# OPTIMIZATION AND USE OF A VOLTAGE CLAMP ASSAY

# WTH INSECT MIDGUT TISSUES

by

DeAnna Lee Borchardt Steiger

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Entomology and Applied Ecology

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**OPTIMIZATION AND USE OF A VOLTAGE CLAMP ASSAY**

**WITH INSECT MIDGUT TISSUES**

by

DeAnna Lee Borchardt Steiger

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# ABSTRACT

Voltage clamp methods, developed to study electrical properties of tissues, were investigated and optimized for use with insect midgut tissues. In optimizing the methodology for use with small tissues, the resistance between electrodes was reduced. The voltage clamp amplifier, ionic strength of the buffer, tissue surface area of the annulus and distance between electrode tips were each investigated and optimized. An optimized voltage clamp chamber was designed and was a major accomplishment of this work. The optimized methods were used to assay *Bacillus thuringiensis* sensitivity in midgut tissues of fifth instar *Lymantria dispar*. Two populations, a laboratory population and Manitowoc, WI population, were subjected to the Bt sensitivity assay. The two populations responded in a dose-dependent manner. At the 1400ng dose, the two populations responded differently, in terms of rates of decay and percentage of short-circuit current remaining. Tissues from a New Jersey population were assayed for the control, but were not subjected to the Bt sensitivity assay due to time and equipment constraints. Midgut tissues extracted from larvae collected from a naturally occurring population in Slovakia did not produce short circuit current like the other populations, suggesting variation in physiology between the native and introduced populations. The toxin binding region of the midgut toxin binding protein showed no amino acid sequence variation between populations. Variation in Bt sensitivity between populations may be due to variation in the toxin binding event or other factors. The optimized voltage clamp assay could elucidate physiological phenomena in insect tissues.

# Chapter 1

## INTRODUCTION

Most Feeding assays used to identify potential insecticides are time and resource intensive. Results from *in vivo* studies are uninformative in elucidating the mechanism of toxicity and are difficult to extrapolate to field populations (Robertson and Preisler 1991). A rapid-response alternative to costly bioassays is necessary. Development of an efficient technique to identify potential insecticides would immediately benefit many research areas.

The mode of action of *Bacillus thuringiensis,* or Bt, toxins permits such a technique. Bt toxins disrupt the electrical potential across the midgut membrane in susceptible insects by creating pores in the membrane of midgut cells. This occurs by insertion of a toxic protein, produced by the bacterium, into the cell membrane. This produces a nonselective pore in the cell and normal physiological activity is disrupted. By monitoring the electrophysiological activity of the midgut tissue, susceptibility can be determined rapidly.

The gypsy moth, *Lymantria dispar* Linnaeus 1758 (Lepidoptera: Lymantriidae), is controlled by Bt applications across its introduced range in the United States. Like many lepidopteran insects, the gypsy moth maintains a highly alkaline midgut environment which is destroyed when Bt toxins are ingested. The gypsy moth has shown varied response to Bt within and between populations (Rossiter *et al.*1990), but the mechanism of this variation has not been identified. A voltage clamp investigation could elucidate the mechanism for varied sensitivity. If variation

between populations is due to the toxin-receptor interaction, results from voltage clamp experiments will show varied toxin response as only the toxin-receptor interaction is required for sensitivity in the experimental setup. Further investigation into the toxin receptor protein on the apical surface of the insect midgut may explain the source of variation in susceptibility as well.

## ***Bacillus thuringiensis***

The gram-positive bacterium *Bacillus thuringiensis* is favored over traditional insecticides due to target pest specificity and safety. Bt is widely used by organic growers to control lepidopteran pests of cotton, fruits and vegetables (Whalon and Wingerd 2003). Significant resources have also been used to develop Bt strains and formulations for forest and public health pests. A fundamental disadvantage of this type of insecticide is that it is necessary for the target insect to ingest enough toxin to cause death. In addition, insecticidal formulations are degrade rapidly once applied (Whalon and Wingerd 2003), and climatic variation may interfere with the timing of pesticide application with insect phenology (Rossiter *et al.* 1990). Despite these limitations, Bt use is widespread within, and beyond, North America.

Bt toxins disrupt physiological processes in the midgut of susceptible insects. Lepidopteran larvae maintain a midgut environment several pH units more basic than the hemolymph (Berenbaum 1980) by the active transport of $K^+$ ions to the lumen of the midgut (Harvey and Wolfersberger 1979). This results in an alkaline midgut environment and an electrical potential across the midgut membrane that may aid in digestion. Bt toxins bind to the membrane and create a pore, allowing $K^+$ ions to move passively from lumen to hemolymph, destroying the pH gradient as well as the potential difference across the midgut membrane.

## ***Bacillus thuringiensis* Classification**

At sporulation, during the stationary phase of growth, *Bacillus thuringiensis* produces a parasporal crystal that possesses insecticidal properties and has been intensely studied (Schnepf *et al.* 1998). These crystals, when ingested by susceptible insects, disrupt the digesive tract causing insect death. As more and more crystal protein genes and their protein products were sequenced and described, standardizing the classification of these genes became more and more important. Two classification schemes were developed.

The first nomenclature system to standardize classification of toxins categorized genes (encoding crystal forming proteins) into four classes based on the size of the protein products and insect host (Höfte and Whiteley 1989). CryI proteins (encoded by the cryI gene) are effective against lepidopteran larvae, CryII against lepidopteran and dipteran larvae, and CryIII towards certain Coleoptera; cryIV genes encode proteins effective against dipteran larvae in the suborder Nematocera. This classification scheme relied on efficacy tests in combination with molecular analysis. Uninformative classifications resulted from cases where efficacy tests suggested one classification while molecular analysis results suggested another (Schnepf *et al.* 1998). CryIIB, for example, was placed among other CryII toxins because molecular investigation suggested the cryIIB gene was homologous to cryIIA yet no toxicity toward Diptera could be demonstrated (Crickmore *et al.*1998).

The more recent nomenclature system eliminated the need to conduct efficacy tests and relied on nucleotide and assumed amino acid sequence analysis to provide phylogenetic relationships (Crickmore *et al*. 1998). The revised nomenclature reflected phylogenetic information about the genes and their proteins while minimizing name changes from the old system. In most cases, genes which were

closely related produced proteins that were effective against the same, or closely related, insects. The authors also addressed the definition of Cry protein. They defined a Cry protein as:

> a parasporal inclusion (crystal) protein from *B. thuringiensis* that exhibits some experimentally verifiable toxic effect to a target organism, or any protein that has obvious sequence similarity to a known Cry protein (Crickmore *et al.* 1998).

Genes with the same primary rank (notated by an Arabic number in the updated scheme) often encoded proteins that affected the same order of insects while genes with different secondary and tertiary ranks (notated by a capitalized letter, and a lower-case letter, respectively) usually encoded proteins that were toxic to different insects within the order (Crickmore *et al*.1998).

## **Mode of Action**

The crystal is comprised of protoxin proteins that must first be solubilized in the midgut and cleaved by proteinases to become active toxin molecules (reviewed by Schnepf *et al.* 1998). Lepidopteran larvae maintain a highly alkaline midgut environment that dissolves the crystal and solubilizes the protoxins (Lightwood *et al.* 2000). Cry1 proteins are water soluble at pH 9.5 (Gill *et al.* 1992). Following solubilization in the midgut, the protoxins pass through the peritrophic membrane and go through a series of cleavage events by proteolytic enzymes (Höfte and Whiteley 1989). For the Cry1 proteins, trypsin-like or chymotrypsin-like proteinases cleave the protoxin protein from the C-terminus toward the N-terminus (Lightwood *et al.* 2000).

The activated Cry1 toxin is a 60-70-kd proteinase-resistant fragment of the N-terminus (Lightwood *et al.* 2000). The activated toxin then binds, first reversibly and then irreversibly, to the receptor on the surface of the insect midgut

membrane cell (Hofmann *et al.* 1988). Reversible binding consists of toxin-receptor recognition and irreversible binding involves insertion into the membrane and pore formation.

Different parts of the toxin protein have specialized functions, as suggested by the structure of each domain. The Cry1A toxins have three domains (Grochulski *et al.*1995). Domain I is comprised of eight helices, domain II of three β-sheets and domain III of two β-sheets in a sandwich formation. Solubility of the molecule may be partially determined by tertiary structure (Gill *et al*. 1992). Domain I, a hydrophobic and amphipathic region, aids in pore formation through insertion of the helical hairpin into the midgut cell membrane (Aronson *et al.* 1992). Site-directed mutagenesis experiments suggested that domain II of the Cry toxin binds reversibly to the apical surface of the midgut cell membrane (Rajamohan *et al.* 1996). The role of domain III has not yet been clearly identified. It may play a role in receptor binding or maintenance of structural integrity (Li *et al*. 1991).

