Research Article

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Short- and long-term effects of commercial formulations of imidacloprid, spirotetramat, and mixtures of these active ingredients on pupae of *Diaeretiella rapae* (Hymenoptera: Braconidae) and its progeny

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Abstract

BACKGROUND: Compatibility studies of insecticides and natural enemies usually focus on short-term lethal effects, without considering the long-term sublethal effects (including progeny). Even less-explored are the effects of commercial insecticides formulated with more than one active product. Short- and long-term lethal and sublethal effects were studied for the first time on the progeny of commercial formulations of spirotetramat, imidacloprid and a commercial mixture of these active ingredients on pupae of *Diaeretiella rapae* (M'ntosh) (Hymenoptera: Braconidae), an endoparasitoid of aphids considered to be a potential biological control agent. Insecticides were exposed topically on aphid mummies in which the parasitoid was in the pupal stage.

RESULTS: Imidacloprid reduced adult emergence by more than 30% and prolonged intra-host development time with respect to control from half the maximum recommended field dose (MFRD). Spirotetramat and commercial mixture only showed significant effects on these endpoints at doses above the MFRD. The tested formulations did not affect adult longevity, sex ratio, and percentage of parasitism in the exposed generation. At low concentrations the active ingredients in the commercial mixture behave synergistically, whereas at medium and high concentrations they behave antagonistically. Considering the 10% lethal dose (LD_{10}), imidacloprid showed the highest hazard coefficient, whereas the commercial mixture was more hazardous when considering the LD_{50} and LD_{90}. The commercial mixture and imidacloprid induced higher adult emergence and altered the sex ratio in the progeny.

CONCLUSIONS: The following order of toxicity on *D. rapae* can be established: imidacloprid > commercial mixture > spirotetramat. Joint use of this species with imidacloprid and commercial mixture should be avoided in integrated pest management programs.

Keywords: endoparasitoid wasps; hazard assessment; insecticide selectivity; toxicity

1 INTRODUCTION

Schizaphis graminum (Rondani) (Hemiptera: Aphididae) is one of the main pests that cause severe damage to cereals, especially in the seedling stage, during the first weeks of crop emergence. This pest causes direct (as sap-sucking insects) and indirect (as vectors of viral diseases) damage to plants, limiting crop productivity.[1]

Among the natural enemies that help maintain aphid densities below economic damage levels, hymenopteran parasitoids stand out as the most efficient biological controllers. In some cases, they are even commercialized for biological pest control.[2] *Diaeretiella rapae* (M'Intosh) (Hymenoptera: Braconidae) is a generalist and cosmopolitan endoparasitoid braconid,[3] considered a potential biological control agent of several aphid species, including *Brevicoryne brassicae* (L.) (Hemiptera: Aphididae),[4] *Diuraphis noxia* (Kurdjumov) (Hemiptera: Aphididae),[5] and *S. graminum*.[6] Consequently, protecting this parasitoid should be a priority when implementing pest management programs.

Despite the existence of biological control agents, the predominant model currently employed to control agricultural pests is the intensive use of synthetic pesticides, disregarding the secondary effects of these compounds on non-target biota[7] and other environmental components.[8] The side effects of pesticides on natural pest enemies are relevant because they alter the natural

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control of pests and, consequently, increase the need for agronomic inputs to avoid losses.

This has led to the development of new active ingredients for controlling agricultural pests classified as 'low risk', which *a priori* have more specific mechanisms of action against pests.[9,10] Neonicotinoids were initially included within this group because of their low toxicity in mammals. However, their selectivity is today discussed because side effects have been reported on different non-target organisms, including pollinators and natural enemies.[11–14] In some countries, such as European Member States, the use of imidacloprid and other neonicotinoids has been almost entirely banned.[15] The US Environmental Protection Agency policy was established to mitigate the acute risk of neonicotinoids to bees.[16] Despite this, the use of neonicotinoids in pest management programs continues to spread, with imidacloprid being one of the most used.[17] Imidacloprid, as well other neonicotinoids insecticides, acts as a central nervous system antagonist by interacting specifically with nicotinic acetylcholine receptors, producing excitement, paralysis, and death.[18]

Spirotetramat belongs to the keto-enol group and acts by interfering with lipid biosynthesis, altering energy balance and endocrine function in pests. Because of its mode of action, it is also considered a low-risk active substance, although there is not much information on its effects on non-target organisms and even less on pest control agents.[19,20]

Many agricultural input companies have developed commercial formulations with more than one active ingredient to increase the action spectrum of pesticides. The toxicity of commercial mixtures cannot be estimated by the sum of the effects of the individual active ingredients because the interaction between them is not always additive and can sometimes be antagonistic or synergistic.[21] Therefore, considering the importance of pest natural enemies to reduce pesticide use, evaluating the potential effects of these mixtures on biological control agents spontaneously present in agroecosystems should also be a priority.

Traditionally, studies on the compatibility of pesticides and natural enemies have been mainly based on evaluating lethal or some sublethal effects in the short term.[22] However, these studies sometimes underestimated the toxic action of pesticides because the assessed parameters were not always associated with the performance of natural enemies[23] or because some effects appeared in the long term, even in generations after the one exposed.[24] Therefore, studies with an ecological input considering sublethal and long-term effects are relevant to make the evaluation more realistic.[25]

In this context, we proposed as a working hypothesis that insecticides formulated with imidacloprid and spirotetramat induce lethal and sublethal short- and long-term effects (including on the progeny) on the parasitoid *D. rapae*, which affect its role as a biological pest control agent. Furthermore, the combined effect of both active ingredients in a commercial mixture is greater than the sum of their individual effects. Within this general framework, we aimed to evaluate the short- and long-term lethal and sublethal effects of commercial formulations of imidacloprid, spirotetramat, and mixtures of these active ingredients on pupae of *D. rapae* and its progeny. Our work provides ecotoxicological information to evaluate the selectivity of these compounds on this parasitoid considered a potential biological control agent for aphids.

