



Artificial selection for emamectin benzoate resistance in the biological control agent *Orius laevigatus*

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HIGHLIGHTS

- Wild populations of *Orius laevigatus* varied in susceptibility to emamectin.
- Four strains were selected for increased emamectin resistance for 41–47 generations.
- All instars expressed resistance, without evidence of cross-resistance to abamectin.
- Resistance remained stable for 18 generations without emamectin exposure.
- Inhibitors of the detoxification enzymes failed to restore susceptibility.

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ABSTRACT

Biological control is occasionally supplemented with insecticides treatments, which may have a significant impact on natural enemies. Typically, selective compounds are chosen to overcome lack of compatibility, but an alternative approach is the use of biocontrol agents resistant to pesticides. *Orius laevigatus* (Fieber) (Hemiptera: Anthoridae) is the main predator used to control thrips and other small pests in greenhouses. The avermectin emamectin benzoate is a bioinsecticide developed for the control of lepidopteran pests, reported as moderately to highly toxic to *O. laevigatus*. Firstly, we studied the variation in susceptibility to emamectin benzoate in 32 wild and commercial populations of *O. laevigatus*. A 62.4-fold variation in response was found (LC_{50} from 0.8 mg L^{-1} to 49.9 mg L^{-1}). The baseline LC_{50} was 4.8 mg L^{-1} . Secondly, we exploited this intraspecific variation to select four distinct emamectin-resistant strains. After 41–47 selection cycles, four resistant strains were successfully obtained ($LC_{50} = 104\text{--}203 \text{ mg L}^{-1}$) compared to the reference population ($LC_{50} = 4.7 \text{ mg L}^{-1}$). The resistance was retained for 18 generations without insecticide exposure and was expressed in all life instars, especially from the 4th nymphal instar to adult. The emamectin-resistant strains did not show cross-resistance to abamectin. Inhibitors of the detoxification enzymes failed to restore susceptibility at the concentrations tested. Fecundity and predation capacity in the resistant strain was similar to those in a commercial population. The resistance obtained may be enough to allow survival of adults and nymphs of *O. laevigatus* exposed to treatments of emamectin benzoate across the crop season.

1. Introduction

Augmentative biological control has proved very effective in many crops, particularly in vegetables grown in greenhouses (van Lenteren et al., 2020). Omnivorous predators are introduced to control key pests, even before pest occurrence, since they can thrive using pollen or other alternative sources as food (Bielza et al., 2020). However, these

integrated pest management (IPM) programs have to be occasionally supported with insecticide applications in order to manage some main and secondary pest outbreaks. In a survey addressed to biological control practitioners in Almería (South-eastern Spain) in 2015, 50% of the growers declared that they had to resort to insecticide applications to control aphids and lepidopteran pests in order to complement biological control (van der Blom, 2015).

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The omnivorous anthocorid *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae) is widely inoculated to control *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) populations in several vegetable crops. This predator is released shortly after transplanting, because its presence before thrips arrival is critical for biological control efficacy (Sánchez et al., 2000). This early introduction increases exposure to pesticide treatments, demanding selective compounds for compatible use. However, there are few or no selective insecticides for certain pests, requiring the application of non-selective compounds to control some pests or to avoid resistance development due to the overuse of very few compatible pesticides (Bielza et al., 2020).

In particular, one group of major pests that may require supplemental insecticide applications are the caterpillars, such as *Helicoverpa armigera* (Hübner), *Spodoptera exigua* (Hübner), *Chrysodeixis chalcites* and *Autographa gamma* (Lepidoptera: Noctuidae). These lepidopterans lack effective natural enemies, but other biological and biotechnical solutions are employed such as entomopathogens, traps and mating disruption. However, chemical compounds are typically applied to manage outbreaks. The diamide chlorantraniliprole is preferably chosen due to a good compatibility with natural enemies (Biondi et al., 2012). Other compounds show moderate toxicity to arthropod biological control agents (BCA), such as indoxacarb and spinosad (Balanza et al., 2021b; Biondi et al., 2012). An additional effective compound is the avermectin emamectin benzoate that was developed for the control of lepidopteran pests (Ishaaya et al., 2002). This compound is a macrocyclic lactone bioinsecticide extracted from the soil bacteria *Streptomyces avermitilis* which belongs to the group Actinomycetes. It causes permanent release of chloride ions into muscle cells, causing suppression of muscle contraction and eventually resulting in death (Ishaaya et al., 2002). This naturally-derived compound rapidly penetrates into the plant tissue and is photodegraded on the surface favouring its selectivity for natural enemies (Ishaaya et al., 2002). Consequently, aged residues of emamectin benzoate are harmless for most predators and parasitoids used as BCAs in vegetable crops in greenhouses (Amor et al., 2012; Bengochea et al., 2012). However, fresh residues or direct spray applications have a significant impact on key predators such as *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae) and *O. laevigatus* (Amor et al., 2012; Bengochea et al., 2012; Dader et al., 2020; van de Veire and Tirry, 2003).

