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A. Badiou-Bénéteau, Aurore Benneveau, F. Géret, Hélène Delatte, Nathalie Becker, J. L. Brunet, Bernard Reynaud, L. Belzunces

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A. Badiou-Bénéteau [a,*], A. Benneveau [b], F. Géret [c], H. Delatte [d], N. Becker [e], J.L. Brunet [a], B. Reynaud [d], L.P. Belzunces [a,*]

[a] *INRA, UR 406 Abeilles & Environnement, Laboratoire de Toxicologie Environnementale, 84914 Avignon Cedex 9, France*
[b] *CIRAD, UMR PVBMT, F-97410 Saint-Pierre, La Réunion, France*
[c] *Université d'Albi, UMR CNRS 5602 Centre Universitaire Jean-François Champollion, Laboratoire GEODE, 81012 Albi cedex 9, France*
[d] *CIRAD, UPR HORTSYS, F-97455 Saint-Pierre, La Réunion, France*
[e] *MNHN, UMR CNRS 7205, Origine, Structure et Evolution de la Biodiversité, 75231 Paris, France*

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A B S T R A C T

The aim of this study was to distinguish the impacts of two different anthropogenic conditions using the honeybee *Apis mellifera* as a bioindicator associated with a battery of biomarkers previously validated in the laboratory. Both the urban (RAV, Ravine des Cabris) and semi-natural (CIL, Cilaos) sites in La Reunion Island were compared in order to assess the impacts of two types of local pollution using the discriminating potential of biomarkers. Hives were placed at the CIL and RAV sites and honeybees were collected from each hive every three months over one year. Honeybee responses were evaluated with respect to several biochemical biomarkers: glutathione-S-transferase (GST), acetylcholinesterase (AChE), alkaline phosphatase (ALP) and metallothioneins (MT). The results showed a significant difference between the localities in terms of GST, AChE and ALP activities, as regarding midgut MT tissue levels. Compared to the CIL site, ALP and MT tissue levels were higher at the RAV site, although AChE activity was lower. GST displayed more contrasted effects. These results strongly suggest that the honeybees based in the more anthropized area were subjected to sublethal stress involving both oxidative stress and detoxification processes with the occurrence of neurotoxic pollutants, amongst which metals were good candidates. A classification tree enabled defining a decision procedure to distinguish the sampling locations and enabled excellent classification accuracy (89%) for the data set. This field study constitutes a strong support in favour of the in situ assessment of environmental quality using honeybee biomarkers and validates the possibility of performing further ecotoxicological studies using honeybee biomarkers.

1. Introduction

La Réunion Island benefits from considerable plant diversity and part of the island was granted the status of a UNESCO World Heritage Site in 2010. The island displays a high degree of endemism and rare terrestrial biodiversity. In view of the important and often irreversible impacts of human activity on this ecosystem, there is an increasing need to develop tools to monitor the impacts of pollution. Bioindicators represent good witnesses of environmental health and their presence, or the structure of their populations, could be considered as highly informative. However, characterization of the physiological integrity and functionality of individuals requires tools to act as biomarkers of exposure to environmental stressors. Biomarkers can be defined as observable or measurable modifications at the molecular, cellular, physiological or behavioural levels which reveal the exposure of an organism to xenobiotics (Lagadic et al., 1997). Biomonitoring programmes are usually based on studying a set of biomarkers in sentinel species of interest (Aguilera et al., 2012; Damiens et al., 2004; Lionetto et al., 2003; Stanic et al., 2006). In the terrestrial environment, the honeybee is a particularly pertinent model for the development of biomarkers in order to assess environmental contamination (Leita et al., 2004; Saifutdinova and Shangaraeva, 1997). Honeybees can constitute reliable indicators of environmental quality because their intense foraging activity brings them into contact with a large number of pollutants within a radius that generally ranges from 1.5 to 3 km around the hive, depending on food abundance (Chauzat et al., 2009). A decline in honeybee populations is currently being seen in many parts of the world, resulting in an active strategy for the monitoring and diagnosis of population health (Nguyen et al., 2009). The honeybee is therefore a species of particular interest in terrestrial ecotoxicology because its physiology, behaviour and ecology have been the subject of extensive study (Alaux et al., 2010; Decourtye et al., 2004; Henry et al., 2012).

The responses of some biochemical parameters, such as alkaline phosphatase, acetylcholinesterase and glutathione-S-transferase, have

* Corresponding authors at: INRA, Laboratoire de Toxicologie Environnementale, UR 406 *Abeilles & Environnement*, 84914 Avignon Cedex 9, France. Tel.: +33 43272 2604; fax: +33 43272 2602.
E-mail addresses: alexandra.badiou@avignon.inra.fr (A. Badiou-Bénéteau), luc.belzunces@avignon.inra.fr (L.P. Belzunces).

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Table 1
General characteristics of the selected sites.

	CILAOS (CIL)	RAVINE DES CABRIS (RAV)
GPS coordinates	E55°27′19.9″ S21°15′45″	E55°28′36.3″ S21°17′12.7″
Anthropogenic pressure	Semi-natural	Dense urban
Altitude (m)	238	250
Sampling period	May 2009 to May 2010	May 2009 to May 2010

been characterized in laboratory studies after the exposure of honeybees to various chemicals (Bounias et al., 1985, 1996; Stefanidou et al., 1996). Their response profiles to chemicals mean that honeybees can be considered as promising tools for use in environmental biomonitoring programmes. However, no in situ validations have been performed to date. During previous studies, we validated the responses to a battery of metabolic and neural biomarkers of *Apis mellifera* honeybees after their exposure to chemicals under laboratory conditions (Badiou-Bénéteau et al., 2012). The purpose of the present study was therefore to validate honeybee biomarkers under field conditions. Urban (RAV, Ravine des Cabris) and semi-natural (CIL, Cilaos) locations in La Reunion Island were compared in order to evaluate the impacts of two types of local pollution using the discriminating potential of biomarkers. The biomarkers chosen for this study included non-specific and specific biomarkers of pollutant toxicity. We focused this study on metabolic biomarkers such as alkaline phosphatase (ALP) and glutathione-S-transferase (GST), a neural biomarker, acetylcholinesterase (AChE), and metal biomarkers such as metallothioneins (MT). Metals and pesticides were also quantified in the honeybees in order to determine the pollutants to which they had been exposed.

2. Materials and methods

2.1. Experimental design

The study sites were located in the south-western part of La Réunion Island and displayed contrasting degrees of anthropisation: a weakly anthropised rural site, CIL (Cilaos, E55°-27′-19.9″; S21°-15′-45″) and a strongly anthropised urban site, RAV (Ravine des Cabris, E55°-28′-36.3″; S21°-17′-12.7″) (Table 1 and Fig. 1). The rural site (CIL) was relatively landlocked between the feet of the Cilaos mountain (the hives being located close to the mountain) and the Cilaos ravine, inducing foraging activity where no industrial contamination could be detected. This site was chosen as the relative reference. The urban site (RAV) was located in the suburbs of Saint Pierre, separated from the CIL site by the cirque de Cilaos. To reduce any variations due to geographical factors (microclimates prevailing on La Réunion Island), the sampling sites were situated within the same ecoregion, separated by a distance of 3.7 km. It was assumed the foraging zones of the bees were relatively independent and restricted to their respective sites because (i) food resources were deemed to be sufficient in the area surrounding the hives, based on the amount of honey produced, and (ii) a broad and deep ravine separates the sites, dissuading the bees from crossing it. Six *A. mellifera* honeybee colonies were placed at the CIL and RAV locations (three colonies per site) and samples were collected every three months over a 1-year period. Foraging *A. mellifera* honeybees were captured at the hive entrance. Sampling for analysis was carried out simultaneously in the colonies of both sites, with approximately 2000 honeybees being collected each time (around 200 g of honeybees) from each hive.

