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SUBLETHAL IMPACTS OF PYRIPROXYFEN ON BIOLOGICAL TRAITS OF NON-TARGET SPECIES, *Drosophila melanogaster* (DIPTERA: DROSOPHILIDAE)

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ABSTRACT

Pyriproxyfen, a juvenile hormone analog (JHA), is considered as reduced-risk alternative to synthetic pesticides for crop protection. It has been frequently used in agriculture and public health to manage insect pests. However, recent studies have reported that pyriproxyfen may have adverse physiological effects on non-target organisms. This study investigated the effects of sublethal doses of the endocrine disrupting insecticide pyriproxyfen on *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) as a non-target and biological model. Results showed that pyriproxyfen had a noticeable effect on developmental stages of the individuals of the exposed generation. Pyriproxyfen treatment significantly shortens adult longevity of both sexes, female and male. Finally, these results suggest that reproduction capacity in *D. melanogaster* is impacted by reducing the number of progeny after the parent's generation treatment with pyriproxyfen. These research findings indicate that sublethal exposure to pyriproxyfen induces adverse physiological effects and affects offspring growth rates in non-target insects of *Drosophila*.
Additional keywords: *Drosophila melanogaster*, longevity, non-target insect, offspring development, pyriproxyfen

RESUMEN

Impactos subletales del piriproxifeno en rasgos biológicos de especies no objetivo, *Drosophila melanogaster* (Diptera: Drosophilidae)

El piriproxifeno, un análogo de la hormona juvenil (AHJ), se considera una alternativa de menor riesgo a los pesticidas sintéticos para la protección de cultivos. Se ha utilizado frecuentemente en la agricultura y la salud pública para controlar plagas de insectos. Sin embargo, estudios recientes han informado que el piriproxifeno puede tener efectos fisiológicos adversos en organismos no objetivo. Este estudio investigó los efectos de dosis subletales del insecticida disruptor endocrino piriproxifeno en *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) como modelo biológico y no objetivo. Los resultados mostraron que el piriproxifeno tuvo un efecto notable en las etapas de desarrollo de los individuos de la generación expuesta. El tratamiento con piriproxifeno acorta significativamente la longevidad adulta de ambos sexos, femenino y masculino. Finalmente, estos resultados sugieren que la capacidad de reproducción en *D. melanogaster* se ve afectada por la reducción del número de progenie después del tratamiento de la generación de los padres con piriproxifeno. Estos hallazgos de la investigación indican que la exposición subletal al piriproxifeno induce efectos fisiológicos adversos y afecta las tasas de crecimiento de las crías en insectos no objetivo de *Drosophila*.
Palabras clave adicionales: Descendencia, *Drosophila melanogaster*, insecto no objetivo, longevidad, piriproxifeno

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INTRODUCTION

In agriculture, insecticides are commonly used to ensure crop protection and optimal yields. However, their injudicious and excessive use often induces harmful effects on non-target ecosystems and humans, as well as the emergence of insect resistance (Barathi et al., 2024).

Therefore, to reduce or eliminate the detrimental effects of synthetic insecticides on ecosystems, biodiversity, and human health, the search for substitute control strategies and

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ecofriendly insecticides with enhanced selectivity has become a significant challenge (Isman, 2020). Insecticides referred as insect growth regulators (IGRs) have gained prominence because they are efficacious against most agricultural pests and environmentally friendly (Wijayaratne et al., 2018; Scudeler et al., 2022).

Pyriproxyfen belongs to the juvenile hormone analogs (JHA) group and has been used as a biorational insecticide (Naeem et al., 2021). It mimics juvenile hormone (JH) activity by competing effectively for JH binding site receptors, disrupting the endocrine system in insects (Sullivan and Goh, 2008). Consequently, this molecule interferes with insect growth by preventing insect metamorphosis to the adult stage (Jindra and Bittova, 2020), as well as promotes morphological and functional aberrations in emerging adults, such as affecting the reproductive system (Maoz et al., 2017). Because it has the characteristics of low mammalian toxicity, as well as having minimal impact on the environment, pyriproxyfen has been frequently employed to manage agricultural insect pests across various orders, including Coleoptera, Diptera, and Hemiptera (Iqbal et al., 2020; Lu et al., 2022) as well as for managing public health pests like mosquitoes and houseflies (Alzahrani, 2021; Fowler et al., 2021).

