**Final Report to Monsanto Canada, Pioneer Hi-Bred Seeds, Syngenta Canada, Dow AgroSciences Canada and the Canadian Food Inspection Agency**

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# BASELINE MONITORING OF Bt - RESISTANCE IN THE EUROPEAN CORN BORER IN ONTARIO AND QUEBEC (2000 – 2005)


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**GOVERNMENT PARTNER:** The Canadian Food Inspection Agency

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## Introduction

The European corn borer (ECB), *Ostrinia nubilalis* (Hubner), is the most destructive pest of corn in Canada and the major target pest for control with transgenic Bt-corn (Fishhoff 1996). Feeding by ECB larvae results in disruption to corn growth, stalk breakage, ear drop, reduced yield and includes direct damage to kernels later in the season. The introduction of Bt-corn has resulted in higher yields and improved quality of grain due to borer-free corn (Rice and Pilcher 1998: Baute et al. 2002). The threat of selection for Bt-resistance in populations of ECB feeding on transgenic corn has highlighted the importance of developing resistance management strategies to prevent or delay the evolution of Bt resistance (Hokkanen and Deacon 1994; Tabashnik 1994: Tabashnik et al. 2003). Risk assessment and consequences of ECB resistance to Bt-endotoxins is summarised in an ILSI publication (Anon 1999). Resistance management strategies require an effective resistance monitoring program, which in turn requires an appropriate bioassay technique to measure baseline susceptibility of ECB populations. With reliable baseline data, potential population-susceptibility changes in response to Bt-toxins can be measured (Fischhoff 1996). This study has been designed to establish this baseline data for Bt-susceptible populations of ECB in Canada and subsequently test this bioassay method.

## Primary Objective

Our primary objective in this project is to see that Bt-corn varieties are used responsibly and in accordance with environmental safety in Canada. All sectors of the agricultural community must manage this transgenic tool responsibly to mitigate the selection of Bt-resistance in ECB. Responsibly conserving this new transgenic technology over a longer period of time will benefit all stakeholders and will postpone the need for development of costly new alternatives to manage ECB. We need to ensure that our Insect Resistance Management (IRM) strategy for Bt-corn in Canada is scientifically based.

At one of the first Bt-Corn Coalition Meetings (21 Nov. 1997), the committee (now called the Canadian Corn Pest Coalition, CCPC) rated the 3 key issues facing the Coalition (in order of importance) as: 1) Baseline Monitoring for ECB Resistance, 2) Bt-free Refugia Studies and 3) Bt-Corn/ECB Education Package. So starting in 1998, AAFC, SCPFRC, London supported by the Coalition and with some finances from the Corn Seed Industry initiated a baseline monitoring study of Bt-toxins, Cry1A(b), Cry1A(c), and Cry9C, against ECB larvae. Using the "University of Nebraska method", bioassays were set up with several Ontario and Quebec field-collected strains of ECB. Probit analysis was used to establish the LC50, LC95, and LC99 values for these susceptible populations. The LC95 or LC99 of a susceptible population is often used as a "discriminating dose", in order to compare susceptibility in other populations (Marcon et al. 2000). Survival of ECB at this discriminating dose would indicate potential resistance development. A significant shift in the LC95 would trigger implementation of the mitigation plan currently being finalized by the CFIA and the CCPC.

## Specific Objectives

1) Validation of our diagnostic/discriminating dose [LC95] against several populations of ECB in Ontario and Quebec;
2) Validation of our data against Blair Siegfried's [University of Nebraska] data by sending ECB egg-masses from a standard Ontario population for analysis in Nebraska;
3) Comparison of several populations of ECB from univoltine and bivoltine regions of Ontario for their susceptibility/variability to Bt-toxins to see if any trends are developing. Also evaluate variability in several populations from the same bivoltine and univoltine areas in order to determine if the variability observed is related to the number of generations or to the area from where it came;
4) Maintain a first-class ECB Rearing Facility in Ontario, at AAFC, SCPFRC, London, in order to be pro-active in Canada's capability to respond to potentially resistant ECB populations in the future and thereby being a centre of expertise where suspect field-collected larvae can be brought, kept alive, reproduced, and tested through our bioassay system.

## Materials and Methods

**ECB collection:**
Following the growing seasons of 1999, 2000, 2001, 2002, 2003 and 2004 corn stalk samples were collected from fields to establish ECB cultures for use in the Bt baseline susceptibility monitoring program. Late instar larvae enter into a diapause state in the fall and will over-winter in corn stalks. Stalks were collected from non-Bt fields (or refugia sites) that exhibited heavy ECB infestation in order to maximize the number of ECB collected. It was hoped that at least 100 larvae would be available from each site to start the culture in order to encompass a wide range of the available gene pool in that area. Based on local infestation rates, the appropriate numbers of stalks were collected in hopes of obtaining the desired 100+ larvae. Most stalks were collected in the fall and were either kept in cold storage (4EC) or in the barn at SCPFRC, London which allowed for natural over- wintering conditions. Some stalk collections were also completed in the spring. Below is a list of sites used for ECB collections:

I. Locations and dates of collection of corn stalks for use in 2001 bioassays:

Quebec - May 4th, 2001 @ Ormstown, QC, 45 08 N 74 00 W
Ottawa - Apr. 24th, 2001 @ Ottawa, ON, AAFC Central Experimental Farm
Prescott - May 2nd, 2001 @ Prescott, ON, 44 43 N 75 35 W
Lambton- Oct. 26th, 1999 @ Wyoming, ON, Highway 21 N and Confederation Line
Middlesex - Nov 14th, 2000 @ London, ON, Highbury and Fanshawe Park Roads
Niagara - Nov 23rd, 2000 @ Niagara County, ON, Balfour and Effingham Roads
Simcoe 1 - Oct. 25th, 1999 @ Bradford, ON, Highway 400 and Highway 88
Simcoe 2 - Oct 11th, 2000 @ Bradford, ON, Highway 400 and Highway 88
Essex - Mar. 23rd, 2000 @ Holiday Beach, ON

