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Multigenerational effects of the insecticide Pyriproxyfen and recovery in *Daphnia magna*

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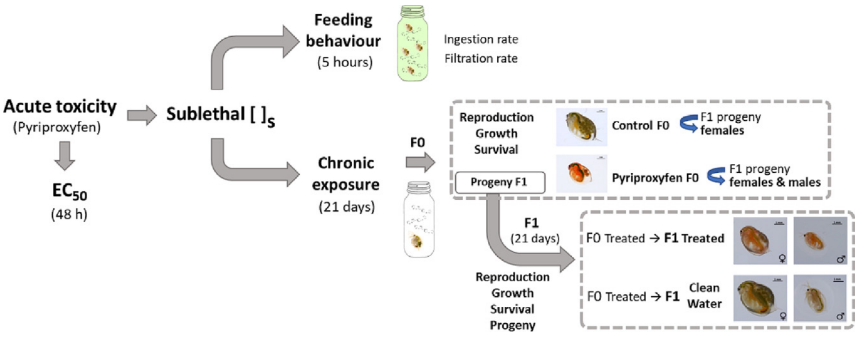
HIGHLIGHTS

- Pyriproxyfen sublethal concentrations induced changes in reproductive parameters.
- Pyriproxifen *D. magna* multigeneration study
- Population recovery process after exposure of mothers to insecticide
- Pyriproxifen induced morphological abnormality and appearance of males in daphnid population.

GRAPHICAL ABSTRACT



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ABSTRACT

In the present study, an ecotoxicological approach to the evaluation of the insecticide Pyriproxifen in the crustacean *Daphnia magna* was done. Acute toxicity tests (48 h), feeding behavior test (5 h) and chronic toxicity test (21 days) were carried out on a parental daphnid generation (F0). Pyriproxifen *D. magna* EC_{50} value in our experimental conditions was 336.47 μg/L. Based on this result, sublethal test concentrations were selected for the feeding study and the F0 chronic experiment. Filtration and ingestion rates of *D. magna* exposed animals did not show any significant difference respect to control daphnids. However, daphnids from the parental F0 generation when exposed to the insecticide during 21 days showed a decreased in all the reproductive parameters tested (mean total neonates per female, mean brood size, time to first brood, and mean number of broods per female) as well as in the population statistic growth rate (r), although survival was not affected. On the other hand, offspring from F0 females exposed to the highest Pyriproxifen concentration (14.02 μg/L) were separated in two F1 generation experiments. One group was transferred during 21 days to a medium free of toxicant (F1 generation-TC group) while the other group was exposed during 21 days to the same insecticide concentration as their mothers (14.02 μg/L) (F1 generation-TT group). Results from both experiments determined a decreased in most of the reproductive parameters which was higher in the F1-TT group, although some of them were recovered in the F1-TC group. On the other hand, the morphological analysis of the daphnids showed that the coloration pattern was altered in the daphnids exposed to the insecticide, together with a significant size decreased, and neonates from F0 progeny with the same morphological abnormality. Finally, we determined that the insecticide caused the appearance of males among the offspring generated by the F_0.

1. Introduction

Pyriproxyfen is an insecticide currently used by many countries to control pests in agricultural crops such as cotton, orange, almond, apple, and

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coffee, among others (Sullivan and Goh, 2008). It is also used indoors to control domestic pests such as the German cockroach (*Blattella germanica*) (Saltzmann et al., 2006) or the common fly (*Musca domestica*) (Biale et al., 2017), and is marketed for topical use in cats and dogs for the prophylactic control of fleas (Stanneck et al., 2002). However, one of its most widespread uses is to control mosquito pests such as the species *Anopheles arabiensis* (Lwetoijera et al., 2014), *Anopheles gambiae* (Jaffer et al., 2015), *Culex quinquefasciatus* (Jambulingam et al., 2008), *Aedes albopictus* (Suman et al., 2018) or *Aedes aegypti* (Tsunoda et al., 2013), insects that through their bites transmit diseases such as malaria, filariasis, yellow fever, dengue or chikungunya, respectively.

This compound is used worldwide in public health programs as an alternative to organophosphate and pyrethroid pesticides. Due to their extensive use, they have generated resistance in target insect populations in various regions of the planet (Lima et al., 2011). It is also used to control multiple pests both in drinking water sources and water bodies, at concentrations of up to 10 and 100 μg/L, respectively (Lajmanovich et al., 2019). Numerous studies show that this insecticide is present in many rivers (Campo et al., 2013; Masiá et al., 2015; Ccanccapa et al., 2016a), reaching concentrations up to 37.74 ng/L detected in the Ebro River (Ccanccapa et al., 2016b), and 99.59 ng/L (equivalent to 1 % with respect to the concentration determined as a safe concentration for drinking water by the World Organization of Health established at 10 μg/L, 2007) in the Júcar River (Moura and Souza-Santos, 2020), both in Spain.

Pyriproxyfen is a juvenile hormone agonist, since its structural formula is similar to it, although much more stable, so it actively competes for its receptors (Sullivan and Goh, 2008). Under natural conditions, the insect stops synthesizing this hormone to give way to the adult state through metamorphosis. Nevertheless, in the presence of analogous, as these continue to bind to the receptors of the juvenile hormone, it prevents the interruption of the process making it impossible to pass from the larval phase to the pupa, altering its natural development and, therefore, making its metamorphosis to the adult stage impossible (Dhadialla et al., 1998).

Therefore, the Pyriproxyfen effects on insects is very variable, among others, its ability to prevent the hatching of eggs (Ohba et al., 2013), affect larval development (Wang et al., 2013), or promote morphological aberrations and reduce fertility in adults has been cited (Maoz et al., 2017). Thus it has also been classified as an endocrine disruptor (Sullivan and Goh, 2008).

