Impacts d'un biopesticide, l'azadirachtine, sur la physiologie et le comportement de Drosophila melanogaster : Reproduction, nutrition et propriétés aversives

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Thèse de doctorat

NNT : 2021UPASB025

Impacts d'un biopesticide, l'azadirachtine, sur la physiologie et le comportement de *Drosophila melanogaster* : Reproduction, nutrition et propriétés aversives

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Thèse présentée et soutenue à Université Badji-Mokhar Annaba, le 26 juin 2021, par

Maroua FERDENACHE

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SOMMAIRE

1 Introduction 1

1.1 Utilisations intensives de pesticides à large spectre 2

1.2 Les biopesticides 4

1.3 L'arbre de Neem 5

1.3.1 Propriétés du Neem (*Azadirachta indica*) 5
1.3.2 L'azadirachtine 6

1.4 Toxicité et risque écotoxicologique de l'azadirachtine 8

1.5 Potentiel insecticide de l'azadirachtine 9

1.5.1 Effets régulateurs de croissance 9
1.5.2 Effets sur le système nerveux 9
1.5.3 Effets sur la reproduction 10
1.5.4 Effets moléculaires 10
1.5.5 Effets anti-appétant 11

1.6 Le système chimiosensoriel et locomoteur comme cible potentielle des pesticides 11

1.7 Le système olfactif chez *D. melanogaster* 13

1.8 Le système gustatif chez *D.* melanogaster 14

1.8.1 L'architecture du système gustatif 14
1.8.2 Les sensilles gustatives du proboscis 16
1.8.3 La structure des récepteurs gustatifs (GR) 17

1.9 Intérêt de l'étude 18

1.10 Objectifs de l'étude 20

2 Matériel et méthodes 21

2.1 Matériel biologique 21

2.2 Présentation de l'insecticide et traitement 23

2.3 Étude de la fécondité et de la préférence d'oviposition 23

2.3.1 Condition de non-choix du milieu de ponte 23
2.3.2 Condition de choix du milieu de ponte 24

2.4 Étude du développement 25

2.5 Analyse de survie des adultes 27

2.6 Étude de l'activité locomotrice des adultes 28

2.7 Étude de la perception olfactive des adultes : Olfactomètre en Y 29

2.8 Test de sensibilité gustative (Proboscis Extension Response : PER) 30
2.9 Étude électrophysiologique 31
2.10 Tests de consommation alimentaire (MultiCAFE : Multi Capillary Feeding) 33
2.11 Analyses statistiques 34
3 Résultats 35
3.1 Effets sur la fécondité et la préférence d'oviposition 35
3.2 Effets sur le développement 40
3.3 Analyse de la survie des adultes 45
3.4 Effets sur l'activité locomotrice des adultes 76
3.5 Effets sur l'orientation à distance vers un substrat traité à l'azadirachtine 78
3.6 Effets sur la perception gustative 108
3.7 Effets sur les réponses électrophysiologiques des adultes 109
3.8 Effets sur la consommation alimentaire 113
4 Discussion 142
4.1 Effets sur la fécondité et le choix d'oviposition 142
4.2 Effets sur le développement et la survie des adultes 146
4.3 Effets sur la perception olfactive et gustative et le comportement locomoteur 150
5 Conclusion et perspectives 157
6 Résumés 159
6.1 Résumé 159
6.2 Abstract 161
6.3 6 ملخص 162
.
3
7 Références bibliographiques 163
8 Annexes 189
8.1 Annexe 1. Production scientifique 189
8.2 Annexe 2. Les molécules testées 191

8.3 Annexe 3. Composition du milieu axénique utilisé pour l'élevage de *Drosophila melanogaster*..... 191

LISTE DES FIGURES

Figure 1. L'arbre de Neem et ses composants (Fernandes et al., 2019). 5

Figure 2. Structure de l'azadirachtine A (PubChem, 2019). 7

Figure 3. Les différents types des sensilles olfactives chez D. melanogaster (de Bruyne & Warr 2006). 13

Figure 4. Organisation du système gustatif chez Drosophila melanogaster. 15

Figure 5. Cartographie des sensilles gustatives sur le proboscis des drosophiles (Weiss et al., 2011). 16

Figure 6. L'expression des Grs dans les neurones répondant à l'amer (B) et dans les neurones répondant aux sucres (S), en fonction du type de sensilles (Weiss et al., 2011). 18

Figure 7. Mâle et femelle de D. melanogaster. 21

Figure 8. Cycle de vie de D. melanogaster. 22

Figure 9. Procédure expérimentale pour l'étude de la fécondité (condition de non-choix du milieu de ponte). 24

Figure 10. Procédure expérimentale pour l'étude de la préférence du site de ponte (En condition de choix). 25

Figure 11. Suivi du développement des différents stades de vie de D. melanogaster. 26

Figure 12. Procédure expérimentale pour l'analyse de survie des adultes de D. melanogaster. 27

Figure 13. Procédure expérimentale pour l'activité locomotrice des adultes de. D. melanogaster. 28

Figure 14. Olfactomètre en Y utilisé pour l'étude de l'orientation des mouches vers des odeurs 29

Figure 15. Schéma des procédures utilisées pour mesurer l'extension du proboscis (PER). 31

Figure 16. Dispositif d'électrophysiologie et étapes d'enregistrements électrophysiologiques à partir de la stimulation d'une sensille gustative du proboscis chez D. melanogaster. 32

Figure 17. Schéma représentant la préparation des mouches pour le test MultiCAFE 33

Figure 18. Schéma du dispositif pour le test alimentaire à choix multiples (MultiCAFE) 34

Figure 19. Effets de l'azadirachtine sur la fécondité des femelles 36

Figure 20. Effets de l'azadirachtine sur la ponte en situation de choix. 37

Figure 21. Effets d'une exposition sur les choix de ponte sur 3 générations 38

Figure 22. Exemples des malformations les plus fréquentes chez D. melanogaster au stade L3. 43

Figure 23. Effets de l'azadirachtine (DL25 et DL50), administré par application topique, chez les larves au début du stade L3 de D. melanogaster, sur le sex-ratio des adultes émergents 44

Figure 24. Effet d'une exposition à l'azadirachtine sur la survie des adultes (P et F1). 46

Figure 25. Effets de l'azadirachtine sur la vitesse de récupération après un choc (réactivité locomotrice) chez la génération parentale 77

Figure 26. Effets de l'azadirachtine sur la vitesse de récupération après un choc (réactivité locomotrice) chez la génération F1. A : Mâles ; B : femelles 78

Figure 27. Réponses des adultes (génération P et F1) à l'odeur de Neem Azal en olfactomètre 80

Figure 28. Réponses d'extension du proboscis à une solution contenant de l'azadirachtine à différentes concentrations chez les adultes 108

Figure 29. Distribution des réponses électrophysiologiques au Neem Azal dans les sensilles du proboscis 110

Figure 30. Réponses des sensilles S5 au Neem Azal en fonction de la concentration et du traitement larvaire (m ± SEM ; n = 12 répétitions). 111

Figure 31. Réponses des sensilles S5 à l'azadirachtine et à la caféine en fonction du traitement larvaire. (m ± SEM ; n = 12 répétitions). Les lettres différentes indiquent une différence significative entre les individus de la même substance (P < 0,05). 112

Figure 32. Exemples de réponses électrophysiologiques à l'azadirachtine, à la caféine et au TCC. 113

Figure 33. Consommation de solutions contenant du Neem Azal chez des individus adultes traités au stade larvaire (A : mâles et B : femelles). 114

Figure 34. *Consommation pendant 24 h d'azadirachtine, de caféine et de saccharose chez des individus traités ou non à l'azadirachtine au début de stadeL3 D. melanogaster (A : Témoins ; B : DL_{25} ; C : DL_{50}), (Aza : 0,25 µg/ml et 0,1 µg/ml ; saccharose : 30 mM ; Caf : 10 mM) (m ± SEM ; n = 32 répétitions).* 115

Figure 35. Évolution des quantités d'hormone juvénile, d'ecdysone et de PTTH au cours des stades de développement (Rewitz et al., 2013). 148

LISTE DES TABLEAUX

Tableau 1 : Principaux constituants chimiques d'Azadirachta indica (Gherida & Goetz, 2014) 6

Tableau 2 : Effet de l'exposition larvaire à l'azadirachtine sur le développement de la génération parentale. 41

Tableau 3 : Effets de l'exposition larvaire à l'azadirachtine sur le développement de la première génération F1. 42

Tableau 4 : Liste des molécules utilisées 191

LISTE DES ABRÉVIATIONS

DDT : Dichlorodiphényltrichloroéthane.

OP : Organophosphate.

HHP : Highly hazardous pesticide.

IGDs : (Insect Growth Disruptors) perturbateurs de croissance des insectes.

HJ : hormone juvénile.

20E : 20-hydroxyecdysone.

PTTH : Prothoracicotrope.

5-HT : Sérotonine.

OA : Octopamine.

DA : Dopamine.

MDA : Malondialdehyde.

SOD : Super-oxyde dismutase.

CAT : Catalase.

GST : Glutathion s-transférase.

LSO : Organe sensoriel labral.

VCSO : Organe sensoriel cibarial ventral.

DSCO : Organes sensoriels cibarial dorsal.

L : Longues.

I : Intermédiaires.

S : Courtes.

GR : Récepteur gustatif.

DL : Dose létale.

OPI : Indice de préférence d’oviposition.

FNO : Nombre final d'organismes.

IO : Indice d’olfaction.

PER : Proboscis Extension Response.

TCC : Tricholinecitrate.

MultiCAFE : Multi Capillary Feeding.

CP : Corps Pédonculés.

SNC : système nerveux central.

PPL1 : Paired Posterior Lateral.

PAM : Protocerebral Anterior Medial.

INTRODUCTION

1 Introduction

Le défi majeur de l'agriculture est d'augmenter la production alimentaire, pour répondre aux besoins de la population mondiale en pleine croissance. Cette population a atteint 7,8 milliards en 2020 et continuera à augmenter pour atteindre les 11,2 milliards en 2100 (Mesterházy *et al.,* 2020).

Pour assurer la sécurité alimentaire mondiale et nourrir 2 milliards de personnes supplémentaires en 2050, la production agricole devrait augmenter de 70% (Mesterházy *et al.,* 2020), alors que la surface de terres cultivables reste limitée (FAO, 2016).

L'une des composantes principales de la gestion de la production agricole, reste la protection des cultures contre les bio-agresseurs. En effets, depuis le début de la culture des plantes, il y a plus de 10000 ans, les agriculteurs sont confrontés au défi de la lutte contre les organismes nuisibles qui endommagent les cultures et réduisent la productivité et la qualité des aliments qu'ils récoltent (Pobanz, 2019).

Les pratiques agricoles actuelles restent dépendantes de l'utilisation des produits phytosanitaires, notamment les pesticides chimiques qui représentent un enjeu économique mais aussi sanitaire majeur, vu leur importance dans l'optimisation des productions agricoles et la lutte contre les maladies vectorielles (Godfray *et al.,* 2010). 40% des récoltes sont perdues du fait de l'action combinée des insectes, des maladies et des mauvaises herbes. Ces pertes pourraient atteindre les 70% en absence de protection efficace (Popp *et al.,* 2013).

Les arthropodes détruisent environ 18 à 26% de la production annuelle des cultures (Culliney, 2014 ; Savary *et al.,* 2019) et les insectes et les acariens représentent la majorité des dommages causés aux cultures (Pimentel, 1997 ; Culliney, 2014).

Selon la FAO, la consommation annuelle mondiale des pesticides est estimée à 4,6 millions de tonnes de matière active. L'utilisation de pesticides chimiques quasi généralisée exerce une pression sur l'environnement, en constituant une masse polluante colossale au plan écotoxicologique, et présente ses limites d'application avec l'apparition de résistance des bioagresseurs, et des effets nocifs sur la santé humaine (FAO, 2019).

Le secteur agricole doit donc répondre aux objectifs de production qualitative et quantitative mais aussi de s'engager dans une transition cruciale en intégrant de nouvelles pratiques répondant aux exigences de développement durable et ce en conciliant les objectifs socio-économiques et environnementaux.

1.1 Utilisations intensives de pesticides à large spectre

Le terme pesticide dérive de « Pest », mot anglais désignant tout organisme vivant (virus, bactéries, champignons, herbes, vers, mollusques, insectes, rongeurs, mammifères, oiseaux) susceptible d'être nuisible à l'homme et/ou à son environnement (Periquet,1986). Selon la FAO un pesticide est une substance ou association de substances destinées à repousser, détruire ou combattre les maladies et les ravageurs, y compris les vecteurs de maladies humaines et animales, et les espèces indésirables de champignons, de plantes ou d'animaux (FAO, 2002). Les pesticides à usage agricole peuvent être désignés de différentes façons : produits phytopharmaceutiques, produits agro-pharmaceutiques ou encore produits phytosanitaires.

La deuxième moitié du XX^{ème} siècle a été marquée par une intensification et une modernisation de l'agriculture qui ont abouti à une utilisation massive des pesticides afin d'obtenir un résultat rapide, avec un maximum d'efficacité.

En effet, une transformation majeure dans la lutte contre les insectes nuisibles a commencé dans les années 1930 – 1950, suite au développement de la chimie de synthèse, avec l'introduction d'insecticides organiques synthétiques tels que le dichlorodiphényltrichloroéthane (DDT), les organophosphates (OP), les cyclodiènes et les N-méthylcarbamates. La fin des années 1940 à 1960 ont été qualifiées d'âge d'or de la découverte des insecticides, car une lutte contre les insectes nuisibles très efficace, fiable et abordable est devenue courante (Casida & Quistad, 1998).

Bien que les pesticides de synthèse aient joué un rôle majeur dans la sécurisation des ressources alimentaire, leur usage répété et intensif a mis en évidence leur limite d'application, avec l'apparition de phénomènes de résistance (Georghiou, 1972 ; Boyer *et al.,* 2012) et une forte contamination des écosystèmes due à leur persistance dans l'environnement (Bilal *et al.,* 2019).

Dès 1962, la biologiste américaine Rachel Carson alerte l'opinion publique sur les effets nocifs des pesticides, en particulier ceux du DDT, sur l'environnement et les espèces non

cibles telles que les oiseaux, les abeilles et les invertébrés aquatiques (Carson, 1962 ; Rose, 2001). Le DDT, un pesticide organochloré, largement utilisé pour ses puissantes propriétés insecticides et acaricides, induit également des effets toxiques pour la santé et l'environnement, accentués par sa forte rémanence. En effet, les produits organochlorés, très lentement dégradés, sont caractérisés par une rémanence ou une persistance forte dans l'environnement. La parution de l'ouvrage de Rachel Carson marque le point de départ du mouvement écologiste et provoquera, en 1973, l'interdiction du DDT aux Etats-Unis, puis un peu partout dans le monde pour son usage dans les cultures (Mehdi *et al.*, 2018).

L'industrie chimique et l'essor de l'agriculture intensive ont permis le développement d'une deuxième génération d'insecticides, représentée par trois grandes familles : organophosphorés, carbamates et pyréthrinoïdes de synthèse. Ces insecticides à action neurotoxique agissent tous au niveau du système nerveux des insectes, soit en bloquant l'acétylcholinestérase (organophosphorés et carbamates) soit en perturbant le fonctionnement des canaux sodium (pyréthrinoïdes) (Haubruge & Amichot, 1998). D'autres molécules ont permis d'enrichir la famille des pesticides telles que les phénylpyrazoles comme le fipronil (Bonmatin *et al.,* 2014) ou encore les néonicotinoïdes comme l'imidaclopride ou le thiaméthoxame, qui sont également des molécules à actions neurotoxique (Christen *et al.,* 2018).

En dépit de leurs effets secondaires sur la santé publique et l'environnement et la multiplication des espèces résistantes (Nasirian, 2010 ; Nauen *et al.,* 2012 ; Harrop *et al.,* 2014), les pesticides considérés comme les plus à risques pour la santé et l'environnement, dits « extrêmement à risque » et classés dans la catégorie HHP (Highly Hazardous Pesticides), par l'organisation mondiale de la santé, dominent encore les ventes au niveau mondial. Les néonicotinoïdes continuent à être largement utilisés et leur impact potentiel sur les organismes non ciblés continue à faire l'objet de recherches très actives (Bass & Field, 2018).

Toutefois, les impératifs environnementaux et les effets nocifs sur les espèces non visées ont conduit à la mise en place de plan d'action visant une réduction de la consommation des pesticides chimiques et leur utilisation de manière compatible avec le développement durable. Dans ce sens, des efforts considérables ont été déployés pour mettre au point des substances alternatives moins persistantes et plus spécifiques comme les perturbateurs de croissance des insectes (IGDs : Insect Growth Disruptors) qui interfèrent avec des processus physiologiques et biochimiques spécifiques aux organismes cibles, soit en mimant l'action des

principales hormones contrôlant le développement et la reproduction des insectes, l'hormone juvénile (JH) et l'ecdysone, soit en inhibant la synthèse du composé majeur de la cuticule, la chitine (Dhadialla *et al.,* 2010 ; Pener & Dhadialla, 2012). De plus, un regain d'intérêt pour les substances naturelles susceptibles d'avoir une activité phytosanitaire (biopesticides) a été observé ces dernières années (Chaudhary *et al*., 2017).

1.2 Les biopesticides

Les pesticides bio-rationnels ou biopesticides, sont des organismes vivants ou produits issus de ces organismes ayant la particularité de limiter ou de supprimer les ennemis des cultures (Deravel *et al*., 2014). Ils peuvent être classés en trois grandes catégories, selon leur nature : les biopesticides microbiens, les biopesticides végétaux et les biopesticides animaux (Chandler *et al.,* 2011 ; Leng *et al.,* 2011).

Aujourd'hui, les molécules naturelles prennent progressivement des parts plus importantes sur le marché des pesticides. Avec un taux de croissance annuel estimé à 14,7%, ce segment de l'industrie continue à croitre pour passer d'une valeur de 4,3 billions de dollars en 2020 et atteindre les 8,5 billion de dollars en 2025 (markets and markets, 2020). On estime que le marché mondial des biopesticides dépassera les 7% des ventes d'ici 2023 (Olson, 2015).

Les biopesticides sont généralement moins toxiques que les pesticides conventionnels, efficaces en faible quantités et possèdent une spécificité accrue vis-à-vis des espèces contre lesquelles ils sont dirigés (Deravel *et al*., 2014). Ils sont composés généralement d'un mélange de principes actifs avec un large spectre d'activité et des cibles d'action multiples réduisant le développement de résistance. De plus, ils sont biodégradables et donc non persistants dans l'environnement (Deravel *et al*., 2014).

Les plantes représentent la source la plus importante de biopesticides, en produisant de nombreux métabolites secondaires biologiquement actifs potentiellement applicables dans les programmes de lutte intégrée. Parmi les pesticides d'origines végétales qui ont eu beaucoup de succès sont ceux qui proviennent de l'arbre du Neem, *Azadirachta indica* A. Juss. (Meliaceae) (Isman & Grieneisen, 2014). En effet, la possibilité d'obtenir des composés actifs variés à partir des feuilles et des graines, évitant ainsi la destruction de la plante entière, représente un avantage majeur (Barrek & Paisse, 2004 ; Maity *et al.,* 2009).

1.3 L'arbre de Neem

Azadirachta indica A. Juss (Synonymes : *Antelaea azadirachta* L. ou *Melia azadirachta* L.), plus communément de son nom vernaculaire margousier ou, en anglais, Neem (Aribi *et al.,* 2020) est un arbre à croissance rapide et à feuilles persistantes. Originaire de l'Inde et du sud-est de l'Asie, sa présence est aujourd'hui répandue dans le monde entier, en particulier dans les pays tropicaux et subtropicaux (Schmutterer, 2002 ; Morgan, 2009 ; Paes *et al.,* 2011 ; Koriem, 2013 ; Chattopadhyay *et al.,* 2014).

Cet arbre est caractérisé par un tronc solide dur, brun foncé avec une croûte brune fissurée de 75 à 150 cm, les feuilles, qui mesurent jusqu'à 25 cm de long, sont constituées de cinq à huit paires de folioles. L'inflorescence en forme de panicules axillaires est constituée de nombreuses fleurs (Fig. 1). La fleur est aromatique et blanche, et le fruit peut atteindre un centimètre de long. Il est vert et devient jaune lorsqu'il mûrit, et a un goût amer de graine (Ketkar, 1976, Radwanski & Wickens, 1981).

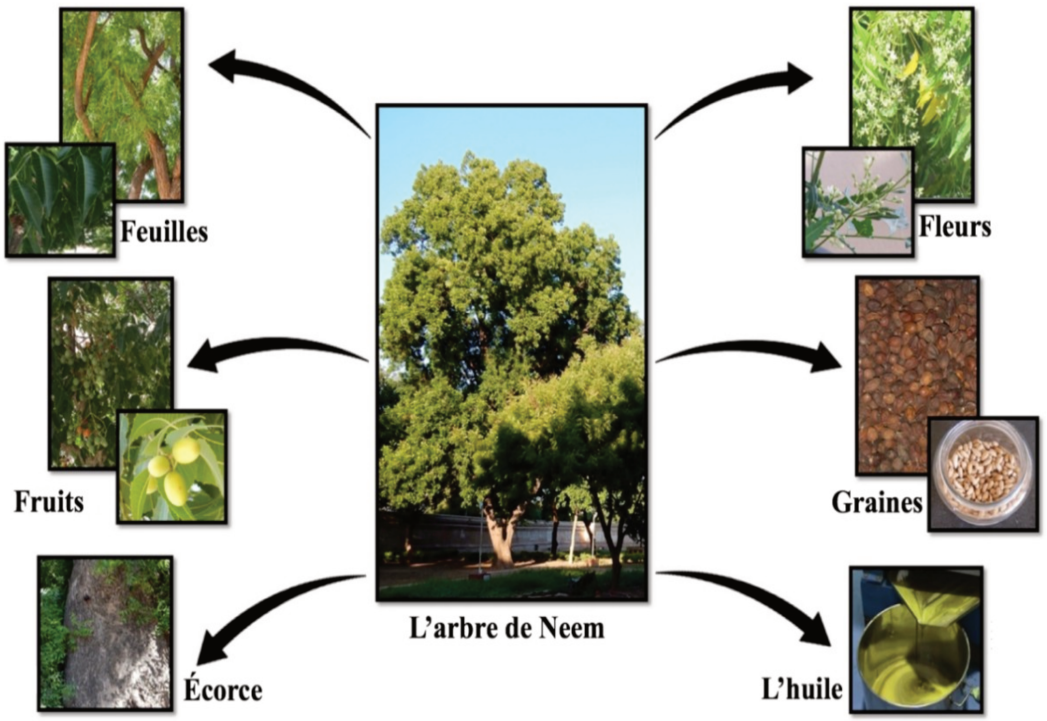

Figure 1. *L'arbre de Neem et ses composants (Fernandes et al., 2019).*

1.3.1 Propriétés du Neem (*Azadirachta indica*)

En Asie, l'arbre de Neem est l'une des principales sources de médicaments naturels, utilisé dans la médecine ayurvédique comme l'un des plus anciens remèdes pour traiter diverses

maladies humaines telles que la malaria, le diabète et les infections de la peau (Biswas *et al.,* 2002 ; Pasquoto-Stigliani *et al.,* 2017). Les extraits de Neem auraient également des effets inhibiteurs sur plusieurs lignées cellulaires cancéreuses (Patel *et al.,* 2016). De plus, les extraits aqueux de la poudre de graines de Neem ont été utilisés comme insecticide en Inde depuis des millénaires pour contrôler les insectes nuisibles (Campos *et al.,* 2016).

Les connaissances empiriques sur les propriétés du Neem ont reçu une validation scientifique dès 1942 grâce aux travaux du chimiste pakistanais Salimuzzaman Siddiqui (1897– 1994) qui ont permis d'isoler trois composés amers dans l'huile extraite des graines, respectivement nimbin, nimbinin et nimbidin (Ghedira & Goetz, 2014).

Plus de 300 composés phytochimiques différents structurellement complexes est appartenant à différentes familles chimiques (Tableau 1) ont été isolés (Biswas *et al.,* 2002 ; Gupta *et al.,* 2017) dont plus de 130 sont des limonoïdes dotés de propriétés médicinales et insecticides (Chen *et al.,* 2018).

Tableau 1 : *Principaux constituants chimiques d'Azadirachta indica (Gherida & Goetz, 2014)*

Familles de constituants chimiques principaux	Constituants chimiques principaux
Limonoïdes (C-seco-tétranortiterpènes)	Azadirachtine (azadirachtine A), 3-tigloylazadirachtol (azadirachtine B),1-tigloyl-3-acétyl-11-hydroxy-méliacarpine (azadirachtine D),11-déméthoxycarbonyl azadirachtine (azadirachtine H), 1-tigloyl-3-acétyl-11-hydroxy-11-déméthoxycarbonyl méliacarpine (azadirachtine I), azadiriadione, azadirachtanine, époxyazadiradione
Protolimonoïdes du groupe gédunine	Nimbine, déacetylnimbine, salannine, azadirachtolide, isoazadirolide, margosinolide, nimbandiol, nimbinène, nimboline A, nimbocinone, nimbocinolide, nimbolide, nimocine, nimocinol
Flavonoïdes	Hyperoside, nimbaflavone, quercitrine, quercétine,rutine
Autres constituants	Tanins β-sitostérol, vilasanine Ca, Mg, P

1.3.2 L'azadirachtine

L'azadirachtine, est un tetranortriterpénoïde naturel hautement oxydé, apparenté à la limonine, et appartenant à la famille des limonoïdes ; il est considéré comme le plus puissant

extrait des graines de Neem (Bennuzzi & Ladurner, 2018). L'azadirachtine est un mélange de différents isomères (A, B, D, E, F, H, I, K, L) contenus dans l'huile de Neem (Bennuzzi & Ladurner, 2017). L'azadirachtine A, est la principale substance active responsable de 72 à 90 % de l'activité biologique du Neem, (Schmutterer, 1990 ; Mordue *et al.,* 2010 ; Wang *et al.,* 2016 ; Gehlot *et al.,* 2017).

L'azadirachtine A possède une structure complexe, et suite à la détermination de sa structure correcte en 1985 (Kraus *et al.,*1985), la première synthèse totale de cette molécule a été publiée deux décennies après la découverte du composé (Ley *et al.,* 2007). En plus de son extraction à partir d'*Azadirachta indica*, l'azadirachtine peut également être isolée à partir de deux autres espèces d'*Azadirachta* : *A. excelsa* et *A. siamensis* (Morgan, 2009 ; Brandyopadhyay *et al.,* 2011).

L'analyse cristallographique aux rayons X a permis de connaître la structure chimique de l'azadirachtine (Fig. 2). L'azadirachtine est un composé hautement oxydé qui présente une structure extrêmement complexe avec une formation rigide due à la présence de liaisons hydrogène intramoléculaires et d'un grand nombre de groupes fonctionnels réactifs en positions extrêmement proches (Mordue & Nisbet, 2000 ; Prakash & Bhojwani, 2002 ; Veitch *et al.,* 2007). L'azadirachtine dérive de la voie de biosynthèse de l'acide mévalonique dans l'arbre de Neem (Aarthy *et al*., 2018).

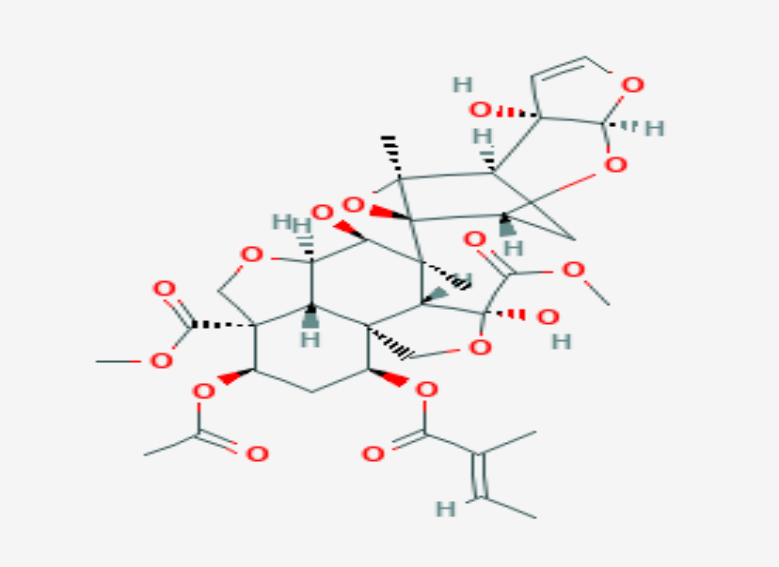

Figure 2. *Structure de l'azadirachtine A (PubChem, 2019).*

L'intérêt croissant pour l'azadirachtine est principalement dû aux propriétés uniques et particulières de ce composé, notamment son large spectre d'activité, même lorsqu'il est présent à l'état de traces. L'azadirachtine possède de nombreuses activités biologiques exploitables aussi bien en tant que bio-pesticide qu'en tant qu'agent thérapeutique (Prakash *et al*., 2002 ; Morgan, 2009 ; Paul *et al*., 2011 ; Hashmat *et al*., 2012).

1.4 Toxicité et risque écotoxicologique de l'azadirachtine

L'azadirachtine est un composé biodégradable qui potentialise son application comme pesticide et présente une très faible toxicité pour les mammifères (Johnson & Morgan, 1997; Morgan 2009 ; Prakash *et al.,* 2002 ; Mordue *et al.,* 2010) ainsi que pour les organismes non cibles (Boeke *et al.,* 2004), avec une DL_{50} de 5000 mg/kg chez le rat, ce qui le classe dans la catégorie des produits "peu susceptibles de provoquer un danger aigu" selon la classification établie par l'OMS (OMS, 2009). La toxicité chronique de l'azadirachtine pour la consommation humaine est de 15 mg/kg (Raizada *et al.,* 2001 ; Boeke *et al.,* 2004).

L'utilisation de fortes doses d'azadirachtine pendant la gestation induit de légères variations du squelette fœtal chez les rats (Braga *et al.,* 2021). Néanmoins, à faibles doses, l'azadirachtine, n'induit aucune modification morphologique, viscérale ou squelettique chez les fœtus du rat (Srivastava, 2000). L'azadirachtine est toxique pour les organismes aquatiques, en particulier lorsque des doses élevées sont administrées (Winkaler *et al.,* 2007 ; Stalin & Kiruba, 2008).

Chez les prédateurs et les parasitoïdes, l'azadirachtine présente des effets toxiques faibles à modérés notamment chez les stades juvéniles (larves, nymphes) et semble être relativement sûr et sélectif pour les adultes (Raguraman & Kannan, 2014).

Néanmoins, des effets toxiques ont été rapportés chez les polinisateurs des cultures après exposition chronique, notamment sur le comportement du bourdon *Bombus terrestris* sous condition de laboratoire (Barbosa *et al.*, 2015). Une réduction de la survie, de la croissance ainsi que du temps de développement a été rapportée chez *Partamona helleri* toujours dans les mêmes conditions (Bernardes *et al.*, 2018). Cependant, l'instabilité de l'azadirachtine et sa faible persistance rendent ces conditions chroniques improbables dans des situations de terrain.

