



# Sub-lethal doses of neonicotinoid and carbamate insecticides reduce the lifespan and alter the expression of immune health and detoxification related genes of honey bees (*Apis mellifera*)

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**ABSTRACT.** Honey bees (*Apis mellifera*) are exposed to sublethal doses of insecticides, but little is known about insecticide effects on their survivorship associated to health-related gene expression. To test the effect of sublethal doses of clothianidin, imidacloprid and carbaryl on the lifespan and health of honey bees, workers were orally and topically exposed to LD5 doses of these insecticides. The survivorship of treated bees was monitored and the expression of three immune-related genes, hymenoptaecin (AmHym), basket (AmBask) and lysozyme (AmLyso2) was analyzed at 24 and 72 hours post treatment (hpt), as well as that of the antioxidant-related gene vitellogenin (AmVit2), the poly-U binding factor (AmPuf68), and the detoxification gene cytochrome P450 (AmCYP9Q3). The three insecticides significantly reduced the length of life of bees but the mode of application did not affect survivorship. AmHym, AmBask and AmVit2 expression was significantly down-regulated at 72 hpt in bees treated with clothianidin and imidacloprid, indicating immunosuppression. However, AmLyso2, AmCYP9Q3 and AmPuf68 were significantly up-regulated. The down-regulation of AmVit2 could have caused decreased resistance to oxidative stress. AmPuf68 expression could be associated with increased protection against xenobiotics. AmCYP9Q3 was up-regulated at 24 and 72 hpt

in oral exposures, but only until 72 hpt in topical exposures, indicating faster sensitivity towards detoxification mechanisms in oral treatments. This study demonstrated detrimental effects of sublethal doses of clothianidin, imidacloprid and carbaryl on honey bee survivorship, immunity and antioxidant mechanisms, and an induction of defense and detoxification responses that could be physiologically costly to the bees.

**Key words:** Honey bees; Neonicotinoid insecticides; Carbamates; Sublethal exposure; Immunity; Gene expression

## INTRODUCTION

A decline in abundance and diversity of wild bees as well as honey bees (*Apis mellifera*) has been recently reported from several regions of the world (Potts et al., 2010). The magnitude of this pollinator crisis is believed to not only have a deep impact on agriculture and its related economy but also on plant diversity. For honey bees in particular, massive losses of colonies have been termed Colony Collapse Disorder (CCD) (Kevan et al., 2007). Parasites and pesticides are among the factors most frequently associated with honey bee colony mortality (Guzman-Novoa, 2016). For pesticides specifically, neonicotinoids insecticides are frequently blamed for honey bee colony losses (Van der Sluijs et al., 2013), but many other insecticides are commonly used in managed ecosystems that may have an impact on honey bee health, such as carbamates (Johnson et al., 2010). Traditionally, measurement of the damage by pesticides to honey bees has relied largely on the determining their toxicity level by estimating their lethal dose (LD<sub>50</sub>). However, estimations of lethal doses may only be a partial measure of the deleterious effects of pesticides. In addition to direct mortality caused by the acute toxicity of pesticides, their sublethal effects on bees' physiology and behavior must be considered for a more comprehensive analysis of their impact. The role of pesticides in honey bee colony losses, with their sublethal effects, has recently regained consideration (Mullin et al., 2010). For example, sublethal levels of neonicotinoids have been shown to impair the learning abilities of honey bees and to possibly inhibit their immune systems (Blacqui re et al., 2012).

A considerable number of genes of the immune system in honey bees have been identified. The honey bee immune system has been found to be very similar to that of *Drosophila* and the same signaling pathways found in *Drosophila* are also at work in *A. mellifera* (Evans, 2006). However, not much is known about how sublethal exposure to different classes of insecticides affect the expression of immune, health and detoxification related genes in the exposed bees. It is likely that exposure to sublethal doses of pesticides might impair the bees' immune, antioxidant and detoxification systems. As a result, bees might not be able to defend themselves from parasites and diseases or could be stressed out, leading to early mortality. Alaux et al. (2010) found physiological evidence that sublethal doses of imidacloprid and the parasitic microsporidian *Nosema* can interact synergistically to affect bee health negatively, including physiological changes initiated by sublethal dose exposure that decreased bee tolerance toward *Nosema* infection.

The objectives of this study were to determine the effects of sublethal doses of three widely used insecticides that have been associated with cases of CCD, clothianidin, imidacloprid and carbaryl, on honey bee survivorship and gene expression related to immunity, antioxidant processes, longevity and detoxification. The immune related genes chosen were *hymenoptaecin* (*AmHym*), *lysozyme* (*AmLyso2*) and *basket* (*AmBask*) (Evans, 2006). *Vitellogenin* (*AmVit2*) was selected because it is associated with antioxidant processes and longevity (Amdam et al., 2004) and the *poly-U binding factor 68 kDa* (*AmPuf68*) linked to pre-mRNA splicing and stem cell proliferation (Wang et al., 2013), was included as health-related gene. *Cytochrome P450* (*AmCYP9Q3*) (Mao et al., 2011), was selected as detoxification gene.

## MATERIALS AND METHODS

### Honey bee sources

Experiments were conducted at the University of Guelph's Honey Bee Research Center in Ontario, Canada. Honey bee colonies containing queens of the Buckfast strain were used as a source of workers. The colonies were free of brood diseases and were not treated to control parasitic mites prior to the experiments because they

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had very low levels of *V. destructor* infestation (<1%). To obtain worker bees of the same age, frames of capped brood from the source hives were incubated at  $32 \pm 2^\circ\text{C}$  and  $50 \pm 10\%$  RH overnight in emergence cages ( $50 \times 7 \times 25$  cm). After 24 h, the newly emerged bees were immediately used for the experiments.