II. Locations and dates of collection of corn stalks for use in 2002 bioassays:

Simcoe - Oct. 18, 2001 @ Bradford, ON, Highway 400 and Highway 88
Niagara - Nov. 21, 2001 @ Niagara County, ON, Highway 3 and Darling Rd.
Essex - Nov. 22, 2001 @ Holiday Beach, ON
Lambton - Oct. 10, 2001 @ Sarnia, ON, Mandaumin Rd. and Confederation Line
Middlesex - Nov. 6 2001 @ Ilderton, ON, New Ontario and Greystead Roads.
Ottawa - Apr. 22, 2002 @ Ottawa, ON, AAFC Central Experimental Farm
Quebec - May 8, 2002 @ Sherington, PQ, ~35km south of Montreal on Rte. 15

III. Locations and dates of collection of corn stalks for use in 2003 bioassays:

Simcoe - Oct. 24, 2002 @ Bradford, ON, Highway 400 and Highway 88
Niagara - Oct. 24, 2002 @ Caledonia, ON, Highway 6 and Third Line
Essex - Oct. 16, 2002 @ Staples, ON, Highway 14 and Highway 77
Lambton - Apr. 15, 2003 @ Sarnia, ON, Perch Creek and Confederation Line
Middlesex - Oct. 16 2002 @ Glencoe, ON, Highway 80
Ottawa - May 28, 2003 @ Ottawa, ON, AAFC Central Experimental Farm
Quebec - May 15, 2003 @ Sherington, PQ, ~35km south of Montreal on Rte. 15
Quebec 2 - Nov. 4, 2002 @ Nicolette, PQ, Bt176 field

IV. Locations and dates of collection of corn stalks for use in 2004 bioassays

Simcoe - Oct. 8, 2003 @ Bradford, ON, Highway 400 and Highway 88
Niagara - Apr. 16, 2004 @ Hagersville, ON, Sandusky Rd.
Essex - Oct. 10, 2003 @ Staples, ON, County Rd. 8 and Highway 77
Lambton - Oct. 9, 2003 @ Sarnia, ON, Brigden Side Road and Confederation Line
Middlesex - Oct. 29, 2003 @ Ilderton, ON, New Ontario and Charlton Roads
Ottawa - May 19, 2004 @ Ottawa, ON, AAFC Central Experimental Farm
Quebec 1- Apr. 12, 2004 @ St.-Denis sur le Richelieu, QC
Quebec 2 - Apr. 28, 2004 @ Monteregie West, QC
Haywood - Oct. 7, 2003 @ Paris, ON, Bt176 field

V. Locations and dates of collection of corn stalks for use in 2005 bioassays:

Simcoe - Oct. 21, 2004 @ Bradford, ON, Highway 400 and Highway 88
Niagara - Oct. 28-29, 2004 @ Hagersville, ON, Sandusky Rd.
Essex - Oct. 20, 2004 @ Staples, ON, County Rd. 7 (#2010 – west of Road 1)
Lambton - Oct. 20, 2004 @ Warwick, ON, Hickory Creek @ Warwick Village
Middlesex - Nov 3, 2004 @ Ilderton, ON, New Ontario and Charlton Roads
Ottawa - May 2, 2005 @ Ottawa, ON, AAFC Central Experimental Farm
Quebec - May 10, 2005 @ St.- Denis sur le Richelieu, QC

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Each spring, having completed their obligatory winter diapause requirements, after one of the above collections, larvae were ready to be extracted from the stalk samples and put into mass rearing conditions (generally 50-150 larvae found per site). Water and diet were made available to the larvae following their extraction from the stalks and subsequent placement into pupation tubs. Pupation occurred within one month, and all adult moths emerged within 2 months of extraction. Regular rearing methods (see appendix A) were implemented until there was sufficient egg production for use in the bioassays. It generally took 2-4 generations before this occurred depending on the number of individuals collected to start a culture.

**Larval Preparation:**

Eggs deposited on wax paper were collected daily from adult rearing cages. Eggs not required for maintenance rearing purposes were used in the bioassays. Egg sheets were labelled with collection date and strain information. Sheets were placed into a sealed plastic sweater box kept in a growth chamber maintained at the same conditions required for adult and larval rearing (16:8 L:D, 27EC photoperiod, 18EC scotoperiod, 75% RH). After approximately four days the eggs had reached the >blackhead= stage and were ready to hatch within a few hours. Eggs in the blackhead stage were transferred to a sealed sweater box kept in a cool 4EC growth chamber (16:8 L:D, 75% RH). These conditions would hold the eggs at the blackhead stage of development for up to a week to increase the number of eggs available on the day bioassays were performed. On the morning that a bioassay was to be performed, blackhead eggs were transferred back into the warmer conditions of the rearing growth chamber to initiate larval eclosion. Egg sheets were loosely scattered within the sweater box to facilitate larval movement upon hatching (compressed sheets hindered movement and increased mortality). Eggs hatched within 2-4 hours of being placed into the warmer rearing growth chamber and first instar larvae were then ready for use in bioassays.

**Bioassay Tray Preparation:**

Diet used for bioassays was formulated in much the same manner as that used for rearing, only on a smaller scale and with a few minor modifications. The diet was mixed in a microwaveable container and dispensed into the bioassay trays with 128 individual wells (Bio-16, CD International) using a media pump. After the diet had solidified in tray wells, the appropriate covers were put into place on the trays. Trays were able to be stored at 4EC for up to 4 weeks until required for bioassays.