Its mode of action in other animal taxa is not known exactly, but there is evidence of its effect on the development of aquatic vertebrates like *Danio rerio* or *Rhamdia quelen*, causing an increase in mortality at low doses (Dzieciolowska et al., 2017; Azevedo-Linhares et al., 2018), malformations, and an enhancement of its effect when associated with microcystin, a toxin produced by cyanobacteria in eutrophic aquatic environments (Azevedo-Linhares et al., 2018). Numerous studies have shown that the effective concentrations used to control mosquito pests produce adverse effects on other terrestrial organisms such as honey bees, or invertebrate aquatic organisms (amphipods, crabs, daphnia), and vertebrates (zebrafish), both in embryos and adults (Truong et al., 2016; Lawler, 2017; Azevedo-Linhares et al., 2018; Maharajan et al., 2018, 2020). Furthermore, several studies have already shown that this compound induces the appearance of males in *Daphnia* broods exposed to this toxicant (Ginjupalli and Baldwin, 2013; Abe et al., 2015; Watanabe et al., 2018).

One of the most widespread and standardized ways to assess the adverse effects of a pesticide is to study the evolution of a daphnid cohort using chronic tests. These kinds of tests allow us to determine their consequences at different levels derived from their exposure, such as the effects on reproduction, development, growth and survival. Complementarily, it could be very useful a feeding behavior study in order to determine the neonate pesticide effect on feeding uptake because it could determine a future effect on reproduction as well as final growth.

In addition, we will get more information if we determine these effects not only to a single daphnid generation but also on several generations. Then, we could have more valuable information about the damage that the pesticide would cause to a daphnid population.

Therefore, the objective of this study focuses on evaluating both the pyriproxyfen chronic effects through the OECD standardized test (reproduction, survival, individual growth) in a parental daphnid generation (F_0) as well as in its progeny (F_1), in order to evaluate the insecticide transfer between generations.

2. Materials and methods

2.1. Test organisms

The animals used in the present study belong to the species *Daphnia magna*, clone K6 (Muyssen and Janssen, 2001), came from a colony which is permanently maintained in our laboratory following the recommendations of the OECD (2012). The animals were kept in 5-litre capacity aquariums with a density of 30 individuals per aquarium and whose culture medium was renewed weekly. Tap water from the municipal supply network was used to keep the daphnid culture (total hardness 210 ± 10 mg/L $CaCO_3$; pH 7.9 ± 0.2; alkalinity 4.1 mmol/L; dissolved oxygen saturation > 90 %) it was previously dechlorinated by aeration and with an oxygen saturation >90 %. Every two days the aquariums were filtered to eliminate the neonates, keeping on only the reproductive females. Constant conditions in the climate chamber were a temperature of 22 ± 1 °C, a light intensity of 64 ft-c, and a photoperiod of 16:8 h light:dark.

The alga *Nannochloris oculata* was used as fresh food, and was supplied to the daphnia colony three times a week at a concentration of 5×10^5 cells/mL (Allen et al., 1995; Taylor et al., 1998).

Daphnia magna colony is tested annually using potassium dichromate at least once as the reference product, in order to assess its sensitivity (Kikuchi et al., 2017). Therefore, we ensure that the individuals used in the experiments keep a constant response to the selected toxicants.

2.2. Test chemical

Pyriproxyfen technical product was supplied by the company Plant Protection Company S.L (PROPLAN, Spain), number CAS 95737-68-1 (>97 % purity). Chemical name is 4-phenoxyphenyl (*RS*)-2-(2-pyridyloxy) propyl ether.

In order to prepare the different stock solutions to be used in the experimental assays, Pyriproxyfen was dissolved in ethanol, with a maximum final ethanol concentration of 0.28 μL/L. Previous experiments carried out in the laboratory showed that this ethanol concentration was safe for *D. magna* and no effect was observed in survival as well as reproduction (Dom et al., 2012).

2.3. Acute toxicity test

To study Pyriproxyfen acute toxicity, immobilization tests were carried out on *D. magna*, following the recommendations of the OECD (2004) "Guideline for Testing of Chemicals, Daphnia sp., Acute Immobilization Test".

The objective of the test was to determine the insecticide concentration necessary to produce 50 % of the immobilization in the study daphnid population for 24 and 48 h which is called Effective Concentration 50 (EC_{50}). Insecticide test concentrations used were 50, 260, 440, 780, and 1000 μg/L for 24 h exposure; and 50, 150, 260, 440, and 780 μg/L for the 48 h acute toxicity assay, plus a control group in each assay. Laboratory conditions were the same as described in chapter 2.1.

10 neonates (<24 h old) from the same brood were placed in glass beakers with 25 mL of each selected concentration plus a control group, consisting in a triplicate per experimental condition. Animals were not fed during the acute test. After 24 and 48 h of exposure, immobilized neonates were recorded.

2.4. Feeding behavior experiments

Daphnia magna neonates (<24 h old) from the culture were exposed for 5 h to the insecticide Pyriproxyfen, using several sublethal concentrations

which were based on the results obtained in the EC_{50} test at 48 h: 3.14, 4.74, 6.12, 8.41 and 14.02 μg/L. These tests were carried out following the recommendations of Ferrando and Andreu-Moliner (1993).

For each test concentration plus a pesticide-free control group, 3 replicates were made, placing 10 neonates in each 25 mL glass container. The animals were fed with the algae *Nannochloris oculata* in a concentration of 5×10^5 cells/mL. All experiments were carried out in darkness for a 5 h period at 22 ± 1 °C. After 5 h of testing, the final algae concentration was determined by counting in a Neubauer blood cell counting chamber. With the data obtained in the experiments the following parameters were calculated, according to the equations of Gauld (1951).

$$F = \frac{V}{n} \cdot \frac{(\ln C_0 - \ln C_t)}{t} - A$$

$$A = \frac{\ln C_0 - \ln C_t'}{t}$$

$$I = F \cdot \sqrt{C_0 \cdot C_t}$$

where F was filtration rate, I was ingestion rate, C_0 initial algal concentration (cells/mL), C_t final algal concentration (cells/mL), t: duration of the experiment (5 h), n number of individuals used, V volume of the medium in μL and A was a corrected control factor.

