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Radia Bezzar-Bendjazia
Laboratory of Applied Animal Biology Department of Biology, Faculty of Sciences, Badji Mokhtar University of Annaba 23000-Annaba, Algeria.

Samira Kilani-Morakchi
Laboratory of Applied Animal Biology Department of Biology, Faculty of Sciences, Badji Mokhtar University of Annaba 23000-Annaba, Algeria.

Nadia Aribi
Laboratory of Applied Animal Biology Department of Biology, Faculty of Sciences, Badji Mokhtar University of Annaba 23000-Annaba, Algeria.

Correspondence
Radia Bezzar-Bendjazia
Laboratory of Applied Animal Biology Department of Biology, Faculty of Sciences, Badji Mokhtar University of Annaba 23000-Annaba, Algeria.

Growth and molting disruption effects of azadirachtin against *Drosophila melanogaster* (Diptera: Drosophilidae)

Radia Bezzar-Bendjazia, Samira Kilani-Morakchi, Nadia Aribi

Abstract
Azadirachtin, a biorational insecticide, known to be an antagonist of the juvenile hormone and 20 hydroxyecdysone (20E), has been used successfully against insect pests; however, its effects on non-target organisms remain contradictory. The current study examined the lethal and sublethal effects of azadirachtin on *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) as biological model. Azadirachtin was applied topically at two dose LD_{25} (0.28 µg) and LD_{50} (0.67 µg) on early third instar larvae. Treatment reduced, significantly pupation and eclosion rates. The increase of doses was directly proportional to reduction in adult emergence. Indeed, pupation rate of 93.33% in controls series drop to 81.33% and 76% respectively for LD_{25} and LD_{50}. The eclosion rates is significantly reduced, after azadirachtin treatment, as compared to controls (93.33%) and the value recorded are 72% and 46.66%, respectively for LD_{25} and LD_{50}. In addition, azadirachtin exhibited various morphological abnormalities on larvae, pupae and adult stages (heavy pigmentation of larvae, pupa-adult intermediate, incomplete/malformed adults), the value recorded was about 13.5 to 27% higher than the controls. Finally, results showed that the compound reduced, at the two tested doses, the body weight of all developmental stages of *D. melanogaster* (larvae, pupae and adults) about 10 to 20%. Thus, our results suggested that a topical application of azadirachtin to early third instar larvae interfered with the endocrine events and disrupted the normal development of this insect. Consequently, this reduced-risk insecticide should be used with caution in integrated pest management programs (IPM).

Keywords: Azadirachtin, *Drosophila melanogaster*; growth; molting disruption

1. Introduction
In recent years, alternatives to synthetic pesticides for effective and environmentally safe pest control have been investigated [1]. Consequently, a drastic re-emergence of interest in the use of natural pesticides, or biopesticides, especially plant-derived compounds was noted [2]. The plant species that is probably best investigated for its insecticidal properties is the neem tree, *Azadirachta indica* A. Juss. (Meliaceae) [3]. All parts of this tropical tree are biologically active, but the most effective one is the neem seed kernel due to their high concentration of azadirachtin, a tetranortriterpenoid produced as a secondary metabolite [4, 5]. Because of its low mammalian toxicity and fast degradation [3], azadirachtin is currently exploited in agriculture for crop protection [6]. In addition azadirachtin has also been reported as safer for non-target organisms than synthetic insecticides [7-9]. Due to its chemical complexity, azadirachtine has many anti-insect properties but no resistance problem; its act as feeding deterrent, insect growth regulator (IGR) and sterilant [10, 11] and is used to control various agricultural pest species, including Coleoptera, Hymenoptera, Diptera, Orthoptera and Isoptera [4]. If considerable progress on the physiological and biological activities and agricultural application of azadirachtin has been achieved, its mechanism of action remains elusive [12, 13]. Furthermore, the earlier perception of azadirachtin's safety towards non target arthropods has been questioned specially in relation with its acute lethal and sublethal effects [14-17]. Here we assessed the potential lethal and sublethal effects of azadirachtin on a model system, *Drosophila melanogaster,* in order to better understand the action of the compound on growth of the different developmental stages, molting process and alteration of some morphological aspects of insect.