2 MATERIALS AND METHODS

2.1 General considerations

Insect colonies and bioassays were carried out in a growth chamber with controlled temperature (25 ± 2 °C), 70% ± 5% relative humidity, and a 16:8 h light/dark photoperiod.

2.2 Insects

A *D. rapae* colony was established from ~50 parasitized aphids (mummies) collected in Brassicaceae crops such as broccoli (*Brassica oleracea* var. *italica* Plenck), cabbage (*Brassica oleracea* var. *capitata* L.) and kale (*Brassica oleracea* var. *sabellica* L.) with no history of pesticide use located in La Plata, Argentina (34°56′04″ S, 58°10′14″ W). Colony maintenance was carried out in the laboratory following existing protocols[26,27] with modifications, using the green cereal aphid, *S. graminum*, as the host.

2.3 Insecticides

Tested insecticide commercial formulations were Movento® (spirotetramat 15% w/v Bayer S.A., Argentina) and Confidor® OD (imidacloprid 20% w/v, Bayer S.A, Argentina). In addition, the commercial mixture Movento® Plus (spirotetramat 12% w/v + imidacloprid 36% w/v, Bayer S.A., Argentina) was tested because the effects of insecticide mixtures on natural enemies are poorly studied. All formulations are authorized and frequently used for the control of sucking pests, including aphids, in Argentina (CASAFE, 2023 https://guiaonline.casafe.org/index.php/welcome/item/.prd=277.mrc=1927.emp=3 accessed 2023) and worldwide.[28] Insecticide solutions were prepared using analytical grade acetone as a solvent, whereas controls were treated only with acetone according to guidelines given by the International Organization for Biological Control (IOBC) for topical treatments. Acetone is commonly used as an organic solvent to facilitate the rapid draying of drop (insecticide acetone solution).[22] For each insecticide, eight doses were evaluated (Table 1), including, in all cases, those that correspond to the maximum field recommended doses regulated in Argentina (MFRD). *Capsicum* sp. (Solanaceae) was the reference species used to establish the MFRD (CASAFE, 2023 https://guiaonline.casafe.org/index.php/welcome/item/.prd=277.mrc=1927.emp=3 accessed 2023).

2.4 Toxicity bioassays—experimental methodology

In each treatment (imidacloprid, spirotetramat, commercial mixture, and control), ~70 aphid mummies of less than 24 h from formation were individually exposed topically to 0.5 µL of each insecticidal solution using a Hamilton® hand-held microapplicator. Previously, aphid mummies were dissected and observed under stereoscopic magnification to corroborate the development stage of the parasitoid, which, in this case, corresponded to the pupal stage. It is relevant to highlight that the egg and larval stages of *D. rapae* occur inside of the aphid body (koinobiont specie), being the aphid mummy the most evident sign of parasitism (change in the aphid cuticle color) that forms ~7 days (at 25 °C) after the aphid was parasitized.[29]

2.4.1 *Short-term effects of insecticides on* Diaeretiella rapae *pupae (exposure generation)*

After application, treated mummies were placed in individual cylindrical plastic containers (2.5 cm long and 3 cm wide) closed at the top. The adult emergence of parasitoids was recorded every 24 h and for 10 consecutive days (the observed lifespan in this species). This parameter was used to estimate the total emergence capacity and the 10, 50, and 90% lethal doses (LD_{10}, LD_{50}, and LD_{90}) 10 days after exposure to the insecticide. Adults that did not emerge from the mummies after 10 days were considered dead. For treatments with adult emergence >30%, the intra-host development time and adult longevity (sublethal parameters) were recorded.

Table 1. Doses of formulated insecticides tested on *Diaeretiella rapae* at pupal stage (inside of aphid host), lethal doses, hazard quotient and estimated toxic units at 10 days from insecticides exposure

Commercial name	Active ingredient (a.i.)	Dose (mg/g)	Lethal dose (mg/g) (µg/individual)[†]			HQ[‡]	TU[§]
Movento®	Spirotetramat 15% w/v (SPI)	6.944	LD_{10}	0.015	0.017	3764.37	
		3.472					
		0.083					
		0.067					
		0.042[††]	LD_{50}	1.222	1.320	47.71	
		0.021					
		0.010					
		0.004	LD_{90}	94.451	104.167	0.60	
Confidor® OD	Imidacloprid 20% w/v (IMI)	9.259	LD_{10}	0.005	0.006	19 767.99	
		0.167					
		0.133	LD_{50}	0.246	0.266	473.66	
		0.083[††]					
		0.042					
		0.021	LD_{90}	10.279	11.102	11.34	
		0.004					

Movento® Plus	Spirotetramat 12% w/v + Imidacloprid 36% w/v	SPI	IMI		SPI	IMI	SPI	IMI	SPI IMI		
		0.155	0.467								
		0.078	0.233	LD_{10}	0.019	0.059	0.021	0.063	1433.86	1416.99	11.21
		0.039	0.117								
		0.019[††]	0.058[††]	LD_{50}	0.055	0.167	0.059	0.179	501.31	500.24	0.72
		0.009	0.029								
		0.005	0.014	LD_{90}	0.158	0.471	0.171	0.509	175.27	176.59	0.05
		0.002	0.007								

[†] Considering the mummies mean weight = 1.08 g.
[‡] HQ (hazard quotient) = Recommended field rate (g a.i/ha)/ LD of insect (µg a.i/individual). HQ < 50, safe; HQ 50–2500, slightly to moderately toxic; HQ >2500, dangerous.
[§] UT (unit toxic) = (DLa mix/DLa alone)/ (DLb mix/DLb alone). UT < 1, antagonistic; UT = 1, additive; TU > 1, synergistic.
[††] Corresponding to the maximum field recommended doses regulated in Argentina (MFRD).