2.5. Chronic toxicity test F_0 generation

All chronic toxicity tests with *D. magna* were carried out following the indications of the OECD 211 (2012). Neonatal individuals (<24 h) from the colony were exposed to different sublethal concentrations: 3.14, 4.74, 6.12, 8.41 and 14.02 μg/L for 21 days. These insecticide concentrations were prepared just before carrying out the daily renewals of the medium.

The experiment consisted of 15 daphnids individually disposed in glass containers with 50 mL of medium for each insecticide concentration and its control. The medium and food (5×10^5 cells/mL *Nannochloris oculata*) were renewed daily, transferring the daphnids to clean containers.

The following parameters were recorded throughout the 21-day chronic assay: time to first clutch, total number of broods per female, number of broods per female, and survival. Since the first clutch, daily neonates were preserved in ethanol after being counted for its subsequent morphological analysis. At the end of the chronic experiment (21-d), adult females were also measured under the stereoscopic microscope. Sex determination in neonates was performed by the presence or absence of the first elongated antennal pair, sexual dimorphism in this species in individuals <24 hour old allows sex differentiation according to OECD 211 (2012).

2.6. Chronic toxicity test F_1 generation

Simultaneously with the F_0 parental chronic test, a second chronic test (F_1) was carried out with daphnids belonging to the second brood of mothers exposed to the highest concentration of insecticide (14.02 μg/L), and from the control group. The purpose of this test was to evaluate the ability to recover of those neonates exposed from their birth to the highest concentration of pyriproxyfen (considered the most adverse condition). The neonates from the mothers exposed to the insecticide were subdivided into two groups, each one composed of 15 individuals. All neonates were selected and placed randomly in the vessels in order to perform the experiment for 21 days.

In the first experiment, the selected neonates were exposed to 14.02 μg/L of pyriproxyfen (the same concentration as their mothers), this group was designated as TT group, while in a second experiment another group of neonates (from the same exposed mothers) was placed in a clean medium, designated as TC group.

Additionally, a control group was also included consisting of neonates from control females recollected the same day as those from pesticide exposed mothers. The same data as in the F_0 parental chronic toxicity test was evaluated during the experiments.

2.7. Statistical analysis

To determine the EC_{50} values (24 and 48 h) and their confidence limits (95 %) in the acute toxicity tests, Probit regression analysis was performed.

ANOVA tests were applied to the feeding assay as well as for chronic test (F_0 and F_1 generations) in order to analyze their statistical relevance, followed by Dunnett's Multiple Comparison posthoc test for feeding and chronic test in F_0 generation, and Bonferroni's Multiple Comparison posthoc test for chronic test in F_1 generation. Normal distribution and equal variance were tested before ANOVA with Kolmogorov-Smirnov and Levene's tests. A GraphPad Prism 6 software (GraphPad Software, USA) was used to perform the different analyses, the significant level was set at $p < 0.05$.

3. Results and discussion

3.1. Acute toxicity assays

Acute toxicity tests results showed that pyriproxyfen insecticide had a higher toxic effect after 48 h of exposure compared to 24 h, since the EC_{50} at 24 h was 479.80 μg/L (388.38–568.96) and was reduced by 30 % at 48 h, 336.47 μg/L (262.69–416.51).

Some authors as Abe et al. (2015) recorded higher Pyriproxyfen toxicity in *Daphnia magna* after 48 h of exposure. They found a quite lower EC_{50}-48 h value of 185 μg/L. These findings could be due to the use of different solvents to prepare insecticide stock solutions. They used dimethylformamide instead of ethanol as a solvent, and they recorded some mortality in the solvent group in long-term experiments whereas non-mortality was registered in any of our long-term experiments.

Pyriproxifen acute toxicity studies in other crustaceans indicated a high variability in the results obtained due mainly to the insecticide chemical characteristics depending on the manufacturing company or the solvent used in the study. Aaen and Horsberg (2016) determined a LC_{50} of 676.6 μg/L in the copepod *Lepeophtheirus salmonis* exposed to 24 h to the insecticide. Similar results were found by Alves et al. (2019) in the crustacean *Artemia salina*, they calculated an EC_{50} value of 500 μg/L when exposed to Pyriproxifen for 48 h.

However, lower acute toxicity results were found in other species exposed to this pesticide as 73.24 μg/L in the copepod *Eurytemora affinis* (Legrand et al., 2017) or 98 μg/L in the shrimp *Leander tenuicornis* (Brown et al., 1996).

3.2. Feeding behavior experiments

No effects were observed on feeding behavior in neonates exposed to the insecticide, compared to the results obtained in the control group, since the rate of ingestion and filtration did not show statistically significant differences ($p > 0.05$) among the experimental groups.

Previous studies have shown how the effect on feeding behavior in the first hours of life was clearly compromised in daphnids exposed to other insecticides. Fernández-Casalderrey et al. (1994) observed how the insecticides endosulfan and diazinon caused a decrease in both parameters when *D. magna* neonates were exposed to different concentrations of these insecticides. Tests carried out with the insecticide tebuconazole on *D. magna* also revealed this decrease in feeding behavior after only 5 h of exposure to increasing concentrations of the toxicant (Sancho et al., 2009).

