall *et al*, 2007). Insecticides that target the VGSC can block the pore of the channel, so preventing flow of ions across a membrane (Indoxocarb) (Dong, 2007) or modify the gating kinetics (pyrethroids, DDT) (Narahashi, 1996; Soderlund, 2010). The more lipophilic a compound, the better it affects the target insect, as it can penetrate the nervous system more easily

1.2.2 DDT

DDT is an organochlorine, a compound very toxic to insects but not to mammals (Longnecker et al*et al*, 1997). DDT or 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (**Fig1-**

6) was found to have insecticidal activity in 1939. The first wide scale use of it reported was for the treatment of lice that transmitted typhus in Italy during World War II. A powder of 10% DDT was used to treat the people infested with lice and the epidemic was quickly under control (Davies *et al*, 2007). After the discovery of DDT, a large number of chlorinated hydrocarbon insecticides were developed: methoxychlor and hexachlorohexane or lindane for example. DDT can also be manufactured in huge quantities for relatively low cost and the insecticidal activity is retained for a long period. The most notable impact of the development of DDT was on malarial vectors from the 1950's (Turusov *et al*, 2002). Malaria was eradicated from Europe and deaths were dramatically reduced in Africa, India and other Far Eastern countries at the time. It was particularly effective because when sprayed onto a surface, the residual surface film was persistently active for a long period. DDT also helped control other diseases such as typhus and yellow fever that are transmitted by insect vectors (Zlotkin, 1999).

DDT is currently banned in most places across the world with a few exceptions for the control of disease vectors, such as malarial mosquitoes. The reason for this ban is the long persistence of DDT in the environment; the p,p'DDT isomer has an estimated half life of 7 years. Its high lipid solubility also allows it to accumulate in the fatty tissue of non-target organisms, and increase in concentration at higher trophic levels in the food chain, possibly causing long term chronic toxicity (Zlotkin, 1999). DDT has also been implicated as an oestrogen mimic (Longnecker *et al*, 1997).

In insects, DDT causes death by its action on the peripheral nervous system. It initially causes random firing of neurons which leads to visible muscle twitches, nicknamed the 'DDT jitters'. Initial depolarization of the membrane leads to an increased frequency of miniature

post-synaptic potentials due to spontaneous release of neurotransmitter. Eventually this leads to paralysis and death due to depletion of neurotransmitter at the neuromuscular junction (Davies *et al*, 2007).

1.2.3 Pyrethroids

Pyrethroids (**Fig1-6**) also target the voltage gated sodium channel of the nervous system and constitute about 17% of the world insecticide market (Davies *et al*, 2007). They are more effective against a wider range of pests than organochlorines, but do not accumulate in the environment like DDT (Vijverberg and Bercken, 1990). Pyrethroids are the synthetic analogues of compounds found in the flowers of *Chrysanthemum cinerariaefolium* (Soderlund and Bloomquist, 1989). The use of Chrysanthemum flowers for insecticidal purposes was first reported in ancient China where the dried flowers were used. By the middle ages, use of dried *C. roseum* flowers was reported in Persia, where the flowers were ground down into 'Persian dust' which was transported to Europe. Since the 19th Century there has been commercial production from the dried flowers, mainly pyrethrins I and II which are still used in household sprays today, but have low stability in light and air.

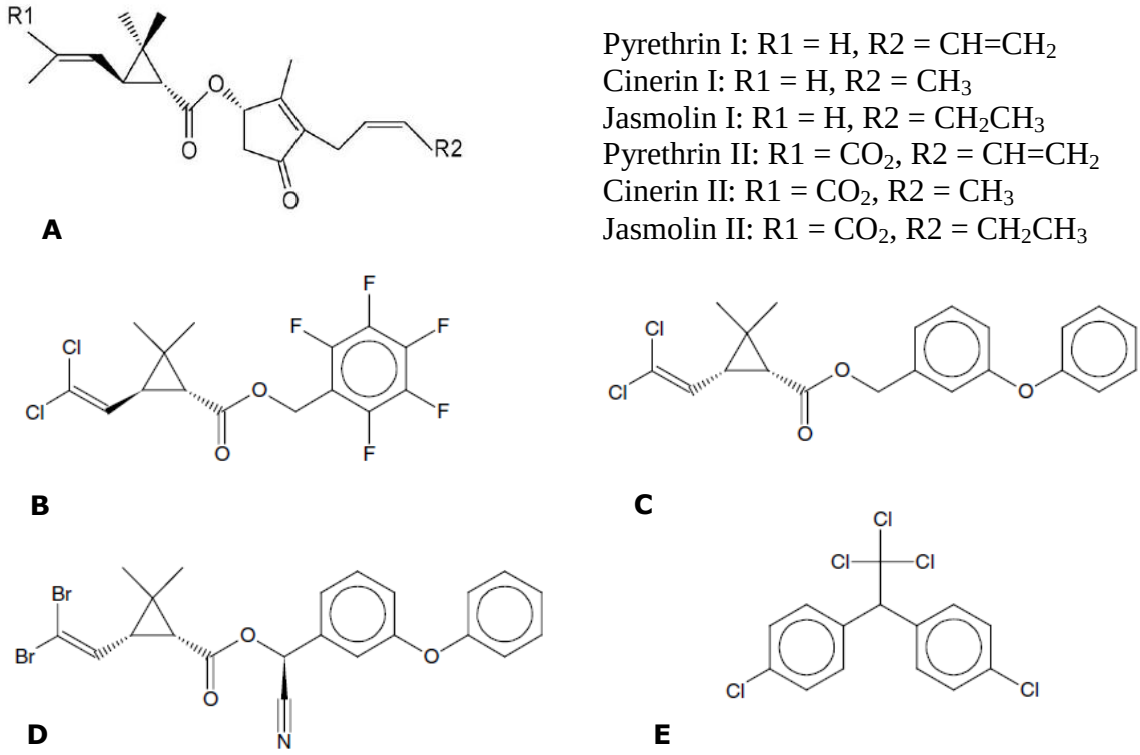

Fig1-6. **A** Natural pyrethrins **B** Fenfluthrin **C** Permethrin **D** Deltamethrin **E** DDT

The main insecticidal compounds found in the flowers are chrysanthemic acid (Pyrethrins I) and pyrethric acid (Pyrethrins II) from the *C. cinerafolis* flowers. The compounds shows three natural variations at the alcohol moiety: pyrethrin I, II, jasmolin I, II and cinerin I,II. Modern day pyrethroids are synthetic analogues of these (Elliott, 1980), for example allethrin is similar to the natural cinerin I. Between 1968 and 1974 more photostable compounds with higher insecticidal activity were developed. These had low mammalian toxicity and limited soil persistence (Davies *et al*, 2007), the main examples being: permethrin, cypermethrin and deltamethrin, which was the most active insecticide ever known at the time. An LD50 of 0.001µg/larva was found when applied to diamondback moth *Plutella xylostella* larvae (Schuler *et al*, 1998).

Pyrethroids affect the central and peripheral nervous systems. Nerve cells first produce repeated discharges, leading to paralysis and death. This occurs in a much shorter time than with DDT. DDT and pyrethroids both only need to affect a small number of total ion channels (~1%) for repetitive discharges to occur (Narahashi, 1996). Pyrethroids also prevent the channel from closing via inactivation or deactivation as they stabilise the channel in its open state (Davies *et al*, 2007; Bloomquist, 1996; Vijverberg and Bercken, 1990). When the pyrethroid molecule binds to the sodium channel it causes the channel to become hyperexcitable, as the activation threshold has been shifted so that it is attained more frequently. This state is relatively stable and causes the sublethal incapacitating effect known as 'knockdown'. After a while, this hyperexcitability overwhelms the cell as the sodium pump is unable to maintain the normal ionic gradients.

Pyrethroids are classified into Type I and Type II compounds. The first is typified by permethrin but also includes the natural pyrethrins (Zlotkin, 1999), and the second by deltamethrin. They have slightly different effects; type I induce the repetitive firing of axons which leads to restlessness, un-coordination and hyperactivity. This leads to prostration and paralysis which gives a good knockdown effect (Davies *et al*, 2007). Also, Type I causes the activation point at which half of the sodium channels are open to be more negative. This causes the channels to be open at their normal resting potential (Vais *et al*, 2001) Type II pyrethroids have an additional cyano group at the α-benzylic position (See **Fig1-6**.) which leads to a pronounced convulsive phase and better kill as the depolarization is irreversible and inactivation is delayed for longer (O'Reilly *et al*, 2006). Depolarization by permethrin lasts only 10-100 milliseconds, whereas with deltamethrin it can last up to a few seconds. During voltage clamping, the effect of pyrethroids on the sodium channel is typified by delayed

inactivation and deactivation, which is seen as a slowly decaying sodium tail current as shown in **Fig1-7** below (Soderlund, 2010).

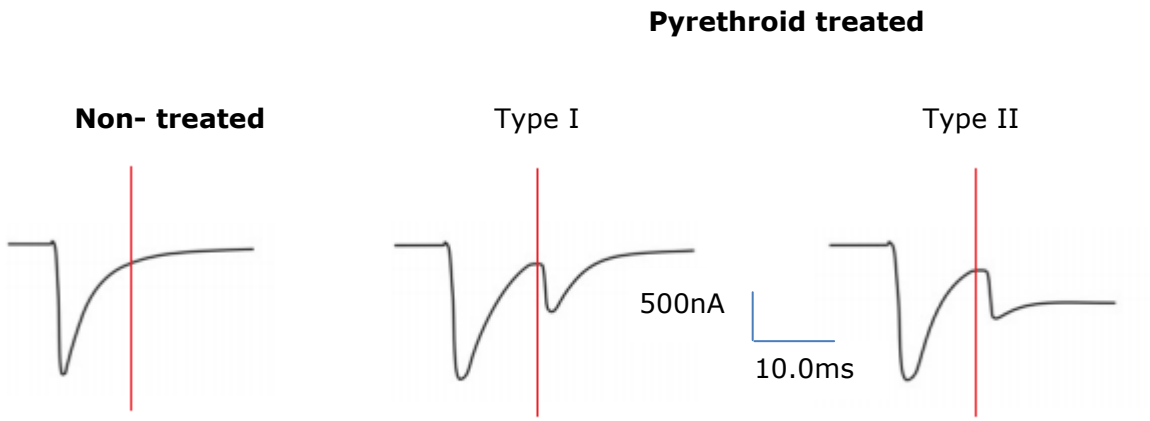

Fig1-7. Shows the tail currents of sodium channels in whole cell recordings of mammalian cells. The tail current is represented by the portion of the curve to the right of the red line. Cells not treated with a pyrethroid (far left) do not show a tail current, whereas those treated with pyrethroids (right) show a tail current. The difference between type I and type II pyrethroids can also be seen, where the type II treated cell's tail current does not decay as quickly. This is a representation of typical tail currents induced by pyrethroids and the scale bars are estimatesFigure adapted from Shafer *et al* (2005).

Pyrethroids typically have 2 or 3 chiral carbon atoms and their toxicity depends on the stereochemistry (Casida *et al*, 1983). In the chrysanthemic pyrethroids the 1R configuration of cyclopropane is needed for insecticidal activity. Both the cis and trans versions can be toxically active but this is difficult to predict as toxicity depends on the alcohol moiety. For example: the cis-3-phenoxybenzyl chrysanthemates are the more insecticidal version but for allethrin analogues the trans version is more toxic. Most pyrethroids are sold as racemic mixtures but deltamethrin is a single 1R cis, S alpha-cyano3-phenoxybenzyl stereospecific isomer (Davies *et al*, 2007). Although the 2D diagrams of pyrethroids appear to show them to

be quite different, when in their 3-dimensional conformation, the shapes are quite similar as are their physical properties. The acid moieties of some pyrethroids are even similar to DDT.

1.2.4 Binding sites

The binding sites of pyrethroids and DDT were determined first; by altering the structure of neurotoxins and studying the effects on their binding to the channel (Yamamoto, 1970). Then, more recently, by studying sodium channels with mutations that reduce or completely remove susceptibility to pyrethroids and/or DDT (O'Reilly *et al*, 2006).

Some pyrethroids bind preferentially to the channel in the open state but it is not mutually exclusive whether they modify the channel in the closed or open conformation (Soderlund, 2010). This was studied using expression in Xenopus oocytes. Smith (1998) expressed the housefly Vssc1/TipE sodium channel in oocytes and looked at the effect of cismethrin using voltage clamping. It was found that this compound did not have enhanced affinity for the open state channel. However, when this was repeated with cypermethrin increased numbers of depolarizing pulses were needed to reach the equivalent level of modification to the channel, it was therefore concluded that this compound binds preferentially to the open channel. A study with deltamethrin on the Para/TipE channel (Vais *et al*, 2001) found that it was more potent after the membrane was depolarized with many brief depolarisations rather than one single pulse. It was concluded that deltamethrin also preferentially binds to the open channel. This was supported by the use of *Anaemonia sulcata* toxin (ATX-II) which prevents fast inactivation and causes deltamethrin to produce a slowly developing current during a

single long depolarizing pulse. Permethrin was found to bind to both the open and closed conformations of the channel but had greater affinity for the open channel (Soderlund, 2010).

More detailed information about the binding of Pyrethroids and DDT was discovered through a homology model of the part of the sodium channel enclosed in the membrane of the housefly, which was created based on the rat potassium channel $K_V1.2$ crystal structure (O'Reilly *et al*, 2006). This model showed a binding site on the *para* sodium channel that potentially bound insecticides with structures like DDT and the pyrethroids. This binding area is the long narrow hydrophobic cavity lined by IIS4-S5 linker and the IIS5/IIIS6 helices and would be accessible to lipophilic insecticides. The model also showed that the binding site for the Type II pyrethroids was formed when the channel was open and activated. This supported observations that deltamethrin binds preferentially to the open channel and is temperature dependent. The channels remain open longer at lower temperature allowing more deltamethrin to bind; it is therefore more potent at lower temperatures, which is possibly one reason for its low mammalian toxicity. Pyrethroid insecticides bind in an extended conformation and stabilise the open state of the channel. Vais *et al* (2001) predicts that deltamethrin has a 1:1 ligand: receptor binding reaction. The exact binding of the pyrethroid is dependent on the structure, type I and II bind slightly differently. DDT occupies a restricted area of the designated binding cavity, hence its lower potency.

It is thought that T929 on the IIS5 helix is key to binding of pyrethroids as it has a high potential for hydrogen bonding with the ester linkage of pyrethroids (O'Reilly *et al*, 2006). This additional point of interaction between the type II pyrethroids and the channel distinguishes them from type I pyrethroids. M918 on the IIS4-S5 linker has a sulphur atom in its side chain which could interact with the second aromatic ring of the pyrethroid alcohol

moiety electrostatically. These interactions, along with Van der Waals forces with hydrophobic residues on the IIS4-S5 linker and IIS5 helix, and electrostatic attraction generated by aromatic residues on IIIS6 helix give strong binding.

The type II pyrethroids with the α-cyano group extend the hydrogen bond interactions with the ester linkage and tightly lock the middle portion of e.g. deltamethrin into the binding cavity. There may also be stronger interactions at the M918 locus due to the optimal position of the alcohol moiety and different distribution of the charge on the benzyl ring of the alcohol moiety. This could be because of the strong electron withdrawing character of the cyano group and/or reduced likelihood of the pyrethroid moving out of the binding pocket.

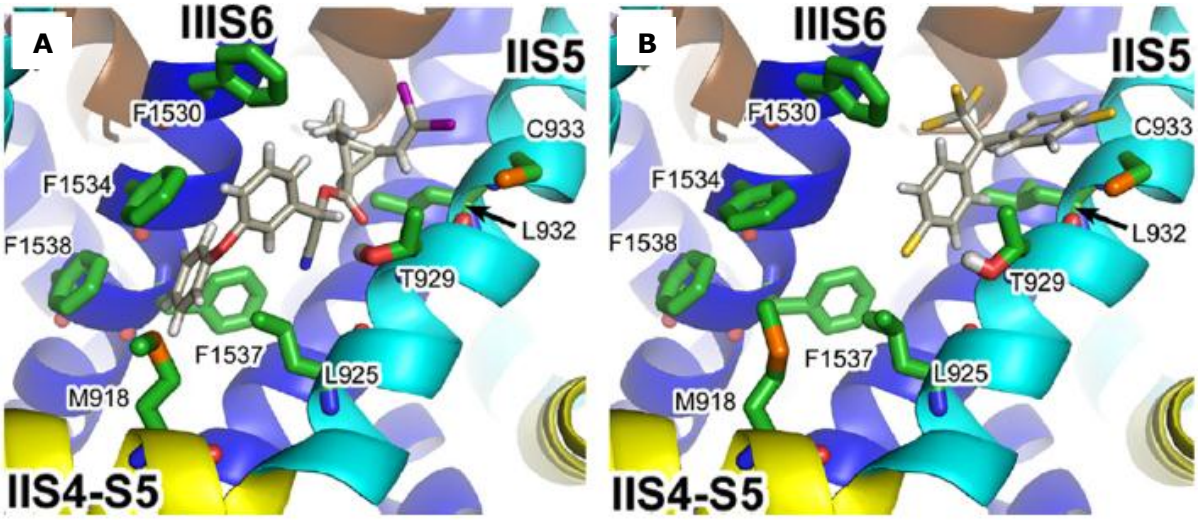

Fig1- 8. **A** Deltamethrin binding. **B** DDT binding (Usherwood *et al*, 2007).

