Evaluation of imidacloprid genotoxicity in *Chrysoperla externa* eggs (Neuroptera: Chrysopidae) through comet assay

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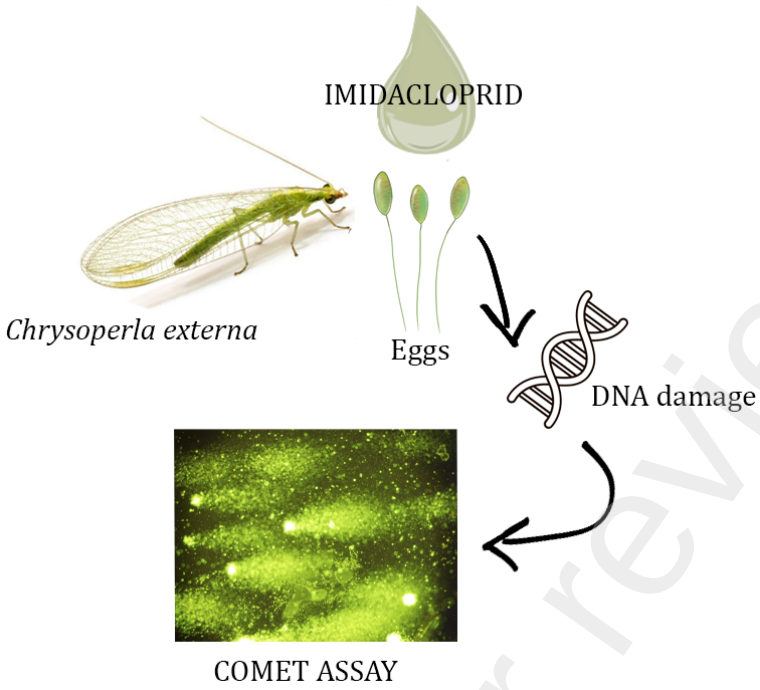

Highlights

- Imidacloprid caused DNA damage in the eggs of the biological control agent *Chrysoperla externa*.
- DNA damage could be quantified using different variables of the comet assay.
- A specific protocol was developed for performing the comet assay for *C. externa* eggs.

Keywords

Pesticides, Neonicotinoid, Predator, lacewings, agrochemical, ADN damage

Abstract

The comet assay allows the analysis of DNA damage caused by different genotoxins. This assay has recently gained interest because of its ease of studying the interactions of xenobiotics with different organisms.

Chrysoperla externa (Hagen, 1861) is a species of great economic relevance because it is a predator of major agricultural pests during its larval stage. Neonicotinoids are the most important chemical class of insecticides introduced into markets. A previous imidacloprid toxicity assessment on C. externa showed that this neonicotinoid insecticide reduced the egg viability. The objective of this study was to analyze the genotoxicity of imidacloprid on the biological control agent *C. externa* at DNA level using the comet assay as an ecotoxicological biomarker. This technic was used for the first time for this organism under laboratory insecticide exposition. For the bioassays, the commercial product formulated Confidor OD® (imidacloprid 20% a.i., LS, Bayer CropScience) was used in two concentrations. The selected eggs were dipped in a Confidor OD® solutions for 15 s. Controls were treated with solvent alone. A comet assay protocol was develop for this species at first time. Variables evaluated in Comet assay were as follow: damage index, % DNA damage and tail length. All statistical analyses were performed using R software. The damage index did not show any significant differences between the different concentrations evaluated, but differences were observed for the tail length, because at higher concentrations of imidacloprid, smaller DNA fragments. The DNA of the cells from treated eggs analyzed at 48 h and 96 h of development showed the same % DNA damage; that is, they had no recovery capacity. Applications of imidacloprid on *C. externa* eggs produce irreparable breaks at the DNA level. The technique adjusted for *C. externa* can be used in other beneficial insects to study pesticide genotoxicity using the comet assay.

1. Introduction

The comet assay allows the analysis of DNA damage caused by different genotoxins both in vivo and in vitro (López et al., 2012). This assay has recently gained interest because of its ease of studying the interactions of xenobiotics with different organisms (Augustyniak et al., 2016). The main benefit of this technique is that it is fast and economical, and various types of cells can be used without prior knowledge of their karyotype, genomic structure, or mitotic

activity (Augustyniak et al., 2016; Cuevas Díaz et al., 2012; López et al., 2012). The comet assay allows for the analysis of single-strand breaks in DNA, incomplete repair sites, and alkali-labile sites. DNA damage determination is commonly performed by counting the affected cells, the length of the comet tail, and using software to measure fluorescence intensity (Cuevas Díaz et al., 2012; López et al., 2012).

