

Impact of the neonicotinoid acetamiprid on immature stages of the predator *Eriopis connexa* (Coleoptera: Coccinellidae)

Marilina N. Fogel · Marcela Inés Schneider ·
Nicolas Desneux · Belén González · Alicia E. Ronco

Accepted: 12 June 2013 / Published online: 22 June 2013
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Abstract *Eriopis connexa* is a native coccinellid predator in the Neotropical Region. In Argentina it is commonly found associated to sucking pests in several crops and among them aphids and whiteflies. These pests are usually controlled with newly developed systemic insecticides, such as the neonicotinoids. However, the compatibility between selective pesticides and natural enemies is required before incorporating them in integrated pest management (IPM) packages. Within this frame, the objective of this study was to evaluate the side effect of various concentrations/doses of one commonly used neonicotinoid in vegetal crops, acetamiprid, on immature stages of *E. connexa* by dipping or topical exposure for eggs and larvae, respectively. Acetamiprid reduced egg hatching from 34 to 100 %. Moreover, the embryogenesis was disrupted by insecticide at early embryo stage at all tested concentrations. Second larval instar was more susceptible to acetamiprid than the fourth one and this susceptibility was positively related with the tested concentrations. On the other hand, the survival reduction at

larval stage reached 100 % from 20 mg a.i./L (10 % of maximum field concentration). Besides, the reproduction of the females developed from topical bioassays on fourth instar larvae was strongly affected, with reduction in fecundity and fertility from 22 to 44 % and from 37 to 45 %, respectively. Overall the results showed a high toxicity of acetamiprid on immature stages of *E. connexa*, demonstrating that this broadly used insecticide could reduce bio-control services provided by this predator and could also likely disturb IPM programs.

Keywords IPM · Sublethal effects · Embryogenesis · Development · Fertility · Fecundity

Introduction

Neonicotinoid-based agrochemicals are widely used to control sucking pests, such as aphids (Hemiptera: Aphididae), mirid bugs (Hemiptera: Miridae) and whiteflies (Hemiptera: Aleyrodidae) (Palumbo et al. 2001; Ishaaya et al. 2007; Liang et al. 2012; Seagraves and Lundgren 2012; Tan et al. 2012). They are very popular insecticides owing to their high efficacy in pest control (namely ovicidal and larvicidal activity), systemic action as well as long lasting effects and environmentally-friendly profiles (Ghanim and Ishaaya 2010). Due to their high specificity, high efficacy and relatively low toxicity to mammals and the environment, these insecticides have been considered a good alternative for the organophosphate insecticides (Tomizawa and Casida 2005; EPA 2012). Indeed, the US Environmental Protection Agency (US EPA) categorizes neonicotinoids as biorational insecticides being compatible with arthropod natural enemies and adequate compounds within Integrated Pest Management (IPM) programs (Ishaaya et al. 2007).

M. N. Fogel · B. González · A. E. Ronco
Centro de Investigaciones del Medio Ambiente (CIMA)
Departamento de Química, Facultad de Ciencias Exactas,
CONICET, Universidad Nacional de La Plata, Calle 47 y 115,
1900 La Plata, Argentina

M. N. Fogel · M. I. Schneider (✉)
Centro de Estudios Parasitológicos y de Vectores (CEPAVE),
CONICET La Plata-UNLP, Calle 2 N° 584, 1900 La Plata,
Argentina
e-mail: mschneider@cepave.edu.ar

N. Desneux
French National Institute for Agricultural Research (INRA),
UMR-ISA, 400 Route des Chappes, 06903 Sophia-Antipolis,
France

One of the major purposes of IPM strategies is the combination of selective pesticides with biological control agents, i.e. predators and parasitoids (Desneux et al. 2006a). Therefore, the evaluation of side effects of pesticides on natural enemies, both lethal and sublethal effects, is essential prior to IPM programs implementation (Desneux et al. 2007; Stark et al. 2007). The Horticultural Green Belt (Gran La Plata Region, Province of Buenos Aires, Argentina) is one of the main producing areas of Argentina, with 7,538 ha of cultivated area (CFHB (Censo flori-hortícola bonaerense) 2005). Pest control is mostly conducted by newly developed synthetic pesticides, being neonicotinoids between the most extensively used for the control of sucking pests, such as aphids and whiteflies, mites, scales and mealybugs in both, field and greenhouse crops (Cappello and Fortunato 2008).

