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# Azadirachtin impact on mate choice, female sexual receptivity and male activity in *Drosophila melanogaster* (Diptera: Drosophilidae)

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ABSTRACT

Azadirachtin, a neem compound (*Azadirachta indica*) with medical and anti-insect properties, is one the most successful botanical pesticides in agricultural use. However, its controversial impact on non-targeted species and its mechanism of action need to be clarified. In addition, Azadirachtin impact on pre- and post-mating traits remains largely undocumented. The current study examined the effects of Azadirachtin on *Drosophila melanogaster* as a non-target and model species. Azadirachtin was applied topically at its $LD_{50}$ (0.63 µg) on the day of adult emergence and its effect was evaluated on several traits of reproductive behavior: mate choice, male activity, female sexual receptivity, sperm storage and female sterility. In choice and no choice conditions, only male treatment reduced mating probability. Female treatment impaired mating probability only when males had the choice. Males' mating ability may have been impaired by an effect of the treatment on their mobility. Such an effect was observed in the actimeter, which revealed that treated males were less active than untreated ones, and this effect persisted over 8 days. Azadirachtin treatment had, however, no effect on the nycthemeral rhythm of those males. Even when mating occurred, Azadirachtin treatment impaired post-mating responses especially when females or both sexes were treated: remating probability increases and female fertility (presence of larvae) decreases. No impairment was observed on the efficiency of mating, evaluated by the presence of sperm in the spermatheca or the ventral receptacle. Male treatment only had no significant effect on these post-mating responses. These findings provide clear evidence that Azadirachtin alters the reproductive behavior of both sexes in *D. melanogaster via* mating and post-mating processes.

## 1. Introduction

Reproductive success depends on a large set of traits that can be broadly classified into pre- and post-copulatory traits. Males searching for females use a wide array of sensory stimuli from visual, olfactory, gustatory, tactile, acoustic and mechano sensory modalities [1,2]. Female sexual attractivity and receptivity also depend on a number of physiological, hormonal and behavioral processes underlying the interactions between sexual partners [3,4]. In addition, environmental conditions may strongly impact mating and reproductive success, as for instance the exposure to natural compounds with pesticidal properties (biopesticides) used in agriculture. Because these natural pesticides may also affect non-target species, their effects need to be more deeply investigated, especially on non-target insects, which may be particularly impacted [5].

Azadirachtin is an effective botanical insecticide isolated from the neem tree *Azadirachta indica* A. Juss (Meliaceae). It is widely used in agriculture and is considered as having a low environmental impact, because it is non-toxic to vertebrates and has no genotoxicity for mammals [6]. Nevertheless, this pesticide shows a strong toxicity for many insect pests of different orders [7,8,9,10,11,12], so that its toxicity to non-target species remains controversial [5,13,14,15,16,17,18]. Azadirachtin is an insect growth disrupter (IGD), which acts by interfering with the insect endocrine signaling molecules, juvenile hormone (JH) and 20-hydroxyecdysone (20E), leading to deleterious effects on development and reproduction (oocyte structure, fecundity, oviposition and egg viability) [7,10,19,20,21,22]. In addition, some studies suggest Azadirachtin impacts on the nervous system [23] and the insulin-signaling pathway [24]. Several works show that Azadirachtin influences the oviposition behavior of various insects [9,13,25,26], but the exact impact on sexual behavior and post-mating responses is poorly understood. In the present study, we evaluated the possible effects of Azadirachtin on several traits of reproductive behavior in *Drosophila melanogaster*, and in particular on post-mating processes. Knowledge of the

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physiology, endocrinology and genetics of *Drosophila* is highly valuable for understanding the mechanisms of action of molecules like pesticides. In addition, post-mating behavioral processes are well known in *D. melanogaster*.

In *Drosophila*, ecdysone, JH and insuline-signaling pathways control the mating process [27,28] in tandem with the brain [2,29,30]. Furthermore, ecdysteroid-signaling is essential for pheromone synthesis [31] and JH has been shown to regulate pheromone maturation and female mating behavior [32]. Mated females show post-mating responses (PMR), including decreased mating receptivity, enhanced oogenesis, changes in sperm storage and use, egg-laying and modulated regulation of JH [33,34,35,36]. These PMR are induced by the male seminal fluid transferred during copulation, which is mainly constituted by accessory gland proteins (Acps) [37]. The purpose of this study was to investigate Azadirachtin effects (i) on *D. melanogaster* sexual behavior through the study of male activity and mating success, and (ii) on several PMR, *i.e.* sperm storage, egg-laying and female receptivity for additional mating.

