Environ Sci Pollut Res (2015) 22:8010–8021
DOI 10.1007/s11356-014-2879-7

Effects of realistic doses of atrazine, metolachlor, and glyphosate on lipid peroxidation and diet-derived antioxidants in caged honey bees (*Apis mellifera*)

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Received: 13 January 2014 / Accepted: 2 April 2014 / Published online: 15 April 2014

Abstract The decline in the population of pollinators is a worrying phenomenon worldwide. In North America, the extensive use of herbicides in maize and soya crops may affect the health of nontarget organisms like the honey bee. In this study, caged honey bees were exposed to realistic doses of atrazine, metolachlor, and glyphosate for 10 days via contaminated syrup. Peroxidation of lipids was evaluated using the thiobarbituric acid reactive substance (TBARS) test, and diet-derived antioxidants—carotenoids, all-*trans*-retinol (*at*-ROH) and α-tocopherol—were detected and quantified using reversed-phase HPLC techniques. Significant increases in syrup consumption were observed in honey bees exposed to metolachlor, and a lower TBARS value was recorded for the highest dose. No relationship was observed between the peroxidation of lipids and the levels of antioxidants. However, β-carotene, which was found to be the most abundant carotenoid, and *at*-ROH (derived from β-carotene) both decreased with increasing doses of atrazine and glyphosate. In contrast, metolachlor increased levels of *at*-ROH without any effects on β-carotene. These results show that the honey bee carotenoid–retinoid system may be altered by sublethal field-realistic doses of herbicides.

Responsible editor: Philippe Garrigues

Electronic supplementary material The online version of this article (doi:10.1007/s11356-014-2879-7) contains supplementary material, which is available to authorized users.

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Keywords *Apis mellifera* · Carotenoids · All-*trans*-retinol · α-Tocopherol · TBARS

Introduction

The declining populations of pollinators are of great concern in many countries (Johnson et al. 2010; Potts et al. 2010). In Canada, from 2007 to 2013, honey bee colony losses over winter were an average of 30.0 %; in the province of Québec, the mean percentage for 2012–2013 was 24.0 % (Boucher 2013). The plight of the honey bee has generated numerous studies delving into the cause of their demise, with most of them focusing on pesticides: fungicides to combat enemies in the hives such as *Nosema apis* and *Varroa destructor* (Mullin et al. 2010; Johnson et al. 2010; 2013; Berry et al. 2013) and other pesticides used in fields, including seeds coated with neonicotinoids (Yang et al. 2008; Maini et al. 2010; Wu et al. 2011; Boily et al. 2013). These reports state that honey bees are repeatedly exposed to phytosanitary products. In the province of Québec, wide-row corn and soya crops are the major productions. In 2012, surface areas devoted to these cultures were the following: maize 385 kha and soya 280 kha (ISQ and MAPAQ 2013). In 2008, 46.0 % of all pesticides commercialized in the province were devoted to these two productions alone (Gorse and Rivard 2011). Glyphosate is a widely used active ingredient in herbicides on large-scale crops, along with atrazine and metolachlor. These herbicides become potentially bioavailable to the foraging bees from pollen, nectar, water, and dusts (Krupke et al. 2012; Tapparo et al. 2011) and are brought back to the hive; atrazine and metolachlor are among the pesticides detected in wax and pollen (Mullin et al. 2010; Johnson et al. 2010). Atrazine, although banned in the European Union, is still widely used in the USA and Canada. This chemical has been shown to induce oxidative stress in fish (Nwani et al. 2010), frogs (Mann et al. 2009), and

rat erythrocytes (Singh et al. 2010). Proteomic studies conducted on *Drosophila melanogaster* suggest that atrazine could disrupt mitochondrial electron transport and induce oxidative stress (Thornton et al. 2010). Oxidative stress is produced in organisms when free reactive oxygen species (ROS) are not neutralized by endogenous antioxidants. This impaired balance may be due to excess ROS or a lack of antioxidants (Waris and Ahsan 2006). ROS can lead to the oxidation of proteins, DNAs, RNAs and the peroxidation of membrane lipids (Weirich et al. 2002). Figure 1 depicts the enzymatic and nonenzymatic components of the antioxidant system known in mammals. In insects, glutathione peroxidase and glutathione reductase are replaced by glutathione-*S*-transferase (GST) with thioredoxin peroxidase and thioredoxin reductase (GSTPX) (Farjan et al. 2012). Catalase (CAT), GST, and superoxide dismutase (SOD) activities were reported in the maize stalk borer (*Busseola fusca*) (George and Gatehouse 2013) and in honey bees (Weirich et al. 2002; Farjan et al. 2012; Hsu and Hsieh 2013).

In mammals, as in all vertebrates, tocopherol and carotenoids are derived from the diet (step 1, Fig. 1). Tocopherol is known to be a powerful antioxidant, and a decreased level of this vitamin can lead to poor fat absorption (Casani 2000). Carotenoid compounds have been identified as antioxidants as well, but their properties vary according to species, tissues, or stage of development (see reviews from Shete and Quadro 2013, and Álvarez et al. 2013). For example, in certain in vitro assays, β-carotene has proven to be a 10 to 25 times more efficient ROS scavenger compared to tocopherol (Mueller and Boehm 2011). In addition to their antioxidant properties, β-carotene, α-carotene, and β-cryptoxanthin can be converted into vitamin A. Central cleavage of carotene by the enzyme β-carotene 15-15′-oxygenase leads to the formation of retinaldehyde (RAL) (step 2, Fig. 1) which is then reduced in retinol (ROH) (step 4). Two dehydrogenations, one reversible (step 5) followed by an irreversible one (step 3), lead to the formation of retinoic acid (RA). These metabolic products are essential for biological functions such as cellular differentiation, vision, reproduction, embryonic development, and immune system response.

