

# Occurrence of pyrethroid resistance mutation in *Cydia pomonella* (Lepidoptera: Tortricidae) throughout Argentina

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## Research Paper

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## Abstract

Pyrethroid insecticides were intensively used against *Cydia pomonella* in the Río Negro and Neuquén valley, main production area of pome fruits in Argentina. Therefore, the first objective was to evaluate lambda-cyhalothrin resistance levels in *C. pomonella* larvae from orchards in this area that are currently under pyrethroids treatments. The second objective was to evaluate the frequency of *kdr* mutation in *C. pomonella* across Argentina. High levels of resistance to lambda-cyhalothrin (resistance ratios > 30) were determined in all the populations evaluated. The L1014F (*kdr*) mutation was evaluated in 355 diapausing larvae collected in 12 orchards from San Juan to Santa Cruz provinces (1690 km away from each other). The highest frequency of *kdr* mutation was determined in larvae from the Río Negro and Neuquén valley (0.61), followed by those from Mendoza (0.36). The *kdr* allele was absent or present at very low frequencies in orchards subjected to low pyrethroid pressure. The frequency of detection of *kdr* mutation in *C. pomonella* from Argentina is related to the use of pyrethroids against this pest in different areas. Target-site insensitivity is, at least, one of the mechanisms involved in resistance to lambda-cyhalothrin in codling moth from the Río Negro and Neuquén valley.

## Introduction

Pyrethroids are a major class of synthetic insecticides which are still used to control agricultural pests (Joseph *et al.*, 2017; Parys *et al.*, 2018) and disease vectors (Smith *et al.*, 2016). Pyrethroids are fast acting insecticides, with high and low insect and mammalian toxicity, respectively (Dong *et al.*, 2014). At a cellular level, pyrethroids disrupt nerve function, causing repetitive discharges, membrane depolarization, and synaptic disturbances (Soderlund 2012; Dong *et al.*, 2014). The primary target site of pyrethroids is the voltage-gated sodium channel that underlies the generation of nerve action potential (Soderlund 2012). The decrease in the sodium channel sensitivity, known as knock-down resistance (*kdr*) mutation, confers resistance to DDT and pyrethroids. Since its initial report in the house fly (Milani, 1956), *kdr* and *kdr*-like resistance have been documented globally in almost all agriculturally important arthropod pests and disease vectors (Soderlund, 2005, 2012; Rinkevich *et al.*, 2013). Study of the mechanism of *kdr* in the past two decades led to the identification of more than 50 sodium channel mutations or combinations of mutations that were associated with pyrethroid resistance in arthropod species (Haddi *et al.*, 2012, 2017; Sierra *et al.*, 2016). In the codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae), the *kdr* mutation reported so far is a single nucleotide polymorphism which results in a substitution of leucine for phenylalanine at position 1014 (L1014F) (Franck *et al.*, 2012).

Codling moth is a severe pest of pome fruits worldwide (Grigg-McGuffin *et al.*, 2015) and the fruit damage is caused by the larval stage. Neonates excavate tunnels and feed on the seed. Infested fruit lose their shape and fall prematurely (Danelski *et al.*, 2017; Husain *et al.*, 2018). Effective *C. pomonella* management mainly rely on chemical insecticides which has led to the development of insecticide resistance to most classes of insecticides (Sauphanor and Bouvier, 1995; Knight *et al.*, 2001; Reyes *et al.*, 2009; Rodriguez *et al.*, 2011; Voudouris *et al.*, 2011; Cichón *et al.*, 2013). Insecticide-resistance in codling moth field populations has been related mainly to increased enzymatic metabolism and target-site mutations (Reyes *et al.*, 2007, 2011; Voudouris *et al.*, 2011), including the sodium channel mutation (Reyes *et al.*, 2009; Franck *et al.*, 2012; Bosch *et al.*, 2018).