However, in the last few years several studies have reported adverse, lethal and sublethal, effects of newly developed pesticides on non-target beneficial organisms (Schneider et al. 2004, 2008; 2009; Desneux et al. 2007; Rimoldi et al. 2008, 2012; Ronco et al. 2008; Benamú et al. 2010; Arnò and Gabarra 2011; Biondi et al. 2012a; Fogel 2012). In particular, the negative impacts of neonicotinoid insecticides were reported towards Coccinellidae predators throughout acute toxicity and physiological and behavioral trait impairments (Grafton-Cardwell and Gu 2003; Youn et al. 2003; Lucas et al. 2004; Papachristos and Milonas 2008; Cabral et al. 2011; He et al. 2012).

Generalist arthropod predators are known worldwide as regulators of insect herbivore populations in agricultural and forest ecosystems (Symondson et al. 2002; Desneux et al. 2006b; Lu et al. 2012); moreover, they are able to establish populations in highly disturbed ecosystems, such as annual cropping systems, by exploiting alternative preys (Harwood et al. 2007; Desneux and O'Neil 2008; Juen et al. 2012). *Eriopsis connexa* (Gemar) (Coleoptera: Coccinellidae) is an indigenous generalist predator in Argentina. It is widely distributed in the Neotropical Region and it is considered a potential control agent of various pests on several crops (Almeida-Sarmiento et al. 2007; Duarte Gómez and Zenner de Polanía 2009). Both larval and adult stages of all coccinellid predator species provide important biocontrol services feeding on different soft-body pests, such as aphids, whiteflies, mites, and lepidopteran eggs and larvae (Obrycki and Kring 1998).

The synthetic neonicotinoid insecticide acetamiprid, (*E*)-N1-[(6-chloro-3-pyridyl)methyl]-N2-cyano-N1-methylacetamide, belongs to a relatively new group of active ingredients and it is characterized by a novel mode of action compared to conventional neurotoxic insecticides (Tomizawa and Casida 2005). This compound acts on the central and peripheral nervous system of insects, specifically interacts with nicotinic acetylcholine receptors

(nAChR), resulting in excitation and paralysis, followed by death (Ghanim and Ishaaya 2010). Mindful of this context, the objective of the present study was to assess the lethal and sublethal effects of various concentrations/doses of acetamiprid under controlled laboratory conditions on immature stages (egg, second and fourth larval instars) of the Neotropical generalist predator *E. connexa*.

Materials and methods

Insects

The *E. connexa* colony was established from samplings collected in infested vegetable crops in La Plata region, Argentina (34° 57'17" S, 57° 53'26" W) in 2008. Adults were collected manually using plastic tubes (10 cm length × 1.5 cm diameter) conditioned and isolated to avoid potential field diseases (fungal, bacterial or viral infections) and/or parasitism. Then, their healthy progeny were used to initiate the predator rearing. Annually, the colony was infused with wild stock (between 50 and 80 adults each) collected from the same geographical area, to maintain its genetic variability.

Rhopalosiphum padi L. (Hemiptera: Aphididae) was used as prey. The aphid colony was initiated from clones obtained from the Faculty of Agricultural and Forestry Sciences (National University of La Plata) and it was reared on pesticide-free wheat seedlings (*Triticum aestivum* L.) (cultivar ACA 901). An artificial diet based on beef liver (Martos and Niemeyer 1990) was offered ad libitum as nutritional supplement for larvae and adults of the predator. Insect colonies and all the bioassays were carried out in a growth chamber with controlled environmental conditions (25 ± 2 °C, 70 ± 5 % HR and 16:8 h L:D).

Toxicity bioassays

Effects on eggs: embryogenesis and development

A commercial formulation of acetamiprid (Mospilan® 10 % p/p, Summit-Agro S.A., Argentina) was used for this experiment. It was tested at its maximum field concentration (200 mg a.i./L) and its half (100 mg a.i./L). The insecticide solutions were diluted in distilled water and, to facilitate adhesion of the insecticide to the egg chorion, a commercial tensioactive (Tween 80®, Merck, Darmstadt, Germany) was added at its label concentrations, i.e. 0.1 ml/L. The untreated control was sprayed only with distilled water plus the tensioactive.

Coetaneous eggs (≤48 hold) were treated by dipping in the solutions for 15 s according to Schneider et al. (2009), left to dry in a fume hood and then maintained in plastic

Petri dishes (9 cm diameter and 1.5 cm high). Each treatment consisted of 3 to 4 replicates with 20–30 eggs per replicate. Embryogenesis and egg hatching were checked daily by a stereomicroscope 24 h after treatment and for seven consecutive days. Hatching generally occurs between 3–4 days after oviposition (Fogel MN, pers. obs.). Therefore, treated and untreated eggs that did not hatch during the 7 days of observations were considered dead. The percentage of eggs hatched was evaluated from the following formula [(Number of eggs hatched/N° initial eggs) × 100]. To assess the effects on the embryos development, from 20 to 30 unhatched eggs were randomly selected from each treatments and were placed in Bouin® solution, then dehydrated in ethanol series of analytical grade (70, 90 and 100 %) and mounted between slide and cover glass in Hoyer's medium and dried in stove at 40 °C.

