Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees

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Large-scale losses of honey bee colonies represent a poorly understood problem of global importance. Both biotic and abiotic factors are involved in this phenomenon that is often associated with high loads of parasites and pathogens. A stronger impact of pathogens in honey bees exposed to neonicotinoid insecticides has been reported, but the causal link between insecticide exposure and the possible immune alteration of honey bees remains elusive. Here, we demonstrate that the neonicotinoid insecticide clothianidin negatively modulates NF-κB immune signaling in insects and adversely affects honey bee antiviral defenses controlled by this transcription factor. We have identified in insects a negative modulator of NF-κB activation, which is a leucine-rich repeat protein. Exposure to clothianidin, by enhancing the transcription of the gene encoding this inhibitor, reduces immune defenses and promotes the replication of the deformed wing virus in honey bees bearing covert infections. This honey bee immunosuppression is similarly induced by a different neonicotinoid, imidacloprid, but not by the organophosphate chlorpyriphos, which does not affect NF-κB signaling. The occurrence at sublethal doses of this insecticide-induced viral proliferation suggests that the studied neonicotinoids might have a negative effect at the field level. Our experiments uncover a further level of regulation of the immune response in insects and set the stage for studies on neural modulation of immunity in animals. Furthermore, this study has implications for the conservation of bees, as it will contribute to the definition of more appropriate guidelines for testing chronic or sublethal effects of pesticides used in agriculture.

Apis mellifera | DWV | NLR (CLR) | neuroimmunity | toxicology

Losses of honey bee colonies have been reported in many regions of the northern hemisphere (1, 2). In the United States these losses have been also attributed to a complex syndrome, denoted as colony collapse disorder (CCD), the basis of which is still poorly understood (3). A specific causal agent has not yet been identified, but there is a wide consensus on the multifactorial origin of colony losses that are often associated with high infection levels of parasites and/or pathogens (4, 5). This indirectly suggests the possible occurrence of reduced immunocompetence in bees challenged by different stress agents. We recently focused on this aspect, showing how the parasitic mite *Varroa destructor* can destabilize the delicate immune balance, keeping under control the covert infections of the deformed wing virus (DWV) (6). Indeed, intense DWV replication is triggered by the immune challenge induced by mite feeding, which calls upon the cellular pool of a nuclear factor-κB (NF-κB) protein family member (Dorsal-1A) and weakens the antiviral response controlled by the Toll pathway (6). This negatively affects different bee functions and may significantly contribute to colony losses (7–12).

Among the other stress factors that appear to play a role in the induction of colony losses, pesticides have received special attention. In particular, neonicotinoid insecticides are currently the subject of intense debate (13). Over the last few years, several countries have restricted their use in agriculture, and they are currently under the close scrutiny of the European Food Safety Authority (14–16); recently, three of them have been temporarily banned by the European Commission (17), based on the growing scientific evidence regarding the negative effects they have on bees. It has been shown that sublethal doses of thiamethoxam can affect the homing capacity of honey bees with negative consequences on colony stability (18). Concurrent studies on bumblebees have provided further confirmation of the hypothesis that neonicotinoids can have a wider negative impact on pollinators (19, 20). Importantly, exposure to neonicotinoids is often associated with a higher pathogenic impact on bees (21–23), although the merely descriptive results reported are somewhat contrasting and do not support any clear epidemiological interpretation, due to significant gaps in our knowledge of if and how these insecticides act on honey bee immunity (24). Here we address this issue, focusing on the mechanism underlying the presumed immunosuppressive activity of neonicotinoids on insects.

Ecotoxicological studies have reported that exposure of *Mytilus galloprovincialis* to sublethal doses of the neonicotinoid insecticide thiacloprid up-regulates the expression of transcription factors that show sequence similarity with members of the CATERPILLER (CLR) protein family in mammals (25), subsequently renamed, along with other aliases, as "Nucleotide-binding domain and Leucine-rich Repeat" (NLR) (26). These proteins play an

Significance

Honey bees are exposed to a wealth of synergistically interacting stress factors, which may induce colony losses often associated with high infection levels of pathogens. Neonicotinoid insecticides have been reported to enhance the impact of pathogens, but the underlying immune alteration is still obscure. In this study we describe the molecular mechanism through which clothianidin adversely affects the insect immune response and promotes replication of a viral pathogen in honey bees bearing covert infections. Our results shed light on a further level of regulation of the immune response in insects and have implications for bee conservation.

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important role in immunity (27), and some members of the NLR (CLR) protein family are known to have negative regulatory functions by inhibiting NF-κB activation (28). Indeed, their down-regulation upon immune challenge is required to trigger a defense response (29). The occurrence in insects, as in mammals, of this negative mechanism of immune regulation, possibly reinforced by exposure to neonicotinoids, as observed in *M. galloprovincialis*, could partly account for the proposed immunosuppressive role exerted by these insecticides. Indeed, based on the stress model we recently proposed (6), we may predict that the resulting negative impact on NF-κB activation could adversely affect a wealth of stress responses depending from this transcription factor.