L'azadirachtine est une molécule faiblement persistante dans les sols en condition aérobie avec une demi-vie de 1,9 à 9,9 jours (EFSA, 2011). Elle est faiblement persistante dans l'eau en condition aérobie avec une demi-vie de 10,6 jours (BPDB, 2013). Selon l'ARLA,

l'hydrolyse est l'une des principales voies de transformation à pH neutre et basique (demi-vie de 19 jours, 13 jours et 2 heures à pH 4, 7 et 10 respectivement). La photolyse au sol est également une voie de transformation importante de l'azadirachtine (demi-vie de 3 à 4 jours) (ARLA, 2012).

1.5 Potentiel insecticide de l'azadirachtine

L'azadirachtine possède une activité insecticide contre les insectes de différents ordres (Mordue *et al.,* 2005 ; Tomé *et al.,* 2013 ; Abedi *et al.,* 2014 ; Poland *et al.,* 2016) avec un mode d'action multiple interférant avec différents processus physiologiques et biochimiques de l'insecte.

1.5.1 Effets régulateurs de croissance

La croissance et le développement des insectes sont régulés par diverses hormones dont les deux principales sont : la 20-hydroxyecdysone (20E), forme active de l'ecdysone, et l'hormone juvénile (JH). L'équilibre de ces deux hormones définit le résultat de chaque transition de développement (Dubrovsky, 2005) et toute interférence avec l'équilibre hormonal entraîne une interruption du développement et est considérée comme une cible spécifique potentielle pour la lutte contre les insectes nuisibles (Penner & Dhadialla, 2012). Chez le stade adulte, ces hormones sont également impliquées dans la régulation de la maturation reproductive (Goodman & Granger, 2005).

L'azadirachtine agit comme antagoniste de ces deux hormones ; son action principale est sa capacité à modifier ou supprimer les taux d'ecdystéroïdes hémolymphatiques et d'hormones juvéniles en inhibant la libération de neurohormones prothoracicotrope (PTTH) et allatotropine à partir des *corpora cardiaca*, induisant ainsi son effet perturbateur de croissance des insectes principalement représenté par une réduction de la nymphose, des malformations et un échec de l'émergence des adultes (Mordue & Blackwel 1993 ; Bezzar-Bendjazia *et al*., 2017).

1.5.2 Effets sur le système nerveux

L'azadirachtine interfère avec le système nerveux central (SNC) par inhibition de la transmission cholinergique excitatrice *via* les canaux calciques (Qiao *et al.,* 2014) mais aussi sur la voie de signalisation de l'insuline (Shao *et al.,* 2016). Cependant, les mécanismes d'action de l'azadirachtine restent encore inconnus.

Les amines biogènes comme la sérotonine (5-HT), l'octopamine (OA) et la dopamine (DA) sont d'importants neuromodulateurs du SNC de *D. melanogatser* (Monastirioti, 1999) qui peuvent influencer l'apprentissage, la mémorisation, l'activité locomotrices ainsi que le comportement sexuel (Majeed *et al.,* 2016). L'azadirachtine réduit significativement les taux hémolymphatique et cérébrale de OA, 5-HT et DA (Awad *et al*., 1997 ; Banerjee & Rembold, 1992) impactant ainsi différents processus cognitifs et comportementaux.

1.5.3 Effets sur la reproduction

Les effets de l'azadirachtine sur les différents paramètres reproducteurs ont été rapportés chez plusieurs espèces d'insectes (Pineda *et al.,* 2009 ; Tine *et al.,* 2011 ; Tomé *et al.,*2013 ; Boulahbel *et al.,* 2015 ; Er *et al.,* 2017 ; Oulhaci *et al.,* 2018). Une réduction de la fécondité et de la fertilité a été enregistrée chez de nombreux insectes et pourrait être due à l'interférence de l'azadirachtine avec la synthèse des protéines du vitellus et/ou son incorporation dans les ovocytes (Boulahbel *et al.,* 2015).

L'azadirachtine inhibe également l'ovogenèse et la spermatogénèse et induit une stérilité des insectes (Chaudhary *et al.,* 2017). En effet, pour un déroulement normal de l'ovogenèse et de la spermatogenèse, un équilibre adéquat entre l'JH et 20E est nécessaire ; l'action antagoniste de l'azadirachtine sur ces deux principales hormones explique les effets délétères sur les paramètres de la reproduction. L'azadirachtine interfère également avec le comportement d'oviposition des insectes et le choix du site de ponte (Bezzar-Bendjazia *et al.,* 2016).

1.5.4 Effets moléculaires

L'azadirachtine impact la transcription et/ou l'expression génique de plusieurs protéines. Aussi, l'azadirachtine est connu pour réprimer l'expression des gènes des protéines cuticulaires et de l'amylase chez *D. melanogaster* (Lai *et al.,* 2014). L'azadirachtine est responsable d'une altération structurale de l'intestin moyen chez les larves de *Spodoptera litura* due à une apoptose caspases-dépendante (Shu *et al.,* 2018). Chez *D. melanogaster,* une dépolarisation de l'actine conduisant à un arrêt du cycle cellulaire et une apoptose caspase-indépendante a également été rapportée après traitement à l'azadirachtine (Anuradha *et al.,* 2007 ; Anuradha & Annadurai 2008).

Le traitement à l'azadirachtine augmente l'activité de la SOD et la teneur en MDA chez *D. melanogaster* et induit l'expression génique des enzymes antioxydantes telles que la SOD,

la CAT et la GST (Zhang *et al.,* 2018). L'azadirachtine inhibe également l'expression des gènes de la ferritine et de la thiorédoxine peroxydase ayant des rôles protecteurs contre le stress oxydatif (Asaduzzaman *et al.,* 2016).

1.5.5 Effets anti-appétant

Parmi les différentes propriétés attribuées à l'azadirachtine, l'activité anti-appétante est la plus documentée (Schmutterer & Singh, 1995 ; Senthil-Nathan *et al.,* 2007). L'azadirachtine stimule les cellules chimioréceptrices, responsables du comportement d'aversion, au niveau des pièces buccales de l'insecte et bloque les sites des récepteurs glucidiques responsables de la stimulation de l'alimentation conduisant ainsi à une inhibition de l'alimentation (Blaney *et al.,*1990 ; Simmonds *et al.,* 1990 ; Mordue (Luntz) *et al.,* 1999). Cette activité représente, l'effet anti-appétant primaire de l'azadirachtine.

Un effet anti-appétant secondaire lié à l'action de l'azadirachtine sur la physiologie de la digestion, notamment en affectant l'activité des enzymes impliquées dans le processus de digestion a également été rapporté et inclut un effet à plus long terme sur la réduction de la consommation de la nourriture (Rharrabe *et al.,* 2008 ; Khosravi & Sendi, 2013 ; Kilani-Morakchi *et al.,* 2017).

1.6 Le système chimiosensoriel et locomoteur comme cible potentielle des pesticides

Les insectes évoluent dans un environnement où ils sont en contact permanent avec des molécules volatiles ou solubles pouvant se révéler nocives. Ils ont développé des systèmes de communication sophistiqués, sensibles, efficaces et adaptés à leur environnement, indispensables à leur survie et leur reproduction. Les systèmes chimiosensoriels gustatifs et olfactifs détectent et transmettent au niveau central les informations chimiques de l'environnement (Hallem *et al*., 2006 ; Wicher & Marion-poll, 2018) afin que l'insecte distingue les sources alimentaires des substances toxiques, reconnaisse ses proies de ses congénères et de ses partenaires sexuels (Ebbs & Amrein, 2007).

La plupart des insectes utilisent l'olfaction pour localiser leur nourriture, leur hôte et trouver leur partenaire sexuel. Le système gustatif permet aux insectes d'identifier les sources de nourriture appropriées et d'éviter les substances toxiques et amères en contrôlant le

comportement de choix alimentaire conduisant à l'acceptation ou le rejet de l'aliment (Scott, 2018).

En plus des signaux olfactifs et gustatifs, la locomotion fait partie intégrante du comportement des insectes, vu son importance pour la recherche de nourriture, l'accouplement et la réaction de fuite (Zhu *et al.,* 2020). Chez *D. melanogaster*, l'activité locomotrice, est un trait de forme physique qui intervient dans une série de comportements associés à des fonctions de base telles que la réponse au stress, la capacité de recherche de nourriture, l'accouplement, la ponte et la reproduction (Baumler & Potter, 2007 ; Khosravi *et al.,* 2010 ; Khosravi & Sendi, 2013).

Chez la drosophile, l'activité locomotrice est classée en deux types (Connolly, 1967), l'activité locomotrice spontanée, qui est indicative d'un état de locomotion à long terme et correspond à l'activité observable présentée par l'insecte sans activation spécifique par des stimuli externes (Zhu *et al.,* 2020) et la réactivité locomotrice qui correspond à une réponse d'activité à court terme qui suit immédiatement un stimulus externe et est déclenchée pour faire face aux changements environnementaux (Zhu *et al.,* 2020 ; Sun *et al.,* 2018).

Si la locomotion et les signaux chimiosensoriels des insectes sont essentiels à leur forme physique et à leur survie, en leur permettant de trouver leur partenaire sexuel et de localiser les plantes hôtes et les sources de nourriture, les insecticides, même à faible dose, agissent comme des perturbateurs de l'information provoquant des réactions inadaptées qui peuvent être exploitées comme stratégies comportementales pour lutter contre les espèces nuisibles (Tricoire-Leignel *et al.,* 2012).

Les perturbateurs de croissance des insectes (IGDs), agissent sur le contrôle endocrinien et la physiologie des insectes en perturbant leur développement et leur reproduction (Mordue *et al.,* 2010). De plus, de nombreux insecticides perturbent la neurotransmission et altèrent de ce fait, l'activité locomotrice, les systèmes sensoriels et les capacités cognitives des insectes (Tricoire-Leignel *et al.,* 2012 ; Benzidane *et al.,* 2010).

L'impact des doses sublétales d'insecticides sur les systèmes sensoriel et nerveux représente aujourd'hui un intérêt certain après la mise en évidence, chez les abeilles mellifères, des effets sublétaux sur le comportement guidé par l'olfaction, l'apprentissage et la mémoire (Desneux *et al.,* 2007 ; Goñalons & Farina, 2018 ; Chmiel *et al*., 2020).

1.7 Le système olfactif chez *D. melanogaster*

D. melanogaster est sensible à une très grande variété d'odeurs. Les molécules en suspension dans l'air peuvent induire des réactions comportementales, notamment d'attraction ou d'évitement.

Les sensilles sont les structures sensorielles olfactives chez les insectes. Ces sensilles sont en forme de poils et sont situées à l'extrémité distale des antennes et sur les palpes maxillaires ou labiaux. Ces sensilles olfactives sont perforées par un grand nombre de petits pores qui sont censés donner accès aux odeurs et peuvent donc être facilement distinguées des autres types de sensilles, comme les sensilles thermo ou hygrosensibles (Stocker, 1994). Elles se présentent sous diverses formes et chaque sensille répond de façon spécifique à une molécule chimique ou à une famille de molécules chimiques.

Sur l'antenne, on distingue trois types morphologiques de sensilles olfactives : les sensilles basiconica, les sensilles trichodea et les sensilles cœloconiques (Fig. 3). Les sensilles basiconiques sont en nombre moindre chez le mâle et les sensilles trichoides plus nombreuses chez la femelle. Par contre, le palpe maxillaire a une structure plus simple que celle de l'antenne et contient un seul type de sensille (basiconique) (Vosshall & Stocker, 2007).

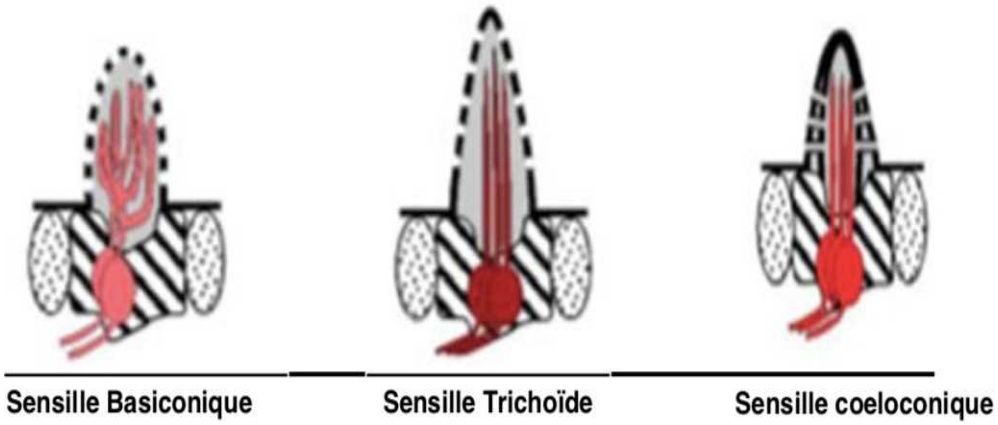

***Figure 3**. Les différents types des sensilles olfactives chez D. melanogaster (de Bruyne & Warr 2006).*

Le système chimiosensoriel de la drosophile est impliqué dans un grand nombre de comportements liés à l'adaptation des insectes à leur environnement, notamment la recherche d'une plante hôte, la recherche et la sélection d'un partenaire sexuel, ou encore dans les relations sociales. Il est aussi indispensable pour la sélection du site de ponte par les femelles.

En effet, les drosophiles sont connues pour être sélectives quant aux choix du site de ponte. Plusieurs études récentes ont montré que ce type de comportement peut être modifié par l'expérience (Dukas, 2008 ; Sarin & Dukas, 2009 ; Abed-Vieillard *et al.,*2013 ; Anderson *et al.,*2013 ; Abed-Vieillard & Cartot, 2016 ; Bezzar-Bendjazia *et al.,*2016).

1.8 Le système gustatif chez *D.* melanogaster

Le sens du goût est essentiel pour évaluer la qualité des aliments, pour cela les cellules sensorielles envoient des informations sensorielles aux centres supérieurs pour stimuler des réponses neurologiques qui sont traitées et décodées pour produire le comportement attractif ou répulsif approprié (Liman *et al.,* 2014). La capacité à identifier les substances attractives et à éviter les substances toxiques est essentielle pour la survie d'un animal. Bien que l'olfaction et la vision contribuent à la détection des aliments, le système gustatif agit comme un point de contrôle final pour cette prise de décision (Scott, 2018).

1.8.1 L'architecture du système gustatif

Le système gustatif chez les insectes est complexe et n'est pas restreint à un seul organe. Il joue un rôle essentiel dans la sélection des aliments et les comportements alimentaires ainsi que dans d'autres activités de la vie des insectes (Miyamoto *et al.,* 2012 ; Jiang *et al.,* 2015). Le comportement alimentaire dépend à la fois de l'apport neural des sens chimiques des insectes (récepteurs gustatifs) et de l'intégration nerveuse centrale de ce code sensoriel (Mordue Luntz, 2005). La détection gustative chez les insectes se fait grâce à des sensilles gustatives, situées au niveau du proboscis, des pattes et des ailes (Fig. 4) (Stocker, 1994 ; Amrein & Thorne, 2005).

La partie externe du proboscis est constituée de deux labelles, également appelés palpes labiaux, qui fusionnent au niveau de la partie proximale du proboscis.

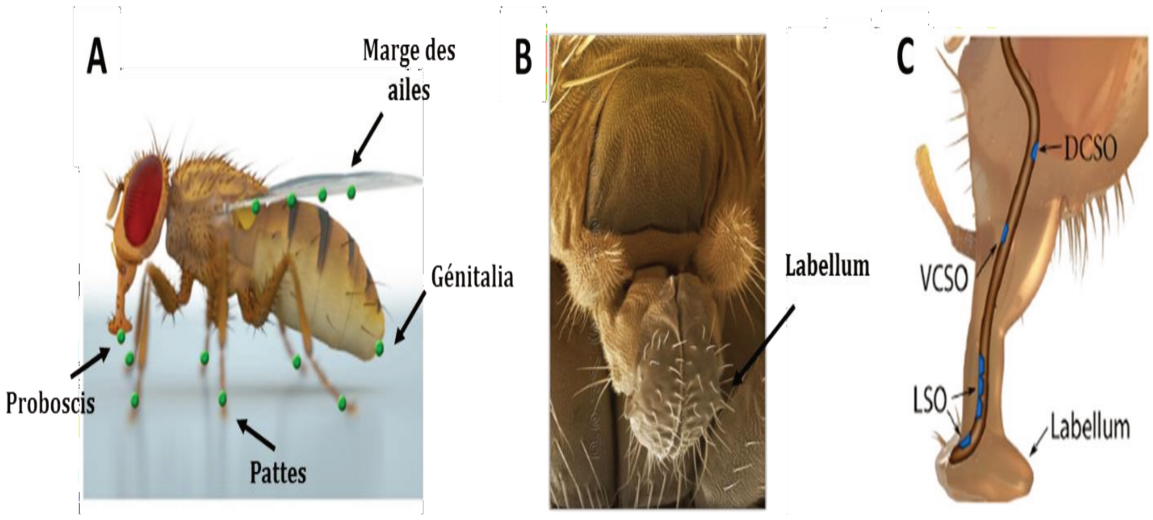

Figure 4. *Organisation du système gustatif chez Drosophila melanogaster.*

(A) Les points verts indiquent la localisation des organes gustatifs externes de la drosophile : le proboscis, le premier tarse des pattes, la bordure antérieure des ailes et l'organe ovipositeur chez la femelle. (B) Le proboscis est le principal organe gustatif chez la drosophile. Il est composé de deux labelles recouverts de sensilles gustatives. (C) Le proboscis de la drosophile contient 3 organes gustatifs internes le long de la paroi du pharynx : le LSO, VCSO et le DCSO impliqués dans l'évaluation de la nourriture et le rejet de substances toxiques. Modifié à partir de (Liman et al., 2014).

Proboscis (trompe) : organe principal du goût chez la drosophile, l'équivalent de la langue chez l'humain. Cet organe contient deux types de structures gustatives distinctes: la première est constituée de sensilles localisées sur la partie externe (labellum), permettant à la drosophile l'évaluation de la qualité de la nourriture avant l'ingestion ; la seconde est située dans la cavité buccale et comprend des sensilles gustatives internes organisées en trois organes (Fig.4). Ceux-ci sont l'organe sensoriel labral (LSO), et les organes sensoriels cibariaux ventraux et dorsaux (VCSO et DSCO) (Fig. 4).

Pattes : toutes les pattes des drosophiles portent des sensilles mécanoréceptrices et gustatives. Il existe un dimorphisme sexuel important à ce niveau, les pattes antérieures des mâles étant équipées d'environ 50 sensilles gustatives alors que celles des femelles n'en possèdent qu'environ 37 (Mitchell, 1999). Ce dimorphisme sexuel est causé par la présence des sensilles spécialisées chez le mâle, dont la fonction est de détecter les phéromones émises par les femelles (Bray & Amrein, 2003). Les sensilles gustatives sont au nombre de 30 pour les pattes du second rang et 32 pour celles du troisième rang, indistinctement pour les deux sexes. Chaque sensille y abrite de 2 à 4 neurones récepteurs gustatifs.

Ovipositeur : les femelles possèdent sur leur partie génitale, 10 sensilles gustatives, qui jouent probablement un rôle important dans le choix des endroits de dépôt des œufs (oviposition) et le réflexe de ponte (Montell, 2009).

Ailes : la bordure de l'aile est équipée de 30 sensilles gustatives essentiellement sur la partie dorsale de la veine costale antérieure. La face ventrale contient 12 autres sensilles gustatives intercalées par des sensilles mécano-sensorielles (Mitchell *et al.,* 1999).

1.8.2 Les sensilles gustatives du proboscis

Les sensilles gustatives, au nombre de 31 sensilles sur chaque côté du proboscis, ont été classifiées en 3 types : longues (L), intermédiaires (I) et courtes (s) en fonction de leur taille (Fig. 5) (Shanbhag *et al.,* 2001). Généralement, les sensilles gustatives renferment, un neurone mécanosensoriel et quatre neurones gustatifs. Parmi les neurones gustatifs, un neurone détecte les sucres ; un deuxième détecte le sel ; un troisième détecte l'eau et un dernier détecte les composés amers. Les sensilles du type (I) ne contiennent que deux neurones, l'un réagissant aux sucres et l'autre aux composés amers (Amrein & Thorne, 2005 ; Montell, 2009).

La plus récente classification des sensilles gustatives du proboscis chez *D. melanogaster* a été proposée par Weiss *et al.* (2011) sur la base de critères fonctionnels, en fonction de la distribution des différents récepteurs gustatifs dans ces sensilles et de leur capacité à répondre à des substances amères. Selon ces critères, les sensilles se distribuent en 5 classes (Fig. 5).

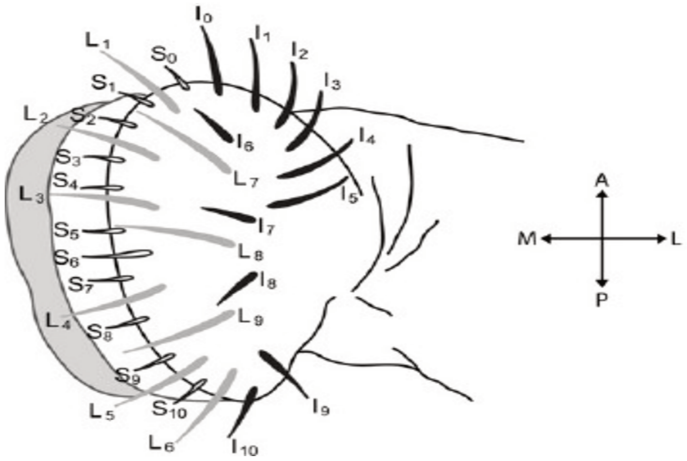

Figure 5. *Cartographie des sensilles gustatives sur le proboscis des drosophiles (Weiss et al., 2011).*

1.8.3 La structure des récepteurs gustatifs (GR)

Les stimuli gustatifs sont reconnus par des récepteurs gustatifs appartenant à une famille de protéines à sept domaines trans-membranaires, et insérés dans la membrane des dendrites des neurones gustatifs (Xu *et al.,*2012 ; Jung *et al.,*2015). En raison de la diversité et de la complexité des récepteurs gustatifs, la plupart de leurs fonctions et mécanismes moléculaires et cellulaires ne sont toujours pas identifiés (Liu *et al.,* 2020). Diverses études ont permis d'identifier 62 gènes codant 68 récepteurs gustatifs par épissage alternatif chez la drosophile. Ces récepteurs gustatifs comprennent 550 à 650 acides aminés et ne présentent que 15 et 25 % d'homologie entre eux (Clyne *et al.,* 2000 ; Dunipace *et al.,* 2001 ; Robertson *et al.,* 2003 ; Scott *et al.,* 2001).

Des études fonctionnelles ont permis de classer les récepteurs gustatifs en 8 familles : les récepteurs aux sucres, aux molécules amères, aux sels, à l'eau (faible osmolarité), au gaz carbonique (CO2), à l'acide, à l'umami et aux phéromones. Les récepteurs gustatifs sensibles aux molécules aversives sont portés par les neurones L2, abrités par les sensilles de types S et I localisées au niveau du proboscis, des pattes et dans le pharynx.

Weiss *et al.* (2011) ont permis d'effectuer une classification plus précise de la localisation des GR répondant aux molécules aversives. En effet, les récepteurs gustatifs portés par le labelle présentent différentes sensibilités vis-à-vis de 16 molécules aversives et 6 groupes de récepteurs gustatifs sensibles aux molécules aversives ont été identifiés : Les GR portés par les sensilles L (groupe 1) répondent très faiblement aux molécules aversives. Les sous-groupes de sensilles I-a (groupe 2) et I-b (groupe 3) répondent à un panel restreint de molécules aversives. Il est probablement possible de mettre en évidence d'autres sous-groupes si ces mêmes expériences sont réalisées avec d'autres composés aversifs (Fig. 6).

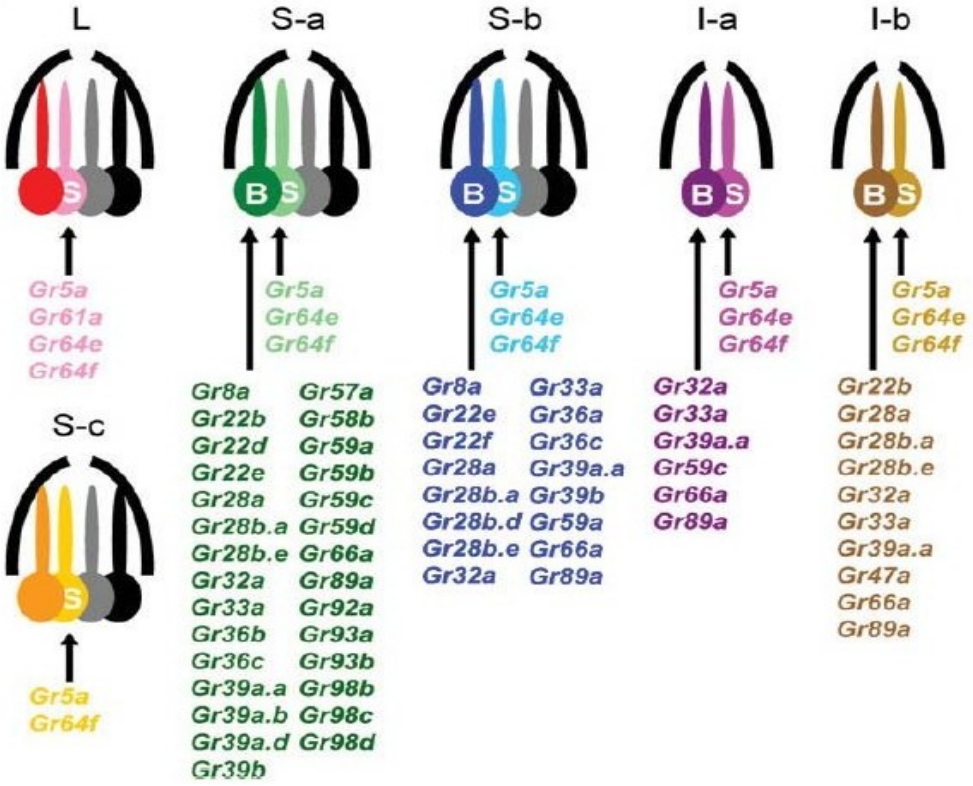

Figure 6. *L'expression des Grs dans les neurones répondant à l'amer (B) et dans les neurones répondant aux sucres (S), en fonction du type de sensilles (Weiss et al., 2011).*

1.9 Intérêt de l'étude

Les nouvelles connaissances en écotoxicologie modifient la manière dont les pesticides et autres substances toxiques sont évalués. Les effets des pesticides et autres substances toxiques sur les organismes ont été traditionnellement évalués en utilisant des mesures de la mortalité aiguë, en tant que paramètre unique, s'appuyant sur la détermination de la dose/concentration létale, ou encore par l'évaluation de la mortalité induite par les doses recommandées sur le terrain (Croft,1990 ; Desneux *et al.,* 2007).

Cependant, en plus de l'effet direct sur la létalité, ces composés peuvent également altérer diverses caractéristiques biologiques essentielles des individus qui survivent à l'exposition, par des effets physiologiques et comportementaux (Desneux, 2007 ; Müller, 2018). Cette toxicité indirecte est associée à des effets sublétaux sur la physiologie de l'insecte (ex : développement, reproduction, longévité…) ou encore sur son comportement (ex : mobilité, recherche de nourriture, communication sexuelle, capacité d'apprentissage, mémorisation..) (Xavier *et al.,* 2015 ; Kilani-Morakchi *et al*., 2017 ; Oulhaci *et al*., 2018). Cependant, tout effet direct ou indirect dépend du stade de développement des insectes, car les

larves ou les nymphes ont tendance à être plus sensibles aux pesticides que les adultes (Desneux, 2007 ; Cloyd & Herrick, 2018).

L'analyse toxicologique qui permet d'estimer l'effet total des insecticides sur les populations, est de plus en plus importante lors du choix de nouveaux pesticides à utiliser pour la lutte intégrée (Banks *et al.,* 2005). La plupart des études concernant les effets sublétaux des insecticides sont liées à une exposition continue ou répétée. Cette exposition provoque un stress généralisé et active une réponse de détoxication telle que la surexpression des gènes du cytochrome P450, qui conduit à long terme à la résistance à l'insecticide (Akhtar *et al.,* 2003).

De plus, ces phénomènes de surexpression constituent également une possibilité d'adaptation environnementale (Terrier, 1984). Pour les insecticides d'origine végétale, un phénomène de désensibilisation à des composés anti-appétant a été rapporté (Akhtar *et al.,* 2003 ; Kool & Wallia, 2009). En effet, suite à une exposition répétée, les insectes deviennent de plus en plus tolérants (Kool & Wallia, 2009). Bomford et Isman (1996) ont également rapporté une habituation à l'azadirachtine pure chez *S. litura,* qui devient moins sensible aux effets anti-appétant de ce composé. Néanmoins, aucun phénomène d'habituation n'est constaté après traitement à l'huile de Neem contenant la même quantité d'azadirachtine (Bomford & Isman, 1996), ce qui suggère une faible probabilité de désensibilisation aux formulations commerciales d'azadirachtine qui renferment d'autres molécules en faibles proportions.

Des études récentes ont révélé que les insectes peuvent moduler leur comportement sur la base d'expériences antérieures en début de vie et que divers changements induits par les insecticides chez la génération directement exposée peuvent persister chez les générations suivantes, non exposées (Da Costa *et al.,*2014 ; Müller *et al.,*2017). Chez *D. melanogaster*, l'exposition des larves à des doses sublétales d'azadirachtine affecte divers aspects de leur physiologie. Cette exposition préimaginale augmente l'aversion à ce composé chez les adultes survivant à l'exposition larvaire (Kilani-Morakchi *et al.,* 2017 ; Bezzar-Bendjazia *et al.,* 2016) traduisant un effet à long terme de sensibilisation (Minoli *et al.,* 2012 ; Crook *et al.,* 2014), induisant un évitement des composés nocifs (Walters *et al.,* 2001).

Dans ce contexte, une évaluation précise des effets physiologiques et comportementaux, induits par les insecticides, est essentielle pour acquérir des connaissances sur leur efficacité globale pour la gestion à long terme des populations d'insectes nuisibles, ainsi que sur leur sélectivité vis-à-vis des espèces non visées (Kilani-Morakchi *et al.,* 2017). En effet, lors des études de la sensibilité des organismes aux insecticides, et en plus des impacts

à court terme sur les individus directement exposés, il est important de prendre en compte l'ensemble du cycle de vie en tant que méthode complète pour évaluer l'effet total sur la population d'insectes, y compris les impacts sur les prochaines générations qui ont des implications importantes pour le succès d'un programme de lutte intégrée (IPM) (Ali *et al.,* 2017 ; Müller , 2018).

1.10 Objectifs de l'étude

Cette thèse s'inscrit dans la continuité des travaux de thèse du Dr. R. Bezzar-Bendjazia (2016) qui a mis en évidence qu'une exposition pré-imaginale chez *Drosophila melanogaster* (Stade L 3) à une formulation commerciale d'azadirachtine, le Neem Azal, affectait différents aspects physiologiques et comportementaux dont la ponte, la préférence du site d'oviposition, et la survie de la génération parentale.