Selection of strains of natural enemies for an enhanced tolerance to insecticides has been highlighted as a main strategy to enhance biological and chemical control compatibility in IPM programs, reinforcing their resilience (Bielza et al., 2020). In the framework of a program for the genetic improvement of *O. laevigatus* to improve key traits for its performance in agrosystems, such as better response when feeding on suboptimal food (Mendoza et al., 2021a) and body size (Mendoza et al., 2021b), selection for increased tolerance to a number of insecticides was carried out. Strains of *O. laevigatus* resistant to neonicotinoids (Balanza et al., 2019), pyrethroids (Balanza et al., 2021a) and spinosyns (Balanza et al., 2021b) have been recently reported. The artificial selection of a strain of *O. laevigatus* resistant to emamectin benzoate would favour their joint use, resulting in a more robust control of lepidopteran pests. In addition, the alternative use of emamectin benzoate will contribute to avoid resistance development to already selective compounds, as has occurred in *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) populations resistant to diamides due to their overuse (Roditakis et al., 2018).

Firstly, this work studied the variation in susceptibility to emamectin benzoate in wild and commercial populations of *O. laevigatus*. Secondly, from this genetic variability, we aimed at selecting a strain of *O. laevigatus* exhibiting tolerance to emamectin benzoate sufficient to minimize its impact in field conditions. In order to maintain genetic variation and benefit from eventual hybrid vigour, we concurrently selected four distinct strains of *O. laevigatus* for resistance to emamectin benzoate and characterized the resistance achieved in each strain. Finally, fitness of the selected strain was checked since selection for

insecticide resistance might involve negative trade-offs.

2. Materials and methods

2.1. Insects

Twenty nine populations of *O. laevigatus* were sampled manually in natural habitats of different Mediterranean countries between 2012 and 2019, trying to avoid areas where commercial populations are released (see Balanza et al., 2019, 2021a, 2021b for details). In addition, commercial populations were provided by the main bioproducers: Agrobio (Spain), Biobest (Belgium), and Koppert (The Netherlands). Insects populations were founded with at least 50 individuals, but normally with >100. Populations were reared in the laboratory by using 1-L plastic containers with lids into which a 6-cm diameter hole screened with filter paper provided ventilation, with ad libitum access to frozen *Ephestia kuehniella* eggs (hereafter *Ephestia* eggs) as food, pieces of green bean pods as moisture source and egg-laying substrate, and black wheat husk as hideout to avoid cannibalism. All these populations were maintained under controlled conditions at 26 ± 1 °C, 65 ± 5 % rh, and L16:D8 light regime. All populations were reproduced in the laboratory for 2–5 generations before bioassays, and maintained with a number of individuals over 1000.

2.2. Insecticides

A commercial formulation of emamectin benzoate (hereafter emamectin) was used (Affirm®, Syngenta Crop Protection). For cross-resistance bioassays, one insecticide of the same mode of action, abamectin (Vertimec®, Syngenta Crop Protection) was tested. The insecticide formulations were dissolved in distilled water plus Tween 80 as wetting agent (0.5%) to obtain different concentrations of the insecticide.

2.3. Bioassay

Dip-bioassays were used according to the method described in Balanza et al. (2019, 2021a, 2021b). Briefly, adults (or nymphs) (<24 h old) of *O. laevigatus* were bioassayed using four-five concentrations and a control (water plus the wetting agent). Three replicates of 10 individuals were used for each concentration. Bean pods were introduced into the insecticide solutions and agitated for 60 s, then air dried and put into the containers. *Ephestia* eggs ad libitum as source of food and buckwheat husk as refuge were added into the containers. Bioassays were maintained at 26 ± 1 °C, 65 ± 5 % R.H. and a 16:8 (L:D) h photoperiod. Mortality was assessed after 72 h. Individuals were considered dead if no movement was observed.

2.4. Selection for resistance

Four groups of 6–9 populations were separated trying to evenly distribute them by geographical origin in order to found four distinct selection lines (Table 1). Each population was selected with emamectin benzoate at 10 mg L^{-1} and the survivors were used as founders of the different resistant lines. The offspring of the survivors from each population within each group made up the first step for each of the four selection lines (hereafter REMA1, REMA2, REMA3 and REMA4).

Each initial line was successively exposed to increasing emamectin concentrations using the same bioassay methodology, maintaining the mortality around 70–90%. In each selection cycle, 700–1200 adults were bioassayed selecting a minimum of 100 individuals per selection line. The highest concentration used was 1000 mg L^{-1} , and 43, 47, 46 and 41 selection cycles were accomplished to obtain the selected strains REMA1, REMA2, REMA3 and REMA4, respectively.

Table 1
Susceptibility data to emamectin in wild and commercial populations of *O. laevigatus*.

