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1 **Transgenerational effects from single larval exposure to azadirachtin on life history**
2 **and behavior traits of *Drosophila melanogaster***

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19 .

26 **Abstract**

27 Azadirachtin is one of the successful botanical pesticides in agricultural use with a
28 broad-spectrum insecticide activity, but its possible transgenerational effects have not been
29 the object of much attention. The effects of sublethal doses of azadirachtin on life-table traits
30 and oviposition behaviour of a model organism in toxicological studies, *D. melanogaster*,
31 were evaluated. The fecundity and oviposition preference of flies surviving to single
32 azadirachtin-treated larvae of parental generation was adversely affected resulting in a
33 reduction of number of eggs laid and increasing aversion to this compound over two
34 successive generations. In parental generation, early exposure to azadirachtin affects adult's
35 development by reducing the number of organisms, delayed larval and pupal development;
36 male biased sex ratio and induced morphological alterations. Moreover, adult's survival of
37 the two generations was significantly decreased as compared to the control. Therefore, Single
38 preimaginal azadirachtin treatment can affect flies population dynamics *via* transgenerational
39 reductions in survival and reproduction capacity as well as reinforcement of oviposition
40 avoidance which can contribute as repellent strategies in integrated pest management
41 programs. The transgenerational effects observed suggest a possible reduce both in
42 application frequency and total amount of pesticide used, which would aid in reducing both
43 control costs and possible ecotoxicological risks.

44 **1. Introduction**

45 The effect of insecticides and other toxicants on insects have been traditionally assessed
46 using measures of the acute mortality as a single endpoint and have relied on the
47 determination of the acute lethal dose/concentration¹. However, in addition to the direct
48 effect on lethality these compounds may also impair various key biological traits of the
49 individuals that survive exposure through physiological and behavioral effects^{1,2}. Among

50 physiological effects, developmental success, morphological deformities, adult longevity, sex
51 ratio, fertility and fecundity are commonly estimated^{3,4}. Behavioral effects may be manifested
52 as impairment in insect mobility, learning ability, host finding, sexual communication as well
53 as feeding and oviposition behavior⁵⁻¹⁰. An accurate assessment of these effects is crucial to
54 acquire knowledge on the overall insecticide efficacy for long-term management of pest
55 insect populations, as well as on their selectivity toward non-target species¹¹. Indeed, when
56 studying susceptibility of organisms towards insecticides, and beside the short term
57 influences on the directly exposed individuals, it is important to take into account the entire
58 life-history as a comprehensive method for evaluating the total effect on insect population,
59 including the impacts on the next generation which have important implications for the
60 success of an Integrated Pest Management (IPM) program^{2,4}.

61 Today and among the insecticides used in sublethal effect studies, the botanical insecticides
62 have been the subject of an increasing number of academic research as a potential option for
63 an environment friendly pest management tools^{12,13} due to their rapid degradation in
64 environment, low mammalian toxicity, low risk of resistance development in target pest
65 populations and good selectivity to non-target arthropods¹⁴⁻¹⁸. Azadirachtin (AZA), a natural
66 tetranortriterpenoid compound extracted from the neem tree, *Azadirachta indica*¹⁹,
67 is considered as one of the most promising plant compounds for pest control in organic
68 agriculture^{14,20}. AZA shows variable effects on insects including the model insect *Drosophila*
69 *melanogaster*^{21,22}. This triterpenoid acts as sterilant, insect growth regulators by disruption of
70 the endocrine system, repellent, oviposition and feeding deterrent by activating bitter
71 sensitive gustatory cells^{23,24}. Larval exposure of *D. melanogaster* to sublethal doses of
72 azadirachtin was found to affects various aspects of their physiology including digestive
73 enzymes²⁵ and this effect is also further observed in the adults¹⁰. This pre-imaginal exposure

74 affects not only the physiology and the fitness of flies but also adults oviposition and feeding
75 preference^{7,10}.