In general, it is accepted that pyriproxyfen has low mammalian toxicity and is considered less environmentally hazardous than synthetic insecticides.

However, the fate of pyriproxyfen in the aquatic and terrestrial ecosystems has been recently reviewed and attracted widespread attention (Devillers, 2020a; Devillers, 2020b), as well as the physiological effects of its trace amounts or residue on non-target species (Lu et al., 2022; Xu et al., 2022; Li et al., 2023a).

Previous research has demonstrated that exposure to low concentrations of pyriproxyfen may interfere with the growth and behavior of aquatic species (Devillers, 2020a; Azevedo et al., 2021), disrupt the normal development of honeybees, and reduce royal jelly production in queen cells (Chen et al., 2016). Similarly, it has been shown that exposure to pyriproxyfen causes damage to the reproductive behaviors and system of beneficial insects such as silkworms and honeybees, as well as affecting ovarian protein expression (Qian et al., 2020; Zhao et al., 2020; Fine et al., 2023).

Consequently, it is imperative to examine the multiple sublethal impacts of pyriproxyfen in non-target organisms as part of an effective pesticide risk assessment. Previously, Bensebaa et al. (2015) demonstrated that third instar larvae exposure to pyriproxyfen can cause hormone disorder by inhibiting the production of 20-hydroxyecdysone (20E) and affecting the secretion of cuticles in adults of *Drosophila melanogaster* (Diptera: Drosophilidae).

The present research aimed to investigate the negative impacts of preimaginal exposure to pyriproxyfen on the growth and longevity of parent generation, as well as on the progeny of surviving adults obtained during the following F1 generation, using *D. melanogaster* as a non-target and biological model to assess the effects of the insecticide.

MATERIALS AND METHODS

Flies. The *D. melanogaster* (Wild-type Canton-S) was raised on a standard *Drosophila* medium containing cornmeal, agar, yeast, and an anti-fungal compound (methyl 4-hydroxybenzoate). *Drosophila* were cultured in a breeding room at a relative humidity of 70 %, a temperature of 25 °C and a 12:12 hour cycle of darkness and light (Boulahbel et al., 2022a).

Treatment and bioassay. Pyriproxyfen (purity of above 95 %) was obtained from Sumitomo Chemical Company. Ltd., Osaka, Japan. The compound was solubilized in acetone and applied topically (1 µl per insect) to the dorsal thoracic region of the third instar larvae of *D. melanogaster*, at two sublethal doses (ID_{25}= 0.10 ng·larva^{-1} and ID_{50}= 0.29 ng·larva^{-1}), using a microsyringe, as previously described by Bensebaa et al. (2015). The control larvae were exposed only to 1 µl of acetone.

Effects of sublethal doses on growth. The growth of *D. melanogaster* of control and treated series (ID_{25} and ID_{50}) was evaluated by measuring the weight using a precision balance (Sartorius AG Gottinger, Germany) and size (millimeter paper) of pupae during all days of the pupal stage (0, 1, 2 and 3 days) and newly emerged adults (0 day) of both gender. Groups of 70 to 100 larvae were exposed to pyriproxyfen treatment to obtain

sufficient survivors pupae and adults. According to the survival rates, 26 to 33 repeats were done for each experiment and each series of treatments.

Measurement of longevity. Measurement of longevity was conducted according to Linford et al. (2013). Newly emerged adults from controls and pyriproxyfen treatment, as described above, were separated by sex and housed in plastic vials containing a standard medium composed of cornmeal, agar, yeast, and an anti-fungal agent (Bezzar et al., 2016). To ensure that the feeding environment for young females is not disrupted by the presence of larvae, live flies were transferred to new vials containing fresh food every two days (Linford et al., 2013). During this process, the number of dead flies was recorded, and animals continued to be moved and kept under observation until all flies were dead. Ten to eleven replicates were set up for each dose, with 5 flies in each vial.

Progeny output obtained from surviving adults. Newly emerged (0-2 h after adult emergence) flies from control (C) and treated groups (ID_{25} and ID_{50}) were collected, and individual female (F) and male (M) were mated according to the following combinations: $M_C x F_C$; $M_{ID25} x F_{ID25}$ and $M_{ID50} x F_{ID50}$. These crosses between females and males that survived the same treatment, allow for the analysis of the reproductive hazards associated with each sublethal dose. Each couple was independently raised in a petri dish containing standard food and maintained in a breeding room under standard conditions, as described elsewhere (Boulahbel et al., 2022b). Two days later, the different couples formed were withdrawn, and the first generation (F1) was observed daily until all adult individuals had emerged. The number of offspring for each stage of development (number of eggs laid, third instar larvae, pupae and adults) was counted. For each combination seven replicates were conducted.