## Insecticides

Three insecticides of technical analytical grade (purity >99%), imidacloprid (1-(6chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine), clothianidin (1-(2-chloro-1, 3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine), and carbaryl (1-naphthyl-N-methyl carbamate), were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Pesticides were dissolved with sterile dH<sub>2</sub>O to prepare stock solutions (1000 ng/ $\mu$ l; pesticide/solvent). To prepare the doses for oral treatments, the stock solutions were diluted in serial dilutions of 50% sucrose syrup. For the doses of contact application, the serial dilutions were made with sterile dH<sub>2</sub>O.

## Experiment 1: Effect of sub-lethal doses of insecticides on the survivorship of honey bees

Worker bees were challenged orally and topically with sublethal doses of clothianidin, imidacloprid, and carbaryl. In previous studies, sublethal doses of pesticides tested were  $5 \times 10^0$  to  $1.5 \times 10^2$  times lower than their LD<sub>50s</sub> (Decourtye et al., 2004; Aliouane et al., 2009), or the LD<sub>5</sub>, LD<sub>10</sub> and LD<sub>25</sub> values (Mackenzie and Winston, 1988). Based on that range of options, in the current study, LD<sub>5</sub> was selected for the sublethal dose to be tested (Table 1). Preliminary studies were conducted to determine LD<sub>5</sub> for the three insecticides (Tarek H, Hamiduzzaman MM, Morfin N and Guzman-Novoa E, Unpublished results).

For oral exposure, newly emerged bees were food deprived for 2 h before administering the insecticides. After that time, each bee was individually fed 10  $\mu$ l of 50% sucrose solution containing a sublethal dose of one of the insecticides (LD<sub>5</sub>) with the aid of a micropipette (Eppendorf, Mississauga, Ontario, Canada) until 30 bees had been fed. Table 1 shows the actual doses of each insecticide received by the bees. Control bees received only 10  $\mu$ l of 50% sucrose syrup. After consuming the solution, each group of 30 bees per treatment was placed in a wooden cage ( $12.7 \times 8.5 \times 14.5$  cm) with a 3 mesh/cm wire screened wall on both sides. The cage was kept at an incubator ( $32 \pm 2^\circ\text{C}$ ,  $50 \pm 10\%$  RH) and the bees were provided with two gravity feeders, one containing 20 ml of a 50% sucrose solution, and the other containing dH<sub>2</sub>O; the bees were permitted to feed *ad libitum*.

For topical exposure, 2  $\mu$ l of each insecticide solution in dH<sub>2</sub>O containing the sublethal dose were administered individually as a single application on the dorsal surface of a worker's thorax using a micropipette. Control bees only received 2  $\mu$ l of dH<sub>2</sub>O. Treated bees were kept in wooden cages as described above.

Treated bees in the cages were observed until all of them died. The number of live and dead bees were recorded at days 0, 7, 14, 21, 27, 33, 35, 37, 39 and 41, and percentages of surviving bees for those days were calculated. Median survival times (MST) of honey bee workers were also calculated using Probit analysis. Three replications per treatment per insecticide were conducted.

**Table 1.** Sub-lethal doses (LD<sub>5</sub>) in ng/bee and median survival time (MST) in days for honey bee workers that were treated orally or topically with three insecticides. Values were calculated by Probit analysis. Treatments with non-overlapping confidence intervals are considered significantly different.

Application mode	Treatment	LD <sub>5</sub> dose* (95% CL**)	MST (95% CL**)
Oral	Control	0.00	30 (29.6-31.3)
	Imidacloprid	39.5 (46.6-29.2)	21 (20.2-22.5)
	Clothianidin	0.63 (0.71-0.59)	26 (25.2-26.8)
	Carbaryl	205.0 (212-196)	28 (24.5-29.4)
Contact	Control	0.00	30 (29.1-31.9)
	Imidacloprid	27.7 (36.5-21.7)	27 (26.2-28.6)

Clothianidin	14.7 (21.3-9.8)	25 (24.5-26.7)
Carbaryl	112.0 (119-107)	23 (21.2-23.7)

(Note: \*Actual doses received by individual bees; \*\*CL: Confidence Limits)

## Experiment 2: Gene expression in honey bees exposed to sublethal doses of insecticides

The expression level of three immune related genes, two health-related genes and one detoxification gene (Table 2) was measured in worker bees at 0, 24 and 72 hours post treatment (hpt) after orally and topically exposing adult bees as described above to the LD<sub>5</sub> doses of the three insecticides (Table 1). For each treatment, 15 workers were obtained from incubating cages and placed in groups of three individuals within each of five, 2 ml eppendorf tubes (Eppendorf, Mississauga, Ontario, Canada). Similarly, untreated bees were collected as a control. Collected samples were stored immediately at -70°C until analysis.

**Table 2.** Primers used for amplification of the target and reference control genes.

Gene product	Gene designation	Primer sequence (5' - 3')	Product length	Source
Hymenoptaein	<i>AmHym*</i>	F: CTCCTCTGTGCCGTTGCATA R: GCGTCTCCTGTCATTCCATT	200 bp	Evans (2006)
Basket	<i>AmBask*</i>	F: AGGAGAACGTGGACATTTGG R: AATCCGATGGAAACAGAACG	243 bp	Evans (2006)
Lysozyme2	<i>AmLyso2*</i>	F: CCAAATTAACAGCGCCAAGT R: GCAATTCTTCACCCAACCAT	166 bp	Evans (2006)
Vitellogenin	<i>AmVit2*</i>	F: ACGACTCGACCAACGACTT R: AACGAAAGGAACGGTCAATTCC	494 bp	Guidugli et al. (2005)
poly-U-binding factor 68 kDa	<i>AmPuf68*</i>	F: CAAGACCTCCAACACTAGCATG R: CAACAGGTGGTGGTGGTG	201 bp	Hamiduzzaman et al. (2012)
CytochromeP450	<i>AmCYP9Q3*</i>	F: GTTCCGGGAAAATGACTAC R: ACTCTCGACGCACATCCTG	296 bp	Mao et al. (2011) This study
Ribosomal Protein S5	<i>RpS5**</i>	F: AATTATTTGGTCTGCGAATTG R: TAACGTCCAGCAGAATGTGGTA	115 bp	Evans (2006)
Glyceraldehyde 3-phosphate dehydrogenase2	<i>GAPD2**</i>	F: GATGCACCCATGTTTGTGG R: TTTGCAGAAGGTGCATCAAC	203 bp	Thompson et al. (2007)

(Note: F, Forward primer and R, Reverse primer. \* Target and \*\* Reference control genes).

