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1     **Phagocytic Activity of Human Macrophages and *Drosophila* Hemocytes after**  
2                                   **Exposure to the Neonicotinoid Imidacloprid**

3

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10

11    **ABSTRACT**

12    Neonicotinoid insecticides are increasingly used in modern pest control and in conventional  
13    agriculture. Their residues are frequently found in our environment and in our food leading to  
14    chronic exposure of pollinating insects and humans. Indeed, evidence has become stronger that  
15    chronic exposure to neonicotinoids might have a direct impact on the immune response of  
16    invertebrates and vertebrates. Therefore, we compared the cellular immune response of human  
17    macrophages (THP-1) and *Drosophila melanogaster* hemocytes (Schneider 2 cells) after exposure to  
18    four different concentrations of the neonicotinoid imidacloprid. Cells were immune activated with  
19    LPS (lipopolysaccharide) of *Escherichia coli* to compare the phagocytic activity of immune activated  
20    and non-activated cells during pesticide exposure. *Drosophila* cells were more strongly affected by  
21    the insecticide than human macrophages. Even though imidacloprid showed an adverse effect on  
22    phagocytosis on both cells while immune activated, it decreased phagocytosis in *Drosophila* cells at  
23    shorter exposure time and without immune activation.

24

25    Keywords

26    Imidacloprid, macrophage, phagocytosis, THP-1, *Drosophila*

27

28    1. INTRODUCTION

29 Having been launched on the market by Bayer Crop science in 1991, Imidacloprid (IMI) was the very  
30 first insecticide of a new group of pesticides: the neonicotinoids (Mason et al., 2013). Neonicotinoid  
31 insecticides are nicotinic acetylcholine receptor agonists in insects and are lethal through disruption  
32 of the nervous system. All neonicotinoids are systematic insecticides with a high solubility in water  
33 leading to the uptake of the pesticide by the plant's tissues. The pesticide's active molecule, which is  
34 spread in the entire plant, thus acts directly on any sucking insect that would feed on the plant. Once  
35 distributed in the whole plant it is also available for non-target insects such as pollinating insects as  
36 they feed on contaminated nectar and pollen (Simon-Delso et al., 2015).

37 In the late 1980's the global insecticide market was dominated by carbamates, organophosphates  
38 and pyrethroids, to which many pest insects have developed increasing resistance (Simon-Delso et  
39 al., 2015). The neonicotinoids rapidly took over the global insecticide sales and by 2010 were  
40 responsible for more than 25 percent of the market share with imidacloprid becoming the second  
41 largest selling pesticide worldwide after Glyphosate (Jeschke et al., 2010). Despite the ban of  
42 imidacloprid, clothianidin and thiamethoxam for outdoor application (they are still authorized in  
43 greenhouses) in the European Union in May 2018 (Official Journal of the European Union, L 132/31,  
44 2018), imidacloprid is still one of the most used insecticides in the world. In fact 75 percent of  
45 neonicotinoids were used in Latin America, North America and Asia. Europe only accounted for 11  
46 percent of the worldwide use (Bass et al., 2015).

47 The most common application and spread of neonicotinoids in agriculture is the use of seed coatings  
48 or dressings which generates toxic dust during the process of sowing and can lead to contamination  
49 of surrounding vegetation (Bonmatin et al., 2015). Further they are also used against sucking insects  
50 in fruit and vegetable cultivations as well as in viticulture and hop growing. Residues of imidacloprid  
51 and other neonicotinoids have been found in honeybees, honey and their pollen (Chauzat et al.,  
52 2009; Codling et al., 2016; David et al., 2016; Hladik et al., 2016; Kasiotis et al., 2014) as well as fruits  
53 and vegetables we consume (Fernández-Alba et al., 2000; Ko et al., 2014; Obana et al., 2002).

54 In the last decade neonicotinoids have been controversially discussed in literature as they have been  
55 associated with a worldwide pollinator decline. While acute toxicity occurs rather seldom the cause  
56 of this decline was associated with chronic pesticide exposure. There is a growing awareness that  
57 neonicotinoids could weaken the immune response of pollinators. Indeed several studies have  
58 shown a weakening effect of neonicotinoids and pathogens to pollinators (Abbo et al., 2017; Alaux et  
59 al., 2010; Fauser-Misslin et al., 2014; Pettis et al., 2012; Vidau et al., 2011). The suppression of the  
60 immune response to infectious diseases by neonicotinoids was not only shown for pollinators  
61 (Brandt et al., 2016; Di Prisco et al., 2013; López et al., 2017) but also hypothesized for insectivorous  
62 vertebrates like amphibians, birds, fish and mammals, when comparing disease outbreaks with the  
63 use of neonicotinoids in the concerned regions and countries (Mason et al., 2013). A study of

64 Sánchez-Bayo and Goka (2005) supports this idea, claiming that medaka fish (*Oryzias latipes*)  
65 exposed to imidacloprid were massively infested by a *Trichodina* ectoparasite when compared to  
66 control groups without pesticides and groups exposed to zinc pyriithione.