Effects on larval survival and development

Second and fourth instar larvae were treated topically with seven concentrations, including the maximum field concentration (see Table 1 for details), of acetamiprid. The weight of the tested larvae averaged 2.66 ± 0.7 mg and 5.7 ± 0.4 mg for L₂ and L₄, respectively. To improve the insecticide dissolution, the solutions were prepared with acetone (ACS analytical grade) diluted in distilled water (80:20 v/v) and used as solvent following the methodology by Youn et al. (2003). The treatments were performed with a manual micro-applicator (Burkard, Rickmansworth, UK) applying 0.5 and 1 µL of insecticide solution to the first abdominal segment of each tested individual. Acetone 80 % was used for untreated controls. Treated larvae were placed into plastic Petri dishes and then were transferred into a growth chamber until adult emergence. They were fed *R. padi* specimens and beef liver-based artificial diet ad libitum (Martos and Niemeyer 1990). Each treatment consisted of three replicates of ten larvae.

Survival and development time of immature stages (from larva to pupa) were checked, every 24 h until adult

emergence. Survival data were used to obtain the reduction of this parameter according to the following formula:

$$\% \text{ survival reduction} = [(SC - ST) \div SC] \times 100$$

where SC is proportion of survivors in the control and ST refers to the proportion of survivors in the treatments.

Effects on reproduction

Sublethal effects on fecundity and fertility were assessed on the adults developed from treated larvae. Because the survival of the treated larvae was very low (see “Effects on larval survival and development” section), the effects on reproduction were assessed on the adults developed from fourth instar larvae treated with the three lower acetamiprid doses, i.e. 0.0001, 0.0008 and 0.0017 µg a.i./g. Newly emerged adults, both males and females, were placed in plastic cylindrical containers (18 cm diameter and 15.5 cm height) and, to help the female ovary development, *R. padi* specimens and artificial diet were offered ad libitum. After 5 days, ten mated females having a large abdomen (i.e. with developed ovaries) per treatment were randomly selected for the reproduction assessment. Females were placed individually in plastic glasses (5 cm diameter and 10 cm height) with a fine mesh net fixed on the upper opening to allow ventilation. The inner walls of the containers were previously covered with untreated paper as substrate for the oviposition, whereas aphids and artificial diet were provided ad libitum. During the following 5 days they were checked for the presence of egg batches and newly laid eggs were collected and placed in Petri dishes. Therefore, fecundity (number of laid eggs) and fertility (number of hatched eggs) were registered for each egg clutch and female.

Statistical analysis

Normality of the data was firstly tested using the Shapiro–Wilk test and homoscedasticity of variances by Bartlett's test. If the assumptions of ANOVA were not met, i.e. the data were not normally distributed, row datasets were transformed [$\log(x + 1)$ or $\arcsine \sqrt{x}$] or a non-parametric test for analysis of data was performed (Kruskal–Wallis test, with the bilateral Dunn test for multiple comparisons in pairs). The parametric test of analysis of variance (ANOVA) and LSD test for mean separation were used to analyze the data of toxicity on eggs. Survival reduction of larval instars values were analyzed by Factorial ANOVA (instars and treatments as main factors). Repeated measures ANOVA was done for fecundity and fertility of *E. connexa* adults survived from treated fourth instar larvae. Means were separated by the LSD multiple range test among the tested doses ($p < 0.05$). All the analyses were performed using the program XLSTAT (Addinsoft XLSTAT for Excel, Paris, France, 2009).

Table 1 Concentrations and doses of acetamiprid evaluated on *Eriopsis connexa* second (L₂) and fourth (L₄) instar larvae

Acetamiprid (mg a.i./L)	L ₂ (µg a.i./g larvae)	L ₄ (µg a.i./g larvae)
200	0.0375	0.0350
100	0.0187	0.0175
50	0.0093	0.0087
20	0.0037	0.0035
10	0.0018	0.0017
5	0.0009	0.0008
1	0.0001	0.0001

