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BIOLOGICAL CONTROL

Toxicity and Metabolism of Zeta-Cypermethrin in Field-Collected and Laboratory Strains of the Neotropical Predator *Chrysoperla externa* Hagen (Neuroptera: Chrysopidae)

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Abstract
Resistance to pesticides has been studied in several insect pests, but information on the natural enemies of pests—including the Neotropical predator *Chrysoperla externa* Hagen (Neuroptera: Chrysopidae), a major biological control agent in South America—is lacking. We report here a comparative study between a field-collected strain of *C. externa* subjected to monthly sprayings of pyrethroids and neonicotinoids and a laboratory strain without exposure to pesticides. The tolerance of both strains against zeta-cypermethrin was similar, and addition of the synergist piperonyl butoxide increased the toxicity by 30% in both strains. Gas-chromatography analyses and mixed-function-oxidase measurements indicated similar values in both strains and also confirmed the key role of oxidative metabolism in this species. Because *C. externa* has maintained a tolerance to zeta-cypermethrin without previous pesticide exposure, this species could potentially be mass-reared and released in fields in the presence of pesticide pressure.

Introduction

Insect-pest control in Argentine fields is based mainly on the use of broad-spectrum pesticides such as pyrethroids, organophosphates, organochlorines, and carbamates (Mugni *et al* 2011, Paracampo *et al* 2012, Lepori *et al* 2013). This extensive use of pesticides has led to failures resulting from the high occurrence of insecticide resistance in pests and the concomitant elimination of their natural enemies (Soleño *et al* 2008, Maggi *et al* 2009, Guedes & Picanço 2012, Onstad 2013). One of the strategies of Integrated Pest Management programs is to combine chemical control with biological control in situations where biological control agents alone are not able to contain a pest population efficiently (Stark *et al* 2007). Within this context, a biological control strategy through mass-reared natural enemies and their subsequent release depends on their survival in insecticide-contaminated fields (van Lenteren 2012).

As the lacewings of the genus *Chrysoperla* Steinmann (Neuroptera: Chrysopidae) have a wide prey range including many undesirable soft-bodied pests such as aphids, whiteflies, thrips, caterpillars, and mites, these Neuroptera species have developed as natural enemies of choice in biological control programs (Silva *et al* 2006, Castilhos *et al* 2011, Flores *et al* 2015, Lavagnini *et al* 2015). The Palearctic species *Chrysoperla carnea* Stephens has also exhibited resistance against pyrethroids and organophosphates, which phenotype was associated with a high activity of detoxification enzymes

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(Ishaaya & Casida 1981, Pree *et al* 1989, Pathan *et al* 2008, 2010). In South America, the Neotropical generalist predator *Chrysoperla externa* Hagen (Neuroptera: Chrysopidae) is considered a major biological control agent, and the massive rearing of that species and its subsequent release in the field have been undertaken in several countries during the last decade (Pappas *et al* 2011, de Fátima *et al* 2013). In recent years, when different developmental stages of this lacewing were observed in and collected from fields having long histories of pesticide use, those populations were found to have low susceptibilities to pyrethroids (Rimoldi *et al* 2008, 2012, Haramboure *et al* 2013). These latest findings indicate that this natural enemy would represent a key feature in any attempt made to progressively diminish the use of pesticides through combination with releases of beneficial insects. This tolerance to pesticides that would enable the survival of the lacewings in the face of insecticide stress could involve a natural immunity of the insect to the chemical or could have resulted from the development of a genetic resistance. To the best of our knowledge thus far, however, no evidence for the existence of resistance mechanisms in *C. externa* has been reported, nor has a comparative study been undertaken between field-collected insects from environments where broad-spectrum pesticides are sprayed during agricultural production and insects that have been mass-reared under laboratory conditions without exposure to pesticides.

Pyrethroids are one of the most effective and widely used groups of insecticides, while cypermethrin represents a large market within that category (Stenersen 2004). Different mechanisms of resistance against pyrethroids have been reported—mainly in the insect pests such as flies, mosquitos, and caterpillars, including those strains harboring mutations in the target site, the gene encoding the *para*-type sodium channel. Those genetic alterations produce a change in the affinity of the insecticide for the binding site in those insects, thus reducing the sensitivity to that compound by increasing its subsequent metabolic detoxification before the drug can reach the target site in the mutants (Zhong *et al* 2013).

