DATA EVALUATION RECORD
HONEY BEE - FIELD TESTING FOR POLLINATORS
§141-5 (OPPTS 850. 3040)

1. **CHEMICAL:** Clothianidin PC Code No.: 044309

2. **TEST MATERIAL:** 1) Prosper FL (9.49% clothianidin, 9.49% thiram, 4.43% carbathiin, 0.316% metalaxyl, 76.247% other ingredients)
2) Poncho 600 FS (48% clothianidin, 52% other ingredients)

3. **CITATION:**

Author: Cutler, C.

Title: An Investigation of the Potential Long-Term Impact of Clothianidin Seed Treated Canola on Honey Bees, *Apis mellifera* L.

Study Completion Date: August 1, 2006

Laboratory: Department of Environmental Biology
University of Guelph
Guelph, Ontario, N1G 2W1

Sponsor: Bayer CropScience
P.O. Box 12014, 2 T.W. Alexander Drive
Research Triangle Park, NC 27709

Laboratory Report ID: 2005-CSD-EBTIX064

DP Barcode: D336888

MRID No.: 469078-01

4. **REVIEWED BY:** Christie E. Padova, Staff Scientist, Dynamac Corporation

Signature: Christie E. Padova **Date:** 9/18/07

APPROVED BY: Teri S. Myers, Ph.D., Senior Scientist, Cambridge Environmental Inc.

Signature: Teri S Myers **Date:** 9/26/07

5. **APPROVED BY:** Anita Pease, Senior Biologist, USEPA/OPP/EFED/ERB6

Signature: APease **Date:** 12-22-10

APPROVED BY: Thomas Steeger, Ph.D., Senior Scientist, USEPA/OPP/EFED/ERB4

Signature: Thomas Steeger **Date:** 12-22-10

6. STUDY PARAMETERS:

Scientific Name of Test Organism: *Apis mellifera* L.

Age or Size of Test Organism at Test Initiation: Queens in all colonies were of the same lineage and *ca.* the same age.

Definitive Study Duration: 130 days (approximately 2 complete life cycles); 21-day exposure period (during peak bloom) followed by a 109-day post-exposure period.

7. CONCLUSIONS:

In a 130-day study (21-day exposure followed by 109-day post-exposure period), the long-term toxicity of clothianidin-treated seed was examined in the European honey bee, *Apis mellifera* L., under open field conditions at four test sites. Each site contained one 1-ha field planted with canola seed, *Brassica napus* var. Hyola 420, that had been treated with a slurry of two end-use products including Prosper® FL (9.49% clothianidin, 9.49% thiram, 4.43% carbathiin, 0.316% metalaxyl, 76.247% other ingredients) at 1250 mL/100 kg seed and Poncho® 600 FS (48% clothianidin, 52% other ingredients) at 417 mL/100 kg seed, delivering clothianidin at a reported rate of 400 g ai/100 kg seed, which according to the study author represents the highest commercial rate for use in Canada. The application rate of 400 g ai/100 kg seed is also the highest commercial rate of clothianidin use on canola in the U.S. In addition to 1-ha field planted with treated seeds, each site also contained one 1-ha control field planted with canola seed that had been treated at the same rate with specially-prepared blanks of Prosper® FL and Poncho® 600 FS formulations (only the clothianidin was removed, but the other active ingredients in the formulations remained). The study authors did not indicate whether a sticking agent was used as part of the seed treatment.

In total, four sites with two fields per site (total of 8 fields) were included in the study; each site consisted of two 1-ha fields, one planted with clothianidin-treated seed and the other planted with control (i.e., formulation blank) seed for a total of eight fields. Negative control fields were not included as part of the study design. Each of the treated and control fields were separated by at least 250 m. All fields received a pre-plant treatment with Treflan EC® (43% trifluralin) at 2.0 L/ha, and with fertilizer (ammonium nitrate, 34-0-0) at 100 kg N/ha which according to the study author was consistent with Ontario canola production recommendations. In addition, all colonies were treated with Checkmite® (10% coumaphos) prior to placement in canola to prevent against varroa and tracheal mites.

Four honey bee colonies were placed in the middle of each of the eight fields (n=32) during a 3-week canola bloom period (Day 1 = July 1, 2005), and thereafter moved to a Fall apiary (Day 21 = July 20/21, 2005) for the remainder of the study (Day 130 = November 7, 2005). Given that four colonies were placed in each of the four treated and four control fields, the study authors consider the number of replicates in both the treatment and control groups to be 16. However, it should be noted that the treatment unit in this study is the canola field and not the individual bee colony; therefore, the number of replicates per treatment group and control is four. Throughout the study, colonies were assessed for bee mortality, worker longevity, and brood development. In addition, samples of honey, beeswax, and worker-gathered pollen and nectar were regularly analyzed for clothianidin residues. Colony weight gain while in the canola fields and honey yield per colony was also determined.

According to the study authors, no other flowering crops or corn grown from seed treated with clothianidin were planted within a 1-km radius of any of the canola test plots, and the availability of alternate forage within 1 km of the test plots was minimal. Potential forage crops including soybean, corn, and alfalfa were located within 1 km of some of the test plots; however none were in bloom while honey bee colonies were in the canola test plots.

According to the study authors, overall, there was no difference ($p>0.05$) in the various measurement parameters between colonies from clothianidin-treated and control fields. Although sporadic treatment or site differences were found on various dates, essentially no differences in worker or drone mortality, worker longevity, or brood development occurred during the study. Colonies in treated fields had similar weight gains and honey yields as those in control fields. However, no raw data were provided in the study to conduct a statistical reanalysis of the study results.

According to the study authors, assessments by experienced bee researchers confirmed that colonies from clothianidin-treated field were as strong and healthy as those from control fields. Although the study authors reported no differences between colonies from clothianidin-treated and control fields, there were differences in emergent canola and flea beetle damage between the treated and control fields. There were more emergent canola seedlings at 13-14 and 20-21 days post-planting in clothianidin-treated fields as compared to control fields. However, development of emerged plants was the same in both; therefore, the bloom period was not impacted. Flea beetle damage was also significantly greater in control fields as compared to treated fields.

Although the majority of treated and control samples collected (>75%) for residue analysis had no detectable levels of clothianidin residues (limit of quantification [LOQ] = 0.5 ng/g; ppb), it is important to note that clothianidin residues were detected in nectar samples collected from the control hives during the exposure and recovery periods. Clothianidin was detected in nectar samples from two control plots including "E1C" at a concentration of 0.535 ppb (LOD=0.5 ppb) on July 7, 2005 and also in control plot "W3C" at concentrations of 0.670 ppb and 0.969 ppb on July 7 and August 11, 2005, respectively. Given the unexpected presence of

clothianidin in nectar samples from control hives, the study authors re-analyzed "back-up" nectar samples from the same control hives (*i.e.*, "E1C" and "W3C") and found similar results with detections of 0.691 to 0.922 ppb in the "E1C" and "W3C" plots, respectively. Based on information provided the study authors, the range of clothianidin residues from hives exposed to treated fields were as follows: honey = 0.501 to 0.928 ppb; nectar = 0.521 to 2.24 ppb; pollen = 0.698 to 2.59 ppb; beeswax = no concentrations detected above the LOQ of 0.5 ppb. Although clothianidin was detected in nectar from control hives at concentrations ranging from 0.535 to 0.969 ppb, it was not detected above the LOQ of 0.5 ppb in honey, pollen, and beeswax from control hives. With the exception of two samples collected during the post-exposure period (one nectar control sample, where clothianidin was detected at a concentration of 0.969 ppb, and one nectar treatment sample, where clothianidin was detected at a concentration of 0.693 ppb), all other measured clothianidin residues in both treatment and control hives were collected during the 21-day exposure period. According to the study authors, the reported levels of clothianidin in honey, nectar, and pollen are approximately 8-fold below the reported field relevant NOAEC of 20 ppb, which is based on feeding experiments using spiked diets.

Despite the findings that clothianidin was detected in <75% of the samples, the study authors concluded that honey bees that forage on clothianidin seed-treated canola will be exposed to clothianidin residues in pollen, nectar, and honey. Although the study authors mention that they confirmed canola pollen from the genus *Brassica* to be present in samples, the percentage of pollen that is canola was not specified; as such, the extent to which the bees actually foraged in the study site is uncertain. Exposure concentrations appeared to be below those required to elicit acute and sublethal effects, although the indication of clothianidin contamination in control hives precludes quantitative comparisons between treated and untreated hives in the period of exposure and overwintering.

An additional confounding factor of the study results is loss of queens that occurred in both clothianidin-treated and control hives. According to the study authors, a total of 6 colonies from clothianidin-treated fields and five colonies from control fields were naturally and/or artificially re-queened during the study. In addition, three of these 11 colonies (two control hives and one treatment hive) were classified as "dead" during the study, and attempts to re-queen these hives were unsuccessful. According to the study authors, data from these hives were omitted from "some" of the statistical analysis; however, the authors considered that loss of these colonies had no impact on the study results.

In a study addendum (MRID 469078-02), the status of 29 of the original 32 over-wintered colonies was assessed on April 19-20, 2006. Observations included the presence/absence of the queen, presence/absence of eggs and larvae, area of sealed brood, number of frames of workers, and overall health of the colony. The Spring assessment found no significant difference in the overall performance of treated and control colonies. Two treated colonies and two control colonies did not survive the winter. Of the 25 colonies that survived the winter, a healthy queen was found in 21, and the presence of eggs and larvae in the remaining four was

considered as indicative that these colonies were queen-right. The study authors did not report whether supercedure may have occurred for the four colonies where no queen was found. There was no statistical difference between treated and control colonies in the amount of sealed brood or in the number of frames with workers. Collectively, the authors classified 24 of the colonies as "healthy", one was classified as "weak" (<4 frames of live bees), and four were "dead" (*i.e.*, two control and two treated colonies).