## RNA extraction and cDNA synthesis

Total RNA was extracted by homogenizing five frozen bees per sample in extraction buffer as per Chen et al. (2000). The homogenates were extracted twice with chloroform, and the RNA was precipitated using LiCl. The amount of total RNA extracted was determined with a spectrophotometer (Nanovue GE Healthcare, Cambridge, UK). For cDNA synthesis, 2 µg of total RNA was reverse-transcribed using Oligo (dT)18 and M-MuLV RT with the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Burlington, Ontario), following the instructions of the manufacturer.

## PCR reactions

All PCR reactions were done with a Mastercycler (Eppendorf, Mississauga, Ontario, Canada). Each 15  $\mu$ l of reaction contained 2  $\mu$ l of cDNA, 1.5  $\mu$ l of 10X PCR buffer (New England BioLabs, Pickering, Ontario), 0.5  $\mu$ l of 10 mM dNTPs (deoxynucleoside triphosphates) (Bio Basic Inc., Markham, Ontario), 0.25-2  $\mu$ l of forward and reverse primers for the honey bee ribosomal protein, *RpS5* as reference gene, or *glyceraldehydes-3-phosphate dehydrogenase2* (*GAPD2*) (Thompson et al., 2007) and 1.5-2  $\mu$ l of each primer for the target gene. Additionally, the reaction contained 0.2  $\mu$ l of 5 U/ $\mu$ l Taq polymerases, and 5.3-7.3  $\mu$ l of sterile dH<sub>2</sub>O to adjust the final volume. *RpS5* was used as reference gene with *AmHym*, *AmBask*, *AmLyso2*, and *AmPuf68*. *GAPD2* was used as reference gene with *AmVit2* and *AmCYP9Q3*. Primers sequences are listed in Table 2. Amplification conditions for *AmHym*, *AmBask*, *AmLyso2* and *AmPuf68* were 94°C for 3 min, followed by 35 denaturing cycles at 94°C for 30 s, annealing at 55°C for 60s and 60s at 72°C, and a final extension step at 72°C for 10 min. Amplification conditions for *AmVit2* and *AmCYP9Q3* were the same, except that the annealing temperature was 58°C.

## Separation and semi-quantification of PCR products

PCR products were separated by electrophoresis in 1.1% agarose gels and stained with ethidium bromide. A 100 bp DNA ladder (Bio Basic Inc., Markham, Ontario) was used to identify the size of the amplicons. The intensity of the amplified bands was measured in pixels using the Scion Image (Scion Corporation, Frederick, MD, USA) as per Dean et al. (2002). The ratio of band intensity between the target gene and the reference gene was calculated to determine relative expression units (REUs) according to Hamiduzzaman et al. (2012). A similar ratio of band intensity between the target gene and the reference gene was observed in all samples. To determine whether quantification at 35 amplification cycles was not affected by signal saturation of the band intensities, randomly selected samples with high, medium and low REUs were also quantified in the same manner with fewer amplification cycles, and the pattern of expression based on the REU values were not significantly different when 25, 30 and 35 amplification cycles were used ( $F_{2,15}=0.30$ ,  $p=0.75$ ). Thus, we chose to analyze results at 35 cycles because in most cases the relationship between the number of cycles and molecules is relatively linear at 35 cycles when semi-quantitative RT-PCR is used, which provides high amplification efficiency.