Codling moth is widely distributed in Argentina where climate conditions are favorable to apple and pear tree development (commercial and wild varieties). The region of Río Negro

**Table 1.** Orchards names and locations.

Province	Locality	Population code	Longitude	Latitude
San Juan	San Juan	SJN	69°25'18"W	31°27'29"S
Mendoza	Mendoza	MZA	68°49'0"W	32°52'60"S
Rio Negro	Cinco Saltos	CSA	68°4'0"W	38°49'0"S
Rio Negro	Guerrico	GUE	67°43'60"W	39°1'0"S
Rio Negro	General Roca	GRC	67°31'60"W	39°2'60"S
Rio Negro	Villa Regina	VRG1	67°04'45.84"W	39°09'41.09"S
Rio Negro	Villa Regina	VRG2	67°03'03.86"W	39°02'42.91"S
Rio Negro	Villa Regina	VRG3	67°10'08.72"W	39°05'50.79"S
Neuquen	San Martin de los Andes	SMA	71°21'15.1"W	40°9'18.32"S
Chubut	Trelew	TRW	65°18'18"W	43°14'56"S
Chubut	Sarmiento	SAR	69°1'21.62"W	45°36'2.21"S
Santa Cruz	Los Antiguos	ANT	71°37'0"W	46°32'60"S

and Neuquén valley, located in northern Patagonia (Argentina), contributes with almost 85% of the country pome-fruit production (especially Río Negro province with 78%), followed by the Uco (Mendoza province) and Calingasta (San Juan province) valleys, among others [https://inta.gob.ar/sites/default/files/script-tmp-inta\\_programa\\_nacional\\_frutales\\_cadena\\_frutales\\_de\\_pe.pdf](https://inta.gob.ar/sites/default/files/script-tmp-inta_programa_nacional_frutales_cadena_frutales_de_pe.pdf). Pyrethroids have been used to control *C. pomonella* in the USA (Mota-Sanchez *et al.*, 2008), France (Bouvier *et al.*, 1998), Greece (Voudouris *et al.*, 2011), Chile (Fuentes-Contreras *et al.*, 2008), etc. In the Río Negro and Neuquén valley, pyrethroids were introduced in 1982 and have been used for almost 20 years against *C. pomonella*. Late 90s, many farmers reported control failures to pyrethroid applications, and its use was discontinued (Soleño *et al.*, 2008). Fruit production area from Mendoza and San Juan provinces has a similar application history but because the pome area is smaller the pesticide pressure is lower. On the other hand, although there are other provinces with pome production, these are of much smaller area and their production is for domestic or family market.

Reyes *et al.* (2009) reported the *kdr* mutation in codling moth obtained in 2005 from two orchards in Argentina. However, these orchards were not georeferenced by the authors and no toxicological data were provided.

Recently, the economic crisis of small farmers has led again to the use of lambda- cyhalothrin in *C. pomonella* control. Therefore, the first objective of the present study was to evaluate the levels of pyrethroid resistance in *C. pomonella* larvae from three orchards that are currently under pyrethroid treatments. The second objective was to evaluate the frequency of *kdr* mutation in *C. pomonella* from the Río Negro and Neuquén valley as well as from other Argentinean areas of pome fruit production.

## Material and methods

### Study area and insect collection

Field populations of diapausing larvae (FCL) were collected in orchards from 31°27'29" N to 46°32'60" S latitudes (1690 km away from each other) (table 1) in 2012. Diapausing larvae were collected from 12 orchards using corrugated cardboard attached to the main branches and trunk trees. Larvae were then

transferred to clean corrugated papers and stored at 4° ± 1°C with 12:12 h L:D regime for three months in order to satisfy chilling requirements (Soleño *et al.*, 2012). The laboratory susceptible strain (LSS) was obtained from INTA Alto Valle (Argentina), which has been reared in the laboratory since 1991 without exposure to any insecticide. From the 12 orchards, FCL for bioassays were from Cinco Saltos (CSA), Guerrico (GUE), and Villa Regina (VRG3) orchards, where pyrethroids are currently used. Both LSS and FCL were transferred to post chilling conditions (25°C, 70% relative humidity and 16:8 L:D) for 48 h before bioassays were performed (Soleño *et al.*, 2008).