Nowadays, only model species or species with great economic relevance have been used to perform comet assay (Augustyniak et al., 2014) due to a few number of insects could be bred and studied under controlled conditions. In this sense, main orders of insects include Diptera, mainly *Drosophila melanogaster* (Meigen), *Aedes aegypti* (L.), *Liriomyza trifolii* (Burgess), lepidopterans such as *Plutella xylostella* (L.), *Spodoptera litura* (F.), and some coleopterans and orthopterans (Augustyniak et al., 2016; Glei et al., 2016; Shetty et al., 2017; Todoriki et al., 2006). It has been also performed on earthworms as a bioindicator of soil contamination (Cuevas Díaz et al., 2012).

Chrysoperla externa (Hagen, 1861) is a species of great economic relevance because it is a predator of major agricultural pests during its larval stage. It has a neotropical distribution and commonly found associated to relevant pest with spontaneous presence in crops in Argentina. Owing to its ease of controlled breeding, it is well produced in biofactories (Souza & Souza Bezerra, 2019).

Although lacewings larvae are polyphagous, they prefers aphids, which are sucking insects that attack all types of plants, mainly in temperate zones, causing physical damage and transmission of viruses of high economic relevance in cultivated plants (Loxdale et al., 2020). Sucking pest chemical management require the use of systemic insecticides such as neonicotinoids, which represent 25% of the global insecticides market (Bass et al. 2015).

Neonicotinoids are the most important chemical class of insecticides introduced into global markets in the 90 decade as less toxic than previous conventional pesticides, mainly the

synthetic pyrethroids, which are not only applied against some arthropods pests in agriculture, but also with use for veterinary and urban pests (Abdel-Halim & Osman, 2020; Nauen et al., 2008). They are chemicals that act at the level of the central nervous system, persistently activating nicotinic receptors that cause the overstimulation of synapses, producing hyperexcitation, paralysis and subsequent death (Puricelli & Arregui, 2008). In relation to its broad spectrum characteristic, these insecticides could affect several non-target organisms such as lacewings, which one habit agroecosystems spraying with these compounds. The most common neonicotinoids used in Argentina correspond to imidacloprid, acetamiprid, thiacloprid, clothianidin, and thiamethoxam (CASAFE, 2023; Puricelli & Arregui, 2008).

A previous imidacloprid toxicity assessment on *C. externa* showed that this neonicotinoid insecticide reduced the egg viability when it or they were or was applied in eggs 24 h-old treated (Pasini et al., 2018). In the case of imidacloprid it was corroborated that it induced DNA damage in *Nile tilapia* (*Oreochromis niloticus* (L.)) and red earthworms (*Eisenia fetida* (Savigny)) (Ansoar-Rodríguez et al., 2015; Wang et al., 2016). However, its genotoxicity and effects on DNA remain unknown for biological control agents of economic relevance such as Chrysopidae according to our knowledgement.

The objective of this study was to analyze the genotoxicity of imidacloprid on the biological control agent *C. externa* at DNA level using the comet assay as an ecotoxicological biomarker. It is relevant to highlight that this technic was used for the first time for this organism under laboratory insecticide exposition.

2. Materials and methods

2.1. Insects

Chrysoperla externa eggs used in this study, which were never exposed to pesticides, were obtained from permanent colonies of the Laboratory of Ecotoxicology, Pesticides and Biological Control of “Centro de Estudios de Parasitología y Vectores” (CEPAVE), “Universidad Nacional de La Plata”- CONICET - CICPBA, located in La Plata, Argentina which were never exposed to pesticides previously. Colonies units were performed from mated adults (around 50-80 females), placed in ventilated transparent plastic containers (5 l, 21 cm diameter, 25 cm high) covered with a fine mesh following the rearing method developed in our laboratory and described in Haramboure et al. (2016). Briefly, considering that Chrysopidae adults have no predator behavior, they were reared on an artificial diet based on honey, wheat germ, and brewer’s yeast, which is commonly used for adults of the species (Vogt et al., 2000), and tap water *ad libitum*. In addition, clean black cardboard (15 cm width × 15 cm height) was added inside containers (the walls of the containers were lined) as an oviposition substrate and replaced periodically. Colonies were maintained in a bioterium at 25 ± 5°C, 70 ± 5% relative humidity, and under a photoperiod of L:D 16:8.