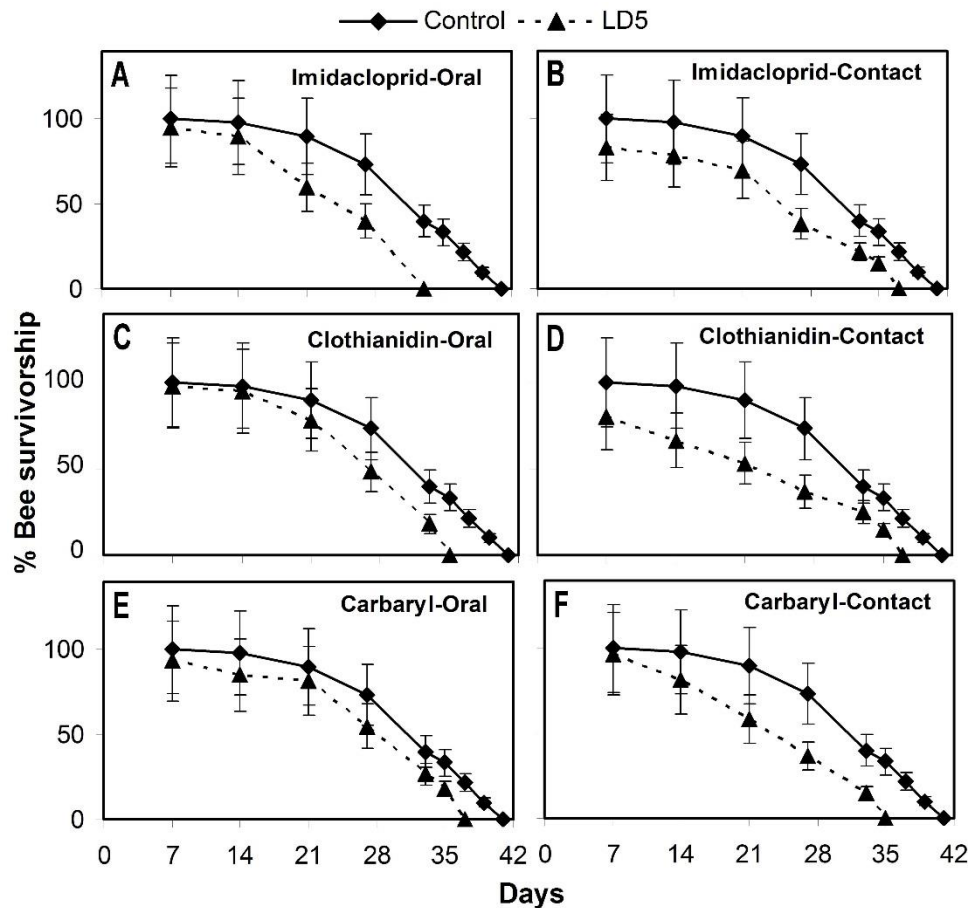
## Statistical analysis

The percent bee mortality caused by the insecticides tested was calculated for the various time points and the data were arcsine square root transformed and subjected to analysis of variance (ANOVA). The median survival time values were determined by Probit analysis using the US Environmental Protection Agency Statistical Program, version 1.5 (USEPA, 1992). Gene expression data of the three insecticides at 24 and 72 hpt were analyzed by ANOVA. These procedures were performed with the package IBM-SPSS v. 23 (SPSS Inc., Chicago, IL, USA).

## RESULTS

### Effect of sub-lethal doses of insecticides on honey bee survivorship

The three insecticides significantly reduced the length of life of exposed bees compared with control bees ( $F_{3, 232}=7.49$ ,  $P<0.0001$ ), but no differences were found between insecticides for this variable. The mode of application did not affect bee survivorship ( $F_{1, 232}=0.89$ ,  $P=0.35$ ) and no significant interactions were detected between treatment and application mode ( $F_{3, 232}=1.23$ ,  $P=0.30$ ). There were significant differences in the percentage of surviving bees over time between control and insecticide treatments ( $F_{3, 232}=90.4$ ,  $P<0.0001$ ). The cumulative mortality rate increased with time in all experimental groups until day 39, which was the day when the last treated bee died; some control bees remained alive until day 41 (Figure 1). Additionally, the LD<sub>5</sub> doses of the three insecticides significantly reduced the MST of treated bees compared to the MST of control bees (Table 1).



**Figure 1.** Mean percentage ( $\pm$  SE) of surviving bees over time (days) after exposure to sublethal doses (LD5) of three insecticides: A) imidacloprid orally-treated bees, B) imidacloprid topically-treated bees, C) clothianidin orally-treated bees, D) clothianidin topically-treated bees, E) carbaryl orally-treated bees, F) carbaryl topically-treated bees. Control bees were not exposed to insecticides and three repetitions of 20 bees were conducted for each treatment.

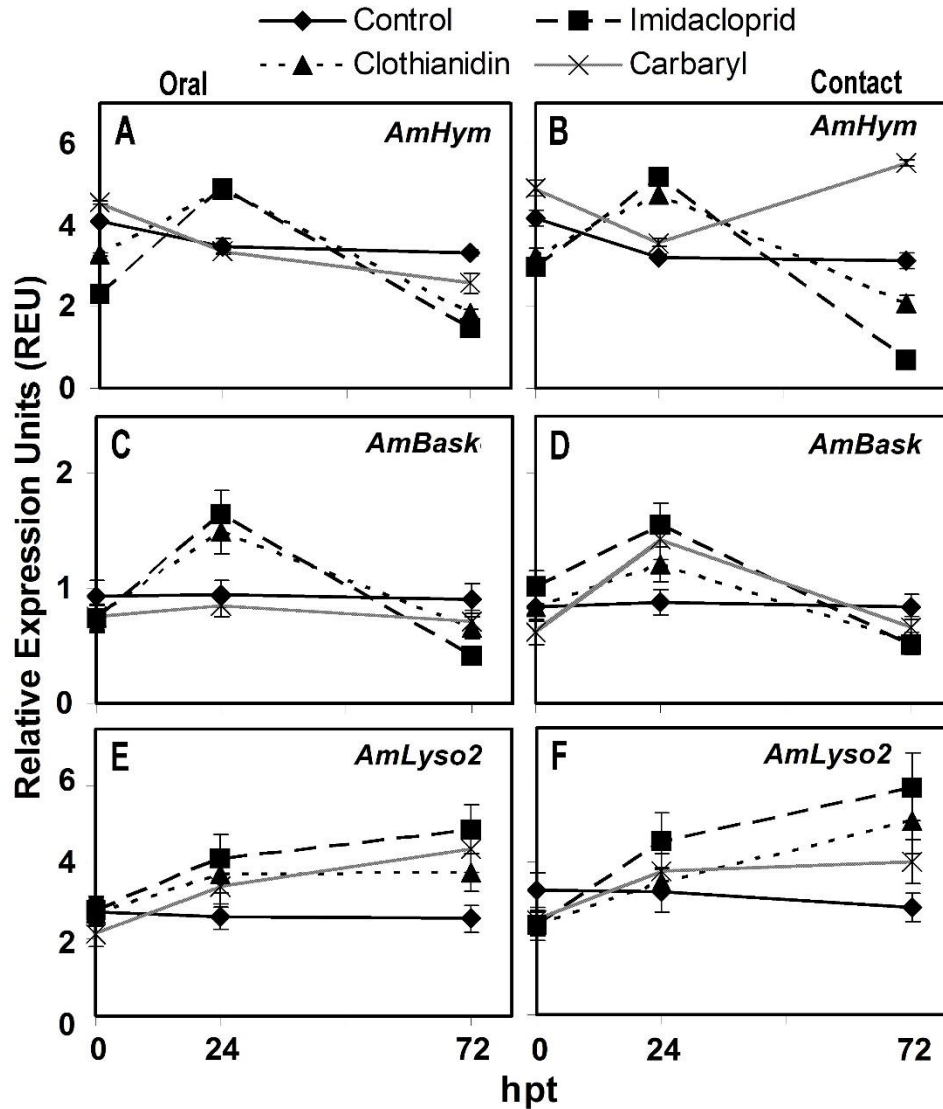
### Immune related gene expression

For control and carbaryl treatments, the expression of *AmHym* was similar at 24 hpt. However, for imidacloprid and clothianidin treated bees, the expression of *AmHym* was significantly higher than that of control bees at 24 hpt in both oral ( $F_{(3,20)}=28.67$ ,  $P<0.0001$ ; Figure 2A) and contact exposures ( $F_{(3,20)}=52.54$ ,  $P<0.0001$ ; Figure 2B). At 72 hpt, again, *AmHym* expression of control bees did not change from that observed at 24 hpt for both exposure modes, but relative to the control treatment, the expression of this gene was significantly down-regulated in clothianidin and imidacloprid treated bees. *AmHym* expression in the carbaryl treatment did not change at 72 hpt when bees were treated orally but was significantly up-regulated when the bees were treated topically ( $F_{(3,20)}=25.65$ ,  $P<0.0001$ ;  $F_{(3,20)}=185.64$ ,  $P<0.0001$ , respectively; Figures 2A and 2B).