Statistical data analysis. The data was presented as the mean ± standard error of the mean (SEM). The homogeneity of the variance of the data was first checked using Levene's test. The experimental groups were analyzed using one-way or two-way ANOVA and Tukey's multiple comparisons test. All statistical computations and illustrations were done on GraPhpad Prism Software version 6.01 and results with $p \leq 0.05$ were considered statistically difference.

RESULTS

The effects of pyriproxyfen at ID 25 and ID 50 doses on pupal weight are given in Figure 1. The mortality rates and the dose-response curve for those doses have already been published by Bensebaa et al. (2015). In the control groups, the average pupal weight recorded at 0 days was 1.32±0.013 mg and remained stable for all duration of pupal development ($p \leq 0.05$). A similar profile was recorded in the treated groups for the two doses tested during pupal development. Pyriproxyfen, at its sublethal doses, resulted in a significant reduction in the average weight of pupae throughout the pupal stage (0, 1, 2, and 3 days) in comparison to the control groups ($F_{(2,333)}=104.5$; $p \leq 0.0001$). However, the two doses tested did not show any significant difference from one another ($F_{(6,333)}=0.235$; $p=0.96$, $p \leq 0.05$), and not significant effects time throughout the pupalstage ($F_{(3,333)}=0.085$; $p=0.96$).

As a consequence of larvae treatment, pyriproxyfen was significantly decreased the weight of adult males and females of *D. melanogaster*, and this effect was not dose-dependent (Figure 2). Moreover, our results also show that the average weight of females is greater than that of males ($p \leq 0.001$). The ANOVA analysis indicated a statistically significant impact of doses ($F_{(2,147)}=36.35$; $p \leq 0.0001$), sex ($F_{(1,147)}=136.9$; $p \leq 0.0001$) and doses-sex interaction ($F_{(2,147)}=4.61$; $p=0.01$).

The pupal size of *D. melanogaster* in the control series was 3.14±0.025 mm at 0 days, decreased to reach a value of 3.02±0.017 mm at 2 days ($p \leq 0.001$) and remained stable on the last day of the pupal stage (3 days) (Figure 3). In treated series, the pupal size stay remained during all pupal development. The comparison among the untreated and treated series demonstrates that pyriproxyfen decreases significantly the pupal size at 0 and 1 day ($p \leq 0.0001$) during pupal development. The ANOVA analysis indicated a statistically significant impact of treatment ($F_{(2,327)}=11.38$; $p \leq 0.0001$) and time ($F_{(3,327)}=14.49$; $p \leq 0.0001$), while the interaction between

treatment and time was not significant ($F_{(6,327)}$=2.003; p=0.064).

The size of both sexes of *D. melanogaster* recorded in the control and treated groups is presented in Figure 4. In males, the average values recorded were 2.96±0.01 mm for controls; 2.83±0.03 mm for ID_{25} and 2.78±0.02 mm for ID_{50}. Concerning the females, the size was 3.29±0.01 mm for controls, 3.14±0.02 mm for ID_{25} and 3.10±0.02 mm for ID_{50}.

It was also shown that the adult male and female size significantly decreased when third-instar larvae were exposed to sublethal doses of pyriproxyfen in comparison to the control groups. However, the two doses tested did not show any significant difference (p≤0.05). The ANOVA analysis indicated a statistically significant impact of treatment ($F_{(2.147)}$=29.83; p≤0 .0001) and sex ($F_{(1.147)}$=246.10; p≤0.001) while the interaction between treatment and sex was not significant ($F_{(2.147)}$=0.08; p=0.92).