At face value, the field study indicates that clothianidin residues were brought back to the bee colonies in both nectar and pollen and residues were detected in comb honey as well; however, residues were not detected in comb wax. Based on the parameters measured, overall, bees foraging on canola grown from clothianidin treated-seed did not differ significantly from bees foraging on fields treated with formulation blanks. Additionally, no statistical differences were detected in overwintering success between the two treatment groups. There are however, confounding effects (see below) in this study limiting its utility in risk assessment.

8. ADEQUACY OF THE STUDY:

A. Classification: Supplemental

B. Rationale: There is uncertainty regarding the ability of this study to discriminate treatment-related effects because clothianidin residues were detected in nectar from formulation control colonies. In addition, no negative control was reported to determine if there were statistically significant effects due to the formulation (actives plus inerts minus clothianidin). The presence of clothianidin residues in control hive nectar at concentrations similar to those detected in nectar from colonies placed in treated fields suggests that at least some workers from control colonies foraged on clothianidin-treated canola. According to the study authors, the minimum distance between the treated and control fields was 250 m and was likely insufficient to prevent cross-foraging of bees between treated and control fields. In addition to the close proximity of treated and control fields, control bees may have foraged in clothianidin-treated fields because the forage in some of the control fields was of lower quality due to insect damage and reduced emergence of canola. Likewise, the extent to which bees in treated fields may have availed themselves to untreated canola is uncertain given the close proximity of the control fields. Additionally, the study did not adequately evaluate the exposure bees to canola via pollen identification analysis. Additional confounding factors associated with overall colony health included the loss of queens from 11 colonies, including three colonies that were classified as "dead" part way through the study and not included in "some" of the statistical analysis. Finally, raw data were not provided in the study to conduct a statistical reanalysis of the study results

Based on the confounding factors associated with potential clothianidin cross-contamination of nectar from control hives and the lack of a negative control, the results of the investigation of the potential long-term impact of clothianidin seed-treated canola on honeybees are not

adequate for use in risk assessment. The data indicate though that under the conditions tested, clothianidin residues were brought back to the honey bee colony through nectar and pollen and were found in honey reserves of the comb; however, clothianidin residues were not detected in comb wax. The study addendum on the assessment of overwintering colonies provides useful information on the subsequent effects of bee colonies in the year following exposure at measured residue concentrations. In summary, this field toxicity study with honeybees (OPP Gdln. No. 141-5; OPPTS 850.3040) is classified as "supplemental".

C. Repairability: N/A

9. GUIDELINE DEVIATIONS: N/A

10. SUBMISSION PURPOSE: This study was submitted to provide data on the toxicity of clothianidin to honeybees in a field test for the purpose of chemical registration (new use).

Specifically, the test was conducted in response to a request by the Canadian PMRA and the U.S. EPA; as a condition for Poncho® registration in these countries, Bayer CropScience was asked to investigate the long-term toxicity of clothianidin-treated canola to foraging honey bees.

11. MATERIALS AND METHODS:

A. Test Organisms

Guideline Criteria	Reported Information
Species: **Species of concern (*Apis mellifera*, *Megachile rotundata*, or *Nomia melanderi*)**	*Apis mellifera* L.
Colony description at beginning of test:	Each colony consisted of a single brood chamber (24 cm deep, 10 frames per super) below a shallow honey super (originally empty, 16.5 cm deep, 9 frames per super). Queens in all colonies were of the same lineage and approximately the same age. A queen excluder was placed between the brood chamber and honey super to retain the queen in the brood chamber. Colonies were adjusted for strength to establish

Guideline Criteria	Reported Information
	similar quantities of food stores (pollen and nectar), brood in all stages of development, and adults in each.
Pre-test health:	Colonies were assessed for presence of Varroa mite, tracheal mite, and infectious honey bee diseases (American Foulbrood, European Foulbrood, and Chalkbrood) prior to placement in canola and throughout the study. Colonies were also treated with Checkmite® (10% coumaphos) prior to placement in canola.
Supplier	Prior to field testing, the honey bee colonies were held at a spring apiary near the Townsend House Bee Research Facility, University of Guelph, Ontario. The study does not provide information on the source or prior exposure history of the bees.
All bees from the same source?	Yes

B. Test System

Guideline Criteria	Reported Information
Exposure Site Location and Establishment:	The four test sites were located in Elora, Ontario, Canada, at the University of Guelph, Elora Research Station (sites E1 and E2), and two neighboring farms owned by Allan and Phillip Wallace (sites W3 and W4). Each test site consisted of two 1-hectare fields, one planted with clothianidin-treated canola seed and the other planted with control seed, giving a total of eight fields. Fields at each site were separated by at least 250 m. Planting of the canola seed occurred on May 20-21, 2005. Seeds were sown to a depth of 4 cm at the highest recommended rate of 15-20 seeds/m

Guideline Criteria	Reported Information
	(8.0 kg/ha). Canola bloomed approximately one month after planting (between June 27 and June 30, 2005).
Site Preparation:	All fields received a pre-plant treatment with Treflan® EC (43% trifluralin) at 2.0 L/ha, and with fertilizer (ammonium nitrate, 34-0-0) at 100 kg N/ha according to Ontario canola production recommendations. Prior to introduction of the colonies, a 10 m x 5 m clearing was mowed in the middle of each canola field to accommodate four colonies.
Number of Replicates/Treatment:	Four colonies per field, with 1 treated and 1 control field per site, and four sites (32 total colonies). According to the study authors, the number of replicates per treatment and control plots was 16 (four hives at four sites for both the treatment and control plots). However, the treatment unit is the individual field and not the colony; technically there are 4 true replicates and 16 psuedoreplicates.
Post-exposure Site Location:	The fall apiary was located at the former University of Guelph, Cambridge Research Station, Ontario, Canada.
Lighting:	Natural; not further described.
Precipitation:	Daily precipitation ranged from 0.0 to 18.8 mm during the exposure period (data obtained from the Canadian weather website cited in the study report; refer to Reviewer's Comments section). The maximum rainfall event occurred on July 16 and 17, when 18.8 and 10.4 mm precipitation occurred, respectively. Total precipitation during the exposure period was 35.4 mm.
Temperature:	Mean daily temperatures ranged from 15.2 to 26.7°C during the exposure period.
Relative humidity:	Not reported

C. Test Design

Guideline Criteria	Reported Information
Range finding test?	None reported
Reference toxicant tested?	No
Duration of Exposure Period	21 days, during canola bloom period
Duration of Post-exposure Period	109 days in the fall apiary
Test Substance(s):	Prosper® FL Formulation Type: flowable suspension Batch No.: 312065M Ai: 9.49% clothianidin + the fungicides thiram (9.49%), carbathiin (4.43%), and metalaxyl (0.316%) Source: Gustafson, McKinney, TX Poncho® 600 FS Formulation Type: flowable suspension Batch No: 407483M Ai: 48.0% clothianidin Source: Bayer CropScience, Kansas City, MO
Control Substance(s):	Prosper FL Blank Lot No.: TAM113:70-1 Ai: thiram (9.49%), carbathiin (4.43%), and metalaxyl (0.316%) Source: Gustafson, McKinney, TX Poncho 600 Blank Lot No.: TAM113:67-1A Source: Gustafson, McKinney, TX
Canola Seed:	Variety A: Hyola 420, supplied from Interstate Payco Seed Company, West Fargo, ND

Guideline Criteria	Reported Information
Application Rate:	Treatment 1: Prosper® FL at 1250 mL/100 kg and Poncho® 600 FS at 417 mL/100 kg, to deliver clothianidin at the rate of 400 g ai/100 kg seed. Treatment 2: Blank Prosper FL (containing thiram, carboxin, and metalaxyl) and Blank Poncho 600 FS at the same ratios as used for Treatment 1.
Verification of Application Rate:	Mean (n=3) of 4175 ppm or 417 g ai/100 kg seed
Method of Seed Coating:	For both treatments, slurries were prepared by combining the appropriate quantities of each formulation. The slurries were applied to the seed using the Gustafson CBT-50 seed treater. Due to the large quantity of seed (100 kg per treatment), each treatment was divided into four 25-kg batches for treatment.
Colony Introduction:	The colonies were moved to the canola fields over a two-night period (June 27/28 and June 29/30), when approximately one-quarter to two-thirds of canola blooms in the test fields had opened (determined by visual estimation). All colonies were positioned so that the entrances faced approximately south. June 30, 2005 was identified as Day 0 of the 3-week exposure period. Honey supers were removed from and added to colonies as needed throughout the study.

Guideline Criteria	Reported Information
Post-exposure:	Colonies were moved at night from the canola fields to the fall apiary on July 20/21, when approximately 20% bloom remained in each field. Colonies remained there until study termination (Day 130; November 7). Control colonies were separated from those from the clothianidin-treated fields by at least 30 m. No other colonies were present at the fall apiary.

D. Biological Assessments

Guideline Criteria	Reported Information
Canola:	- Seedling emergence rates (determined on June 3 and June 7/8) - Development rates - Crucifer flea beetle (*Phyllotreta cruciferae* Goeze) and striped flea beetle (*Phyllotreta striolata* (F.) damage
Weight Gain:	Colonies were weighed on Days -1 (the night of transport to the canola fields) and Day 21 (the night colonies were moved to the fall apiary).
Honey Yield:	Honey yield per colony by weight. Determined by weighing empty honey supers before placement on colonies and after removal from colonies.
Adult Mortality:	Dead workers and drones were collected and counted *ca.* every 7 days from Days 0 to 130. Mortality was assessed using Gary Dead Bee Traps (DBT) or 1 x 2 m white sheets placed on the ground extending out from the hive entrance. As only eight DBT units were available, one randomly selected colony at each field was fitted with a DBT, while the entrance sheet method was used for the remaining three colonies.

