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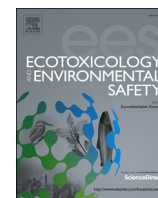
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Superparasitism by a parasitoid wasp: The absence of sublethal effects from the neonicotinoid insecticide imidacloprid enlightens the specificity of the cholinergic pathway involved

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ABSTRACT

Imidacloprid is an insecticide that is used globally and is suspected to be at least partly responsible for the decrease in the number of pollinator insects. The effects of an LC20 of imidacloprid on the parasitic behavior of the parasitoid wasp *Leptopilina boulardi* were investigated. Two genetically identical *L. boulardi* strains were used for the experiments. The strains differed in that one was infected by LbFvirus and the other was not. LbFvirus is a virus that induces an increase in the superparasitism behavior of the wasp. Results of two previous works have shown that the organophosphorus insecticide chlorpyrifos induces an increase in the superparasitism rate of *L. boulardi* through its specific action on cholinergic nervous pathways. Imidacloprid targets receptors implicated in cholinergic nervous pathways and thus it was expected that imidacloprid would also increase the superparasitism rate of *L. boulardi*. However, the results of the present experiment demonstrate that imidacloprid does not interfere with the parasitic behavior of *L. boulardi* and does not increase the rate of superparasitization. It can then be concluded that the major target of imidacloprid, namely type 1 α -bungarotoxin resistant nicotinic acetylcholine receptors (nAChR1), which imidacloprid is an agonist of, and the minor target, type D α -bungarotoxin sensitive nicotinic acetylcholine receptors (nAChRD), which imidacloprid is an antagonist of, are not involved in the superparasitism behavior by *L. boulardi*. Therefore, the superparasitism behavior of the parasitoid wasp is controlled by cholinergic pathways that do not involve nAChR1 or nAChRD subtype receptors. These findings may enable a better understanding of the mechanisms by which the LbFvirus acts, and contribute to a better evaluation of the potential environmental impact of imidacloprid use.

1. Introduction

Imidacloprid is the first homologated neonicotinoid insecticide. It was introduced to the market in 1991 by Bayer (Tomizawa and Casida, 2011). Imidacloprid has been extensively used as a systemic insecticide for some time. For example, 20,000 tons of imidacloprid were produced worldwide in 2010, including 13,620 tons produced in China (Simon-Delso et al., 2015). Imidacloprid is persistent in the environment with a half-life in soil that ranges from 1 to 3 years (Wood and Goulson, 2017). Since neonicotinoids are systemic insecticides, they have been found in all parts of treated plants, including pollen and nectar (Bonmatin et al., 2007). Consequently, they can impact beneficial insects, such as pollinators, and residues of imidacloprid and other neonicotinoids have been found in honey from all over the world (Mitchell et al., 2017). Exposure to neonicotinoids can have serious effects on honeybee navigation and survival (Fischer et al., 2014; Henry et al., 2012), thus

neonicotinoids are suspected to be at least partly responsible for colony collapse disorders of honeybees and bumblebees. The outdoor use of these insecticides was banned in the European Union and Ontario, Canada in 2018. Goulson and 232 signatories (2018) have raised a “call to restrict neonicotinoids” in the hope of extending the ban.

Parasitoid wasps are important species because they regulate the demographic development of other insect populations. The wasp *Leptopilina boulardi* Barbotin, Carton, and Kelner-Pillaut (Hymenoptera: Figitidae) is a parasitoid of *Drosophila* larvae. Female wasps lay their eggs into *Drosophila* larvae. Eventually the larvae are killed and a parasitoid adult emerges. Only one parasitoid egg can successfully develop inside a *Drosophila* larva.

Female *L. boulardi* find their host by probing the substrate with their ovipositor. They discriminate between parasitized and unparasitized host larvae (Vet and Bakker, 1985; Visser et al., 1992) through the use of gustatory receptors they have on their ovipositor (Le Ralec et al.,

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1996). Furthermore, *L. heterotoma*, a species closely related to *L. boucardi*, can discriminate between host larvae parasitized only once and those parasitized twice (Ruschioni et al., 2015). These perceptions are dependent on nervous transmissions from the gustatory receptors in the central nervous system (Ruschioni et al., 2015) and can be impeded by neurotoxic insecticides that act by interfering with the nervous transmissions of insects.