67 Also other reviews support this hypothesis and Gibbons et al. (2015) stated that imidacloprid and  
68 clothianidin exert sublethal effects on vertebrates ranging from genotoxic and cytotoxic effects, and  
69 impaired immune function, to reduced growth and reproductive success. Mostafalou and Abdollahi  
70 (2013) mentioned human chronic diseases like cancer, Alzheimer, Parkinson or autoimmune diseases  
71 that could be linked to sublethal pesticides exposure, even though they did not mention imidacloprid  
72 in their work. Though Cimino et al. (2016) cited several studies of chronic exposure to imidacloprid  
73 which showed adverse health effects on humans, still not much is known about the effect of  
74 neonicotinoids on human health.

75 Innate immune responses in mammals and insects do show several functional similarities. Cells,  
76 especially phagocytes, of these innate immune systems are comparable both in terms of morphology  
77 and reactivity towards pathogen recognition (Browne et al., 2013). An important role in the primary  
78 immune response is given to phagocytes. These cells are responsible among others for the  
79 recognition and removal of foreign pathogens and the recruitment of other immune cells through  
80 the release of cytokines (Chanput et al., 2014). Insect hemocyte's main function is phagocytosis of  
81 pathogens and apoptotic material (Feldhaar and Gross, 2008) therefore being of major importance to  
82 the organism. The most dominant hemocytes in *Drosophila* are plasmatocytes which are highly  
83 motile and resemble monocytic macrophages in humans (Feldhaar and Gross, 2008). Insect  
84 hemocytes are able to change their state from non-adherent to adherent upon pathogen recognition  
85 and respond by "spreading" which enhances phagocytosis of small antigens (Negri et al., 2013). In  
86 contrast to human monocytes, insect hemocytes are able to perform nodulation and encapsulation  
87 of larger foreign objects like parasites as they adhere and spread over the surface of the foreign  
88 object until they form overlapping layers (Negri et al., 2013). The encapsulation is often followed by  
89 melanisation of the antigen (Schmid et al., 2008) and hemocytes are also able to secrete cytotoxic  
90 compounds into the capsule (Negri et al., 2013). Imidacloprid could have a suppressive effect on this  
91 cellular immune response of invertebrates as well as of vertebrates.

92 Hence in this study we investigate the impact of imidacloprid (IMI) on the phagocytic activity of  
93 immune system cells of one invertebrate and one vertebrate species. We chose the cell lines  
94 Schneider-2 from *Drosophila melanogaster* and THP-1, a human monocytic cell line that can be  
95 differentiated into macrophages. To simulate an immune reaction we activated the cells with  
96 lipopolysaccharide (LPS) of *Escherichia coli*.

97

## 98 2. MATERIALS AND METHODS

## 99 2.1. Cell culture and exposure

100 Both human THP-1 (Ref. ACC 16) and *D. melanogaster* Schneider-2 (Ref. ACC 130) cells were  
101 purchased from Leibnitz – Institute DSMZ- Deutsche Sammlung von Mikroorganismen und  
102 Zellkulturen GmbH. THP-1 cells were maintained in RPMI 1640 with 10 percent heat inactivated FBS  
103 (fetal bovine serum), 2mM L-Glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin at 37 °C and 5  
104 percent CO<sub>2</sub>. Schneider cells were kept in 44.75 percent Schneider's insect medium, 44.75 percent  
105 TC-100 insect medium, 10 percent heat inactivated FBS, 50 U/mL penicillin and 50 µg/mL  
106 streptomycin at 20 °C.

107 Imidacloprid (37894 Sigma-Aldrich, powdery analytical standard) was dissolved in PBS (pH 7.4) at 300  
108 µg/mL prior to experiments. Imidacloprid was further diluted in RPMI or Schneider medium in order  
109 to obtain the following concentrations: 1; 25; 50 and 100 µg/mL IMI. During each experiment all cell  
110 solutions have been tested with HPLC to determine the actual concentration of IMI that has been in  
111 contact with cells to avoid measuring errors. An analytical standard of Imidacloprid solution (46341  
112 Sigma-Aldrich) of 100 µg/mL in acetonitrile was used to obtain a standard curve for HPLC analyses.  
113 Cells were incubated with imidacloprid at the cell particular conditions for 5 or 24 hours. Cell viability  
114 was examined after 5 and 24 hours with a trypan blue solution (2 g/L in 0.9 percent NaCl solution).  
115 Trypan blue is a diazo dye used for vital staining of cells (Tolnai, 1975). After the exposure to  
116 imidacloprid cells were washed with PBS and incubated for 16 hours with LPS (lipopolysaccharide)  
117 from *Escherichia coli* at concentrations of 0.1 µg/mL or 1 µg/mL for THP-1 and 1 µg/mL for Schneider  
118 cells. LPS is an endotoxin and a component of the outer membrane of Gram-negative bacteria  
119 resulting in a strong inflammatory response of the immune system.

## 120 2.2. Phagocytosis

121 Phagocytosis of THP-1 and Schneider cells was examined by using amine-modified polystyrene,  
122 fluorescent yellow-green latex beads (1 µm mean particle size) after slight modifications of the  
123 methodology of Bennisroune et al. (2012).