However, the alteration of these parameters can be determined by the action of the compound itself, since the success in feeding filter feeders is given by the individual's ability to mobilize the appendages and thus, by filtering the algae (Day and Kaushik, 1987). If the toxicant alters the nervous system and reduces coordination, or causes total or partial paralysis, ingestion and filtration rates will be reduced. Even certain compounds such as the insecticides permethrin or fenvalerate can cause the adhesion of food

Table 1
D. magna (F_0 generation) survival, fecundity, and body length after exposure to Pyriproxyfen for 21 days.

	Brood size	Number of broods per female	Days to first brood	Number of neonates per female	Longevity (days)	Body length (mm)
Control	23.52 ± 1.51	5.07 ± 0.13	7.80 ± 0.4	119.33 ± 9.49	21.00 ± 0.00	4.30 ± 0.13
3.14 μg/L	4.68 ± 0.30*	3.40 ± 0.25*	12.60 ± 0.44*	15.87 ± 1.07*	21.00 ± 0.00	3.57 ± 0.11*
4.74 μg/L	4.31 ± 1.07*	3.07 ± 0.13*	13.07 ± 0.39*	13.20 ± 3.09*	21.00 ± 0.00	3.54 ± 0.15*
6.12 μg/L	3.74 ± 0.24*	3.07 ± 0.25*	13.20 ± 0.54*	11.60 ± 1.16*	21.00 ± 0.00	3.55 ± 0.03*
8.41 μg/L	3.29 ± 0.18*	2.67 ± 0.21*	14.27 ± 0.65*	8.86 ± 0.69*	21.00 ± 0.00	3.52 ± 0.07*
14.02 μg/L	2.60 ± 0.15*	2.73 ± 0.25*	13.87 ± 0.78*	7.20 ± 0.83*	21.00 ± 0.00	3.37 ± 0.07*

* $p < 0.05$.

particles to the appendages, making their movement difficult (Christensen et al., 2005; Reynaldi et al., 2006).

Daphnia feeding is a complex behavior, as it is regulated by the interaction of physiological mechanisms, including both the coordination of intestinal function with the filtering apparatus (Lampert, 1987; Hartman and Kunkel, 1991). Low concentrations of metals such as cadmium or copper are known to impair feeding in case of intestinal poisoning in daphnia (Barata et al., 2005).

However, in others studies, the effect begins to be noticeable after 24 h of exposure (Araujo et al., 2019), or using juveniles or adults instead of neonates (Li et al., 2020). Therefore, it is possible that we did not find a significant reduction in the feeding rates of Pyriproxifen exposed daphnids because of a short 5 h exposure period.

3.3. Chronic toxicity test in F_0 generation

During the chronic toxicity study, both survival and reproduction data were recorded throughout the 21 days of the experiment. At the end of the study, all the individuals were photographed in order to take measurements of body length.

Table 1 and Fig. 1 show the results obtain in the chronic toxicity study using F_0 generation females. All of the test daphnids survived the 21 days

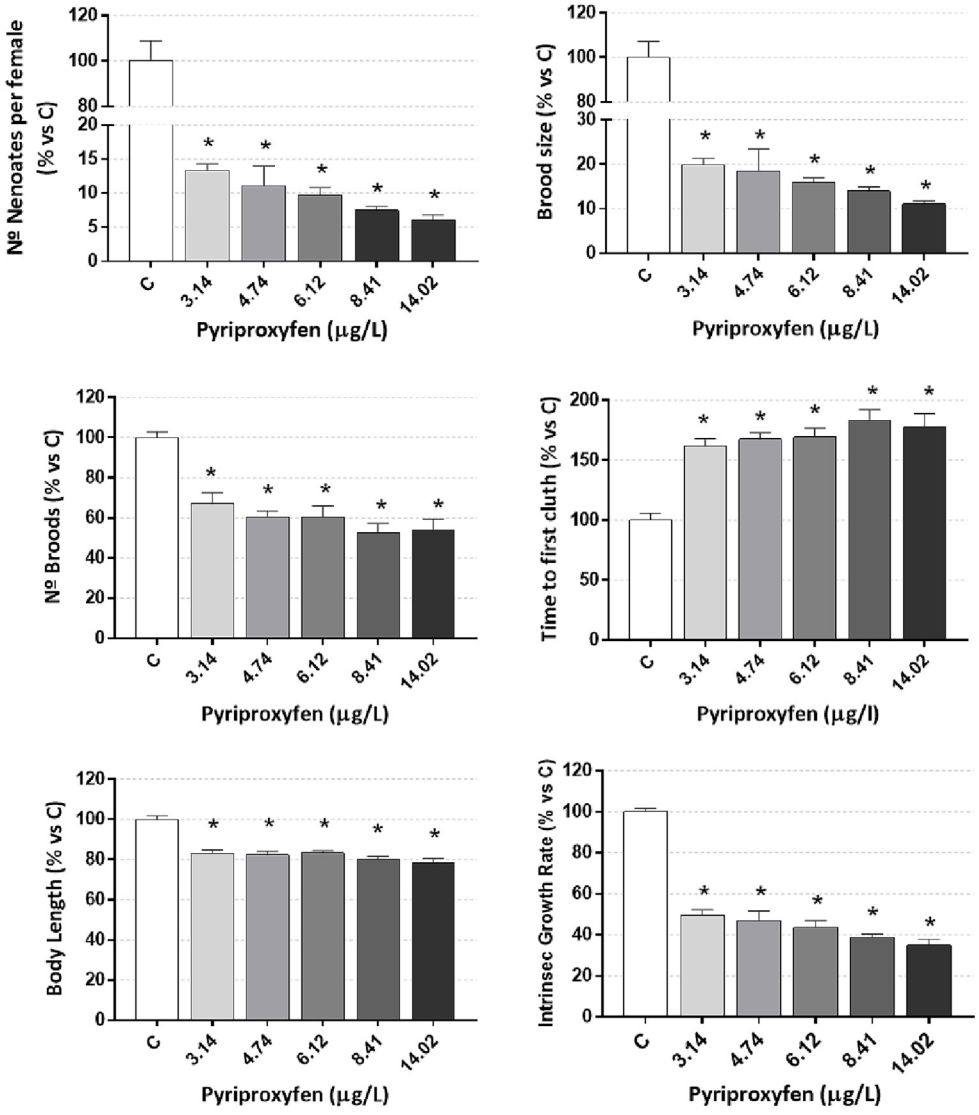

Fig. 1. *D. magna* chronic toxicity experiment in F_0 generation exposed to Pyriproxyfen for 21 days. * $p < 0.05$ statistical differences vs control group.

