Effects of pesticides and electromagnetic fields on honeybees: a field study using biomarkers

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Abstract:	The effect of pesticide mixtures and electromagnetic fields were evaluated on honeybees in three experimental sites located in northern Italy: a control site far from anthropogenic stress sources, a semi-natural site close to a high voltage electric line and an agricultural site with intensive pesticide treatments. From each experimental site, young workers and foraging bees were taken monthly from May to October and analysed for four enzymatic biomarkers: acetylcholinesterase (AChE), catalase (CAT), glutathione S-transferase (GST) and alkaline phosphatase (AP). Results revealed time- and site-specific effects in respect to control site, confirming the role of biomarkers as diagnostic and early-warning tools for multi-stress sources on honeybees. In the electromagnetic-stress site an effect of an over-activation of all analyzed biomarkers was observed at the end of the season. According to other literature findings, this event was related to a behavioral over-activation in a period in which bees should prepare themselves to overwintering. This finding poses potential problems to winter survival. In the pesticide-stress site, different pesticide-induced responses were identified. We demonstrated in the field that pesticide mixtures, currently used in agriculture, were able to greatly affect biochemical parameters of bees (with both enzymatic under- and over-activations).
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ABSTRACT

The effect of pesticide mixtures and electromagnetic fields were evaluated on honeybees in three experimental sites located in northern Italy: a control site far from anthropogenic stress sources, a semi-natural site close to a high voltage electric line and an agricultural site with intensive pesticide treatments. From each experimental site, young workers and foraging bees were taken monthly from May to October and analysed for four enzymatic biomarkers: acetylcholinesterase (AChE),

catalase (CAT), glutathione S-transferase (GST) and alkaline phosphatase (AP). Results revealed time- and site-specific effects in respect to control site, confirming the role of biomarkers as diagnostic and early-warning tools for multi-stress sources on honeybees. In the electromagnetic-stress site an effect of an over-activation of all analyzed biomarkers was observed at the end of the season. According to other literature findings, this event was related to a behavioral over-activation in a period in which bees should prepare themselves to overwintering. This finding poses potential problems to winter survival. In the pesticide-stress site, different pesticide-induced responses were identified. We demonstrated in the field that pesticide mixtures, currently used in agriculture, were able to greatly affect biochemical parameters of bees (with both enzymatic under- and over-activations).

Keywords: bees; biomarkers; pesticides; electromagnetic fields; stress effects.

1. INTRODUCTION

The general bee decline registered in many countries all over the world is a problem of great concern. Since the late ’90s, a complex pathology (Colony Collapse Disorder – CCD), described by Underwood and vanEngelsdorp (2007) and vanEngelsdorp et al. (2009), was linked to to widespread events of honeybee disappearance especially in the U.S. (vanEngelsdorp et al. 2007; Lee et al. 2015) and in Europe (Potts et al. 2010). Often, it was not possible to relate them to a specific cause and several authors proposed that they should be attributed to different stresses or to a combination of them (Maini et al. 2010; Nazzi et al. 2012; Goulson et al. 2015; Porrini et al. 2016). Poor nutrition, depending to vegetation health status, can affect bee resistance and exacerbate the effect of other stresses (Naugh 2009; Huang 2012). Recurrence of old and new

pathologies (Berthoud et al. 2010) may be responsible of CCD events and of the general honeybee decline (Simon-Delso et al. 2014). Varroosis recrudescence (Le Conte et al.2010) and the emergence of new pathologies such as *Nosema ceranae* (Higes et al. 2009) and Israeli acute paralysis virus (IAPV) (Ribiere et al. 2008) are among the most studied biotic adversities. Urbanization, agricultural intensification and habitat fragmentation have strongly reduced natural areas for food foraging throughout the year. Pollinated crops are also subject to pesticide treatments and consequently bees are exposed to many pesticides during their development and their adult life (Halm et al. 2006; Johnson et al. 2009). Contamination from pesticides, such as neonicotinoid insecticides (Goulson 2013), are among the most cited causes but also the combined effects of more contaminants has to be considered (Gill et al. 2012). Emerging contaminants such as pharmaceuticals or nanoparticles should not be excluded even if not yet sufficiently inquired. General environmental stresses, including climate change, can also have important effect on honey-bee colonies at different levels as it can directly influence bee behaviour and physiology or it can alter the quality and quantity of plants in the foraging area (Le Conte and Navajas 2008). Man induced electromagnetic fields are among the potential causes of stress to honeybees (Favre 2011). It is known that honeybees possess magnetite crystals in their fat body cells able to respond to very small changes in the constant local geomagnetic field intensity. Korall (1987) observed a change in honeybees behaviour induced by electromagnetic fields.

In order to better understand bee decline phenomena, different networks across European and Northern America countries were set up (e.g. Genersch et al. 2010). In Italy, since 2011 the network BeeNet recorded data from approximately 3,000 colonies from 303 apiaries on a) pathogen status of the colonies; b) pollen sources and its nutritional content; c) pesticide contamination; d) colony mortality (Porrini et al. 2016). In this context, this research proposes a field approach for analysing the effects of electromagnetic fields and pesticides on honeybees using a biomarker approach.

Biomarkers are considered as promising prognostic and diagnostic tools in many species (Galloway et al. 2004) but studies in honeybees are still limited. Most of them have been performed in

laboratory (Badiou et al. 2008; Badiou- Bénéteau et al. 2012; Carvalho et al. 2013; Boily et al. 2013; Badawy et al. 2015) less in the field (Badiou-Bénéteau et al. 2013; Boily et al. 2013; Wegener et al. 2016), looking mainly at the effects of pesticides (Stefanidou et al. 1996; Boily et al. 2013; Carvalho et al. 2013; Badawy et al. 2015). Boily et al. (2013) reported that AChE activity increased in response to neonicotinoids. They supposed that, as neonicotinoids occupy the binding-site of acetycholine, these compound tend to accumulate in the synapses, stimulating the action of AChE by a typical substrate-induced response. Other classes of pesticides are able to alter the AChE activity. Badiou et al. (2008) in laboratory experiments reported an important increase of AChE activity in surviving bees following deltamethrin exposure. In addition, this increase was not abolished by pirimicarb treatment, which is a typical AChE inhibitor. Claudianos et al. (2006) reported that honeybees have low levels of xenobiotic detoxifying enzymes (GST, P450 and carboxyl/cholinesterases) in relation to others insects, being so more sensitive to pesticides. Wegener et al. (2016) used a large battery of biomarker and behavioural indicators for studying in the field the chronic effects of the carbamate fenoxycarb and the neonicotinoid imidacloprid.

Effects of electromagnetic radiations emitted by antennas, mobile phones, high-voltage transport lines have been studied in humans (e.g. Leszezynski et al. 2002; Gandhi and Singh 2005; Hardell and Sage 2008), rats (e.g. Lai and Singh 1996), bats (e.g. Nicholls and Racey 2007) birds (e.g. Everaert and Bauwens 2007), frogs (e.g. Balmori 2016) and insects (e.g. Weisbrot et al. 2003). In bees, several authors studied the effect of high frequency radiations (0.8-3 GHz) typical of mobile phones and mobile-phone transmission antennas (Sharma nd Kumar 2010; Favre 2011;Vilić et al. 2017), as well as those of Extremely Low Frequency (ELF) typical of high voltage electric transmission lines (Greenberg et al. 1981; Martin et al. 1988; Kirschvink et al. 1997; Bindokas et al. 1988).

In the present work, the effects of electromagnetic fields and pesticides were studied on honeybees by means of three experimental sites: a ‘control’ site without stress sources, an exposure site with a direct source of electromagnetic fields (electromagnetic stress site) and a third site characterized by

a specific pesticide exposure (pesticide-stress site). In each site, foraging and in-hive working bees were analysed by a battery of biomarkers: acetylcholinesterase (AChE), catalase (CAT), glutathione-S-transferase (GST), and alkaline phosphatase (AP). This research aims to characterize the physiological variation of the analysed biomarker in foraging and in-hive working bees in 'natural' (control site) and 'stress' conditions (exposure sites), in order to test their sensitivity toward these stress sources.

2. MATERIALS AND METHODS

2.1. Experimental sites

The three experimental sites and the land use around them are shown in Fig1. Control-site was located at Ramponio Verna (45° 59' 22.64" N of latitude and 9° 4' 2.64" E of longitude) in a forested mountain area near Lake of Como far from direct stress sources (urban centers or intensive agriculture areas).

Electromagnetic-exposure site was located at Cantello (45° 48' 51.63'' N of latitude and 8° 53' 21.62'' E of longitude) 20 km far the city of Varese in a hilly area below the Alps. In this site, the experimental hives were positioned just below a high-voltage transmission line (identified by the electric authority as 'n° 525 Cagno-Induno Olona' transmission line). The power line was always active with the following characteristics: 132 kV and frequency of 50 Hz. All around mixed forest and permanent lawns surround small villages. In the area there is also a small extension of high income horticultural crop pollinated by bees, such as an asparagus cultivar well known in the area.

Pesticide-exposure site was located at Arcagna (45° 20' 18.20" N of latitude and 9° 27' 5.07" E of longitude), 20 km south of Milan, inside a farm of the Agricultural Faculty of the University of

Milan, where an orchard with a cultivar collection of different fruit species is maintained for experimental, teaching and productive purposes. Different cultivars with a different period of production are present in every fruiting crop: apple (1.32 ha), pear (1.3 ha), peach (2.5 ha), apricot (1 ha), plum (0.8 ha) and cherry (0.15 ha). During the year, 121 treatments were performed (77 with fungicides and 44 with insecticides) using 32 commercial products containing 34 active ingredients. In total 249 kg of a.i. were used (94 kg of fungicides and 155 kg of insecticides). Most of this huge amount is composed by few products such as white mineral oil (148 kg of a.i.) used as insecticide (95% of the total insecticide amount) or sulfur and copper oxychloride (22 and 20 kg of a.i., respectively) used as fungicides (45% of the total fungicide amount). 27 active ingredients were used at the level of 1 kg or less. Table 1 reports the pesticide list and the amount used and Figure S-1 shows their time schedule during 2015. The applied pesticides were indexed, in terms of their potential impact towards bees, by the so called 'toxicity ratio' (the ratio between the used amount divided by the LD_{50} on bees). LD_{50} literature values and the resulting toxicity ratios are reported in Table S-1, as described in supplementary information. Used pesticides showed toxicity ratios ranging over more than 5 order of magnitude from 0.0008 for the plant regulator NAA (1-naphthylacetic acid) to 351 for the insecticide imidacloprid. Figure S-2 shows the time sequence of the pesticide treatments expressed as toxicity ratios.

Daily mean temperatures and cumulated daily precipitations for the three experimental sites are shown in Figures S-3, S-4 and S-5. Calculated mean monthly temperatures and cumulated monthly precipitations were reported in Table S-2. Data were obtained by the meteorological network of the Regional Environmental Protection Agency, as described in Supplementary Information. The comparison among the meteorological data in the three experimental sites is reported in Supplementary Information too.

2.2. Experimental design and sampling modalities

In each experimental site, three hives were positioned during spring 2015; all hives were healthy and with a young and productive queen of the same age and they were regularly checked for general health conditions during the trial.

From June to October 2015, 5 sampling campaigns were performed in each site on a monthly basis. For logistic reasons, samplings in Cantello and Ramponio-Verna were performed the same day (18th of June, 16th of July, 26th of August, the 22 of September, 19th of October), while those in Arcagna the day after (19th of June, 17th of July, 27th of August, the 23 of September, 20th of October). At every sampling date and in every experimental site, 20 forage bees and 20 young workers were sampled taking them equally from the different hives present in each site. Forage bees were manually collected among those at the entrance of the hive (excluding guard bees), and young workers from those on the comb near the brood (excluding foraging bees recognisable for their dancing behaviour). Bees were put in single vials, immediately frozen in liquid nitrogen, and carried to the laboratory for biomarker analyses.