2.2. Determination of honeybee races

In the subtropical island of La Réunion, the dominant race of honeybee is *A. mellifera unicolor* (Ruttner, 1975, 1988; Schneider, 1989), although several European races of *A. mellifera (carnica, ligustica, mellifera)* are known to have been introduced in the past (Schneider, 1989). It was therefore necessary to verify the races of the honeybee populations used during this study, and the homogeneity of our samples. Two workers per colony were taken from the samples collected for the biomarker study, and preserved in alcohol before extraction of their DNA. The mtDNA region including the tRNAleu gene, the COI-COII intergenic region and the 5′ end of the COII subunit gene were PCR-amplified according to a protocol detailed by Garnery et al. (1993). A fraction of the PCR product was run on 1% agarose gel for total size determination and the remaining product was restricted

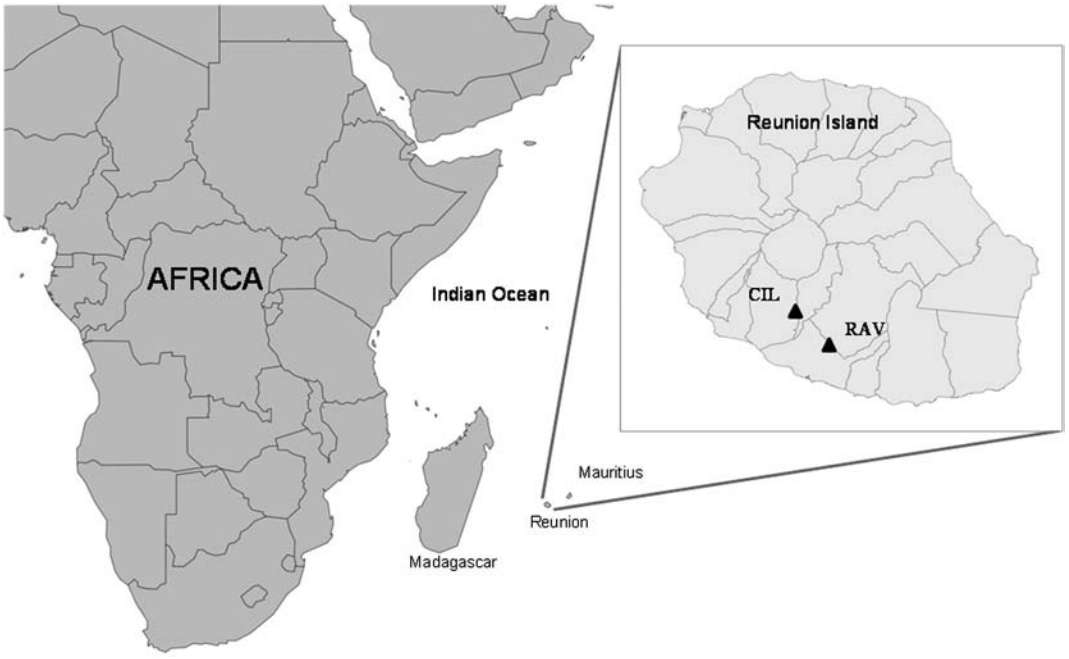

Fig. 1. Location of honeybee colonies at the CIL (Cilaos) and RAV (Ravine des Cabris) sites in Reunion Island (three colonies per site). These sites of interest were located in the south-western part of La Réunion Island and displayed contrasting degrees of anthropization: a slightly anthropized site (CIL) and a strongly anthropized site (RAV). CIL was chosen as the relative reference.

with DraI prior to electrophoresis, according to the detailed protocol used by Franck et al. (2001). Restriction profiles were read and the sizes compared to those published by Franck et al. (2001) for the south-western Indian Ocean region.

2.3. Enzyme extractions

To prevent any animal suffering, the honeybees were anaesthetised with carbon dioxide and their tissues sampled immediately. The head was removed first, followed by the midgut. All tissues and honeybees were stored at −80 °C until analysis. AChE was extracted from the honeybee head and GST and ALP were extracted from the midgut. Each tissue extract was prepared by homogenising five heads or midguts in an appropriate buffer to make a 10% (w/v) extract. For enzyme quantification, twelve extracts were performed and assayed in triplicate. Two homogenisation media were used, depending on the type of enzyme to be studied: 10 mM NaCl and 40 mM sodium phosphate, pH 7.4, for soluble enzymes (GST and ALP), and 1% Triton X-100, 10 mM NaCl and 40 mM sodium phosphate, pH 7.4, for the membrane enzyme (AChE). The homogenization media contained a mixture of 5 $\mu g.mL^{-1}$ of antipain, leupeptin and pepstatin A, 25 $units.mL^{-1}$ aprotinin and 0.1 $mg.mL^{-1}$ soybean trypsin as protease inhibitors. Tissue homogenisation was performed using a high speed TissueLyser II homogeniser (Qiagen®) for three periods of 30 s at 30-second intervals, and the extracts were then centrifuged at 4 °C for 20 min at 15,000 *g*. The resulting supernatants were split and frozen at −20 °C for subsequent analysis.

2.4. Enzyme assays

Biochemical analyses were performed using a Bioblock dual-beam microplate UV-Visible spectrophotometer. Enzyme assays were performed at 25 °C except for GST, which was assayed at 37 °C according to the method described by Roméo et al. (2003) with minor modifications. GST was measured at 340 nm in a medium containing 1 mM EDTA, 2.5 mM GSH (reduced glutathione), 1 mM 1-chloro-2,4-dinitrobenzene as the substrate and 40 mM sodium phosphate pH 7.4. Protein concentrations were estimated using the method described by Bradford (1976), with bovine serum albumin as the standard. AChE activity was measured at 412 nm according to the technique described by Ellman et al. (1961) with modifications from Belzunces et al. (1988). ALP was monitored at 410 nm in a medium containing 20 μM $MgCl_2$, 2 mM p-nitrophenyl phosphate as the substrate and 100 mM Tris–HCl pH 8.5 (Bounias et al., 1996). For AChE and ALP, one unit of enzyme activity was defined as the quantity of enzyme that hydrolysed 1 μmol of substrate per min, under these assay conditions. For GST, one unit of activity corresponded to the quantity of enzyme conjugating 1 μmol of GSH per min.

2.5. Analysis of metals

Twenty-one metals were analysed in the head and midgut of the honeybees (Table 2). The metals were analysed using Inductively Coupled Plasma/Mass Spectrometry (ICP-MS) according to the NF EN ISO 17294 standard. A quantity of 0.1 g of bees was put in a reactor with a mixture containing 38% nitric acid, 10% hydrogen peroxide and 4% HCl. The reactors were then placed in a Multiwave 3000 microwave oven (Anton Paar) and subjected to the following treatment cycle: a 0–600 W gradient for 15 min, a plateau at 600 W for 20 min, and a 600–0 W gradient for 15 min.

2.6. Analysis of metallothioneins

The heads and midguts were separated from the honeybees, weight and homogenised in 10 volumes of buffer (10 mM β-mercaptoethanol, protease inhibitors (SigmaFAST Protease Inhibitor, SIGMA) and 20 mM Tris–HCl pH 7.4). The homogenates were centrifuged at 4 °C for 30 min at 30,000 *g*. Supernatants were subjected to heat-denaturation for 15 min at 75 °C and centrifuged for 15 min at 10,000 *g*. The newly obtained supernatants were frozen at −80 °C until metallothionein (MT) quantification. MT levels in heat-denatured supernatants were estimated by differential pulse polarography (DPP) (Thompson and Cosson, 1984). The amount of MT in the supernatants was quantified using the standard addition method, with rabbit liver metallothioneins (COGER, ALX-202-072-MO) as the reference material. MT concentrations were expressed as $\mu g.mg^{-1}$ of proteins.

