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The effect of temperature on candidate gene expression in the brain of honey bee *Apis mellifera* (Hymenoptera: Apidae) workers exposed to neonicotinoid imidacloprid

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ABSTRACT

Neonicotinoid insecticides are potent agonists of nicotinic acetylcholine receptors and are a major factor in the decline of pollinators worldwide. Several studies show that low doses of this neurotoxin influence honey bee physiology, however, little is known about how insecticides interact with other environmental variables. We studied the effects of two neonicotinoid Imidacloprid doses (IMD, 0, 2.5, and 10 ppb), and three temperatures (20, 28, and 36°C) on gene expression in the brains of worker honey bees (*Apis mellifera*). Using qRT-PCR we quantified the expression of eight key genes related to the nervous system, stress response, and motor and olfactory capacities. Gene expression tended to increase with the low IMD dose, which was further intensified in individuals maintained in the cold treatment (20°C). At 20°C the octopamine receptor gene (*oa1*) was underexpressed in bees that were not exposed to IMD, but overexpressed in individuals exposed to 2.5 ppb IMD. Also, heat shock proteins (*hsp70* and *hsp90*) increased their expression at high temperatures (36°C), but not with IMD doses. These results suggest that despite the low insecticide concentrations used in this study (a field-realistic dose), changes in gene expression associated with honey bee physiological responses could be induced. This study contributes to the understanding of how neonicotinoid residual doses may alter honey bee physiology.

1. Introduction

There are scientific concerns regarding to ecosystemic service of pollination due to the decline of insect pollinators (Ollerton et al., 2011; Sánchez-Bayo and Wyckhuys, 2019; Steffan-Dewenter et al., 2005). Studies in the honey bee *Apis mellifera* indicate that the synergistic effects of several environmental stressors drive this phenomenon, in particular the role of agrochemical exposure (Potts et al., 2010).

Neonicotinoid insecticides are widely used in agriculture. They are highly efficient at controlling pest insects through potent agonist effects on the postsynaptic nicotinic acetylcholine receptors within the insect central nervous system (Jeschke et al., 2011). This agonist over-stimulates excitatory synapses, causing uncoordinated signaling, and thus hyperactivity, tremors, paralysis, and death in insects (Christen et al., 2016; Suchail et al., 2001). In honey bees, sublethal doses of neonicotinoids are associated with beehive weakening (Trapp et al., 2017; Woodcock et al., 2017), health and behavioral changes including impairments of the immune system (Brandt et al., 2016), reproduction (Chaimanee et al., 2016; Whitehorn et al., 2012), energy metabolism (Hatjina et al., 2013; Vergara-Amado et al., 2020), synapsin levels (Tavares et al., 2019), and olfactory associative abilities and memory/learning pathways (Armengaud and Lambin, 2002; Farooqui, 2013; Williamson and Wright, 2013).

Changes in gene expression were reported in larvae and worker honey bees fed with residual doses of neonicotinoids, where genes related to lipid, carbohydrate, xenobiotic metabolism, stress response, and miRNAs were altered (Derecka et al., 2013; Koo et al., 2015; Potts et al., 2010; Siede et al., 2012). Furthermore, Koo et al. (2015) showed that gene expression of *hsp70*, *hsp90,* and *grp78*, which have chaperon functions to overcome stresses, negatively correlated with increasing

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neonicotinoid doses in worker honey bees.

Neonicotinoid residue levels in the environment are extremely variable. However, an interesting residual concentration range of a specific class of neonicotinoid (Imidacloprid, IMD) is around 2.5 ppb (2.5 μg/L^{-1}). This concentration was reported as a very low field-realistic dose to which bees are exposed by the pollen and nectar (Carreck and Ratnieks, 2014; Mullin et al., 2010). Also, unpublished data (master thesis, Himelreichs, 2017) detected transcriptomic and proteomic alterations in the brain of adult worker bees when exposed to a residual dose of 2.5 ppb IMD in semi-field conditions. Because IMD has a high affinity for insect brain nicotinic acetylcholine receptors (Déglise et al., 2002; Ihara et al., 2015; Tomizawa and Casida, 2003) which are located primarily in the brain of insects (Moffat et al., 2015), we selected the brain as the organ of focus for this study.

Little is known regarding how neonicotinoids interact with other biotic and abiotic variables. Pesticides and pathogens can have synergistic effects which lead to increased honey bee mortality (Aufauvre et al., 2014; Pilling and Jepson, 1993; Vidau et al., 2011). Low concentrations of neonicotinoid pesticides compromise locomotor abilities related to thermoregulation behavior (Williamson et al., 2014), and alter the thoracic temperatures of honey bees and bumblebees (Potts et al., 2018; Tosi et al., 2016). Worker bees with lower thermoregulatory capacity would form hives with greater internal thermal fluctuation; this being critical for the correct development of the brood. Therefore, studying the effect of temperature on honey bees exposed to IMD is key to understanding how these variables interact and affect honey bee health.