The aim of this work was therefore to perform a comparative study on cypermethrin susceptibility between two strains of *C. externa*, one reared in the laboratory in the absence of insecticides and the other collected from a field within the so-called horticultural green belt in the Buenos Aires Province (La Plata, Argentina), where weekly sprayings of pyrethroids are routinely done. First, we tested the susceptibility to cypermethrin in the two strains; second, we determined by gas chromatography the non-metabolized cypermethrin with and without the addition of the synergist piperonyl butoxide (PBO). Finally, we established the impact of the pesticide on oxidative metabolism in the lacewings by measuring the total mixed-function-oxidase (MFO) activity with *p*-nitroanisole as substrate. All this information will enable us to establish whether the field strain has developed cypermethrin resistance or whether either or both strains harbor an innate tolerance to the pesticide.

Material and Methods

*Origin and Maintenance of **C. externa** Strains*

The field strain was collected on vegetable crops in the horticultural green belt of La Plata, where weekly sprayings of pyrethroids (cypermethrin, deltamethrin, and lambda-cyhalothrin) and neonicotinoids (imidacloprid, thiamethoxam, acetamiprid) are done for pest control. The laboratory strain collected from an experimental field belonging to the School of Agricultural and Forestry Sciences (National University of La Plata, Argentina) has been reared in the Laboratory of Ecotoxicology (Centro de Estudios Parasitológicos y de Vectores (CEPAVE) Argentina) from 2006 on without exposure to pesticides.

For the experiments, the insects of both strains were maintained under controlled conditions in the laboratory (25 ± 1°C, 70 ± 5% relative humidity, and 16:8-h light-to-dark cycle) and maintained in ventilated plastic containers (15 cm diameter, 9 cm height) covered with a fine-mesh cloth.

Adults were fed on an artificial diet (Vogt *et al* 2000), and larvae were maintained on an *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) colony as prey. Experiments on the field-collected strain were done within the F1 generation after collection.

Toxicity of Zeta-Cypermethrin to Larval Stages

The insecticide assays were done with third-instar larvae less than 24 h old (both the field-collected and the laboratory strains) that were collected from the respective colonies. The insects were treated topically with PBO alone, cypermethrin alone, PBO + cypermethrin, and acetone for controls, applied with a microsyringe (Hamilton, Switzerland) as reported before (Medina *et al* 2001). Acetone was used as the solvent for all solutions. The cypermethrin formulation used was Fury 100 EW, which product contains 10% (*w*/*v*) zeta-cypermethrin ([S-a-cyano-3-phenoxybenzyl-(1RS)-*cis*-*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate; Belchim, Belgium) and whose maximum recommended field concentration corresponds to 6 mg of active ingredient (a.i.)/L. The insecticide synergist PBO (Sigma, Belgium) was applied 6 h before the addition of the insecticide and was dosed at 60 mg/L, which concentration is 10 times that of the insecticide (Smagghe *et al* 1998). For each treatment, we performed three biological repetitions with each one comprising 10 insects. After 6 h of treatment, the insects were scored and the mortality recorded, with those without movement upon gentle stimulation with a brush being considered dead (Medina *et al* 2001). A

6-h treatment was chosen since our own preliminary tests had indicated a mortality after treatment of 100% at 3 h and 0% at 9 h.

Amounts of Non-metabolized Cypermethrin Determined by Gas Chromatography

For gas-chromatographic analysis, third-instar larvae (<24 h old) were selected from the colonies of the two strains and treated with cypermethrin (25% HPLC purity; Gleba, Argentina), either alone or in combination with PBO. After 9 h of treatment, 30 mg of *C. externa* larvae (the weight of the complete body mass of 12 individuals at the start of the experiment) were crushed with a spoon in 20 ml of hexane and then agitated at 35 kHz for 2 min in an ultrasonic shaker (Transsonic 700, Techspan; ELMA, Germany). The mixture was sieved through a Whatman N° 2 filter paper, and the filtrate evaporated and redissolved in 2 ml of hexane. The extraction efficacy was 60%. The gas chromatography of non-metabolized cypermethrin, performed with electron-capture detection (GC-μECD; Agilent Technologies 6890 N), was carried out in accordance with the optimized in-house protocol described by Ouattara *et al* (2013).

