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Immune-related gene expression in nurse honey bees (*Apis mellifera*) exposed to synthetic acaricides

Paula Melisa Garrido [a,*], Karina Antúnez [b], Mariana Martín [c], Martín Pablo Porrini [a], Pablo Zunino [b], Martín Javier Eguaras [a]

[a] Laboratorio de Artrópodos, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata – CONICET, Mar del Plata, Buenos Aires, Argentina
[b] Departamento de Microbiología, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay
[c] Centro de Investigaciones Biológicas, CEBB-MdP-INBA, Fundación para Investigaciones Biológicas Aplicadas (FIBA) – CONICET, Mar del Plata, Argentina

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ABSTRACT

The mite *Varroa destructor* is an ectoparasite affecting honey bees worldwide. Synthetic acaricides have been among the principal tools available to beekeepers for its control, although several studies have shown its negative effects on honey bee physiology. Recent research suggests that those molecules strongly impact on immune signaling cascades and cellular immunity. In the present work, LC_{50} in six-day-old bees were determined for the following acaricides: tau-fluvalinate, flumethrin, amitraz and coumaphos. According to this obtained value, a group of individuals was treated with each acaricide and then processed for qPCR analysis. Transcript levels for genes encoding antimicrobial peptides and immune-related proteins were assessed. Flumethrin increased the expression of hymenoptaecin when comparing treated and control bees. Significant differences were recorded between coumaphos and flumethrin treatments, while the first one reduced the expression of hymenoptaecin and abaecin, the last one up-regulated their expressions. No significant statistically changes were recorded in the expression levels of vitellogenin, lysozyme or glucose dehydrogenase among bees treated with acaricides and control bees. This work constitutes the first report, under laboratory conditions, about induction of immune related genes in response to synthetic miticides.

1. Introduction

Varroa destructor is an ectoparasite affecting honey bees worldwide, causing economic damage on beekeeping. This mite is a new parasite of the honey bee *Apis mellifera*, for that reason, there is not a balanced host–parasite relationship and beekeepers do not have long-term experience in dealing with this pest (Rosenkranz et al., 2010). However, colony survival in the presence of varroa mites depends on their intervention, because an unmanaged colony is likely to succumb (Boecking and Genersch, 2008). There are many discrepancies in the regulation of treatments among various countries (Wallner, 1999). The most noted synthetic acaricides against *V. destructor* are the organophosphate coumaphos, the pyrethroids tau-fluvalinate and flumethrin, as well as the formamidine amitraz (reviewed in Rosenkranz et al., 2010).

Researchers of different parts of the world have found residuals of synthetic acaricides and its metabolites in different apicultural matrices (Bogdanov et al., 1997; Lodesani et al., 2003; Maver and Poklukar, 2003; Martel et al., 2007; Chauzat and Faucon, 2007; Mullin et al., 2010; Orantes-Bermejo et al., 2010; Pareja et al., 2011). Furthermore, another important problem that generates this kind of treatments is the recorded phenomena of mite resistance (Elzen et al., 1999; Elzen and Westervelt, 2002; Maggi et al., 2009, 2010, 2011; Milani, 1995).

There are many documented studies showing the negative effects that produce synthetic acaricides on honey bee health (Cox and Wilson, 1984; vanBuren et al., 1992a; Duff and Furgala, 1992; Currie, 1999; Rinderer et al., 1999; Wallner, 1999; Westcott and Winston, 1999; Ellis et al., 2001; Fell and Tignor, 2001; Skinner et al., 2001; Haarmann et al., 2002; Collins et al., 2004; Pettis et al., 2004; Burley, 2007; Bevk et al., 2012; Gregorc and Ellis, 2011; Wu and Anelli, 2011).