Nous avons donc envisagé un travail complémentaire qui répond à ses perspectives et qui se découpe en deux parties distinctes :

Une première partie concerne l'évaluation des effets sur la reproduction et le développement des adultes de *D. melanogaster* après une exposition unique à l'azadirachtine (DL_{50} : 0,67 µg et DL_{25} : 0,28 µg des stades immatures), des larves L3 et ce sur les différentes générations successives.

La seconde partie, traite de l'impact de cette exposition unique sur la prise de nourriture et les propriétés aversives de la molécule sur les adultes ayant survécu au traitement, en évaluant les effets sur différents paramètres comportementaux (olfaction, gustation, locomotion) ainsi que sur les réponses des sensilles gustatives grâce à une étude électrophysiologique des récepteurs gustatifs.

Matériel & méthodes

2 Matériel et méthodes

2.1 Matériel biologique

Drosophila melanogaster (Meigen, 1830) (Fig. 7) communément appelée mouche du vinaigre, appartient à la famille des drosophilidae. Son génome entièrement séquencé (Adams, 2000), sa facilité et son faible coût d'élevage, son cycle de reproduction court (10 à 12 jours à 25° C), sa petite taille (2-3 mm) ainsi que la disponibilité de nombreux outils génétiques et moléculaires font de cette mouche un modèle d'étude privilégié en recherche scientifique et médicale.

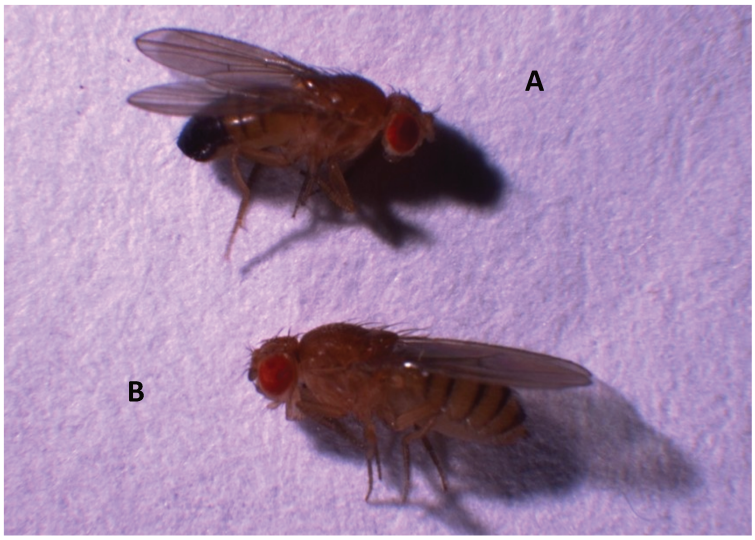

Figure 7. *Mâle et femelle de D. melanogaster.*

A : Mâle, Gr : x 11,66 ; B : Femelle, Gr : x 11,42.

Dès le début du 20ème siècle, Thomas Hunt Morgan a été le premier à comprendre l'importance de la drosophile en tant que modèle animal ; ce qui lui a valu en 1933, le prix Nobel de physiologie/médecine pour ses recherches sur la relation entre le chromosome et l'hérédité.

L'importance de la drosophile pour la santé humaine a été reconnue plus récemment par l'attribution du prix Nobel de médecine en 1995 pour les travaux sur la génétique du développement embryonnaire. Le dernier prix Nobel a été obtenu en 2017 par Jeffrey C. Hall et ses collaborateurs pour leurs études sur les mécanismes moléculaires qui contrôlent le rythme circadien.

Dans le domaine des neurosciences, la drosophile a été largement étudiée, puisque malgré sa petite taille, son système nerveux central (100.000 neurones) semble fonctionner selon des mécanismes moléculaires identiques à ceux des mammifères y compris l'homme.

Le cycle de vie de *D. melanogaster* comprend 4 stades : embryonnaire, larvaire, pupal et adulte. À 25°C, il ne faut que 10 jours à la drosophile pour passer de stade œuf à adulte. La femelle peut pondre jusqu'à 400 œufs de 0,5 mm de taille dans un lieu de ponte favorable. Au bout de 24 heures après la ponte, les œufs donnent naissance aux larves du premier stade.

Le stade larvaire se compose de trois stades (L1, L2 et L3 d'une durée respective de 24 h, 24 h et 48 h). Après 4 jours d'alimentation vorace, la larve du 3ème stade s'encapsule dans un puparium où se déroule la métamorphose.

La période pupale dure 3 jours et demi environ, au terme desquels, les structures adultes sont mises en place (Fig. 8). L'adulte de *D. melanogaster* émerge par l'opercule du puparium et devient mature sexuellement.

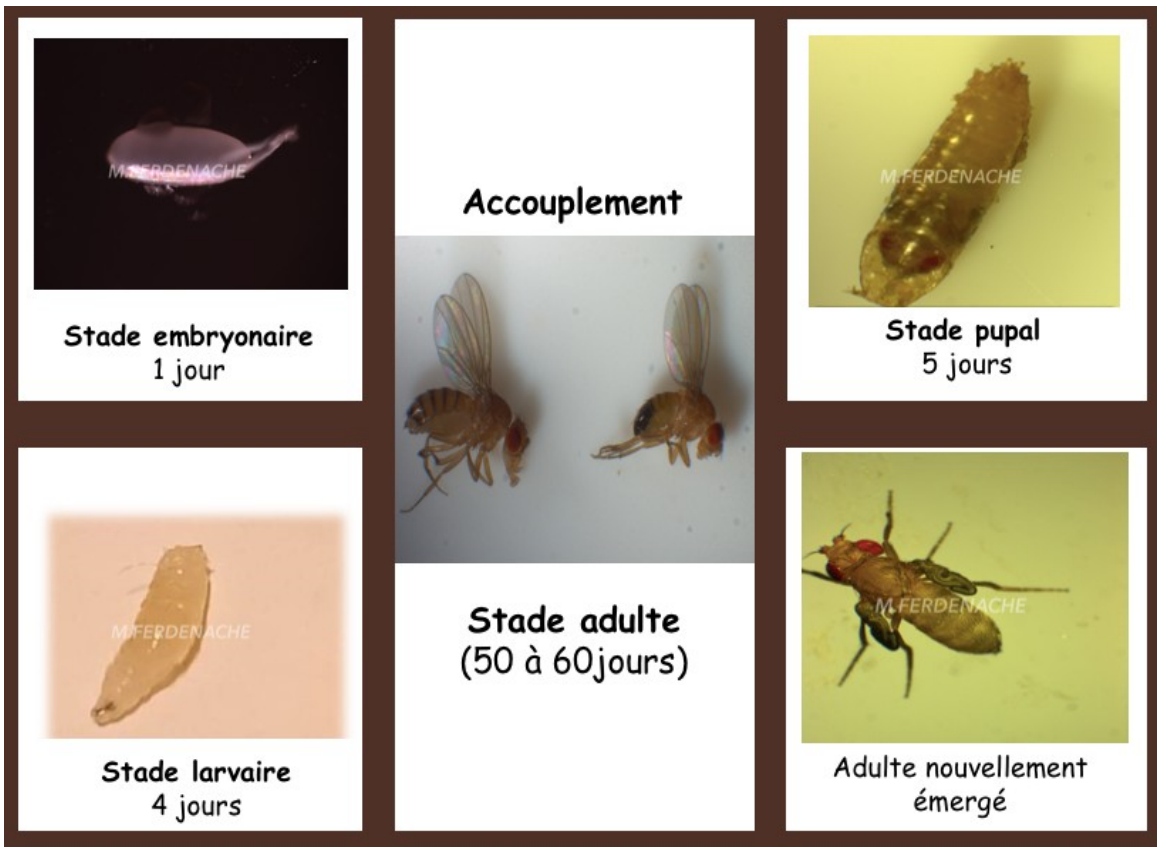

Figure 8. *Cycle de vie de D. melanogaster.*

Les mouches utilisées pour nos expériences appartiennent à la souche Canton S et sont élevées au laboratoire sur un milieu nutritif standard (voir annexe 3), sous un cycle jour-nuit de 12 h, une température de 25°C et une humidité relative de 70%. Afin d'éviter le problème de compétition larvaire, les mouches sont transférées dans des tubes contenant un nouveau milieu tous les 3 jours.

2.2 Présentation de l'insecticide et traitement

Le NeemAzal-T/S est une formulation commerciale d'un insecticide d'origine naturelle dont la matière active est l'azadirachtine (1% azadirachtine A ; Émulsion Concentrée (EC) ; firme : trifolio-M GmbH, Lahnau, Allemagne) issue d'une extraction huileuse des graines de l'arbre de Neem, *Azadirachta indica*. L'azadirachtine a été dissous dans l'acétone et administré par application topique sur des larves de *D. melanogaster* au début du troisième stade larvaire (L3) à deux doses, 0,28 µg et 0,67 µg correspondant respectivement aux DL_{25} et DL_{50} des stades immatures (Bezzar-Bendjazia *et al.,* 2016).

Toutes les expériences ont été réalisées sur des adules ayant survécu au traitement larvaire. Les insectes témoins ont été exposés uniquement au solvant (1 µl d'acétone) selon Bensebaa *et al* (2015) et toutes les mouches ont été conservées dans les mêmes conditions que celles mentionnées ci-dessus.

2.3 Étude de la fécondité et de la préférence d'oviposition

Afin d'évaluer si une exposition unique à l'azadirachtine au début de stade L3 de *D. melanogaster* affecte l'oviposition des femelles, nous avons étudié les performances de ponte en utilisant un test de fécondité en conditions de choix ou de non-choix du milieu de ponte.

2.3.1 Condition de non-choix du milieu de ponte

Trois femelles vierges âgées de 3 jours préalablement accouplées avec des mâles du même âge (Bezzar-Bendjazia *et al.,* 2016), sont utilisées pour les tests de fécondité. Les femelles des séries témoins et traitées (azadirachtine au début de stade L3 : DL_{25} et DL_{50}) ont été mises à pondre pendant 24 h dans une boîte de Petri (Ø = 65 mm) remplie de 3 ml de milieu nutritif contenant de l'azadirachtine à deux concentrations : 0,1 µg/ml ou 0,25 µg/ml (Fig. 9). Un milieu nutritif contenant de l'acétone a été utilisé comme milieu contrôle. À la fin du test, les mouches ont été retirées et le nombre d'œufs pondus sur chaque milieu a été comptabilisé.

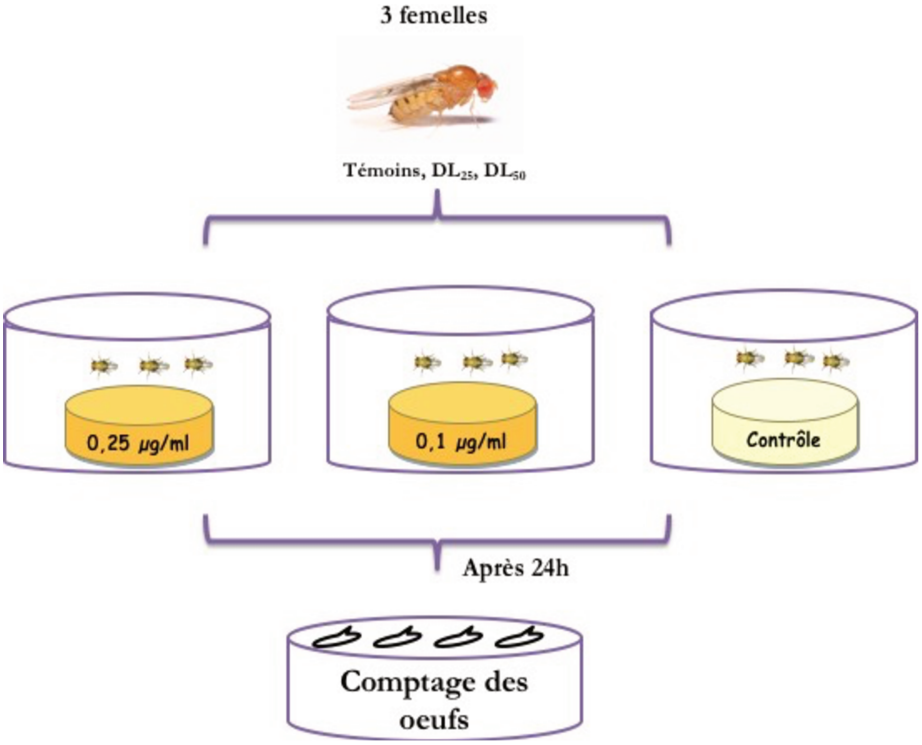

Figure 9. *Procédure expérimentale pour l'étude de la fécondité (condition de non-choix du milieu de ponte).*

2.3.2 Condition de choix du milieu de ponte

Des expériences similaires ont été réalisées afin d'étudier la préférence du site de ponte en condition de choix. Trois femelles fécondées âgées de 3 jours issues des séries témoins et traitées (DL_{25} et DL_{50}) ont été mises à pondre pendant 24h dans un dispositif de ponte (pondoir) à choix binaire contenant deux boîtes de Petri l'une remplie de milieu contrôle (acétone) et l'autre d'un milieu traité (azadirachtine à 0,1 µg/ml ou 0,25 µg/ml) (Fig. 10). Après 24h, la préférence de ponte a été évaluée en comptant le nombre d'œufs pondus sur chaque milieu.

Les tests réalisés dans les deux conditions (choix ou non-choix) ont été effectués sur trois générations successives (génération parentale, F1 et F2), avec 12 répétitions pour chaque milieu, chaque condition et chaque génération. Aucune mortalité des femelles n'a été enregistrée au cours de nos expérimentations et ce durant la durée d'exposition (24h).

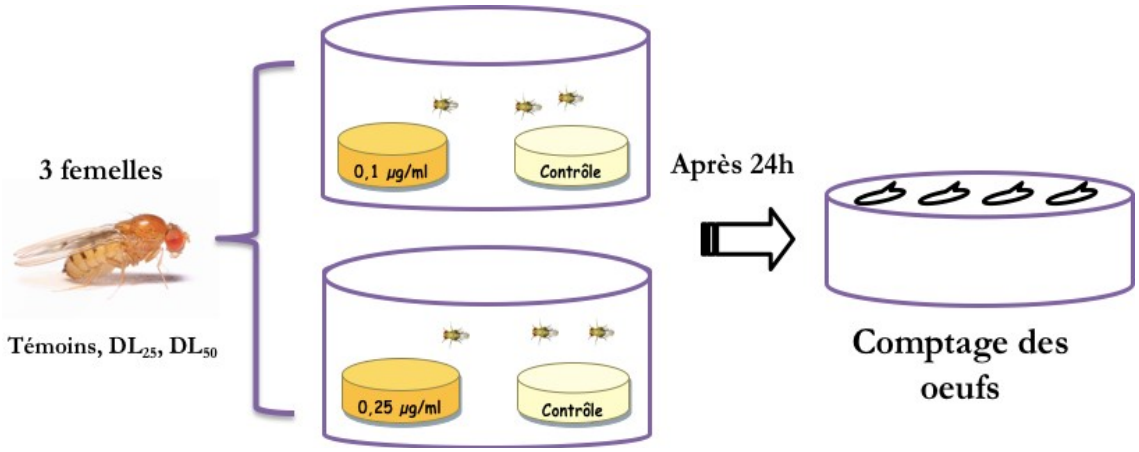

Figure 10. *Procédure expérimentale pour l'étude de la préférence du site de ponte (En condition de choix).*

À l'issue du dénombrement des œufs déposés sur chaque pondoir, un indice de préférence d'oviposition (OPI) est calculé comme suit : (nombre d'œufs pondus sur le milieu d'azadirachtine - nombre d'œufs pondus sur le milieu témoin) / nombre total d'œufs. Cet indice peut varier de -1 (aversion pour l'azadirachtine) à +1 (attirance envers l'azadirachtine).

2.4 Étude du développement

Afin d'évaluer l'impact d'une exposition unique à l'azadirachine des stades L3 sur le développement de *D. melanogaster* au cours des générations successives ; dix femelles, des séries témoins ou traitées à l'azadirachtine (DL_{25} et DL_{50}), appartenant à la génération parentale et âgées de 3 jours, ont été mises à pondre pendant 8h dans un dispositif de ponte contenant deux pondoirs (boite de Petri) remplis de milieu témoin (acétone) ou traité (azadirachtine : 0,1 ou 0,25 µg/ml).

À l'issu du test, les mouches ont été retirées et un pool de 100 œufs, pour chaque condition, a été transféré dans une nouvelle boîte de Petri contenant le même milieu. L'impact sur le développement de chaque stade (larve, pupe et adulte) a été évalué, en comptabilisant le nombre de larves de troisième stade, de pupes et d'imagos (Fig. 11). Le sex-ratio exprimé par le nombre de mâles divisé par le nombre total d'insectes émergés a également été évalué.

Les mouches de la génération parentale ont été croisées et les mêmes expérimentations ont été répétées pour la génération suivante (non exposée : F1), en comptabilisant les mêmes paramètres.

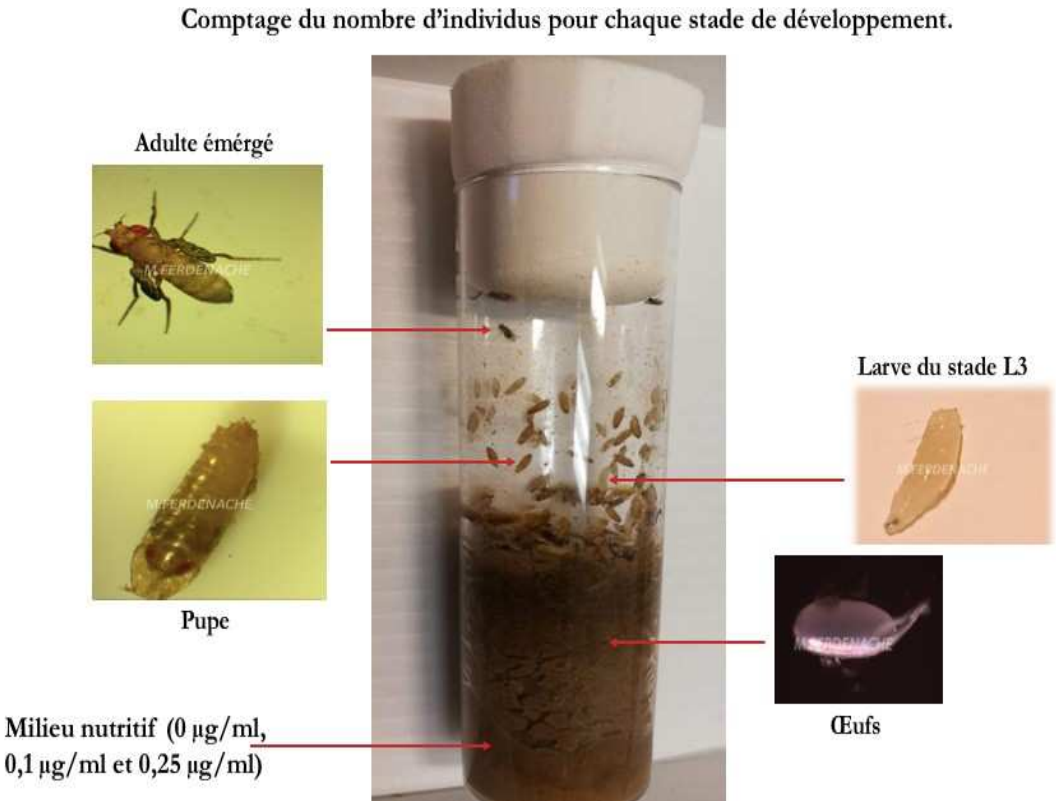

Figure 11. *Suivi du développement des différents stades de vie de D. melanogaster.*

La durée de développement pour chaque stade a été enregistrée pour les deux générations testées et exprimées par le T_{50} (temps en heures, lorsque 50% de la population a atteint le stade larvaire, pupal ou imaginal dans les flacons). Un stéréomicroscope à zoom (Leica Z16 APO) a été utilisé afin de détecter d'éventuelles distorsions morphologiques et des photographies ont été prises.

Les résultats ont été comparés en utilisant un facteur (FNO) décrivant le nombre final d'organismes en comparaison aux témoins (Ventrella *et al.,* 2016). Le FNO a été calculé comme suit : $FNO = \frac{!t\#\$}{\$}\% \times 100$

T = nombre final d'organismes sur le milieu traité.

C = nombre final d'organismes sur le milieu témoin.

Des valeurs positives de FNO révèlent un nombre d'organismes plus élevé sur le milieu traité, alors que des valeurs négatives signifient que le nombre d'individus est plus élevé sur le milieu témoin.

2.5 Analyse de survie des adultes

Une analyse de survie a été réalisée afin d'évaluer les effets de l'unique exposition au stade larvaire sur la longévité des adultes et ce sur deux générations successives (P : exposés, F : non exposés) en utilisant le protocole de Linford *et al.* (2013).

Les adultes nouvellement émergés ayant survécu au traitement préalable au stade larvaire (DL_{25} et DL_{50}) ont été séparés selon leurs sexes dans des flacons en plastique (15 mâles et 15 femelles par flacon) contenant 3ml de milieu standard. Les insectes sont maintenus à 25°C (photopériode : 12 : 12h) et transférés dans un nouveau flacon tous les 2 jours. Les mouches ont été gardées en observation pendant 15 jours au cours desquels la mortalité a été évaluée toutes les 24h (Fig. 12). Une série témoin a été réalisée en parallèle dans les mêmes conditions. Dix répétitions ont été effectuées pour chaque dose, sexe et génération.

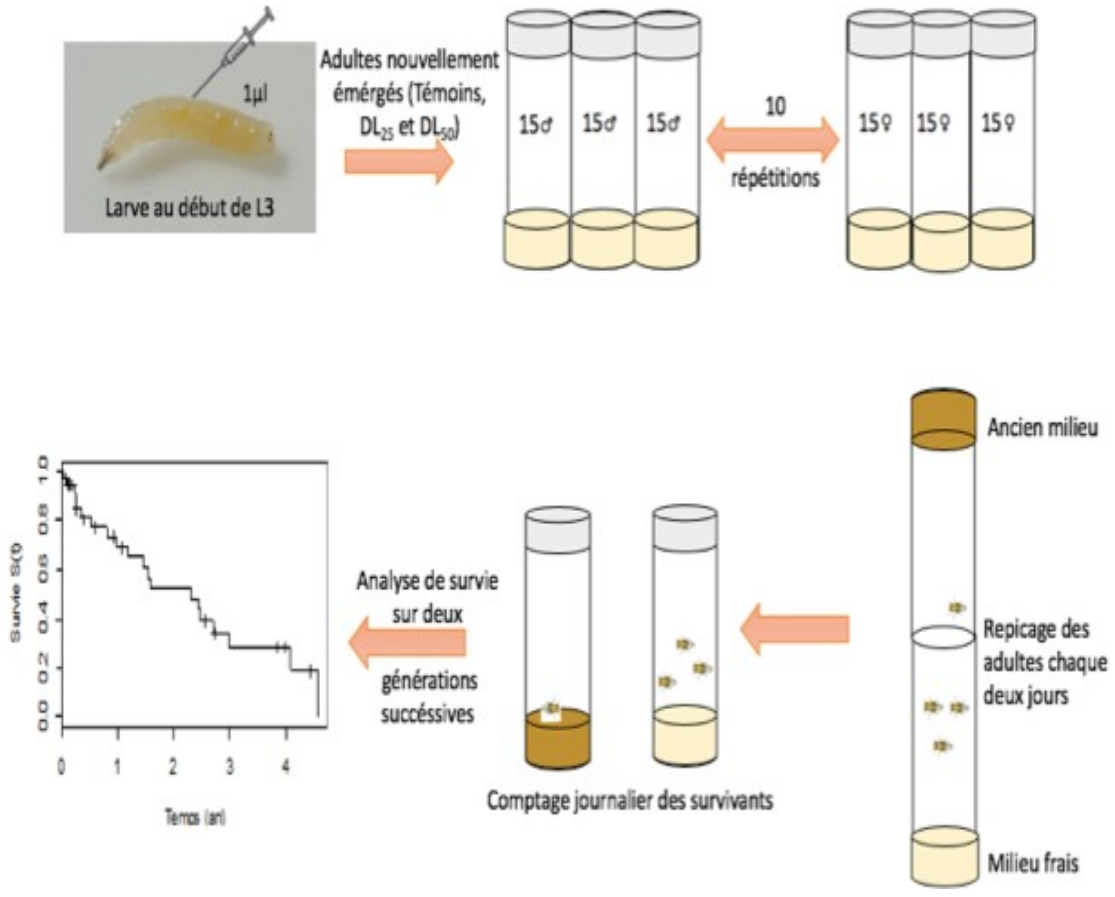

Figure 12. *Procédure expérimentale pour l'analyse de survie des adultes de D. melanogaster.*

2.6 Étude de l'activité locomotrice des adultes

Dans le but d'évaluer les effets de l'azadirachtine administré au début du stade L3 de *D. melanogaster* sur l'activité locomotrice des adultes survivants au traitement, les mouches ont été testées selon le protocole comportementale de géotaxie négative réalisé sous lumière inactinique rouge (Ali *et al*., 2011).

Dix mouches (mâles ou femelles séparément) âgées de trois jours des séries témoins et traitées (DL_{25} et DL_{50}) ont été placées, sans anesthésie préalable, dans un tube en plastique vide (18 cm de long x 2 cm de diamètre). Après 15 minutes sous lumière inactinique, les tubes sont doucement tapés durant 3 secondes de manière à ce que toutes les mouches se retrouvent au fond du tube. Le nombre de drosophiles capables d'atteindre le haut du tube en 15 secondes est comptabilisé.

Cinq répétitions à 5 min d'intervalle ont été effectuées pour chaque dose, sexe et génération (P et F1) pour une durée totale de 25 min. Les résultats sont présentés en pourcentage de mouches ayant atteint le haut du tube (1cm avant le bouchon) (Fig. 13).

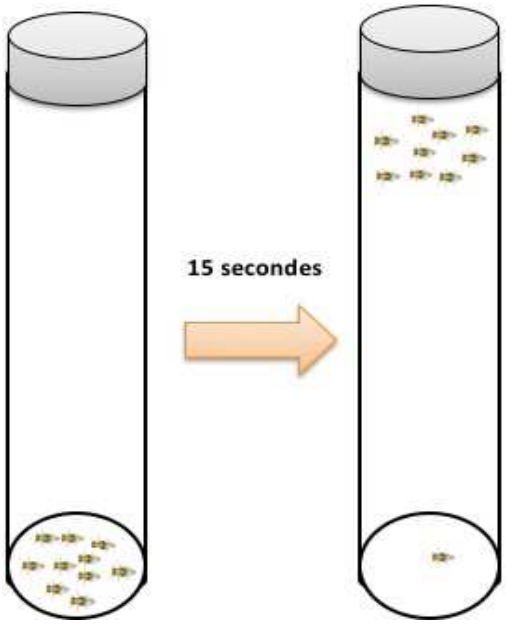

Figure 13. *Procédure expérimentale pour l'activité locomotrice des adultes de. D. melanogaster.*

2.7 Étude de la perception olfactive des adultes : Olfactomètre en Y

Afin d'évaluer l'impact de l'expérience pré-imaginale à l'azadirachtine sur le choix olfactif (attirance/répulsion) des mouches adultes, nous avons utilisé un labyrinthe en forme de Y présentant un choix olfactif binaire.

Les adultes (10 mâles, 10 femelles), âgés de 3 jours, des séries témoins et traitées (DL_{25} et DL_{50}) séparés selon leur sexe, sont mis à jeûner 17 heures avant le test dans un tube contenant un papier humidifié, et maintenus à 25° C.

On dépose sur un papier filtre (Whatmann, N° 41), 50µl de solution d'azadirachtine à 0,25µg/ml, et sur un autre papier filtre 50µl d'acétone (utilisé comme témoin). On laisse évaporer et on humidifie les papiers filtre avec 1ml d'eau distillée.

On découpe alors les zones de façon à obtenir un carré mesurant 5 x 5 mm, on place ces papiers filtre dans deux tubes d'élevage en plastique (longueur : 9,3 cm, Ø 2,2cm).

Les tubes sont reliés entre eux par un tube en Y (Fig. 14). Afin de piéger les mouches qui entrent dans chaque tube, le tube en Y est relié à un cône sectionné, traversant un bouchon de mousse. La troisième branche du tube en Y est reliée à un troisième tube, contenant les 10 adultes à tester.

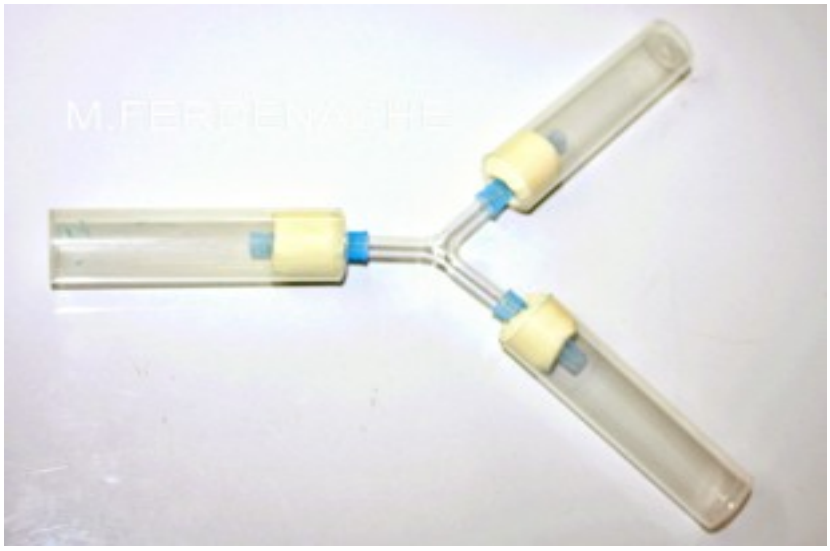

Figure 14. *Olfactomètre en Y utilisé pour l'étude de l'orientation des mouches vers des odeurs.*

Les tests sont effectués sous lumière rouge afin d'éviter les stimuli visuels autant que possible (Simonnet *et al.,* 2014). Après 2 heures, on compte le nombre de drosophiles dans chaque tube et un indice d'olfaction (IO) est calculé comme suit :

IO = (nombre de drosophiles dans le tube contenant l'azadirachtine – nombre de drosophiles dans le tube contenant l'acétone) / nombre total de mouches utilisées.

2.8 Test de sensibilité gustative (Proboscis Extension Response : PER)

Nous avons utilisé une approche comportementale appelée extension du proboscis (PER) afin de vérifier la détection de l'azadirachtine par les mouches. 30 mâles et femelles, âgés de 3 jours, préalablement traités (DL_{25} et DL_{50}) au stade L3 ainsi qu'une série témoin (tubes ne contenant que du papier filtre imbibé d'eau distillée) sont mis à jeûner 20 h avant le test et maintenus à 25°C).

Deux heures avant le test, les mouches sont anesthésiées en les plaçant dans un tube de verre plongé dans de la glace (1-2 minutes) puis disposées sur du patafix (UHU stick), et immobilisées sur le dos grâce à une bandelette de scotch placée sur le thorax (Fig.15).