Because only one *L. boucardi* egg can successfully develop inside a *Drosophila* larva, females generally lay only one egg per host larva. However, it can occur that females lay more than one egg per host larva. This behavior is called superparasitism. Delpuech (2017) showed that female *L. boucardi* that survived dried residues exposure to a lethal concentration 20% (LC20) of chlorpyrifos, an organophosphorus insecticide, superparasitized their host larvae at a higher rate than control females. It was concluded that this effect was either due to the general over-stimulation of nervous transmissions generated by the insecticide or to the specific effect of the insecticide on cholinergic nervous pathways (transmissions involving the neurotransmitter acetylcholine). In contrast, exposure to an LC20 of endosulfan, a cyclodiene organochlorine insecticide that over-stimulates nervous transmissions through the gabaergic pathway, had no significant effect on superparasitism rate. Therefore, it was concluded that the increase in the superparasitism behavior induced by chlorpyrifos was due to its specific effect on the cholinergic pathways rather than its general effect of nervous hyperstimulation (Delpuech, 2019).

The parasitic behavior of *L. boucardi* can be modified by the virus *Leptopilina boucardi filamentous virus* (LbFvirus). Varaldi et al. (2003, 2006) have shown that females infected with LbFvirus superparasitized their host larvae at a higher rate than uninfected females. Two strains of *L. boucardi* were used in this experiment, one infected by LbFvirus and the other not infected. The noninfected strain, which does not superparasitize its host in control conditions, is best suited to evidence a potential increase in the rate of superparasitism; whereas the strain infected by LbFvirus, which does superparasitize its host at a high rate in control conditions, is best suited to evidence a potential decrease in the rate of superparasitism.

In the present experiment, the sublethal effects of exposure to the LC20 of imidacloprid on the parasitic behavior of two strains of the parasitoid wasp *L. boucardi*, one infected by LbFvirus (S strain) and the other not infected by LbFvirus (NS strain), were evaluated. In the nervous system of insects, imidacloprid targets and binds to the post-synaptic nicotinic acetylcholine receptors that induce nervous stimulations (Casida and Durkin, 2013). Therefore, imidacloprid induces an over-stimulation of nervous transmissions by acting through cholinergic pathways, as chlorpyrifos does. Thus, it is expected that imidacloprid will also induce an increase in the rate of superparasitism by *L. boucardi*.

2. Materials and methods

2.1. Parasitoid strains

Two strains of *L. boucardi*, kindly provided by J. Varaldi, were used in this experiment, one non-superparasitizing (a strain whose females generally laid only one egg per *Drosophila* larvae), called NS strain, and one superparasitizing (a strain whose females frequently laid more than one egg per *Drosophila* larvae), called S strain. *L. boucardi* of the NS strain were trapped in the vicinity of Sienna, Italy and were inbred for eight generations (brother-sister mating) to homogenize the strain. This resulted in 82% homozygosity (Varaldi et al., 2003). The S strain was created from females of the NS strain that were contaminated with the LbFvirus that induces the superparasitism behavior. For the contamination, *Drosophila* larvae were parasitized by females from the NS strain and then injected with extracts containing the LbFvirus, enabling the contamination of the parasitoid larva inside the *Drosophila*, creating the S strain (0.03 µL of the extract was injected in second instar parasitized *Drosophila* larvae; the extract was obtained by crushing long

glands from 25 females of an *L. boucardi* superparasitizing strain originating from Gotheron (near Valence, France) in 15 µL of 1% PBS solution, see Varaldi et al. (2006) for details). Both *L. boucardi* strains (NS and S) were genetically identical (82% homozygosity) and differed only by the presence or absence of LbFvirus.