124 THP-1 cells were differentiated to their macrophage phenotype with 5 ng/mL of PMA (phorbol 12,  
125 myristate 13-acetate) for 48 hours and washed once with PBS prior to pesticide exposure. Schneider  
126 cells and differentiated THP-1 cells were grown on glass coverslips at a concentration of  $7 \times 10^5$   
127 cells/mL in six-well or twelve-well plates. Cells were exposed to different concentrations of  
128 imidacloprid from 1 µg/mL to 100 µg/mL, including a negative control without IMI, for either 5 or 24  
129 hours at the particular cell conditions. 4 hours prior to the phagocytosis measure, 100 latex beads  
130 per cell diluted in the cell particular medium were added to each well. After 4 hours of incubation  
131 cells were washed with 3 percent BSA (Bovine serum albumin) dissolved in PBS in order to eliminate

132 latex beads which have not been absorbed by the cells (Komiyama et al., 2003). The glass coverslips  
133 of differentiated THP-1 cells and Schneider cells were dipped three times in the BSA solution before  
134 being dipped into PBS. Glass coverslips were then mounted on object plates and phagocytosis was  
135 measured under a fluorescent microscope. A minimum of 250 cells were counted for each sample.  
136 Phagocytosis was calculated as follows:

$$137 \quad \text{cells (phagocytosis)} = \frac{\text{number of cells ingesting at least 1 bead}}{\text{number of total cells}} * 100$$

138

### 139 2.3. Statistical analyses

140 All data was analysed using Addinsoft (2019) XLSTAT statistical and data analysis solution, Long  
141 Island, NY, USA. For all tests a probability level of 0.05 was considered to be statistically significant.  
142 After checking for normality and variance homogeneity using the Shapiro-Wilk's test and the  
143 Bartlett's test, a one-way ANOVA was performed to identify differences between different IMI  
144 groups of each LPS treatment, followed by a Dunnett's test for comparison of different IMI groups to  
145 the control group or a Tukey's test of multiple comparison between different IMI groups of the same  
146 LPS treatment. Data for viability of human THP-1 cells after 5 hours exposure time were log  
147 transformed in order to fit parametric conditions. As parametric conditions were not given for the  
148 viability of human THP-1 cells after 24 hours exposure time, even after log or arcsine transformation,  
149 we used a non-parametric Kruskal-Wallis test.

150 Results were expressed as means of all replicates  $\pm$  SE (standard error).

151

## 152 3. RESULTS

### 153 3.1. *D. melanogaster* Schneider cells

154 Cell viability in Schneider cells of *D. melanogaster* was higher after 5 hours than after 24 hours  
155 exposure to IMI. Cell viability varied between  $88.4 \pm 0.47$  percent and  $90.9 \pm 0.96$  percent after 5  
156 hours and between  $84.6 \pm 1.73$  percent and  $80.4 \pm 1.84$  percent after 24 hours. No statistically  
157 significant differences were found between the control without IMI and different IMI concentrations  
158 neither after 5 hours (ANOVA  $p=.233$  with  $n=9$ ) nor after 24 hours (ANOVA  $p=.480$  with  $n=9$ ) (Figure  
159 1).

160 Phagocytosis of *D. melanogaster* Schneider cells was tested after 5 and 24 hours of exposure to IMI  
161 with or without immune activation with  $1 \mu\text{g/mL}$  LPS of *Escherichia coli*.

162 Phagocytosis after 5 hours and no LPS activation showed a decline of phagocytosis with increasing  
163 concentrations of IMI.  $24.84 \pm 1.71$  percent of cells from the control group were able to phagocytize  
164 a minimum of one latex bead. Percentages decreased continuously with increasing pesticide  
165 concentrations until phagocytosis reached only  $5.02 \pm 0.86$  percent at the highest IMI concentration,  
166 which was statistically significant from the control (Dunnett  $p=.035$ , with  $n=3$ ). When immune  
167 activated with  $1 \mu\text{g/mL}$  LPS, phagocytosis increased slightly up to  $30.74 \pm 3.26$  percent in the control  
168 group. IMI treated groups revealed decreasing percentages of phagocytosis compared to the control.  
169 Statistically significant differences were determined between 25, 50 and  $100 \mu\text{g/mL}$  IMI and the  
170 control group (Tukey  $p=.009$ ;  $p=.025$ ;  $p=.004$  respectively). Phagocytosis in these three treatment  
171 groups varied from  $21.37 \pm 1.15$  percent to  $18.11 \pm 0.95$  percent.

172 After 24 hours of exposure to IMI and no immune activation phagocytosis ranged from  $42.90 \pm 4.99$   
173 percent in the control group to  $27.85 \pm 1.38$  percent after IMI exposure, showing no statistical  
174 significant difference between control and IMI (ANOVA  $p=.222$  with  $n=3$ ).

175 After immune activation with  $1 \mu\text{g/mL}$  LPS for 16 hours following a 24 hours exposure to IMI we  
176 observed a statistically significant decrease of phagocytosis with increasing pesticide concentrations  
177 upon the lowest used IMI concentration (Tukey  $p=.033$ ,  $p=.0001$ ,  $p=.0001$ ,  $p<.0001$  for 1; 25; 50 and  
178  $100 \mu\text{g/mL}$  IMI respectively). Values varied between  $31.55 \pm 1.92$  percent in the control group and  
179  $12.92 \pm 1.19$  percent at the highest IMI concentration. At the lowest used IMI concentration of  $1$   
180  $\mu\text{g/mL}$  only  $24.05 \pm 1.01$  percent of cells were capable of phagocytosis, which is about a quarter less  
181 compared to the control group. At  $25 \mu\text{g/mL}$  the ability of cells for phagocytosis was reduced by one  
182 third and at  $50 \mu\text{g/mL}$  it was already reduced by more than half compared to the control (Figure 2, b).

