

Acetylcholinesterase in honey bees (*Apis mellifera*) exposed to neonicotinoids, atrazine and glyphosate: laboratory and field experiments

Monique Boily · Benoit Sarrasin · Christian DeBlois ·
Philippe Aras · Madeleine Chagnon

Received: 19 October 2012 / Accepted: 11 February 2013 / Published online: 27 February 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract In Québec, as observed globally, abnormally high honey bee mortality rates have been reported recently. Several potential contributing factors have been identified, and exposure to pesticides is of increasing concern. In maize fields, foraging bees are exposed to residual concentrations of insecticides such as neonicotinoids used for seed coating. Highly toxic to bees, neonicotinoids are also reported to increase AChE activity in other invertebrates exposed to sub-lethal doses. The purpose of this study was therefore to test if the honey bee's AChE activity could be altered by neonicotinoid compounds and to explore possible effects of other common products used in maize fields: atrazine and glyphosate. One week prior to pollen shedding, beehives were placed near three different field types: certified organically grown maize, conventionally grown maize or non-cultivated. At the same time, caged bees were exposed to increasing sub-lethal doses of neonicotinoid insecticides (imidacloprid and clothianidin) and herbicides (atrazine and glyphosate) under controlled conditions. While

increased AChE activity was found in all fields after 2 weeks of exposure, bees close to conventional maize crops showed values higher than those in both organic maize fields and non-cultivated areas. In caged bees, AChE activity increased in response to neonicotinoids, and a slight decrease was observed by glyphosate. These results are discussed with regard to AChE activity as a potential biomarker of exposure for neonicotinoids.

Keywords *Apis mellifera* · Biomarker · Neonicotinoids · Acetylcholinesterase · Maize cultivation

Introduction

In the last few years, important losses of honey bee (*Apis mellifera* L.) colonies have been reported worldwide, especially in Europe (Potts et al. 2010) and in North America (van Egmond et al. 2009). Although several factors are involved in these losses, including numerous pathogens and parasites, the extensive use of pesticides is regularly cited as a potential contributor (Johnson et al. 2010; Wu et al. 2011). Of growing concern are neonicotinoid insecticides such as imidacloprid, clothianidin and thiamethoxam as several studies have provided evidence that these products can be harmful to honey bees (Yang et al. 2008; Maini et al. 2010). Largely used for seed coating of many crops, especially corn and soya, these systemic insecticides protect the seeds and young plants from pests. In Québec, coated seeds count for more than 99 % of maize crops and 30 % to 50 % of soya crops (Giroux and Pelletier 2012). The flying bees, beehives or flowers attracting bees may come into contact with the dust resulting from the pneumatic action of sowing machines (Tremolada et al. 2010). More often, honey bees are exposed to residual neonicotinoid concentrations in

Responsible editor: Markus Hecker

M. Boily (✉) · M. Chagnon
Département des Sciences Biologiques, Université du Québec à Montréal, Succursale Centre-Ville, C.P. 8888, Montréal, QC H3C 3P8, Canada
e-mail: boily.monique@uqam.ca

B. Sarrasin · C. DeBlois
Ministère du Développement durable, de l'Environnement, de la Faune et des Parcs du Québec, Centre d'expertise en analyse environnementale du Québec, 2700 rue Einstein, Local B2-104, Sainte-Foy, QC G1P 3W8, Canada

P. Aras · M. Chagnon
Centre de recherche en sciences animales de Deschambault, 120-A, Chemin du Roy, Deschambault, QC G0A 1S0, Canada

plants and flowers—the pesticide originating in coated seeds (Bonmatin et al. 2005; Chauzat et al. 2006).

Neonicotinoids and their metabolites are agonists of acetylcholine (Nauen et al. 2001). They bind to the post-synaptic nicotinic receptors *nAChERs* triggering a continuous signal leading to the death of the insect. Their high efficiency is attributed to the irreversible link with the *nAChERs* receptors (Thany 2009) recognized as the most abundant in insects (Gauthier 2010) and the partial inhibition of γ -aminobutyric acid (GABA) receptors known to protect the cell from overstimulation (Déglyse et al. 2002). This dual mechanism results in the lowest LD₅₀ values (0.003 to 0.006 µg/bee, oral exposure) tested on bees among all currently used pesticides (Decourtye and Devillers 2010). Moreover, several neonicotinoid metabolites, e.g. imidacloprid, retain the toxicological characteristics of the parent molecules in terms of receptor affinity and toxicity (Suchail et al. 2001). The neonicotinoids, unlike pyrethroid insecticides (cypermethrin and deltamethrin), are not generally known to alter the AChE activity. However, increased AChE activity has been reported in the German cockroach after exposure to the neonicotinoid acetamiprid (Morakchi et al. 2005).

Neonicotinoids are not the only source of pesticides posing a threat to honey bees. The annual monitoring of surface waters in agricultural areas by the Ministry of Environment of Québec showed that atrazine and glyphosate (RoundUp®) are among the most abundant products detected (Giroux and Pelletier 2012). In the most contaminated areas, several adverse physiological effects have been found in resident frogs (Bérubé et al. 2005; Boily et al. 2005, 2009) including a high AChE activity in plasma (Marcogliese et al. 2009). It is possible that these contaminants may also affect honey bees. Atrazine has been linked to oxidative stress damage (Thornton et al. 2010) and altered activities of cytochrome P450 and glutathione-S-transferase (GST) in *Drosophila melanogaster* (Le Goff et al. 2006), while reduced longevity has been observed in Coccinelidae *Eriopis connexa* Germar (Mirande et al. 2010) and the wolf spider *Pardosa milvina* (Evans et al. 2010) treated with different formulations of glyphosate. Such effects have not been investigated in field studies with bees due to limitations in methods. Whereas in vertebrate species, oxidative stress and enzyme-based biomarkers have been developed, no such tools exist for honey bees. The objectives of this study were therefore to explore the effects of the main pesticides used in maize fields on the honey bee and to evaluate AChE activity as a potential biomarker for neonicotinoids exposure. Honey bees were exposed by placing their hives near organically grown or conventionally grown cornfields and were compared with those located near non-cultivated areas. Lab experiments, conducted in parallel to the field studies, exposed different groups of bees to increasing sub-lethal doses of imidacloprid, clothianidin, atrazine and glyphosate. AChE activity, protein concentration and

body mass were used for comparison between the in-field and laboratory conditions.

Materials and methods

Chemicals

Triton X-100 (*t*-octylphenoxy polyethoxyethanol), acetylthiocholine iodide, DMSO (dimethyl sulfoxide), DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)), sodium phosphate monobasic, Trizma base (tris[hydroxymethyl]aminomethane), technical carbaryl (Sevin, 99.8 % pure), diazinon (99.4 % pure) and clothianidin ((E)-1-(2-chloro-5-thiazolylmethyl)-3-methyl-2-nitroguanidine, 99.9 % pure) were purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada). BSA (bovine albumin serum) and Pierce BCA protein assay kits were from Fisher Scientific (Montreal, QC, Canada). Commercial formulation of pesticides imidacloprid (Admire® 240F), atrazine (Aatrex® 480) and glyphosate (Weathermax® 240) were provided by Centre de Recherche en Science Animale de Deschambault (CRSAD, QC, Canada) and represent commonly applied pesticide formulations in Québec.

AChE solubilization

All the following steps were carried out between 0 °C and 4 °C, and all media were newly prepared. The AChE solubilization was based on Badiou et al. (2008) with slight modifications. The method was developed with bees from a healthy beehive, supplied from CRSAD. Eight to ten bees were individually weighed prior to severing the heads from the body in weighing dishes disposed on dry ice. Heads were pooled (>100 mg of tissues) and immediately homogenized with a glass Potter homogenizer in 1.0 ml of LST buffer (10 mM NaCl, 1 % w/v Triton X-100, 15 mM sodium phosphate), pH 7.3 in order to make a 10 % (w/v) extract. The homogenates were centrifuged at 100,000×g for 1 h at 4 °C (rotor SW 50.1, Beckman Counter Optima L-100 XP). The supernatants were set aside, and the pellets were rinsed without agitation using 200 µl LST buffer, which was recovered and combined with the supernatant. The pellets were subjected to a second homogenization with 500 µl of LST buffer and processed as above. The combined supernatants and rinsing volumes (±1,700 µl) were used for AChE assay and protein analysis.