To explore if different doses of IMD have an accentuated effect under suboptimal temperature conditions, we evaluated how temperature affected the expression of a reduced number of genes encoding proteins that are putatively associated with different functions in worker honey bees (*Apis mellifera*). Specifically, the selected genes are related to brain ionic channels, response to stimuli, and the response to stress. We expected that under less favorable conditions (suboptimal temperatures and IMD exposure) gene expression would present significant changes compared with optimal conditions (intermediate temperature and IMD-free diet). The genes selected for this study provide insight into how IMD affects honey bee health.

2. Material and methods

2.1. The origin of honey bees and laboratory conditions

Three healthy beehive nuclei (hives younger than one year) of *Apis mellifera* from two locations (six nuclei in total) were obtained from commercial apiaries on the northwest coast of Colombia (Magdalena and Sucre departments). The queens of the nuclei came from a single queen per locality. Beehives were transported and maintained (as a sample source) in a field close to the Laboratorio de Ecofisiología of the Universidad de Ibagué in July 2016 (Fig. 1a) for one month in equal environmental conditions before collecting the worker bees for the study. Approximately one hundred worker bees were collected from each nucleus, specifically from the brood frames, and were kept in wooden cages (12 × 12 × 3.5 cm) in a climatic incubator at 27 ± 1°C with 70 ± 10% relative humidity in constant darkness and a piece of cotton moistened with a syrup feeding solution composed by organic honey, sugar, and water at 2:1:1 *ad libitum* (Fig. 1b). These initial conditions in the laboratory were kept for 12 h before starting the insecticide/temperature experiment.

2.2. Exposure of honey bees to experimental conditions

Commercially neonicotinoid Imidacloprid (IMD) from Sigma-Aldrich (St. Louis, MO) was diluted into the feeding syrup and were used at three different concentrations for IMD exposure: a control condition of the IMD dose (D0, 0 ppb IMD), a residual dose (D1, 2.5 ppb IMD), and a higher dose (D2, 10 ppb IMD). A pilot test was conducted to select the maintenance or "control" temperature based on the minimum mortality and normal activity of honey bees. We established 28°C as the control temperature, and two suboptimal temperatures including 20°C as the "cold treatment" and 36°C as the "warm treatment".

The experiment was a factorial design with temperature range and insecticide doses as the categorical factors. Treatments were compared using orthogonal factors, where the combination of D0 and 28°C was

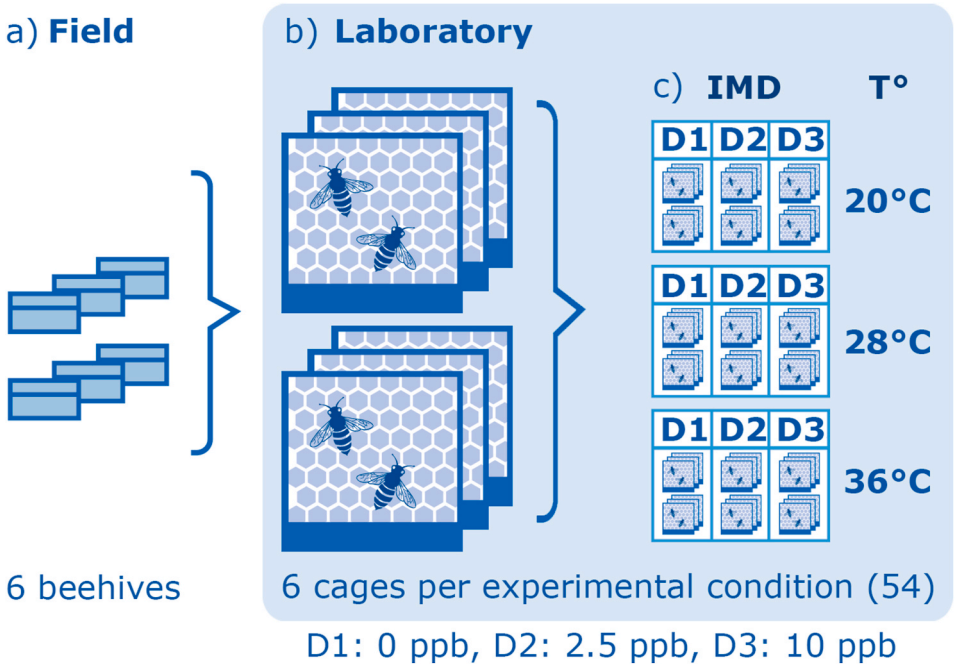

Fig. 1. Experimental design. (a) Six beehives of *Apis mellifera* arranged together in the common field as a source of samples. (b) 54 cages with bees distributed for experimental conditions (six cages per condition) were maintained in the laboratory at 27°C. (c) Experimental conditions with three different temperatures (20, 28, and 36°C) and three Imidacloprid insecticide concentrations: D0 (0 ppb), D1 (2.5 ppb), and D2 (10 ppb).

