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The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (*Apis mellifera* L.)

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ABSTRACT

A strong immune defense is vital for honey bee health and colony survival. This defense can be weakened by environmental factors that may render honey bees more vulnerable to parasites and pathogens. Honey bees are frequently exposed to neonicotinoid pesticides, which are being discussed as one of the stress factors that may lead to colony failure. We investigated the sublethal effects of the neonicotinoids thiacloprid, imidacloprid, and clothianidin on individual immunity, by studying three major aspects of immunocompetence in worker bees: total hemocyte number, encapsulation response, and antimicrobial activity of the hemolymph. In laboratory experiments, we found a strong impact of all three neonicotinoids. Thiacloprid (24 h oral exposure, 200 μg/l or 2000 μg/l) and imidacloprid (1 μg/l or 10 μg/l) reduced hemocyte density, encapsulation response, and antimicrobial activity even at field realistic concentrations. Clothianidin had an effect on these immune parameters only at higher than field realistic concentrations (50–200 μg/l). These results suggest that neonicotinoids affect the individual immunocompetence of honey bees, possibly leading to an impaired disease resistance capacity.

1. Introduction

Honey bees provide vital pollination services to crops and wild plants and are thus important components for food security and the maintenance of biodiversity (Bascompte et al., 2006; Fontaine et al., 2006; Klein et al., 2007). Recent reports on global pollinator declines (Biesmeijer et al., 2006; Potts et al., 2010a; Cameron et al., 2011) are alarming, especially with respect to the increasing demands for pollination services (Klein et al., 2007; Aizen and Harder, 2009). Honey bees are the most economically valuable pollinators (Klein et al., 2007). However, the number of managed honey bees decreased by one fourth in Europe between 1985 and 2005 (Potts et al., 2010b; Goulson et al., 2015) and by more than one half in North America between 1947 and 2005 (vanEngelsdorp et al., 2008; Goulson et al., 2015; National Research Council, 2007).

Several stress factors are suspected to negatively affect the survival of honey bee colonies. There is consensus on the involvement of multiple causal factors, parasites and pathogens are among the main candidates (Genersch et al., 2010), but diet quantity, quality, and diversity (Alaux et al., 2010; Brodschneider and Crailsheim, 2010; Di Pasquale et al., 2013) as well as exposure to pesticides may also affect colony survival (Sandrock et al., 2014; Goulson et al., 2015). In particular the application of neonicotinoid insecticides, which has increased substantially on a global scale over the last decade (Elbert et al., 2008; Mullin et al., 2010; Jeschke et al., 2011; van der Sluijs et al., 2013; Goulson et al., 2015), has been suspected to represent a major threat to honey bee survival (Desneux et al., 2007; Goulson, 2013; Pisa et al., 2015; Vanbergen and the Insect Pollinators Initiative, 2013).

Neonicotinoids are neurotoxins that act as agonists of the nicotinic acetylcholine receptor by disrupting the neuronal cholinergic signal transduction, leading to abnormal behavior, immobility and death of target insect pests (Matsuda et al., 2001; Tomizawa and Casida, 2005; Elbert et al., 2008). Frequently, non-target insects, like honey bees, come into contact with these insecticides (Pisa et al., 2015). Neonicotinoids are referred to as "systemic" as they are absorbed by plants and spread to all tissues through their vascular system (Elbert et al., 2008). Thus, pollen, nectar and also guttation fluids can contain neonicotinoids (Desneux et al., 2007; Cresswell, 2011; Blacquière et al., 2012; Goulson, 2013; van der Sluijs et al., 2013; EASAC, 2015). Thus, forager bees can come into contact with neonicotinoid-contaminated pollen and nectar and transport them to the hive, where they are frequently detected in honey and bee bread (Genersch et al., 2010; Blacquière et al., 2012; Rosenkranz et al., 2014).

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Direct lethal effects of neonicotinoids, caused by accidental exposure of forager bees to acute toxic concentrations of neonicotinoids, occur only rarely (Pistorius et al., 2009). More commonly, honey bees are exposed to lower concentrations of neonicotinoids, leading to sublethal effects, like impaired learning or homing behavior (Yang et al., 2008, 2012; Han et al., 2010; Henry et al., 2012; Decourtye et al., 2004). Some neonicotinoids like thiacloprid are applied as sprays on flowering crops, e.g. oil seed rape. Others, like imidacloprid, clothianidin, or thiamethoxam are mainly applied as seed dressings or soil applications. Recently, these latter three neonicotinoids have been temporarily banned by the European Commission (2013), because of growing concerns about the risk they may pose on honey bees and other pollinators (Gross, 2013; EFSA, 2013a,b,c, 2014).