2.2 Biossays

2.2.1 Insecticides and Treatments

For the bioassays, the commercial product formulated Confidor OD® (imidacloprid 20% ai, LS, Bayer CropScience, S.L Argentina) was used.

The two most recommended field rates of this insecticide for pest control in vegetable crops in Argentina were used (CASAFE, 2023).

Field rates of 50 and 90 cm3/hl equivalent to 100 and 180 mg/l of active ingredient, respectively were chosen for bioassays and distilled water was used as solvent to prepare the insecticide solutions. Black cartons with lacewing eggs less than 24 h old were removed from the plastic containers of the *C. externa* colonies and cartons with groups of 60 eggs were randomly selected for each treatment. The selected eggs were dipped in a Confidor OD® solutions for 15 s. Controls were treated with solvent alone. After the application, the cards with eggs were kept under a hood until completely dry and were placed in ventilated petri dishes in a bioterium at 25 ± 5°C, 70% ± 5% relative humidity and under a photoperiod L:D 16: 8 until preparation of comet trials.

2.2.2 Set up of the comet assay

The first step in this study was to develop and standardize a specific protocol for comet assay for *C. externa* eggs, due to there were not available previous ones for similar arthropods. Briefly, a single-cell gel electrophoresis was performed using the alkaline version described by Singh et al. (1988) with some modifications. To set up the comet assay, tests were performed combinations of lysis solutions, and electrophoresis run times. The protocol that yielded the best results was that use of whole eggs, from which the intrachorion material was extracted in a macerate with 30 µL of distilled water. The cell solution was suspended in 170 ul of low melting point agarose (0,5%). This material was seeded on two slides covered with 100ul of normal melting point agarose (0,5%). Each sample was covered with a coverslip and refrigerated at 4 °C for 10 min. The coverslip was then removed and placed in a lysis solution (2.5 MNaCl, 100 mM Na_2EDTA, 10 mM Tris, 0,5% Triton X-100, pH 10). The slides were placed in a horizontal electrophoresis tank covered with a buffer solution (pH > 13) composed of distilled water, NAOH, and EDTA for 15 min at 4 °C at 25 volts and 250 mA. Once the run was complete, the slides were washed three times with Tris-HCl buffer solution (pH 7.5) for one minute at room temperature, then with distilled water, and placed in 96% ethanol for 10

153 min. Finally, the samples were dried at room temperature. The preparations were stained with
154 SYBR Green dye and observed by fluorescence microscopy with a 40x Olympus BX51
155 objective and an Olympus DP71 camera, and images were obtained with the DP controller
156 3.3.1.292 and DP manager 3.3.1.222 program.
157

158 ### 2.2.3 Variables evaluated in Comet assay

159 Chrysoperla externa 24 hs old eggs were treated with imidacloprid solutions and evaluations
160 were done at 24 and 96 hs after insecticide applications, when the embryo begins to manifest
161 within the egg (24 hrs) or when it is fully formed (96 hrs) (Fernandez Acevedo et al., 2022).
162 Treatments were named according to egg age and insecticide concentrations applied.
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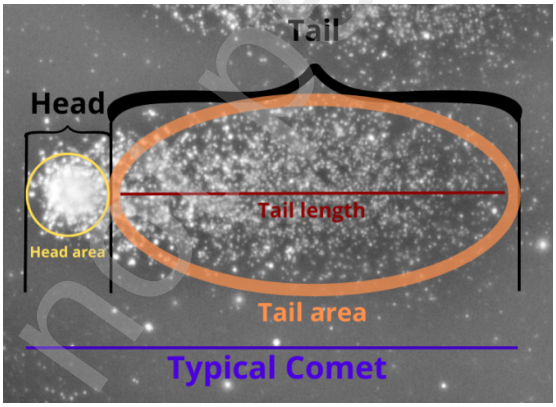


164
165 Figure 1: Typical comet description. Cells are composed of a head and tail. Variables
166 analyzed to determine DNA damage. Comet tail width (red), comet tail area (orange) and
167 comet head area (yellow).
168

169 The comet from C. externa eggs were observed having into account the typical comet
170 description (Figure 1). The damage index (DI) was calculated according to Collins (2004); 100
171 comets were randomly counted per slide and this procedure was replied twice. The comets

were classified into five categories or degrees according to the damage (Figure 2). Each image was assigned with a value between 0 and 4 and the DI was calculated using the formula:

DI= (1x Nd1 + 2 x Nd2 + 3 x Nd3 + 4 x Nd4) / Total comets observed.