established as the control condition to be compared with all other combinations (Fig. 1a). Diets were administrated by changing the moistened cotton daily, and experimental conditions were maintained during four days (96 h) to ensure that the insecticide reached its site of action (Suchail et al., 2004).

2.3. RNA extraction and cDNA synthesis

Three bees from each cage were randomly selected and beheaded, then kept in 600 µL of RNAlater solution from Invitrogen Thermo Fisher Scientific (Carlsbad, CA) following the manufacturer's recommendations. Samples were kept at −20°C. The pool of three worker bee brains were dissected (proboscis, antennae, and cuticle were removed from each head) on a freeze tray to avoid thawing, and total RNAs were immediately extracted using the NucleoSpin RNA-RNA kit from Macherey-Nagel GmbH Co. KG (Düren, Germany). RNA concentrations were verified using a DQ300 Hoefer fluorometer and a Quant-iT™ RiboGreen® kit from Life Technologies Thermo Fisher Scientific (Carlsbad, CA). Then, RNA integrity (RIN) was verified by capillary electrophoresis in a Fragment Analyzer Systems from Agilent Technologies, Inc (Sta. Clara, CA).

Reverse transcription (RT) reactions were carried out using a Reverse Transcriptase System ImProm-II kit from Promega (Madison, WI), which included 4 µL of total RNA as a template for cDNA with 1 µL of oligo (dTs) as a primer, 6 µL of nuclease-free water, 4 µL of buffer, 3 µL of MgCl2, 1 µL of dNTPs mix, and 1 µL Improm-II Reverse Transcriptase for each reaction. The RT reaction was conducted by incubating all samples at 25°C for 5 min, 42°C for 60 min, and 70°C for 15 min.

2.4. Candidate genes and qRT-PCR

Real-time Quantitative PCR (qRT-PCR) primers for *hsp90*, *hsp70*, *nalcn*, *kcnh8*, *para*, *unc-80*, *tyr1*, and *oa1* were designed using sequences based on the literature (Table 1), taking into account genes with altered expression under IMD exposure. Primers for candidate reference genes (*gapdh*, *rps5*, *ief4*, and *actin*) were designed using public sequences (see Table 1), and for primer design we considered an amplicon size from 125 to 175 pb, primer size from 18 to 22 pb, melting temperature from 56 to 62°C and %GC in range of 45–55%.

qRT-PCR amplifications were conducted in a 10 µL reaction volume containing 1 µL of cDNA (0.75 ng/uL), 0.2 µL of gene-specific primers at a concentration of 10 µM, 5 µL of Kapa Sybr Fast Universal from Sigma-Aldrich (St. Louis, MO), and 3.6 µL of nuclease-free molecular biology grade water (HyPure). Real-time fluorescence reactions were carried out in a Rotor-Gene RG-3000 Thermocycler by Corbett Research from QIAGEN (Venlo, Netherlands). Reaction conditions were set up using specific annealing temperatures (*Ta*) for each primer (see Table 1), and following the next amplification protocol: 1 cycle (95°C for 1 min), and 40 cycles (95°C for 15 s, *Ta* for 30), and the melting curve (from 65–95 °C in 0.5 °C/5s increments) to know which primers showed non-specific amplification products and therefore be discarded.

Primer efficiencies (forward and reverse) were estimated from a serial dilution threshold cycle (Ct) curve using the Rotor-Gene software. Then free online Reffinder tool (Xie et al., 2012) was used to rank reference gene amplification using default settings and the BestKeeper, NormFinder, and Genorm algorithms. Relative quantifications of gene expression among treatments and the control condition (hereafter Fold-Change or FC) were calculated using the Pfaffl (2001) method with the better reference gene and their efficiency values (Pfaffl, 2001). The $\log_2$ of relative expression (Log_2FC) was calculated to ease graphical representations.