Colony losses are often associated with high infection levels of pathogens and parasites (Neumann and Carreck, 2010; Ratnieks and Carreck, 2010). This suggests a causal link between external stress factors and reduced immune function (Gregory et al., 2005; Yang and Cox-Foster, 2005). The immune defense depends on several internal and external factors such as the nutritional state or the age of honey bees (Wilson-Rich et al., 2008; Di Pasquale et al., 2013; Mao et al., 2013; Frias et al., 2015; Negri et al., 2015). Moreover, there is evidence that the invasive ectoparasite *Varroa destructor* Anderson and Trueman (2000) impairs the immune defense of honey bees by reducing the expression of immune-relevant genes and boosting viral replication, thereby affecting lifespan and disease resistance (Yang and Cox-Foster, 2005; Nazzi et al., 2012).

The immune defense of honey bees may also be affected by pesticides (for review see James and Xu, 2012). The exposure to sublethal dosages of neonicotinoids is often associated with a higher pathogenic impact, including the prevalent gut-parasite *Nosema* spp. and viruses typically associated with *V. destructor*, such as deformed wing virus (DWV) (Alaux et al., 2010; Aufauvre et al., 2012; Pettis et al., 2012; Fauser-Misslin et al., 2014; Doublet et al., 2015). Di Prisco et al. (2013) demonstrated that the neonicotinoid clothianidin adversely affects a member of the gene family NF-κB and promotes the replication of the deformed wing virus in honey bees bearing a covert infection. Due to its central role in insect immunity (Evans et al., 2006; Schlüns and Crozier, 2007), pesticide induced changes in NF-κB-related signaling may also affect other immune responses, like encapsulation, wound healing, or antimicrobial defense. However, the effect of neonicotinoids on these functional traits of honey bee immunity has not been investigated so far (for review see Collison et al., 2015).

In this study, we examined whether general immune defense mechanisms of adult worker honey bees are affected by sublethal concentrations of neonicotinoids. Neonicotinoid exposure was performed in laboratory cage experiments, including field realistic concentrations found in bee products. Since disease resistance is difficult to measure (Luster et al., 1993; Keil et al., 2001; Adamo, 2004; Rantala and Roff, 2005; Wilson-Rich et al., 2008), we selected three established parameters of immunity to analyze honey bee immunocompetence, namely total hemocyte count, encapsulation response, and antimicrobial activity of the hemolymph.

2. Material and methods

2.1. Neonicotinoid exposure in laboratory cage experiments

Worker bees were collected from six *A. m. carnica* colonies. All colonies were regularly inspected for symptoms of diseases. Prior to the experiments, samples of adult bees from each colony were tested for the presence of Chronic Bee Paralysis Virus (CBPV) as described by Amiri et al. (2014), and deformed wing virus (DWV), acute bee paralysis virus (ABPV), and sacbrood virus (SBV; Genersch et al., 2010; Rosenkranz et al., 2014). Only healthy colonies were used. For all experiments, single frames of late stage capped brood were brought to the laboratory and incubated in the dark at 32 °C (Binder, Tuttlingen, Germany; humidity provided by open water jars). Newly emerged bees (⩽24 h) were collected and transferred to standard metal cages (8.5 × 6.5 × 4 cm, 10 bees per cage) containing water and pollen (collected at the Bee Institute Kirchhain or obtained from Imkereibedarf Bährle, Aschaffenburg, Germany), and *ad libitum* sugar syrup (Apiinvert, Mannheim, Germany) diluted to a 60% solution with distilled water in a 5 ml syringe (Carl Roth, Karlsruhe, Germany). Cages were kept in an incubator in the dark at 32 °C (Williams et al., 2013).

Neonicotinoid stock solutions were diluted in sugar syrup (60% Apiinvert) and fed *ad libitum* in the following concentrations: 200 μg/l thiacloprid, 2000 μg/l thiacloprid, 1 μg/l imidacloprid, 10 μg/l imidacloprid, 10 μg/l clothianidin, 50 μg/l clothianidin, 100 μg/l clothianidin, and 200 μg/l clothianidin. Control bees received sugar syrup *ad libitum* (60% Apiinvert) containing the same concentration of the solvent (acetone) as the neonicotinoid-treated groups. Worker bees in each cage were exposed to one of these concentrations for 24 h. On the next day, their immunocompetence was evaluated by one of the methods: quantification of hemocytes, antimicrobial activity of the hemolymph, or encapsulation response.

2.2. Hemolymph collection

Worker bees were anesthetized on ice before hemolymph was collected by inserting a microinjection needle (Hartenstein, Würzburg, Germany) into the proximal abdomen. Any fluid which appeared yellow or brown was discarded and excluded from further analysis as this was likely not hemolymph but gastric fluid (Wilson-Rich et al., 2008).