Although tau-fluvalinate is considered to have a low toxicity to honey bees (Stoner et al., 1984; Pettis et al., 1991), there has been recent concern among beekeepers that it may have an impact on queen performance (Sokol, 1996) and Rinderer et al. (1999) determined that it could impact on sexual competitiveness of drones. Tau-fluvalinate is a subset of isomers of fluvalinate; its mode of action is as an agonist of the voltage-gated sodium channel (Wang et al., 2002). But it has been demonstrated that some

* Corresponding author. Address: Laboratorio de Artrópodos-Grupo Acarología y Entomología Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata – CONICET, Deán Funes 3350, Mar del Plata (7600), Buenos Aires, Argentina. Tel.: +54 0223 475 2426x450.
E-mail address: pmgarrid@mdp.edu.ar (P.M. Garrido).

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pyrethroids (cypermethrin and fenitrothion), which act mainly on the voltage dependent Na^+ channels of the nerve cell membrane, can have secondary effects underlying the neurotoxicity like effects on acetylcholinesterase (AChE) activity (Bandyopadhyay, 1982; Bendahou et al., 1999). Furthermore, Locke et al. (2012) found direct effects of tau-fluvalinate on honey bee increasing host susceptibility to deformed wing virus infection; they suggest that those molecules could debilitate physiology and/or immune system response.

Nielsen et al. (2000) observed that flumethrin treatment led to increased Glutathione S-transferase activity in nurse bees and may result in smaller worker adults.

Coumaphos can act as a systemic agent and can also be distributed through trophallaxis (vanBuren et al., 1992b). This substance or its oxon metabolite inactivate AChE, thereby interfering with nerve signaling and function, leading to the death of the insect. Recently, a research work about the effects of acaricides on gene expression was performed by Boncristiani et al. (2012), at field conditions. They employed commercial formulations and found that coumaphos altered some metabolic responses, including immune and detoxification gene expression pathway.

Amitraz, is an octopaminergic agonist, it can impact learning and cognition in honey bees. Colonies treated with amitraz and flumethrin showed an induction in the AChE activities due to toxic stress in emerged and nurse bees. (Loucif-Ayad et al., 2008).

Since the honey bee genome was sequenced, it has been suggested that honey bees are relatively immunologically deficient (i.e., express fewer immune response proteins) compared to non-social insects, moreover, they have about half detoxifying enzymes than pesticide resistant insects (Claudianos et al., 2006). This immunological deficiency can further reduce their ability to response against infections. Also, target site resistance mutations have not yet been described in this species, although there are reports of race-based differences in sensitivity to pyrethroids (Danka et al., 1986; Elzen et al., 2000; Claudianos et al., 2006). Currently, a highly accepted explanation for honey bee susceptibility is that social immunity provides significant protection for honey bee colonies.

Nevertheless, there is an overall lack of immunological studies of synthetic acaricides treated honey bees under laboratory conditions. For that reason, to gain a more comprehensive understanding of the impacts of those molecules, we studied their effects on the expression of immune related genes, in the same cohort of honey bees.

2. Materials and methods

2.1. Chemicals

Technical grade tau-fluvalinate, flumethrin, amitraz and coumaphos were purchased from Sigma–Aldrich.

2.2. Insects

This work was performed with local hybrid colonies (*Apis mellifera mellifera*/*Apis mellifera ligustica*) in January of 2012. Capped brood frames were collected from healthy colonies located in the experimental apiary J. J. Nágera coastal station, placed on route 11 km 32 (38°10′06″S, 57°38′10″W) and kept under incubator conditions during the experiment (32 °C ± 0.79; 40% ± 3.3 HR). Mite infestation levels were monitored in our colonies using the natural mite fall method (Rosenkranz et al., 2010). The phoretic *Varroa* mite infestation rates were determined by washing samples of 200 bees with soapy water to dislodge the mites (Fries et al., 1991). In order to evaluate the presence of *Nosema* spp. spores, 60 bees from each colony were macerated and examined using light microscopy.

Newly emerged bees, which are free from *Nosema ceranae* infection (Higes et al., 2007; Suwannapong et al., 2010), were placed in wooden cages with a plastic mesh ($11 \times 9 \times 6\ cm^3$) in groups of 100–200. Also were provisioned with candy (powdered sugar and glucose syrup) and fresh bee bread during 6 days.