2.5. Statistical analysis

The effects of the fixed factors temperature, IMD dose, and their interaction were evaluated for each gene independently. We checked the normal distribution of FC data by visual analysis of FC histograms, and statistically significant differences of expression ratios were determined through parametric statistics using a factorial ANOVA test at $p \leq 0.05$, including the original location of beehives as a random effect. Then, to identify the combination of experimental treatments with significant effects on gene expression that are specifically related to the control condition (D0 at 28°C), the FCs of the control condition were compared with the 8 remaining combinations. Model coefficients were used to identify these significant differences. All statistical analyses were carried out using R version 3.5.1.

3. Results

3.1. Reference gene selection for normalization

Among the five analyzed reference genes (*gapdh*, *rps5*, *ief4*, and *actin*)

Table 1
mRNA primers. Oligonucleotide sequences with the annealing temperatures (Ta) used for qRT-PCR analysis of *Apis mellifera*.

Gene	Synonim	Target gene	Primer sequence	*Ta* (°C)	Accession	Reference
nalcn	*LOC413947*	sodium leak channel non-selective protein	F-TGCCACATCTCACAGAGAGC R-ATGCCAACTGTTCCTTCCAC	53.4	XM_026444169.	
kcnh8	*LOC409845*	potassium voltage-gated channel H member 8	F-CCACTGTCACCTGTTGTTGG R-CGCGCTAAATCTGTGCAATA	54	XM_006557868.	
para	*GB12929*	sodium channel protein paralytic	F- TGGTGTTCGATCCATTCGTA R- AGCTTCAACGTTGCTTCGAT	53.4	XM_006561545	
unc-80	*LOC726032*	protein unc-80 homolog	F-GTCCGGTTATCGTGAGTCGT R-GCTTCCTTCGAAAACGTGAG	56	XM_026444202	
hsp70	*hsp70ab-like*	heat shock protein 70	F-CGCCTTCACGGACACAGA R-TTCATTGCGACCTGATTTTTG	54	NM_001160072.	Koo et al., 2015
hsp90	*Hsp83*	heat shock protein 90	F- AGGACGTCACCATGGCTAAT R- TGTGCAATTTCAGCTTGGAA	53.4	NM_001160064.	Koo et al. (2015)
tyr1	*TyrR*	tyramine receptor	F-GAAGAGGAAACGGGCTCGAA R-GATACGAAGGGGCCTGTACG	57	NM_001011594.	
oa1	*OaR*	octopamine receptor	F-ATCCGTGTATCTACGCGCTG R-CTTGGGCTCCGATCGTTTCT	53.4	NM_001011565.	
gapdh	*LOC410122*	glyceraldehyde-3-phosphate dehydrogenase 2	F-GATGCACCCATGTTTGTTTG R-TTTGCAGAAGGTGCATCAAC	54	XM_393605.6	De Smet et al. (2017)
actin	*Arp1*	actin related protein 1	F-TGCCAACACTGTCCTTTCTG R-AGAATTGACCCACCAATCCA	54	NM_001185146.	Derecka et al. (2013)
eif3	*eIF3-S8*	eukaryotic translation initiation factor 3 subunit C	F-TGAGTGTCTGCTATGGATTGC R-TCGCGGCTCGTGGTA	53.4	XM_006564593.	De Smet et al. (2017)
rps5	*RPs5*	40S ribosomal protein S5	F-AATTATTTGGTCGCTGGAATTG R-TAACGTCCAGCAGAATGTGGTA	53.4	XM_006570237.	Aufauvre et al. (2014)

for data normalization, the best performing reference gene, when comparing a stable gradient of ΔCts across insecticide and temperature treatments, were *rps5*. The Cq values of *rps5* in the present study were 24 ± 1.26. Also, *rps5* has previously been used for qRT-PCR normalization of honey bees exposed to IMD (Aufauvre et al., 2014).

3.2. qRT-PCR data: Transcriptional levels

All qRT-PCR assays generated specific products with single melting peaks. Significant differences in gene expression were evaluated using a linear model independently with the interaction of both categorical factors (IMD insecticide and temperature). The results of the analysis of variance for gene expression shows that both factors affected the amount of mRNAs from the studied genes in the brain of worker honey bees ($p < 0.05$) (see Table 2). The same model was evaluated in all the studied genes, with a sample size of n = 54 for *para*, *hsp70-90*, *tyr1* and *oa1* genes; n = 53 in *kcnh8*; and n = 51 in *unc-80*. Specifically, after the four day period, IMD effects were detected in the gene expression pattern of *nalcn* ($F_{2,45} = 3.43$; $p < 0.05$), *kcnh8* ($F_{2,44} = 6.01$; $p < 0.05$), *hsp90* ($F_{2,45} = 3.39$; $p < 0.05$), and *oa1* ($F_{2,45} = 5.59$; $p < 0.05$). Temperature affected the gene expression of heat shock proteins, *hsp70* ($F_{2,45} = 7.31$; $p < 0.05$) and *hsp90* ($F_{2,45} = 10.82$; $p < 0.05$). An interaction effect between IMD and temperature was detected only in *unc-80* ($F_{4,42} = 3.790$; $p < 0.05$). No significant effects were detected in the transcriptional levels of *para* and *tyr1* genes.