Ingested, solubilized, proteolytically processed toxins bind to receptors on the apical surface of midgut cells. Much research has focused on identifying and describing these receptors. In *L. dispar*, the Cry1Ab toxin binds to a 210 kDa protein distinct from the APN receptor protein for Cry1Ac (Lee *et al.* 1996a). In *M*. *sexta* the 210 kDa protein, named BT-R (Bt receptor), is a cadherin protein containing 12 extracellular repeat domains, a membrane-proximal extracellular domain, a membrane-spanning domain and a cytoplasmic domain (Hua *et al.* 2004). The 169-amino acid sequence of BT-R adjoining the membrane-proximal domain is capable of binding Cry1A toxins (Dorsch *et al.* 2002). This toxin binding region (TBR) is found on cadherin repeat 11. Cadherin repeat 12, the membrane-proximal extracellular

domain, was the essential domain for Cry1Ab toxicity (Hua *et al.*2004). Cadherin repeats 11 and 12 appear to be essential to Cry1Ab toxicity.

The reversible binding event of toxin-receptor recognition is followed by an irreversible binding event. During irreversible binding, domain I of the toxin is inserted into the apical surface of the midgut membrane cell (Aronson *et al.* 1992). Voltage clamp studies confirm that toxin-receptor irreversible binding results in nonspecific pore formation in membrane cells (Harvey and Wolfersberger 1979).

There are two models describing how toxin insertion results in a non-selective channel. The umbrella model suggests that a helical hairpin of domain I of the toxin inserts into the midgut membrane cell as other toxins aggregate (Li *et al.* 1991). This results in a toxin conformation change, which creates a shape similar to an opening umbrella that produces a non-selective pore allowing ions to flow into the cell, resulting in lysis. The pen-knife model suggests that helices of domain I of the toxin insert into the cell while the rest of the toxin molecule remains outside (Hodgman and Ellar 1990). Both models agree that nonspecific pores allow water and ions to enter cells according to an ionic gradient, causing swelling and eventual cell lysis. Midgut tissue is destroyed by the toxin, causing insect starvation and death.

Variability in receptor sites is a key component of Bt specificity. Insects that are susceptible to Bt have high affinity binding of toxin to receptor sites on the brush border membrane whereas resistant insects can lack these sites (van Rie *et al*. 1990). Surprisingly, in some cases, there is no correlation between binding affinity and toxin efficacy (Schnepf *et al*. 1998). Binding affinity is thought to be a part of reversible binding, while toxin efficacy is the result of irreversible binding.

## ***Lymantria dispar***

The European gypsy moth, *Lymantria dispar*, is an invasive species that continues to defoliate forested areas in New England and the Mid-Atlantic region of the U.S. In recent years, areas of the Mid-West have been invaded. Forest tracts containing high percentages of oak trees are most susceptible to defoliation and tree death (Campbell and Valentine 1972 as cited in Davidson 2001).

The relationship between gypsy moth larvae and their hosts is complex. Oak leaves produce tannins which may be a defense mechanism against herbivory. The tannins, when ingested by larvae, block digestion by creating large macromolecules, preventing nutrient absorption (Feeny 1970). Like other herbivorous insects, (particularly Lepidoptera), gypsy moth larvae maintain a highly alkaline midgut environment to prevent the formation of these macromolecules (Berenbaum 1980). As described previously, the highly alkaline midgut environment plays an important role in the activation of Bt toxins.

Gypsy moth larvae were shown to exhibit variation in susceptibility to Bt both within and between populations in *in vivo* studies (Rossiter *et al.* 1990). Within three wild populations collected in Pennsylvania and one laboratory population (Pest Survey Detection and Exclusion Laboratory Otis ANGB, MA), families exhibited variation in their response to Bt. There were also significant differences between in Bt sensitivity between populations. Also worth noting was the important finding that the lab strain had an $LC_{50}$ approximately twice that of wild populations.

Variation in susceptibility to Bt may be due to several components that are impossible to elucidate with *in vivo* studies. Altered proteolytic processing or modified binding of the toxin to midgut membrane cells can cause variation in susceptibility or resistance (Whalon and Wingerd 2003). Shedding or rapid repair of

midgut membrane cells in response to Bt intoxication can also result in altered sensitivity (Ferre´ and Van Rie 2002). Although resistance has not been detected in the gypsy moth, field populations have shown reduced and varied susceptibility to Bt applications (Gimble and Lewis 1985).

**Voltage clamp**

Voltage clamp methods have been used to study insect physiology and susceptibility to *Bacillus thuringiensis* insecticidal toxins (Peyronnet *et al.* 1997; Harvey *et al.* 1990; Rajamohan *et al.* 1996; Lee *et al.* 1996a; Lee *et al.* 1996b). The voltage clamp assay allows investigators to monitor the ability of the midgut tissue to transport ions and maintain a gradient (Harvey *et al.* 1990). Although this technique has been used in several investigations, few authors have described the detailed specifications of their materials and methods. The single most thorough description of the technique came from Harvey *et al.* (1990).

In voltage clamp experiments, midgut tissue is excised from larvae and placed on an annulus which is bathed in physiological buffer (Figure 1). The annulus is mounted between two half cells of a chamber, with buffer bathing it on both sides. On either side of the tissue, a current electrode and a voltage electrode monitor changes in the buffer as the tissue transports ions. The electrodes are connected to an amplifier which allows investigators to record difference in potential across the midgut tissue, current generated under open-circuit conditions, or current generated under short-circuit conditions.

In an open-circuit situation, the active transport of $K^+$ is accompanied by passive movement of $Cl^-$ ions across the ionic gradient (Harvey *et al.* 1990). This results in a gradient between the hemolymph and lumen sides of the tissue. Under

short-circuit conditions, however, the concentration of $K^+$ ions and $Cl^-$ ions on hemolymph and lumen sides are equal. This results in no potential difference or gradient between the two halves of the chamber. In this situation, there can be no passive movement of $Cl^-$ ions. The current electrodes move $Cl^-$ ions from the electrode pellet to the buffer solution on the lumen side and from the buffer solution to the pellet on the hemolymph side, thus maintaining electroneutrality between the two sides of the chamber and creating a current flow as the tissue actively pumps $K^+$ ions from hemolymph side to the lumen side. The current generated by $Cl^-$ movement is equal to the amount of $K^+$ transport by the gut tissue. Thus, short-circuit conditions allow investigators to monitor the ability of the midgut tissue to actively transport $K^+$. When Bt toxin is added to the buffer on the lumen side, the toxins produce pores on the surface of midgut cells, allowing $K^+$ ions to move by ionic gradient toward the hemolymph side. Although the tissue is still able to pump $K^+$ ions, the ions move passively down their ionic gradient and no gradient can be maintained by the tissue sample.

Voltage clamp methods have been used to quantify Bt susceptibility in agricultural insect pests (Karim *et al.* 2000). These investigations demonstrated that midgut tissues respond in a dose-dependent manner and also that voltage clamp technology can be used to quantify toxin sensitivity.

Previous investigations using voltage clamp methods with insect models are few. One reason may be in the inherent difficulty of working with small tissue samples. Voltage clamp chambers that are available for purchase are made for larger tissue samples so in order to use voltage clamp methods for insects, a specialized chamber must be constructed. Previous investigations have used the chamber designed

by Dow *et al.* (1985). This chamber has a lollipop-like apparatus on which the tissue sample is mounted. The lollipop is then inserted into the chamber in a tight-fitting slot. After learning about voltage clamp methods and attempting to use both the lollipop and commercially available chamber, I decided to optimize my set-up by designing a new chamber that employs the strengths of both models and does not lose sight of accessory availability.

## Objective

Voltage clamp methods were developed and modified to investigate physiological activity of *L. dispar* midgut tissues. Because *in vivo* studies showed that Bt sensitivity varies widely between and among populations of *L. dispar,* I hypothesized that voltage clamp methods could be used to quantify differences in susceptibility between populations to the *Bacillus thuringiensis* toxin Cry1Ab. To determine if this variation in susceptibility was due to differences in the Bt receptor, nucleotide and amino acid sequence variation in the toxin binding region of the Bt receptor were investigated.

## Chapter 2

## MATERIALS AND METHODS

### Larvae and Tissues

Fifth instar *L. dispar* were randomly selected from a laboratory population maintained by the Beneficial Insects Research Laboratory in Newark, DE. Egg masses were collected by volunteers in Slovakia and reared in quarantine according to BIRL protocol. Egg masses were also collected from Manitowoc, WI and southern NJ, USA and reared according to BIRL protocol. $F_1$ fifth instars from Slovakia, and fifth instars from New Jersey, Wisconsin, and the laboratory population were used in the voltage clamp study. *L. dispar* were collected from regions where Bt has, and has not, been used as a control method. Bt has not been used in Slovakia, but has been widely used in New Jersey, USA. Bt has not been used in Manitowoc, WI as this is the leading edge of the invasion. Fifth instars were randomly selected from each population for molecular analysis of the Bt toxin receptor. From those individuals, whole midgut tissue was excised as described for the voltage clamp assay and used for RNA extraction.