2.3. LC_{50} Bioassays

Six-d-old worker bees were used for all bioassays. All compounds were dissolved in 1 ml of ethanol 96%.

Honey bees were treated with of coumaphos, tau-fluvalinate, flumethrin, amitraz in accordance with Ruffinengo et al. (2005), at doses of 10, 100, 1000, 2000 ppm for all acaricides. Groups of twelve bees were placed per Petri dish. Four replicates were done for each concentration and control.

Very low mortality was observed in control bees that received only the solvent. In this case, Abbott method (Abbott, 1925) was used to correct natural mortality.

Mortality was recorded at 17, 24, 36, 48 and 72 h after treatment.

Bees were provisioned with a piece of candy during treatment and maintained in an incubator (28 °C; 30% HR).

Calculations of LC_{50} values and 95% fiducial limits, as established by USEPA (1986), were conducted using EPA software (version 1.5) as recommended by Lindberg et al. (2000a).

2.4. Treatments

Once obtained the LC_{50} values, treatments were repeated using these values as exposure doses. The time of exposure for each acaricide was 24 and 17 h only for Flumethrin (due to the high mortality caused by this treatment).

2.5. Manipulation of bee samples

When the treatment had finished, bees were placed in clean Petri dishes.

Nine bees belonging to each experimental group (control or treated with amitraz, coumaphos, tau-fluvalinate and flumethrin) were individually frozen at −20 °C during approximately 20 min, sliced in pieces and merged in 1 ml of RNA later® (Ambion, Inc. Austin, TX) in order to avoid the degradation of mRNA. Samples were immediately sent to Department of Microbiology, IIBCE, Uruguay, and analyzed within 24 h of received.

2.6. RNA isolation and cDNA synthesis

Six hundred μl of RLT buffer (Qiagen) were added to samples conserved in RNA later, and they were disrupted and homogenized using a sterile glass rod and a sterile plastic tube.

Total RNA was isolated from each individual bee using the RNeasy Plus Mini Kit (Qiagen), following to manufacturer's instructions. This kit includes a column for elimination of genomic DNA. The total RNA recovered was immediately used to generate first strand cDNAs using the QuantiTec Reverse Transcription Kit (Qiagen), according to the manufactureŕs instructions. Reaction mixture consisted in 2 μl of 7× gDNA Wipeout Buffer, 5 μl of RNA and 7 μl of RNase-free water. It was incubated at 42 °C during 2 min in order to remove contaminating genomic DNA, and placed immediately on ice. Then, 6 μl of Master mix containing 1 μl of Quantiscript Reverse Transcriptase, 4 μl of 5× Quantiscript RT Buffer and 1 μl of RT Primer Mix (mixture of oligo-dT and random primers) was added. RNA was subjected to retrotranscription by incubation at 42 °C for 15 min. Enzymes were inactivated by

Table 1
Primers used for the amplification of immunity related genes.

Amplification target	Primer	Sequence	T (°C)	Efficiency	Reference
Ribosomal protein S5	RPS5-F RPS5-R	5′-AATTATTTGGTCGCTGGAATTG-3′ 5′-TAACGTCCAGCAGAATGTGGTA-3′	75.8	2.01	Evans (2006)
Antibacterial peptide abaecin	Abaecin-F Abaecin-R	5′-CAGCATTCGCATACGTACCA-3′ 5′-GACCAGGAAACGTTGGAAAC-3′	79.3	2.06	Evans (2006)
Antibacterial peptide defensin	Defensin-F Defensin-R	5′-TGTCGGCCTTCTCTTCATGG-3′ 5′-TGACCTCCAGCTTTACCCAAA-3′	82.3	2.05	Yang and Cox-Foster (2005)
Antibacterial peptide hymenoptaecin	Hymenopt-F Hymenopt-R	5′-CTCTTCTGTGCCGTTGCATA-3′ 5′-GCGTCTCCTGTCATTCCATT-3′	80.5	2.82	Johnson et al. (2009)
Stress protein vitellogenin	VgMC-F VgMC-R	5′-AGTTCCGACCGACGACGA-3′ 5′-TTCCCTCCCACGGAGTCC- 3′	83	2.01	Johnson et al. (2009)
Glucose dehydrogenase	GLD-F GLD-R	5′-CTGCACAACCACGTCTCGTT-3′ 5′-ACCGCCGAAGAAGATTTGG-3′	86.5	1.55	Yang and Cox-Foster (2005)
(Prophenol-) phenoloxidase	PO-F PO-R	5′-AATCCATTACCTGAAATTGATGCTTAT-3′ 5′-TAATCTTCCAACTAATTCATACGCTCTT-3′	75.7	1.82	Yang and Cox-Foster (2005)
Lysozyme	LYS-F LYS-R	5′-ACACGGTTGGTCACTGGTCC-3′ 5′-GTCCCACGCTTTGAATCCCT-3′	84.5	2.36	Yang and Cox-Foster (2005)