## 2. Material and methods

### 2.1. Insect rearing and treatment

*D. melanogaster* (Canton-S) was reared on standard corn-meal food at 25 ± 2 °C, 70% relative humidity with a 12 h light/dark photoperiod. Flies were transferred every three days to avoid larval competition and to regularly provide abundant progeny for testing.

The commercial formulation of Azadirachtin, NeemAzal (1% Azadirachtin; TrifolioM GmbH; Lahnau, Germany), was dissolved in acetone and topically applied on adults at the lethal dose or $LD_{50}$ (0.63 µg) evaluated previously by Oulhaci et al. [38]. All insects were subjected to a topical application: “control” insects were treated with solvent (acetone) alone [38].

### 2.2. Mating assays

Newly-emerged male and female flies (< 6 h post-emergence) were separated and treated topically with Azadirachtin. Then control ($M_C$, $F_C$) and treated ($M_T$, $F_T$) males and females were placed in vials (25 mm in diameter, 95 mm in height) containing a standard corn-meal medium. After 48 h, males and females (survivors) were mated in choice and no-choice assays. In the no-choice assay, one male and one female were placed together in individual vials with food. Four conditions were tested: $M_C + F_C$, $M_C + F_T$, $M_T + F_C$, $M_T + F_T$. In the choice assay, one male and two females or one female and two males were placed together in individual vials. Four conditions were tested: $M_C + F_C/F_T$, $M_T + F_C/F_T$, $F_C + M_C/M_T$, $F_T + M_C/M_T$.

Before Azadirachtin treatment, flies were anesthetized on ice and marked under a binocular microscope by punching a tiny hole with a needle on the posterior part of the right wing. This marking procedure allowed treated and untreated flies to be differentiated. Statistical analysis did not show any effect of this marking on reproductive success (Student's *t*-test, $p > 0.11$ for all conditions). Mating was monitored for 3 h in the morning. For each condition, the number of matings was counted and then expressed as a mating percentage.

### 2.3. Male activity

In order to understand the impact of Azadirachtin on the males' activity, their motility was analyzed using a tubular actimeter, commonly used for monitoring locomotor activity rhythms in adult fruit flies (*Drosophila* Activity Monitor DAM2-5, Trikinetics, Waltham, MA, USA). Recently emerged males (< 6 h) were separated and treated topically with Azadirachtin at the $LD_{50}$ (0.63 µg). Then, control and treated males (survivors) were placed separately in vials containing standard corn-meal medium. After 48 h, control and treated males were disposed individually in the tubes of the actimeter containing diet at one end, and their motility was monitored for 8 days (Fig. 1). Displacement of individual *Drosophila* trapped in the tubular unit was detected each time the insect was caught in an infrared beam. The detection events perceived by each cell were transmitted to a computer equipped with the DAMSystem collection software that allows the number of passages to be counted. A comparison of the males' motility in the two groups was performed.

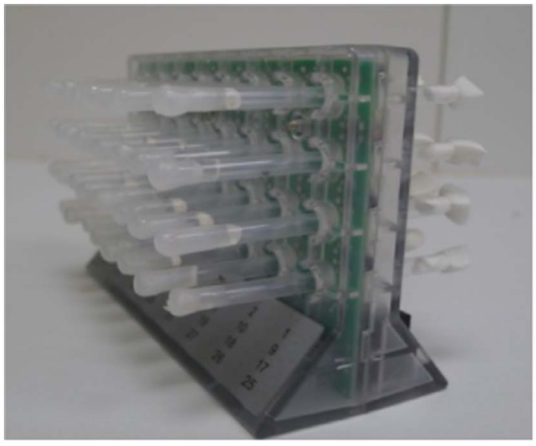

**Fig. 1.** Actimeter for testing male motility. The DAM actimeter is a monitor made up of a grid pierced with 32 holes. A 65 × 5 mm polycarbonate tube (5 mm i.d.) is inserted into each hole, which is equipped with an infrared photoelectric detector. This configuration allows for automatic, continuous, and simultaneous recording of the displacements of 32 individuals. At one end of the tube, standard corn-meal medium was inserted and the tube was closed with paraffin.