period. The brood size was already reduced by 80 % at the lowest insecticide concentration (3.14 μg/L), reaching almost 90 % at the highest (14.02 μg/L). The number of broods was also altered in *D. magna* exposed to all insecticide concentrations compared to the control group. Therefore, the control group had an average of 5 broods, while in the lowest concentration tested (3.14 μg/L) this parameter was reduced to 3–4 times (decrease of 33 %), reaching only 2–3 broods in the highest tested concentration tested (14.02 μg/L), leading to a decrease of almost 50 % on average compared to the control group.

On the other hand, the parameter time to the first brood was also altered on daphnids exposed to all the concentrations tested. The first clutch in the control group took place on day 8, while at the lowest concentration of insecticide, 3.14 μg/L, it was delayed until day 12, reaching up to 14 days in daphnids exposed to concentrations of 8.41 and 14.02 μg/L, which meant an increase from 60 % to 80 % in the lowest concentration and highest concentration respectively.

The total number of neonates per female was the most sensitive parameter. A decrease of 87 % in the case of *D. magna* exposed to the lowest tested concentration (3.14 μg/L) was observed. Those daphnids exposed to the highest insecticide concentration reached a 94 % of decrease in this parameter.

Regarding to body length, it was also altered in all the tested concentrations, producing a reduction in *D. magna* size from 17 % in those daphnids exposed to 3.14 μg/L of Pyriproxifen, to 23 % in the ones exposed to 14.02 μg/L.

The population statistic growth rate (r) was chosen as a parameter because it integrates survival as well as reproduction, and this parameter takes in consideration the great importance of the early reproduction for the population growth as was demonstrated by Daniels and Allan (1981). On the other hand, several studies demonstrated that in *D. magna* the r value at day 21 correlated in a 99 % to the r calculated for the entire life table (Daniels and Allan, 1981; van Leeuwen et al., 1985).

In the present study, the intrinsic growth rate (Fig. 1) was reduced in the daphnids Pyriproxifen treated groups up to a maximum of 0.11 in the highest concentration used, compared to the values obtained in the control group (0.32), assuming a maximum reduction of 65 %.

Daphnids constant exposure during 21 days to Pyriproxifen was enough to alter their offspring production and growth from the lowest to the highest concentration. A high depletion in all the reproductive parameters was observed specially in the number of neonates per female and brood size which could be considered as a good indication of the strong impact that Pyriproxifen had on *D. magna* population.

Our results agree with those found by Jordao Jordão et al. (2016) who observed that a concentration of 6.01 μg/L of Pyriproxifen affected the development of *D. magna*, decreased reproduction rate, inhibiting moult and increased male offspring production. This result reflects the high sensitivity of *D. magna* at low concentrations of this toxic agent.

Similar results were found by Kim et al. (2008) and Friberg-Jensen et al. (2010) when exposed *D. magna* to another insecticide as cypermethrin for 21 days and observed similar sublethal reproduction effects, such as a reduced total brood number, reduced brood size, increased time to first brood, and a high decreased in the total number of neonates produced. And several authors (Naddy et al., 2000; Zalizniak and Nugegoda, 2006) observed that insecticides such as chlorpyrifos induced a reduction in reproductive parameters when *Daphnia magna* were exposed to concentrations between 0.005 and 0.5 μg/L of the insecticide.

Fig. 3. Malformed progeny from F_0 generation parental females exposed to Pyriproxyfen, caudal spine is not completely extended.

Therefore, we can say from our results that while daphnids were able to survive for 21 days, exposure to the insecticide Pyriproxifen seriously affected *D. magna* reproductive parameters, which could be a serious problem for the population equilibrium as derivated from the reduction in the population growth rate (r) found in the present study.

The morphological analysis of the daphnids (Fig. 2) showed that the coloration pattern was altered in the daphnids exposed to the insecticide, going from the typical brown color in the control group (left side) to an orange-pink pattern (right side) in the daphnids exposed to the highest insecticide concentration (14.02 μg/L) on day 21. The different coloration detected in exposed daphnids was also correlated with the significant size decreased observed (Table 1 and Fig. 2).

On the other hand, when we looked at the F_0 progeny, we detected that all the neonates analyzed presented the same morphological abnormality (Fig. 3), with an incomplete extension of their caudal spine, and also, regarding to the sex ratio, >90 % of the offspring were male, although it did not follow a proportional increase to the exposure concentration (Table 2).

Palma et al. (2009), tested the effect of the insecticide chlorpyrifos on *D. magna* at concentrations of 0.01 and 0.03 μg/L and did not observe

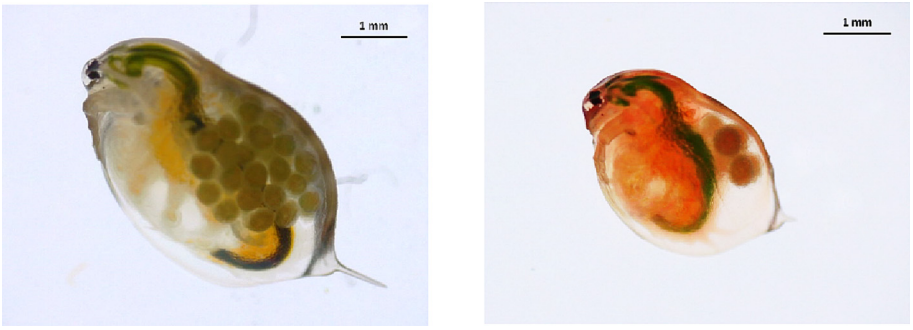

Fig. 2. Optical microscope photography of a control female (left side) with a typical phenotype of a normal individual at 21 days of age, and a female exposed during 21 days to 14.02 μg/L Pyriproxyfen (right side).