incubation at 95 °C for 3 min. Obtained cDNA was stored at −20 °C. Negative controls were run in parallel for each step (RNA extraction and reverse transcription reactions).

2.7. Gene and primer selection

In order to evaluate the effect of insecticides in the immune response of bees, the transcript levels for the genes encoding the antimicrobial peptides abaecin (ABA), hymenoptaecin (HYM), defensin (DEF), the immunity related enzymes phenoloxidase (PO), glucose dehydrogenase (GLD), lysozyme (LYS) and vitellogenin (VG) were assessed using primers previously described (Evans, 2006; Cox-Foster et al., 2007; Yang and Cox-Foster, 2007). Transcript levels for the ribosomal protein RPS5 and β-actin, two moderately expressed housekeeping genes were used as reference genes (Evans, 2006). According to suggestion of Vandesompele et al. (2002), the geometric mean between the Cq of both genes was used for analysis.

Table 1 summarizes the primers used.

2.8. Real-time PCR

Real time PCR reactions were carried out using QuantiTec SYBR PCR Kit (Qiagen), according to manufactures recommendations, and specific primers for the amplification of different genes (Table 1).

Reaction mixture consisted in 1× QuantiTect SYBR Green PCR Master Mix, 0.5 μM of each primer (one pair of primers per reaction), RNAse free water and 5 μl of 1:10 diluted cDNA in a final volume of 25 μl. No template controls were carried out excluding nucleic acids from the reaction.

PCR reactions were carried out using a Rotor Gene 6000 (Corbett Research) and cycling program consisted in an initial activation step at 50 °C for 2 min and 95 °C for 15 min, and 40 cycles of 94 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s. Fluorescence was measured in the elongation step and negative controls (without DNA) were included in each reaction run. Specificity of the reaction was checked by analysis of the melting curve of the final amplified product (from 65 to 95 °C).

2.9. Normalization of the real-time data and statistical analysis

The amplification results from the different genes were expressed as the threshold cycle (Cq) value, which represented the number of cycles needed to generate a fluorescent signal greater than a predefined threshold. The expression ratio between each target gene and the geometric mean of references genes (RPS 5 and β-actin) was calculated according to the method described by Pfaffl (2001). The variation in gene transcript levels between different groups was evaluated by the non-parametric Kruskal Wallis test. Comparisons between different treatments were performed by multiple comparisons of mean ranks, and *p*-values were determined by using Bonferroni adjustment.

p-Values below 0.05 were considered significant.

Table 2
LC_{50} estimated for each acaricide against *Apis mellifera*. LC_{50} values (lethal concentration that kills 50% of the exposed individuals) are expressed in ppm.

	LC_{50} honeybees + (95% confidence limits)
Flumethrin	7.89 (2.09–16.75)[a]
Tau-fluvalinate	61.43 (23.77–115.98)
Coumaphos	751.07 (343.19–1603.18)
Amitraz	282.91[b]

[a] Only 17 h of exposure due to its highly toxicity.
[b] Confidence limits could not be recorded due to significant heterogeneity.

