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Imidacloprid intensifies its impact on honeybee and bumblebee cellular immune response when challenged with LPS (lippopolysacharide) of *Escherichia coli*

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ABSTRACT

Insect hemocytes play an important role in insects' defense against environmental stressors as they are entirely dependent on their innate immune system for pathogen defense. In recent years a dramatic decline of pollinators has been reported in many countries. The drivers of this declines appear to be associated with pathogen infections like viruses, bacteria or fungi in combination with pesticide exposure. The aim of this study was thus to investigate the impact of imidacloprid, a neonicotinoid insecticide, on the cellular immune response of two pollinators (*Apis mellifera* and *Bombus terrestris*) during simultaneous immune activation with LPS (lipopolysaccharide) of *Escherichia coli*. For this purpose the phagocytosis capacity as well as the production of H_2O_2 and NO of larval hemocytes, exposed to five different imidacloprid concentrations *in vitro,* was measured. All used pesticide concentrations showed a weakening effect on phagocytosis with but also without LPS activation. Imidacloprid decreased H_2O_2 and increased NO production in honeybees. Immune activation by LPS clearly reinforced the effect of imidacloprid on the immune response of hemocytes in all three immune parameters tested. Bumblebee hemocytes appeared more sensitive to imidacloprid during phagocytosis assays while imidacloprid showed a greater impact on honeybee hemocytes during H_2O_2 and NO production.

1. Introduction

In recent years pollinating insects, especially honeybees, have been the main target of many research investigating the cause of sudden colony losses around the world (Genersch et al., 2010; Neumann and Carreck, 2010). This phenomenon has been especially monitored on honeybees (*Apis mellifera*) due to their cultural and economic importance (Allsopp et al., 2008) and generally has been referred to as the colony collapse disorder (CCD) (Bromenshenk et al., 2010; vanEngelsdorp et al., 2009). This disorder is characterized by the sudden loss of bee colonies where bee hives appear to have been abandoned by its workers leaving ample resources and healthy breed in the hive (Bromenshenk et al., 2010).

However, not only honeybees but pollinators worldwide are affected by the development of CCD leading to a loss of biodiversity (Potts et al., 2015). As specialist bee species rely on fewer plant species than generalist pollinators like honeybees, the former are often more affected by these losses. Many assumptions have been made on the cause of this decline mentioning climate change, habitat loss, pathogens and pesticide exposure, while there is still no true evidence that one of these alone might be the ultimate cause (Potts et al., 2010). Wild pollinators such as bumblebees (*Bombus spp.*) are further threatened by domesticated pollinators as they can act as a link of pathogen transmission. Indeed honeybee pathogens were proven to be infectious to wild bumblebees and parasites have been transferred to wild bee populations (Fürst et al., 2014).

In effect the exposure to pathogens like bacteria, viruses, fungi and parasites has increased in bee colonies in recent years, having thought to be a major cause of the decline (Bromenshenk et al., 2010; Gisder et al., 2010). The Israeli acute paralysis viruses (IAPV) was thought to be a potential cause of CCD, but was found to have been present in the USA long before CCD appeared on the continent (Bromenshenk et al., 2010). The parasitic mite *Varroa destructor,* which is one of the main pests of bees (Martin, 2001; Rinkevich et al., 2017), and the American foulbrood, also an important bee diseases (Yue et al., 2008), did not indicate that one of them alone is responsible of CCD (Nazzi et al., 2012). An important impact on CCD was also described for the microsporidian species *Nosema* sp., a high evolved bee-pathogenic fungi (Gisder et al., 2011, 2010). In Europe and the US the spread of *Nosema ceranea,* which was believed to be a pathogen only to the Asian

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honeybee *Apis ceranea*, has been documented in the recent years (Chen et al., 2008; Paxton et al., 2007). While scientists in Spain correlated the colony collapse to the infection with these fungi (Higes et al., 2008), it could not be correlated to the decline in the US (Bromenshenk et al., 2010).

Apart from pathogens the most influencing threat on honeybees and other pollinating invertebrates is the widespread use of pesticides in agriculture today (Chauzat et al., 2009; Goulson et al., 2015). Studies have shown that especially during the period of pesticide application on fields bees can suffer acute toxicity (Marzaro et al., 2011; Sanchez-Bayo and Goka, 2016), nevertheless during the whole foraging period, in which bees are exposed to especially systemic pesticides, mortality due to ingestion is either low (Cresswell, 2011) or could not be demonstrated. This leads to the assumption that not only one stressor is responsible for the decline of pollinators but more likely a combination of multiple agents (Alaux et al., 2010; VanEngelsdorp et al., 2010).