The binding site of pyrethroids and DDT was further defined by a mutational study in the DIIS5 and the DIIS4-S5 linker of *D.melanogaster para* sodium channel (Usherwood *et al*, 2007). The pyrethroids: permethrin, deltamethrin and fenfluthrin were studied along with DDT. The substitutions used were those that are known to give resistance to pyrethroids and/ or DDT pesticides in certain pest species and substitutions that represent differences between

the *para* channel and that of other insects and the mammalian channel. The mutations on the IIS4-S5 linker were L914F, L914I and M918T. On the S5 segment the substitutions were L925I, N927I, T929I, L932F, C933A and I936V. It was found that all of these mutations decrease deltamethrin potency. M918T, L925I and T929I decreased permethrin potency. T929I, L925I and I936V decreased fenfluthrin potency. Interestingly, the M918T mutation had no affect on the binding of DDT yet T929I completely abolished this interaction (Usherwood *et al*, 2007). L925I, L932F and I936V also reduced DDT potency. This suggests that at least part of DDT's binding domain is on DIIS5. This data supports O'Reilly's model of binding. **Fig1-8** shows 3D images of the binding of deltamethrin and DDT in the VGSC.

When looking at specific mutations it was found that the M918T mutation reduced deltamethrin potency ~370-fold compared with only a ~20-fold decrease for permethrin. This suggests that deltamethrin binds significantly to the DIIS4-S5 linker where the 918 position is found (Usherwood *et al*, 2007). The α-cyano group on the deltamethrin molecule results in reduced flexibility in the positioning of the alcohol moiety compared with permethrin. This essentially means that the interaction with the M918 residue is maximised for deltamethrin. The L914I mutation also showed a significant decrease in susceptibility to deltamethrin. C933A greatly reduced deltamethrin potency by ~400 fold, possibly because it eliminates the potential interaction of deltamethrin with the nucleophilic thiolate anion of C933. These results are consistent with deltamethrin being more effective at immobilizing the gating charge of the channel due to strong interaction with M918, and so keeping the channel open for longer. M918 is found on the IIS4-S5 linker, which has an important role in gating of the channel. L925 is thought to have a role with either the second aromatic ring on the alcohol moiety of the pyrethroid or in influencing the position of the DIIS4-S5 linker during interaction with the alcohol moieties.

1.3 Insecticide Resistance

Insects persistently exposed to insecticides are under a selection pressure to develop resistance. Any insect with a gene alteration which gives a phenotype that reduces the potency of the insecticide will be at an advantage in the population, and so this mutation will spread if the insecticide is continually used. Over use and mis-management of pesticides intensifies this problem, therefore, the effective management of pesticide use is extremely important (Coustau *et al*, 2000). Correct management of pesticides also extends the period that they can be used for before a build up of resistance makes them ineffective.

Resistance can be said to be metabolic or target site (Hemingway and Ranson, 2000). Examples of metabolic resistance are: an over expression of detoxifying enzymes, gene amplification and transcriptional regulation. Target site resistance involves a change in structure of the target receptor/ion channel/enzyme. Coustau *et al* (2000) divided resistance into four mechanisms. The first of these is constitutive overproduction, for example, of toxin inactivating enzymes, transporters or drug target proteins. The second is constitutive underproduction of the drug target or drug activating enzyme. Third, an inducible change in gene regulation, and finally; the modification of the target or receptor. It was previously believed that the highest resistance was obtained through target site changes. More recently, very high levels of resistance to neonicotinoids has been found in *Myzus persicae* aphids through gene copy amplification and increased transcription of detoxifying enzymes (Puinean *et al*, 2010). Target site resistance is discussed next in the context of pyrethroid and DDT resistance.

1.3.1 Target site resistance to Pyrethroids and DDT

All of the mutations referred to are cited by the numbering they are given in the VGSC sequence of the housefly genome. Resistance to DDT and pyrethroids was first described in the housefly (*Musca domestica*) in 1951 (Busvine). These flies were described as being knockdown resistant (kdr) and showed resistance to the initial paralytic effects of DDT and pyrethroids (Soderlund, 2008). Resistance was through reduced neuronal sensitivity. Some of the houseflies were found to have an even higher level of resistance and were termed super-kdr. Kdr flies have similar levels of resistance to DDT and pyrethroids but the super-kdr flies had resistance that varied from ~ 4-fold to ~ 300-fold, with a particularly high resistance to potent pyrethroids such as deltamethrin. The resistance was known not to be metabolic, as synergists that inhibit esterases and monooxygenases involved in metabolic resistance had no effect on abolishing resistance.

The kdr and super-kdr phenotypes were linked with the housefly sodium channel (vssc1) gene using oligonucleotide primers based on parts of the para sodium channel to amplify a small section of the housefly sodium channel gene (Williamson *et al*, 1993a; 1993b). Probes were then used to isolate larger segments of the gene for use in genetic analysis (Soderlund, 2008). The link between the resistant phenotypes and the channel was then confirmed using discriminating dose bioassays and strain specific restriction fragment length polymorphisms markers within the housefly vssc1 gene (Williamson et al, 1994).

The original kdr mutation found in the housefly (Busvine, 1951) was the L1014F found in domain II, segment 6 of the voltage gated sodium channel. The kdr mutation gives a 10- fold reduction in target site sensitivity to permethrin when expressed in the para/TipE sodium

channel in Xenopus oocytes. This gene is found on chromosome 3 in the housefly genome and is a recessive allele (Soderlund and Knipple, 2003). L1014F gives cross resistance to pyrethroids, pyrethrins and DDT, but not other classes of insecticide. It is thought that this mutation was originally selected for in populations during the time when DDT was widely used before it was banned. As the mutation is recessive it can persist at low levels in the population and may be reselected for by use of modern pyrethroids.

A second mutation was also found in *Musca domestica* on the IIS4-S5 linker. This involves a M918T alteration and the phenotype was named super-kdr as it gave much higher levels of resistance to pyrethroids. In fly populations M918T was never found without L1014F. The link between the kdr and super-kdr mutation and the resistant phenotype was confirmed by expression of the mutant channel in Xenopus oocytes.

The L1014F and M918T mutations originally described in the housefly (Williamson *et al*, 1993b; Knipple *et al*, 1994; Liu and Pridgeon, 2002) have since been identified in many other pest species. The L1014F mutation has been found in many pest species, including the African malarial mosquitos (Martinez-Torres *et al*, 1998), the German cockroach, *Blattella germanica* (Dong and Scott, 1994; Dong, 1997) and the cat flea, *Ctenocephalides felis* (Bass *et al*, 2004). The L1014F along with M918T has been found in the horn fly, *Haematobia irritants* (Guerrero *et al*, 1997), the South American leaf miner, *Liriomyza huidobrensis* (Nordhus *et al*, 2006) and the Peach potato aphid, *Myzus persicae* (Eleftherianos *et al*, 2002).

Other mutations have also been identified in the S4/S5 linker and S5 and S6 helixes of domain II of the sodium channel. Many of these are referred to as a kdr or super-kdr mutation

depending on the level of resistance that they convey. Some corresponding mutations have also been found in domains I and III (See **Fig1-9** for all mutations).

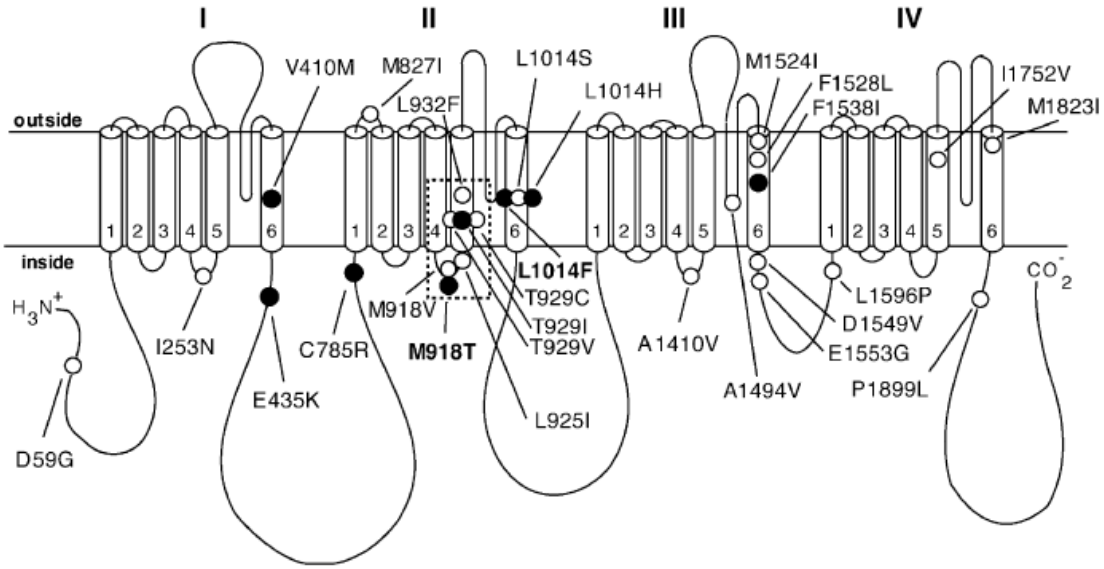

Fig1-9. Shows kdr and super-kdr mutations (Soderlund, 2008)

The M918T and L1014F mutations give a much higher level of resistance when expressed together than either expressed individually, as was demonstrated in *Xenopus laevis* oocytes (Soderlund, 2008). M918T alone was also found to have no effect on resistance to DDT. Mutations in the DII S4-S5 linker are associated with higher levels of resistance. The mutations that give a higher level of resistance, particularly to pyrethroids are named super-kdr and include mutations at the positions on domain II: M918 on the S4-S5 linker, T929 and L925 on S5 (O'Reilly *et al*, 2006) and F15381I on the IIS6 helix (Davies *et al*, 2007). In *Leptinotarsa decemilineata* and *Bemisia tabaci* (Whitefly) the DNA of the sodium channel was sequenced and variants in these regions (M918V and L925I) were linked to resistant phenotypes that gave >100-fold resistance to fenpropathrin (Lee *et al*, 2000; Morin *et al*, 2002).

The kdr mutations on Domain II are at L1014 and L932. Vais *et al* (2001) found T929I mutation in combination with L1014F reduced sensitivity to deltamethrin 10,000-fold compared to the wild type channel. Corresponding mutations on the S4-S5 linker and S5 and S6 segments in domains I and III include those at positions V410 and F1538 which convey insensitivity to pyrethroids at a similar level to kdr mutations.

Not all resistance mutations physically interact with the insecticide molecule, some may indirectly alter the structural design of the binding site by changing the conformational flexibility of the channel protein (Soderlund, 2008). L1014F gives resistance to both pyrethroids and DDT, it was therefore thought that this residue was not located at the site of binding of the insecticides, but instead in a region that conveys the conformational changes of insecticide binding to the gating kinetics (Davies *et al*, 2007). The L1014F mutation has been shown to shift the voltage dependence of gating to a more positive potential by approximately 5mV in the Drosophila recombinant sodium channel (Vais *et al*, 2001). It also promotes closed state inactivation, meaning 70-80% of channels never open (Davies *et al*, 2007). As pyrethroids bind preferentially to the open state of the channel, the reduction in the number of open channels limits the number of high affinity binding sites available (O'Reilly *et al*, 2006). L1014F is close to a proposed gating hinge position and could therefore impede the bending of S6 needed for channel opening. This is consistent with evidence that this mutation shifts the equilibrium to a closed state of the channel. Another explanation could be that the altered residue modifies packing of S6 and S5, so the amino acids which bind the insecticide on these segments of domain II are displaced (O'Reilly *et al*, 2006). In oocytes this mutation caused a ~20-fold reduction in sensitivity to deltamethrin.

Type II pyrethroids may be more easily displaced by mutations that produce small changes in the shape of the binding cavity due to the second aromatic ring of these pyrethroids giving such a close fit to the binding site in the cavity (O'Reilly *et al*, 2006). The super-kdr mutations give the highest resistance to Type II pyrethroids and are therefore sensitive to the structure of the binding molecule. It was deduced that they must be located at the actual binding site of the insecticide. The super-kdr M918T and T929I mutations alone had no effect on voltage dependence in the Drosophila channel but increased the rate of onset and recovery from inactivation especially around the threshold for activation. Is thought that they convey resistance via elimination of polar interactions with the pyrethroid molecule (O'Reilly *et al*, 2006), so, if the insecticide cannot bind at the M918 and T929 positions it cannot lock into the channel's open confirmation where it wedges the IIS5 and IIIS6 segments open. M918T has not been found on its own in any insect, it is probable that its accompanying mutation (usually L1014F) counteracts a functional deficit (Soderlund, 2008).

It is generally thought that the kdr and super-kdr mutations are recessive, and therefore both copies are needed for the resistant phenotype (Soderlund and Knipple, 2003). However, there is evidence that heterozygous individuals of certain species exhibit some levels of resistance. Eleftherianos *et al* (2008) found that heterozygotes for the super-kdr mutation M918T showed increased levels of resistance compared to homozygous wild-type individuals. This work was done on *M. persicae*. Clones that were heterozygous for both L1014F and M918T were shown to have a higher resistance to pyrethroids than homozygotes for L1014F. This appears to show that only one copy of the M918T mutation was needed to convey some increased resistance above that of the kdr level. All individuals homozygous for L1014F and M918T showed strong pyrethroids resistance.

If only one copy of the M918T resistance allele is needed to convey increased resistance to pyrethroids, then this could also be the case for other super-kdr mutations such as the T929I.

1.3.2 Detection of resistance alleles

The detection of resistance in insect pest species is important to reduce the ineffective use of pesticides which wastes time, money and causes damage to other wildlife (Anstead *et al*, 2004). Traditionally, pests carrying the kdr and super-kdr phenotypes were identified using *in vivo* bioassays on live insects or through DNA sequencing, processes that take days to get results. Modern PCR based allelic discrimination assays use fluorescent dye labelled probes to detect mutant alleles that give rise to the resistant phenotypes, these give much faster and more reliable results (Ginzinger, 2002; Livak, 1994). A similar system using quantitative assays was developed for detection of resistance in strobilurin resistant fungi (Fraaije *et al*, 2002). Fluorescent PCR can be used on any life stage of the pest from eggs to dead individuals. Anstead *et al* (2004) used a fluorescent taqman bioassay to look for the M918T mutation in the peach potato aphid *M. persicae* (Anstead *et al*, 2008). This method had previously been used to determine kdr and super-kdr in *M. persicae* by Guillemaud *et al* (2003).

Over the past few years the fluorescent TaqMan diagnostic assay has been developed for the use of distinguishing between mosquito species and detecting resistance to insecticides in those that vector the malarial parasite (Bass *et al*, 2007a; 2007b; 2008a; 2008b; 2010). These studies have also compared the TaqMan assay to other diagnostic techniques, proving it to be the most sensitive assay with the greatest specificity. One such study compared a TaqMan

assay and a high resolution melt assay (HRM) which uses Eva green dye with 4 older techniques for kdr detection. The newer techniques were found to be more reliable, with the TaqMan technique the most sensitive and specific, as HRM had a higher rate of failure and incorrect scores (Bass *et al*, 2007a). Bass *et al* (2010) developed a higher throughput real time PCR TaqMan diagnostic assay and compared this and the HRM assay with PCR-RFLP. The new TaqMan technique was found to have the greatest specificity and sensitivity again, and was cheaper than the traditional PCR method.

The TaqMan and HRM techniques are known as 'closed tube' as they require only one contained step. TaqMan has a higher throughput and is less prone to failure of amplification than standard PCR (Bass *et al*, 2007b). Real time PCR was developed in the 1990's and is significantly more reliable than traditional PCR as the whole amplification profile is known. It is also faster and easier to perform with no post-PCR processing necessary (Wilhelm and Pingoud, 2003). The 'closed tube' also means there is a lower risk of contamination.

In the TaqMan diagnostic assay the 5' nuclease activity of Taq polymerase is used to cleave a probe during the extension phase of PCR, a dual labelled fluoreogenic hybridization probe is used (Heid *et al*, 1996). The TaqMan probe is an oligonucleotide, 5'-terminally labelled with a reporter fluorophor like fluorescein and labelled internally or 3'-terminally with a quencher (Wilhelm & Pingoud, 2003). These are included in the mix with forward and reverse primers that amplify a specific area of DNA. The use of different reporter dyes means that in a single PCR, cleavage of different allele specific probes can be detected (Livak, 1999). During the extension phase of PCR, the probe which is complimentary to the amplicon sequence, binds to the single stranded PCR product. The Taq DNA polymerase, when it reaches the probe, sheers and endonucleolytically cuts the probe. This releases the quencher and the fluorophor

can now fluoresce (Wilhelm & Pingoud, 2003). The probes are only cleaved if they have hybridised to a specific target sequence. The close proximity of the quencher in uncleaved probes means the fluorescence signal is reduced.