**Bioassays:**

Bioassay trays were removed from the 4EC cabinet and warmed to room temperature the morning on which a bioassay was to be performed. Blackhead eggs were also moved from the cool cabinet into the warmer rearing cabinet at this time. Tray covers were labelled with date, Bt toxin concentration, and larval strain information. The covers were then folded back to expose the diet (using a paper towel, any excess moisture was removed from the underside of the covers). The appropriate concentration of Bt toxin was then transferred (30$\Phi$l[1]) onto the diet

surface of each well. The diet for control larvae was treated with 0.1% Triton X-100. Trays were repeatedly tilted in all directions to cover the entire surface area of the diet with the Bt toxin. Trays were then left to sit in a fumehood until the solvent component of the toxin solution had evaporated. At this point, larvae were individually transferred from the plastic sweater box to the wells of the bioassay trays (one per well) using a fine-point paint brush. Following infestation of the larvae, tray covers were replaced and then vented by piercing with a pin several times over each well. Trays were then placed into the rearing growth chamber and covered with paper (thus blocking overhead light which helped to keep the larvae on the diet and prevent condensation build-up). After 7 days, larval mortality and development[2] were recorded. Severely stunted larvae that were alive but weighed less than 0.1 mg were considered dead for the purposes of probit analysis. Results from these experiments were statistically processed using SAS probit analysis to generate the lethal concentration and discriminating dose ($LC_{95}$) data used for baseline monitoring and relative susceptibility studies.

[1] The quantity of toxin used per well was based on a calculation that determines the coverage of toxin per square centimetre of diet. Bt-toxin solutions were serially diluted from a stock solution using 0.1% Triton X-100. Five concentrations plus a control were used for each bioassay with the actual concentrations encompassing a range of 0-100% mortality. For the Cry1A(b) bioassays, concentrations of 0.125, 0.25, 0.5, 1.0 and 2.0 ng/cm$^2$ were used. For the Cry1F bioassays, concentrations of 1, 2, 5, 10 and 15 ng/cm$^2$ were used. For each bioassay, 36 larvae were treated per concentration of each toxin as well as a control. Each bioassay was then repeated 3 times to enhance statistical significance (n=540 + control).

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[2] Larval weights were originally recorded but this practice was discontinued due to inconsistencies between the results generated by growth inhibition and probit analyses. Larval development (instar) was subsequently recorded and this data used to illustrate the growth inhibition caused by Bt proteins.

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## Results

The susceptibility of various populations of ECB to Cry1A(b) and Cry IF, as measured by $LC_{50}$, $LC_{95}$ and $LC_{99}$ is presented in Tables 1 and 2, respectively. Table 3 shows the discriminating dose test ($LC_{95}$) that was validated for both Cry1A(b) and Cry1F using the apparently "least susceptible strain" (Essex County) and the apparently "most susceptible strain" (Simcoe County).

**Table 1. 2001 vs. 2002 vs. 2003 v. 2004 vs. 2005 susceptibility of populations of European corn borer neonate larvae to Cry1Ab. Lethal concentration (LC) with 95% confidence limits (CL) in brackets.**

| Population | n | $LC_{50}$ (95% CL) ng a.i./cm$^2$ | $LC_{95}$ (95% CL) ng a.i./cm$^2$ | $LC_{99}$ (95% CL) ng a.i./cm$^2$ |
|---|---|---|---|---|
| | | | | |
| Ormstown $F_2$-$F_3$ - 2001 | 540 | 0.23 (0.19-0.27) | 1.19 (0.94-1.66) | 2.37 (1.70-3.84) |
| Quebec $F_2$ - 2002 | 540 | 0.12 (0.08-0.15) | 0.94 (0.72-1.40) | 2.21 (1.47-4.13) |
| Quebec $F_3$ - 2003 | 540 | 0.24 (0.04-0.46) | 1.87 (0.84-107) | 4.39 (1.46-2101) |