In addition, emerged adults were sexed, and the sex ratio was estimated in the control treatment and those corresponding to the MFRD of each insecticide.

The toxic unit (TU) model was used to determine the toxicity of each active ingredient in the commercial mixture (mix) compared with the toxicity of the same ingredient in each individual formulation (imidacloprid and spirotetramat). The TU estimation was performed as follows.[30]

$$TU = \frac{DLa(mix)}{DLa(alone)} + \frac{DLb(mix)}{DLb(alone)}$$

Where a and b are the active ingredients imidacloprid and spirotetramat, respectively; DLx (mix) is the lethal dose (LD_{10}, LD_{50}, and LD_{90}) of each active ingredient in the binary mixture; and DLx (alone) is the lethal dose (LD_{10}, LD_{50}, and LD_{90}) of the active ingredients in the individual formulation. Therefore, according to the model, if TU = 1, the toxicity is additive; if TU < 1, the toxicity is antagonistic; and if TU > 1, the toxicity of the mixture is synergistic.[31]

In addition, based on the LD_{10}, LD_{50}, and LD_{90} values at 10 days of exposure, the hazard quotient (HQ) of each insecticide was calculated according to the following formula.[32]

$$HQ = \frac{\text{Recommended field rate}(\text{g a.i.}/\text{ha})}{\text{LD of insect}\,(\mu\text{g a.i.}/\text{individual})}$$

Where a hazard ratio for a pesticide <50 is considered safe, 50–2500 is slight to moderately toxic, and >2500 is hazardous.[33] The LD used in this algorithm were estimated in µg a.i./individual and not in µg/mg.

2.4.2 *Long-term effects of insecticides on* Diaeretiella rapae *(effects on the progeny of exposed organisms)*

Seven pairs of *D. rapae* adults less than 24 h old emerging from aphid mummies treated with the MFRD of each insecticide solution (imidacloprid, spirotetramat, and commercial mixture) and control (treated with acetone alone) were placed in cylindrical plastic containers (10 cm long and 8 cm wide) covered with voile to allow gas exchange and fed *ad libitum* with a honey solution. Males and females were paired for copulation only for 24 h. The male was then removed, and the female remained in the container until death. During the first 5 days, every 24 h, wheat seedlings with 40 aphids of different development stages randomly chosen were offered to females as a parasitism source. After 24 h of contact with the female parasitoids, the aphid seedlings

were isolated and checked daily for 11 days to record the number of mummies and estimate the parasitism rate of females that emerged from treated mummies. The mummies found (progeny of females emerged from treated mummies) were placed individually in cylindrical plastic containers (2.5 cm long and 3 cm wide) closed at the top, and the adult emergence and sex ratio were recorded for 5 days.

2.5 Statistical analysis

Results are presented as means ± standard deviation (SD). Data normality and variance homoscedasticity were tested with Shapiro–Wilks and Levene tests. Normal data with homogeneous variances were analyzed with a one-way analysis of variance (ANOVA) to test for differences between treatments. The least significant difference test was used to evaluate differences between pairs of treatments ($\alpha \leq 0.05$). To normalize the data expressed as percentages, arc-sine-square root transformation was used.[34] When data were not adjusted to ANOVA premises, the Kruskal–Wallis and Dunn tests were used. LD_{10}, LD_{50}, and LD_{90} values were estimated by Probit regression using all doses that caused effects on survival between 0% and 100%. A computer program developed by Chi was used for this analysis.[35,36] The sex ratio was compared with the control using a χ^2 test. Comparisons between the emergence percentage of the exposed generation and the following one (progeny) for each treatment were performed using Student's *t*-test. The bilateral non-parametric Mann–Whitney test was used to compare the sex ratio between the two generations for each treatment. Survival analysis estimated through the Kaplan–Meier method was used to determine the mean intra-host development time for each treatment. Associations between adult emergence and longevity, intra-host development time, and application doses were performed by simple correlations using Pearson's coefficient. All analyses were performed with XLStat (Addinsoft XLStat for Excel, Paris, France, 2009 http://xlstat.softonic.com) and InfoStat software.

3 RESULTS

3.1 Effects of insecticides on pupae of *Diaeretiella rapae* (exposed generation)

The three tested insecticide formulations showed a high and significant negative correlation between exposure doses and the total percentage of adult emergence from exposed mummies (imidacloprid: correlation coefficient = −0.9096, $p = 0.0045$; spirotetramat: correlation coefficient = −0.9011, $P = 0.0022$; mixture commercial: correlation coefficient = −0.8593, $P = 0.0132$). Adult total emergence was significantly reduced from the application dose corresponding to half the MFRD ($F = 9.38$; $df = 7,15$; $P = 0.0003$) in the imidacloprid treatment (Fig. 1(a)). With spirotetramat and the commercial mixture, this parameter was significantly inhibited only at exposure doses above the MFRD. Spirotetramat had significant effects on emergence with respect to control from doses near to two orders of magnitude higher than the MFRD ($H = 16.87$; $P = 0.0243$) (Fig. 1(b)), whereas in the commercial mixture, significant effects with respect to the control were observed from the treatment corresponding to double the MFRD ($F = 25.8$; $df = 7,16$; $P < 0.0001$) (Fig. 1(c)).