### 183 3.2. Human THP-1 cells

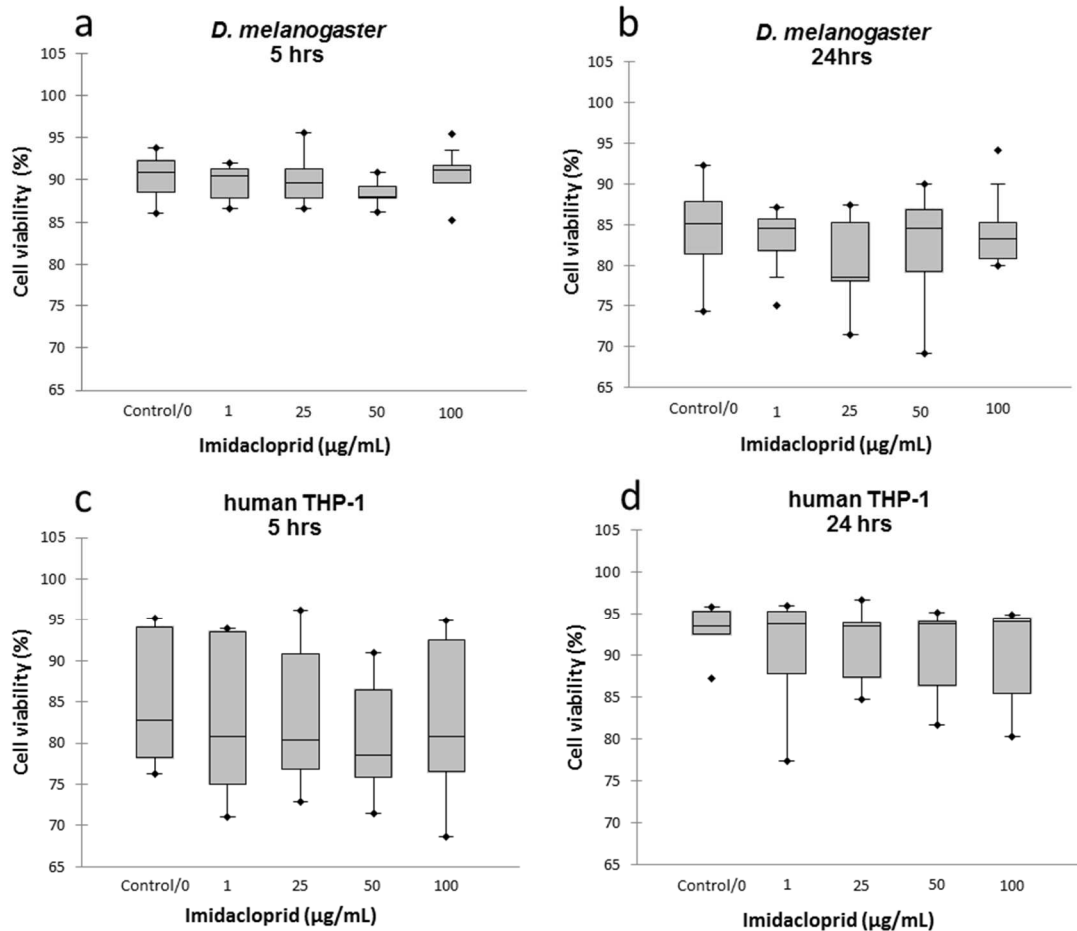
184 Cell viability in THP-1 was measured after 5 and 24 hours exposure to IMI. Viability in human THP-1  
185 cells was between  $92.9 \pm 1.06$  and  $90.3 \pm 2.05$  percent after 24 hours exposure while it ranged  
186 between  $84.9 \pm 2.61$  percent and  $80.6 \pm 2.27$  percent after 5 hours. No statistical significant  
187 differences in THP-1 viability were found between the control group and any tested IMI  
188 concentration at 24 hours (Kruskal-Wallis  $p=.857$  with  $n=12$ ) or 5 hours (ANOVA  $p=.88$  with  $n=9$ )  
189 exposure to IMI (Figure 1).

190 Phagocytosis of differentiated THP-1 cells was stimulated by immune activation of either  $0.1 \mu\text{g/mL}$   
191 or  $1 \mu\text{g/mL}$  LPS. Percentages of phagocytizing cells of the same population increased in average  
192 about 16.4 percent while stimulated with LPS. Phagocytosis measured after an exposure of 5 hours  
193 to IMI and no immune activation with LPS was ranging between  $37.3 \pm 5.02$  percent and  $55.3 \pm 4.78$   
194 percent. Phagocytosis increased with immune activation and varied between  $62.5 \pm 4.31$  percent to

195 72.2 ± 4.76 percent at 0.1 µg/mL LPS and between 52.1 ± 1.62 percent and 68.3 ± 3.21 percent at  
196 1µg/mL LPS activation. No statistically significant difference between the control group and the  
197 treatment groups was found for all tests after 5 hours exposure without, with 0.1 µg/mL or 1 µg/mL  
198 LPS (ANOVA, p=.161, p=.624 and p=.070 respectively, with n=3).