AChE assay

Acetylcholinesterase assay was carried out following the method of Ellman using acetylthiocholine as the substrate. A reactive medium was made for each sample by mixing 2.87 ml Tris-HCl buffer (0.01 M, pH 7.6) in a sterile glass tube, along with 100 µl DTNB (1.5 mM Tris-HCl, pH 7.6) and 30 µl

supernatant. Blank reaction tubes consisted of all reagents except supernatants. A volume of 20 μ l of the medium composed of 0.3 mM acetylthiocholine iodide and 1.5 mM DTNB, Tris-HCl, pH 8.0, was deposited in a non-sterile 96-well microplate (three replicates per sample). The microplate was covered, and the reaction rate was monitored at 10 and 35 min on a spectrophotometer (model Infinite M1000 Quadruple Monochromator Microplate Reader, Tecan Group Ltd., Durham, NC, USA) set at 412 nm. Analyses (6 to 15) were performed for each dose of pesticides tested and their control group. Values of blank reaction tubes were subtracted to provide specific AChE activity. The expression of the activity was based on substrate degradation either by micro-moles per hour per gram of tissue or nanomoles per hour per milligram protein and is referred in the text as AChE activity.

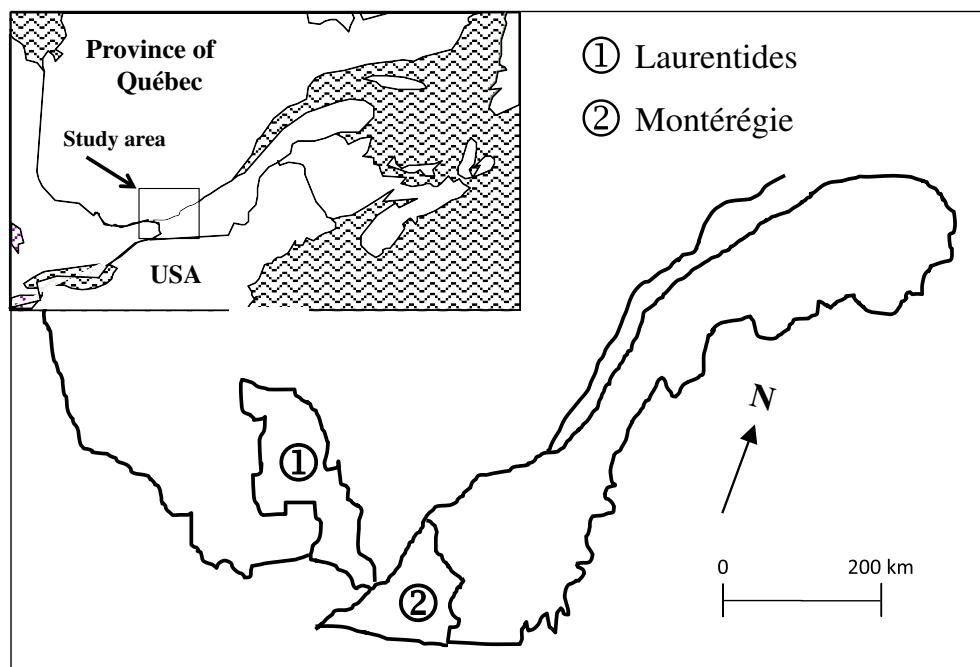
Inhibition study

The AChE activity was inhibited in vitro by two insecticides: diazinon and carbaryl. Tested separately, each compound was dissolved in DMSO, which never exceeded 2 % in the reaction medium. Diazinon and carbaryl at molar concentrations ranging from 10^{-10} to 10^{-5} were added to solutions resulting from AChE solubilization. The extraction and analysis (three replicates per dose per pool) were carried out as described above using two different pools of bees from the control groups.

Protein concentration

The protein content in each sample was determined using Pierce BCA protein assay kit.

Fig. 1 Location of field study regions in southern Québec, Canada



Field study

Field observations took place in two regions in Québec, Canada: Laurentides and Montérégie (Fig. 1). The proximity of the Laurentian Mountains limits the oleaginous and cereal cultivations to the southern area of the Laurentides region, where agricultural activities are dominated by bovine and milk productions (MDEIE 2012). Located in southwest Québec, the Montérégie is the most productive region in terms of corn-soya cultivations. This territory represents 33 % of all agricultural activities of the province with nearly 60 % of the total corn-soya production (MAPAQ 2006). In 2009, both regions had approximately 26 % bee loss (Boily et al. 2009; Bilan de la mortalité hivernale 2008–2009 au sein des colonies d’abeilles du Québec d’après le sondage postal effectué au printemps 2009, <http://www.agriresseau.qc.ca/apiculture/navigation.aspx?sid=938&p=3>, URL verified on July 10, 2012). In each region, three field types, *condition*, were tested: organically grown maize, conventionally grown maize and non-cultivated fields (situated at least 3 km away from any crop using coated seeds). A total of 18 honey bee colonies (3 hives per condition \times 3 conditions \times 2 regions) were investigated. All colonies were equalized in brood surface (larvae) and bee population before the beginning of the experiment. The hives were randomly placed a few days before maize pollen shedding, from August 5 to September 5 (2009), and six of them (one per field) were equipped with a pollen trap. Once a week, all colonies were checked for diseases, pests and mortality, which was assessed by counting the number of dead bees accumulated on a 1 \times 1-m white sheet placed in front of the hive. At the same time, approximately 30 to 40 active foragers were captured in front of each hive, using a

collector's net, immediately euthanized by placing the sample on dry ice, and then kept in a freezer at -80°C until analysis. Eight to 15 pools of ten bee heads were analyzed for each hive, the hives being the experimental units. Once a week, pollen traps were emptied, and the content was transferred to a plastic bag and kept at -20°C . Sub-samples of pollen (2 sub-samples per condition \times 3 conditions \times 4 weeks = 24 total) were sorted in the laboratory as suggested by Louveaux et al. (1978) using the glycerine-gelatine dyed with fuchsin method. The pollen grains were then examined under the microscope ($\times 1,000$) to identify pollen from maize plants. Samples having maize pollen were analyzed for neonicotinoid content.

Chronic exposure (cage experiments)

Honey bees were taken on frames without brood from a healthy beehive (directly supplied by CRSAD) and placed in acrylic cages ($13 \times 14 \times 18$ cm) in groups of 30. They were maintained in a temperature-controlled room (darkness, $25 \pm 1^{\circ}\text{C}$ with $55 \pm 5\%$ relative humidity) and fed with a sucrose solution (50 % *w/w*) ad libitum for 24 h. Prior to pesticide exposures, tests were conducted to estimate the daily mean consumption of syrup per bee. The highest selected doses represented 5 % or less than the published LD₅₀ values in order to respect the sub-lethal domain. For example, the highest doses for atrazine and glyphosate (10 ng/bee) were 0.01 % and 0.04 % of the LD₅₀ values associated with these pesticides, 97 and 28 $\mu\text{g}/\text{bee}$, respectively. The oral LD₅₀ value for imidacloprid, 6.12 ng/bee, was previously determined in our laboratory (data not shown). The lowest exposure levels were based on analyzed in-field pollen and nectar concentrations (Bonmatin et al. 2005; Chauzat et al. 2006) in order to represent field-realistic exposures. Bees were offered sugar solutions spiked with nominal concentrations of imidacloprid (0.08, 0.16, 0.24 and 0.30 ng per bee), clothianidin (0.03, 0.06, 0.12 and 0.24 ng per bee), atrazine (1.25, 2.5, 5.0 and 10 ng per bee) and glyphosate (1.25, 2.5, 5.0 and 10 ng per bee). Bees in the control groups were fed a sugar solution without pesticides. Only the technical compound clothianidin was first dissolved in DMSO prior to be mixed with the sugar solution. The DMSO in contaminated syrup never exceeded 0.02 %. An additional solvent control group was set up for clothianidin with sugar solution containing 0.02 % DMSO. The doses for imidacloprid, atrazine and glyphosate were based on the quantity of active matter (a.m.) of their respective commercial formulation. The sugar solutions of all cages (three replicates per dose and controls) were changed daily for the 10-day duration of the test. The control and contaminated sugar solutions were prepared 10 days prior of