2.3. Biomarker analyses

Four enzymatic biomarkers were measured on each single bee: acetylcholinesterase (AChE), catalase (CAT), glutathione S-transferase (GST) and alkaline phosphatase (AP). Sampled animals were suspended and homogenized with nine volumes of ice cold Hepes–Tris 10 mM, pH 7.5, containing 50 mM mannitol and 1 mM dithiothreitol. The homogenate was filtered at 4 °C through surgical gauze to remove tissue debris. The crude extract was then centrifuged at 15,000 × *g* (4 °C) for 30 min to eliminate mitochondria. The supernatant was used to measure cytosolic enzyme activities: alkaline phosphatase, glutathione-*S*-transferase and esterases other than acetylcholinesterase (AChE). For the AChE assay, bees were homogenized in a sodium phosphate buffer (20 mM, pH 7.4), containing 250 mM sucrose and 1% Triton X-100, and processed as above. Enzymes were assayed spectrophotometrically. Glutathione-*S*-transferase (GST) was assayed according to Habig et al. (1974) through the measurement of glutathione-1-chloro-2, 4-

dinitrobenzene conjugate production. AChE was assayed at 412 nm in the presence of 0.5 mM acetylthiocholine iodide as substrate, as reported by Berra et al. (2004). Alkaline phosphatase (AP) was assayed at 405 nm using *p*-nitrophenylphosphate as substrate. All enzyme assays were performed in triplicate at 30 °C using sample volumes varying from 5 to 40 µl in 1 ml test cuvettes and a Cary3 UV–vis spectrophotometer. Enzyme activities were analyzed by Cary Win UV application software for Windows 2000, expressed as international units (U) in µmol min^{-1} mL^{-1} and referred to protein concentration (mg ml^{-1}) as determined according to Bradford (1976) using bovine serum albumin as standard. Catalase activity was determined according to Bergmeyer and Grassl (1983) using H_2O_2 12 mM as substrate.

2.4. Electric and Magnetic field measurements

Generated field intensities were measured with a tri-axial field meter PMM 8053. During bee sampling (May-October 2015), every sampling date, a 24-hours measurements of the electric and magnetic fields were performed, at the hive level, with a time-resolution of 5 min. In addition, in order to characterize the decreasing gradient as a function of the distance, in a free-field area (not shielded by vegetation), measurements of the magnetic and electric fields were performed at 1.5 m height at various distance from the transmission line. In all sites, we also monitored for the presence of high frequency electromagnetic field (HF-EMF) sources in the frequency range of 100 kHz-2.5 GHz with a Chauvin Arnoux C.A. 43 field meter.

2.5. Statistical analyses

Biomarker activities were analyzed after Log transformation, because of the significant shift from normal distribution, considering all data (n=600) and each experimental site separately (n=200) (Kolmogorov-Smirnov test, $P<0.002$). On the contrary, Log transformed data, especially within experimental site, approached normal distribution ($p>0,05$) and outliers, identified by box-plot

analysis, were reduced to few cases, not excluded by graphical and statistical analyses because they were near the edges of the distribution boxes.

Generalized Linear Models (GLM) of Log transformed data was performed, using enzymatic activities as dependent variables, and 'site', 'date' and 'bee-type' as factors. Bonferroni's post-hoc test was used in order to establish significant differences between groups. Correlation analyses were performed using Pearson's coefficient. Box plot and statistical analyses were performed using the program SPSS v. 15.0.

3. RESULTS

3.1. Electromagnetic field exposure

In control and pesticide-stress sites, all measurements of electric and magnetic fields revealed background levels. On the contrary, in the electromagnetic-stress site, hives were subject to a mean magnetic field of 0.42±0.06 μT with mean peak intensity of 0.65±0.07 μT. At the hive position, the electric field was almost completely shielded by the presence of a bush all around the hive, while in the surrounding free-field area both magnetic and electric fields were present. The magnetic field is subject to time variation according to the line load (depending on electricity necessity), whereas the electric field is mainly determined by the transmission line characteristics. Under the electric line and when vegetation shield was present, the electric field was 0.58 V/m, while without vegetation it was around 1500 V/m (Fig2). The daily variation of the magnetic field and the decreasing gradient of the magnetic and electric field as a function of the distance from the transmission line is shown in Fig2 (graph above and below, respectively). Magnetic field reached negligible values at distances of about 50 m, whereas the electric one presented a bit slower decay.

In the three sites, measures of high frequency electromagnetic field (HF-EMF) in the frequency range of 100 kHz-2.5 GHz showed background values.

3.3 Biomarker results in the control site

In the control site, using 'bee type' and 'date' as factors and biomarker values as dependent variable, GLM showed that 'date' and the interaction between 'date' and 'bee type' had a significant effect on each biomarker ($p<0.001$ and $p<0.002$, respectively), while 'bee type' had not ($p>0.087$). Generally, young workers and forager bees showed the same enzymatic activity (Figure 3), only in some dates differences between the two bee types were present: enzymatic activities in forager bees were generally lower than those in young workers, but without a significant difference. On the contrary, the seasonal trends of all analyzed biomarker were evident (Fig3). AChE, CAT and AP showed a clear decreasing trend from June to October, while GST presented an initial increase between June and July followed by a plateau and then by a final slight decrease. Temporal trends found in the control site were mainly interpreted as physiological variations related to the seasonal cycle of the bee activity, which is higher in summer, because of food sources and brood rearing, and lower in Autumn, because bees are preparing themselves to overwintering. Seasonal trends of the analysed biomarkes were evaluated in the original unit, retransforming the marginal means of the seasonal trends from the Log values to the original one. AChE activity was halved from June to October from 0.5 to 0.25 U/mg prot., in the same period CAT activity strongly decreased from 17.8 to 2.1 U/mg prot. and AP activity from 0.045 to 0.005 U/mg prot., while GST activity varied between 0.1 and 0.2 U/mg prot.

The decreasing trends of AChE, CAT and AP from June to October suggest a relationship between enzymatic activity and the temperature. In order to test this thesis, we set a temperature value representative of the sampling period, comparing the mean temperatures of each sampling date with those of 3 and 6 days before. Among these data, high correlation exists ($r>0.955$; $n=15$; $P<0.001$), so the intermediate period of 3 days before sampling was chosen as representative value of the temperature condition of each sampling date. AChE, CAT and AP showed a highly significant correlations between the enzymatic activities and the mean temperature of each sampling period ($n=200$; $P<0,001$) with positive correlation coefficients ($r = 0.60$, 0.73 and 0.53 for AChE, CAT and

AP, respectively). On the contrary, GST showed a negative correlation with temperature (r = -0.28; n=200; P<0.001). Plotting the enzymatic activities of AChE, CAT and AP as a function of the mean temperature of the sampling period, we did not obtain regular increasing trends, but mainly two levels of activity: a higher activity over a mean temperature of 15-17°C (such as in summer) and a lower activity below 15°C (such as in October). This finding is consistent with the social-physiology of bees. They are ectothermic organism, but they are able to finely regulate the inside temperature of the hive (they reduced temperature excesses in summer by wing ventilation, while they warm the hive microclimate in winter by muscular activity without wing movements). By this behavior they are not strictly dependent from temperature, anyway they present a typical cycle of activities linked to seasonal cycle. Biomarker activities are probably more related to the physiological cycle of the hive activities than directly to the specific temperature of the sampling period. In this way, 3-day-before-sampling mean temperatures do not predict directly the enzymatic activities, but it is the season and therefore the date of sampling a better predictor of such activities. The decrease of the biomarker activities at the end of the season may be related to the reduced hive activities at that time. In Autumn, bees are preparing themselves to overwintering, stopping the reproduction activities and the brood breeding until the next spring. If biomarker activities are more related to the seasonal cycle than to the specific temperature of the sampling period, the site comparability will be reinforced, despite temperature differences among them (up to 4°C between pesticide stress site and control one, Table S-1). At the sampling dates, the seasonal cycle of bee activity was highly comparable among the three sites.

3.4 Biomarker results in the stress sites

Acetylcholinesterase (AChE)

The seasonal comparison of AChE activity in the three sites is graphically shown in Fig4a. For clarity, young workers and forager bees are plotted separately, but in statistical analyses they were analyzed together (GLM with ‘site’, ‘date’ and ‘bee-type’ as factors) in order to highlight

differences between bee types too. AChE levels in the three sites was significantly different (GLM, $P<0.001$), in the pesticide-stress site, general mean inhibition of 22% was calculated in respect to control site (marginal mean difference). On the contrary, in the electromagnetic-stress site, a general over-activity of 14% in respect to control was found. The 'date' and 'bee' factors were highly significant ($P<0.001$) too, as well as their interactions. Fig4a visually shows these interactions: for example in the electromagnetic-stress site, AChE activity was lower than the control in the first two sampling, while later it was much higher (September and October). In addition only forager bees showed a high inhibition in June, while in July the inhibition was present in both bee types, on the contrary higher AChE activity in September and October was observed in both bee types. This evident interaction among all experimental factors, should be taken into account in the interpretation of the results. Electromagnetic-site is subject to a near constant stress (regardless intraday variations), especially to bees working inside the hive. In June only foraging bees presented a very large inhibition of AChE activity, which, a month later, was present on both types but with lower intensity. This effect can be better related to an external stress, taken up by forager bees via food collection and then transferred to the hive. A plausible hypothesis can be a contamination by AChE- inhibitors pesticides used nearby. The area is mainly characterized by wood and forage crops but, in the foraging area, there is also an asparagus crop which is pollinated by bees, and on which the use of insecticides (dimetoate, deltamethrin and spinosad) are indicated in the pest management guidelines. Then, bee exposure to AChE-inhibitor pesticides such as dimethoate has been possible. Electromagnetic field effects were not evident in the first period, while, at the end of the season, AChE levels, instead of decreasing, as in control and pesticide–stress sites, rose up.

In the pesticide-stress site, we observed an AChE inhibition in June only in forager bees and, a month later, in both bee types, like in electromagnetic-stress site. In pesticide-stress site, most of the insecticide treatments were performed before June, including the organophosphorus chlorpyrifos.

Later, at the end of the season, AChE levels in pesticide-stress site were exactly those observed in control site.

Catalase (CAT)

The seasonal comparison of CAT activity (in logarithm) in the three sites for young workers and forager bees is shown in Fig4b. CAT activity in the three sites was significantly different (GLM with 'site', 'date' and 'bee-type' as factors, $P<0.001$), with the lowest activity in the pesticide-stress site (5.62 with 95% confidence interval of 5.27-5.98) and similar mean levels in the other two (marginal means of 7.50 and 7.33 with 95% confidence interval of 7.03-7.98 and 6.87-7.80, for control and electromagnetic sites, respectively). The 'date' and 'bee type' factors were highly significant ($P\leq0.001$) too, as well as their interactions. As shown in Fig4b, in the electromagnetic-stress site, CAT activity was inhibited in June and July in both bee-types, while it was over-activated in October. October levels in the electromagnetic-stress site were similar to maximum levels in control site (summer levels). In pesticide-stress sites, CAT activity was largely inhibited in June and July, and over activated in August and in October in both bee-types.

Glutathione-S-transferase (GST)

The seasonal comparison of GST activity (in logarithm) in the three sites for young workers and forager bees is shown in Fig4c. The three sites showed significantly different levels of GST activity (GLM, $P<0.001$): pesticide-stress site presented the lowest activity (marginal mean of 0.137 with 95% confidence interval of 0.130-0.144), electromagnetic-stress site the highest one (0.213 with 95% confidence interval of 0.206-0.220), and the control site was in between (0.163 with 95% confidence interval of 0.156-0.170). 'Bee type' didn't affect GST activity ($P=0.42$). On the contrary, the interactions between 'date' and 'site', between 'date' and 'bees' and among 'date', 'site' and 'bees' were highly significant ($P<0.001$), meaning that seasonal trends were different among sites in general and among sites depending on bee type (young workers or forager bees).

In electromagnetic-stress site, GST activity was over-activated in young workers in June, inhibited in both bee-type in July and, at the end of the season, a large over-activation was evident in both bee-types. In pesticide stress site, GST activity was inhibited in July in both bee types.

Alkaline phosphatase (AP)

The seasonal comparison of AP activity (in logarithm) in the three sites for young workers and forager bees is shown in Fig4d. AP activity in the three sites was significantly different (GLM, $P<0.001$), with the lowest activity in the control site (0.015 with 95% confidence interval of 0.014-0.016) and higher mean levels in the two stress sites (marginal means of 0.022 and 0.021 with 95% confidence interval of 0.021-0.023 and 0.020-0.022, for electromagnetic- and pesticide-stress sites, respectively). The interactions between 'date' and 'site', between 'site' and 'bees' and among 'date', 'site' and 'bees' were highly significant ($P<0.001$), meaning that seasonal trends were different among sites and between bee type depending on sites (Fig4d).

In electromagnetic-stress site, AP activity was inhibited in June in young workers and over-activated, at the end of the season, in both bee-types. In pesticide stress site, AP activity was inhibited in June in foraging bees and over-activated in August and October in both bee-types.