### 2.4. Sperm storage within females

The effect of Azadirachtin on the success of sperm storage in the female genital tract was assessed in both ventral receptacle and spermatheca. Recently emerged adults (< 6 h) were separated and treated topically with Azadirachtin at $LD_{50}$ (0.63 µg). Males and females of the control and treated series (survivors) were placed separately in plastic vials containing standard corn-meal medium. After 48 h, four mating conditions were tested: $M_C + F_C$, $M_C + F_T$, $M_T + F_C$, $M_T + F_T$. Three hours after mating, females were frozen and dissected under a stereo microscope. The number of females exhibiting sperm within the spermatheca or the ventral receptacle was noted and the results were expressed in percentages.

### 2.5. Female sexual receptivity to remating

Azadirachtin was tested on the receptivity of females to assess their capacity to remate after a first mating with treated or untreated males. Recently emerged adults (< 6 h) were separated and treated topically with Azadirachtin at the $LD_{50}$ (0.63 µg). Males and females of the control and treated series (survivors) were placed separately in vials containing standard corn-meal medium. After 48 h, four conditions were tested: $M_C + F_C$, $M_C + F_T$, $M_T + F_C$, $M_T + F_T$. Seven days after the first mating, which is less than the time needed by females to recover their sexual receptivity [39], a new 4 day-old male was proposed to the female for remating. The number of remating was noted and results were expressed in percentages.

### 2.6. Female sterility

Newly emerged adults (< 6 h) were separated and treated topically with Azadirachtin at the $LD_{50}$ (0.63 µg). Males and females of the control and treated series (survivors) were placed separately in vials containing standard corn-meal medium. After 48 h, four conditions

were tested: $M_C + F_C$, $M_C + F_T$, $M_T + F_C$, $M_T + F_T$. Two days later, females without larvae were counted as sterile and results were expressed in percentage.

### 2.7. Statistical analysis

In all experiments except for the one evaluating male activity, the recorded data were dichotomousas 0 and 1 (mating, sperm storage, remating and female offspring percentages). Most experiments contained one control group ($M_C + F_C$) in which both male and female were untreated, and three test groups in which at least one sex was treated: $M_C + F_T$, $M_T + F_C$ and $M_T + F_T$. In these experiments, each treated group was compared to the control group using Fisher's exact test. In the two choice mating experiments, insects had a choice between one treated and one untreated individual of the opposite sex. Mating success, defined as the proportion of trials in which the focal animal mated with one of the choice animals, was compared between groups using Fisher's exact test. Within each group, the proportion of mating with the control and with the treated individual was compared to a 50% theoretical distribution (equal attractiveness) using the exact binomial test.

In the experiment evaluating male activity, actimeter data provide numbers of passages per male per day. For statistical analysis, the data were square-roots transformed to achieve normality (tested using Shapiro-Wilk's test) and homoscedasticity (tested using Brown-Forsythe's test). Then a repeated-measure ANOVA was used on the transformed data, with *Day* as repeated measure and *Treatment* as between-group factor. Statistical analyses were performed using GraphPad Prism (v 6.01 for Windows), Statistica 10 (Statsoft) and R 3.3.1 (RDevelopment Core Team, 2008).

## 3. Results

### 3.1. Mating tests in choice and no-choice conditions

In *D. melanogaster*, Azadirachtin applied topically ($LD_{50}$) the day of adult emergence induced, 48 h after treatment, clear effects on mating success in all tested conditions (Fig. 2A, B and C).

In the no-choice condition (Fig. 2A), the mating percentage, compared to controls ($M_C + F_C$, 43.2%), declined when only males ($M_T + F_C$, 16.8%, Fisher's exact test, $p < 0.001$) or when both sexes were treated ($M_T + F_T$, 13.8%, Fisher's exact test, $p < 0.001$). However, results showed no effect when only the females were treated ($M_C + F_T$, 39.4%, Fisher's exact test, $p = 0.55$).