exposure, kept at -20°C and thawed at room temperature before use. Mortality and hyperactivity (tumbling and trembling) were recorded every 12 h. After 10 days, bees were anesthetized/euthanized by placing the cages in an insulated container with dry ice for 5 min. After this time, bees exposed to the same dose (four cages) were randomly mixed, transferred to identified plastic bags and stored at -80°C until analyses. Seven to ten pools of bee heads were analyzed for each dose of the compounds tested.

Neonicotinoids analysis

Pollen samples (from beehives with maize pollen) were tested for neonicotinoids based on the method of Garcia et al. (2007). Pollen (3 g) tissue samples were thawed in a glass container. Tebuthiuron (99 % pure; Accustandard, New Haven, CT, USA) and 30 ml acetone were added to the samples, which were transferred to an ultrasonic bath for 10 min. The organic extract was filtered through Celite (0.5 g; Supelco, Bellefonte, PA, USA) in a Buchner funnel, and the residue was rinsed with 10 mL acetone. A second extraction using 25 ml of acetone was performed on an Eberbach reciprocal shaker followed by filtration on Celite and the addition of 50 ml coagulating solution consisting of aqueous ammonium chloride 1 % and orthophosphoric acid 2 % in water. After 30 min, the solution was filtered through a new portion of Celite (0.5 g) and washed with 10 mL of acetone:water (40:60, *v/v*). The filtered solution was transferred into a separation funnel with 50 ml of NaCl 2 % along with 100 ml dichloromethane, and the compounds were extracted by shaking by hand for 2 min. The extraction procedure was repeated using 50 ml dichloromethane. The resulting organic phases were combined and evaporated to dryness by rotary evaporation (30°C). The extract was transferred to a 10-ml tube, and the remaining solvent was evaporated to dryness under a gentle stream of nitrogen gas. The extract was redissolved in 1 mL of formic acid (0.1 %), acetonitrile (5 %) in water and internal standard was added (Atrazine-D5, Cambridge Isotope Laboratories Inc, Andover, MA, USA). The extract was filtered through a 0.45- μm PTFE filter, and 30 μl was injected in the LC-MS/MS system. Analysis was carried out on an API-3000 PE SCIEX LC-MS/MS with the turbo spray source in positive mode. The mass spectrometer was coupled to an Agilent 1100 quaternary pump and autosampler. A column Zorbax Eclipse XDS-C8 (150 \times 4.6 mm, with 5- μm particle) was used. The gradient program, with a flow rate of 0.2 ml/min, was: A, 100 % acetonitrile and B, 0.1 % formic acid in water. A linear mobile phase gradient was initiated from B (90 %) to B (57 %) for 3 min. After 4.5 min, B was reduced to 20 % and held from 8 to 14 min. The run ended by a column equilibration at initial conditions from 14.5 to

30 min. Eight neonicotinoids and metabolites were analyzed with quantitative limits of detection of 0.0003 µg/g and recoveries varying between 28 % and 54 % depending upon the compound. The analytes are the following: imidacloprid, imidacloprid urea, thiamethoxam, acetamiprid, fenamidone metabolite, fenamidone, azoxystrobin and clothianidin.

Statistical analysis

Otherwise noted, AChE activity was tested with both units: micromoles per hour per gram of tissue and nanomoles per hour per milligram protein. For the field experiment, differences between time points for AChE activity, protein and weight (mean values for each hive, 8 to 15 pools of 10 bees) were compared between conditions (organic, conventional and non-cultivated fields) using two-way repeated measures ANOVA, 3×2 design (treatment \times region). Significant models ($p < 0.05$) were submitted to contrast procedures or post-hoc tests using Bonferroni correction. In case of non-respect of sphericity, Greenhouse–Geisser statistic was used. Mortality (total count of dead bees) was tested between regions with Fisher's exact test, while χ^2 test was used to compare the field conditions within the same region. Relationship between AChE activity and protein concentration was explored within conditions at each week using Pearson correlations. In the case of chronic exposures to imidacloprid, clothianidin, atrazine and glyphosate, means data (7 to 10 pools of 10 bees) for AChE activity, protein concentration and weight were compared using a GLM one-way analysis of variance. The significant models ($p < 0.05$) were followed by a Dunnett *t* test in relation to the control group. In the case of unequal variances, a T3 Dunnett test was applied. All multiple comparisons tests were submitted to Bonferroni correction in order to control the rate of false positives. In certain cases, e.g. exposure to atrazine, a Jonckheere–Terpstra analysis (JT) was used in order to detect the influence of increasing doses of pesticides, based on the medians ordered in a particular direction. Pearson correlations were performed to explore the relationship between the honey bee protein concentration and AChE activity. The values for hyperactivity and survival (percent) were computed from the means ($n=4$) of the total counts of observations per cage, after 10 days. Exposed groups were compared to the control group using Fisher's exact test. For the highest dose of imidacloprid exposure, survival data were submitted to non-linear regression analysis to determine LC₅₀ value. AChE activity, protein concentration and weight values for clothianidin were compared between the two control groups (with and without DMSO) with a Student's *t* test. All statistical analyses were performed using SPSS® Statistic 18.0 software (IBM® Corporation, Armonk, NY, USA).

Results

AChE activity in honey bee heads and enzyme inhibition study

A standard AChE assay procedure using the Ellman reaction was adapted to honey bee head tissue. Basic AChE activity (means and SD) is depicted in Fig. 2a. Under the conditions established in our assay, the yield of hydrolyzed substrate in unexposed bees varied between 0.91 and 1.13 µmol/h/g of tissue or 24.4 and 51.9 nmol/h/mg protein. In all trials and routine assays, the coefficient of variation was lower when the AChE activity was expressed in micromoles per hour per gram of tissue (CV=10.5 %) instead of nanomoles per hour per milligram of protein (CV=25.6 %). The AChE activity (micromoles per hour per gram of tissue) was tested in vitro in the presence of pesticides representing two major categories of AChE inhibitors: the organophosphate diazinon and the carbamate carbaryl (Fig. 2b). Both insecticides clearly inhibited AChE activity.