The most commonly used group of insecticides are the neonicotinoids (Bonmatin et al., 2015) and residues of them have been found in pollinators like honeybees, their pollen, and honey as well as in bumblebees, their pollen and solitary bees (Chauzat et al., 2009; Codling et al., 2016; David et al., 2016; Hladik et al., 2016; Kasiotis et al., 2014). Even if these environmental concentrations are rather small compared to concentrations for acute toxicity they can still lead to sublethal effects in the exposed organism (Chaimanee et al., 2016; Chauzat et al., 2009; Desneux et al., 2007).

Several studies have already shown a weakening effect of neonicotinoids on pollinators when confronted with pathogens (Abbo et al., 2017; Alaux et al., 2010; Fauser-Misslin et al., 2014; Pettis et al., 2012; Vidau et al., 2011). Indeed Brandt et al. (2016), Christen et al. (2016), Di Prisco et al. (2013) and López et al. (2017) provided evidence that neonicotinoid pesticides affect the immunocompetence of honeybees. However the effect of pesticides on the individual immune response of pollinators and a simultaneous immune reaction to pathogens is still not well understood (Collison et al., 2016).

Unlike mammals insects are entirely dependent on their innate immune system in pathogen defense (Strand, 2008). Innate immune defense consists of humoral and cellular responses (Censoplano et al., 2014). The humoral defense reacts to all macromolecules, like soluble antimicrobial peptides and production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that can be found in extracellular humour while the cellular defense is based on cells circulating in the hemolymph of insects (Marmaras and Lampropoulou, 2009). These immune cells, so called hemocytes, play an important role in insect's defense against pathogens (Lavine and Strand, 2002). They have diverse activities like phagocytosis, nodulation and encapsulation and their function depends on the developmental stage of the animal (Feldhaar and Gross, 2008). Hence, cellular immune competence is of vital importance for the organism.

As pathogens are often put in relation with the CCD alongside pesticides (Sánchez-Bayo et al., 2016), pesticides might directly interfere with immune system cells leading to a weaker immune response of the host organism and hence to a greater impact of pathogens.Thus, in this study we examined the immunocompetence of honeybees (*Apis mellifera*) and bumblebees (*Bombus terrestris*) when confronted with imidacloprid (IMI), a neonicotinoid insecticide, and a simultaneous immune response to lipopolysaccharide (LPS) from *Escherichia coli*. Bee's hemocytes were exposed to IMI *in vitro* and their phagocytosis capacity as well as their production of H_2O_2 a ROS and NO a RNS were measured with or without an immune activation with LPS.

2. Material and methods

2.1. Larval collection

Honeybee larvae (*Apis mellifera*) were obtained from colonies held at an urban field site in Metz, Lorraine/France and a rural field site in Meuse, Lorraine/France. After collection combs were incubated over night at 25 °C and 65% humidity prior to larval extraction.

4 boxes of Natupol excel bumblebees (*Bombus terrestris*) were purchased from Koppert France S.A.R.L. and held in the garden protected from sun and rain. Bumblebee larvae were collected when required just prior to experiments.

2.2. Hemolymph extraction

Hemolymph was extracted from fifth instar larvae by puncturing the soft cuticle with a sterile needle and collecting the resulting bubble with a micropipette (Negri et al., 2014a). 25 µL were collected from individual honey bee larvae and 40 µL from individual bumble bee larvae. Hemolymph was immediately diluted in either PBS (phosphate buffer saline) or WH-2 medium. WH-2 medium was prepared after the description of Hunter (2010).

2.3. Cell culture and exposure

Imidacloprid (37894 Sigma-Aldrich, powdery analytical standard) was dissolved in PBS (pH 7.4) at 300 µg/mL prior to experiments. Imidacloprid was further diluted in either PBS or WH-2 medium in order to obtain the following concentrations: 1; 10; 25; 50 and 100 µg/mL IMI. These concentrations were chosen according to literature.

Bal et al. (2010) and Benzidane et al. (2011) used concentrations from 0.25 µg/mL to 250 µg/mL in *in vitro* exposures of neuronal cells (vertebrate and invertebrate) to IMI. Benzidane et al. (2011) found IMI to negatively impact the cell viability of cockroaches neuronal Kenyon cells at 12.75 µg/mL after 8 h of exposure. As IMI is a nicotinic acetylcholine receptor agonist we assumed that the impact of IMI on neuronal cells will be stronger than on immune cells. Benzidane et al. also concluded that toxicity depends on the cell type. As the cell exposure to IMI was conducted *in vitro,* we are missing detoxification processes of the organism. Metabolites of IMI might play a stronger role on chronic toxicity than on acute toxicity of bees' hemocytes. We thus choose to add higher concentrations as environmentally relevant as we wanted to test rather acute toxicity than chronic toxicity in an *in vitro* model.