Table 2
D. magna F_0 progeny analyzed in the third brood onwards (percentage of males and females in each experimental group).

	Total neonates analyzed	Females (%)	Males (%)
Control	200	100	0
3.14 μg/L	39	5	95
4.74 μg/L	45	3	97
6.12 μg/L	33	3	97
8.41 μg/L	22	0	100
14.02 μg/L	13	15	85

any reduction in the reproduction of the exposed daphnids, however they found an increased in neonates malformations which were similar to those found in the present study.

Our results are similar as those found by other authors like Ginjupalli and Baldwin (2013) when exposed *D. magna* neonates (3–10 days old) to Pyriproxifen. As a consequence of the insecticide exposure a high amount of males were observed in the parental daphnids offspring together with a reduction in the broods size, and they found only few females in the offspring as in the present study. However, other authors as Matsumoto et al. (2008) and Abe et al. (2015) confirmed a 100 % of males prevalence in *D. magna* offspring after exposure to 0.1 μg/L and 1 μg/L of Pyriproxifen, respectively.

Different alterations in the offspring sex ratio females/males after *D. magna* exposure to Pyriproxifen were found by other authors as Olmstead and LeBlanc (2006) or Watanabe et al. (2018).

3.4. Chronic toxicity test in F_1 generation

Results from the chronic toxicity test carried out with daphnids from F_1 generation, coming from F_0 insecticide exposed (14.02 μg/L) mothers were shown in Table 3 and Fig. 4. As we can see in Table 3, all the control F_1 daphnids as well as F_1 daphnids from exposed mothers (TC and TT groups) survived 21 days.

F_1 generation daphnids in free pesticide medium during 21 days (TC group) showed a significant ($p < 0.05$) alterations in some reproductive parameters compared to control values. The number of broods per female decreased in a 19 % and time to first reproduction increased from 7.4 days in the controls to 10.67 days, however no changes were observed in the number of neonates per female, brood size or body length (Table 3 and Fig. 4), which indicated that F_1 generation daphnids from a parental F_0 generation exposed to Pyriproxifen showed a good recover in most of the studied parameters when the daphnids are transferred to clean water free of toxicant during 21 days.

On the other hand, F_1 generation daphnids transferred to a medium with Pyriproxifen (14.02 μg/L) (TT group) showed a significant ($p < 0.05$) alteration in all the reproductive parameters as well as in body length. A highest decreased was observed in both brood size (87 %) and number of neonates per female (94 %) respect to the controls. The number of broods per female also decreased in a 55 % respect to the control value and a high increased was observed in the time to first reproduction which changed from 7.4 days in the control group to 14.25 days in the exposed F_1 daphnids (Table 3 and Fig. 4). Relative to the body length of F_1 daphnids at day 21, females from TT group showed a decrease of 23 % with respect to the control group.

As we can see in Fig. 4, the intrinsic rate of natural growth (r) decreased significantly ($p < 0.05$) in both TC and TT groups with respect to control values, however, this decreased was quite higher in those F_1 daphnids exposed to 14.02 μg/L (TT group) due to the highest effect of Pyriproxifen in the reproductive parameters even survival was not affected.

On the other hand, when we compared the sex ratio of the offsprings in *D. magna* for both F_1 generation groups, those belonging to TC group were entirely females, while in the TT group we found also males. Therefore, F_1 females transferred to an insecticide free medium (TC group) but coming from parental females previously exposed to Pyriproxifen had only females in their broods, so it could be said that there wasn't a multigenerational effect on the sex determination induced by the pesticide.

Similar results were found by Sánchez et al. (1999) when exposed several *D. magna* generations to the insecticide diazinon. Reproduction was still reduced in F_1 generation daphnids from parentals (F_0) exposed to diazinon. Other authors as Villarroel et al. (2000) exposed *D. magna* to the acaricide tetradifon in a multigenerational study and their results indicated that reproduction was still reduced in F1 generation daphnids from parentals (F0) exposed tetradifon even after 21 days in clean water. However, survival was not different in those F1 offsprings from parentals pre-exposed to the acaricide.

On the other hand, some authors as Martins and Guilhermino (2018), studied the recovery ability of several *D. magna* generations after exposure to some microplastics. They found a recovery of several parameters when the daphnids were transferred to toxicant free medium (survival, number of broods per female and time to first brood) while others (brood size or number of neonates per female) needed several generations to complete recovery. Similar results were found by Völker et al. (2013), when studied the recovery of several *Daphnia* generations previously exposed to different silver nanoparticles. *D. magna* needed 4 consecutive generations in free toxicant medium to recover all the reproductive studied parameters.

Fig. 5 shows *D. magna* adult photographs from F_1 generation (females and males) at the end of the experiment (21 days). The determination of males was made based on the size of the adult individual at the end of the test, the elongation of the first antennal pair, and the shape of the valves (in females, the valves are symmetrical and rounded, while in the male, the upper part forms almost a 90° angle).

Several studies associated changes in the coloration of daphnids mainly as a consequence of exposure to hypoxic environments, going from a pale to pinkish and even reddish hue, due to the increase in haemoglobin in animals to counteract the low content of dissolved oxygen in the medium (Fox et al., 1951; Pirow et al., 2001). The structural formula of Pyriproxyfen does not present any group that justifies that result, so a possible explanation would be that the insecticide presence into the medium put the daphnids in a stress situation so they couldn't take enough oxygen from the water, causing them a deficit of oxygen and a consequent color change by increasing the haemoglobin content.