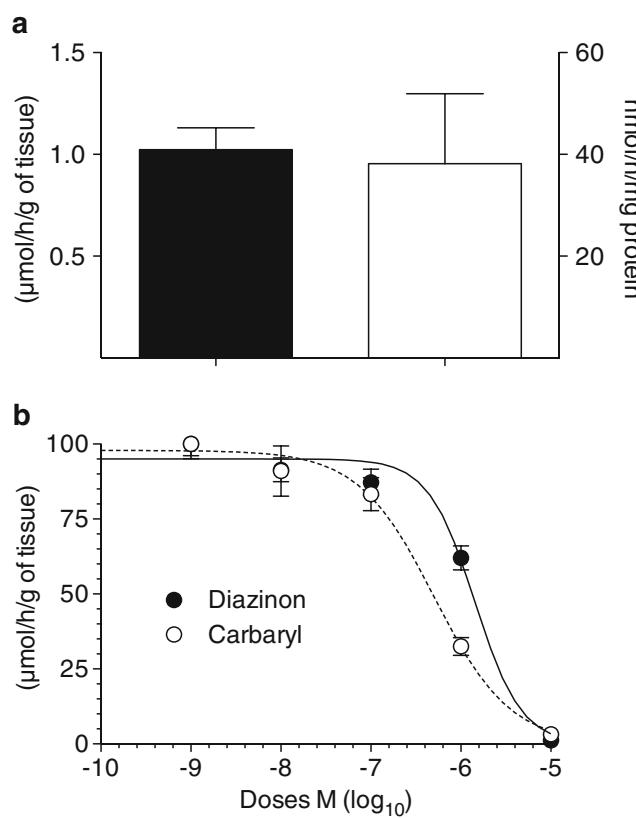


Fig. 2 AChE activity in honey bee heads. **a** Yield of acetylthiocholine hydrolyzation in unexposed bees by micromoles per hour per gram of tissue (black bars, left y-axis) and by nanomoles per hour per milligram protein (clear bars, right y-axis; means and SD, $n=10$ pools of 10 bees). **b** In vitro inhibition procedure of AChE activity (micromoles per hour per gram of tissue) using technical diazinon (black dots) and carbaryl (clear dots) as inhibitors (means and SD, $n=3$ pools of 10 bees)

Field experiment

One week after the beehives were placed in the field, the AChE activities were slightly lower than those found in bees from our preliminary assays and mostly varied between 0.5 and 0.8 $\mu\text{mol/h/g}$ of tissue (Fig. 3a) and 21.0 and 26.1 nmol/h/mg protein (Fig. 3b). It appeared that the term *region* was not a significant factor for AChE activity (micro-moles per hour per gram of tissue, $F_{3,36}=2.03$, $p=0.13$, or nanomoles per hour per milligram protein, $F_{3,36}=2.28$, $p=0.14$) protein content ($F_{3,36}=0.56$, $p=0.65$) and bee weight ($F_{3,36}=1.79$, $p=0.17$) when the design (3×2 ; field conditions \times regions) was used in ANOVA two-way repeated measures. Data from both regions were then combined, and only the influence of field conditions was used in one-way repeated measured ANOVA. This test showed that AChE micromoles per hour per microgram of tissue were significantly influenced by field conditions ($F_{6,45}=6.89$, $p<0.001$; Fig. 3a) as well as AChE nanomoles per hour per milligram protein ($F_{6,45}=13.79$, $p<0.001$; Fig. 3b) pointing week 2 as significantly different from all other time points ($p<0.001$). When solely tested by GLM one-way analysis of variance, the difference was confirmed for AChE micromoles per hour per microgram tissue ($F_{2,15}=13.91$, $p<0.001$): the conventionally grown fields being significantly higher than both organically grown fields ($p<0.001$) and the non-cultivated fields ($p<0.01$). Similar results were obtained for AChE nanomoles per hour per milligram protein activity ($F_{2,15}=7.17$, $p<0.01$): the conventionally grown fields being significantly higher than both

organically grown fields ($p<0.01$) and the non-cultivated fields ($p<0.01$). The protein contents were also influenced by the time points (two-way repeated measured ANOVA, $F_{3,51}=2.98$, $p<0.05$; Fig. 3c). When tested against field conditions (Bonferroni post-hoc test), the protein content in organically-grown fields at week 4 was significantly higher than values associated with conventionally-grown fields ($p<0.05$). This increased protein content was negatively related to AChE activity (nmol/h/mg of protein) in a significant manner ($r=-0.65$; $p<0.05$; $n=13$). The repeated measures test with a Greenhouse–Geisser correction determined that mean bee weight differed statistically between time points ($F_{1,9, 30,8}=3.89$, $p<0.05$; Fig. 3d). Significant differences were established between weeks 1 and 2 in organic ($p<0.05$) and conventional fields ($p<0.05$) but not for non-cultivated fields ($p=0.38$).

The total weekly dead bee count in front of the hives showed a significantly higher mortality for the Laurentides region, particularly at weeks 1 and 3 (Fisher's exact test, $p<0.001$; Table 1). In the Montérégie region, only week 4 showed a higher number of dead bees (Fisher's exact test, $p<0.05$). In the Laurentides, the mortality was significantly lower in the organic fields compared to both conventional and non-cultivated conditions ($\chi^2=32.47$, $p<0.01$, $n=191$). Results differed in Montérégie where the mortality was higher in conventional condition than either organic or non-cultivated fields ($\chi^2=101.58$, $p<0.01$, $n=115$).

Using collected pollen samples, the weekly inputs of maize pollen to the hives equipped with pollen traps (two per condition) were examined. In conventional and non-

Fig. 3 Parameters (means and SD, $n=8$ –15 pools of 10 bees) measured in honey bees from hives located near organic (clear bars) and conventional (black bars) maize fields and non-cultivated fields (striped bars) for 4 weeks. Means were compared using two-way repeated measures ANOVA followed by Bonferroni correction (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). See “Materials and methods” section for details. **a** AChE activity (micromoles per hour per gram of tissue). **b** AChE activity (nanomoles per hour per milligram protein). **c** Protein concentration. **d** Weight

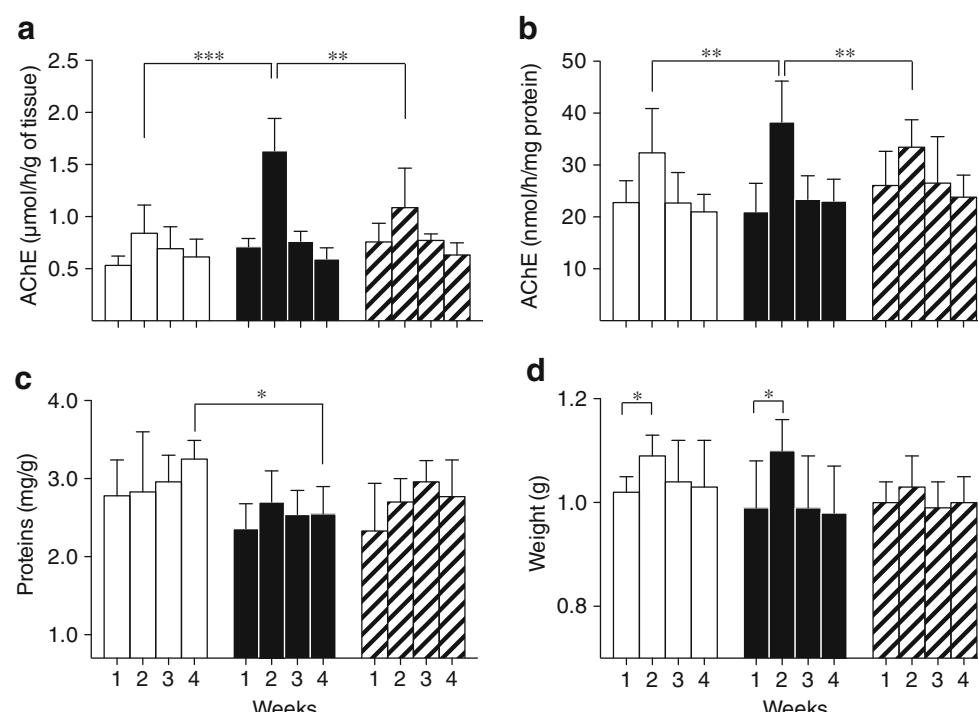


Table 1 Summary of dead bees accumulated in front of the hive (counts per week) from the two regions during the 4 weeks (1–4) of the field experiment

Field conditions	Laurentides					Montérégie				
	1	2	3	4	Total	1	2	3	4	Total
Conventional	120	170	141	80	511	125	100	35	150	410****
Organic	104	50	15	34	203***	60	75	45	95	275
Non-cultivated	396	50	213	56	715	75	50	30	73	228
Total	620*	270	369*	170	1,429*	260	225	110	318**	913

* $p<0.001$; ** $p<0.05$ (week comparisons between regions, Fisher's exact test); *** $p<0.01$; **** $p<0.05$ (comparisons between field conditions within the same region, χ^2)

cultivated fields, maize pollen was present in traps at weeks 1 and 2 (Table 2), while in organic fields maize pollen was present in the trap at week 2 and at week 4. No maize pollen was found in weeks 3 and 4 for conventional and non-cultivated conditions. No neonicotinoids were detected in the pollen samples at concentrations above the detection limit of 0.0003 µg/g.