76 Most studies concerning the sublethal effects of insecticides are related to continuously or
77 repeated exposure. This exposure provokes a generalized stress and activating a
78 detoxification response such as up-regulated of cytochrome P450 genes which may lead to the
79 detoxification of insecticide and even the development of resistance²⁶. Moreover, the up-
80 regulation is thought to provide versatility in environmental adaptation²⁷. In botanical
81 insecticide the potential fast desensitization to a feeding deterrent was reported^{28,29}.
82 Individual insects initially deterred by feeding inhibitor become increasingly tolerant due to
83 repeated or continuous exposure²⁹. Bomford and Isman¹⁵ reported an habituation to pure
84 azadirachtin in the tobacco cutworms which become less sensitive to the antifeedant
85 properties of azadirachtin, but not to a neem containing a same absolute amount of
86 azadirachtin. This might have an important implication to avoid desensitization to
87 commercial neem-based insecticides which contains additional non AZA-compounds¹⁵.
88 Larval exposure to Neem Azal, a commercial Azadirachtin-rich based formulation, was
89 found to enhance avoidances of this compound in adults of *D. melanogaster* surviving from
90 previously treated larvae^{10,25}. This long-lasting avoidance is related to conditioned aversion
91 and may be related to another mechanism such as sensitization^{30,31} which also generally
92 occurs after long term or repeated exposure and may increase avoidance to noxious
93 stimulus³². Moreover, increasing evidence has highlighted the critical role of early life
94 experience in adult physiology and behavior in insect³³. Recent studies have revealed that
95 insect can modulate their behavior on the basis of previous experiences early life and that
96 various insecticide-mediated changes in the directly exposed generation can persist into the
97 subsequent non-exposed generations^{34,35}. Previously, we focused on the impact of larval
98 exposure to azadirachtin on adult's fitness (fecundity, survival) and oviposition site

99 preference of the parental generation of *D. melanogaster* as model organism for testing
100 insecticide activity⁷. Current study aimed to evaluate, the possible adverse effects of this
101 prior single exposure to azadirachtin experienced by the preceding generations on life table
102 and oviposition site preference of the filial generations. We monitored the oviposition site
103 preference, fecundity, development, sex ratio, survival and morphological abnormalities of
104 exposed and non-exposed generations. All these parameters were investigated over
105 generations until their restoration to predict the outcome of azadirachtin use on pest
106 management practices.

107 **Results**

108 **Fecundity and oviposition site preference**

109 Azadirachtin, topically applied on the 3rd instar larvae (LD₂₅ and LD₅₀ of immature stages)
110 affected fecundity of females by a significant reduction of the number of eggs laid as
111 compared to controls (KW= 24.73; $p < 0.001$). This reduction was observed over two
112 successive generations (parental and F1), however, the total eggs laid was higher in the
113 unexposed generation (F1) than in parental (P) ones (KW= 50.89; $p < 0.001$) (Fig.1). Full
114 restoration of affected fecundity was noted in the second generations (F2).

115 Results of oviposition preference in the no choice experiments (Fig.1) revealed a clear
116 preference for oviposition on untreated medium than in azadirachtin-treated ones.

117 For parental generation, Kruskal-Wallis test revealed significant effects in medium 0.1 µg/ml
118 (KW= 29.42; $p < 0.001$) and medium 0.25 µg/ml (KW= 24.73; $p < 0.001$). In the first
119 generation, a significant effect was also noted for medium 0.1 µg/ml (KW= 22.95; $p < 0.001$)
120 and medium 0.25 µg/ml (KW= 27, 93; $p < 0.001$).

121 Results concerning the dual choice experiments (Fig. 2) revealed an oviposition preference
122 for control medium than treated medium in all tested generations (P, F1 and F2).
123 Furthermore, flies previously exposed to azadirachtin (early 3rd instar larvae) showed a

124 highest aversion to this substance compared to naïve flies and led fewer eggs for the two first
125 generations (P and F1) with a more marked effects for parental generation ($P < 0.001$).

126 The oviposition preference index (OPI) of adult females of *D. melanogaster* exposed, or not,
127 to azadirachtin at larval stage of parental generation were always negative in all generations
128 (Fig. 3).

129 In the generation P, statistical analysis showed significant differences between OPI of
130 previously treated flies and controls flies with a dose-dependent response (fig. 3). In addition,
131 for medium 0.1 $\mu\text{g/ml}$, Mann-Whitney revealed significant effects between LD_{25} of the
132 parental generation and the first generation (Mann-Whitney test $U = 8$; $P < 0.001$), LD_{25} of
133 parental and second generation (Mann-Whitney test $U=20$; $P=0.0018$) but there was no
134 difference between the first and the second generations (Mann-Whitney test $U=42$;
135 $P=0.0887$). Similar results were observed for the LD_{50} , with significant effects observed
136 between the parental generation and the F1 (Mann-Whitney test $U=19$; $P=0.0014$), also
137 between P and F2 (Mann-Whitney test $U=34$; $P=0.0284$) but no difference between F1 and
138 F2 (Mann-Whitney test $U=58$; $P=0.4428$). For control, there was no difference between all
139 tested generations.