Our results agree with those found by Rider et al. (2005) who described how certain compounds as Pyriproxifen (23–64 ng/L) or Methyl farnesoate, induced a stress response in the crustaceans *D. magna* and *D. pulex* and as a consequence of that they found an increase in the haemoglobin content together with changes to pink color and a highest male production in the offspring.

On the other hand, the synthesis of haemoglobin induced by the insecticide Pyriproxyfen would act as a defensive mechanism against the toxic

Table 3
D. magna (F_1 generation) survival, fecundity and body length after exposure to Pyriproxyfen for 21 days (TT group), or in a free insecticide medium (TC group), and the control group.

	Brood size	Number of broods per female	Time to first brood (days)	Number of neonates per female	Longevity (days)	Body length (mm)
Control	20.06 ± 4.10	4.93 ± 0.26	7.40 ± 0.51	98.93 ± 20.93	21.00 ± 0.00	4.30 ± 0.27
TC group	22.17 ± 3.88	4.00 ± 0.00*	10.67 ± 0.58*	88.67 ± 15.50	21.00 ± 0.00	4.23 ± 0.10
TT group	2.71 ± 0.35*,$	2.25 ± 0.50*,$	14.25 ± 1.50*,$	6.00 ± 0.82*,$	21.00 ± 0.00	3.30 ± 0.05*,$

* $p < 0.05$ statistical differences vs control group.
$ $p < 0.05$ statistical differences between both groups (TT and TC).

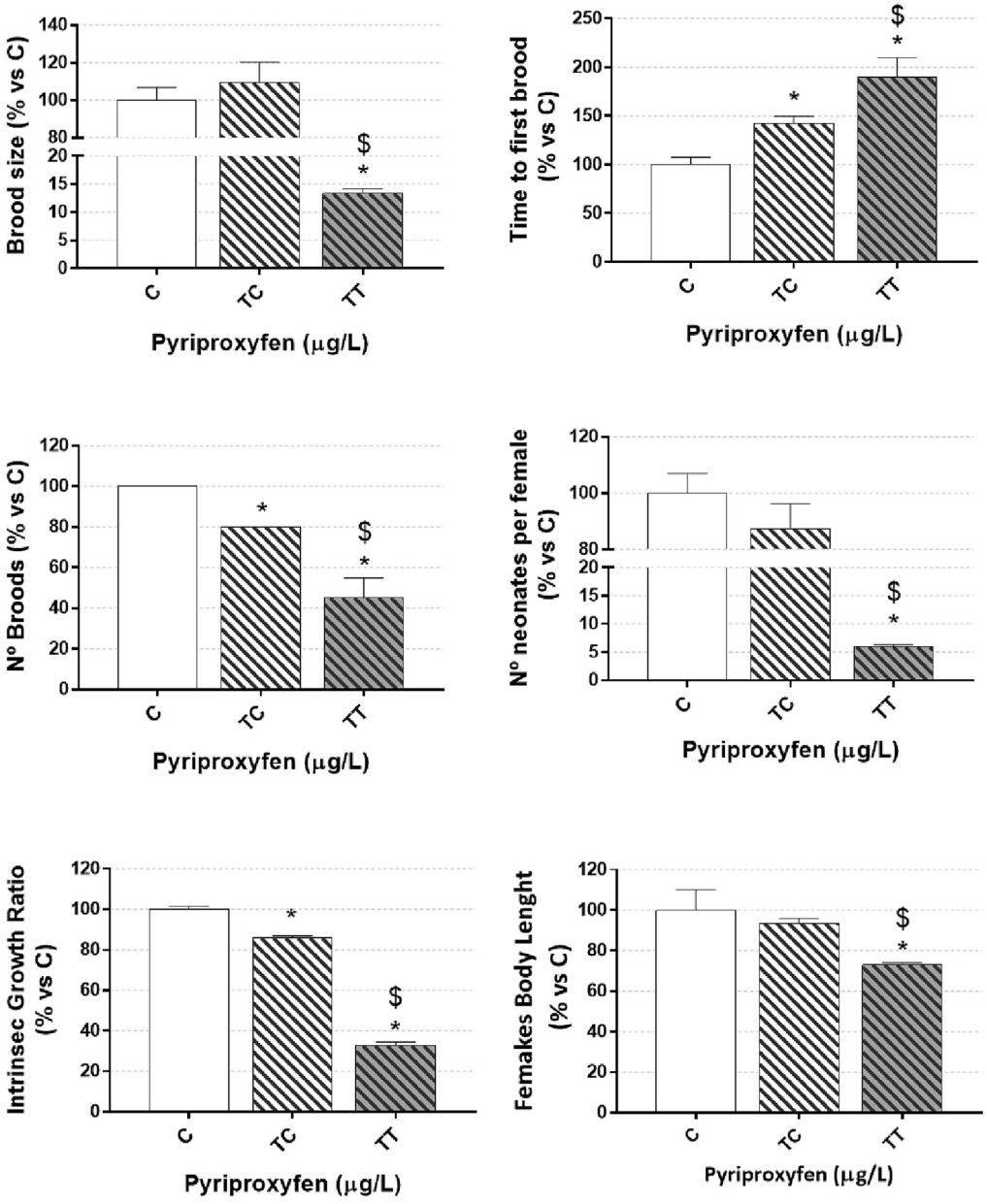

Fig. 4. *D. magna* F_1 generation chronic toxicity experiment for 21 days. (TT) means F_1 daphnids exposed to Pyriproxifen, (TC) means F_1 daphnids in free insecticide medium. *$p < 0.05$ statistical differences vs control group. $ $p < 0.05$ statistical differences between both groups (TT and TC).

stress as Eytcheson and LeBlanc (2018) hypothesized when they demonstrated that exposure to low concentrations of this insecticide (96 ng/L) for 48 h reduced the toxicity of sodium nitrite present in the medium as a consequence of the activation of haemoglobin synthesis in *D. magna*, thus acting as a defence mechanism against environmental contamination.

