



Elicitation of superparasitization behavior from the parasitoid wasp *Leptopilina boulardi* by the organophosphorus insecticide chlorpyrifos



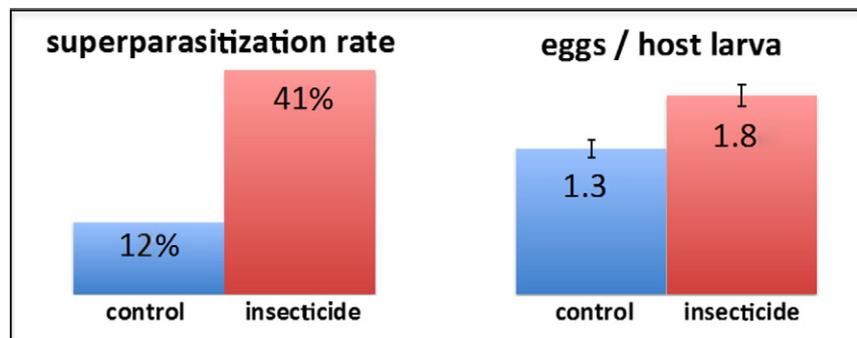
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HIGHLIGHTS

- Parasitoids are key species because they control insect populations.
- Chlorpyrifos is one of the most used insecticides worldwide.
- Chlorpyrifos increased the rate of superparasitization by parasitoids.
- This effect involves acetylcholine concentration and is equivalent to that of LbFV.
- This effect may jeopardize the equilibrium of wild insect communities.

GRAPHICAL ABSTRACT



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ABSTRACT

Chlorpyrifos is an organophosphorus insecticide that largely contributes to environmental pollution. Parasitoids, as any other non-target species, can be exposed to insecticides through environmental pollution. Parasitoids are key species because they regulate natural populations of other insects. The hymenopterous parasitoid *Leptopilina boulardi*, whose larvae develop inside *Drosophila* larvae, is a solitary parasitoid; thus, only one larva can successfully develop per host. Therefore, females generally lay only one egg per host because any increase in the number of eggs laid will decrease its fitness. The effects of an LC20 of chlorpyrifos on the parasitization behavior of two strains (NS and S) of *L. boulardi* were evaluated. The NS and S strains were genetically identical but differed in that the S strain was infected by a virus, LbFV, which modifies the parasitization behavior of the parasitoid. In control conditions, parasitoid females from the NS strain rarely superparasitized (laid more than one egg per host) their host whereas females from the S strain frequently superparasitized their host. When parasitoids were exposed to an LC20 of chlorpyrifos, the rates of host larvae superparasitized by females and the mean numbers of eggs laid per host larva increased for both NS and S strains. Therefore, both the insecticide and the virus induced an increase in the superparasitization of the host. The effect of the insecticide on the superparasitization behavior of the parasitoid is discussed according to its mode of action.

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1. Introduction

Hymenopteran parasitoids are ecologically important natural enemies because they control the development of the populations of other insect species by killing the host inside of which their larvae

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develop. Some hymenopteran parasitoid species are strictly proovigenic. This is the case of *Leptopilina boulardi* Barbotin, Carton and Kelnner-Pillaut (Hymenoptera: Figitidae) whose females emerge with their total stock of eggs and do not mature more eggs afterwards (Jervis et al., 2008). Therefore, the management of their egg load is an important feature of their fitness. To maximize their fitness, females must maximize the number of eggs that will successfully develop into adults. However, different factors may modify the fecundity, and therefore, the fitness of these parasitoids.

The larvae of the hymenopteran parasitoid *L. boulardi* develop inside *Drosophila* larvae. *L. boulardi* females, which find their host larvae by probing the host substratum with their ovipositor (Vet and Bakker, 1985; Visser et al., 1992), can discriminate if the host larva is already parasitized or not through gustatory receptors present on their ovipositor (Le Ralec, 1991; Le Ralec et al., 1996). The capacity to detect if the host larva has already been parasitized enables *L. boulardi* females to avoid parasitized hosts (Van Lenteren, 1981); this behavior, called host discrimination, is an important trait because *L. boulardi* is a solitary parasitoid, i.e. only one parasitoid per host can successfully develop.