2.7. Analysis of pesticides

Sixty-four pesticides were screened using a multi-residue analysis based on a modified QuEChERS method, followed by gas chromatography coupled with Time of Flight mass spectrometry (GC-ToF) or liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS), depending on their properties. This analytical approach had been developed specifically by Wiest et al. (2011) to detect pesticides in honeybees. Briefly, the QuEChERS method combines a salting-out liquid-liquid extraction with acetonitrile (ACN) and a dispersive-SPE clean up. Five grammes of honeybees were extracted in ACN/water/hexane and citrate QuEChERS salts (10/3/3) and analysed using LS-MS/MS or GC-ToF. Samples were prepared in 90/10 of the mobile phase (0.3 mM ammonium formate and 0.05% formic acid/ACN) and their analytical volume was 10 μl. GC-ToF analysis was performed with a 6890 Agilent gas chromatograph (Agilent Technologies, Avondale, USA) coupled with a Time of Flight (ToF) mass spectrometer GCT Premier from Waters. Samples (1 μL) were injected into a 30 m chromatographic column with helium as the carrier gas. The initial temperature of 80 °C was increased to 320 °C. Using a combination of LC–MS/MS and GC-ToF, the active substances analysed were: Aldrin, Amitraz, Benalaxyl, Bifenthrin, Bitertanol, Bromopropylate, Bupirimate, Buprofenzin, Piperonyl butoxide, Cadusaphos, Carbaryl, Carbofuran, Chlorpyrifos, Chlorpyrifos methyl, Clothianidin, Coumaphos, Cyfluthrin, Cypermethrin, Cyproconazole, DDT, Deltamethrin, Diazinon, Dichloran, Dichlorvos, Dicofol, META, Dieldrin, Dimethoate, Endosulfan alpha, Endosulfan beta, Endosulfan sulphate, Esfenvalerate, Ethoprofos, Fenarimol, Fenitrothion, Flusilazole, HCB, Imidacloprid, Lindane, Malathion, Metamidiphos, Methoxychlor, Myclobutanil, Paclobutrazide, Parathion, Penconazole, Permethrin, Phenthoate, Phosalone, Phosmet, Prochloraz, Propargite, Propiconazole, Pyriproxyfen, tau-Fluvalinate, Tebuconazole, Tetradifon, Tolclofos methyl, Thiamethoxam, Triphenylphosphate and Vinclozoline. The limit of detection (LOD) was around 1 ng/g and the limit of quantification (LOQ) around 10 ng/g.

2.8. Statistical analysis

Statistical analyses were performed with R software on untransformed data. Firstly, in order to compare the mean levels of metals found at the RAV and CIL sites, Student's *t*-Test was used. Two-way ANOVA was then used to test the site and season effects and the interaction of both effects on the activity of each biomarker. The construction of a classification tree was performed using the "Tree" package, grown by binary recursive partitioning. At each stage, the split that produced the most homogeneous classes was chosen and the process was then repeated. Splitting continued until the terminal nodes were too small or too few to be split. The leave-one-out method was used to choose the optimal leaf number by minimising the misclassification rate, and to calculate the real error rates.

3. Results

3.1. Analysis of metals and pesticides in the honeybees

The levels of different metals were analysed in bees from each site (Table 2). A test of the equality of means between bees from the CIL and RAV sites was performed for each metal. The profiles of these

Table 2
Quantities of metals in honeybees from the two sites. Data are expressed as mg/g of honeybees for the first group of metals (Al to Zn) analysed and as μg/g of honey bees for the second group (As to V). * indicates values statistically different at $p < 0.1$.

	May			August			November		
	CIL	RAV	p	CIL	RAV	p	CIL	RAV	p
Al	114.69 ± 16.7	110.07 ± 31.33	0.757	79.61 ± 11.18	116.74 ± 56.44	0.17	128.83 ± 32.59	460.41 ± 435.99	0.091*
B	174.64 ± 35.21	210.76 ± 40.47	0.13	191.64 ± 24.91	182.72 ± 31.77	0.6	181.72 ± 17.98	127.64 ± 53.77	0.039*
Ca	9889.96 ± 976.43	10,269.35 ± 830.2	0.485	10,114.31 ± 910.15	10,544.76 ± 689.85	0.378	10,103.37 ± 959.13	11,622.29 ± 2886.6	0.229
Cu	226.59 ± 18.98	226.81 ± 5.1	0.979	239.96 ± 16.86	228.62 ± 8.89	0.176	225.56 ± 20.51	197.51 ± 18.96	0.026*
Fe	1201.67 ± 157.86	1180.78 ± 169.06	0.829	1170.39 ± 71.34	1209.14 ± 26	0.256	1151.76 ± 92.56	1949.46 ± 977.76	0.074*
K	17,779.13 ± 1134.41	17,587.04 ± 1376.62	0.797	17,192.42 ± 656.92	17,755.31 ± 1790.33	0.496	16,875.86 ± 1500.63	14,600.08 ± 1999.7	0.043*
Mg	9288.43 ± 641.23	8926.74 ± 368.65	0.259	9126.76 ± 239.97	9170.86 ± 223.93	0.749	9052.62 ± 705.56	8781.59 ± 1124.22	0.621
Mn	1239.51 ± 293.53	1398.43 ± 205.9	0.303	1501.84 ± 157.39	1452.23 ± 306.45	0.732	1285.41 ± 88.56	908.26 ± 594.29	0.146
Na	4833.71 ± 299.39	4470.09 ± 389.87	0.1*	4588.18 ± 101.27	4731.22 ± 232.86	0.211	4362.16 ± 528.49	4293.67 ± 217.34	0.776
Si	556.4 ± 74.22	555.58 ± 96.53	0.987	586.31 ± 102.62	632.12 ± 66.88	0.381	608.79 ± 53.63	1046.83 ± 499.28	0.059*
Zn	1302.34 ± 57.82	1333.33 ± 148.45	0.649	1333.13 ± 125.87	1262.74 ± 74.93	0.266	1195.84 ± 31.38	1278.31 ± 102.6	0.082*
As	357.02 ± 57.42	331.16 ± 19.2	0.335	312.44 ± 37.34	374.72 ± 60.21	0.057*	335.47 ± 39.31	497.77 ± 171.67	0.047*
Cd	3654.57 ± 441.55	4399.27 ± 1132.91	0.181	4336.48 ± 126.76	4244.9 ± 1042.84	0.839	4347.66 ± 291	3136.43 ± 1379.26	0.06*
Co	1117.42 ± 147.38	1259.14 ± 147.04	0.126	1273.06 ± 170.98	1247.04 ± 133.73	0.775	1112.75 ± 37.37	1463.05 ± 426.81	0.073*
Cr	6835.71 ± 1506.2	9731.8 ± 1079.24	0.003*	8369.91 ± 1761.01	9240.18 ± 425.15	0.287	7849.12 ± 1329.58	9572.88 ± 1876.23	0.087*
Ni	2484.15 ± 234.61	3131.57 ± 707.18	0.077*	2614.26 ± 274.44	2901.75 ± 152.11	0.049*	2993.54 ± 295.17	7287.26 ± 6069.58	0.111
Pb	816.06 ± 330.27	712.28 ± 254.13	0.555	534.39 ± 58.73	583.92 ± 65.23	0.197	585.41 ± 161.6	1343.13 ± 1008.31	0.095*
Sb	41.45 ± 19.21	270.81 ± 455.41	0.272	53.52 ± 59.27	29.06 ± 4.79	0.359	25.83 ± 2.67	159.95 ± 157.13	0.065*
Se	968.42 ± 187.78	1577.55 ± 1017.44	0.205	877.3 ± 229.69	850.41 ± 105.86	0.8	798.42 ± 177.76	1536.78 ± 898.85	0.074*
Ti	180,279.31 ± 10,342.08	205,040.55 ± 34,090.69	0.14	187,846.41 ± 19,091.61	183,253.95 ± 6786.35	0.598	174,537.76 ± 7871.31	226,973.61 ± 77,000.29	0.122
V	187.21 ± 225.46	511.57 ± 212.54	0.028*	349.2 ± 297.56	616.95 ± 134.45	0.072*	479.38 ± 297.65	1737.51 ± 1483.6	0.067*