Le test consiste à toucher le proboscis avec une goutte de la substance à tester. En cas de réponse positive à la substance testée, la mouche étendra son proboscis pour se nourrir (Fougeron *et al.,* 2011). Afin d'éviter les faux positifs, avant chaque stimulation, l'insecte est stimulé avec de l'eau. En cas de réponse positive, la mouche ne sera pas testée. Si la mouche n'a aucune réaction, on teste alors une goutte de saccharose (100 mM) afin de vérifier que l'extension du proboscis peut se faire correctement. Si aucune extension n'est visible, la mouche ne sera pas testée.

Différentes concentrations d'azadirachtine ont été testées (0,1 µg/ml, 0,25µg/ml, 1µg/ml, 10 µg/ml et 100 µg/ml). Les réponses comportementales observées sont l'extension du proboscis (PER : proboscis étendu pendant 5 s) ou l'absence de réponse.

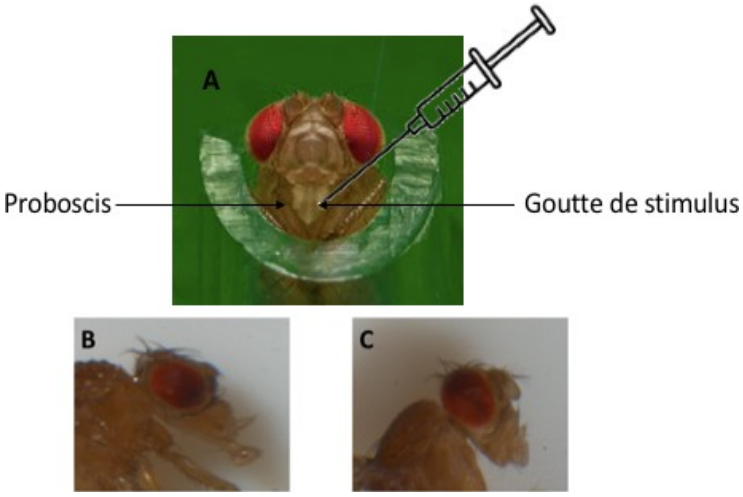

Figure 15. *Schéma des procédures utilisées pour mesurer l'extension du proboscis (PER).*

(A) : *Fixation de la mouche et présentation du stimulus,* ***(B) :*** *Extension du proboscis,* ***(C) :*** *Proboscis au repos.*

2.9 Étude électrophysiologique

Afin d'évaluer la sensibilité des mouches préalablement traitées (DL_{25} et DL_{50}) à l'azadirachtine au début du stade L3 vis-à-vis de cette molécule ; des enregistrements électrophysiologiques ont été réalisés sur des femelles âgées de 3 à 5 jours ayant survécu au traitement larvaire. Les expériences consistent à coiffer l'extrémité d'une sensille gustative avec un capillaire en verre contenant une solution stimulante et un électrolyte pendant 2 s.

Après avoir été anesthésiée dans un tube de verre plongé dans de la glace pilée (1 à 2 min), une mouche est fixée sur un support malléable (Patafix UHU stick, ®) et immobilisée avec 2-3 bandelettes de papier adhésif (Scotch crystal) de 0,2-0,5 mm x 1 cm, et placée de manière à exposer les sensilles gustatives du proboscis (Fig. 16). Une électrode de référence, constituée d'un fil d'argent placée contre la mouche et mise en contact avec celle-ci à l'aide d'une goutte de gel d'électrocardiographie (Redux Gel, Parker Laboratories, Fairfields NJ, USA).

La mouche fixée est ensuite amenée sous un microscope (Leica MZ16), et correctement orientée de façon à ce que les sensilles sur le proboscis soit accessible à la stimulation (Fig. 16) Pour la stimulation, nous avons utilisé des capillaires en verre borosilicaté (taille de la pointe d'environ 10 µm ; Harvard Apparatus LTD, EdenBridge, UK), remplis de la solution de stimulation. Cette électrode est connectée à un amplificateur de goût (TastePROBE DT-02)

(Marion-Poll & Van der Pers, 1996) qui déclenche au contact, un enregistrement de 2 secondes avec une carte d'acquisition de données 16 bits (DT9803, Data Translation, USA) échantillonnant les données à 10 kHz, sous le contrôle d'un programme personnalisé dbWave (Marion-Poll, 1996). Les données ont ensuite été amplifiées (×500) et filtrées (10-2800 Hz) avec un amplificateur CyberAmp 320 (Axon Instruments, USA).

Les solutions stimulantes utilisées sont dissoutes dans de l'eau distillée contenant du tricholinecitrate à 30 mM (TCC ; Sigma-Aldrich, France), qui est un électrolyte inhibant l'activité de la cellule à l'eau (Wieczorek & Wolff, 1989). Les solutions testées sont : L'azadirachtine (0,1µg/ml, 0,25µg/ml, 1µg/ml, 10µg/ml), la caféine (10mM) et le saccharose (30mM). Le TCC à 30mM a été utilisé comme contrôle.

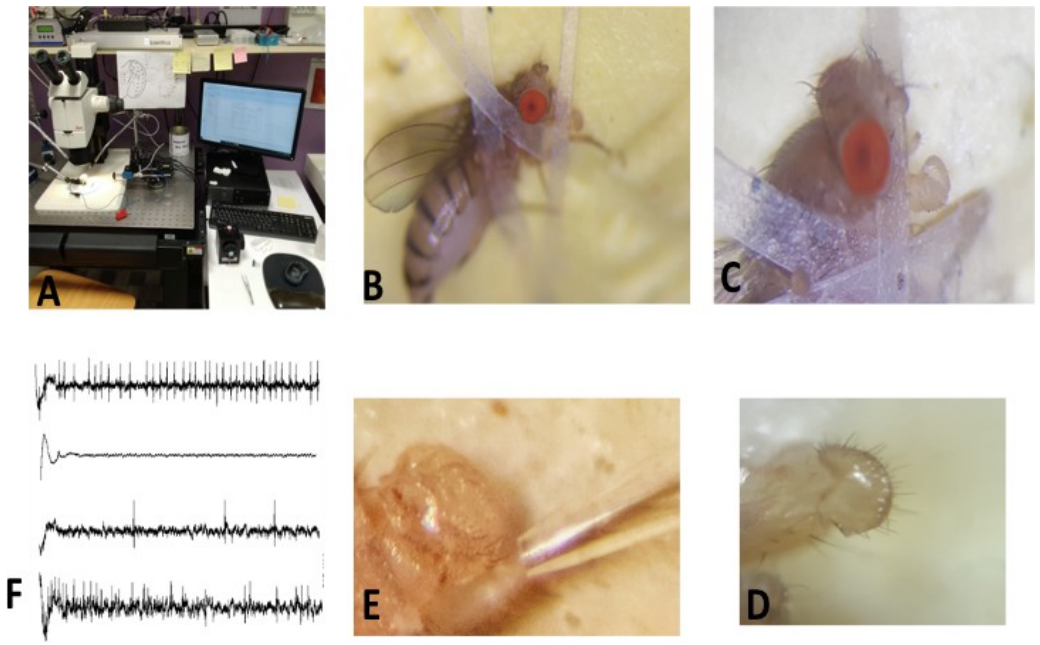

***Figure 16**. Dispositif d'électrophysiologie et étapes d'enregistrements électrophysiologiques à partir de la stimulation d'une sensille gustative du proboscis chez D. melanogaster.*

***(A) :** Poste d'électrophysiologie de gustation comprenant une loupe, un éclairage par lumière froide, un système d'amplification et d'acquisition de données et la préparation placée sur un plot magnétique, posé sur un bras articulé permettant de changer son orientation. **(B) :** Vue d'ensemble de la mouche immobilisée par des bandelettes de papier adhésif transparent. **(C) :** Vue du proboscis bien ouvert afin d'accéder facilement aux sensilles gustatives. **(D) :** Vue d'ensemble des sensilles. **(E) :** Procédure de stimulation d'une sensille : Le proboscis est maintenu immobile et la position des sensilles est identifiée. Un capillaire de verre contenant la solution stimulante et relié à un amplificateur par un fil d'argent est approché sous contrôle visuel grâce à un micromanipulateur afin de coiffer la sensille choisie. **(F) :** Exemple de type d'enregistrement.. La trace du haut représente le signal enregistré pendant 2 s.*

2.10 Tests de consommation alimentaire (MultiCAFE : Multi Capillary Feeding)

Pour l'estimation de la quantité de liquide consommé, un test de capillaires multiples (MultiCAFE) a été réalisé. 32 adultes mâles et femelles, âgés de 3 jours, ayant survécu au traitement (DL_{25} et DL_{50}) larvaire, sont séparés selon leur sexe, et mis à jeûner, dans des tubes contenant un coton humidifié, pendant 20 à 22 h (Fig. 17).

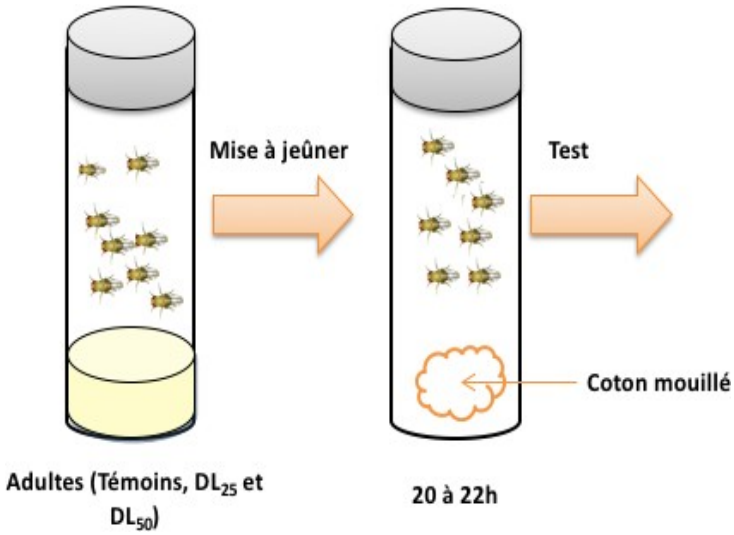

Figure 17. *Schéma représentant la préparation des mouches pour le test MultiCAFE.*

Les mouches sont ensuite transférées dans des cages individuelles rectangulaires dans lesquelles elles ont accès à 2 capillaires de 5 µl (Hirschman Laborgewäre, VWR), remplis d'une solution test. Chaque cage fait partie d'une boîte comprenant 10 cages individuelles dont 2 ne contiennent pas de mouches, et qui servent à évaluer l'évaporation (Fig. 18). Un papier filtre humidifié est placé dans les boites afin de maintenir un niveau d'humidité élevé durant l'expérience. Ces boîtes sont alors mises dans une étuve à 25°C où une webcam Logitech HC920 est utilisée pour capturer une image par minute pendant une durée de 2 h.

A la fin de l'expérience, les images sont analysées grâce à un plugin (développé par Pr. F. Marion-Poll) développé en Java qui fonctionne sous le logiciel d'analyse d'image ICY (Chaumont *et al.,* 2012), afin de mesurer la quantité de liquide consommé ainsi que le taux d'évaporation. Deux concentrations d'azadirachtine (0,1 µg/ml et 0,25µg/ml) mélangées avec 30mM de saccharose ont été testées. Des séries témoins ont été réalisées en utilisant uniquement du saccharose à 30mM. Afin d'évaluer la spécificité de réponse à l'azadirachtine,

une seconde molécule amère, la caféine (CAF à 10 mM) a été utilisée. Toutes les solutions ont été colorées avec du bleu brillant R pour faciliter la quantification de la consommation.

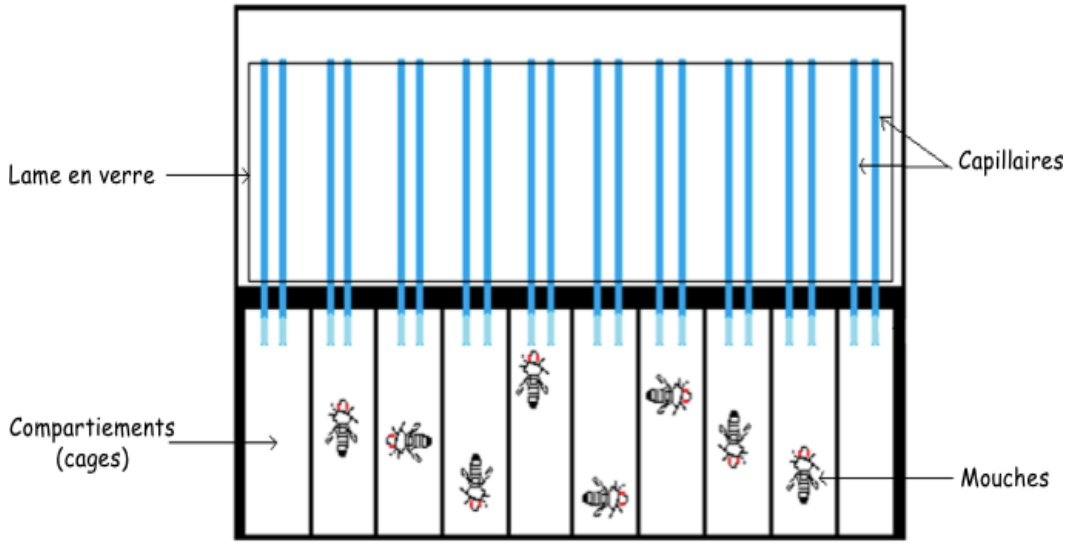

Figure 18. *Schéma du dispositif pour le test alimentaire à choix multiples (MultiCAFE).*

2.11 Analyses statistiques

Toutes les analyses statistiques ont été effectuées par R studio version 3.5.0 pour Mac OS. Les résultats ont été exprimés sous la forme de moyennes ± SEM pour chaque série d'expériences. L'homogénéité des variances a été vérifiée à l'aide du test de Bartlett. Le test statistique de Shapiro-Wilk a été utilisé pour tester la normalité des données.

Les données relatives à la préférence de ponte, à l'indice d'oviposition, sex-ratio, la réactivité locomotrice et la réponse olfactive ont été analysées par le test de Kruskal-Wallis suivi par des comparaisons multiples par la méthode de Dunn's. Les résultats du développement ont été analysés par l'ANOVA, suivie par le test post-hoc HSD de Tukey. Les résultats de l'analyse de survie ont été soumis au test de survie de Kaplan-Meier.

Les données de la réponse d'extension du proboscis (PER) ont été soumises au test du chi square (χ^2). Les résultats de test de préférence alimentaire et les réponses éléctrophysiologiques ont été analysés par le test de Mann-Whitney.

Résultats

3 Résultats

3.1 Effets sur la fécondité et la préférence d'oviposition

Une étude de fécondité en condition de choix et non-choix du milieu de ponte a été réalisé *via* un comptage du nombre d'œufs pondus pour chaque groupe de 3 femelles, sur chaque milieu (Témoin, 0,1 et 0,25 µg/ml), sur trois générations successives.

L'azadirachtine, testé par application topique (DL_{25} et DL_{50} des stades immatures) sur les larves au début du stade L3 affecte la fécondité des femelles par une réduction significative du nombre d'œufs pondus comparativement aux témoins (KW = 24,73 ; P < 0,001). En effet, on enregistre une réduction de 27% pour les individus traités à la DL_{25}, cette réduction atteint 41% pour les individus traités à la DL_{50}.

Cette réduction observée chez les femelles de deux générations successives (parentale : exposée et F1 : non exposée) reste plus marquée chez la génération parentale (P) (KW = 50,89 ; P < 0,001) (Fig. 19). Une restauration complète de la fécondité a été enregistrée chez la génération F2.

Chez la génération P, le test de Kruskal-Wallis révèle des effets significatifs pour les milieux 0,1 µg/ml (KW = 29,42 ; P < 0,001) et 0,25 µg/ml (KW = 24,73 ; P < 0,001). En effet, la comparaison multiple par le test de Dunn's révèle des différences significatives entre les différents insectes testés (Témoins, DL_{25} et DL_{50}) pour les milieux témoin (P < 0,001) et traités à 0,1 µg/ml d'azadirachtine (P < 0,001). En ce qui concerne le milieu traité à 0,25 µg/ml, aucune différence n'est trouvée entre les deux doses testées (DL_{25} et DL_{50}) (P = 0,7802).

Pour la génération F1, un effet significatif du milieu a également été noté (milieu 0,1 µg/ml : KW = 22,95 ; P < 0,001 et milieu 0,25 µg/ml : KW = 27, 93 ; P < 0,001). En effet, la comparaison multiple par le test de Dunn's révèle des différences significatives entre les différents insectes témoins et traités pour les milieux témoins (P < 0,001), traités à 0,1 µg/ml d'azadirachtine (P < 0,001) et traités à 0,25 µg/ml d'azadirachtine. Néanmoins, aucun effet dose n'est enregistré et ce pour les trois milieux testés (P > 0,05).

Pour la génération F2, aucune différence n'est enregistrée entre les traitements (Témoins, DL_{25} et DL_{50}) et ce pour les trois milieux (0 µg/ml, 0,1 µg/ml et 0,25 µg/ml).

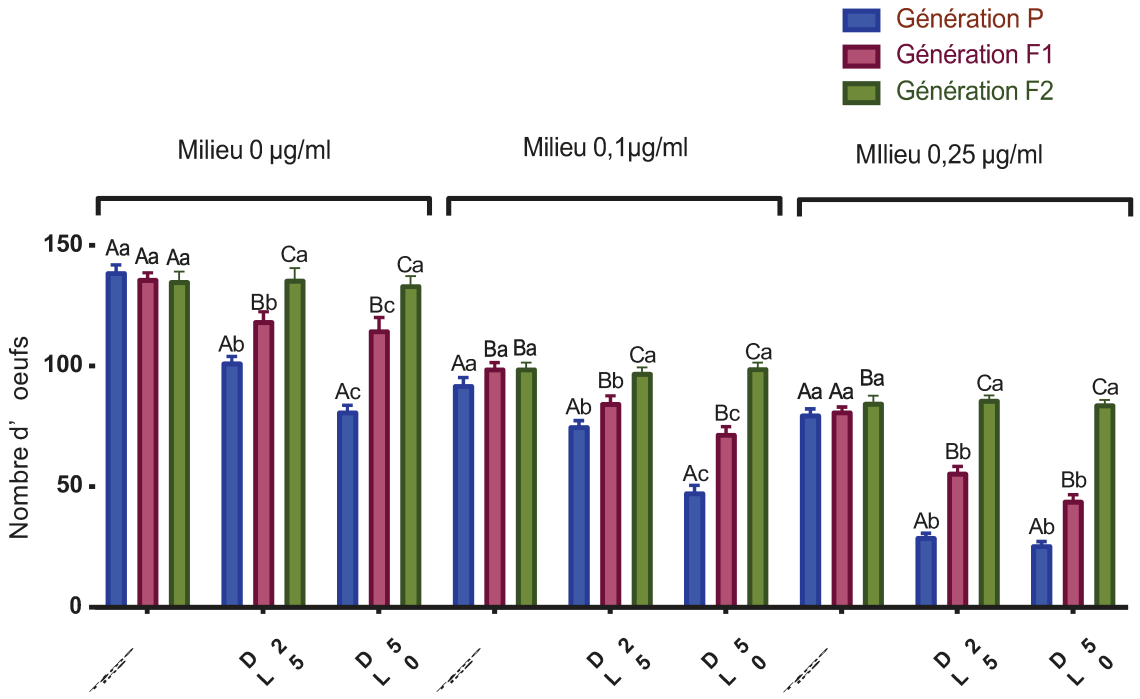

Figure 19. *Effets de l'azadirachtine sur la fécondité des femelles.*

m ± SEM, n = 12 répétitions de trois femelles chacune. Les moyennes suivies d'une même lettre ne sont pas significativement différentes (p > 0,05). Les lettres minuscules différentes indiquent une différence significative entre les individus témoins et les individus traités pour un même milieu (P < 0,05). Les lettres majuscules différentes indiquent une différence significative entre les générations pour un même milieu (P < 0,05).

Les résultats concernant la préférence d'oviposition en condition de choix (Fig. 20) révèlent une nette préférence de ponte sur le milieu témoin et ce pour toutes les générations testées (P, F1 et F2). De plus, les mouches précédemment exposées à l'azadirachtine (L3 de début de stade) présentent une aversion plus marquée envers cette substance en comparaison aux mouches "naïves", avec un nombre d'œufs pondus très réduit pour les deux premières générations (P et F1) et des effets plus marqués chez la génération parentale (P < 0,001).

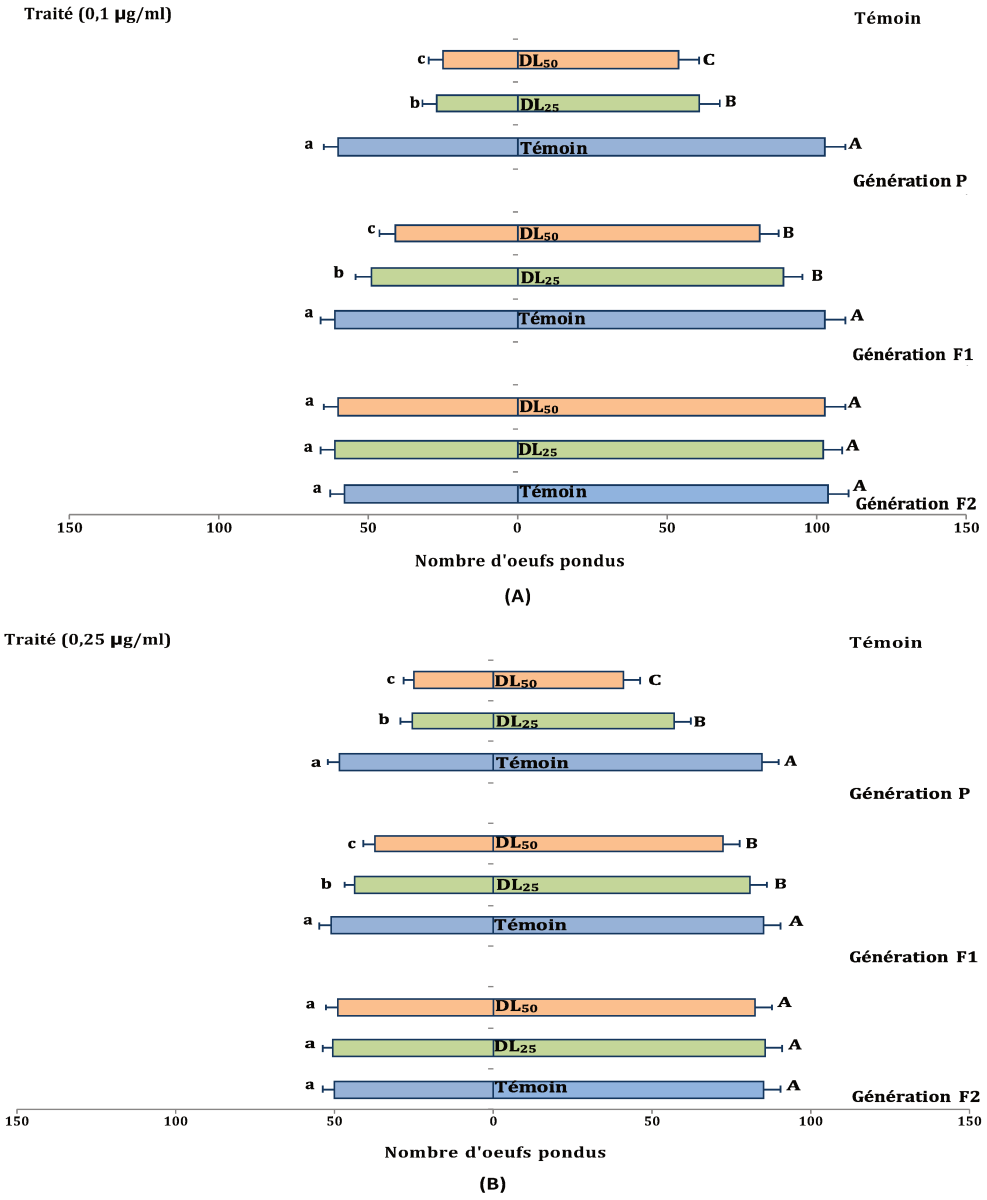

Figure 20. *Effets de l'azadirachtine sur la ponte en situation de choix.*

A : Milieu témoin vs milieu 0,1 µg/ml d'azadirachtine ; B : Milieu témoin vs milieu 0,25 µg/ml d'azadirachtine. (m ± SEM ; n = 12 répétitions de trois femelles chacune). Les moyennes suivies d'une même lettre ne sont pas significativement différentes (p > 0,05). Les lettres minuscules différentes indiquent une différence significative entre les générations pour un même milieu (P < 0,05). Les lettres majuscules différentes indiquent une différence significative entre les individus témoins et les individus traités pour un même milieu (P < 0,05).

Les indices de préférence d'oviposition (IPO) des femelles, exposées ou non à l'azadirachtine au stade larvaire (L3) de la génération parentale, présentent des valeurs négatives et ce pour toutes les générations testées (Fig. 22). Les moyennes des valeurs de l'IPO

obtenues respectivement pour les adultes témoins, DL_{25} et DL_{50} sont pour le milieu 0,1 µg/ml chez la génération parentale, de -0,27, -0,39 et -0,37, chez la génération F1 elles sont de -0,25, -0,31 et -0,32 ; chez la génération F2 elles sont de -0,28, -0,26 et -0,25.

Pour le milieu 0,25 µg/ml, les moyennes des valeurs de l'IPO obtenues respectivement pour les adultes témoins, DL_{25} et DL_{50} sont de -0,27, -0,38, -0,37 chez la génération parentale, de -0,25, -0,30, -0,32 chez la génération F1, de -0,26, -0,26 et -0,25 chez la génération F2.

En ce qui concerne le milieu 0,1 µg/ml et pour la génération P, l'analyse statistique révèle des différences significatives entre l'IPO des mouches ayant survécu au traitement préalable au stade larvaire et des mouches témoins et ce sans effet dose-dépendant (Fig. 21).

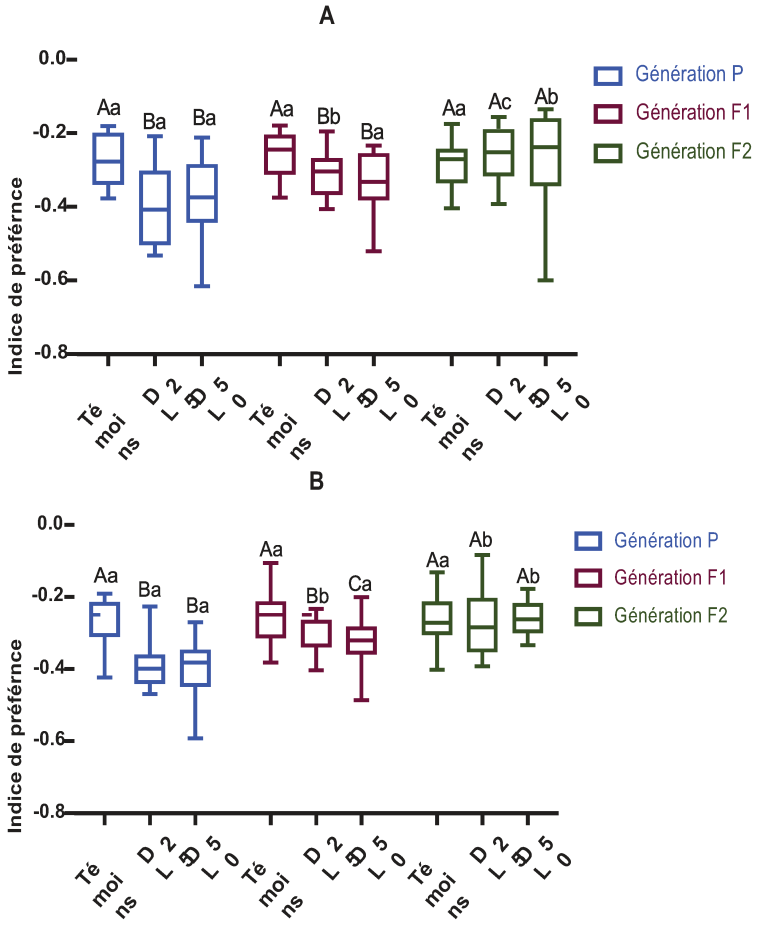

Figure 21. *Effets d'une exposition sur les choix de ponte sur 3 générations.*

Indice de préférence d'oviposition (m ± SEM ; n=12 répétitions) des femelles de D. melanogaster en condition de choix du milieu de ponte (A : 0,1 µg/ml ; B : 0,25 µg/ml). Les moyennes suivies d'une même lettre ne sont pas significativement différentes (P > 0,05). Les lettres en minuscules indiquent une différence significative entre la

même dose des différentes générations. Les lettres en majuscules indiquent une différence significative entre les différentes doses testées pour une même génération (P < 0,05).

Le test de Mann-Whitney révèle des effets significatifs entre la DL_{25} de la génération parentale et la première génération F1 (U = 8 ; P < 0,001), la DL_{25} de la génération parentale et la deuxième génération (U = 20 ; P = 0,0018) mais aucune différence n'est enregistrée entre la première et la deuxième génération F2 (U = 42 ; P = 0,0887).

Des résultats similaires sont observés pour la DL_{50}, avec des effets significatifs entre la génération parentale et la F1 (U = 19 ; P = 0,0014), entre les générations P et F2 (U = 34 ; P = 0,0284) mais aucune différence entre la F1 et la F2 (U = 58 ; P = 0,4428).

En ce qui concerne les séries témoins, aucune différence n'est relevée entre les générations testées (P > 0,05).

Des résultats similaires sont obtenus pour le milieu 0,25 µg/ml. Le test de Mann-Whitney révèle des effets significatifs entre les individus traités à la DL_{25} de la génération parentale et de la F1 (U = 25 ; P = 0,0045), de la génération parentale et la deuxième génération F2 (U = 24 ; P = 0,0045) mais aucune différence entre la première et la deuxième génération (U = 66 ; P = 0,5512).

Pour la DL_{50}, des différences significatives sont observées entre la génération parentale et la F2 (U = 25,50 ; P = 0,0025), entre la F1 et la F2 (U = 34 ; P = 0,0028) mais aucune différence n'est observée entre la F1 et la génération P (U = 49 ; P = 0,1974). Aucune différence n'est enregistrée entre les témoins des différentes générations (P > 0,05).

L'application topique de l'azadirachtine au stade L3 de *D. melanogaster* affecte donc la fécondité des femelles par une réduction significative de nombres d'œufs pondus des adultes (DL_{25} et DL_{50}) avec un effet dose-dépendant comparativement aux témoins. Cette baisse a été retrouvée chez la génération parentale P (exposée) et la génération suivante F1 (non-exposée) avec un effet plus marqué chez la génération P. La fécondité des mouches est totalement restorée chez la génération F2. Cependant, en condition de choix, les mouches évitent de pondre dans le milieu traité à l'azadirachtine (0,1 et 0,25 µg/ml) pour toutes les générations testées.

3.2 Effets sur le développement

Un suivi du développement de *D. melanogaster* a été réalisé afin d'évaluer la sensibilité des différents stades à l'exposition unique à l'azadirachine au stade L3 et ce sur deux générations successives.