2.2. Sensitivity to imidacloprid

L. boucardi from the NS and S strain were reared on *Drosophila melanogaster* larvae from the Sainte Foy strain (*Drosophila* trapped in the vicinity of Lyon, France) and were grown in a thermostatic cabinet (12:12 h light/dark, 60% relative humidity) at 25 °C. At emergence, parasitoids were placed in plastic vials (9.5 cm long with a 2.5 cm diameter) containing 10 mL of sugared (0.1 g/mL) agar-agar (0.02 g/mL) medium with nipagin (3 mg/mL) and maintained at 14 °C in a thermostatic cabinet (12:12 h light/dark, 60% relative humidity). Female *L. boucardi* (1–4 days old) were exposed to imidacloprid, in groups of ten, in glass vials (7.5 cm long with a 12 mm diameter) by feeding on a tiny drop of honey contaminated with a given concentration of insecticide. The honey was contaminated with imidacloprid (95% certified purity from Cluzeau Info Labo, Sainte-Foy-La-Grande, France), which was first diluted in distilled water. 3 µL of the dilution was mixed with 28 mg ± 1 mg of honey previously deposited on the wall of the vial. Pure distilled water was used in place of the imidacloprid dilution as a control. Female *L. boucardi* from each strain (NS and S) were exposed to five concentrations of imidacloprid and a control. For each treatment, three vials containing ten females were used. The vials were stored in a thermostatic cabinet (12:12 h light/dark, 60% relative humidity) at 25 °C for 24 h before mortality was recorded. The experiment was repeated three times and data were pooled to calculate the regression line of mortality by the log-probit program of Raymond (1985) based on Finney (1971) for each strain. Results obtained from the NS and S strain were not statistically different; therefore, they were pooled together to calculate the regression line of mortality and the LC20 value to be used in the parasitization experiment.

2.3. Exposure to imidacloprid and parasitization

Female *L. boucardi* were either exposed to the LC20 of imidacloprid or to a control solution following the same procedure as described previously (2.2. Sensitivity to imidacloprid). Females surviving after 24 h of exposure to imidacloprid or to the control solution were placed in plastic petri dishes (1.3 cm tall with a 5.5 cm diameter) containing 10 mL of sugared (0.1 g/mL) agar-agar (0.02 g/mL) medium with nipagin (3 mg/mL). A drop of live baker's yeast (0.1 mL) and 12 to 20 *Drosophila* eggs were deposited in each petri dish 24 h before a single *L. boucardi* female was added to each dish. Female *L. boucardi* were left in the petri dishes for 24 h to allow the parasitization of *Drosophila* larvae. Petri dishes were maintained at 21 °C from the deposit of *Drosophila* eggs until the removal of female *L. boucardi* (48 h period). Petri dishes containing the parasitized larvae were maintained at 21 °C for three more days, then frozen (−20 °C) and were later dissected to determine how many *L. boucardi* eggs had been laid in each *Drosophila* larvae and their encapsulation status.

2.4. Statistical analysis

Results were statistically analyzed by performing two-way analysis of variance (ANOVA) with Statistica software (StatSoft Inc., Tulsa, OK, USA). Values recorded as percentages were arcsine square root (p/100) transformed before statistical analyses. Values recorded as numbers were not transformed. Values reported in tables were not transformed even if a transformation was performed for their statistical analysis.

Table 1

(A) Mean percentage of *Drosophila* larvae parasitized by female parasitoids (Parasitized) and mean percentages of *Drosophila* larvae that were Mono- or Super-parasitized within parasitized larvae for both *L. bouhardi* strains (NS and S) under control conditions or exposure to an LC20 of imidacloprid. (B) Two-way ANOVA on arcsine square root ($\sqrt{p/100}$) transformed values. Abbreviations: N: number of female parasitoids; SEM: standard error of the mean.

A								
Strain	Treatment	N	Parasitized ± SEM	Mono ± SEM	Super ± SEM			
NS	Control	51	50.8 ± 3.2	90.9 ± 2.0	9.1 ± 2.0			
	LC20	65	46.5 ± 3.1	90.6 ± 2.1	9.4 ± 2.1			
S	Control	56	73.4 ± 3.2	41.6 ± 4.1	58.4 ± 4.1			
	LC20	59	74.8 ± 3.2	37.1 ± 3.7	62.9 ± 3.7			

B								
Effect	Parasitized				Super			
	F	df 1	df 2	P	F	df 1	df 2	P
Strain (NS/S)	66.8	1	227	< 0.001	247	1	227	< 0.001
Treatment (Control/LC20)	0.02	1	227	0.90	0.32	1	227	0.57
Strain/Treatment	0.94	1	227	0.33	0.36	1	227	0.55

3. Results

3.1. Imidacloprid concentration-effect line

The concentration-effect line (log-probit regression) obtained from the combined mortality tests of the NS and S strains was Y (in probit) = $2.68 \log(X) + 2.30$. From this equation, the concentration inducing 20% mortality (LC20) was calculated to be 4.93 ng/mg (weight of imidacloprid (active ingredient) per weight honey solution) with a 95% confidence interval ranging from 3.76 to 5.86 ng/mg.