When comparing each combination of experimental conditions with the control condition (D0 at 28°C), in general, we found greater transcriptional variations of FC in the IMD treatment with the residual dose (D1) of 2.5 ppb compared to the higher dose (D2) of 10 ppb of IMD (see Fig. 2). The transcriptional level of *nalcn* (Fig. 2a) increased in the residual dose (D1) treatment and this increase was significant in the cold condition group (20°C) by 3-fold relative to the control (1.7 Log_2FC; $p = 0.030$). The *kcnh8* gene expression pattern (Fig. 2b) increased in the dose D1 group. Higher expression levels were observed in D1 (3-fold; 1.6 Log_2FC) at 20°C and lower expression levels in D2 (0.5-fold; −1.1 Log_2FC) at 20°C. The pattern of *hsp70* (Fig. 2c) and *hsp90* (Fig. 2d) gene expression levels were affected by both factors (IMD and temperature). Specifically, *hsp70* overexpression reached 5.3-fold (2.4 Log_2FC; $p = 0.017$) in the D1 group with warm conditions (36°C). Also at warm conditions *hsp90* reached a 4.6-fold increase (2.2 Log_2FC; $p = 0.003$) in the D2 group and 4-fold (2.0 Log_2FC; $p = 0.007$) in the D1 group. For *oa1* (Fig. 2e), the gene expression pattern was affected by the IMD factor, with the highest overexpression of 2.8-fold (1.5 Log_2FC) in the D1 group, and decreased expression of 0.5-fold (−1.1 Log_2FC) in the D2 group. An interaction effect for both factors was only detected for the *unc-80* expression pattern (Fig. 2f), where at each evaluated temperature the response to IMD doses showed different patterns. The significant interaction trend of *unc-80* is presented in Fig. 2g. In D1 opposite responses were observed at the warm (36°C) and cold (20°C) temperature conditions. Therefore, *unc-80* was 3.5-fold overexpressed (1.75 Log_2FC) in warm conditions and was underexpressed 0.8-fold (- 0.33 Log_2FC) in cold conditions, although important variations between biological replicates were observed for this data. Also, in the cold condition *unc-80* expression levels decreased as the dose of IMD increased and an opposite trend was observed at the other two temperatures. Expression levels of *para* and *tyr1* genes presented no statistically significant differences between the experimental conditions, and these data varied widely (see Fig. S1, a and b).

Table 2
F-statistics as F-value (*p*-value) from the gene Fold Change variations induced by different Imidacloprid (IMD) doses and temperatures in honey bees. Genes correspond to *nalcn, kcnh8, para, unc-80, hsp70, hsp90, tyr1, oa1*, and *rps5* which was used as the normalizer gene. The number of samples (n) is indicated for each gene, and the significant F-value is in bold.

Gene	IMD Dose (D)	Temperature (T)	D*T
***nalcn* (54)**	**3.436(0.041)****	0.053 (0.950)	0.925 (0.458)
***kcnh8* (53)**	**6.012(0.004)****	0.193 (0.824)	0.675 (0.613)
***para* (54)**	1.213 (0.3070)	1.428 (0.2510)	0.585 (0.6750)
***unc-80* (51)**	0.332 (0.7191)	1.290 (0.2859)	**3.790(0.0102)***
***hsp70* (54)**	2.608 (0.0848)	**7.311(0.0018)****	0.154 (0.9601)
***hsp90* (54)**	**3.391(0.0420)****	**10.821(0.0001)*****	0.919 (0.4612)
***tyr1* (54)**	1.353 (0.2690)	2.074 (0.1380)	0.933 (0.4530)
***oa1*(54)**	**5.592(0.0068)***	2.799 (0.0715)	1.239 (0.3081)

p-value: ***$p < 0.001$, **$p < 0.05$, *$p < 0.01$.

4. Discussion

We studied gene expression in the brains of adult worker bees exposed to residual field-realistic (D1: 2.5 ppb) and high (D2: 10 ppb) Imidacloprid (IMD) doses under different thermal environments: an intermediate temperature (28°C) and two suboptimal temperatures, a cold temperature (20°C) and a warm temperature (36°C).