The NLR (CLR) proteins in vertebrates are remarkably similar in structure to disease resistance proteins that mediate immune responses in plants (30, 31). In contrast, just limited structural remnants of their tripartite domain architecture are found in insects and nematodes, which show weakly related sequences to members of the NLR (CLR) family, characterized by only individual domains like NACHT (NAIP, CIITA, HET-E, TP1) or leucine-rich repeat (LRR) (27). Here we report that LRR proteins of insects act as negative regulators of NF-κB–dependent immune responses. Then, we show that the neonicotinoid clothianidin up-regulates their expression and results in lower immune competence. This determines an uncontrolled viral replication in honey bees bearing covert DWV infections, similarly induced by the neonicotinoid imidacloprid, but not by the organophosphate chlorpyriphos, which does not affect NF-κB signaling.

Results and Discussion

Identification of a Negative Modulator of NF-κB Activation in Insects. To identify *NLR(CLR)*-related genes in invertebrates with a potential negative impact on immunity, we first focused on the *Drosophila melanogaster* gene *CG1399*, hereafter denoted as *Dmel\LRR*. This gene shows the highest sequence similarity with *CLR16.2* (*NLRC3*), a negative modulator of NF-κB activation in human T cells (29), and contains only an individual LRR domain (Fig. S1*A*). When fly larvae were immune-challenged, with a tungsten needle previously dipped into a concentrated cell suspension of *Saccharomyces cerevisiae*, *Dmel\LRR* was down-regulated in a time-dependent manner over the first 30 min ($P < 0.05$; Fig. 1*A*), showing, in that time interval, a transcriptional pattern which inversely matched that of genes encoding the antimicrobial peptides Drosomycin ($\rho = 0.952$, $n = 8$: $P < 0.001$; Fig. 1*B*) and Defensin ($\rho = 0.976$, $n = 8$: $P < 0.001$; Fig. 1*B*), both of which are under control of the Toll pathway (32–34). The subsequent change of this relationship suggests that other regulatory mechanisms, likely controlled by persisting pathogen associated molecular patterns (32), may become predominant at later times after the onset of infection, whereas LRR rapidly resumes to functional levels.

Moreover, fly larvae expressing the *UAS-CG1399-RNAi* transgene, under control of the ubiquitous driver *Act-GAL4*, showed a significant knockdown of the *Dmel\LRR* gene ($U = 0, n_1 = 4, n_2 = 4$: $P < 0.05$; Fig. 1*C*), which was associated with a significant increase in the basal transcription rate of the *drosomycin* and *defensin* genes ($U = 0, n_1 = 4, n_2 = 4$: $P < 0.05$ for both genes; Fig. 1*D*).

These results demonstrate that *Dmel\LRR*, like *CLR16.2* (*NLRC3)* in humans, exerts an inhibitory action on NF-κB activation, and its expression is down-regulated to allow an inducible immune response. It is interesting to note that these proteins, showing only limited sequence similarity (Fig. S1), exert a similar function. NLR (CLR) proteins play an important role in immunity, cell death, and disease in vertebrates, and their close counterparts exert a similar action in plants (27); our results indicate that the negative regulatory functions shown in mammals by some members of this protein family are conserved across distant evolutionary lineages and associated with different proteins, which share the presence of a LRR domain. This is not surprising, as LRR domains are one of the most commonly occurring domains in proteins associated with innate immunity (35, 36); moreover, the function of many LRR domains is to provide a structural framework for protein–protein interactions (37). The down-regulation of these inhibitory proteins upon immune challenge is required to trigger a defense response in mammals and likely plays a pivotal role in preventing an overzealous reaction that could be detrimental to the host (27). Our data indicate the occurrence in insects of similar control patterns of the immune response, which, so far, have been less intensively investigated compared with activation pathways (32, 38, 39). However, an effective down-regulation of the immune reaction is as important as its activation, not only to prevent dysfunctional overreactions against foreign invaders, but also to finely orchestrate the gut–microbe homeostasis by shaping the commensal community while efficiently eliminating unwanted pathogens (40, 41).