Selection line	Population	Country/commercial	Slope (\pm SE)	LC ₅₀ mg L ⁻¹ (95% FL) ^a	RF ₅₀ ^b	MFR(%)	Class ^d
1	Perin	Spain	1.0 \pm 0.2	13.6 (6.2–24.3)	17.0	51.4	2
	Cabo de Palos	Spain	1.4 \pm 0.3	4.3 (1.8–7.1)	5.4	76.6	2
	Portonovo	Spain	1.3 \pm 0.3	3.5 (0.5–7.6)	4.4	79.6	2
	La Zenia	Spain	2.9 \pm 1.0	49.9 (20.1–76.4)	62.4	4.6	1
	Islantilla	Spain	1.4 \pm 0.3	3.5 (1.3–5.8)	4.4	79.6	2
2	Arroyo de San Servan	Spain	1.8 \pm 0.3	5.8 (2.9–9.1)	7.2	76.6	2
	Corrubedo	Spain	1.3 \pm 0.3	1.3 (0.2–2.7)	1.6	92.3	3
	Moreira	Spain	1.8 \pm 0.4	8.2 (4.1–13.1)	10.2	66.2	2
	Cabo de Gata	Spain	1.9 \pm 0.6	3.9 (0.6–7.0)	4.9	86.3	3
	Cuevas de Almanzora	Spain	3.4 \pm 1.0	32.8 (14.6–46.7)	41.0	11.7	1
	Hellin	Spain	0.7 \pm 0.2	0.8 (0.0–2.1)	1.0	77.6	2
	Acate	Italy	1.7 \pm 0.4	1.1 (0.2–2.0)	1.4	97.1	3
	Policoro	Italy	2.8 \pm 1.0	10.2 (2.6–15.2)	12.7	64.9	2
	Samaria	Greece	1.9 \pm 0.4	23.5 (11.8–36.3)	29.3	31.4	2
	Koppert	Commercial	1.0 \pm 0.2	1.5 (0.1–4.3)	1.8	83.5	3
3	Catadoiro	Spain	1.7 \pm 0.3	4.0 (2.0–6.1)	5.0	82.4	3
	Cazorla	Spain	1.5 \pm 0.3	5.9 (2.6–9.7)	7.4	71.7	2
	Carmona	Spain	1.0 \pm 0.2	10.1 (4.2–18.0)	12.6	54.6	2
	Cabrils	Spain	1.9 \pm 0.4	5.2 (2.1–8.3)	0.2	80.6	3
	Teruel	Spain	2.2 \pm 0.4	6.0 (3.4–8.8)	7.5	79.0	2
	Logroño	Spain	1.0 \pm 0.2	6.5 (2.0–12.1)	8.1	61.6	2
	Mentrida	Spain	2.0 \pm 0.4	5.9 (3.0–8.8)	7.4	78.3	2
	Ruidera	Spain	1.5 \pm 0.3	4.6 (2.1–7.2)	5.7	76.4	2
	Biobest	Commercial	1.4 \pm 0.3	5.7 (2.2–9.6)	7.1	72.0	2
	Puerto de la Mora	Spain	3.7 \pm 0.9	5.7 (3.3–7.8)	7.1	93.1	3
4	Alcobendas	Spain	1.8 \pm 0.5	3.1 (0.9–4.8)	3.9	88.4	3
	Guadalupe	Spain	1.3 \pm 0.3	6.7 (2.0–12.7)	8.4	65.1	2
	Merida	Spain	1.3 \pm 0.3	5.4 (2.1–9.3)	6.7	69.5	2
	Cefalu	Italy	1.2 \pm 0.2	3.2 (1.0–5.6)	4.0	78.6	2
	Palermo	Italy	1.5 \pm 0.3	5.5 (2.2–9.1)	6.9	72.7	2
	Rethymno	Greece	1.9 \pm 0.3	4.8 (2.5–7.4)	6.0	81.7	3
	Agrobio	Commercial	0.8 \pm 0.1	4.7 (1.3–19.9)	5.8	66.0	2
	Baseline			1.2 \pm 0.1	4.8 (3.8–5.8)	5.9	72.2

a Concentration of insecticide killing 50% of individuals and its 95% fiducial limits.

b Resistant factor = LC₅₀ of each population/LC₅₀ of susceptible reference population.

c Mortality at the maximum field rate (15 mg L⁻¹).

d Toxicity classes: class 1 (harmless): effect < 30%; class 2 (slightly harmful): 30–79 % effect; class 3 (moderately harmful): 80–99% effect; class 4 (harmful): effect > 99%. (Sterk et al., 1999).

2.5. Characterization of resistance

The level of resistance in the different life stages, resistance stability, cross-resistance pattern and involvement of metabolic resistance mechanisms were characterized in the new selected strains. Emamectin resistance in the immature instars of the selected strains were compared to that in the commercial population Agrobio, used as reference population. In addition, stability of emamectin resistance was assessed after 18 generations without exposure to the insecticide.

Cross-resistance to other registered avermectin (abamectin) was assayed in both the resistant strains and the reference population.

The role of metabolic resistance was studied testing the addition of the inhibitors of detoxification enzymes PBO (piperonyl butoxide), DEF (S,S,S-tributyl phosphotriothioate), and DEM (diethyl maleate). Bioassays with synergists were similar to those with insecticides alone, except that first bean pods were dipped into synergist solutions for 60 s, and the individuals were exposed for 24 h. Then, those individuals were recovered and a bioassay with the insecticide solutions was carried out. Synergist concentrations were 100 mg·L⁻¹ for DEF, 300 mg·L⁻¹ for PBO and 1000 mg·L⁻¹ for DEM. These were the maximum concentrations of the synergists that could be used without any deleterious effects on the adults of the susceptible strain.

2.6. Resistance costs

In order to test reproductive fitness of the resistant strains, fecundity was studied in the strain showing the highest resistance level, REMA4,

without and under exposure to the maximum recommended field rate of emamectin (15 mg·L⁻¹) (sublethal effect) compared with the commercial reference population Agrobio. Freshly emerged adults (<24 h old) from each population were collected and introduced in a rearing container. In the case of testing sublethal effects, the bean pods were previously treated with 15 mg·L⁻¹ as in the bioassays abovementioned. After 4 days during which mating and pre-oviposition period took place, surviving adults were sexed and females isolated to test fecundity and longevity. Forty females per treatment were placed individually into small polypropylene cups (45 mL) with ventilated lids with a piece of green bean pod end-sealed with paraffin wax as an egg-laying substrate, and *Ephestia* eggs as food. Eggs counts were carried out every 2–3 days, switching females to a clean piece of bean pod and adding fresh food. Fecundity was assessed until females' death.