Les résultats sont présentés sur les tableaux 1 et 2, respectivement pour la génération parentale (exposée) et la génération F1 (non exposée). Le traitement des larves au début du troisième stade (L3) à deux doses (DL_{25} et DL_{50}) a conduit à une réduction dose-dépendante du nombre de larves, de pupes et d'adultes chez la génération parentale avec un facteur FNO toujours négatif, traduisant un nombre d'individus plus élevé sur le milieu témoin.

Le développement de la génération suivante (F1) ne semble pas être affecté par le traitement larvaire (L3) de la génération parentale. Cependant, le nombre final d'organismes (FNO) reste significativement plus faible sur les milieux traités et ce pour toutes les mouches testées (Témoins, DL_{25}, DL_{50}). Aucune différence dans le nombre d'organismes ayant atteint le stade pupal ou imaginal n'est enregistrée entre les deux générations (P et F1).

Le traitement des larves du stade L3 affecte également le développement de *D. melanogaster* en prolongeant de manière significative ($P < 0,001$), et avec une relation dose-réponse, la durée du développement larvaire et pupal exprimée par le T_{50} (Tableau 2), et ce uniquement pour la génération parentale (exposée). Aucun effet du milieu testé n'a été enregistré sur la durée de développement des différents stades ($P > 0,05$).

Tableau 2 : *Effet de l'exposition larvaire à l'azadirachtine sur le développement de la génération parentale.*

Les lettres en minuscules indiquent une différence significative entre les différentes doses testées pour un même milieu et pour chaque stade de développement (P < 0,05). Les lettres en majuscules indiquent une différence significative entre les mêmes doses testées pour différents milieux (P < 0,05). (m ± SEM ; n = 15 répétitions).

Milieu	**Doses**	**Larves**			**Pupes**			**Imagos**		
		N individus	T_{50} (h)	Malformations (%)	N individus	T_{50} (h)	Malformations (%)	N individus	Malformations (%)	FNO
Témoin	Témoins	93,73± 1,3 A a	41,93± 0,25 A a	0,00± 0,00 A a	92,66± 1,41 A a	150,86± 1,43 A a	0,00± 0,00 A a	90,8± 1,48 A a	0,00± 0,00 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	3,60± 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
Azadirachtine 0,1 µg/ml	Témoins	89,93± 0,64 A a.	42,06± 0,6 A a	0,00± 0,00 A a	86,26± 01,00 A a	151,46± 0,89 A a	0,00± 0,00 A a	81,60± 1,1 B a	0,00± 0,00 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± 1,10 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
Azadirachtine 0,25 µg/ml	Témoins	80,13± 1,74 B a	43,86± 0,90 A a	0,00± 0,00 A a	74,8± 1,67 B a	150,8± 0,75 A a	0,00± 0,00 A a	73,53± 1,93 C a	0,00± 0,00 A a	0
	DL_{25}	72,53± 1,56 B b	54,13± 1,10 B b	7,06± 1,10 C b	63,00± 1,48 B b	159,06± 0,89 A b	09,06± 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

Tableau 3 : *Effets de l'exposition larvaire à l'azadirachtine sur le développement de la première génération F1.*

Les lettres en minuscules indiquent une différence significative entre les différentes doses testées pour un même milieu et pour chaque stade de développement (P < 0,05). Les lettres en majuscules indiquent une différence significative entre les mêmes doses testées pour différents milieux (P < 0,05). (m ± SEM ; n = 15 répétitions).

Milieu	**Doses**	**Larves**			**Pupes**			**Imagos**		
		N individus	T_{50} (h)	Malformations (%)	N individus	T_{50} (h)	Malformations (%)	N individus	Malformations (%)	FNO
Témoin	**Témoins**	97,53± 0,80 A a	42,93± 0,46 A a	0,00± 0,00 A a	97,2± 0,80 A a	150,4± 1,22 A a	0,00± 0,00 Aa	96,8± 0,76 A a	0,00± 0,00 Aa	0
	DL_{25}	97,2± 0,82 A a	43,73± 0,85 A a	0,00± 0,00 Aa	96,26± 0,94 Aa	152,6± 1,05 A a	0,00± 0,00 Aa	94,00± 0,95 A a	0,00± 0,00 Aa	-2,85
	DL_{50}	97,73± 0.85 A a	42,93± 0,69 A a	0,00± 0,00 Aa	96,13± 0,97 A a	152,00± 0,80 A a	0,00± 0,00 Aa	93,93±1,17 A a	0,00± 0,00 Aa	-2,90
Azadirachtine 0,1 µg/ml	**Témoins**	89,62± 1,80 A a	43,06± 0,50 A a	0,00± 0,00 A a	87,86± 1,84 B a	150,86± 0,57 A a	0,00± 0,00 Aa	85,60± 1,59 B a	0,00± 0,00 Aa	0
	DL_{25}	84,20± 1,30 B a	42,6± 0,77 A a	0,00± 0,00 Aa	82,4± 1,37 B a	152,73± 1,12 A a	0,00± 0,00 Aa	82,51± 1,36 B a	0,00± 0,00 Aa	-4.44
	DL_{50}	84,60± 1,32 B a	42,86± 0,79 A a	0,00± 0,00 Aa	81,26± 1,48 B a	151,86± 1,19 A a	0,00± 0,00 Aa	80,86± 1,57 B a	0,00± 0,00 Aa	-5.30
Azadirachtine 0,25 µg/ml	**Témoins**	86,53± 1,97 A a	42,64± 0,83 A a	0,00± 0,00 Aa	78,73± 2,17 B a	149,46± 0,94 A a	0,00± 0,00 Aa	78,73± 2,17 B a	0,00± 0,00 Aa	0
	DL_{25}	83,66± 1,73 B a	41,8± 0,82 A a	0,00± 0,00 Aa	76,40± 1,23 B a	150,33± 0,71 A a	0,00± 0,00 Aa	73,2± 1,48 C a	0,00± 0,00 Aa	-6.41
	DL_{50}	78,8± 1,52 B b	42,00± 0,81 A a	0,00± 0,00 Aa	76,86± 1,38 B a	151,4± 0,60 A a	0,00± 0,00 Aa	73,73± 1,55 B a	0,00± 0,00 Aa	-5.37

Différentes malformations conduisant à la mort de l'insecte (Fig. 22) ont été enregistrées, après traitement des larves du début du stade L3 à l'azadirachtine (DL_{25}, DL_{50}), chez la génération parentale, et ce pour les différents stades de développement. Les aberrations morphologiques les plus couramment observées sont des mues incomplètes, des adultes malformés (abdomen et ailes déformés) avec une forme de corps courbée et plus petite, des larves brûlées (hyperpigmentées) ainsi que des adultes morts à l'intérieur des pupes.

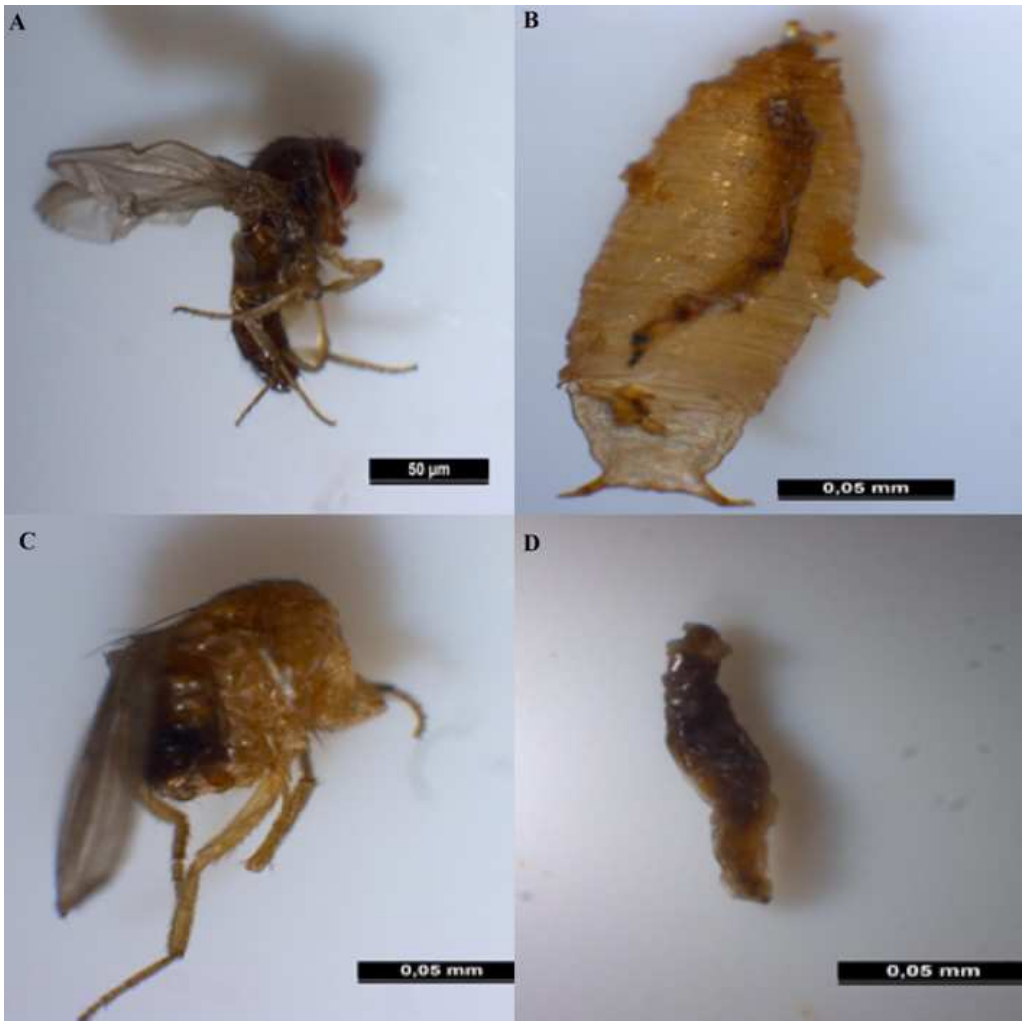

Figure 22. *Exemples des malformations les plus fréquentes chez D. melanogaster au stade L3.*

A : abdomen et ailes malformés, forme du corps courbée et plus petite ; B : adulte mort à l'intérieur d'une pupe ; C : adulte malformé ; D : larve brûlée.

L'exposition pré-imaginale à l'azadirachtine affecte le sex-ratio avec une domination du nombre de mâles et ce uniquement pour la génération parentale (Fig. 23). Le test de Kruskal-Wallis révèle des effets significatifs entre les différents insectes testés (Témoins, DL_{25} et DL_{50}) dans un milieu non traité (KW = 9,30 ; P = 0,0095), un milieu à 0,1 µg/ml d'azadirachtine (KW = 8,02 ; P < 0,0181) et un milieu à 0,25 µg/ml d'azadirachtine (KW = 18,85 ; P < 0,001). Aucun effet n'a été enregistré sur le sex-ratio de la première génération (F1).

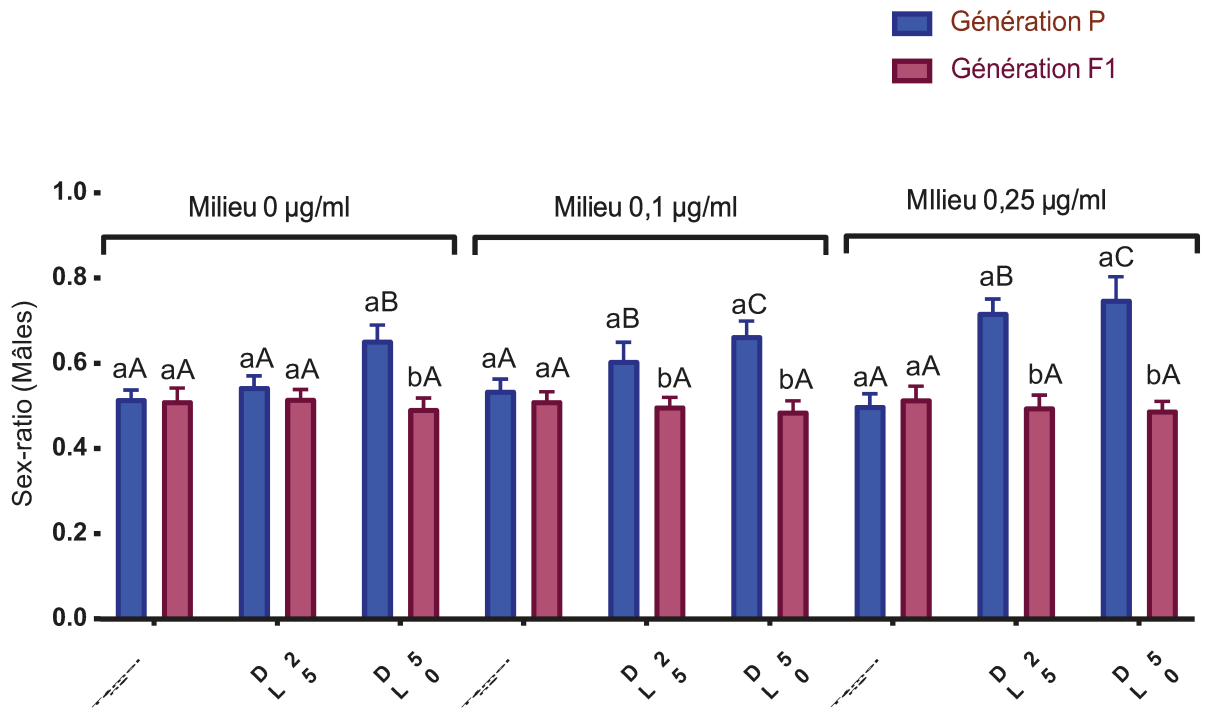

***Figure 23**. Effets de l'azadirachtine (DL25 et DL50), administré par application topique, chez les larves au début du stade L3 de D. melanogaster, sur le sex-ratio des adultes émergents.*

Les différentes lettres en minuscules indiquent une différence significative entre les générations pour un même milieu (P < 0,05). Les différentes lettres en majuscules indiquent une différence significative entre les individus témoins et traités pour un même milieu (P < 0,05). (m ± SEM ; n = 15 répétitions).

L'application topique de l'azadirachtine affecte le développement de *D. melanogaster* avec une réduction significative du nombre d'individu pour chaque stade (larvaire, pupal et adulte). Plusieurs malformations ont été retrouvées ainsi qu'un sex-ratio biaisé en faveur des mâles. Ces résultats ont été noté uniquement chez la génération parentale (exposée).

3.3 Analyse de la survie des adultes

L'évaluation des effets d'une unique exposition larvaire au stade L3 sur la longévité des adultes a été réalisé et ce sur deux générations successives, jusqu'à la restauration de cet effet.

Un suivi de la survie des adultes durant les 15 premiers jours de la vie imaginale révèle une réduction significative du nombre d'adultes, survivants au traitement larvaire, chez la génération P (Mâles : test de Kaplan-Meier, $\chi^2 = 184$, df = 2, $P < 0{,}001$; Femelles : test de Kaplan-Meier, $\chi^2 = 214$, df = 2, $P < 0{,}001$). Une mortalité plus faible a été enregistrée pour la génération F1 (non exposée) (Mâles : test de Kaplan-Meier, $\chi^2 = 39{,}1$, df = 2, $P < 0{,}001$; Femelles : test de Kaplan-Meier, $\chi^2 = 63{,}1$, df = 2, $P < 0{,}001$). La mortalité enregistrée est dépendante de la dose administrée et les femelles semblent plus sensibles au traitement.

Aucune mortalité n'a été enregistrée chez les témoins et ce pour les deux générations testées. L'azadirachtine à la dose la plus faible (DL_{25}) réduit la survie des adultes à 49% pour les mâles et 36% pour les femelles de la génération P contre 94% pour les mâles et 84% pour les femelles de la génération F1. La dose la plus élevée (DL_{50}) induit des effets plus marqués sur la survie des adultes avec des taux de 27% pour les mâles et 16% pour les femelles de la génération P et 81% pour les mâles et 64% pour les femelles de la génération F1. Une survie de 100 % a été constatée pour les mâles et les femelles de la génération F2 (Fig. 24).

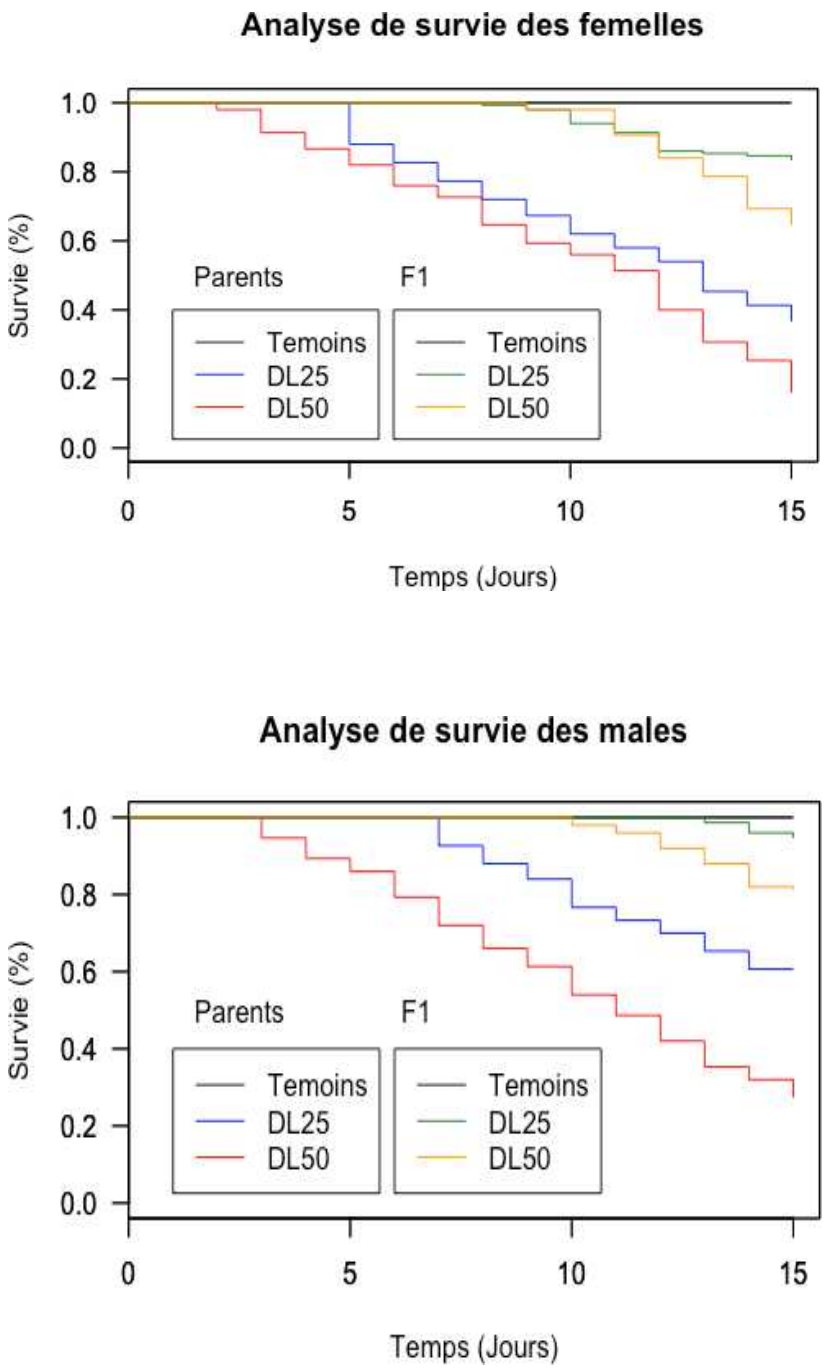

Figure 24. *Effet d'une exposition à l'azadirachtine sur la survie des adultes (P et F1).*

L'exposition pré-imaginale à l'azadirachtine, affecte la survie des adultes avec une mortalité plus marquée chez les femelles que les mâles chez la génération parentale (exposée). La mortalité est moins maquée chez la génération F1 (exposée). l'azadirachtine ne semble pas affecté la survie de la génération F2.

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

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Abstract

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

1. Introduction

The effect of insecticides and other toxicants on insects have been traditionally assessed using measures of the acute mortality as a single endpoint and have relied on the determination of the acute lethal dose/concentration[1]. However, in addition to the direct effect on lethality these compounds may also impair various key biological traits of the individuals that survive exposure through physiological and behavioral effects[1,2]. Among physiological effects, developmental success, morphological deformities, adult longevity, sex ratio, fertility and

fecundity are commonly estimated[3,4]. Behavioral effects may be manifested as impairment in insect mobility, learning ability, host finding, sexual communication as well as feeding and oviposition behavior[5–10]. An accurate assessment of these effects is crucial to acquire knowledge on the overall insecticide efficacy for long-term management of pest insect populations, as well as on their selectivity toward non-target species[11]. Indeed, when studying susceptibility of organisms towards insecticides, and beside the short term influences on the directly exposed individuals, it is important to take into account the entire life-history as a comprehensive method for evaluating the total effect on insect population, including the impacts on the next generation which have important implications for the success of an Integrated Pest Management (IPM) program[2,4] .

Today and among the insecticides used in sublethal effect studies, the botanical insecticides have been the subject of an increasing number of academic research as a potential option for an environment friendly pest management tools[12,13] due to their rapid degradation in environment, low mammalian toxicity, low risk of resistance development in target pest populations and good selectivity to non-target arthropods[14–18]. Azadirachtin (AZA), a natural tetranortriterpenoid compound extracted from the neem tree, *Azadirachta indica*[19], is considered as one of the most promising plant compounds for pest control in organic agriculture[14,20]. AZA shows variable effects on insects including the model insect *Drosophila melanogaster*[21,22]. This triterpenoid acts as sterilant, insect growth regulators by disruption of the endocrine system, repellent, oviposition and feeding deterrent by activating bitter sensitive gustatory cells[23,24]. Larval exposure of *D. melanogaster* to sublethal doses of azadirachtin was found to affects various aspects of their physiology including digestive enzymes[25] and this effect is also further observed in the adults[10]. This pre-imaginal exposure affects not only the physiology and the fitness of flies but also adults oviposition and feeding preference[7,10].

Most studies concerning the sublethal effects of insecticides are related to continuously or repeated exposure. This exposure provokes a generalized stress and activating a detoxification response such as up-regulated of cytochrome P450 genes which my lead to the detoxification of insecticide and even the development of resistance[26]. Moreover, the up-regulation is thought to provide versatility in environmental adaptation[27]. In botanical insecticide the potential fast desensitization to a feeding deterrent was reported[28,29]. Individual insects initially deterred by feeding inhibitor become increasingly tolerant due to repeated or continuous exposure[29]. Bomford and Isman[15] reported an habitation to pure azadirachtin in the tobacco cutworms which become less sensitive to the antifeedant properties of azadirachtin, but not to a neem containing a same absolute amount of azadirachtin. This might have an important implication to avoid desensitization to commercial neem-based insecticides which contains additional non AZA-compounds[15]. Larval exposure to Neem Azal, a commercial Azadirachtin-rich based formulation, was found to enhance avoidances of this compound in adults of *D. melanogaster* surviving from previously treated larvae[10,25]. This long-lasting avoidance is related to conditioned aversion and may be related to another mechanism such as sensitization[30,31] which also generally occurs after long term or repeated exposure and may increase avoidance to noxious stimulus[32]. Moreover, increasing evidence has highlighted the critical role of early life experience in adult physiology and behavior in insect[33]. Recent studies have revealed that insect can modulate their behavior on the basis of previous experiences early life and that various insecticide-mediated changes in the directly exposed generation can persist into the subsequent non-exposed generations[34,35]. Previously, we focused on the impact of larval exposure to azadirachtin on adult's fitness (fecundity, survival) and oviposition site preference of the parental generation of *D. melanogaster* as model organism for testing insecticide activity[7]. Current study aimed to evaluated, the possible adverse effects of this prior single exposure to azadirachtin experienced by the preceding generations on life table and oviposition

site preference of the filial generations. We monitored the oviposition site preference, fecundity, development, sex ratio, survival and morphological abnormalities of exposed and non-exposed generations. All these parameters were investigated over generations until their restoration to predict the outcome of azadirachtin use on pest management practices.

Results

Fecundity and oviposition site preference

Azadirachtin, topically applied on the 3rd instar larvae (LD_{25} and LD_{50} of immature stages) affected fecundity of females by a significant reduction of the number of eggs laid as compared to controls (KW= 24.73; $p < 0.001$). This reduction was observed over two successive generations (parental and F1), however, the total eggs laid was higher in the unexposed generation (F1) than in parental (P) ones (KW= 50.89; $p < 0.001$) (Fig.1). Full restoration of affected fecundity was noted in the second generations (F2).

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

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

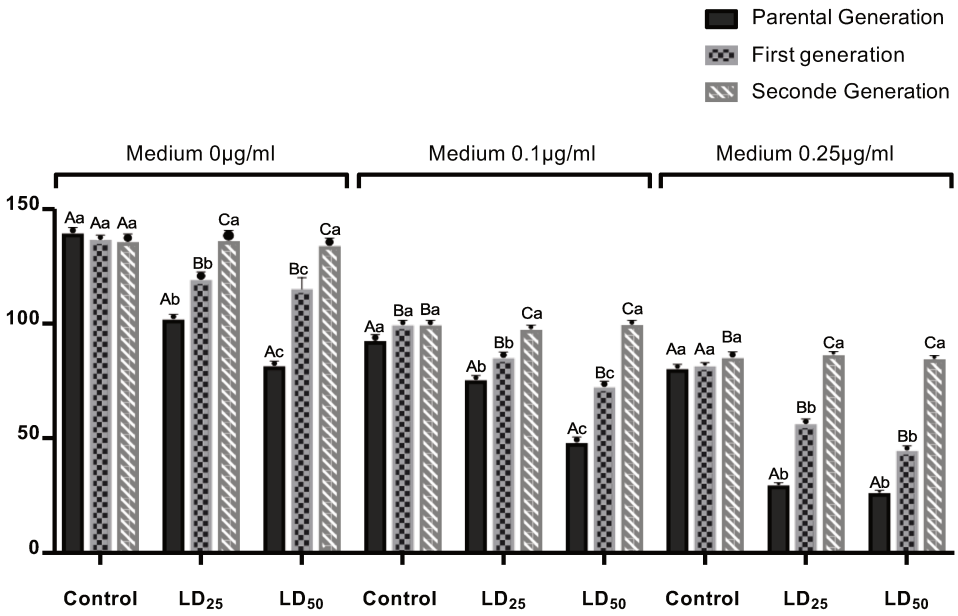

Figure 1. Effect of azadirachtin (LD_{25} and LD_{50}), topically applied on early third instars larvae of *D. melanogaster* on fecundity of females (number of eggs laid) subjected to non-choice experiments (m ± SE; n =12 replicates of 3 flies). Different small letters indicate a significant difference between control and treated individuals of the same medium ($P<0.05$). Different capital letters indicate a significant difference between generations of the same medium ($P<0.05$).

Results concerning the dual choice experiments (Fig. 2) revealed an oviposition preference for control medium than treated medium in all tested generations (P, F1 and F2). Furthermore, flies previously exposed to azadirachtin (early 3rd instar larvae) showed a highest aversion to this substance compared to naïve flies and led fewer eggs for the two first generations (P and F1) with a more marked effects for parental generation ($P <0.001$).

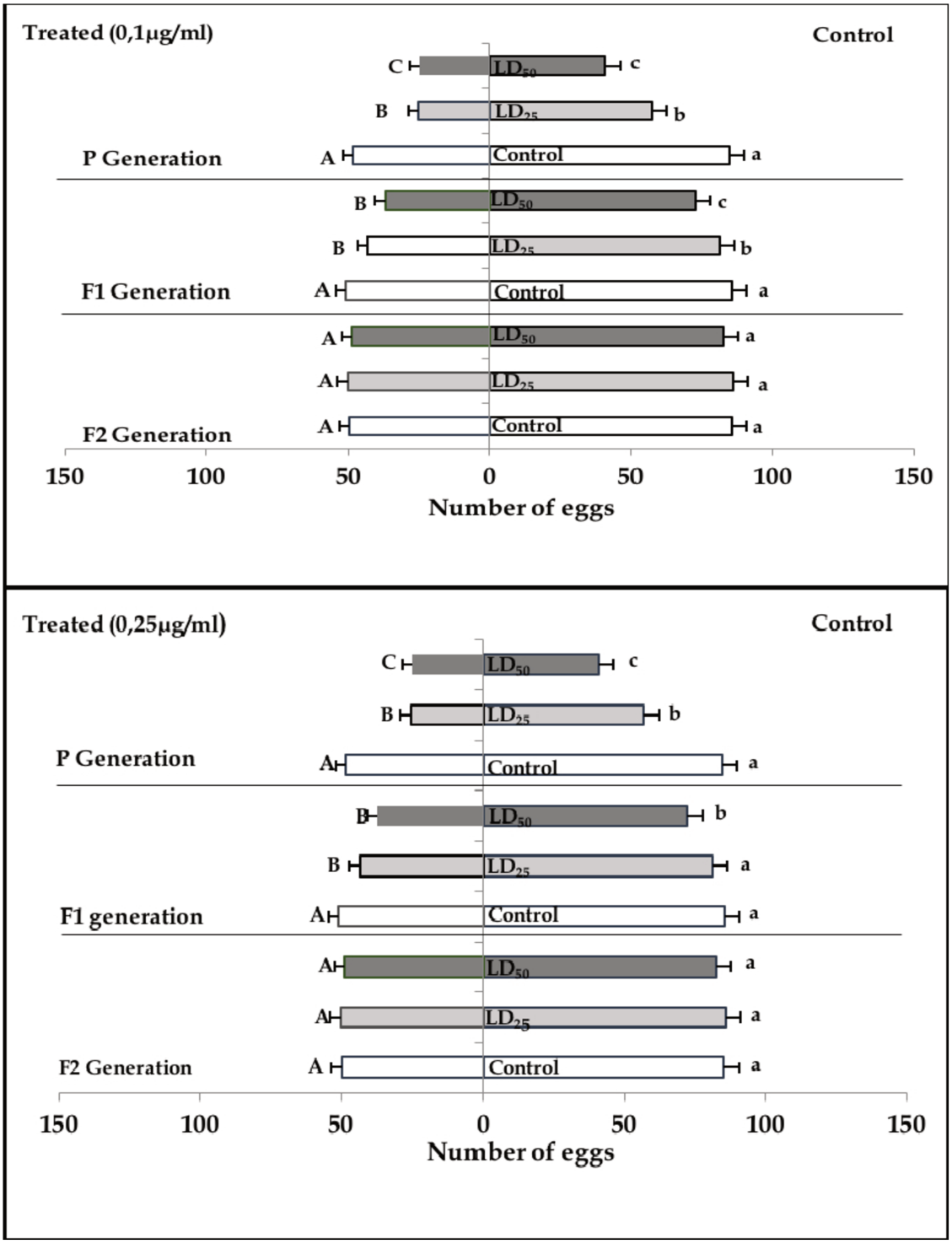

Figure 2. Egg-laying preference (m ± SE; n=12 replicates) of female adults of *D. melanogaster* subjected to a free-choice test on food treated with azadirachtin at two doses (0.1µg/ml and 0, 25µg/ml). Different small letters indicate a significant difference between control and treated individuals of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

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

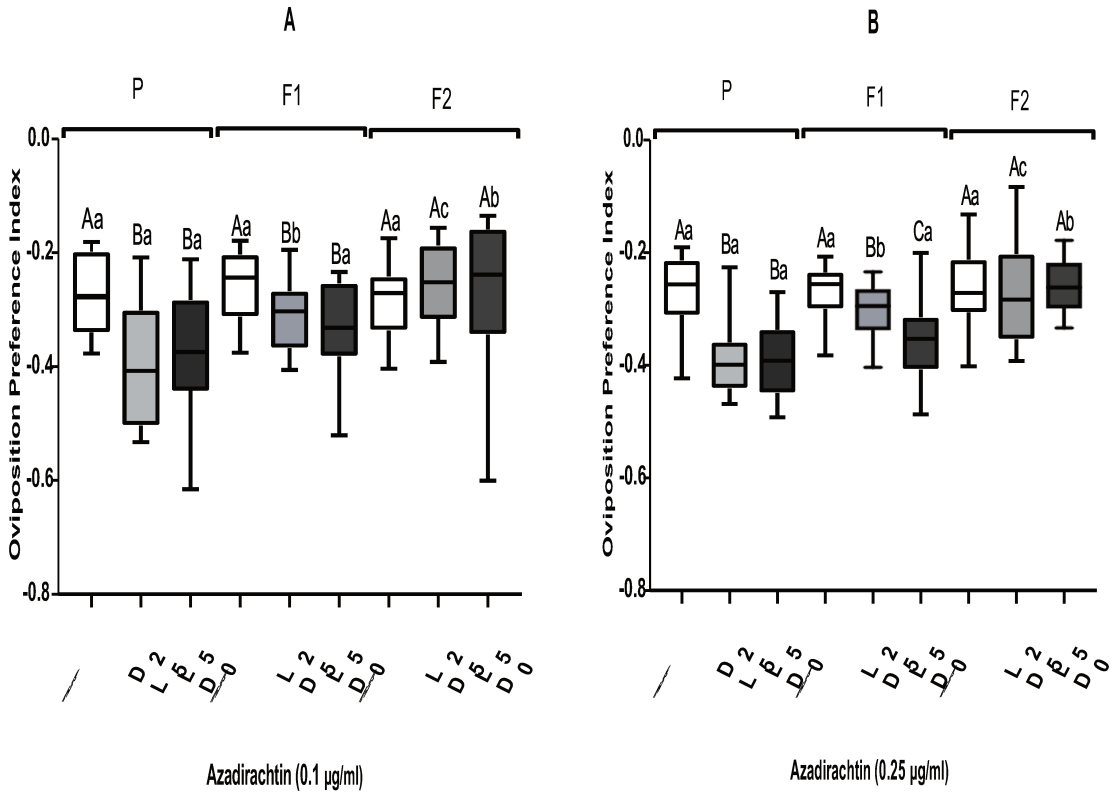

Figure 3. Oviposition preference index (m ± SE; n=12 replicates) of female adults of *D. melanogaster* subjected to a free-choice test on food treated with azadirachtin at two doses (A: 0, 1 µg/ml; B: 0, 25µg/ml). Different small letters indicate a significant difference between the same dose of different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

In the generation P, statistical analysis showed significant differences between OPI of previously treated flies and controls flies with a dose-dependent response (fig. 3). In addition, for medium 0.1 µg/ml, Mann-Whitney revealed significant effects between LD_{25} of the parental generation and the first generation (Mann-Whitney test $U = 8$; $P < 0.001$), LD_{25} of parental and

second generation (Mann-Whitney test U=20; P=0.0018) but there was no difference between the first and the second generations (Mann-Whitney test U=42; P=0.0887). Similar results were observed for the LD_{50}, with significant effects observed between the parental generation and the F1 (Mann-Whitney test U=19; P=0.0014), also between P and F2 (Mann-Whitney test U=34; P=0.0284) but no difference between F1 and F2 (Mann-Whitney test U=58; P=0.4428). For control, there was no difference between all tested generations.