The taqman assay for the presence of the T929I mutations contains two probes: one probe is complementary for the wild type allele and the other for the mutant allele. The mutant probe is labelled with the dye FAM and the wild-type with VIC, along with the quenchers (**Fig1-10**). If the wild-type allele is present, the quencher will be removed from VIC and result in fluorescence. If it is the mutant allele that is present then the FAM will fluoresce. If a heterozygote is present then there will be an increase in fluorescence by both dyes.

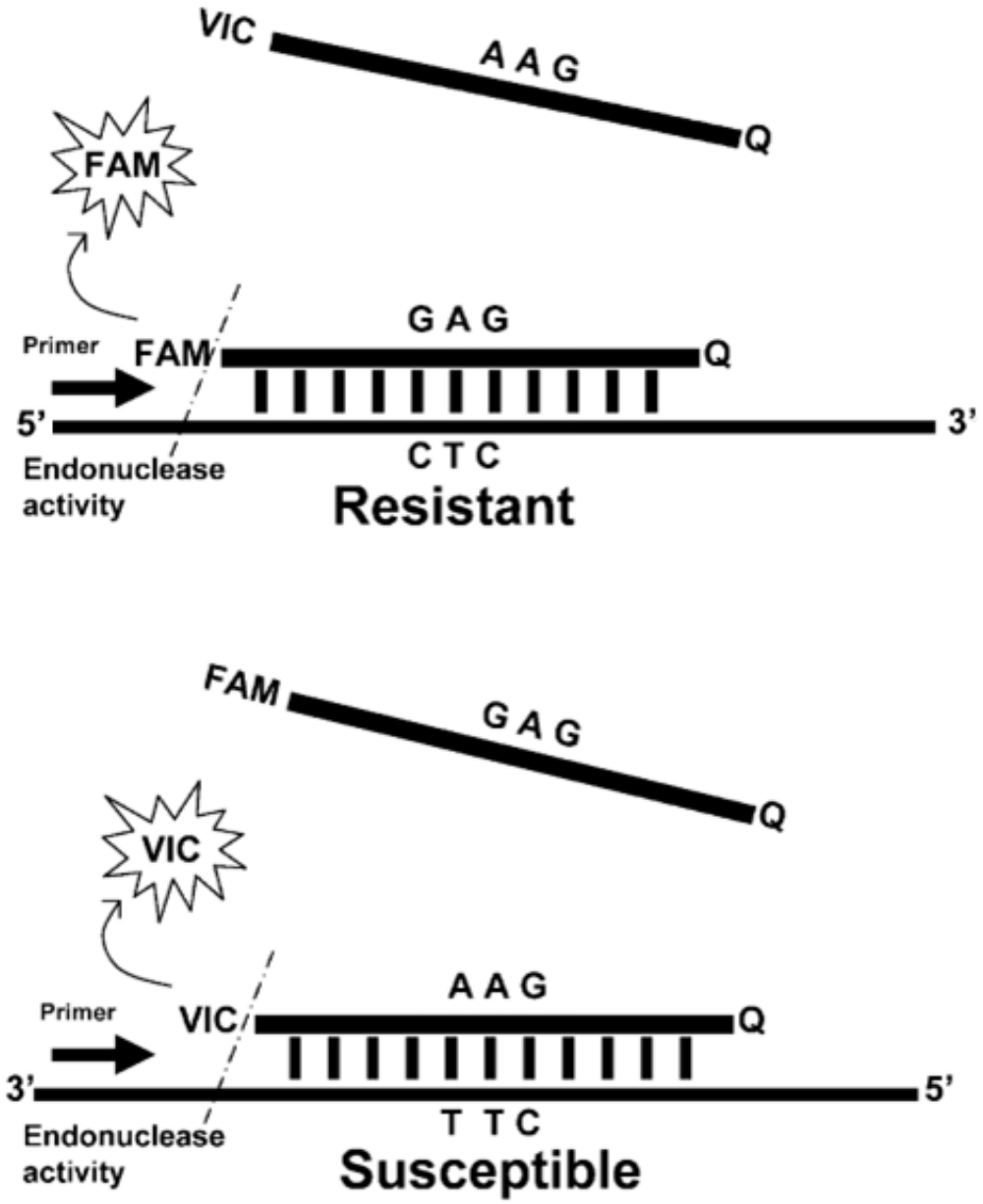

Fig1-10. During amplification each probe binds specifically to its complementary PCR product, which has been amplified by a specific set of primers. DNA polymerase then cleaves the reporter dye so it becomes separated from the quencher. This cleavage occurs every cycle of the PCR so the increase in fluorescence is proportional to the amount of PCR product. Adapted from Anstead *et al* (2004).

1.3.3 Expression of sodium channels in *Xenopus laevis* oocytes and Electrophysiology

The *para* VGSC can be expressed in oocytes from *Xenopus Laevis*, the South African clawed frog (Dumont, 1972), as shown in **Fig1-11**. The reproductive cycle of this species can be initiated at anytime of the year using hormones. This means that the *Xenopus* oocytes are available all year round. The advantages of using the *Xenopus* oocyte expression system are: that it is more efficient than a cell free system and can translate mRNA with high efficiency and for long periods (Gurdon *et al*, 1971). There is also little specificity for the species which the mRNA comes from. The oocytes are large making them easy to study and are relatively easily available. When mRNA is injected it associates with polyribosomes attached to endoplasmic reticulum in the cytoplasm and is translated in the same way as the oocytes own mRNA. The oocyte is also able to carry out post translational modifications such as glycosylation and assembly of the channel subunits. The channel is then transported to, and expressed in the cell membrane (Lane *et al*, 1979).

Fig1-11. Showing the *Xenopus laevis* African clawed frog and oocytes (Svenningsen *et al*, 2009)

The insect voltage gated sodium channel generally used for expression studies in oocytes is from the *para* gene of *D. melanogaster*. This is because the Drosophila *para* gene has been

cloned and the entire cDNA sequence determined (Loughney *et al*, 1989). The Drosophila *para* sodium channel shares ~ 50% homology with the α- subunit of vertebrate sodium channels. Alone, the *para* mRNA gives poor expression of sodium channels in the oocytes (Warmke *et al*, 1997; Feng *et al*, 1995). When injected in combination with mRNA coding for another protein called TipE, robust sodium currents can be elicited.

1.3.3.1 Two electrode voltage clamp technique

The membrane potential of oocytes can be measured with a voltage clamping technique that uses to microelectrodes to measure the potential and inject current (Anderson and Stevens, 1973). The idea is to fix the membrane potential at a certain value and measure the current to maintain that potential. The voltage clamping technique has been developed since the 1940's and one of the first descriptions comes from Hodgkin *et al* (1952). Patch clamping and whole cell techniques have been developed more recently (Neher and Sakmann, 1992; Hamill *et al*, 1981).

Two electrode voltage clamping (TEVC) is used to measure the current across the membranes of the *Xenopus* oocytes. Voltage clamping refers to the voltage across the membrane being fixed (Revest and Longstaff, 1998). The fixed voltage is called the command voltage (Vc) and is fed into the positive input of a voltage clamp amplifier. Two microelectrodes impale the oocyte. One of them measures the voltage across the membrane (Vm) and the other injects a current. The Vm is compared to the Vc, and if there is a difference between them, for example when ion channels open, a current that is equal and opposite to the potential produced is injected into the oocyte. In effect, it is a negative

feedback loop. The aim is to keep Vm equal to Vc. When an ion channel opens the membrane potential changes and so the injected current changes to counteract this. This method can be used to test for successful expression of ion channels in the oocytes. A depolarizing pulse is used to activate the voltage gated sodium channels and a current will be visible if the oocyte has expressed the sodium channels. When pyrethroids are applied they should interact with the open state of the channel, preventing it from inactivating and deactivating and so producing a tail current. This illustrates that the channel expressed is susceptible to pyrethroids.

1.4 The Maize weevil

The maize weevil *Sitophilus zeamais* (Coleoptera: Curcilionidae) is a pest of stored crop products, primarily maize (*Zea mays*) (**Fig1-12**). It is closely related to two other weevil pest species; *S. granaries* (Grain weevil) and *S. oryzae* (Rice weevil). Until the 60's it was thought that *S. zeamais* and *S. oryzae* were the same species as they are externally identical, however, they show behavioural differences and can be distinguished on dissection and examination of the male genitals. *S.zeamais* can breed in a wider range of moisture contents than either the grain or rice weevil and is therefore able to infect grain in the field before storage and can also become a pest of some fruits (Rees, 2003; Longstaff, 1981). Maize weevils are attracted to the maize crop by the odours produced (Walgenbach *et al* 1987) and infection is facilitated by the ability to fly; the wings are under the elytra.

Fig1-12. *Sitophilus zeamais* on the left and infected maize on the right.

Sitophilus zeamais inhabit warm, humid areas of the world and are particularly common where maize is grown. This species causes damage to the grain by feeding activities, the development of immature larvae inside the maize kernels and by the production of frass. Frass is a mixture of grain dust, weevil faeces and exuviae (Longstaff, 1981). **Fig1-12** shows maize infected with the maize weevil. The weevils can also cause damage indirectly through the production of heat. It has been estimated that 15% of the total weight of the stored product is lost due to infection by the maize weevil (Santos *et al*, 1986). The 9th international working conference on stored product protection reported that in the state of Minas Gerais in Brazil, 36.4% of maize stored on the cob in the period May to November, suffered damage due to insect pests. In other states a similar level of damage was found, in Espirito Santo 36%, Sao Paulo 36.2%, Rio Grande do Sul 36.2%. Distribution of this pest has been found all over the world, from USA, Canada and South America to Europe, Asia, Africa and Australia (Rees, 2003).

1.4.1 Life cycle

The adults of the Sitophlius species range from 2.5-4mm in length. The adults of *S. zeamais* can live from 3 to 6 months or even longer in cooler temperatures and a female can lay up to 150 eggs in her lifetime (Rees, 2003). *Sitophilus zeamais* that are living in stored maize will move to the surface of the maize when they are ready to breed in order to attract mates (Longstaff, 1981). The females release sex pheromones to attract the males (Mason, 2003). Once fertilised the female will excavate a hole in a maize kernel and deposit an egg. Occasionally more than one egg may be placed in the same kernel, although it is unlikely that both will develop to adulthood due to cannibalism. The female then secretes a mucilaginous plug which, when it dries and leaves a raised spot, is the only evidence that the maize is infected. Some grains initially excavated for oviposition become feeding holes instead (Campbell, 2002).

Inside the grain the progeny go through 4 larval instar stages (Longstaff, 1981). During the first, the larva burrows through the tissue of the grain. At the end of the 4^{th} stage, the larva seals the end of the burrow using a mixture of frass and larval secretions. It then assumes a prepupal form before becoming a pupa inside the pupal cell which it has created. Before formation of the pupal cell, if the carbon dioxide exceeds 5% then the larvae will create a hole in the grain and push out most of the frass. Once the pupa has developed into an adult weevil it will remain inside the grain for a period of time whilst its cuticle hardens and matures. The length of this period varies with temperature.

The time taken for development of an adult weevil from time of oviposition varies due to humidity and temperature. Immature development time has been found to vary from 36.2

days at 27˚C (Sharifi and Mills, 1971) to 40.3 days at 25˚C (Howe, 1952). Optimal conditions of 30˚C and 75%RH showed maximum egg production at 6.7 eggs per female in 24 hours. The duration of development and number of progeny were also optimal in these conditions with immature development taking <30 days. Lower and upper temperature limits for egg development were found to be 15.6˚C and 32.5˚C respectively (Longstaff, 1981). It was found (Okelana and Osuji, 1985) that below 70% relative humidity oviposition was reduced. At 90%RH the highest number of eggs were laid but weevils were unable to develop due to growth of bacteria and fungi. Khare and Agrawal (1963) found that maximum productivity was achieved at 30˚C and 75%RH.

1.4.2. Maize weevil control

Initially, DDT was the most common chemical for the control of weevil infestations (Gallo *et al*, 1978), which was popular until 1985, when it was banned. Organophosphates then became popular, particularly malathion (Guedes *et al*, 1995). However, resistance suddenly appeared all over Brazil and its use was discontinued (Santos *et al*, 1986; Guedes *et al* 1995). Pirimiphos-methyl, deltamethrin and permethrin then became the most common chemicals for control, but resistance began to emerge (Guedes *et al* 1995). The most recent insecticide used for the control of *Sitophilus zeamais* is a mixture of organosphosphate and pyrethroid insecticides (Corréa *et al*, 2010b). At present, the popular mix employed is that of Fenitrothrin and esfenvalerate (Braga *et al*, 1991). New studies (Chaisaeng *et al*, 2010) are looking into the use of the parasitoid *Anisopteromalus calandrae* for control of the maize weevil. However, on a large scale this may be impractical.

1.4.3. Insecticide resistance in maize weevils

Resistance to insecticides was first detected in *Sitophilus zeamais* by Champ and Dyte in 1978 to organophosphates. In 1995 resistance to DDT and pyrethroids was reported by Guedes *et al* (1995) in Brazilian populations of the maize weevil. However, this resistance appears to be limited to only a few grain storage units in Brazil (Fragoso *et al*, 2003) and there is no special pattern evident. It is therefore likely that the spread of resistance is due to the grain trade within Brazil and local selection through the use of pesticides. The resistance to pesticides is commonly associated with their overuse (Perez-Mendoza, 1999), pesticides have been heavily relied upon to control the maize weevil in Brazil. This has lead to environmental concerns (Ribeiro *et al*, 2003).

A common resistance mechanism to pyrethroids and DDT is a change at the target site of the pesticide, in this case the voltage gated sodium channels. Secondary resistance is thought to be due to detoxification by Glutathione S-transferases and phosphotriesterases (Ribeiro *et al*, 2003; Fragoso *et al*, 2003; 2007). The target site mutation that conveys resistance has recently been found to be a super-kdr T929I change in S5 of Domain II in the insect sodium channel, this was found in two lab populations originally collected from Jacarezinho county and Juiz de Fora in Brazil (Araujo *et al*, 2011). Although this target site resistance causes insensitivity to pyrethroids in these populations of maize weevil, other mechanisms or factors may be involved in different populations. Other possible factors in the resistance to pyrethroids in some populations of *S.zeamais* are: increased levels of proteinases (Araujo *et al*, 2008) and also behavioural resistance. Feeding plasticity describes the avoidance of maize sprayed with deltamethrin by known resistant maize weevils. The behaviour was found to be significantly different to that of non resistant*S.zeamais* (Guedes *et al*, 2009). The maize

weevil has also been found to show hormesis under low concentrations of deltamethrin. Hormesis is seen when a low dose of a toxin that is inhibitory at higher doses is shown to be stimulatory (Guedes *et al*, 2010). Under low exposure to deltamethrin, females increase their reproductive output at the cost of a reduced lifespan.

The T929I mutation was found by first sequencing the DIIS4-S6 of the *para* sodium channel gene obtained from the maize weevil cDNA. Then primers were designed for the amplification of the same region from the genomic DNA. The T929I change was found on DIIS5 in the transmembrane segment of the sodium channel, in which no accompanying L1014F mutation or other kdr mutation was found in combination (Araujo, 2010). The T929I mutation has been reported to be found on its own in one other insect species; the onion Thrip *Tabaci lindeman* by Toda and Morishita (2009).

When the single mutation T929I was studied in the *para* VGSC of *D.melanogaster* in oocytes, it was found that for deltamethrin this mutation reduced the potency ~100-fold compared to the wild type channel. DDT potency was reduced 10,000-fold in the T929I mutant (Usherwood *et al*, 2007).

1.4.4 The T929 site

The T929I mutation has been found in combination with L1014F in the diamondback moth, *Plutella xylostella* (Schuler *et al*, 1998), and in combination with M827I and L932F in the headlouse, *Pediculus humanus capitis* Deg (Lee *et al*, 2000). Also at the 929 location is the T929C mutation, found in combination with the L1014F in the Western Flower Thrip,

Frankliniella occidentalis (Forcioli *et al*, 2002). T929V with the mutation L1014F has been found in the cat flea, *Ctenocephalides felis* Bche, on its own in the cottontail strain (Bass *et al*, 2004) and on its own or with L925I in the Tobacco whitefly, *Bemisia tabaci.* Here the T929V mutation in combination with L925I gave a resistance ratio of 1900 to Fenopropathrin compared with only 250 for L925I alone (Alon *et al*, 2006). Insect species showing resistance conveying mutations at the T929 site with or without accompanying mutations are shown in the **Table1-1** below.

Table 1-1

Amino acid substitutions at the T929 site and their accompanying mutations

T929 site and accompanying mutations	Common name	Species	Reference
T929I + L1014F	Diamnondback moth	*Plutella xylostella*	Schuler *et al*, 1998
T929I + M827I + L932F	Headlouse	*Pediculus humanus capitis* Deg	Lee *et al*, 2000
T929C + L1014F	Western Flow Thrip	*Frankliniella occidentalis*	Forcioli *et al*, 2002
T929V + L1014F T929V alone	Cat flea Cottontail strain	*Ctenocephalides felis* Bche	Bass *et al*, 2004
T929V +/- l929I	Whitefly	*Bemisia tabaci*	Alon *et al*, 2006

As mentioned above, the T929V mutation with L925I and no accompanying kdr mutation have been found in the whitefly *Bemisia tabaci* (Roditakis *et al*, 2006). Therefore, insects with these super-kdr mutations are viable on their own, as supported by Bass *et al* (2004). In these whitefly populations, resistance was found to persist without selection, suggesting a low fitness cost under laboratory conditions. This is supported by Alon *et al* (2006) who found

that *B.tabaci* populations with both T929V and L925I and also wild-type insects did not deviate from Hardy Weinberg equilibrium. This equilibrium states that the allele and genotype frequencies of a population remain constant. If a resistance cost was present the allele frequencies would be expected to favour the wild type genotype. The T929V mutation in the Drosophila *para* sodium channel expressed in oocytes reduced the deltamethrin sensitivity by 2600-fold (Atkinson, 2002).