4 DISCUSSION

4.1 Biomarkers in bees: the control site

A crucial point in the use of biomarker is their physiological variability, which typically depends on the environmental conditions (seasonal variability) (Ippolito et al. 2016) and on the individual characteristics (stage, age and sex) (Scarduelli et al. 2017). Belzunces et al. (1992) studied changes in AChE activity during post embryonic development, and Polyzou et al. (2017) during pupal development. In the control site of the present study, the four enzymatic activities did not significantly differ between the two different stages of adult bees (young workers and foraging

bees), while they greatly change according to the season. Badiou-Bénéteau et al. (2013) and Pfeifer et al. (2005) reported that biomarkers present a typical seasonal variability, due to a combination of climatic features and physiological characteristics of the life cycle. In the present work, enzymatic activities seemed to be more related to the season than to temperature. The lowest enzymatic activities were reached in October the higher in June (at least for AChE, CAT and AP). In October, bees interrupt their reproduction and prepare to overwintering, reducing their metabolism in order to safe food stocks (Goodman and Fisher 1991). AP is a metabolic biomarker involved in absorption processes (Vlahović et al. 2009), so its decrease can be related to reduced nutrition needs during low activity periods. CAT is an antioxidant enzyme which can be activated by stress-induced Reactive Oxygen Species (ROS) (Carvalho et al. 2013) as well as by metabolism-induced ROS production (ROS are increased by the intensity of the metabolism, Jimenes and Gilliam 1996). Therefore, the decrease of the CAT activity in October can be explained to the metabolic slowdown in this period. AChE activity followed the same seasonal trend as CAT and AP but within a lower gradient. This neurotransmitter enzyme is more linked to basal metabolism than CAT and AP, which appear to be modulated more efficiently. Differently from the other enzyme, GST presented a peculiar seasonal trend with a negative correlation with the temperature. This enzyme is a phase-II detoxifying enzyme, localized in bees mainly in the midgut region (Badiou- Bénéteau et al. 2012). Its activity can be induced by several classes of contaminants, including metals, poliaromatic hydrocarbons (PAHs) and polichlorinated byphenils (PCBs) (Badiou-Bénéteau et al. 2013). Among them, PAHs are linked to combustion processes, including vehicular traffic. The control site is localized in a mountain area where in summer anthropogenic activities grow up mainly for tourism (especially in the Como Lake which is 10 km far). High touristic flux increases traffic emissions (e.g. PAHs), and emitted contaminants could be transferred to bees trough their foraging activity, inducing GST for metabolic decontamination processes. According to this interpretation, in June, GST activities in foraging bees were higher to those in young workers, suggesting a transfer of contaminants from outside to inside the hive, when anthropogenic activities began to increase. The

opposite can be observed in September when anthropogenic contaminant sources are reduced and GST levels in foraging bees reduced as well. By this interpretation, the control site too appear to be, at least partially, involved by anthropogenic-stress sources. This fact is unavoidable because also mountain area, at least for atmospheric long-range transport, are subject to environmental contamination (Tremolada et al. 2015), and, in mountain areas, touristic activities caused a limited, but not negligible, summer impact (Tremolada et al. 2009). Nevertheless, mountain area must be taken as the most pristine areas in high industrialized countries, such as Italy. In this way, mountain environment can be considered the best control site for studying enzymatic activities under low human pressure.

4.2 Effects of electromagnetic fields

Electromagnetic fields of this study (transmission line of 50 Hz and 132 kV, producing a mean magnetic field of 0.45 μT and electric field of 1.7 kV/m under the line) was less severe than that of Greemberg et al. (1981), and produced no evident behavioral effects at the population level (monitored by periodic inspections). Experiments of Greemberg et al. (1981) revealed that exposure to ELF electromagnetic fields (transmission line of 60 Hz and 765 kV, producing an electric field of 7 kV/m) was associated to an increase of the bee activity, an increase of the inside temperature, a weight loss of the hive, an increased queen losses and abnormal real cell production, a reduced operculated brood and finally a reduced winter survival. The others studies experimented more intense electromagnetic stresses, not comparable with those of the present study. Despite we did not observed effects at the population level, biomarker activities appeared to be altered. Excluding the AChE inhibition in June, interpreted by a possible local contamination effect by pesticides, the most consistent effect was the over-activation of all the enzymes in both bee types at the end of the season, when normally bees prepare themselves to overwintering. In electromagnetic-stress site AChE, CAT, GST and AP activities in October were still hich as in June when bee activities were actually at maximum. On the contrary in control site and also in pesticide-stress one, enzymatic

activities in October were much lower, indicating that bees reduced their metabolism for overwintering. This enzymatic over-activation at the end of the season, accords to the behavioral over-activation (higher bee activity), observed by Greemberg et al. (1981). Others literature studies on different species accord to indicate that ELF electromagnetic fields induce a large-spectrum enzymatic over-activation due to an increase of oxidative stress. Todorović et al. (2012) measured an increase of CAT and SOD (superoxide dismutase) activities in the larvae of the insect *Baculum extradentatum* exposed to magnetic field of 6 mT, and Regoli et al. (2005) revealed the presence of oxidative stress in the snail (*Helix aspersa*) in the presence of ELF electromagnetic fields comparable to ours (0.75 μT).

4.3 Effects of pesticides

In pesticide-stress site, AChE activity was inhibited in June only in foraging bees and in July in both bee types. This effect suggests an indirect contamination via foraging bees, transferred later to the hive system. AChE inhibition is specifically caused by organophosphorous and carbamate pesticides (Stefanidou et al. 1996), and, indeed, a chorpyrifos treatment occurred the 7th of May. The pyrethroid deltamethrin and the neonicotinoid inidacloprid were used few days before sampling and one month before, respectively. Both pirethroid and neonicotinoid insecticides are known to induce AChE over-activation (Badieu et al. 2008; Boily et al. 2013), but we did not observe it. Glyphosate herbicide, which was used repeatedly for weed control within rows, is known to produce AChE inhibition (Boily et al. 2013) and it can have contributed to the observed inhibition. In parallel to the effects on AChE, we observed an evident inhibition of CAT activity in June only in foraging bees and in July in both bee types. It is known that ROS species act as activator of the anti-oxidant defenses until a threshold, but over it ROS are able to inhibit anti-oxidant enzymes and many pesticides are a well-known ROS activator. There are evidences that AChE activity can be generically inhibited by high ROS levels (preferentially H_2O_2), and therefore AChE and CAT can be inhibited by an increase of ROS induced by pesticide treatments. In August and October AP

activity was over-activated in both bee types, before these two months two treatment with the insecticide spinosad were performed (the 22nd of August and the 8th of October). In literature, an over-activation of AP is reported for this insecticide, together with an over-activation of CAT activity and an inhibition of AChE one (Carvalho et al. 2013). In parallel to the AP activation in both bee types, we observed an over-activation of CAT (in both bee types too), but not an inhibition of AChE. In August, there was treatments with deltamethrin, which may have masked the spinosad's effect on AChE activity.

5 CONCLUSION

Biomarker results in the control site revealed a high seasonal variability mainly related the physiological cycle of honeybee activities. From the methodological point of view, the results in the control site have several implications:

- bees might present very different enzymatic activities depending on the period and/or physiological status of the colony;
- mountain areas too, can be impacted by anthropogenic contamination sources; touristic activities in such areas, which typically peak in July and August, can be considered a potential source of contamination as pointed out by several monitoring studies;
- enzymatic activities can be measured successfully in both young workers and forager bees, but, between the two bee types, foraging bees appeared as more sensitive to stress factors, being more exposed to external stress sources.

In the electromagnetic site, the most relevant observed effect was the wide-spectrum over-activation of the four enzymatic activities at the end of the season. This enzymatic over-activation was interpreted as a symptom of a behavioral over-activation of bees, according to the literature findings in which bees exposed to electromagnetic fields revealed a behavioral over-activation. This phenomenon at the end of the season may pose survival problems of the colony during

overwintering, because an excess of activity will cause an excess of food consumption (early stock exhaustion). The biochemical signal of the enzymatic over-activation in October can be interpreted as an early warning signal of a more severe effect which can happen later at the population level. In the same site, the severe inhibition of AChE activity in foraging bees in June was interpreted as specific exposure to AChE-inhibitor compounds, such as phosphorganic and carbamate pesticides, not prevented in the experimental plan (diagnostic tool).

In the pesticide-stress site two main effects were observed: in June and July, a prevalent inhibition of AChE and CAT was present, interpreted as a specific pesticide-induced response or via ROS-excess induction. Secondly, in August and October, a specific over-activation of AP and CAT was observed and it was interpreted as a consequence to spinosad treatments occurred before the two sampling dates. The complexity of the pesticide exposure in our experimental site makes the interpretation of the results very difficult and the cause-effect relation only speculative. However, it demonstrate that field exposure to pesticide mixtures, used currently in agriculture, was able to greatly affect biochemical parameters of bees (with both enzymatic under- and over-activations).

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TABLE CAPTION

Table 1 - List of commercial products and their active ingredients used in the pesticide-stress site (Arcagna) together with the amount used for each orchard crops.

SI-TABLE CAPTION

Table S-1 - Toxicity data, amount used and toxicity ratio values of the different pesticides in the Arcagna's site.

Table S-2 - Monthly mean temperature and monthly cumulated precipitation in the three experimental sites.

FIGURE CAPTION

Figure 1 - Map of the Lombardy, Italy with the indication of the three experimental sites and the soil use around them (3 km radius): Ramponio Verna's site (control site) is characterized by forest (green), lake (blu), forage crops (orange) and few urbanised area (grey), Cantello's site (electromagnetic-stress site) by forest (green), large urbanised area (grey) and few forage crops (orange) and Arcagna's site (pesticide-stress site) by mais (yellow) forage crops (orange) and urbanised area (grey).

Figure 2 - Daily variation of the magnetic field in the proximity of the bee-hive in electromagnetic-stress site (graph above) and magnetic and electric field gradient as a function of the distance from the transmission line (placed at the origin of x-coordinates) in an area adjacent the bee-hive not shielded by vegetation (graph below).

Figure 3 - Box-plots of the analysed biomarkers, in logarithm, in control site (Ramponio-Verna) for young workers and foraging bees in function to the sampling date.

Figure 4 - Box-plots of the analysed biomarkers, in logarithm, in the three experimental sites (control, electromagnetic- and pesticide-stress sites) for young workers and foraging bees separately (left and right, respectively) in function to the sampling date. (a) refers to Log AChE results; (b) to log CAT results; (c) to Log GST results; (d) to Log AP results.

SI-FIGURE CAPTION

Figure S-1 – Time schedule of the pesticide amounts in the pesticide-stress site (Arcagna).

Figure S-2 - Time schedule of the toxicity ratio of the pesticide used in the pesticide-stress site (Arcagna).

Figure S-3 – Daily mean temperature with minimum and maximum hourly mean temperature and daily cumulated precipitations in the control site (Ramponio-Verna) during the experimental trial. Arrows indicate the sampling dates.

Figure S-4 - Daily mean temperature with minimum and maximum hourly mean temperature and daily cumulated precipitations in the electromagnetic-stress site (Cantello) during the experimental trial. Arrows indicate the sampling dates.

Figure S-5 - Daily mean temperature with minimum and maximum hourly mean temperature and daily cumulated precipitations in the pesticide-stress site (Arcagna) during the experimental trial. Arrows indicate the sampling dates.