Guideline Criteria	Reported Information
Brood:	The area of sealed brood was determined on Days -2, 1/2, 14/15, 33/34, and *ca.* every 14 days up to Day 98 (refer to Reviewer's Comments section). The area of sealed brood was estimated by placing an empty template brood frame that was divided into six quadrants over each test brood frame, and estimating the percent sealed. Estimates were performed on both sides of each frame, for all 10 frames of each colony. Presence/absence of eggs and unsealed larvae were also noted.
Worker Longevity:	Tagged worker bees were counted on Days 5 and 9 (post-introduction assessments), 14 and 15, and thereafter at *ca.* 14-day intervals up to Day 98. On Day 4 (allowing for a 3-day colony acclimation to the canola fields), newly-emerged (<24 hours) worker bees (from spare colonies maintained at the Townsend House Bee Research Facility) were marked with Opalith® colored/numbered thoracic tags (Graze, Bienenzuchtgeräte), and 50 marked workers were then introduced to each colony. Assessments on Day 5 indicated unsuccessful introductions in six colonies (three control and three treatment), and therefore a re-introduction was performed on Day 8. On Day 70, a second set of tagged workers was added to all colonies. At that time, 25 colonies had no tagged workers left, four colonies had one tagged worker, one colony had five tagged workers, and one colony still had 12 tagged workers. Following each reintroduction of tagged workers, those from subsequent introductions were disregarded during data collection.

Guideline Criteria	Reported Information
Queen Assessments:	Queen assessments were conducted at the time of brood assessment, *i.e.*, Days -2, 1/2, 14/15, 33/34, and *ca.* every 14 days up to Day 98. Queens were located and visually inspected to ensure normal physical health and behavior. When queens were not located, the presence of eggs confirmed the presence of a laying queen in the colony within the last 3 days. Inspections were also conducted at these times for queen supercedure cells (elongate cells in which a new queen is reared). Most often, these cells were opened to verify the presence of a larva, and then destroyed. If the queen was absent in a colony, however, in some cases supercedure cells were left to allow a new queen to be reared by the colony. In other cases, marked queens were collected from spare colonies and introduced to the experimental colonies (refer to Reviewer's Comments section for further detail).

E. Residue Analysis

Guideline Criteria	Reported Information
Nectar Collection:	A 5-g pooled sample of nectar (when available) was collected from colonies at each field on Days -3/-1, 7, 14/15, 42, and thereafter at *ca.* 21-day intervals up to Day 83. Nectar was extracted from cells using a disposable syringe, or removed by gently shaking a brood frame over a sheet of waxed paper.

Guideline Criteria	Reported Information
Honey Collection:	A 5-g pooled sample of honey was collected from colonies at each field using a small disposable spatula on Days -3/-1, 7, 13, 40, and thereafter at *ca.* 21-day intervals up to Day 102.
Pollen Collection:	A 10-g pooled sample of pollen was collected from colonies at each field on Days -3/-1, 7, 14/15, 42, and thereafter at *ca.* 21-day intervals up to Day 106. Pollen was collected over a 24-hour period using an OAC pollen trap. Approximately 5 g of each sample was analyzed under a light microscope to confirm the bees foraged on canola. The remainder was used for residue analysis.
Beeswax Collection:	A 3-cm^2 pooled sample of brood and food-free beeswax was collected from colonies at each field on Days -3/-1, 7, 13, 40, and thereafter at *ca.* 21-day intervals.

Guideline Criteria	Reported Information
Storage of Samples:	All samples collected for residue analysis were held in a freezer at -20°C until shipment to the laboratory, and were shipped on dry ice. Conditions of storage (samples and extracts) once at the laboratory were not reported. Intervals of storage (reviewer-determined): Honey: 157 days prior to extraction, and 49 days prior to analysis. Nectar: 283 days prior to extraction, and 26 days prior to analysis. Pollen: 212 days prior to extraction, and 44 days prior to analysis. Beeswax: 273 days prior to extraction, and 22 days prior to analysis. **Storage stability assessments were apparently not performed.**
Extraction/Analysis:	The residue method for clothianidin in pollen, honey, and nectar was based on Bayer Method 00554 with minor modifications. For wax, a short summary supplied by Ralf Schoning of Bayer for imidacloprid was used as the basis for extraction. For all matrices, concentrations of clothianidin were determined using LC/MS/MS, and the limit of quantitation was 0.5 ppb (ng/g).

12. REPORTED RESULTS:

Guideline Criteria	Reported Information
Quality assurance and GLP compliance statements were included in the report?	Yes (see Reviewer's Comments section for details regarding non-GLP sections).
Raw data included?	No, raw data were generally not provided for any measured parameter. Summarized data tables (means) were provided for worker and drone mortality, and worker longevity. In addition, residue data for honey, nectar, pollen, and beeswax were provided as means of the residue data from the 4 hives for each of the treated and control plots at the designated sampling intervals.
Signs of toxicity (if any) were described?	Yes

Canola Emergence:

Canola emergence, development, and flea beetle damage were compared by treatment and site. There were significantly more emerged plants per meter in clothianidin seed-treated fields than in untreated fields on both June 3 and June 8 ($p<0.0001$). Comparison among treated sites indicated that emergence was greatest at site E2 and lowest at site W4 at both intervals, although the difference was only significant on June 3 ($p = 0.0029$). On both sampling days, however, a significant site-treatment interaction was found, with generally greater emergence in treated fields ($p = 0.026$ on June 3 and $p = 0.0025$ on June 8).

Although there were generally more emerged canola plants per meter in clothianidin-treated fields than control fields, development of emerged plants was the same in both. The growth stage of emerged plants did not differ with treatment or site, and there was no significant interaction of those effects.

Flea beetle damage was significantly greater in control fields on both June 3 and June 8 ($p<0.0001$). Although flea beetle damage did not vary with site on June 3, a difference between sites was found on June 8 ($p = 0.014$).

Weight Gain:

There was no significant difference in weight gain of colonies from control and clothianidin-treated fields (**Figure 1**). In both treatments, colony weights increased approximately 23-24 kg during the 3-week exposure period in canola fields. In addition, differences in colony weight gain were not significant among sites, and there was no significant treatment-site interaction.

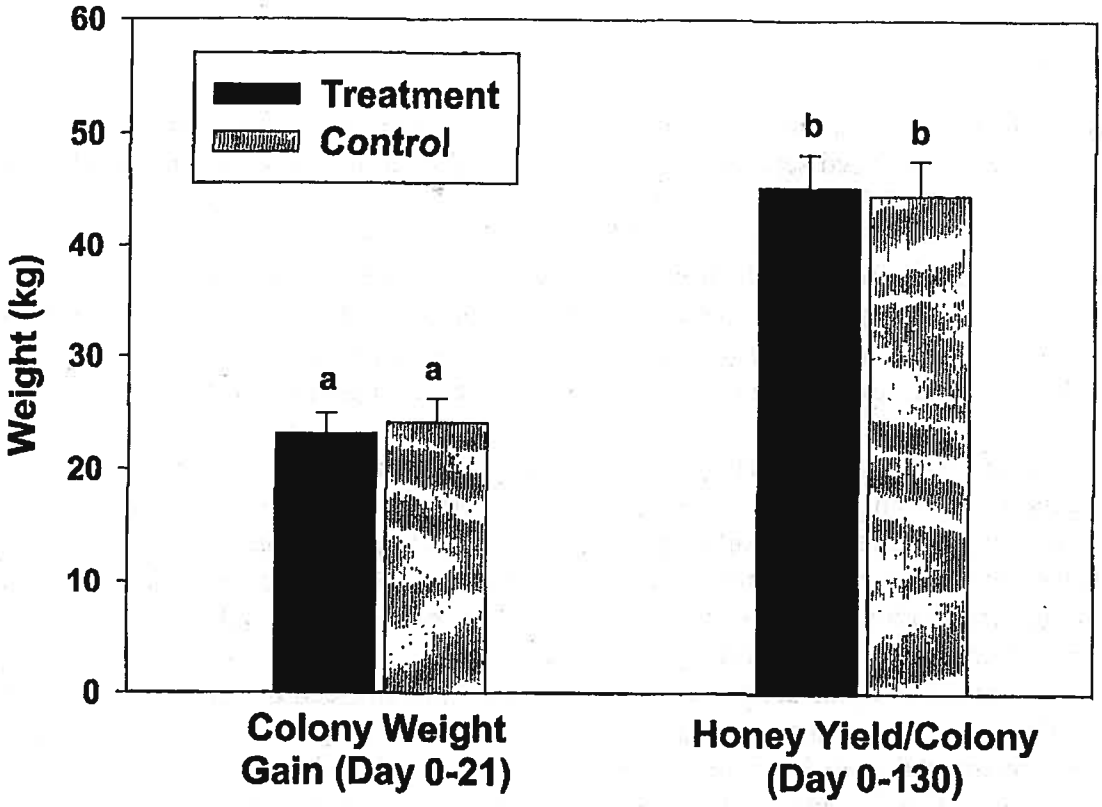

Figure 1: *Mean (± SEM) honey bee colony weight gain and honey yield after exposure to clothianidin-treated (n=16) and control (n=15) canola. Colonies were in canola fields for 21 days during bloom (Days 0-21), and thereafter moved to a fall apiary, approximately 35 km away, where they were maintained for another 109 days.*

Honey Yield:

There was no significant difference in honey yield from colonies from control and clothianidin-treated fields (**Figure 1**). A mean of 45.3 kg and 44.7 kg of honey was harvested from treated and control fields, respectively, over the 130 days of the experiment. Values were comparable to the 2005 Ontario honey yield average of 46.6 kg. In addition, differences in honey yield were not significant among sites, and there was no significant treatment-site interaction for honey yield.