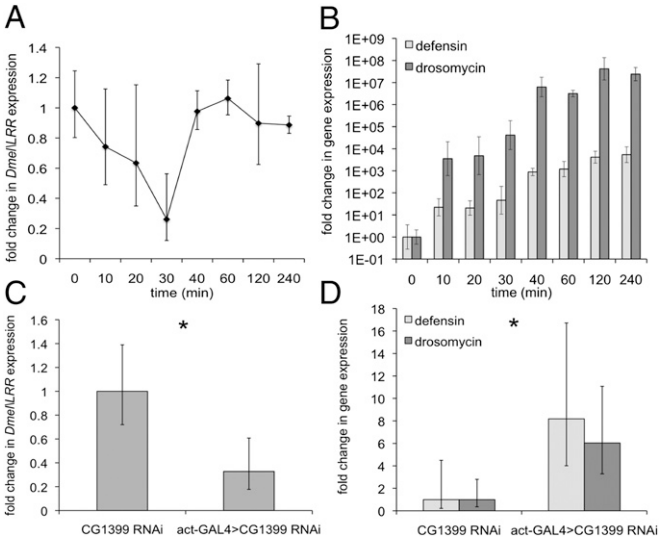

Fig. 1. *Dmel\LRR* inhibits NF-κB activation and negatively modulates the immune response. The transcriptional down-regulation of *Dmel\LRR* upon immune challenge (*A*) was associated with a concurrent increase of the transcription rate of the genes encoding the antimicrobial peptides Defensin and Drosomycin, under the control of the Toll pathway (*B*). The RNAi-mediated knockdown of *Dmel\LRR* (*C*) determined a significant increase in the basal level of transcription of the *defensin* and *drosomycin* genes (*D*). The mean ± SD of the fold change in gene expression is represented. The observed trends for *Dmel\LRR* (*A*) and *defensin* and *drosomycin* (*B*) are significant at $P < 0.05$, $P < 0.001$, and $P < 0.001$, respectively; significant ($P < 0.05$) differences between treatments (*C* and *D*) are shown with an asterisk.

Effect of the Neonicotinoid Insecticide Clothianidin on NF-κB Signaling and Immune Response. We assessed whether exposure to clothianidin enhanced the expression of *Dmel\LRR* and the NF-κB inhibitory pathway under its control, as observed in *M. galloprovincialis* for related *NLR (CLR)* genes (25). The level of NF-κB activation was determined by monitoring the expression of the *drosomycin-GFP* reporter gene that is under the control of the Toll pathway (32) in a transgenic strain of *D. melanogaster* exposed by topical treatment to a standard LD_{50} dose of the neonicotinoid clothianidin (40 ng per larva, Fig. S2*A*) or of the organophosphate chlorpyriphos (15 ng per larva; Fig. S2*B*) and subsequently infected with *S. cerevisiae.* This immune challenge was performed to activate the Toll pathway (32) and to measure the degree of NF-κB activation as affected by insecticide exposure. In transgenic fly larvae treated with clothianidin, the percentage of experimental individuals showing intense fluorescence in response to immune challenge, as a result of NF-κB activation and downstream-enhanced transcription of the GFP-fused reporter gene, was significantly lower than that seen in controls [Mantel-Haenszel (M-H) $\chi^2 = 90.32$, df = 3: $P <$

0.001; Fig. 2*A*], demonstrating that NF-κB activation is impaired by clothianidin exposure. In contrast, the fluorescence response observed in experimental fly larvae treated with chlorpyriphos was not significantly different from controls (M-H $\chi^2 = 0.015$, df = 1: N.S.; Fig. 2*A*). Interestingly, this clothianidin-induced suppression of NF-κB signaling was associated with a significant up-regulation of the *Dmel\LRR* transcription rate ($U = 0$, $n_1 = 4$, $n_2 = 4$: $P < 0.05$; Fig. 2*B*) that was not observed after treatment with chlorpyriphos ($U = 7$, $n_1 = 4$, $n_2 = 4$: N.S.; Fig. 2*B*).

To test whether a similar negative impact of clothianidin on NF-κB signaling occurs in bees, we monitored the transcription level of the gene encoding the antimicrobial peptide Apidaecin as a measure of Toll pathway activation (42). In honey bees exposed to a standard LD_{50} dose of the neonicotinoid clothianidin (21 ng per bee; Fig. S2*C*) and subsequently infected with *S. cerevisiae*, the *apidaecin* transcript level in response to infection was significantly lower than that recorded for control bees [$Q_{\text{Van Elteren}}$ (Q_{VE}) = 3.960, $n_1 = 15$, $n_2 = 15$: $P < 0.05$; Fig. 3*A*], again indicating that the NF-κB signaling is impaired. In contrast, this was not the case for treatments with the LD_{50} dose (320 ng per bee; Fig. S2*D*) of the organophosphate chlorpyriphos ($Q_{VE} = 0.004$, $n_1 = 15$, $n_2 = 15$: N.S.; Fig. 3*A*). The negative impact of clothianidin on NF-κB signaling was associated with a remarkable up-regulation ($Q_{VE} = 20.455$, $n_1 = 15$, $n_2 = 15$: $P < 0.001$; Fig. 3*B*) of the honey bee gene (accession no. XP003251213) showing the highest sequence similarity (53%; Fig. S1 *B* and *C*) with *Dmel\LRR* and hereafter denoted as *Amel\LRR*. Instead, a much smaller effect was noted using chlorpyriphos (comparison with control: $Q_{VE} = 6.113$, $n_1 = 15$, $n_2 = 15$: $P < 0.05$; Fig. 3*B*; comparison between insecticides: $Q_{VE} = 18.331$, $n_1 = 15$, $n_2 = 15$: $P < 0.001$), in agreement with the lack of significant effects on the transcription level of *apidaecin*. Moreover, a significant up-regulation over time of *Amel\LRR* was recorded after clothianidin treatment, even in the absence of immune challenge ($\rho = 0.886$, $n = 6$: $P < 0.05$; Fig. 3*C*). This demonstrates that in honey bees, as in flies, the insecticide clothianidin interferes with the NF-κB signaling by up-regulating the transcription of a *LRR* gene. Therefore, this agonist of the nicotinic acetylcholine receptor is able to exert a negative modulation of the immune response.