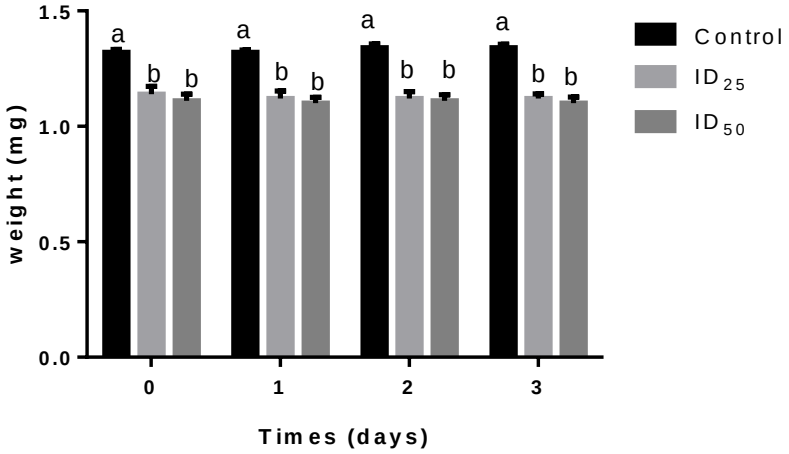

Figure 1. Pupal weight, as a function of time, following exposure of *D. melanogaster* third instar larvae to ID_{25} and ID_{50} of pyriproxyfen by topical application (mean±SEM; n=26-30). Mean values followed by different small letters between control and treated individuals are statistically different according to Tukey's test at p≤0.05.

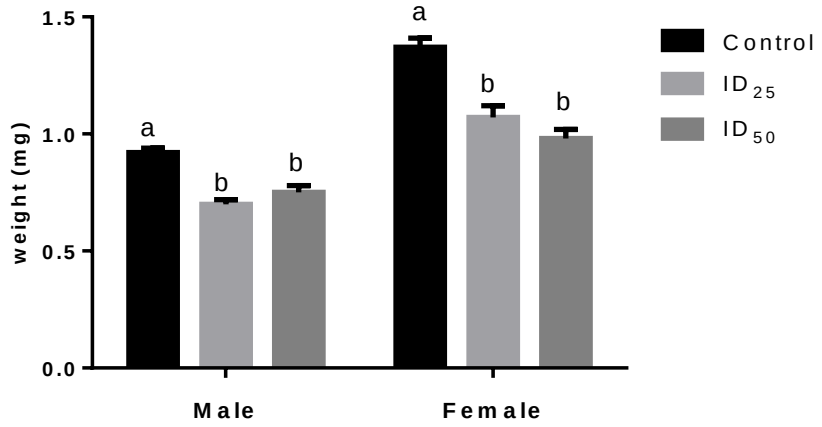

Figure 2. Weight of adults male and female (mg) following exposure of *D. melanogaster* third instar larvae to ID_{25} and ID_{50} of pyriproxyfen by topical application (mean± SEM; n=26-30). Mean values followed by different small letters between control and treated individuals are statistically different according to Tukey's test at p≤0.05.

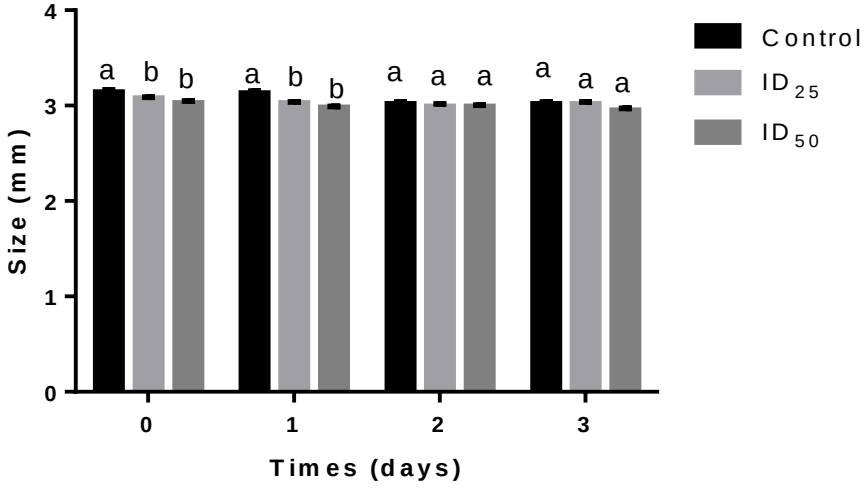

Figure 3. Pupal size, as a function of time, following exposure of *D. melanogaster* third instar larvae to ID_{25} and ID_{50} of pyriproxyfen by topical application (mean± SEM; n=26-31). Mean values followed by different letters between control and treated individuals are statistically different according to Tukey's test at $p \leq 0.05$.