Where Nd represents the number of comets at each value. Besides, DNA damage has been estimated through the relationship between tail and head areas using the formula:

%DNA damage= tail area / (tail area + head area) X 100

Direct measurements were performed to calculate tail length in µm. Both variables were counted in 100 comets per treatment and measured using the Image J software.

2.3 Statistical Analyses

All statistical analyses were performed using R software. Permutation two-way ANOVA with interaction. Eta squared (η^2) was used as a measure of effect size. Post-hoc analyses were performed considering the variables analyzed with adjustment of the level of significance using the Sidak method ($p<0.05$).

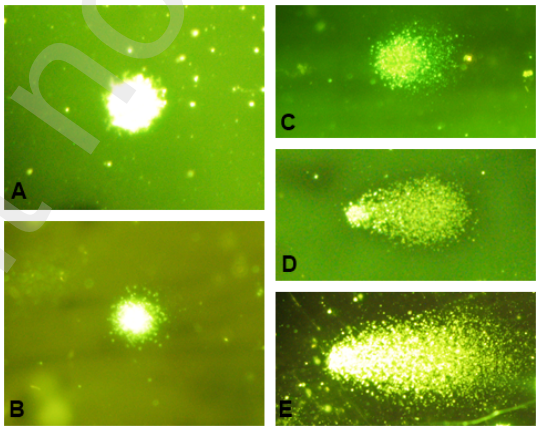


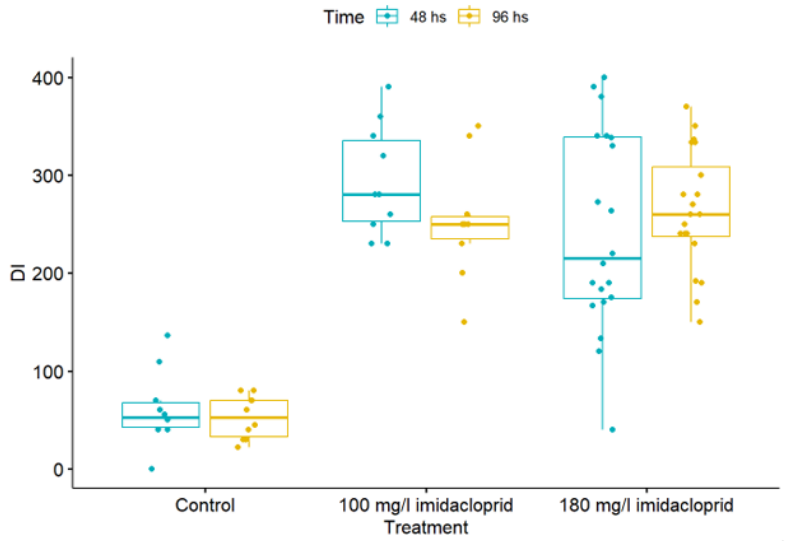
Figure 2: Classification degrees of comets. A: grade 0. Comets without halo around them. B: grade 1. Comets with tails smaller than the nucleus diameter C: Grade 2. Comets with a tail between one and two diameters of nucleus. D: Grade 3. Comets with tails between 2 and 3

192 times the diameter of the nucleus. E: Grade 4: Comets with a tail larger than three core
193 diameters with wide spread of DNA.
194

195 3. Results

196 The damage index (DI) caused by imidacloprid was observed at both concentrations evaluated
197 and it was similar at both development time of embryos developing inside of eggs. In this
198 sense, the damage index registered in the treated samples for both eggs development times
199 was much higher than that of the control (Figure 3). These differences were marked by
200 insecticide treatment *(F=67.11; df=2;* $p < 1x10^{-8}$*)* whereas both age of egg evaluated were
201 similar (*F=0.01; df=1; p =0.905*). The η^2 for the treatment reaches 0.63, where this 63% of
202 DNA damage (ID) ID is explained by the applied product.