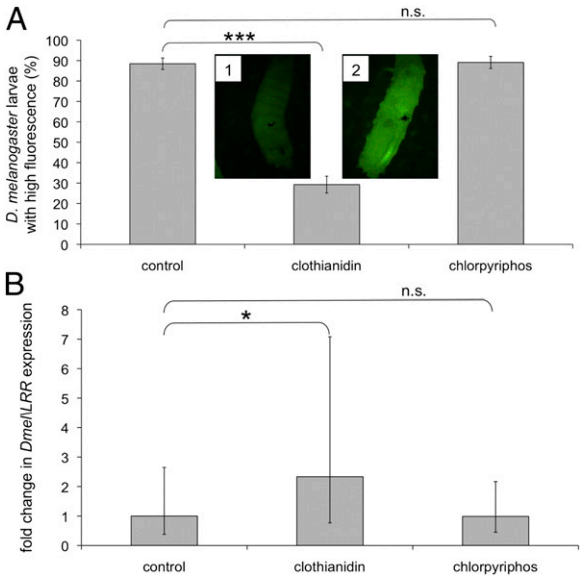

Fig. 2. Effect of insecticides on NF-κB signaling in *D. melanogaster*. The Toll pathway activation in the larvae of the transgenic strain expressing *drosomycin-GFP* was assessed by scoring the fluorescence as low (*Inset 1*) or high (*Inset 2*) in experimental individuals treated with the neonicotinoid clothianidin or the organophosphate chlorpyriphos, at a standard LD_{50} dose, and immune-challenged with *S. cerevisiae*. The large majority of the larvae exposed to clothianidin showed low or barely visible fluorescence, whereas the opposite was observed both for controls and for larvae treated with chlorpyriphos. The mean percentage of *D. melanogaster* larvae with high fluorescence ± normalized SD is represented; significant differences are shown with asterisks (***$P < 0.001$) (*A*). The *DmelΛLRR* transcript was up-regulated in the experimental larvae exposed to clothianidin. The mean ± SD of the fold change in *DmelΛLRR* expression is represented; significant differences are shown with asterisks (*$P < 0.05$) (*B*).

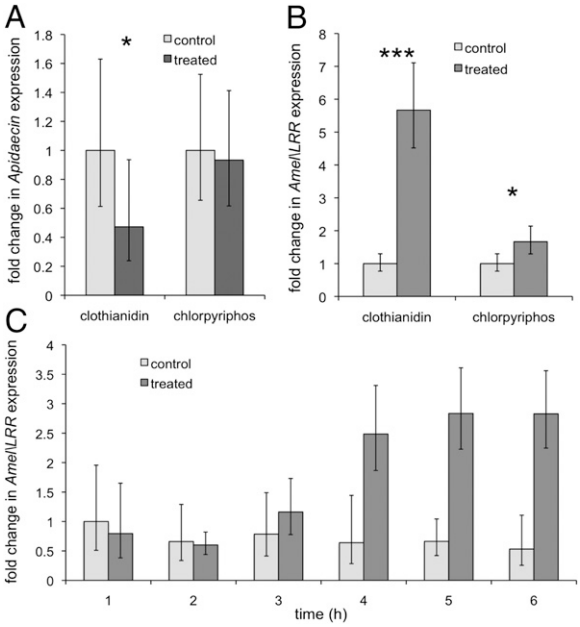

Fig. 3. Effect of insecticides on NF-κB signaling in *A. mellifera*. The Toll pathway activation, assessed by measuring the transcript level of the antimicrobial peptide Apidaecin gene, was evaluated in honey bees treated with the neonicotinoid clothianidin or the organophosphate chlorpyriphos, at a standard LD_{50} dose, and immune-challenged with *S. cerevisiae*. Clothianidin treatment significantly reduced the transcription level of *apidaecin* gene, which, in contrast, was not affected by chlorpyriphos. The mean ± SE of the fold change in *apidaecin* expression is represented; significant differences are shown with asterisks (*$P < 0.05$) (*A*). The immune-challenged larvae exposed to clothianidin showed an increased transcription of the gene *AmelΛLRR*, which has 53% sequence identity with *DmelΛLRR*. The mean ± SE of the fold change in *AmelΛLRR* expression is represented; significant differences are shown with asterisks (***$P < 0.001$) (*B*). A sustained transcriptional up-regulation of *AmelΛLRR* was evident in honey bees starting from 4 h after the treatment with clothianidin and in absence of immune challenge. The mean ± SD of the fold change in *AmelΛLRR* expression is represented; the observed trend is significant at $P < 0.05$ (*C*).