Table 1 shows the lethal doses estimated 10 days from exposure, TU values, and HQ values. For all treatments, the LD_{50} values were above the MFRD of each insecticide. Comparing the single formulations, imidacloprid induced greater acute lethal effects than spirotetramat because of lower $LD_{10,50,90}$ values for the neonicotinoid insecticide. In the commercial mixture, the LD_{50} and LD_{90} values of each active ingredient were lower than those of the individual formulations. The LD_{10} value for spirotetramat practically did not differ between the mixture and the individual formulation, and for imidacloprid, the individual formulation was an order of magnitude lower than the mixture. The toxic unit model estimated from the LD_{10} value corroborated a synergic effect of the mixture at low doses, whereas from LD_{50} and LD_{90} the effect was antagonistic, but in the case of LD_{50} the value of TU is close to 1, which would correspond to additive pattern effect.

Considering the HQ values estimated from the LD_{10} value, imidacloprid proved to be the most hazardous insecticide for this species when applied topically on parasitized aphid mummies. However, when the HQ values were estimated from the LD_{50} and LD_{90}, the commercial mixture proved to be the most dangerous insecticide among those evaluated. By contrast, considering the hazard classification,[33] all three formulations were moderately toxic, except for spirotetramat and imidacloprid in the individual formulations, which were classified as hazardous when HQ values were estimated from LD_{10}. Also, these insecticides were considered safe when QH values were estimated from LD_{50} and LD_{90} for spirotetramat and from LD_{90} for imidacloprid (Table 1).

The application doses and intra-host development time in the individual formulations of imidacloprid and spirotetramat showed no significant correlation (imidacloprid: correlation coefficient = 0.1046, $P = 0.8233$; spirotetramat: correlation coefficient = 0.1936, $P = 0.6459$). Thus, although spirotetramat did not induce any effect on this parameter compared with the control (log-rank 5.8327; $P = 0.4422$), imidacloprid showed significant effects at 0.167, 0.083 and 0.042 mg a.i./g (log-rank 22.2807; $P = 0.0011$). In the commercial mixture, intra-host development time correlated positively (correlation coefficient = 0.8144, $P = 0.0257$) with application doses. We observed significant prolongation in intra-host development time of the parasitoid compared with the control at the highest doses tested (0.233 mg a.i./L imidacloprid; 0.078 mg a.i./L spirotetramat) (log-rank 14.0671; $P = 0.0242$) (Table 2).

Table 2 also shows the longevity of adults (days) emerging from the treated mummies in the spirotetramat, imidacloprid, commercial mixture, and control treatments. The parasitoid longevity and application doses did not correlate significantly (imidacloprid: correlation coefficient = −0.3553, $P = 0.4343$; spirotetramat: correlation coefficient = −0.4010, $P = 0.3248$; commercial mixture: correlation coefficient = −0.1906, $P = 0.6823$). Also, this parameter showed no significant effects compared with the control at any of the treatments evaluated ($F = 21.322$; $df = 5,1$; $P = 0.163$). However, adult longevity correlated negatively with intra-host developmental time (imidacloprid: correlation coefficient = −0.7875, $P = 0.0355$; spirotetramat: correlation coefficient = −0.7983, $P = 0.0175$; commercial mixture: correlation coefficient = 0.2498, $P = 0.5890$) in individual formulations, suggesting that an increase in intra-host developmental time reduces adult survival time. Moreover, the MFRD of each insecticide did not significantly affect the sex ratio compared with the control. Concerning the parasitism percentage, we did not observe significant effects for any treatment (Table 2).

3.2 Effects of insecticides on the progeny of *Diaeretiella rapae*

Long-term effects on the progeny of *D. rapae* were only evaluated for the MFRD of each insecticide and the control. None of the