Chronic pesticides exposure

When honey bees were exposed to four common products used in maize cultivation, marked results were obtained for imidacloprid: for all tested doses, AChE activity was significantly higher than in the control group (GLM one-way analysis of variance, followed by Dunnett *t* test): AChE micromoles per hour per gram of tissue ($F_{3,21}=24.38$; $p<0.001$) and AChE nanomoles per hour per milligram protein ($F_{3,21}=18.04$; $p<0.001$; Fig. 4a). Survival counts at the highest dose of imidacloprid (0.30 ng/bee) were too low (2.5 % after 10 days) to properly test these individuals for AChE activity. A significant GLM one-way analysis of variance was computed for protein ($F_{3,22}=7.16$; $p<0.01$). At the dose 0.24 ng/bee, the mean value was significantly lower than the control group (Dunnett *t* test; Fig. 4a) resulting in significant negative relationships between protein and AChE micromoles per hour per microgram of tissue ($r=-0.519$, $p<0.01$, $n=25$) and between protein and AChE nanomoles per hour per milligram

protein ($r=-0.778$, $p<0.01$, $n=25$). Bee weight values associated with the doses 0.16 and 0.24 ng/bee were significantly lower than the control group (GLM one-way analysis of variance followed by Dunnett *t* test, $F_{3,22}=3.88$; $p<0.05$).

The clothianidin exposure also led to higher AChE micromoles per hour per microgram of tissue with doses 0.12 and 0.24 ng/bee ($F_{4,52}=11.4$; $p<0.001$) and AChE nanomoles per hour per milligram protein with the highest dose, 0.24 ng/bee ($F_{4,52}=9.12$; $p<0.01$; Fig. 4b). Protein contents varied greatly among doses, and despite a significant model (GLM one-way analysis of variance, $F_{3,51}=4.51$; $p<0.01$), no tested doses were statistically different from the control group. Similar results were obtained testing the weight (GLM one-way analysis of variance, $F_{3,53}=4.53$; $p<0.01$), with no doses statistically different from the control group. For clothianidin, there was a significant negative relationship between protein concentration and AChE micromoles per hour per microgram of tissue ($r=-0.463$, $p<0.01$, $n=55$) or AChE nanomoles per hour per milligram protein ($r=-0.741$, $p<0.01$, $n=55$). A significant model was computed (GLM one-way analysis of variance, $F_{4,43}=2.59$; $p<0.05$) when atrazine was tested absent of any significant comparisons between doses and the control group for AChE micromoles per hour per gram of tissue. However, a significant negative linear trend was found with increasing doses (Jonckheere-Terpstra test=308.0, $p<0.05$, $n=49$; Fig. 4c). A significant model was computed for AChE nanomoles per hour per milligram protein (GLM one-way analysis of variance followed by Dunnett *t* test, $F_{4,43}=9.29$; $p<0.001$) showing a tendency for the dose 1.25 ng/bee to be higher ($p<0.1$) and the dose 2.5 ng/bee to be lower ($p<0.05$) than the control group. Atrazine had no effect on protein concentration ($F_{4,43}=1.81$; $p=0.14$) or weight of bees ($F_{4,40}=0.81$; $p=0.13$). Significant lower AChE micromoles per hour per microgram of tissue values were associated with 2.5, 5 and 10 ng/a.m./bee of glyphosate (GLM one-way analysis of variance followed by Dunnett *t* test, $F_{4,44}=11.12$; $p<0.001$), while only doses 2.5 and 5 led to lower AChE nanomoles per hour per milligram protein concentration

Table 2 Maize pollen (percent) found in traps of six beehives (two per condition) during the 4 weeks of the field experiment

Field conditions	Maize pollen (%)	Week
Organic	0–1	2
	0–42	4
Conventional	0.4–5.5	1
	5–6	2
Non-cultivated	0–5.5	1
	0–0.8	2

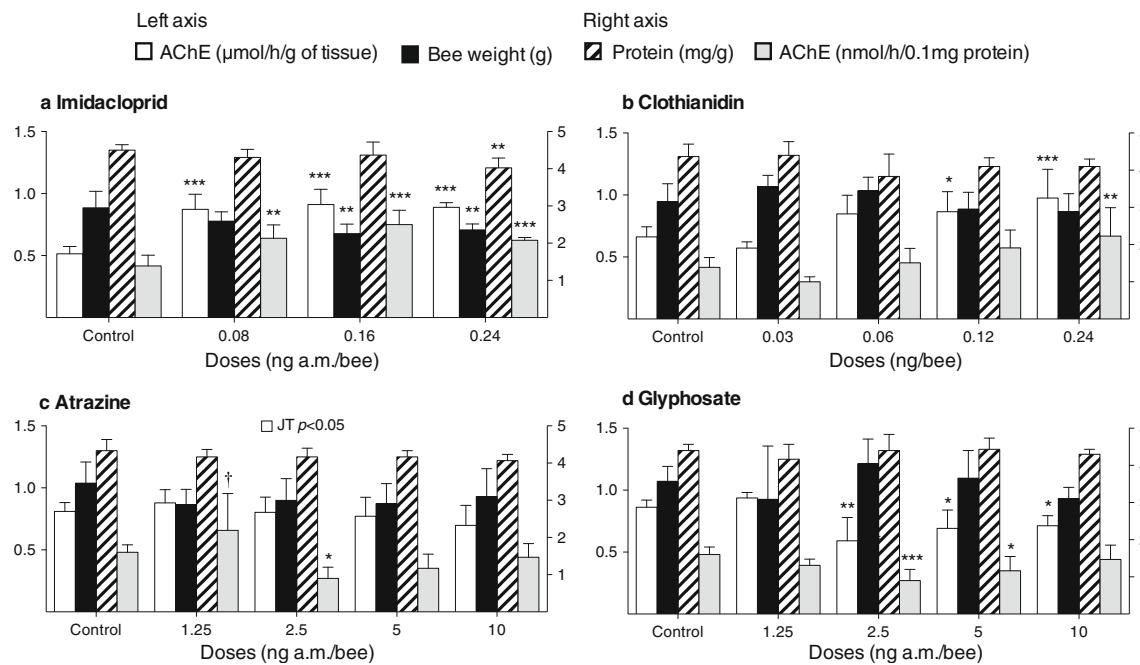


Fig. 4 Means and SD ($n=7$ – 10 pools of 10 bees) for AChE activity (micromoles per hour per gram of tissue: clear bars, left y-axis; nanomoles per hour per milligram protein: gray bars, right y-axis), weight (black bars, left y-axis) and protein concentration (striped bars, right y-axis) in honey bees exposed for 10 days. **a** Imidacloprid (Admire® 240F), **(b)** clothianidin (technical grade), **(c)** atrazine

(Aatrex® 480) and **(d)** glyphosate (Weathermax® 240). Means were compared using GLM one-way analysis of variance followed by the Dunnett *t* test against the control group (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). AChE activity for atrazine was tested with Jonckheere–Terpstra analysis ($p<0.01$). See “Materials and methods” section for details

Discussion

The dose–response studies using diazinon and carbaryl demonstrated that the AChE assay for honey bees provided a suitable quantitative measure of AChE activity. The literature is rife with studies showing the inhibition of cholinesterase activities by carbamate and organophosphate pesticides, whereas increased AChE is seldom reported. The increased AChE activity found in fields was not expected due to known biochemical modes of action of the major pesticides used in Québec for corn production. Based on the pollen traps, maize pollen was available to all beehives during the two first weeks, whatever the field condition: organic, conventional and non-cultivated fields (Table 2). It is possible that the higher AChE activity found in bees during week2 corresponded with the maize pollen emission peak. This duration (1 to 2 weeks) concurs with previous observations. More specifically, corn plants typically shed pollen for 5 to 6 days, whereas a whole field may take 10 to 14 days to completely shed due to the natural variation in growth (Nielsen 2010, “Tassel emergence & pollen shed”; <http://www.agry.purdue.edu/ext/corn/news/timeless/Tassels.html>, URL verified on October 10, 2012).