Adult Mortality:

In analyzing changes in the number of adult dead workers or drones over time, DBT (dead bee trap) and sheet data were analyzed separately, and the GLM (general linear model statistical platform; SAS Institute, Cary, NC 2003) incorporated effects of day, treatment, and the interaction of these terms. For analyses on individual days, the GLM in most cases was able to simultaneously incorporate analysis of effects of site, treatment, and method of dead bee collection. In some cases, however, inclusion of one of these parameters resulted in a significant Lack of Fit (LOF) in the model, while itself not contributing significantly to the model. In such cases, the parameter causing the LOF was removed, resulting in a simpler but more robust model (**Table 1**).

There were significant changes over time in the number of dead workers recovered from colonies with both the DBT (trap) and the sheet methods ($p<0.001$). As shown in **Table 1**, there were statistically significant effects on worker mortality associated with treatment on days 77-79, 92, and 120; however, there were no consistent trends in worker mortality between treatment and controls. All remaining observation dates showed no significant differences in mortality in the clothianidin seed treatment compared to the control seed treatment. In addition, there were no significant differences in the mortality of drones resulting from clothianidin seed treatment compared to the control seed treatment. For many observation dates, mortality in worker bees was higher in the control seed treatment than in the clothianidin seed treatment. Although the recovery of dead drones changed over time with the entrance sheet method ($p<0.001$), no change over time was found with DBT.

Table 1: Honey bee adult mortality in colonies located in clothianidin-treated (n=16) and control (n=16) canola fields. At each of four sites (one treatment, one control field per site) three colonies equipped with a white entrance sheet and one colony fitted with a dead bee trap (DBT). Effects of site, treatment, dead bee assessment method, and their interaction were determined using a general linear model platform (SAS Institute 2003). Statistically-significant effects ($\alpha = 0.05$) are in bold.

Day	Dead Workers		Dead Drones	
	Effects Test	Statistics	Effects Test	Statistics
7[1]	Site	P = 0.45	Site	P = 0.11
	Treatment	P = 0.75	Treatment	P = 0.44
	Site*Trt	P = 0.51	Site*Trt	P = 0.45

Day	Dead Workers		Dead Drones	
	Effects Test	**Statistics**	**Effects Test**	**Statistics**
13	Site[2]	P = 0.40	Site	P = 0.69
	Treatment	P = 0.13	Treatment	P = 0.61
	Site*Trt	P = 0.18	Method	P = 0.92
			Site*Trt	P = 0.39
			Site*Method	P = 0.56
			Trt*Method	P = 0.58
18	Treatment[3]	P = 0.07	Treatment[3]	P = 0.18
	Method	**P < 0.001**	Method	P = 0.06
	Trt*Method	P = 0.13	Trt*Method	P = 0.12
21	**Colonies moved from canola fields to fall apiary**			
28	Site	P = 0.77	Site	P = 0.97
	Treatment	P = 0.31	Treatment	P = 0.38
	Method	P = 0.34	Method	P = 0.32
	Site*Trt	P = 0.71	Site*Trt	P = 0.57
	Site*Method	P = 0.27	Site*Method	P = 0.31
	Trt*Method	**P = 0.03**	Trt*Method	P = 0.70
35[1]	Site	P = 0.51	Site	P = 0.41
	Treatment	P = 0.35	Treatment	P = 0.88
	Site*Trt	P = 0.06	Site*Trt	P = 0.45
42[1]	Site	P = 0.20	Site	P = 0.59
	Treatment	P = 0.46	Treatment	P = 0.35
	Site*Trt	P = 0.50	Site*Trt	P = 0.67
49	Treatment[3]	P = 0.15	Site	P = 0.93
	Method	P = 0.09	Treatment	P = 0.60
	Trt*Method	**P = 0.002**	Method	P = 0.32
			Site*Trt	P = 0.57
			Site*Method	P = 0.81
			Trt*Method	P = 0.71

Day	Dead Workers		Dead Drones	
	Effects Test	**Statistics**	**Effects Test**	**Statistics**
64	Site	P = 0.96	Site	P = 0.87
	Treatment	P = 0.31	Treatment	P = 0.23
	Method	P = 0.32	Method	P = 0.97
	Site*Trt	P = 0.56	Site*Trt	P = 0.26
	Site*Method	P = 0.42	Site*Method	P = 0.37
	Trt*Method	**P = 0.03**	Trt*Method	P = 0.90
71	Site	P = 0.39	Site	P = 0.67
	Treatment	P = 0.07	Treatment	P = 0.22
	Method	P = 0.52	Method	P = 0.84
	Site*Trt	P = 0.33	Site*Trt	P = 0.28
	Site*Method	P = 0.13	Site*Method	P = 0.95
	Trt*Method	P = 0.79	Trt*Method	P = 0.73
77 / 79	Site	**P = 0.002**	Site	P = 0.72
	Treatment	**P = 0.02**	Treatment	P = 0.97
	Method	**P < 0.001**	Method	P = 0.82
	Site*Trt	P = 0.98	Site*Trt	P = 0.54
	Site*Method	**P = 0.004**	Site*Method	P = 0.68
	Trt*Method	**P = 0.004**	Trt*Method	P = 0.47
89	Site	P = 0.50	Site	P = 0.99
	Treatment	P = 0.62	Treatment	P = 0.47
	Method	P = 0.46	Method	P = 0.29
	Site*Trt	P = 0.37	Site*Trt	P = 0.12
	Site*Method	P = 0.91	Site*Method	P = 0.88
	Trt*Method	P = 0.18	Trt*Method	P = 0.70
92	Site	**P = 0.03**	Site	P = 0.86

Day	Dead Workers		Dead Drones	
	Effects Test	**Statistics**	**Effects Test**	**Statistics**
	Treatment	**P < 0.001**	Treatment	P = 0.57
	Method	**P < 0.001**	Method	P = 0.89
	Site*Trt	**P = 0.04**	Site*Trt	P = 0.99
	Site*Method	**P = 0.002**	Site*Method	P = 0.35
	Trt*Method	**P = 0.004**	Trt*Method	P = 0.87

Day	Dead Workers		Dead Drones	
	Effects Test	**Statistics**	**Effects Test**	**Statistics**
99	Site[4]	P = 0.19	Site	P = 0.16
	Method	**P < 0.001**	Treatment	P = 0.47
	Site*Method	P = 0.17	Method	**P = 0.04**
			Site*Trt	P = 0.06
			Site*Method	**P = 0.03**
			Trt*Method	P = 0.96
106	Site	P = 0.07	Treatment[3]	P = 0.07
	Treatment	P = 0.54	Method	P = 0.08
	Method	P = 0.38	Trt*Method	P = 0.10
	Site*Trt	P = 0.49		
	Site*Method	P = 0.08		
	Trt*Method	P = 0.89		
112	Site	**P < 0.001**	Treatment[3]	P = 0.07
	Treatment	P = 0.13	Method	**P = 0.05**
	Method	**P < 0.001**	Trt*Method	P = 0.07
	Site*Trt	P = 0.81		
	Site*Method	**P < 0.001**		
	Trt*Method	**P = 0.02**		
120	Treatment[3]	**P = 0.008**	Treatment[3]	P = 0.20
	Method	**P < 0.001**	Method	**P < 0.001**
	Trt*Method	**P = 0.009**	Trt*Method	P = 0.24

Day	Dead Workers		Dead Drones	
	Effects Test	**Statistics**	**Effects Test**	**Statistics**
127	Site	**P < 0.001**	Site	**P < 0.001**
	Treatment	P = 0.82	Treatment	P = 0.34
	Method	**P < 0.001**	Method	**P < 0.001**
	Site*Trt	P = 0.22	Site*Trt	P = 0.60
	Site*Method	**P < 0.001**	Site*Method	**P < 0.001**
	Trt*Method	P = 0.26	Trt*Method	P = 0.72

[1] DBT data unsuitable for analysis.
[2] Method parameter resulted in a significant Lack of Fit and therefore was omitted from the model.
[3] Site parameter resulted in a significant Lack of Fit and therefore was omitted from the model.
[4] Treatment parameter resulted in a significant Lack of Fit and therefore was omitted from the model.

On various collection dates, there were significant differences due to site, dead bee recovery method (DBT or sheet), treatment, and the interaction of these terms. However, there were no consistent trends in effect of these variables during the experiment. Recovery of dead bees using DBT was usually significantly greater than that with entrance sheets, although a significant method-site interaction was often found, indicating high variability in the number of dead bees recovered among the colonies fitted with a DBT. From a total of 18 sampling dates over 130 days, only one data set (Day 56) showed a statistically significant increase in worker mortality in treated colonies, whereas on three sampling dates (Days 77/79, 92, and 12) mortality from control colonies was statistically higher, Thus, there was overall no relevant difference between treatment and control colonies in worker mortality. In general, more dead workers than drones were recovered throughout the experiment, regardless of the collection method (**Table 2; Figure 2**). As shown in **Figure 2**, mortality in worker bees appeared to be substantially higher in clothianidin-treated colonies at days 13-64 with the DBT, although this trend is not apparent in the entrance sheet method. The reported statistical analysis did not reflect the difference in worker bee mortality with the DBT method, possibly due to high variability in the number of dead worker bees. Effects on the number of dead drones recovered were minimal, but were occasionally found near the end of the experiment. As expected, the number of dead workers increased near the end of the experiment (*e.g.*, Day 99) as colonies prepared for over-wintering.