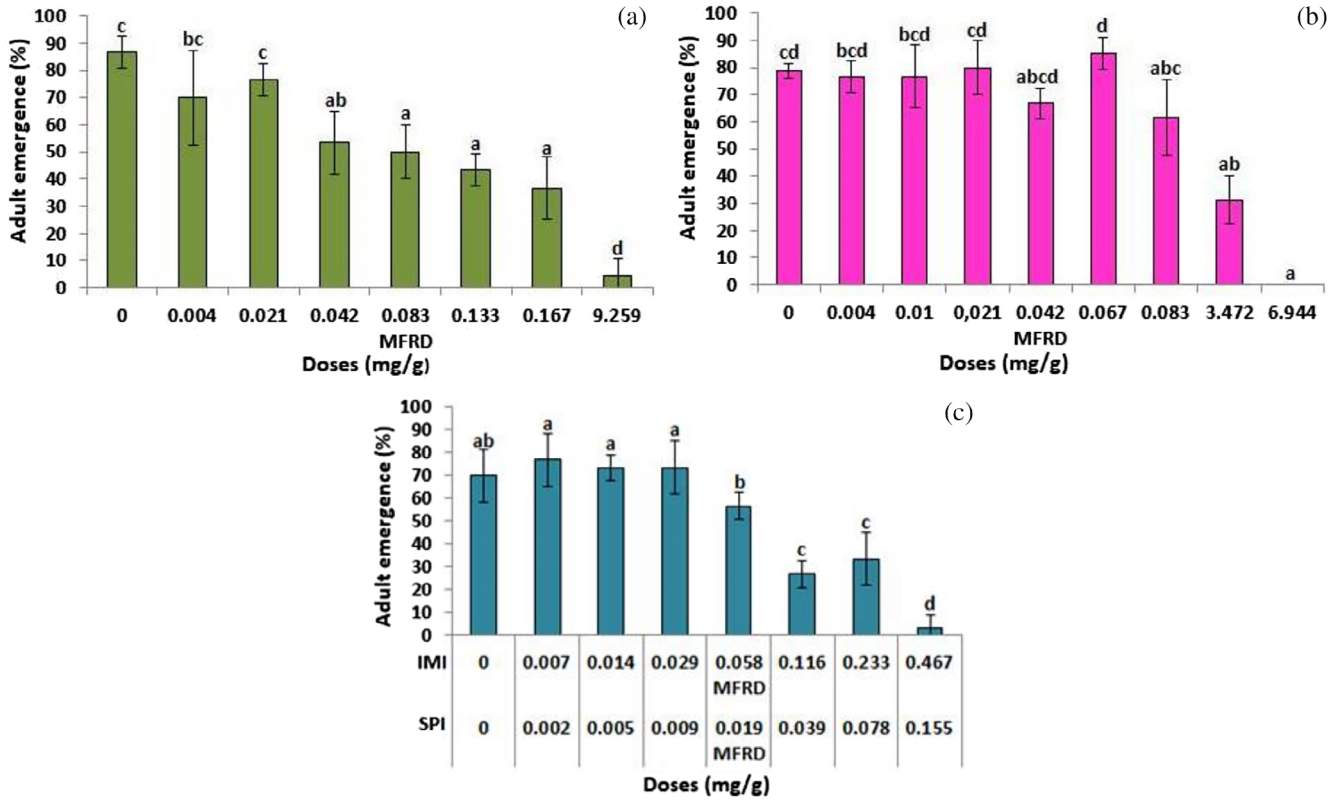

Figure 1. Emergence of adult parasitoids of *Diaeretiella rapae* (Hymenoptera: Braconidae) from aphid mummies exposed to different doses of imidacloprid (a), spirotetramat (b), and a commercial mixture of these active ingredients (c) (Exposure generation). The exposure was performed topically on aphid mummies in which the parasitoid was in the pupal stage. MFRD, maximum field recommended doses regulated in Argentina. Data are given as mean ± SD. Different letters denote significant differences between treatments ($\alpha \leq 0.05$).

insecticides tested significantly inhibited adult emergence compared with the control in the post-exposure generation, even in the treatments with imidacloprid and the commercial mixture the emergence percentages were higher than in the control ($F = 9.74$; $df = 3,3$; $P = 0.0007$). Comparing the percentages of emergence obtained in exposure generation and the progeny for each treatment, imidacloprid and the commercial mixture showed a significantly higher percentage of adult emergence in the progeny than in the exposed generation (imidacloprid: $t = 2.41$, $P = 0.039$; commercial mixture: $t = 3.05$, $P = 0.014$). By contrast, the percentage of emergence in the control and spirotetramat treatment did not differ significantly between the two generations (spirotetramat: $t = -0.45$, $P = 0.65$; control: $t = -1.04$, $P = 0.17$) (Fig. 2).

Imidacloprid and the commercial mixture disrupted the sex ratio of adults that emerged in the progeny of exposed organisms compared with the control, with a higher proportion of females observed in the treatments with these insecticides ($X^2 = 22.033$, $P = 0.0002$) (Fig. 3). However, none of the insecticides showed significant differences in the sex ratio of adults in the progeny compared with the exposure generation (imidacloprid: $U = 19.000$, $P = 0.5541$; spirotetramat: $U = 18.500$, $P = 0.6111$; commercial mixture: $U = 14.000$, $P = 0.7589$; control: $U = 28.000$, $P = 0.3163$).

4 DISCUSSION

Although the side effects of pesticides on several natural pest enemies have been documented in recent years,[12,13,37] the intergenerational effects of pesticides on surviving progenies remain poorly explored,[38,39] especially those related to the impact of commercial formulations with more than one active ingredient.[40]

Because of its vital role as a biological controller of different pests, *D. rapae* has previously been used as a model species to evaluate the secondary effects of insecticides on natural enemies,[41,42] including the short-term effects of other imidacloprid formulations.[43,44] However, to our knowledge, no study has assessed the effects on the progeny of organisms exposed to imidacloprid or the compatibility of spirotetramat and the mixture of these active ingredients on this species.