Measurement of MFO Enzyme Amount

For preparation of the enzyme source, we selected the abdomens of five third-instar larvae as described by Smagghe *et al* (2003). After extraction and measurement of proteins, the MFO enzyme amount was measured with *p*--nitroanisole as substrate. The larval abdomens were homogenized in potassium phosphate buffer (0.1 M, pH 7.4), the resulting homogenate centrifuged for 5 min at 1,000*g*, and that supernatant further centrifuged for 15 min at 12,000*g*. The final supernatant was used as the source of enzyme amount. The reaction mixture consisted of 75 μl of enzyme solution in potassium-phosphate buffer, 115 μl of 2 mM *p*--nitroanisole, and 10 μl of 0.5 mM NADPH. The reaction was started by the addition of the *p*-nitroanisole and was ran for 10 min at 27°C. The absorbance was measured at 400 nm with a spectrophotometer (Powerwave X340, BioTek Instruments, Inc., Winooski, VT) and the MFO amount expressed as millimolar of enzyme per milligram of protein. For each strain, we performed three biological repetitions.

Statistics

For the insect-toxicity and gas-chromatography data, a factorial analysis of variance (ANOVA) was used with the factors of treatment and strains. The different treatments for the toxicity assays were control (acetone), PBO alone, zeta-cypermethrin alone, and PBO + zeta-cypermethrin and for the gas-chromatography data were cypermethrin alone and PBO + cypermethrin. The different strains were the laboratory and field specimens. For the toxicity assays, the mortality of the individuals after 6 h of exposure to the different treatments was analyzed. For the gas-chromatography data, the amount of non-metabolized cypermethrin was measured and the values in the presence and absence of PBO compared. Finally, least-significant-difference *post-hoc* tests were carried out. The MFO-activity data were analyzed by the Kruskal-Wallis H test and the medians expressed in the form of a box-and-whisker plot. XLSTAT (2013) was used with a $P < 0.05$ being considered significant.

Results

Toxicity to Larval Stages

Contact with cypermethrin in the field-collected and laboratory strains killed the larval stages to a similar extent at 65–70% of the larvae (Fig 1a). The susceptibility analysis (ANOVA) confirmed that the treatment factor was significantly different between the two strains ($F = 398.79$; $df = 3$; $P \leq 0.001$), but the factors of strain and the interaction treatment × strain were not significant (factor strain, $F = 0.2743$; $df = 1$; $P = 1.24$; treatment x strain, $F = 0.6871$; $df = 3$; $P = 0.5$). In addition, when PBO was combined with cypermethrin, the mortality increased to 80–90% in both the field-collected and laboratory strains.

Amounts of Non-metabolized Compound as Determined by Gas Chromatography

The combined treatment of PBO + cypermethrin increased the amounts of non-metabolized cypermethrin above those measured without the synergist, with the effect being similar in both strains (Fig 1b). The difference with PBO was significant in both strains (field-collected strain, $F = 245.93$; $df = 2.6$; $P < 0.001$; laboratory strain, $F = 101.07$; $df = 2.6$; $P < 0.001$), confirming a deleterious effect on oxidative metabolism. Without PBO, the amounts of cypermethrin in the field-collected and laboratory strains were similar ($F = 6.14$; $df = 1.4$; $P = 0.068$).

*MFO Enzyme Amount in Strains of **C. externa***

With the substrate *p*-nitroanisole for MFO enzyme catalysis, the incubations of field-collected and laboratory-reared insects demonstrated an enzyme amount of 1.69 ± 0.27 and 1.5 ± 0.14 mM/mg protein, respectively (Fig 1c). These enzyme amounts were statistically equivalent ($H = 0.6$; $P = 0.4385$, with protein concentrations of 1.93 and 1.88 mg/ml in the laboratory and field strains, respectively).