In the male choice condition (Fig. 2B), two groups were tested, with either an untreated male (two left bars) or a treated male (two right bars), which each had a choice between a treated and an untreated female. When assessing mating success of the focal male, *i.e.* the proportion of replicates in which mating was successful with either one of the females, we found that treated males mated less often than control males (30.7% *vs* 61.6% respectively, Fisher's exact test, $p < 0.001$). Generally, males tended to mate more often with the untreated female than with the treated female. The comparison was highly significant for treated males ($M_T$ choosing $F_C$, 23.5% *vs* $M_T$ choosing $F_T$, 7.2%, Exact binomial test, $p < 0.001$) and on the verge of significance for control males ($M_C$ choosing $F_C$, 39.5% *vs* $M_C$ choosing $F_T$, 22.1%, Exact binomial test, $p = 0.053$).

In the female choice condition (Fig. 2C), two groups were also tested, with either an untreated female (two left bars) or a treated female (two right bars), which had a choice between a treated and an untreated male. When assessing the mating success of the focal female (proportion of replicates in which mating was successful with either one of the males), we found that treated and control females mated just as frequently (59.0% *vs* 46.7% respectively, Fisher's exact test, $p = 0.15$). In both conditions, males mated more often with the untreated female than with the treated female ($F_C$ choosing $M_C$, 36.0% *vs* $F_C$ choosing $M_T$, 10.7%, Exact binomial test, $p < 0.01$ and $F_T$ choosing $M_C$, 48.7% *vs* $F_T$ choosing $M_T$, 10.3%, Exact binomial test, $p < 0.001$).

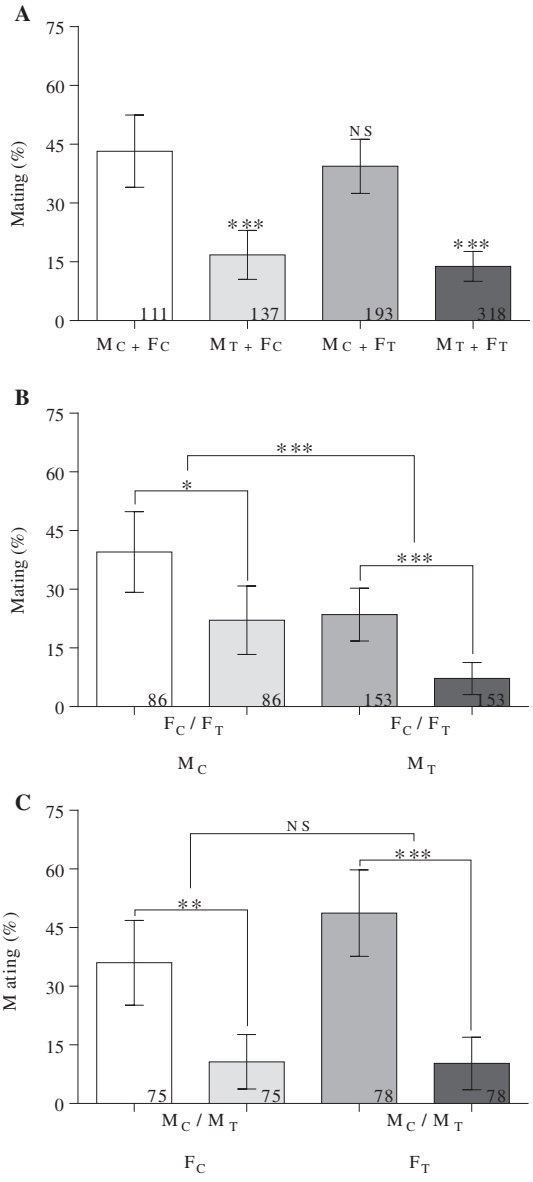


**Fig. 2.** Effects of Azadirachtin on mating observed 48 h after treatment (0.63 µg) by topical application on the day of adult emergence of *D. melanogaster* (mean with 95% confidence intervals; $n$ = 75–318; $M_C$: male control; $F_C$: female control; $M_T$: male treated; $F_T$: female treated). Mating percentages in the no-choice condition (A); the male choice condition (B); the female choice condition (C). Numbers within each bar indicate the number of repetitions; * indicates significant difference at $p \leq 0.05$; ** indicate significant differences at $p \leq 0.01$; *** indicates significant differences $p \leq 0.001$.