The most reasonable explanation accounting for such an effect is the possible occurrence in insects of neural circuits controlling immunity, as reported in mammals (43). The inflammatory reflex circuit described in mammals is essential to ensure a neural coordination of the defense response to have a sufficient protection while preventing damages deriving from an overzealous reaction (44, 45). This is achieved through a cholinergic anti-inflammatory pathway, which inhibits cytokine-producing immune cells expressing nicotinic acetylcholine receptors (44, 45). Exposure of these cells to acetylcholine or nicotine results in the down-regulation of the nuclear translocation of NF-κB, which is required for the production of proinflammatory cytokines (43–45). Here we have observed that neonicotinoids interfere with insect immune response, likely through a similar mechanism, as indicated by the reduced NF-κB activation in insects exposed to these insecticides. Unraveling the functional and molecular bases underlying this overlooked integration between the nervous and

immune systems in insects will likely promote a better understanding of the neural circuits involved in the regulation of immunity in animals (43, 46), which appears to be an ancient homeostatic mechanism present also in distant evolutionary lineages such as nematodes (47, 48).

Neonicotinoid Effects on DWV Replication. Based on our recently proposed honey bee stress model (6) and the observed negative impact of clothianidin on NF-κB activation, it is possible to predict that this insecticide may promote the replication of DWV in bees bearing covert infections, an extremely frequent occurrence in a large number of apiaries all over the world (10). To test this hypothesis, we applied increasing doses of clothianidin and measured the number of DVW genome copies in experimental bees. Indeed, this showed a dose-dependent positive response ($T_{5,25} = 10.681$: $P < 0.001$; Fig. 4*A*). Viral replication was similarly promoted by treatments with imidacloprid ($T_{4,20} = 10.056$: $P < 0.001$; Fig. 4*A*), another neonicotinoid insecticide with a different chemical structure (i.e., ring system vs. noncyclic structure of clothianidin) (49). In contrast, this viral-promoting effect was not observed in bees exposed to the organophosphate chlorpyriphos ($T_{6,30} = 0.505$: $P = 0.898$; Fig. 4*B*). It is worth noting that the lower sublethal doses used here allowed the survival of the experimental bees for several days (Fig. S3). Moreover, for clothianidin and imidacloprid, which induced DWV proliferation at the experimental doses considered, we reiterated daily the topical treatment using much lower doses, ranging from 1/1,000 to 1/10 of the LD_{50} (i.e., 21 ng per bee for both clothianidin and imidacloprid; Fig. S2 *C* and *E*). In this further analysis, we observed again a significant enhancement of viral replication ($T_{4,16} = 3.222$: $P < 0.01$ and $T_{4,16} = 3.863$: $P < 0.01$ for clothianidin and imidacloprid, respectively; Fig. S4).

To test the potential effects of a prolonged exposure to sublethal doses of experimental insecticides under more realistic conditions, we fed ad libitum honey bees with a sucrose solution containing the tested molecules at concentrations ranging from 0.1 to 10 ppb, thus matching contamination levels occurring under field conditions (13, 50, 51). We found that both neonicotinoids were active in promoting DWV proliferation ($T_{4,16} = 2.666$: $P = 0.022$ and $T_{4,16} = 2.282$: $P = 0.055$ for clothianidin and imidacloprid, respectively; Fig. 4 *C* and *D*), whereas chlorpyriphos had only a negligible effect ($T_{4,16} = 1.332$: $P = 0.324$; Fig. 4*E*). In contrast, viral loads of control bees did not change over time (Fig. 4 *C*–*E* and Fig. S4).

Collectively, our data demonstrate that two neonicotinoid insecticides, each representing one of two alternative structure types in the group of nitroguanidines, actively promote DWV replication. The analysis of the possible impact on honey bees can be attempted in the framework of the model for bee colony collapse we recently proposed (6). Indeed, these insecticide molecules may act as an additional stress factor which, by negatively affecting the activation of NF-κB, reinforces and exacerbates the negative impact on honey bee immunity of the transcriptional down-regulation of NF-κB observed in bees exposed to the *Varroa*-DWV association (6) (Fig. S5). Furthermore, this insecticide-induced alteration of the innate immune response may also influence gut microbial pathogens. Indeed, innate immunity in the gut epithelia of *Drosophila* actively controls gut–microbe homeostasis through a subtle modulation of NF-κB signaling (40, 41). The recent analysis of the gut microbiota in honey bees (52) sets the stage for future research aiming to define the role that gut commensal bacteria have in nutrition and pathogen control. The observed immunosuppressive activity of the studied neonicotinoids may likely interfere with these complex microbial interactions, further affecting honey bee health. Moreover, we cannot rule out that prolonged exposure to sublethal levels of insecticide may have additional negative effects on bee physiology and development which may enhance bee immunosuppression. The emerging multifaceted scenario lends further support to the widely accepted hypothesis of the multifactorial origin of colony losses and CCD, with neonicotinoids being one of the many environmental challenges that may potentially influence bee colony health and survival (Fig. S5). However, field studies are necessary to carefully evaluate their real impact under different environmental conditions.