2.3. Total hemocyte count

For total hemocyte counts, 1 μl of hemolymph was transferred to a PCR-tube (Biozym, Hessisch Oldendorf, Germany) containing 3 μl PBS (Sigma, pH 7.4) and 1 μl of DAPI-staining solution (4′,6-diamidino-2-phenylindole; 1:100 dilution of an 5 mg/ml DAPI stock solution; lifetechnologies). Immediately after collection, the diluted hemolymph solution was transferred to a Bürker hemocytometer chamber (Carl Roth, Karlsruhe, Germany), where hemocytes were counted (average of five chambers per bee) under a phase contrast/fluorescent microscope (Leica DMIL, Leica camera DFC 420C). To verify the cellular character of the observed structures, the DAPI staining was used as counterstaining of nuclear DNA. On rare occasions, obviously cell-like structures, which did not contain a DAPI-stained nucleus, were observed. These cell-like structures were included in the total hemocyte count. Each experiment was repeated at least three times with 30–45 individuals per treatment group.

2.4. Encapsulation response

We provoked an encapsulation response by inserting a nylon filament into the abdomen, thus mimicking the behavior of *V. destructor* (Cox-Foster and Stehr, 1994; Allander and Schmid-Hempel, 2000; Wilson-Rich et al., 2008). A nylon fishing line (0.2 mm diameter, Nexos, Naila, Germany) was cut with a razor blade into approximately 2 mm long segments and sterilized in 100% pure ethanol (Roth). Honey bees were first anesthetized on ice, and a nylon filament was implanted in the abdomen through the intersegmental membrane between the 3rd and 4th tergum

(Allander and Schmid-Hempel, 2000; Wilson-Rich et al., 2008). This provokes the encapsulation of the filament within the hemocoel as well as the closure of the wound. The strength of the immune reaction was measured by the degree of melanization on the filament. Nylon filaments were implanted in such a way that approximately 1 mm of the filament remained outside the body wall. After implantation, bees were transferred to a 2 ml microcentrifuge tube (Eppendorf) with holes poked through cap and sidewalls. This prevented bees from grooming themselves such that the implant remained in place, while maintaining access to air. A small amount of sugar candy (Apiinvert) was placed inside the cap to provide food during the four-hour incubation period. Afterwards, the nylon filament was removed, fixed in Formaldehyde (4% in PBS, Carl Roth), rinsed three times in PBS, counterstained in DAPI-staining solution and mounted in Aquapolymount (Polysciences). Each experiment was repeated at least three times (11–58 individuals per treatment group).

A segment of each explanted filament was photographed at 100× magnification using a Leica phase contrast/fluorescence microscope and image capturing software (Leica, LASV4.4). Three pictures per explant were taken at different focal depths to quantify a three-dimensional mechanism using two-dimensional tools (Rantala et al., 2000; Rantala and Kortet, 2003; Contreras-Garduño et al., 2006; Kapari et al., 2006; Wilson-Rich et al., 2008). The mean grey value per filament served as a measure of melanization and was quantified for the inserted portion of the filament using image analysis software (Allander and Schmid-Hempel, 2000; Rantala et al., 2000; Wilson-Rich et al., 2008; ImageJ 1.34s, National Institutes of Health, USA). Mean grey values of the inserted portions were subtracted from the mean grey value of an unimplanted filament which served as background value (Allander and Schmid-Hempel, 2000; Rantala et al., 2000; Wilson-Rich et al., 2008).

2.5. Inhibition-zone assay

The worker bees were exposed to neonicotinoids for 48 h. After the first 24 h, the immune system was challenged by the injection of 1 µl of heat-inactivated *Escherichia coli* (grown to OD 0.5). For inhibition-zone assays, 2–3 µl of hemolymph were collected, stored in PCR-tubes and kept frozen at −20 °C until the assay was conducted. Antibacterial test plates (ø 9 cm) were prepared by adding 0.3 ml of live *Micrococcus flavus* bacteria suspension (OD 0.5) to 150 ml of sterile broth medium (48 °C, 1.5 g Agar No. 1, Oxoid; 3.75 g nutrient broth, Applichem). Per test plate, five holes (ø 1 mm) were punched into the medium and 1 µl of hemolymph solution was added to each one. The plates were then incubated at 38 °C overnight and the diameter of inhibition zones were measured with a digital slide caliper. The areas of these zones of inhibition were used as a measure of the strength of antibacterial activity in the hemolymph. Each experiment was repeated at least three times with 30–40 individuals per treatment group.