### Buffer Solutions

The buffer solution for the voltage clamp experiments with *L. dispar* tissue consisted of 32 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 240 mM sucrose and 5 mM Tris HCl (Harvey *et. al.* 1990). The pH was adjusted to 8.3 by addition of 3.5%

NaOH. The solution was perfused with $O_2$ for twenty minutes before use and continuously throughout the experiment.

For voltage clamp experiments without tissues, two additional buffer solutions were prepared to investigate fluid resistance. Complex buffer, similar to that of Chamberlin (1990) contained 2mM Hepes, 5mM $MgCl_2$, 1 mM $CaCl_2$, 5.8 mM KOH, 9 mM NaOH, 3.0 mM sodium methylsulfphate, 7.7 mM potassium citrate, 2.8 mM sodium succinate, 5.6 mM malic acid, 2.0 mM glucose, 27.0 mM trehalose, 9.4 mM glutamine, 8.9 mM serine, 7.4 mM proline, 12.8 mM glycine, 4.6 mM threonine, 3.6 mM alanine and 23.1 mM N-methyl-D-glucamine. Also, 3M KCl was prepared and used to investigate fluid resistance.

**Toxins**

Activated Cry1Ab Bt toxins were generously prepared and provided by Gang Hua and Mike Adang at University of Georgia, Athens, GA.

**Voltage Clamp Set-Up**

The DVC 1000 voltage clamp, EK1 Ag/AgCl electrodes, Ussing chamber stand and gas lift circulation reservoir were purchased from World Precision Instruments, Incorporated (Sarasota, FL). AcqKnowledge acquisition and data analysis software from Bio Pac Systems, Incorporated (Goleta, CA) was used. The chamber and annulus (Figure 1) were fabricated by the Chemical Engineering machine shop at University of Delaware (Newark, DE) from acrylic purchased from United States Plastics Corporation (Lima, OH). High Vacuum Grease (Dow Corning Corporation, Midland, MI) was used to prevent leaking at the electrode/chamber interface. The circulation reservoir was connected to the chamber with male luer and

hose barb adapter fittings from Cole-Parmer (Vernon Hills, IL) and 3/16" inner diameter Tygon class VI tubing.

## Methods

### Midgut Tissue

After the weight of an individual larva was recorded, it was dissected. A dorsal longitudinal cut exposed the gut tissue and all remaining internal connections were severed. The whole gut was removed and opened longitudinally. The midgut was placed on the annulus, held in place by the annulus cover, and excess tissue was removed. The annulus was then mounted between the two halves of the voltage clamp chamber and the chamber was filled with buffer solution.

### Tissue Surface Area

To investigate the affect that surface area of exposed tissue has on resistance between electrodes, three annuli of varying tissue surface area were made and fluid resistance was measured using the DVC 1000 voltage clamp according to the manufacturer's instructions. Annuli one, two and three had openings 4.71, 7.07 and 12.57 $mm^2$, respectively. These annuli exposed midgut tissue equal in area to these holes. The complex buffer solution (Chamberlin 1990) was used throughout these experiments.

### Distance Between Electrodes

To design a chamber that could reduce the amount of resistance between the electrodes, the effect of distance between voltage measuring electrodes was investigated. To determine the relationship between resistance and distance between

the electrodes, two voltage measuring electrodes were placed in complex buffer (Chamberlin 1990). The electrode tips were held close together, then moved slowly apart.

**Ionic Strength of Buffer**

Three buffers of varying KCl concentration, as described previously, were prepared and the resistance compensation necessary for each was measured by the DVC 1000 voltage clamp. An annulus with a tissue surface area of 4.71 $mm^2$ was used in this experiment.

**Voltage Clamp Data Analysis**

Data were collected for 60 minutes at a rate of one sample per second. Samples were then averaged to produce the average short-circuit current for each minute. For each dose, the percentage of average short-circuit current remaining was calculated beginning at minute 20 (time of toxin addition) with minute 20 being considered 100%. Average percentages were compared between doses and between populations at different times using analysis of variance and t-test pairwise comparison with SAS software (SAS Institute 1982). SAS was also used to determine the slope for each dose for each population with the general linear model procedure and arcsin transformation. The average percentage of $I_{sc}$ remaining was compared by probit analysis. To determine if differences in slopes were statistically significant, the Z values were calculated according to Keil and Othman (1988). Z values $\geq$ 1.96 were considered significant, $p \leq 0.05$.

**Molecular Analysis**

To investigate population differences in nucleotide sequence differences of the gene encoding the Cry1Ab Bt receptor, BT-R, midgut tissue was excised from fifth instars in each population and RNA was extracted according to Qiagen RNeasy Midi/Maxi RNA Extraction Kit protocol for animal tissues (Valencia, CA). For New Jersey, Manitowoc, WI and Slovakia populations, n=3. For the laboratory population, n=2. RNA concentrations were quantified with a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). 19-mer primers were designed from published sequences of the BTR from *Lymantria dispar* (GenBank accession number AF317621) and *Manduca sexta* (GenBank accession number AF319973). The amplified region corresponds with the toxin binding region within cadherin repeats 11 and 12 on the toxin receptor (Dorsch *et al.* 2002; Hua *et al.* 2004). Primers Lym-F (5'-3') CTTCCACGATCCAGTGTTC and Lym-R GCACTATCCGTACTGATGTC were produced by Sigma Genosys (The Woodlands, TX). RNA samples were then reverse transcribed and amplified by a one-step reaction according to Qiagen OneStep RT-PCR Kit protocol. Products were purified according to QIAquick PCR Purification Kit protocol using a microcentrifuge. cDNA products were then quantified with the NanoDrop ND 1000 and separated on 0.8% agarose gel. Sequencing reactions were carried out by the Allen Laboratory at University of Delaware (Newark, DE). Forward and reverse sequence products were then trimmed and aligned with DNAStar EditSeq software (Madison, WI). The amino acid sequence for each sequence was then predicted. Predicted amino acid sequences from all four populations were aligned and compared to the published *Lymantria dispar* BtR and *Manduca sexta* amino acid sequences. Similarity in the 67 amino acid toxin binding region was determined with DNAStar.

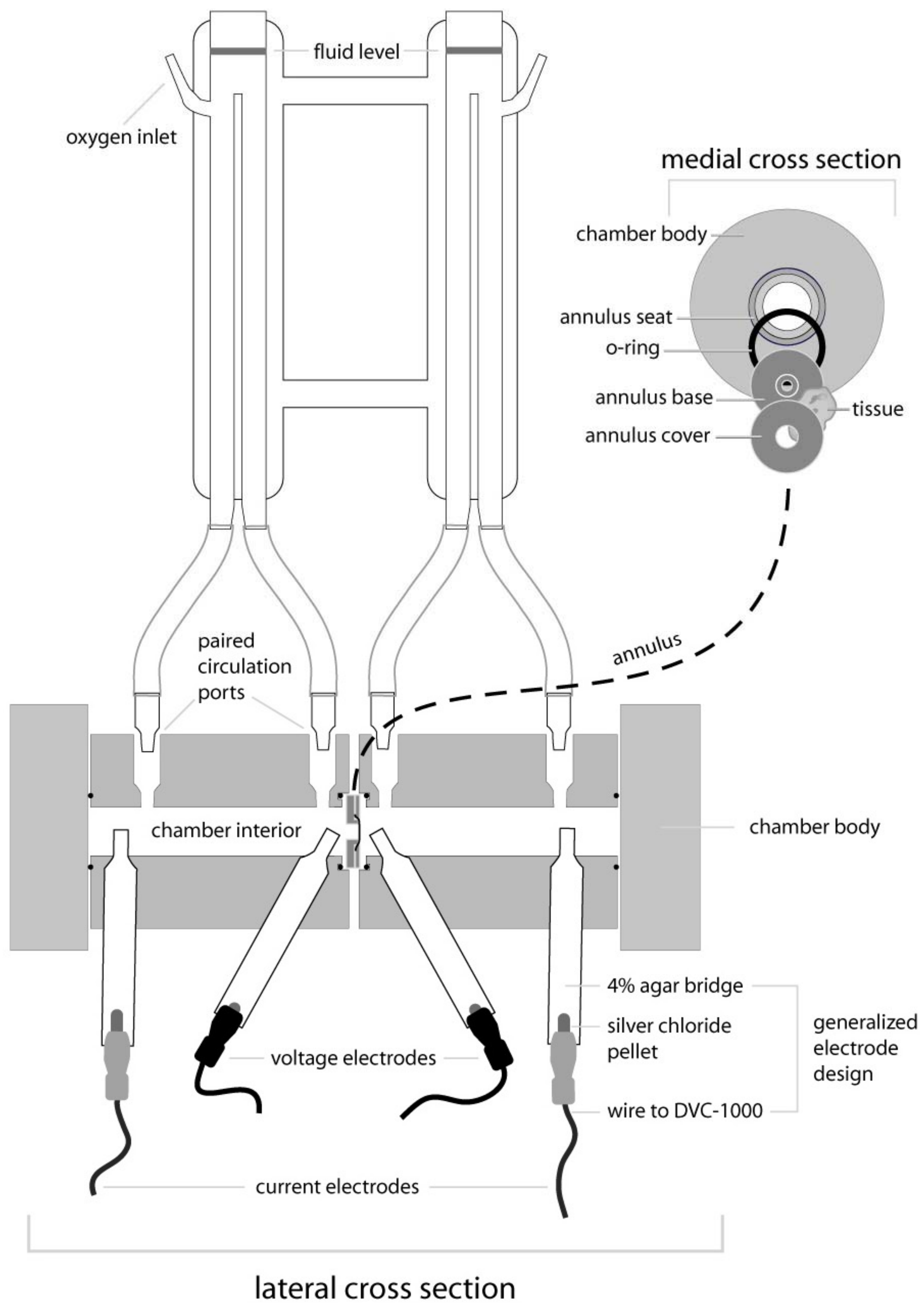
fluid level
oxygen inlet
medial cross section
chamber body
annulus seat
o-ring
annulus base
tissue
annulus cover
annulus
paired circulation ports
chamber interior
chamber body
4% agar bridge
silver chloride pellet
generalized electrode design
voltage electrodes
wire to DVC-1000
current electrodes
lateral cross section