140 Similar results were obtained for medium 0.25 $\mu\text{g/ml}$, Mann-Whitney test revealed
141 significant effects between LD_{25} of the parental generation and the first generation (Mann-
142 Whitney test $U = 25$; $P =0.0045$), LD_{25} of parental and second generation (Mann-Whitney
143 test $U=24$; $P=0.0045$) but there was no difference between the first and the second
144 generations (Mann-Whitney test $U=66$; $P=0.5512$). For the LD_{50} , significant effects were
145 observed between the parental generation and the F2 (Mann-Whitney test $U=25.50$;
146 $P=0.0025$), also between F1 and F2 (Mann-Whitney test $U=34$; $P=0.0028$) but no difference
147 was observed between F1 and P (Mann-Whitney test $U=49$; $P=0.1974$). There was no
148 difference between controls for all generations.

149 **Analyses of development**

150 Results from development analysis of *D. melanogaster* are given in tables 1 and 2,
151 respectively for parental (exposed) and F1 (non-exposed) generation. Treatment of early third
152 instar larvae at two tested doses (LD₂₅ and LD₅₀) decreased the number of larvae, pupae and
153 the final number of organisms of parental generation with a dose-dependent relationship as
154 expressed by the FNO which is always negative for the treated series. The development of F₁
155 *D. melanogaster* doesn't seem to be affecting by the early treatment of the parental
156 generation. However, the FNO of tested flies (LD₂₅, LD₅₀ and control) in treated medium was
157 significantly lower than in the control medium for both generations. There is no difference
158 between the number of organisms reached the pupae stage and the final number of organism
159 in both generations. In addition, treatment of early third instar larvae increased significantly
160 (p<0.001) the duration of larval and pupal development as expressed by T₅₀, with dose-
161 dependent manner only for the Parental generation (exposed) as compared to controls. There
162 is no difference between the T₅₀ of the tested flies in both treated and untreated medium.

163 Larvae, pupae and imagoes of the parental generation showed several types of malformations
164 and anomalies followed by death at each stage of development of *D. melanogaster*. The most
165 prominent malformations detected are incomplete and malformed imagoes (malformed
166 abdomen and wings), curved and smaller body shape, burned larvae, dead adults inside pupae
167 (Fig.4).

168 Pre-imaginal exposure of azadirachtin induced a male-biased sex ratio only for the parental
169 generation with a dose-dependent relationship (Fig.5). Kruskal-Wallis test revealed
170 significant effects between the different tested insect (Control, LD₂₅ and LD₅₀) in untreated
171 medium (KW = 9.30; p =0.0095), medium 0.1 µg/ml (KW = 8.02; p < 0.0181) and medium
172 0.25 µg/ml (KW= 18.85; p < 0.0001) for the parental generation.

173

174 **Survival analysis of adults**

175 A survival analyses during the 15 first days of adults previously treated with azadirachtin as
176 3rd instars larvae (Fig.6) revealed a rapid reduction of adult surviving of the generation P
177 (Male: Kaplan-Meier test, $\chi^2 = 184$, $df = 2$, $P < 0.001$; Female: Kaplan-Meier test, $\chi^2 = 214$,
178 $df = 2$, $P < 0.001$). Lower mortality was noted for the generation F1 compared to parental.
179 (Male: Kaplan-Meier test, $\chi^2 = 39.1$, $df = 2$, $P < 0.001$; Female: Kaplan-Meier test, $\chi^2 = 63.1$,
180 $df = 2$, $P < 0,001$). Flies mortality was dose-dependent and the females were more affected by
181 the treatment.

182 For the control series no mortality was recorded for both tested generations. For the treated
183 series, the lowest dose (LD₂₅) decline the adult's survival to 49% for males and 36% for
184 females of the P generation versus 94% for males and 84% for females of the F1 generation.
185 The highest dose (LD₅₀) induced more marked effects on adult's survival with 27% for males
186 and 16% for females of the P generation and 81% for males and 64% in females for the F1
187 generation. Survival of 100% was noted for males and females of the F2 generation.