	May 2010		
	CIL	RAV	p
Al	180.69 ± 15.21	299.04 ± 116.16	0.016*
B	66.3 ± 11.12	68.08 ± 25.28	0.85
Ca	8679.09 ± 723.85	8215.32 ± 1622.16	0.45
Cu	168.75 ± 23.41	144.4 ± 13.61	0.016*
Fe	837.15 ± 90.72	909.44 ± 85.4	0.101
K	12,731.71 ± 1512.59	8276.15 ± 623.66	0*
Mg	7861.1 ± 490.95	7268.25 ± 187.45	0.007*
Mn	199.79 ± 34	227.16 ± 29.7	0.088*
Na	4021.52 ± 503.75	3541.63 ± 406.47	0.041*
Si	513.25 ± 72.29	573.38 ± 132.85	0.25
Zn	428.79 ± 65.55	488.96 ± 50.74	0.045*
As	296.36 ± 67.3	480.17 ± 84.56	0*
Cd	286.36 ± 48.73	520.97 ± 58.17	0*
Co	637.15 ± 59.04	837.95 ± 137.53	0.002*
Cr	7254.86 ± 989.02	7952.76 ± 1218.8	0.201
Ni	4842.88 ± 354.61	5216.77 ± 1414.54	0.461
Pb	2172.98 ± 465.77	516.76 ± 227.66	0*
Sb	96.4 ± 13.44	78.57 ± 26.1	0.093*
Se	233.45 ± 125.07	303.57 ± 78.42	0.173
Ti	247,363.94 ± 19,466.26	259,453.22 ± 20,523.3	0.218
V	811.5 ± 85.17	1258.24 ± 393.68	0.009*

metals were then subjected to an evolution during the observation year. In May 2009, significant differences between the two sites were found for four metals ($p < 0.1$), Na, Cr, Ni and V, higher levels being found at the RAV site except for Na. In August 2009, As, Ni and V exhibited significant differences with higher levels at the RAV site ($p < 0.1$). In November 2009, the number of metals whose levels differed significantly between the two sites increased markedly. Significant differences were found for Al, B, Cu, Fe, K, Si, Zn, As, Cd, Co, Cr, Pb, Sb, Se and V ($p < 0.1$), with higher levels at the RAV site for Al, Fe, Si, Zn, As, Co, Cr, Pb, Sb, Se and V. In May 2010, large number of metals differed significantly between the two sites. It was possible to observe a difference for Al, Cu, K, Mg, Mn, Na, Zn, As, Cd, Co, Pb, Sb and V ($p < 0.1$), with higher levels at the RAV site for Al, Mn, Zn, As, Cd, Co and V. None of the pesticides screened were detected in the bees at any of the sampling time points (data not shown).

3.2. Biological variability

Biological variability is known to affect biomarker responses and could be characterized with satisfactory precision. Although seasonal variability is a type of biological variability, we chose to analyse it separately (see below). Numerous precautions were taken when choosing the honeybees in order to limit biological variability: all the hives possessed a queen of one year, were carefully controlled regarding their sanitary state, and were equivalent in terms of population and development, and the honeybees collected were only foragers sampled at the hive entrance after their return flight. Moreover, all the honeybees possessed the same maternal origin, as only one mitochondrial profile was found in the six hives. This profile was the same as the A1 profile found by Franck and colleagues in that region, and referred to as belonging to a race of the African lineage *A. mellifera unicolor* (Franck et al., 2001). We were able to estimate two types of biological variability, related to: (i) the activities of honeybee biomarkers within each hive (intra-hive) and (ii) the mean of honeybee activity from one hive to another (inter-hive). In order to compare these sources of variability, coefficients of variation were calculated (Table 3). Intra-hive variability was described by the dispersion of the honeybee activity values for each hive at each sampling period. Inter-hive variability corresponded to a comparison of average honeybee activity in each hive at a given sampling period. The results revealed specific intra-hive and inter-hive variabilities for each biomarker, independently of the month, in the following order: AChE < GST < ALP. Unlike other biomarkers, MT displayed more heterogeneous intra-hive variability between months. Moreover, inter-hive variability was generally slightly higher than intra-hive variability, probably because of the internal characteristics of the hive and the slightly different foraging areas covered. The main outstanding question was whether biological variability introduced a confounding factor in the discrimination of sites.

Table 3
Intra and inter-hive biological variability regarding biomarker activities at each month. Coefficients of variation (SD/mean) were calculated for each hive and the means for hives were calculated to assess intra-hive variability, n = 360. Coefficients of variation were calculated on the mean values of hives in order to assess inter-hive variability (n = 30).

Sampling period	Type of biological variability	AChE (%)	ALP (%)	GST (%)	Head MT (%)	Midgut MT (%)
May 2009	Intra-hive	8.62	24.99	14.44	19.48	21.69
	Inter-hive	25.19	45.45	29.78	15.13	15.13
August 2009	Intra-hive	8.75	22.98	18.85	15.01	12.24
	Inter-hive	24.64	33.11	58.07	12.55	12.55
November 2009	Intra-hive	8.70	19.20	14.98	8.65	8.65
	Inter-hive	8.21	17.90	41.32	14.24	10.94
February 2010	Intra-hive	8.80	17.13	14.82	_	_
	Inter-hive	11.48	10.76	26.57	_	_
May 2010	Intra-hive	5.85	10.35	15.37	19.12	8.05
	Inter-hive	11.07	14.55	5.64	8.57	8.57

3.3. Profiles of biomarker response

The evolution of biomarkers in bees from the CIL and RAV sites was followed for one year by sampling the honeybees every three months (Fig. 2). The temporal profiles of ALP, GST and AChE differed markedly at the two sites. For AChE and ALP, activity was lower in the urban RAV site throughout the observation period (Fig. 2A, B). Compared to May 2009, considered here as the baseline reference, ALP displayed a gradual decline at both sites, reaching 49.7% and 87.6% of activity in May 2010, respectively. Whereas ALP at the CIL site tended to return to baseline activity, RAV ALP appeared to reach stability at a lower value. AChE activity decreased at the RAV site from 33.6% in November 2009 to 22.9% in May 2010, whereas CIL AChE activity appeared to be steadier during the observation period. For GST, an enzyme induced rapidly by pollutants, activity was similar at the two sites during the first three months and then rose at the RAV site (Fig. 2C). In May 2010, GST showed an increase of 43.0% compared to May 2009 at the CIL site, whilst a marked increase of 139.6% was observed at the RAV site. After a slight fall in August 2009, MT levels in the honeybee head appeared to remain steady at the reference level in the bees from the CIL site (Fig. 2D). At the RAV site, MT levels remained steady between May and November 2009 and then displayed an increase of 53.6% over baseline in May 2010. In the midgut, MT levels increased between May and August 2009 to reach 140.5% and 143.4% of baseline at the CIL and RAV sites, respectively (Fig. 2E). MT levels then remained stable until May 2010 at both sites. Despite the fact that MT levels tended to follow the same trend at both the CIL and RAV locations, a significant difference was noted in May 2010 between the two sites, with contrasting profiles in the midgut and head.

The activities and concentrations of the biomarkers are presented in Tables 4 and 5. The tissue and specific activity responses of ALP, AChE and GST appeared to be statistically different at the CIL and RAV sites at each sampling period and for all biomarkers ($p < 0.01$), except for ALP and AChE in February 2010 (Table 4). Midgut MT tissue levels were also statistically different between the CIL and RAV sites at each sampling period ($p < 0.05$), whereas head MT levels appeared to be statistically different only in May and November 2009 (Table 5). Compared to the CIL site, RAV displayed lower AChE activities (except in May 2009), higher ALP activities and midgut MT tissue levels (except in May 2010) and higher GST activities in November 2009 and February 2010. For ALP, AChE and GST, the specific and tissue response patterns were similar. For MT, the profiles of specific concentrations did not always follow the profiles of tissue concentrations. And for specific concentrations, head MT levels only appeared to be statistically different between the CIL and RAV sites in August and November 2009 ($p < 0.05$).