199 No statistically significant differences in phagocytosis between different IMI concentrations and the  
200 control were found after 24 hours of exposure and no immune activation with LPS (ANOVA p=.102  
201 with n=3). Values varied between 84.07 ± 2.15 percent and 70.23 ± 2.65 percent. However,  
202 differences between different IMI concentrations were found after immune activation with either 0.1  
203 µg/mL or 1 µg/mL LPS. After activation with 0.1 µg/mL LPS phagocytosis decreased after 24 hours IMI  
204 exposure in treated groups compared to the control group for up to 17.1 percent. The biggest  
205 decreases in phagocytosis were found at 25 µg/mL and 100 µg/mL IMI which was also confirmed by  
206 statistical analyses (Tukey p=.038 and p=.044 respectively). 81.33 ± 1.43 percent of cells of the  
207 control group were capable of phagocytizing at least one latex bead while this percentage decreased  
208 with increasing IMI concentrations except for cells at 50 µg/ml IMI which were able to phagocytize  
209 more latex beads than cells at 25 µg/mL IMI. Phagocytosis after 24 hours and later activation with 1  
210 µg/mL LPS (Figure 2) ranged from 81.21 ± 4.69 percent at the control group to 67.2 ± 4.99 percent at  
211 50µg/mL IMI, which was statistically different from the control group (Tukey p=.034).





212

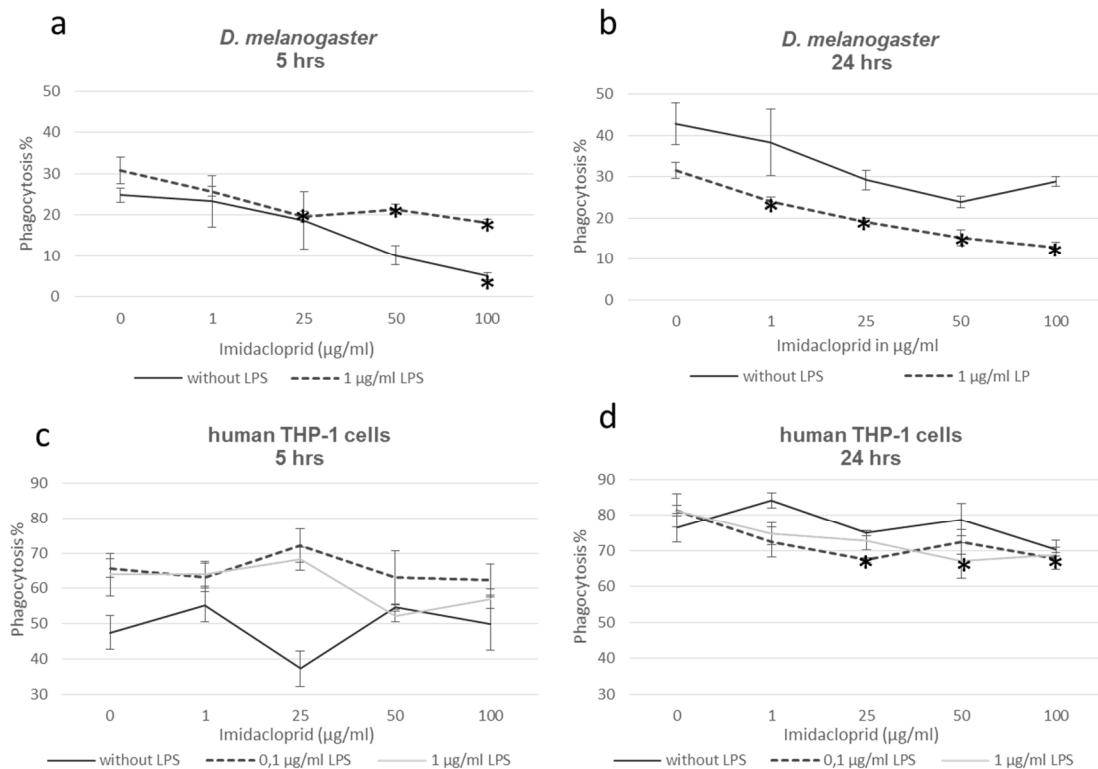
213 **Figure 1: Viability of *D. melanogaster* Schneider cells (a,b) and human THP-1 cells (c,d) after 5 and 24**  
 214 **hours exposure to IMI. No statistical differences were found between the control group without IMI and**  
 215 **the IMI concentrations neither for Schneider after 5 and 24 hours nor THP-1 after 5 and 24 hours (ANOVA:**  
 216 **p=.233; p=.480; p=.880; Kruskal-Wallis p=.857 and n=9; n=9; n=9; n=12 respectively).**