Table

Product	Use	Active Ingredient	Culture						Tot
			Apple	Pear	Peach	Abricot	Plum	Cherry	
		Crop surface (ha)	1.32	1.3	2.5	1	0.8	0.15	
			kg a.i.	kg a.i.	kg a.i.	kg a.i.	kg a.i.	kg a.i.	**kg a.i.**
Actara® 240 SC	insecticide	thiamethoxam a.i. 216 g/Kg		0.22					**0.22**
Affirm®	insecticide	emamectina benzoate a.i. 9.5 g/kg	0.1						**0.1**
Aliette®	fungicide	fosetyl-aluminium a.i. 800 g/kg		14.4					**14.4**
Alsystin® SC	insecticide	triflumuron a.i. 480.7 g/L	0.24						**0.24**
Applaud® Plus	insecticide	buprofezin a.i. 250 g/kg	0.25	0.75					**1**
Caddy	fungicide	cyproconazole a.i. 10%			0.18				**0.18**
Calypso®	insecticide	thiacloprid a.i. 80 g/L			0.36				**0.36**
Confidor® 200 SL	insecticide	imidacloprid a.i. 200 g/L	0.2	0.15					**0.35**
Coragen®	insecticide	chlorantraniliprole a.i. 200 g/L	0.08	0.08					**0.16**
Crittam WG®	fungicide	ziram a.i. 760 g/kg			14.8	1.9	1.5	1.1	**19.3**
Decis® Jet	insecticide	deltamethrin a.i. 15 g/L			0.05		0.015	0.0075	**0.073**
Delan® 70 WG	fungicide	dithianon a.i. 700 g/kg	4.9	2.1					**7**
Dodina® 65 WG	fungicide	dodina a.i. 650 g/kg						0.33	**0.33**
DursbanTM 75 WG	insecticide	chlorpyrifos a.i. 750 g/kg		0.75					**0.75**
Enovit metile® FL	fungicide	thiophanate-methyl a.i. 417 g/kg	0.21	0.62					**0.83**
Epik SL	insecticide	acetamiprid a.i. 46.7 g/kg		0.19				0.11	**0.3**
Fixormon®	plant regulator	NAA (1-naphthylacetic acid) a.i. 85 g/L	0.013						**0.013**
Flint® Max	fungicide	trifloxystrobin a.i. 250 g/kg	0.125						**0.125**
		tebuconazole a.i. 500 g/kg	0.25						**0.25**
Intrepid®	insecticide	methoxyfenozide a.i. 240 g/L			0.91				**0.91**
Iperion®	fungicide	copper oxychloride a.i. 60-70%	1.1	2.6	9.4	1.9	3.8	1.1	**19.8**
Laser™	insecticide	spinosad a.i. 480 g/L	0.24	0.17				0.096	**0.5**
Mitran S	insecticide	amitraz a.i. 192 g/L		0.77					**0.77**
Movento 48 SC	insecticide	spirotetramat a.i. 48 g/L	0.24			0.12	0.12		**0.48**
Scala®	fungicide	pyrimethanil a.i. 400 g/L	0.6	1					**1.6**
Signum®	fungicide	boscalid a.i. 267 g/kg			0.53				**0.53**
		pyraclostrobin a.i. 67 g/kg			0.13				**0.13**
Sipcamol E	insecticide	whilte mineral oil a.i. 80%	12	28	68		20	20	**148**
Sweel WDG	fungicide	sulphur a.i. 800 g/kg	0.8	0.8	12.8	2.4	1.6	1.2	**22**
Tiovit JET®			1.2	1.2					
Switch®	fungicide	cyprodinil a.i. 375 g/kg				0.24			**0.24**
		fludioxonil a.i. 250 g/kg				0.16			**0.16**
Tebusip 46	fungicide	tebuconazole a.i. 46 g/L	0.18	0.18	0.64		0.37	0.37	**1.74**
Teldor® Plus	fungicide	fenhexamid a.i. 500 g/L			1.5	0.5	1.1	2.4	**5.5**
Trebon® UP	insecticide	etofenprox a.i. 280 g/L	0.6	0.18			0.056		**0.84**
		Fungicide treatment n°	14	16	19	6	9	13	**77**
		Insecticide treatment n°	12	10	11	1	5	5	**44**
		Total treatment n°	26	26	30	7	14	18	**121**
		Fungicide kg a.i.	9	23	40	7	8	7	94
		Insecticide kg a.i.	14	31	69	0.12	20	20	**155**
		Pesticide kg a.i.	23	54	109	7	29	27	**249**

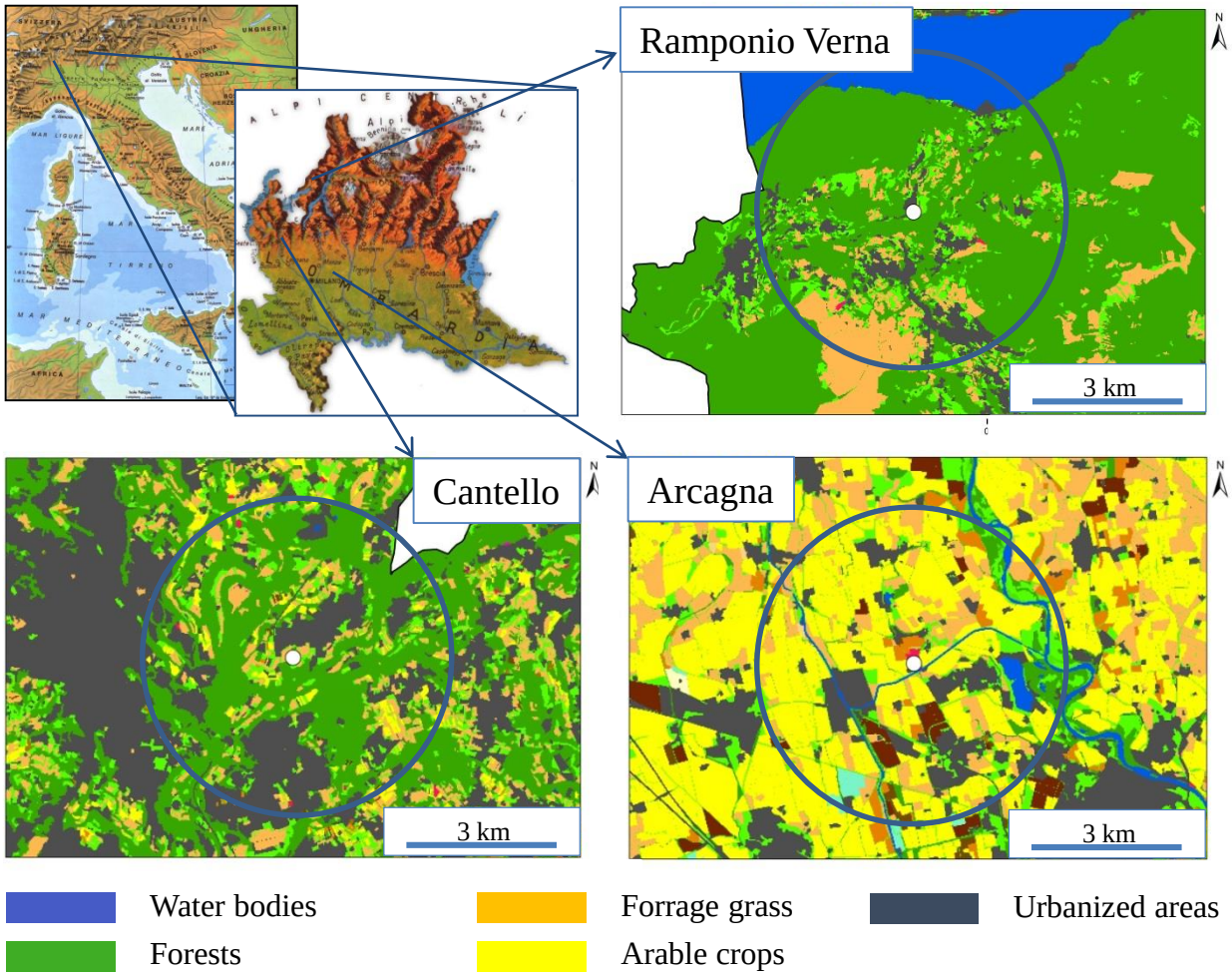
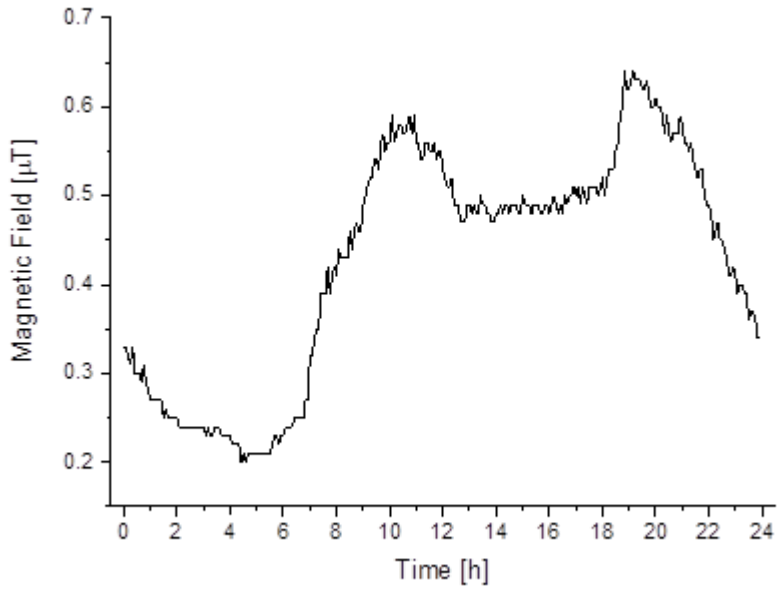


FIG. 1


Magnetic Field [μT]
0.7
0.6
0.5
0.4
0.3
0.2
0
2
4
6
8
10
12
14
16
18
20
22
24
Time [h]

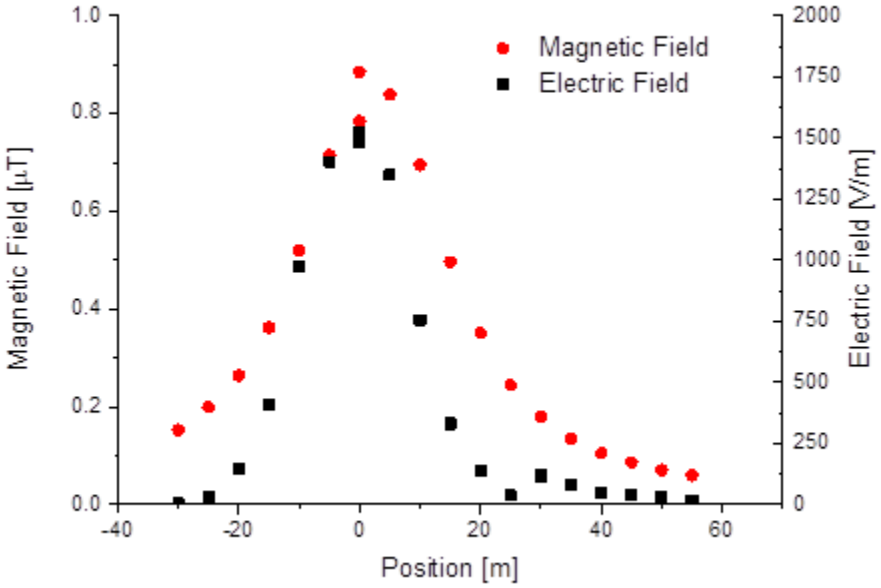

Magnetic Field
Electric Field
Magnetic Field [μT]
Electric Field [V/m]
1.0
0.8
0.6
0.4
0.2
0.0
2000
1750
1500
1250
1000
750
500
250
0
-40
-20
0
20
40
60
Position [m]

FIG. 2

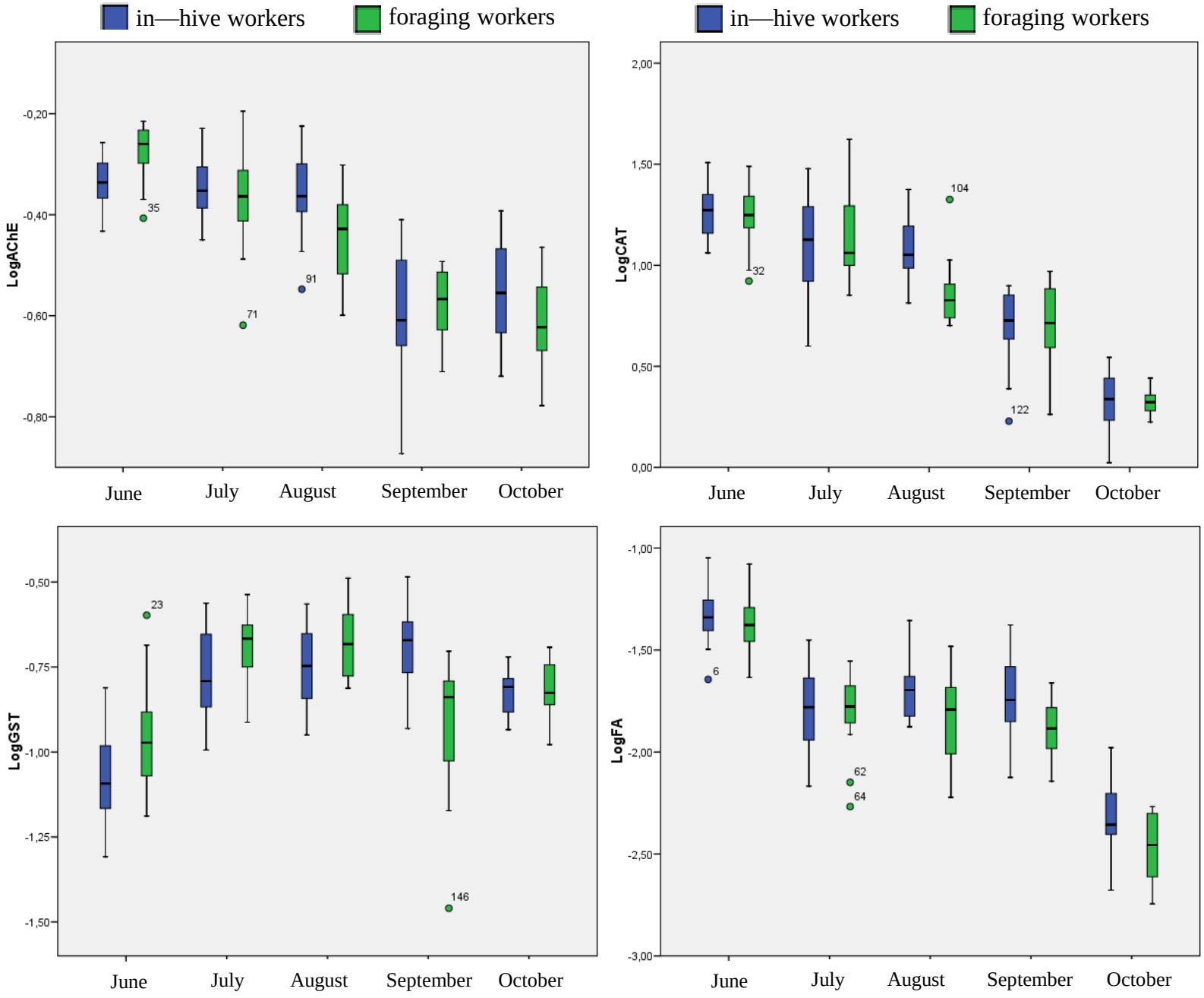

in—hive workers
foraging workers
LogAChE
-0,20
-0,40
-0,60
-0,80
35
71
91
June
July
August
September
October
in—hive workers
foraging workers
LogCAT
2,00
1,50
1,00
0,50
0,00
104
32
122
June
July
August
September
October
LogGST
-0,50
-0,75
-1,00
-1,25
-1,50
23
146
June
July
August
September
October
LogFA
-1,00
-1,50
-2,00
-2,50
-3,00
6
62
64
June
July
August
September
October