2.6. Statistical methods

Total hemocyte counts, melanization/mean grey values and mean diameters of inhibition zones were not normally distributed and hence non-parametric statistics were used. Each immunocompetence measure was compared between groups treated with neonicotinoids and untreated control bees using Kruskal–Wallis tests followed by post hoc pairwise comparisons with Mann–Whitney *U* tests. All statistical tests were run with the computer program SPSS for Windows (v. 20).

3. Results

3.1. Total hemocyte counts

Total hemocyte counts (THC) were performed as an indirect measurement of baseline cellular immunocompetence (Wilson et al., 2002; Lee et al., 2006; Wilson-Rich et al., 2008). Exposure to all three neonicotinoids significantly reduced the total hemocyte counts of young adult worker bees (Fig. 1A–C). The median total hemocyte counts were lower in thiacloprid treated worker bees than in control bees (Fig. 1A, K.–W. test, $p < 0.0001$). Untreated control bees displayed a higher hemocyte density than bees treated with 200 µg/l thiacloprid (M.–W. *U* test, $p = 0.003$; control: median = 8200 hemocytes/µl (h/µl), n = 37; 200 µg/l thiacloprid: median = 6200 h/µl, $n = 45$), or treated with 2000 µg/l thiacloprid (M.–W. *U* test, $p < 0.0001$, median = 3100 h/µl, $n = 34$). The exposure to thiacloprid reduced the hemocyte density in a dose dependent manner: THC was significantly lower in worker bees exposed to 2000 µg/l than in bees exposed to 200 µg/l thiacloprid (M.–W. *U* test, $p = 0.007$). Total hemocyte counts of bees treated with imidacloprid was lower than in control workers (Fig. 1B, K.–W. test, $p = 0.049$), with control bees displaying a higher hemocyte density than bees treated with 1 µg/l imidacloprid (M.–W. *U* test, $p = 0.035$; control: median = 6835 h/µl, $n = 34$; 1 µg/l imidacloprid: median = 3800 h/µl, $n = 35$), or treated with 10 µg/l imidacloprid (median = 4500 h/µl, $n = 34$, M.–W. *U* test, $p = 0.032$).

However, clothianidin reduced THC only when applied in higher than field relevant concentrations (Fig. 1C; 100 µg/l). Untreated control bees displayed a higher hemocyte density than bees treated with 100 µg/l clothianidin (K.–W. test, $p = 0.029$, M.–W. *U* test, $p = 0.002$; control: median = 6835 h/µl, $n = 34$; 100 µg/l clothianidin: median = 3200 h/µl, $n = 18$), but not than bees treated with 50 µg/l clothianidin (median = 5800 h/µl, $n = 22$), or 10 µg/l clothianidin (median = 4600 h/µl, $n = 34$). The THC was lower in bees exposed to 100 µg/l than in bees exposed to 50 µg/l clothianidin (M.–W. *U* test, $p = 0.041$).

3.2. Encapsulation response

Compared to control bees, the encapsulation response of bees treated with neonicotinoids was significantly reduced (Fig. 2A–C). The encapsulation response was reduced in thiacloprid treated bees (Fig. 2B, K.–W. test, $p = 0.013$; M.–W. *U* test, control vs. 200 µg/l thiacloprid: $p = 0.028$, control vs. 2000 µg/l thiacloprid: $p = 0{,}004$; control: median = 115.01% grey value (gv), $n = 39$; 200 µg/l thiacloprid: median = 52,93% gv, $n = 38$; 2000 µg/l thiacloprid: median = 58.38% gv, $n = 43$). Encapsulation responses of workers that were treated with imidacloprid were lower than in control bees (Fig. 2C, K.–W. test, $p < 0.0001$).

Control bees showed a stronger melanization reaction than bees treated with imidacloprid (M.–W. *U* test, control vs. 1 µg/l imidacloprid: $p = 0.016$, control vs. 10 µg/l imidacloprid: $p < 0.0001$; control: median = 113.02% gv, $n = 34$; 1 µg/l imidacloprid: median = 68.62% gv, $n = 34$; 10 µg/l imidacloprid: median = 35% gv, 14, $n = 25$).

Encapsulation response was also reduced in clothianidin treated worker bees (Fig. 2D, K.–W. test, $p < 0.001$) with melanization of control bees being significantly higher than of bees fed with 50 µg/l and 200 µg/l, but not of bees exposed to 10 µg/l clothianidin (M.–W. *U* test, control vs. 50 µg/l clothianidin: $p = 0.08$, control vs. 200 µg/l clothianidin: $p < 0.0001$; control: median = 115.01% gv, $n = 58$; 10 µg/l clothianidin: median = 113.05% gv, $n = 27$; 50 µg/l clothianidin: median = 51.05% gv, $n = 23$; 200 µg/l clothianidin: median = 27.21% gv, $n = 63$).