3.4. Effect of site and season on biomarker activity

In order to clarify whether variations in biomarkers could be ascribed to the anthropogenic environment despite seasonal variations, r^2 contributions were calculated to quantify that part of biomarker variability due to the site and/or season. The results showed that the season was the main effect observed for all biomarkers: the r^2 contribution of season was higher than the r^2 contribution of site (Table 6). This effect could be graded as follows: ALP (0.462) > MT (0.438 – 0.392) >AChE (0.232) > GST (0.115). However, the results also confirmed the overall influence of site for AChE, GST, ALP and midgut MT ($p < 0.05$; Table 6). The analysis also indicated a significant interaction between the site and season effects on each biomarker ($p < 0.05$; Table 6). The contribution of the site effect was greater in August for AChE (0.450), ALP (0.644), GST (0.507) and head MT (Table 7).

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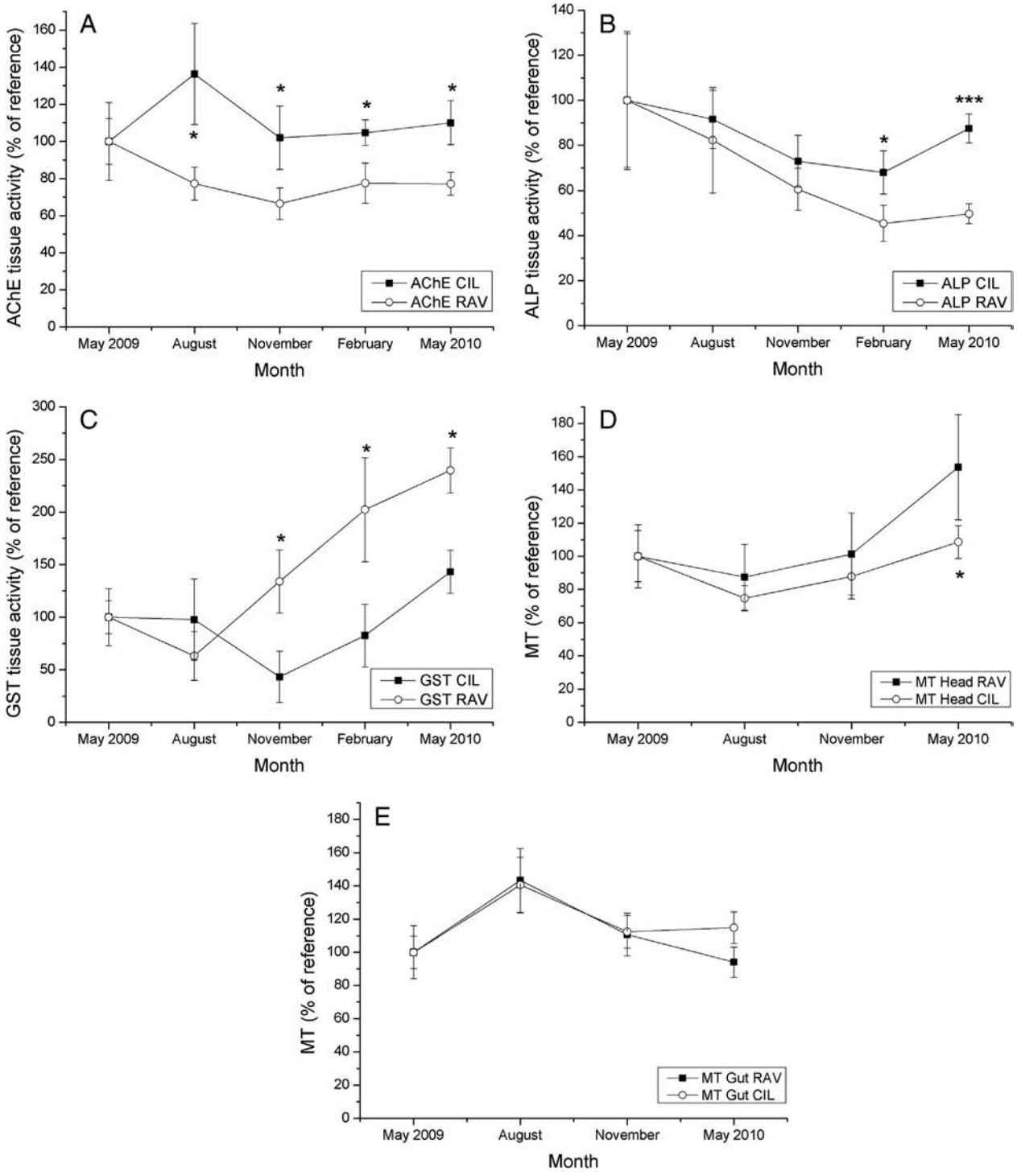

Fig. 2. Profiles of biomarker responses in honeybees sampled every three months at the CIL and RAV sites over 1 year. Biomarker levels were expressed as a percentages of initial activities in May 2009 (reference). (A) ALP, (B) GST, (C) AChE, (D) midgut MT, (E) head MT. The data correspond to means ± SD of 12 repetitions performed in triplicate. Asterisks indicate a significant difference versus the reference at $p \leq 0.05$.

3.5. Decision procedure to distinguish sampling sites

A classification tree was implemented to generate a set of decision rules to determine the site belonging to each honeybee sample. Only three of the biomarkers, ALP, AChE and GST, were present at the nodes as partitioning criteria (Fig. 3). The classification tree has nine pure leaves containing only honeybees from a single site: six for CIL and three for RAV. The CIL pure leaves were characterized by a low to moderate level of ALP and a high level of AChE; a low to moderate level of ALP and a low to moderate level of AChE; a low level of ALP, a moderate to high level of AChE and a high level of GST; a moderate to high level of ALP, a high level of AChE and a low level of GST; a moderate to high level of ALP, a low to moderate level of AChE and a high level of GST; a moderate level of ALP and a moderate to high level of GST. The RAV pure leaves were only characterized by three profiles: a high level of ALP; a moderate to high level of ALP; a low level of ALP, a moderate to high level of AChE and a moderate to high level of GST. The other terminal nodes contained honeybees from more than one site.

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Table 4
Honeybee biomarker responses at the RAV and CIL sites. Honeybees were collected every three months at the CIL and RAV sites over 1 year and then the biomarkers were analysed. Biomarker levels were expressed as tissue (nmol/min/g of tissue) and specific (nmol/min/mg of proteins) activities. Data corresponded to the mean ± SD of 12 repetitions performed in triplicate (180 honeybee samples per site).