2. Material and methods

2.1 Insect rearing

D. melanogaster (Canton-S wild-type), a widely used laboratory strain, was reared in glass vials containing a yeast/cornmeal/agar laboratory medium with an anti-fungal agent and kept in a breading room at 25 °C with 70% humidity on a 12:12 h light/dark cycle. Flies were transferred every three days to avoid larval competition and regularly provide abundant progeny for testing.

2.2 Insecticide and treatment

Neem Azal-TS, a commercial formulation of azadirachtin (1% EC; Trifolio-M GmbH, Lahnau, Germany) was dissolved in acetone and topically administrated (1 µl per insect according to Bensebaa *et al*. [18]) on early third-instar larvae of *D. melanogaster* (< 3hours) at two doses, 0.67 µg and 0.28 µg corresponding respectively to LD_{50} and LD_{25} (doses that caused respectively a mortality of 50% and 25% of immature stages). Control insects were treated with acetone (1µl). To obtain larvae of a synchronized age, they were collected from vials where gravid females had been transferred every two hours [19].

2.3 Pupation and adult eclosion rates

Early third-instar larvae were treated as above with two doses (LD_{25} and LD_{50}). The rates of pupation and adult eclosion were determined for controls and treated series. All tests were monitored until all adults emerged (~ 6 days) or until no live larvae or pupae were observed. Each experiment used 15 individuals and was replicated five times.

2.4 Morphological study

Morphological analyses were conducted in order to detect possible abnormalities after azadirachtin treatment. The morphological analyses were done on third-instar larvae (24h after treatment), pupae (0, 24, 48, and 76 h after pupation) and newly emerged adults (0 day) using a Leica DM500 microscope equipped with a Leica ICC50 HD camera. Abnormalities were observed 24h post- exposure of early third instar larvae of *D. melanogaster* to azadirachtin until adult emergence. The percentages of each observed phenotype were calculated. Three repeats of 15 insects are used for each series.

2.5 Body weight

Larvae from controls and treated series (LD_{25} and LD_{50}) were collected and transferred to a plastic tube containing fresh food. The larval weight was recorded after 24 h, using a precision balance. The weight of insect was then followed up for the pupal stage (0, 24, 48, and 76 h after pupation) and adults of both gender 24 h after emergence, 30 replicates were used for each doses.

2.6 Statistical analysis

Results are given as means ± standard errors (SE).The homogeneity of variances was checked using Bartlett's and Brown-Forsythe tests. Data were subjected to one -way or two - way analysis of variance (ANOVA) followed by a post-hoc HSD Tukey test. The statistical analysis for the pupation and emergence rates as well as the proportions of abnormalities was done on the number of each studied parameter. All statistical analyses were performed using GraphPad prism version 6.01 for Windows (GraphPad software, La Jolla California, U.S.A., www.Graphpad.com).

3. Results

3.1 Effect of azadirachtin on pupation and eclosion rates

As a consequence of larval treatment, the pupation rates and adult emergences decreased (Fig. 1). Controls larvae (93.33%) initiated pupation at day 2 after treatment. Azadirachtin reduced the pupation rate to 81.33% and 76% respectively for LD_{25} and LD_{50} ($P<0.0001$) (Fig. 1A). Adult eclosion of pupated individuals in the control group was 93.33%. The eclosion rates in treated series is significantly reduced as function the doses and the value recorded are 72% and 46.66%, respectively for LD_{25} and LD_{50} ($P<0.0001$) (Fig. 1B).