**Figure 1. Optimized Voltage Clamp Chamber Assembly.**

# Chapter 3

# RESULTS

## Voltage Clamp Optimization

Initial attempts to compensate for fluid resistance were unsuccessful; resistance was too great to be compensated for by the amplifier. Resistance between voltage-measuring electrodes was investigated in order to design an optimized voltage clamp chamber that would hold the tissue sample and buffer solution and reduce resistance. Three factors influenced resistance in the voltage clamp chamber; distance between the electrodes, ionic strength of the buffer, and tissue surface area of the annulus which separated the electrodes. As the electrode tips were moved apart, the resistance between them increased. The ionic strength of the buffer also affected the resistance between electrodes. For 3M KCl, the buffer with the strongest KCl concentration, 30mV of compliance voltage was necessary in order to compensate for the resistance of the buffer. For 96 mM buffer, 700mV of compliance voltage was required. The voltage clamp was unable to produce enough compliance voltage to compensate for the resistance of the 10 mM buffer, and so returned a value of "resistance overload". The surface area of exposed tissue on the annulus was also investigated. The voltage clamp was unable to compensate for the resistance between electrodes when the tissue surface areas exposed by the annuli were 4.71 and 7.07 $mm^2$. The annulus that exposed a tissue surface area of 12.57 $mm^2$ required 780 mV of compliance voltage to compensate for the resistance. The annulus with the largest

exposed tissue surface area required the least compensation and was within the operating parameters of our equipment.

The results of these experiments led us to design an optimized chamber with the electrode tips as close together as possible (Figure 1). The tissue surface area of the annulus was maximized and the buffer solution was chosen based on the KCl content and viability of the tissue. The resulting chamber consisted of two halves held in place with pins and with the annulus base and annulus cover mounted between the two halves in a recessed cavity. The voltage electrodes were inserted into the chamber at an angle so the tips nearly touched, but allowed room for the annulus to be mounted between them. Ports were drilled perpendicular to the electrode ports for connection to the gas lift circulation reservoir. This optimized setup allowed for successful, repeatable voltage clamp experiments with *L. dispar* midgut tissues.

**Voltage Clamp Assay**

Midgut tissues from fifth instars from a laboratory population were assayed by voltage clamp. Control tissues were allowed to produce short-circuit current for 60 minutes without the addition of toxin. After 60 minutes, midgut tissues from the laboratory strain of gypsy moth (n=7) were able to produce an average of 61.63% of the $I_{sc}$ they produced at minute 20. Tissues from the Manitowoc, WI population (n=5) were able to produce an average of 79.10% of the short-circuit current produced at minute 20.

After 20 minutes of stabilization under short-circuit conditions, Bt was added to the lumen side of the chamber with a micro syringe. Doses ranged from 0 ng to 1800ng of Cry1Ab toxin (Figure 2). The tissues responded to activated Bt toxin in a dose-dependent manner. The $I_{sc}$ response to eight different doses was investigated

with tissues from the laboratory population and, from those data, four were designated as the informative doses that would also be applied to the Wisconsin population. For both the laboratory (Figure 3) and the Wisconsin populations (Figure 4), increased dosages resulted in greater reduction in short-circuit current. Higher dosages also resulted in more rapid inhibition of short-circuit current than lower dosages. Differences between populations were seen at the 1400ng dose of toxin (Table 1).

Addition of 400ng of Cry1Ab reduced short-circuit current to 93.24% for the laboratory population (n=8) and 86.19% for the Wisconsin population (n=5) after 5 minutes. At 35 minutes (15 minutes after addition of 400ng of Cry1Ab), the average $I_{sc}$ produced by the tissues from the laboratory population (n=8) was 55.57% of that produced at the time of toxin addition. The average $I_{sc}$ was 59.47% for the tissues sampled from the Wisconsin population (n=5). $I_{sc}$ was reduced to 85.22% in the laboratory population and 89.78% in the Wisconsin population 5 minutes after the addition of 1400ng of Cry1Ab. $I_{sc}$ was further reduced to 32.44% and 57.15% for the laboratory and Wisconsin populations respectively, 15 minutes after the addition of toxin. $I_{sc}$ was reduced to 88.41% five minutes after the addition of 1800ng of toxin and 25.54% after 15 minutes in the laboratory population. Tissues from the Wisconsin larvae produced 84.89% of the $I_{sc}$ produced at the time of toxin addition 5 minutes after the addition of 1800ng. After 15 minutes, the tissues produced 31.33% of the short-circuit current produced at the time of toxin addition.

Differences between the two populations in mean percentage $I_{sc}$ remaining were calculated and compared (Table 1). The rates of decay were also computed using the general linear model procedure in SAS (Table 2). Z values were calculated to determine if the slopes at each dose differed between populations (Table 2). The Z

value of the 1400ng dose was 5.43, indicating that the two slopes were significantly different between populations at that dose. All other Z values were insignificant.

The responses of the two populations were also evaluated with probit analysis. Plotting the probit value of the average percent $I_{sc}$ remaining at 35 minutes against the dose ($\log_{10}$) suggested a similar amount of variation in each of the two populations (Figure 5). The two populations responded similarly to 400ng and 1800ng of toxin. Plotting the slope (computed by the SAS general linear model procedure) against the dose ($\log_{10}$) suggested that the responses of the Wisconsin and laboratory populations were different, as was suggested by the Z value calculation (Figure 6).

Midgut tissues from fifth instars from New Jersey (n=7) produced $I_{sc}$ similar to the laboratory and Wisconsin tissue samples. After 25 minutes of stabilization, the $I_{sc}$ produced was 97.58% of the $I_{sc}$ produced at 20 minutes (Figure 7). At 35 minutes, 90.25% of the $I_{sc}$ produced at minute 20 remained. After completion of the assay for the control group as described, the $O_2$ used for circulating the buffer was depleted and could not be replenished in time to continue with the Bt assay. The larvae pupated before the assay could be completed.

Midgut tissues from fifth instars from Slovakia were assayed by voltage clamp with surprising results. Under the described experimental conditions, only 4 tissues out of 12 assayed were able to produce short-circuit current over the 60 minute experiment (Figure 8). None of the tissues produced enough $I_{sc}$ within the 20 minutes of stabilization to evaluate sensitivity to Bt. Sensitivity to Bt could not be assessed under the described methods.

**Molecular Analysis of the Bt Midgut Receptor**

RNA was extracted from midgut tissue of fifth instar individuals according to Qiagen protocol and amplified by RT-PCR (Table 3). Complete sequences from each population were trimmed and assembled. The corresponding amino acid sequence was then predicted for each population (Appendix) and compared to the published amino acid sequence for *L. dispar* Bt-R to confirm the validity of the sequence products. The laboratory population amino acid sequence was 99.7% identical to the published Bt-R sequence. The New Jersey, Slovakia and Wisconsin population amino acid sequences were 98%, 92.8% and 99.1% identical, respectively, compared to the published *L. dispar* sequence for Bt-R (GenBank accession number AF317621), confirming that our products corresponded to the sequence of interest. Within the amplified region, only two amino acids were variable. These two amino acids were outside of the 67 amino acids that interact with the Bt toxin during binding (Dorsch *et al.*2002). There was no variability within the amino acid sequence that interacts with the Bt toxin in the four populations we investigated.

The predicted amino acid sequences for each population were also compared to the published sequence considered the Bt-R toxin binding region for *Manduca sexta*. The laboratory population amino acid sequence was 56.9% identical to the *M. sexta* toxin binding region sequence. The New Jersey, Slovakia and Wisconsin population amino acid sequences were 59.5%, 58.1% and 59.2% identical to the *M. sexta* sequence.