During each experiment all cell solutions have been tested with HPLC to determine the actual concentration of IMI that has been in contact with the hemocytes to avoid measuring errors. An analytical standard of Imidacloprid solution (46341 Sigma-Aldrich) of 100 µg/mL in acetronitril was used to obtain a standard curve for HPLC analyses. Hemocytes were incubated at 22 °C in the dark for 3, 5 or 24 h. Cell viability was examined after 24 h in WH-2 medium with a trypan blue solution (2 g/L in 0.9% NaCl solution). Trypan blue is a diazo dye used for vital staining of cells (Tolnai, 1975). A minimum of 200 cells were counted for each replicate. To induce an immune reaction hemocytes were treated with LPS (lipopolysaccharide) from *Escherichia coli*. LPS is an endotoxin and a component of the outer membrane of Gram-negative bacteria resulting in a strong inflammatory response of the immune system. Hemocytes were treated with either 0; 1 or 10 µg/mL LPS (Tafalla et al., 2003; Tauszig et al., 2000).

2.4. Phagocytosis

Phagocytosis of hemocytes was examined by using amine-modified polystyren, fluorescent yellow-green latex beads (1 µm mean particle size) (L1030 Sigma) after modifications of the methodology of Bennasroune et al. (2012). 25 µL extracted hemolymph was diluted in 250 µL WH-2 medium. 40 µL of cells exposed to the range of IMI concentrations indicated above were distributed on sterile glass coverslips. Each glass coverslip was placed in a petri disk containing cotton drenched in PBS to ensure humidity during incubation. Hemocytes were incubated at 22 °C in the dark for either 5 or 24 h. 4 h prior to examination approximately 100 fluorescent yellow-green latex beads per cell were added to each drop. After incubation hemocytes were

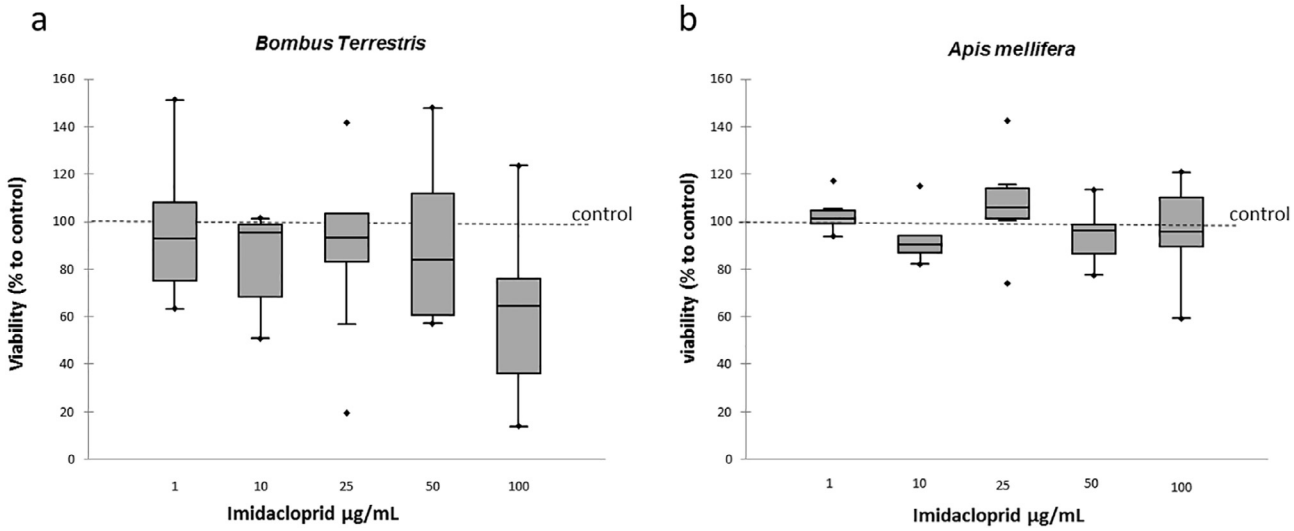

Fig. 1. Cell viability of bumblebee hemocytes (a, n = 10) and honeybee hemocytes (b, n = 6) after 24 h exposure to Imidacloprid. No significant differences were found between IMI concentrations and the control group without IMI for neither bumblebee (one-way ANOVA, p = .155) nor honeybee hemocytes (Kruskal-Wallis, p = .182).

washed with 3% BSA (bovine serum albumin) dissolved in PBS and with PBS alone and were mounted on a fluorescent microscope. A minimum of 250 hemocytes were counted for each sample. Phagocytosis was calculated as follows:

$$Phagocytosis = \frac{cells\ ingesting\ at\ least\ 1\ bead}{total\ number\ of\ cells} * 100$$

2.5. Hydrogen peroxide

The concentration of H_2O_2 was determined with an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (invitrogen, Cat. No. A22188) for H_2O_2 determination. Amplex Red is a colorant that can be used to detect extracellular H_2O_2 (Kalyanaraman et al., 2012). In the presence of peroxidase, the Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product resorufin. The methodology was following the protocol provided with the purchased product. Hemocytes were incubated in 120 µL of PBS for 3 h. Supernatants were centrifuged and stored at -20 °C for later analysis.