FIG. 3

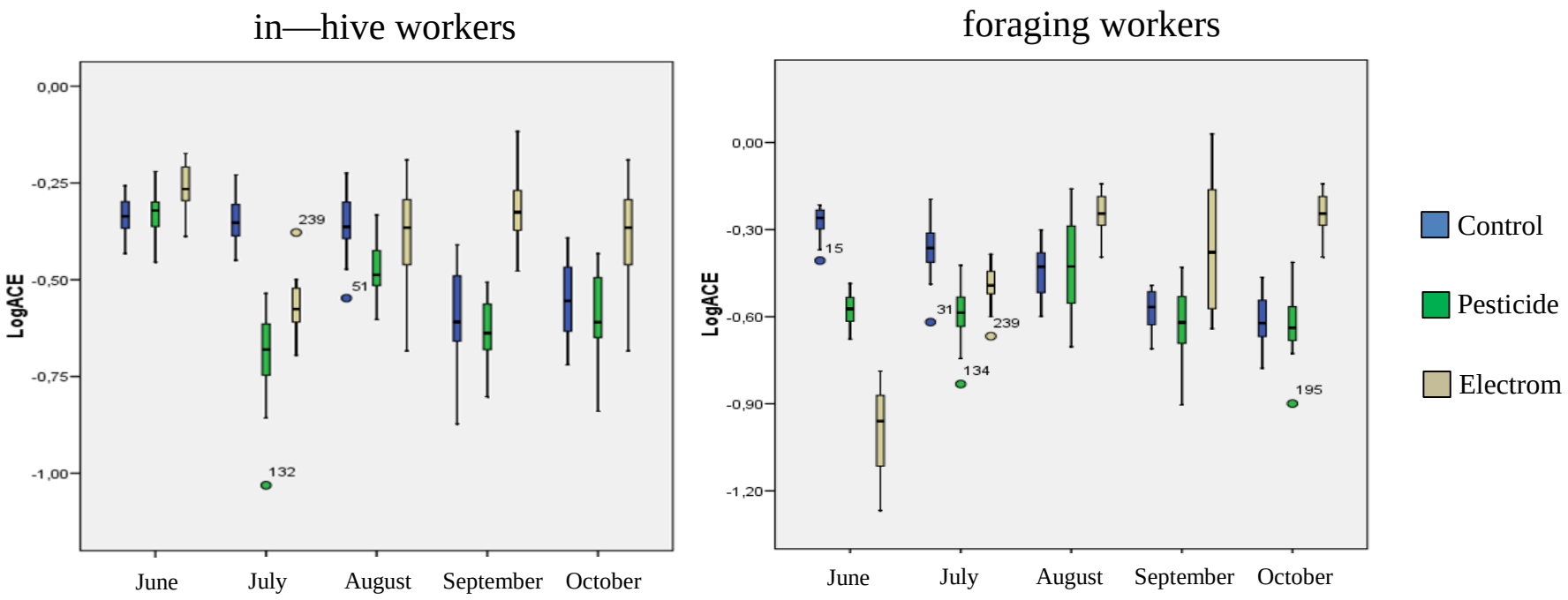

in—hive workers
foraging workers
LogACE
0,00
-0,25
-0,50
-0,75
-1,00
-0,30
-0,60
-0,90
-1,20
239
51
132
15
31
134
195
June
July
August
September
October
Control
Pesticide
Electrom

FIG. 4a

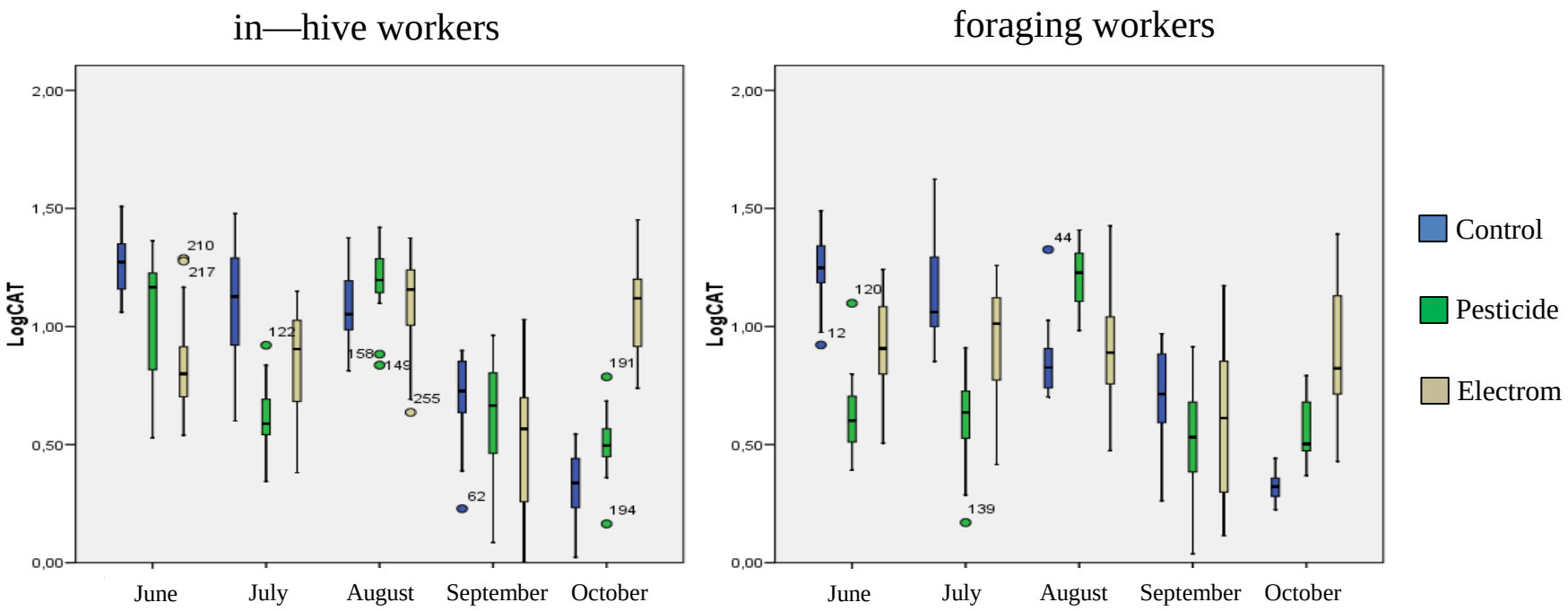

in—hive workers
foraging workers
LogCAT
2,00
1,50
1,00
0,50
0,00
June
July
August
September
October
Control
Pesticide
Electrom

FIG. 4b

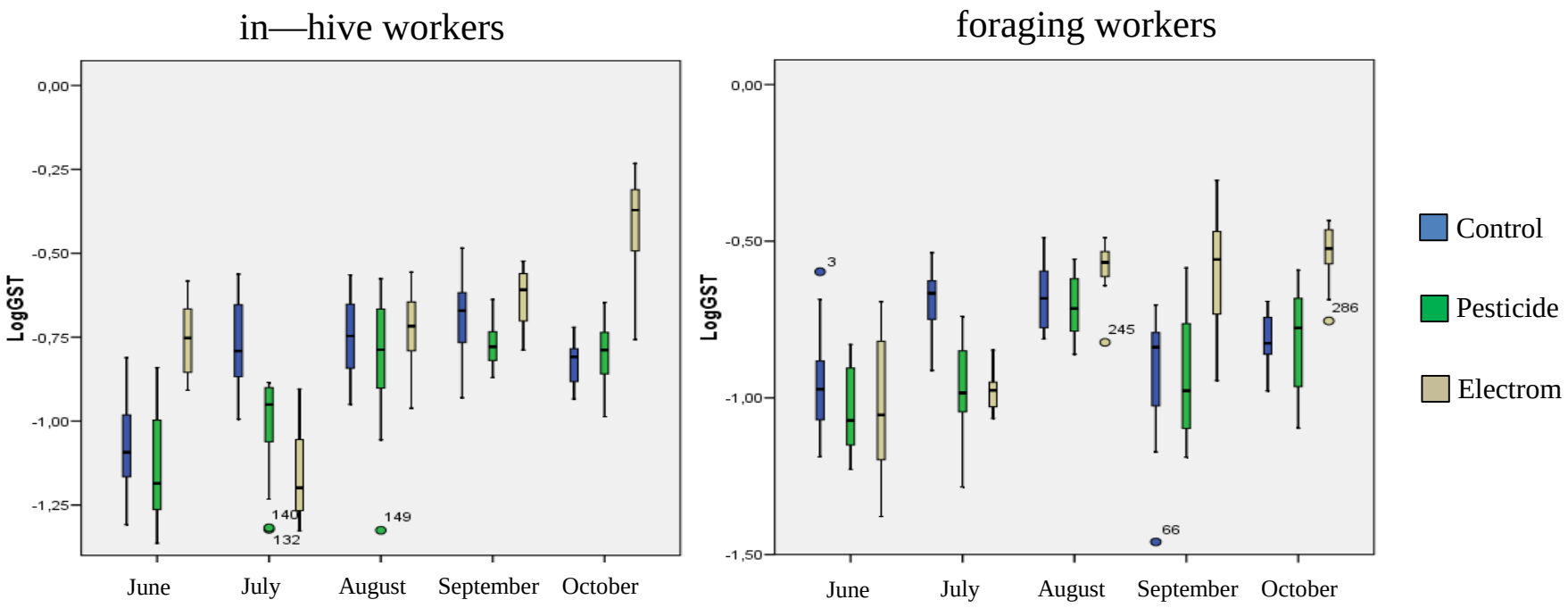

in—hive workers
foraging workers
LogGST
0,00
-0,25
-0,50
-0,75
-1,00
-1,25
-1,50
June
July
August
September
October
140
132
149
3
245
66
286
Control
Pesticide
Electrom