For bees, the source of vitamin A could be β-carotene as this compound is abundant in the pollen stored in hives and is used to feed larvae, the queen, drones, and older workers (Di Pasquale et al. 2013). Studies of carotenoids conducted on *Drosophila* revealed that a transmembrane protein, NinaD, expressed in the midgut (Wang et al. 2007), is responsible for the absorption of carotenoids. In honey bees, carotenoids are stored in adipose tissues such as fat bodies (Kayser 1982) and are converted into retinoids by a carotenoid isomeroxygenase, NinaB, responsible for both cleavage and isomerization of carotenoids (Wang et al. 2007; Álvarez et al. 2013) to produce essential compounds for vision: 11-*cis* and 3-OH-11-*cis*-RAL (Smith and Goldsmith 1991; Arshavsky 2009). As in vertebrates, unbalanced retinoids (e.g., RA) have led to developmental malformations experienced with the tick (*Rhodnium prolixus*) (Nakamura et al. 2007) and *Drosophila*

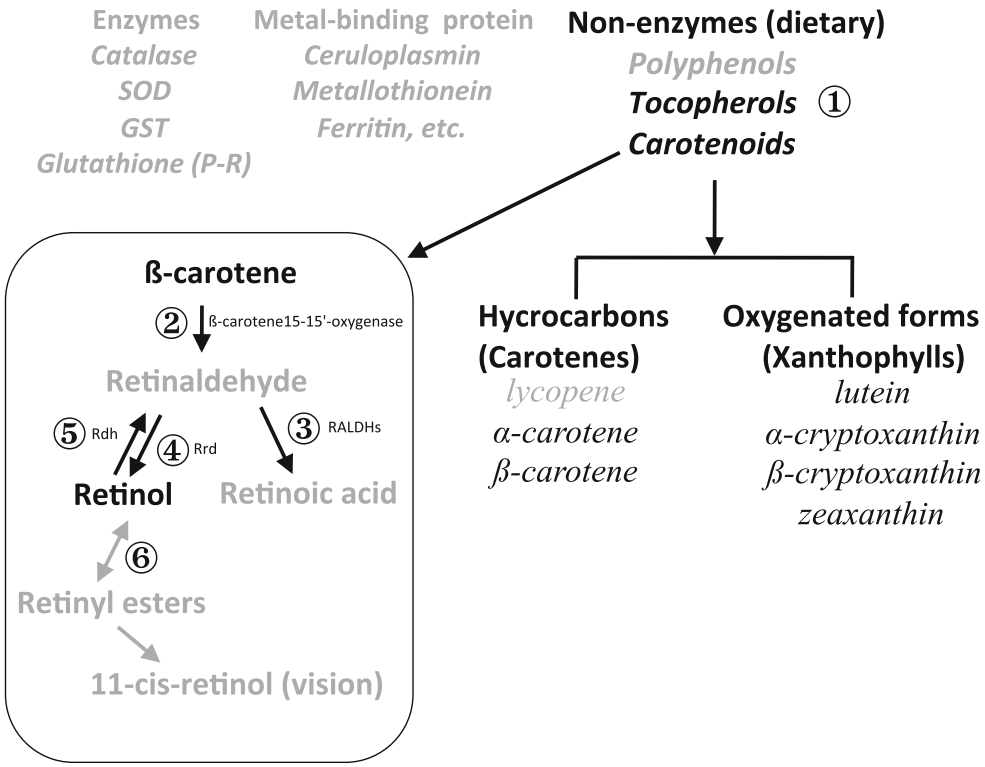

Fig. 1 Partial endogenous responses to oxidative stress as known in mammals. Superoxide dismutase (*SOD*), glutathione-*S*-transferase (*GST*), peroxidase–reductase (*P-R*). *1* Absorption from diet, *2* β-carotene 15-15′-oxygenase, *3* retinal dehydrogenases (*RALDHs*), *4* retinal reductase (*Rrd*), *5* retinol dehydrogenase (*Rdh*), *6* storage (esterification, hydrolization). Items written in bold were targeted in this paper

(Halme et al. 2010). Blindness in *Drosophila* has been shown to be a result of mutations in the gene responsible for encoding the β-carotene 15,15′-dioxygenase enzyme (von Lintig et al. 2001). Carotenoid and retinoid systems including uptake, absorption, and transport are crucial for the honey bees (larval development and vision and antioxidant capacities) but, if unbalanced, may affect honey bee health and contribute to the decline in bee populations.

Retinoid levels have been shown to be affected by organochlorine contaminants, and detrimental effects have been reported for turtles (Sleeman et al. 2008), fish (Ndayibagira and Spear 1999; Široká and Drastichová 2004), as well as birds (Murvoll et al. 2007). Furthermore, the impact of agricultural runoff on the retinoid system has been shown in different frog species (Boily et al. 2009; Spear et al. 2009; Lenkowski and McLaughlin 2010). Despite a few studies conducted on *Drosophila* that have reported the positive influence of carotenoids on vision (Giovannucci and Stephenson 1999; Wang et al. 2007), of vitamin A on tissue regeneration (Halme et al. 2010), and of vitamin E on increased life span (Bahadoroni et al. 2008), these components have not been investigated in insects. Considering that pollen from herbicide-treated crops is largely available for honey bees due to expansive monocultures, there is a need to develop tools to assess honey bee health in agricultural environments. Therefore, evaluating the risk associated with the herbicides found most frequently in agricultural ecosystems is paramount.

The objectives of our study were to adapt methods of carotenoid and retinoid detection in *Apis mellifera*, to estimate the average levels of the dominant carotenoids and retinoids detected in honey bees, and finally to assess whether atrazine, metolachlor, and glyphosate may affect the concentration of these compounds and lipid peroxidation. Caged honey bees were then exposed to realistic environmental doses of commercial formulations of atrazine, metolachlor, and glyphosate.

Material and methods

Chemicals

Butylated hydroxytoluene (BHT), malonaldialdehyde (MDA), carotenoids (lutein, zeaxanthin, α-cryptoxanthin, β-cryptoxanthin, α-carotene, and β-carotene), and vitamins A (all-*trans*-retinol) and E (α-tocopherol) standards were purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada). Bovine serum albumin (BSA), ascorbic acid, sodium deoxycholate, trichloroacetic acid, dodecyl sulfate, nitric acid, HPLC-grade solvents, acetone, and hexane were from Fisher Scientific (Saint-Laurent, QC, Canada); methanol (MeOH) and ethyl acetate were obtained from EMD Millipore Corporation (Billerica, MA, USA), and acetonitrile (ACN) was obtained from Caledon Laboratory Ltd., Georgetown, Ontario, Canada. Commercial formulations of the pesticides atrazine (Aatrex® 480), metalochlor (Dual® 915), and glyphosate (Credit Extreme® 240) were purchased from Les Moulins Mondou (Mirabel, QC, Canada) and represent commonly applied pesticide formulations in Québec.

Chronic exposure (cage experiments)

Honey bees were taken from frames without brood in a healthy beehive and placed in acrylic cages (13 cm×14 cm×18 cm) in groups of 30. They were maintained in a temperature-controlled room (darkness, 25 °C±1 with 55±5 % relative humidity) and fed a sucrose solution (50 % *w*/*w*) ad libitum for 24 h. Prior to pesticide exposures, the estimated daily mean syrup consumed per bee was determined to be 41 μl.