Table 2: Honey bee adult worker and drone mortality in colonies located in clothianidin-treated (n=16) and control (n=16) canola fields. At each of four sites (one treatment, one control field per site) three colonies equipped with a white entrance sheet, and one colony fitted with a dead bee trap (DBT).

Day	Mean No. Dead per Colony			
	Dead bee trap (DBT)		Entrance Sheet	
	Treated	Control	Treated	Control
Worker Mortality				
7	--	--	43.1	39.8
13	70.5	4.0	22.1	14.1
18	213.5	123.3	26.4	18.8
28	66.8	17.8	18.7	37.8
35	--	--	34.1	44.9
42	--	--	24.9	34.4
49	174.5	54.8	47.6	95.5
56	128.5	40.0	29.9	35.6
64	72.3	27.8	28.1	44.8
71	19.1	63.0	11.6	44.8
77	117.5	188.9	37.2	29.0
89	54.8	107.5	72.8	47.8
92	24.7	86.8	15.7	20.9
99	356.3	333.5	79.8	113.0
106	255.3	225.3	208.3	160.8
112	117.3	148.0	16.7	9.4
120	141.0	291.8	4.8	6.3
127	87.3	109.6	18.1	2.9
Drone Mortality				
7	--	--	4.2	5.4
13	10.8	3.0	7.1	7.3
18	56.8	14.3	6.0	8.8
28	0.2	5.8	6.3	8.5
35	--	--	8.5	7.8

Day	Mean No. Dead per Colony			
	Dead bee trap (DBT)		Entrance Sheet	
	Treated	Control	Treated	Control
42	--	--	2.7	5.0
49	7.3	9.3	15.5	27.3
56	6.8	12.0	14.8	19.0
64	4.5	14.5	5.8	13.8
71	1.8	4.0	1.4	5.3
77	6.0	13.7	11.6	3.2
89	6.9	36.3	45.2	54.1
92	3.4	4.8	3.3	5.8
99	42.0	38.0	29.7	25.0
106	9.5	143.8	6.2	11.9
112	4.0	93.8	0.3	0.3
120	4.3	2.3	0.3	0.2
127	37.8	36.0	2.0	1.2

There was a noticeable spike in the number of dead workers recovered in DBT from clothianidin-treated and control colonies on Day 18 (**Table 2; Figure 2**). In the week preceding collection of dead bees on Day 13, the mean maximum temperature was 30.9°C. Under these conditions, it is possible that DBT (essentially large metal boxes covering the colony entrance) caused poor ventilation and over-heating, resulting in an increased number of dead workers on this day.

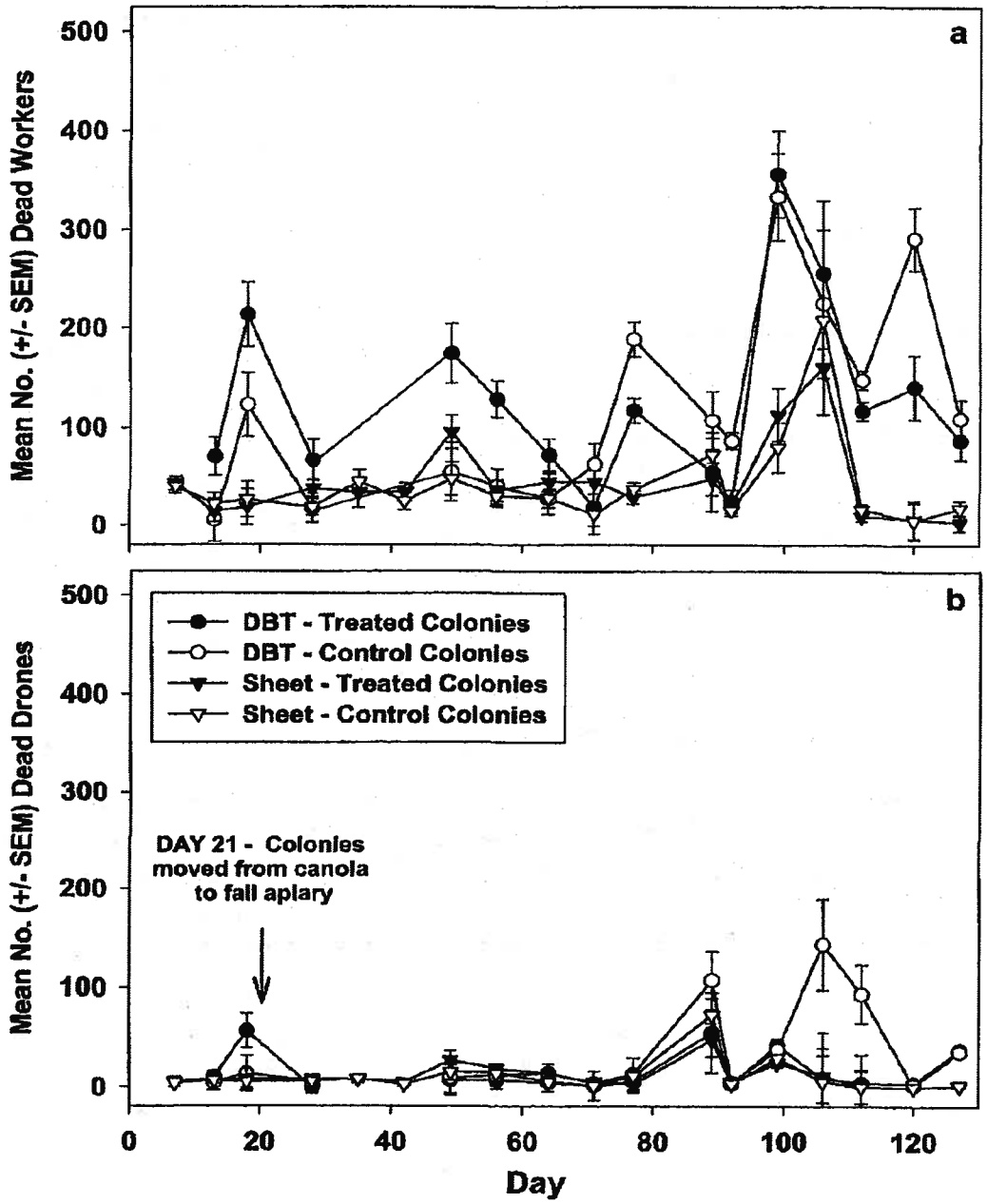

Figure 2. Honey bee adult worker (a) and drone (b) mortality in colonies exposed to clothianidin-treated (n=16) and control (n=16) canola fields. At each of four sites (one treatment, one control field per site) three colonies equipped with a white entrance sheet, and one colony fitted with a dead bee trap (DBT).

Brood Assessment:

Although brood assessments were to be conducted up to Day 130, it was evident by Day 112 that there was no or minimal sealed brood in colonies in preparation for over-wintering. Therefore, the final brood assessment was conducted on Day 97/98.

The amount of sealed brood per colony changed significantly over time in colonies from control and clothianidin-treated canola fields ($p<0.0001$). However, on most days there was no effect of site, treatment, and/or sampler (the individual determining the amount of sealed brood), or the interaction of these terms (**Figure 3; Table 3**). On Day 1/2, the amount of sealed brood per colony differed significantly across sites and on Day 33/34, the amount of sealed brood differed with the sampler. At no time during the experiment did the amount of sealed brood in colonies from clothianidin-treated field differ significantly from that found in colonies from control fields.

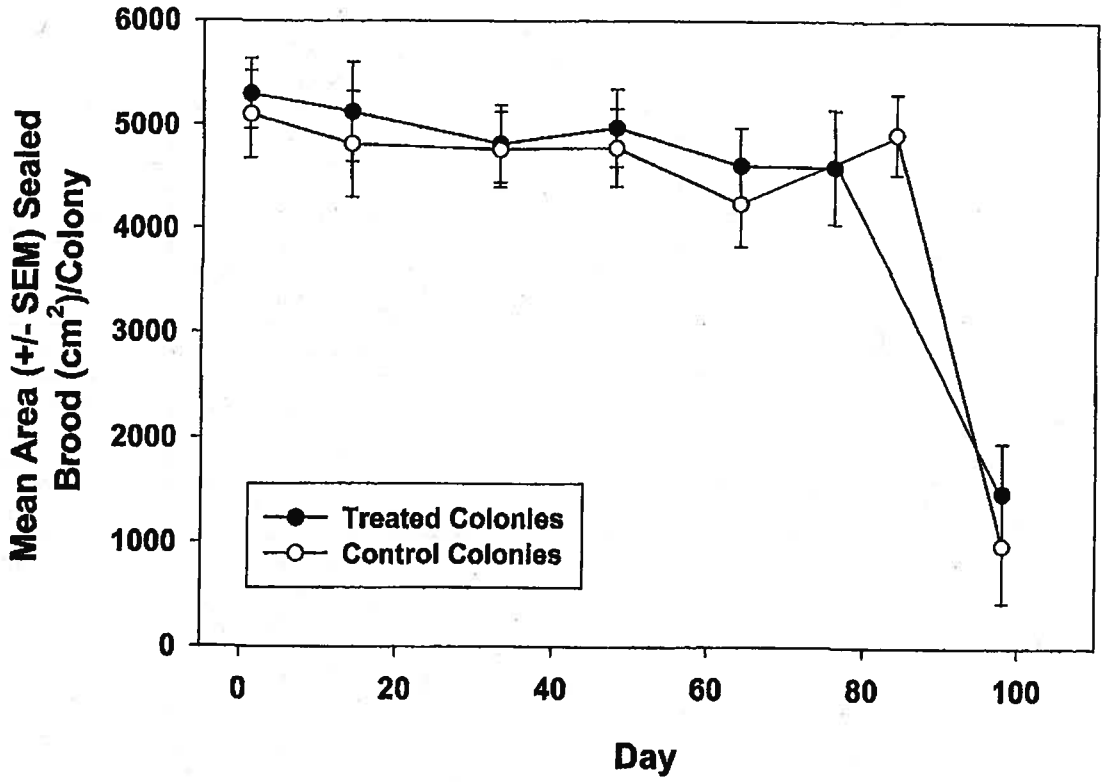

Figure 3: Mean area (±SEM) of sealed brood in honey bee colonies in clothianidin-treated (n=16) and control (n=16) canola fields.