203 In the comparisons between different treatments, the control showed differences both with the
204 lowest and he highest concentration ($p < 1x10^{-8}$). The DI tended to have a greater difference
205 than the control at the lower concentration, influenced by the high proportion of damage at 48
206 h of development of embryon. However, there were no significant differences between the
207 concentrations with respect to the DNA damage caused (p=0.62).



Treatment	Time	media	sd	n
Control	48 hs	61.1	37.93	10
Control	96 hs	52.7	21.94	10
100 mg/l imidacloprid	48 hs	294.0	55.82	10
100 mg/l imidacloprid	96 hs	253.0	58.70	10
180 mg/l imidacloprid	48 hs	242.7	101.77	20
180 mg/l imidacloprid	96 hs	263.7	61.12	20

208

209 Figure 3: Genotoxicity of Imidacloprid on *Chrysoperla externa* eggs. Damage Index (DI)
210 measured in arbitrary units. The Damage Index evaluation times were 48 hours (blue) and 96
211 hours (yellow) of embryo development inside the egg. The concentrations of the treatments
212 were 0 (controls), 100 nd 180 mg/l active ingredient of imidacloprid. Boxes indicate mean
213 values and standard deviations (sd). Points represent all samples tested.

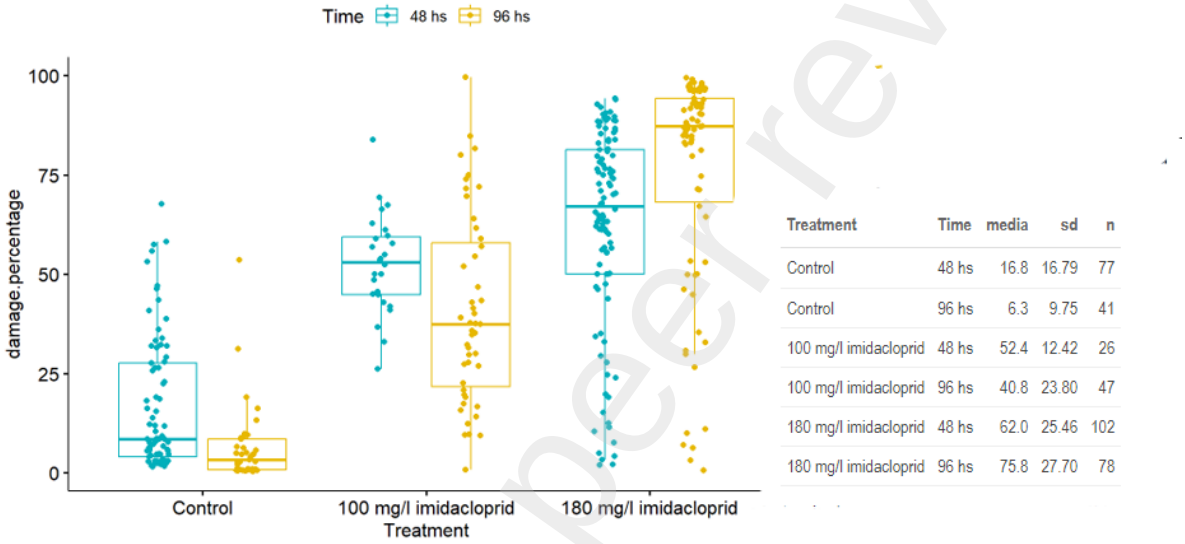
214

215 The percentage of damage presented increasing mean values with respect to the
216 concentration regardless of the development time, with the highest value being the eggs at 96
217 h of development that correspond to the highest dose of imidacloprid (75.8%, se=27.70)
218 (Figure 4).

219 There was an interaction between the treatment and age of development regarding the
220 percentage of damage (*F=13.53; Df=2*; $p = 2.1 \times 10^{-6}$). Similar to the previous parameters,
221 the percentage of damage was mainly influenced by imidacloprid treatment ($\eta^2 = 0.52$) and not
222 by the age at egg development ($\eta^2 = 0.03$).