217

#### 218 4. DISCUSSION

219 In the last decade neonicotinoid insecticides have been associated with a worldwide pollinator  
 220 decline as they might act as an immune suppressor in insects. But evidence has become stronger  
 221 that they might not only weaken the immune response in insects but also in insectivorous  
 222 vertebrates (Mason et al., 2013). Further residues of neonicotinoids have also been detected in  
 223 human urine as we are exposed to contaminated food (Nomura et al., 2013; Taira et al., 2013). A few  
 224 studies exist on human chronic exposure to neonicotinoids but still not much is known on their  
 225 impact on human immune cells (Han et al., 2018). To investigate this effect, macrophage-like  
 226 immune system cells of two different species, *Drosophila melanogaster* and human, were tested *in*  
 227 *vitro* on their cellular reactivity when confronted with four different concentrations of IMI (1, 25, 50  
 228 and 100 µg/mL). The cells of the two organisms had similar characteristics and were able to

229 phagocytize particles. Thus we measured the phagocytic capacity of the two cell lines after pesticide  
 230 exposure. Further cells were exposed to LPS of *Escherichia coli*, which acts as a pathogen associated  
 231 molecular pattern (PAMP). Hence cells confronted with LPS were immune activated leading to an  
 232 immune reaction or an inflammatory response. We were then able to compare the effect of IMI on  
 233 immune activated and non-activated cells.

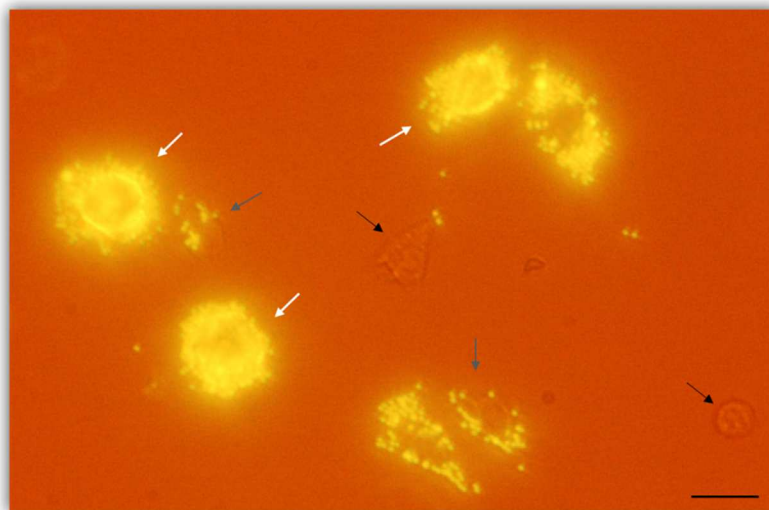


234

235 **Figure 2: Phagocytosis of *D. melanogaster* Schneider cells (a,b) and human THP-1 cells (c,d) after 5 and 24 hours exposure**  
 236 **to IMI and immune activation with LPS. Stars indicate statistically significant differences from the control group without**  
 237 **IMI (ANOVA  $p < .05$  with  $n = 3$ ).**

238 Without immune activation IMI significantly decreased phagocytosis of Schneider cells after 5 hours  
 239 of exposure at the highest tested concentration. Immune activation with LPS increased this inhibiting  
 240 effect leading to a significant decrease of phagocytosis upon 25 µg/mL IMI. These results match with  
 241 results of Di Prisco et al. (2013) who found clothianidin, another neonicotinoid insecticide, to  
 242 negatively affect the activation of the Toll-pathway of *Drosophila melanogaster*. The Toll-pathway in  
 243 turn plays an important role on immune responses including the activation of phagocytosis (Valanne  
 244 et al., 2011). Similar results on phagocytosis were also obtained after an exposure of 24 hours. IMI  
 245 significantly decreased phagocytosis in Schneider cells after immune activation upon our lowest  
 246 tested concentration of 1 µg/mL. It thus seems that the immune activation with LPS enforces the  
 247 toxic effect of IMI in *Drosophila* cells as immune stress increases.

248 As for human macrophages IMI significantly decreased phagocytosis only after 24 hours exposure at  
249 higher IMI concentrations when cells were immune activated with either 0.1 µg/mL or 1 µg/mL LPS.  
250 No significant alteration of phagocytosis was found in macrophages after the shorter exposure time  
251 of 5 hours with or without immune activation. Several studies have shown a dose-dependent  
252 suppression of phagocytosis of phagocytic vertebrate cells alongside other pollutants (Bennasroune  
253 et al., 2012; Couleau et al., 2012; Dunier et al., 1991; Roszell and Anderson, 1994) suggesting that  
254 concentrations used were not high enough to induce effects on phagocytosis of THP-1 cells after  
255 short time exposure. In THP-1 cells LPS results in an activation of the NF-κB transcription factor  
256 (Chanput et al., 2014). Di Prisco et al. (2017) tested clothianidin on the THP-1 cell line showing that  
257 NF-κB was down regulated by the neonicotinoid insecticide. However, it is to mention that we were  
258 only differentiating between cells that were able to phagocytise at least one fluorescent bead and  
259 those who did not phagocytize any beads at all. We did not consider the number of beads that were  
260 ingested by the cells. As shown in Figure 3 cells were able to engulf many beads, making it impossible  
261 to count their number. We did though see cells that clearly ingested less beads than others when  
262 observed under the microscope. Therefore, it is possible that with our method we might not have  
263 been able to detect an effect of IMI on the efficiency of phagocytosis. IMI could be influencing  
264 different aspects of the phagocytic process. With our method we cannot clearly say if IMI can  
265 influence the rate of the uptake of bacteria or the degradation of bacteria once engulfed.