The exposure levels were based on the herbicides analyzed in crops or vegetation near maize production in order to represent field-realistic exposures. The highest reported concentrations of atrazine (1,133 μg/kg) and metolachlor (295 μg/kg) were for dandelions near maize fields (Krupke et al. 2012). These concentrations represent less than 1 % of the mean lethal dose (LD_{50}) values for the honey bee. For example, the oral LD_{50} value for atrazine, 97 μg/bee (ARLA 2007), corresponds to 746 μg/g, based on a mean bee mass of 130 mg (our study). The highest concentration of atrazine in dandelions, 1,133 μg/kg or 1.133 μg/g, represents 0.15 % of the LD_{50} value (1.133 μg/g ÷ 746 μg/g). The doses for atrazine, metolachlor, and glyphosate were based on the quantity of active matter (am) in their respective commercial formulations and the daily consumption of syrup of 41 μl to obtain concentrations less than 1 % of the LD_{50}. Nominal concentrations were the following: 1.25, 2.50, and 5.0 ng/bee for atrazine and glyphosate and 5.0, 10.0, and 20 ng/bee for metolachlor. The highest dose of each compound was verified by chemical analysis by the Laboratoire d'expertises et d'analyses alimentaires, Sous-ministériat à la santé animale et à l'inspection des aliments, MAPAQ (Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec), Québec (QC, Canada). The measured doses for the nominal 5.0 ng/bee for atrazine and glyphosate were 4.42 and 4.46 ng/bee, respectively, while for metolachlor, the highest nominal dose of 20 ng/bee was in fact 21.9 ng/bee. Nominal concentrations have been used in the text. Bees in the control groups were fed a sugar solution without herbicides. The control and contaminated sugar solutions were prepared 10 days prior to exposure, stored at −20 °C, and thawed at room temperature before use. The sugar solutions of all cages (five replicates per dose and controls) were changed daily for the duration of the 10-day test. Cages were equipped with two containers for syrup (1.5-ml capacity each). The containers were weighed before and after daily changes to estimate the

consumption of syrup per cage/day adjusted for dead bees. Mortality was recorded every 24 h.

After 10 days, the surviving bees were anesthetized/euthanized by placing the cages in an insulated container with dry ice for 5 min. The bees were then transferred to identified plastic bags and stored at −80 °C until analysis. Pools of bees (n=7 to 10; randomly mixed between replicates for each dose of herbicide) were analyzed for *at*-ROH, α-tocopherol, carotenoids, peroxidation of lipids (thiobarbituric acid reactive substance (TBARS)), and protein content.

at-ROH, α-tocopherol, and carotenoid analysis

Six whole bees were weighed and homogenized on ice in a glass tube (12 ml screw top borosilicate) with four volumes of water (Nanopure) containing 0.5 % ascorbic acid. A volume of 2 ml of MeOH (0.1 % BHT) was added and the tube was vortexed for 30 s. The homogenate was extracted twice with 4 ml hexane (0.1 % BHT)/acetone (50:50). After each addition of the hexane/acetone solution, the tube was vortexed for 90 s and centrifuged for 5 min at 3,000×*g*. The supernatant (1.6 ml) was taken and evaporated for≈15 min in a vacufuge (Eppendorf™, Fisher Scientific, Ottawa, Canada) at 45 °C. A second extraction was performed, with the supernatant (1.6 ml) being added to the first extraction and evaporated for an additional 15 min at 45 °C. The residue was resuspended in 200 μl MeOH (consisting of 0.1 % BHT). The tube was saturated with inert gas (N_2), promptly closed, vortexed for 10 s, and centrifuged for 1 min. The supernatant (≈200 μl) was then transferred to a 5-ml glass tube sealed under N_2 and kept at −20 °C until analysis. Prior to injection, the sample was equilibrated to room temperature for 10 min. Two injections were performed simultaneously in two reversed-phased HPLC systems (Water Corporation, Milford, MA) connected to Empower Pro software, version 5.0, a model 510 pump and a model 7725i Rheodyne injector. Honey bee tissue extraction and chromatography were conducted under yellow incandescent light to prevent isomerization of the compounds.

Chromatographic procedures

HPLC 1–Carotenoids A volume of 30 μl from the sample extract was injected into the system equipped with a model 2774 absorbance detector set at 445 nm. Compounds were separated on a Chromegabond C30 analytical column 4.6× 250 mm, 5-μm particles size (Canadian Life Science, Peterborough, ON, Canada), using gradient elution based on the method developed by García-Plazaola and Becerril (1999) for the determination of antioxidants in plants. The initial solvent A: ACN–MeOH–H_2O (84:9:7) changed linearly over a 12-min period to the final solvent B: MeOH–ethyl acetate (68:32). After 11 min on solvent B, a 1-min linear gradient was used to return to 100 % solvent A. The flow rate was constant at 1.5 ml/min for the total 35-min elution time. Under these conditions, the retention times for lutein, zeaxanthin, α- and β-cryptoxanthin, and α- and β-carotene were 10.3, 10.6, 14.7, 15.1, 20.4, and 21.1 min, respectively (Fig. 2a). The peaks were identified with commercial standards.

HPLC 2–at-ROH and α-tocopherol The detection was performed with the injection of 80 μl of the sample extract. The system used was equipped with a model 486 detector set at 325 nm. The wavelength was changed to 292 nm between 19 and 26 min for the detection of vitamin E. The vitamins were separated on an Ace C18 analytical column, 4.6×150 mm, 5 μm (Canadian Life Science), using gradient elution with the initial solvent A: MeOH–H_2O (88:12) changing linearly over a 20-min period to the final solvent B: MeOH (100 %). After 5 min on solvent B, a 1-min linear gradient was used to return to 100 % solvent A. The flow rate was 1.25 min/ml for the total 35-min elution time. Under these conditions, the retention times for retinol and vitamin E were 11.3 and 25.9 min, respectively (Fig. 2b). The peaks were identified with commercial standards.