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

Analyses of development

Results from development analysis of *D. melanogaster* are given in tables 1 and 2, respectively for parental (exposed) and F1 (non-exposed) generation.

Table1. Effect of larval exposure to azadirachtin on development of parental generation (exposed) of *D. melanogaster*. Letters indicate a significant difference between the different tested doses of the same medium for each stage of development (P<0.05). Different capital letters indicate a significant difference same doses tested of different medium (P<0.05). (m ± 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 A c	7.6±1.21 B c	66.93±0.81 B b	167.06±0.91 A c	3.73±0.72 b A	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 b C	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 c B	53.13±1.82 B b	24.13±1.76 B b	-26.94

Table2. Effect of larval exposure to azadirachtin on development of first generation (non-exposed) of *D. melanogaster*. Letters indicate a significant difference between the different tested doses of the same medium for each stage of development (P<0.05). Different capital letters indicate a significant difference same doses tested of different medium (P<0.05). (m ± 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
DL_{25}	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
DL_{50}	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
DL_{25}	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
DL_{50}	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
DL_{25}	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
DL_{50}	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

Treatment of early third instar larvae at two tested doses (LD_{25} and LD_{50}) decreased the number of larvae, pupae and the final number of organisms of parental generation with a dose-dependent relationship as expressed by the FNO which is always negative for the treated series. The development of F_1 *D. melanogaster* doesn't seem to be affecting by the early treatment of the parental generation. However, the FNO of tested flies (LD_{25}, LD_{50} and control) in treated medium was significantly lower than in the control medium for both generations. There is no difference between the number of organisms reached the pupae stage and the final number of organism in both generations. In addition, treatment of early third instar larvae increased significantly ($p<0.001$) the duration of larval and pupal development as expressed by T_{50}, with dose-dependent manner only for the Parental generation (exposed) as compared to controls. There is no difference between the T_{50} of the tested flies in both treated and untreated medium. Larvae, pupae and imagoes of the parental generation showed several types of malformations and anomalies followed by death at each stage of development of *D. melanogaster.* The most prominent malformations detected are incomplete and malformed imagoes (malformed abdomen and wings), curved and smaller body shape, burned larvae, dead adults inside pupae (Fig.4).

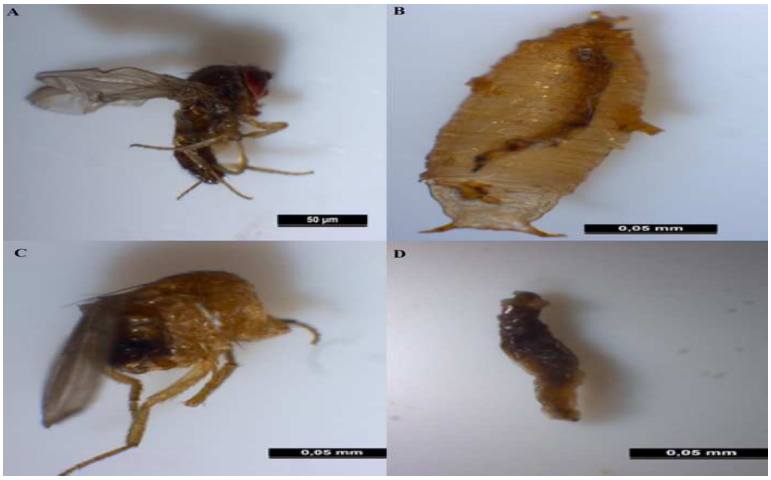

Figure 4. Examples of the most frequent malformations of *D. melanogaster* (n=50). A) Malformed abdomen and wings curved and smaller body shape; B) dead adults inside pupae; C) malformed adult; D) burned larvae.

Pre-imaginal exposure of azadirachtin induced a male-biased sex ratio only for the parental generation with a dose-dependent relationship (Fig.5).

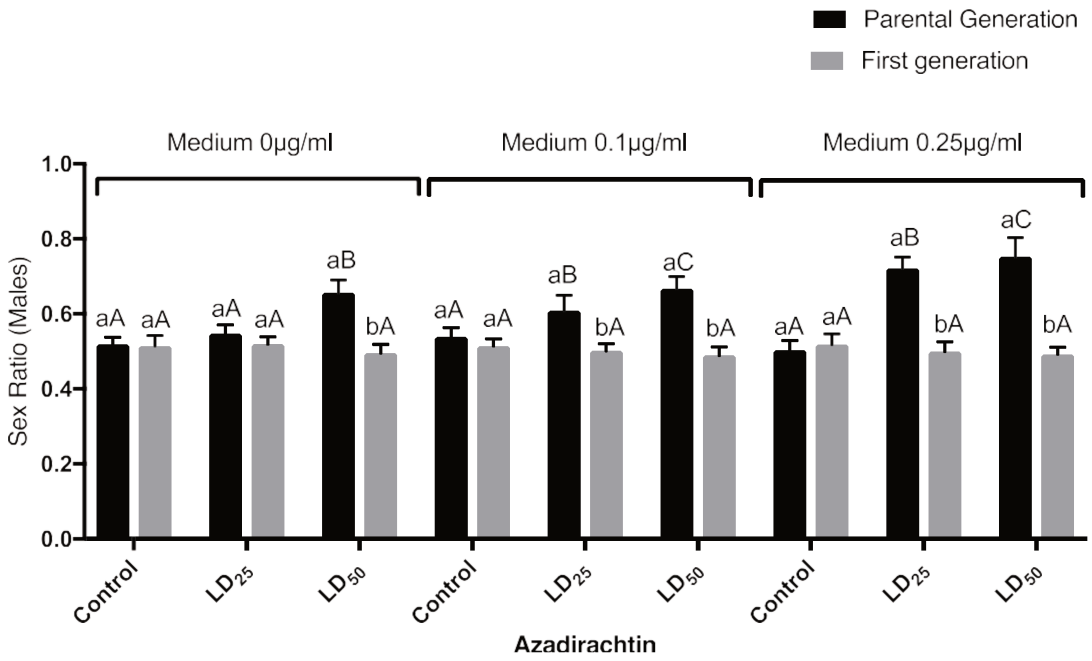

Figure 5. Effect of azadirachtin (LD_{25} and LD_{50}), topically applied on early third instars larvae of *D. melanogaster* on sex ratio of adults emerged. Different small letters indicate a significant difference between generations of the same medium ($P<0.05$). Capital letters indicate a significant difference between control and treated individuals of the same medium ($P<0.05$). (m ± SE; n =15 replicates).

Kruskal-Wallis test revealed significant effects between the different tested insect (Control, LD_{25} and LD_{50}) in untreated medium (KW = 9.30; p =0.0095), medium 0.1 µg/ml (KW = 8.02; p < 0.0181) and medium 0.25 µg/ml (KW= 18.85; p < 0.0001) for the parental generation.

Survival analysis of adults

A survival analyses during the 15 first days of adults previously treated with azadirachtin as 3rd instars larvae (Fig.6) revealed a rapid reduction of adult surviving of the generation P (Male: Kaplan-Meier test, $\chi2 = 184$, df = 2, P < 0.001; Female: Kaplan-Meier test, $\chi2 = 214$, df = 2, P < 0.001). Lower mortality was noted for the generation F1 compared to parental. (Male: Kaplan-Meier test, $\chi2 = 39.1$, df = 2, P < 0.001; Female: Kaplan-Meier test, $\chi2 = 63.1$, df = 2,

P < 0,001). Flies mortality was dose-dependent and the females were more affected by the treatment.

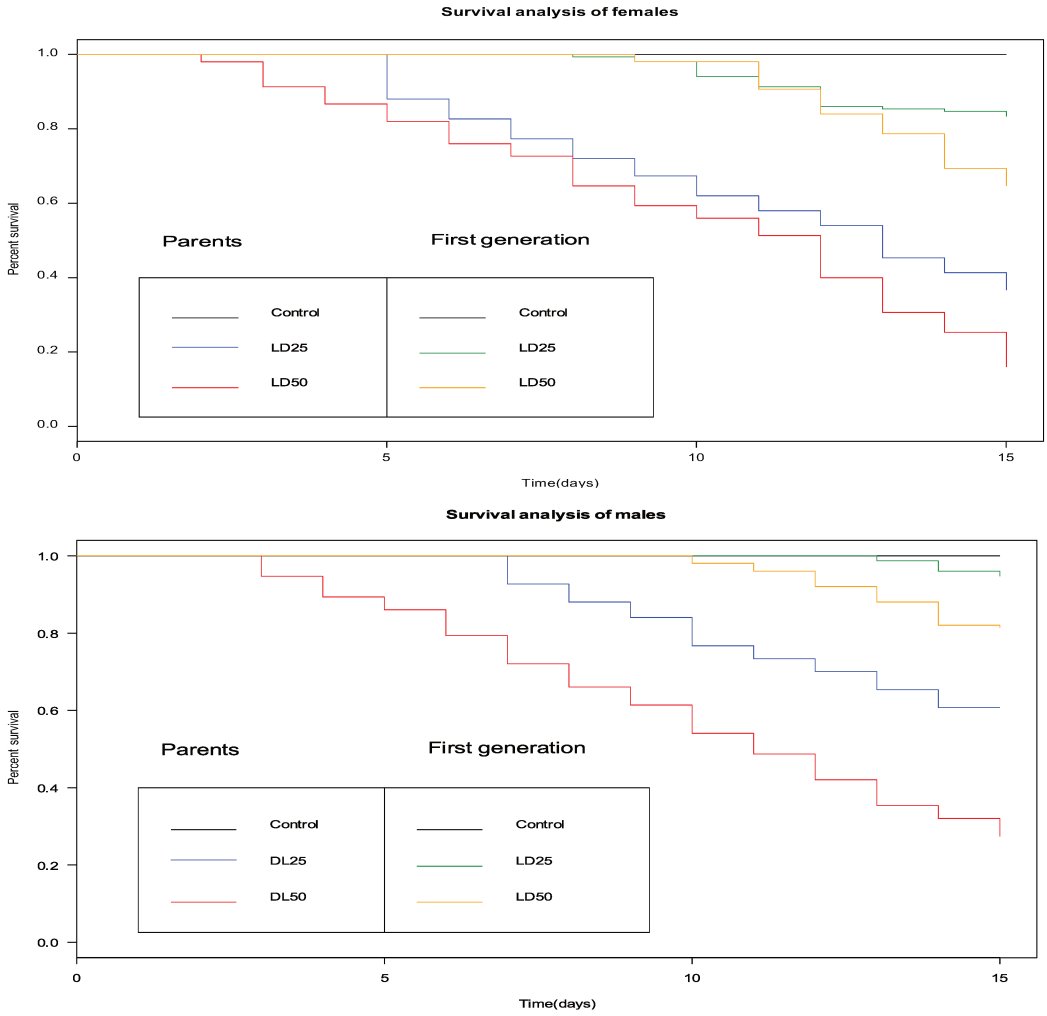

Figure 6. Effect of azadirachtin (LD_{25} and LD_{50}), topically applied on early third-instar larvae of *D. melanogaster* on the adult's survival (male and female) of two generations tested ($p<0.05$).

For the control series no mortality was recorded for both tested generations. For the treated series, the lowest dose (LD_{25}) decline the adult's survival to 49% for males and 36% for females of the P generation versus 94% for males and 84% for females of the F1 generation. The highest dose (LD_{50}) induced more marked effects on adult's survival with 27% for males and 16% for females of the P generation and 81% for males and 64% in females for the F1 generation. Survival of 100% was noted for males and females of the F2 generation.

Discussion

Azadirachtin impact on reproduction have been reported on different insect species[21,41–45]. Our study has demonstrated that a single azadirachtin treatment (LD_{25}/LD_{50}) of *D. melanogaster* larvae reduced eggs number affecting negatively the fecundity of surviving females, not only through direct sublethal effects in exposed individuals, but also through transgenerational effects on F1individuals that were never directly exposed to the insecticide.

Oviposition is a complex and critical activity in the life cycle of an insect with a variety of factors that influence both physiology and subsequent behavior, that lead to egg deposition by an insect which tries to ensure safety to their progeny. Reduced fecundity and fertility after azadirachtin treatment has been reported in many insects including *Spodoptera littoralis*, *D. melanogaster, Galleria mellonella, Dysdercus cingulatus, Tuta absoluta* and *Helicoverpa armigera*[17,41,43–46] and may be correlated to the negative action of azadirachtin on yolk protein synthesis and/or its uptake into oocytes[21].

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

In *Anopheles stephensi*, azadirachtin treatment led to abnormal ovaries structure with a complete arrest of oogenesis, vitellogenesis and vitelline envelope formation impairment and follicle cells degeneration [48]. Ovaries of azadirachtin-treated females of *Heteracris littoralis* also showed complete shrinkage with suppression of oocyte growth[49], disintegration and destruction in follicular cells and mitochondria[49]. In addition, Azadirachtin reduce mating success in *D. melanogaster* flies and negatively affect cyst and oocyte numbers and size [45].

Azadirachtin treatment also affect the amount of food intake in this species and digestive enzyme activity in the midgut[10], which may affect oogenesis and vitellogenesis since ecdysone and JH rates are affected by nutrient availability which acts as positive regulator on insulin pathway to confer to ovaries the signalling necessary for a normal oogenesis[50,51].

In addition, flies of all tested generations preferred control medium for oviposition avoiding the azadirachtin ones for the two tested doses and conditions (no-choice and free choice). A low oviposition rate of non-exposed (naïve) flies in azadirachtin-treated areas may be due to the known repellent effect, deterrent effect and locomotor stimulation effect of azadirachtin and other neem based insecticides which were reported by Silva *et al.*[52] in medflies *Ceratitis capitata*. Valencia-Botín et al.[53] also suggest that the repellent property of neem extracts is the major factor responsible for the reduction of eggs numbers of *Anastrepha ludens* (Loew)[53]. The ovipository behavior inhibition may have a valuable impact in pest control.

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

Here, we have reported for the first time that the negative effects of a single larval exposure to azadirachtin can also be passed on to the F1 generation (transgenerational effects). Environmental toxicants such as insecticide are able to provoke epigenetic alterations which

can be inherited to next generations[59]. This may explain the reduced fecundity and oviposition avoidance in our non-exposed generation (F1).

Our study has also demonstrated that azadirachtin applied during the third larval instar of Parental generation (LD_{25} and LD_{50}) can negatively affect various life traits of *D. melanogaster*, with a dose-dependent manner, by significantly reducing, larval, pupation and emergence rate of the exposed generation. The biopesticide also significantly prolong the larval and pupation period of development inducing an important delays in immature stages development and affect sex ratio (with fewer females in the offspring) of the same generation. Additionally, the treatment induced morphological alterations of larvae, pupae and adults only on the exposed generation (P generation). The most prominent abnormalities are burned larvae, larva-pupa intermediate, pupa-adult intermediate, deformed wings, smaller body size and deformed abdomen. The recorded malformations finally result to insect dead. Similar results were noted in *D. melanogaster*[37], *Hyalomma anatolicum excavatum*[60] and *Spodoptera litura*[22]. Finally, a reducing of adult's survival was noted for the two successive generations with more marked effects among the P generation.

Azadirachtin is known to reduce pupation and eclosion rates of many insects like *Aphis glycines*[61], *Plodia interpunctella*[62], *Aedes aegypti*[63] and *D. melanogaster*[21]. A negative impact of azadirachtin on the immature stages was expected in view of its insect's growth disruptor (IGD) action by suppressing haemolymph ecdysteroid and JH peaks[25,64]. Furthermore, azadirachtin is known to cause nucleus degeneration in the different endocrine glands (prothoracic gland, *corpus allatum* and *corpus cardiacum*) controlling insects moulting and ecdysis which may act as generalised disruption of neuroendocrine system[24]. Azadirachtin by altering the growth and molting process of several insects compromise their survival[7,20,43,65,66]. Lai *et al.*[67] reported that azadirachtin down regulated expression of different genes linked to

hormonal regulation which may explain the developmental aberrations observed in our results. Azadirachtin also affect *Drosophila* nutrient intake and metabolism compromising the nutritional signals which result in a decrease in insect weights and growth rates resulting in smaller body size and impacting survival[10,25,37,66]. The male biased sex ratio under azadirachtin treatment was also reported in literature[67,68].

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

Results demonstrate that a single azadirachtin application can significantly reduce the survival of flies over two successive generations (P: exposed and F1: unexposed) while insects showed clear recovery in the survival rates in the second generation (F2). These findings reflect a long term and delayed effects through developmental stage and generations. This consistent effect over the two first generations may be considered as advantage on pest control by compensate the well known fast degradation by sunlight and low persistence of azadirachtin in environment (half-life DT_{50}: 1.7- 25d)[23, 69] and suggest a possible reduce both in application frequency and total amount of pesticide used.

Moreover, the decreased fecundity and survival in P and F1 generations indicated an absence of induction of the resurgence in offspring, even after full restoration in F2, when parental flies were treated translating an absence of hermetic effect, which is considered as serious problem of exposure to sublethal doses in agriculture.

In addition, the treatment increases the aversive effect induced by azadirachtin over two successive generations which may contribute as push-pull strategies increasing its insecticidal effects in integrated pest management programs.

Material and methods

Flies

Wild-type Canton-S strain of *D. melanogaster* flies were reared on artificial fly food (cornmeal/agar/yeast) at 25°C, 70% humidity and 12D-12 L cycle[10].

Treatment

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

Fecundity and oviposition site preference

We assessed the egg-laying performances of the females of *D. melanogaster* using a no-choice test. Three mated females (3 days old) that were pre-exposed to azadirachtin at the larval stage (LD_{25} and LD_{50}) were tested for 24 h in a petri dish (Ø=65mm) filled with 3 ml medium containing azadirachtin at two concentrations 0.1 and 0.25 µg/ml according to Bezzar-Bendjazia et al.[37]in addition to acetone as control medium**.** These concentrations were not lethal with the short exposure time (24 h) used. At the end of the test, flies were removed, and the number of eggs laid on each medium was counted. The control medium was used to test the possible effect of azadirachtin on female fecundity. The experiment was repeated 12 times for each medium and each generation. Oviposition site preference was measured by means of dual choice experiments. Three fertilized females (3 days old) from controls and treated series (LD_{25} and LD_{50}) were allowed to oviposit for 24h in a free choice egg-laying device. This device

consisted of a two petri dishes either filled with control medium (acetone) or treated medium (azadirachtin: 0.1, 0.25 µg/ml). After 24h, the egg-laying preference was assessed by counting the number of eggs laid in each medium. The test was performed for two successive generations with 12 replicates for each medium and generation.

Oviposition preference index (OPI) defined as (number of eggs on azadirachtin medium – number of eggs on control medium)/total number of eggs was calculated[38].

Development assays

Ten controls or pre-exposed (LD_{25} and LD_{50}) mated females (3 days old), named parental generation, were released into an oviposition box containing petri dishes filled with control (acetone) or treated medium (azadirachtin: 0.1 or 0.25 µg/ml) and left to lay eggs for 8 hours. At the end of the test, the flies were removed and a pool of 100 eggs for each experiment was transferred to a new petri dish containing the same medium. For all groups, we monitored the time course of larval development from egg to adult emergence by counting the number of third instar larvae, pupae, imagoes and their sex ratio, expressed as the number of males divided by the total number of emerged insects.

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

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

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

$$FnO = \frac{\mathrm{T} - \mathrm{C}}{\mathrm{C}} \times 100$$

T = final number of organisms counted in treated medium.

C = final number of organisms counted in control medium.

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

Survival analysis of adults

Survival analysis was performed according to Linford et al.[40]. For each generation (P: exposed (LD_{25} and LD_{50}), F1: non-exposed) newly emerged adults were sexed and housed separately into a plastic vials (15 flies per vial) containing fresh food. Insects were transferred to new vial every 2 days. The flies were kept under observation for 15 days during which mortality was assessed every 24h. Ten replicates were done for each dose and generation.

Statistical analyses

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

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

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3.4 Effets sur l'activité locomotrice des adultes

Le comportement locomoteur des adultes mâles et femelles de *D. melanogaster* a été évalué en utilisant un test de géotaxie négative. Les résultats, montrent que l'azadirachtine (DL_{25}, DL_{50}), administré par application topique chez les larves L3 du début de stade, affecte l'activité locomotrice des adultes et ce comparativement aux témoins avec un effet dose et sexe dépendant, uniquement pour la génération parentale (Fig.25).

L'activité locomotrice enregistrée chez les mouches témoins de la génération parentale à 5 minutes est de 98% et 96% respectivement pour les mâles et les femelles. L'activité locomotrice diminue ensuite avec le temps pour atteindre des valeurs moyennes de 54% pour les mâles et 62% pour les femelles à 25 min.

En ce qui concerne les mouches exposées préalablement à l'azadirachtine (DL_{25} et DL_{50}), les résultats montrent pour les individus traités à la DL_{25} que l'activité locomotrice enregistrée à 5 min est de 72 % pour les mâles et 54 % pour les femelles. Une diminution en fonction du temps est notée pour atteindre à 25 min, des valeurs moyennes de 44% pour les mâles et 38% pour les femelles.

En ce qui concerne les individus traités à la DL_{50}, l'activité locomotrice des mouches à 5 minutes est de 38% pour les mâles et 28% pour les femelles. Une diminution est enregistrée avec le temps pour atteindre, 8% et 6%, respectivement pour les mâles et les femelles à 25 min.

Chez les mâles, l'azadirachtine réduit de manière significative le pourcentage de mouches capables d'atteindre le sommet du tube ($KW = 9,96$; $P = 0,001$) et ce sans effet dose (Fig. 25 A), (test de Dunn $P > 0,05$). Des résultats similaires sont observés pour les femelles avec une réduction significative du pourcentage de mouches capables d'atteindre le sommet du tube chez les mouches traitées (DL_{25} et DL_{50}), toujours sans effet dose ($P > 0,05$) (Fig. 26 B). ($KW = 12,57$; $P < 0,001$).

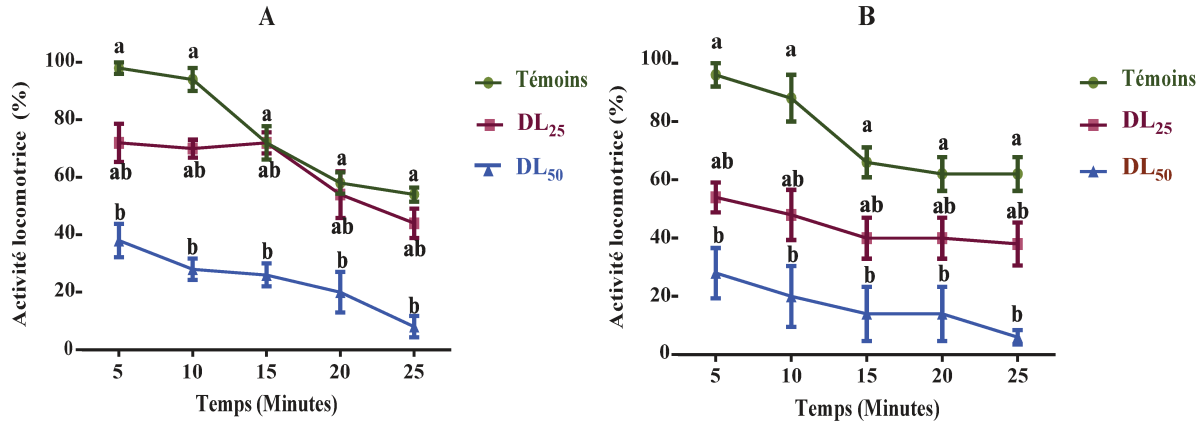

Figure 25. *Effets de l'azadirachtine sur la vitesse de récupération après un choc (réactivité locomotrice) chez la génération parentale.*

A : Mâles ; B : femelles. Les différentes lettres indiquent une différence significative entre les témoins et les traités (P < 0,05) pour chaque temps.

L'activité locomotrice enregistrée chez les mouches témoins de la génération F1. (Fig. 26) à 5 min est de 90% aussi bien pour les mâles que pour les femelles. L'activité locomotrice diminue ensuite avec le temps pour atteindre des valeurs moyennes de 56% pour les mâles et 68% pour les femelles à 25 min.

Pour les individus traités à la DL_{25}, l'activité locomotrice enregistrée à 5 min est de 94% pour les mâles et 88% pour les femelles. Une diminution en fonction du temps est notée pour atteindre à 25 min des valeurs moyennes de 64% pour les mâles et 68% pour les femelles.

En ce qui concerne les individus traités à la DL_{50}, l'activité locomotrice des mouches à 5 min est de 86% pour les mâles et 80% pour les femelles. Une diminution est enregistrée avec le temps pour atteindre, 66% et 46%, respectivement pour les mâles et les femelles à 25 min.

Chez les mâles, l'azadirachtine réduit de manière significative le pourcentage de mouches capables d'atteindre le sommet du tube (KW = 0,18 ; P = 0,9236) et ce sans effet dose (Fig. 26 A), (test de Dunn P > 0,05). Des résultats similaires sont observés pour les femelles avec une réduction significative du pourcentage de mouches capables d'atteindre le sommet du tube chez les mouches traitées (DL_{25} et DL_{50}), toujours sans effet dose (P > 0,05) (Fig. 26 B) (KW = 0,98 ; P = 0,6396).

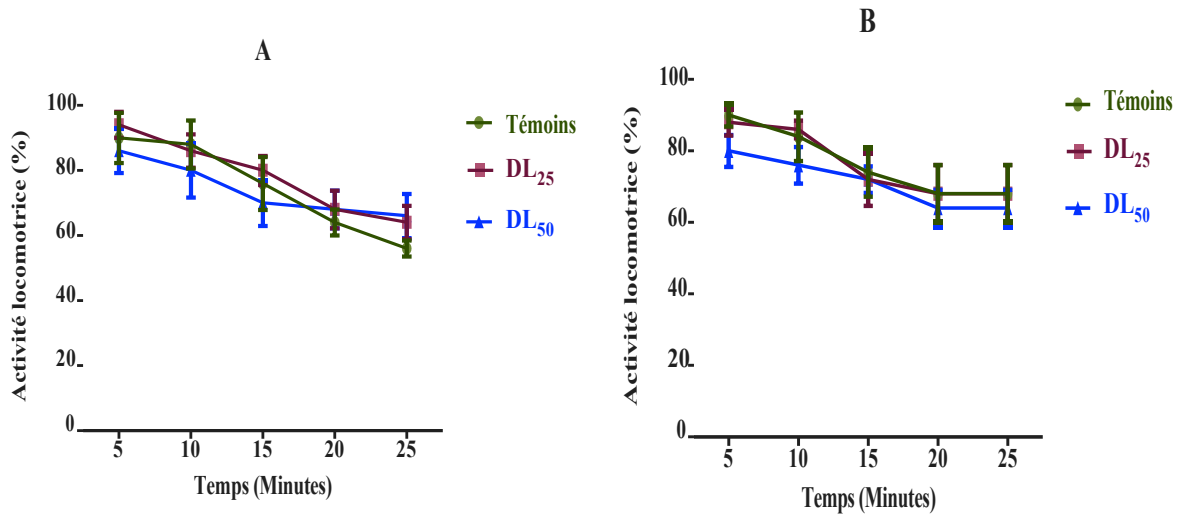

***Figure 26**. Effets de l'azadirachtine sur la vitesse de récupération après un choc (réactivité locomotrice) chez la génération F1.* A : Mâles ; B : femelles.