3. Results

3.1. Bees

Colonies did not present any visible clinical symptoms of any disease (i.e., American Foulbrood or Chalkbrood). *Nosema* spp. spores were not detected. During the assays, infestation rates of varroa were 1-2%.

3.2. LC_{50} values

LC_{50} and fiduciary confidence limits are shown in Table 2. During the assay, behavioral alterations could be observed in all treatments except for control.

A total of 7 immune-genes covering immune-related pathways were studied in order to evaluate the direct effect of different synthetic acaricides on individual honey bees. Transcripts encoding for different genes were successfully retrotranscribed and amplified. Specificity of the amplified product was confirmed by single peaks in the melting curve analysis and confirmation of Tm values (Table 1).

Variability between samples was observed, however in some cases significant differences were recorded between treatments.

3.3. Effects of synthetic acaricides on expression of the genes encoding immune-related proteins

The patterns of gene expression of vitellogenin and lysozyme had a similar trend. No significant changes were detected in the expression levels of VG ($H = 1.60$; df = 4; $N = 45$; $p = 0.81$) (Fig. 1A), LYS ($H = 5.25$; df = 4; $N = 44$; $p = 0.26$) (Fig. 1B) or GLD ($H = 7.66$; df = 4; $N = 31$; $p = 0.10$) (Fig. 1C). In spite of a down-regulation of this gene under amitraz treatment and an up-regulation under flumethrin treatment were observed, they were not statistically significant. It may be due to the few amplified samples.

Phenoloxidase mRNA levels were significantly affected by the exposure to acaricides ($H = 9.53$; df = 4; $N = 39$; $p = 0.05$) (Fig. 1D). Its expression increased in response to flumethrin ($z = 2.71$; $p = 0.06$) when comparing with nontreated bees (Fig. 1D), but differences were not statistically significant since the p-value exceeds narrowly the acceptance limit.

3.4. Effects of synthetic acaricides on expression of the genes encoding antimicrobial peptides

In response to synthetic acaricides treatment, the expression patterns of defensin and abaecin (Fig. 2A and B) had the same trend, no significant expression changes were detected between bees treated with acaricides and control bees. However, significant differences were found in the expression of abaecin gene between coumaphos versus flumethrin and fluvalinate treatments ($H = 13.80$; df = 4; $N = 45$; $p = 0.008$), coumaphos reduced its expression but flumethrin and fluvalinate raised it ($z = 3.10$; $p = 0.01$ and $z = 3.30$; $p = 0.02$ respectively). When this comparison was made respect on defensin levels no significant differences were found ($p = 0.10$).

The mRNA level of hymenoptaecin (Fig. 2C) was also affected by treatments with acaricides ($H = 26.31$; df = 4; $N = 43$; $p \leqslant 0.0001$). It was significantly increased by flumethrin exposure as compared with the nontreated control bees ($z = 4.19$; $p \leqslant 0.001$) while other acaricides did not alter its expression. Significant differences were found in the expression of this gene between coumaphos and flumethrin treatments ($z = 4.47$; $p \leqslant 0.001$).

4. Discussion

This work demonstrated that median lethal concentration of the synthetic acaricides flumethrin, fluvalinate and coumaphos could impact on individual honey bee immunity by modifying the transcription of some genes encoding antimicrobial peptides and immunity-related enzymes.

Bioassays performed in adult worker bees in this study demonstrated that flumethrin increased hymenoptaecin and an up-regulation trend in phenoloxidase mRNA levels. This revealed that this acaricide may be impacting cellular and humoral immunity and immune signaling cascades. We suggest that these peptides could be involved in detoxification pathway, although further experimental