3.2. Effects of imidacloprid on parasitization

Female *L. bouhardi* from the NS strain parasitized a significantly lower percentage of *Drosophila* larvae than female *L. bouhardi* from the S strain (Table 1). However, the insecticide, imidacloprid, at LC20, had no significant effect on this parasitization rate, and no significant interaction between the insecticide and the strains was observed (Table 1 B). Female *L. bouhardi* from the S strain superparasitized many more *Drosophila* larvae than female *L. bouhardi* from the NS strain, but the LC20 of imidacloprid had no significant effect on the percentage of *Drosophila* larvae superparasitized. There was no significant interaction between the parasitoid strains and exposure to the imidacloprid.

Female *L. bouhardi* from the S strain laid significantly more eggs per *Drosophila* larva than females from the NS strain (Table 2). Imidacloprid had no significant effect on the mean number of eggs laid per *Drosophila* larva, either for the S or NS strain (Table 2 B). There was no significant interaction between the effect of the insecticide and the *L. bouhardi* strain. Graphically, this resulted in similar distributions of the number of parasitoid eggs per host larva, whether female *L. bouhardi* were exposed to imidacloprid or not. In the NS strain, a high percentage of host larvae were parasitized only once and a very low percentage of larvae were parasitized more than once by female parasitoids (Fig. 1). In the S strain, a low percentage of host larvae were parasitized only once, and the percentage of larvae parasitized two to 9+ times regularly decreased (Fig. 2).

3.3. Effects of imidacloprid on the encapsulation reaction

There was no significant difference in the percentage of encapsulated eggs between NS and S strains (Table 3). Encapsulation was

Table 2

(A) Mean number of eggs laid per parasitoid female per parasitized host larva for *L. bouhardi* NS and S strains when females were under control conditions or exposed to a LC20 of imidacloprid. (B) Two-way ANOVA on values. Abbreviations: N: number of female parasitoids; SEM: standard error of the mean.

A				
Strain	Treatment	Mean ± SEM	N	
NS	Control	1.1 ± 0.04	51	
	LC20	1.2 ± 0.05	65	
S	Control	2.4 ± 0.2	56	
	LC20	2.5 ± 0.2	59	

B				
Effect	F	df 1	df 2	P
Strain (NS/S)	126	1	227	< 0.001
Treatment (Control/LC20)	0.33	1	227	0.57
Strain/Treatment	0.06	1	227	0.81

relatively low, between 9 and 14%. Imidacloprid had no significant effect on encapsulation and there was no significant interaction between *L. bouhardi* strains and imidacloprid exposure (Table 3 B).

4. Discussion

Contrary to what was observed with chlorpyrifos (Delpuech, 2017), an organophosphorus insecticide stimulating cholinergic nervous pathways (Casida and Durkin, 2013), imidacloprid, the neonicotinoid insecticide used in this study, which also stimulates cholinergic nervous pathways, had no significant effect on superparasitism by the parasitoid *L. bouhardi*. In fact, female *L. bouhardi* exposed to an LC20 of imidacloprid superparasitized *Drosophila* larvae at a percentage not significantly different from that of control females. The absence of sublethal effects by imidacloprid on the superparasitism rate was observed regardless of infection by LbFvirus, a virus that drastically increases the superparasitization behavior of infected females. Furthermore, imidacloprid, at LC20, had no significant effect on the mean number of *L. bouhardi* eggs laid per *Drosophila* larvae by female parasitoids who were infected or not infected by LbFvirus, nor did it have an effect on the encapsulation by *Drosophila* larvae. Whereas, chlorpyrifos, when administered to *L. bouhardi* at its LC20, induced an increase in both the superparasitization rate and the mean number of parasitoid eggs laid per host larvae (Delpuech, 2017).