2.6. Nitric oxide

NO was measured by means of the fluorescent dye DAF-FM-DA following protocols of Negri et al. (2017, 2014b, 2013). 50 mM stock solutions of DAF-FM-DA were prepared in DMSO and kept frozen at -20 °C. Hemocytes were incubated in 96-well glass bottom plates in PBS containing 5 µM DAF-FM-DA (molecular probes D-23842). Fluorescence was measured with a fluorimeter from 15 min until 4 h exposure to IMI. Fluorescence excitation and emission maxima were at 495 and 515 nm.

2.7. Statistical analyses

As hemocyte activity varies between different individuals all results are expressed as the percentage of the treated groups to the control group (no IMI). Hemocytes of one larva were contaminated with one of the three LPS concentrations (0, 1 or 10 µg/mL) and all 5 concentrations of IMI plus a negative control without IMI, in order to determine the individual effects of IMI on hemocytes of the same larvae. Hemocytes of different larvae were not pooled together as mixing would alter an immune reaction between hemocytes of different larvae. All data were analysed using XLSTAT base. For all tests a probability level of 0.05 was considered to be statistically significant. In order to obtain parametric conditions data of bumblebee hemocytes viability were arcsine transformed prior to analyses. After checking for normality and variance homogeneity using the Shapiro-Wilk test and the Bartlett's test, a one-way ANOVA was performed for cell viability of bumblebee hemocytes. For all other parameters parametric conditions, normality and homogeneity of variance, were not given even after log and arcsine transformation. We used a non-parametric Kruskal-Wallis test followed by a pairwise comparison of Dunn to compare different IMI concentrations of one treatment group to the control of each group. Results were expressed as means of all replicates ± SE (standard error).

3. Results

3.1. Cell viability of hemocytes after IMI exposure

Hemocyte viability was examined after 24 h of exposure to IMI in WH-2 medium. No statistical significant differences between the tested IMI concentrations and the control without IMI were found neither for bumblebee nor for honeybee hemocytes. Cell viability for honeybee hemocytes for all IMI concentrations and the control was around 59.8 ± 2.4 percent. Cell viability of bumblebee hemocytes was at 56.5 ± 4.8 percent for the control group without IMI and decreased with 1, 10, 25, 50,100 µg/mL IMI to 48.8 ± 5.5, 53.8 ± 5.6, 50.4 ± 6.4, 50.4 ± 6.1 and 32.3 ± 5.7 percent respectively (Fig. 1). At the highest IMI concentration tested cell viability decrease by 42.8 percent compared to the control group.

3.2. Effect of IMI on phagocytosis capacity of hemocytes from Apis mellifera and Bombus terrestris

The capability of phagocytising latex beads decreased significantly in bumblebee larval hemocytes after 5 h exposure to IMI from 10 µg/mL to 100 µg/mL when cells were not challenged with LPS ($p < .0001$) (Fig. 2a). When cells were challenged with either 1 or 10 µg/mL LPS IMI already reduced phagocytosis compared to the control by more than 25% from the lowest concentration of 1 µg/mL imidacloprid to more than 45% at the highest concentration of 100 µg/mL IMI ($p < .0001$). No significant differences were found between low and high LPS treatment groups.

The inhibiting effect of IMI on phagocytosis in bumblebee hemocytes was even stronger after 24 h exposure to IMI with phagocytosis