For control and carbaryl treatments, the expression of *AmBask* was similar at 24 hpt. However, for imidacloprid and clothianidin treated bees, the expression of *AmBask* was significantly higher than that of control bees at 24 hpt in both oral ( $F_{(3,20)}=14.93$ ,  $P<0.0001$ ; Figure 2C) and contact exposures ( $F_{(3,20)}=34.96$ ,  $P<0.0001$ ; Figure 2D). At 72 hpt, again, *AmBask* expression of control bees did not change from that observed at 24 hpt for both exposure modes, but relative to the control treatment, the expression of this gene was significantly down-regulated in clothianidin and imidacloprid treated bees. *AmBask* expression in the carbaryl treatment decreased significantly at 72 hpt when bees were treated orally but did not change when bees were treated topically ( $F_{(3,20)}=10.56$ ,  $P<0.0001$ ,  $F_{(3,20)}=4.95$ ,  $P=0.010$ , respectively; Figures 2C and 2D).

The expression of *AmLyso2* did not differ among treatments at 24 hpt in both oral ( $F_{(3,20)}=2.23$ ,  $P=0.116$ ) and contact applications ( $F_{(3,20)}=0.70$ ,  $P=0.561$ ; Figures 2E and 2F). At 72 hpt, *AmLyso2* expression of control bees

did not change from that observed at 24 hpt for both exposure modes, but the expression of this gene was significantly up-regulated in clothianidin and imidacloprid treated bees. *AmLyso2* expression in the carbaryl treatment increased significantly at 72 hpt when bees were treated orally but did not change when bees were treated topically ( $F_{(3,20)}=35.45$ ,  $P<0.0001$ ,  $F_{(3,20)}=4.16$ ,  $P=0.019$ , respectively; Figures 2E and 2F).



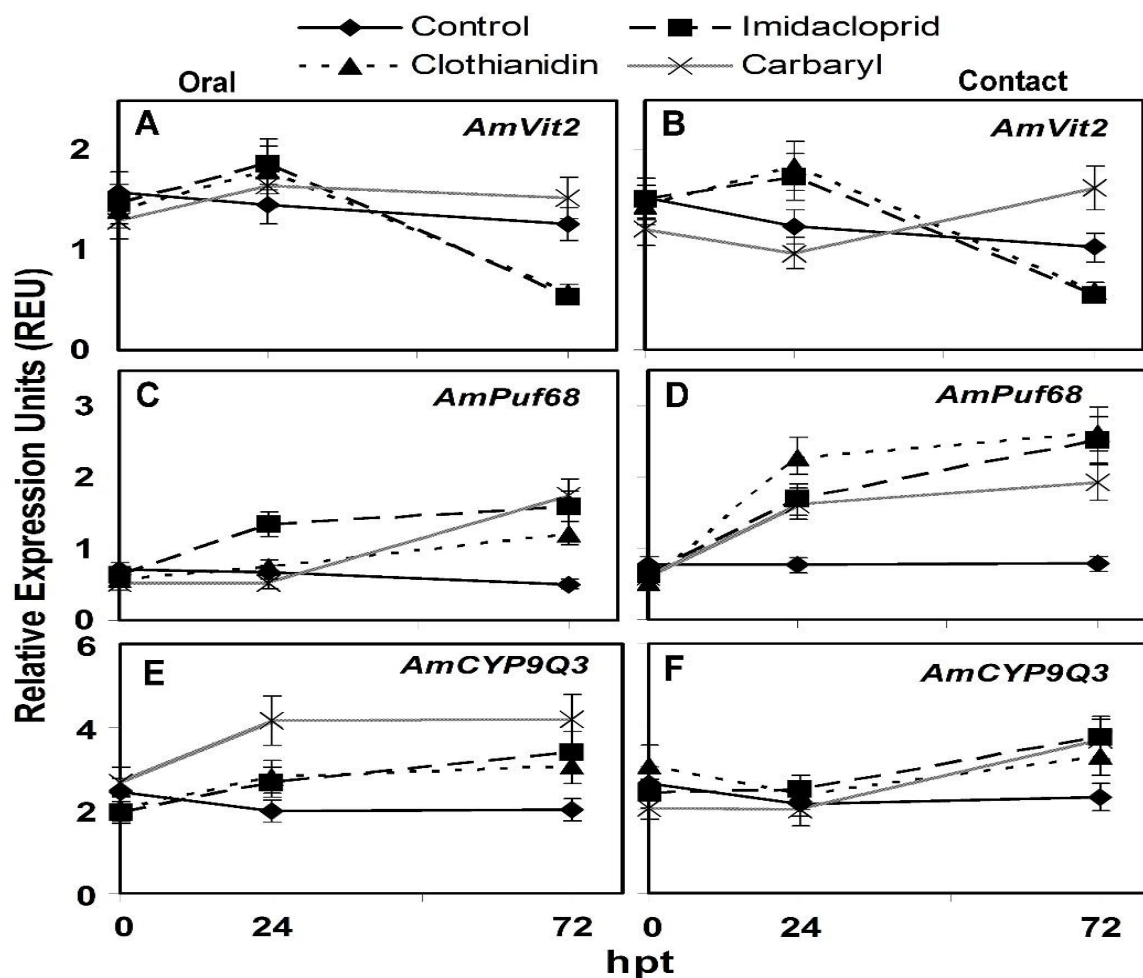
**Figure 2.** Mean relative expression units (REU  $\pm$  SE) of immune related genes in worker bees treated orally or topically with sublethal doses (LD5) of three insecticides from 0 to 72 hours post treatment (hpt): A) *AmHym* for orally-treated bees, B) *AmHym* for topically-treated bees, C) *AmBask* for orally-treated bees, D) *AmBask* for topically-treated bees, E) *AmLyso2* for orally-treated bees, F) *AmLyso2* for topically-treated bees. Control bees were not exposed to insecticides and three repetitions of 20 bees were conducted for each treatment.