In this work, we exposed aphid mummies in which the parasitoid was in the pupal stage (protected stage) to insecticides. The aphid cuticle constitutes an additional barrier to the entry of insecticides into the parasitoid body.[45,46] In general, high penetration of an insecticide through the cuticle is associated with a low molecular mass and a high beeswax–water partition coefficient of the compound,[47] which correlates positively with the octanol–water partition coefficient (log P).[48] The active ingredient imidacloprid has a higher octanol/water partition coefficient and molecular mass (log $K_{ow} = 0.57$; molecular mass = 255.66 g/mol) than spirotetramat (log $K_{ow} = 2.51$, molecular mass = 301.38 g/mol) (PubChem, 2023 https://pubchem.ncbi.nlm.nih.gov/compound/Imidacloprid-d4 access). Therefore, according to the partition coefficient, imidacloprid would have a higher penetration capacity than spirotetramat, and if we consider the molecular mass vice versa. However, the properties of the adjuvants, which

Table 2. Side effects of spirotetramat, imidacloprid, and a mixture commercial of these active ingredients on several life parameters of *Diaeretiella rapae* after insecticides exposure at their maximum field recommended doses

Commercial name	Active ingredient (a.i.)	Dose (mg/g)	Intra-host development time (days)	Adults longevity (days)	Sex ratio Male/ Female	Parasitism (%)
Control		0	4.57 (4.80–4.33)	3.17 (4.05–2.29)	0.46/0.54 (±0.05)	25.24 ± 15.32
Movento®	Spirotetramat 15% w/v (SPI)	3.472	4.62 (5.16–4.08)	2.4 (4.67–0.13)		
		0.083	4.84 (5.23–4.44)	1.83 (2.41–1.24)		
		0.067	4.43 (4.80–4.06)	3.44 (4.57–2.31)		
		0.042†	4.36 (4.69-4.04)*	3.20 (4.42–1.98)	0.60/0.40 (± 0.20)	24.61 ± 12.97
		0.021	4.66 (5.17–4.16)	3.40 (4.58–2.22)		
		0.010	4.53 (5.06–4.00)	2.87 (4.44–1.30)		
		0.004	4.13 (4.48–3.78)	3.56 (4.59–2.53)		
Confidor® OD	Imidacloprid 20% w/v (IMI)	0.167	5.40 (5.80–4.99)*	1.18 (1.82–0.54)		
		0.133	4.66 (5.08–4.25)	3.18 (4.07–2.29)		
		0.083†	5.07 (5.49-4.64)*	1.33 (2.02–0.65)	0.39/0.61 (±0.21)	24.37 ± 8.58
		0.042	5.96 (6.47–5.46)*	1.50 (2.34–0.66)		
		0.021	5.00 (5.53–4.46)	2.04 (2.69–1.39)		
		0.004	5.1 (5.68–4.52)	2.52 (3.09–1.95)		
Movento® Plus	Spirotetramat 12% w/v + Imidacloprid 36% w/v	SPI	IMI			
		0.078	0.233	6.27 (6.72–5.81)*	2.50 (3.64–1.36)	
		0.039	0.117	4.87 (5.00–4.77)*	1.37 (2.00–0.74)	
		0.019†	0.058†	4.87 (5.35-4.38)	1.88 (2.48–1.28)	0.44/0.56 (± 0.24) 13.54 ± 10.60
		0.009	0.029	5.23 (5.71–4.75)	2.04 (2.55–1.54)	
		0.005	0.014	4.66 (5.32–4.00)	2.54 (3.20–1.89)	
		0.002	0.007	5.16 (5.85–4.48)	2.91 (3.59–2.24)	

Note: Data correspond to mean times (upper and lower limit). Data correspond to mean ± SD. All the parameters evaluated correspond to the exposure generation.
† Corresponding to the maximum field recommended doses regulated in Argentina (MFRD).
*Statistical differences are noted compared with the control.

generally modify the partitioning of the active ingredient, are unknown in each formulation. So, the more noticeable effects on emergence observed for imidacloprid compared with spirotetramat in the individual formulations could be related to higher penetration of this insecticide associated with the partition coefficient. Like us, other authors have also recorded the effects of this and other neonicotinoids on adult emergence when exposures were made at the pupal stage of *Eretmocerus mundus* (Mercet) (Hymenoptera: Aphelinidae) and *Aphidius gifuensis* (Ashmead) (Hymenoptera: Braconidae)[19,49,50] parasitoids, but no effects were reported on *E. mundus* and *Aphelinus mali* (Haldeman) (Hymenoptera: Aphelinidae) at the MFRD of spirotetramat.[19,51] Regarding commercial mixtures, studies that evaluated the mortality of chrysopids and spiders showed that the lethal effects of the insecticide Movento Energy® (spirotetramat + imidacloprid) were lower than those recorded for other commercial mixtures, such as Fountain® (fipronil + imidacloprid) and Concept Plus (pyriproxyfen, fenpyroximate + acephate).[40] Likewise, we also found a relatively low toxicity of the spirotetramat + imidacloprid commercial mixture, which was safe at environmentally relevant doses. However, laboratory studies showed a decreased viability of *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) eggs exposed by immersion to the MFRD of this commercial mixture.[52] Thus, for imidacloprid, we corroborated part of the working hypothesis, in which we proposed that the insecticides induced short-term lethal effects on *D. rapae*, whereas for spirotetramat and the commercial mixture this hypothesis was rejected.