La comparaison de l'activité locomotrice entre les deux générations testées révèle chez les femelles, des différences hautement significatives entre les DL_{25} de la génération P et la F1 ($U = 2$; $P < 0{,}001$), ainsi qu'entre les DL_{50} des deux générations ($U = 0$; $P < 0{,}001$). Aucune différence n'est enregistrée entre les témoins des deux générations ($U = 11{,}50$; $P = 0{,}8968$). Chez les mâles, des différences hautement significatives entre la génération P et la F1 sont enregistrées seulement entre les individus traités à la DL_{50} ($U = 0$; $P < 0{,}001$).

L'exposition pré-imaginale à l'azadirachtine du stade L3 de *D. melanogaster* réduit significativement la réactivité locomotrice chez les adultes mâles et femelles de la génération parentale (exposée). Aucun effet n'est enregistré chez les générations non-exposées

3.5 Effets sur l'orientation à distance vers un substrat traité à l'azadirachtine

Le choix olfactif des adultes mâles et femelles de *D. melanogaster* a été évalué en utilisant un olfactomètre en Y. L'azadirachtine (DL_{25}, DL_{50}), administré par application topique chez les larves L3 au début de stade, affecte la perception olfactive des adultes et ce comparativement aux témoins. Chez la génération parentale, les résultats révèlent, chez les mouches préalablement traitées au stade L3, une aversion plus marqué à l'azadirachtine, Aucun effet n'est enregistré chez les mouches de la génération F1.

Les valeurs de l'indice d'olfaction (IO), permettant de comparer les réponses obtenues pour chaque condition (adultes exposés ou non à l'azadirachtine au début du stade larvaire L3) sont toujours négatives pour les insectes traités de la génération parentale, et positives pour les insectes témoins de cette même génération (Fig. 27). Chez les mâles, les moyennes des valeurs de l'IO obtenues sont de 0,08, -0,16 et -0,21 ; chez les femelles elles sont de 0,15, -0,22 et -0,23 et ce respectivement pour les adultes témoins, DL_{25} et DL_{50}.

À l'opposé, les IO obtenus pour la génération F1 sont toujours positifs aussi bien pour les insectes témoins que traités. Les moyennes des valeurs de l'IO obtenues pour les mâles sont de 0,09, 0,10 et 0,20 et pour les femelles de 0,09, 0,13, 0,25, respectivement pour les témoins, DL_{25} et DL_{50}.

L'analyse statistique révèle des différences significatives entre l'IO des mouches qui ont survécu au traitement préalable au stade larvaire et des mouches témoins (Fig. 27). Chez les mâles, le test de Kruskal-Wallis révèle des différences significatives entre les différents insectes testés (Témoins, DL_{25} et DL_{50}) pour la génération P ($KW = 24,02$; $P < 0,001$) ainsi que pour la génération F1 ($KW = 7,92$; $P < 0,0190$). Des résultats similaires sont obtenus pour les femelles des générations P ($KW = 23,66$; $P < 0,001$) et F1 ($KW = 16,03$; $P < 0,001$).

La comparaison entre les deux générations testées a permis de mettre en évidence des différences significatives, chez les mâles, entre la DL_{25} de la génération P et la F1 ($U = 0,50$; $P < 0,001$), la DL_{50} de la génération P et la F1 ($U = 0$; $P < 0,001$); aucune différence n'est enregistrée entre les témoins des deux générations ($U = 67$; $P= 0,77$). Des résultats similaires sont enregistrés chez les femelles traitées à la DL_{25} ($U = 0$; $P< 0,001$) et à la DL_{50} ($U = 0$; $P < 0,001$) avec des effets hautement significatifs entre les deux générations (P et F1). Chez les témoins, aucune différence n'est enregistrée entre les deux générations ($U = 4,50$; $P > 0,05$).

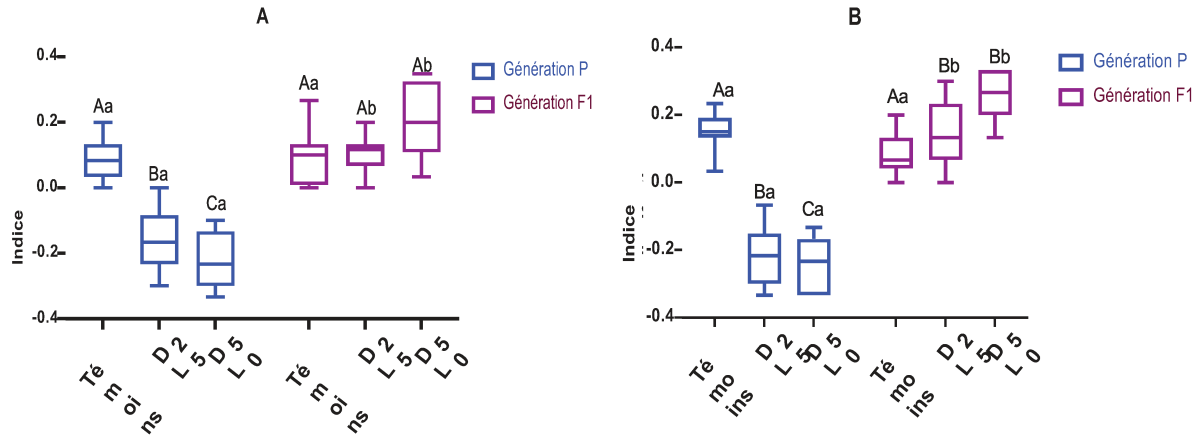

***Figure 27**. Réponses des adultes (génération P et F1) à l'odeur de Neem Azal en olfactomètre.*

Mâles : A et femelles : B (m ± SEM ; n=12 répétitions). Les différentes lettres en minuscules indiquent une différence significative entre une même dose chez différentes générations (P < 0,05). Les différentes lettres en majuscules indiquent une différence significative entre les différentes doses testées chez une même génération (P < 0,05).

Le traitement pré-imaginale à l'azadirachtine induit une aversion marqué envers cette molécule des adultes de la génération P. Aucun effet n'a été noté pour la génération F1.

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Preimaginal exposure to azadirachtin affects food selection and digestive enzymes in adults of *Drosophila melanogaster* (Diptera : Drosophilidae)

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Abstract

Among the plant derived product, azadirachtin, a neem-based insecticide, is exceptional in having a broad range of bioactivity including toxicity, growth, development and reproduction effects, repellency and antifeedancy. If considerable progress on the physiological and biological activities and agricultural application of azadirachtin has been achieved, its exact mechanism of action remains uncertain. In this study, we aimed at assessing the lethal and sublethal behavioral and physiological 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. Results showed that flies preferentially ingested control medium rather than azadirachtin-treated medium. Pre-imaginal exposure (L3) to azadirachtin increased aversion to this substance suggesting a memorability of the learned avoidance. In addition, all tested flies revealed a clear preference for solvent odour rather than azadirachtin odour. Moreover, azadirachtin treatment decreased significantly the amount of food intake in the adults of both sexes. Finally, azadirachtin was found to affect digestive enzyme activities in the midgut of flies. Indeed, an inhibition of α-amylase, chitinase, and protease activities and an increase of lipasic activity were noted. These results may reflect interference of azadirachtin with regulation of feeding and metabolism, and provide some evidence of a long term antifeedancy and delayed effects through developmental stage which may reinforce the insecticidal activity of this bioinsecticide.

Keywords: Azadirachtin, *Drosophila melanogaster*, Feeding behavior, olfactory response, Food intake, Digestion.

1. Introduction

Plants with pesticidal properties have been investigated for decades as alternatives to synthetics for developing sustainable agricultural practices and promote human and environmental safety [1, 2]. The prominent botanical pesticides that have received the best of attention are those issued from the neem tree, *Azadirachta indica* A. Juss. (Meliaceae) [3]. Azadirachtin, a tetranortriterpenoid, is the most effective compound produced as a secondary metabolite of the neem tree [1]. This compound acts as repellent, oviposition and feeding deterrence, sterilant and as insect growth regulator (IGR) by interfering with both juvenile hormone and ecdysone titers, the two major hormones responsible for insect growth, development and reproduction [1, 4, 5].

Due to its short persistence and low mammalian toxicity, azadirachtin has been tested for various pests and is currently exploited in agriculture for crop protection against different phytophagous insects [6, 4, 7]. The compound has also been reported as safer for non-target organisms; however, this earlier perception has been recently challenged specially in relation with natural enemies and pollinators [8, 9, 10, 11]. Despite these problems neem derived insecticides still valuable components in integrated pest management and its use is recommended for insecticide resistance control and organic production pest control [12, 13].

The impairment of insect development, defined as reduced pupation, malformation and failure of adult emergence remain the most frequently reported effects of azadirachtin [14, 15, 16, 17]. However, its behavioral effects still more controversial being somewhat dependent upon the insect species, time and/or method of application, and concentration used [14, 18, 7, 17].

Insect use olfaction system to search and locate potential food [19] and thereafter contacting chemoreception, called primary antifeedancy, could confirm its quality and provide a basis for food selection and discrimination [20, 21].

The antifeedant effect of azadirachtin was reported on large number of pest insects, including hemipterans [22], lepidopterans [23, 24], ortheopterans [25] and coleopterans [26]. A secondary antifeedant of azadirachtin consisting on a disruption in the physiology of the insect and its ability to digest food was also reported [27, 28, 24].

In insects such as the fruit fly *Drosophila melanogaster*, the ability to discriminate chemicals by taste and avoid toxins in food is critical to the survival and reproduction, and accordingly gustatory information were used to assess the suitability of potential food sources and calorie sensing to make critical decisions of feeding choices that maximizes fitness [29, 30]. Previously, azadirachtin, applied topically to third instars larvae, was reported to affect fitness and eggs laying preference in adults of *D. melanogaster* suggesting an avoidance learning memory which may reinforce its insecticidal activity [17]. Here, we investigated the influence of previous experience on adults food preference and assessed whether larval exposure can affect food consumption and digestive enzymes of adults of *D. melanogaster*.

2. Material and methods

2.1. Flies

The wild-type *D. melanogaster* Meigen flies (Diptera: Drosophilidae) were raised on standard fly medium made of cornmeal, yeast and agar at 25 °C and 70% relative humidity under a 12-h light:12-h dark cycle.

2.2. Insecticide and treatment

A commercial azadirachtin-bases insecticide formulation was used for the experiments (Neem Azal-TS, 1 % EC; Trifolio-M GmbH, Lahnau, Germany) and dissolved in acetone for topical application (1 µl per insect) [17]. Azadirachtin was applied on early third-instar larvae of *D. melanogaster* at two doses, 0.28 µg and 0.67 µg corresponding respectively to LD_{25} and LD_{50}

(doses that caused respectively a mortality of 25% and 50% of immature stages) [17]. Controls were exposed only to the solvent (1µl acetone) and all flies were kept under the same conditions as cites above.

2.2 Two-choice food preference test

The food preference of adultes males and females three-day-old were tested in binary choice using a 2-dye marker method blue-red [31] in 96-well microplates (Fig.1A). The blue food-dye was FD&C Blue Dye no.1, used at a concentration of 0.5 mg/ml. The red food-dye was acid red, used at a concentration of 2 mg/ml. These food-dyes, at the above concentrations, were ascertained to be neither toxic nor were they metabolized [31]. The wells in the micro test plate were alternately filled with 10 µl azadirachtin treated food (0.25 µg/ml) marked with blue or red food-dye or control food containing the alternate food dye.

In each experiment, flies of both sexes (10 males and 10 females, tested in single sex groups) emerged from previously treated early 3rd instar larvae (LD_{25} and LD_{50}) were starved for 24 h and placed in plate. After being left to feed for 1 h at 25 C° in the dark, the flies were conserved in -10 C° during 24 h. A control series was done in parallel. Flies were classified into four groups according to abdominal colouring, i.e. blue (B), red (R), mixed (M) and uncoloured (Fig.1). The fraction of the uncoloured flies was usually below 5%. Five replicates were used for each test. A preference index (PI) was calculated, according to the formula:

$$PI = [(N^b + N^m/2) / (N^b + N^m + N^r)] * 100$$

Paired control plates were prepared similarly, using standard food containing the alternate food dye, and used to test the possible effect of dyes on flies preference.

2.3. Olfactory response

Groups of adults three day-old of both sexes surviving from previously treated larvae (LD_{25} and LD_{50}) were tested in Y-maze assay according to Simonnet et al. [32] with slight modifications. The flies were starved for 17 h at 25 °C in glass tubes containing wet paper towel before testing. Before connecting the "trap" vials, a filter paper (~ 5mm) was placed in each vial. 50 µl of azadirachtin (0.25 µg/ml) were deposited on one filter paper, and 50 µl of acetone on the second filter paper. Flies were tested individually, to avoid group effects, and placed into the third vial. Forty replicates were used for each sex and each dose. Percentage of attraction was calculated of each group.

2.4. Quantification of food intake

Quantification of food intake was performed as previously described by Wong et al. [33]. For each experiment five 3-day-old adults males or females surviving from previously treated larvae (LD_{25} and LD_{50}) were provided fresh food medium containing 2.5% (w/v) blue food dye (FD &C Blue no 1). After 30 minutes of feeding, flies were decapitated, to prevent eye pigment from interfering with the absorbance spectrum of the dye. Only the flies with blue abdomen were selected.

Flies were homogenised in 200 µl of distilled water. A further 800 µl of distilled water was added and the suspension cleared by centrifugation at 13,000 g for 15 min. The absorbance of the supernatant was measured at 629 nm. Age-matched flies exposed to non-dyed food were used as the baseline during spectrophotometry. The absolute amount of food intake was calculated from a standard curve made by serial dilution in water of a sample of blue food.

2.5. Enzymatic determination

2.5.1. Preparation of enzyme extract

3-days old adult males or females surviving from previously treated larvae (LD_{25} and LD_{50}) were used to quantify the activity of digestive enzyme. Individual's midgut were dissected out under a stereomicroscope and rinsed in ice cold saline buffer (0.15 M NaCl). A pool of ten midguts were homogenized in 1 ml of universal buffer (pH 7) and centrifuged at 13,000 g for 15 min. The supernatant was used as the enzyme source. Six replicates were used for each dose. The protein concentrations in each sample were determined, according to Bradford [34], and used to calculate the specific activity.

2.5.2. Assay of α- amylase activity

α-amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure [35], using 1% soluble starch as substrate. 20 µl of the enzyme were incubated for 30 min at 35°C with 100 µl universal buffer (pH 7) and 40 µl soluble starch. The reaction was stopped by adding 100 µl DNS and heating in boiling water for 10 min. After cooling at room temperature, 100 µl of distilled water were added prior to reading the absorbance at 540 nm. One unit of *α*-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35°C.

2.5.3. Assay of lipase activity

The activity of lipase was determined by the method of Tsujita et al. [36]. Ten µl of the enzyme was mixed with 18 µl p-nitrophenyl butyrate (50 mM) as a substrate, and 172 µl of universal buffer solution (pH 7). After incubation at 37°C for 30 min, the absorbance was read at 405 nm. One unit of lipase activity was defined as the amount of enzyme necessary to liberate 1 µmol of *p*-nitrophenyl per minute under assay conditions.

2.5.4. Assay of protease activity

Protease activity was measured determined using casein as the substrate [37]. The reaction mixture consisting of 200 µl of casein (casein dissolved in PBS to obtain a solution at 1%), 100 µl enzymes and 100 µl universal buffer (pH 7) was incubated at 37°C for 60 min. Proteolysis was stopped by the addition of 800 µl of 5% trichloroacetic acid (TCA). The mixture was centrifuged at 8000 g for 15 min and the absorbance was recorded at 280 nm. The activity was calculated from a standard curve using tyrosine with a known concentration.

2.5.5. Assay of chitinase

- *Preparation of colloidal chitin*

Colloidal chitin was prepared according to Shen et al. [38]. Fore grams of chitin powder (Crab shells, Sigma) was added slowly to 40 ml of concentrated HCl and kept at 4° C for 1 h under vigorous stirring. The mixture was added to 1 L of ice-cold distilled water with rapid stirring and kept overnight at 35° C. After filtration, the substrate was washed several times with sterile distilled water until the colloidal chitin became neutral (pH 7). One gram of the colloidal chitin (1% w/v) was dissolved in citrate phosphate buffer (0.05 M, pH 6.6).

- *Enzyme assay*

Chitinase activity was determined with colloidal chitin as the substrate according to Miller [39]. 250 µl of enzyme solution was added to 250 µl of 1% colloidal chitin, after incubation for 30 min at 50°C with continuous shaking (120 rpm), the mixture was heated in a boiling-water bath for 5 min, cooled at room temperature and then centrifuged at 5000 g for 5 min. The reducing sugar released was measured by the DNS method at 540 nm using N-acetyl-D-glucosamine (GlcNAc) as standard. One unit of chitinase activity was defined as the amount of enzymes that produces 1 µmol of reducing sugar per hour under reaction conditions.

2.6. Statistical analyses

All statistical analyses were performed using Prism v 6.01 for Windows (GraphPad Software Inc., www.graphpad.com). Results are given as means ± standard errors (SE). The homogeneity of variances was checked using Bartlett's and Brown-Forsythe tests. Data on food preference index was assessed using Mann-Whitney test. Olfactory response was analyzed using the Chi-square (χ^2) test. Data of Quantification of food intake and activity of digestive enzymes were subjected to one-way analysis of variance (ANOVA) followed by a post-hoc HSD Tukey test.

3. Results

3.1. Two-choice food preference test

The sensitivity of adults of *D. melanogaster,* surviving from previously treated larvae (LD_{25} and LD_{50}), to azadirachtin was tested using 2-dye marker to discriminate the preferential ingestion of flies. Results show that flies preferentially ingested control medium rather than azadirachtin treated medium ($P<0.01$). The marked food dyes also affected the choice of flies. Indeed, flies preferentially ingested the medium marked with the blue food-dye (53.76%) than the medium marked with the red food-dye (39%).

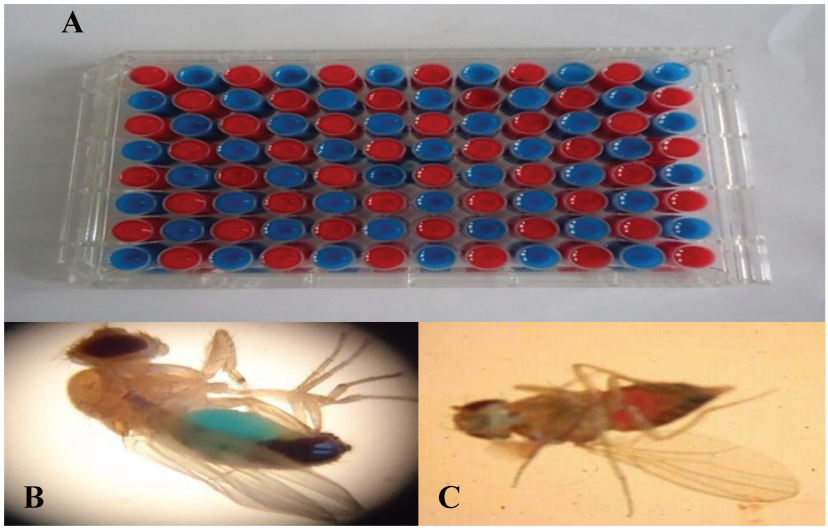

Figure.1. Feeding preference test: (**A**) 96-well test plate filled with food marked with blue or red food-dye. ***(B)*** Abdomen colored in blue. (**C**) Abdomen colored in red.

The preference index (PI) of adult males and females of *D. melanogaster* (Controls, LD_{25} and LD_{50}) are shown in Fig. 2.

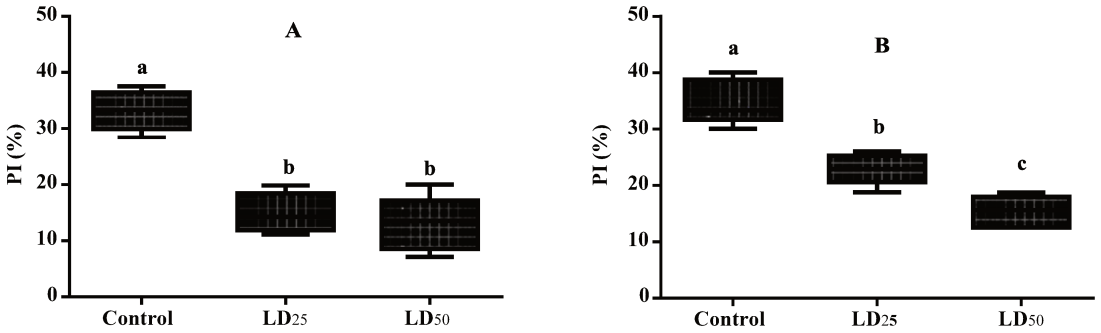

Figure.2. Preference index (PI) of adults of *D.melanogaster* subjected to two-choice test on azadirachtine-treated food (0.25 µg/µl) marked with the blue food-dye and control medium marked with red food-dye. **A**: Male, **B**: Female. Different small letters indicate a significant difference between control and treated individuals.

For males, the means values of PI recorded in azadirachtin-treated medium (0.25µg/µl) marked with the blue food-dye were 33.21%, 15.00% and 13.00% respectively for controls, LD_{25} and LD_{50}. For females, the PI was 34.83%, 22.91% and 22.31% respectively for controls, LD_{25} and LD_{50}. Furthermore, the values of PI recorded in azadirachtin-treated medium (0.25µg/µl)

marked with the red food-dye were always null for both sexes from controls and treated (LD_{25} and LD_{50}) series. Mann-Whitney test showed significant differences ($P<0.01$) between PI of previously treated flies and controls flies and no significant effects between the two tested doses ($P>0.05$) (Fig.2).

3.2. Effect on olfactory responses

Effects of azadirachtin on fly olfactory response were evaluated using Y-maze. The behavioural test showed a clear preference of fly to enter trap vial containing the solvent rather than azadirachtin. There was no difference between sexes and so data were pooled. Proportion of flies choosing the solvent was 90% for controls, 83.75% for LD_{25} and 77.75% for LD_{50}. Statistic analysis revealed no significant difference between the tested groups ($P = 0.1$).

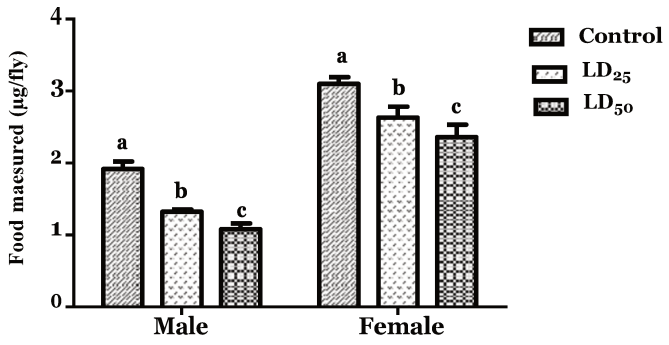

Figure.3. Effect of Azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on feeding rates (µg/fly) of adults male and female, (m ± SE; n = 5 replicates of 5 insects). Different small letters indicate a significant difference between control and treated series ($P<0.05$).

3.2. Effect of azadirachtin on food intake

Amounts of ingested food in controls and treated series (DL_{25} and DL_{50}) were quantified using spectrophotometry and shown in Fig. 3. In control series the amount of ingested food was 1.91 ± 0.10 µg/fly for males and 3.10 ± 0.095 µg/fly for females. Azadirachtin treatments of early third instars larvae caused a significant reduction of food intake with a dose dependent relationship (Fig.3). Indeed, the mean values recorded in treated series were 1.32 ± 0.029 µg/fly

for males and 2.36 ± 1.15 µg/fly for females at the lowest dose (LD_{25}) and 1.08 ± 0.083 µg/fly for males and 2.36 ± 0.017 µg/fly for females at the highest dose (LD_{50}). ANOVA indicated significant effects of doses (P<0.001), sex (P<0.001) but no significant (P>0.05) in doses-sex interaction.

3.3. Effect of azadirachtin on digestive enzyme

3.3.1. Effect on *α*- amylase activity

Results showed that azadirachtin treatment of early third instars larvae of *D. melanogaster* affected *α*- amylase activity of adult of both sex as compared to controls (Fig.4).

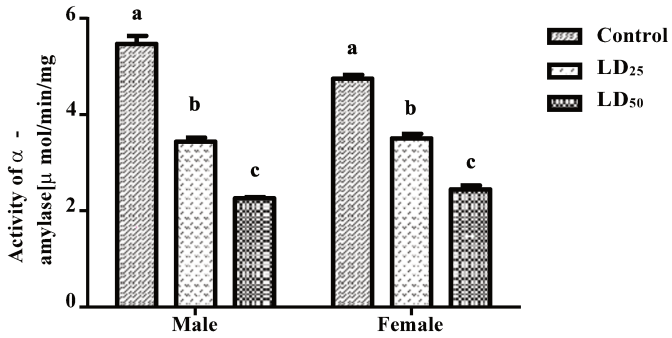

Figure.4. Effect of Azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of *α*-amylase of adults male and female, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series (P<0.05).

In males the mean values recorded were 5.47 ± 0.16 µmol/min/mg proteins for the controls, 3.43 ± 0.08 µmol/min/mg proteins for the LD_{25} and 2.26 ± 0.02 µmol/min/mg proteins for the LD_{50}. Similar results was observed for female with mean values of 4.47 ± 0.08 µmol/min/mg proteins for the controls, 3.51 ± 0.09 µmol/min/mg proteins for the LD_{25} and 2.44 ± 0.08 µmol/min/mg proteins for the LD_{50}. ANOVA indicated significant effects of doses (P<0.001), and doses-sex interaction (P<0.001) but no significant of sex (P>0.05).

3.3.2. Effect on lipase activity

The activity of lipase increased in adult's flies surviving from previously treated early third instars larvae. The highest activities were observed in females treated with LD_{50} (Fig.5).

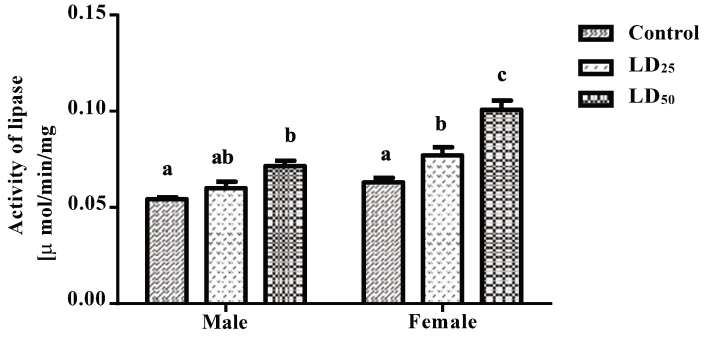

Figure.5. Effect of Azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of lipase of adults male and female, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series ($P<0.05$).

The mean values recorded in the males were 0.054 ± 0.001 µmol/min/mg proteins for the control, 0.060 ± 0.003 µmol/min/mg proteins for the LD_{25} and 0.071 ± 0.003 µmol/min/mg proteins for the LD_{50}. In the female the mean values were 0.063 ± 0.002 µmol/min/mg proteins for the control, 0.077 ± 0.004 µmol/min/mg proteins for the LD_{25} and up to 0.101 ± 0.005 µmol/min/mg proteins for the LD_{50} (Fig.6). ANOVA indicated significant effects of doses ($P<0.001$), sex ($P<0.001$) and doses-sex interaction ($P = 0.016$).

3.3.3. Effect on protease activity

Results showed that azadirachtin treatment of early 3rd instar larva (DL_{25} and DL_{50}) affected protease activity of adults of both sexes by a significant reduction compared to controls ($P<0.001$) (Fig.6).

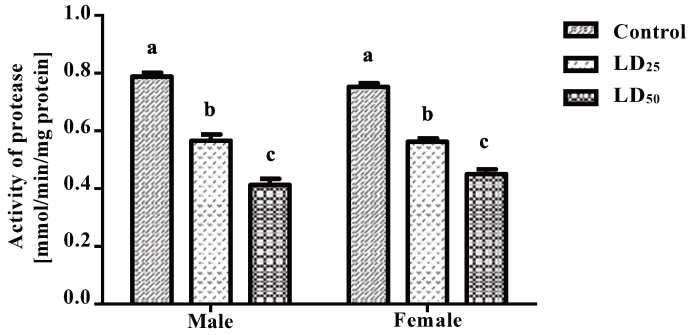

Figure.6. Effect of Azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of protease of adults male and female, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series (P<0.05).

In control series the mean values recorded were 0.78 ± 0.014 mmol/min/mg proteins for males and 0.75 ± 0.012 mmol/min/mg proteins for females. The activity of protease for the lowest dose (LD_{25}) was 0.56 ± 0.022 mmol/min/mg proteins for males and 0.56 ± 0.011 mmol/min/mg proteins for females. At the highest dose (LD_{50}) the value recorded dropped to 0.41 ± 0.021 mmol/min/mg proteins for males and 0.45 ± 0.017 mmol/min/mg proteins for females (Fig.7). ANOVA indicated significant effects of doses (P<0.001), but no significant effects of sex (P>0.05) and doses-sex interaction (P>0.05).

3.3.4. Effect on chitinase activity

As a consequence of larval treatment, the chitinolytic activity of adult of both sex of *D. melanogaster* decreased significantly as compared to controls (P<0.001) (Fig.7).

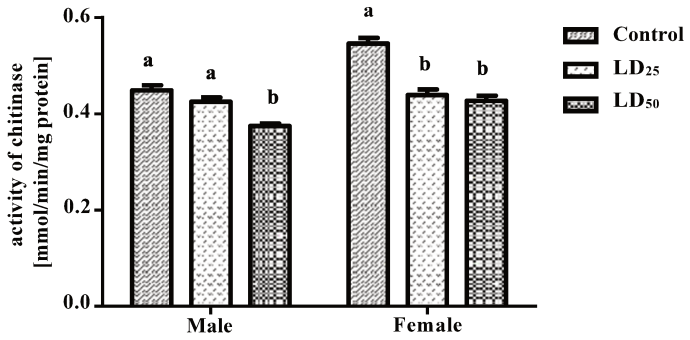

Figure.7. Effect of Azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of chitinase of adults male and female, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series (P<0.05).

ANOVA revealed significant effects of dose (P<0.001), sex (P<0.001) and dose-sex interaction (P<0.001). The mean values for controls series were 0.44 ± 0.01 mmol/min/mg proteins and 0.54 ± 0.012 mmol/min/mg proteins respectively for males and females. In treated series the mean values recorded were 0.42 ± 0.009 mmol/min/mg proteins for the LD_{25} and 0.37 ± 0.005 mmol/min/mg proteins for the LD_{50} for males and 0.43 ± 0.012 mmol/min/mg proteins for the LD_{25} and 0.42 ± 0.010 mmol/min/mg proteins for the LD_{50} for females (Fig.7).

4. Discussion

In this study, we evaluated the effects of azadirachtin treatment of early third instar larvae on feeding behavior and digestive enzymes of adults of *D. melanogaster*. In our results, flies preferentially ingested dyed control food rather than azadirachtin-treated food. Dyes appear to affect the choice of flies, with a clear preference for blue food-dye than the red food-dye. This preference for blue food-dye was reported by Fougeron [40]. Recently, Bantel & Tessier, [41] reported the impact of these dyes concentration on food preference in *D. melanogaster*. In addition, our results revealed that the antifeeding effect of azadirachtin was more marked in previously treated flies (third instar larvae) as compared with naïf flies suggesting that previous experience plays a major role in the feeding preference of adults of *D. melanogaster*.