FIG. 4c

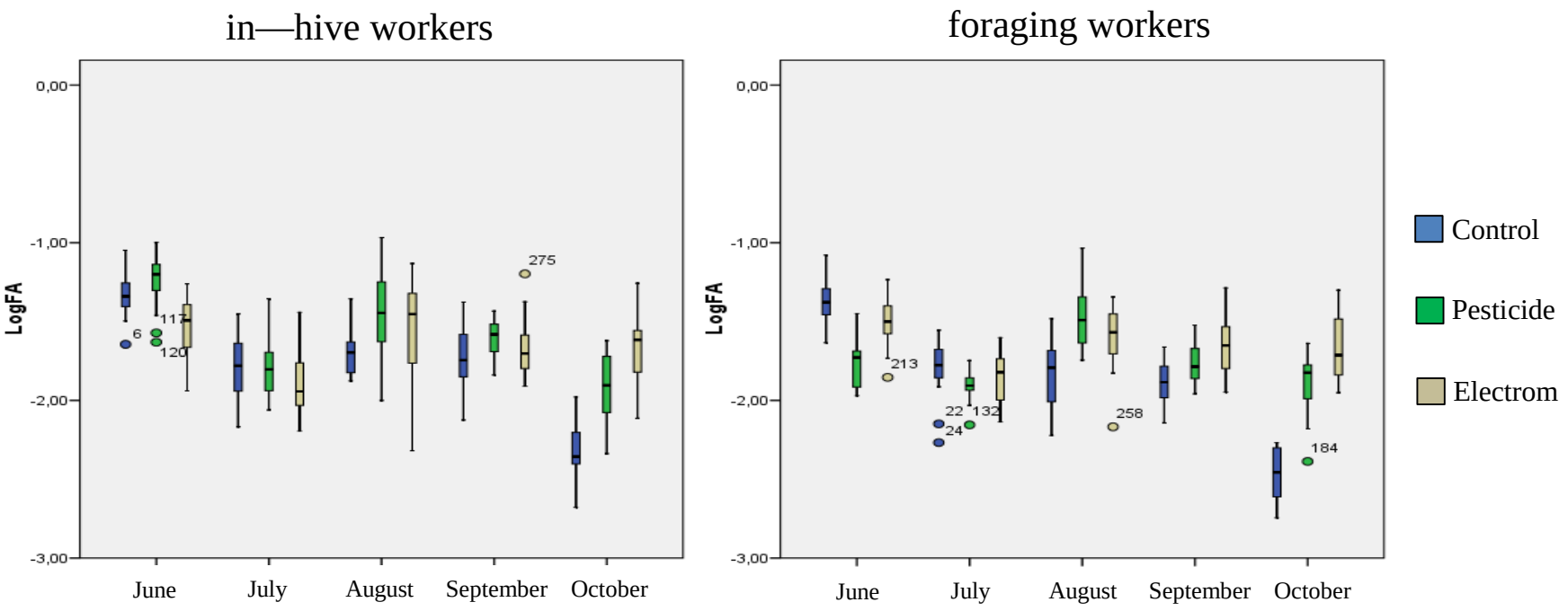

in—hive workers
foraging workers
LogFA
0,00
-1,00
-2,00
-3,00
June
July
August
September
October
6
117
120
275
213
22
24
132
258
184
Control
Pesticide
Electrom

FIG. 4d

SUPPLEMENTARY INFORMATION

Meteorological data

Meteorological parameters (temperature and precipitations) in the three experimental sites were obtained by the meteorological network of the Regional Environmental Protection Agency.

For control site (Ramponio Verna), two meteorological stations ware selected because altitude differences between the experimental site (705 m a.s.l.) and the two nearest meteorological stations: Porlezza with an altitude of 291 m a.s.l. and 8 km far from the experimental site and Cavargna with an altitude of 1100 m a.s.l. and 11 km far from the experimental site. Correlation of temperature data between Porlezza and Cavargna were good only from March to September, with the higher station colder than the lower one. During this period, the mean temperature decreasing gradient (at daily basis) was 0.44 °C every 100 m of altitude (0.11-1.0 °C /100 m as min-max interval). On the opposite, in winter the higher station was frequently warmer than the lower one, because of the thermal inversion phenomena. For example in January increasing temperature gradients vs. altitude up to 0.76 °C/100 m were often observed together with typical decreasing gradients up to 0.81 °C/100 m. Because of this climatic phenomenon (thermic inversion) temperatures in the experimental site were calculated on a daily basis, considering day by day the specific temperature gradient existing between the two meteorological stations. Daily temperatures were calculated proportionally to the altitude difference between the higher meteorological station (Cavargna) and the experimental site (446 m of altitude difference). Calculated mean daily temperatures in the experimental site resulted generally higher than those registered in Cavargna's meteorological station because of the lower altitude, but, when the thermal inversion was present, temperatures in the experimental site resulted lower than those registered in Cavargna's meteorological station, although this last is located at a higher altitude.

24 h-cumulated precipitation data from the two reference stations (Porlezza and Cavargna) were well correlated ($P_{Cavargna}=1.0778 * P_{Porlezza} + 0.3658$; n=365; $R^2 =0.85$), with the last one showing

slightly higher precipitations, because of the orographic precipitation gradient. Because our experimental site (Ramponio Verna) was in between the two reference stations, mean values of the 24 h-cumulated precipitation data of the two stations were considered.

For electromagnetic stress site (Cantello), Arcisate's meteorological station was chosen because of its proximity; it was 5.5 km far and 30 m of altitude below that of the experimental site, so that spatial and altitudinal differences were considered negligible.

For pesticide stress site (Arcagna), two meteorological stations were selected (Tavazzano, 1.3 km far and Landriano, 15 km far). Both of them were at the same altitude than the experimental site, but the nearest one (Tavazzano) recorded data only until the 14th of June 2015. For this period, temperature and precipitation from that station were used, but for the second period (from the 16th June to the 31st of December 2015) those of the second station were taken. Between the two stations, the correlations of temperature and precipitation data were analysed during the first period in which data from both stations were available. Between the 1st of January and the 15th of June, hourly mean temperatures in Landriano were 1.0361 those of Tavazzano - 1.1158 (R^2=0.988). Basing on this relationship, hourly mean temperatures in Tavazzano during the missing period were calculated from those in Landriano. For precipitation data the same scheme was followed: during the first period (1st of January and the 15th of June), 24 h-cumulated precipitation data from the two stations (Landriano and Tavazzano) were compared and their correlation was very good (n=161; R^2 =0.91). Basing on this evidence, precipitation from Landriano were taken as surrogate for the second period (from the 16th June to the 31st of December 2015).

Daily mean temperatures and cumulated daily precipitations in the three experimental sites are shown in the figures S-3, S-4 and S-5. For an easier comparison, mean monthly temperatures and cumulated monthly precipitations were reported in Table S-2. Daily mean temperatures in the two stress sites were highly correlated with those in the control site (r=0,980; n=365; P= <0,001 and r=0,968; n=365; P=<0,001 for electromagnetic and pesticide stress sites *vs* control one, respectively), as well as 24-h cumulated precipitations (r= 0,780; n=364; P<0,001 and r= 0,383;

n=361; P<0,001 for electromagnetic and pesticide stress sites *vs* control one, respectively). Meteorological conditions were much more similar between electromagnetic and control site than between pesticide and control one. This is due to the lower distance and the lower altitude difference of the formers. Annual mean temperature in the control site (12.4 °C) was quite identical to that in the electromagnetic one (12.5 °C), while that in the pesticide stress site was higher (14.9 °C), according to the lower elevation. Minimum- maximum interval of the hourly mean temperature varied between -3.3°C to 33.9 °C (in January and July), between -5.2 °C to 35.2 °C (in January and July), and between -4.3°C to 37.8 °C (in February and July) in control, electromagnetic and pesticide stress sites, respectively. Even if the control site is located at a higher altitude in a pre-alpine environment, its climate is warmer in winter (because of the thermic inversion phenomenon and the mitigation effect of the Lake of Como near it) and cooler in summer (because of the altitude). Total annual precipitation were 1361, 1390 and 720 mm, in the control, electromagnetic and pesticide stress sites, respectively. These data reflect the climatic similarity of the electromagnetic and control sites and the decreasing precipitation gradient in function to the distance from the mountains for the pesticide-stress site.

Pesticide exposure

In the pesticide-stress site, chemical exposure is directly affected by the treatment performed in the orchard farm, where the experimental hive were positioned. Pesticide list and treatment schedule are reported in Table 1 and Figure S-1, respectively). In the surrounding agricultural area, manly arable crops (maize and soybean) were present, which can be a secondary source of pesticide exposure. On maize, insecticides are used during sowing at the end of march. Seeds treated with neonicotinoids were forbidden since 2008, instead of them mainly pyrethroids, such as tefluthrin, are actually used (e.g. 5 g of tefluthrin /ha). Others insecticides are used during the growing season against the Lepidopteran *Ostrinia nubilalis* and the Coleopteran *Diabrotica virgifera* but their

occurrence is limited. On soybean mainly herbicides are used both in pre- and post-emerging phase, while miticide against the mite *Tetranychus urticae*, are occasionally used. Despite these possible pesticide sources, orchard crops constitute a pesticide exposure.

In Europe, Pesticide risk assessment toward pollinators is currently evaluated according to EFSA Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees) (EFSA 2013). Here, as a first approximation, the hazard quotient (HQ) or Toxicity Exposure Ratio (TER) concept of the EFSA approach was extremely simplified by the ratio between the actual application rate (μg a.i. cm^{-2}), as exposure index, and the acute toxicity (μg a.i. bee^{-1}), as toxicity measure. Between oral and contact acute toxicity data, the lower LD_{50} value was chosen, according to the precautionary principle. The application rate was expressed in μg a.i. cm^{-2} to conform it to the LD_{50} unit which is usually expressed in μg a.i. bee^{-1}. In fact, the ratio between the amount used in μg a.i./cm^2 and the toxicity in μg a.i. bee^{-1} results in the unit of bee cm^{-2}. Considering that the projection surface of a bee can be roughly approximated to 1 cm^2, the ratio of 1 conceptually means that the dispersed dose is equal to that able to cause a lethal effect on the 50 % of the cases. A ratio >1 means that used amount are x-time higher than the lethal doses of that pesticide, while the opposite for a ratio <1. We are conscious that the used approach is an extreme simplification of the current evaluation procedure (EFSA 2013), but we believe that is sufficient for indexing the toxicity of the pesticide used in the area (Table S-1) and we called it 'toxicity ratio'. Used pesticides showed toxicity ratios ranging over more than 5 order of magnitude from 0.0008 for the plant regulator NAA (1-naphthylacetic acid) to 351 for the insecticide imidacloprid. Figure S-2 shows the time sequence of the pesticide treatments expressed as toxicity ratios. From 15 of April to 8 of October, 15 treatments with active ingredients highly toxic toward bees were performed: 2 with Imidacloprid (toxicity ratio of 351) the 5th and the 16th of May; 1 with thiamethoxam (toxicity ratio of 340) the 25th of July; 1 with chlorpyrifos (toxicity ratio of 98) the 7th of May; 8 with deltamethrin (toxicity ratio of 65) the 15th of April, the 30th of May, the 13th and

the 26 of June, the 10th, the 17th and the 27th of July, and the 7th of August; and 3 with spinosad (toxicity ratio of 49) the 25th of May, the 22th of August, and the 8th of October.

Chemical classs	Active Ingredient	Use	LD_{50} contact acute-48*h* µg/bee
Alkanes	whilte mineral oil a.i. 80%	insecticide	>3814
Amidine	amitraz a.i. 192 g/L	insecticide	50
Anilinopyrimidine	cyprodinil a.i. 375 g/kg	fungicide	>784
	pyrimethanil a.i. 400 g/L	fungicide	>100
Anthranilic diamide	chlorantraniliprole a.i. 200 g/L	insecticide	>4
Avermectine	emamectina benzoate a.i. 9.5 g/kg	insecticide	-
Benzinidazole	thiophanate-methyl a.i. 417 g/kg	fungicide	>100
Benzoylurea	triflumuron a.i. 480.7 g/L	insecticide	>200
Carbamate	ziram a.i. 760 g/kg	fungicide	>100
Carboxiamide	boscalid a.i. 267 g/kg	fungicide	>200
Diacylhydrazine	methoxyfenozide a.i. 240 g/L	insecticide	>100
Guanidine	dodina a.i. 650 g/kg	fungicide	>100
Hydroxyanilide	fenhexamid a.i. 500 g/L	fungicide	>200
Inorganic compound	copper oxychloride a.i. 60-70%	fungicide	-
	sulfur a.i. 800 g/kg	fungicide	>100
Neonicotinoid	acetamiprid a.i. 46.7 g/kg	insecticide	8.09
	imidacloprid a.i. 200 g/L	insecticide	0.081
	thiacloprid a.i. 80 g/L	insecticide	38.82
	thiamethoxam a.i. 216 g/Kg	insecticide	0.024
Organophosphate	chlorpyrifos a.i. 750 g/kg	insecticide	0.059
	fosetyl-aluminium a.i. 800 g/kg	fungicide	>1000
Phenylpyrrole	fludioxonil a.i. 250 g/kg	fungicide	>100
Pyrethroid	deltamethrin a.i. 15 g/L	insecticide	0.0015
	etofenprox a.i. 280 g/L	insecticide	>0.13
Quinine	dithianon a.i. 700 g/kg	fungicide	>100
Strobilurin	pyraclostrobin a.i. 67 g/kg	fungicide	>100
	trifloxystrobin a.i. 250 g/kg	fungicide	>200
Synthetic auxin	NAA (1-naphthylacetic acid) a.i. 85 g/L	plant regulator	>120
Tetramic acid	spirotetramat a.i. 48 g/L	insecticide	>100
Triazole	cyproconazole a.i. 10%	fungicide	>100
	tebuconazole a.i. 46 g/L	fungicide	>200
	tebuconazole a.i. 500 g/kg	fungicide	>200
Unclassified	buprofezin a.i. 250 g/kg	insecticide	>200
	spinosad a.i. 480 g/L	insecticide	0.05