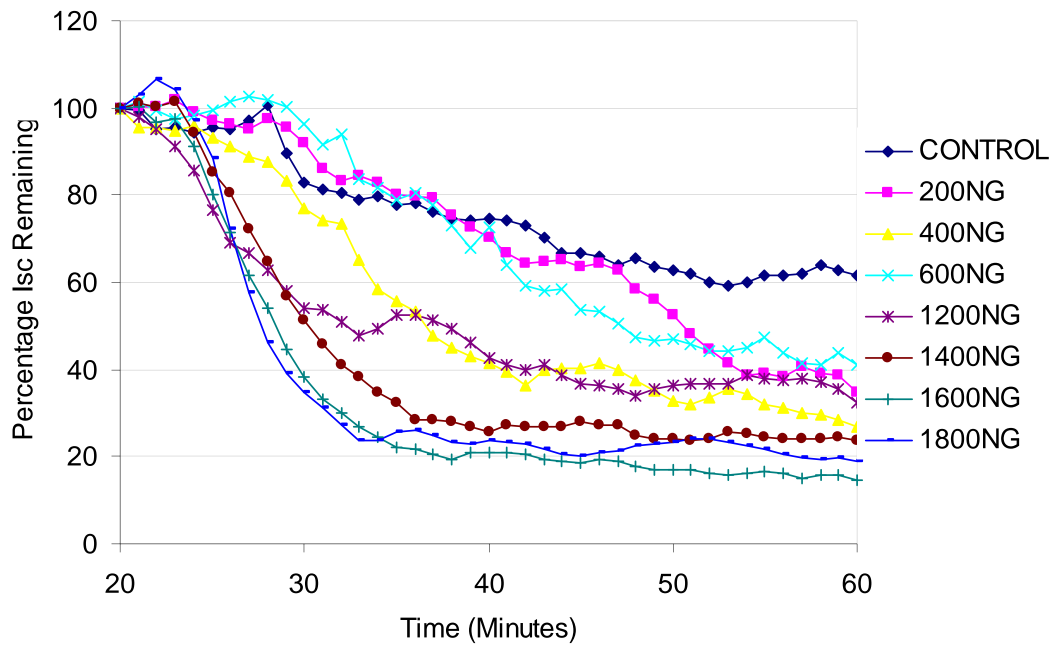


**Figure 2.** **Laboratory Population Response to Cry1Ab Toxin. Average percentage of $I_{sc}$ remaining was calculated for each minute for each dose of toxin. $I_{sc}$ at 20 minutes was considered 100% and was the time toxin was added.**

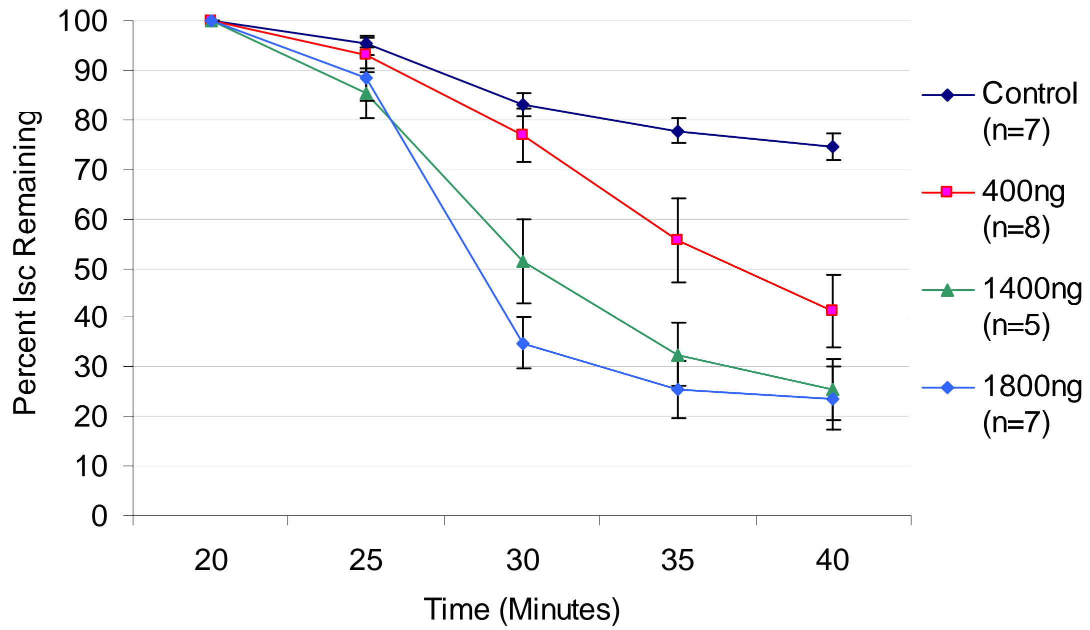


**Figure 3. Laboratory Population Response to Cry1Ab Toxin. Average percentage of $I_{sc}$ remaining was calculated at five minute intervals for each dose of Cry1Ab toxin. $I_{sc}$ at 20 minutes was considered 100% and was the time toxin was added.**

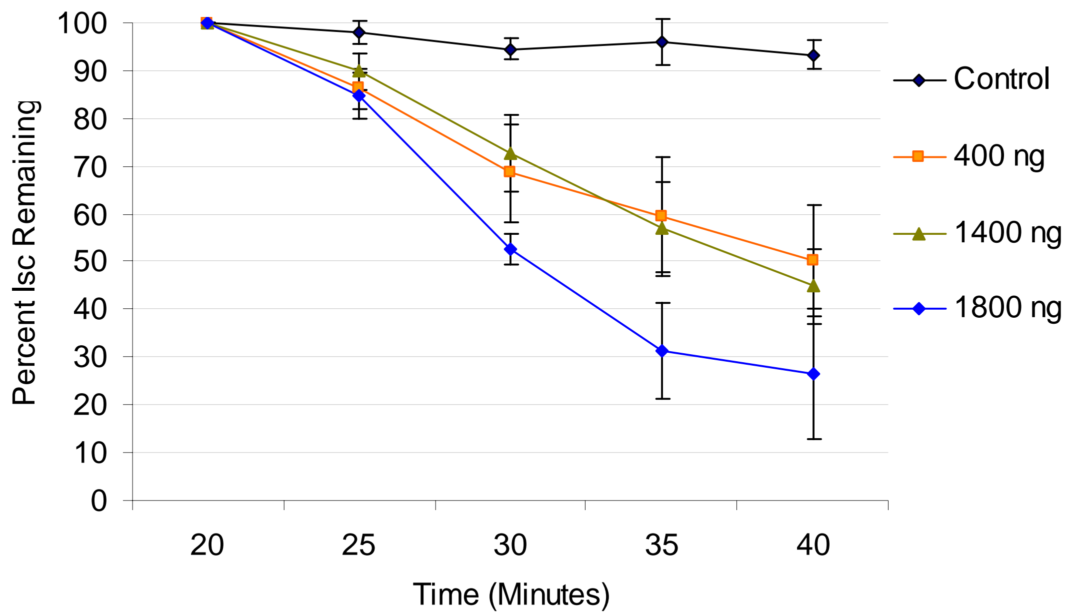


**Figure 4.** **Wisconsin Population Dose Response to Cry1Ab Toxin. Average percentage of $I_{sc}$ remaining was calculated at five minute intervals for each dose of Cry1Ab toxin. $I_{sc}$ at 20 minutes was considered 100% and was the time toxin was added. N=5 for all doses.**

**Table 1. Mean Differences in $I_{sc}$ Remaining Between Two Populations of *Lymantria dispar*. Mean differences in percentage of $I_{sc}$ remaining at different times after the addition of Cry1Ab toxin between the Laboratory and Wisconsin populations were computed with the t-test procedure in SAS (1982).**

| | Dose | | | | | |
|---|---|---|---|---|---|---|
| | 400ng | | 1400ng | | 1800ng | |
| Time (minutes) | 25 | 35 | 25 | 35 | 25 | 35 |
| Mean difference | 7.06 | 3.89 | 4.56 | 24.72 | 3.52 | 14.03 |
| Standard Error | 5.84 | 14.33 | 6.28 | 11.47 | 6.99 | 9.67 |
| p-value | 0.28 | 0.80 | 0.49 | 0.06 | 0.61 | 0.19 |

**Table 2. Rate of $I_{sc}$ Decay Per Minute by Dose and Population. The general linear model was used to compute the average percentage inhibited per minute. The highest dose, 1800 ng inhibited the $I_{sc}$ at the fastest rate (11% average reduction per minute for the laboratory population and 9% average reduction for the Wisconsin population)[1]**

| Dose | Population | | Z Value |
|---|---|---|---|
| | Laboratory | Wisconsin | |
| 400 ng | -0.06 ± 0.003 | -0.05 ± 0.005 | 0.68 |
| 1400 ng | -0.10 ± 0.006 | -0.06 ± 0.003 | 5.43 |
| 1800 ng | -0.11 ± 0.010 | -0.09 ± 0.005 | 1.75 |

[1] The Z value was calculated to determine if the differences between slopes was significant. Z values $\geq$ 1.96 were considered significant (Keil and Othman 1988).