Host discrimination relies on the perception of stimuli (odors) by receptors that transmit their stimulation through nervous pathways (Ruschioni et al., 2015). Because neurotoxic insecticides interfere with nervous transmission (Casida and Durkin, 2013), the perception of these stimuli may be modified. It was shown, for example, that the organophosphorus insecticide chlorpyrifos interferes with pheromonal communications within hymenopteran parasitoid species (Delpuech et al., 1999). Chlorpyrifos has also been shown to interfere with the discrimination of sexual pheromones between species of hymenopterous parasitoids (Dupont et al., 2010). By interfering with the perception of odors, insecticides may interfere with host discrimination. Delpuech and Leger (2011) and Delpuech and Delahaye (2013) have shown that chlorpyrifos and the pyrethroid insecticide deltamethrin inhibited the perception by the hymenopteran parasitoid *Trichogramma brassicae* Bezdenko (Hymenoptera, Trichogrammatidae) of an already parasitized host. Organophosphorus insecticides may interfere with host discrimination not only because they perturb nervous transmissions along neurons but also because these perturbations are caused by an increase in the concentration of acetylcholine in synapses (Casida and Durkin, 2013). Indeed, it has been shown that acetylcholine intervenes in odor perceptions (D'Souza and Vijayaraghavan, 2014) and odor discriminations (Chapuis and Wilson, 2013).

Besides insecticides, any other factor interfering with the parasitization process by parasitoids may also modify their fecundity and, therefore, their fitness. Viruses capable of manipulating the parasitization behavior of parasitoids are such factors. Varaldi et al. (2003, 2006a) have analyzed the parasitization behavior of parasitoid females from two different *L. boulardi* strains. The two strains were genetically identical and only differed by the fact that one was infected by a virus named *L. boulardi* Filamentous Virus (LbFV) and the other was not. Authors demonstrated that the virus induced an increase in the superparasitization behavior (i.e. laying more than one egg per host larva) of female parasitoids.

In this paper, the possible effect of the organophosphorus insecticide chlorpyrifos on the parasitization behavior of the parasitoid *L. boulardi* was tested. For this, the previously mentioned strains were used. The strain not infected by LbFV (NS strain) rarely superparasitized its hosts in control conditions whereas the strain infected by LbFV (S strain) frequently superparasitized the host larvae in control conditions. Therefore, the use of these two strains enables to evidence a possible effect of the insecticide either toward an increase or a decrease in the parasitization behavior of the parasitoid. The results obtained with the strain infected by LbFV will also possibly bring useful knowledge for further investigation and understanding of the mode of action of the virus in increasing the superparasitizing behavior of *L. boulardi*. Furthermore, because every organophosphorus insecticide has the same mode of action (inhibition of acetylcholinesterase in the central nervous system

of insects), the results obtained with chlorpyrifos may also be valid for any other organophosphorus insecticide.

2. Materials and methods

2.1. Biological material

Two strains of *L. boulardi* were used for the experiments, a non-superparasitizing strain, called NS, and a superparasitizing strain, called S. The NS strain was an inbred line (8 generations of brother-sister mating, 82% of homozygosity, origin: Sienna, Italy) (Varaldi et al., 2006a). The S strain was derived from the NS strain by injecting extracts containing the LbFV, inducing superparasitization, into *Drosophila* larvae previously parasitized by the NS strain (Varaldi et al., 2006b). Thus, the two parasitoid strains, NS and S, shared the same genetic background, but were either uninfected (non-superparasitizing phenotype, NS) or infected (superparasitizing phenotype, S) by LbFV. Parasitoid strains were reared on larvae of a *Drosophila melanogaster* strain Sainte Foy.