Sampling period	Activities	AChE			ALP			GST		
		CIL	RAV	p	CIL	RAV	p	CIL	RAV	p
May 2009	Tissue	3016.8 ± 371.2	3913.8 ± 824.3	1.10^{-6}	237.1 ± 70.2	362.7 ± 111.2	1.10^{-6}	4925.0 ± 1333.7	2651.5 ± 415.0	1.10^{-8}
	Specific	22.9 ± 0.7	27.7 ± 8.9	0.003	4.5 ± 0.6	9.0 ± 3.1	1.10^{-11}	96.8 ± 20.3	68.6 ± 11.4	1.10^{-5}
August 2009	Tissue	4110.2 ± 821.9	3024.1 ± 349.8	1.10^{-7}	217.1 ± 30.7	298.5 ± 84.9	1.10^{-5}	4816.3 ± 1896.7	1673.6 ± 614.9	1.10^{-13}
	Specific	29.5 ± 6.1	21.1 ± 2.6	1.10^{-9}	5.1 ± 0.6	9.1 ± 1.1	1.10^{-16}	114.9 ± 44.4	48.8 ± 18.0	1.10^{-11}
November 2009	Tissue	3074.9 ± 513.9	2600.5 ± 332.3	1.10^{-4}	173.0 ± 27.4	219.6 ± 33.9	1.10^{-5}	2138.1 ± 1202.1	3551.8 ± 797.3	1.10^{-6}
	Specific	30.5 ± 2.1	27.8 ± 1.2	1.10^{-3}	5.4 ± 1.2	6.9 ± 0.4	1.10^{-6}	60.9 ± 13.9	108.3 ± 34.3	1.10^{-9}
February 2010	Tissue	3157.6 ± 205.6	3031.9 ± 221.5	0.20	161.3 ± 23.5	164.7 ± 34.5	0.366	4071.5 ± 1465.6	5361.4 ± 2353.3	0.001
	Specific	23.2 ± 1.1	20.1 ± 2.2	1.10^{-6}	2.8 ± 0.3	2.9 ± 0.4	0.550	67.9 ± 21.5	90.8 ± 16.0	1.10^{-6}
May 2010	Tissue	3320.1 ± 359.2	3019.1 ± 241.6	0.002	207.6 ± 15.1	180.1 ± 16.1	1.10^{-4}	7047.2 ± 1007.6	6352.1 ± 567.6	0.026
	Specific	28.3 ± 1.6	24.1 ± 2.3	1.10^{-11}	3.5 ± 0.6	3.0 ± 0.2	0.004	114.4 ± 6.9	105.5 ± 0.1	0.003

The confusion matrix resulting from the "leave-one-out" test showed an overall classification accuracy of 89%. The analysis of MT and other biomarkers (AChE, ALP and GST) was performed on the same honeybees from a same hive. At the time of sampling, the honeybees were divided into two groups (one for AChE, ALP and GST and a second for MT). Consequently, decision rules were applied to mean biomarker activities per hive and per month, in order to integrate MT in the classification tree (Fig. 4). The results produced a simpler classification tree. Two biomarkers appeared to be highly discriminating: ALP and MT. They were the two complementary biomarkers that determined, with a low error rate (17%), the site to which the hives belonged. Hives displaying ALP >6.82 nmol/min/mg were correctly classified at the RAV site and hives displaying MT <21.78 μg/mg were correctly identified as belonging to the CIL site. The final terminal node corresponding to hives displaying ALP <6.82 nmol/min/mg and MT >21.78 Ug/mg did not clearly identify the site of origin but tended to correspond to hives located at the more contaminated site, RAV. In summary, these decision rules satisfactorily classified the RAV site with 100% accuracy. Hives from the CIL site were well classified as belonging to this site, with 67% of accuracy.

4. Discussion

The aim of this study was to determine the potential for the use of honeybee biomarkers to assess environmental quality. This is the first study to have explored the responses of a battery of biomarkers in the honeybee collected in different anthropogenic contexts. The results revealed significant differences in the evolution of neural and metabolic biomarkers (AChE, GST, ALP and MT) in bees sampled at different locations. These biomarkers displayed specific profiles that could enable the discrimination of weakly and markedly anthropised sites.

To assess environmental health, any modulation of biomarkers must be attributable to the effect of pollutants and not to natural variations linked to developmental, physiological or genetic parameters. It is widely accepted that the effects of pollutants can differ as a function of the metabolic status and physiology of individuals. For example, a clear gender effect was observed regarding metabolic biomarker responses with high values in a study on *Gasterosteus aculeatus* L. males (Sanchez et al., 2007). Moreover, physiological effects are the most pronounced during the breeding period, as observed with GST activity levels in *Anguilla anguilla* and *Mugul cephalus* (Gorbi et al., 2005). In the honeybee, it has been shown that significant variability may be due to the developmental stage of both workers and the queen (Polyzou et al., 1997). However, compared to other species, biological variability can easily be reduced in the honeybee by sampling foragers only. This enables a reduction in variations linked to: (i) gender, (ii) spawning period and (iii) age, which markedly modulates polyethism in the colony. Moreover, foragers represent primarily exposed individuals in a colony and their use can increase the pertinence of potential biomarker responses. According to Deviller et al. (2005), GST exhibited greater biological variability than ALP and AChE. This result could be explained in part by the role of biomarkers. Metabolic biomarkers are directly involved in the detoxification process and display greater variability than neural biomarkers because organisms are often in contact with numerous pollutants. For all biomarkers, biological variability can be attributed in part to the type of biomarker, the existence of half sibs in a colony due to the fecundation of the queen by different males, the age of foragers (which can range from 18 to 24 days in the summer) (Abou-Donia et al., 2004; Dukas, 2008) and the foraging area of honeybees, which can vary between individuals and between colonies. In the present study, intra-hive and inter-hive variabilities appeared to be equivalent, except for AChE which displayed lower intra-hive variability. This result enabled a conclusion as to the correct homogeneity of the colonies studied. It therefore appeared more pertinent to consider the responses of several colonies, which offered better coverage of the areas under study. Moreover, honeybees are subjected to seasonal variations which cause changes to various physiological

Table 5
MT levels in honeybees sampled at the RAV and CIL sites. Honeybees were collected every three months at the CIL and RAV sites over 1 year and the biomarkers were analysed. MT levels are expressed in μg/g of tissue and mg/g of proteins. Data corresponded to the mean ± SD of 12 repetitions performed in triplicate (180 honeybee samples per site).

Sampling period	MT levels	Head MT			Midgut MT		
		CIL	RAV	p	CIL	RAV	p
May 2009	Tissue	378.7 ± 71.8	295.7 ± 45.5	0.01	776.7 ± 123.8	868.3 ± 84.4	0.031
	Specific	10.8 ± 1.3	15.5 ± 7.1	0.114	96.7 ± 13.6	112.7 ± 15.7	0.002
August 2009	Tissue	283.0 ± 28.6	258.6 ± 58.3	0.114	1091.2 ± 129.3	1244.8 ± 166.4	0.008
	Specific	15.4 ± 2.5	22.4 ± 8.4	0.043	149.2 ± 25.9	166.8 ± 29.3	0.1
November 2009	Tissue	332.7 ± 50.9	299.5 ± 73.0	0.018	873.2 ± 76.0	961.6 ± 112.0	0.019
	Specific	29.1 ± 7.2	24.4 ± 4.6	0.031	152.1 ± 15.9	141.6 ± 12.0	0.019
February 2010	Tissue	–	–		–	–	
	Specific	–	–		–	–	
May 2010	Tissue	411.4 ± 37.5	454.3 ± 93.9	0.448	892.3 ± 74.1	816.6 ± 79.4	0.01
	Specific	21.7 ± 4.9	19.1 ± 5.3	0.146	152.3 ± 36.2	167.0 ± 35.2	0.184

Table 6
Effects of site and season on biomarkers in *Apis mellifera* honeybees from the CIL and RAV sites. Analysis of variance was used to assess interactions between the activity of biomarkers and the site and season. Analyses were performed on 360 honeybee samples for each biomarker. SS, sum of square; df, degree of freedom; F, Fisher value; p, p value; r^2 ctb, r^2 contribution.