TBARS (lipid peroxidation)

The measurement of oxidative stress, adapted from Ohkawa et al. (1979), was estimated by the levels of TBARS as by-products of lipid peroxidation. Two or three whole bees (≈360 mg) were homogenized in 2 ml PBS buffer (pH 7.0). A volume of 50 μl was taken for protein dosage, and the homogenate was centrifuged at 3,000×*g* for 10 min at 4 °C. A volume of 200 μl of the supernatant was mixed with 100 μl of sodium deoxycholate (0.15 %*w*/*v*), and 800 μl of nanopure water was added. The resulting solution was vortexed and incubated for 10 min at room temperature. After an addition of 100 μl of trichloroacetic acid (72 %*w*/*v*), the vial was vortexed and centrifuged at 9,000×*g* for 15 min. Supernatant (300 μl) was placed in a glass tube previously washed in 10 % nitric acid. Then, 20 μl of sodium dodecyl sulfate (SDS) (10 %), 150 μl of acetic acid (20 % pH 3.5), and 150 μl of thiobarbituric acid (TBA) (0.6 % in 0.05 N NaOH) were added. The test tube was vortexed, filled with nitrogen gas, sealed with a glass bead and parafilm, and incubated at 100 °C for 1 h. Tubes were then removed and placed in an ice bath to stop the reaction. A volume of 400 μl of butanol/pyridine was added, vortexed, and centrifuged at 1,000×*g* for 10 min at 4 °C. A volume of 200 μl was then transferred into a 96-well quartz microplate and read on a spectrophotometer (Infinite M1000 Quadruple Monochromator Microplate Reader, Tecan Group Ltd., Durham, NC, USA) in fluorescence mode at an excitation wavelength of 532 nm and an emission wavelength of 553 nm. The mean values were corrected for solutions without tissue homogenates (blanks), and the resulting fluorescence

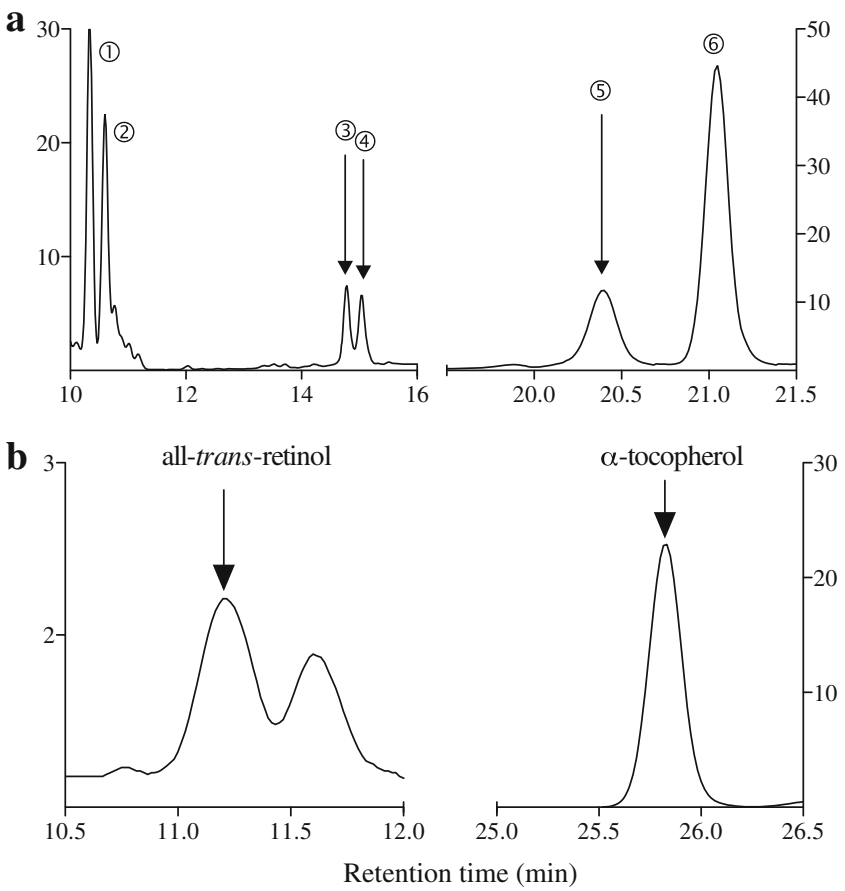

Fig. 2 HPLC chromatograms of representative extract of whole honey bees (Y axes units= mAUFs). **a** Carotenoids. Peaks are lutein (*1*), zeaxanthin (*2*), α-cryptoxanthin (*3*), β-cryptoxanthin (*4*), α-carotene (*5*), and β-carotene (*6*). **b** Peaks of all-*trans*-ROH and α-tocopherol. All peaks were identified with commercial standards

was estimated according to an MDA linear standard curve. Typical parameters of the curve were the following: R^2= 0.997; $p<0.001$. Lipid peroxidation based on nanomole equivalent of MDA is referred to in the text as TBARS/gram of tissue. Analyses (n=8 to 10 pools of two or three bees) were performed for each dose of herbicides tested and their control group.

Protein concentration

The protein content in each sample (supernatant of the homogenate following the first centrifugation) was determined by the Bradford method (Bradford 1976).

Statistical analysis

Consumption of syrup was tested using general linear model (GLM) two-way repeated measures (time and treatment design including a "time×treatment" interaction term). When sphericity (homogeneity of variance in repeated measures tests) was violated, the Greenhouse–Geisser correction was applied. When significant, the time was further tested by comparing the consumption between days (multiple comparison test followed by Bonferroni correction), while significant models for treatment were subjected to the Dunnett t test ($p<0.05$) (doses against the control group). For each herbicide, the survival of bees after 10 days was compared between the doses and the control group (five cage replicates) using the chi-square test. The survival was defined as the number of bees at day 1 minus the survival bees at day 10 and is expressed as percentage relative to day 1. Mean concentrations of *at*-ROH, α-tocopherol, carotenoids (lutein, zeaxanthin, α-cryptoxanthin, β-cryptoxanthin, and β-carotene), TBARS, protein, and bee mass were compared for each herbicide by GLM one-way analysis of variance, followed by the Dunnett t post hoc test against the control group. When transformations failed to normalize the distribution of data, nonparametric tests were used. Jonckheere–Terpstra analysis (JT; based on the medians ordered in a particular direction, $p<0.1$) was computed to detect the influence of increasing doses of pesticides, and variables were compared between herbicides using the Kruskal–Wallis (KW), followed by Dunn's multiple comparison test. Spearman correlations were used to assess the relationships between variables in order to observe factors likely to have influenced the results. All statistical tests were performed with SPSS® Statistic 18.0 software (IBM® Corporation, Armonk, NY, USA).