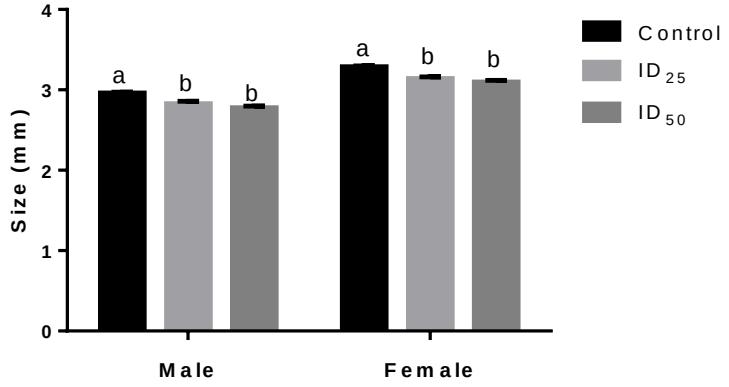

Figure 4. Size of adults male and female (mm) following exposure of *D. melanogaster* third instar larvae to ID_{25} and ID_{50} of pyriproxyfen by topical application (mean± SEM; n=26-30). Mean values followed by different letters between control and treated individuals are statistically different according to Tukey's test at $p \leq 0.05$.

Effects of sublethal doses on adult longevity. The results revealed that pyriproxyfen exposure to third instar larvae of *D. melanogaster* could significantly affect adult longevity of both sexes females and males compared with the longevity of those in the control series (Figure 5). Flies mortality was not dose-dependent. As shown in the figure, significantly lower longevity levels were found in the males than females for all treatment. Analysis of the variance indicated a significant impact of doses ($F_{(2,294)}$=88.20; $p \leq 0.0001$), sex ($F_{(1\ ,294)}$=100; $p \leq 0.0001$) and doses-sex interaction ($F_{(2\ ,294)}$ =5.05; p=0.0069).

Effects of sublethal doses on the progeny output obtained from surviving adults. Offspring from surviving adults following larvae treatment has been assessed and all developmental stages (Eggs; third instar larvae, pupae and adults) of various couples formed from control individuals ($M_C x F_C$), individuals treated with the ID_{25} ($M_{ID25} x F_{ID25}$) and ID_{50} ($M_{ID50} x F_{ID50}$) have been considered (Table 1 and Table 2).

Results showed that pyriproxyfen significantly reduced the fecundity ($p \leq 0.001$) of treated females mated with treated males comparatively to couples from controls individuals ($M_C x F_C$), with a dose response relationship (Table 1).

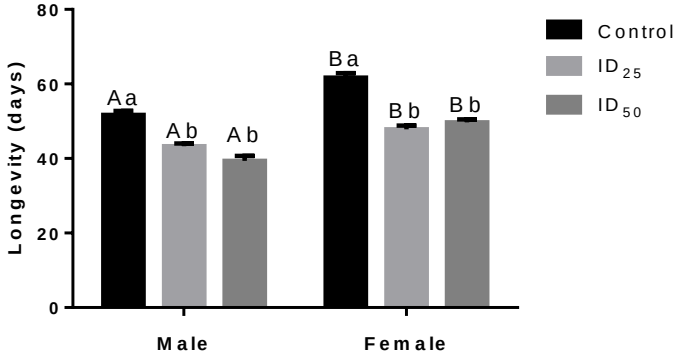

Figure 5. Longevity of adults male and female following exposure of *D. melanogaster* third instar larvae to ID_{25} and ID_{50} of pyriproxyfen by topical application (mean± SEM; n=50-55). Mean values followed by different small letters between control and treated individuals for the same sex are statistically different, while mean values followed by different capital letters for the same treatment between both sexes are statistically different according to Tukey's test at $p \leq 0.05$.

Table 1. Impact of pyriproxyfen (ID_{25} and ID_{50}) topically administered to larvae of *D. melanogaster* at the end of the third instar on fecundity (eggs/female) in control and treated females (Mean ± SEM; n = 7 repeats each corresponding to one couple). Mean values followed by different letters between control and treated individuals are statistically different according to Tukey's test at $p \leq 0.05$.