Maize pollen may contain residual concentration of pesticides, e.g. imidacloprid: 2 to 3 ng/g (Bonmatin et al. 2005), 1.2 ng/g (Chauzat et al. 2006) or less than 1 ng/g (Donnarumma et al. 2011). In the present study, pollen from beehives equipped with traps was analyzed for

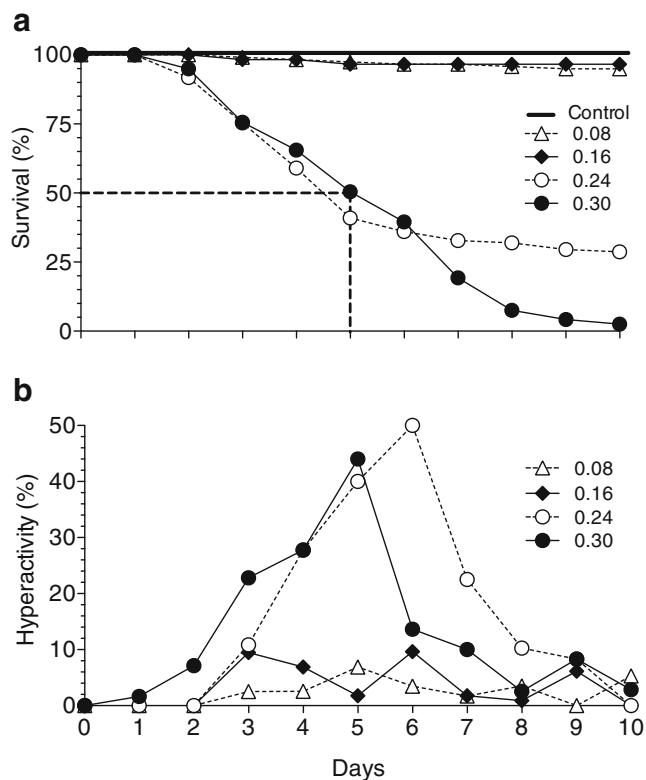


Fig. 5 Effects of chronic exposure (10 days) of imidacloprid on honey bee's survival and hyperactivity. **a** Survival. Values represent the cumulated number of events (death) of four replicates (cages) per dose and the control group $\times 100$. Survival for each dose was compared to the control group using χ^2 test. **b** Hyperactivity. Values represent the cumulated behaviour observations (tumbling and trembling) of four replicates (cages) per dose $\times 100$. Hyperactivity was compared to the control group using Fisher's exact test

neonicotinoids content, but no such compound was found, at least above the detection limit ($0.0003 \mu\text{g/g}$). Due to the low percentage of maize pollen in the samples, these results were not unexpected. It is suggested that in further studies, a collection of maize pollen directly from the plants also be taken for analysis. In theory, the collected samples of our study could not contain neonicotinoid concentrations higher than 0.3 ng/g . This value, corrected by the highest percentage of recovery (54 %), would give an effective limit of 0.5 ng/g pollen. Suchail et al. (2001) reported bee mortality with imidacloprid or its metabolites in syrup at concentration of $0.1 \mu\text{g/L}$ or 0.1 ng/g , suggesting that the adverse effects of imidacloprid on honey bees may occur at very low doses, often impossible to detect by chemical analysis. Despite the general tendency that maize pollen was limited to the first 2 weeks of the experiment, one beehive from the organic condition still contained 42 % maize pollen at week 4. This availability of maize pollen at week 4 could be attributed to different crop managements, time of seeding and cultivars, thus offering a longer exposure period. Curiously, a closer examination of this hive revealed that

AChE activity value was in the same range as other hives of the same group and time ($0.74 \pm 0.05 \mu\text{mol/h/g}$ of tissue, $n=6$). An explanation could be that AChE activity did not increase in this case because bees from this hive foraged mainly organic maize pollen, in which the conditions susceptible to increase the AChE activity in bees did not prevail.

Because neonicotinoids may persist in soils (Liu et al. 2011), they could have migrated and have been absorbed by other flowering plants or shrubs near our study fields. For example, Krupke et al. (2012) found clothianidin (1.1 to 9.4 ng/g) in dandelions near a maize field as well as metolachlor (5.7 to 295 ng/g) and atrazine (125 to 1,133 ng/g). The fact that neonicotinoid effects could be enhanced by the presence of other compounds (active matter, metabolites and surfactants from commercial formulations) cannot be excluded. In laboratory experiments, Key et al. (2007) found more potent effects in grass shrimp larvae when these organisms were exposed to imidacloprid along with atrazine, demonstrating synergistic effects. In another study, Iwasa et al. (2004) found a similar relationship between neonicotinoids and fungicides: the neonicotinoid thiacloprid, less toxic than imidacloprid, was 559 times more toxic when combined with propiconazole, a fungicide known to inhibit the P450 activity. If such conditions were to be encountered in our field study, the neonicotinoids could alter AChE activity at a very low concentration. Lately, in Québec, the use of fungicides has increased to improve maize, soya and cereal crop yields (Giroux and Pelletier 2012), and propiconazole is among the products suggested by agrochemical companies (CRAAQ and MAPAQ 2011). Several fungicides from the triazole family (propiconazole, ketoconazole, clotrimazole, etc.) are known to alter cytochrome P450 activity: inhibition in *Spodoptera eridania* caterpillar (Brattsten et al. 1994) and increase in black fly larvae (Kenneke et al. 2009). The P450 system is highly involved in detoxification processes, and disturbances could heighten the adverse effects of contaminants, along with the fact that the honey bee appears to be deficient in comparison to other insects; according to Claudiános et al. (2006), the honey bee would have half the encoding xenobiotic detoxifying enzymes (GST, P450 and carboxyl/cholinesterases) of *D. melanogaster* and *Anopheles gambiae*. This short supply may influence the sensitivity of the honey bees to pesticides.

In most studies based on vertebrate organisms, body weight and protein concentration are associated with fitness and homeostasis conditions. In our field study, bees associated with organic fields demonstrated a steady increase of protein concentration compared to bees from other field conditions, especially conventionally grown fields (Fig. 3c). The significant increase of weight between the first and second weeks (Fig. 3d) could be due to the abundance of maize pollen, as this effect was not observed in non-cultivated fields. When

apiaries are located near large corn culture areas, bees feed almost exclusively on this pollen source (Decourtey et al. 2011). While the protein value associated to maize pollen is still a question of debate, bees submitted to maize pollen diet demonstrated reduced brood productivity and lifespan (Höcherl et al. 2012). A more detailed protein profile could help monitor the honey bee's physiological status while also adding to current knowledge of its action mechanisms, such as the possibility of decreased fitness.

There is no clear pattern or trend regarding the in-field mortality. While the mortality was more important in the Laurentides region compared to the Montérégie, the count of dead bees in front of the hive varied greatly among weeks and in-field conditions. Further studies should include a report of hive variation pre-treatment in order to control the detectable differences between the hives. In healthy beehives, worker bees normally dispose of dead bees preventing infection or diseases. However, a weekly cumulated high number of dead bees in front of the hives could suggest a symptom of poisoning or colony disorder. According to Johansen and Mayer (1990), the dead bee count in front of the hive could represent 1 % to 20 % of the total (real) mortality (in-field and inside the hive).