### 3.2. Male activity

The number of male passages was observed for 8 days, starting 48 h after Azadirachtin treatment. Fig. 3 shows the average profiles of male activity throughout the day in control and treated flies (average of 8 days). While both groups showed the same activity profile, treated males displayed lower activity between 3 and 21 h (Fig. 3). When evaluating male activity throughout the 8 days of the experiment (Fig. 4), we found a general reduction in the total number of passages made by treated males compared to controls (Fig. 4, repeated-measure ANOVA, *Treatment effect*, $F_{1,\ 581} = 8.66$, $p < 0.01$). While there was a significant effect of the day considered (*Day effect*, $F_{7,\ 581} = 5.90$,

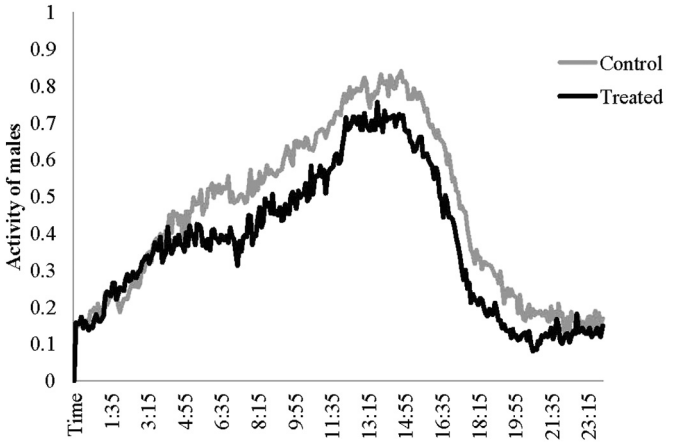


**Fig. 3.** Effects of Azadirachtin applied topically (0.63 µg) on the day of *D. melanogaster* male emergence on cumulative activity profiles for 8 days. The activity profiles were observed 48 h after treatment ($n$ = 42–43).

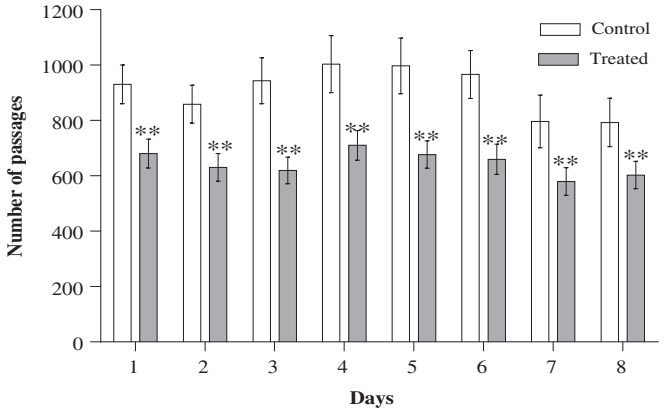


**Fig. 4.** Effects of Azadirachtin applied topically (0.63 µg) on the day of emergence on male activity for 8 days. The number of passages was observed 48 h after topical application (mean ± SE; n = 42–43). ** indicate significant differences at $p \leq 0.01$ between controls and treated for each day.

$p < 0.001$), the interaction between *Day* and *Treatment* was not significant ($F_{7,\ 581} = 0.75$, $p = 0.65$). We conclude that treated males were less active than control males throughout the 8 days of the experiment.

### 3.3. Sperm storage within females

Azadirachtin topical application (0.63 µg) on the day of adult emergence did not produce any effects on the number of females storing sperm in the ventral receptacle (Fig. 5A) and the spermatheca (Fig. 5B). Treated and control females were able to store sperm similarly. Indeed, no significant differences appeared between the treated groups ($M_C + F_T$, $M_T + F_C$ and $M_T + F_T$) and the control group ($M_C + F_C$), both in the spermatheca (Fisher's exact test, $p = 0.36$) and in the ventral receptacle (Fisher's exact test, $p = 0.25$).