Overall, our results showed a general trend of increased gene expression alterations in individuals exposed to D1 compared to those exposed to D2, and these variations were intensified in bees maintained in the cold condition (except for *hsp70-90*). However, the transcriptional levels of some genes displayed large variances, and this was expected because there are factors associated to transcriptomic variations among ontogenic stages (Garcia et al., 2009), and large genetic diversity within and between honey bee colonies (McAfee et al., 2016) that were not considered for this study. Neonicotinoid reaches pharmacological relevance when these neurotoxins arrive at their site of action in the bee brain (Eiri and Nieh, 2012; Moffat et al., 2015). The selected genes related to brain nicotinic acetylcholine receptors (*nalcn*, *kcnh8*, *unc-80*, *para*) have altered expression in worker honey bees.

Our results show significant overexpression of *nalcn* (3.2-folds) in worker bees at D1 and 20°C which could trigger an increase of the NALCN membrane complex involved in voltage-independent Na^+ transport (Cochet-Bissuel et al., 2014; Lu et al., 2010). This complex is responsible for the depolarization from a resting potential and is a key complex in neuronal excitability (Lu et al., 2007; Ren, 2011). Increased depolarization in worker bees will increase the probability of opening Na^+ voltage-dependent channels, triggering higher excitability and/or nervous impulse transmission. We observed overexpression in worker bees exposed to D1 at all temperatures, but this was significantly accentuated at cold conditions. In insects, the effects caused by the cold are attributed to a lack of ionic balance in the haemolymph, which strongly suppress maximal muscle force production and muscle cell paralysis (Findsen et al., 2014; MacMillan et al., 2016). Thus overexpression of *nalcn* may be one of the mechanisms involved in this stress response. Also, IMD appears to accentuate these effects, which is interesting because IMD could be an agent that amplifies the effects of cold in beehives and could determine the success of bees exposed to neonicotinoids over winter (Blacquiere et al., 2012).

As for *nalcn*, cold conditions increased *kcnh8* expression (2.8-folds) at D1 but not at D2. This gene codes for a protein that forms part of a K^+ transport voltage-dependent channel, and has an important role in nervous impulse repolarization, by moving K^+ to the extracellular space (Gutman et al., 2003; Zou et al., 2003). Overexpression of K^+ channels should trigger hyperexcitability by facilitating neuronal voltage repolarization during the nervous impulses. D2 triggered *kcnh8* underexpression at all temperatures, and this prompted impairments in K^+ ionic balance due to the lack of voltage repolarization, with potential effects of continuous and uncoordinated impulse, paralysis, or lack of movement.

The interaction between IMD and temperature on the expression of *unc-80* accounted for the complex manner in which IMD affects worker bee physiology in combination with other environmental variables. In cold conditions, *unc-80* decreased its expression level, accentuating the decrease in the dose of IMD greater and in the warm condition, the expression pattern was similar to that observed in the other genes with a