In addition, predation capacity on their natural prey *F. occidentalis* was compared between the REMA4 strain and the commercial reference strain Agrobio. Either 2nd-instar larva or adult individuals were aspirated, with the aid of a slight vacuum, into 5 mL-plastic vials with a rectangular section (30 × 5 mm) of sweet pepper leaf inside and covered by a carefully punctured lid. In the case of 2nd-instar thrips, densities of 50 individuals were assessed, as well as a negative control. For adults, densities of 30 individuals were provided and a control was also evaluated. After 24-hour contact between adult *O. laevigatus* and the prey and 24-hour fasting, one single *Orius* female was transferred to each plastic vial. There were ten replicates per population and prey life instar. After 24 h, the predators were removed from experimental arenas and the number of killed prey was recorded.

2.7. Data analysis

Data were corrected for mortality observed in the control (Abbott, 1925). Data were analysed using the program POLO-PC for Probit analysis (Russell et al., 1977). To compare any two LC₅₀ values, the LC₅₀ ratio and the 95% confidence limits for this ratio were calculated using the program POLO-PC (Russell et al. 1977). If the 95% confidence interval includes 1, then the LCs are not significantly different (Robertson et al. 2007). In addition, mortality at the maximum field rate (MFR) (15 mg L⁻¹) was calculated using the dose-mortality relationship obtained for each population and insecticide.

Mortality was categorized by the toxicity classes developed for the “worst case” initial contact toxicity laboratory test: class 1 (harmless): effect < 30%; class 2 (slightly harmful): 30–79 % effect; class 3 (moderately harmful): 80–99% effect; class 4 (harmful): effect > 99% (Sterk et al., 1999).

Differences in fecundity and longevity between the selected and the reference populations were analysed by a two-way ANOVA test, with population and insecticide treatment as factors. Females who didn't lay eggs were excluded from the analysis. Predation capacity was analysed by a two-way ANOVA test, with population and prey instar as factors. Assumptions of normality (Shapiro–Wilk test) and homogeneity of variances (Levene test) were checked prior analysis. When significant differences between populations were observed, means were separated using Tukey's HSD test.

3. Results

3.1. Variation among populations

Toxicity data of wild and commercial populations of *O. laevigatus* from Spain, Italy and Greece resulted in a 62.4-fold variation in response, with a minimum LC₅₀ value of 0.8 mg L⁻¹ (Hellin) and a maximum of 49.9 mg L⁻¹ (La Zenia), with significant differences between them (Table 1). The LC₅₀ value for the baseline was 4.8 mg L⁻¹, slightly lower than the maximum field rate (MFR) of this compound (15 mg L⁻¹). Mortality at the MFR was variable between populations, reaching a maximum value of 97.1%, being toxicity classes 2 and 3 (slightly and moderately harmful, respectively) for all the populations, except for La Zenia and Cuevas de Almazora, in which class 1 (harmless) was observed. The mortality at the MFR in the commercial populations ranged from 66.0 to 83.5% (classes 2 to 3). These results show that there is variability among populations, emamectin being mostly slightly to moderately harmful to *O. laevigatus*.

3.2. Selection for resistance

Selection considerably improved the resistance to emamectin (LC₅₀ = 104–203 mg L⁻¹) in adults of the selected lines REMA1, REMA2, REMA3 and REMA4 to 22-, 37-, 25- and 43-fold, respectively, compared with the reference population (LC₅₀ = 4.7 mg L⁻¹) (Table 2). Although the LC₅₀ values did not differ significantly among the selected lines, the value for REMA4 (203 mg L⁻¹) almost doubled that for REMA1 (104 mg L⁻¹) (Table 2).

3.3. Resistance levels and their characterization

For the Agrobio reference population there were no significant differences in susceptibility to emamectin among the different instars (LC₅₀ 1.2–4.7 mg L⁻¹), only N1 showing increased susceptibility (LC₅₀ = 0.1 mg L⁻¹) (Table 2). The mortalities at the MFR were moderate from N3 to adults, rating in class 2 (slightly harmful), and very high for youngest instars N1 and N2, rating 3 (moderately harmful).

The selected strains REMA 1 to 4 showed higher resistance to emamectin in all life instars than the reference population (Table 2). For all the resistant strains, the two first nymphal instars (N1 and N2) were

Table 2

Emamectin benzoate susceptibility data in adults and nymphal instars of resistant and commercial populations.