188 **Discussion**

189 Azadirachtin impact on reproduction have been reported on different insect species^{21,41-45}.
190 Our study has demonstrated that a single azadirachtin treatment (LD₂₅/LD₅₀) of *D.*
191 *melanogaster* larvae reduced eggs number affecting negatively the fecundity of surviving
192 females, not only through direct sublethal effects in exposed individuals, but also through
193 transgenerational effects on F1 individuals that were never directly exposed to the insecticide.
194 Oviposition is a complex and critical activity in the life cycle of an insect with a variety of
195 factors that influence both physiology and subsequent behavior, that lead to egg deposition
196 by an insect which tries to ensure safety to their progeny. Reduced fecundity and fertility
197 after azadirachtin treatment has been reported in many insects including *Spodoptera*
198 *littoralis*, *D. melanogaster*, *Galleria mellonella*, *Dysdercus cingulatus*, *Tuta absoluta* and

199 *Helicoverpa armigera*^{17,41,43-46} and may be correlated to the negative action of azadirachtin
200 on yolk protein synthesis and/or its uptake into oocytes²¹.

201 Ecdysteroids, JH and insulin/insulin-like growth factor signalling (IIS) regulation are crucial
202 for reproduction of *D. melanogaster*⁴⁷. Vitellogenesis in females is stimulated under JH
203 action and led to oocytes development, JH synergic action with 20E and IIS controls the
204 nutrient-sensitive checkpoint necessary for oocytes formation⁴⁷. Consequently, reduced
205 fecundity may be related to the antagonist action of azadirachtin on major hormones
206 controlling the reproductive process (JH/ecdysteroids)⁷.

207 In *Anopheles stephensi*, azadirachtin treatment led to abnormal ovaries structure with a
208 complete arrest of oogenesis, vitellogenesis and vitelline envelope formation impairment and
209 follicle cells degeneration⁴⁸. Ovaries of azadirachtin-treated females of *Heteracris littoralis*
210 also showed complete shrinkage with suppression of oocyte growth⁴⁹, disintegration and
211 destruction in follicular cells and mitochondria⁴⁹. In addition, Azadirachtin reduce mating
212 success in *D. melanogaster* flies and negatively affect cyst and oocyte numbers and size⁴⁵.

213 Azadirachtin treatment also affect the amount of food intake in this species and digestive
214 enzyme activity in the midgut¹⁰, which may affect oogenesis and vitellogenesis since
215 ecdysone and JH rates are affected by nutrient availability which acts as positive regulator on
216 insulin pathway to confer to ovaries the signalling necessary for a normal oogenesis^{50,51}.

217 In addition, flies of all tested generations preferred control medium for oviposition avoiding
218 the azadirachtin ones for the two tested doses and conditions (no-choice and free choice). A
219 low oviposition rate of non-exposed (naïve) flies in azadirachtin-treated areas may be due to
220 the known repellent effect, deterrent effect and locomotor stimulation effect of azadirachtin
221 and other neem based insecticides which were reported by Silva *et al.*⁵² in medflies *Ceratitis*
222 *capitata*. Valencia-Botín *et al.*⁵³ also suggest that the repellent property of neem extracts is

223 the major factor responsible for the reduction of eggs numbers of *Anastrepha ludens*
224 (Loew)⁵³. The ovipository behavior inhibition may have a valuable impact in pest control.

225 In addition, flies who have already been treated (third instar larvae of P generation) showed
226 an increased aversion to azadirachtin in comparison to the naïf flies and this for two
227 successive generations (P and F1). When oviposition sites were treated with azadirachtin or
228 other neem-based compounds, oviposition repellency, deterrence, or inhibition occurred in
229 several insects' species which can detect the bioinsecticide on the treated surface^{7,14,43,54,55}.
230 The capacity of insects to retain memory from early life exposure affecting the adult response
231 was reported^{38,56-58}. In *D. melanogaster* females avoid oviposition on sites containing
232 azadirachtin after larval exposure to the bio-insecticide⁷.

233 Here, we have reported for the first time that the negative effects of a single larval exposure
234 to azadirachtin can also be passed on to the F1 generation (transgenerational effects).
235 Environmental toxicants such as insecticide are able to provoke epigenetic alterations which
236 can be inherited to next generations⁵⁹. This may explain the reduced fecundity and
237 oviposition avoidance in our non-exposed generation (F1).