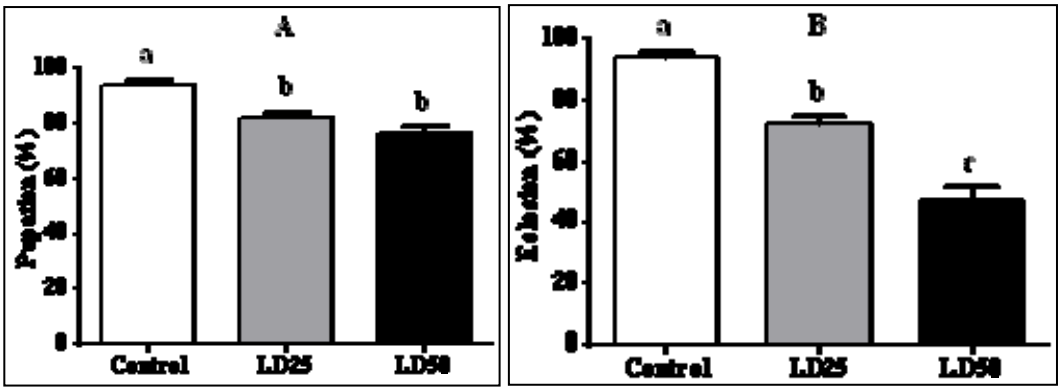

Fig 1: Effect of azadirachtin (LD_{25} and LD_{50}) topically applied on early third-instar larvae on the pupation (A) and adult eclosion (B) rates. (m ± SE, n= 5 replicates of 15 insects). Different small letters indicate a significant difference between control and treated individuals ($p < 0.05$).

3.2 Morphological analyses of puparia, larvae and adults

Several forms of morphological malformations resulted from treatment of early third instars larvae with azadirachtin (Fig. 2). The larval abnormalities are represented by heavy pigmented and twisted larvae, as well as larval-pupal intermediates. The pupal and adult aberrations were: pupa-adult intermediates, incomplete adult emergence, malformed adult (absence or deformed wings) and total inhibition of adult emergence. With regard to morphological abnormalities (Fig. 3), the different aberrations noted are significantly only at the highest dose (LD_{50}) as compared to controls ($P<0.0001$). However, the rates of incomplete/malformed adults and successfully emerged adults differ with dose-dependent manner from controls. Accordingly, azadirachtin caused significant level of growth disturbance and exhibited deformities.