Results

Effects on eggs: embryogenesis and development

Acetamiprid treatment at the maximum field concentration (200 mg a.i./L) caused a 100 % mortality of the predator at the egg stage ($F = 52.9$; $df = 3,4$; $p < 0.0001$; Fig. 1a). Unhatched eggs became black 48 h after treatment and some of them got dehydrated. For acetamiprid treatment at 50 % of the maximum field concentration (100 mg a.i./L), the eggs hatching rate was significantly lower than in the control ($F = 52.9$; $df = 2,3$; $p = < 0.012$; Fig. 1a). However, the neonate larvae that were able to emerge showed a significant reduction of survivorship compared to the control ($F = 47.1$; $df = 2,3$; $p = 0.0001$), with values of 10.0 ± 8.6 and 90.0 ± 3.4 %, for acetamiprid (100 mg a.i./L) and control, respectively. Development time of the eggs (embryos development) was significantly longer in the acetamiprid treatment (100 mg a.i./L) compared to the control ($K = 3.84$, $p = 0.034$); reaching 2.5 ± 0.28 days from treatment (0.5 days more than control) (Fig. 1b). In those where no larvae emerged, the embryos mortality was corroborated by observation under the stereo microscope preparations (Fig. 2). Embryos of the untreated control (Fig. 2a, b) were fully developed at 48 h post-treatment (72–96 h from oviposition), being observed abdominal segments, legs, setae and mandibles. However in treated

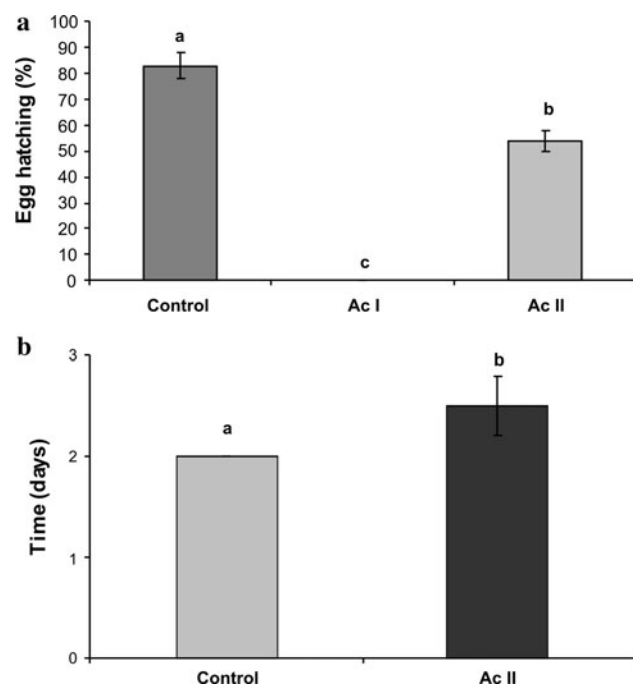


Fig. 1 Means (\pm SE) of acetamiprid effects on egg hatching **a** and length of embryogenesis **b** of *Eriopsis connexa*. Bars with different letters are significantly different [**a** ANOVA; **b** Kruskal–Wallis ($p < 0.05$)]. Ac I = 200 mg a.i./L; Ac II = 100 mg a.i./L

ones, the vitelline membrane is the only detectable and the developed embryos showed just mandibles (Fig. 2d, e) while abdominal segments, setae and legs were not visible. Overall, these studies have verified the interruption of embryogenesis at both tested acetamiprid concentrations (200 mg a.i./L; Fig. 2c, d and 100 mg a.i./L; Fig. 2e, f).

Effects on larval survival and development

The effects of acetamiprid on *E. connexa* larvae survival were significantly different according to the larval instar being exposed (*instar* factor) and to the insecticide concentration applied (*dose* factor). Moreover, the interaction between these two factors was also significant (Table 2). Indeed, second instar larvae were significantly more susceptible to acetamiprid than the fourth instar ones at all the tested concentrations (Fig. 3a). The reduction of larval survival was more evident at higher concentrations of acetamiprid (200, 100, 50 and 20 mg a.i./L) than at lower ones (10, 5 and 1 mg a.i./L) (Fig. 3b). The development time of fourth instar larvae to adults in acetamiprid treatments did not differ significantly from the control ones reaching values of 7.3 ± 0.21 ; 7.0 ± 0.19 ; 6.9 ± 0.34 ; 7.15 ± 0.22 days for control and acetamiprid doses of 0.0001, 0.0008 and 0.0017 μ g/g, respectively ($K = 7.81$, $p = 0.504$). At higher doses this parameter was not evaluated due to high mortality recorded at second day from treatment. On the other hand, the development time to adults could not be recorded for the treated second instar larvae, since all the specimens died few hours after treatment regardless of the insecticide concentration considered.

Effects on reproduction

Reproduction of *E. connexa* just was evaluated at lower doses of acetamiprid and in adults emerged from fourth instar larvae because at second instar larvae and higher doses no survivors were obtained. Acetamiprid effects on fecundity and fertility were evaluated taking into account the time (*time* factor) and the pesticide dose (*doses* factor) (Table 3). Acetamiprid at 0.0017 μ g a.i./g significantly reduced the daily fecundity of *E. connexa* females emerged from treated fourth instar larvae ($p = 0.001$) (Fig. 4a). Analyzing data according to *time* factor, a tendency in the reduction of the fecundity after the first oviposition day was detected. The fecundity was significantly affected at day five compared to first day, regardless of the concentration considered ($p = 0.007$) (Fig. 4b). The acetamiprid doses of 0.0017, 0.0008 and 0.0001 μ g a.i./g significantly reduced the number of daily hatched eggs (fertility) compared to controls ($p < 0.0001$) (Fig. 4c). By contrast, no significant differences were observed among the five oviposition days in the portion of the hatched eggs (Fig. 4d).