Treatment	Fecundity
Control (C)	75.14 ± 2.14 a
Pyriproxyfen (ID_{25})	66.28 ± 1.75 b
Pyriproxyfen (ID_{50})	44.28 ± 2.11 c

Table 2. Impact of pyriproxyfen (ID_{25} and ID_{50}) topically administered to larvae of *D. melanogaster* at the end of the third instar on the number of progeny (Third instar larvae, Pupae, Adults) of adults that survived from treated larvae (Mean ± SEM; n=7 repeats each corresponding to one couple). Mean values followed by different small letters for each stage of development are statistically different according to Tukey's test at $p \leq 0.05$.

Treatment	Third instar larvae	Pupae	Adults
Control (C)	73.57 ± 2.23 a	68.14 ± 1.43 a	66.42 ± 1.06 a
Pyriproxyfen (ID_{25})	64.42 ± 1.58 b	62.57 ± 1.34 b	60.42 ± 1,32 b
Pyriproxyfen (ID_{50})	42 ± 1.87 c	39.71 ± 1.65 c	37.28 ± 1.37 c

ANOVA: (Larvae: $F_{(2,18)} = 71.75$, $p < 0.0001$; Pupae: $F_{(2,18)}=102.6$, $p < 0.0001$; Adults $F_{(2,18)}=148.6$, $p \leq 0.0001$).

Pyriproxyfen treatments of third instars larvae significantly reduced the number of progeny (larvae, pupae and adults) of the first generation (F1) for all couples formed from treated groups ID_{25} ($M_{ID25}xF_{ID25}$) and ID_{50} ($M_{ID50}xF_{ID50}$) comparatively to couples from controls individuals ($M_C x F_C$) (Table 2).

Furthermore, a dose-dependent effect was noted for all developmental stage, and a more significant reduction was observed when the two partners (female and male) were treated with the ID_{50} dose of pyriproxyfen.

DISCUSSION

In the present research, the application of pyriproxyfen at sublethal doses to the third larval instar had a harmful impact on the developmental stages of the exposed generation, as it significantly reduced the weight and size of pupae and adults of both sexes. The adverse effects on various growth stages under pyriproxyfen treatment were reported in several insect pests (Khan, 2021; Bakr et al., 2021).

In insects like Drosophila, the two primary endocrine hormones are JH and ecdysteroids (20E) (Li et al., 2023b). Throughout the insect life cycle, these hormones modulate diverse biological processes, such as growth and development, molting and metamorphosis (Jindra, 2019; Santos et al., 2019; Zhu, 2022). Certain processes are controlled by one of the two hormones, while others depend on the action of both hormones (Zhu, 2022). For example, 20E coordinates all major developmental transitions of the insect life cycle such as larval-larval molting and larval-pupal-adult metamorphosis, but it is an interaction with JH that transduces the 20E pulses into stage-specific responses (Riddiford et al., 2000; Dubrovsky, 2005). Indeed, JH has been shown to modulate ecdysteroid signaling in various insects (Riddiford, 1996). According to the classical dogma of insect endocrinology, the dynamic balance between 20E and JH defines the outcome of each developmental transition (Dubrovsky, 2005). Consequently, maintaining an appropriate equilibrium between these two hormones is crucial for normal insect development.

Recent research has demonstrated that trace residues or amounts of pyriproxyfen can disrupt hormone balance, resulting in an elevated JH level and a decrease in ecdysone titer, suggesting that trace residues of pyriproxyfen cause abnormal and defects of metamorphosis in non-target insect like silkworm (Li et al., 2021; Xu, 2022). A deleterious effect of pyriproxyfen on the development of *D. melanogaster* was expected due to its insect's growth disruptor (IGD) action by suppressing the ecdysteroid titer in the hemolymph during the pupal stage (Bensebaa et al., 2015).

Our results revealed that a single pyriproxyfen treatment of *D. melanogaster* larvae could significantly shorten the longevity of *Drosophila* adults in both sexes, mainly in males than in females. Similarly, Li et al. (2023b) reported that exogenous application of JH III or methoprene, a JH analog, significantly shortened the lifespan in the adulthood of male silkworms. According to the literature, decreased adult longevity after pyriproxyfen treatment has been documented in several insects (Ghasemi et al., 2010; Khan., 2021; Grisales et al., 2021). Longevity is influenced by a variety of determining parameters, including genetics, differences in environmental conditions and diet (Bin et al., 2022; Hall et al., 2019). The insulin signalling pathway is one of the most well-known associated with lifespan regulation (López et al., 2013; Wątroba et al., 2017).