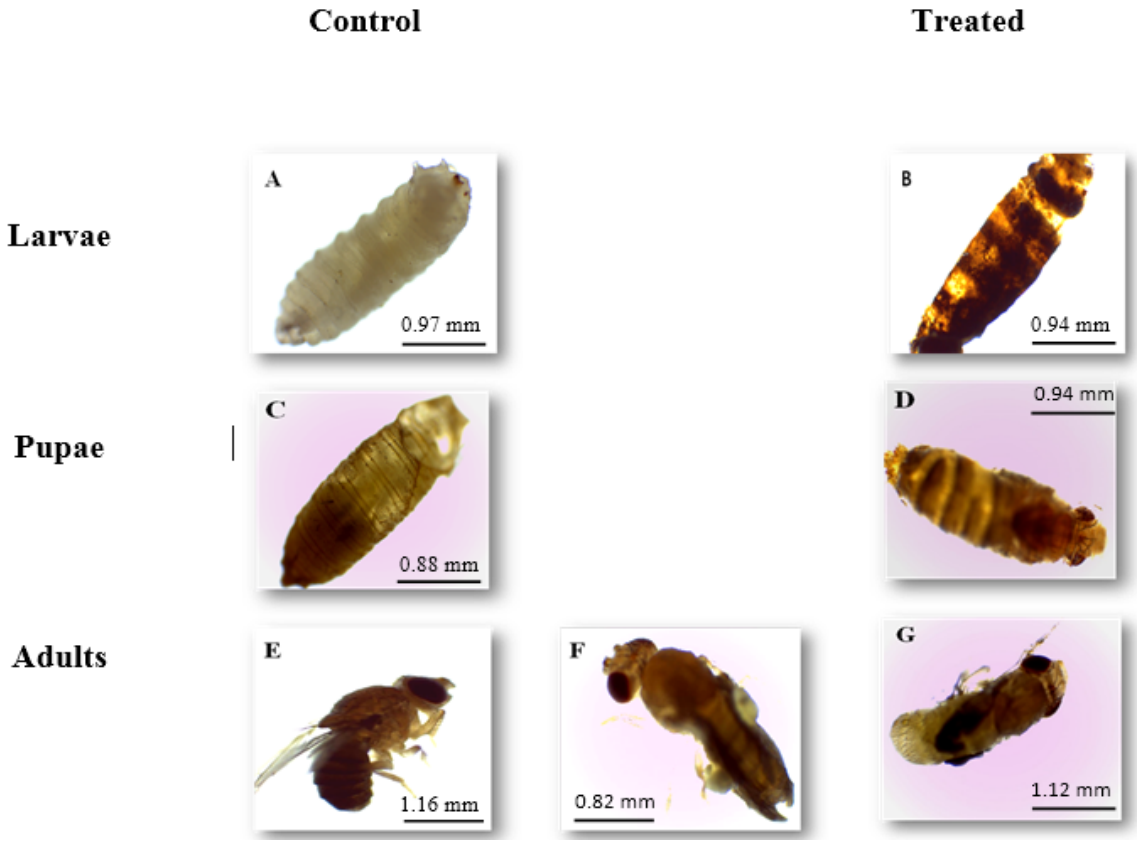

Fig 2: Photographs showing morphological abnormalities in *D. melanogaster*, induced after early third instar treatment with azadirachtin: (A) normal larva, (B) larvae showed heavy pigmentation, (C) normal pupa, (D) pupa-adult intermediate, (E) normal adults, (F) and (G) incomplete/malformed adults.

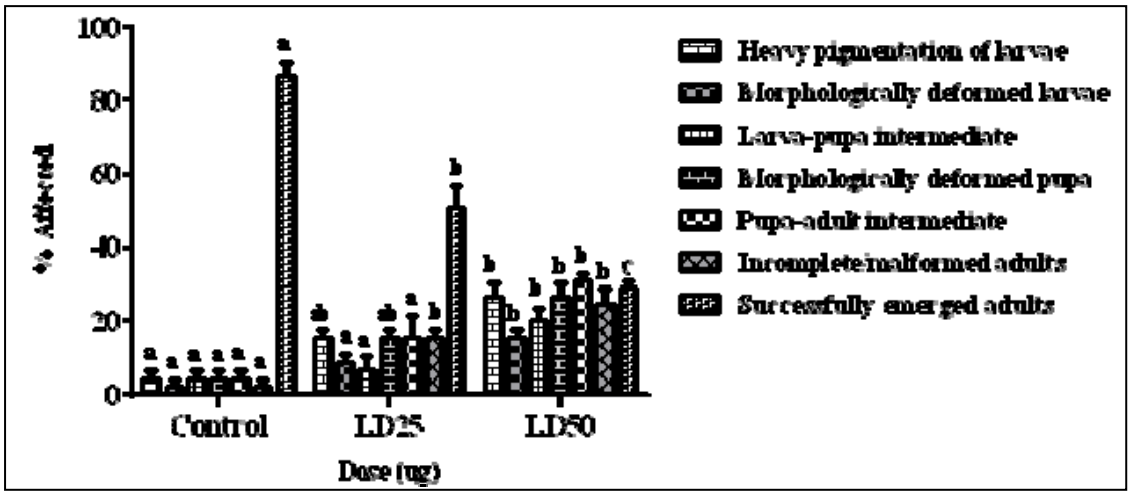

Fig 3: Proportion of abnormalities in *D. melanogaster* after Azadirachtin (LD_{25} and LD_{50}) topical application on early third instars larvae. (m ± SE, n= 3 replicates of 15 insects). Different small letters indicate a significant difference between control and treated individuals ($p < 0.05$).