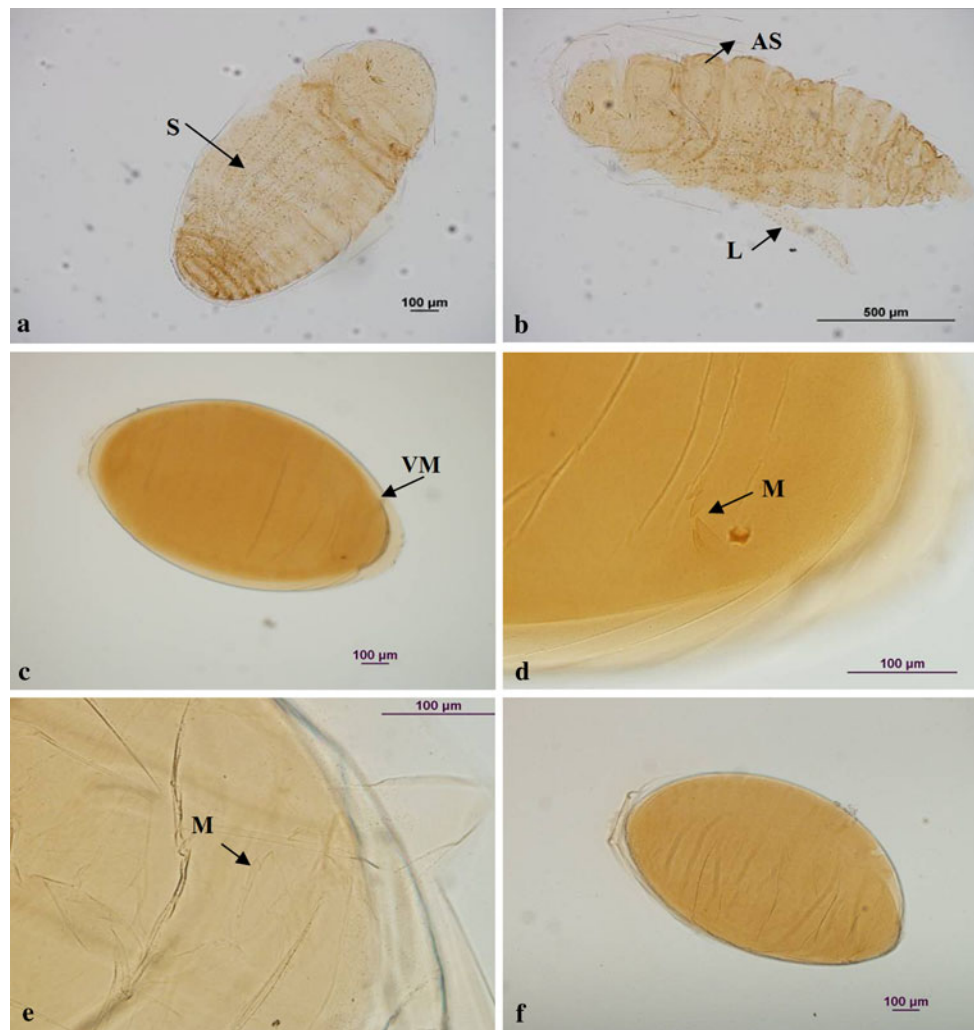


Fig. 2 Embryogenesis disorders in eggs of *Eriopis connexa* exposed to acetamiprid. Control **a, b**; Acetamiprid 200 mg i.a./L **c** and **d**; Acetamiprid 100 mg i.a./L **e, f**. AS abdominal segments; M mandibles; VM vitelline membrane; L legs; S setae

Table 2 Factorial ANOVA (main factors: instars and acetamiprid treatments) for survival reduction of *Eriopis connexa* larvae, from topical bioassay on second (L_2) and fourth (L_4) instar larvae

Factor	df	<i>F</i>	<i>p</i> value
Instars	1	159.6	<0.0001
Treatment	6	62.9	<0.0001
Instars × treatment	6	33.1	<0.0001

Discussion

Exposure of beneficial arthropods to pesticides can result in a wide range of effects, including the simultaneous occurrence of multiple sublethal effects (Desneux et al. 2007; Stark et al. 2007; Biondi et al.; 2012b). Besides the short term effects (acute toxicity), the long-term ones could also strongly impact on natural enemies reproduction, leading to a reduction in offspring, affecting the population

growth rates (Stark et al. 2004). In the present study we observed both lethal and sublethal effects of various concentrations/doses of acetamiprid on immature stages of *E. connexa*.