Usherwood *et al* (2007) studied the T929I mutation alone *in vitro* in the sodium channel of *D. melanogaster* expressed in Xenopus oocytes. They found that this mutation alone reduced the potency of deltamethrin ~ 100 fold and completely eliminated DDT potency. When they applied 100µm DDT to an oocyte carrying the T929V mutation, they also failed to elicit a tail current. Vais *et al* (2001) found the T929I mutation in combination with L1014F reduced the channel sensitivity to deltamethrin by over 10,000-fold. When comparing the T929I channel with the wild type channel there was no difference in half activation or half inactivation that could suggest a fitness cost.

1.4.5 Fitness cost of resistance

It was originally assumed that the resistance to pesticides would carry a cost to the maize weevil (Coustau *et al*, 2000). However a population from Jacarezinho (JA) county in Brazil have been maintained in the lab with no selection, and have been found to maintain their resistance mutation at the T929 site without selection suggesting the resistance mutation is fixed (Fragoso *et al*, 2005, Oliveira *et al*, 2005). Another population from Juiz de Fora (JF) lose their mutation when not selected with insecticide due to a fitness cost (Araujo *et al*,

2008). Through research on these two populations, it is now thought that the Jacarezinho population has overcome the fitness cost of resistance by having a higher body mass with higher energy reserves (Guedes *et al*, 2006). The higher number of fat cells (Trophocytes) allows the weevils to be resistant as well as maintaining development and reproduction. Araujo *et al* (2008) found that the resistance without fitness cost is associated with a greater accumulation of total proteins and carbohydrates. It was also found that the activity of the proteinases: serine, cysteine and cellulase was lower in the susceptible populations, but it was found that serine proteinases were most active in the resistant cost strain. This suggested that the cysteine and cellulase activities were most important in mitigating the cost of resistance. Lopes *et al* (2010) also suggests enhanced α-amylase activity as playing a role in removing the cost of resistance traits in this species. Further, Guedes *et al* (2010) found that lower concentrations of pyrethroids can cause hormesis (wherein low concentrations of a toxin show beneficial effects) in weevil populations, thus posing more problems for the appropriate control of this species.

In Brazil there has been a low spread of resistance in the maize weevils, thought to be due to a fitness cost of the resistance in many populations (Guedes *et al*, 2006). However, the JA population carries no fitness cost, and if the T929I mutation gives resistance as a heterozygote, the existence of this mutation even at low levels in the population may be a problem. Therefore, it is important to be able to detect this mode of resistance in the field.

1.4.6 Bioassays

The T929I mutation in the VGSC of *S.zeamais* has previously only been studied closely *in vitro*. The *in vivo* study of this single mutation in live insects has not been very extensive. There are different methods to carry out insecticide bioassays. One method involves applying the insecticide indirectly by coating the inside of vials with the insecticide solution at different concentrations, and exposing insects for a length of time in the vials, then assessing the number of dead individuals (Ribeiro *et al*, 2003; Correa *et al*, 2010). A similar method involves impregnating filter paper with the insecticide and leaving the insects on it before assessing mortality (Guedes *et al*, 1995). Another method is topical bioassays, which allows the study of exact concentrations of insecticide on individual insects. Drops of insecticide are applied to either the larval forms of the pest or the adult; mortality is assessed after a number of hours (Schuler *et al*, 1998). Although this method is more time consuming, the exact concentration of insecticide that the insect is exposed to is known.

1.5 Aims and Objectives

The three main aims of this study are:

1. To select a discriminating concentration of DDT/deltamethrin that could be used for resistance selection in populations of *Sitophilus zeamais by:*

- Performing bioassays on populations of the maize weevil with known resistant and susceptible phenotypes.
- Attempt to re-select lab-maintained field collected strains of resistant *S. zeamais*

2. To establish the potential use of the TaqMan diagnostic assay for testing for the T929I super-kdr mutation in maize weevils in the field by:

- Running the test on field samples of *Sitophilus zeamais* that have been previously selected for resistance using the discriminating concentration of insecticide.

3. To extend studies on the resistance conveyed by changes at the T929 site of the VGSC.

- The wildtype and mutant forms of the *para* VGSC will be expressed in *Xenopus laevis* oocytes.
- The TEVC technique will be used to study the effects of pyrethroids on these channels.

2. Methodology

2.1 Maize weevil populations

The 3 populations of maize weevil maintained at Nottingham originally came from the Ecotoxicology laboratory of the federal university of Viçosa, Minas Gerais, Brazil. The standard susceptible population is from Sete Lagoas county, it has been maintained in the lab for over 20 years without insecticide exposure. This population was provided by the national centre of maize and sorghum from the Brazilian agricultural research corporation (EMBRAPA Milho e Sorgo). The susceptibility of this population was first classified by Guedes *et al* (1995), they found that 100% of the population from Sete Lagoas died following treatment with a concentration of DDT known to cause 100% mortality to the standard susceptible population used in their study.

The two pyrethroid and DDT resistant populations originally came from: Jacarezinho county in the late 1980's and Juiz de Fora county in 1999. Their resistance has been well documented in the literature (Guedes *et al*, 1994; Ribeiro *et al*, 2003; Fragoso *et al*, 2005; Oliveira *et al*, 2007; Araujo *et al*, 2008).

Twelve field populations of *Sitophilus zeamais* were also obtained from different areas of Brazil (**Fig2-1**) and were originally collected in 2007, their resistance is relatively unknown. Maize weevil populations were maintained in jars of whole maize grain (13% moisture content) at a controlled temperature of 25 ± 2°C, RH of 70 ± 5% and a photoperiod of 12hr light 12hr dark.

Fig2-1. Map showing the original locations from Brazil of the 12 field populations of unknown resistance. (**1**) Guarapua, (**2**) Piracicaba, (**3**) Espirito Santo Do Pinhal, (**4**) Votuporanga, (**5**) Sâo José Do Rio Pardo, (**6**) Machado, (**7**) Viçosa, (**8**) Nova Erà, (**9**) Sacramento, (**10**) Rio Verde, (**11**) Sâo Joâo, (**12**) Dourados Linha Barreirinha.

2.2 Bioassays

Insecticides used were of analytical standard: DDT (Aldrich), Deltamethrin (Fluka Analytical, Sigma-Aldrich).

2.2.1 Topical bioassays

Topical bioassays to assess the resistance of the 3 original lab populations to deltamethrin and DDT *in vivo* were performed on unsexed adult weevils. 1µl of the compound was applied topically to each insect using a Gilson pipette. All insecticides were diluted in acetone. Deltamethrin was applied to the susceptible Sete Lagoas population in concentrations ranging from 0-3mg/ml and to the two resistant populations from 0-100mg/ml. DDT was applied to all populations in a range from 0-100mg/ml as it was impractical to go above 100mg/ml due to precipitation. The treated weevils were placed in petri dishes and kept at 25 ± 2°C for 24-48 hours and mortality was recorded at 24 and 48 hrs for deltamethrin and 48hrs for DDT treated insects. The insects were recorded as dead if they could not walk when prodded with a fine paint brush. For each concentration used, 3 repeats of 20 insects were performed for each population.

2.2.2 Vial bioassays

Bioassays were also performed using a vial application method. In this case, 400µl of the insecticide in acetone were pipetted into 20ml glass vials and placed on a roller to evenly distribute the solution on the inner surface of the vial and allow the acetone to evaporate. When the acetone had evaporated (up to 2 hours) 20 insects were placed in each vial, again 3 repeats per concentration were performed and they were kept under the same conditions as the topical bioassays. This was only performed using deltamethrin on the Sete Lagoas population and the Jacarezhino population. Mortality was assessed at 48 and 72hrs.

Fig2-2. Demonstrates the two bioassay methods employed in this study. Vial bioassays on the left, where the inside of the vial is coated with insecticide. The topical application is shown on the right wherein insects had a microlitre of insecticide pipetted onto them and were contained in a petri dish.

2.2.3 Analysis

The results were analyzed by calculating the LD_{50} using Probit analysis in SPSS-16 and populations were compared using univariate ANOVAs in SPSS-16 and post-hoc Tukeys HSD test.

2.2.4 Selection bioassays

Assays were performed on the 12 field populations to select for resistant individuals potentially carrying the T929I super-kdr mutation. 400µl of DDT in acetone was pipetted into 20ml glass vials and placed on a roller to distribute the solution and allow acetone to

dissolve. Concentrations of 1 and 0.5mg/ml were used, taken from data recorded by R.A.Araujo (personal communication). Depending on the number of insects in each field population, 50-80 individuals were placed in each vial and exposed for 72hrs. The live insects after this time were taken and placed into a new population in fresh maize, in separate tubs for each field population. They were then left for 4-6 weeks to allow them to breed according to studies which give an estimated immature development time of 36.2 days at 27˚C (Sharifi and Mills, 1971) .

2.3 Diagnostic assay

2.3.1 Preparation of 96 sample reference plate

After selection for resistance 5 out of the 12 populations appeared to show some level of resistance to DDT and so were selected for the TaqMan diagnostic assay. Twenty insects were taken from each strain, before and after selection. In total 200 insects were tested from the field populations. Individuals from the Sete Lagoas and Jacarezhino populations were also tested as controls and water was used as a blank negative control. A standard 96 well plate was used.

Total DNA was extracted from each individual maize weevil using DNAzol reagent at 200µl per insect. After extraction and purification using ethanol the DNA was re-suspended in 20µl of sterile nuclease free water. The concentration and purity of DNA in the samples was determined using a NanoDrop spectrophotometer (NanoDrop Technologies) which

determines the absorption at 260nm. 1µl of DNA from each individual was used in the TaqMan assay.

2.3.2 TaqMan SNP genotype assay

The IIS4-IIS6 region of the *para* sodium channel gene in many insects has an intron close to the kdr and super-kdr sites (Bass *et al*, 2007). This intron can vary between insects of the same species and so inhibit performance of an assay that uses primer binding sites within this region. Previously Araujo (2010) designed primers/probes using a conserved region found in the super-kdr area of the sodium channel gene. Forward and reverse primers and 2 minor groove binding (MGB) probes (Applied Biosystems) were designed using Primer Express^{TM} software version 2.0. The minor groove binder raises melting temperature (T_M) between matched and mis-matched probes and so gives better allelic discrimination (Afonina *et al*, 1997). Primers weevil-TtoV Forward (5'-ACCATGGGTGCCTTGGG-3') and weevil-TtoV Reverse (5'-GCATACCCATCACGGCGAATATAAA-3') were standard oligonucleotides with no modification. The probe weevil-TtoVV2 (5'-ACAACACAAAGGTCAGGTT-3') labelled with VIC at 5' end for detection of WT and probe weevil-TtoVM2 (5'-ACAACACAAAGATCAGGTT-3') labelled with 6-FAM to detect the s-kdr allele. A 3' non-fluorescent quencher and a minor groove binder were also attached to each probe as the 3' end. The forward and reverse weevil-ToV primers were used in the assay with the probes weevil-TtoVV2 for wild type specification and weevil TtoVM2 for super-kdr detection (**Fig2-3**).

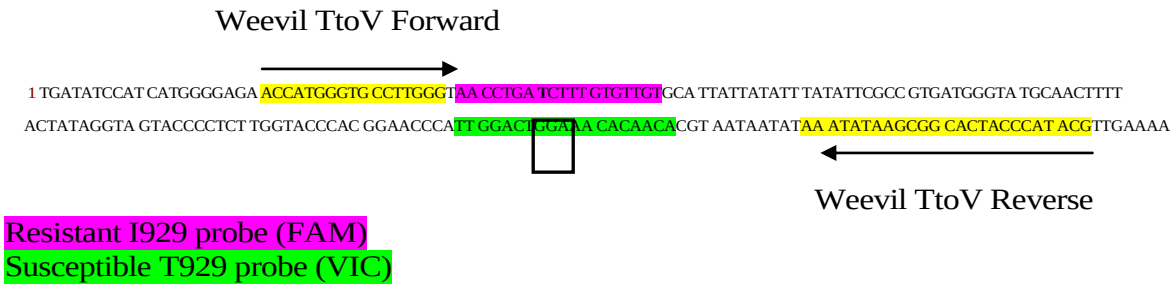

Fig2-3. Gene fragment from *Sitophilus zeamais para* sodium channel gene showing the primers and probes used in the diagnostic TaqMan assay.

The real-time PCR reaction mixtures (20µl) contained 1µl of genomic DNA, 10µl of SensiMix DNA kit (Bioline), 0.5µl of the primer probe and 8.5µl of nuclease free water. The Rotor-gene 6000^{TM} (Corebett Research) was used to run the samples under the temperature cycling conditions: 95°C for 10 minutes, 42 cycles of 95°C for 10 seconds and 60°C for 45 seconds. The increase in the different reporter dyes (VIC and FAM) was recorded in real time on each cycle on the yellow (530nm excitation and 555nm emission) and green channel (470nm excitation and 510nm emission) of the Rotor-gene.

2.3.3 Analysis

The results were analysed by auto scaling the fluorescence graphs and comparing them to the positive and negative controls. The endpoint values of fluorescence for each dye were also plotted in bivariate scatter plots, these showed obvious clustering of the different genotypes, allowing easy scoring.

2. 4 Electrophysiology using *Xenopus Laevis* oocytes

2.4.1 Xenopus oocytes

The oocytes used for injection of RNA came from female Xenopus laevis (The European Xenopus Resource Centre, University of Portsmouth). Collagenase (Sigma, St. Louis, MO) was used at 2.5mg in 5ml of Ca^{2+} free GTP (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM Na-pyruvate, 0.5 mM theophylline, 50 mg/l gentamicin, pH 7.5) to break down connective tissue and remove the follicle layer around the oocytes. Oocytes were gently shaken in an incubator at 18°C for approximately 1 hour before being transferred to Barth's GTP solution (**Fig2-4**) (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM Na-pyruvate, 0.5 mM theophylline, 50 mg/l gentamicin, pH 7.5).

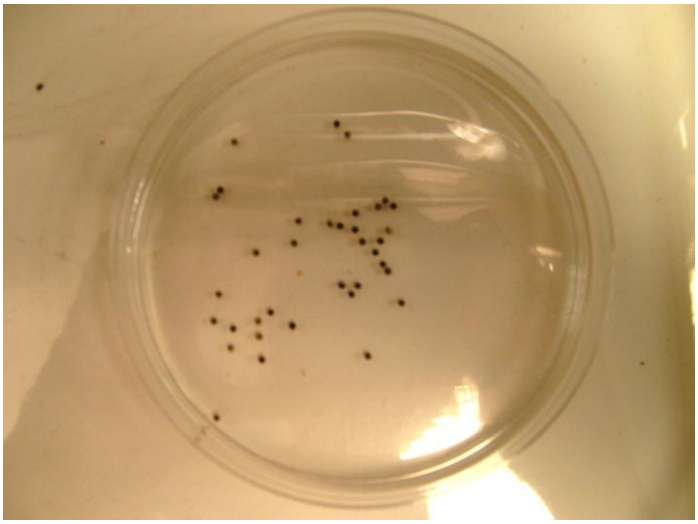

Fig2-4. Shows Xenopus oocytes after separating with collagenase.

2.4.2 Injection

Large, similar sized and healthy oocytes were selected for injection. Pipettes for injection were pulled from glass capillaries (3.5 NANOLTR, World Precision instruments) by a 700C vertical pipette puller (David Kopf instruments). The fine end was gently snapped off on a sterile surface to create a slanted opening at the end of the pipette 1-2µm in diameter. The pipette was then filled with filtered paraffin oil and fastened to the injecting needed of a Nanoliter injector (World Precision instruments).

The RNA coding for the Drosophila *para* sodium channels was prepared at approximately 0.5µg/µl and 1µl of this was mixed with 1µl of TipE RNA (0.5µg/µl) and 3µl distilled water (5µl total solution). The RNA constructs were obtained from Rothamsted Research (Hertforsdshire, UK). This solution was slowly sucked into the pipette with care taken to prevent the uptake of air bubbles.

The oocytes were injected on a wire lined petri dish to keep them from moving during injection. Oocytes were injected with 50.6nl of RNA solution. After injection, oocytes were kept in Barth's GTP solution at 18°C for 48 hours when they were ready for testing. During this time the Barth's GTP was refreshed after 24 hours. Oocytes were maintained in a fridge at 5°C and the solution was changed every day. The oocytes were able to be used for testing for up to 7 days.

2.4.3 Two Electrode Voltage Clamp

Electrophysiology was carried out on the oocytes 2-5 days after injection. The sodium currents were measured using the two electrode voltage clamp technique, carried out at room temperature.