223 Eggs 48 hours old show a higher percentage of damage in both treatments with imidacloprid
224 compared to control eggs ($p < 1 \times 10^{-8}$) but not between the evaluated concentration

(p=0.266). The 96-h-development eggs presented not only a higher percentage of damage in those treated with imidacloprid ($p< 1\times10^{-8}$), but also, between concentrations at a higher dose of active ingredient, the damage increases ($p< 1\times10^{-8}$). The 180 mg/l of imidacloprid produces a higher percentage of damage in eggs with 96 hours of development than in eggs with 48 hours of development ($p=1.3\times10^{-4}$), that is to say, the variable increased with time.



Treatment	Time	media	sd	n
Control	48 hs	16.8	16.79	77
Control	96 hs	6.3	9.75	41
100 mg/l imidacloprid	48 hs	52.4	12.42	26
100 mg/l imidacloprid	96 hs	40.8	23.80	47
180 mg/l imidacloprid	48 hs	62.0	25.46	102
180 mg/l imidacloprid	96 hs	75.8	27.70	78

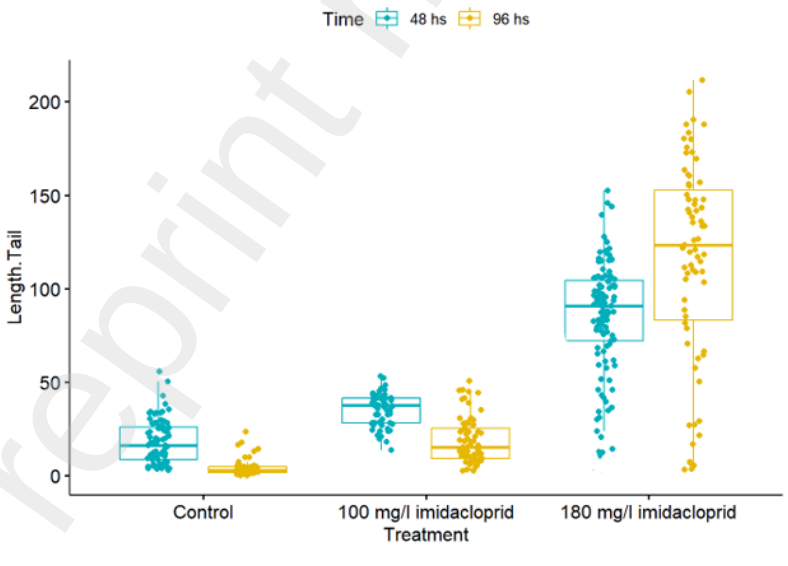
Figure 4: Genotoxicity of Imidacloprid on *Chrysoperla externa* eggs. Damage percentage measured in percentage. The evaluation times of the damage percentage were 48 hours (blue) and 96 hours (yellow) of embryo development inside the egg. The concentrations of the treatments were 0 (controls), 100 and 180 mg/l active ingredient of imidacloprid. Boxes indicate mean values and standard deviations (sd). Points represent all samples tested

The tail length parameter of comets presents an interaction between the evaluated variables. DNA damage evaluation through the comets tail length showed that both concentrations of imidacloprid assayed and development times of eggs caused variations in the length of tail comets ($F=33.93$; $df=2$; $p < 1\times10^{-8}$) (Figure 6). At the lowest concentration assayed tail length were greater in eggs at 48 h of development, with a mean of 35.2 µm (sd= 8.81 µm) (following

a pattern C of Figure 2) than in eggs at 96 h of development with 18.7 µm (sd= 12.17 µm) (following a pattern B of Figure 2) (Figure 5). At the highest concentration of imidacloprid, eggs at 96 h of development had a greater mean tail length, with mean values of 115.3 µm (sd= 54.33 µm) corresponding to comets grade E (Figure 2) than eggs at 48 h of development, with mean values of 84.9 µm (sd=30.29 µm) (comets grade F of Figure 2) (Figure 5). Besides, when measuring the size of the effect, treatment ($\eta^2 = 0.61$) was the factor that most influenced variability, and its effect was much higher than that of development time ($\eta^2 = 0.05$).

At 48 hours of development, the longest tail was present at the highest concentration evaluated ($p < 1\times10^{-8}$), an intermediate value at the lowest concentration, and the lowest value in the control. The same situation that occurred in the eggs of 96 hours of development ($p < 1\times10^{-8}$). That is, the higher the concentration, the greater the length of the comet tail, regardless of the development time (Figure 6).