LD_{50} oral acute-48*h* µg/bee	Treatment dose Kg a.i. /ha	Treatment dose µg a.i. /cm^2	Toxicity ratio bee/cm^2
1474	25	250	**0.17**
-	0.59	5.9	**0.12**
112.5	0.12	1.2	**0.011**
>100	0.31	3.1	**0.031**
>104.1	0.061	0.61	**0.15**
-	0.04	0.4	**-**
>100	0.16	1.6	**0.016**
>226	0.18	1.8	**0.009**
-	2.6	26	**0.26**
100	0.21	2.1	**0.021**
>100	0.18	1.8	**0.018**
>200	2.2	22	**0.22**
>102.07	1.3	13	**0.13**
12.1	2.6	26	**2.15**
>106.8	2.2	22	**0.22**
14.53	0.27	2.7	**0.33**
0.0037	0.13	1.3	**351**
17.32	0.14	1.4	**0.081**
0.005	0.17	1.7	**340**
0.25	0.58	5.8	**98**
462	2.2	22	**0.048**
>100	0.081	0.81	**0.0081**
0.074	0.0098	0.098	**65**
0.27	0.095	0.95	**3.52**
>25.4	0.89	8.9	**0.35**
>73.1	0.054	0.54	**0.0074**
>200	0.047	0.47	**0.0024**
-	0.0097	0.097	**0.00080**
>107.3	0.15	1.5	**0.015**
>100	0.0087	0.087	**0.00087**
>83.05	0.15	1.5	**0.018**
>83.05	0.82	8.2	**0.098**
>163.5	0.38	3.8	**0.023**
0.049	0.24	2.4	**49**

Period	Reference site (Ramponio-Verna)		Electromagnetic site (Cantello)		Pesticide site (Arcagna)	
	Temperature (°C)	Precipitation (mm)	Temperature (°C)	Precipitation (mm)	Temperature (°C)	Precipitation (mm)
January	3.8	149.2	3.1	124.8	4.3	39
February	3.5	100.2	3.3	149.4	4.7	152.9
Marc	7.8	24.4	8.2	39.2	10.3	48.7
April	11.8	103.7	12.4	105.6	14.8	82
May	15.4	193.5	16.4	194.4	19.9	81.4
June	19.6	111.9	20.9	152.8	23.7	69.8
July	24.4	86	25.2	83	28	5.8
August	20.8	133.1	21.6	161.2	24.2	100.4
September	15	289.4	16.3	165.2	19.3	59.2
October	11.2	166.6	11.2	208	14	70.4
November	9.1	1.7	6.9	3	8.9	6.4
December	5.5	1.3	3.4	3	5.7	3
Year mean	12.4	1361	12.5	1389.6	14.8	719

Supplementary Material

Click here to access/download;Supplementary Material;FIG-SI Bees2019.pptx

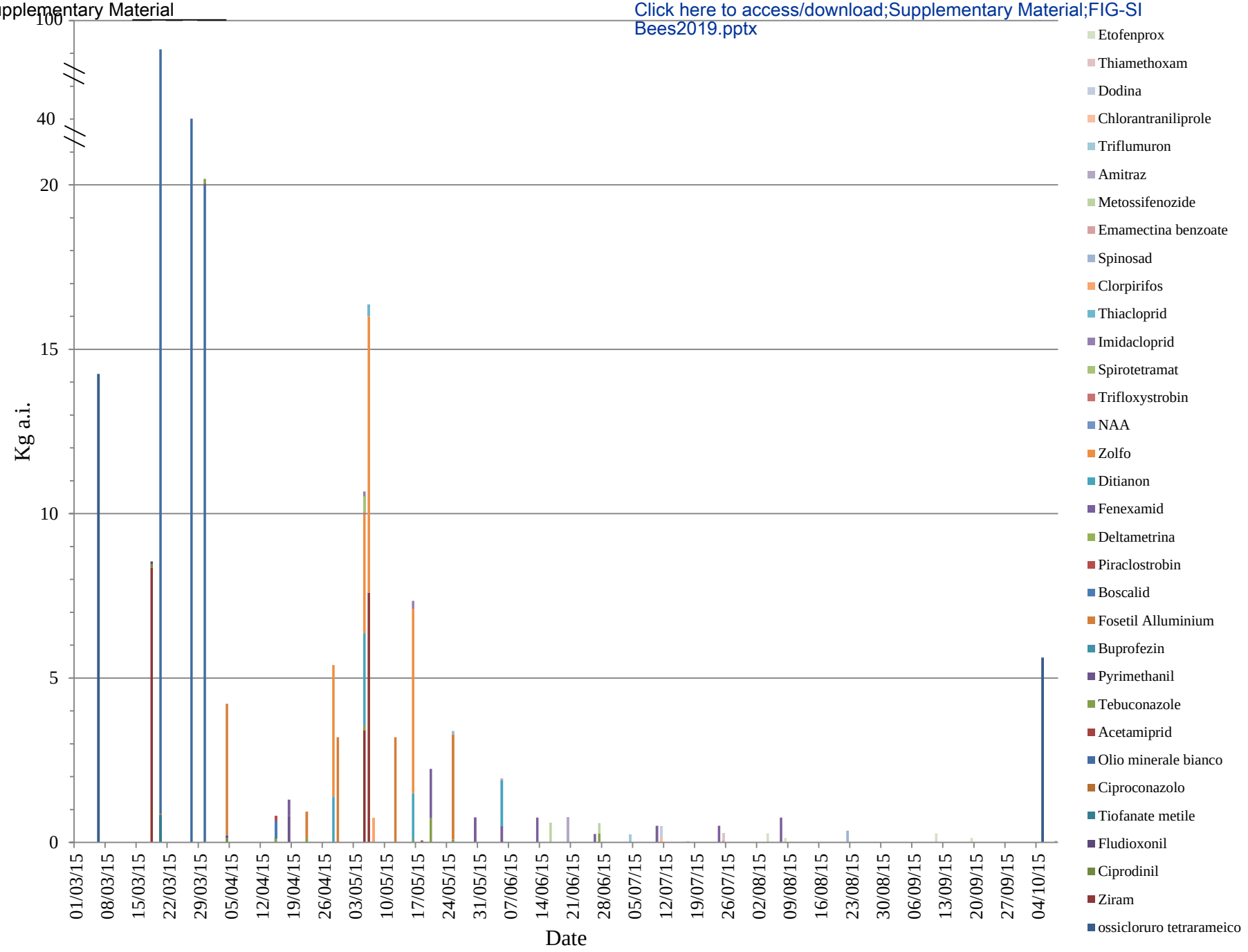


FIG. S-1

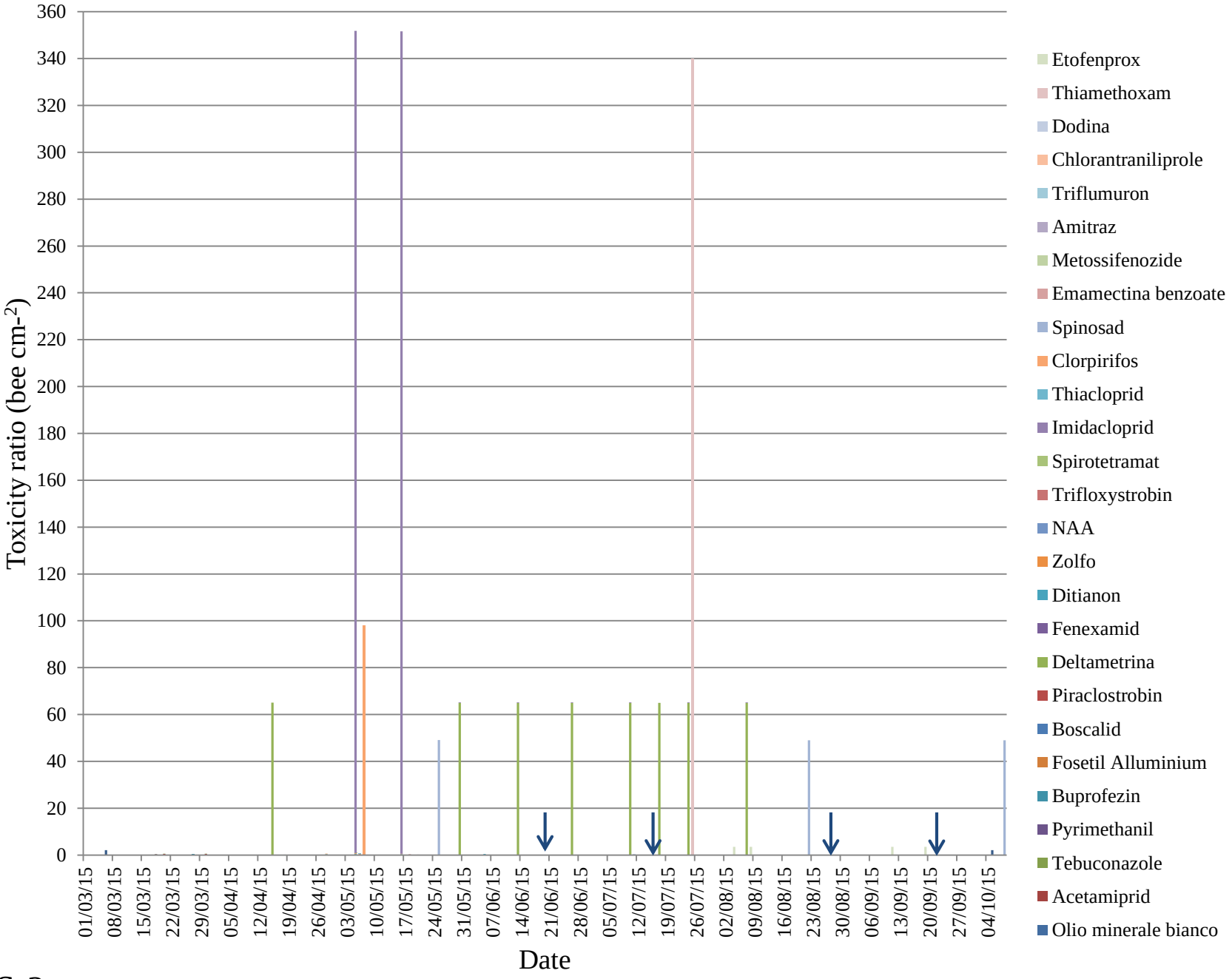

Toxicity ratio (bee cm-2)
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
01/03/15
08/03/15
15/03/15
22/03/15
29/03/15
05/04/15
12/04/15
19/04/15
26/04/15
03/05/15
10/05/15
17/05/15
24/05/15
31/05/15
07/06/15
14/06/15
21/06/15
28/06/15
05/07/15
12/07/15
19/07/15
26/07/15
02/08/15
09/08/15
16/08/15
23/08/15
30/08/15
06/09/15
13/09/15
20/09/15
27/09/15
04/10/15
Date
Etofenprox
Thiamethoxam
Dodina
Chlorantraniliprole
Triflumuron
Amitraz
Metossifenozide
Emamectina benzoate
Spinosad
Clorpirifos
Thiacloprid
Imidacloprid
Spirotetramat
Trifloxystrobin
NAA
Zolfo
Ditianon
Fenexamid
Deltametrina
Piraclostrobin
Boscalid
Fosetil Alluminium
Buprofezin
Pyrimethanil
Tebuconazole
Acetamiprid
Olio minerale bianco