Electrodes were pulled from borosilicate glass capillaries (GC150TF-10, Harvard Apparatus) using a P-97 Flaming Brown micropipette puller (Sutter instruments Co.) to give a resistance of ≈ 1MΩ. This low resistance allows faster clamping speeds. The electrodes were filled with a solution of 0.7M potassium chloride plus 1.7M potassium citrate. The electrodes were tested for resistance before clamping the oocyte.

The oocyte to be tested was placed in a disposable bath in 2ml of bath solution (Barth's GTP minus sodium pyruvate, theophyline and gentamycin). The oocyte was impaled with both electrodes and the unclamped membrane potential measured. Ideally, oocytes used for recording had resting membrane potentials more negative than -20mV, however oocytes with membrane potentials more positive than this were used in some cases. Both the electrodes should show equal values.

Current was injected into the oocyte via the intracellular electrode to maintain the desired potential difference between the intracellular voltage electrode and the agar bridge in the surrounding bath. The oocytes were clamped to -70mV (Clampator one, CA-1B High Performance Oocyte clamp, DAGAN). The gain was turned up to maximum. Test pulses were generated by PULSE software (Heka Elektronic, Germany) and recorded on a PC. The leakage current was automatically subtracted by PULSE using a P/N-5 protocol, and the

capacitive current. Therefore, any alteration in leak current caused by the mutation or permethrin would not be seen.

2.4.4. Voltage protocols

2.4.4.1 Activation voltage protocol

The activation of the VGSC was studied with and without the presence of pyrethroids. The oocyte was initially clamped at -70mV for 20ms then this voltage was increased by 5mV to -65mV for 32ms. It was then returned to -70mV for a further 5ms to allow for recovery from inactivation before the next step depolarisation. This was continued, increasing the voltage by a further 5mV at each cycle up to +45mV (a total of 24 steps with a 5ms gap between each step) (Burton et al, 2011). The diagram below (**Fig2-5**) represents this protocol.

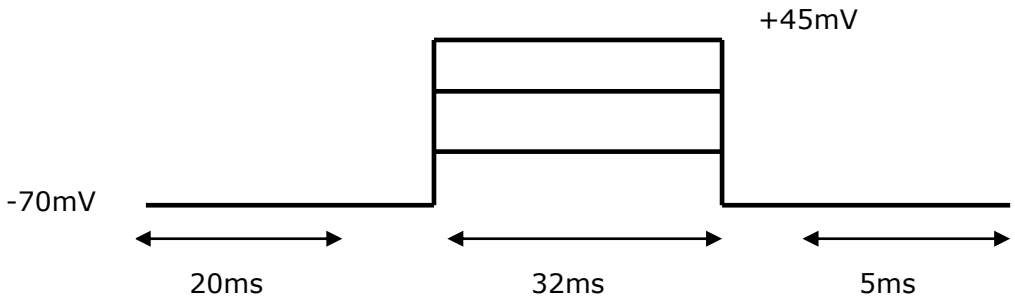

Fig2-5. Illustrates the activation protocol, initiating at -70mV. Each line indicates a 5mV increment as the voltage is progressively increased to +45mV

The current amplitude for each of the test voltages (V_T) from -70mV to +45mV was measured and plotted against V_T to give a current-voltage relationship. The data was then transformed and normalised to account for the variation between oocytes. This data was then fitted with a modified Boltzmann equation.

$I_{peak} = I_{max}*((V_T-V_{rev})/(1+\exp(-(V_T-V_{50})/k)))$ – equation 1

I_{peak} represents the peak sodium current amplitude at V_T. I_{max} represents the maximal peak current and V_{rev} is the reversal potential. The V_{50} is the voltage at half the maximal current, i.e. when half the channels are activated. *k* is the slope factor in millivolts.

The data was then transformed using:

$G=I_{peak}/(V_T-V_{rev})$ – equation 2

G represents conductance, I_{peak} is the peak current, V_T is the step depolarisation value and V_{rev} is the reversal potential for the sodium current determined by the I-V fit with equation 1. Following this, the data was again normalised and averaged to give a conductance-voltage relationship before being fitted with a Boltzmann sigmoidal function to estimate V_{50} and k. Data analysis was carried out using Graphpad Prism 5 software.

2.4.4.2 Inactivation voltage protocol

The steady state inactivation can be studied using this protocol. The oocyte was first clamped to a holding potential of -70mV. A 200ms inactivation pre-pulse was then applied starting at -90mV and increasing to -20mV in 5mV increments. After each pre-pulse a test pulse to -10mV for 32ms was applied, this would cause immediate channel activation unless all or some of the channels were inactivated by the prepulse. **Fig2-6** represents these voltage steps. The more positive the pre-pulse potential, the more channels there would be in the inactivated state and so there would be a smaller sodium current.

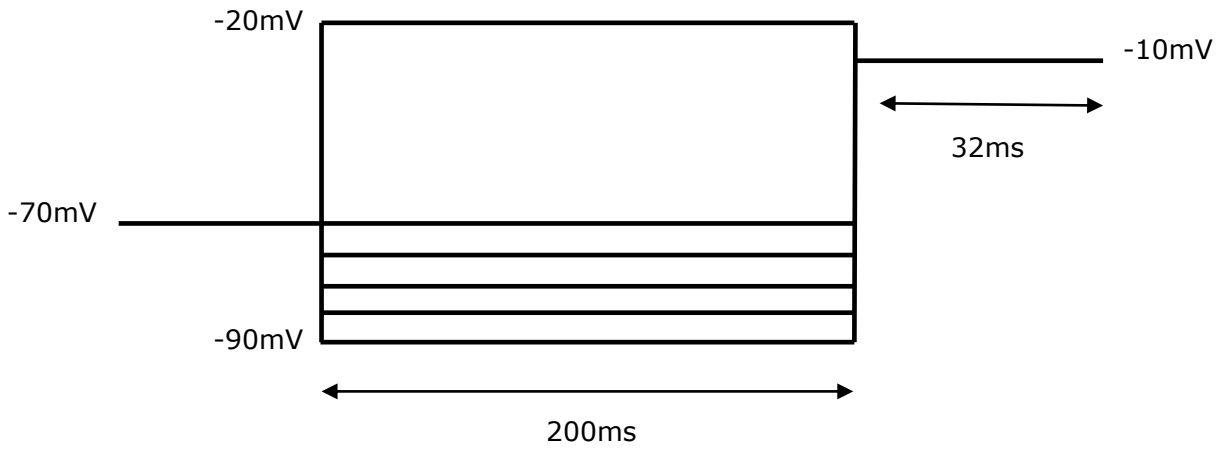

Fig2-6. Illustrates the inactivation protocol, which starts by clamping the oocytes at -70mV. An inactivating pre-pulse is applied, the first of these at -90mV, these increase to -20mV in 5mV increments represented by each line. After each pre-pulse a test pulse to -10mV is applied

The peak sodium current at each -10mV test depolarisation was plotted against the pre-pulse potential. These data were transformed and normalised before being fitted with a simplified

Boltzmann relationship (equation 3). The midpoint potentials were estimated to give a test potential at which 50% of the channels are inactivated.

$I_{peak}/ I_{max}=1/(1+\exp(-(V_T-V_{50})/k))$ – equation 3

I_{peak} represents the peak sodium current for a single test pulse of -10mV following an inactivating pre-pulse of V_T. I_{max} is the maximal peak current for a series of test pulses. V_{50} is the voltage at which 50% of the channels are inactivated, k is the slope factor in millivolts.

2.4.4.3 Tail current voltage protocol

This protocol looks at the characteristic feature of VGSC affected by pyrethroids and DDT: the tail current. Pyrethroids are known to slow channel inactivation and deactivation (Vais *et al* 2001). Both type I and type II pyrethroids bind more efficiently to the open channel. Therefore, their effect can be seen more clearly after pre-conditioning pulses to open and close the channel before the protocol is applied (Vais *et al*, 2000)

The protocol produces 100 conditioning pre-pulses (5ms, 66Hz) to 0mV from the holding potential of -70mV. 10ms intervals were given between each pulse to allow for recovery from open state inactivation. A single 12 second repolarising pulse to -110mV was then applied (**Fig2-7**). This should mean that all VGSC were closed, so any residual current indicated modification by the pyrethroids. This is known as the tail current.

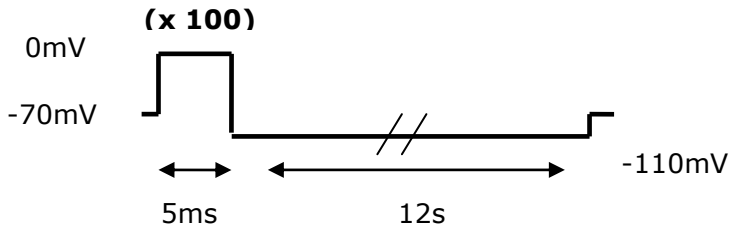

Fig2-7. Shows the tail current protocol with the 100 conditioning pre-pulses followed by a 12 second repolarising pulse.

The tail currents were initially examined using:

$M(\%)=\{[I_{tail}/V_{tail}-V_{rev})]/G_{max}\}\times 100$ - equation 4

M(%) is the estimate of the percentage of channels that have been modified (Vais *et al*, 2001; Vais *et al*, 2000). I_{tail} is the peak amplitude of the tail current recorded at the membrane potential V_{tail} (-110mV). The V_{rev} is the reversal potential of the VGCS for the individual oocyte. G_{max} represents the maximum sodium conductance of the cell. V_{rev} and G_{max} are obtained by applying equation 1 to the IV data for the individual oocyte tested. The peak amplitude of the tail current was measured relative to the zero 'baseline' before pyrethroids were applied.

Equation 4 does not account for the length of the tail current, it was therefore modified and termed integral modification (M_I) (Usherwood *et al*, 2007):

$M_I = \{[I_{tail,integ}/(V_{tail} - V_{rev})]/G_{max}\} \times 100$ - equation 5

$I_{tail,integ}$ represents the multiplication of the peak amplitude of the tail current with the time constant value (s) of an exponential fit to the decaying tail current. Equation 5 is generally considered a more accurate representation of the total charge moving across the membrane.

2.4.5 Application of permethrin

Permethrin (cis, trans isomer mix, PESTANAL, Sigma-Aldrich) was diluted in dimethyl sulphoxide (DMSO, Sigma, St Louis, MO) to create serial dilutions from 10^{-2} to 10^{-6} M. The final concentrations of DMSO in the bath did not exceed 1%, a concentration previously shown to have no effect on the oocyte (Usherwood *et al*, 2007). Permethrin was originally added to the bath to give final concentrations that ranged from 0.001-100µM. However, results were difficult to obtain as sodium channel expression and oocyte quality was poor. This has been found previously in oocytes between May to August (Burton, 2008). As the oocytes were not able to be clamped for long periods it was found best to apply a single dose of permethrin of 1µM to obtain results. The insecticide was allowed to diffuse in the bath for 5 minutes, initially lightly blowing on the surface of the solution to aid dispersal. If time had permitted this was to be repeated with deltamethrin for comparison.

3. Bioassays

3.1 Introduction

The purpose of carrying out the bioassays on populations of *Sitophilus zeamais* was, firstly, to determine a dose of insecticide that could be used to discriminate between resistant and susceptible individuals. This would then be used to select field populations of the maize weevil for insecticide resistance before carrying out a diagnostic assay to look for the presence of T929I previously found in 2 resistant populations of the maize weevil. Secondly, bioassays using a range of concentrations would enable comparisons with other resistant populations carrying the T929I and other mutations. This would contribute to a resistance profile of the pest. The T929I mutation has been investigated in Xenopus oocytes, where it conveyed very high levels of resistance to deltamethrin and DDT (Usherwood *et al*, 2007). *In vivo* the T929I mutation has only been investigated in combination with L1014F in the diamondback moth (Schuler *et al*, 1998) where the combination of mutations gave >10,000-fold decrease in sensitivity to deltamethrin and >100–fold to DDT. It was therefore predicted that the maize weevil would show high levels of resistance to deltamethrin and DDT in the bioassays.

The Taqman diagnostic assay to be carried out after the lab bioassays will determine the presence of the T929I mutation. If this is not reported in the field populations, resistance may still be present due to another mechanism. Therefore a simple bioassay on the field populations comparing them to the lab susceptible population would be useful. A single concentration of insecticide was used for this.

3.2 Bioassay techniques

The most common procedures for bioassays are: 1. topical application of the insecticide to adult or larval stages of the pest; 2. to coat the insecticide onto the inside of glass vials and expose a number of insects in them; 3. filter paper impregnated with insecticide or coated onto a leaf (know as leaf-dip). This study initially used the vial approach to assess resistance in *Sitophilus zeamais*, but as the Jacarezhino populaion are so highly resistant it was difficult to reach concentrations high enough to be able to obtain the LD_{50} value. The method of topical application to the adult insect was then chosen as it is known exactly how much insecticide each insect receives and a better kill is achieved. The use of two different bioassay methods allows assessment of the advantages of each method. See **2.2.1** for bioassay methods.

3.3 Resistance to Deltamethrin and DDT

The LD_{50} values for the 3 populations of *Sitophilus zeamais*: Sete Lagoas, Jacarezhino and Juiz de Fora exposed to deltamethrin and DDT were calculated. The results are shown in the graphs and tables below. The population names may be abbreviated to SL (Sete Lagaos), JA (Jacarezhino) and JF (Juiz de Fora) to simplify the results. Graphs were created using Graphpad prism 5 software.

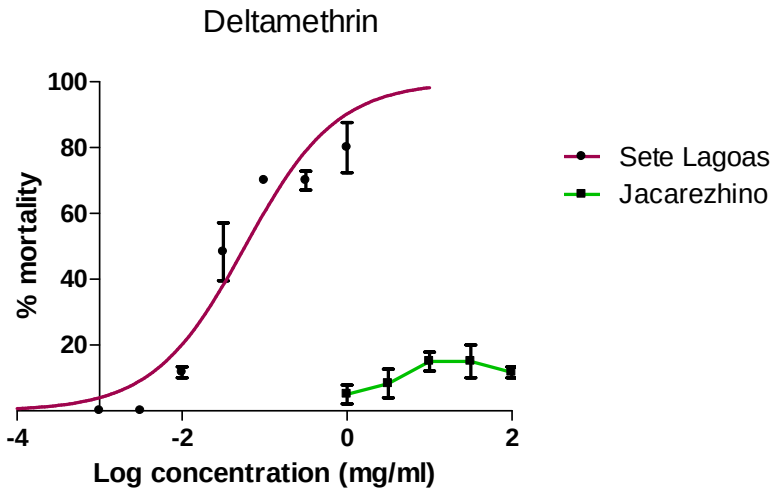

Fig3-1. Mortality graphs representing the resistance levels to deltamethrin of Jacarezhino and Sete lagoas populations of the maize weevil carried out using vial bioassays. Each point represents the mean mortality as a percentage for that concentration with the Standard error calculated from three repeats.

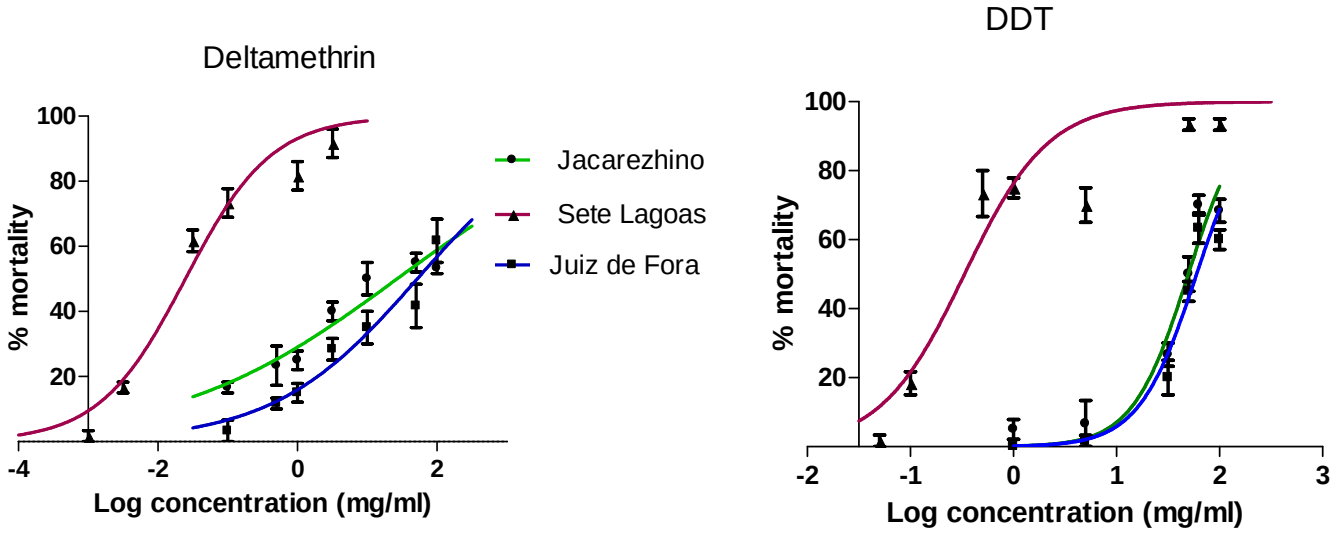

Fig3-2. Mortality graphs showing the difference in resistance between the maize weevil populations from Sete Lagoas and Jacarezhino to deltamethrin and DDT from the topical bioassays.