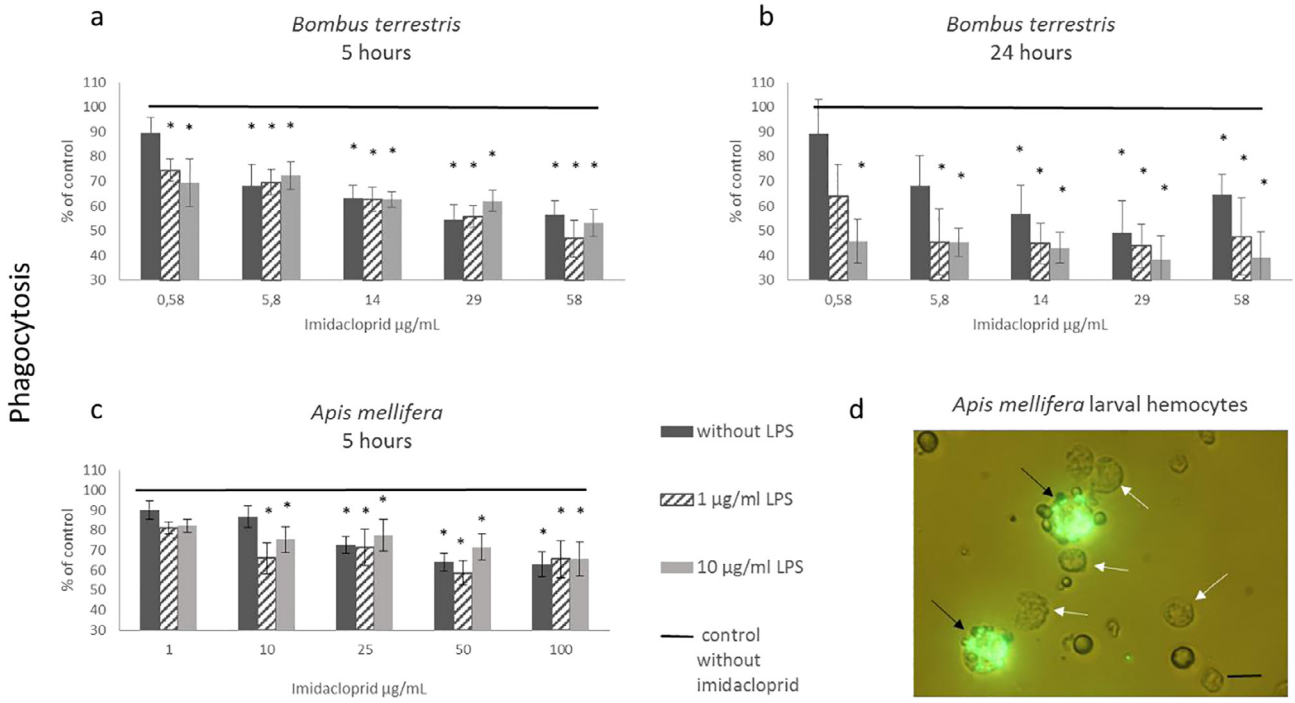

Fig. 2. Phagocytosis of bumblebee larval hemocytes after 5 h (a) and 24 h (b) and honeybee larval hemocytes after 5 h (c) of exposure to 1; 10; 25; 50 and 100 µg/mL of IMI (all n = 9). Hemocytes were incubated for four hours with fluorescent latex beads with 1 µm diameter prior to cell counting. Asterisks show statistically significant differences between the different IMI concentrations and the control group (no IMI). The picture (d) shows honeybee hemocytes that phagocytized fluorescent latex beads (black arrows) and those who did not phagocytize any beads (white arrows). The scale bar is 10 µm.

dropping down from 69% after five hours to 45% at the lowest IMI concentration and high LPS treatment compared to the control group (Fig. 1b). When challenged with a high LPS concentration hemocytes significantly reduced phagocytosis as of the lowest (1 µg/mL) IMI concentration ($p < .004$) while during low LPS challenge IMI only showed an important impact after 10 µg/mL ($p < .006$) and without LPS challenge after 25 µg/mL ($p < .012$).

In all three LPS treatment groups imidacloprid significantly decreased the phagocytosis capacity of honeybee larval hemocytes (Kruskal-Wallis, post hoc Dunn, all p-values $< .001$) after 5 h exposure with increasing concentrations (Fig. 1c). While phagocytosis activity significantly decreased from 25 µg/mL to 100 µg/mL imidacloprid when cells were not immune activated it already decreased at 10 µg/mL imidacloprid when cells were immune activated with either 1 or 10 µg/mL LPS. Honeybee hemocytes showed to be less adherent to the glasscover slips after 24 h of IMI exposure. Cells were lost during the washing process to remove excess fluorescent latex beads, therefore results for honeybee hemocytes are given only after 5 h.

3.3. Reactive oxygen species production in Apis mellifera and Bombus terrestris hemocytes after exposure to IMI

No significant differences in the hydrogen peroxide production of bumblebee hemocytes between the control group and the two treatment groups of no immune activation and low immune activation with LPS was detected after 3 h of exposure to five different IMI concentrations (0 LPS $p = .913$; 1 µg/ml LPS $p = .832$, $n = 10$) (Fig. 3a). However IMI significantly reduced hydrogen peroxide production of bumblebee hemocytes at concentrations of 10; 50 and 100 µg/mL during strong immune activation with 10 µg/mL LPS ($p = .0001$, $n = 10$). At 50 and 100 µg/mL H_2O_2 production was reduced by more than 30 percent.

Honeybee hemocytes showed a stronger sensitivity to IMI in their hydrogen peroxide production compared to bumblebee hemocytes as production significantly decreased at 50 µg/ml IMI and no immune activation ($p = .002$) and 100 µg/mL with low LPS treatment ($p = .006$, $n = 10$)(Fig. 2b). As with bumblebees IMI had a greater impact on honeybee hemocytes when exposure occurred during stronger immune activation with LPS ($p = .0001$) with significant differences to the control as of 25 µg/mL of IMI as production was reduced by more than 20 percent at 25 µg/mL until 57.7 percent compared to the control group.