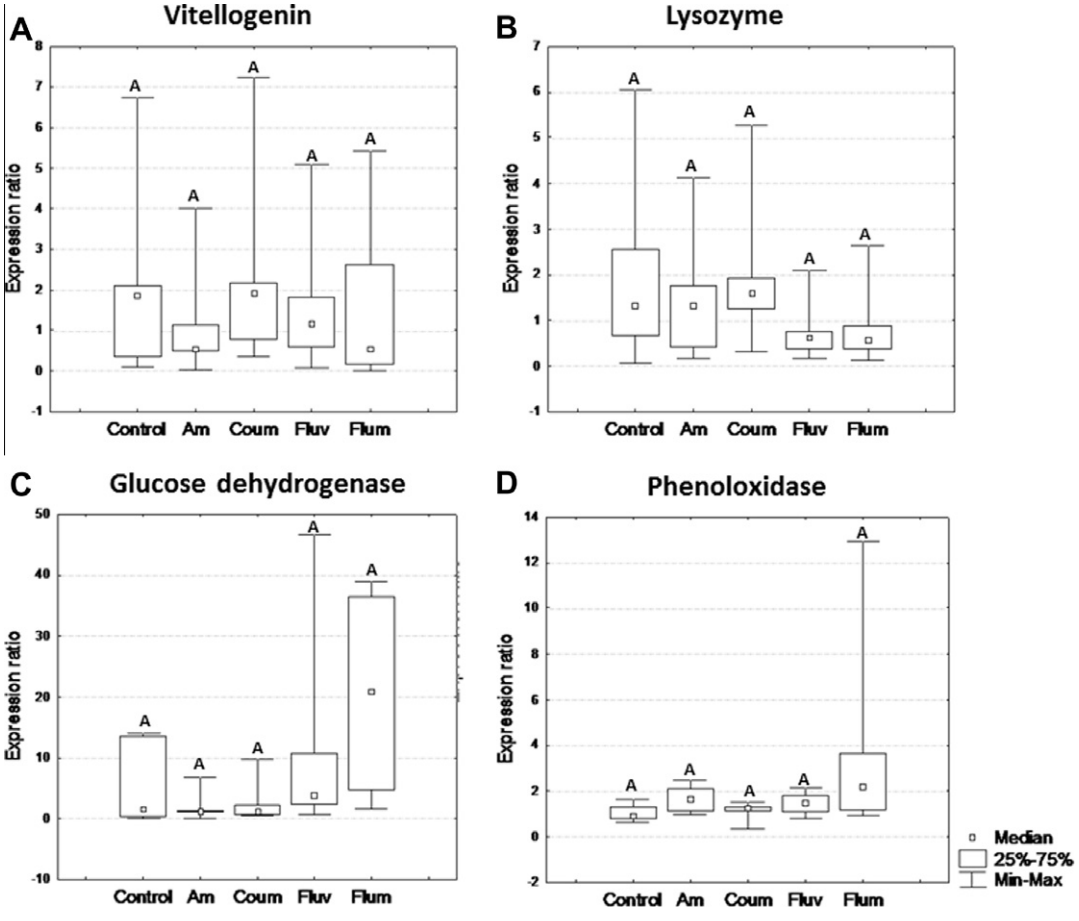

Fig. 1. The effects of acaricidal treatments on the expression of genes encoding the immunity-related proteins in 6-d-old worker bees. C refers to the control treatment. AM: bees exposed to Amitraz. COUM: Coumaphos treatment. FLUM: Flumethrin treatment. FLUV: Tau-fluvalinate treatment. The box plots with different letters are significantly different.