Table 3-1

Vial bioassays (48 hours)

Population	LD_{50} deltamethrin (mg/ml)	Slope ± SE
Sete Lagoas (susceptible)	0.026 (0.017-0.40)	0.738 ± 0.072
Jacarezhino (resistant)	100mg/ml = 11.65% kill	

Table 3-2

Topical bioassays (48 hours)

Population	LD_{50} deltamethrin (mg/ml)	Slope ± SE	LD_{50} DDT (mg/ml)	Slop ± SE
Sete Lagoas (susceptible)	0.032 (0.016-0.061)	0.85 ± 0.084	0.593 (0.281-1.149)	0.86 ± 0.076
Jacarezhino (resistant)	26.661 (11.907-91.694)	0.39 ± 0.065	47.268 (32.159-74.220)	1.30 ± 0.151
Juiz de Fora (resistant)	48.780 (26.384-115.642)	0.61 ± 0.076	62.221 (51.983-76.575)	2.06 ± 0.301
RF (JA/SL)-(JF/SL)	**833.16- 1524.38**		**79.71-104.93**	

As can be seen in **table3-1**, the vial bioassays were limited as to the level of mortality that could be reached with the resistant population. However, more extensive data was obtained for the Sete Lagoas population, but when compared to the topical data, there was not found to be a significant difference in the results obtained via the different bioassays (Univariate Analysis of Variance, test of between-subjects effects, F=0.236, p=0.630).

A clear difference can be seen between the susceptible population and the two resistant ones in the topical bioassays (**Fig3-1;Fig3-2;Table3-2**). The difference between the 3 populations was tested using a univariate ANOVA in SPSS followed by a *post hoc* comparison, two separate tests were performed for deltamethrin and DDT. **Fig3-1** plainly illustrates the difference in resistance between a maize weevil population susceptible to DDT and deltamethrin and a resistant population.

The ANOVA results for the deltamethrin bioassays showed there to be a significant difference between 2 or more of the populations ($F = 55.751$, $p < 0.001$). A Tukeys HSD *post-hoc* comparison showed that this difference lies between Sete Lagoas and Jacarezhino ($p=0.001$), Sete Lagoas and Juiz de Fora ($p < 0.001$) and that there was no significant difference in mortality between Jacarezhino and Juiz de Fora ($p = 0.065$).

Results for the ANOVA with DDT bioassay data again showed there to be a significant difference between 2 or more populations ($F = 58.008$, $p < 0.001$). The *post-hoc* Tukeys HSD test showed there to be a significant difference between Sete Lagoas when compared with both Jacarezhino and Juiz de Fora (both p-values < 0.001) and there was no difference between the two resistance populations: Jacarezhino and Juiz de Fora ($p = 0.377$).

A further bioassay was performed, in which 1mg/ml of DDT was tested using the vial approach, chosen because of the speed and ease of this method. This was carried out on the 12 field populations of the maize weevil and the lab susceptible population from Sete Lagoas. A significant difference was found to be present between the Sete Lagoas population and 1 or more of the field populations (Univarate analysis of variance, $F = 6.886$, $p < 0.001$). A Post hoc test showed this difference to lie between Sete Lagoas and Dourados Linha Barreirinha ($p=0.038$). On observing the data it could be seen that this difference was due to the Dourados Linha Barreirinha population showing higher mortality to the dose of DDT. After 48 hours exposure to 1mg/ml DDT Sete Lagoas showed 27% mortality, Dourados Linha Barreirinha showed 58% mortality.

3.4 Discussion

Study of the super-kdr T929I mutation alone has not before been comprehensively carried out *in vivo*. Vial and topical bioassays were carried out on known resistant and susceptible populations of *Sitophilus zeamais*. It was found that the resistant populations carrying the T929I mutation gave a 833-1524-fold decrease in sensitivity to deltamethrin and 80-105 fold for DDT. This was as expected from studies *in vitro* of the T929I mutation. There was no difference in resistance found between the 2 resistant populations where one carries a fitness cost and the other does not. However there was some suspicion that the susceptible Sete Lagoas population had become contaminated with resistant individuals as an LD_{50} of 0.032mg/ml for deltamethrin and 0.593mg/ml for DDT seemed very high relative to original bioassays on this population. Initial bioassays had been carried out in the populations on their arrival from Brazil via vial application. These showed that a concentration of 0.4mg/ml DDT

resulted in 91.65% mortality in the susceptible Sete Lagoas strain; R.A. Araujo (personal communication). The suspicions of some resistant individuals in the susceptible populations was tested with the TaqMan diagnostic assay which detects the T929I mutation (discussed further in the next chapter) and 2 resistant and 3 heterozygote individuals were found in a sample of 12 weevils. Therefore, the resistance factor given in this study from LD50 studies is likely to be an under-estimate of the resistance difference between maize weevils carrying the T929 allele and the I929 allele.

The presence of resistance in the susceptible population had to be taken into account when choosing a discriminating concentration for the selection of resistant individuals from the 12 field populations. It was therefore decided to take concentrations of DDT used in the original bioassays by R.A. Araujo (personal communication) as a guide to a discriminating concentration. The original bioassays showed that a concentration of 0.04mg/ml of DDT where 0.4ml was coated onto the inside of a 20ml glass vial gave a mortality of 28.3%, 0.4mg/ml gave 91.65% mortality and 4mg/ml gave 98.3% mortality. Using these data two discriminating concentrations were chosen of 0.5mg/ml and 1mg/ml to be applied using the vial method.

The resistance of maize weevils to deltamethrin and DDT has been studied previously. Maize weevils from the sites that the lab populations in this study originated from, may therefore have carried the same mutation, but this is not confirmed. Studies by Correa *et al* (2010) and Ribeiro *et al* (2003) used a bioassay method of coating the inside of 20ml glass vials with different concentrations of insecticide and placing 20 unsexed insects into the jars then recording how many were dead after a number of hours. Guedes *et al* (1995) used the filter paper approach on a maize weevil population susceptible to insecticides. A summary of the results from these studies is shown in the table below (**Table3-3**) and show very high levels

of resistance, supporting our findings of the presence of resistance in populations of the maize weevil from these areas at the time the *Sitophilus zeamais* samples were collected.

Table 3-3

Bioassay	**Ref.**	**Insecticide**	**Exposure time**	**Pop**	**LD_{50}**		**RF**
					µg/cm^2		
Glass vials	Correa *et al*, 2010	Deltamethrin	48 hours	SL JA	0.06 318.43	0.4 ml put in vials	5307.2
	Ribeiro *et al*, 2003	Deltamethrin	24 hours	SL JA	0.08 0.648=0%kill	1.0ml put in vials	
					mg/l		
Insecticide impregnated filter paper	Guedes *et al*, 1995	Deltamethrin DDT	6 hours 24 hours	S	0.063 0.531	Volume applied not indicated	

SL – Sete Lagoas JA – Jacarezhino S – Susceptible

A topical bioassay was carried out on the diamondback moth (Schuler *et al*, 1998) which was found to carry the T929I mutation along with the L1014F kdr mutation. The insecticides were applied to fourth instar larvae. Shuler's bioassaydata suggest that the T929I mutation in combination with L1014F gives much increased resistance to deltamethrin compared to DDT (**Table3-4**). Our results found that T929I alone in the maize weevil did not give such high levels of resistance and showed a smaller difference between the resistance to deltamethrin and DDT.

Table 3-4

Bioassay	Ref.	Insecticide	Exposure	Pop	LD_{50} (µg/larva)	RF
Topical	Schuler *et al*, 1998	Deltamethrin	5 days	Susceptible	0.001	>10,000
				Resistant	10µg=6%kill	
		DDT		Susceptible	0.92	>10
				resistant	10µg=1%kill	

The bioassays carried out on the field populations only showed a significant difference between the "susceptible laboratory" Sete Lagoas population and one other field population. When looking at the data it could be seen that this difference was due to a higher mortality in the field population. This further supports the presence of resistance in the lab susceptible populations and also means low levels of resistance could be present in the field populations but were not detected.

Recently, a study was carried out on resistance in the maize weevil against the current mix of insecticides used in their control. Correa *et al* (2010b) studied the resistance of field samples of *Sitophilus zeamais* to a mix of an organophosphate (fenitrothion) and a pyrethroid (esfenvalerate). Low levels of resistance were reported, ranging from 1-5-fold at the LC_{50} value. The populations tested included the 3 lab populations in this study and the 12 field populations. In Correa's study the Jacarezhino population was found to have only a 4.06-fold level of resistance to the insecticide mix. This is probably due to the use of organophosphates rendering the T929I mutation ineffective at providing resistance to this insecticide mix. The low levels of resistance present could suggest the emergence of metabolic resistance.

In summary, due to unexpected resistance in the lab "susceptible" population Sete Lagoas, a discriminating concentration was elected using data previously collected on this population. The concentrations to be used are 0.5 and 1.0mg/ml DDT to be applied in vials. The resistance factor reported for the T929I mutation in the maize weevil, when resistance in the susceptible population is accounted for, was similar to that found in bioassays previously performed on these populations (Correa *et al*, 2010), and shows high levels of resistance to deltamethrin and DDT. It is also shown that the T929I mutation alone does not give as high levels of resistance as it does when found in combination with L1014F in the diamondback moth (Schuler *et al*, 1998). No resistance was reported in the 12 field populations of the maize weevil.

4. Diagnostic assay for the detection of T929I

4.1 Introduction

The detection and monitoring of resistance in the field is essential in the control of insect pest species. Early detection of target site resistance also aids the effective use of insecticides as, once resistance is detected the insecticide used can be switched to one which has a different target site. This removes the selection pressure for the resistance mutation, hopefully leading to its decline. Alternating the use of different insecticides extends their shelf life. These experiments were designed to establish the feasibility of early detection of the T929I mutation in Brazilian field samples of the maize weevil: *Sitophilus zeamais*, which conveys resistance to pyrethroids and DDT. Resistance has been reported in this species since the mid 90's (Guedes *et al*, 1995) and the T929I mutation was identified in two resistant lab populations in 2010, where is was found to be the primary form of resistance (Araujo *et al*, 2011). If this mutation is found to be present in many field populations, a diagnostic test applicable in the field could be a useful tool in detecting and controlling resistance. Twelve field populations of *Sitophilus zeamais* were obtained from Brazil to be tested for the T929I mutation using a taqman diagnostic assay 20 individuals from each population were tested (Araujo, 2010). Out of 240 insects only two heterozygotes and no homozygous individuals were found. Only one of the populations where the resistance allele was found to be present were tested in the current set of 12 field populations.

The low frequency of the resistant allele previously found could have been due to the populations being maintained for a long period in the lab without selection. This could lead to a reduced frequency of the resistance allele due to a fitness cost. The relatively small sample size of 20 in Araujo's (2011) study may therefore have not picked up on the presence of this mutation if it was at very low levels. It is therefore reasonable to try re-selecting these

populations for the resistant allele and re-running the taqman assay to detect if higher levels of resistance are present after selection. If this were the case, it would suggest that even low levels of the resistance allele present in field populations of the maize weevil pose a possible threat to pesticide management if pyrethroids are over used. If selection for the resistance in the maize weevil populations does not lead to an increased frequency in the T929I allele it is likely that either this mode of resistance is not a big problem in the field (even though it may have been in the past) or that keeping the populations unselected in the lab has caused them to lose their resistance due to a fitness cost. Carrying out the taqman diagnostic assay will also allow us to draw conclusions on whether it would be a valuable tool in the field for resistance detection.

4.2 Selection of field populations

Field samples of *Sitophilus zeamais* were obtained from different areas of Brazil: Sacramento, Votuporanga, Sâo José do Rio Pardo, Espirito Santo Do Pinhal, Machado, Novaera, Piracicaba, Viçosa, Dourados Linha Barreirinha, Sâo Joâo, Guarapuava and Rio Verde. These were allowed to breed in the lab for approximately a month to allow numbers to build up and were then selected for resistance with DDT. 50-80 individuals (depending on numbers in each population) were placed in glass vials where the interior was coated with 0.4ml DDT solution. After 72 hours, the surviving individuals were removed from the vials and placed into fresh maize to allow them to reproduce. An apparent difference could be seen in survival rates between the different populations, suggesting some level of resistance in a few of the populations.

This suggested resistance was assessed using a bioassay of 1mg/ml applied in vials. However, the only significant difference found showed a population from Dourados Linha Barreirinha to be more susceptible to DDT than the "susceptible" Sete Lagoas population.

The selected populations were assessed after one month. However, it was found that little breeding had occurred, therefore the individuals originally selected were the ones used to carry out the TaqMan assay. In five out of the twelve field populations there were enough surviving individuals after selection to carry out the assay (São Joâo, Dourados Linha Barreirinha, São José, Votuporanga, Viçosa). 20 individuals from these selected populations and also 20 from the populations before selection were taken to perform the taqman assay. This was in order to compare levels of the resistant allele present before and after selection.

4.3 The TaqMan assay

The TaqMan diagnostic assay is based on single nucleotide polymorphism (SNP) genotyping. The technique uses Real time PCR where the fluorescence of specific reporter dyes attached to sequence specific probes is assessed in real time during the extension phase of PCR (Wilhelm & Pingoud, 2003). It is much more accurate and less labour intensive than traditional PCR techniques where post-PCR steps are required. The increased accuracy comes from the whole amplification profile being known and so increases precision (Wilhem & Pingoud, 2003).

The TaqMan on the *Sitophilus zeamais* used two dyes attached to allele specific probes to determine the presence of either the mutant I929 or the wild-type T929 in DIIS5 of the *para*

sodium channel. The Wild-type probe was labelled with VIC dye and the mutant with FAM. The presence and frequency of these alleles was assessed by looking at the cycling graphs for both dyes. **Fig4-1** shows the discrimination of the resistant allele I929.

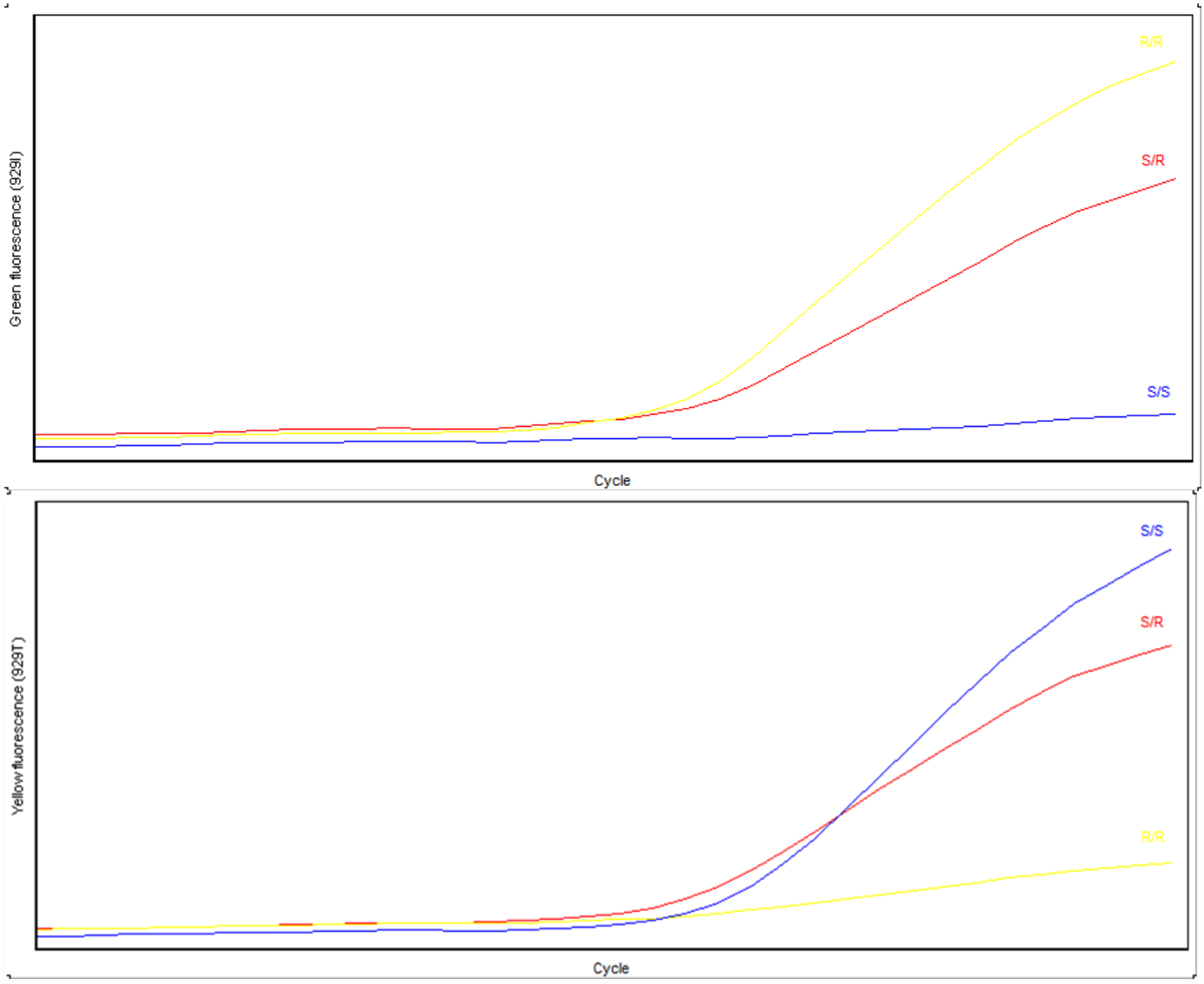

Fig4-1. The top figure shows the cycling for the FAM labelled probe specific for the 929I mutant allele and the bottom figure shows the cycling for the VIC labelled probe specific for the 929T wild-type allele. The traces labelled R/R represent an individual homozygous for the 929I super-kdr allele, the traces labelled S/S represent an individual homozygous for the 929T wild-type allele and traces labelled S/R represent heterozygous individuals

As can be seen in the figure above, a large increase in FAM fluorescence and no increase in VIC fluorescence indicates a homozygous resistant individual, and a homozygous susceptible individual is shown by a large increase in VIC fluorescence and no increase in FAM fluorescence. An intermediate increase in both signals shows a heterozygote individual.