Population	Instar	Slope (±SE)	LC ₅₀ mg L ⁻¹ (95% FL) ^a	LC ₅₀ ratio ^b	MFR (%) ^c	Class ^d
Agrobio	Nymph 1	0.4 ± 0.1	0.1 (0.0–0.2)	1.0	84.9	3
	Nymph 2	1.7 ± 0.3	1.2 (0.7–2.4)	1.0	97.0	3
	Nymph 3	0.8 ± 0.1	2.7 (1.2–4.2)	1.0	72.2	2
	Nymph 4	0.8 ± 0.1	2.9 (0.9–5.9)	1.0	74.4	2
	Nymph 5	0.7 ± 0.1	3.8 (1.4–6.9)	1.0	66.0	2
	Adults	0.8 ± 0.1	4.7 (2.3–7.8)	1.0	65.8	2
REMA1 (43 cycles)	Nymph 1	0.7 ± 0.2	5.4 (2.1–9.5)	68*	63.2	2
	Nymph 2	0.5 ± 0.1	11.6 (5.8–16.7)	9*	52.4	2
	Nymph 3	0.7 ± 0.2	20.8 (14.1–33.5)	7*	45.6	2
	Nymph 4	0.8 ± 0.2	40.6 (21.0–65.9)	13*	36.1	2
	Nymph 5	0.9 ± 0.2	102.7 (61.7–148.6)	26*	21.8	1
	Adults	0.7 ± 0.2	104.3 (54.5–147.5)	22*	26.2	1
REMA2 (47 cycles)	Nymph 1	0.7 ± 0.2	3.9 (1.8–7.3)	50*	65.8	2
	Nymph 2	0.6 ± 0.1	25.0 (14.2–40.7)	19*	44.6	2
	Nymph 3	0.6 ± 0.1	61.0 (34.3–87.1)	22*	34.2	2
	Nymph 4	0.8 ± 0.2	89.1 (53.5–142.6)	30*	24.6	1
	Nymph 5	0.7 ± 0.2	129.9 (81.2–223.0)	33*	23.5	1
	Adults	1.3 ± 0.3	176.7 (98.5–292.7)	37*	7.1	1
REMA3 (46 cycles)	Nymph 1	0.8 ± 0.2	3.6 (2.1–7.8)	45*	69.8	2
	Nymph 2	0.7 ± 0.1	20.6 (14.7–39.5)	16*	45.9	2
	Nymph 3	0.6 ± 0.1	59.4 (25.12–85.0)	22*	13.5	1
	Nymph 4	0.8 ± 0.2	64.58 (20.3–98.8)	21*	29.4	1
	Nymph 5	0.8 ± 0.2	89.9 (60.9–158.7)	22*	27.0	1
	Adults	0.9 ± 0.2	116.7 (78.8–236.6)	24*	19.3	1
REMA4 (41 cycles)	Nymph 1	0.6 ± 0.1	3.0 (1.3–6.8)	38*	67.9	2
	Nymph 2	0.5 ± 0.1	18.5 (8.8–42.8)	14*	48.1	2
	Nymph 3	0.7 ± 0.2	21.8 (14.3–39.7)	8*	45.1	2
	Nymph 4	0.9 ± 0.2	65.3 (18.1–108.2)	22*	28.4	1
	Nymph 5	0.8 ± 0.2	102.9 (73.1–145.5)	26*	23.3	1
	Adults	1.3 ± 0.4	202.8 (145.2–303.7)	42*	6.9	1

a Concentration of insecticide killing 50% of individuals and its 95% fiducial limits.

b LC₅₀ of each population/LC₅₀ of susceptible reference population. *: significant 95%.

c Mortality at the maximum field rate (15 mg L⁻¹).

d Toxicity classes: class 1 (harmless): effect < 30%; class 2 (slightly harmful): 30–79 % effect; class 3 (moderately harmful): 80–99% effect; class 4 (harmful): effect > 99%. (Sterk et al., 1999).

significantly less tolerant to emamectin ($LC_{50} = 3\text{--}25 \text{ mg L}^{-1}$) than the last instar (N5) and adult stages that were the most resistant ones ($LC_{50} = 90\text{--}203 \text{ mg L}^{-1}$). The mortalities at the MFR were low for the last instars, 7 to 27 % in N5 and adult, with toxicity classes of 1 (harmless).

No cross-resistance to abamectin was observed in the emamectin-resistant strains REMA1 to 4 (Table 3). After rearing without selection pressure for 18 generations, the selected strains exhibited similar resistance levels ($LC_{50} = 86\text{--}170 \text{ mg L}^{-1}$), being 18–36 times more resistant than the commercial reference population and retaining a class 1 toxicity, with 1.4–25% mortality at the MFR (Table 4). The addition of the synergists did not have a significant impact in the tolerance to emamectin in the resistant strain tested (Table 5).

3.4. Resistance costs

There was no interaction between the factors population and insecticide treatment, both for fecundity ($F = 0.67$, $df = 1/128$, $P > 0.05$) and longevity ($F = 0.00$, $df = 1/128$, $P > 0.05$). Life-time fecundity was similar in the selected and reference population (Table 6). However, the females of the emamectin-resistant population lived longer than those of the reference population (Table 6). No reduction in fecundity nor longevity were detected due to sublethal effects when the females were exposed to the maximum field rate (15 ppm) of emamectin benzoate (Table 6).

Similarly, there was no difference between the selected and the reference populations in predation rate, but there was between prey instars, killing significantly more larvae (38 larvae) than adults (18 adults) (Table 7). No interaction between population and prey instar was detected ($F = 0.42$, $df = 1/36$, $P > 0.05$).