Results

Consumption of syrup and survival

The daily consumption of syrup by caged honey bees is depicted in Fig. 3. With the atrazine experiment, the consumption of syrup was low at the beginning, but starting at day 4, the consumption increased steadily (Fig. 3a). After 10 days, the consumption of syrup had significantly increased with time ($F_{3.91,\ 62.49}$=15.76; p<0.001; GLM two-way repeated measures with a Greenhouse–Geisser correction). No significant differences were observed for the time×dose interaction term ($F_{11.72,\ 62.49}$=1.28, p=0.25) nor between doses (the three doses and the control group) ($F_{3,\ 16}$=0.19, p=0.89). Significant results for time were found in the consumption of syrup for bees exposed to metolachlor ($F_{4.07,\ 65.09}$=67.46, p<0.001) (Fig. 3b). However the time×dose interaction was not significant ($F_{27,\ 65.09}$=0.63, p=0.61). Further analysis showed that the significant result for time was due to the high increase in syrup consumption between days 8 and 9, with the day 9 being significantly different from all other days (p<0.001; multiple comparison test followed by Bonferroni correction). While all groups seemed to follow the same fluctuating pattern, a significant difference was found when comparing the doses ($F_{3,\ 16}$=3.84, p<0.05). The 10 and 20 ng/bee doses were significantly higher than the control group (p<0.05; Dunnett t test). The experiment with glyphosate-spiked syrup along with the control group demonstrated a significant decrease in consumption over time ($F_{4.68,\ 74.81}$= 23.99, p<0.001) (Fig. 3c). Day 10 was identified as the day with the lowest consumption of syrup compared to all other days (p<0.001; multiple comparison test followed by Bonferroni correction). No significant results were computed for the time×dose interaction term ($F_{14.02,\ 74.81}$=1.32, p= 0.15) or when comparing the doses against the control group ($F_{3,\ 16}$=0.24, p=0.87).

No significant results were found when the survival of dosed bees was compared to their respective control group after 10 days of exposure (Table 1). Metolachlor exposure including the control group was characterized by lower survival percentages with higher SD values for the two highest doses, 10 and 20 ng/bee.

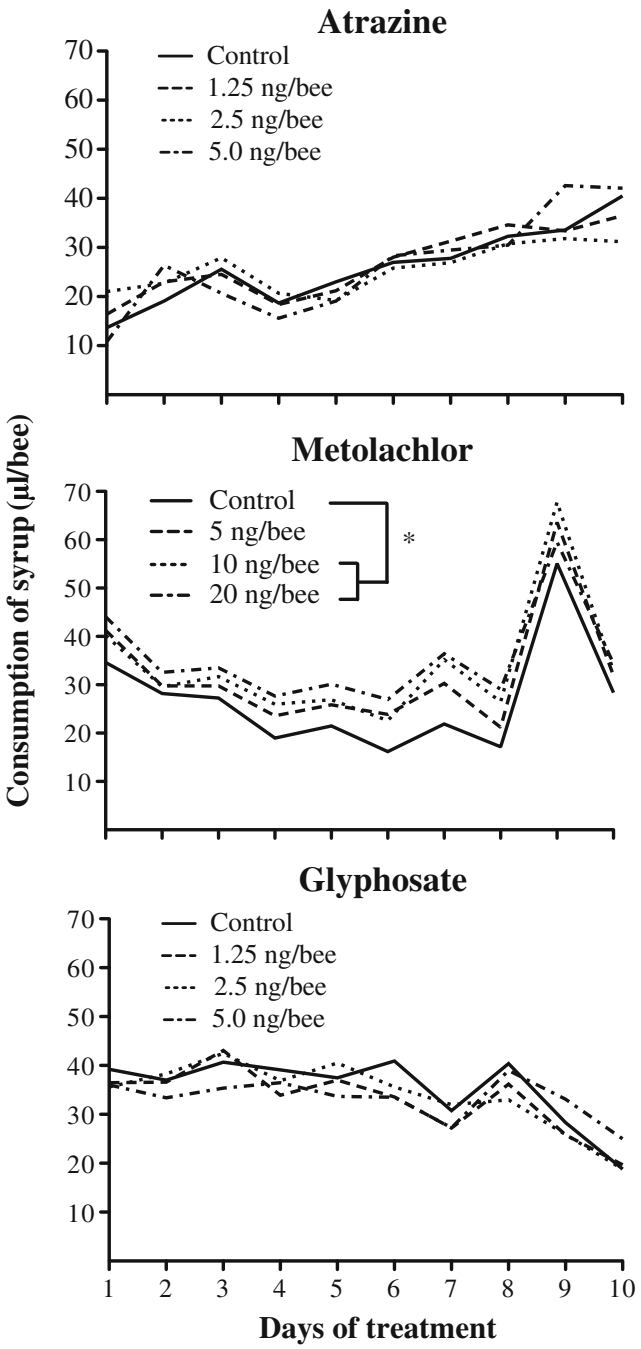

Fig. 3 Daily consumption of syrup per honey bee exposed for 10 days to commercial formulation of atrazine, metolachlor, and glyphosate. Consumption over time was tested using GLM two-way repeated measures analysis with Greenhouse–Geisser correction. Doses were compared against the control group with Dunnett t test *p<0.05

Carotenoids, *at*-ROH, and α-tocopherol

When the honey bee extracts were analyzed, six distinct carotenoid compounds were detected and quantified (Fig. 2a). While the method adapted for our study allowed the detection of α-carotene, this compound was not consistently detected in all samples. The best detection was obtained for metolachlor exposure where levels varied greatly; mean (all doses combined)=756 (120–2440) nmol/g of tissue. For this reason, values for α-carotene were not subjected to statistical analysis. Means and SD of carotenoids measured in bees exposed to the three herbicides are reported in Table 2. When atrazine-dosed bees were compared to the control group (GLM one-way analysis of variance followed by Dunnett t test), significant differences were found for β-carotene ($F_{3,34}$=4.66; p<0.01), with the highest doses, 2.5 and 5.0 ng/bee, leading to lower values. Metolachlor increased

Table 1 Survival and chi-square statistic for bees exposed to herbicides for 10 days

Herbicide	Nominal dose (ng/bee)	Survival[a] (%)	χ^2 Statistic[b]	Probability
Atrazine	Control	97.6±1.4	0.60	p=0.89
	1.25	97.2±3.2		
	2.5	98.1±1.7		
	5.0	98.1±2.8		
Metolachlor	Control	89.8±5.7	1.55	p=0.67
	5.0	86.5±11.0		
	10.0	86.6±17.9		
	20.0	83.7±18.9		
Glyphosate	Control	92.5±7.9	6.28	p=0.10
	1.25	97.0±3.7		
	2.5	97.1±2.1		
	5.0	96.9±2.1		

[a] Values represent the means±SD of five caged bees replicates (see "Material and methods" section for details)

[b] Chi-square statistic was computed from the number of bees at day 10 to the number of bees at day 0. Comparisons were made between the three doses and their relative control group for each herbicide

the level of lutein with increasing doses (JT=412.0; p<0.01; n=40). For this herbicide, the α-cryptoxanthin value at the 5.0 ng/bee dose showed a tendency to be lower than that at the control group ($F_{3,36}$=3.22; p<0.1). In the glyphosate group, a significant comparison between carotenoid contents was found only for β-carotene. Levels of this compound significantly decreased with increasing doses (JT=195.5; p<0.05; n=40).