The observed deleterious effects were highlighted by the significant reduction of survival, both on egg and larval stages exposed to the recommended field and to lower concentrations. Moreover, the lower tested concentrations not only affected the survival, but also decreased the fecundity, fertility and embryogenesis of the developed adults. Indeed, high mortality of embryos and of the newly hatched larvae after the direct exposure of eggs to acetamiprid was observed. Although the chorion surface layer is a sclerotized protein membrane providing mechanic resistance with non-permeable properties (Nation 2008), some chemicals can pass through it affecting (i) the embryo development (ii) or, in the case of successful hatching, the individual may die when feeding the insecticide-contaminated

Fig. 3 Means (\pm SE) of acetamiprid effects on survival reduction on *Eriopis connexa* larvae. **a** Larval Instar factor; **b** Acetamiprid dose factor. Treatments with different letters are significantly different. Factorial ANOVA ($p < 0.05$)

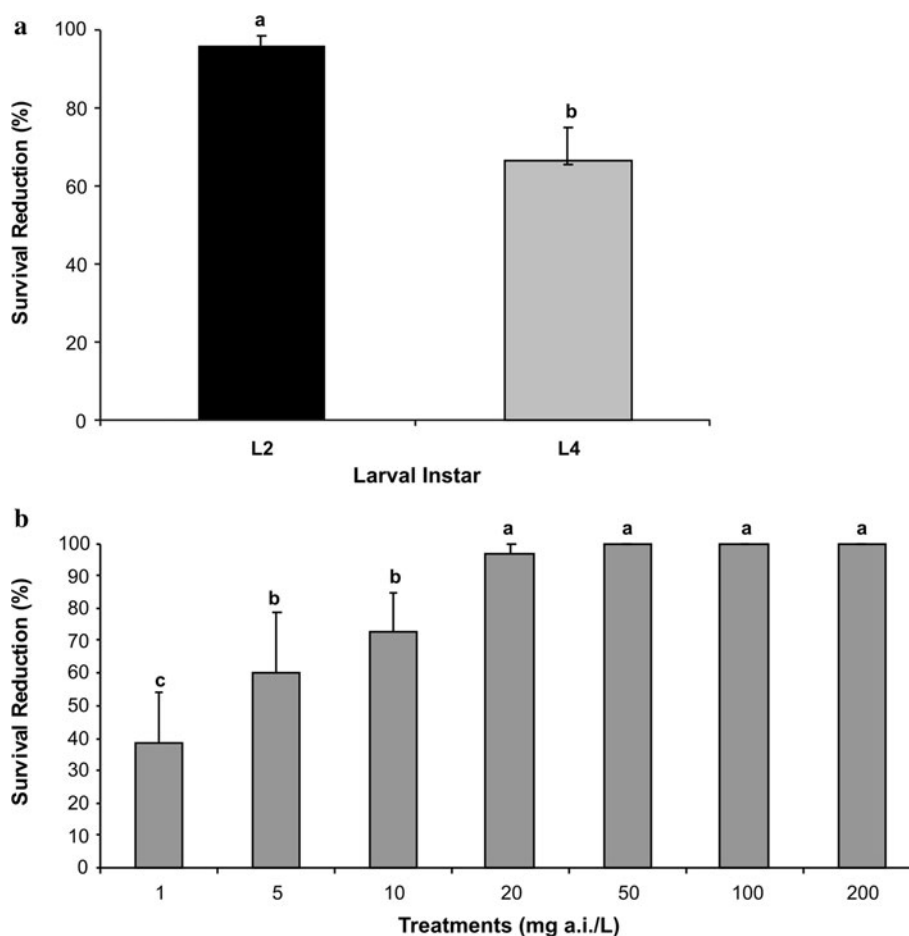


Table 3 Repeated measures analysis of variance (ANOVA) for fecundity and fertility of *Eriopis connexa* adults, from topical bioassay on fourth (L_4) instar larvae

	df	F	p value
Fecundity			
Time	4	1.696	0.157
Dose	3	1.432	0.238
Time \times dose	12	1.545	0.122
Fertility			
Time	4	0.364	0.833
Dose	3	2.727	0.048
Time \times dose	12	2.008	0.032

chorion (Trisyono et al. 2000; Consoli et al. 2001; Galvan et al. 2005; Rimoldi et al. 2008). Coccinellidae eggs have a ring of micropyles useful for the fecundation process and for oxygen diffusion inside the eggs (Nedvěď and Honěk 2012). Furthermore, the treatment with 50 % of acetamiprid maximum field recommended concentration induced a lengthening of the embryogenesis of the predator eggs. According to our studies it could be hypothesized that acetamiprid was able to trespass the chorion of *E. connexa* eggs or to penetrate through the ring of micropyles, blocking the embryo development.