### 3.4. Female sexual receptivity to remating

Azadirachtin applied topically ($LD_{50}$) on the day of adult emergence had an effect on female remating (Fig. 6). Compared to the control ($M_C + F_C$, 15.3%), the proportion of remating was significantly higher when both sexes were treated ($M_T + F_T$, 32.7%, Fisher's exact test, $p < 0.05$). The effect was near significant when only females were treated ($M_C + F_T$, 28.4%, Fisher's exact test, $p = 0.078$). No difference appeared however when only males were treated ($M_T + F_C$, 15.8%,

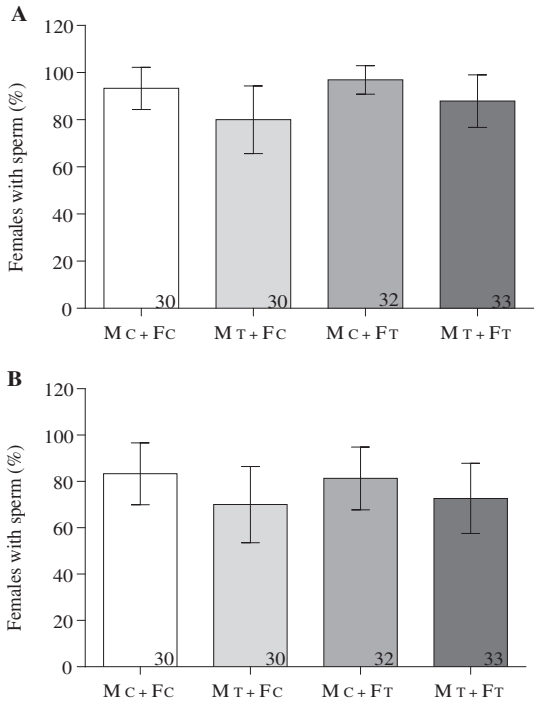


**Fig. 5.** Effects of Azadirachtin applied topically (0.63 µg) on the day of adult emergence on sperm storage in ventral receptacle (A) and spermatheca (B). The females with sperm were observed 3 h after mating ($M_C$: male control; $F_C$: female control; $M_T$: male treated; $F_T$: female treated) occurring 48 h after treatment (mean with 95% confidence intervals; $n$ = 30–33). Numbers within each bar indicate the number of repetitions; for all treated series, no significant differences for ventral receptacle ($p = 0.25$) and for spermatheca ($p = 0.36$) with respect to controls.

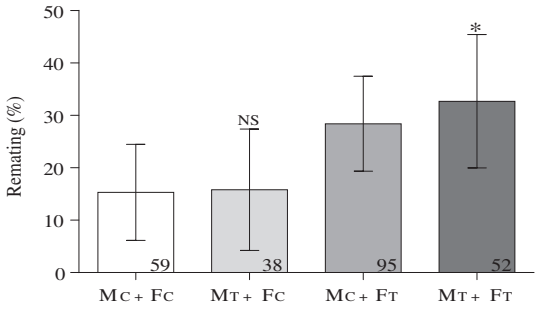


**Fig. 6.** Effects of Azadirachtin applied topically (0.63 µg) on the day of adult emergence on female remating. Remating percentages were measured 7 days after the first mating ($M_C$: male control; $F_C$: female control; $M_T$: male treated; $F_T$: female treated), occurring 48 h after topical application (mean with 95% confidence intervals; $n$ = 38–95). Numbers within each bar indicate the number of repetitions;* indicates significant difference with respect to controls at $p \leq 0.05$.

Fisher's exact test, $p = 1.0$).

### 3.5. Female offspring

Azadirachtin applied topically ($LD_{50}$) also had an effect on female sterility (Fig. 7). The percentage of mated females without larvae in spawning increased when both sexes were treated (26.9%) compared to the controls (8.5%, Fisher's exact test, $p < 0.05$). When only the males (5.3%) or the females (18.9%) were treated, no significant effect was observed compared to controls (Fisher's exact test, respectively $p = 0.70$ and $p = 0.10$).