Nitric oxide was measured between 15 min and 4 h. Results are given as the consolidated production during this time. IMI slightly reduced nitric oxide production in bumblebee hemocytes at 1 µg/mL by 4 percent followed by an induction by five and four percent at 10 and 25 µg/mL and again a reduction at higher concentrations when cells were not immune activated ($p < .0001$, $n = 171$) (Fig. 2c). While during slight immune activation ($p < .0001$, $n = 131$) NO levels increased by 5 percent they decreased by ten percent during strong immune activation ($p < .0001$, $n = 131$) at the lowest tested concentration of 1 µg/mL. This decrease of NO was equally observed for both immune activations at 10 and 25 µg/mL IMI. NO levels rose again to control levels at 50 µg/mL for both immune activations before they dropped again at the highest IMI concentration of 100 µg/mL.

Honeybee hemocytes showed a stronger reaction to IMI concerning the NO production compared to those of bumblebee hemocytes (Fig. 2d). At smaller IMI concentrations (1 and 10 µg/mL) honeybee hemocytes produced more NO during slight immune activation ($p < .0001$, $n = 300$) than without ($p < .0001$, $n =$, $n = 225$) or high immune activation ($p = .0001$, $n = 300$). At higher IMI concentrations (25; 50 and 100 µg/mL) NO decreased by 4 and 8 percent for 25 and 50 µg/mL and increased for one percent at 100 µg/mL without LPS while it increased by 1, 9 and 15 percent during slight immune activation and 11, 17 and 27 percent during strong immune activation respectively.

4. Discussion

In this paper we assess the impact of imidacloprid (IMI) on the

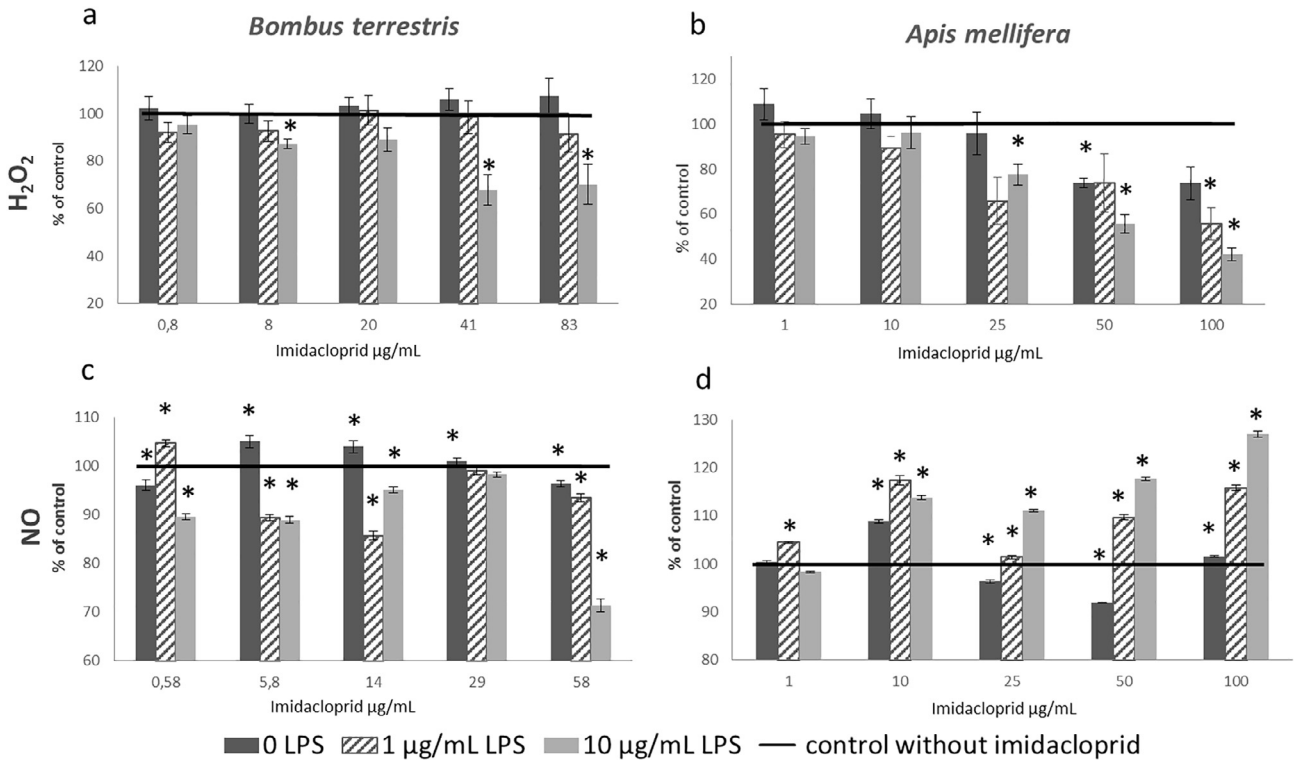

Fig. 3. Production of H_2O_2 of bumblebee (a) and honeybee (b) hemocytes after three hours exposure to 1; 10; 25; 50 and 100 µg/mL IMI (n = 10). NO production of bumblebee (c) and honeybee (d) larval hemocytes during 15 min and 4 h exposure to 1; 10; 25; 50 and 100 µg/mL IMI and 0 (n = 171, n = 225); 1 µg/mL (n = 131, n = 300) and 10 µg/mL LPS (n = 131, n = 300). NO was measured with the fluorescent dye DAF-FM DA. Asterisks show statistically significant differences to the control group (Kruskal-wallis $p < .001$, mean ± SE).