### Bioassays

Topical concentration–response assays on diapausing larvae were performed by the application of 1 µl of lambda-cyhalothrin (AccuStandard Inc., New Heaven, USA, 98% purity) to the dorsum of each larva using a Hamilton microsyringe. The insecticide was dissolved in acetone and the concentration range assayed was from 1 to 50,000 mg/l (8–10 concentrations). Bioassays were conducted on 3–5 groups of 20 larvae placed in petri dishes according to the available insects. Batches of larvae treated with acetone were used as control. Larvae were subsequently placed under controlled conditions (25°C, 70% RH and 16:8 h L:D photoperiod) for 48 h. Before scoring mortality, larvae were removed and placed in the cap of the corresponding petri dish. Larvae were considered moribund if, after a brush touch, they presented one of the following: they continued lying on their side or in the dorsal position or were unable to move in a coordinated manner. Categories of dead and moribund were combined to assess the percentage of mortality (Soleño *et al.*, 2008).

### Detection of the *kdr* mutation

Total DNA was individually extracted from half of the diapausing larvae with 150 µl of Chelex 100 (Bio Rad, California, USA). Briefly, tissues were digested 30 min at 56°C and, after boiling for 8 min they were centrifuged at 12,000 rpm during 5 min. The supernatants were used as DNA templates for PCR reaction. DNA concentration was spectrophotometrically determined at

**Table 2.** Toxicity of lambda-cyhalothrin in field populations and a laboratory susceptible strain of diapausing larvae of *C. pomonella*.

Population	N	LC <sub>50</sub> (µg a.i./larva) (95% CL)	Slope (± se)	χ <sup>2</sup>	P <sup>a</sup>	df	RR
LSS	473	0.197 (0.137–0.282)	1.02 (±0.086)	5.78	0.055	2	1
CSA	542	6.000 (3.981–8.836)	1.04 (±0.13)	3.81	0.80	7	30.55
GUE	378	7.381 (4.516–12.739)	0.82 (±0.12)	3.95	0.41	4	37.54
VRG3	408	7.217 (4.943–10.678)	0.87 (±0.10)	3.14	0.79	6	36.70

RR, resistance ratio = LC<sub>50</sub> field population/LC<sub>50</sub> laboratory susceptible strain.

<sup>a</sup>Goodness of fit ( $P < 0.05$ )

**Table 3.** Distribution of genotypes and *kdr* frequency in *C. pomonella* samples from different orchards throughout Argentina.

Population code	N	Observed genotype			<i>kdr</i> frequency	H.W. χ <sup>2</sup>	P
		R/R	R/S	S/S			
LSS	16	0	0	16	0	–	–
SJN	21	0	0	21	0	–	–
MZA	38	3	21	14	0.36	1.62	0.20
CSA	17	0	17	0	0.50	17.00	< 0.001
GUE	28	7	16	5	0.54	0.62	0.43
GRC	28	3	13	12	0.34	0.04	0.85
VRG1	31	10	18	3	0.61	1.55	0.21
VRG2	16	2	7	7	0.34	0.015	0.90
VRG3	34	3	14	17	0.24	0.0024	0.96
SMA	40	0	5	35	0.063	0.18	0.67
TRW	16	2	12	2	0.50	4.00	0.046
SAR	32	0	0	32	0	–	–
ANT	34	0	3	31	0.044	0.072	0.79

N, diapausing larvae number; R/R, homozygous for *kdr*; R/S, heterozygous for *kdr*; S/S, homozygous wild-type; H.W. χ<sup>2</sup>; P: values for χ<sup>2</sup> tests for deviation from Hardy–Weinberg equilibrium ( $P < 0.05$ )