238 Our study has also demonstrated that azadirachtin applied during the third larval instar of
239 Parental generation (LD₂₅ and LD₅₀) can negatively affect various life traits of *D.*
240 *melanogaster*, with a dose-dependent manner, by significantly reducing, larval, pupation and
241 emergence rate of the exposed generation. The biopesticide also significantly prolong the
242 larval and pupation period of development inducing an important delays in immature stages
243 development and affect sex ratio (with fewer females in the offspring) of the same
244 generation. Additionally, the treatment induced morphological alterations of larvae, pupae
245 and adults only on the exposed generation (P generation). The most prominent abnormalities
246 are burned larvae, larva-pupa intermediate, pupa-adult intermediate, deformed wings, smaller

247 body size and deformed abdomen. The recorded malformations finally result to insect dead.
248 Similar results were noted in *D. melanogaster*³⁷, *Hyalomma anatolicum excavatum*⁶⁰ and
249 *Spodoptera litura*²². Finally, a reducing of adult's survival was noted for the two successive
250 generations with more marked effects among the P generation.

251 Azadirachtin is known to reduce pupation and eclosion rates of many insects like *Aphis*
252 *glycines*⁶¹, *Plodia interpunctella*⁶², *Aedes aegypti*⁶³ and *D. melanogaster*²¹. A negative impact
253 of azadirachtin on the immature stages was expected in view of its insect's growth disruptor
254 (IGD) action by suppressing haemolymph ecdysteroid and JH peaks^{25,64}. Furthermore,
255 azadirachtin is known to cause nucleus degeneration in the different endocrine glands
256 (prothoracic gland, *corpus allatum* and *corpus cardiacum*) controlling insects moulting and
257 ecdysis which may act as generalised disruption of neuroendocrine system²⁴. Azadirachtin by
258 altering the growth and molting process of several insects compromise their
259 survival^{7,20,43,65,66}. Lai *et al.*⁶⁷ reported that azadirachtin down regulated expression of
260 different genes linked to hormonal regulation which may explain the developmental
261 aberrations observed in our results. Azadirachtin also affect *Drosophila* nutrient intake and
262 metabolism compromising the nutritional signals which result in a decrease in insect weights
263 and growth rates resulting in smaller body size and impacting survival^{10,25,37,66}. The male
264 biased sex ratio under azadirachtin treatment was also reported in literature^{67,68}.

265 In summary, the present study indicated that pre-imaginal exposure to sublethal doses of
266 azadirachtin would affect the fecundity, oviposition preference and survival of *D.*
267 *melanogaster* of parent generation as well as the F1 non exposed generation. The treatment
268 would also trigger life history traits variation at the P generation.

269 Results demonstrate that a single azadirachtin application can significantly reduce the
270 survival of flies over two successive generations (P: exposed and F1: unexposed) while
271 insects showed clear recovery in the survival rates in the second generation (F2). These

272 findings reflect a long term and delayed effects through developmental stage and generations.
273 This consistent effect over the two first generations may be considered as advantage on pest
274 control by compensate the well known fast degradation by sunlight and low persistence of
275 azadirachtin in environment (half-life DT_{50} : 1.7- 25d)^{23, 69} and suggest a possible reduce both
276 in application frequency and total amount of pesticide used.
277 Moreover, the decreased fecundity and survival in P and F1 generations indicated an absence
278 of induction of the resurgence in offspring, even after full restoration in F2, when parental
279 flies were treated translating an absence of hermetic effect, which is considered as serious
280 problem of exposure to sublethal doses in agriculture.
281 In addition, the treatment increases the aversive effect induced by azadirachtin over two
282 successive generations which may contribute as push-pull strategies increasing its insecticidal
283 effects in integrated pest management programs.

284 **Material and methods**

285 **Flies**

286 Wild-type Canton-S strain of *D. melanogaster* flies were reared on artificial fly food
287 (cornmeal/agar/yeast) at 25°C, 70% humidity and 12D-12 L cycle¹⁰.

288 **Treatment**

289 Neem Azal-TS (1 % azadirachtin A, Trifolio-M GmbH, Lahnau, Germany) was solubilised
290 in acetone for topical application (1 µl/ larvae according to Bensebaa et al.³⁶). The
291 bioinsecticide was applied on *D. melanogaster* early third-instar larvae using two lethal
292 doses of immature stages, 0.28 µg (LD_{25}) and 0.67 µg (LD_{50})³⁷. Controls received 1µl acetone
293 (solvent) and all flies were kept under the same conditions as cited above. All experiments
294 were performed over two consecutive generations, the exposed (parental generation: P) and
295 non-exposed (first generation: F1) generation.