2.2. Determination of lethal concentrations

Newly emerged parasitoids (males and females) were maintained in vials (9.5 cm long, 2.5 cm of diameter) containing 10 ml of agar medium with sugar and nipagin at 25 °C, 70% relative humidity, 12 h:12 h light:dark until they were exposed to the insecticide. Parasitoid females, one to 4 days old, were exposed to the insecticide in groups of ten in glass vials (length 7.5 cm, width 12 mm) containing a piece of paper (length 5 cm, width 8 mm) on which 12 µl of the insecticide diluted in acetone were deposited (pure acetone was used for controls). The pieces of paper were left 1 h on the lab bench for evaporation of the acetone before placing in vials. A small drop of honey was placed on the side of the vial to feed the parasitoids and vials were kept at 25 °C, 70% relative humidity, 12 h:12 h light:dark. The mortality of *L. boulardi* was assessed after 24 h of contact with the treated piece of paper. For each strain (NS and S), 5 different concentrations of insecticide were used and, for each concentration, 50 adults were exposed to the insecticide. The lethal concentration for 20% of the parasitoids (LC20) was estimated from the regression line of mortality determined by the log-probit program of Raymond (1985), based on Finney (1971) after two replicates. The insecticide used was chlorpyrifos (99% certified purity; Cluzeau Info Labo, Sainte-Foy-La-Grande, France).

2.3. Exposure to the insecticide and parasitization

Newly emerged parasitoids were maintained and exposed to the LC20 as described in Section 2.2 (pure acetone was used for controls). After 24 h of exposure to the LC20 of insecticide, females that survived were separately placed in petri dishes. One female was deposited per petri dish (1.3 cm height, 5.5 cm of diameter) that contained 10 ml of agar medium (with sugar and nipagin), a drop of about 0.1 ml of living bakery yeast deposited on the center of the agar medium and 12 *Drosophila* eggs deposited in each petri dish 24 h before parasitoid females. The delay of 24 h was sufficient enough for allowing eggs to hatch into larvae before parasitoid females were placed inside the petri dishes. Parasitoid females were allowed to parasitize *Drosophila* larvae in the petri dishes during 24 h, and then, were removed. From the moment *Drosophila* eggs were deposited to the moment parasitoid females were removed, the petri dishes were stored at 21 °C. After that, the petri dishes were stored at 14 °C until the dissection of *Drosophila* larvae performed the following 3–7 days to count the parasitoid larvae and encapsulated parasitoid eggs and larvae per *Drosophila* larva used for the calculation of the percentage of encapsulation (i.e. the number of encapsulated eggs and larvae over the total number of parasitoid eggs and larvae present in the *Drosophila* larva).

The results were analyzed performing two-way ANOVAs with the software Statistica (Statsoft Inc.). The variables analyzed were the mean percentages of *Drosophila* larvae parasitized by parasitoid females, the mean percentages of *Drosophila* larvae that were superparasitized within parasitized larvae, the mean numbers of eggs laid per parasitoid female per parasitized host larva and the mean percentages of encapsulation of parasitoid eggs and larvae per parasitoid female per host larva. The factors were the strain (NS and S) and the treatment (control conditions and exposed to an LC20 of chlorpyrifos). When the variable analyzed was a percentage, values were arcsine square root ($p/100$) transformed before test. Values were not transformed when they were numbers. Means reported in the tables were values obtained without transformation even if a transformation was performed for their statistical analysis.

3. Results

3.1. Sensitivity to the insecticide

The linear regression equation obtained from the observed mortalities of females from both NS and S strains was Y (in probit) = $5.57 \log(X) + 0.066$. The theoretical concentration inducing 20% mortality (LC20) was 5.43 ng cm^{-2} (active ingredient) $4.75\text{--}5.98 \text{ ng cm}^{-2}$ (95% confidence interval).