| | | | | |
|---|---|---|---|---|
| Quebec 1 $F_2$ - 2004 | 540 | 0.19 (0.16-0.22) | 0.74 (0.60-0.96) | 1.30 (0.99-1.90) |
| Quebec 2 $F_2$ - 2004 | 540 | 0.04 (0.01-0.07) | 0.31 (0.24-0.48) | 0.71(0.47-1.76) |
| Quebec - 2005* | ---- | ---------- | ---------- | ---------- |
| | | | | |
| Nicolette $F_5$ - 2003 | 540 | 0.13 (0.10-0.16) | 0.60 (0.48-0.83) | 1.12 (0.81-1.86) |
| Paris $F_4$ - 2004 | 540 | 0.09 (0.06-0.12) | 0.34 (0.28-0.47) | 0.58 (0.43-1.00) |
| | | | | |
| Ottawa $F_2$-$F_4$ - 2001 | 540 | 0.25 (0.21-0.29) | 1.16 (0.93-1.56) | 2.17 (1.60-3.32) |
| Ottawa $F_2$ - 2002 | 540 | 0.12 (0.09-0.15) | 0.79 (0.61-1.13) | 1.71 (1.18-3.01) |
| Ottawa $F_3$ - 2003 | 540 | 0.11 (0.08-0.14) | 0.80 (0.61-1.17) | 1.80 (1.21-3.32) |
| Ottawa $F_3$ - 2004 | 540 | 0.03 (0.00-0.06) | 0.20 (0.15-0.29) | 0.41 (0.28-1.28) |
| Ottawa $F_8$ - 2005 | 540 | 0.11 (0.08-0.14) | 0.59 (0.47-0.84) | 1.17 (0.83-2.04) |
| | | | | |
| Prescott $F_2$-$F_4$ - 2001 | 540 | 0.18 (0.14-0.21) | 0.91 (0.72-1.27) | 1.80 (1.29-2.95) |
| | | | | |
| Lambton $F_9$-$F_{11}$ - 2001 | 540 | 0.35 (0.17-0.56) | 2.62 (1.32-18.2) | 6.00 (2.35-99.0) |
| Lambton $F_4$ - 2002 | 540 | 0.18 (0.15-0.22) | 1.13 (0.88-1.59) | 2.38 (1.67-3.96) |
| Lambton $F_5$ - 2003 | 540 | 0.08 (0.05-0.11) | 0.48 (0.38-0.68) | 0.98 (0.68-1.82) |
| Lambton $F_3$ - 2004 | 540 | 0.04 (0.00-0.12) | 0.66 (0.32-2512) | 2.11 (0.70-239326321) |
| Lambton $F_4$ - 2005 | 540 | 0.10 (0.07-0.13) | 0.65 (0.50-0.94) | 1.38 (0.95-2.53) |
| | | | | |
| Middlesex $F_2$-$F_4$ - 2001 | 540 | 0.34 (0.30-0.39) | 1.56 (1.25-2.09) | 2.93 (2.17-4.39) |
| Middlesex $F_2$ - 2002 | 540 | 0.25 (0.13-0.38) | 1.48 (0.87-5.12) | 3.08 (1.49-19.10) |
| Middlesex $F_5$ - 2003 | 540 | 0.21 (0.18-0.24) | 0.68 (0.57-0.87) | 1.11 (0.87-1.56) |
| Middlesex $F_4$ - 2004 | 540 | 0.08(0.05-0.10) | 0.30 (0.25-0.43) | 0.52 (0.38-0.95) |
| Middlesex $F_8$ - 2005 | 540 | 0.14(0.11-0.16) | 0.64 (0.51-0.88) | 1.21 (0.88-1.96) |
| | | | | |
| Niagara $F_2$-$F_4$ - 2001 | 540 | 0.24 (0.20-0.29) | 2.05 (1.52-3.13) | 4.98 (3.25-9.17) |
| Niagara $F_2$ - 2002 | 540 | 0.15 (0.11-0.19) | 1.02 (0.78-1.50) | 2.26 (1.53-4.03) |
| Niagara $F_3$ - 2003 | 540 | 0.09 (0.01-0.25) | 0.94 (0.40-1.75) | 2.51 (1.34-5.41) |
| Niagara $F_4$ - 2004 | 540 | 0.15 (0.11-0.19) | 1.20 (0.88-1.90) | 2.83 (1.80-5.76) |
| Niagara $F_8$ - 2005 | 540 | 0.17 (0.14-0.20) | 0.67 (0.55-0.90) | 1.19 (0.89-1.81) |
| | | | | |
| Essex $F_9$-$F_{11}$ - 2001 | 540 | 0.53 (0.26-1.01) | 3.01 (1.38-56.58) | 6.18 (2.24-369.66) |
| Essex $F_{14}$-$F_{15}$ - 2001 | 540 | 0.87 (0.25-1.21) | 2.28 (1.52-59.85) | 3.38 (1.95-501.26) |
| Essex $F_3$ - 2002 | 540 | 0.11 (0.08-0.14) | 0.90 (0.69-1.33) | 2.14 (1.42-4.03) |
| Essex - 2003** | ---- | ---------- | ---------- | ---------- |
| Essex $F_2$ - 2004 | 540 | 0.08 (0.05-0.12) | 0.77 (0.57-1.25) | 1.95 (1.22-4.39) |
| Essex $F_4$ - 2005 | 540 | 0.15 (0.12-0.18) | 0.80 (0.63-1.14) | 1.62 (1.15-2.73) |
| | | | | |
| Simcoe $F_9$-$F_{11}$ - 2000 | 540 | 0.29 (0.25-0.34) | 1.62 (1.29-2.23) | 3.31 (2.40-5.12) |
| Simcoe $F_2$-$F_4$ - 2001 | 540 | 0.17 (0.13-0.20) | 0.96 (0.76-1.35) | 2.00 (1.42-3.27) |
| Simcoe[1] $F_8$ - 2000 | 512 | 0.22 (0.16-0.27) | 0.46 (0.36-0.71) | 0.84 (0.58-1.94) |
| Simcoe $F_2$ - 2002 | 540 | 0.10 (0.07-0.13) | 0.56 (0.44-0.79) | 1.15 (0.81-2.00) |
| Simcoe $F_4$ - 2003 | 540 | 0.27 (0.10-0.46) | 1.83 (0.91-17.3) | 4.00 (1.57-111) |

| | | | | |
|---|---|---|---|---|
| Simcoe $F_3$ - 2004 | 540 | 0.08 (0.05-0.10) | 0.39 (0.31-0.55) | 0.75 (0.53-1.37) |
| Simcoe $F_4$ - 2005 | 540 | 0.06 (0.03-0.09) | 0.76 (0.55-1.30) | 2.19 (1.28-5.77) |
| | | | | |

* Quebec colony was lost during over-wintering diapause
** Essex colony was lost during over-wintering diapause
[1] Bioassays ran for comparison at the University of Nebraska by Dr. Siegfried's group.

**Table 2. 2001 vs. 2002 vs. 2003 vs. 2004 vs. 2005 susceptibility of populations of European corn borer neonate larvae to Cry1F. Lethal concentration (LC) with 95% confidence limits (CL) in brackets.**