296 **Fecundity and oviposition site preference**

297 We assessed the egg-laying performances of the females of *D. melanogaster* using a no-
298 choice test. Three mated females (3 days old) that were pre-exposed to azadirachtin at the
299 larval stage (LD₂₅ and LD₅₀) were tested for 24 h in a petri dish (Ø=65mm) filled with 3 ml
300 medium containing azadirachtin at two concentrations 0.1 and 0.25 µg/ml according to
301 Bezzar-Bendjazia et al.³⁷; in addition to acetone as control medium. These concentrations were
302 not lethal with the short exposure time (24 h) used. At the end of the test, flies were removed,
303 and the number of eggs laid on each medium was counted. The control medium was used to
304 test the possible effect of azadirachtin on female fecundity. The experiment was repeated 12
305 times for each medium and each generation. Oviposition site preference was measured by
306 means of dual choice experiments. Three fertilized females (3 days old) from controls and
307 treated series (LD₂₅ and LD₅₀) were allowed to oviposit for 24h in a free choice egg-laying
308 device. This device consisted of a two petri dishes either filled with control medium (acetone)
309 or treated medium (azadirachtin: 0.1, 0.25 µg/ml). After 24h, the egg-laying preference was
310 assessed by counting the number of eggs laid in each medium. The test was performed for
311 two successive generations with 12 replicates for each medium and generation.

312 Oviposition preference index (OPI) defined as (number of eggs on azadirachtin medium –
313 number of eggs on control medium)/total number of eggs was calculated³⁸.

314 **Development assays**

315 Ten controls or pre-exposed (LD₂₅ and LD₅₀) mated females (3 days old), named parental
316 generation, were released into an oviposition box containing petri dishes filled with control
317 (acetone) or treated medium (azadirachtin: 0.1 or 0.25 µg/ml) and left to lay eggs for 8 hours.
318 At the end of the test, the flies were removed and a pool of 100 eggs for each experiment was
319 transferred to a new petri dish containing the same medium. For all groups, we monitored the

320 time course of larval development from egg to adult emergence by counting the number of
321 third instar larvae, pupae, imagoes and their sex ratio, expressed as the number of males
322 divided by the total number of emerged insects.

323 Next, ten parental flies from each condition (controls or treated) were crossed and the
324 experiments were repeated for the non-exposed first generation (F1) as cited above with the
325 same parameters recorded.

326 Furthermore, the developmental duration of each stage was recorded for the two tested
327 generations expressed by T_{50} (time in hours, when 50% of population reached larval, pupal
328 and imaginal developmental stage in vials). All insects were observed under stereo zoom
329 microscope to find any morphological distortions and photographs were taken with Leica
330 Z16 APO.

331 A factor describing the final number of organisms in comparison to control (FNO) according
332 to Ventrella et al.³⁹ was determined to compare the results:

333

$$FNO = \frac{T - C}{C} \times 100$$

334 T = final number of organisms counted in treated medium.

335 C = final number of organisms counted in control medium.

336 Positive values of FNO show that number of organisms was higher in tested groups than
337 within control, negative values mean that the number of individuals was higher in control
338 than in exposed groups.

339 **Survival analysis of adults**

340 Survival analysis was performed according to Linford et al.⁴⁰. For each generation (P:
341 exposed (LD₂₅ and LD₅₀), F1: non-exposed) newly emerged adults were sexed and housed
342 separately into a plastic vials (15 flies per vial) containing fresh food. Insects were transferred

343 to new vial every 2 days. The flies were kept under observation for 15 days during which
344 mortality was assessed every 24h. Ten replicates were done for each dose and generation.

345 **Statistical analyses**

346 Data analysis was performed by R studio version 3.5.0 for Mac OS. The results were
347 expressed as the means \pm SE of each series of experiments. The homogeneity of variances
348 was checked using Bartlett's test. The Shapiro-Wilk statistic test was used for testing the
349 normality.

350 Data from egg-laying preference and oviposition index preference was subjected to Kruskal–
351 Wallis test and pairwise multiple comparisons using Dunn's method. Development test were
352 analysed with ANOVA followed by a post-hoc HSD Tukey test. Sex ratio was analysed using
353 Kruskal–Wallis test and the FNO was calculated and shown. The results of the survival
354 analysis were subjected to Kaplan–Meier survival test.

355

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552 **Author Contributions**

553 S.K.M. designed the experiment; M.F and R.B.B. performed the experiments; M.F. and
554 S.K.M analyzed the data and wrote the manuscript; S.K.M. and F.M.P reviewed and
555 approved the manuscript.

556 **Additional Information**

557 **Competing Interests:** The authors declare no competing interests.