### Health and detoxification related gene expression

For control and carbaryl treatments, the expression of *AmVit2* was similar at 24 hpt. However, for imidacloprid and clothianidin treated bees, the expression of *AmVit2* was significantly higher than that of control bees in both oral ( $F_{(3,20)}=4.77$ ,  $P=0.011$ ; Figure 3A) and contact exposures ( $F_{(3,20)}=10.53$ ,  $P<0.0001$ ; Figure 3B). At 72 hpt, *AmVit2* expression of control bees did not change from that observed at 24 hpt for both exposure modes, but relative to the control treatment, the expression of this gene was significantly down-regulated in clothianidin and imidacloprid treated bees. However, *AmVit2* expression in the carbaryl treatment increased significantly at 72 hpt when the bees were treated topically ( $F_{(3,20)}=27.46$ ,  $P<0.0001$ ; Figure 3B).

The expression of *AmPuf68* was significantly up-regulated at 24 hpt for the oral application in imidacloprid treated bees compared to the other treatments ( $F_{(3,20)}=64.54$ ,  $P<0.0001$ ; Figure 3C). However, for the contact application, the expression of *AmPuf68* was significantly higher in imidacloprid, clothianidin and carbaryl treated bees than in control bees ( $F_{(3,20)}=42.90$ ,  $P<0.0001$ ; Figure 3D). At 72 hpt, *AmPuf68* expression of control bees did not change from that observed at 24 hpt for both exposure modes, but it was significantly up-regulated in clothianidin, imidacloprid and carbaryl treated bees ( $F_{(3,20)}=44.13$ ,  $P<0.0001$ ,  $F_{(3,20)}=213.12$ ,  $P<0.0001$ , respectively; Figures 3C and 3D).

The bees orally treated with the three insecticides showed significant up-regulation of *AmCYP9Q3* at 24 hpt, with the highest expression observed in carbaryl treated bees ( $F_{(3,20)}=146.48$ ,  $P<0.0001$ ; Figure 3E). However, the expression of this gene did not differ among treatments at 24 hpt for contact applications ( $F_{(3,20)}=0.66$ ,  $P=0.586$ ; Figure 3F). At 72 hpt, *AmCYP9Q3* expression of control bees did not change from that observed at 24 hpt for both exposure modes, but the expression of this gene was significantly up-regulated in imidacloprid, clothianidin and carbaryl treated bees for both oral and contact application treatments ( $F_{(3,20)}=133.52$ ,  $P<0.0001$ ,  $F_{(3,20)}=40.50$ ,  $P<0.0001$ , respectively; Figures 3E and 3F).



**Figure 3.** Mean relative expression units (REU  $\pm$  SE) of health and detoxification related genes in worker bees treated orally or topically with sublethal doses ( $LD_5$ ) of three insecticides from 0 to 72 hours post treatment (hpt): A) *AmVit2* for orally-treated bees, B) *AmVit2* for topically-treated bees, C) *AmPuf68* for orally-treated bees, D) *AmPuf68* for topically-treated bees, E) *AmCYP9Q3* for orally-treated bees, F) *AmCYP9Q3* for topically-treated bees. Control bees were not exposed to insecticides and three repetitions of 20 bees were conducted for each treatment.



## DISCUSSION

### Honey bee survivorship

Worker bees treated orally or topically with LD<sub>5</sub> doses of the three insecticides lived significantly less than control bees, which was measured in different ways (length of life, MST and survival over time). The length of life of treated bees decreased between 2 and 8 d (7-27% reduction). These findings coincide with what has been reported in the literature for carbaryl and other insecticides. For example, bees treated with a LD<sub>5</sub> of diazinon, carbaryl, and resmethrin lived between 7% and 25% shorter lives than control bees (Mackenzie and Winston, 1988). Also, Wu et al. (2011) found that adult longevity decreased 4d in bees exposed to residues of imidacloprid, clothianidin, and aldicarb in contaminated brood comb during development, although compared to our study, Wu et al. (2011) used doses that were at least five times higher than those used by us for clothianidin and imidacloprid. López et al. (2017) reported that different pesticides, including clothianidin, could negatively affect bee survivorship, but again, these authors used doses of clothianidin 12 times higher than those used in this study. Williamson et al. (2014) observed that bees fed clothianidin suffered significantly greater mortality than control bees, but once more, the doses used in their study, were five times higher compared to this study. Therefore, this study confirms results of detrimental effects of neonicotinoids and carbaryl on the lifespan of bees, but using the lowest doses so far tested of these insecticides.

### Immune related gene expression

The expression of *AmHym* in treated bees was significantly higher at 24 hpt and significantly lower at 72 hpt than that of control bees when they were treated with both neonicotinoid insecticides regardless of mode of exposure. This temporal variance in *AmHym* expression has been reported from other studies using biotic factors as inducers. For example, the expression of *AmHym* was increased within the first hour when honey bees or bumble bees were challenged with *E. coli*, but after 24 h, the expression level of the gene was similar or lower to that of the control treatment (Erler et al., 2011). Similarly, a long-term reduction in the expression of *AmHym* was found when honey bees were challenged with *V. destructor* mites (Hamiduzzaman et al., 2012). Other studies of bees exposed to insecticides and pathogens have found contradictory results. For example, Collison et al. (2017) found an up-regulation of *AmHym* in bees treated for 2 to 168 h with imidacloprid and then challenged with lipopolysaccharides from *E. coli*. In another study, no effect on *AmHym* regulation was found in bees treated with thiacloprid, but a down-regulation was observed in bees exposed to the insecticide and then challenged with *P. larvae* (Siede et al., 2017).