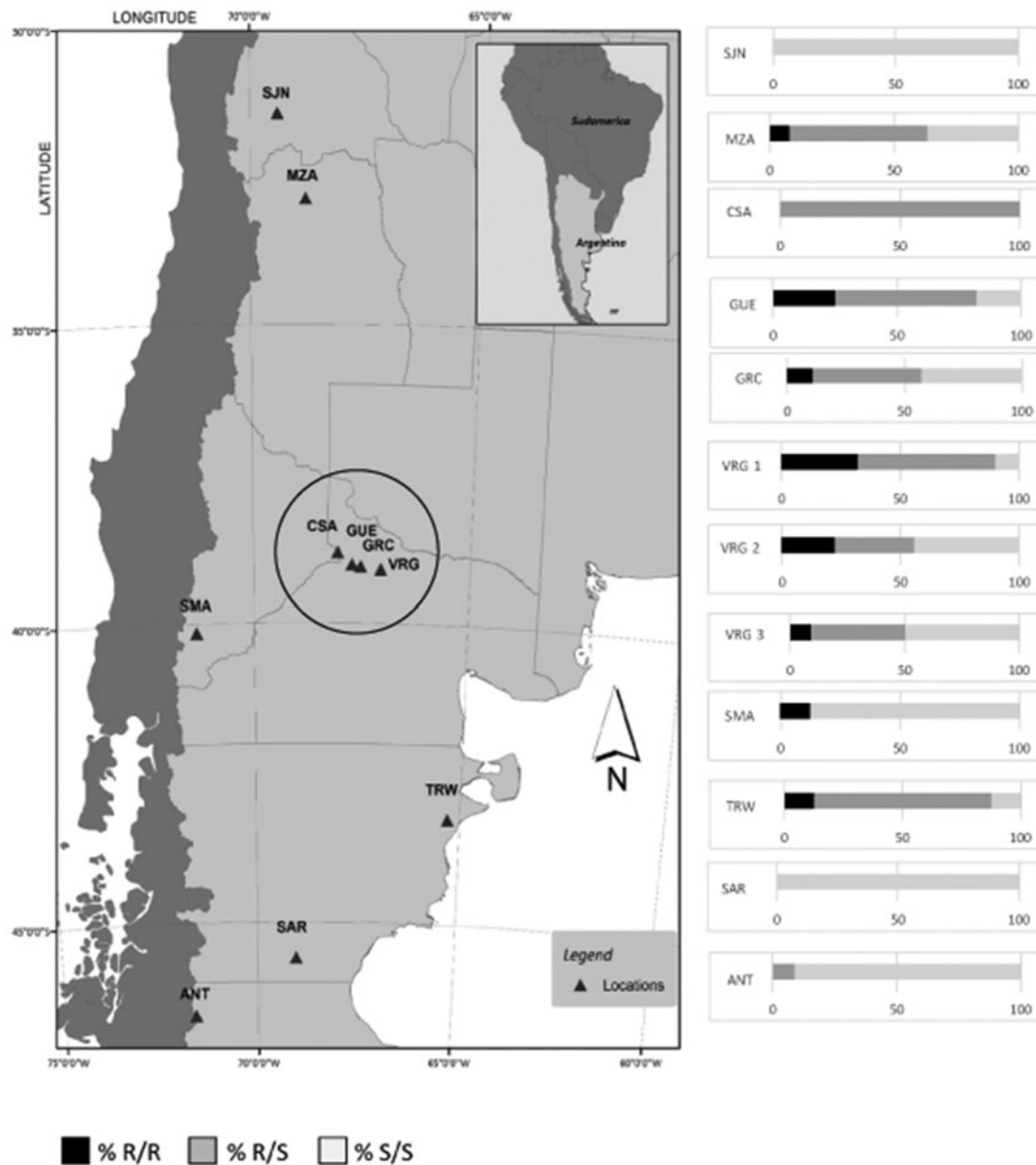
260/280 nm. The genetic variability was analyzed in a 169-bp fragment of the sodium channel gene by polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) from 351 larvae. This fragment encompasses the molecular target linked to pyrethroid resistance (leucine-to-phenylalanine replacement at position 1014 in trans-membrane segment IIS in the amino acid sequence) (Brun-Barale *et al.*, 2005). PCR amplifications were carried out in a 25 µl reaction volume with concentrations according to the manufacturer (Thermo Fisher Scientific, Massachusetts, USA). Specific primers were CpNaF (5' TAGAGAGCATGTGGGATTGC 3') and CpNaR (5' AATTTTCGTAGCCCTTGATCG 3') (Franck *et al.*, 2007). The sequence of the voltage-gated sodium channel from *C. pomonella* Rv strain is noted in the GenBank database under the reference AY763097. DNA amplification product was then subjected to overnight treatment with MluCI enzyme (New England BioLabs, Massachusetts, USA). Restriction enzyme MluCI cuts specifically at the AATT site, which is specific for the *kdr* allele associated with pyrethroid resistance in *C. pomonella* (Brun-Barale *et al.*, 2005). After digestion, DNA fragments of 77 and 112 bp identified *kdr* and the susceptible alleles, R y S respectively. DNA fragments were visualized in a 4% agarose gel with GelRed™ (Biotium, Fremont, California, USA).