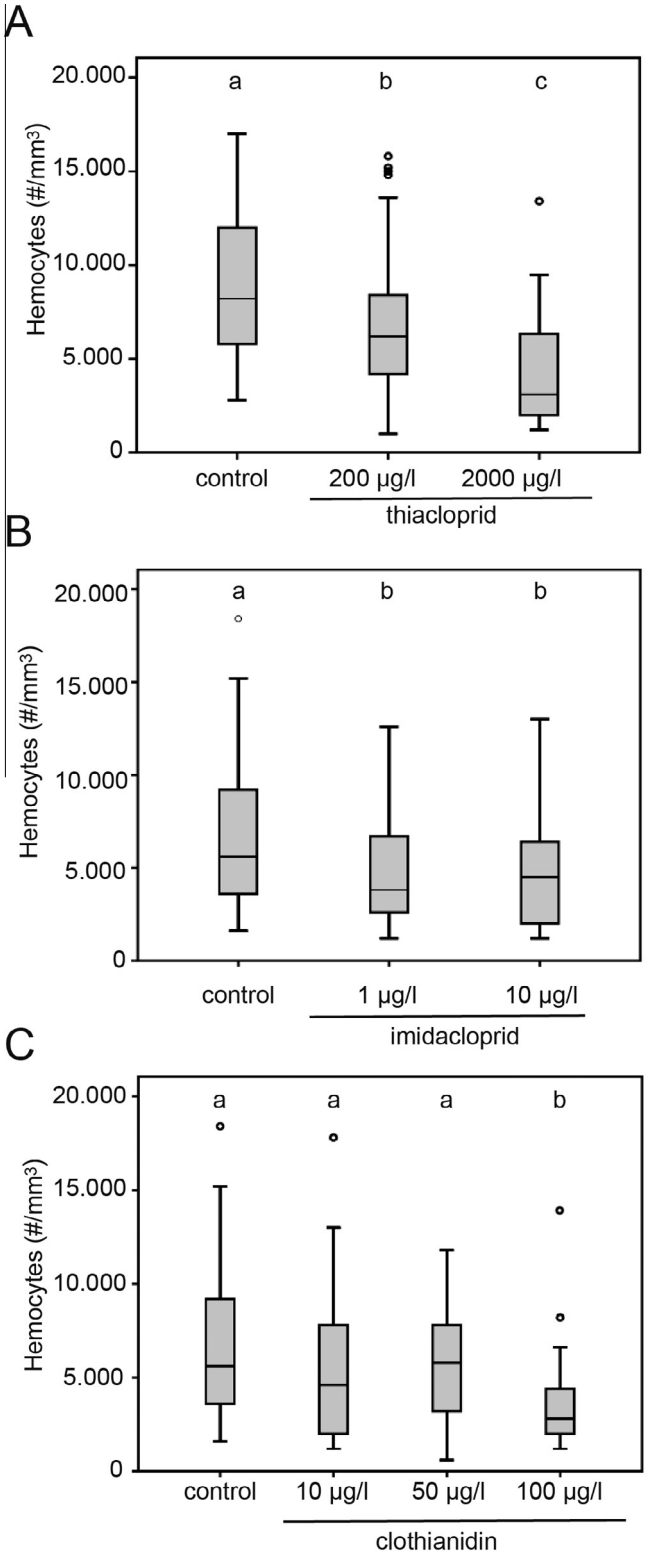

Fig. 1. Cage experiments: exposure to neonicotinoids reduces total hemocyte counts. The 24 h-treatment of newly hatched worker bees with thiacloprid (A; control: $n = 37$, 200 mg/l: $n = 45$, 2000 mg/l: $n = 34$), imidacloprid (B; control: $n = 34$; 1 µg/l: $n = 35$; 10 µg/l $n = 34$), or clothianidin (C; control: $n = 34$; 10 µg/l: $n = 34$; 50 µg/l: $n = 22$; 100 µg/l: $n = 18$) reduced the total hemocyte counts compared to control bees. Boxes show 1st and 3rd interquartile range with black lines denoting medians. Whiskers encompass 95% of the individuals, beyond which outliers (circles) reside. Treatments with different letters differ significantly from each other.