Table 3: Mean area of sealed brood in honey bee colonies in clothianidin-treated (n=16) and control (n=16) canola fields. Colonies were placed in canola fields on July 1 (Day 1) and were moved to a fall apiary on July 21 (Day 21) for the remainder of the experiment (to Day 130). Effects of site, treatment, sampler (individual determining the amount of sealed brood), and their interaction were determined using a general linear model platform (SAS Institute 2003). Statistically-significant effects ($\alpha = 0.05$) are in bold.

Day	Mean Area (cm^2) Sealed Brood per Colony		Effect	Statistics
	Treated	Control		
1[1]	5304.2	5104.3	Site	**P = 0.007**
			Treatment	P = 0.06
			Site*Trt	P = 0.09
14	5126.14	4814.7	Site	P = 0.32
			Treatment	P = 0.61
			Sampler	P = 0.27
			Site*Trt	P = 0.48
			Site*Sampler	P = 0.39
			Trt*Sampler	P = 0.56
33/34[2]	4816.8	4762.0	Sampler	**P = 0.05**
			Treatment	P = 0.84
			Sampler*Trt	P = 0.88
48/49[2]	4975.0	4780.3	Sampler	P = 0.36
			Treatment	P = 0.69
			Sampler*Trt	P = 0.16
63/64[2]	4612.1	4238.6	Sampler	P = 0.17
			Treatment	P = 0.93
			Sampler*Trt	P = 0.14
76/84[1]	4597.4	4917.6	Site	P = 0.53
			Treatment	P = 0.49
			Site*Trt	P = 0.79
97/98[1]	1179.9	819.6	Site	P = 0.54
			Treatment	P = 0.15
			Site*Trt	P = 0.07

[1] Could not incorporate 'sampler' effect into model.

[2] Could not incorporate 'site' effect into model.

Worker Longevity:

The number of tagged workers decreased over time in colonies from both clothianidin-treated and control canola fields ($p<0.0001$). The GLM found no significant effect of site or treatment on longevity of tagged workers. There also was no significant ($p>0.05$) date*site, date*treatment, or site*treatment interaction effects. Throughout the experiment, there was no significant difference in the number of tagged workers found in colonies from clothianidin-treated and control fields on any give day (**Figure 4; Table 4**). That is, workers lived as long in colonies in treated fields as in control fields.

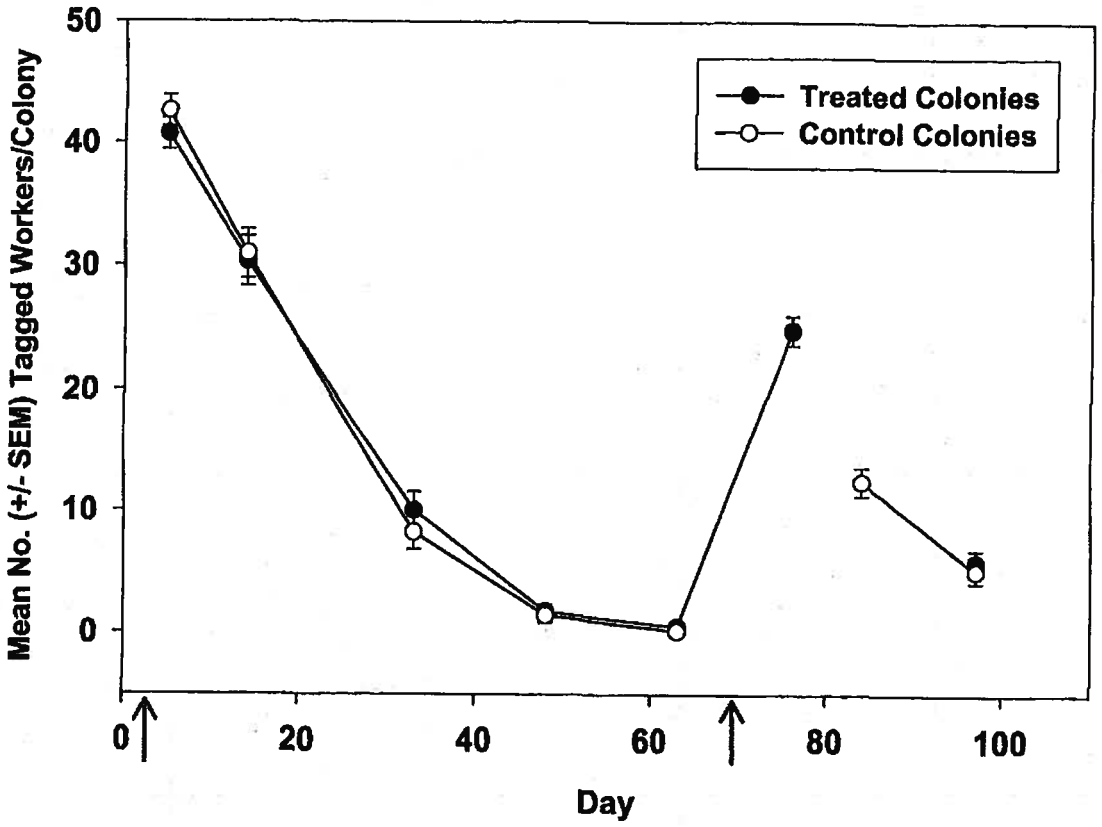

Figure 4: Honey bee worker longevity in colonies in clothianidin-treated (n=16) and control (n=16) canola fields. Workers (n=50) tagged with colored/numbered thoracic tags were added to each colony (denoted by arrows on X-axis) on Day 4 (July 4); a second tagged worker introduction (n=50) was made on Day 70 (Sept. 8). The number of tagged workers in each colony was assessed on ca. 14-day intervals, but was not determined on Day 76-77 and Day 88 for control and clothianidin-treated colonies, respectively.

Table 4: Honey bee worker longevity in colonies in clothianidin-treated (n=16) and control (n=16) canola fields. Workers (n=50) tagged with colored/numbered thoracic tags were added to each colony on Day 4 (July 4); a second tagged worked introduction (n=50) was made on Day 70 (Sept. 8). Effects of site, treatment, and their interaction were determined using a general linear model platform (SAS Institute, Cary, NC. 2003).

Day	Mean No. Tagged Workers		Effect	Statistics
	Treated	Control		
5/9	40.7	42.6	Site	P = 0.87
			Treatment	P = 0.30
			Site*Trt	P = 0.12
14/15	30.3	30.3	Site	P = 0.81
			Treatment	P = 0.98
			Site*Trt	P = 0.34
33/34	9.7	8.2	Site	P = 0.54
			Treatment	P = 0.48
			Site*Trt	P = 0.08
48/49	1.7	1.4	Site	P = 0.72
			Treatment	P = 0.70
			Site*Trt	P = 0.26
63/64	0.5	0.1	Site	P = 0.77
			Treatment	P = 0.22
			Site*Trt	P = 0.55
76/77	24.8	NA[1]	Site	--
			Treatment	--
			Site*Trt	--
84	NA[1]	12.4	Site	--
			Treatment	--
			Site*Trt	--
97/98	5.8	5.1	Site	P = 0.71
			Treatment	P = 0.62
			Site*Trt	P = 0.27

[1] Data not collected.

Disease:

Incidence of disease was low throughout the study. Colonies were treated with Checkmite® (10% coumaphos) prior to placement in canola. As a result, the incidence of varroa and tracheal mites was very low throughout the study; in the majority of colonies, no varroa or tracheal mites were detected. The bacterial diseases American foulbrood (*Paenibacillus larvae ssp.*) and European foulbrood (*Melissococcus plutonius*) were not found in any colonies during the study. The fungal disease Chalk brood (*Ascosphaera apis*) was sporadically detected at very low levels (*i.e.*, 5-10 cells/colony) throughout the study. However, as workers routinely remove chalkbrood mummies, the disease never affected the overall health of colonies. Larval mummies were not included in the number of dead bees counted in the DBT and entrance sheets; therefore, the results were used only to monitor the number of dead adult bees.

Queen Losses and Overall Colony Health:

The presence of eggs and larvae were observed in colonies throughout the study. Due to losses of the queen, some colonies at some observations had low numbers of eggs and larvae. According to the study authors, loss of queens from colonies was expected given the intense amount of data collection, movement of colonies, and large number of colonies in the study. During the experiment, personnel replaced queens in eight colonies (**Table 5**). In five colonies (E2Cc, W3Cc, W3Cd, E2Tb, and E2Tc), original marked queens that died or were killed were naturally replaced by a virgin queen (*i.e.*, the colony replaced the original queen on its own). Colonies W3Cc and E2Tc were both artificially and naturally re-queened. Therefore, a total of six colonies from clothianidin-treated fields and five colonies from control fields were naturally and/or artificially re-queened during the study. Colony W3Ta was problematic throughout the study. It was found to be queenless on July 8 (likely killed during the move of colonies to canola fields), and subsequently did not accept artificially re-introduced queens. However, the colony was found to be successfully naturally re-queened on Day 63 and thereafter.