3.2. Differences between NS and S strains and effects of the insecticide on the parasitization of the host by the parasitoid

The mean percentages of *Drosophila* larvae parasitized by parasitoid females were significantly different between the NS and S strain (Table 1, Parasitized, Strain effect: $F_{1,186} = 4.99 P = 0.03$), the exposure to the insecticide had no significant effect on these percentages (Treatment effect: $F_{1,186} = 0.36 P = 0.55$), but there was a significant interaction between the strains and the insecticide (Interaction Strain/Treatment: $F_{1,186} = 4.92 P = 0.03$), the exposure to the insecticide tending to induce an increase in the percentage of parasitization by the NS strain and a decrease for the S strain (Table 1).

The mean percentages of *Drosophila* larvae that were superparasitized were significantly higher for the S strain than for the NS strain (Table 1, Strain effect: $F_{1,186} = 201 P < 0.001$). The exposure to the insecticide induced a shift in the distribution of parasitoid eggs for both strains (Figs. 1 and 2) by decreasing the percentage of *Drosophila* larvae monoparasitized and increasing the percentage of *Drosophila* larvae superparasitized (Table 1). These differences induced by the exposure to the insecticide were very significant (Treatment effect: $F_{1,186} = 29.6 P < 0.001$). There was a significant interaction between the strains and the exposure to the insecticide (Interaction Strain/Treatment: $F_{1,186} = 4.45 P = 0.04$) that corresponds to a higher increase for the NS strain than for the S strain of the percentage of *Drosophila* larvae superparasitized.

The mean numbers of eggs laid per parasitoid female per parasitized host larva was significantly higher for the S strain than for the NS strain (Table 2, Strain effect: $F_{1,186} = 96.5 P < 0.001$). The exposure to the insecticide induced a significant increase in the mean numbers of eggs laid per parasitoid female for both strains (Table 2, Treatment effect:

$F_{1,186} = 4.46 P = 0.04$). There was no significant interaction between the strains and the exposure to the insecticide (Interaction Strain/Treatment: $F_{1,186} = 0.32 P = 0.57$).

3.3. Differences between NS and S strains and effects of the insecticide on the percentage of encapsulation

The mean encapsulation percentages of parasitoid eggs and larvae per parasitoid female per host larva (cf. Materials and methods part 2.3 for the mode of calculation) were significantly different between both strains. Eggs and larvae from the S strain were significantly more encapsulated by host larvae than those from the NS strain (Table 3, Strain effect: $F_{1,186} = 12.7 P < 0.001$). In contrast, the exposure to the insecticide had no significant effect on the percentage of encapsulation ($F_{1,186} = 0.01 P = 0.93$) and there was no significant interaction between the strains and the exposure to the insecticide (Interaction Strain/Treatment: $F_{1,186} = 0.49 P = 0.49$).

4. Discussion

The exposure of *L. boulandi* parasitoids to an LC20 of the insecticide chlorpyrifos induced a significant stimulation of the superparasitization behavior of parasitoids from both NS (non-superparasitizing, LbFV negative) and S (Superparasitizing, LbFV-infected) strains. Under control conditions, females from the NS strain tended to lay only one egg per host larva whereas females from the S strain tended to lay several eggs per host larva because of their infection by LbFV. The insecticide chlorpyrifos induced an increase of superparasitism and an increase in the mean numbers of eggs laid per host larva for parasitoid females from both the NS and S strains. Therefore, both the insecticide and the virus induced an increase, with seemingly-similarity, in the superparasitization of host larvae by the parasitoid *L. boulandi*.

A possible explanation of this increase in superparasitization by parasitoids exposed to chlorpyrifos is the inhibition of the perception of the host larvae as being already parasitized due to an overstimulation of nervous pathways. Indeed, the mode of action of organophosphorus insecticides such as chlorpyrifos is to induce an overstimulation of cholinergic nervous pathways by irreversibly binding to acetylcholinesterase, an enzyme whose function is to stop nervous transmissions in synapses by hydrolyzing acetylcholine (Casida and Durkin, 2013). When the enzyme and the insecticide are bound, the functions of the enzyme are inhibited, acetylcholine is no longer hydrolyzed, and nervous stimulations are prolonged and increased leading to an overstimulation of nervous pathways (Casida and Durkin, 2013).