According to the literature, it has been proven that JH also regulates the adult lifespan in certain insect species (Yamamoto et al., 2013). Indeed, the ablation of the corpora allata (place of synthesis of JH) could extend the lifespan of *D. melanogaster* (Yamamoto et al., 2013). At the same time, it was shown that the takeout gene plays a role in determining longevity by specifically increasing adult takeout expression and prolonging lifespan in insects (Bauer et al., 2010; Chamseddin et al., 2012), and exogenous application of JH III or its analog methoprene was found to shorten longevity in a crucial economic insect, silkworm, by suppressing the FoxO-takeout axis (Li et al., 2023b). This could explain the adverse effects of pyriproxyfen on adult longevity.

The results of the current investigation demonstrate that the use of pyriproxyfen at its sublethal doses has a detrimental impact on the reproductive capabilities of non-target insects, like *D. melanogaster*. Additionally, the delayed effects of pyriproxyfen on the F1 generation of *D. melanogaster* showed a decrease in fertility and fecundity by reducing the number of offspring (eggs, larvae (L3), pupae and adults) after parent's generation treatment with pyriproxyfen. The depressive effect seems to be more marked when both partners (female and male) received the

highest dose ID_{50} of pyriproxyfen ($M_{ID50}xF_{ID50}$). Pyriproxyfen has been documented to affect many insect pests' reproductive capacity, including fecundity and fertility (Yadav et al., 2019; Khan, 2021; Cremonez et al., 2023).

Similarly, it has been found that pyriproxyfen and its residues can reduce reproductive capacity in non-target species such as *Eretmocerus mundus* (Francesena and Schneider, 2018); *Bombyx mori* (Qian et al., 2020) and *Hippodamia convergens* (Iftikhar et al., 2020). In insect, successful reproduction of adult females partially depends on effective vitellogenesis (Zhang et al., 2023). During insect vitellogenesis, the vitellogenin (Vg) is mainly synthesized in the fat body, is then transported through circulating hemolymph to the ovary, and absorbed via endocytosis mediated through Vg receptor (VgR), which is stored by mature oocytes (Upadhyay et al., 2016; Wu et al., 2021; Zhang et al., 2023). These events are governed by two primary hormones, namely JH and ecdysone and by the nutritional signaling pathway regulated by neuropeptides.

In females, JH is the primary gonadotropic hormone regulating insect vitellogenesis and oogenesis (Zheng et al., 2022). JH synergic action with 20E and insulin/insulin-like growth factor signalling (IIS) regulates the nutrient-sensitive checkpoint required for oocytes development (Toivonen and Partridge, 2009). Based on previous work, pyriproxyfen application on beneficial insects deter vitellogenesis (Pinto et al., 2002). Similarly, pyriproxyfen has been shown to cause a significant decrease in yolk protein synthesis, as well as affecting the expression levels of Vg, the absorption of nutrients and egg formation, thus leading to damage the normal reproductive function in insects (Qian et al., 2020). Additionally, Qian et al. (2020) indicated that this compound down regulated the transcription levels of different genes that are associated with ovary development and hormone regulation, resulting reproductive disorders and sterility of the insects. In *P. interpunctella*, pyriproxyfen treatment interferes with the growth and development of ovaries, resulting in severe morphological alterations in the ovaries of emerged adults (Ghasemi et al., 2010). Moreover, diminished reproductive potential may occur by the action of pyriproxyfen on the reproductive system, where it causes morphological and morphometric changes in both ovarioles and testes, mainly in the ovarian structure and disarrangement of the nurse cells as reported for *Euschistus heros* (Cremonez et al., 2023).

CONCLUSIONS

The results obtained in this experiment demonstrated that preimaginal exposure to sublethal doses of pyriproxyfen affects growth parameters and longevity in parents exposed as well as the offspring development rate of the non-exposed (F1 generation) of a non-target species *D. melanogaster*. Thus, it reflects that the sublethal effects of pyriproxyfen acting in the long term through developmental stages and/or generations, which could be helpful for pest management. However, if pyriproxyfen was reported to be relatively safe for humans and the environment, these prolonged and transgenerational effects should be considered to assess the environmental risk and totally validate the favorable ecotoxicological profile of this biorational insecticide. Further investigations on this active ingredient's sublethal and residual effects under field conditions are required to understand their long-term impact on non-target organisms better.

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