4. Discussion

The compatibility of synthetic and natural insecticides with predators and parasitoids is critical for successful IPM programmes (Bielza et al., 2009). However, emamectin has been reported as highly toxic for many natural enemies, including *O. laevigatus*. For instance, this insecticide produced > 90% mortality when *O. laevigatus* was exposed 3 days to tomato plants with 1-h old or 7-d old pesticide residues (Biondi et al., 2012), classifying emamectin as moderately harmful (class 3) when using IOBC toxicity categories. Therefore, the joint use of avermectin-based biopesticides, abamectin and emamectin, and predators such as *O. laevigatus* was not recommended for pest management programs (Biondi et al., 2012). Emamectin was harmful up to 21 days after application in direct spray on a sweet pepper crop, killing 100% of the

Table 3

Cross resistance with abamectin in the commercial population (Agrobio) and the emamectin benzoate-resistant strains REMA1 to 4 of *Orius laevigatus*.

Insecticide	Population	Slope (\pm SE)	$LC_{50} \text{ mg L}^{-1}$ (95% FL) ^a	LC_{50} ratio ^b	Class ^c
Abamectin	Agrobio	0.3 \pm 0.1	0.036 (0.005–0.098)	1.0	3
	REMA1	3.6 \pm 0.1	0.075 (0.024–0.166)	2.1 ns	3
REMA2	REMA2	0.3 \pm 0.1	0.047 (0.011–0.142)	1.3 ns	3
	REMA3	0.3 \pm 0.1	0.199 (0.087–0.292)	5.5 ns	2
REMA4	REMA4	0.8 \pm 0.2	0.143 (0.072–0.172)	4.0 ns	3

a Concentration of insecticide killing 50% of individuals and its 95% fiducial limits.

b LC_{50} of each population/ LC_{50} of susceptible reference population. ns: no significant.

c Toxicity classes: class 1 (harmless): effect < 30%; class 2 (slightly harmful): 30–79 % effect; class 3 (moderately harmful): 80–99% effect; class 4 (harmful): effect > 99%. (Sterk et al., 1999).

Table 4

Susceptibility data of emamectin benzoate in the susceptible (Agrobio) and resistant strains of *Orius laevigatus* after 18 generations without insecticide pressure.

Population	Generation ^a	Slope (\pm SE)	$LC_{50} \text{ mg L}^{-1}$ (95% FL) ^b	LC_{50} ratio ^c	Class ^d
Agrobio	–	0.8 \pm 0.1	4.7 (1.3–13.9)	1.0	2
REMA1	43	0.7 \pm 0.2	104.3 (64.5–207.5)	22.1*	1
	61	0.9 \pm 0.2	85.8 (39.67–152.42)	18.1*	1
REMA2	47	1.3 \pm 0.3	176.7 (98.5–232.7)	37.3*	1
	65	2.0 \pm 0.5	169.9 (92.1–220.5)	35.8*	1
REMA3	46	0.9 \pm 0.2	116.7 (68.9–206.7)	24.6*	1
	64	1.5 \pm 0.4	106.7 (41.8–186.2)	22.5*	1
REMA4	41	1.3 \pm 0.3	202.8 (115.2–383.7)	42.8*	1
	59	1.1 \pm 0.2	132.3 (50.2–178.1)	27.9*	1

a Generations without insecticide application from the selected population.

b Concentration of insecticide killing 50% of individuals and its 95% fiducial limits.

c LC_{50} of each population/ LC_{50} of susceptible reference population. *: $p < 0.05$.

d Toxicity classes: class 1 (harmless): effect < 30%; class 2 (slightly harmful): 30–79 % effect; class 3 (moderately harmful): 80–99% effect; class 4 (harmful): effect > 99%. (Sterk et al., 1999).

Table 5

Susceptibility data for emamectin benzoate alone and in mixture with inhibitors of detoxification enzymes in adults of the selected strain REMA3 of *Orius laevigatus*.

Population	Treatment	Conc (mg L ⁻¹) ^a	Slope (\pm SE)	$LC_{50} \text{ mg L}^{-1}$ (95% FL) ^b	LC_{50} ratio ^c
REMA3	Emamectin	–	1.4 \pm 0.2	98.4 (38.3–184.2)	1
	+ PBO	300	1.2 \pm 0.2	94.5 (39.4–171.4)	0.9 ns
	+ DEF	100	1.0 \pm 0.3	70.7 (16.6–155.0)	0.7 ns
	+ DEM	1000	1.3 \pm 0.2	83.4 (35.7–148.4)	0.8 ns

a Synergist concentration applied.

b Concentration of insecticide killing 50% of individuals and its 95% fiducial limits.

c LC_{50} with the synergist/ LC_{50} with the insecticide alone. ns: no significant.

individuals of *O. laevigatus* (Amor et al., 2012). However, it was classified as slightly harmful (class 2) to *O. laevigatus* in field trials on sweet pepper when exposed to residues on plants after spray applications (Amor et al., 2012; Dader et al., 2020), which is in agreement with our results.

For another *Orius* species, *O. insidiosus*, Studebaker and Kring (2003) reported that fresh residues of emamectin in laboratory and extended laboratory tests were moderately harmful to harmful for nymphs and adults. Also for a different *Orius* species, *O. albidipennis*, it was found that direct application of emamectin was harmful for nymphs and adults of the predator (Atwa et al., 2017).

Like most works on side-effects of insecticides on biological control agents, these studies on the effect of emamectin on *Orius* species were carried out using a single population, no considering the variation in

Table 6

Lifetime fecundity and longevity (mean \pm SE) of adult females of *Orius laevigatus* of the commercial population (Agrobio) and the emamectin-resistant strain (REMA4) non-exposed and exposed to the maximum field rate of emamectin benzoate (15 ppm).