For bees exposed to imidacloprid (lab experiment), neurotoxic symptoms and mortality occurred at the lowest dose tested, 0.08 ng/bee or 1.1 µg/L. These results were also in agreement with values published by Suchail et al. (2001). The hyperactivity effects were not observed in bees exposed to clothianidin. This difference could be attributed to a slightly different action mechanism or to the fact that clothianidin was a pure compound and not a commercial formulation. When Franklin et al. (2004) exposed *Bombus impatiens* to both products, trembling was observed with imidacloprid but not with clothianidin. The negative relationship between AChE activity and protein concentration found in caged bees exposed to neonicotinoids and in organic field conditions at week 4 suggest indirect effects or specific protein synthesis. Studies focusing on specific genes and protein responses (e.g. RT-PCR) could indicate if the high AChE activity that we reported here has been induced by neonicotinoid exposure.

Our laboratory study showed that exposure to neonicotinoids increased AChE activity in honey bees and that hyperactivity and decreased survival were closely linked. In the fields, the increased AChE activity seemed to be associated to the maize pollen availability. Further research will be necessary to detect and identify the sources of neonicotinoids as well as other compounds susceptible to influence the AChE expression as deltamethrin. In a laboratory experiment where the honey bees were exposed to deltamethrin, AChE activity increased in surviving bees and decreased in dead bees (Badiou et al. 2008). Pyrethroid insecticides are rarely used in maize crops, but they could

have been used in nearby productions of vegetables, fruits or even sweet corn. Herbicides though are commonly used. Reduced protein concentration, P450 enzymes and AChE activity have been reported in aquatic midge exposed to metolachlor (Ying et al. 2008). When Modesto and Martinez (2010) exposed the fish *Prochilodus lineatus* to Roundup Transorb (1 m/L), they observed ROS (reactive oxygen species) damages after 24 h and inhibition of brain AChE activity after 96 h. Without presuming direct comparison between vertebrates and invertebrates, a reduced AChE activity was also observed when we exposed bees to a commercial formulation of Roundup Weathermax.

At this point, the exact action mechanisms triggering adverse effects in bees exposed to agricultural contaminants are not known. In our study, we had not anticipated that neonicotinoids primary action on nAChERs would elicit heightened cholinesterase activity, as clearly demonstrated by our laboratory experiment. We can only presume that because neonicotinoids occupy the binding-site of acetylcholine, this compound tends to accumulate in the synapses, stimulating the action of AChE, in a typical substrate-enzyme cellular response.

Conclusions

To our knowledge, this is the first time that an increased AChE activity is reported for both in-field and laboratory data implicating honey bees. While many laboratory experiments have been conducted to test the toxicity of several chemicals on honey bees, the real impact of sub-lethal exposures to a mixture of contaminants faced by the bees in wild environments is yet to be discovered. The precise concentration triggering the observed effects is difficult to predict. The results of the chronic exposure under controlled conditions suggest that the NOEL (no observable effects level) for imidacloprid alone is less than 0.08 ng per bee. The age of the bees, their genetic background and their potential for detoxification are unknown factors that may have a profound influence on toxicological studies. Furthermore, the mixture of contaminants in the wild environment, the use of various surfactants in commercial formulation products, the trophallaxis practices and the possible repellent character of certain contaminated syrup used for cage experiments prevent the exact amount of product absorbed by the bees to be known.

While the dose–effect relationships are yet to be established for many pesticides used in agricultural areas, in-field data and measures of effects, other than mortality, are expressly needed. AChE activity could be useful for monitoring effects associated with neonicotinoids or other neurotoxic compounds. Parameters like bee weight and protein concentration should not be overlooked. Considering weight and

protein concentration, bees in organic fields of our study appeared to have a slight advantage from other field conditions. We can only speculate on the long-term impact of altered nervous transmission, unbalanced protein synthesis and possible deficient detoxification mechanisms faced by honey bees on an individual or collective basis. With the continuing development of phytosanitary products and the tendency for increasing cultivation of wide-row crops, one may expect greater environmental hazards for the honey bee and for other pollinators.

Acknowledgments The authors would like to thank Isabelle Ferland, Geneviève Beaunoyer, Cynthia Franci, Karine Dufresne, Dieynaba Diop, Hannan Alami and Arnaud Villier for their technical assistance. We also thank Émile Houle and Michael Benoit from CRSAD and the beekeepers and farmers involved in the field study. We acknowledge the contribution of Stéphane Laramée, André Pettigrew and François Gouin-Legault for fieldwork. We are grateful to TOXEN-CIRE (Centre interinstitutionnel de recherche en toxicologie de l'environnement—Centre Interinstitutionnel de recherche en écotoxicologie) for the use of laboratories and analytical equipment. The authors thank Dr. Philip Spear and Stephanie Hedre-Helmer, M.Sc. for reviewing the manuscript. This study was supported by Programme de soutien à l'innovation en agroalimentaire (PSIA) from Ministère de l'Agriculture, des pêcheries et de l'alimentation du Québec (MAPAQ), Conseil pour le développement de l'agriculture du Québec (CDAQ) from Agriculture et Agroalimentaire Canada and Canadian Pollinator Initiative (CANPOLIN), grants attributed to M. Chagnon.