266

267 *Figure 3 : Phagocytized fluorescent latex beads by Schneider 2 cells of D. melanogaster. White arrows show cells that*  
268 *engulfed a high number of beads, grey arrows show cells that engulfed a smaller number of beads and black arrows show*  
269 *cells that did not phagocytise any fluorescent latex bead. The scale bar is 10 µm.*

270

271 IMI already showed an effect on Drosophila Schneider cells even when cells were not stimulated with  
272 LPS whereas effects in human THP-1 cells only occurred if cells were confronted with LPS and thus  
273 initiating an immune reaction. Having in mind that IMI is an insecticide, with insects being the

274 desired target, it is not surprising that Schneider cells are reacting more sensitive to it than human  
275 THP-1 cells. Cresswell *et al.* (2012) interpreted in their results that temperature might play a role  
276 during IMI intoxication in honey and bumblebees. Schneider cells as well as bee hemocytes were  
277 incubated at 22 °C while THP-1 cells were incubated at 37 °C. These temperature differences are due  
278 to different cell characteristics but it is not to neglect that this may play a role in the intoxication  
279 processes in the two cell lines. Camp and Buchwalter (2016) and Song *et al.* (1997) found higher  
280 chronic toxicity of IMI on aquatic invertebrates at lower temperatures while Van den Brink *et al.*  
281 (2016) found that summer populations of aquatic insects were stronger effected by IMI than winter  
282 populations. Apart from intoxication processes temperature also plays a role during the immune  
283 response. Indeed Linder *et al.* (2008) suggested that colder temperatures had rather beneficial  
284 effects on the immune response of *Drosophila* as heat shock proteins may improve immune function.  
285 According to our results IMI did not reduce viability of human THP-1 nor *Drosophila*'s Schneider cells  
286 at any tested concentration from 1 µg/mL to 100 µg/mL IMI. We were thus working with sublethal  
287 doses in our experimental conditions. Hence EC<sub>50</sub> values are dependent on cell and tissue type as it  
288 was already assumed by Benzidane *et al.* (2011). Concentrations used during experiments were  
289 ranging from 1 µg/mL to 100 µg/mL, which were quite high when compared to environmental  
290 concentrations. Chauzat *et al.* (2009) and Kasiotis *et al.*(2014) detected 5.7 to 73.9 ng/g imidacloprid  
291 in pollen while LD<sub>50</sub> values for honey bees varied from 4.5 ng – 81 ng/bee for oral ingestion (Nauen *et*  
292 *al.*, 2001; Cresswell, 2011) and from 18 ng – 102 ng/bee during contact (Iwasa *et al.*, 2004; Nauen *et*  
293 *al.*, 2001). In *Drosophila* the LD<sub>50</sub> at oral ingestion was estimated at 0.41 - 1.39 µg/g (Arain *et al.*,  
294 2014). It is, though, to mention that IMI has a very short half-life in organisms. IMI showed a similar  
295 metabolism in honey bees as in *Drosophila melanogaster*. In bees the half-life of IMI accounts of 4.5  
296 hours (Chauzat *et al.*, 2009) and is reduced to several metabolites like 6-chloronicotinic acid and urea  
297 derivative (Suchail *et al.*, 2004). Consequently concentrations of IMI in honeybees could be way  
298 higher than the actual ones found, depending on when the bee has ingested contaminated nectar for  
299 the last time. Further these metabolites might have other toxicological effects on the host organisms.  
300 Indeed Suchail *et al.* (2004) claims that the mortality of bees is more due to the action of the  
301 metabolites than the actual parent molecule. The EC<sub>50</sub> value of honey bee Kenyon cells was  
302 estimated at around 6.3 µg/mL IMI (Déglise, *et al.*, 2002). As IMI is an agonist on the nicotinic  
303 acetylcholine receptors effects on neuronal cells are expected to be higher than on immune system  
304 cells. It is therefore not implausible that effects on the two cell types used appeared at higher  
305 concentrations. These indications should be kept in mind when analysing *in vitro* toxicity test as we  
306 are missing metabolic processes.

307 To conclude we can summarize that the concentrations from 1 µg/mL to 100 µg/mL of IMI did reduce  
308 phagocytic activity in both human macrophages and *Drosophila* hemocytes after immune activation

309 with LPS of *Escherichia coli*. LPS clearly intensified the inhibiting effects of IMI on cell phagocytic  
310 activity. Phagocytosis in *Drosophila* cells was more strongly inhibited than in human macrophages.  
311 While we observed no effect of IMI on phagocytosis of human macrophages without immune  
312 activation, IMI did reduce phagocytosis in *Drosophila* cells after even short exposure and no immune  
313 activation.