DNA was extracted from 351 larvae from 12 orchards distributed through Argentina along with laboratory susceptible individuals. The assay allowed homozygous resistant (R/R), heterozygous (R/S), and homozygous susceptible individuals (S/S) to be distinguished. Samples with known genotypes were processed in all assays as controls. Codling moth positive controls of *kdr* mutation were gently donated by Eduardo Fuentes-Contreras of the Molecular Ecology and Evolutionary Applications Center in Agroecosystems of the University of Talca, Chile.

### Data analyses

Data from concentration–response bioassays were subjected to PROBIT analysis and the regression lines were compared by Likelihood Ratio χ<sup>2</sup> Test. LC<sub>50</sub> and LC<sub>95</sub> values and their 95% confidence limits (CL) were calculated with Dr Sakuma's PriProbit NM software (USDA, 2019). LC<sub>50</sub> and LC<sub>95</sub> values for LSS and FCL were significantly different if their 95% CL did not overlap. Resistance ratios (RR) were calculated as the ratio of LC<sub>50</sub> FCL/LC<sub>50</sub> LSS.

Deviations from Hardy–Weinberg equilibrium (HWE) at each sampling site were analyzed with the χ<sup>2</sup> test ( $P < 0.05$ ) according to the methodology described in Freeland (2005).



**Figure 1.** Sampling sites of the field populations and *kdr* percentages throughout Argentina. Samples inside the circle belong to the Río Negro and Neuquén upper valley.

## Results

### Bioassay

Diapausing larvae were exposed to lambda-cyhalothrin from 1 to 50,000 mg/l for 48 h. The  $LC_{50}$  values obtained from the PROBIT analysis are presented in table 2. The  $LC_{50}$  between the LSS and each FCL was significantly different based on their 95% CL. Diapausing larvae from CSA, GUE, and VRG3 populations showed a high level of resistance to lambda-cyhalothrin, especially GUE ( $LC_{50}$  7.381  $\mu$ g/larva) and VRG3 ( $LC_{50}$  7.217  $\mu$ g/larva) compared to susceptible strain ( $LC_{50}$  0.197  $\mu$ g/larva). The resistance ratios at  $LC_{50}$  level from CSA, GUE, and VRG3 populations ranged from 30 to 37. Moreover, both GUE and VRG3 showed not only the highest  $LC_{50}$  values, but also the lowest slope values. Population genetic heterogeneity is evidenced by low slope values showing an increase of resistant genotypes.

Lambda-cyhalothrin concentration-mortality data from the LSS and all three FCL fitted the PROBIT model as indicated by the goodness-of-fit test ( $P > 0.05$ ).

### Detection and frequency of *kdr* mutation in *C. pomonella*

The L1014F (*kdr*) mutation was detected in ten out of the twelve samples analyzed (table 3) as homozygous resistant (R/R), heterozygous (R/S) and homozygous susceptible individuals (S/S). Larvae from SJN and SAR were homozygous wild type. All samples from the Río Negro and Neuquén valley carried the *kdr* allele with frequencies ranging between 0.24–0.61, although the proportion of homozygotes for *kdr* alleles within each sample was generally low (fig. 1). The frequency of homozygous *kdr* genotypes from all samples in this production region was 0.16. The highest frequency of *kdr* alleles (0.61) was found in VRG1 sample, where

almost 30 and 60% of the sampled individuals were homozygotes and heterozygotes for *kdr* mutation, respectively.

Samples from CSA and TRW showed a significant departure from Hardy–Weinberg equilibrium, and the 100 and 75% of the individuals were heterozygotes, respectively.