FIG. S-2

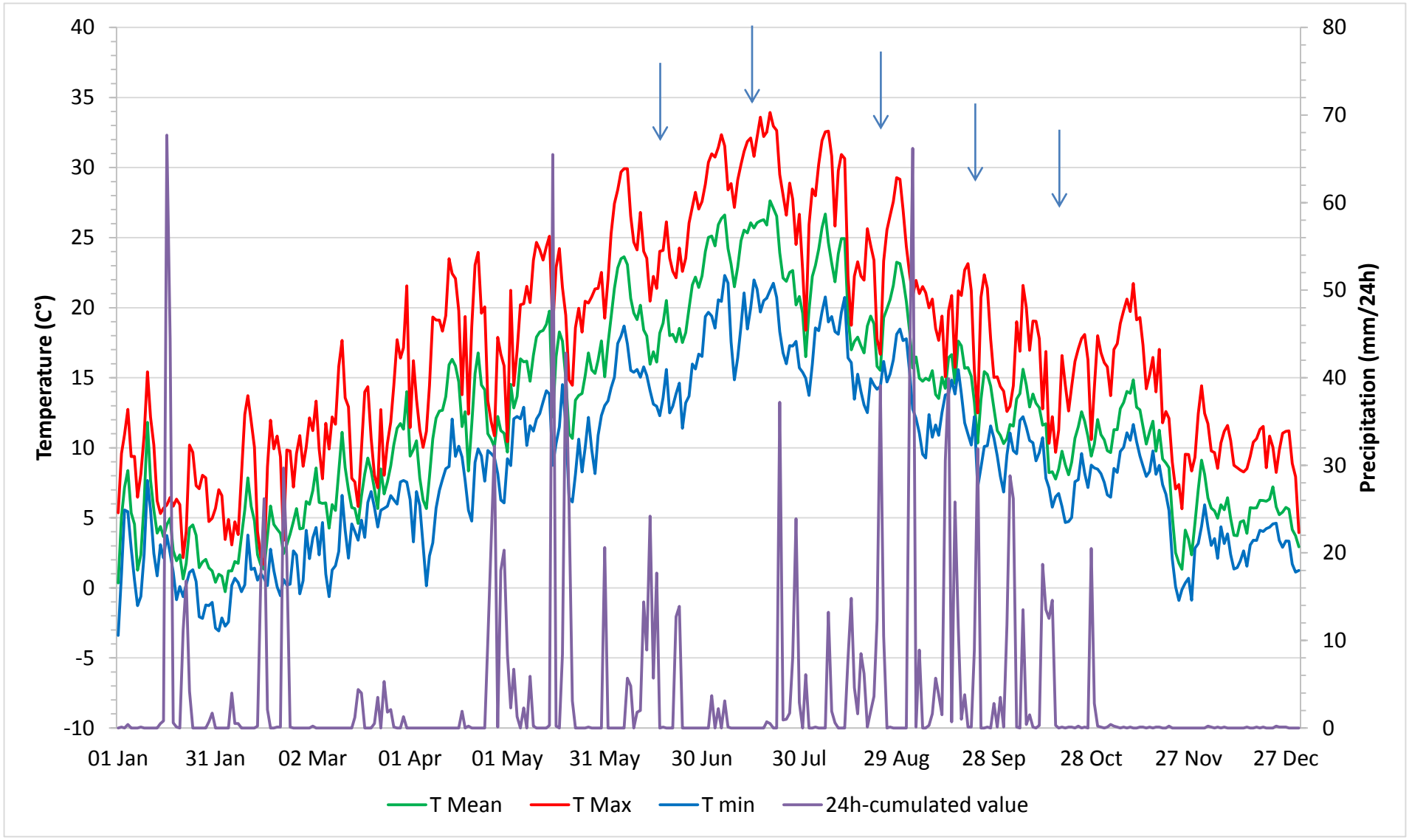

RAMPONIO VERNA
Temperature (C°)
Precipitation (mm/24h)
40
35
30
25
20
15
10
5
0
-5
-10
80
70
60
50
40
30
20
10
0
01 Jan
31 Jan
02 Mar
01 Apr
01 May
31 May
30 Jun
30 Jul
29 Aug
28 Sep
28 Oct
27 Nov
27 Dec
T Mean
T Max
T min
24h-cumulated value

FIG. S-3

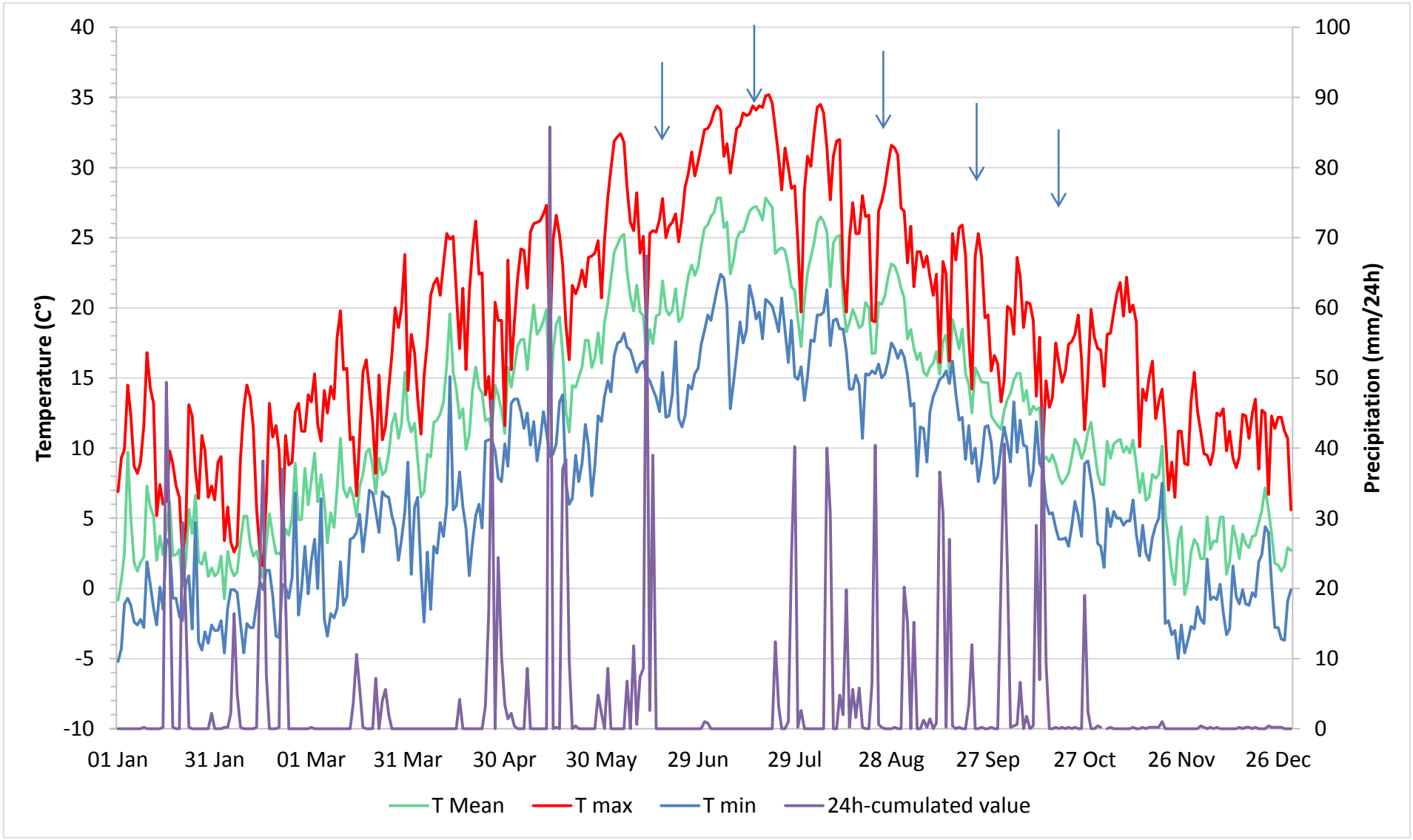

CANTELLO
Temperature (C°)
Precipitation (mm/24h)
40
35
30
25
20
15
10
5
0
-5
-10
100
90
80
70
60
50
40
30
20
10
0
01 Jan
31 Jan
01 Mar
31 Mar
30 Apr
30 May
29 Jun
29 Jul
28 Aug
27 Sep
27 Oct
26 Nov
26 Dec
T Mean
T max
T min
24h-cumulated value

FIG. S-4

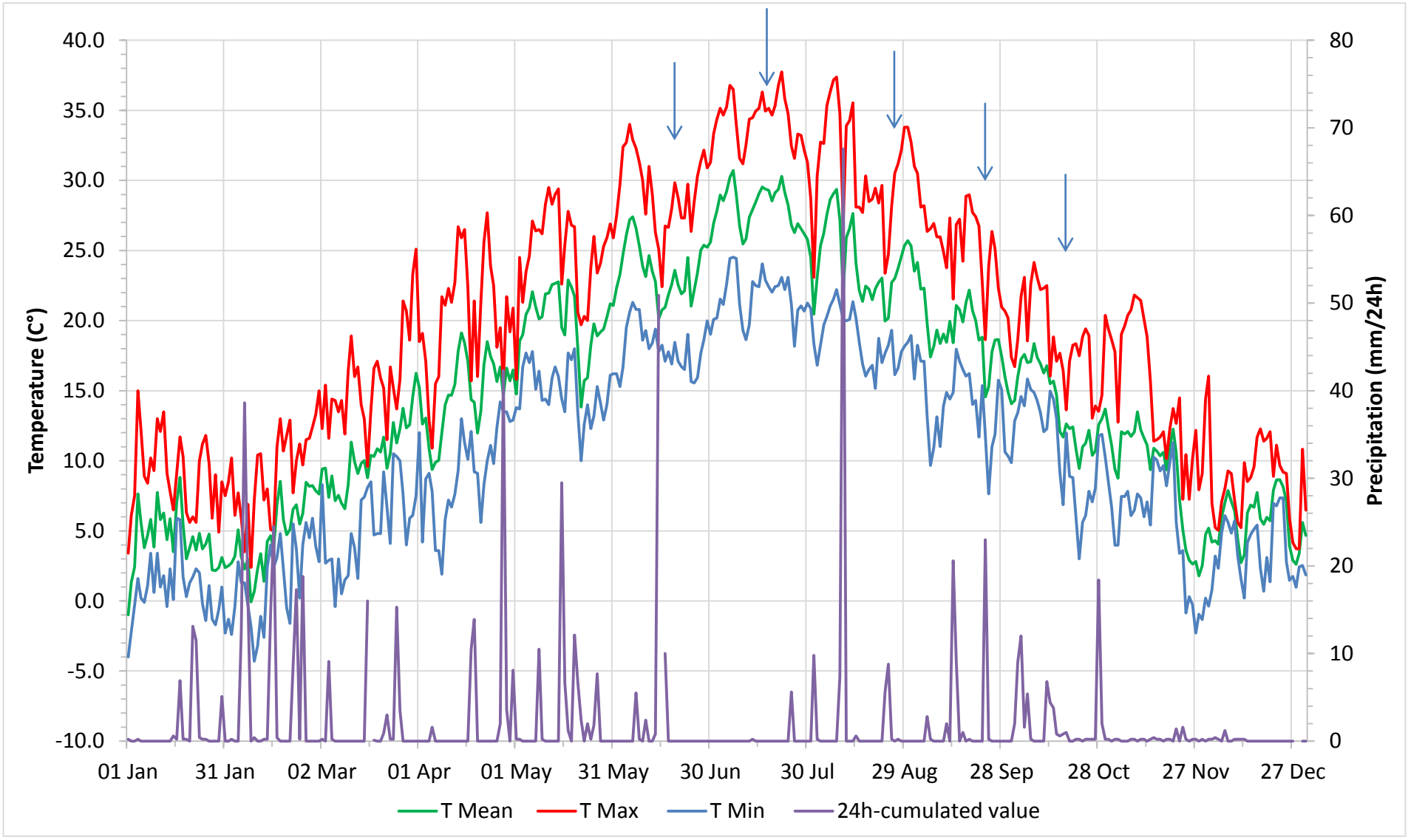

ARCAGNA
Temperature (C°)
Precipitation (mm/24h)
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
-5.0
-10.0
80
70
60
50
40
30
20
10
0
01 Jan
31 Jan
02 Mar
01 Apr
01 May
31 May
30 Jun
30 Jul
29 Aug
28 Sep
28 Oct
27 Nov
27 Dec
T Mean
T Max
T Min
24h-cumulated value

FIG. S-5

Highlights

Bees are subject to many stress-factors. Here the effects of electromagnetic fields and those of pesticides were evaluated by means of a battery of enzymatic biomarkers. In the electromagnetic-stress site, an effect of an over-activation of all analyzed biomarkers was observed at the end of the season, posing potential problems to winter survival. In the pesticide-stress site, pesticide mixtures, currently used in agriculture, were able to greatly affect biochemical parameters of bees (with both enzymatic under- and over-activations).

UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI SCIENZE E POLITICHE AMBIENTALI
DEPARTMENT OF ENVIRONMENTAL SCIENCE AND POLICY

Milan, 5th of July 2019

Dear Prof. M. Ardestani and M.H. Niksokhan
Editors in chief of International Journal of Environmental Research

The submitted manuscript 'Effects of pesticides and electromagnetic fields on honeybees: a field study using biomarkers' presents interesting data on stress factors for pollinators using biomarkers as sensible stress markers. Pollinator health status is one the most relevant environmental problem in many countries, especially in developed countries because of anthropogenic stress factors. We found that pesticide and electromagnetic field effected enzymatic activities on honeybees under actual field conditions and that biomarkers were a very useful diagnostic and early-warning tool on honeybees. The most important result coming up from this research is the over-activation of all analyzed biomarkers at the end of the season following electromagnetic field exposure. This event was related to a behavioral over-activation in a period in which bees should prepare themselves to overwintering, posing potential problems to winter survival.
By our advice, the obtained results are of scientific interest also from a methodological point of view: they show a marked seasonal cycle of the enzymatic activities under natural conditions (control site) that should be taken into account in interpreting biomarker data.
We hope to meet your interest.
Sincerely yours

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