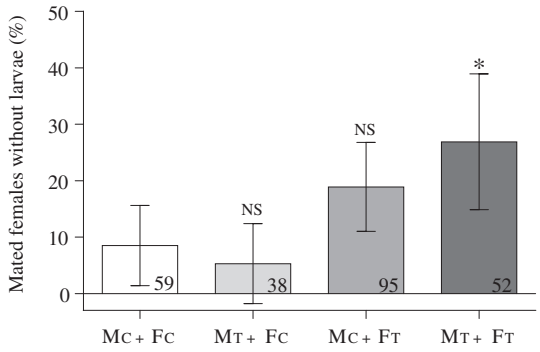


**Fig. 7.** Effects of Azadirachtin applied topically (0.63 µg) on the day of adult emergence. The number of females without larvae in spawning was counted 2 days after mating ($M_C$: male control; $F_C$: female control; $M_T$: male treated; $F_T$: female treated) occurring 48 h after topical application (mean with 95% confidence intervals; $n$ = 38–95). Numbers within each bar indicate the number of repetitions; * indicate significant difference with respect to controls at $p \leq 0.05$.

## 4. Discussion

Our results show that reproduction in a non-targeted insect like *D. melanogaster* is impacted by Azadirachtin. This botanical insecticide reduced mating probability and motility in the treated males, but had no effect on the nycthemeral rhythm. When mating occurred, Azadirachtin treatment impaired female fertility, as noticed by an absence of larvae but increased remating probability. No impairment of the efficiency of mating was observed (presence of sperm in the spermatheca and ventral receptacle).

Topical application of Azadirachtin induced a reduction of mating success in all choice conditions. Previous reports using other IGDs, like ecdysteroid agonists, demonstrated decrease infertility with a reduction in mating success in several insects [40,41,42,43]. Male mating behavior in *D. melanogaster* appears to be controlled by an insulin/insulin-like growth factor and ecdysone-signaling pathways [27] but JH also plays an important role in mating regulation processes [28]. Consequently, the observed impact of Azadiracthin on mating can be explained by the widely documented inhibition of JH and ecdysteroids by Azadirachtin action [7] and their interaction with insulin-signaling [44]. In choice and no choice conditions, only male treatment reduced mating probability in *D. melanogaster*. When females had the choice, they mated less frequently with treated males. However, female treatment also impaired mating probability, because when males had the choice, they mated less frequently with treated females. Males' mating ability may have been impaired by an effect of the treatment on their motility or activity. Such an effect was observed in the actimeter, which revealed that the treated males were less active than untreated ones, in spite of similar nycthemeral rhythms. These results are in agreement with those of Lima et al. [16], who reported similar results in *Neoseiulus baraki* (Acari). These authors also showed that while the pesticide impairs the overall activity of the males, the search for females is not affected [16]. Thus, the observed differences between all tested conditions may be attributable both to the female physiological state and male motility. Indeed, since mating is a negotiation between the two sexes, the behaviors of both are likely to interact and influence mating outcomes [45]. Consequently, changes in females' sexual attractivity can be explained by Azadirachtin antagonist action on major hormones (JH and ecdysteroids), in the reproduction process [46] and can be linked to the observed infertility induced by the pesticide [7,21,38]. It is well established that feedback/release of ecdysone and JH depend on a neural regulation [47,48]. Moreover, Azadirachtin is known for its neurotoxic action by blocking of voltage-gated calcium channels [23]. For courtship and mating success, many sensory modalities, including olfaction, are needed by *D. melanogaster* males to find their mates [2,29,30]. Thus, a mating decrease, observed in all conditions, may also be due to an impact of Azadirachtinon these sensory modalities in the treated males.