## Discussion

Pyrethroid insecticides were intensively used during the 90s decade against codling moth in apple and pear orchards from the Río Negro and Neuquén valley (Soleño *et al.*, 2008). The intense use of chemical insecticides produced a severe selection pressure on insects, favoring the increase in pesticide resistance genotypes. In fact, non-target *Simulium* spp larvae from irrigation channels in this area developed extremely high levels of fenvalerate resistance (400-fold) (Montagna *et al.*, 2012). Results from the present study showed high levels of resistance to lambda-cyhalothrin in the three populations evaluated ( $RR > 30$ ). Previously, low levels of resistance to the organophosphate azinphos methyl and the neonicotinoids acetamiprid and thiacloprid have been observed on field populations from the Río Negro province and resistance to these two insecticide families was highly correlated to esterase and ECOD activities, respectively (Soleño *et al.*, 2008, 2012; Cichón *et al.*, 2013). The increased activities of both enzymes might confer cross-resistance to pyrethroids. Indeed, increased activity of CYP450 and esterase has been related to pyrethroids resistance in codling moth (Sauphanor *et al.*, 1997) as well as in other several lepidopteran species (Gunning *et al.*, 1996; Kranthi *et al.*, 2001; Chen *et al.*, 2005; Yang *et al.*, 2006; Sonoda, 2010).

Besides metabolic resistance, the *kdr* mutation in the voltage-gated sodium channel has been also linked to pyrethroid resistance in codling moth (Brun-Barale *et al.*, 2005; Franck *et al.*, 2012) and in other insect species (Kasai *et al.*, 2017; Zibae *et al.*, 2018).

The single point substitution L1014F in the voltage-gated sodium channels was analyzed in 12 samples of *C. pomonella* from apple and pear orchards throughout Argentina. All samples from the Río Negro and Neuquén valley (CSA, GUE, GRC, VRG1, VRG2, and VRG3) and the one from MZA showed the *kdr* allele with frequencies varying between 0.29–0.61. The frequencies of the homozygous *kdr* genotypes from larvae collected in Río Negro and Neuquén valley were generally low, with the exception of the sample from VRG1 (0.61). The discontinuation for many years of pyrethroid applications, and the immigration of susceptible individuals from surrounding abandoned and organic orchards could explain the low of *kdr* frequency in this region. Since the *kdr*-type resistance allele is partially or completely recessive (Bouvier *et al.*, 2001; Gomes *et al.*, 2017), results from this study suggest that *kdr* mutation in populations from Río Negro and Neuquén valley may not explain by itself the lambda-cyhalothrin resistance levels. The present and previous studies (Soleño *et al.*, 2008; Cichón *et al.*, 2013) would indicate that lambda-cyhalothrin resistance in *C. pomonella* from CSA, GUE, and VRG3 is conferred by multiple-resistance mechanisms including *kdr* mutation in the sodium channel and increased activities of CYP450 and esterases. In French *C. pomonella* populations, a negative correlation between CYP450 activity and the proportion of homozygous *kdr* genotypes was found (Franck *et al.*, 2012). The authors hypothesized that metabolic resistance should be sufficient for the codling moth to resist pyrethroid treatments, limiting the selection of sodium channel target mutations in the absence of strong pyrethroid selection.

CSA population showed a significant deviation from HWE, evidencing violation for at least one of the HWE assumptions. We consider that because CSA is the population that showed one of the highest R/S frequencies and no S/S genotype is under mutation and has an effect on allele frequencies.

The *kdr* mutation was not identified in samples from SJN and SAR and its frequency was very low in ANT (0.044) and SMA (0.063). Neither ANT nor SMA showed the homozygous *kdr* genotype. Fruit production, including apples and pears, from the irrigated valleys in Chubut (TRW and SAR) and Santa Cruz (ANT) provinces is characterized by family orchards, and the control of *C. pomonella* usually involve pyrethroids and low toxic pesticides such diatomaceous earth. The sample of *C. pomonella* from SMA was collected from wild apple trees non-treated with pesticides. According to the low or null pyrethroid selection pressure at these sites (ANT and SMA), it was not expected to found the *kdr* mutation. Since this pest has low dispersal range (150–300 m) (Basoalto *et al.*, 2010), it is possible that resistant alleles from codling moths from the Río Negro and Neuquén valley had spread to distant sites by the transport of fruits in wooden packages infested with diapausing larvae.

## Conclusions

The *kdr* mutation in *C. pomonella* is detected in a geographical wide spectrum in Argentina. Moreover, *kdr* frequency mutations in the Río Negro valley area are higher than in other tested areas. The *kdr* frequency is possibly related to the use of pyrethroids against this pest. Target-site insensitivity is, at least, one of the mechanisms involved in resistance to lambda-cyhalothrin in codling moth from the Río Negro and Neuquén valley.

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