Interesting results for *at*-ROH were obtained with the three herbicides; increasing doses led to decreased values when the bees were exposed to atrazine (JT=185.9; p<0.05; n=37) and glyphosate (JT=229.5; p<0.1; n=40). However, increased values were observed when the bees were exposed to metolachlor (JT=341.0; p<0.05; n=37). When tested against their respective control group, a significant result for α-tocopherol was found only for the herbicide glyphosate ($F_{3,33}$=5.12; p<0.01) where the 1.25 ng/bee dose showed a higher mean value (p<0.05).

Levels of α-tocopherol, α-cryptoxanthin, β-cryptoxanthin, and β-carotene were higher in atrazine-treated bees (both dose and control groups) compared to those in other herbicide-exposed bees (KW=71.92, 68.03, 72.56, 74.59, respectively; p<0.001; n=118), while higher zeaxanthin values were measured in bees exposed to glyphosate (KW=52.51; p<0.001; n=120). Several positive relationships between carotenoids were observed (Table 3). Only β-carotene and zeaxanthin were negatively related. Significant positive correlations were shown between *at*-ROH and lutein, α-cryptoxanthin, β-cryptoxanthin, and α-tocopherol, as well as between α-

Table 2 Means±SD for parameters measured in honey bees exposed for 10 days to increasing doses of atrazine, metolachlor and glyphosate

Herbicide	Dose (ng/bee)	*at*-ROH (pmol/g tissue)	α-Tocopherol (nmol/g tissue)	Lutein pmol/g tissue)	Zeaxanthin (pmol/g tissue)	α-Cryptoxanthin (pmol/g tissue)	β-Cryptoxanthin (pmol/g tissue)	β-Carotene (nmol/g tissue)	Protein[a] (mg/g)	Bee mass (g)
Atrazine	Control	172.3±49.86	130.9±71.45	501.4±121.7	185.0±67.26	303.9±68.99	1027±272.9	27.47±8.17	40.92±5.78	0.135±0.004
	1.25	177.4±22.69	162.9±97.13	559.2±134.7	168.2±65.20	307.0±105.9	942.2±385.2	19.89±9.56	41.69±7.64	0.133±0.006
	2.5	155.2±36.28	116.1±54.64	453.8±91.20	152.2±54.14	246.8±95.70	684.3±322.0	16.73±11.14*	44.41±5.90	0.132±0.005
	5.0	143.0±24.10§	125.4±98.21	517.1±120.2	222.1±162.5	363.3±139.0	940.1±480.8	11.80±7.02**	41.55±6.52	0.129±0.006*
Metolachlor	Control	136.6±54.85	24.73±6.28	441.9±127.0	110.9±69.54	117.0±30.57	234.9±54.01	0.96±0.52	39.98±4.74	0.132±0.004
	5.0	167.5±82.47	23.02±6.21	453.2±133.4	144.0±98.05	76.00±35.02†	156.4±111.6	0.80±0.76	38.87±8.07	0.135±0.007
	10.0	197.1±79.41	22.62±6.86	568.0±129.5	309.5±304.2	155.0±89.97	239.2±94.91	0.88±0.52	43.57±10.0	0.138±0.005
	20.0	198.4±35.23§	24.66±5.56	581.5±146.3¶	240.0±256.2	142.0±69.25	213.1±57.44	0.97±0.97	35.15±7.40	0.132±0.007
Glyphosate	Control	275.0±280.1	27.02±10.97	598.0±183.3	491.8±220.2	91.30±64.31	219.0±104.0	2.07±1.27	44.38±6.17	0.128±0.011
	1.25	251.0±236.3	34.54±12.85*	758.7±291.5	595.5±283.0	117.8±97.74	254.2±106.4	1.41±0.80	42.88±8.29	0.127±0.010
	2.5	133.0±63.61	21.43±6.61	602.4±214.3	479.2±175.4	77.10±38.96	184.1±76.76	1.60±0.98	35.32±4.89	0.129±0.005
	5.0	140.0±56.96‡	24.28±12.89	651.4±273.3	506.5±293.0	80.40±45.74	202.6±137.3	0.96±0.51§	39.26±3.64§	0.130±0.008

†p<0.1; *p<0.05; **p<0.01. One-way analysis of variance (GLM followed by Dunnett t test)

‡p<0.1; §p<0.05; ¶p<0.01. Medians oriented to one direction with increasing doses (Jonckheere-Terpstra nonparametric test)

[a] Protein levels quantified from TBARS test (see "Material and methods" section for details)

Table 3 Spearman significant correlation coefficients (Rho) for carotenoids, *at*-ROH, α-tocopherol, and TBARS measured in honey bee tissue (n=117–120)

	Zeaxanthin	α-Cryptoxanthin	β-Cryptoxanthin	β-Carotene	*at*-ROH	α-Tocopherol	TBARS
Lutein	0.416**	0.286**	0.296**		0.407**		
Zeaxanthin				−0.233*			
α-Cryptoxanthin			0.886**	0.530**	0.267**	0.682**	0.406**
β-Cryptoxanthin				0.743**	0.284**	0.786**	0.428**
β-Carotene						0.690**	0.383**
at-ROH						0.277**	
α-Tocopherol					0.254**		0.363**

*p<0.05; **p<0.01

tocopherol and all carotenoids except zeaxanthin and lutein (Table 3).

TBARS, protein levels, and bee mass

When the honey bees were exposed to increasing concentrations of atrazine, no effect was observed in tissue TBARS content ($F_{3,36}$=0.48; p=0.69) (Fig. 4a). The TBARS test proved to be significant when the bees were exposed to metolachlor ($F_{3,34}$=6.09; p<0.01): The highest dose, 20 ng/bee, led to values lower than the control group (Dunnett t test, p<0.05) (Fig. 4b). Glyphosate did not significantly modify TBARS compared to the control group at any dose ($F_{3,35}$=0.42; p=0.74) (Fig. 4c). When herbicides were compared for TBARS, atrazine had a significantly higher rank distribution than metolachlor and glyphosate (KW=26.06; p<0.001; n=117). Positive relationships were obtained between TBARS and α-cryptoxanthin, β-cryptoxanthin, β-carotene, and α-tocopherol (Table 3).