immunocompetence of two pollinating insects (*Apis mellifera* and *Bombus terrestris*) during a simultaneous immune activation induced by LPS, which acts like a pathogen-associated molecular pattern causing a strong inflammatory response. In order to characterize the individual immunocompetence we examined three immune parameters *in vitro* of the two insects' immune cells. More precisely we examined the phagocytosis capacity as well as the production of hydrogen peroxide and nitric oxide of larval hemocytes.

Hemocytes are the basis of the cellular immune response of insects. While in embryos and pupae they play an important role in the removal of apoptotic cells or degenerated tissues, their main function in larvae and adults might be phagocytosis of pathogens (Feldhaar and Gross, 2008). This uptake and digestion of pathogens through phagocytosis is an important step in immune defense (Kocks et al., 2005). Our results show that IMI had an inhibiting effect on phagocytosis in both bumblebee and honeybee hemocytes upon our lowest tested concentration of 1 µg/mL. Di Prisco et al. (2013) found clothianidin, another neonicotinoid insecticide, to negatively affect NF-$_K$B signaling in the Toll-pathway of *Drosophila melanogaster* and *Apis mellifera*. The Toll-pathway in turn plays an important role on immune responses including the activation of phagocytosis (Valanne et al., 2011). However phagocytosis is not only activated through the Toll-pathway but also through the JAK/STAT pathway (Evans et al., 2006). Thus IMI could reduce phagocytosis by inhibiting more than one immune pathway who activates phagocytic activities in hemocytes. Indeed Christen et al. (2016) found that IMI upregulated defensin-1 an antimicrobial peptide of the Toll-pathway in honeybees assuming that IMI might rather act through the JAK/STAT pathway. When comparing phagocytic activity of the two pollinators, bumblebee hemocytes showed to be more sensitive to IMI compared to honeybee hemocytes. Cresswell et al. (2012) also stated that bumblebees are more sensitive to dietary imidacloprid than honeybees. They presumed that the difference could be found in the different metabolic detoxification of the two species. The half-life of IMI in honeybees is four hours (Suchail et al., 2004b) while the half-life in bumblebees was stated to be 10 h (Cresswell et al., 2012). In our study we tested phagocytosis after 5 and 24 h exposure to IMI. Time of exposure indeed played a role in the intoxication process. IMI had a stronger impact on hemocytes during longer exposure times. Phagocytosis in bumblebee hemocytes at the lowest IMI concentration was reduced by more than 50 percent after 24 h compared to 30 percent after 5 h. While imidacloprid showed no strong impact on honeybee hemocytes viability after 24 h of *in vitro* exposure, it did reduce viability of bumblebee hemocytes by 42 percent at our strongest tested concentration of 100 µg/mL of IMI. Honeybee and bumblebee hemocytes cannot be held in continuous cell lines (Goblirsch et al., 2013) therefore cells have to be extracted from larvae just prior to the experiment. As cell viability suffers from time, longer exposure times during *in vitro* experiments could interfere with the actual effect of the pesticide. We thus used short exposure times with high IMI concentrations when compared to IMI concentrations which can be found in the environment (up to 101 ng/g IMI in pollen) (Dively and Kamel, 2012). We did see a stronger impact of IMI with time assuming that lower concentrations than the ones tested here might already lead to an alteration of hemocytes activity given longer incubation time. However, as intoxication was performed *in vitro* we are missing a metabolic degradation of IMI in our experiment to simulate realistic conditions. Especially concerning is the toxicity of IMI metabolites that are formed during degradation of the parent molecule (Suchail et al., 2004a). Either way as neonicotinoids are systemic pesticides pollinators are rather exposed to a continuous low intoxication than high punctual intoxication, thus it is likely that bees keep a certain pesticide concentration in their body over a certain period of time because of continuous pesticide intake.

The additive effect of neonicotinoids and pathogens have been demonstrated in many studies (Abbo et al., 2017; Collison et al., 2016; Czerwinski and Sadd, 2017; López et al., 2017). While most of these studies focused on neonicotinoids and pathogen effects concerning bee