The expression pattern of *AmBask* was almost identical to that of *AmHym*. *AmBask* transcription was significantly increased at 24 hpt and significantly decreased at 72 hpt for workers treated orally and topically with imidacloprid and clothianidin. To the best of our knowledge, the impact of neonicotinoid insecticides on the expression of this gene is reported for the first time. Like in the case of *AmHym*, similar patterns of *AmBask* expression have been observed when insects are challenged with biotic agents. For example, a significant down-regulation of *AmBask* was observed within 8 hpt when Erler et al. (2011) injected *E. coli* to bumble bees (*B. terrestris*). For abiotic factors, acaricides like thymol and coumaphos caused decreased expression of *AmBask* in honey bees 30 days post application of the acaricides (Boncristiani et al., 2012).

*AmBask*, as part of JNK signaling pathway, can activate melanization and the production of antimicrobial peptides when challenged with pathogens (Evans, 2006). Similarly, *AmHym* regulates the production of hymenoptaecin, an antimicrobial peptide synthesized after activation of the Toll and Imd pathways, which also leads to activation of components of the JNK signaling pathway (Broderick et al., 2009). The findings of the current study suggest that the JNK pathway might have been induced by a chemical molecule in the same manner that microorganisms induce it. Reduction in the expression of *AmBask* could perhaps contribute to reduced longevity of honey bees by decreasing their ability to activate immune signaling pathways and to defend themselves against pathogens through the synthesis of AMPs. Also, it is possible that the drop in expression observed in *AmHym* and *AmBask* at 72 hpt may have been due to the effect of metabolite compounds derived from the insecticides' molecules, which are produced after insecticides are metabolized. Some metabolites of imidacloprid, such as 4-hydroxy imidacloprid and olefin-imidacloprid have insecticidal properties which are distinct from those of the parent compound. For instance, olefin-imidacloprid was about 16 times more active than imidacloprid against the cotton aphid (Nauen et al., 1998). Additionally, many synthetic insecticides increase oxidative stress, and this could have severe impacts on the production of some AMPs in insects (James and Xu, 2011). Therefore, metabolite compounds of neonicotinoids may act synergistically becoming more toxic. It is possible that in this study, they may have similarly affected the expression of *AmHym*

and *AmBask* over time. It seems that initially (24 hpt), the exposed bees responded with an up-regulation of these genes to produce AMPs as a defense mechanism, but at 72 hpt, the neonicotinoid insecticides ended up suppressing the expression of the genes, which may have resulted in immunosuppression and reduced survivorship.

The pattern of expression of *AmHym* in bees orally treated with carbaryl was very similar to that in control bees, whereas in topical applications, the gene was up-regulated at 72 hpt relative to the control treatment. Similarly, *AmBask* expression was not affected in bees treated orally with carbaryl, but responded temporarily to the insecticide in topical applications with elevated expression at 24 hpt. Clearly, carbaryl induced the activation of *AmBask* more by contact application than by oral ingestion, probably because of the topical nature of its mode of action (Simon, 2014). Degradation of carbaryl by detoxification enzymes could be rapid when ingested and thus, the insecticide may have been at very low levels after ingestion to induce *AmBask* or *AmHym* expression in the bees.

The up-regulation of *AmLyso2* was triggered by neonicotinoid insecticides, particularly imidacloprid at 72 hpt, although a similar effect but to a lesser degree, was caused by carbaryl. The difference in up-regulation levels between neonicotinoid and carbaryl treatments in topical applications may have been due to the possibility that molecules of neonicotinoids rapidly reach their sites of action compared with the carbaryl molecule. This might have led to a faster and stronger induced activity in *AmLyso2*. Most insecticides are metabolized by oxidative reaction, and *AmLyso2*, as a cellular immune gene, is associated to phagocytosis, which involves the production of reactive oxygen species (ROS). ROS plays an important role in the oxidative reaction to insecticide toxicity (Broderick et al., 2009), which could explain differences in expression levels due to different insecticides tested. In addition, as mentioned before in this study, metabolizing neonicotinoid insecticides produce more toxic compounds, which may become an additional inducer of the gene. Despite the above differences in expression levels, all insecticides tested share in common that all up-regulated this gene to some degree. *AmLyso2* is expressed after the activation of the Imd and Toll pathways, and its activity is related to cellular responses mainly against microbes (Evans, 2006). Therefore, the up-regulation of *AmLyso-2* by sublethal doses of insecticides could be associated with a transient activation of immune responses, potentially protecting bees against bacterial infections. However, the energetic cost of a triggered immune response could affect bees by limiting long term responses due to exhaustion of energy resources.