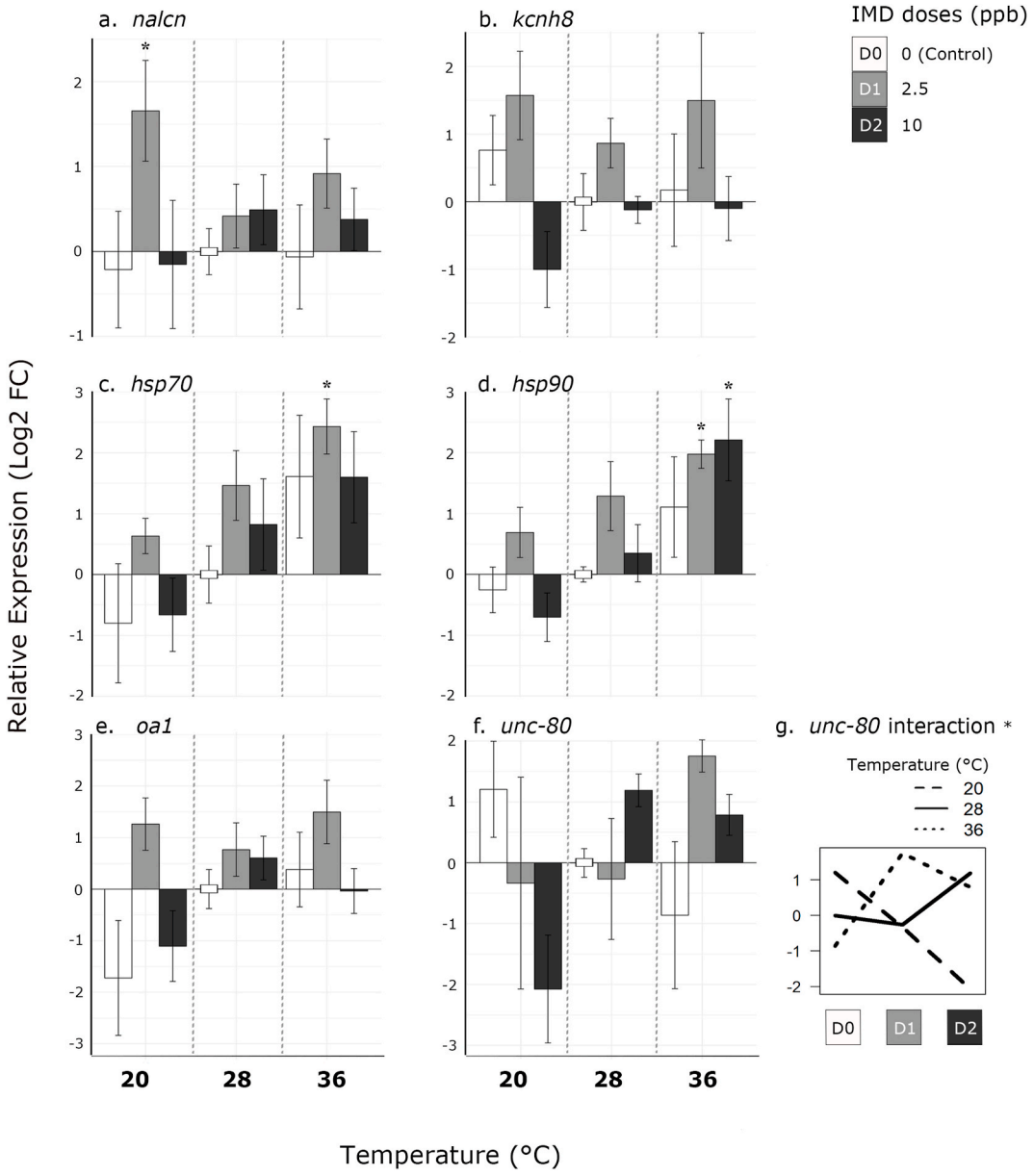

Fig. 2. Effects of the Insecticide (IMD) and temperature (T) treatments on the gene expression of *Apis mellifera*. The expression levels ($n = 6$, mean and SE) relative to the control condition are presented as Log2 fold change of *nalcn* (a), *kcnh8* (b), *hsp70* (c), *hsp90* (d), *oa1* (e), and *unc-80* (f), and in the interaction plot from *unc-80* (g). Each gene was normalized with *rps5* following the Pfaffl method. Dotted lines limit the temperature treatment and the color legend indicates the insecticide dose (D0: 0 ppb, D1: 2.5 ppb, and D2:10 ppb). Asterisks represent statistically significant effects ($p < 0.05$) among IMD doses. The white rectangle represents the control condition used as a reference of comparison (D0 at 28°C).

higher FC at D1 than D2. Regulation of this gene also compromises the transmission of nervous impulses, since *unc-80* interacts with the NALCN complex, through the stabilization and localization of the ionic channel, as reported in *Caenorhabditis elegans* and *Drosophila melanogaster* (Jospin et al., 2007; Lear et al., 2013; Yeh et al., 2008). Thus, regardless of the residual dose to which honey bees are exposed, the physiological effect triggered by IMD exposure could also depend on other environmental variables, such as temperature: indicating the importance of the field conditions.

The heat shock protein (HSP) family is well studied and corresponds to one of the first known mechanisms of the cellular stress response (Feder and Hofmann, 1999). Insecticide tolerance (Bagchi et al., 1996) and thermal stress (Neven, 2000) are also associated with over-expression of *hsp70* and *hsp90*. The transcriptional level variation of *hsp70* and *hsp90* showed a similar pattern response induced by temperature and IMD, with a tendency towards increased gene expression as the temperature increases, but not a clear tendency with IMD alone. Bees in warm conditions increased both *hsp70* and *hsp90* expression, with significant increases when individuals were exposed to the residual dose (D1) of IMD. Temperature-mediated chaperone activation could be a relevant mechanism in healthy bees exposed to IMD, for cellular protection against damage by different stress sources. The results show that the residual IMD doses emphasize changes in *hsps* expression levels, which I could contribute to increase the fitness costs attribute to excess consumption of energy caused by over production of *hsps* enzymes (Hoekstra and Montooth, 2013; Zhang et al., 2015).