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568 **List of figure captions**

569 **Figure 1.** Effect of azadirachtin (LD₂₅ and LD₅₀), topically applied on early third instars
570 larvae of *D. melanogaster* on fecundity of females (number of eggs laid) subjected to non-
571 choice experiments ($m \pm SE$; n =12 replicates of 3 flies). Different small letters indicate a
572 significant difference between control and treated individuals of the same medium (P<0.05).
573 Different capital letters indicate a significant difference between generations of the same
574 medium (P<0.05).

575 **Figure 2.** Egg-laying preference ($m \pm SE$; n=12 replicates) of female adults of *D.*
576 *melanogaster* subjected to a free-choice test on food treated with azadirachtin at two doses
577 (0.1µg/ml and 0, 25µg/ml). Different small letters indicate a significant difference between
578 control and treated individuals of medium untreated and treated (P<0.05). Different capital
579 letters indicate a significant difference between individuals of the same dose in the different
580 medium (P<0.05).

581 **Figure 3.** Oviposition preference index ($m \pm SE$; n=12 replicates) of female adults of *D.*
582 *melanogaster* subjected to a free-choice test on food treated with azadirachtin at two doses
583 (A: 0, 1 µg/ml; B: 0, 25µg/ml). Different small letters indicate a significant difference
584 between the same dose of different generations (P<0.05). Different capital letters indicate a
585 significant difference between difference tested doses of the same generation (P<0.05).

586 **Figure 4.** Examples of the most frequent malformations of *D. melanogaster* (n=50). A)
587 Malformed abdomen and wings curved and smaller body shape; B) dead adults inside pupae;
588 C) malformed adult; D) burned larvae.

589 **Figure 5.** Effect of azadirachtin (LD₂₅ and LD₅₀), topically applied on early third instars
590 larvae of *D. melanogaster* on sex ratio of adults emerged. Different small letters indicate a
591 significant difference between generations of the same medium (P<0.05). Capital letters

592 indicate a significant difference between control and treated individuals of the same medium
593 ($P < 0.05$). ($m \pm SE$; $n = 15$ replicates).

594 **Figure 6.** Effect of azadirachtin (LD_{25} and LD_{50}), topically applied on early third-instar
595 larvae of *D. melanogaster* on the adult's survival (male and female) of two generations tested
596 ($p < 0.05$).

597 **List of tables captions**

598 **Table1.** Effect of larval exposure to azadirachtin on development of parental generation
599 (exposed) of *D. melanogaster*. Letters indicate a significant difference between the different
600 tested doses of the same medium for each stage of development ($P < 0.05$). Different capital
601 letters indicate a significant difference same doses tested of different medium ($P < 0.05$). ($m \pm$
602 SE ; $n = 15$ replicates).

603 **Table2.** Effect of larval exposure to azadirachtin on development of first generation (non-
604 exposed) of *D. melanogaster*. Letters indicate a significant difference between the different
605 tested doses of the same medium for each stage of development ($P < 0.05$). Different capital
606 letters indicate a significant difference same doses tested of different medium ($P < 0.05$). ($m \pm$
607 SE ; $n = 15$ replicates).

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617 **Table1.** Effect of larval exposure to azadirachtin on development of parental generation
618 (exposed) of *D. melanogaster*. Letters indicate a significant difference between the different
619 tested doses of the same medium for each stage of development ($P<0.05$). Different capital
620 letters indicate a significant difference same doses tested of different medium ($P<0.05$). ($m \pm$
621 SE; n=15 replicates).