Our findings add a significant piece of information to the ongoing and unresolved discussion of the possible role of neonicotinoid insecticides in the induction of colony losses and have important implications for toxicology and risk assessment studies. Indeed, pathogen proliferation induced by insecticides can cause additional mortality, even at sublethal doses, and may contribute to the observed negative influence of some insecticides, or their mixtures, on bee longevity and colony stability (53, 54). The results we report clearly indicate the need for longer-term toxicity tests, aiming at assessing how the pathogen progression in honey bees is influenced by insecticide residues and by their cumulative effects, both on adults and larvae. A comprehensive and thorough assessment of insecticide impact on bees will significantly contribute to their conservation and to the development of more sustainable protocols of intensive agriculture.

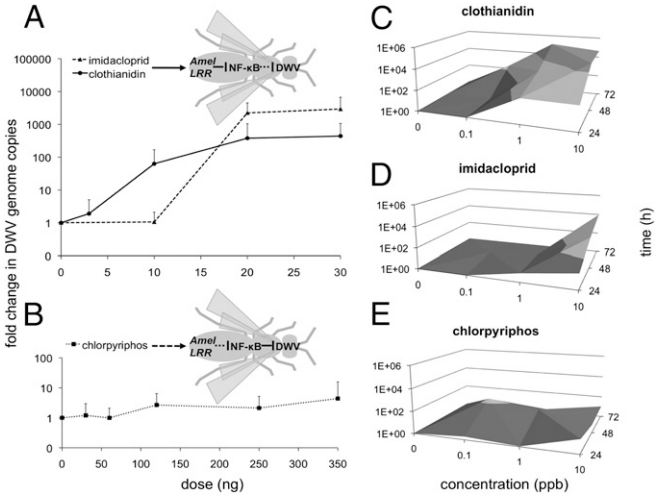

Fig. 4. Effect of insecticides on DWV replication in honey bees bearing covert infections. The number of DWV genome copies was assessed in honey bees treated with increasing amounts of different insecticides; topical application (*A* and *B*) and oral uptake by feeding (*C*–*E*) were adopted to deliver insecticides to the experimental honey bees. In bees treated topically, viral replication was assessed after 24 h; in bees treated orally, viral replication was assessed over time, at 24-h time intervals. Viral replication was promoted, in both cases, in a dose-dependent manner by treatments with the neonicotinoid insecticides clothianidin and imidacloprid (*A*, *C*, and *D*), whereas this was not the case for chlorpyriphos (*B* and *E*). The ratio: (DWV genome copies at dose *x*)/(DWV genome copies at dose 0) ± SD is represented. The graphical schemes summarize how neonicotinoids, unlike chlorpyriphos, up-regulate the transcription of *Amel\LRR*, which results in the reduced activation of NF-κB and of the downstream antiviral barriers. Arrows indicate positive (e.g., stimulation or up-regulation) interactions; bar-headed lines mark negative interactions (e.g., inhibition or down-regulation); and dashed lines mark reduced effects.

Materials and Methods

Insect Material. Honey bees used in this study were from *Apis mellifera ligustica* colonies, maintained in the experimental apiary of the Università degli Studi di Napoli Federico II. Newly emerged bees used in all of the experiments were obtained from brood frames taken from the experimental hives and kept in an incubator at 34 °C, 80% relative humidity (RH), for 12 h. *D. melanogaster* stocks were raised on standard cornmeal/yeast/agar medium at 21 °C. The Canton-S stock was used for the assessment of clothianidin and

chlorpyriphos toxicity. The *drosomycin-GFP* stock was kindly provided by Jean-Luc Imler (Department IBMC UPR9022 'Immunologie et Developpement des Insectes' CNRS, Strasbourg, France). The *UAS*-CG1399-RNAi line (no. 24826) was obtained from the Vienna *Drosophila* RNAi Center (VDRC) (55). The *Act-GAL4; UAS-GFP/ In(2LR)Gla, wg [Gla-1] Bc [1]* stock derived from a stock carrying the *Act-GAL4; UAS-GFP* transgenes kindly provided by Bruno Lemaitre (Global Health Institute, FSV Ecole Polytechnique de Lausanne, Lausanne, Switzerland) (56).