| Population | n | $LC_{50}$ (95% CL) ng a.i./$cm^2$ | $LC_{95}$ (95% CL) ng a.i./$cm^2$ | $LC_{99}$ (95% CL) ng a.i./$cm^2$ |
|---|---|---|---|---|
| | | | | |
| Ormstown $F_2$-$F_3$ - 2001 | 540 | 2.09 (1.76-2.43) | 8.89 (7.21-11.72) | 16.20 (12.21-23.87) |
| Quebec $F_2$ - 2002 | 540 | 0.55 (0.01-1.38) | 16.58 (6.83-1043) | 68.25 (17.17-115776) |
| Quebec $F_3$ - 2003 | 540 | 1.60 (1.29-1.91) | 7.84 (6.27-10.57) | 15.12 (11.12-23.25) |
| Quebec 1 $F_2$ - 2004 | 540 | 0.76 (0.53-0.95) | 3.52 (2.81-4.99) | 6.63 (4.73-11.69) |
| Quebec 2 $F_2$ - 2004 | 540 | 0.67 (0.44-0.88) | 3.75 (2.95-5.42) | 7.65 (5.32-14.12) |
| Quebec - 2005* | ---- | ---------- | ---------- | ---------- |
| | | | | |
| Nicolette $F_5$ - 2003 | 540 | 0.67 (0.42-0.88) | 3.82 (2.98-5.57) | 7.88 (5.44-14.71) |
| Paris $F_4$ - 2004 | 540 | 0.49 (0.24-0.71) | 2.97 (2.30-4.49) | 6.28 (4.23-13.42) |
| | | | | |
| Ottawa $F_2$-$F_4$ - 2001 | 540 | 1.58 (1.26-1.90) | 10.17 (7.90-14.34) | 21.97 (15.39-36.36) |
| Ottawa $F_2$ - 2002 | 540 | 1.03 (0.81-1.24) | 4.88 (3.91-6.69) | 9.29 (6.77-14.93) |
| Ottawa $F_3$ - 2003 | 540 | 0.72 (0.50-0.91) | 3.74 (2.98-5.25) | 7.39 (5.26-12.87) |
| Ottawa $F_3$ - 2004 | 540 | 0.73 (0.51-0.92) | 3.11 (2.51-4.39) | 5.67 (4.09-9.94) |
| Ottawa $F_8$ - 2005 | 540 | 0.75 (0.51-0.97) | 3.85 (3.03-5.58) | 7.56(5.28-13.82) |
| | | | | |
| Prescott $F_2$-$F_4$ - 2001 | 540 | 1.05 (0.79-1.30) | 5.88 (4.61-8.34) | 11.99 (8.44-20.39) |
| | | | | |
| Lambton $F_9$-$F_{11}$ - 2001 | 540 | 2.90 (2.44-3.37) | 13.94 (11.15-18.74) | 26.69 (19.70-40.47) |
| Lambton $F_4$ - 2002 | 540 | 0.90 (0.62-1.18) | 7.65 (5.80-11.40) | 18.56 (12.27-34.86) |
| Lambton $F_5$ - 2003 | 540 | 0.47 (0.21-0.68) | 2.34 (1.86-3.53) | 4.55 (3.13-10.17) |
| Lambton $F_3$ - 2004 | 540 | 0.71 (0.48-0.90) | 3.14 (2.52-4.46) | 5.83 (4.17-10.40) |
| Lambton $F_4$ - 2005 | 540 | 0.59 (0.35-0.79) | 3.07 (2.43-4.53) | 6.10 (4.22-11.99) |
| | | | | |
| Middlesex $F_2$-$F_4$ - 2001 | 540 | 1.71 (0.78-2.67) | 11.15 (6.28-45.77) | 24.22 (11.07-199.77) |
| Middlesex $F_2$ - 2002 | 540 | 1.69 (0.94-2.46) | 8.43 (5.30-21.87) | 16.41 (8.84-66.45) |
| Middlesex $F_5$ - 2003 | 540 | 1.73 (1.46-2.01) | 6.76 (5.54-8.77) | 11.87 (9.09-17.11) |
| Middlesex $F_4$ - 2004 | 540 | 0.43 (0.18-0.65) | 2.65 (2.05-4.09) | 5.64 (3.76-13.14) |
| Middlesex $F_8$ - 2005 | 540 | 1.02 (0.81-1.21) | 4.04 (3.27-5.48) | 7.13 (5.29-11.30) |
| | | | | |

| | | | | |
|---|---|---|---|---|
| Niagara $F_2$-$F_4$ - 2001 | 540 | 1.29 (0.99-1.58) | 10.22 (7.83-14.75) | 24.11 (16.41-41.86) |
| Niagara $F_2$ - 2002 | 540 | 0.74 (0.47-1.01) | 6.40 (4.83-9.66) | 15.60 (10.22-30.51) |
| Niagara $F_3$ - 2003 | 540 | 0.79 (0.50-1.05) | 4.94 (3.8-6.99) | 10.63 (7.44-18.23) |
| Niagara $F_4$ - 2004 | 540 | 0.75 (0.49-0.99) | 4.27 (3.31-6.32) | 8.77 (6.00-16.55) |
| Niagara $F_8$ - 2005 | 540 | 0.57 (0.32-0.77) | 2.44 (1.95-3.60) | 4.45 (3.14-9.04) |
| | | | | |
| Essex $F_9$-$F_{11}$ - 2001 | 540 | 3.50 (0.21-11.83) | 31.26 (10.17- | 77.41 (17.47- |
| Essex $F_{14}$-$F_{15}$ - 2001 | 540 | 4.07 (1.29-16.62) | 18.46 (10.38-149.23) | 34.52 (16.07-831.99) |
| Essex $F_3$ - 2002 | 540 | 0.81 (0.55-1.06) | 7.04 (5.37-10.38) | 17.21 (11.44-32.08) |
| Essex - 2003** | ---- | ---------- | ---------- | ---------- |
| Essex $F_2$ - 2004 | 540 | 0.53 (0.29-0.76) | 3.95 (3.03-5.92) | 9.08 (6.03-18.42) |
| Essex $F_4$ - 2005 | 540 | 0.83 (0.59-1.05) | 5.45 (4.27-7.73) | 11.89 (8.28-20.70) |
| | | | | |
| Simcoe $F_9$-$F_{11}$ - 2000 | 540 | 2.31 (2.03-2.61) | 8.88 (7.39-11.22) | 15.50 (12.15-21.32) |
| Simcoe $F_2$-$F_4$ - 2001 | 540 | 1.60 (1.33-1.87) | 8.01 (6.43-10.73) | 15.61 (11.53-23.72) |
| Simcoe[1] $F_8$ - 2000 | 512 | 2.34 (1.97-2.72) | 5.53 (4.63-7.06) | 11.17 (8.48-16.80) |
| Simcoe $F_2$ - 2002 | 540 | 0.76 (0.52-0.99) | 5.17 (4.02-7.44) | 11.43 (7.85-20.54) |
| Simcoe $F_4$ - 2003 | 540 | 1.53 (1.20-1.86) | 9.57 (7.46-13.41) | 20.46 (14.43-33.57) |
| Simcoe $F_3$ - 2004 | 540 | 0.61 (0.36-0.82) | 3.20 (2.52-4.74) | 6.36 (4.39-12.51) |
| Simcoe $F_4$ - 2005 | 540 | 0.26 (0.01-0.51) | 1.39 (0.99-2.13) | 2.78 (1.91-12.37) |

* Quebec colony was lost during over-wintering diapause

** Essex colony was lost during over-wintering diapause

[1] Bioassays ran for comparison at the University of Nebraska by Dr. Siegfried's group.