When comparing the different concentrations, the eggs dipped in 100 mg/l a.i. of imidacloprid had a longer tail at 48 hours of development than at 96 hours of development (p= 0.0027) and the eggs dipped in 180 mg/l a.i. of imidacloprid of insecticide had a longer tail at 96 hours than at 48 hours of development ($p < 1\times10^{-8}$) (Figure 5 and 6).



Treatment	Time	media	sd	n
Control	48 hs	18.0	11.69	78
Control	96 hs	4.5	4.99	46
100 mg/l imidacloprid	48 hs	35.2	8.81	59
100 mg/l imidacloprid	96 hs	18.7	12.17	69
180 mg/l imidacloprid	48 hs	84.9	30.29	117
180 mg/l imidacloprid	96 hs	115.3	54.33	71

Figure 5. Genotoxicity of Imidacloprid on *Chrysoperla externa* eggs. Comet tail length measured in micrometers. The tail length evaluation times were 48 hours (blue) and 96 hours (yellow) of embryo development inside the egg. The concentrations of the treatments were 0 (controls), 100 nd 180 mg/l active ingredient of imidacloprid. Boxes indicate mean values and standard deviations (sd). Points represent all samples tested

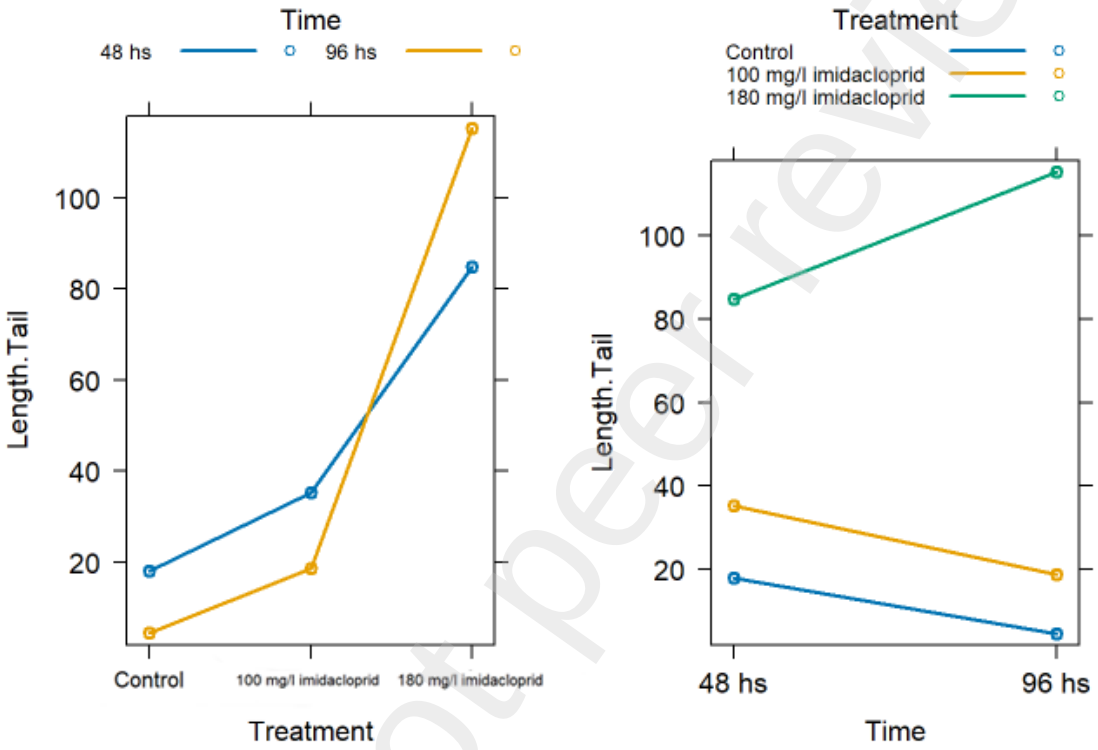


Figure 6. Genotoxicity of Imidacloprid in *Chrysoperla externa* eggs. Effects of the interaction between egg development time and treatments. Comet tail length was measured in micrometers. Left graph: effect of treatment for two developmental periods. The tail length assessment times were 48 h (blue) and 96 h (yellow) of embryonic development within the egg. The concentrations of the treatments were 0 (control), 100, and 180 mg/l of the active ingredient, imidacloprid. Right graph: effect of treatment development time. The treatments were as follows: control (blue), 100 mg/l of active ingredient imidacloprid (yellow), and 180 mg/l of active ingredient imidacloprid (green).