## Health and detoxification related gene expression

Responses of health and detoxification related genes differed with the type of insecticide bees were exposed to. *AmVit2* responded to neonicotinoid exposure similarly to *AmHym* and *AmBask*, for both, oral and contact applications. That is, there was a transient up-regulation of *AmVit2* at 24 hpt, but a strong down-regulation by 72 hpt. Similarly, another study showed that *AmVit2* was also down-regulated in the long term, when honey bees were exposed to acaricides such as thymol and coumaphos (Boncristiani et al., 2012). Down-regulation of *AmVit2* toward neonicotinoids after 72 h might have been due to the synergistic effect of metabolite compounds as mentioned before. The decreased *AmVit2* expression caused by neonicotinoid insecticides may have led to the reduction in the length of life observed in the treated bees. This conclusion is supported not only by the results of our survivorship experiments, but also by previous studies that have shown a positive correlation between *AmVit2* and lifespan of honey bees (Seehuus et al., 2006; Corona et al., 2007). *AmVit2* regulates the production of vitellogenin, the yolk protein in the bee's fat body that acts as an antioxidant agent in insects (Corona et al., 2007). Yolk protein is considered the most important protein in insects because it has multiple functions related to development, longevity, immunity, and general health (Amdam et al., 2003). Thus, the reduction of *AmVit2* levels might cause immune senescence (aging) in honey bees (Amdam et al., 2005). In addition to the above, high vitellogenin levels in the body of an insect provides protection to cells against ROS because vitellogenin is a potent antioxidant agent, which enhances cell tolerance to oxidation reaction (Seehuus et al., 2006). Therefore, suppressed or reduced expression of *AmVit2* in worker bees may lead to decreased resistance to oxidative stress, and consequently, longevity might be reduced. Contrary to our results, Christen et al. (2016), found that exposure of honey bees to imidacloprid and clothianidin, up-regulated *AmVit2*. Differences in *AmVit2* expression between their study and our study were possibly related to the tissue used for RNA extraction; whole bodies were used in this study whereas in the study by Christen et al. (2016) brains and thoraces were used. Additionally, Christen et al. (2016) exposed foraging bees of unknown ages to the neonicotinoid insecticides *ad libitum* in sugar syrup, whereas this study used newly emerged bees that were treated once with a single dose of the neonicotinoid insecticide. Uniform protocols are thus needed to make results of studies on the effects of insecticides on gene expression comparable.

Conversely to the above results, *AmVit2* expression did not vary relative to the control when bees were treated orally with carbaryl, but significantly increased by 72 hpt in workers that were treated topically with the insecticide. Increased expression of this gene in topical treatments might be related to the mode of action of carbaryl. Carbaryl kills insects mainly by contact rather than by ingestion. This may have resulted in the up-regulation of *AmVit2*, as a protective mechanism against the action of carbaryl molecules, but it may have taken time for the pesticide to reach the sites of action. The fact that carbaryl did not affect the expression of *AmVit2* in oral treatments may have been related to a fast degradation of the insecticide when ingested by the bees; degradation would have reduced carbaryl concentration to a level not high enough to trigger *AmVit2* expression.

*AmPuf68* exhibited a strong response to all insecticides. The expression of this gene was significantly increased at 24 hpt in response to one insecticide provided orally to the bees (imidacloprid) and to all of them at 72 hpt in bees treated with all insecticides using both application methods. To the best of our knowledge, this study is the first to examine the expression of *AmPuf68* in response to insecticides. Previous studies have reported an effect on the regulation of *AmPuf68* in bees parasitized by pathogens such as *V. destructor* (Hamiduzzaman et al., 2012; Koleoglu et al., 2017) or by entomopathogenic fungi (Hamiduzzaman et al., 2012). Parasitism by *V. destructor* mites inhibits *AmPuf68* expression, whereas entomopathogenic fungi increase it. Clearly, the expression of *AmPuf68* is differentially affected by insecticides compared to how it is affected by some pathogens such as mites, but reflects that of other pathogens such as fungi. It is known that *pUf68* regulates mRNA splicing in a subset of genes in *D. melanogaster* (Van Buskirk and Schumbach, 2002). Thus, the effect of insecticides on the expression of *AmPuf68* could be associated with the regulation of mRNA, increasing its expression, which could result in better protection against xenobiotics. Nevertheless, further investigation of the mechanisms that are regulated by *AmPuf68* and how they impact honey bee health are warranted.

*AmCYP9Q3* regulates the production of detoxification enzymes that metabolize and enhance tolerance of honey bees to pesticides (Claudianos et al., 2006). The results of this study showed significant up-regulation of *AmCYP9Q3* as a response to sublethal doses of imidacloprid, clothianidin, and carbaryl, for oral and contact applications, particularly at 72 hpt. Similarly, a high expression of this gene was observed by Mao et al. (2011) when they topically exposed honey bees to 10 µg/µl of the pyrethroid tau-fluvalinate, and De Smet et al. (2017) also found the gene to be up-regulated in bees exposed orally to imidacloprid for 40 days. Considering that imidacloprid's half-life ranges between 4.5 and 5 h in bees exposed to a concentration of 20 ppm to 50 ppm (Suchail et al., 2003; the doses used in this study fall within this range), the up-regulation of *AmCYP9Q3* by secondary metabolites, such as oleofin and 5-hydroxyimidacloprid, cannot be discarded. The results of this study also revealed that routes of exposure had an influence on the expression of *AmCYP9Q3*. In oral applications, *AmCYP9Q3* was up-regulated at 24 and 72 hpt responding to all insecticides. By comparison, in contact applications, the up-regulation of *AmCYP9Q3* was delayed until 72 hpt for all insecticides. There are two factors that might explain these results. First, a high level of expression of *AmCYP9Q3* is common in the midgut compared to the hemolymph of honey bees (Claudianos et al., 2006). The higher concentration of the insecticide in the midgut of bees treated orally, might have accelerated the response of this gene to the chemicals. Second, the ingestion route makes it faster for the insecticides to reach the sites of action than when the product is applied topically, resulting in a faster activation of the gene. In topical applications, wax, lipid, and lipoprotein layers in the cuticle of the insects might have slowed down the penetration of the insecticides, taking longer to reach their sites of action. This would have delayed the response of *AmCYP9Q3*. The increase in expression of this gene caused by all insecticides suggests that the bees were able to activate detoxification mechanisms.

## CONCLUDING REMARKS

Honey bees are not a target for agricultural pesticides, yet, they are constantly exposed to sublethal doses of them by different means. This study demonstrated that sublethal doses of neonicotinoid and carbamate insecticides significantly shorten the life span of honey bees and induce or inhibit the expression of immune, health and detoxification related genes of these insects, which could have negative or beneficial implications. Regardless of the benefits or negative effects in the exposed insects, responses of the different systems associated with these genes are costly and may end up being traded off against other physiological functions, such as reduction in the host's lifespan, susceptibility to pathogens or productivity. Further studies are warranted to better understand how sublethal exposure to neonicotinoid and carbamate insecticides impact biological pathways and the physiological costs incurred by the affected bees.

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