**Table 3. Validation of our discriminating dose [LC95] against 2 populations of ECB in Ontario**

| Population | Bt toxin | concentration | number tested | number affected | % affected |
|---|---|---|---|---|---|
| Essex | none | check | 48 | 1 | 2.08 |
| Essex | Cry1F | 21ng/cm2 | 216 | 213 | 98.61 |
| Essex | Cry1Ab | 3.5ng/cm2 | 216 | 203 | 93.98 |
| Simcoe | none | check | 48 | 1 | 2.08 |
| Simcoe | Cry1F | 21ng/cm2 | 216 | 208 | 96.30 |
| Simcoe | Cry1Ab | 3.5ng/cm2 | 216 | 206 | 95.37 |

**Figure 1. The average weight of instars 1, 2 and 3 of European corn borer**

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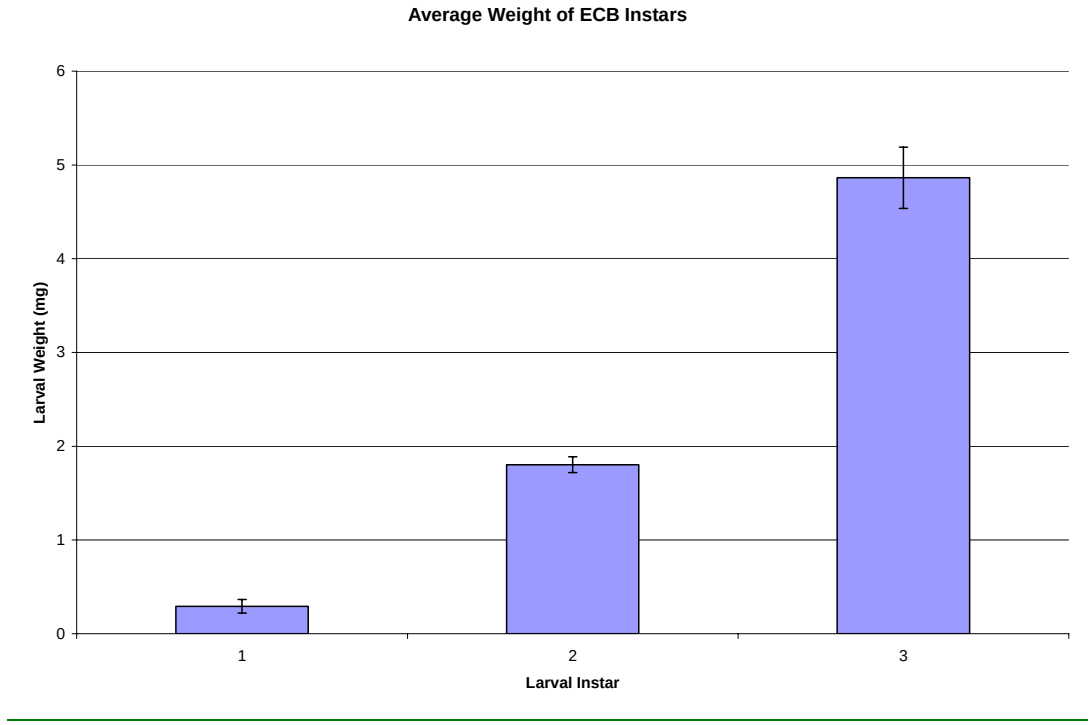


**Figure 2: Average development after 7 days exposure to 5 concentrations of Cry1A(b) for Lambton County ECB 2003 compared to non-treated check.**

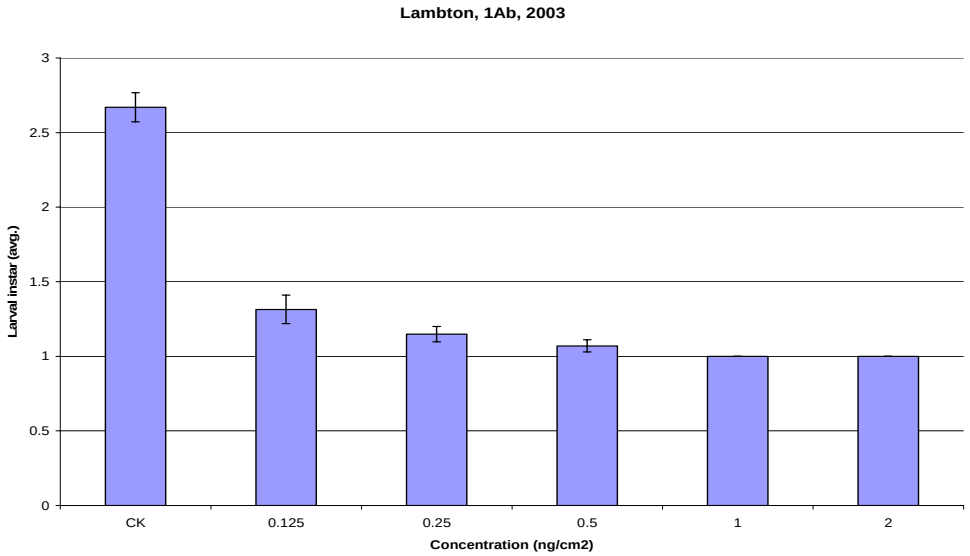
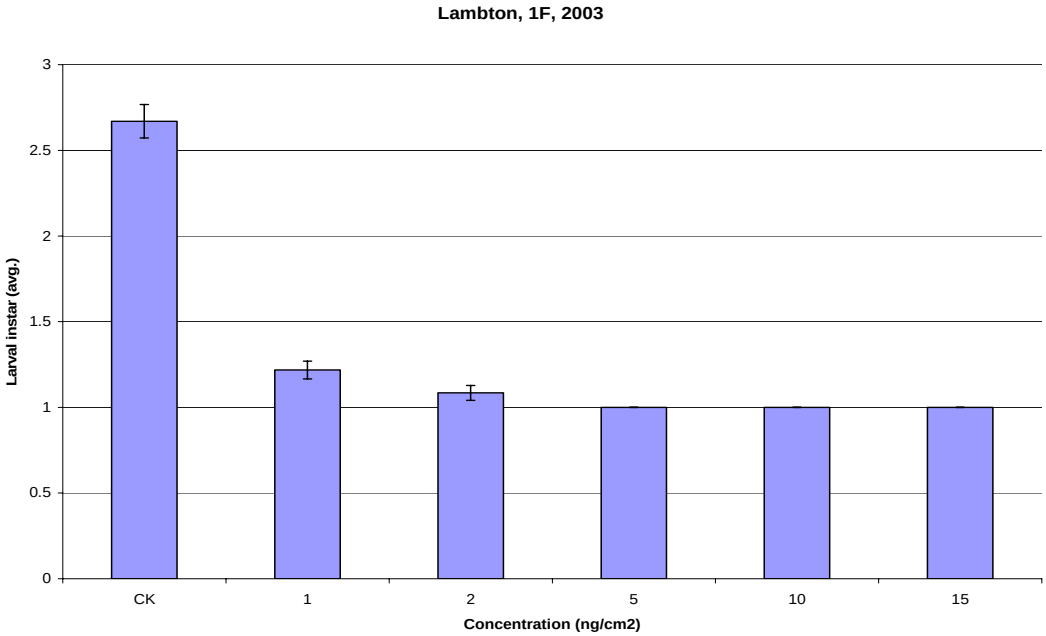