4. Discussion

Comet assay analysis protocolized for first time for C. externa eggs was useful to corroborate DNA damage due to imidacloprid exposition at both concentrations studied. The lowest concentration evaluated corresponds to one of the lowest recommended field rates for pest control in agroecosystems in Argentina. These results indicate that the use of these concentrations was detrimental to *C. externa*, which is a non-target organism.

First, it is important to highlight that the controls presented basal damage because complete individuals were being analyzed and not only independent tissues, as is usually done in these assays, which entails differential electrophoretic damage in the tissues. Ecotoxicological assays on erythrocytes of *Rana nigromaculata Hallowell* (Anura: Ranidae) showed a direct relationship between comet grade and imidacloprid concentration (Feng et al., 2005), but not in studies on *Oreochromis niloticus* (Perciformes: Cichlidae) and *Misgurnus anguillicaudatus* exposed to imidacloprid, in which DNA damage was independent of the dose used (Ansoar-Rodríguez et al., 2015; Xia et al., 2016). In our study, the damage index did not show significant differences for the different doses, but differences were marked for tail length, because at higher doses of imidacloprid, smaller DNA fragments were produced as a result of strand breaks, alkali-labile sites, and delayed repair sites, similar to that reported by Xia et al. (2016).

With a single exposure to the lowest dose, the DNA of the cells analyzed at 48 h and 96 h of development showed the same damage (% damage); that is, they had no recovery capacity. In addition, the cells analyzed at 48 h presented shorter DNA fragments than those analyzed at 96 h.

Ecological studies carried out on earthworms (*Eisenia fetida*) with subchronic exposure to analytical-grade imidacloprid showed damage not only at the DNA level but also lipid peroxidation due to the increase in reactive oxygen species and the involvement of antioxidant

enzymes (Wang et al., 2016). Studies have concluded that imidacloprid favors the production of reactive oxygen species (ROS) at the cellular level (Abdel-Halim & Osman, 2020; Duzguner & Erdogan, 2012; EL-Gendy et al., 2010; Ge et al., 2015). .

In these studies, the main hypothesis of damage to *C. externa* eggs was that exposure to low doses of imidacloprid produces an increase in the amount of ROS, and it would seem that the antioxidant system fails to compensate for this increase causing an imbalance that would lead to an increase in DNA breaks that are maintained throughout the development time. The main difference in the length of the fragments could be that, at 48 h of development, there is high cell division, with a greater number of okasahi fragments; therefore, the basal DNA damage is greater at this time. These fragments did not form a complete DNA chain because of the addition of ROS to the structures. On the other hand, at the highest dose, the more advanced the stage of egg development, the more DNA damage was observed. The ROS production rate was so high that it appeared that the antioxidant complex weakened over time. This would cause an imbalance, and the unbalanced ROS would cause widespread breaks in the cells and DNA.

The use of imidacloprid in insects not only causes direct damage at the central nervous system level, but also causes damage at the cellular level, damaging biological molecules of utmost importance. Specific applications of *C. externa* eggs produce breaks at the DNA level, which cause irreparable damage to organisms in the short and medium term. This situation could be repeated in other non-target organisms, causing environmental damage in agricultural fields that is not being taken into account. It is extremely important to know what these synthetic molecules are produced in the environment, to estimate the environmental damage caused by their release, not only in wildlife, but also their involvement in human health and nutrition.

5. Conclusions

The new comet assay protocol for *C. externa* is novel and has not been previously described. It was concluded that Confidor OD® (imidacloprid 20% ai) and its concentrations related to the maximum field recommended concentrations (MFRC) caused DNA damage in *C. externa* eggs regardless of the evaluated concentration and eggs age.

The comet assay as a tool for the evaluation of genetic damage in non-target organisms of agriculture is something new and easy to use in the terrestrial ecotoxicology studies.

6. Author contributions

Victoria Fernandez Acevedo: Conceptualization, Methodology, Investigation, Formal analysis, Writing - Original draft preparation; **AnaliaSeoane y Sergio Rodriguez Gil**: Conceptualization, Methodology, Supervision, Investigation, Formal analysis, Writing; **Graciela Minardi**: Statistical analysis; **Marcela Inés Schneider**: Conceptualization, Methodology, Supervision, Resources, Funding acquisition, Writing - Reviewing and Editing.

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