Delpuech (2017) concluded that the effect of chlorpyrifos on superparasitization could either be due to a general and non-specific increase in nervous stimulations induced by the insecticide or to the specific increase in nervous stimulations by cholinergic pathways. A test with another insecticide that also induces an increase in nervous transmissions, but through a different pathway, enabled a choice between these two hypotheses. Delpuech (2019) showed that endosulfan, a cyclodiene organochlorine insecticide that increases nervous stimulations by acting on gabaergic pathways (Casida and Durkin, 2013), did not increase the rate of superparasitization by *L. bouhardi*. The fact that both chlorpyrifos and endosulfan induce an increase in nervous transmissions, but only chlorpyrifos increases the superparasitization rate, demonstrate that the effect of chlorpyrifos on superparasitism is not due to a general nervous stimulation, but to its specific mode of action through nervous cholinergic pathways.

Both chlorpyrifos and imidacloprid act through cholinergic pathways (Casida and Durkin, 2013), but only chlorpyrifos induced an increase in the superparasitization behavior of female *L. bouhardi* (Delpuech, 2017). This can be explained by the difference in their specific targets. During a normal nervous transmission between an excitatory neuron and a receptor neuron through a cholinergic synapse,

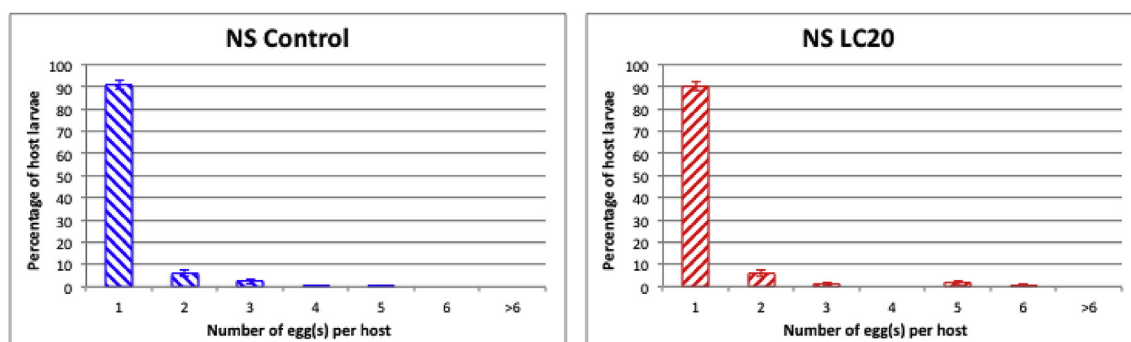


Fig. 1. Distribution of the number of parasitoid eggs per host larvae when NS strain female parasitoids of *L. boucardi* were in control conditions (NS Control, N = 51) or exposed to an LC20 of imidacloprid (NS LC20, N = 65). Error bars are standard errors of the mean.

the action potential arriving to the synapse induces the release of the neurotransmitter, acetylcholine, that binds to post-synaptic nicotinic acetylcholine receptors and generates a new action potential in the receptor neuron. As soon as acetylcholine is liberated into the synapse, it is degraded by the enzyme acetylcholinesterase to stop the effect of acetylcholine once the nerve impulse is transmitted to the receptor neuron. Chlorpyrifos binds permanently to acetylcholinesterase and impedes the action of the enzyme to degrade acetylcholine, leading to prolonged nervous stimulation, each time acetylcholine is liberated into the synapse, by increasing its concentration. Therefore, chlorpyrifos increases nervous transmissions through all the receptors activated by acetylcholine. Imidacloprid binds directly to only 2 types of nicotinic receptors (Salgado and Saar, 2004; Bodereau-Dubois et al., 2012). It binds to- and is an agonist of type 1 α -bungarotoxin-resistant nicotinic acetylcholine receptors (nAChR1) and it binds to- and is an antagonist of type D α -bungarotoxin-sensitive nicotinic acetylcholine receptors (nAChRD); however, it does not bind to other types of nicotinic receptors (nAChR2, nAChRN, $\alpha_3\beta_4$ receptors, etc.) nor to muscarinic acetylcholine receptors, contrary to acetylcholine (Bodereau-Dubois et al., 2012; Salgado and Saar, 2004). Therefore, we can conclude that the nervous pathways involved in the superparasitism behavior of the parasitoid wasp *L. boucardi* are cholinergic pathways that do not involve nAChR1 or nAChRD receptors.