**Figure 3: Average development after 7 days exposure to 5 concentrations of Cry1F for Lambton County ECB 2003 compared to non-treated check.**



**Discussion and Conclusions**

Following each summer growing season, corn stalks containing ECB larvae were collected for the past 5 years of this project. ECB strains were collected from the same growing areas on a yearly basis in order to monitor any change in that population's susceptibility. This is important given the wide range of natural susceptibility between different geographic strains. Originally, the locations were chosen to encompass the growing area of SW Ontario (Lambton, Essex, Middlesex, Simcoe, and Niagara counties) plus the outlying Ottawa and Quebec regions. It may be more prudent to focus on the most corn-intensive growing areas in the SW Ontario region. This was discussed at the last Canadian Corn Pest Coalition (CCPC) meeting and these new areas should be phased in for our collections in future years. A maximum of 10 collection sites would be possible given time and space constraints (growth chamber space for rearing and space for over-wintering corn stalks). Following an obligatory diapause period, each strain was then tested for Bt (Cry1A(b) and Cry1F) susceptibility using our standard ECB bioassay. This data was then processed using probit analysis to determine the susceptibility of each strain for that year's collection. This data was then compared to determine relative susceptibility of different geographic strains as well as any change in a given strain's susceptibility over time (i.e. the potential development of resistance). A discriminating dose ($LC_{95}$) was also determined for both Cry1A(b) and Cry1F in order to simplify bioassay procedures for potentially resistant strains that may be sent to SCPFRC, London for analysis.

To date there has been no significant increase in Bt-tolerance [based on $LC_{95}$ values] when comparing ECB populations from Ontario and Quebec collected in 1999, 2000, 2001, 2002, 2003 or 2004 [and tested in the subsequent year]. In fact there appears to be a trend towards more susceptibility to the 2 Bt-toxins over time, although this variability is in the range of 5-fold and not considered significant. We did find natural variability between-populations in the same year of up to 5-fold and between-generations of the same population of up to 2-fold [see Essex 2001]. Larval size and instar data has also been collected during the bioassays to determine if growth inhibition occurred within the low number of ECB survivors. There was in fact a strong anti-feedant effect with significant decrease in the size of larvae that were able to survive exposure to the Bt toxins in the bioassays (see Figs 1, 2 and 3 from Lambton County as representative data from all Counties tested).

When ECB eggs from Ontario were sent to Dr. Siegfried's lab in Nebraska for validation of our data in 2001, their $LC_{95}$ value was 3.5-fold lower for Cry1A(b) and 1.6-fold lower for Cry1F than in our bioassay. However this statistically insignificant difference was likely due to the slightly weakened state of the neonate larvae used in Nebraska after a slightly longer egg storage time. The bivoltine populations of ECB in Ontario [e.g. Niagara County] were not more susceptible than the univoltine (e.g. Simcoe County) or mixed populations (e.g. Lambton County) when $LC_{95}$ values were compared for the 2 Bt-toxins.
Our discriminating dose ($LC_{95}$) was validated [Table 3] for both Cry1A(b) and Cry1F using the apparently "least susceptible strain" (Essex County) and the apparently "most susceptible strain" (Simcoe County) with between 93.9-98.6% mortality recorded.

Therefore we conclude that after 5 years of study there is no evidence of any decrease in susceptibility of European corn borer from Ontario or Quebec to the Bt-toxins, Cry1A(b) or

Cry1F.

Using similar lab bioassay techniques in Spain, Farinos et al. (2004) found that although the susceptibility of ECB populations showed minor fluctations from year to year (1999-2002) there was no trend towards higher levels of Bt tolerance. Marcon et al. (2000) established a discriminating dose for Cry1A(b) and validated this using field-collected ECB populations from across the USA, with no evidence of resistance.

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**Appendix A**

European Corn Borer (*Ostrinia nubilalis*) Rearing Procedures

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AAFC, SCPFRC, London, Ontario
November 2001

Diet: Use the meridic diet recipe attached. Sterilize larval dishes with benzalkonium chloride solution. (Pour approximately 1 Tbsp. into a sink of hot water. Make sure to wear gloves as it's very corrosive!). You will be able to make about 10 dishes with ~1 ½ inch of diet in each from this recipe. After pouring, diet is left to set and vent the volatile mould inhibitors (dishes are covered with brown paper to prevent dessication of the diet). After 24hrs, dishes are placed in the fridge in heavy plastic bags.