No significant changes were observed in protein levels following atrazine and metolachlor exposures, whereas the values from the glyphosate experiment led to a significant decrease with increasing doses (JT=209.9; p<0.05; n=40) (Table 2). Metolachlor and glyphosate did not significantly affect bee mass, yet in the highest dose of atrazine, 5.0 ng/bee, a significant difference in mass compared to the control group was observed ($F_{3,36}$=2.96; p<0.05 followed by Dunnett t test, p<0.05).

Discussion

Protocols based on contaminated food for animal subjects are common in toxicological studies, but the "real" dose received by each individual is hard to determine precisely. In our study, even knowing the concentration of herbicide active matter as well as the volume of consumed syrup, the exact estimation of individual dose values remained impossible. Factors such as the age of the bees, trophallaxis practices, and the stress caused by the absence of hive activity could have influenced the consumption of syrup and, consequently, the doses received by individual caged bees. Although imperfect, the estimation of consumption of syrup in our study is important.

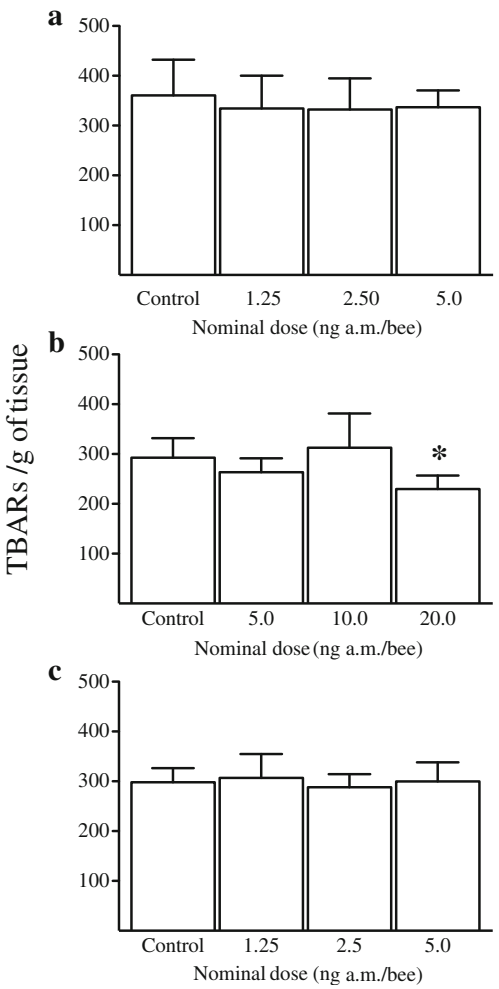

Fig. 4 TBARS measured in bees exposed to increasing doses of **a** atrazine, **b** metolachlor, and **c** glyphosate. Herbicide-treated bees were compared to the control group using GLM one-way analysis of variance followed by Dunnett t test *p<0.05

The results for the consumption of syrup confirmed that the bees were exposed to nonlethal doses for each of the three herbicides. In order to respect the nominal doses, the volume of syrup consumed per day should have been 41 μl/bee. Daily syrup consumptions for metolachlor and glyphosate were close to 41 μl/bee, but this theoretical consumption volume for the syrup containing atrazine was reached only around day 8. The low consumption of atrazine-contaminated syrup observed during the first days (between 15 to 25 μl/bee) could be explained, at least in part, by the capture of bees at the end of the summer season (August–September in Québec). At this time of the year, worker and nurse bees have more stocked nutrients than any other time of the year (Otis et al. 2004; Hahn and Denlinger 2007). We may then suppose that bees were less eager to use the cages' feeders resulting in lower syrup consumed for all doses including the control group. Atrazine exposure was then probably lower than expected. The high consumption of syrup at day 9 for metolachlor exposure is difficult to explain. It is well documented that insect metabolic rate (e.g., flight activity) can be influenced by abiotic factors such as temperature, humidity, and barometric pressure (Rousse et al. 2009). In our study, the consumption of syrup between days 8 and 9 could not be related to variations in temperature or relative humidity (data not shown), and the barometric pressure was not taken into account. While the study of honey bee behavior is beyond the scope of this study, the significantly higher quantity of syrup consumed by honey bees exposed to metolachlor compared to the control group is also intriguing. This result could have been in "response" to metabolic effects not included in our experimental design. In a study by Dobšíková et al. (2011), carp (*Cyprinus carpio* L.) exposed to metolachlor demonstrated a significant increase in glucose and lactate dehydrogenase levels in the plasma as well as a decrease in chloride and triglyceride levels when compared to a control group. The effect on triglyceride metabolism is interesting and could explain the lower TBARS value obtained when the bees were exposed to the highest doses of metolachlor. Using ultracentrifugation techniques, Bonnefont et al. (1989) demonstrated that 43 % of TBARS was located in lipoproteins. Since the TBARS test is based on MDA formation following the peroxidation of lipids, a decrease in lipoproteins (namely triglycerides) could lead to a lower production of MDA and lower TBARS values. Lower lipoprotein levels could have triggered higher food consumption and, as in our study, a higher consumption of syrup. Our results with metolachlor suggest that higher foraging activities and consequently higher energy expenditure could affect honey bees exposed to this herbicide in the wild. Because atrazine- and glyphosate-induced oxidative stress are commonly reported for several species (Nwani et al. 2010; Jasper et al. 2012), significant TBARS levels in honey bees exposed to these herbicides compared to those in their respective control group would have been expected. The TBARS test has been widely criticized over the years due to its lack of sensitivity and specificity. A large number of secondary oxidation products and the presence of other compounds such as sugar and amino acids may contribute to the determined values (Grotto et al. 2009). Moreover, in vitro assays performed by Rael et al. (2004) demonstrated that saturated fatty acids (e.g., linoleic acid and arachinodic acid) are natural inhibitors of TBARS formation. These authors also stated that TBARS measurement should be fatty acid standardized to get a more appropriate estimation of the level of lipid peroxidation. Although other tests can be used to measure the peroxidation of lipids, we selected TBARS because of its extensive utilization in pathologies and toxicology (Grotto et al. 2009) and also because it has been used successfully in an in vitro study to evaluate oxidative stress following exposure to chlorpyrifos in the honey bee (Rehman et al. 2012). In future studies with honey bees, we could investigate energetic metabolism pathways linked to glycogen, lipids, or electron transport, and the TBARS could be standardized with fatty acid levels. The level of metal-binding protein as well as enzyme activities linked to CAT, SOD, and GST would be interesting to quantify in order to obtain a more complete picture of the antioxidant responses in the honey bee.