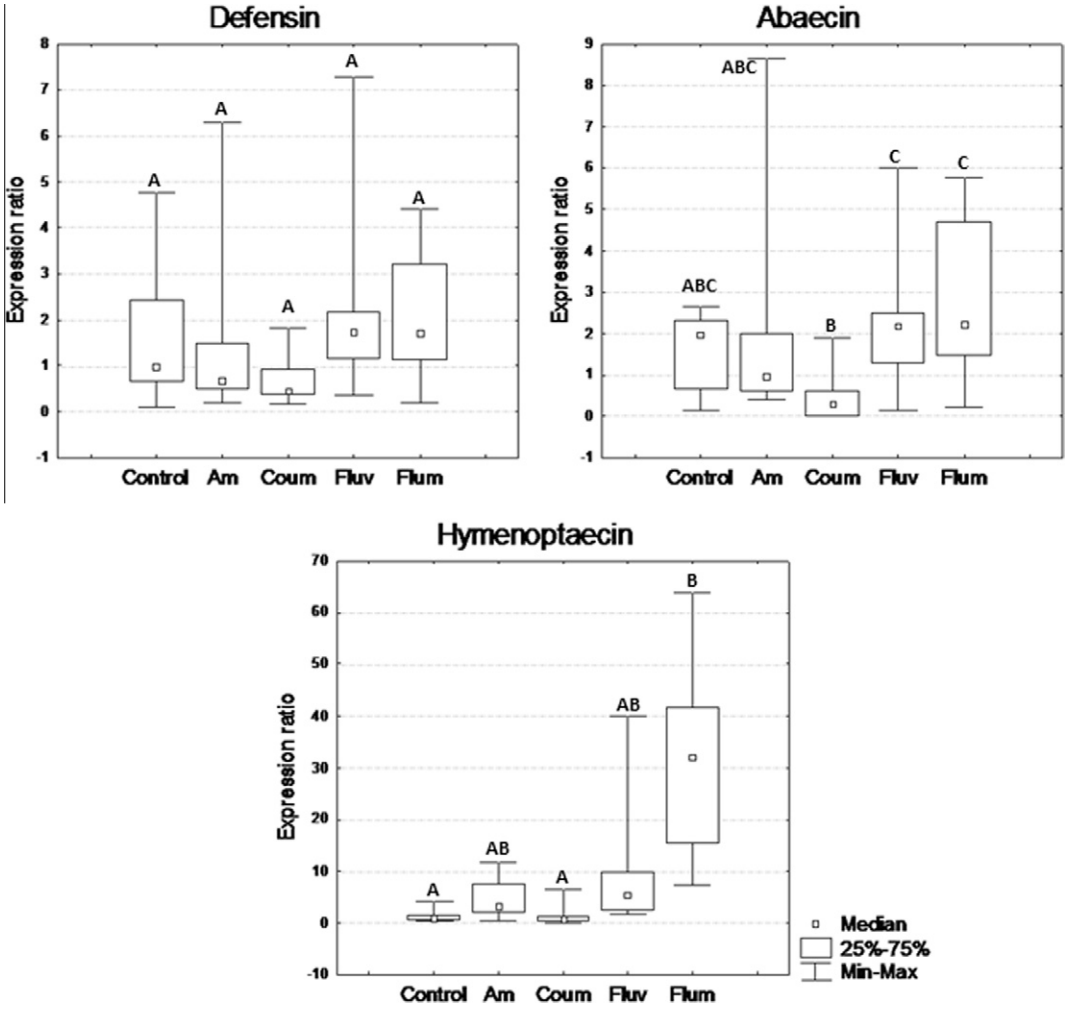

Fig. 2. The effects of acaricidal treatments on the expression of genes encoding the antimicrobial peptides in 6-d-old worker bees. C: control treatment. AM: Amitraz treatment. COUM: Coumaphos treatment. FLUM: Flumethrin treatment. FLUV: Tau-fluvalinate treatment. The box plots with different letters are significantly different.

assays must be performed to explain this up-regulation. Hymenoptaecin is an antimicrobial peptide, which is highly expressed in the bee hemolymph after challenge with bacterial infections (Casteels et al., 1993; Chan et al., 2009). The function of phenoloxidase is thought to be a part of the recognition system of foreignness in insect and is involved in cellular immunity (Decker and Jaenicke, 2004). Nevertheless, until this work, phenoloxidase and hymenoptaecin never have been proposed as participants in detoxifying cascades which could be involved in the tolerance to flumethrin in honey bees. It will be very interesting to test this hypothesis in further research works.