Other comparative studies on the lethal effects of pesticide interaction and individual active ingredients showed contradictory results. Some studies evidenced synergistic effects of thiamethoxam (Th) + λ-cyhalothrin (λ-cy) and thiamethoxam (Th) + abamectin (Ab) insecticidal mixtures on the mortality of *Apis mellifera ligustica* (Spinola) (Hymenoptera: Apidae). By contrast, a Th + β-cypermethrin (β-cy) mixture showed additive behavior.[53] On the other hand, binary mixtures of acetamiprid, fipronil, and ivermectin showed synergistic and antagonistic effects depending on the combination of active ingredients.[21] Other studies also compared the effects of commercial mixtures of spirotetramat + imidacloprid (CMT-560) and individual formulations on field populations of the predator *O. insidiosus*. These studies indicated that imidacloprid had a lower effect on the abundance of this species than spirotetramat and the commercial mixture of both active ingredients.[54] Our study also showed a disparity in the effects of the commercial mixture compared with the individual active ingredients, confirming the hypothesis that the interaction of the active ingredients in the commercial mixture induces effects that differ from those of the sum of the individual active products. Note, this behavior was dose-dependent, because the

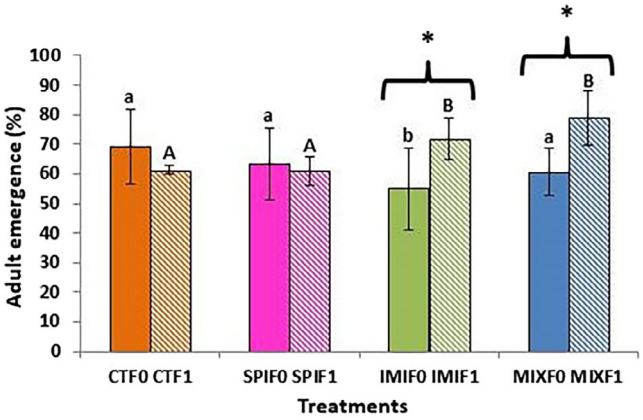

Figure 2. Emergence of adult parasitoids of *Diaeretiella rapae* (Hymenoptera: Braconidae) in the treatments: control (CT), spirotetramat (SPI), imidacloprid (IMI), and a commercial mixture of these active ingredients (MIX) at the maximum field recommended doses regulated in Argentina (MFRD). The exposure was performed topically on aphid mummies in which the parasitoid was in the pupal stage. Solid-filled bars correspond to exposure generation, and textured bars correspond to adult emergence in the progeny of exposed organisms. Data are given as mean ± SD. Different lowercase letters denote significant differences in exposure generation between insecticide treatments and control. Different capital letters denote significant differences in the progeny between insecticide treatments and control. *Statistical differences are noted between exposure generation and progeny.

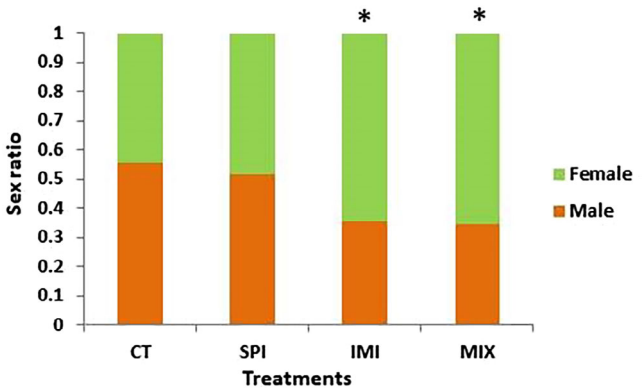

Figure 3. Sex ratio of adult parasitoids of *Diaeretiella rapae* (Hymenoptera: Braconidae) in the progeny of organisms exposed to maximum field recommended doses regulated in Argentina of imidacloprid (IMI), spirotetramat (SPI), and a commercial mixture of these active ingredients (MIX). CT, control treatment. The exposure was performed topically on aphid mummies in which the parasitoid was in the pupal stage. *Statistical differences are noted compared with the control.

mixture induced synergistic effects at low doses and antagonistic effects at high doses.

HQ analysis is a simple measure to assess pesticide safety against natural enemies. The relationship between the lethal effects induced by an insecticide and its recommended application dose for field use, allows us to know how close an insecticide is to inducing significant effects on an organism at probable concentrations in the environment.[34] We found that spirotetramat was the least hazardous insecticide for *D. rapae*, making it compatible for use in the presence of aphid mummies parasitized with this species. The highest hazard value was recorded for imidacloprid when the HQ was estimated from the LD_{10}, whereas the mixture was higher than the individual formulations when the HQ values were estimated from LD_{50} and LD_{90}. Although we did not find other studies analyzing the hazard of spirotetramat or the commercial mixture, imidacloprid seems to be particularly dangerous for *D. rapae* compared with its effect on other species such as *Cyrtorhinus lividipennis* (Reuter) (Hemiptera: Miridae), *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae),[34] and *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae)[55] for which it was safe, and *Encarsia formosa* (Gahan) (Hymenoptera: Aphelinidae),[56] *Trichogramma dendrolimi* (Matsumara) (Hymenoptera: Trichogrammatidae), and *Trichogramma ostriniae* (Pang & Chen) (Hymenoptera: Trichogrammatidae)[57] for which it was mildly to moderately toxic.

From an ecological perspective, altering the parasitoid's developmental time could affect the host-parasitoid interaction scheme.[25] Some studies documented a prolonged developmental time of immature stages of *E. mundus* exposed to acetamiprid, imidacloprid and thiamethoxam (neonicotinoid neurotoxicants), spinetoram (naturally occurring), and sulfoxaflor (non-neonicotinoid neurotoxicant),[58] and of *E. formosa*[59] and *Aenasius arizonensis* (Girault) (Hymenoptera: Encyrtidae)[60] exposed to imidacloprid. In contrast, abamectin, lambda-cyhalothrin, tebufenozide, and teflubenzuron reduced the development time of *Trichogramma pretiosum* (Girault) (Hymenoptera: Encyrtidae).[61] We observed no effect on the intra-host development time of the parasitoid with spirotetramat, but we recorded an increase in this end-point at some tested doses of imidacloprid and the commercial mixture (but at doses above MFRD); however, whereas in the first case, it did not correlate with the exposure doses, in the commercial mixture, it did.