Population	Insecticide treatment	Fecundity		Longevity			
Agrobio	Control	116.18 \pm 13.60	A	a	25.50 \pm 3.11	B	a
	Emamectin benzoate	100.19 \pm 9.25		a	24.00 \pm 2.04		a
REMA4	Control	111.62 \pm 9.08	A	a	32.72 \pm 3.35	A	a
	Emamectin benzoate	111.50 \pm 7.32		a	32.79 \pm 2.88		a

Values followed by different uppercase letters between populations for fecundity ($F = 0.12$, $df = 1/128$, $P > 0.05$) and longevity ($F = 8.85$, $df = 1/128$, $P < 0.01$) and lowercase letters within populations for fecundity ($F = 0.69$, $df = 1/128$, $P > 0.05$) and longevity ($F = 0.28$, $df = 1/128$, $P > 0.05$) are significantly different.

Table 7

Larval and adult *Frankliniella occidentalis* (mean \pm SE) killed by an adult female of *Orius laevigatus* in 24 h when offered in excess by the commercial population (Agrobio) and the emamectin-resistant strain (REMA4).

Predator Population	Prey					
	Instar	Offered	Number killed	% Killed		
Agrobio	Larva	50	38.10 \pm 1.53	A	a	76.20 \pm 3.06
	Adult	30	18.70 \pm 1.29		b	62.33 \pm 4.30
REMA4	Larva	50	38.30 \pm 1.57	A	a	76.60 \pm 3.14
	Adult	30	17.10 \pm 1.08		b	57.00 \pm 3.60

Values followed by different uppercase letters between populations ($F = 0.26$, $df = 1/36$, $P > 0.05$) and lowercase letters within populations ($F = 215$, $df = 1/36$, $P < 0.001$) are significantly different.

susceptibility among populations within a species (Balanza et al. 2019, 2021a, 2021b). In this study, a significant interspecific variability (62-fold) in emamectin susceptibility was observed in wild populations of *O. laevigatus*. Emamectin was found moderately harmful for 9 populations, slightly harmful for 21 populations, but harmless for a couple of populations at the MFR. Regarding commercial populations, widely used for most toxicological studies, emamectin was classified as slightly harmful for two populations but moderately harmful for another. Similarly, other studies also reported a significant variation in susceptibility to neonicotinoids (Balanza et al., 2019), pyrethroids (Balanza et al., 2021a) and spinosyns (Balanza et al., 2021b) in wild and commercial populations of *O. laevigatus*. These findings underline the importance of using several populations of different origin of a species of natural enemy when assessing the susceptibility to a pesticide (Luna et al., 2018, Balanza et al., 2019, 2021a, 2021b).

One of the main challenges for an effective biological control is the selective use of pesticides. Therefore, availability of BCAs showing compatibility with synthetic pesticides and biopesticides will foster a wider use of biological control in spite of corrective insecticide applications to manage main or secondary pests (Bielza, 2016; Bielza et al., 2020). In fact, pesticide resistance is one of the first traits suggested for genetic improvement in natural enemies (Hoy, 1986) since the effect of this selection is readily observed in the field by biocontrol practitioners (Bielza et al., 2020). Indeed, in a recent review the authors observed that most selection studies in BCA select for insecticide resistance (Lirakis and Magalhães, 2019). Already in the 80 s the phytoseiid mite *Metaseiulus occidentalis* was artificially selected for insecticide resistance and introduced in orchards (Hoy, 1986). Recently, different strains of *O. laevigatus* resistant to neonicotinoids, pyrethroids and spinosyns have been also artificially selected using the natural variation in wild populations never exposed to pesticides (Balanza et al., 2019, 2021a,

2021b).

In the present work, four strains of *O. laevigatus* were successfully selected for resistance to emamectin, mainly used to control caterpillars. In the predator *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), resistance to emamectin has also been further selected from field populations already showing resistance (Mansoor et al., 2013). Insecticide resistance have been repeatedly reported in lacewings when exposed to selection pressure in the field (Bielza, 2016). By contrast, similarly to the cases of neonicotinoid, pyrethroid and spinosyn resistance (Balanza et al., 2019, 2021a, 2021b), our emamectin-resistant strains were obtained exploiting the variation found in natural populations never exposed to insecticides. Therefore, these findings suggest the presence of genetic background and variability in *O. laevigatus* populations that can result in resistance evolution when subjected to selection pressure. Thus, our results support the hypothesis that the absence of resistance cases in field populations of some predators is not due to lack of genetic potential or enough variability but to lack of continuity of selection pressure over sufficient generations because of the release every season of susceptible predators from mass-rearing (Balanza et al., 2021a).