314

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#### 317 Conflict of interest

318 The authors report no conflicts of interest to be declared.

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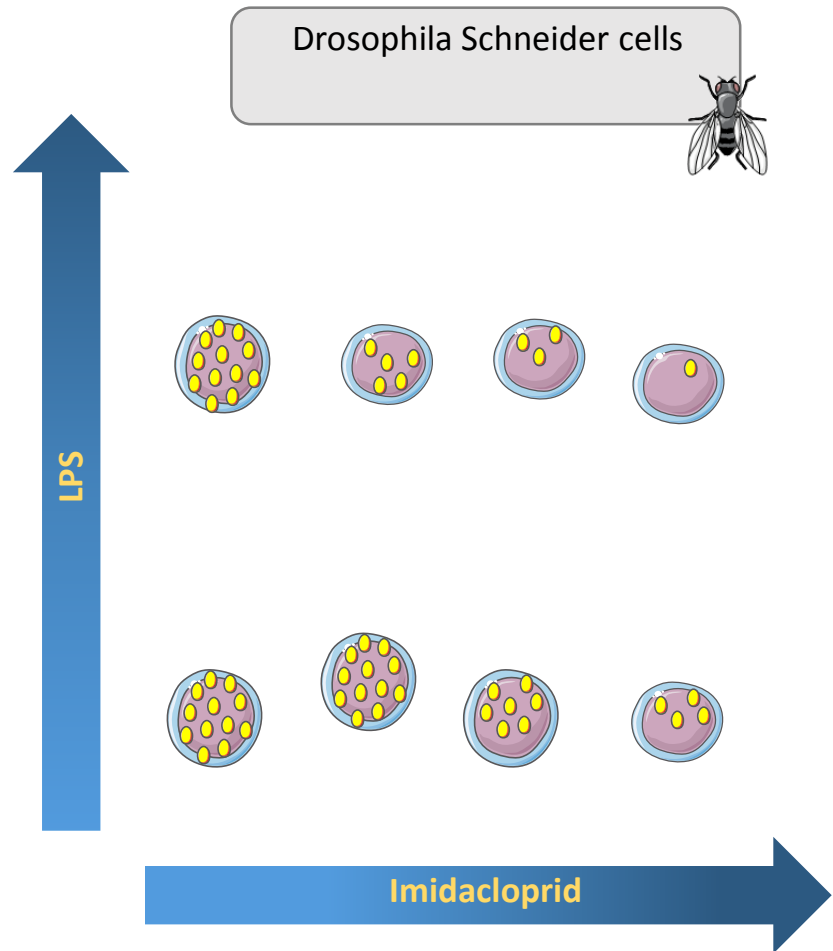
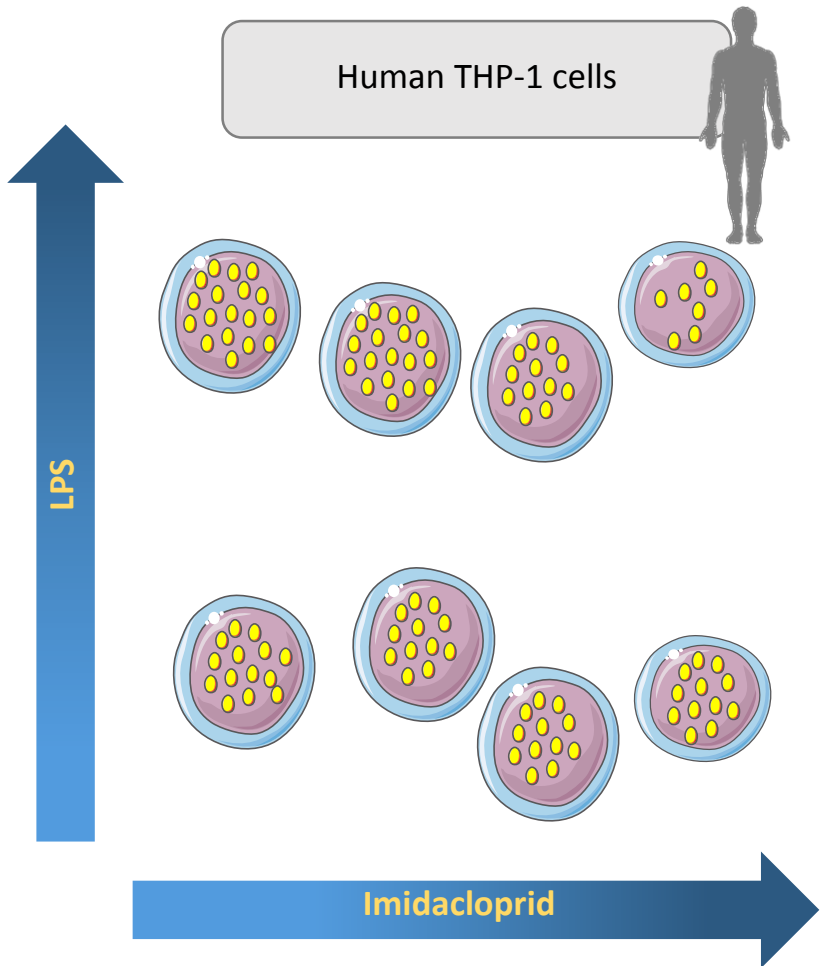
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Phagocytosis of latex beads after immune activation with LPS

Phagocytic cell

Fluorescent latex bead

Detailed description: A legend box with rounded corners. It contains a title 'Phagocytosis of latex beads after immune activation with LPS'. Below the title are two items: a purple circular cell with a blue outline, labeled 'Phagocytic cell', and a small yellow dot, labeled 'Fluorescent latex bead'.