4. Conclusions

It is very important to study long-term effects of insecticides on aquatic organisms since they are present in the environment at very low concentrations. Constant exposure of sublethal Pyriproxifen concentrations on *D. magna* will impact these populations as indicated by the reproduction results.

On the other hand, the results found in the multigeneration study indicated that an F_1 generation daphnid from parental females exposed to Pyriproxifen would be able to recover in most of the reproduction parameters after 21 days in free toxicant medium while those F_1 daphnids transferred to a medium with the same insecticide concentration as their mothers would be very affected. The fact that the offspring are mostly males should not be overlooked, so even if the females do recover, the net reproductive capacity of the population would be affected.

More studies are needed regarding the environmental fate of Pyriproxifen in aquatic organisms, especially on the effects of sublethal concentrations. The information obtained in the present study will be useful to predict Pyriproxifen toxic effects in the aquatic ecosystems, although the environmental levels of this insecticide were very low to induce the direct mortality.

CRediT authorship contribution statement

Conceptualization: B.S; methodology and validation: B.S.; formal analysis: B.S; software: B.S; investigation: B.S; resources: D.F, E.S, J.T; data curation: B.S; visualization: B.S, E.S, D.F; writing—original draft preparation: B.S; writing—review and editing: B.S, D.F; supervision: E.S, D.F, J.T; funding acquisition: D.F, E.S, J.T. All authors have read and agreed to the published version of the manuscript.

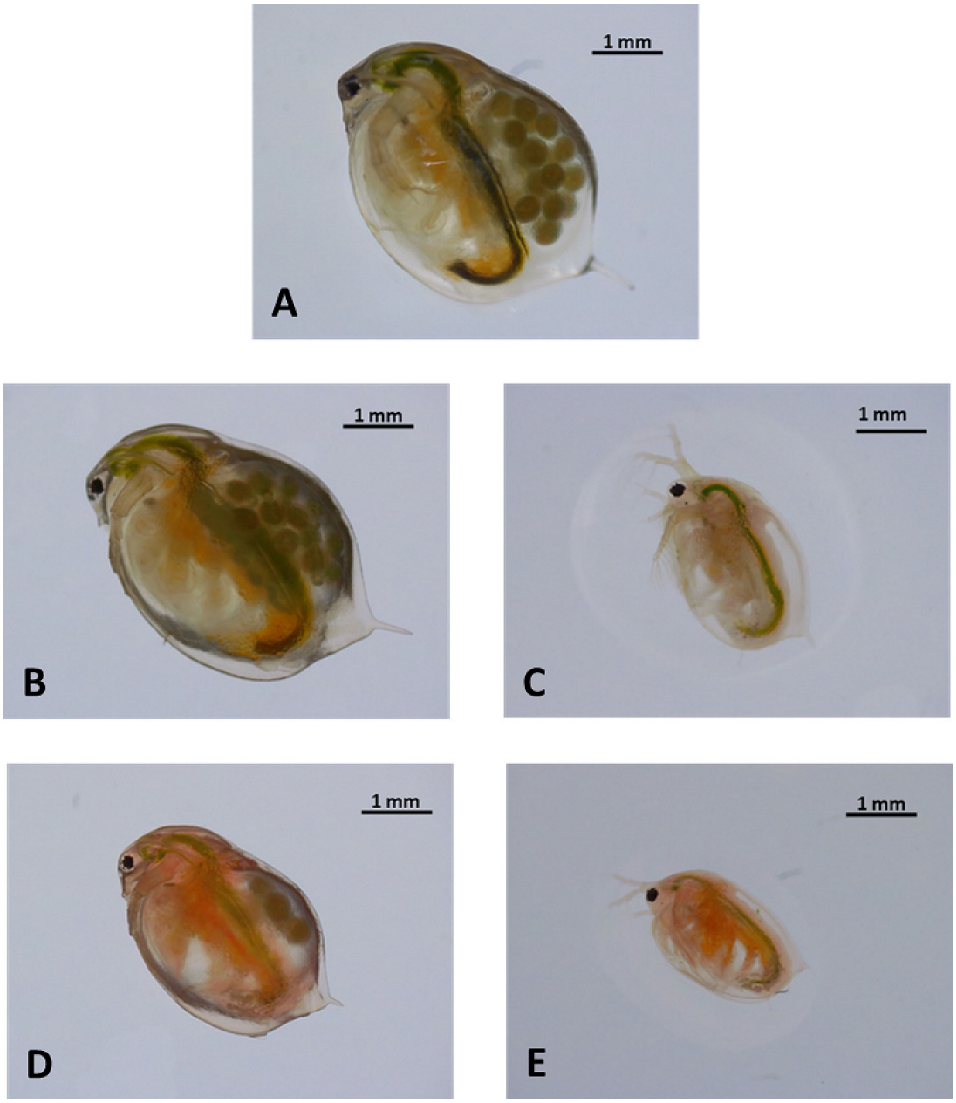

Fig. 5. 21-Day-old F_1 individuals from F_0 mothers exposed to Pyriproxyfen (14.02 μg/L). A: *D. magna* female from the control group. B: *D. magna* female from TC group C: *D. magna* male from TC group. D: *D. magna* female from TT group. E: *D. magna* male from TT group.

Data availability

No data was used for the research described in the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors would like to express their gratitude to the Fundación Universidad Católica de Valencia San Vicente Mártir and the University of Valencia for their support.

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