An inhibition of host discrimination was already observed on *Trichogramma* with the same insecticide, chlorpyrifos (Delpuech and Leger, 2011) and with a pyrethroid insecticide, deltamethrin (Delpuech and Delahaye, 2013). Authors of these papers showed that insecticides inhibited the antennal perception of already parasitized hosts. Antennal perceptions were inhibited because they were regularly solicited and because the insecticides prolonged nervous stimulations each time antennae were solicited; therefore, after several nervous stimulations, concerned nervous pathways were permanently overstimulated and no longer able to discriminate between parasitized and not parasitized hosts. The parasitoid *L. boulandi* neither finds host larvae nor discriminates between parasitized and unparasitized host larvae by antennal perceptions but by probing the substratum with its ovipositor (Vet and Bakker, 1985; Visser et al., 1992) on which it has mechano- and gustatory receptors (Le Ralec, 1991; Le Ralec et al., 1996). Van Lenteren et al. (2007) have shown that *L. heterotoma* (Thomson, 1862) (Hymenoptera: Figitidae), a species closely related to *L. boulandi*, also detects its hosts with its ovipositor that also carries gustatory sensilla (seven) at the distal end, all innervated by six neurons. Furthermore, Ruschioni et al. (2015) have shown that *L. heterotoma* is able to discriminate between un-parasitized, one time parasitized and two-times parasitized hosts through the electrophysiological activity of the

Table 1

Mean percentages of *Drosophila* larvae parasitized by parasitoid females (Parasitized), mean percentages of *Drosophila* larvae that were Mono- or Super- parasitized within parasitized larvae for both *L. boulandi* strains (NS and S) in control conditions or exposed to an LC20 of chlorpyrifos. N: Number of parasitoid females. SEM: Standard Error of the Mean.

Strain	Treatment	N	Parasitized \pm SEM	Mono \pm SEM	Super \pm SEM
NS	Control	41	75.9 \pm 4.0	88.0 \pm 3.9	12.0 \pm 3.9
	LC20	40	86.1 \pm 2.8	59.4 \pm 5.7	40.6 \pm 5.7
S	Control	54	90.0 \pm 2.1	22.2 \pm 3.6	77.8 \pm 3.6
	LC20	55	83.1 \pm 3.6	11.0 \pm 2.7	89.0 \pm 2.7

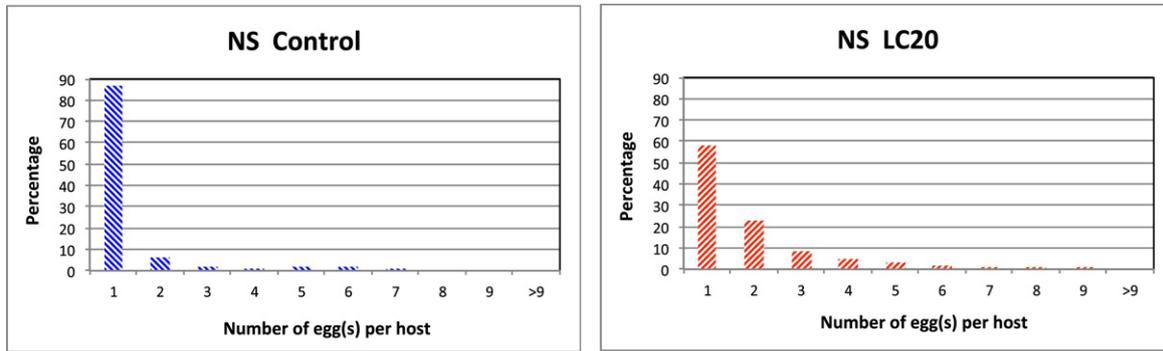


Fig. 1. NS strain (non-superparasitizing, not infected by LbFV), *L. bouleari* parasitoid. Distribution of the number of parasitoid eggs per host larvae when parasitoid females were in control conditions (Control, 40 females) or exposed to an LC20 of chlorpyrifos (LC20, 41 females).