Octopamine and its precursor tyramine are biogenic amines that play an important role in modulating a variety of physiological and behavioral processes in insects (Verlinden et al., 2010); such as metabolic state (Pflüger and Duch, 2011), motor rhythms (Brembs et al., 2007; Vierk

et al., 2010), and learning and memory (Schwaerzel et al., 2003). Octopamine and tyramine receptor mRNAs (here *oa1* and *tyr1*) are widely expressed in the honey bee brain and present an overlapping patterns of expression (Mustard et al., 2005). Alterations in octopamine and tyramine receptors expression can affect how incoming mechanosensory information is perceived, thus, modifying processes such as neuromuscular junctions, sucrose response threshold, vision, and behavioral responses (Roeder, 2005). Although until recently it was thought that tyramine was thought only to be the precursor of octopamine, studies over the last decade have suggested opposite effects of both amines in insects (Fussnecker et al., 2006; Saraswati et al., 2004).

Particularly, increases in the neurotransmitter octopamine are related to increased flying behavior, whereas tyramine showed a decrease in flying in honey bees (Fussnecker et al., 2006). Similarly, tyramine elevated and octopamine reduced has been related to limited locomotor capacity in *Drosophila* (Saraswati et al., 2004). In complement, overexpression of octopamine receptors have been associated to neonicotinoid exposure, with consequences of hyperexcitation and quick energy exhaustion in *Aedes aegypti* (Ahmed et al., 2015), and in honey bees with oxidative stress, neuroinflammation and olfactory dysfunction (Farooqui, 2013). In complement, overexpression of octopamine receptors have been associated to neonicotinoid and another biogenic amines insecticide exposure, with consequences of hyperexcitation and quick energy exhaustion in *Aedes aegypti* (Ahmed et al., 2015) and with oxidative stress, neuroinflammation and olfactory dysfunction in honey bees (Farooqui, 2013).

Proboscis extension reflex, olfactory conducts, and orientation are altered in honey bees exposed to insecticides (Giurfa and Sandoz, 2012; Matsumoto et al., 2012; Scheiner et al., 2002). Increases in the neurotransmitter octopamine levels are related to increased flying behavior in honey bees (Fussnecker et al., 2006), and decreased transmission has been related to limited locomotor capacity in *Drosophila* (Saraswati et al., 2004). Overexpression of octopamine receptors are associated with neonicotinoid exposure, with consequences of hyperexcitation and quick energy exhaustion in *Aedes aegypti* (Ahmed et al., 2015), and in honey bees with oxidative stress, neuroinflammation, and olfactory dysfunction (Farooqui, 2013).

No effect of the treatments was found on the *tyr1* expression (Table 2; Fig. S1b), but it was detected IMD effect in *oa1* (Table 2; Fig. 2e) with duplicated and triplicate expression at D1 in cold and warm conditions respectively, suggesting that exposure to IMD may alter octopamine/tyramine pathways in honey bees with greater effects in unfavorable temperatures. This result also supports the study by Tosi and collaborators who report depression of thermoregulatory capacity in beehives under IMD chronic and field-realistic conditions (Tosi et al., 2016). In fact, in honey bees the exposition to insecticides trigger abnormal functioning in the proboscis extension reflex, olfactory conducts, and orientation during flight and thermoregulation function (Scheiner et al., 2002; Giurfa and Sandoz, 2012; Matsumoto et al., 2012), and the molecular mechanism by which these behaviors are affected are not yet clear at all, but future studies should board the phenotipic alterations related with octopamine/tyramine signaling under neonicotinoid exposure.

In this study, we emphasize that beehives require maintenance of a stable temperature range (Stabentheiner et al., 2010) and that thermoregulation is depressed in honey bees exposed to IMD (Tosi et al., 2016). Therefore, the beehives under the combined effect of residual doses of IMD and unfavorable temperatures may suffer a complex and unpredictable response: effects that are gene-dependent, dose-dependent, and temperature-dependent. Also, despite the low concentration of insecticide administered, this is perceived and can induce a physiological and transcriptional response. The most alarming and novel finding of this study is that the greatest transcriptional gene alterations was under the almost imperceptible dose of neonicotinoid venom.

5. Conclusions

Extremely low but field-realistic doses of Imidacloprid (IMD) neonicotinoid insecticide produced changes in the gene expression response. We observed a dose-dependent IMD response on gene expression in the brain of honey bees. Also, we detected a relationship between IMD doses and temperature in the expression of our targeted genes, especially at 20°C. This study contributes to our understanding of how extremely low doses of neonicotinoids influence honey bee physiology when exposed to different temperatures.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2020.102696.

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