3.3 Effect on the body weight

Effects of the different doses of azadirachtin on body weight of larvae, pupae and adults are shown in Fig. 4. In control series the larval weight was 1.96±0.03 mg, azadirachtin treatments of early third instars larvae caused a significant reduction ($F_{2, 87} = 38.97$; $p < 0.0001$) of the larval weight. The mean values recorded in treated series were 1.77 ± 0.03 mg for the (LD_{25}) and 1.52 ± 0.04 mg for the (LD_{50}) treated larvae. Larval weight reduction reached 9.69% for LD_{25} and 22.44% for LD_{50} (Fig.4.A). Azadirachtin (LD_{25} and LD_{50}) decreased the pupal weight during pupal development (0, 24, 48 and 76 h) compared to control series (Fig.4.B). ANOVA revealed significant effects of dose ($F_{2, 384} = 28.77$; $p < 0.0001$), time ($F_{3, 348} = 7.93$; $p < 0.0001$) and non-significant effects of dose-time interaction ($F_{6, 384} = 0.407$; $p = 0.847$). There was no significant ($p > 0.05$) difference between the two tested doses. Azadirachtin also reduced the weight of adults of both sexes of *D. melanogaster* without dose-dependent effect (Fig.4.C). ANOVA indicated significant effects of doses ($F_{2, 174} = 21.54$; $P < 0.0001$), sex ($F_{1, 174} = 246.4$; $P<0.0001$) but no significant ($F_{2, 174} = 0.568$; $P = 0.567$) in doses-sex interaction.

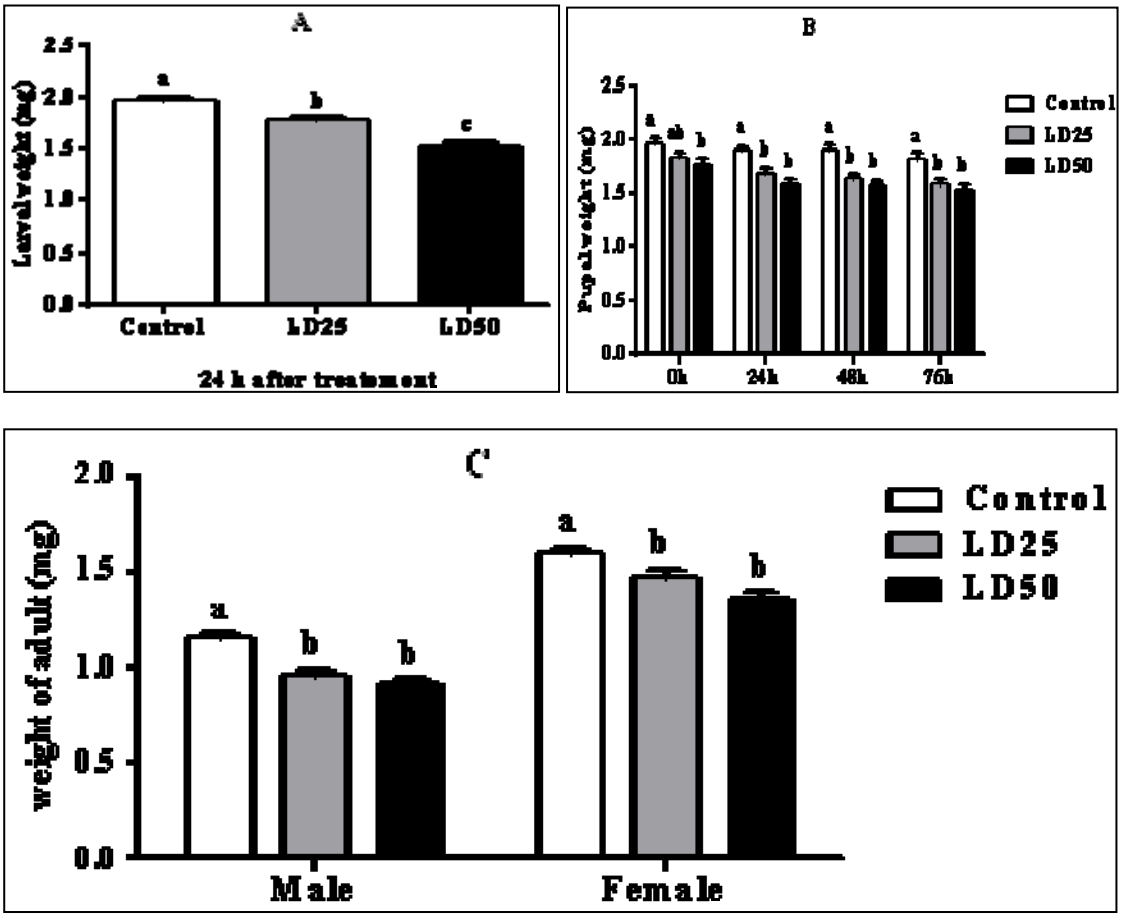

Fig 4: Effect of Azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on the body weight of A: larvae, B: pupae, C: adults male and female (24 h post emergence), (m ± SE; n = 30 replicates). Different small letters indicate a significant difference between control and treated series ($p < 0.05$).