Assessment of Insecticide Toxicity. The toxicity of the neonicotinoids clothianidin and imidacloprid and the organophosphate chlorpyriphos was assessed on adult honey bees as reported below. Pure insecticide compounds were obtained from Sigma-Aldrich. Newly emerged bees were treated by using a micropipette to apply 1 μL of an acetone solution containing the insecticide to be tested, or acetone alone as a control, to the thorax. Subsequently, groups of 30 bees, all receiving the same treatment, were transferred into plastic cages and kept at 34 °C, 80% RH, as described by Evans et al. (57). After 24 h, cages were inspected, and dead bees were counted and removed. The following dosages per bee were used: 10, 20, 30, 40, and 50 ng of clothianidin; 2.5, 5, 10, 20, 30, 40, and 50 ng of imidacloprid; and 15, 30, 60, 125, 250, 350, and 500 ng of chlorpyriphos. These ranges were across the LD_{50} values provided by the producers or available in the literature (58, 59). For *D. melanogaster,* the toxicity of the insecticides clothianidin and chlorpyriphos was assessed on third instar larvae. Groups of 30 experimental larvae were treated with the following doses per individual: 1.5, 3, 6, 12, 25, 50, 70, and 100 ng of clothianidin; 2.5, 5, 15, 60, 125, and 250 ng of chlorpyriphos. Treated larvae were kept for 24 h at 21 °C and 60% RH in an incubator with food available, and their survival rate was then assessed. Groups of 30 individuals for each of the different doses were used; the experiment was replicated three times both with bees and fly larvae. Probit analysis and LD_{50} values are reported in Fig. S2, whereas the relative survival curves are reported in Fig. S3.

Transcriptional Analysis of Dmel\LRR, Defensin, and Drosomycin Genes After Immune Challenge in *Drosophila* Larvae. Third instar larvae from the *drosomycin-GFP* stock were pricked with a thin tungsten needle previously dipped in a concentrated culture of *S. cerevisiae* and incubated at 21 °C for 0, 10, 20, 30, 40, 60, 120, and 240 min. The experiment used groups of three larvae for each time point and was replicated three times. RNA was extracted and used for qRT-PCR using the primer pair as in Table S1.

Insecticide Effect on the Expression of the Drosomycin-GFP Reporter Gene and CG1399 (Dmel\LRR) in *Drosophila* Larvae. To assess the possible effect of the experimental insecticides on NF-κB signaling, we analyzed the response to an immune challenge of third instar larvae of a *D. melanogaster* stock carrying the *drosomycin-GFP* reporter gene (60) and scored the fluorescence after the artificial infection. Clothianidin and chlorpyriphos insecticides were dissolved in acetone at a concentration of 40 and 15 ng/μL, respectively, using 1 μL of this solution for topical treatment of experimental larvae or 1 μL of pure acetone for controls. This allowed us to deliver a dose per larva that corresponded to the calculated LD_{50} (Fig. S2 *A* and *B*). After treatment, the larvae were aligned manually on a strip of double-sided Scotch tape mounted on a microscope slide. The systemic response was triggered by pricking the larvae with a thin tungsten needle previously dipped in a concentrated culture of *S. cerevisiae*. Each slide contained ∼15 larvae and was placed in a wet Petri dish and incubated at 21 °C for 4.5 h before observation. Following the incubation period, the slides were placed in a freezer (−15 °C) for 1 min to immobilize the larvae. Then the larvae were carefully examined under epifluorescent illumination with a stereomicroscope. The experiment was repeated four times; overall, a total of 123, 110, and 130 fly larvae were treated with clothianidin, chlorpyriphos, and acetone, respectively. To quantify the relative transcription level of the CG1399 gene (*Dmel\LRR)*, groups of five larvae from the experiment described above were used for qRT-PCR using the primer pair as in Table S1. The experiment was replicated three times.

Knockdown of Dmel\LRR Gene. *UAS-CG1399-RNAi* females were crossed at 25 °C with *Act-GAL4; UAS-GFP/ In(2LR)Gla, wg [Gla-1] Bc [1]*. The third instar larvae of the *UAS-CG1399-RNAi/ Act-GAL4; UAS-GFP* genotype were selected by scoring the GFP fluorescence with epifluorescent illumination with a stereomicroscope. The levels of the *CG1399* (*Dmel\LRR*), *defensin*, and *drosomycin* transcripts obtained from the *UAS-CG1399-RNAi/Act-GAL4; UAS-GFP* larvae were compared with those of the larvae expressing only the *UAS-CG1399-RNAi* construct. The experiment, using groups of five larvae per genotype, was replicated four times. The *CG6194* gene is reported as a potential off-target gene for the *CG1399-RNAi* construct (see ref. 55 for VDRC's methods of detecting off-target genes). Therefore, to exclude any effect on this potential off-target gene, we compared the level of *CG6194* transcript detected in the UAS-*CG1399-RNAi/Act-GAL4* larvae with that obtained from the larvae expressing only the *UAS-CG1399-RNAi* construct by qRT-PCR with the primer pair reported in Table S1 (Fig. S6).