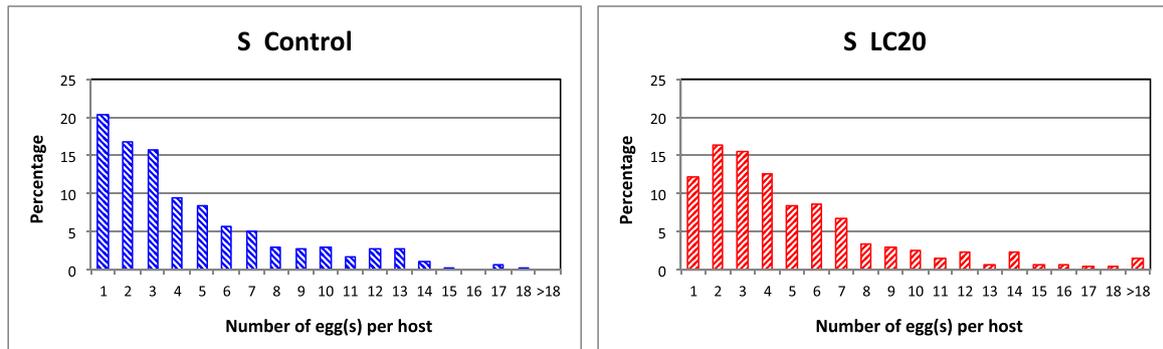


Fig. 2. S strain (Superparasitizing, LbFV-infected), *L. bouleari* parasitoid. Distribution of the number of parasitoid eggs per host larvae when parasitoid females were in control conditions (Control, 54 females) or exposed to an LC20 of chlorpyrifos (LC20, 55 females).

coeloconic gustatory sensillum of its ovipositor in contact with the haemolymph of *Drosophila* larvae. Therefore, when *L. bouleari* parasitoid females exposed to the insecticide were detecting host by probing the substrate, it is probable that, after several encounter with hosts, nervous pathways from their ovipositor were permanently over stimulated because of the effect of the insecticide that increased and prolonged nervous stimulations. Therefore, parasitoid females were no longer able to differentiate between healthy and parasitized larvae. Then, already parasitized host larvae were parasitized one or several more times, leading to an increase in the superparasitization of host larvae.

The inhibition of the capacity to identify host larvae as being already parasitized was likely accelerated by the effect of the insecticide on the activity of the parasitoid. Indeed, Rafalimanana et al. (2002) have shown that the insecticide chlorpyrifos induces a stimulating effect on host searching by the parasitoid *L. heterotoma*. Chlorpyrifos increased the percentage of females probing the substrate in search of hosts and females exposed to the insecticide found and oviposited in host larvae more quickly than control females. Therefore chlorpyrifos, by increasing the encounter rate of parasitoid females with host larvae, would accelerate the saturation of nervous pathways enabling the discrimination of already parasitized larvae. By this mechanism, the insecticide would accelerate the occurrence of the inhibition of host discrimination.

Table 2

Mean numbers of eggs laid per parasitoid female per parasitized host larva for NS and S strains when females were under control conditions or exposed to an LC20 of chlorpyrifos. SEM: Standard Error of the Mean. N: Number of parasitoid females.

Strain	Treatment	Mean \pm SEM	N
NS	Control	1.3 \pm 0.1	41
	LC20	1.8 \pm 0.1	40
S	Control	4.3 \pm 0.3	54
	LC20	5.2 \pm 0.4	55