Table 5: Honey bee colonies to which new queens were introduced.

	July 8	August 4	August 18
Treated Colonies			
E2Ta		X	
E2Tc		X	
W3Ta	X	X	X
W4Tc	X	X	
W4Td			X
Control Colonies			
W3Cc			X

W4Ca		X	
W4Cb	X		

Three colonies (W3Cc, W4Cb, and W4Td) were classified as "dead" part way through the study. These colonies were artificially or naturally re-queened during the experiment, but failed to successfully establish a queen. Although data from these colonies may have inadvertently been collected (prior to status was realized), these data were omitted from some statistical analyses. Although the study authors indicate that the three colonies (W3Cc, W4Cb, and W4Td) were omitted from "some" statistical analyses, including sealed brood analyses near the end of the experiment), it is unclear the extent to which data from these hives were removed from other statistical analysis presented in the study. Given that adequate data were collected from these colonies through much of the experiment, and that there were a large number of replicates in total, the loss of these colonies had no impact on the study overall according to the study authors.

As a general observation, experienced beekeepers/researchers qualitatively assessed colonies from clothianidin-treated and untreated canola fields throughout the study and found no differences in overall colony health and vigor.

Residue Analysis:

Clothianidin was detected in treated seed at the prescribed level at an average of 417 g ai/100 kg seed. In-phase recovery (± SD) of clothianidin residues from spiked samples of honey, nectar, pollen, and beeswax was 93 ± 13.0, 89 ± 13.3, 87 ± 13.8, and 104 ± 21.6%, respectively.

The majority of samples (>75%) collected had no detectable levels of clothianidin residues (LOQ = 0.5 ppb), whether from colonies in treated or control fields (**Table 6**). Clothianidin was detected at concentrations ranging from 0.501 to 0.928 ppb in honey collected from colonies in clothianidin-treated fields, and < LOQ (<0.5 ppb) in honey collected from colonies in control fields. In nectar collected from colonies in treated fields, clothianidin was detected at concentrations ranging from 0.521 to 2.24 ppb; nectar from colonies in control fields contained clothianidin concentrations ranging from 0.535 to 0.969 ppb. Clothianidin residues in pollen from treated fields ranged from 0.698 to 2.59 ppb and <LOQ (<0.5 ppb) in control canola fields. In beeswax, clothianidin concentrations were < LOQ (<0.5 ppb) in samples collected from colonies in clothianidin-treated and control fields.

Regarding the cross-contamination of clothianidin in nectar samples collected from colonies in control fields, analyses conducted in January 2006 detected residues in three samples (field E1C, July 7; field W3C, July 7; and field W3C, August 11). Subsequent analyses of back-up nectar samples detected residues in two control colonies (field E1C, July 7; and field W3C, July 7), suggesting that workers in control colonies may have foraged on clothianidin-treated canola. This may have occurred because the separation between some pairs of control and treated fields was insufficient or because the forage in some control fields was of lower quality (due to insect damage

and lower rates plant emergence), which may have lured workers from control fields to the treated fields. Clothianidin was also detected in two nectar samples collected from colonies during the post-exposure period when they were not in canola fields (field W3T, June 27; field W3C, August 11).

Table 6: Clothianidin residues in honey, nectar, pollen, and beeswax collected from honey bee colonies in clothianidin-treated and control canola fields. Pooled samples were collected at each site approximately every 21 days.

Matrix	Treatment	Total No. Samples	Samples with Residues Detected	Residue Detected (ng ai/g or ppb)
Honey	Clothianidin	28	W4T, July 07 W3T, July 07 E2T, July 07 E1T, July 07 W3T, July 07	0.501 0.647 0.510 0.928 0.507
	Control	28	--	<0.5[1]
Nectar[2]	Clothianidin	15 (Jan.)	E1T, July 07 E2T, July 07 W3T, July 07 W4T, July 07	0.521 0.979 1.17 0.855
		23 (Mar.)	W3Td, July 27 E1T, July 07 E2T, July 07 W3T, July 07 W4T, July 07	0.693 2.24 0.717 1.74 1.14
	Control	15 (Jan.)	E1C, July 07 W3C, July 07 W3C, August 11	0.535 0.670 0.969
		23 (Mar.)	E1C, July 07 W3C, July 07	0.691 0.922
Pollen	Clothianidin	19	E1T, July 07 E2T, July 07 W3T, July 07 E1T, July 14	1.05 0.698 2.59 1.40
	Control	19	--	<0.5
Beeswax	Clothianidin	0	--	<0.5
	Control	0	--	<0.5

[1] 0.5 ng/g = LOQ

[2] Residue analyses conducted in January 2006 unexpectedly detected clothianidin residues in nectar samples collected from control colonies. Therefore, back-up nectar samples were sent to the laboratory in March 2006 for re-analysis.

Reported Statistical Results:

Plant emergence, development, and flea beetle damage were compared among treatments and sites using a general linear model (GLM) platform (SAS Institute, 2003). Colony weight gain during exposure, honey yield during exposure, worker and drone mortality, brood area per colony, and tagged worker longevity (based on the number of tagged workers recorded each collection day) were also compared over time using a GLM platform.

13. SUPPLEMENTAL ASSESSMENT OF OVERWINTERED COLONIES:

A supplemental report documenting further assessments of the colonies during over-wintering was concurrently-submitted [MRID 469078-02; Cutler, C., and C. Scott-Dupree. 2006. Spring 2006 Assessment of Overwintered Colonies Studied in an Investigation of the Potential Long-Term Impact of Clothianidin Seed Treated Canola on Honey Bees, *Apis mellifera* L. Unpublished report conducted by the University of Guelph, Ontario, Canada, and sponsored by Bayer CropScience, Research Triangle Park, NC. Report submitted July 12, 2006]. Data presented in the addendum report were not collected in accordance with GLP requirements, and raw data were not submitted.

As the colonies prepared for over-wintering beginning in late October, each colony was administered *ca.* 30 g of a mixture of oxytetracycline and icing sugar. Colonies were then provided access to 150 L of a sucrose:water (2:1) solution. In mid-November, colony entrances were reduced, an upper entrance was provided, and insulation was placed between the inner cover and the colony lid. On April 19-20, 2006, the status of over-wintered colonies was assessed for the presence/absence and health of queen, presence/absence of eggs and larvae, area of sealed brood, number of frames of workers, and overall health based on a collective assessment of all data per colony. Colonies were classified as "healthy" if they had ≥4 frames of live bees, and "weak" with <4 frames of live bees.

Overall, the spring assessment found no significant differences in the health of treated versus control colonies. Of the initial 32 colonies, three were classified as "dead" at the end of the fall 2005 data collection, and an additional four colonies (two from treated fields and two from control fields) did not survive the winter. It was reported that a loss of 10-15% of colonies in an apiary over winter is not uncommon in Canada. Of the 25 colonies that survived winter, a healthy queen was found in 21. The presence of eggs and larvae, however, confirmed that the remaining four colonies were queen-right. There was no difference between control and clothianidin-treated colonies in amount of sealed brood ($p = 0.56$) or in the number of frames of workers ($p = 0.95$). Collectively, 24 colonies were classified "healthy", one was classified "weak", and four were "dead".

14. REVIEWER'S VERIFICATION OF STATISTICAL RESULTS:

Replicate data were not provided to statistically verify the results of this study. The reviewer visually verified the reported results; the statistical analyses are consistent with the study author's assessments, with the understanding that individual colonies are not the treatment unit but for the sake of this analysis, they are treated as such.

15. REVIEWER'S COMMENTS:

Based on the information provided in the study report, the reviewer's conclusions agreed with those of the study author, which were reported using the individual colonies as a replicate rather than the individual field. However, as previously mentioned, no raw data were provided in the study to conduct a statistical reanalysis of the study results. Although sporadic differences between treatment and formulation control colonies were found on various dates, there were no consistent differences in bee mortality, worker longevity, or brood development between treated and formulation control colony performance measures over the course of the study. In addition, colonies in clothianidin-treated field gained as much weight and yielded as much honey as those in control fields.

Although the study utilized a formulation blank for both Prosper FL (containing thiram, carbathiin and metalaxyl) and Poncho 600 FS where all inerts and actives (minus clothianidin) were included, the study did not include a negative control. As such, there is no way to determine whether the formulation blank had an effect on the bees' performance.

No residues of clothianidin were detected (LOQ = 0.5 ppb) in the majority (>75%) of samples (honey, nectar, pollen, and beeswax) collected for residue analysis. Based on information provided by the study authors, the range of detected concentrations of clothianidin from hives exposed to treated fields were as follows: honey = 0.501 to 0.928 ppb; nectar = 0.521 to 2.24 ppb; pollen = 0.698 to 2.59 ppb; beeswax = no concentrations detected above the limit of quantitation (LOQ) of 0.5 ppb. In contrast, clothianidin was detected in control hives at concentrations ranging from 0.535 to 0.969 ppb in nectar; however, it was not detected above the LOQ of 0.5 ppb in honey, pollen, and beeswax. Further analysis of the nectar residue data indicates that four out of 33 (12%) samples collected across controls were at or above the LOQ of 0.5 ppb. One contaminated sample was from control plot E1, while the remaining three samples were from control plot W3. The one contaminated sample from plot E1 was slightly above the LOQ (0.535 ppb). The remaining three contaminated samples from plot W3 ranged from 0.67 to 0.969 ppb. In contrast, eight of 33 (24%) from clothianidin-treated fields contained residues ranging from 0.521 to 2.24 ppb. The maximum concentration of clothianidin in any sample was 2.59 ppb in pollen collected from a colony exposed to treated fields on Day 7. Based on the reported nectar/pollen oral NOAEC from a spiked diet feeding study for honey bees of 20 ppb (Schmuck and Keppler, 2003), the maximum concentration of clothianidin detected in any sample during this study was nearly 8-fold below the reported oral NOAEC, indicating a high margin of safety according to the study authors. It should be noted, however, that the honey bee field study associated with the reported NOAEC value of 20 ppb (MRI 45422440) has been reviewed and classified as "supplemental" because only one replicate hive per

treatment level was tested; as such, the ability of the study to statistically test for a NOAEC is limited.