Larvae: Filter papers with egg masses are placed inside the larval diet dish, on top of the pupal ring. A circular mesh screen made of plastic floss is placed between the ring and the eggs to keep the filter papers from touching the diet. Larval dishes are then placed in the following conditions:

Growth cabinet: 16:8 L:D, $27^0$C day, $18^0$C night, 75% RH
Life Cycle: approximately one month from eggs to egg-laying adults

Remove the filter papers and plastic mesh from the diet dish after 1 week when all egg masses have hatched and the larvae have moved onto the diet. Generally four diet dishes are used per generation (one started every week for a month).

Pupae: After larvae have fed on the diet for approximately 2 ½ weeks, they start to climb up into the cardboard pupal ring and will pupate within a week. The pupal ring is removed after >50% of the cells are filled. Pupal rings are placed into the adult cage (30cm x 30 cm x 60 cm, mesh ceilings) where the adults will emerge. A second ring can be added to tubs to maximize the number of adults used for oviposition. This may be necessary for cultures coming out of diapause where the larvae are not pupating at the same time. Larval tubs are then placed into a freezer to kill the remaining larvae. Diet is removed from the dishes which are then washed in hot, soapy water. Do not sterilize them until they are to be used for diet.

Adults: Expect adults within a week adding the first pupal ring to the cage. White plastic margarine tubs with square holes cut out of the lids are used for the water wicks. Four paper towels are swirled up and then placed evenly through the hole in the lid of the tub. One end of the towels is placed into the water filling the dish. Open up the 2-3 inches of towel left sticking out of the dish and dampen with water, to give maximum surface area for the adults to drink from. Adults are kept under the same growth cabinet conditions as the larvae. Make sure the paper towel wicks and the sides of the adult cages are sprayed with water daily (spray cage until mesh sides are saturated with water). Replace the paper towels in the water wick if mould starts to develop.

Eggs: Wax paper strips are placed under the black plastic sheets on top of the cage. These sheets are changed daily. If eggs are to be kept for rearing, individual egg masses are cut from the sheets and placed into a petri dish lined with a moistened filter paper. This allows for selection of the larger, healthier egg masses. Petri dishes are labelled with collection date (and strain information if necessary) and then placed into a growth cabinet with the same conditions as required for adult and larval rearing. After approximately four days the eggs will have reached 'blackhead' stage and are then ready to be placed into the larval rearing tubs. At this point, eggs may be stored at $4^0$C for up to a week without affecting eclosion or development. Generally three days worth of eggs are kept for rearing while the rest are kept for bioassays if required. Egg sheets kept for bioassays are cut into eight equal squares and then stapled together. These are labelled and placed into a sealed plastic sweater box lined with moistened paper towels. The sweater box is placed into the same conditions as the eggs used for rearing. After blackhead stage is reached these sheets are transferred to a sweater box that is kept at $4^0$C. These eggs can be kept under these conditions for up to a week.

Method for Preparation of Meridic Diet for European Corn Borer (ECB), Ostrinia nubilalis
(from Guthrie et al, Handbook of Insect Rearing Vol. II, Singh and Moore [editors])

A. Sterilization

Before diet is made, the ECB diet dishes must be sterilized. Wash dishes in hot, soapy water, then rinse. In a sink full of hot water, pour roughly 1 Tbsp. of benzalkonium chloride in the water. Wash the dishes again in this mixture, taking care to wear gloves. Do not rinse the dishes after washing; just let them air dry. After dishes are sterilized, the media can be made.

B. Diet Preparation

1. Place in steam kettle: 280g Agar
   6.665L Tap water

Turn the kettle on high heat (setting 10) and start the stirrer on low, increasing the speed as the mixture thickens.
The kettle will reach $85^0$C - $87^0$C in ~ 20 min.

2. When the kettle reaches as close to $90^0$C as possible, add:
   4L chilled Tap water (placing in fridge the night before)
   520g Wheat germ

Turn the heat off at this point, but leave the stirrer on.

3. Measure blender ingredients:
   400g Dextrose
   440g Casein
   32g B-Sitosterol

144g Wesson's salt
92g Vitamin Supplement - #F8095 Bio-Serv
(NB: kept in fridge until needed)
120g Ascorbic Acid
1.05g Streptomycin Sulphate
(NB: kept in fridge until needed)
7g Fumadil - B *
21g Methyl-p-hydroxybenzoate
8g Sorbic Acid

* Fumidil - B component is tripled every 3-4 generations in order to keep Nosema contamination in check

4. Keep checking the temperature of the mixture in the kettle, inserting a thermometer in it each time. When the mixture reaches $58^0$C (~30 min.), it is time to add the blender ingredients. Do the steps in this order:

| | |
|---|---|
| to the blender, add: | 86ml Proprionic /Phosphoric Acid |
| | 7ml Formalin (or Formaldehyde) |
| | 1L Tap Water |
| then add: | all the measured powdered ingredients |
| then add: | 1.25L Tap Water on top |

Cover and blend for 3 min. Be careful because this mixture is very volatile and can erupt in the blender, oozing out through the top and sides.

5. Add all this from the blender into the kettle, stirring constantly. The kettle can't stir all this very well, so some of the mixture must be decanted and put back into the blender for 30 sec. or so. When mixed, it is added back into the kettle, and the blender is filled again with more mixture. This is repeated 3-5 times so that all the mixture in the kettle is mixed well and is uniform.

6. Place one ECB diet dish on a metal cart under the kettle and pour the diet into the bottom of the dish at a depth of ~ 1½ in (will make 10 dishes). When all the dishes are poured, they are left on a bench covered only with brown paper sheet and the lids placed loosely on top of the paper (to keep it in place). This procedure must be done so that the formalin can vent for 24hrs.

7. Once all the diet has been poured out of the kettle, add hot, soapy water to the kettle and scrub excess diet away. Decant the dirty water and keep repeating until the kettle is clean.

8. The next day, remove the brown paper, cover the diet dishes and place them in a heavy plastic bag, unsealed, in the fridge. Diet can be stored in this manner for ~4 weeks.