The absence of effects of neonicotinoid insecticides on longevity observed in our study has also been reported by other authors when exposing *A. gifuensis* pupae to thiamethoxam.[46] However, some studies have reported that other neonicotinoids, such as acetamiprid, decreased this parameter in *Eretmocerus sp.*[62,63] and *E. formosa*[56] parasitoids when pupae were exposed by immersion. As we observed in *D. rapae*, spirotetramat did not significantly affect the longevity of the parasitoids *Aphelinus certus* (Yasnosh) (Hymenoptera: Aphelinidae),[64] *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae),[65] *Aphytis melinus* (DeBach) (Hymenoptera: Aphelinidae),[66] and *Anagyrus* sp. near *pseudococci* (Girault) (Hymenoptera: Encyrtidae).[67] Therefore, this parameter does not seem sensitive to this insecticide exposure.

Some reports warn about the disruption of the parasitoid sex ratio induced by insecticides exposed to the host.[38] Ecologically, effects on this parameter could alter the population dynamics of the species and, consequently, its role as a biological control agent. We did not observe significant effects on this parameter for any of the insecticides evaluated. Similarly, other studies did not observe any effect of imidacloprid on the adult sex ratio of *E. mundus* at exposures beyond the pupal stage.[68] We did not find any previous studies on the impact of spirotetramat and the mixtures analyzed here on this parameter in *D. rapae*, highlighting the need to focus future studies on this topic to increase our knowledge about the toxicological profile of these insecticides. Thus, if we take into account the sublethal effects evaluated on the exposed generation, the hypothesis of induction of short-term sublethal effects should be accepted for imidacloprid and rejected for spirotetramat and the commercial mixture. Note that the effects observed on intra-host developmental time in the commercial mixture were at concentrations above the MFRD.

Insecticide compatibility studies with natural enemies often do not assess the effects on the progeny of exposed organisms, even though they may negatively impact the ecological services these organisms provide.[25,69] In this sense, the predator *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) significantly reduced progeny survival when exposed to imidacloprid and sulfoxaflor.[70] Other authors exposed eggs parasitized by *T. pretiosum* to thiodicarb, chlorfenapyr and flupyradifuron, and found reduced adult emergence in the post-exposure generation (progeny). Still, the insecticide growth regulator teflubenzuron caused no effect compared with the control.[38]

In our study, treatments with imidacloprid and the commercial mixture had higher adult emergence in the post-exposure generation than the control and the exposed generation, whereas spirotetramat showed no significant effects. The induction of stimulatory effects (hormesis) of pesticides on non-target biota is a phenomenon that has been previously reported even in beneficial insects.[71–74] However, interpreting this effect as beneficial should be carefully considered because a greater understanding of the principles and mechanisms by which this phenomenon occurs is still needed.[71] It is known that certain moderate levels of stress can trigger adaptive responses in organisms that result in an increase in some of their biological parameters.[75] It has even been demonstrated for some insects that there are epigenetic molecular mechanisms that act in response to pesticide exposure by regulating gene expression without modifying genetic sequences and giving rise to the expression of different stress responses, as well as compensatory mechanisms.[76] Thus, the hormetic responses observed in treatments with imidacloprid and the commercial mixture in the percentage of adult emergence of progeny could be explained as a compensatory mechanism associated with exposure to these compounds.

In most Hymenoptera, the sex ratio is close to 1:1; however, it can be affected by different environmental factors[77] and, consequently, alter the population dynamics of the species. Female Hymenoptera can regulate the sex of their offspring because of their haploid genetic system, in which fertilized eggs become females (diploid) and unfertilized eggs become males (haploid).[78] This phenomenon leads us to consider that the sex ratio could be altered in response to either natural or anthropogenic stress factors, which would explain the higher female proportion in the progeny observed in the treatments with imidacloprid and the commercial mixture. By contrast, other authors found a lower proportion of transgenerational females in *T. pretiosum* exposed to thiodicarb.[38] The offspring sex ratio was not affected in *E. mundus*,[19] *A.* sp. near *pseudococci*, and *M. mediator*[65,67] pupae exposed to spirotetramat, and of *Tamarixia triozae* (Burks) (Hymenoptera: Eulophidae) exposed to minimal imidacloprid doses.[79] Thus, the higher percentage of emergence and the higher proportion of females recorded with imidacloprid and the commercial mixture corroborate the hypothesis that these treatments induce sublethal effects in the progeny of exposed organisms, whereas in the case of spirotetramat the hypothesis should be discarded.

In conclusion, among the insecticides tested, imidacloprid was the most toxic to *D. rapae*, causing significant short-term lethal effects at environmentally relevant doses. Imidacloprid and the commercial mixture altered some long-term parameters, demonstrating that they could act as stressors that continue to manifest in the progeny. Our results highlight the relevance of including the assessment of long-term effects in pesticide compatibility studies with natural enemies. The working hypothesis was fully corroborated for imidacloprid and partly for the mixture, because this commercial formulation did not induce short-term lethal and sublethal effects at environmentally relevant concentrations but effects on progeny, whereas it should be rejected for spirotetramat.

The scarce information on the side effects of commercial mixtures of active ingredients on natural enemies stimulates us to continue collecting baseline information to assess the risk of using these products in integrated pest management programs.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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