3.3. Antimicrobial activity of the hemolymph

The antimicrobial activity of the hemolymph, measured as the size of the inhibition zones, was significantly reduced in bees treated with thiacloprid, imidacloprid, or clothianidin compared to control bees (Fig. 3, K.–W. test, $p < 0.0001$). The post hoc pairwise analysis revealed that the control group was significantly different to all neonicotinoid treatments and concentrations (M.–W. *U* test: $p < 0.0001$; control: median = 20.8 mm, $n = 40$; thiacloprid 200 µg/l: median = 18.5 mm, $n = 38$; thiacloprid 2000 µg/l: median = 17.2 mm, $n = 40$; imidacloprid 1 µg/l: median = 18.0 mm, $n = 38$; imidacloprid 10 µg/l: median = 19.2 mm, $n = 40$; clothianidin 10 µg/l: median = 15.8 mm, $n = 37$; clothianidin 200 µg/l: median = 15.6 mm, $n = 30$). For additional statistical analysis see Supp. Table 1.

4. Discussion

In this paper we report effects of three neonicotinoids on general immune parameters of honey bees. As measures of individual immunocompetence we used three different aspects of honey bee immunity, total hemocyte count, encapsulation/wound healing response, and antimicrobial activity of the hemolymph. Our results indicate that all three aspects of immunity are affected by sublethal concentrations of neonicotinoids.

Total hemocyte counts provide an indirect measure of basal cellular immunocompetence. In our cage experiments, exposure to thiacloprid and imidacloprid resulted in significant effects on total hemocyte counts, even in concentrations as low as those reported from pollen samples collected by bees (highest concentrations of thiacloprid: 498 µg/kg and 240 µg/kg, in 2012 and 2013 respectively (Rosenkranz et al., 2014); imidacloprid 5.7 µg/kg, (Chauzat, 2006); clothianidin: 2.59 µg/kg, (Cutler and Scott-Dupree, 2007)). Although the lethal dosages of imidacloprid and clothianidin are in the same order of magnitude, exposure to clothianidin reduced total hemocyte counts only at much higher than field-realistic concentrations (100 µg/l). On the other hand, the profound impact of thiacloprid at field realistic concentrations was unexpected, since its acute toxicity is much lower than that of imidacloprid or clothianidin (thiacloprid: oral acute $LD50_{48h}$ = 17.32 µg/bee, imidacloprid: oral acute $LD50_{48h}$ = 0.0037 µg/bee, clothianidin: oral acute $LD50_{48h}$ = 0.004 µg/bee; University of Hertfordshire, 2013).

The strong effects of imidacloprid on total hemocyte counts in our experiments are in contrast to a previous study reporting no significant effect of exposure to this neonicotinoid (Alaux et al., 2010). However, in this previous study all experimental bees, including control bees, were already infected with low levels of *Nosema* spp. spores. This may indicate that honey bees bearing an infection react differently to neonicotinoids compared to healthy ones. Further studies are needed to investigate the effect of pesticides on diseased honey bees (Collison et al., 2015).

Hemocytes are key components of cellular immune defense of insects, since they are responsible for phagocytosis and participate in the encapsulation of pathogens and in the closure of wounds (Gupta, 1986; Tanada and Kaya, 1993; Alaux et al., 2012). An altered hemocyte density following neonicotinoid exposure could thus influence immune defense and increase a bee's susceptibility towards pathogens. By measuring THC, we only investigated the effect of neonicotinoids on the overall number of free ranging hemocytes, without specifying subclasses in detail (Van Steenkiste, 1988). It would be interesting to find out whether a differential effect on the subclasses of hemocytes exists. Hemocyte density of worker bees also varies with development (Schmid et al.,