The TaqMan assay was carried out on 5 field samples of the *Sitophilus zeamais*, on populations before and after selection for resistance with an insecticide (Sâo Joâo, Dourados Linha Barreirinha, Sâo José Do Rio Pardo, Votuporanga, Viçosa). 20 individuals from each populations were assayed, so 200 individuals in total. 12 individuals from the known resistant and susceptible populations (JA and SL) were initially run on the TaqMan to provide positive controls for each of the dyes. DNA was extracted individually from each insect and concentrations tested from a few samples. The average amount of DNA extracted from each weevil was 61.89 ± 5.8 ng/µl at a purity of 2.38 ± 0.10 at 260/280 when 2µl of the suspended DNA was tested from 10 individuals.

An easy way of analysing large samples is to use the Rotor-Gene analysis function that arranges the data into a scatter plot by taking the endpoint fluorescent values for the two dyes, autocorrecting them for background and plotting them against each other in bi-directional scatter plots (**Fig4-2**).

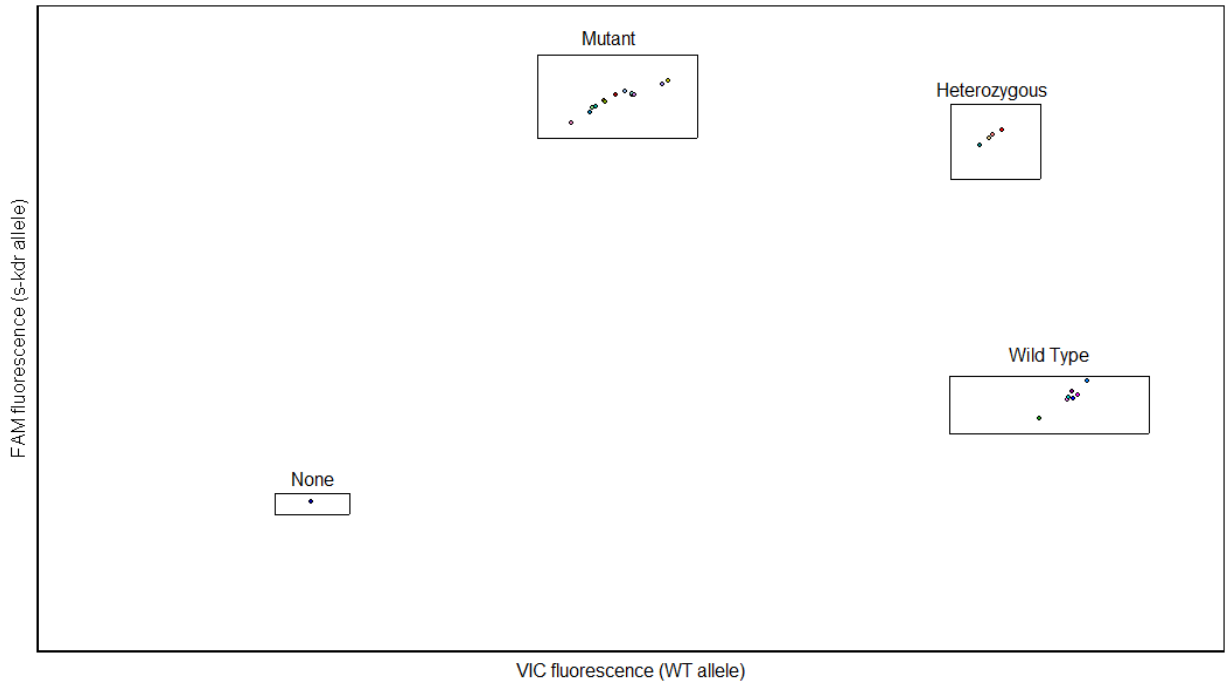

Fig4-2. Scatter plot analysis of TaqMan fluorescence data of positive controls. Displays 24 different DNA samples from JA and SL populations and a blank of water. The clusters are easily identifiable as mutants carrying the I929 allele showing more FAM fluorescence, Wild-type carrying T929 allele expressing more VIC fluorescence and those in between that are heterozygous for both alleles. The cluster labelled none, is the control water blank.

None of the weevils from the field samples were found to carry the resistant allele in either the homozygous or heterozygous form, the resistant samples shown in the figures are the positive control from the JA population. All individuals from the field samples tested as homozygous for the Wild-type T929 allele.

From the original 2 lab populations tested (Sete Lagoas and Jacarezhino) the susceptible population was found to have the resistance allele present: 2 individuals categorized as homozygous resistant, 3 as heterozygotes and 7 as homozygous susceptible. For the known resistant population from JA; 11 individuals were homozygous resistant and 1 was heterozygote.

4.4 Discussion

The fast detection of resistance in insect pest species is important to prevent the spread of resistance and to allow effective management of the insecticides used in control. Two lab populations were found to carry the T929I super-kdr mutation in S5 of DII of the *para* sodium channel. It is therefore likely that this mutation may be present in field populations of the maize weevil where resistance to pyrethroids has been reported. Even if this mutation is only present at low levels in the field, it is still important to manage insecticide use to prevent the selection for the mutation.

The TaqMan diagnostic assay was carried out on 12 field samples of *Sitophilus zeamais,* none of which showed any presence of the resistance allele. This may be because farming practices have changed in Brazil, and the pesticides in most frequent use are organophosphates; usually in combination with pyrethroids (Correa *et al* 2010b). Another possibility is that the populations had lost their resistance through being stored in a lab without re-selection. This could cause the resistance allele to be lost due to a fitness cost that has been found to be present in the Juiz de Fora population (Araujo *et al*, 2008). When the TaqMan was previously run on 12 field samples from Brazil, only 2 heterozygote individuals for I929 were detected (Araujo, 2010). This could suggest that the resistance was present and has been lost over time. Previously DNA from 4 out of the 5 field populations used in the assays in this study have had the DNA sequences analysed and no other mutations or variations in the nucleotide sequence were found (Araujo, 2010). Only the target site resistance was tested for, therefore other forms of resistance could potentially be present. A noticeable difference in survival between the populations was observed on selection with DDT, suggesting that some form of resistance may be present. Metabolic resistance may be

present in some of these field populations. Fragoso *et al* (2003) found that a field sample from Viçosa showed increased resistance to pyrethroids when compared the standard susceptible population of Sete Lagoas using discriminating concentrations. It was found that there was higher esterase and glutathione S-transferase activity in this population than the susceptible. Jacarezhino and Juiz de Fora were also found to have higher levels of activity of glutathione S-transferases. The Jacarezhino and Juiz de Fora populations showed higher resistance than the population from Viçosa. Target site resistance was not examined in this study. Ribeiro *et al* (2003) also reported resistance in a population from Viçosa, but only to DDT, not to pyrethroids. This could suggest a form of resistance other than target site alteration. However, a single dose bioassay on the 12 field populations did not show any significantly lower mortality levels when compared with the Sete Lagoas lab susceptible population. It should be noted that the I929 allele was found to be present in this population using the taqman assay. 17.5% were found to be homozygous resistant and a further 25% were heterozygous for the resistance allele. Therefore, this bioassay does not rule out the possibility of low levels of non-target site resistance.

Correa *et al* (2010b) carried out a study on maize weevil populations from states across Brazil, including from all of those that our 12 field populations and 3 lab populations had originally been collected from. They looked for resistance to the organophosphate and pyrethroid mix of fenitrothion and esfenvalerate. Low levels of resistance were found from 1 to 5-fold when compared to a susceptible population.

The rapid detection and monitoring of resistance assists effective control and use of insecticides. In the case of *Sitophilus zeamais* the assay for the presence of T929I super-kdr mutation has not been proven to be an effective tool to be taken into the field for resistance

monitoring. In a previous study on many of the same field populations of the maize weevil (Araujo, 2010) only 2 heterozygotes were reported, and in the present study, the 929I allele was not reported at all. It may be that this mutation used to be a problem but changes in pest control strategies have led to its decline.

Although, this particular assay has not been found to be useful, the ease of use and speed of gaining results in the TaqMan assay means it is an important tool for the management of resistance. Results from Correa *et al* (2010b) could suggest an emerging problem of resistance to the combined organophosphate/pyrethroid mix currently preferred for maize weevil control in Brazil.

5. Electrophysiology

5.1 Introduction

The 929 position in the VGSC is found in domain II, segment 5. Three different amino acid changes have been discovered in insects at this position. The wild type amino acid is threonine. Threonine has been mutated to Isoleucine in populations of the diamondback moth, *Plutella xylostella* (Schuler *et al*, 1998) and headlouse, *Pediculus humanus capitis* Deg (Lee *et al*, 2000) and also in the maize weevil, *Sitophilus zeamais* (Araujo *et al* 2011). In populations of the Western flower thrip, *Frankliniella occidentalis* threonine has been switched to cysteine (Forcioli *et al*, 2002). The third change at this site is to valine. This mutation has been found in the cat flea, *Ctenocephalides felis* Bche (Bass *et al*, 2004) and the whitefly, *Bemisia tabaci* (Alon *et al*, 2006).

The T929I and T929V mutated channels have been studied in oocytes previously. The T929I mutation showed a ~100-fold decrease in sensitivity to deltamethrin, ~80-fold decrease to permethrin and eliminated DDT potency (Usherwood *et al*, 2007). Usherwood also found that 100μM DDT could not elicit a tail current on oocytes expressing the T929V mutation. Atkinson (2002) found T929V conveyed high levels of resistance to deltamethrin. The T929C mutation has not been expressed in oocytes.

Type I and Type II pyrethroids have slightly different effects on the VGSC. Type I pyrethroids are generally slightly less potent than Type II. The depolarising effects of type II pyrethroids last much longer (several seconds) than type I (10-100milliseconds) (O'Reilly *et al*, 2006). Both shift the activation potential to a more negative value, causing the channels to open at more negative potentials.

This part of the study aimed to examine the different mutations at the 929 site of the Drosophila *para* VGSC using the Xenopus expression system. However, time did not allow the production of the T929C mutant transcripts and the T929I mutant had previously been studied more extensively than the T929V mutant, therefore it was the T292V mutant channel that was focused on. Deltamethrin and DDT had previously been tested on this channel, so here a type I pyrethroid (permethrin) was used.

The objective was to look at the effect of permethrin on the T929V channel. This was done by expressing the channel in Xenopus oocytes. Data on the T929V channel would be compared with the wild-type *para* sodium channel and to published data on T929I. The results would also be compared with the previously published data on the effects of deltamethrin and DDT on the T929V mutant channel.

Time was not the only problem encountered in the study using Xenopus oocytes. It has been previously reported that between May and August the oocytes are often of poor quality (Burton, 2008). This meant that the lipophilicity of the pyrethroids became a problem as it caused the oocytes to become excessively leaky, particularly if they were already damaged or unhealthy. Because of this it was decided that only one concentration (1μM) of permethrin would be tested on each oocyte.

5.2 Activation

Whole cell recordings of oocytes were made during application of an activation protocol described in **2.4.4.1**. The peak inward current of each oocyte was recorded at each

depolarising step in the protocol. **Fig5-1** shows a typical collection of traces from a single oocyte.

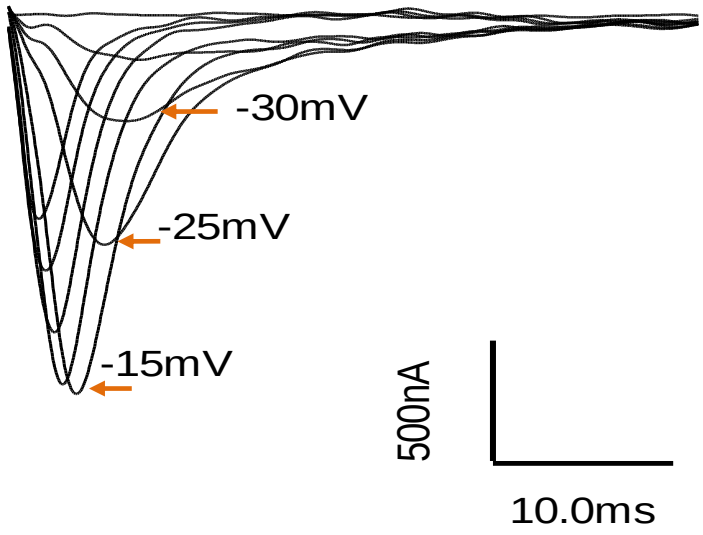

Fig5-1. Illustration of sodium currents induced from a single oocyte by the activation protocol in **2.4.4.1**. Some traces are omitted to give a clearer image. Traces ranging from -65mV to +25mV are illustrated.

These data were transformed and normalised to allow all oocyte recordings to be analysed together. A modified Boltzmann function was then applied (equation 1) to give a current-voltage relationship (**Fig5-2**). This was performed for the T929V and wild type channels both in the presence and absence of permethrin. The initial voltage dependence of activation for the channels was determined by calculating half activation (V_{50}).

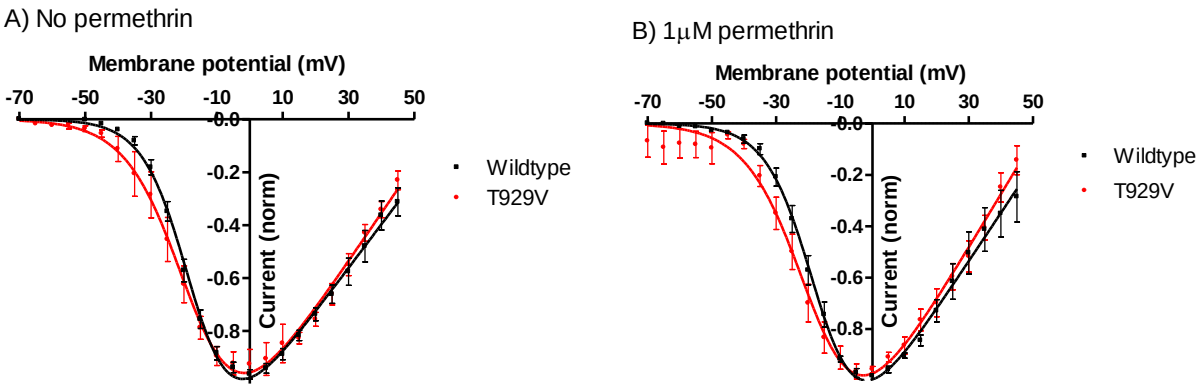

Fig5-2. Graphs showing the current-voltage relationship for wildtype VGSC and the T929V mutant in the absence of insecticide (A), **wildtype**: V_{50} = -16.91 ± 0.6, V_{rev} = 64.05 ± 2.0, *k* = 6.814 ± 0.4, n = 14. **T929V**: V_{50} = -18.35 ± 1.3, V_{rev} = 64.48 ± 3.8, *k* = 8.435 ± 0.8, n = 10. and in the presence of 1µM permethrin (B), **wildtype**: V_{50} = -16.33 ± 0.1, V_{rev} = 58.58 ± 2.4, *k* = 7.224 ± 0.6, n = 9. **T929V**: V_{50} = -17.72 ± 1.2, V_{rev} = 53.29 ± 2.0, *k* = 8.954 ± 0.7, n = 7.

The IV relationship gives an initial comparison of V_{50} values, a more accurate value can be obtained by looking at the conductance-voltage relationship (**Fig5-3**). This is calculated by extrapolating the V_{rev} value (voltage at which there is no net movement of ions across the membrane) from the IV curve when the current equals 0. This part of the curve is linear and so the extrapolation of the V_{rev} value is considered accurate. The V_{rev} value is then used to calculate conductance (equation 2, section **2.4.4.1**). This is considered a more accurate way of obtaining the V_{50} value.