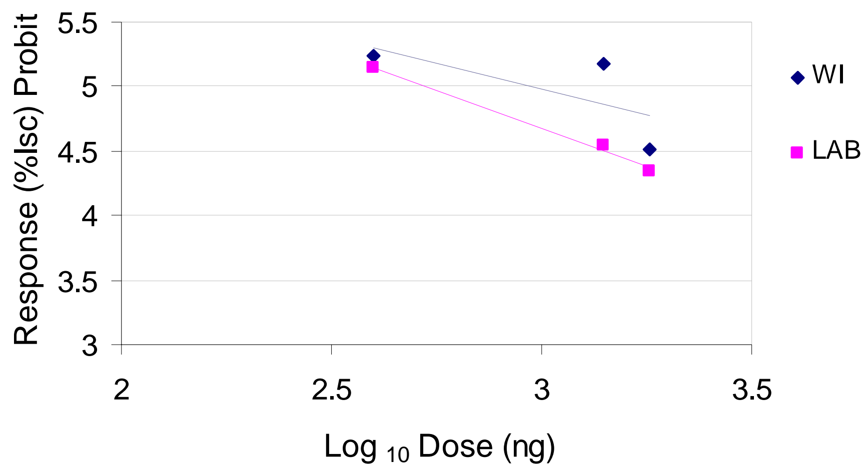


**Figure 5.** **Dose-Dependent Response (%$I_{sc}$) to Cry1Ab Toxin. The probit responses were similar for the two populations at the 400ng and 1800ng doses.**

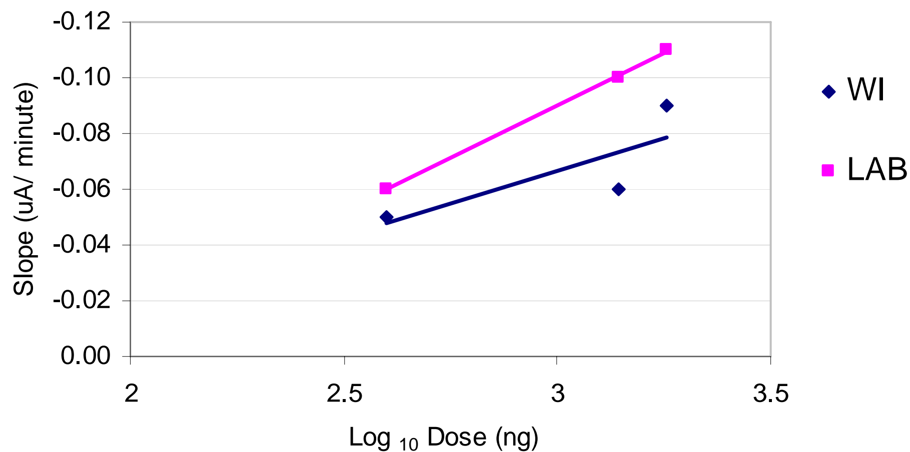


**Figure 6.** **Dose-Dependent Response (Slope) to Cry1Ab Toxin. The rate of decay was dose-dependent in both populations. Only the slopes at the 1400ng dose were significantly different.**

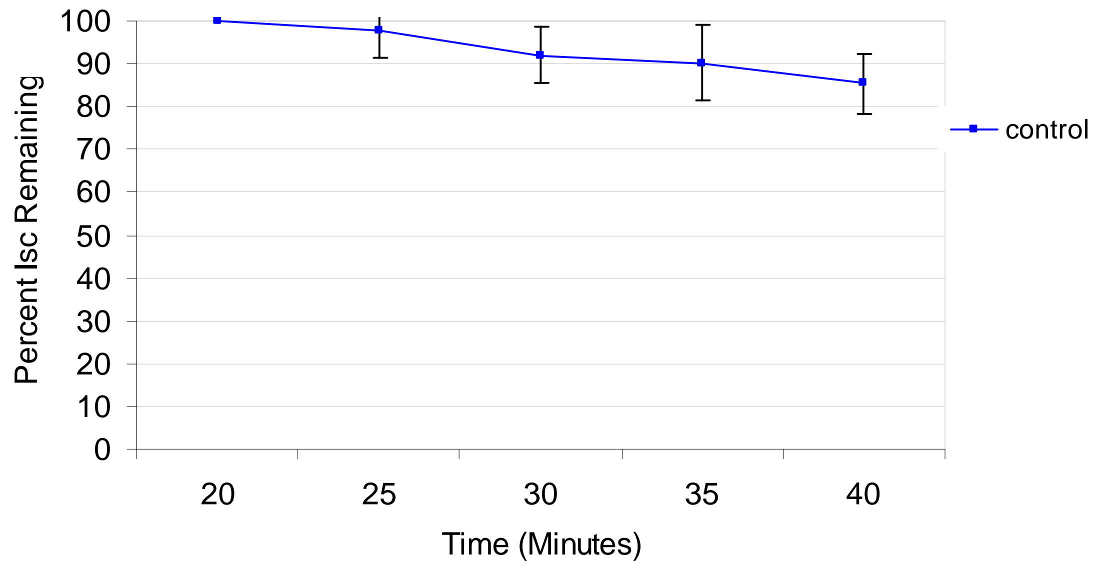


**Figure 7. Average Percent of $I_{sc}$ Remaining in New Jersey Population Larval Midgut Tissues. N=7.**

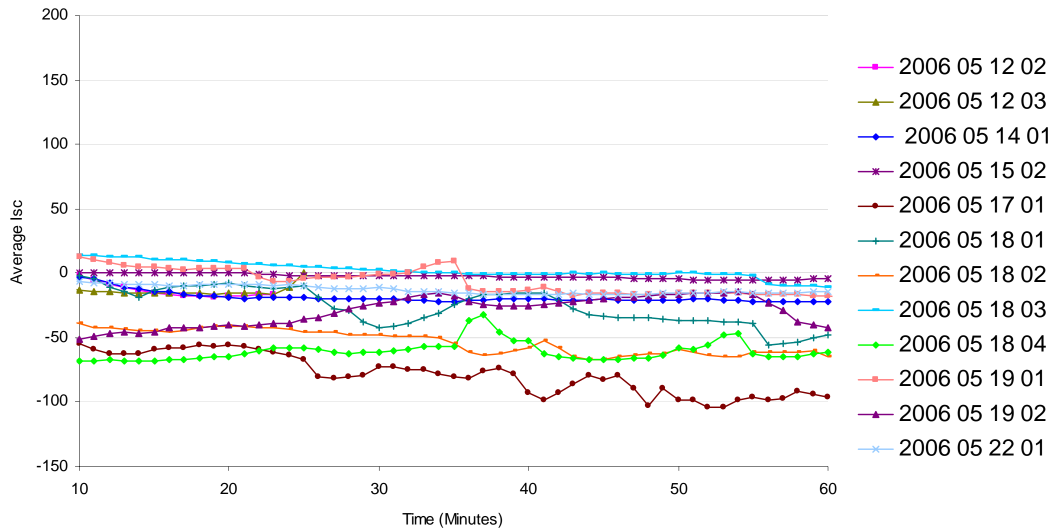


**Figure 8.** **Average $I_{sc}$ Produced By Gypsy moth Larval Midgut Tissues from Slovakia.**

**Table 3. Concentrations of RNA Extraction and RT-PCR Reactions (Products ng/uL).**

| | Population | | | |
|---|---|---|---|---|
| | Laboratory | Slovakia | Wisconsin | New Jersey |
| RNA Extraction | 373.5 | 254.5 | 481.4 | 460 |
| cDNA RT-PCR Products | 73.1 | 70.3 | 123.4 | 85.8 |

# Chapter 4

# DISCUSSION

## Voltage Clamp Optimization

Because few authors have contributed details of strengths, limitations and specifications of the technique to the literature, development and modification of the voltage clamp methods proved to be difficult and time-consuming. An even smaller number addressed a biological audience rather than one specialized in physics and circuitry. Because of this void, fundamental tenets of the technique had to be established before data collection could begin, and the voltage clamp experimental assembly needed to be optimized to work with small tissues.

When first learning the strengths and limitations of the technique, there was difficulty in compensating for the fluid resistance of the buffer used. The Ussing chamber was not compatible with the EVC 4000 amplifier. A new, more powerful amplifier was needed to replace the EVC 4000. More compliance voltage, the voltage available to the amplifier to compensate for resistance, was needed. The EVC 4000 amplifier works in the same way as the more powerful DVC 1000, but has only 32 V of compliance voltage, whereas the DVC 1000 is capable of 100 V of compliance voltage. Modification of the Ussing chamber to accommodate the small tissue samples of even large gypsy moth larvae increased the fluid resistance in the system beyond the compensatory capabilities of the EVC 4000. More compliance voltage was needed and the resistance between the electrodes needed to be reduced. Communicating with

other scientists more familiar with the fundamentals of the technique confirmed our results. A DVC 1000 voltage clamp was purchased and a new chamber was designed that would accommodate the small tissue size and weak buffer by reducing the resistance between voltage electrodes.