Biomarkers	Site effect					Season effect					Site × season				
	SS	df	F	p	r^2 ctb	SS	df	F	p	r^2 ctb	SS	df	F	p	r^2 ctb
AChE	678.5	1	47.2	0.000	0.077	2042.5	4	35.5	0,000	0.232	1353.5	4	23.5	0.000	0.154
ALP	344.0	1	199.4	0.000	0.143	1113.8	4	161.4	0.000	0.462	357.8	4	51.9	0.000	0.148
GST	4206.0	1	6.9	0.009	0.011	45,240.0	4	18.5	0.000	0.115	138,007.0	4	56.4	0.000	0.351
MT in head	44.0	1	1.4	0.241	0.005	3365.5	3	35.4	0.000	0.392	857.8	3	9.0	0.000	0.100
MT in intestine	3228.0	1	5.3	0.023	0.020	71,374.0	3	38.7	0.000	0.438	4811.0	3	2.6	0.054	0.030

parameters such as neurotransmitter levels, protein metabolism or juvenile hormone titres which might modify the effects of xenobiotics (Crailsheim, 1986). In this study, all metabolic and neural biomarkers were subject to seasonal variations. Moreover, the site effect could differ depending on the season for all biomarkers. GST activity displayed a stronger interaction between site and season ($r^2 = 0.351$, Table 6). Despite biological variations due to the seasonal and internal characteristic of colonies, a site effect was clearly identified during this study, and was confirmed by two-way factorial analysis. The similarity of the seasonal patterns at the RAV and CIL sites, observed with respect to GST, ALP and AChE during a year, may have contributed to the distinctiveness of the sites. Interestingly, all biomarkers displayed a stronger site effect in August, probably due to the greater abundance of pollutants at that period (Table 7). According to Jolly et al. (2012), the response profiles of the biomarkers could be explained by the seasonality of pollution due to local practices in the areas under investigation.

Several authors have demonstrated a high degree of GST activity induction following exposure to various contaminants such as metals, pesticides, PAH and PCB (Garner and Di Giulio, 2012; Papadopoulos et al., 2004; Yu et al., 2012). Sanchez et al. (2006) also showed a 75% induction of the control in the stickleback after exposure to Agral 90® (polyethoxylated nonylphenol) for 21 days at 1000 μg/L. However, after combined exposure to Agral 90® and the herbicide Diquat (1000:444 μg/L), induction was more marked and represented 300% of the control activity. Amongst the numerous insecticides that we tested under laboratory conditions, maximum GST induction in the honeybee was moderated. For example, after exposure to thiamethoxam at a dose of 2.6 ng.bee^{-1}, the induction of specific activity represented 20% of the control activity (Badiou-Bénéteau et al., 2012). However, the evolution profile of GST under environmental conditions showed a maximum of induction of approximately 140% of initial activity at the RAV site in May 2010. This revealed that the induction potential of GST under environmental conditions was much greater than that expected in the laboratory. Similarly, under laboratory conditions, no effect was observed on ALP after exposure to thiamethoxam at 2.6, 5.1 and 51.2 ng.bee^{-1} (Badiou-Bénéteau et al., 2012), and only limited effects were observed after exposure to fipronil (unpublished data) at 0.58 and 0.27 ng.bee^{-1} whereas considerable induction was observed at the RAV site. Maximum induction of around 200% was observed between May 2009 and May 2010. By contrast, the maximum response of AChE, observed at the RAV site, appeared to be moderate (a reduction of 20% in May 2010 compared to May 2009, $p > 0.01$) when compared to GST and ALP, but its activity was more stable and its natural variability lower. Consequently, although its modulation was moderate, AChE remained the better biomarker in the honeybee because of its stable activity and low variability (see also Badiou et al., 2008; Badiou-Bénéteau et al., 2012). The results showed that the induction potential of honeybee biomarkers could be revealed partially under laboratory conditions. However, differences in biomarker responses could be seen between controlled and natural conditions. At similar levels of exposure, these differences could in part be explained by the presence of other contaminants and interactions with these latter.

The evolution profiles of MT displayed marked differences between the head and midgut. In the midgut, the initial MT concentrations are lower at the CIL site than at the RAV site. MT concentrations remained lower between May 2009 and February 2009 but then rose in May 2010. However, there was no great difference between the relative evolution profiles in bees from the CIL and RAV sites. Conversely, in the head, MT concentrations were higher at the CIL site, except in May 2010. Relative MT values were higher at the RAV site, and the relative increase in MT concentrations observed from August 2009 was more pronounced at the RAV site, leading to a higher absolute level at the RAV site after one year. The very similar relative evolution profiles of midgut MT levels observed in bees from the RAV and CIL sites were somewhat surprising because the digestive tract is the first site to be exposed to pollutants following the ingestion of contaminated food. This contrasts with the ability of insect gut MT to bind non-essential trace elements such as cadmium (Hensbergen et al., 2000). Conversely, the higher relative head MT values observed at the RAV site may have reflected a greater ability of the brain to respond to exposure to non-essential trace elements. This could be explained by the fact that the brain is a critical organ whose defences against stressors are particularly efficient. MT could therefore be involved in the detoxification of non-essential trace elements, and especially of heavy metals that induce neurotoxicity (Dallinger, 1996). This is consistent with the involvement of MT in preventing or repairing injuries in a brain subjected to different types of impairments (Arellano-Ruiz et al., 2012; Kim et al., 2012; Leung et al., 2012; Sohn et al., 2012.

The volcanic activity of La Reunion island gives rise to high metal concentrations in the environment that increase the probability of metals being recovered from bioindicator species, as observed during this study. The difference of metal profiles between the CIL and RAV sites evolved during the year of observation. As a function of time, the number of metals displaying significant differences between the two sites increased gradually to reach a maximum in May 2010, a month during which the greatest differences between the two sites were observed for almost all biomarkers. It may therefore be legitimate to correlate the evolution of metal profiles to those of biomarkers, especially MT. This is particularly true if we consider that most of the metals for which significant differences were seen between the CIL and RAV sites are linked to human and/or volcanic activities. However, although the impacts of metals on different physiological systems are relatively well documented in the literature (e.g. Hensbergen et al., 2000; Yu

Table 7
Effect of the site on biomarkers *Apis mellifera* honeybees from *the* CIL and RAV sites during the observation period. Analysis of variance was used to assess interactions between the activity of biomarkers and the site as a function of the month and the r^2 contribution of the site. Analyses were performed on 72 honeybee samples for each biomarker, each month.

Month	AChE	ALP	GST	MT	
				Head	Midgut
May 2009	0.150	0.545	0.247	0.180	0.239
August	0.450	0.644	0.507	0.255	0.097
November	0.168	0.272	0.431	0.140	0.127
February	0.276	0.006	0.286	–	–
May 2010	0.477	0.114	0.122	0.061	0.043

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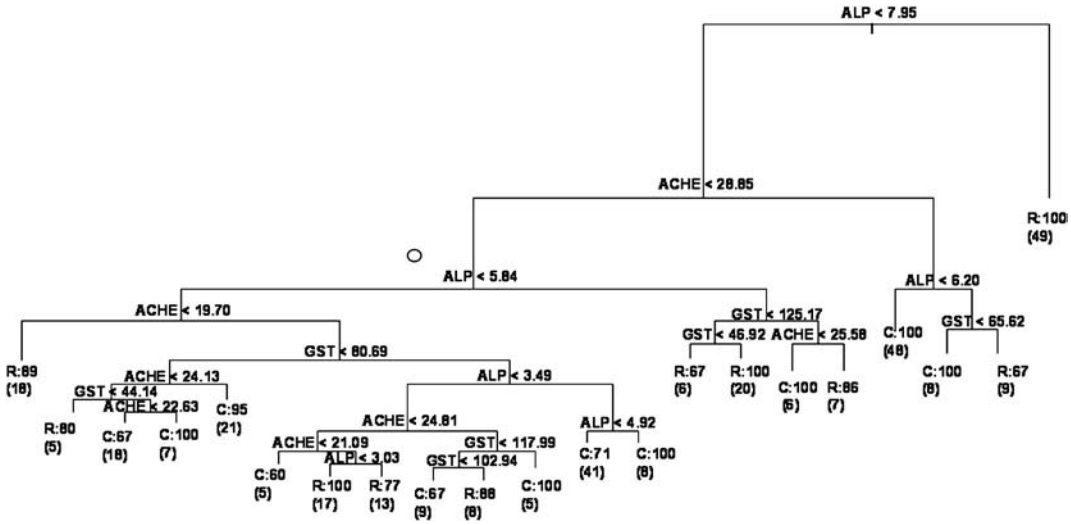

Fig. 3. Classification tree of biomarker responses obtained from 328 honeybee samples collected every three months at the CIL and RAV sites over 1 year (all hives and months taken together). Terminal nodes identify the site or dominant site with its corresponding associated percentage and number of honeybees.

et al., 2012), changes to the biomarker profiles may also have been due to other pollutants such as xenobiotic organic substances, which were not investigated during this study. In fact, the most important piece of information is that honeybee colonies placed in different anthropogenic contexts may display significant differences in terms of their biomarker profiles that could be accentuated by the time spent in these different environments.