Concentration	Larvae			Pupae			Imagoes		
	N° of individuals	T ₅₀ (h)	Malformations (%)	N° of individuals	T ₅₀ (h)	Malformations (%)	N° of individuals	Malformations (%)	FNO
Control Medium									
Control	93.73±1.31 A a	41.93±0.25 A a	0.0±0.0 A a	92.66±1.41 A a	150.86±1.43 A a	0.0±0.0 A a	90.8±1.48 A a	0.0±0.0 A a	0
DL ₂₅	85.86±1.22 A b	49.8±0.63 A b	2.53±0.96 A b	79.4±1.37 A b	159.4±0.35 A b	1.53±0.70 A b	78.4±1.52 A b	17.86±2.65 A b	-13.41
DL ₅₀	80.20±2.24 A b	60.93±0.61 A c	3.6±1.03 A b	75.06±2.50 A b	166.2±0.53 A c	4.4±2.53 A c	73.2±2.53 A c	20.33± 2.65 A b	-19,25
Medium treated with azadirachtin 0.1µg/ml									
Control	89.93±0.64 A a	42.06±0.61 A a	0.0±0.0 A a	86.26±1 A a	151.46±0.89 A a	0.0±0.0 A a	81.60±1.15 B a	0.0±0.0 A a	0
DL ₂₅	79.93±1.27 A b	49.6±1.64 A b	4.06±0.93 B b	69.4±0.98 B b	160.13±0.50 A b	4.20±0.92 B b	67.4±1.1 B b	15.86± 2.06 A b	-17.08
DL ₅₀	77.46±1.52 A b	62.53±1.68 A c	7.6±1.21 B c	66.93±0.81 B b	167.06±0.91 A c	3.73±0.72 A b	65.66±1.37 A c	16.13± 1.85 A b	-19.34
Medium treated with azadirachtin 0.25 µg/ml									
Control	80.13±1.74 B a	43.86±0.90 A a	0.0±0.0 A a	74.8±1.67 B a	150.8±0.75 A a	0.0±0.0 A a	73.53±1.93 C a	0.0±0.0 A a	0
DL ₂₅	72.53±1.56 B b	54.13±1.10 B b	7.06±1.10 C b	63±1.48 B b	159.06±0.89 A b	9.06±0.97 C b	60.64±1.77 B b	19.4±2.15 A b	-17.31
DL ₅₀	66.2±2.18 B b	61.6±0.98 A c	10.86±0.91 B c	54.13±1.85 C c	171.06±0.69 A c	11.86±1.07 B c	53.13±1.82 B b	24.13±1.76 B b	-26.94

622

623 **Table2.** Effect of larval exposure to azadirachtin on development of first generation (non-
624 exposed) of *D. melanogaster*. Letters indicate a significant difference between the different
625 tested doses of the same medium for each stage of development ($P<0.05$). Different capital
626 letters indicate a significant difference same doses tested of different medium ($P<0.05$). ($m \pm$
627 SE; n=15 replicates).

Concentration	Larvae			Pupae			Imagoes		
	N° of individuals	T50 (h)	Malformations (%)	N° of individuals	T50 (h)	Malformations (%)	N° final organisms	Malformations (%)	FNO
Control Medium									
Control	97.53±0.80 A a	42.93± 0.46 A a	0.0±0.0 A a	97.2±0.80 A a	150.4±1.22 A a	0.0±0.0 A a	96.8±0.76 A a	0.0±0.0 A a	0
DL₂₅	97.2±0.82 A a	43.73±0.85 A a	0.0±0.0 A a	96.26±0.94 A a	152.6±1.05 A a	0.0±0.0 A a	94.00±0.95 A a	0.0±0.0 A a	-2.85
DL₅₀	97.73±0.85 A a	42.93±0.69 A a	0.0±0.0 A a	96.13±0.97 A a	152±0.80 A a	0.0±0.0 A a	93.93±1.17 A a	0.0±0.0 A a	-2.90
Medium treated with azadirachtin 0.1µg/ml									
Control	89.62±1.80 A a	43.06±0.50 A a	0.0±0.0 A a	87.86±1.84 B a	150.86±0.57 A a	0.0±0.0 A a	85.60±1.59 B a	0.0±0.0 A a	0
DL₂₅	84.2±1.3 B a	42.6±0.77 A a	0.0±0.0 A a	82.4±1.37 B a	152.73±1.12 A a	0.0±0.0 A a	82.51±1.36 B a	0.0±0.0 A a	-4.44
DL₅₀	84.60±1.32 B a	42.86±0.79 A a	0.0±0.0 A a	81.26±1.48 B a	151.86±1.19 A a	0.0±0.0 A a	80.86±1.57 B a	0.0±0.0 A a	-5.30
Medium treated with azadirachtin 0.25 µg/ml									
Control	86.53±1.97 A a	42.64±0.83 A a	0.0±0.0 A a	78.73±2.17 B a	149.46±0.94 A a	0.0±0.0 A a	78.73±2.17 B a	0.0±0.0 A a	0
DL₂₅	83.66±1.73 B a	41.8±0.82 A a	0.0±0.0 A a	76.40±1.23 B a	150.33±0.71 A a	0.0±0.0 A a	73.2±1.48 C a	0.0±0.0 A a	-6.41
DL₅₀	78.8±1.52 B b	42±0.81 A a	0.0±0.0 A a	76.86±1.38 B a	151.4±0.60 A a	0.0±0.0 A a	73.73±1.55 B a	0.0±0.0 A a	-5.37