The widespread use of neurotoxic insecticides contributes to environmental pollution, and has shown negative effects on hymenopteran parasitoids of both ecological- and economic importance. For examples, insecticides have been shown to interfere with the sex-pheromonal communications of the parasitoid wasp *Trichogramma brassicae* (Delpuech et al., 1998a, 1999a, 2012, 1998b, 1999b; Dupont et al., 2010). Insecticides have been shown to interfere with the kair-omonal communications (Delpuech et al., 2005; Komeza et al., 2001) and circadian rhythms (Delpuech et al., 2015) of *Leptopilina* parasitoids. In this work, it is shown that the neonicotinoid imidacloprid does not modify the parasitism behavior of *L. boucardi*, contrary to what was

Table 3

(A) Mean encapsulation percentage of parasitoid eggs and larvae per parasitoid female per host larva for the *L. boucardi* NS and S strains under control conditions or exposed to an LC20 of imidacloprid. (B) Two-way ANOVA on arcsine square root ($p/100$) transformed values. Abbreviation: N: number of female parasitoids; SEM: standard error of the mean.

A				
Strain	Treatment	Mean \pm SEM	N	
NS	Control	9.2 \pm 1.8	51	
	LC20	14.0 \pm 3.4	65	
S	Control	12.6 \pm 2.9	56	
	LC20	9.7 \pm 2.0	59	

B				
Effect	F	df 1	df 2	P
Strain (NS/S)	0.01	1	227	0.91
Treatment (Control/LC20)	0.00	1	227	0.99
Strain/Treatment	1.82	1	227	0.18

expected. The absence of a sublethal effect enables us to narrow the possible subtypes of cholinergic receptors involved in this behavior. This can also lead to a better understanding of the possible mechanisms enabling the LbFvirus to manipulate the parasitic behavior of *L. boucardi*. Furthermore, the finding of unaltered superparasitism behavior of *L. boucardi*, after exposure to imidacloprid dried residues, adds to our knowledge of the potential impact of this commonly used insecticide on nontarget organisms.

Credit author statement

Jean-Marie Delpuech: Writing - original draft, review and editing, Experimental work, Data acquirement and analysis.

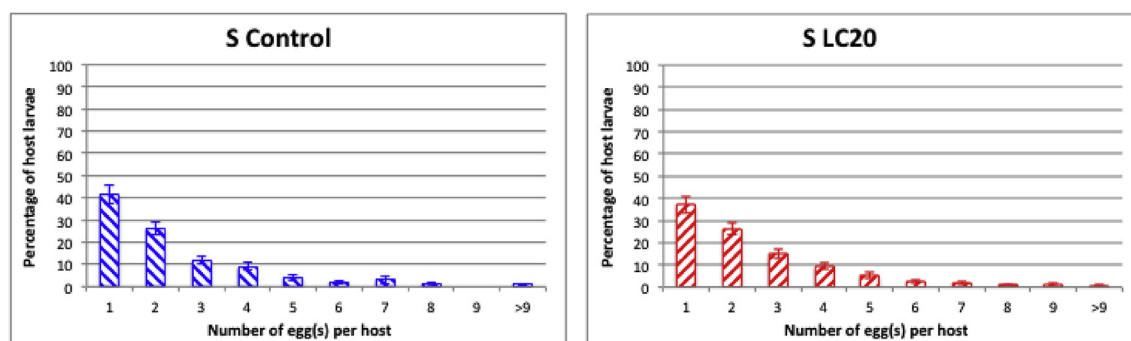


Fig. 2. Distribution of the number of parasitoid eggs per host larvae when S strain female parasitoids of *L. boucardi* were in control conditions (S Control, N = 56) or exposed to an LC20 of imidacloprid (S LC20, N = 59). Error bars are standard errors of the mean.

Declaration of competing interest

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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