According to our results, herbicide exposure could influence carotenoid or retinoid metabolism. Decreased β-carotene in honey bees exposed to atrazine and glyphosate could be the result of a stimulatory effect of these herbicides on β-carotene 15-15′-monooxygenase activity (step 2, Fig. 1). Considering the metabolism cascade shown in Fig. 1, higher levels of RAL, RA, and *at*-ROH (retinol in Fig. 1) would have been expected. RAL and RA were not analyzed in our study, but the *at*-ROH levels are reported in Table 2. For the two herbicides, *at*-ROH levels decreased and did not increase. We may then suppose an additional effect on Rdh activity (step 5, Fig. 1). The latter could increase, depleting *at*-ROH and increasing the RAL and RA levels. A different result was associated with metolachlor exposure. In this case, *at*-ROH contents increased, but β-carotene levels did not change, suggesting a positive effect on Rrd activity (step 4, Fig. 1). These changes observed in *at*-ROH levels could be the effect on specific enzymes responsible for the reversible conversion of RAL to ROH along with enzymes leading to the formation of RA and RA metabolites (CYP26). In vertebrates, this metabolic pathway is often tested with luminescent reporter gene assays (RA receptor plasmid activator) such as performed by Paganelli et al. (2010) where increased RA activity has been observed in *Xenopus laevis* embryos exposed to glyphosate-based herbicides. Other enzymes associated with the storage (step 6, Fig. 1) and mobilization of retinoids have been studied in frogs (Boily et al. 2009) and fish (Ndayibariga and Spear 1999), but in insects, retinyl esters (e.g., palmitate, oleate, and stearate) were not found (Wang et al. 2007). Processes linked to the absorption, metabolization, and isomerization of

carotenoids (step 1, Fig. 1) should not be overlooked as well as protein levels and mass for which slight decreases were observed in bees exposed to glyphosate (protein) and atrazine (bee mass) (Table 2). Mass and protein level depletions may have an impact on "fitness" and lead to the deterioration of physical state of an organism. Also, the fact that our results could have been enhanced by the presence of unknown compounds (e.g., surfactants) from commercial formulations cannot be excluded.

The carotenoids have been well studied in *Drosophila* (Wang et al. 2007; von Lintig 2012) in the context of vision and carotenoid contents that have been reported for the eyes or the head of the insects. However, Giovannucci and Stephenson (1999) quantified carotenoids and xanthophylls in sequential development stages (larva, pupa, and imago). Imago levels for total carotenoids (α-carotene+β-carotene) were≈60 pmol/fly (fruit fly weight is 1.0 mg), while total xanthophylls (lutein+zeaxanthin+β-cryptoxanthin) were≈ 44 pmol/fly. Considering that the mean honey bee mass value in our study was≈130 mg, the concentration of total carotenoids would be slightly higher in *Drosophila* compared to that in honey bees. However, direct comparison with Giovannucci and Stephenson's study is difficult as the fruit flies were raised on agar media supplemented with cornmeal, carotenoids, or carrot juice. An interesting fact in this study is the comparison of carotenoid levels through the developmental stages in *Drosophila*; higher carotenoid values were found in the larval stage (≈ 900 pmol/fly), followed by the pupa and imago stages. Xanthophyll levels were rather low in the larval and pupal stages but increased in the imago stage. Carotenoid congeners found in *Drosophila* were also found in our honey bee samples, but RAL and RA could not be determined with our HPLC method (#2). Solvents for tissue extraction and mobile phases were selected to detect and quantify *at*-ROH and α-tocopherol, but these selections prevented us from recovering RAL and RA in the samples. Future analysis could include oxime derivation to detect and quantify RAL (Giovannucci and Stephenson 1999) and the use of a slightly acidic mobile phase to determine the RA levels in honey bees (Kane et al. 2008).

To our knowledge, this is the first study reporting distinct carotenoids, *at*-ROH, and α-tocopherol levels in the whole honey bee. Compared to other carotenoids, β-carotene was the most abundant (Table 2), and the levels of both β-carotene and α-tocopherol were higher in bees from the atrazine group, while higher zeaxanthin levels were observed with exposure to glyphosate. These differences could be linked to the different capture time for cage tests: mid-July for glyphosate, mid-August for metolachlor, and the beginning of September for atrazine. While all bees for the three experiments came from the same hive, they were raised with different pollen and nectar sources in the summer and fall seasons. Nutrient contents vary greatly according to pollen sources (Chantarudee et al. 2012; Di Pasquale et al. 2013). High carotenoid contents have been found in flowers from the Asteraceae family. For example, β-carotene levels may range from 0.31 to 1.1 mg/g (fresh plant material) (Hanganu et al. 2012; Horváth et al. 2010). These flowers are in full bloom in southern Québec during the end of summer and could have contributed to the high carotenoid levels in bees at this time of the year.

Conclusions

In this study, we examined the antioxidant parameters that derived from the diet, namely, carotenoids, *at*-ROH, and α-tocopherol. While no relationship was found between these antioxidants and the peroxidation of lipids, our experiments using sublethal field-realistic doses of herbicides on caged bees provide a method for carotenoid, *at*-ROH, and α-tocopherol analysis of the honey bee and possibly other pollinators or insect indicator species. The results obtained with *at*-ROH and β-carotene at very low doses (the nominal doses for both atrazine and glyphosate could be doubled and remain realistic) provide important stepping stones for future research focusing on the state of health of the honey bee and the development of biomarkers.

The combination of atrazine, metolachlor, and glyphosate deserves to be further investigated with caged lab experiments to better represent field conditions where honey bee colonies are exposed to various mixtures of agricultural contaminants.

Acknowledgments The authors would like to thank Maxime Gauthier for HPLC technical assistance. We also thank Dr. Diana Averill for generous advices in TBARS analysis. We are grateful to Dr. Philip Spear for providing access to his laboratory and TOXEN (Centre de recherche en toxicologie de l'environnement) for the use of analytical equipment. This study was supported by the Programme de soutien à l'innovation en agroalimentaire (PSIA) from Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ), attributed to M. Boily (#811175).

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