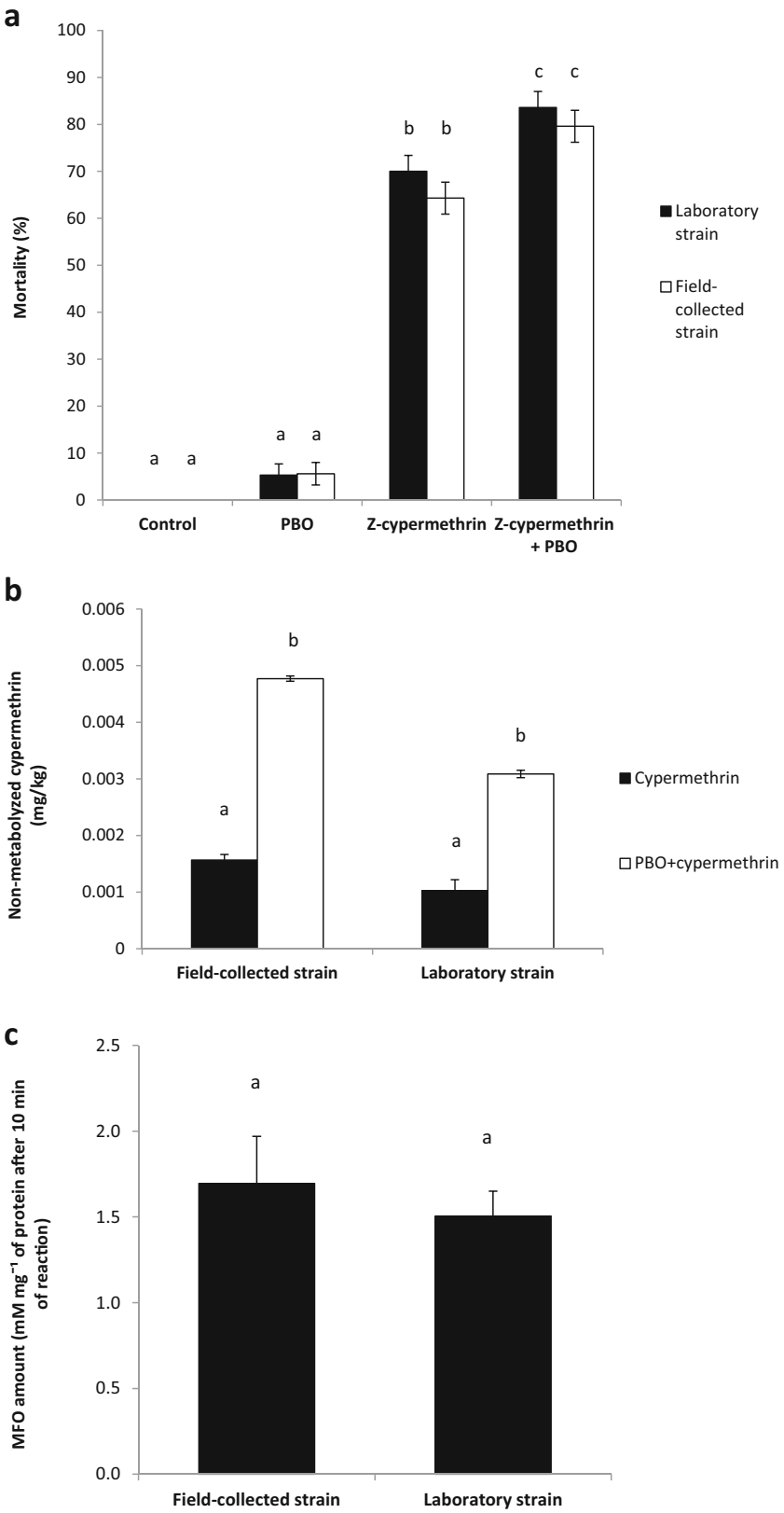
a
Mortality (%)
100
90
80
70
60
50
40
30
20
10
0
a a
a a
b b
c c
Control
PBO
Z-cypermethrin
Z-cypermethrin + PBO
Laboratory strain
Field-collected strain
b
Non-metabolyzed cypermethrin (mg/kg)
0.006
0.005
0.004
0.003
0.002
0.001
0
a
b
a
b
Field-collected strain
Laboratory strain
Cypermethrin
PBO+cypermethrin
c
MFO amount (mM mg⁻¹ of protein after 10 min of reaction)
2.5
2.0
1.5
1.0
0.5
0.0
a
a
Field-collected strain
Laboratory strain