Mating decisions are controlled by a balance of excitatory and inhibitory drives onto central courtship-promoting neurons [49]. Neuromodulators like serotonin (5-HT), octapamine (OA) and dopamine (DA) have central effects on the adult *Drosophila* brain in relation with learning and behavior and their actions can influence the decision to engage in locomotion, fight or courtship activities [50,51,52]. In *Drosophila*, the sexually dimorphic circuit (motor neurons, interneurons and mechanosensory neurons) controls reproductive processes such as sperm ejection in males, ovipositor extension, sperm storage and egg-laying decisions in females [53,54,55]. Furthermore, DA interacts differentially with JH depending on the sex of the animal [56] and is known to mediate ovarian development, sexual receptivity and fertility [57,58,59]. Recent studies show that the insulin-signaling pathway regulates JH and DA metabolism [60]; consequently, Azadirachtin impact on insulin and JH signaling as well as also its neurotoxic action could explain the greater sensitivity of *D. melanogaster* treated females noted in our experiments; this, in spite of similar survival to Azadirachtin between males and females [38]. Indeed, no significant effect was observed on post-mating responses in treated males. Mating stimulates female germline stem cell (GSC) proliferation in *D. melanogaster via* ecdysteroid-signaling [61]; GSC activity is coordinately regulated by the neuroendocrine system to sustain the reproductive success in response to mating [48,61]. In addition, ovogenesis, vitellogenesis, egg maturation and oviposition are controlled by ecdysteroid and JH [45,46]. Therefore, a wide range of possible mechanisms could be involved in the observed impact of Azadirachtin on female fertility. Results obtained on sterility in treated females show higher values comparatively to those obtained in treated males but they are not significantly different. This could be linked with impairment of oviposition inducing considerable variability in oviposion delay between individuals. We might have observed a significant difference during a longer period of experimentation. When mating occurred, however, no impairment was observed on the efficiency of mating, evaluated by the presence of sperm in female genital tracts (spermatheca and ventral receptacle). In *D. melanogaster* females, sperm release is regulated by neuromodulators like OA [62]. At this level, Azadirachtin could have impacted sperm use through a potential blockage in the genital tracts *via* the neurotoxic action.

In our results, female sterility was positively correlated with a remating increase. In untreated females, the decrease of productivity or production of in fertile eggs can induce new mating [63]. Consequently, Azadirachtin impact on female fertility could explain the remating increase when both sexes were treated. Remating increase can also be linked to an impact of Azadirachtin on protein synthesis in the male accessory glands (for instance on the Sex peptide) *via* JH involvement [64]. Such potential modifications of the seminal fluid could favor female remating receptivity. Furthermore, the *D. melanogaster* female remating rate can be modulated by JH action *via* regulation of the sex pheromones and hydrocarbon production [32,65]. Like JH, ecdysteroid-signaling is essential in pheromone synthesis for the maintenance of cuticular lipids and oenocytes throughout adulthood, [31] and these parameters may alter both the reproductive physiology and behavior of treated flies. Finally, in *D. melanogaster* an optimal nutritional level is required to maximize reproductive success through the initiation of effective pre- and post-mating responses in females [66,67]. Thus, an effect of Azadirachtin on nutrition [68] and on insulin-signaling [24] could also negatively impact several reproductive traits. Indeed, Azadirachtin induces, in *D. melanogaster*, a decrease in food intake, biochemical effects (decrease in α-amylase, chitinase, proteases and lipases), [69] and detrimental impacts on various tissues [7]. In addition, oxidative stress induced by Azadirachtin in *D. melanogaster*[21] can interact with ecdysteroid and insulin-signaling pathways [70].

*D. melanogaster* males and females showed a similar survival at $LD_{50}$

of Azadirachtin [38]. This effect was also found in other species like *Blatta orientalis* treated with Azadirachtin [71] or the *Blattella germanica* treated with Spinosad (another natural pesticide) [72] However, in the *Blattella germanica* and for several other pesticides (Bendiocarb, Chlorpyrifos, Cyfluthrin, Cypermethrin, Fenvalerate, Hydramethylnon, Malathion, Propetamphos, propoxur, and pyrethrins) a difference of toxicity at $LD_{50}$ was observed between males and females, but also between females, gravid or not [73]. Thus pesticides may act differently within the same species. Indeed, in *Trichogramma chilonis* (Hymenoptera) Beta-cypermethrin exposure ($LD_{20}$) induced a decrease of male-specific sex pheromone production and mating rate but without decreasing locomotor activity of treated males, whereas Spinosad exposure ($LD_{20}$) caused a significant decrease in male locomotor activity of *T. chilonis*, but did not affect male-specific sex pheromone production or mating rate [74].

In conclusion, our study documents several impairments of reproductive traits in *D. melanogaster* by topical application of Azadirachtin. These effects can be explained by a direct and/or an indirect action or through domino effects on the endocrinal, neuroendocrinal and neuronal complex [44,60,75]. Studying the sublethal effects of pesticides (like Azadirachtin or others molecules) is particularly important because it allows to better understand toxicological and physiological mechanisms induced by the treatment; consequently, the choice of pesticides and their use (single or combined treatment) in integrated pest management may be facilitated. Future work will aim to decipher which of the proposed effects actually play a crucial role in the observed reproductive defects.

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