Even though neonicotinoids exhibit the same mode of action targeting the nicotinic acetylcholine receptor, ovi-cidal activity and effects on embryos is variable between compounds of this chemical group (Hoffmann et al. 2008). Our results match with those reported by Youn et al. (2003), which observed no larvae emergence when dipping the eggs of *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) in the two neonicotinoids, acetamiprid and imidacloprid. While another insecticide belonging to this family, thiametoxam, did not cause any deleterious effect (Youn et al. 2003). Additionally, Kim et al. (2006) observed that, although acetamiprid-treated eggs of the predator *Deraeocoris brevis* Uhler (Hemiptera: Miridae) did not evidence effects on hatching; a significant reduction of survival of emergent nymphs was observed. Furthermore, studies on the exposure of egg of the predator *Podisus maculiventris* (Heteroptera: Pentatomidae) to imidacloprid did not cause any egg mortality, although a significant reduction of emergent larvae was recorded (Cutler et al. 2006). Whereas, imidacloprid caused lengthening in the embryogenesis of *Apolygus lucorum* Meyer-Dur (Hemiptera: Miridae) in eggs laid by treated females (Tan et al. 2012).

It is well known that susceptibility of natural enemies toward pesticides varies with the development stage of

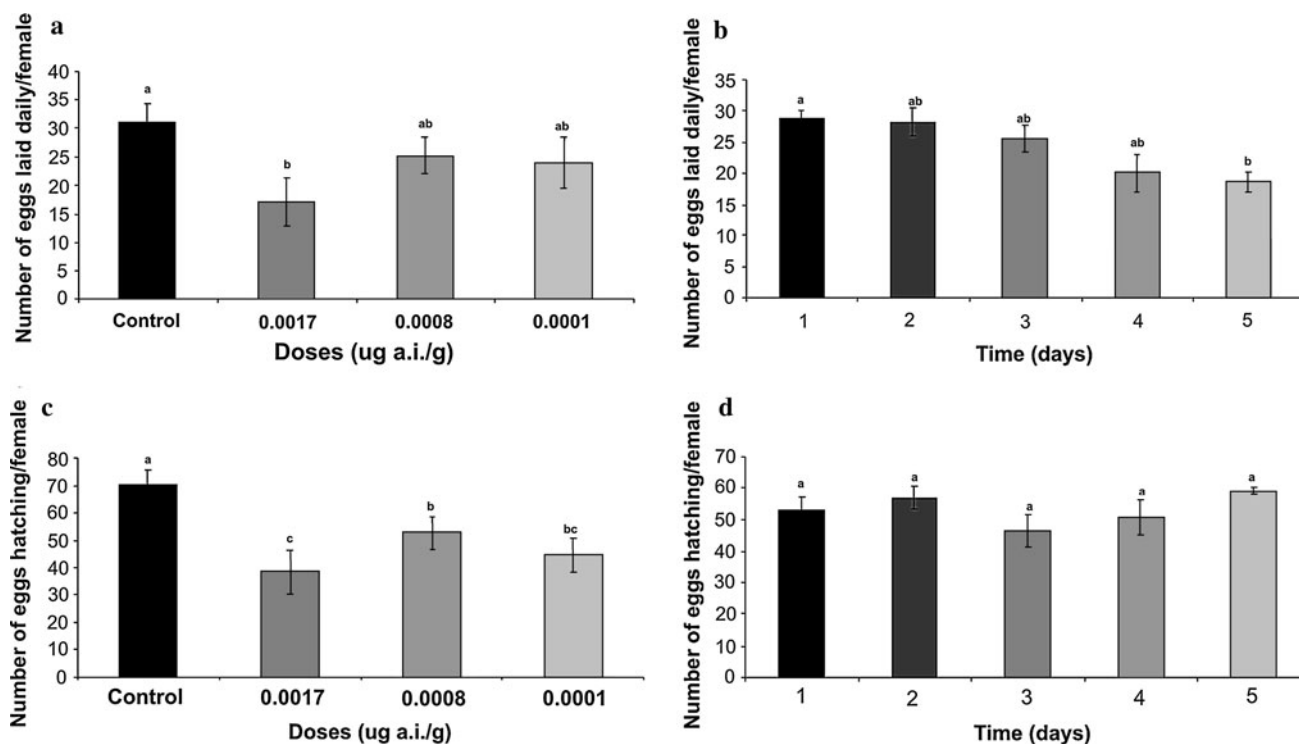


Fig. 4 Means (\pm SE) of sublethal effects of acetamiprid at 0.0017, 0.0008, and 0.0001 (μ g a.i./g) on fecundity (number of eggs laid daily/female) and fertility (number of eggs hatching/female) of *Eriopsis connexa* emerged from treated fourth instar larvae. **a** and **b** Effects on