The environmental significance of biomarker responses seems easy to assess when biomarker activity is modulated as was generally observed in the contaminated area, such as such as an increase for GST and a decrease for AChE, for example. The relationship between the presence of contaminants and the effects observed is more difficult to establish when deviations from the usual modulation patterns are observed for biomarkers. Variations in activity may depend on the characteristics of each study, and inhibition or activation (or even both) have been reported in tissues from different organisms (Costa et al., 2008). In our work, it was noteworthy that the selected biomarkers were modulated according to the usual responses. Stronger effects on ALP, AChE and midgut MT tissue levels were observed at the RAV site, with a decrease of AChE and an increase in ALP and MT. A reduction of AChE activity is known to be an indicator of direct neurotoxic effects, particularly following exposure to organophosphate and carbamate pesticides (Galgani and Bocquene, 1990; Payne et al., 1996). In addition, metals, detergents and complex mixtures of pollutants can also decrease AChE activity, so that AChE constitutes a general biomarker of neurotoxic effects (Bandyopadhyay, 1982; Frasco et al., 2005). The relationship between exposure to neurotoxic compounds and a reduction in AChE activity has been demonstrated in numerous studies (Fulton and Key, 2001). The low AChE activity seen in honeybees collected at the RAV site thus suggested the presence of more neurotoxic compounds at RAV site than at CIL. However, the decrease in AChE activity was not a systematic response to pollutants and, depending on the pollutant, a marked increase of AChE might also occur, as has been observed with pyrethroids (Badiou et al., 2008). Moreover, ALP, GST and MTs are involved in cellular defence processes that are typically reflected by an increase of their activities after exposure to xenobiotics (Bounias et al., 1996; Durou et al., 2007). Their respective functions imply that responses occur with many different contaminants, such as metals (Bounias et al., 1996; Martín-Díaz et al., 2008; Stone et al., 2002). For example, MT is considered to be a potential biomarker of exposure to heavy metals in the terrestrial environment (Hensbergen et al., 2000). Similarly to AChE, ALP activity between May 2009 and February 2010 and midgut MT between May 2009 and November 2009 exhibited typical profiles with higher levels of activity at RAV than at the CIL site. The results appeared to be more contrasted for GST, where lower levels of activity were observed in May and August 2009 and in May 2010 at the RAV site. However, the results obtained with biomarkers strongly suggested that honeybees based in an anthropized area were subjected to sublethal stresses involving both oxidative stress and detoxification processes, with the occurrence of neurotoxic compounds. Moreover, the results of metals analyses revealed a prevalence of metals at the RAV site, suggesting that metals represent good candidates to modulate biomarkers.

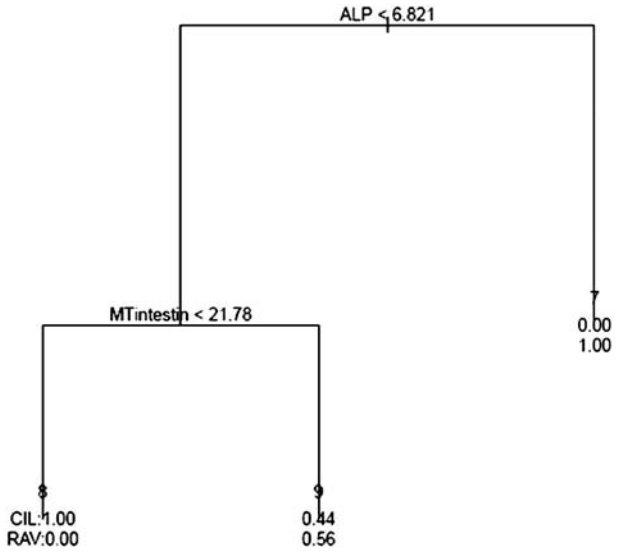

Fig. 4. Classification tree of biomarker responses obtained on samples from each hive every three months at the CIL and RAV sites over 1 year. The mean biomarker responses for each hive at each month were used to build the classification tree (n = 24). Terminal nodes identify the site or dominant site with its corresponding associated percentage and number of hives.

The translation of biological data into environmental information is sometimes problematic because it is difficult to distinguish between the effects of temporal and spatial variability on variations in biomarker levels. A pertinent approach can consist in integrating these parameters into a site discrimination model to reveal site-specific contamination patterns that are reliably discriminated by biomarker responses (Narbonne et al., 2005). Discriminant analysis (DA) and principal component analysis are widely recognised as multivariate statistical tools to investigate differences amongst sites and summarize responses

(Falfushynska and Stolyar, 2009). However, these statistical analyses require that an unexplained part of data variation should be normally distributed, a condition rarely respected during field studies. Rule-based classification models appear to be more appropriate for a broad range of data distributions. These models were extensively described by Chèvre et al. (2003a). They are simple to express, invariant under monotonic parameter transformations, they account for qualitative factors and provide efficient classifiers. Instead of forming a minimal set of rules based on linear combinations of parameters (as is the case with DA), rule-based methods build logical combinations of single parameter conditions. During the present study, different discrimination methods were tested and especially two rule-based methods: a rough set (data not shown) and a classification tree (CT). Although these two methods produced a satisfactory classification, a better classification of honeybee biomarkers from the two sites was provided by the classification tree with accuracy reaching 89%, which was very similar to that observed by Chèvre et al. (2003b). CT analysis showed that the set of biomarkers with marked significance included ALP and MT. The greater weight of ALP was underlined by the CT, insofar as honeybees exhibiting high ALP levels were rapidly identified as belonging to the more anthropogenic site, RAV: pure leaves were characterised by activities higher than 7.97 nmol/min/mg of protein (higher than 6.82 nmol/min/mg if an analysis of mean biomarker activities per hive was performed).

The data collected during this study enabled a discrimination between the two sites. Such discrimination constitutes the first step in the development of a honeybee index that could integrate data derived from numerous sites and applicable to both spatial and temporal studies. The design of honeybee risk assessment studies needs to be based on a model which integrates the variability of environmental pressures and honeybee physiology. Development of this index will facilitate the comparison of different sites and the identification of contaminated terrestrial sites so that environmental health can be assessed using the honeybee. The addition of other study sites and biomarkers should improve the discriminating power of the biomarkers and the robustness of the index (Sanchez et al., 2008; Schiedek et al., 2006). In this way, the development of specific biomarkers from biological systems or tissues specifically targeted by pollutants needs to be pursued in the honeybee and validated in different environmental contexts. As a result, it will be possible to propose a battery of biomarkers as a new tool for use under a multi-parametric approach in routine terrestrial biomonitoring programmes.

5. Conclusion

The purpose of this study was to evaluate two types of anthropogenic contexts using the discriminating potential of honeybee biomarkers previously validated under laboratory conditions. Urban and semi-natural sites in La Reunion Island were studied for one year and the results clearly indicated local and seasonal differences in pollutant levels. Despite biological variations due to the seasonal and intrinsic characteristics of the honeybee colonies studied, the neural and metabolic response profiles of the biomarkers enabled good discrimination between the sites. These findings therefore validate the use of such biomarkers under field conditions. When compared to the CIL site only slightly affected by human activities, the urban RAV site displayed profiles of biomarker response that enable a conclusion regarding the environmental pressure to which the honeybees were subjected and the occurrence of neurotoxic compounds, amongst which metals were good candidates. A decision procedure was developed in order to distinguish the sampling sites and enabled excellent classification accuracy (89%) for the data set. This field study constitutes the first report on use of the honeybee for the in situ assessment of environmental health using a multi-biomarker approach.

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