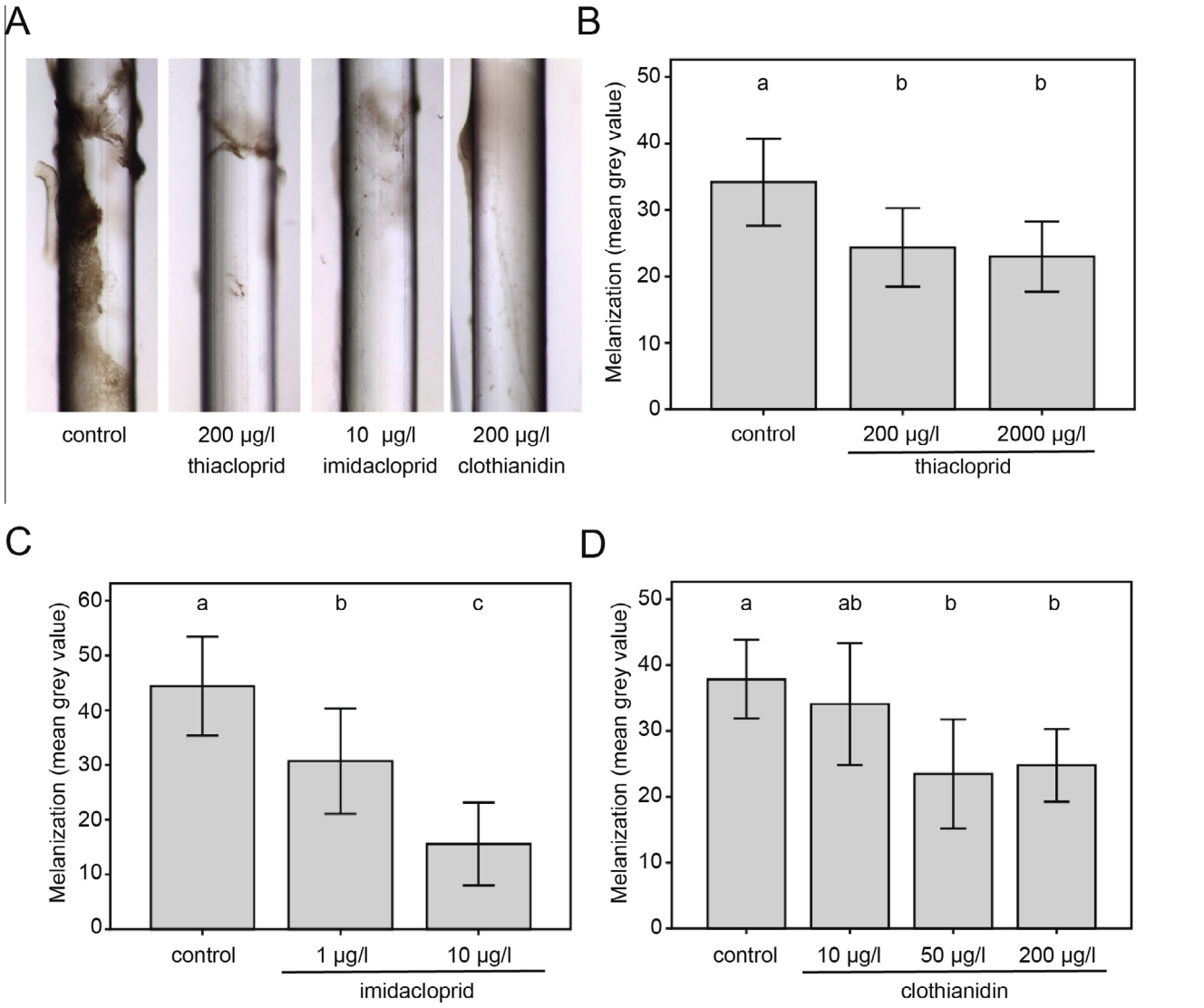

Fig. 2. Encapsulation response is reduced by neonicotinoids. (A) Implanted nylon filaments were encapsulated by dark brown melanin (melanization). (B–D) The 24 h-treatment with neonicotinoids in cage experiments reduced melanization. (B) Thiacloprid (control: $n = 39$; 200 µg/l: $n = 38$; 2000 µg/l: $n = 43$). (C) Imidacloprid (control = 34; 1 µg/l: $n = 38$; 10 µg/l $n = 40$). (D) Clothianidin (control: $n = 58$; 10 µg/l: $n = 27$; 50 µg/l: $n = 23$; 200 µg/l: $n = 63$). Error bars denote standard deviations; treatments with different letters differ significantly from each other.

2008; Wilson-Rich et al., 2008), infection status (Gilliam and Shimanuki, 1967), or diet (Szymaś and Jędruszuk, 2003). The consequences of altered hemocyte density for the survival and the disease susceptibility of honey bees as a reaction towards external stress factors are not yet fully understood.

One central immune defense mechanism mediated by hemocytes is the encapsulation and melanization of intruding pathogens. The melanization reaction is catalyzed by phenoloxidase, whose precursor (prophenoloxidase) is produced by hemocytes and activated by serine proteases (Evans et al., 2006). In our study, we observed a significantly reduced encapsulation response after treatment with all three neonicotinoids and all tested concentrations, except clothianidin 10 µg/l. A reduced encapsulation response may be caused by (a) reduced numbers of total hemocytes, (b) a reduced proportion of hemocytes that engage in aggregation, (c) a reduced production of prophenoloxidase, or (d) a combination of all three.

A possible molecular link between neonicotinoids and immunity was reported by Di Prisco et al. (2013) showing an immunosuppressive effect of clothianidin by up-regulating an inhibitor of a member of the gene family NF-κB within the TOLL pathway and promoting the replication of the deformed wing virus in honey bees. Since the NF-κB gene family is involved in central aspects of insect immunity, e.g. the transcriptional regulation of AMP expression (abaecin, hymenoptaecin; Schlüns and Crozier, 2007), as well as in the clotting reaction of hemocytes, and in melanization of foreign objects (Evans et al., 2006), its inhibition could be a possible explanation for our results regarding reduced encapsulation.