4. Discussion

In this study we evaluated the topical application of azadirachtin to early third instar larvae of *D. melanogaster*. Results revealed that azadirachtin applied during the larval stage affected moulting and induced a range of anatomical abnormalities in *D. melanogaster*. The pupation and eclosion rates were significantly reduced at the two tested doses (LD_{25}, LD_{50}) compared to controls. Larvae that did moult did not complete the process properly, and the defects intensified with the sequence of moults into the later instars. Azadirachtin is known to reduce pupation and eclosion rates of many insects like *Aphis glycines* [20], *Plodia interpunctella* [21, 22], *Aedes aegypti* [23]. Recently, Boulahbel *et al*. [24] demonstrated that topical application of azadirachtin on newly emerged pupa of *D. melanogaster* inhibit adult emergence with a dose-dependent manner. In the current experiments, azadirachtin induced morphological alterations of larvae, pupae and adults of *D. melanogaster*. Many abnormalities are observed like heavy pigmentation of larvae, larva-pupa intermediate, pupa-adult intermediate, malformed adults (absence or deformed wings). Similar effects were obtained when last-instar larvae of *Spodoptera litura, Spodoptera mauritia, Ephestia kuehniella* Zell, *Manduca sexta* and *Pericallia ricini* were subjected to azadirachtin action [25-28]. The deleterious effects of azadirachtin could be attributed to a disruption of ecdysteroid and juvenile hormone resulting of defective metamorphosis [29-32]. Indeed, Insect ecdysone induces the larval-pupal metamorphosis in the absence of JH, but the presence of antagonist to JH during the JH sensitive periods (larval period) could lead to a new larval stage at ecdysis, or to the development of larval-pupal or larval-adult intermediates that are unable to give rise to normal adults [33]. Furthermore, Lai *et al*. [13] showed that azadirachtin provoked potent growth inhibitory effects in *Drosophila* larvae by regulating the gene of cuticular protein which might be related to the deleterious metamorphic effects observed in our results. Moreover, Qiao *et al*. [34] Reported the neurotoxic effect of azadirachtin which might interfere with different endocrinological and physiological actions in insects.

The effects of azadirachtin were also expressed as a reduction in the weight of larvae, pupae and adults of *D.melanogaster*. This is probably due to the known antifeeding effect of azadirachtin [35]. Indeed, treated larvae may have eaten less than larvae from the control. Indeed, growth and nutrient intake are function-ally linked processes in development and growth and body mass are directly affected by nutrient uptake, which are governed by the insulin/insulin-like growth factor signalling (IIS) pathway during insect growth [36]. Lai *et al*. [13] showed that azadirachtin inhibited growth and development, which was consistent with those caused by disruption of the IIS pathway. In addition, bad nutrition, starvation, or restriction of food during insect larval stages may force pupation before the achievement of an ideal species specific weight given rise to smaller adult individuals [37, 38]. The reduction of insect weight under azadirachtin treatment was reported in *D. melanogaster* [13], *Helicoverpa armigera* [39] and *Periplaneta americana* [40]. Furthermore, Boulahbel *et al.* [24] showed a reduction in pupal weight after azadirachtin topical

application on newly emerged pupae (non feeding stage). This finding support the putative mechanism of insect growth inhibition reported by Tai Jun *et al*. [41] stipulated that azadirachtin may inhibit the growth of *D. melanogaster* by induction of apoptosis.

5. Conclusion

This work showed that topical application of azadirachtin on early third instar larvae clearly provoked potent growth inhibitory effects in *D. melanogaster* and resulted in irreversible damage to physiological processes associated with molting and metamorphosis. However, Disruption of growth and development in flies and other insect species under azadirachtin treatment have yet to be fully investigated.

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