Also a trend of up-regulation produced by flumethrin and a down-regulation by amitraz exposure was observed in GLD expression, a gene involved in the cellular response during encapsulation reaction, but they were not statistically significant because the low sample size obtained during the amplification step, a repetition was made but we obtained the same result. It is not clear how much GLD influence has on immunity in honey bees but in lepidopterans, has been shown that its activity is detected in immune-activated plasmatocytes (Cox-Foster and Stehr, 1994). Thus, it may be used as a marker indicating initial activation of the cellular immune response has occurred (Lovallo and Cox-Foster, 1999). Our results demonstrated that cellular immunity may be compromised under a high exposure to amitraz.

Tau-fluvalinate belongs to the same family compound and has the same mode of action than flumethrin; however, different results were observed for this molecule. This may be due to flumethrin acts through an alternative pathway. Our results agree with those obtained by Boncristiani et al. (2012) in which the gene expression was not significantly different by tau-fluvalinate exposure. But it is important to notice that different doses were used.

Boncristiani et al. (2012) found, at field conditions, that antimicrobial peptides (abaecin, apidaecin, defensin, and hymenoptaecin) were not affected by acaricidal treatments (coumaphos, amitraz and tau-fluvalinate). We have demonstrated the same results in our local *A. mellifera* hybrid. However, it is important to notice that coumaphos and flumethrin/fluvalinate treatments seem to have opposite effects in the expression levels of the antimicrobial peptides, abaecin and hymenoptaecin. On the other hand, they also found that vitellogenin gene presented significant down regulation when bees were exposed to coumaphos, but in our results its expression was not influenced by any of the acaricides employed. It remains unclear the explanation of why they obtained that difference, perhaps the key to understand this response is the wide range of age of nurse bees collected from brood combs, when the experiment is performed in the field. The analysis of vitellogenin from pooled individuals is complex since the expression of these genes might also vary according to the age related task of worker

bees (Amdam and Omholt, 2003) even nurse bees are not as immunologically competent as foraging bee (Wilson-Rich et al., 2008), season (Dainat et al., 2012), presence of some pathologies (Amdam et al., 2004a; Antúnez et al., 2009) and moreover, the protein synthesis depends on the nutritional status (Cremonez et al., 1998). In our study, we employed nurse bees with the same age to keep under control these variables. In addition, the sensitivity to many acaricidal compounds also varies developmentally (vanBuren et al., 1992b). Vitellogenin is a storage protein that is utilized by workers for various metabolic purposes including the synthesis of brood food, ageing (Amdam and Omholt, 2002), and is involved in immunity through hormonal regulatory pathways (Munch et al., 2008). Furthermore is a zinc carrier (reviewed by Falchuk, 1998), in which zinc affects the number of functional haemocytes in honeybees. Zinc deficiency induces oxidative stress and apoptosis in several cell lines in mammals, including nerve and immune cells (reviewed by Mocchegiani et al., 2000; Amdam et al., 2004b). For that reason, Vg can act as a free-radical scavenger and decrease levels of oxidative stress (Amdam et al., 2004b; Seehuus et al., 2006). Lysozyme is known to be important in insect immunity against bacteria (Daffre et al., 1994; Lavine and Strand, 2002).

In this experiment we provided a pollen diet. Recently Alaux et al. (2011) have demonstrated that this nutrient had a positive influence on genes affecting longevity and the production of some antimicrobial peptides. We think that this is a variable to take into account to design future assays in laboratory conditions. However, since the same pollen diet was applied to treated and control bees, we think this point does not affect the obtained results.

Due to the lack of information about the effects of synthetic acaricides on the immune system at the beginning of our experiments, LC_{50} was employed as a ceiling to our analysis. However, they only provide information about the impact of the overexposure to a miticide.

These results added to the potential of honey bee colonies to accumulate acaricide residues and their metabolites at levels that may be detrimental to honey bee health and productivity, we consider that there is very important to test sublethal effect of pesticides to gain information about the long-term effects that produce these widely used compounds in beekeeping.

V. destructor has a negative impact on colony health and it is undisputable that its control is necessary to avoid colony losses. However, there is no consensus on the best control method that reduces mite fitness, but being minimally harmful on its host with low persistence along time to avoid synergistic or secondary effects in the future. Our results provide data to expand knowledge on this research line.

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