With the DVC 1000, simple experiments were conducted to suggest how to optimize the chamber. Three buffers, three annuli, and the distance between the electrodes were investigated. Results of these simple experiments suggested an optimized chamber design with the electrode tips as close together as possible, the tissue sample surface area maximized, and the KCl content of the buffer as high as possible without harming the tissue. The resulting chamber design incorporated the strengths of the two previous designs as well as the results of the resistance experiments.

The commercially available Ussing chamber is suited to tissue samples much larger than insect tissues (frog tissues) and is widely used in university physiology classrooms. This chamber is composed of two half-cells that snap together with 3 pins holding the tissue sample between the two halves. This apparatus could have been modified simply by mounting our annulus with the insect tissue between the two halves, but the electrode tips were not close enough together, resulting in large resistance values when an annulus exposing a small tissue surface area was used. Because of the fragility of midgut tissue, a chamber that could be set up quickly and easily was desired. This chamber was easily assembled for experimentation and also could be easily and thoroughly cleaned between experiments. Another important consideration was that the Ussing chamber was meant to be used with cartridge electrodes that easily plug into the chamber and also a circulation reservoir for

perfusion of the buffer with oxygen during the experiment. Because this model was easily purchased, and widely used, technical assistance was also available.

This chamber design is not without limitations. Because the chamber is meant for larger tissue samples, electrode tips are not placed close together. When the surface area of the tissue was downsized, the resistance between electrodes rapidly increased, resulting in resistance so great it could not be compensated for with any standard amplifier.

The second model, the lollipop model (Dow *et al.* 1985), was designed for use with insect tissues. Like the Ussing chamber, the lollipop chamber consists of two half-cells separated by the tissue. The tissue was tied onto a lollipop apparatus that inserted into the chamber, thus separating the two halves. Strengths of this model are that it accommodates small tissue sizes, had been used by other entomologists, and places electrode tips as close together as possible thus reducing resistance in the system. Several limitations, however, prevented us from using this model. The lollipop model used electrodes that were difficult to fill with agar and insert into the chamber and must be specifically made for the lollipop chamber, rather than the cartridge electrodes that can be easily purchased. The lollipop apparatus is also problematic in that the tissue sample is mounted and tied onto the lollipop mount with string. This was not only difficult, but also slow. Another factor making the lollipop model difficult to use is the challenge of removing any bubbles in the buffer. The chamber is also difficult to clean thoroughly after an experiment. In addition, accessories that fit this model, such as a circulation reservoir, are not available commercially and would need to be designed specifically for this chamber.

With an understanding of the strengths and limitations of the two models in use, an optimized chamber for voltage clamp experiments with insect tissue samples was designed and fabricated (Figure 1). The optimized chamber has two halves that hold the tissue sample between them by compression. The tissue sample is held on an annulus base with an annulus cover. The annulus is placed between the two halves of the chamber in a recessed seat cushioned and sealed with an o-ring on either side. The two halves of the chamber are aligned by pins (much like the Ussing chamber model) and held together by compression by the chamber stand. The voltage cartridge electrodes are inserted into the chamber body at an angle so that the tips are as close together as possible without touching the tissue sample. The current cartridge electrodes are lateral to the voltage electrodes, but are not inserted at an angle. To prevent leaking, electrodes are first lightly coated with high vacuum grease (Dow Corning, Corporation, Midlind, MI) before insertion into the chamber. The chamber body is constructed of 1.5" (3.81 cm) diameter clear acrylic with 2.0" (5.08 cm) diameter end caps held on with screws and sealed with o-rings. The entire chamber is 4.5" (11.43 cm) in length. The chamber interior has a diameter of 3/8" (0.94 cm) and each side has a volume of 4 mL. This chamber is quick and easy to assemble and is also easy to clean between experiments.

With the new chamber, we attempted to conduct an experiment with midgut tissue, but we were still unable to monitor the physiological activity of the tissue. It was clear that the buffer needed to be perfused with $O_2$ not only before the experiment, but also during the experiment to maintain viability of the tissue. A gas lift circulation reservoir was added to the chamber assembly. The gas lift circulation reservoir is connected to the chamber with 3/16" (0.46 cm) inner diameter tubing and

male luer and hose barb fittings that insert into the two circulation ports on either side of the tissue. In our design, the circulation ports are perpendicular to the electrode ports. The circulation reservoir is held directly above the chamber. The tubing that connects the reservoir and the chamber must all be of equal length to allow continuous, equal circulation on both sides of the chamber. Unequal tubing lengths results in a pressure differential between the two sides, preventing simultaneous circulation. Making all tubing lengths equal solved this problem. Even after the circulation reservoir was added to the experimental configuration we were still unable to conduct a successful experiment. It appeared that the gut was viable but as the tissue was bathed in buffer, it was being poisoned.

The literature revealed that the buffer solution had to be adjusted to pH 8.3 (Lee *et al.*1996a). It was surprising that the buffer adjusted to the pH of the hemolymph maintained viability of the tissue considering the high pH in the midgut of these insects (Berenbaum 1980). When the buffer was adjusted to pH 8.3, equivalent to the hemolymph, the tissue was able to maintain viability over the one-hour period of data collection.

**Voltage Clamp Assay**

The optimized voltage clamp system was used to assay Bt sensitivity in midgut tissues from the laboratory population. The midgut tissues responded in a dose-dependent manner, as in previous studies (Liebig *et al.* 1995, Lee *et al.*1996a, Karim *et al.* 2000). The highest dose, 1800ng of Cry1Ab toxin, resulted in the most drastic loss of $I_{sc}$, while the lowest dose, 400ng, caused the least severe response and allowed for wide variation in response between individuals. The average $I_{sc}$ remaining diminished most rapidly at the highest dose (Table 2). At the highest doses of 1600ng

and 1800ng of toxin, the responses were very similar (Figure 2), suggesting saturation as previous authors have reported (Liebig *et al.* 1995). The assay has a predictive value within the ranges tested for the laboratory population (Figure 5).

Midgut tissues from the Manitowoc, WI population were also assayed according to the same methods. Again, the tissues responded in a dose-dependent manner and the rate of decay was greatest in the trials using the highest dose of toxin (1800ng). The probit response data was more variable in the 1400ng dose, suggesting variation between individuals in their response at that dose.

The 1400ng dose of Cry1Ab toxin proved to be an informative dose. The two populations analyzed, the laboratory and Wisconsin populations, had statistically significant differences in the average $I_{sc}$ remaining at minute 35 (Table 1) suggesting variation in sensitivity. Also, the slopes of $I_{sc}$ decay were different between the two populations at this dose (Table 2) providing additional evidence that the two populations vary in their sensitivity to Bt toxin. It is likely that assaying the response of the tissues to additional doses of 800ng and 1000ng of toxin would also be informative in elucidating variation between populations in their response to toxin.

Overall, the data suggested a variation in response to toxin over the range of doses tested. Although we could not conclude from this data that variation in Bt sensitivity *in vivo* was due to the specific interaction of the toxin with the midgut tissue, we could validate the assay for detecting variation between individuals as well as differences in physiological qualities.

Midgut tissues extracted from the Slovakian population responded differently to the assay, suggesting physiological variation between the native and introduced populations (Figure 8). The data suggested two responses. One group of

tissues was able to produce approximately 50 uA of $I_{sc}$ while the other group produced 0 uA. During the attempts to assay $I_{sc}$ response in midgut tissues from the Slovakian gypsy moth population, the pH of the buffer was measured, corrected, and confirmed on several occasions, and several pairs of electrodes were used thus confirming the response of the tissues to the assay rather than a procedural error. Because the tissues did not produce sufficient $I_{sc}$, response to Bt toxin could not be assessed. A comparison of midgut pH between populations may elucidate the differences in physiology suggested by the voltage clamp data. Considering the relationship between the insect and its hosts, it would be surprising to find pH variation between populations, but this may lead to further investigations to elucidate this physiological phenomenon.

**Molecular Analysis**

In order to investigate variation in the toxin binding region of the Bt receptor, the predicted amino acid sequences of the region containing and surrounding the toxin binding region were compared between populations. The amino acid sequence of the toxin binding region in the four populations contained little variability (Appendix). Two amino acids within the amplified region, but outside of the toxin binding region, were variable among the four populations studied. The actual toxin binding region (Dorsch *et al.* 2002) contained no variability.

Although the toxin binding region contained no variation between populations, variation in Bt sensitivity could still be due to the toxin binding event. It is still unclear whether the interaction between the toxin and the amino acids comprising the toxin binding region was reversible or irreversible. Because Bt toxicity requires both reversible and irreversible binding of the toxin to the receptor, variation

in susceptibility could have been due to either, or both, of those events. Separation of the two events could explain variability in sensitivity to toxins and further the understanding of the mechanism of toxicity. Future investigations should also confirm the interaction of this toxin binding region with the Cry1Ab toxin in *L. dispar*.