References

- Badiou A, Meled M, Belzunces LP (2008) Honeybee *Apis mellifera* acetylcholinesterase—a biomarker to detect deltamethrin exposure. *Ecotoxicol Environ Saf* 69:246–253
- Bérubé V, Boily MH, DeBlois C, Dassylva N, Spear PA (2005) Plasma retinoid profile in bullfrogs, *Rana catesbeiana*, in relation to agricultural intensity of sub-watersheds in the Yamaska River drainage basin, Québec, Canada. *Aquat Toxicol* 71:109–120
- Boily M, Bérubé VE, Spear PA, DeBlois C, Dassylva N (2005) Hepatic retinoids of bullfrogs in relation to agricultural pesticides. *Environ Toxicol Chem* 24:1099–1106
- Boily M, Thibodeau J, Bisson M (2009) Retinoid metabolism (LRAT, REH) in the liver and plasma retinoids of bullfrog, *Rana catesbeiana*, in relation to agricultural contamination. *Aquat Toxicol* 91:118–125
- Bonmatin JM, Marchand PA, Charvet R, Moineau I, Bengsch ER, Colin ME (2005) Quantification of imidacloprid uptake in maize crops. *J Agric Food Chem* 53:5336–5341
- Brattsten LB, Berger DA, Dungan LB (1994) In vitro inhibition of midgut microsomal P450s from *Spodoptera eridania* caterpillars by demethylation inhibitor fungicides and plant growth regulators. *Pest Biochem Physiol* 49:234–243
- Chauzat MP, Faucon JP, Martel AC, Lachaize J, Cougoule N, Aubert M (2006) A survey on pesticide residues in pollen loads collected by honey bees (*Apis mellifera*) in France. *J Econ Entomol* 99:253–262
- Claudianos C, Ranson H, Johnson RM, Biswas S, Schuler MA, Berenbaum MR, Feyereisen R, Oakeshott JG (2006) A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. *Insect Mol Biol* 15:615–636
- CRAAQ and MAPAQ (2011) Répertoire 2011, Traitements de protection des grandes cultures, Québec, Centre de référence en agriculture et agroalimentaire du Québec et ministère de l'Agriculture, des pêcheries et de l'Alimentation du Québec, 571 p
- Decourtey A, Devillers J (2010) Insect nicotinic acetylcholine receptors. In: Thany SH (ed) *Insect nicotinic acetylcholine receptors*. Springer, Berlin, pp 85–96
- Decourtey A, Alaux C, Odoux J-F, Henry M, Vaissière BE, Le Conte Y (2011) Why enhancement of floral resources in agro-ecosystems benefit honeybees and beekeepers? In: Grillo O, Venora G (eds) *Ecosystems biodiversity*. In Tech, Chapter 16, pp 371–388
- Déglyse P, Grünewald B, Gauthier M (2002) The insecticide imidacloprid is a partial agonist of the nicotinic receptor of honeybee Kenyon cells. *Neurosci Lett* 321:13–16
- Donnarumma L, Pulcini P, Pochi D, Rosati S, Lusco L, Conte L (2011) Preliminary study on persistence in soil and residues in maize of imidacloprid. *J Environ Sci Health B* 46:469–472
- Evans SC, Shaw EM, Rypstra AL (2010) Exposure to a glyphosate-based herbicide affects agrobiont predatory arthropod behaviour and long-term survival. *Ecotoxicol* 19:1249–1257
- Franklin MT, Winston ML, Morandin LA (2004) Effects of clothianidin on *Bombus impatiens* (Hymenoptera: Apidae) colony health and foraging ability. *J Econ Entomol* 97:369–373
- Garcia MDG, Galera MM, Valverde RS, Galanti A, Girotti S (2007) Column switching liquid chromatography and post-column photochemically fluorescence detection to determine imidacloprid and 6-chloronicotinic acid in honey bees. *J Chromatogr A* 1147:17–23
- Gauthier M (2010) State of the art on insect nicotinic acetylcholine receptor function in learning and memory. In: Thany SH (ed) *Insect nicotinic acetylcholine receptors*. Springer, Berlin, pp 97–115
- Giroux I, Pelletier L (2012) Présence de pesticides dans l'eau au Québec: bilan dans quatre cours d'eau de zones en culture de maïs et de soya en 2008, 2009 et 2010, Québec, ministère du Développement durable, de l'Environnement et des Parcs, Direction du suivi de l'état de l'environnement, ISBN 978-2-550-64159-9, 46 pp +3 annexes
- Höcherl N, Siede R, Illies I, Gätschenberger H, Tautz J (2012) Evaluation of the nutritive value of maize for honey bees. *J Insect Physiol* 58:278–285
- Iwasa T, Motoyama N, Ambrose JT, Roe MR (2004) Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Prot* 23:371–378
- Johansen C, Mayer D (1990) Pollinator protection: A bee and pesticide handbook. Wicwas, Cheshire
- Johnson RM, Ellis MD, Mullin CA, Frazier M (2010) Pesticides and honey bee toxicity. *Apidologie* 41:312–332
- Kenneke JF, Mazur CS, Kellock KA, Overmyer JP (2009) Mechanistic approach to understanding the toxicity of the azole fungicide triadimefon to a nontarget aquatic insect and implications for exposure assessment. *Environ Sci Technol* 43:5507–5513
- Key P, Chung K, Siewicki T, Fulton M (2007) Toxicity of three pesticides individually and in mixture to larval grass shrimp (*Palaeomonetes pugio*). *Ecotoxicol Environ Saf* 68:272–277
- Krupke CH, Hunt GJ, Eitzer BD, Andino G, Given K (2012) Multiple routes of pesticide exposure for honey bees living near agricultural fields. *PLoS One* 7:e29268. doi:10.1371/journal.pone.0029268
- Le Goff G, Hillioub F, Siegfried BD, Boundya S, Wajnberg E, Soferb L, Audantb P, ffrench-Constant RH, Feyereisenb R (2006) Xenobiotic response in *Drosophila melanogaster*: sex dependence of P450 and GST gene induction. *Insect Biochem Mol Biol* 36:674–682
- Liu Z, Dai Y, Huang G, Gu Y, Ni J, Wei H, Yuan S (2011) Soil microbial degradation of neonicotinoid insecticides imidacloprid, acetamiprid, thiacloprid and imidaclothiz and its effect on the

- persistence of bioefficacy against horsebean aphid *Aphis craccivora* Koch after soil application. Pest Manag Sci 67:1245–1252
- Louveaux J, Maurizio A, Vorwohl G (1978) Methods of melissopalynology. Bee World 59:139–162
- Maini S, Medrzycki P, Porrini C (2010) The puzzle of honey bee losses: a brief review. Bull Insectol 63:153–160
- MAPAQ (2006) Profil agroalimentaire de la Montérégie. Ministère de l'Agriculture, des pêcheries et de l'alimentation du Québec, 102 p
- Marcogliese DJ, King KC, Salo HM, Fournier M, Brousseau P, Spear P, Champoux L, McLaughlin JD, Boily M (2009) Combined effects of agricultural activity and parasites on biomarkers in the bullfrog, *Rana catesbeiana*. Aquat Toxicol 91:126–134
- MDEIE (2012) Profil régional - Laurentides. Ministère du Développement économique, de l'innovation et de l'exportation, 12 p
- Mirande L, Haramboure M, Smagghe G, Piñeda S, Schneider MI (2010) Side-effects of glyphosate on the life parameters of *Eriopsis connexa* (Coleoptera: Coccinellidae) in Argentina. Commun Agric Appl Biol Sci 75:367–72
- Modesto KA, Martinez CB (2010) Effects of roundup transorb on fish: hematology, antioxidant defenses and acetylcholinesterase activity. Chemosphere 81:781–787
- Morakchi S, Maïza A, Farine P, Aribi N, Soltani N (2005) Effects of a neonicotinoid insecticide (acetamiprid) on acetylcholinesterase activity and cuticular hydrocarbons profil in German cockroaches. Commun Agric Appl Biol Sci 70:843–848
- Nauen RU, Ebbinghaus-Kintzsch U, Schmuck R (2001) Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in *Apis mellifera* (Hymenoptera: Apidae). Pest Manag Sci 57:577–586
- Potts SG, Roberts SPM, Dean R, Marrs G, Brown MA, Jones R, Neumann P, Settele J (2010) Declines of managed honey bees and beekeepers in Europe. J Api Res 49:15–22
- Suchail S, Guez D, Belzunce LP (2001) Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in *Apis mellifera*. Environ Toxicol Chem 20:2482–2486
- Thany SH (2009) Agonist actions of clothianidin on synaptic and extrasynaptic nicotinic acetylcholine receptors expressed on cockroach sixth abdominal ganglion. Neurotoxicol 30:1045–1052
- Thornton BJ, Elthon TE, Cerny RL, Siegfried BD (2010) Proteomic analysis of atrazine exposure in *Drosophila melanogaster* (Diptera: Drosophilidae). Chemosphere 81:235–241
- Tremolada P, Mazzoleni M, Saliu F, Colombo M, Vighi M (2010) Field trial for evaluating the effects on honey bees of corn sown using Cruiser and Celest xl treated seeds. Bull Environ Contam Toxicol 85:229–234
- van Eaglesdorp D, Evans JD, Saegerman C, Mullin C, Haubruege E, Nguyen BK, Frazier M, Frazier J, Cox-Foster D, Chen Y, Underwood R, Tarpy DR, Pettis JS (2009) Colony collapse disorder: a descriptive study. PLoS ONE 4:64–81
- Wu JY, Anelli CM, Sheppard WS (2011) Sub-lethal effects of pesticide residues in brood comb on worker honey bee (*Apis mellifera*) development and longevity. PLoS ONE 6:e14720. doi:10.1371/journal.pone.0014720
- Yang EC, Chuang YC, Chen YL, Chang LH (2008) Abnormal foraging behavior induced by sublethal dosage of imidacloprid in the honey bee (Hymenoptera: Apidae). J Econ Entomol 101:1743–1748
- Ying JC, Anderson TD, Zhu KY (2008) Effect of alachlor and metolachlor on toxicity of chlorpyrifos and major detoxification enzymes in the aquatic midge, *Chironomus tentans* (Diptera: Chironomidae). Arch Environ Contam Toxicol 54:645–652