Fig. 1 Results from exposure of larvae from field-collected and laboratory strains of *Chrysoperla externa* to zeta-cypermethrin with or without piperonyl butoxide (PBO). **a** Mortality with zeta-cypermethrin alone, the synergist PBO alone, and the combination of PBO + zeta-cypermethrin. In the figure, the percent larval mortality is plotted on the *ordinate* for each of the experimental groups indicated on the *abscissa*. *Black squares*, laboratory strain; *white squares*, field-collected strain. **b** Larval-tissue levels of metabolized and non-metabolized cypermethrin with and without previous exposure to PBO as measured by gas chromatography. In the figure, the tissue concentration of zeta-cypermethrin in milligrams per kilogram is plotted on the *ordinate* for the two *C. externa* strains indicated on the *abscissa*. **c** Cytochrome P450 activities with *p*-nitroanisole as substrate. In the figure, the mixed-function-oxidase (MFO) amount in millimeters per milligram of protein after 10 min incubation is plotted on the *ordinate* for the two *C. externa* strains indicated on the *abscissa*. In all three panels, the data are means ± SE. The *same letters* denote statistical equivalence between strains (for **a** and **b**, ANOVA and the post hoc Fisher's least-significant-difference test were used at a $P < 0.05$ and, for **c**, the Kruskal-Wallis box-and-whisker–plot test at a $P < 0.05$).

Discussion

The terms tolerance and resistance have been used indiscriminately throughout the scientific literature. Tolerance can be defined as a natural tendency of any species or even a life stage toward an insensitivity to a chemical, while resistance involves the selection of specific heritable traits in a population in response to the contact with a chemical (Coles & Dryden 2014). Tolerance or resistance to pesticides in a natural enemy could therefore be a favorable feature leading to organisms physiologically prepared to confront toxic conditions in an agroecosystem and thus be able to continue with their predation.

Accordingly, resistance or tolerance against pesticides in beneficial arthropods has currently been documented, and the release of these beneficial organisms could be implemented in biological programs in fields treated with pesticides. An ideal biological control agent would be one that is tolerant to synthetic insecticides (El-Wakeil *et al* 2013) since under certain circumstances, the use of pesticides cannot be avoided. Inundative releases of *C. carnea* have been effective in controlling populations of pest complexes in several crops (Simmons & Abd-Rabou 2011); and the use of this species in biological control has increased within the last decades because of the advantage of a relatively broad resistance to many insecticides, particularly during the larval and cocoon stages (Medina *et al* 2001, 2003, Pathan *et al* 2008, 2010). All the stages of *C. carnea* can survive applications of many synthetic pyrethroids, botanicals, microbial insecticides, insect-growth regulators, fungicides, herbicides, and acaricides (Sayyed *et al* 2010, Mansoor *et al* 2013, 2017, Abbas *et al* 2014).

In the example of pyrethroids, Grafton-Cardwell & Hoy (1985) have suggested that the survival of the lacewing was probably a result of the species' natural tolerance. With respect to the Neotropical species *C. externa*, less information is available, but that particular lacewing is well known for its generalist foraging behavior and strong adaptability to different ecosystems (de Moura *et al* 2011, de Fátima *et al* 2013). The appearance of *C. externa* in pesticide-treated fields could be a result of pesticide resistance as opposed to tolerance. For instance, de Moura *et al* (2011)—having found a low mortality rate of *C. externa* with sulfur, abamectin, and trichlorphon—concluded that the mechanism involved could be one of resistance. In the current toxicological study, we found no significant differences in the sensitivity of either strain in the presence of the PBO synergist, which observation is indicative of an equivalent involvement of cytochrome-P450 amount, as previously reported by Sayyed *et al* (2010) in Pakistan. In agreement with this hypothesis, the gas-chromatography data indirectly confirmed the role of MFOs in cypermethrin metabolism because significantly higher non-metabolized amounts were found in the larvae of both strains after exposure to PBO.

Our results demonstrated that the Neotropical lacewing *C. externa* maintained the ability to survive in the presence of cypermethrin, even after 6 years in the laboratory without exposure to insecticides. A fresh field-collected lacewing population manifested the same insecticide insensitivity and resistance to metabolism as the laboratory population had evidenced. This species could therefore possess a natural tolerance to cypermethrin. Since in a mass-rearing program the individuals are not exposed to pesticides during their breeding and because all agricultural fields are (or at least were) exposed to chemicals, this species would appear to be a promising natural enemy of unwanted pests and could be useful in biological control programs, especially in areas with (heavy) applications of pesticides. Moreover, the further exploration of the reasons behind this natural tolerance in *Chrysoperla* species is intriguing, and new technologies (such as next-generation DNA sequencing and the i5k initiative to sequence the genomes of 5000 insects/arthropods) will reveal new insight into pesticide-tolerance mechanisms and evolutionary interactions in agriculturally relevant species.

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