females' fecundity respect to *dose* and *time* as main factors, respectively. **c** and **d** Effects on females' fertility respect to *dose* and *time* as factors, respectively. Treatments with *different letters* are significantly different. Repeated- measures ANOVA ($P < 0.05$)

tested organisms, being generally the immature stages more susceptible than adults (Delbeke et al. 1997; Michaud 2002; Grafton-Cardwell and Gu 2003; Schneider et al. 2004; Galvan et al. 2005; Cutler et al. 2006). The difference could be associated to a thinner and more permeable cuticle of immature stages with lower chitin content added of less active enzymatic detoxifying processes in comparison to adult stage (Stark et al. 2004). According to the results of the toxicity bioassays with second and fourth instar larvae of *E. connexa*, it is evident that both instars are highly susceptible to acetamiprid but the effect was even clearer on second instar larvae bioassays. However, the survival reduction in fourth instar larvae was observed at concentrations between 20 and 200 mg a.i./L. Other authors have reported similar results for neonicotinoids on various beneficial insects. Acetamiprid caused 100 % mortality of *H. axydiris* fourth instar larvae and imidacloprid was more toxic to the second instar larvae than the fourth instar one (Youn et al. 2003). Furthermore, acetamiprid and imidacloprid caused 100 % mortality on second instar larvae of the coccinellid predator *R. cardinalis* by residual exposure (Grafton-Cardwell and Gu 2003). Likewise, Lucas et al. (2004) observed 100 % mortality of the third instar larvae of *Coleomegilla maculata* (De Geer) (Coleoptera: Coccinellidae) exposed to

imidacloprid. Moreover, carbofuran and imidacloprid reduce survival of *Hippodamia undecimnotata* (Schneider) (Coleoptera: Coccinellidae) larvae by ingestion of treated preys (Papachristos and Milonas 2008). Similar results were also observed on larval stages of *Orius laevigatus* Fieber (Heteroptera: Anthracoridae), *Macrolophus caliginosus* Wagner (Heteroptera: Miridae) (Van de Veire and Tirry 2003), *D. brevis* (Kim et al. 2006) and *Picromerus bidens* L. (Heteroptera: Pentatomidae) (Mahdian et al. 2007).

Reproductive capacity of females emerged from fourth instar larvae survivors was significantly affected by very low doses of acetamiprid. These results agree with those reported by Sohrabi et al. (2012), where the fecundity and fertility of *Encarsia inaron* (Walker) (Hymenoptera: Aphelinidae) adults developed from imidacloprid-treated larvae were significantly affected. Likewise, Grafton-Cardwell and Gu (2003) observed a fertility reduction of *R. cardinalis* females exposed to acetamiprid, imidacloprid and thiametoxan. Similarly, fecundity reduction of *H. undecimnotata* females was observed when this predator was fed with aphid treated with sublethal concentrations of imidacloprid and carbofuran (Papachristos and Milonas 2008). On the contrary, no adverse effects on fecundity and fertility were observed on treated females of *D. brevis* under topic exposure of predator's nymphs (Kim et al.

2006). Likewise, this insecticide did not induce any side effects on reproductive parameters of the predator *P. bi-dens* (Mahdian et al. 2007).

Taken as whole, although they should be confirmed with field studies, the results from our laboratory experiments demonstrated that acetamiprid would strongly reduce the *E. connexa* population development throughout the impairment of crucial physiological processes in the juvenile development. This could result in limiting the predator biocontrol services in the crops where this insecticide is broadly used, with negative implications for the Integrated Pest Management programs. Finally, this research provides new insights into side-effects of acetamiprid in the embryogenesis and highlights the importance of incorporating juvenile stages in pesticide risk assessments.

Acknowledgments This research was funded by a PICT 0891-BID and PICT 1752-BID projects from the Argentine National Agency for the Promotion of Science and Technology (ANPCyT-FONCYT) granted to Alicia Ronco and Marcela. I. Schneider, respectively. M. Fogel is grateful to National Council of Scientific and Technical Research (CONICET) for doctoral fellowship granted. The authors thank Ing. Armando Junquera (Asociación Cooperativas Argentinas) for wheat seeds ACA 901, Dr. Mónica Ricci (Faculty of Agronomic and Forest Sciences, National University of la Plata, UNLP) for *R. padi* clones to initiate our aphid colonies, and Summit-Agro S.A., Argentina for providing samples of acetamiprid.

Conflict of interest The authors declare that they have no conflict of interest.

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