Analysis of the Transcription Level of the Antimicrobial Peptide Apidaecin and XP00325113.1 (Amel\LRR) Genes in Honey Bees As Affected by Insecticides. Newly emerged bees were treated with insecticides as described above. Clothianidin and chlorpyriphos were used at a dose of 21 and 320 ng per bee, respectively; this corresponds to the LD_{50} value determined above (Fig. S2 *C* and *D*). One hour after the insecticide treatment, experimental bees were immune-challenged by inserting a needle previously dipped in a concentrated culture of *S. cerevisiae* at the base of the forewing. Experimental bees, in groups of 30 individuals, were then placed into cages at 34 °C, 80% RH, as described above. After 6 h, five bees were sampled to assess the transcript level of *apidaecin* and of the *XP00325113.1* gene (*Amel\LRR*) by real-time PCR, using the primer pairs reported in Table S1. This experiment was replicated three times.

Expression of XP00325113.1 Gene (Amel\LRR) in Clothianidin-Treated Honey Bees. Newly emerged bees were treated with clothianidin as described above. Experimental bees, divided into groups of 30 individuals each, were then placed into cages at 34 °C, 80% RH, as described above. After 0.5, 1, 2, 4, 8, and 24 h, five bees were used for the transcription-level analysis of the *XP00325113.1* gene (*Amel\LRR*) by qRT-PCR, using the primer pair reported in Table S1.

DWV Replication in Bees As Affected by Insecticide Exposure. Newly emerged bees were treated by using a micropipette to apply 1 μL of acetone containing the insecticide under study to the thorax. The following doses were used: 0, 3, 10, 20, and 30 ng per bee for clothianidin; 0, 10, 20, and 30 ng/bee for imidacloprid; and 0, 30, 60 120, 250, and 350 ng per bee for chlorpyriphos. Bees were then transferred into cages at 34 °C, 80% RH, as described by Evans et al. (57). Cages contained groups of 30 bees, each group receiving the same treatment. After 24 h, six bees from each cage were sampled for qRT-PCR analysis as described below. This experimental design was replicated three times; in all cases the starting number of DWV genome copies in control bees ranged between 10^8 and 10^{11}. In a second experiment, newly emerged bees, maintained as described above, were treated by applying daily on the thorax, with a micropipette, 1 μL of acetone containing 0.02, 0.2, and 2 ng of either clothianidin or imidacloprid, corresponding to 1/1,000, 1/100, and 1/10 of LD_{50}. Acetone was used as control. Bees that received the same treatment were maintained into plastic cages with 1:1 sugar syrup and kept at 34 °C, 80% RH. After 24, 48, and 72 h, five bees for each treatment were collected and flash frozen at −80 °C. DWV genome copies in control bees, at the beginning of the experiment, were 2.2×10^5. To reproduce realistic levels of insecticide exposures occurring under field condition, newly emerged bees, maintained as described above, were fed ad libitum with 1:1 sugar syrup solution containing the experimental insecticides at the following concentrations: 0.1, 1.0, and 10 ppb. Sugar syrup without insecticides was used as control. After 24, 48, and 72 h, five bees for each treatment were collected and flash frozen at −80 °C. The starting viral load of control bees was 1.2×10^7 DWV genome copies.

qRT-PCR Analysis. Quantitative RT-PCR was carried out using standard methods described in detail in *SI Materials and Methods*.

Statistical Analysis. A randomization test was used to test the significance of the observed time-dependent pattern of *Dmel\LRR* expression after immune challenge (61). Briefly, the labels of the data points (i.e., readings from real-time PCR analysis) were rearranged randomly, and the resulting ranking of the mean expression values at each time was calculated. The *P* value of the test was calculated as the proportion of sampled permutations such that the resulting ranking matched that observed. The analysis was carried out using a computer program written specifically for the purpose. Time- and dose-dependent responses were tested by assessing the significance of the Spearman rank correlation coefficient. The LD_{50} and 95% of confidence limits were calculated from the survival curves by probit analysis (62) using Statgraphics Plus v5 software. Gene expression data (as the difference of C_T value between target and housekeeping gene) under different treatments were compared by means of the nonparametric Mann-Whitney test or the van Elteren's test in case of stratified data. The proportions of transgenic *Drosophila* larvae showing intense fluorescence upon immune challenge in groups treated or not with insecticides were compared using the Mantel-Haenszel χ^2 test. To test the effect of increasing doses of pesticides on DWV replication, the Bretz type of the Williams test for trend (63) was applied using Stat4tox software on the log-transformed number of DWV genome copies; for the analysis, data from different experimental replicates were pooled.

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