fitness and health or cellular responses to pesticides alone, only few of these concerned cell reactivity next to pesticides and pathogens. Indeed, we found that stress through immune activation with LPS intensified the impact of IMI on hemocytes activity in all three immune parameters tested. We thus showed here that the combined effect of IMI and pathogens is additive at a cellular level. While H_2O_2 and NO production altered between high and low immune activation, phagocytosis of hemocytes did not vary between the two different LPS concentrations. As Moret and Schmid-Hempel (2000) claimed in their study on phagocytosis in bumblebees, LPS and latex beads may induce different pathways of the immune system. This could explain the LPS dose dependent increase and decrease of NO and H_2O_2 when challenged with IMI but no difference between the low and high LPS concentration during phagocytosis activity. LPS did though induce a stronger reduction in phagocytosis when hemocytes were simultaneously exposed to IMI. Cells have to react to multiple stress and activate several immune pathways at the same time. However it does not trigger the same immune pathway thus the effect of higher LPS doses are neglectable for plastic bead phagocytosis. Detoxification is a highly energy consuming process and can thus lead to a weakening effect on the organism's metabolism. Indeed Abbo et al. (2017) evidenced in a recent study that IMI in environmental concentrations reduced the titer of vitellogen, an egg yolk precursor which is often linked to energy homeostasis. Immune efficiency of phagocytosis can further be impacted by IMI as hemocytes also secret oxygen and nitrogen radicals upon immune activation to disable engulfed microbes. Tafalla et al. (2003) suggested that NO might play a role in the inhibition of phagocytosis in carpet shell clam (*Ruditapes decussatus*) hemocytes. However we found an enhancement of NO production in honeybees while we observed a decrease of NO in bumblebees. Yet phagocytosis was stronger inhibited by IMI in bumblebees. Interestingly IMI showed only slight interactions on either H_2O_2 or NO production when cells were exposed to IMI alone. While IMI alone had no significant impact at any concentration on bumblebee H_2O_2 production it was reduced in honeybee cells at very high concentrations. This is surprising as toxicants induced H_2O_2 in human immune cells in a time and dose dependent matter rather than inhibiting it (Baulig et al., 2003; Klestadt et al., 2002; Qin, 2012). Due to their lifestyle bees are exposed to increasing loads of oxidative stress as nectar, pollen and propolis contain allelochemicals which produce reactive oxygen species. ROS and NOS are not only toxic to microbes but also to bee cells thus they have developed a highly effective antioxidant enzymatic system to remove oxygen free radicals (Korayem et al., 2012). IMI might alter this enzyme system enhancing the reduction of H_2O_2. LPS enforced the observed reduction of H_2O_2 by IMI from 30 percent without LPS to almost 60 percent during high immune activation. Results for NO showed the opposite effect in honeybees leading to an increase of NO. Interestingly bumblebees and honeybees responded completely different to IMI when tested on NO production. While IMI slightly increased NO at medium IMI concentrations without immune activation in bumblebees, it decreased at low and high LPS activation. In honeybees NO increased dose dependent with IMI and LPS concentrations. LPS and IMI had a significant additive impact on NO production. NO plays an important role in insect host defense mechanisms against viruses, bacteria and parasites and is produced as an immune effector molecule functioning both as a signaling molecule and a cytotoxic component (Rivero, 2006). NO was shown to play a major part in the beginning of immune activation in honeybee hemocytes upon non-self recognition and hemocyte spreading (Negri et al., 2013). Further it is a ubiquitous pathogen-killing non-specific response to almost any pathogen, thus of relevance in the process of phagocytosis. However these cytotoxic characteristics can also harm the organism itself as high secretion of NO leads to oxidative stress in cells (Rivero, 2006). While NO acts as an inducer of immune response in phagocytes, large amounts of NO produced by hemocytes can trigger apoptosis in nearby cells. Thus, maintaining its homeostasis is critical as changes in its secretion might lead to detrimental immune responses.

5. Conclusion

Imidacloprid clearly affected the immune competence of honeybee and bumblebee hemocytes. The phagocytic activity was reduced and ROS and RNS excretion was either enhanced or decreased, thus altering the homeostasis of the immune system. This effect was further intensified when cells were immune activated with LPS which was simulating a pathogen-like immune response in hemocytes. Results thus showed an additive interaction of pesticide and pathogens to hemocytic activity. Though this additive affect was observed in both pollinator hemocytes, the cell reactivity of both species clearly differed from each other depending on the assessed immune parameter. Both species hemocytes showed differing sensitivities to IMI upon phagocytosis or nitric oxide and hydrogen peroxide synthesis. While IMI decreased NO in bumblebee hemocytes it was strongly enhanced in honeybee hemocytes, suggesting different metabolic intoxication processes in the two pollinator species that might date back to different evolutionary development (Cresswell et al., 2012). In bumblebees a shift in the relative abundance of several species has been observed in Europe and northern America. This decline in species richness is especially affecting specialist bumblebee species with narrow ecological niches. In contrast, generalist species, like *Bombus terrestris,* seem to have benefited from this lack of competition and rather replaced other bumblebee species (Feldhaar, 2016). The impact of pesticides and pathogens should thus also be evaluated on other bumblebee species as it could be one cause of this decline as immune reaction clearly differ upon species.

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Conflict of interest

The authors report no conflicts of interest to be declared.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jinsphys.2018.05.002.

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