In greenhouse vegetable crops, emamectin is mainly used to control lepidopteran pests. These pests are generally kept under economic thresholds using mating disruption and semiochemical baited traps. However, outbreaks are controlled by insecticidal treatments. The diamide chlorantraniliprole is the main insecticide used since it is selective with BCAs such as *O. laevigatus* (Biondi et al., 2012). Spinosad is also applied to control lepidopteran pests but is slightly toxic to *O. laevigatus* impacting predator populations (Biondi et al., 2012; Balanza et al., 2021b). Likewise, emamectin has a strong effect on *Orius* populations. As a result, growers tend to use the diamide rather than spinosad or emamectin, not following a critical resistance management recommendation such as the alternation of mode of actions. This overuse may result in the evolution of insecticide resistance as has been reported in several lepidopterans (Guedes et al., 2019; Haddi et al., 2017; Roditakis et al., 2018). The use of our insecticide-resistant strains will permit the rotation of different compounds without a significant effect in *O. laevigatus* biocontrol action. On the other hand, the global impact of a pesticide on the performance of a natural enemy depends on the effect upon the life stage instars due to differences in tolerance to insecticides (Balanza et al., 2021b). In both our reference and resistant strains, a progressive increase in tolerance to emamectin was evident from first nymphal instar to adult. The same pattern was found in *O. albidipennis* when tested with emamectin (Atwa et al., 2017). This trend was also observed for susceptible and pyrethroid- and spinosyn-resistant strains of *O. laevigatus* (Balanza et al., 2021a, 2021b).

Differential expression of insecticide resistance among developmental stages is well known in crop pests, being the imago the most resistant in some cases (Nauen et al., 2008) and the juvenile instars in others (Contreras et al., 2010). The progressive increase of body size throughout the life instars may have a significant effect, but this age-specific expression of tolerance among developmental stages has also been attributed to differential action of detoxification enzymes and variation in cuticle permeability (Van de Veire et al., 2002).

Moreover, the resistance to emamectin was retained in the REMA3 strain for at least 18 generations without insecticide exposure. Therefore, the selected trait will be maintained over enough time to warrant the level of tolerance to emamectin during a whole crop cycle (4–8 months), even if the selected strain is introduced soon after transplanting. Stability of resistance has also been found in *O. laevigatus* to spinosad (Balanza et al., 2021b) and other insect species, like the thrips *F. occidentalis* (Bielza et al., 2008).

Surprisingly, the resistant strains, selected for resistance to emamectin, exhibited no cross-resistance to abamectin, within the same mode of action. This result would suggest that the resistance mechanism is rather metabolic than target site mediated. Cross-resistance between emamectin and a pyrethroid was reported in a pyrethroid-resistant

strain of *O. laevigatus* (Balanza et al., 2021a). However, none of the inhibitors of detoxification enzymes was able to restore susceptibility to emamectin in the resistant strain REMA3.

Selection for insecticide resistance may lead to detrimental changes in other important traits for biocontrol efficacy, such as reproduction, life span and predatory rate. However, no trade-offs in these traits were observed in the emamectin-resistant strain. Not only did the artificially selected strain exhibit similar fecundity and predation rate to that of the commercial population, but also a superior longevity. Moreover, *O. laevigatus* strains genetically improved for other traits (body size, better fitness feeding on pollen) didn't show any reduction in reproductive fitness either (Mendoza et al., 2021a, 2021b), and even presented enhanced predation rate (Mendoza et al., 2022).

Most studies on trade-offs of insecticide resistant strains of insects have reported reduced fitness compared to susceptible ones in the absence of selective pressure (see Freeman et al., 2021 for a recent review). However, no detrimental effects on fitness or even superior performance have been observed in some cases, both for insect pests and natural enemies (Bielza et al., 2008; Mendoza et al., 2021a, 2021b). The common green lacewing *C. carnea* is an important predator used to control various insect pests. Different strains of this predator selected for resistance to pyrethroids, organophosphates and spinosyns have been found to show increased fitness and predation rate (Khan Pathan et al., 2010; Abbas et al., 2014). Moreover, a strain of this predator artificially selected for resistance to emamectin exhibited superior reproductive traits, including higher development rate, hatchability and fecundity (Mansoor et al., 2013). Similarly to our study, fitness attributes not related to resistance may be selected in the resistant strains throughout the selective breeding, resulting in the integration of resistance and fitness factors. Nevertheless, there might be trade-offs for other traits important for the overall biocontrol function of the *O. laevigatus* strains, therefore further trials under real field conditions are needed.

5. Conclusions

The continuous presence of BCA in crops is considered key for biological control success (Bielza et al., 2020). Therefore, omnivorous predators, such as *O. laevigatus*, are released in crops shortly after transplanting for them to be able to build up a sufficient population before pest appearance. But at the same time, this early introduction and continuous presence render these predators particularly exposed to pesticide applications. This is especially serious when the pesticide sprayed is highly toxic to BCAs, such as emamectin. This insecticide is very useful to manage severe pests like caterpillars when other non-chemical control measures are not effective enough. The achievement of strains of *O. laevigatus* resistant to emamectin will allow the joint use of this valuable compound with this key predator for augmentative biological control in greenhouse vegetable crops.

Moreover, as far as we know, this is the first time that different strains of the same species of a natural enemy have been selected for resistance to the same insecticide. Rearing separately these strains and crossing them before industrial scaling up in biofactories will allow to maintain genetic variation, avoid inbreeding and benefit from eventual hybrid vigour.

CRedit authorship contribution statement

Virginia Balanza: Methodology, Formal analysis. **Estefanía Villafra:** Formal analysis, Investigation. **José Enrique Mendoza:** Methodology, Investigation. **Carolina Grávalos:** Methodology, Investigation. **Amador Rodríguez-Gómez:** Investigation, Data curation. **Dina Cifuentes:** Formal analysis, Data curation. **Pablo Bielza:** Conceptualization, Formal analysis, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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