## Conclusions

Voltage clamp methods were optimized and used to assay variation in sensitivity to Bt toxins. This optimized system was sensitive enough to detect variation between individual tissues as well as temporal variation at different dose levels. These methods would be useful to continuing studies of variation in toxin sensitivity between populations as well as elucidating the physiological variation between populations. Previous authors have used voltage clap methods to investigate relative toxicities of different Bt toxins (Lee *et al.*1996a, Karim *et al.* 2000). This is the first use of voltage clamp methods to quantify variation in response between populations of the same species to a single toxin. Investigators should not only use the described methods, but also publish the strengths and limitations for the benefit of fellow biological researchers.

## Appendix

## AMINO ACID SEQUENCE OF TOXIN BINDING REGION[2]

VNGLLVTTQLEFLERIRATDEDGLHAGIVTFHVEGNAEAVQFFEV 50

SNDGENAGSLILLNTFDEDIR**R**F**T**VVIRGTDGGTEPGTRHTDCSVRLVFV 100

PTQGEPTFSENTASVAFFELEGGMTEQFQLPLADDPKNYLCEDDCFSTYY 150

LIIDGNADGHFAVNPVTNVIYLVEELDREVEETYTILVAASNSPDSVNA 200

LPSNTLTVTVNVREANPRPMFTSEEYMAGISTSDNINRVLLTVQATHSEG 250

APVTYEIDHSTMIVDPTLEAVKDTAFVLNSQTGVLTLNMQPTAFMHGNFE 300

FKVVATDPSEATDRAAVKIYLISSLNRVTFTFRNTLQQINDN 342

[2] Amino acids in blue were variable between the four populations. The underlined sequence is the toxin binding region and contained no variation.

## REFERENCES


Aronson, A., D. Wu and C. Zhang. 1992. Mutagenesis of specificity and toxicity regions of a *Bacillus thuringiensis* protoxin gene. J. Bacteriol. 177: 4059-4065.

Berenbaum, M. 1980. Adaptive significance of midgut pH in larval Lepidoptera. Am. Nat. 115: 138-146.

Campbell, R. W. and T. H. Valentine. 1972. Tree condition and mortality following defoliation by the gypsy moth. Res. Pap. NE-236. Upper Darby, PA: U. S. Department of Agriculture, Forest Service, Northeastern Forest Experiment Station. 331p.

Chamberlin. M. 1990. Luminal alkalinization by the isolated midgut of the Tobacco hornworm (*Manduca sexta*). J. Exp. Biol. 150: 467-471.

Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, J. van Rie, D. Lereclus, J. Baum and D. H. Dean. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62: 807-813.

Davidson, C. B., K. W. Gottschalk and J. E. Johnson. 2001. European gypsy moth (*Lymantria dispar L.*) outbreaks: a review of the literature. Gen. Tech. Rep. NE-278. Newtown Square, PA: U. S. Department of Agriculture, Forest Service, Northeastern Research Station. 15p.

Dorsch, J., M. Candas, N. Griko, W. Maaty, E. Midboe, R. Vadlamudi and L. Bulla, Jr. 2002. Cry1A Toxins of *Bacillus thuringiensis* Bind specifically to a region adjacent to the membrane-proximal extracellular domain of BT-$R_{1a}$ in *Manduca sexta*: involvement of a cadherin in the entomopathogenecity of *Bacillus thuringiensis.* Insect Biochem. Mol. Biol. 32: 1025-1036.

Dow, J., W. Harvey M. Wolfersberger and B. Boyes. 1985. An improved chamber for the short-circuiting of epithelia. J. Exp. Biol. 114: 685-689.

Feeny, P. 1970. Seasonal changes in Oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. Ecology. 5: 565-581.

Ferre`, J. and J. van Rie. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 47: 501-533.

Gill, S. S., E. A. Cowles and P. V. Pietrantonio. The mode of action of *Bacillus thuringiensis* endotoxins. 1992. Annu. Rev. Entomol. 37: 615-636.

Gimble, D. G. and F. B. Lewis. 1985. Proceedings, symposium: Microbial Control of Spruce budworms and Gypsy Moths: April 10-12, 1984; Windsor Locks, Conn. General Technical Report NE-100. USDA, Forest Service, Northeastern Forest Experiment Station, Broomall, Pa.

Grochulski, P., L. Masson, S. Borisova, M. Pusztai-Carey, J. Schwartz, R. Brousseau and M. Cygler. 1995. *Bacillus thuringiensis* Cry1A(a) insecticidal toxin: crystal structure and channel formation. J. Mol. Biol. 254: 447-464.

Harvey, W. R., and M. G. Wolfersberger. 1979. Mechanism of inhibition of active potassium transport in isolated midgut of *Manduca sexta* by *Bacillus thuringiensis* endotoxin. J. Exp. Biol. 83:293–304.

Harvey, W. R., D. N. Crawford and D. D. Spaeth. 1990. Isolation, voltage clamping, and flux measurements in Lepidopteran midgut. Methods Enzymol. 192: 599-608.

Hodgman, T. C., and D. J. Ellar. 1990. Models for the structure and function of the *Bacillus thuringiensis* delta-endotoxins determined by compilational analysis. DNA Sequencing 1: 97-106.

Hofmann, C., P. Luthy, R. Hutter, and V. Pliska. 1988. Binding of the δ-endotoxin from *Bacillus thuringiensis* to brush-border membrane vesicles of the cabbage butterfly (*Pieris brassicae*). Eur. J. Biochem. 173:85–91.

Höfte, H., H. R. Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53: 242-255.

Hua, G., J. Jurat-Fuentes and M. Adang. 2004. Bt-$R_{1a}$ extracellular cadherin repeat 12 mediates *Bacillus thuringiensis* Cry1Ab binding and cytotoxicity. J. Biol. Chem. 279: 28051-28056.

Karim, S., F. Gould and D. Dean. 2000. *Bacillus thuringiensis* δ-endotoxin proteins show a correlation in toxicity and short circuit inhibition against *Helocoverpa zea.* Current Microbiol. 41: 214-219.

Keil, C. B. and M. H. Othman. 1988. Effects of Methoprene on *Lycoriella mali* (Diptera: Sciaridae).J. Econ. Entomol. 81: 1592-1597.

Li, J., J. Carroll and D. J. Ellar. 1991. Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. Nature 353: 815–821.

Liebig, B., D. Stetson and D. Dean. 1995. Quantification of the effect of *Bacillus thuringiensis* toxins on short-circuit current in the midgut of *Bombyx mori*. J. Insect Phys. 41(1): 17-22.

Lee, M. K., A. Curtiss, E. Alcantara and D. H. Dean. 1996a. Synergistic effect of the *Bacillus thuringiensis* toxins CryIAa and CryIAc on the gypsy moth, *Lymantria dispar*. Appl. and Environ. Microbiol. 62(2): 583-586.

Lee, M. K., T. H. You, B. A. Young, J. A. Cotrill, A. P. Valaitis and D. H. Dean. 1996b Aminopeptidase N purified from gypsy moth brush border membrane vesicles is a specific receptor for *Bacillus thuringiensis* CryIAc toxin.. Appl. Environ. Microbiol. 62: 2845-2849.

Lightwood, D., D. Ellar and P. Jarrett. 2000. Role of proteolysis in determining potency of *Bacillus thuringiensis* Cry1Ac δ-endotoxin. Appl. Environ. Microbiol. 66: 5174-5181.

Peyronnet, O., V. Vachon, R. Brousseau, D. Baines, J. Schwartz and R. Laprade. 1997. Effect of *Bacillus thuringiensis* Toxins on the membrane potential of Lepidopteran insect midgut cells. Appl. Environ. Microbiol. 63: 1679-1684.

Rajamohan, F., O. Alzate, J. A. Cotrill, A. Curtiss and D. H. Dean. 1996. Protein engineering of *Bacillus thuringiensis* δ-endotoxin; mutations at domain II of CryIAb enhance receptor affinity and toxicity toward gypsy moth larvae. Proc. Natl. Acad. Sci. U.S.A. 93: 14338-14343.

Robertson, J.L., and H.K. Preisler. 1991. Pesticide Bioassays with Arthropods. CRC Press, Boca Raton. 127 pp.

Rossiter, M., W. G. Yendol and N. R. Dubois. 1990. Resistance to *Bacillus thuringiensis* in gypsy moth (Lepidoptera: Lymantriidae): genetic and environmental causes. J. Econ. Entomol. 83: 2211-2218.

Schnepf, E., N, Crickmore, J. van Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigler and D. H. Dean. 1998. *Bacillus* thuringiensis and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62: 775-806.

Van Rie, J., W. H. McGaughey, D. E. Johnson, B. D. Barnett and H. van Mellaert. 1990. Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. Science 247: 72-74.

Whalon, M. and B. Wingerd. 2003. Bt: Mode of Action and Use. Arch. Insect Biochem. Physiol. 54: 200-211.