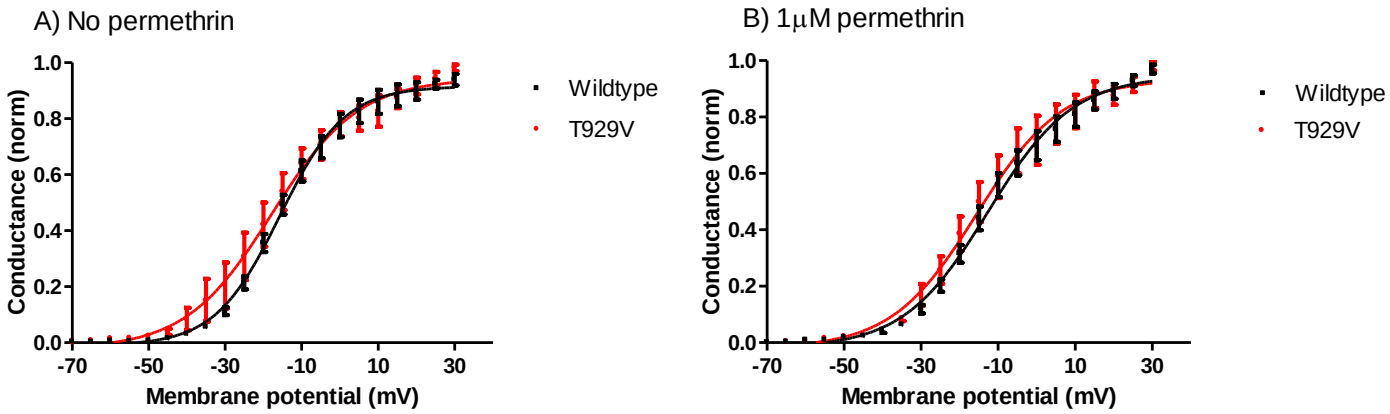

Fig5-3. Graphs showing the conductance-voltage relationship for wildtype and T929V mutant VGSC with and without permethrin. Without permethrin, **wildtype**: V_{50} = -15.72 ± 0.7, *k* = 8.478 ± 0.6, n = 14. **T929V**: V_{50} = -18.38 ± 1.6, *k* = 9.295 ± 1.5, n = 10. With 1μM permethrin, **wildtype**: V_{50} = -12.66 ± 0.9, *k* = 11.00 ± 0.9, n = 9. **T929V**: V_{50} = -15.52 ± 1.4, *k* = 11.09 ± 1.4, n = 7

To find out if there was a significant difference in activation between the wild type and T929V channels, two tailed t-tests were performed. Comparisons were also made between the each channel with and without permethrin.

No significant difference was found in activation between the wild type and T929V channel when permethrin was absent. (V_{50} comparison, F = 2.925, p = 0.0879. *k* comparison, F = 0.3112, p = 0.5773).

No significant difference was found in activation between the wildtype and T929V channel after application of 1μM of permethrin. (V_{50} comparison, F = 2.804, p = 0.0951. *k* comparison, F = 0.003, p = 0.9551).

When comparing the channels with and without permethrin, a significant difference was found between V_{50} values for the wildtype *para* channel (wildtype: V_{50} comparison, F = 6.306, p = 0.0124. *k* comparison, F = 1.929, p = 0.1656). No significant difference was found in the T929V channel (T929V: V_{50} comparison, F = 1.506, p = 0.2207, *k* comparison, F = 0.6052, p = 0.4372).

5.3 Inactivation

The protocol described in the methods **2.4.4.2** enables the study of steady state inactivation. The peak sodium current was measured after the -10mV depolarising pulse for each pre-pulse value.

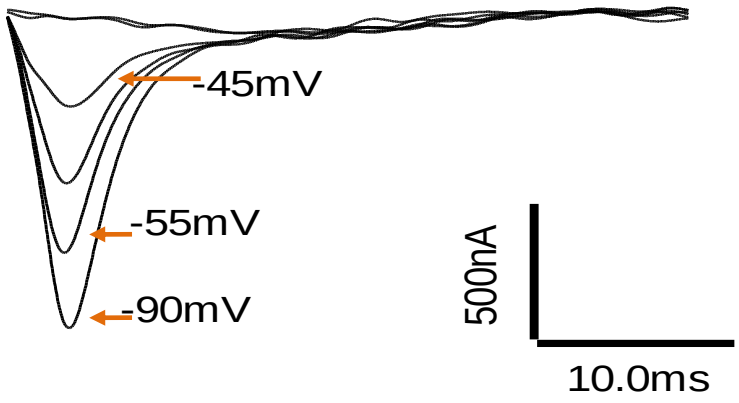

Fig5-4. Illustrates the sodium currents induced by the inactivation protocol (**2.4.4.2**) on a single oocyte. Traces shown range from -90mV to -20mV.

These data were transformed and normalised to analyse all oocytes together. Data points were then fitted with a simplified Boltzmann relationship (equation 3), **Fig5-5** illustrates a graph of

these data. The test potential that caused 50% of channels to be inactivated was estimated from the midpoint potentials.

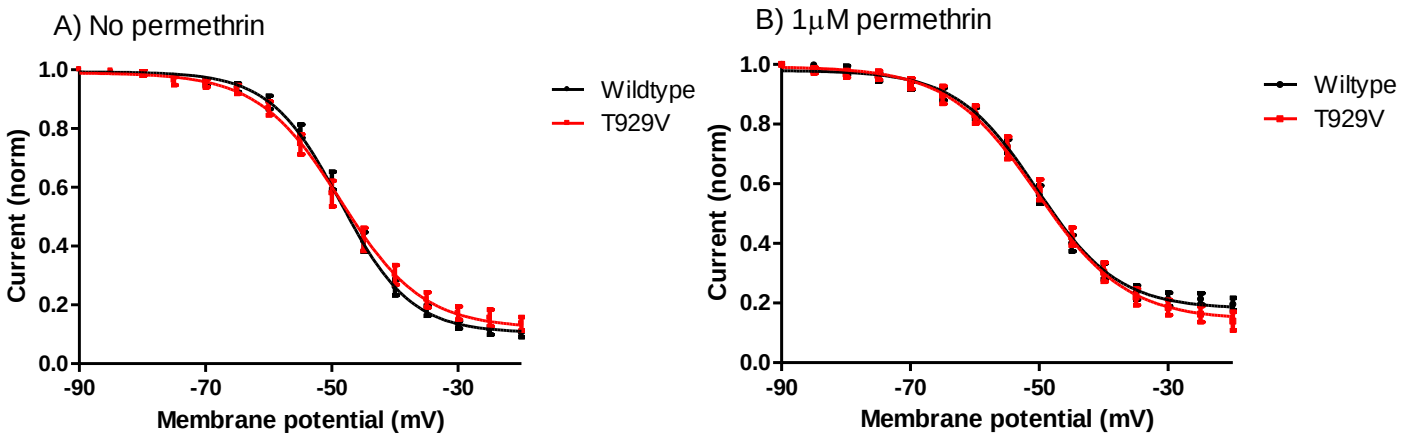

Fig5-5. Represents the current-voltage relationship for wild type and mutant channels with and without permethrin. In the absence of permethrin, **wildtype**: V_{50} = -48.18 ± 0.4, *k* = 5.618 ± 0.3, n = 8. **T929V**: V_{50} = -49.28 ± 0.6, *k* = -6.364 ± 0.5, n = 8. In the presence of 1µM permethrin, **wildtype**: V_{50} = -50.91 ± 0.5, *k* = 6.211 ± 0.5, n = 10. **T929V**: V_{50} = -49.50 ± 0.7, *k* = 7.018 ± 0.6, n = 8.

To find out if there was a significant difference in inactivation between the wild type and T929V channels, two tailed t-tests were performed. Comparisons were also made between each channel with and without permethrin.

There was no significant difference found between the wild type channel and the T929V channel either in the absence of permethrin or in its presence (no permethrin, V_{50} comparison: F = 2.664, p = 0.1040. *k* comparison: F = 1.446, p = 0.2305. 1µM permethrin, V_{50} comparison: F = 3.166, p = 0.0764. *k* comparison: ambiguous result).

There was no significant difference found between the wild type channel before and after permethrin application (V_{50} comparison: F = 2.214, p = 0.1380. *k* comparison: F = 0.0126, p

= 0.9108). This was also found to be the case for the T929V channel (V_{50} comparison: F = 0.06312, p = 0.8019. *k* comparison: F = 0.6319, p = 0.4275).

5.4 Tail currents

Section **2.4.4.3** describes the protocol used to elicit tail currents in VGSC. The slowing of channel inactivation and deactivation can give rise to these characteristic traits. They can therefore be used as a measure of the channels sensitivity to pyrethroids. **Fig 5-6** demonstrates the effect of permethrin on single wild type and T929V oocytes. A clear tail current can be seen to be present in the wild-type trace. Equation 4 was used to establish the percentage modification from the tail current amplitude. Equation 5 goes further by including the length of the tail current in the equation; because of this it is considered a more accurate analysis of the raw tail current data. As no tail current could be illicit on the T929V channels, the equations did not prove useful tool in comparing the modification of the two channel by 1µM permethrin.

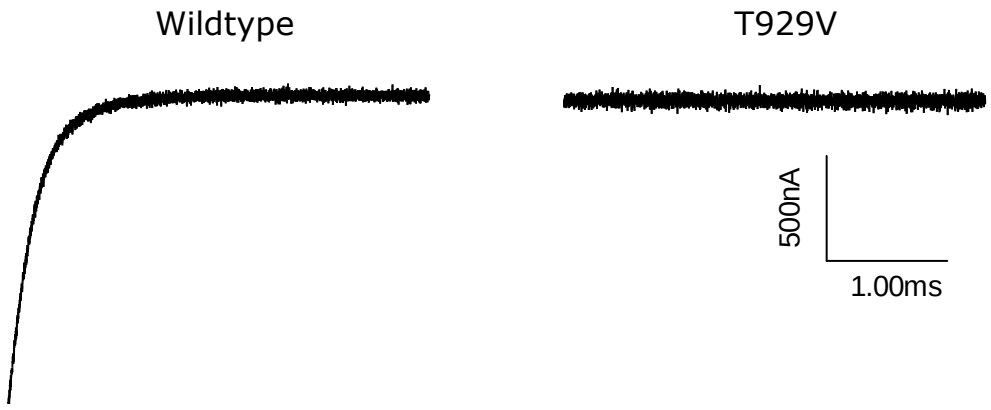

Fig5-6. Illustrates the tail currents elicited by 1µM permethrin on the wildtype and T929V channels. The difference in sensitivity to this type I pyrethroid can clearly be seen. No tail current was able to be elicited in the T929V channel using 1µM permethrin.

5.5 Discussion

The 929 site is found on domain II, segment 5 of the VGSC, at the binding site of pyrethroids and DDT (O'Reilly *et al*, 2006). Mutations at this site have been found to convey high levels of resistance to both pyrethroids and DDT *in vitro* and *in vivo* (Schuler *et al*, 1998; Atkinson, 2002; O'Reilly *et al*, 2006; Usherwood *et al*, 2007). The maize weevil, *Sitophilus zeamais* carried the T929I mutation. Usherwood *et al* (2007) previously found this mutation to convey high levels of resistance to deltamethrin, permethrin and to completely eliminate DDT sensitivity. This chapter examined the T929V mutation, which had previously been studied with deltamethrin and DDT. The potency of permethrin had not been examined on this channel.

Atkinson (2002) had found that the T929V channel showed a 5mV hyperpolarizing shift in activation when compared with the wild type para channel ($p = 0.011$). Here, a ~3mV shift in the negative direction was found for T929V ($V_{50} = -18.38 \pm 1.6$ mV) when compared with the wild type *para* channel ($V_{50} = -15.72 \pm 0.7$mV). However, this difference was not found to be significant when tested with a two tailed t-test ($p = 0.0879$). Atkinson also found there to be no significant difference between the inactivation V_{50}'s of the T929V channel and that of the wild type, this supports the results gained in this study.

O'Reilly *et al* (2006) included the T929I in their study of insecticide potencies of mutant sodium channels. They found that the T929I mutation has no effect on voltage dependence in the Drosophila channel. They concluded that the mutation conveys resistance by eliminating the polar interactions with pyrethroids, preventing them from locking in the open conformation of the channel where they wedge the IIS5 and IIIS6 open.

Regarding the sensitivity to pyrethroids, in this study it was found that 1μM permethrin could not elicit a tail current on the T929V channels, but gave a clear tail current on the *para* wildtype channels. Atkinson (2002) showed that 1μM deltamethrin was able to cause a tail current in the T929V channels, a decrease in sensitivity of ~2600-fold was estimated. This was probably due to the differences between type I and II pyrethroids. Type I pyrethroids are considered less potent than type II (O'Reilly *et al*, 2006). Usherwood *et al* (2007) found that the T929I mutant channel showed a ~100-fold decrease in sensitivity to deltamethrin, ~80-fold decrease to permethrin and completely obliterated DDT sensitivity. Usherwood was also unable to elicit a tail current on the T929V channel using DDT. The present study was unable to elicit a tail current in the T929V mutant using 1μM permethrin.

A difference in activation V_{50} was found in the wild type channel when permethrin was applied. A 3mV shift from -15.72 ± 0.7 when permethrin was absent to -12.66 ± 0.9 in the presence of 1μM permethrin ($p = 0.0124$). This was not as expected. It is predicted that pyrethroid modification would shift the half activation in a more negative direction, causing the channel to open at lower potentials and therefore more frequently (O'Reilly *et al*, 2006). Burton *et al* (2011) found a change in activation V_{50} from -17.3 in the wildtype to -21.3 when modified with 30nM permethrin.

The results found here support those reported in chapter 3, where *in vivo* studies on the maize weevil show that mutations at the 929 site convey high resistance to pyrethroids. Mutations at this super-kdr site pose a risk for resistance control when pyrethroids alone are used in control. As a recent study has shown (Correa *et al*, 2010b), the efficiency of this resistance mutation can be removed by combining a pyrethroid with an insecticide that has a different target within the nervous system.

6. Summary

6.1 Summary of the project

This study has covered target site resistance in the maize weevil, *Sitophilus zeamais*. The initial aims (**1.5**) stated that the objectives were: 1. To find a discriminating concentration of a pyrethroid or DDT to select for resistance in field populations of the maize weevil. 2. To determine the use of a taqman diagnostic assay in detecting the T929I mutation in the field, and 3. To use electrophysiology to study mutations at the T929 site *in vitro*.

The bioassay results showed that the T929I mutations conveyed a 833-1524-fold decrease in sensitivity to deltamethrin and a 80-105-fold decrease to DDT. This result was supported by other bioassays on weevil populations (Guedes *et al*, 1996; Ribeiro *et al*, 2003; Correa *et al*, 2010) and by a bioassay on the diamondback moth which carries the T929I mutation with the L1014F kdr mutation (Schuler *et al*, 1998). The vial bioassays were shown to be limited, in that it was difficult to reach high levels of mortality in the resistant populations, topical bioassays where the insecticide was applied directly to the insect gave more extensive results.

The lab susceptible population from Sete Lagoas showed higher LD_{50}'s than was expected from previous data. It was therefore decided that the discriminating dose would be taken from initial bioassays performed on the population by R.A Araujo. 0.5 and 1mg/ml DDT were selected to be applied in vials to the 12 field populations of unknown resistance.

After selection for resistance, samples from the field populations which appeared to show resistance to DDT were tested for the T929I mutation using a taqman diagnostic assay. No resistant homozygous or heterozygous individuals were found in the field populations. The lab susceptible Sete Lagaoas populations scored as having 2 homozygous resistant

individuals, 3 heterozygotes and 7 homozygous susceptible individuals. This confirmed the suspicions raised by the bioassays.

To determine whether another form of resistance, such a metabolic resistance, was present in the field populations a bioassay using 1mg/ml DDT was performed on them and the lab "susceptible" population. None of the field populations showed a significantly lower mortality level than the lab susceptible population. However, one population did show a significantly higher mortality than the Sete Lagoas population. This supported the presence of resistance in Sete Lagoas susceptible population further and also means that low levels of metabolic resistance in the field population may not have been detected.

The lack of the resistance allele in the field is probably due to the current mix of insecticides used. A pyrethroid is applied in combination with an organophosphate (Correa *et al*, 2010b). The T929I mutation would be useless against the organophosphate, which targets AChE. This may have led to the decline in the resistant allele due to a fitness cost (Coustau *et al*, 2000; Araujo *et al*, 2008). Although the taqman diagnostic assay did not prove useful in detecting the T929I resistant allele in the field, it could still be a useful field tool to detect other forms of resistance as it is very quick and easy to perform.

The electrophysiological work on the T929V mutant channel showed this mutation to convey high levels of resistance to permethrin. 1µM permethrin was unable to elicit a tail current in this mutant. Thus, supporting previous findings that mutations at the T929 position of the VGSC give high resistance to pyrethroids.

6.2 Future work

The work here could be taken forward in a couple of ways. It would be interesting to compare more extensively the differences in mutations at the T929 site. The T929C *para* VGSC transcript could be created. It would be interesting to see the effect the different amino acids at this position have on activation, inactivation and the level of resistance they convey to pyrethroids and DDT.

Secondly, the taqman work could be taken forward as low levels of resistance to the new combination of pyrethroid and organophosphate insecticides has been reported (Correa *et al*, 2010b). It is likely that this is due to metabolic resistance, as any target site resistance to the pyrethroid would be ineffective against the organophosphate. Therefore, a diagnostic assay for metabolic resistance could be useful in the field. However, the taqman assay may not be appropriate for this as metabolic resistance is often conveyed by an over expression of detoxifying enzymes, gene amplification and transcriptional regulation. These would be more difficult to detect than a single amino acid change which causes target site resistance.

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