The ability to encapsulate a foreign body correlates positively with the resistance to viral infections (Washburn et al., 1996; Trudeau et al., 2001), parasitoids (Carton and David, 1983; Kraaijeveld et al., 2001) and parasites (Doums and Schmid-Hempel, 2000). Wound closure involves similar mechanisms as encapsulation and plays an important role for reducing virus transfer between bees (Chen, 2011). Antimicrobial peptides (AMPs) which combat pathogens are produced by fat body cells and their production is triggered by the TOLL and Imd pathways (Evans et al., 2006; Schlüns and Crozier, 2007). In our cage experiments, we showed that challenge with thiacloprid, imidacloprid, or clothianidin significantly reduced the antimicrobial activity of the

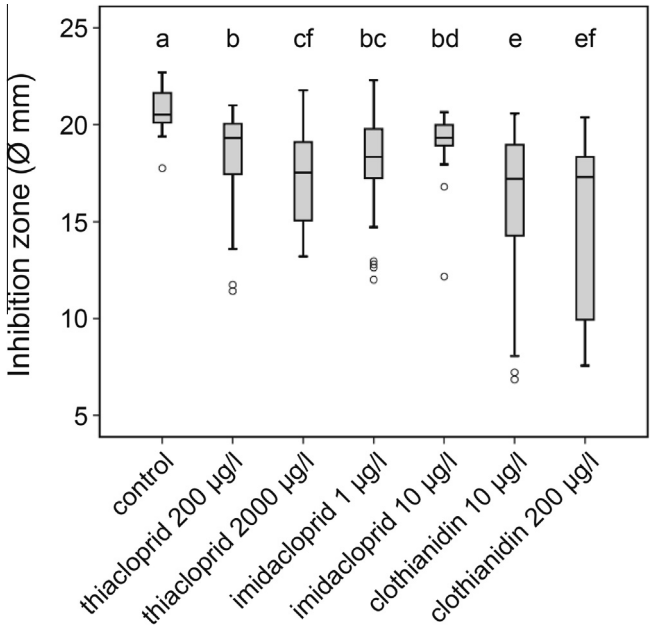

Fig. 3. Neonicotinoid exposure reduced antimicrobial activity of hemolymph. The hemolymph inhibited the growth of grampositive bacteria (*M. flavus*) on agar plates. The 24 h-treatment with thiacloprid (200 µg/l: $n = 38$; 2000 µg/l: $n = 40$), imidacloprid (1 µg/l: $n = 38$; 10 µg/l $n = 40$), or clothianidin (10 µg/l: $n = 37$; 200 µg/l: $n = 30$) reduced the antimicrobial activity of the hemolymph, the diameter of the inhibition zones being smaller than in control bees ($n = 40$). Boxes show 1st and 3rd interquartile range with black lines denoting medians. Whiskers encompass 95% of the individuals, beyond which outliers reside (circles). Significant differences indicated with letters.

hemolymph, together with a decrease of the encapsulation response. These findings may be interpreted as impairment of disease resistance capacities of honey bees in consequence of exposure to neonicotinoids. Our results may be especially important in light of the continuous threat to the health of honey bees by the parasitic varroa mite, in particular due to its central role as a vector of viruses (Genersch et al., 2010; Le Conte et al., 2010; Rosenkranz et al., 2010; Di Prisco et al., 2011; Nazzi et al., 2012). The investigation of additional parameters of immunocompetence, like fat body weight or immune gene expression profiles (Schmehl et al., 2014) and of other developmental stages (larvae and pupae, Gätschenberger et al., 2013) and castes (drones and queens) would add to a comprehensive understanding of the effect of neonicotinoids.

4.1. Conclusion

This study shows a clear impact of neonicotinoids at field realistic concentrations on immunocompetence in adult worker honey bees. However, it remains to be shown whether the observed alterations of the immune system have consequences for the disease resistance capacity of honey bees. Two of the neonicotinoids tested, imidacloprid and clothianidin, are temporarily banned by the EU moratorium until the end of 2015. The third substance, thiacloprid, is frequently being used as spray application on flowering crops and was found in more than 50% of bee bread samples (Genersch et al., 2010; Rosenkranz et al., 2014). Interestingly, thiacloprid, which is classified as "not harmful for bees" due to its much lower acute toxicity, showed similar sublethal effects on immune parameters at a field realistic concentration. Our findings add a significant piece of information to the ongoing discussion of the role of neonicotinoid insecticides in colony losses. The results we report clearly indicate the need for more detailed laboratory and long-term field studies, aiming to assess how insecticides interfere with pathogen propagation and disease susceptibility.

Conflict of interest

The authors have no financial and personal relationships that might bias or be seen to bias their work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2016.01.001.

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