Parasitoid eggs and larvae from the S strain were significantly more encapsulated by host larvae than parasitoid eggs and larvae from the NS strain. The results obtained with chlorpyrifos demonstrate that the difference in the percentage of encapsulation between both strains is not caused by the fact that the S strain superparasitizes its hosts more than the NS strain does. Indeed, the exposure to the insecticide induced an increase in the superparasitization behavior for both strains but it did not induce an increase in the percentage of encapsulation neither for the S strain nor for the NS strain. Because the only difference between the NS and S strain is their status of viral infection, it is most likely that the increase in the percentage of encapsulation of the S strain is induced by the presence of LbFV. This would correspond to a trade-off between the presence of the virus and the induction of the host immune reaction leading to encapsulation. It is worth noting that an increase in the percentage of encapsulation does not necessarily leads to an increase in the survival of parasitized *Drosophila* larvae. Indeed, it is sufficient that only one parasitoid larva, among those present inside the host, evades the encapsulation reaction for the *Drosophila* larva to die. The success of the encapsulation reaction (corresponding to the efficient encapsulation, i.e. the total encapsulation of every parasitoid larva present into a host, leading to its survival) was not measured in my experiments. However, Martinez et al. (2012) showed that *Drosophila* parasitized by

Table 3

Mean encapsulation percentages of parasitoid eggs and larvae per parasitoid female per host larva and standard errors of the mean (SEM) for the NS and S strains in control conditions or exposed to an LC20 of chlorpyrifos. N: number of parasitoid females.

Strain	Treatment	Mean \pm SEM	N
NS	Control	22.4 \pm 5.3	41
	LC20	19.7 \pm 3.9	40
S	Control	34.6 \pm 4.0	54
	LC20	37.9 \pm 4.3	55

parasitoids infected by LbFV had a less efficient encapsulation rate than when parasitized by parasitoids not infected by LbFV.

With *Trichogramma*, Dupont et al. (2010) showed that the same insecticide, chlorpyrifos, at a dose inducing the same mortality, an LD20, induced confusion in males in discriminating female sexual pheromones used for mate finding between two closely related *Trichogramma* species. This confusion, being able to lead to interspecific matings between the two *Trichogramma* species, would decrease the fecundity of the parasitoid because the interspecific fertilization of the parasitoid eggs leads to their degeneration (Delpuech et al., 2010). In this work, I showed that chlorpyrifos increased the behavior of superparasitizing host larvae likely through an inhibition of host discrimination. This sublethal effect of the insecticide also corresponds to a decrease in the fecundity of the parasitoid. Indeed, *L. boulandi* is a proovigenic species. Females possess their total stock of eggs when emerging from the host and no more eggs can be matured after emerging. Therefore, laying several eggs in the same host larva corresponds to the loss of all laid eggs but one because only one egg can successfully develop per host larva. However, when parasitoid eggs are encapsulated by the host, superparasitism may be beneficial for parasitoids because it may enable them to overcome encapsulation by exhausting host capacity to encapsulate (Van Alphen and Visser, 1990). If this would be the case, the efficient encapsulation rate would be decreased, however this rate was not measured in my experiments. The impact of such an exposure to chlorpyrifos on the community composed of the parasitoids and its host will also depend on the sublethal effect of the insecticide on the fitness of its host, the *Drosophila*, which is not known for the moment.

Several pesticides have been shown to induce sublethal effects that could potentially modify the equilibrium of interactive species such as parasitoids and their hosts. For example, the organochlorine endosulfan has been shown to modify the circadian rhythms of the two sympatric species *L. boulandi* and *L. heterotoma* (Delpuech et al., 2014). Another work has revealed the impact of fungicides on the abundance and the species diversity of the natural populations of hymenopterous parasitoids and their hosts (Delpuech and Allemand, 2011). The pyrethroid insecticide deltamethrin was shown to jeopardize the specific discrimination of sex pheromones in two sympatric *Trichogramma* species (Delpuech et al., 2012), *Trichogramma* species that are also hymenopterous parasitoids controlling lepidopterous populations several of which are agricultural pests. The pesticide chlorpyrifos is intensively used worldwide for crop protection and actively participates to environmental pollution (Lavin et al., 2012). Non-target species as parasitoids are therefore most likely to be exposed to this pesticide. Because parasitoid species control the development of insect populations that they parasitize, the sublethal effects of chlorpyrifos evidenced in this paper on the parasitoid *L. boulandi* may have important consequences on the equilibrium of the wild insect communities composed of these interacting species.

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