Contamination of the control hives suggests that workers in control colonies may have foraged on clothianidin-treated canola. According the study authors, this may have occurred because the separation between some pairs of control and treated fields was insufficient or because the forage in some control fields was of lower quality (due to insect damage and lower rates plant emergence), which may have lured workers from control fields to the treated fields. If contamination occurred in the control hives due to foraging on treated fields, it is plausible to assume that treated colonies foraged in control plots as well. In this case, the potential for exposure is reduced by the availability of control plots to provide an alternative source of forage. However, given that clothianidin was detected only in nectar from control hives, and not pollen, honey, or beeswax, the mechanism by which clothianidin was transported in canola to nectar only is uncertain.

All fields received a pre-plant treatment with Treflan® EC (43% trifluralin) at 2.0 L/ha, and with fertilizer (ammonium nitrate, 34-0-0) at 100 kg N/ha according to Ontario canola production recommendations. In addition, all colonies were treated with Checkmite® (10% coumaphos) prior to placement in canola to guard against varroa and tracheal mites. Although the study authors indicated that soil samples were taken from all test fields to determine soil type, no pesticide residue analyses of the soil from test plots and/or canola plants were collected as part of the study design. In addition, the soil type test results were not included in the study report.

The flowable suspension Prosper® FL nominally contains 9.49% clothianidin, 9.49% thiram, 4.43% carboxin, and 0.316% metalaxyl; carboxin, thiram and metalaxyl are classified as fungicides. The respective CAS Numbers are 210880-92-5, 137-26-8, 5234-68-4, and 57837-19-1. A Certificate of Analysis was not provided for this test substance, and only the actual percentage of the active ingredient of interest, *i.e.*, clothianidin at 9.64%, was reported in the in Appendix 4 – Seed Treatment Phase Report of the study report. The actual percentages of the other active components were not reported. In addition, the study report did not contain sufficient information to confirm a seedling rate of 400 b ai/100 kg seed based on the reported rates of Poncho® 600 FS at 1250 ml/100 kg seed and Prosper® FL at 417 ml/100 kg seed.

The study authors state that "approximately 5 g of pollen was analyzed under a light microscope, which confirmed that bees foraged on canola…". This type of identification indicates that canola was present in the pollen samples; however, it does not quantify the proportion of canola pollen present in the sample and/or adequately characterize the foraging behavior of bees in this study. It is possible that the bees in treated fields could have foraged disproportionately on other uncontaminated sources relative to bees in the control fields. To the knowledge of the study author, no other flowering crops or corn grown from seed treated with clothianidin were planted within a 1-km radius of the canola test plots. According to the study authors, the availability to bees of alternative forage within 1 km of their colonies while situated in canola fields was minimal. Although potential forage crops (*e.g.*, soybean, corn, alfalfa) were within 1 km of some fields, none of these were in bloom while honey bee colonies were in the canola fields. However, honeybees

have been shown to forage up to 6 km (Visscher and Seeley, 1982) or even twice that in instances when no competing forage is available (Ratnieks, 2000); therefore, a description of available forage within a radius of 1-km is inadequate to describe the surrounding area.

It was reported that brood assessments required opening the colony supers for *ca.* 60 minutes, sometimes under very hot, no-shade conditions, and that this procedure was very stressful for the bees. Coupled with the additional stress of moving the colonies to the fall apiary (which took several hours), it was decided not to conduct brood assessments on the day of colony removal from the canola fields. According to the study authors, it was decided to wait a week after the move before continuing with the brood assessments, to allow the colonies to acclimatize to their new surroundings. Once the hives were moved to the fall apiary on Day 21 (July 20/21) as part of the post-exposure phase of the study, brood assessments were conducted on Days 33 and 34 (August 2/3; approximately 12 days after being moved) and every 14 days thereafter until Day 98 (October 6).

Originally, brood assessments were to include the presence/absence of eggs, unsealed larvae, and sealed brood for each colony. However, the study author reported it was apparent during the Day 1 assessment that it would not be possible to make all assessments for all 32 colonies [due to lack of time and/or adequate sunlight (fall apiary assessments only)]. Therefore, it was decided to determine the amount of sealed brood only, which would reflect development of egg and larval stages. It was reported that since normal, healthy, unsealed brood eventually are sealed by workers, effects of clothianidin on brood development could still be detected.

According to the study author, queen assessments occurred, "when possible" during brood assessments. It was reported that queens were lost in some colonies during the experiment, *e.g.*, they were accidentally killed during data collection, moving colonies, or rejected by the colony over time. During the experiment, study personnel replaced queens in 8 colonies from clothianidin-treated (E2Ta, E2Tc, W3Ta, W4Tc, and W4Td) and control (W3Cc, W4Cc, and W4Cb) colonies. In such cases, marked queens from the same/similar lineage were collected from spare colonies at the Townsend House Bee Research Facility and introduced to the experimental colonies. In other cases, a new queen was allowed to emerge from a supercedure cell to replace the old queen. While the number of colonies to which new queens were introduced, either artificially or naturally, was higher for treated colonies relative to control colonies, it appears that re-queening was necessary in three out of the four test fields. Re-queening occurred for both control and treated hives in fields W3 and W4, while only treated hives required new queens in field E2.

Swarm cells (queen cells usually found on the bottom of the combs before swarming) when found were destroyed to prevent swarming.

While the entrance sheet method of collecting dead bees resulted in no technical difficulties or inadequate data, the operation of the DBT (traps) was occasionally unreliable during the experiment. For example, on various collection dates, traps were left partially open, came loose from the colony, or became partially filled with water after heavy rainfall events. Another colony (E1Tc) was mistakenly thought to be "dead" on September 27-30; however, no data are provided in the report to

substantiate the author's perceptions of the colony's status. Therefore, adult mortality data from some colonies fitted with a DBT were not used on some data collection dates. Data were excluded if the number of dead bees in the DBT were unusually low (*i.e.*, 0-1 dead bees) because, for example, the trap was not tightly fitted to the colony, or if the DBT was partially filled with water due to rain, which may have caused some live bees to drown. On sampling dates where there were less than three treated and three control colonies with DBT from which data could be used, analysis comparing dead bee collection methods were not performed. In such cases, the "method" parameter (DBT vs. entrance sheet) was not used in the GLM analyses. A total of n=18 collection dates incorporated entrance sheet dead bee collection data, while n=15 collection dates incorporated DBT collection data.

For the 3 years prior to the field studies, the sites had been planted with alfalfa, corn, soybean, barley, and wheat in 2002; soybean, barley, and corn in 2003; and soybean, corn white bean, and corn/barley in 2004. The extent to which clothianidin or other pesticides may have been used to treat these crops is uncertain because no previous soil residue analysis was provided as part of the study report.

The study timetable was as follows:

Seed Treatment Phase	Experiment Start: April 27, 2005 Test and Control Item Receipt: May 4, 2005 Report Completion: November 22, 2005
Field Study Phase	Seed planted May 20-21, 2005 Experiment Start (Day 1): July 1, 2005 Experiment Completion (Day 130): November 7, 2005 Final Report Completion: August 1, 2006
Residue Analysis Phase	Experiment Start (first samples received): August 30, 2005 Analytical Initiation Date: December 14, 2005 Analytical Completion: May 15, 2006 Report Completion: July 5, 2006

The GPS Coordinates for the canola fields were -80.4 X-coordinate and 43.6 Y-coordinate (combined and reduced to three significant figures), and for the fall apiary were -80.3 X-coordinate and 43.4 Y-coordinate.

Signed and dated GLP, Quality Assurance and "No Data Confidentiality" statements were provided. The test was conducted in compliance with the OECD and EPA Principles of Good Laboratory Practice. However, the following field study phase data were non-GLP compliant:

- Seed storage – before planting, seed was stored for *ca.* 2 weeks in a 10°C walk-in refrigerator at 39% relative humidity. Although no fluctuations in temperature or relative humidity were observed, the refrigerator maintenance and data logging was non-GLP compliant.
- Planting and maintenance (fertilizer and herbicide application) of fields.
- GPS coordinates of treatment (canola fields), pre-treatment (Airport apiary), and post-

treatment (former University of Guelph – Cambridge Research Station) sites.
- Ground truthing.
- Weather data were obtained from Environment Canada weather stations in close proximity to sites at which colonies were maintained. Data are available on-line at http://www.climate.weatheroffice.ec.gc.ca/climateData/canada_e.html.
- Sample refrigeration temperatures.
- Statistical Analysis – the software used for statistical analysis (JMP Version 5.1, SAS Institute) was not GLP validated.

Based on the confounding factors associated with potential clothianidin cross-contamination of the control hives and the absence of a negative control, the results of the investigation of the potential long-term impact of clothianidin seed-treated canola on honeybees are not adequate for use in risk assessment. The data indicate though that under the conditions tested, clothianidin residues were brought back to the honey bee colony through nectar and pollen and were found in honey reserves of the comb; however, clothianidin residues were not detected in comb wax. The study addendum on the assessment of overwintering colonies provides useful information on the subsequent effects of bee colonies in the year following exposure at measured residue concentrations. In summary, this field toxicity study with honeybees (OPP Gdln. No. 141-5; OPPTS 850.3040) is classified as "supplemental".

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