The effects of azadirachtin on olfactory response were also evaluated on adults of *D. melanogaster*. All flies tested in dual choice revealed a clear preference for solvent odour rather than azadirachtin odour. Pre-imaginale exposure of flies to the bioinsecticide had no significant effects on insect olfactory orientation.

In insect, feeding preingestive inhibitors affect insect orientation, searching, and food selection by interfering with olfactory or gustatory chemosensillas [42]. A signal to the Central Nervous System (CNS) causes avoidance from further approach or feeding [42]. In *Drosophila* species attractive odorants have been studied extensively, but little is known about avoidance pathways [43]. However, the conservation of olfactory avoidance was reported in *Drosophila* species [43] which may be exploited as new behavioral strategies for controlling pest's species.

Previous studies have demonstrated that azadirachtin caused a strong antifeeding effects in several insects species [44, 24, 45]. This primary antifeeding effect of azadirachtin seems to be mediated by contact chemoreceptors and linked to an inhibitory effect on the rate of firing of sugar sensitive cells of the gustatory chemoreceptors [46]. Indeed, several species have been reported to be extremely sensitive to primary antifeedancy of azadirachtin, which prefer to starving to death rather than ingesting the biopesticide [47]. In addition, *D. melanogaster* seems to be able to discriminate food texture and integrate the chemical and physical qualities of food [48] which may contribute to avoiding azadirachtin-treated food.

Different recent studies support the hypothesis that a pre-imaginal experience can change the adult response and generate learning memory through developmental stages [49, 50, 51, 17]. This learning avoidance of azadirachtin was reported in *D. melanogaster* oviposition sites selection after larval exposure (third instar larvae) to the bioinsecticide [17].

The primary antifeedancy is mediated by gustation and involved in discriminating between edible and toxic substance. However, a secondary antifeedant effect including a long-term

reduction in food intake, and direct detrimental effects on different insect tissues such as muscles, fat body and gut epithelial cells [47].

In the current experiments, azadirachtin reduced significantly food intake of flies surviving to azadirachtin-treated third insatr larvae (LD_{25}, LD_{50}) as compared to the controls. This long-term feeding reduction was reported in several insects' species and the level of food consumption differs upon species and was correlated to the concentration used [28, 24]. In addition, azadirachtin was reported to affect negatively weight gain in *Spodoptera eridania* [24], *Plodia interpunctella* [52] and adults surviving from larval exposure of *D. melanogaster* [53]. 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 [54]. Lai *et al*. [55] reported that the inhibition of growth and development caused by azadirachtin in *D. melanogaster,* was consistent with those caused by disruption of the IIS pathway.

Azadirachtin was reported to cause disruption in insect physiology and its ability to digest food [24]. Our results showed a clear disruption of digestive enzymes activities responsible of the broken down of dietary components before its absorption by the intestinal epithelium. Similar results were reported in several insects species [56, 57, 58]. In the current study, azadirachtin reduced significantly the activity of α-amylase and chitinase of adults of *D. melanogaster* surviving to azadirachtin-treated third instar larvae (LD_{25}, LD_{50}).

Midgut α-amylases are involved in starch and other carbohydrate metabolism and its activities depends on feeding diet [59]. Insects Chitinases hydrolyze the β-(1-4) bonds of the residues of N-acetyl-β-D-glucosamine and are essential for moulting process as well as digestive enzymes compartimentalization because they are involved in the turn-over of the peritrophic matrices lining the gut epithelium [60]. The reduction of α-amylase activity under azadirachtin treatment was reported in *Periplaneta Americana* [44], *Glyphodes pyloalis* [28]. Moreover, Lai *et al.* [55],

reported that azadirachtin reduced the activity of α-amylase in midgut of *D. melanogaster* by repressing expression of α-amylase gene. Azadirachtin was also reported to affect the periotophic memebrane, leading to damage of cellular surfaces of the midgut [28]. In addition, in insects the expression of chitinase gene was regulated by 20E [61], which may explain the effect observed in our results, since azadirachtin act as an antagonist of the 20E.

Lipases are responsible for hydrolysis the outer links of fat molecules. These enzymes play an important role in different physiological process such as storage and lipid mobilization, reproduction, growth and defence against pathogens [62]. In this study, lipase activity was significantly increased in azadirachtin-treated insects as compared to controls and the females showed the highest activity. This result is different from other reports where reduction in lipase is mostly reported after azadirachtin treatment; however, the observed effect was dependent on the chemical formulation and concentration of azadirachtin [28]. In our results the increase in lipase activity could be attributed to its esterase activity needed for detoxification [62]. However, the highest activities observed in female may be a result of a increasing of energy demand for reproduction [63].

Proteases have crucial role in food digestion by broken down proteins into amino acids [62]. Our results showed that azadirachtin caused a significant reduction of the protease activity in *D. melanogaster.* Low protease activity in the midgut of insects treated with azadirachtin was reported in *Periplaneta americana* [44] and *Glyphodes pyloalis* [28]. Moreover, Timmins & Reynolds [64] reported that azadirachtin reduces trypsine activity in the midgut of *Spodoptera litura.*

Our result supports the secondary antifeedancy action of azadirachtin which included a long-term reduction in food consumption and digestive efficiency. The precise effects of azadirachtin on food metabolism are not well known. However, different recent studies reported that azadirachtin interfere with activities of different enzymes such as α- amylase, lipase, α - and β

-glucosidases, proteases, lactate dehydrogenase and glutathione S-transferase [59, 57, 58, 16]. Jbilou *et al*. [65] and Almeida *et al*. [66] found that azadirachtin affect the midgut epithelial cells, resulting in inhibition of digestive enzymes, efficiency of digestion and nutrient absorption which reduce access of nutrients for biological requirements. Furthermore, this reduction in efficiency of conversion of ingested and digested food may be a result of an energy conversion from biomass production into induction enzymes involved in detoxification of azadirachtin [24].

In conclusion, preimaginal exposure to azadirachtin affects the behavioral and physiological processes that underlie food selection and digestion in *D. melanogaster.* Theses finding suggest a long term antifeedancy action which may reinforce behavioral avoidance of azadirachtin and contribute as push-pull strategies in integrated pest management programs using antifeedants as a safer alternative crop protectants.

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3.6 Effets sur la perception gustative

Afin de déterminer si l'impact de l'exposition pré-imaginale à l'azadirachtine pouvait influencer la reconnaissance gustative des adultes de *D. melanogaster*, nous avons réalisé un test d'extension du proboscis (PER).

L'azadirachtine (DL_{25}, DL_{50}), administré par application topique chez les larves L3 du début de stade, affecte la gustation des mouches en augmentant de manière significative. ($p < 0,001$) l'aversion pour cette molécule avec une relation dose-dépendante (Fig. 28).

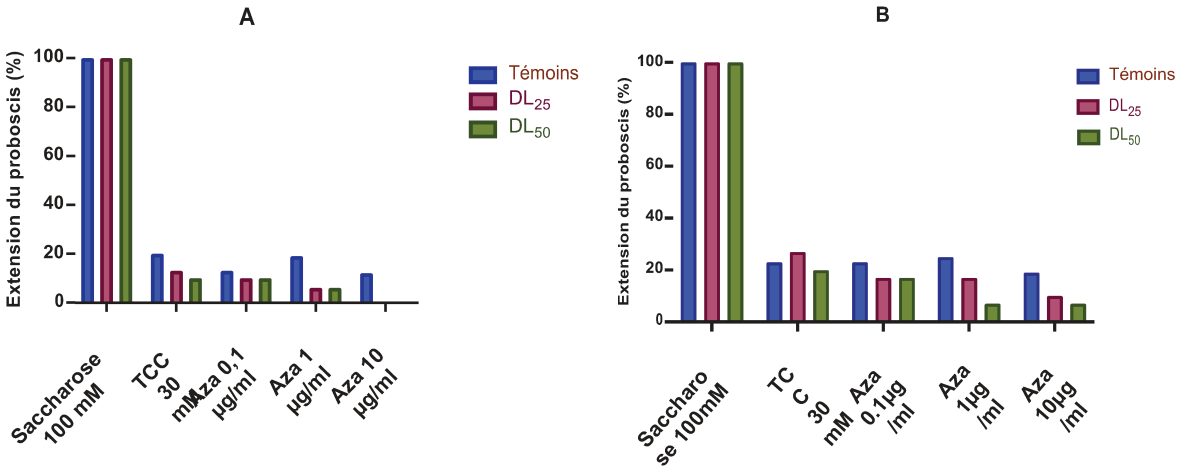

Figure 28. *Réponses d'extension du proboscis à une solution contenant de l'azadirachtine à différentes concentrations chez les adultes.*

(A : femelles ; B : mâles) témoins et traités (DL_{25} et DL_{50}) de D. melanogaster. (m ± SEM ; n = 30 répétitions).

Chez les mâles et femelles, les pourcentages d'extension du proboscis obtenus pour le saccharose à 100 mM sont de 100% pour toutes les séries de mouches testées (Témoins, DL_{25} et DL_{50}).

Les pourcentages obtenus pour les réponses à l'azadirachtine à 0,1µg/ml, sont de 20%, 13,33% et 10% respectivement pour les adultes femelles témoins, traitées à la DL_{25} et traitées à la DL_{50}. Les valeurs obtenues pour les mâles sont de 23,33%, 16,66% et 16,66% respectivement pour les adultes témoins, traitées à la DL_{25} et traitées à la DL_{50}.

Les pourcentages de l'extension du proboscis pour l'azadirachtine à 0,25 µg/ml sont de 26%, 20% et 10% pour les femelles contre 28%, 10% et 10% respectivement pour les mâles témoins, traitées à la DL_{25} et traitées à la DL_{50}.

En ce qui concerne l'azadirachtine à 1 µg/ml, les valeurs enregistrées sont de 33,33%, 6,66% et 6,66% respectivement pour les adultes femelles témoins et traitées à la DL_{25} et traitées à la DL_{50}. Les valeurs obtenues pour les mâles sont de 30%, 16,66% et 6,66% respectivement pour les adultes témoins, traitées à la DL_{25} et traitées à la DL_{50}.

Chez les femelles, l'azadirachtine à la plus forte dose (10 µg/ml) induit une extension du proboscis de 40% uniquement chez les individus témoins. Chez les mâles les valeurs enregistrées sont de 43,33%, 10% et 6,66% respectivement pour les adultes témoins, traitées à la DL_{25} et traitées à la DL_{50}

D'une façon générale, les analyses statistiques révèlent que le traitement larvaire à l'azadirachtine affecte l'extension du proboscis aussi bien chez les femelles ($\chi^2 = 83,03$; $P < 0.001$) que chez les mâles ($\chi^2 = 41,10$; $P < 0.001$) de *D. melanogaster*.

Les résultats obtenus montrent que le traitement larvaire augmente l'aversion envers l'azadirachtine avec une relation dose-dépendante de la concentration.

3.7 Effets sur les réponses électrophysiologiques des adultes

Dans un premier temps, nous avons procédé à l'identification des sensilles gustatives répondant à la formulation commerciale d'azadirachtine, le Neem-Azal, utilisée pour nos expérimentations. Pour cela, nous avons examiné systématiquement les réponses électrophysiologiques de 31 sensilles gustatives présentes sur le proboscis de *D. melanogaster* (Fig. 29) après stimulation avec une solution à 1 µg/ml (concentration donnant les meilleures réponses en PER).

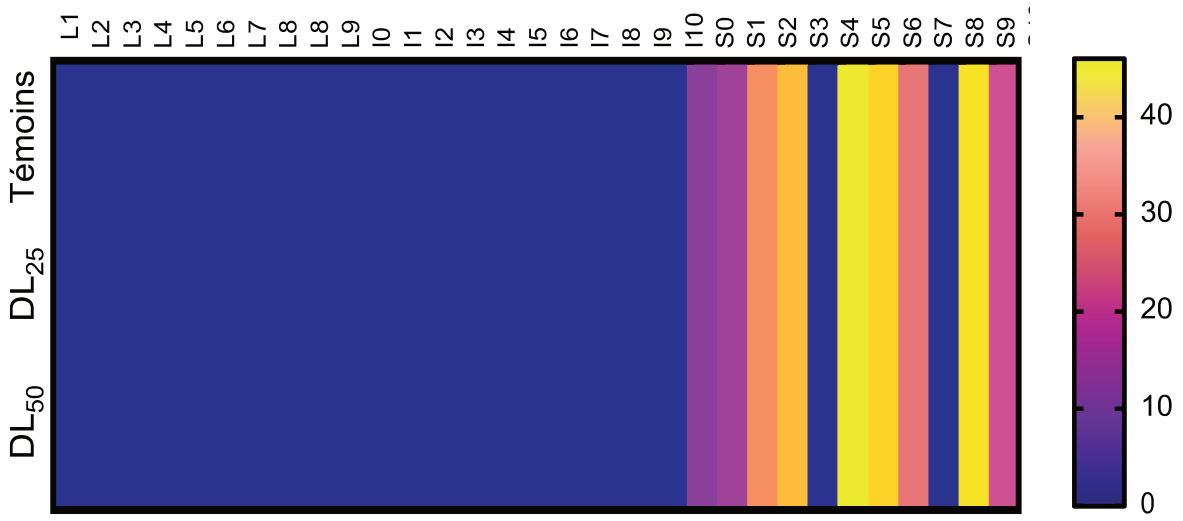

Figure 29. *Distribution des réponses électrophysiologiques au Neem Azal dans les sensilles du proboscis.*

La carte de chaleur montre les réponses électrophysiologiques, à l'azadirachtine (1 µg/ml) des sensilles gustatives du proboscis de D. melanogaster afin d'identifier les sensilles qui répondent à la molécule (n = 10).

Les sensilles longues (L) et intermédiaires (I) ne présentent aucune réponse physiologique à notre molécule. Cependant, toutes les sensilles de type (S) répondent à l'azadirachtine mais avec des différences d'intensités à l'exception des sensilles S4 et S8.

Dans un second temps, nous avons testé plusieurs concentration d'azadirachtine sur les femelles de *D. melanogaster* (Témoins, DL_{25} et DL_{50}) afin d'évaluer l'intensité de réponse de chaque mouche. Les enregistrements sont réalisés pour la sensille S5, qui répond le mieux à l'azadirachtine. Les résultats sont représentés sur la figure ci-dessous (Fig. 30).

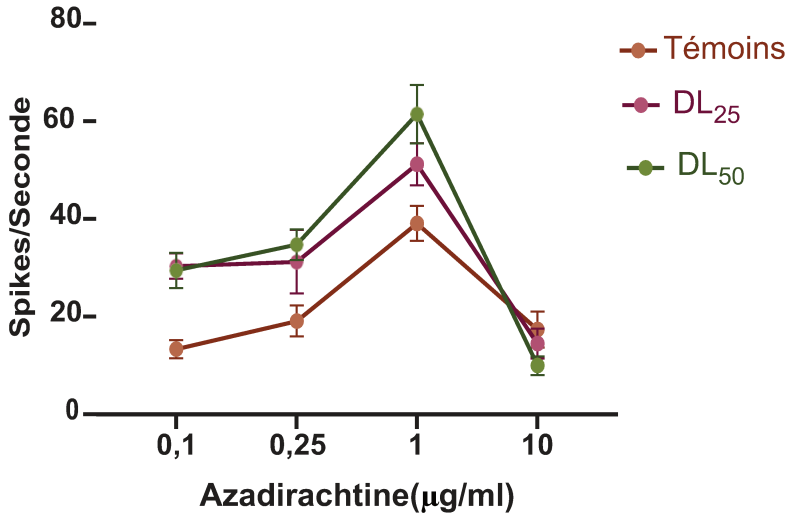

Figure 30. *Réponses des sensilles S5 au Neem Azal en fonction de la concentration et du traitement larvaire (m ± SEM ; n = 12 répétitions).*

Les courbes obtenus montrent que l'intensité de réponse des mouches est dépendante de la concentration d'azadirachtine. Une diminution drastique de l'intensité de réponse est enregistrée pour la concentration de 10 µg/ml.

Pour la concentration de 0,1 µg/ml, le test de Mann Whitney révèle des effets significatifs entre témoin et la DL_{25} ($U = 1$; $p < 0,0003$) et entre témoin et la DL_{50} ($U = 4,5$; $P = 0,0020$), aucun effet trouvé entre les adultes traités à la DL_{25} et DL_{50} ($U = 32$; $P > 0,05$). En ce qui concerne la concentration de 0,25 µg/ml, un effet significatif est enregistré uniquement pour la DL_{50} ($U = 6,5$; $P = 0,0045$).

Pour la concentration de 1 µg/ml, une différence significative trouvée entre témoin et DL_{25} ($U = 14$; $P = 0,00601$) et entre témoin et DL_{50} ($U = 6$; $P = 0,0042$). Un effet significatif a également été noté entre les deux doses testés (DL_{25} et DL_{50}) ($U = 22,6$; $P = 0,00424$).

Aucune différence trouvée entre les mouches témoins et traités (DL_{25} et DL_{50}) pour la concentration 10 µg/ml ($P > 0,05$)

Enfin, et dans le but de de vérifier si les réponses, plus marquées, obtenues chez les séries traitées étaient spécifique à l'azadirachtine, nous avons testé une autre molécule connue pour ses propriétés aversive, la caféine (CAF). Les tests sont réalisés sur la sensille (S5) qui est la sensille qui répond le mieux aussi bien à l'azadirachtine qu'à la caféine. La caféine est testée à une concentration de 10 mM, concentration à partir de laquelle on obtient une réponse chez les mouches (Weiss *et al.,* 2011) (Fig. 31).

Les résultats obtenus, pour la caféine, ne révèlent aucune différence significative entre les réponses des mouches témoins et traités à l'azadirachtine (KW = 0,28 ; P = 0,8653). Les valeurs moyennes enregistrées sont de 36,00 ± 4,14 ; 31,08 ± 2,16 et 32,67 ± 3,62 respectivement pour les témoins, DL_{25} et DL_{50}.

Les résultats pour la concentration de 1µg/ml d'azadirachtine montrent une forte réponse des mouches traitées par rapport aux témoins (KW = 11.63 ; P = 0.0030) mais sans effet dose (test de Dunn's P > 0,05). Les moyennes obtenues pour les témoins, DL_{25} et DL_{50} sont respectivement de 41,08 ± 3,94, 55,83 ± 3,02 et 61,00 ±3,31.

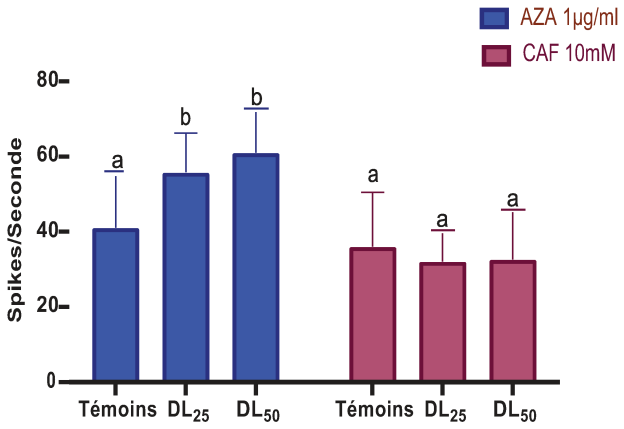

Figure 31. *Réponses des sensilles S5 à l'azadirachtine et à la caféine en fonction du traitement larvaire. (m ± SEM ; n = 12 répétitions). Les lettres différentes indiquent une différence significative entre les individus de la même substance (P < 0,05).*

La figure 32 représente un exemple des réponses d'enregistrements à l'azadirachtine pour la sensille S8 (pas de réponse) et la réponse de la sensille S5 à l'azadirachtine (1 µg/ml), TCC (30 mM) et CAF (10 mM).

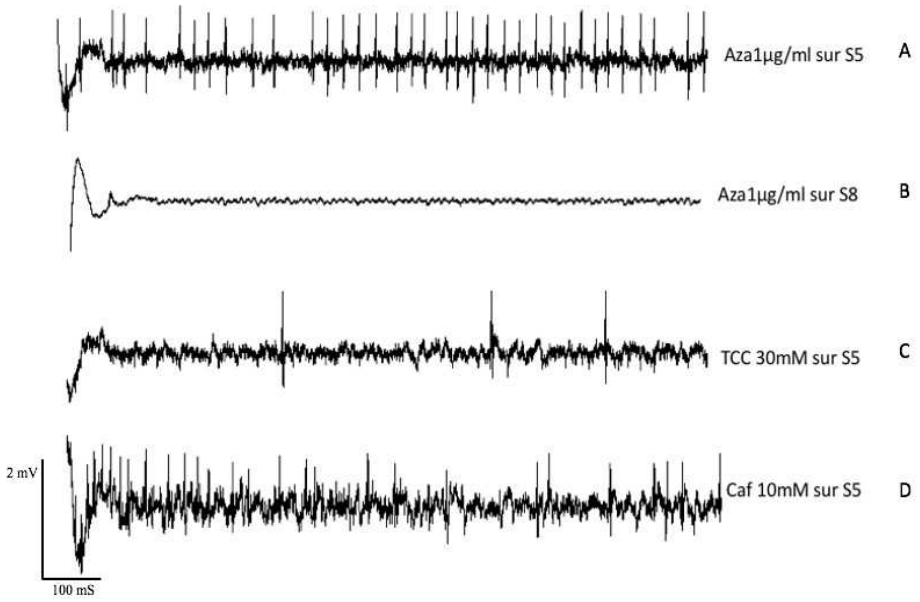

Figure 32. *Exemples de réponses électrophysiologiques à l'azadirachtine, à la caféine et au TCC.*

Exemples d'enregistrements obtenus. ***A :*** *Réponse de la sensille (S5) à l'azadirachtine,* ***B :*** *une sensille (S8) qui ne répond pas à l'azadirachine,* ***C :*** *Réponse de la sensille S5 au TCC utilisé comme contrôle,* ***D :*** *Réponse de la sensille (S5) à la caféine.*

3.8 Effets sur la consommation alimentaire

Le comportement alimentaire des adultes mâles et femelles de *D. melanogaster* a été évalué en utilisant la technique de MultiCAFE (Capillary Feeder), décrite précédemment. Nous avons évalué dans un premier temps, l'effet de la concentration en azadirachtine (0,1 µg/ml ou 0,25 µg/ml) sur la consommation alimentaire des mouches des séries témoins et traités (DL_{25} et DL_{50}) (Fig. 33). Les résultats révèlent un effet significatif avec une consommation plus élevée pour la plus faible (0,1 µg/ml).

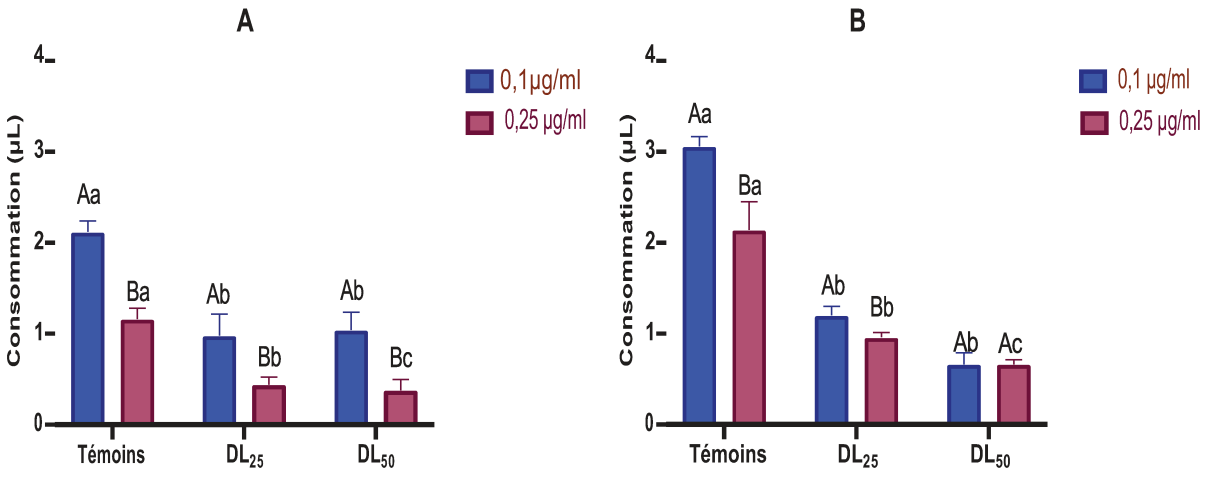

Figure 33. *Consommation de solutions contenant du Neem Azal chez des individus adultes traités au stade larvaire (A : mâles et B : femelles).*

m ± SEM ; n = 32 répétitions. Les différentes lettres en minuscules indiquent une différence significative entre les doses testées (P < 0,05). Les différentes lettres en majuscules indiquent une différence significative entre les différents milieux testés (P < 0,05).

Chez les mâles, le test de Mann-Whitney révèle des effets significatifs entre les deux milieux testées (0,1 µg/ml et 0,25 µg/ml) chez les témoins (U = 1 ; P= 0,0003), DL_{25} (U = 12,89 ; P = 0,0016) et DL_{50} (U = 4,5 ; P = 0,0020). Chez les femelles, des différences significatives sont enregistrées seulement chez les insectes témoins (U = 6,5 ; P = 0,0048) et traités à la DL_{25} (U = 6 ; P = 0,0042).

L'évolution en fonction du temps de la consommation des mouches (Témoins, DL_{25} et DL_{50}) à l'azadirachtine (0,1 µg/ml), révèle une consommation plus importante chez les femelles, probablement corrélée à leurs taille légèrement plus importante que celle des mâles.

Afin de vérifier si la baisse de consommation des mouches traitées est spécifique ou non à l'azadirachtine, nous avons évalué en utilisant la même technique en évaluant l'effet du traitement larvaire (début de stade L3) sur la consommation de l'azadirachtine (0,1 µg/ml ou 0,25 µg/ml) et la caféine (10 mM) des mouches des séries témoins et traités (DL_{25} et DL_{50}) (Fig. 34). Le saccharose (100 mM) a été utilisé comme témoin (Fig. 34).

Chez les femelles témoins, aucune différence significative entre les différentes concentrations de molécules amères testées (Aza et Caf) (P > 0,05). Une différence a été noté uniquement chez les mâles entre les deux doses testés d'azadirachtine (0,1 µg/ml et 0,25 µg/ml) et entre Aza (0,25 µg/ml) et la caféine (P < 0,001).

Chez les femelles traitées à la DL_{25}, une différence significative trouvée entre l'azadirachtine à 0,1 µg/ml et 0,25 µg/ml ($P < 0,001$) et entre la cafeine 10 mM. Aucune différence enregistrée entre la concentration la plus élevée d'azadirachtine (0,25 µg/ml) et la cafeine ($P > 0,05$). Chez les mâles, une différence significative est enregistrée uniquement entre l'azadirachine (0,25 µg/ml) et les différentes doses testées ($P < 0,001$). Des résultats similaires ont été obtenus chez les insectes mâles et femelles traités à la DL_{50}

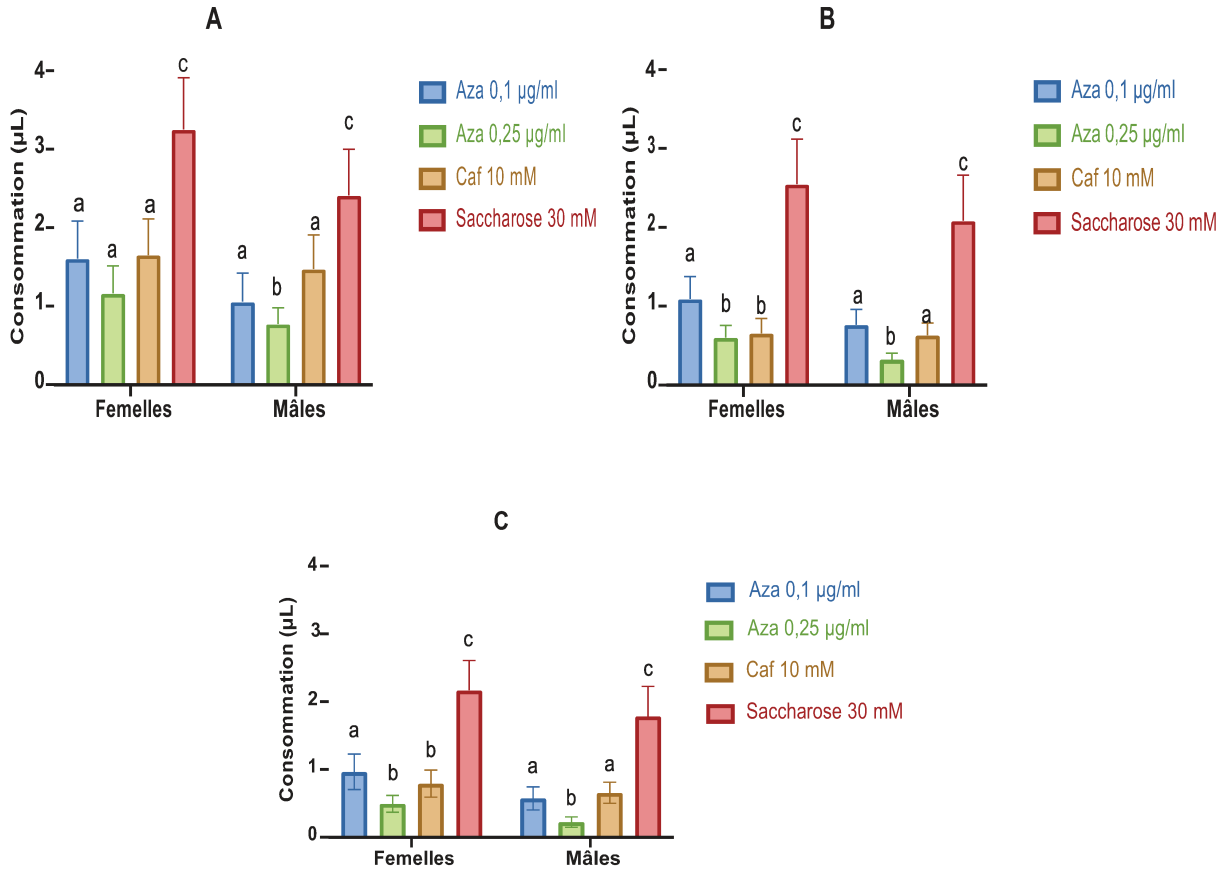

Figure 34. *Consommation pendant 24 h d'azadirachtine, de caféine et de saccharose chez des individus traités ou non à l'azadirachtine au début de stadeL3 D. melanogaster (A : Témoins ; B : DL_{25} ; C : DL_{50}), (Aza : 0,25 µg/ml et 0,1 µg/ml ; saccharose : 30 mM ; Caf : 10 mM) (m ± SEM ; n = 32 répétitions).*

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Azadirachtin induced larval avoidance and antifeeding by disruption of food intake and digestive enzymes in *Drosophila melanogaster* (Diptera: Drosophilidae)

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Abstract

Botanical insecticides are a promising alternative to reduce the harmful effects of synthetic chemicals. Among the botanical biopesticides, azadirachtin obtained from the Indian neem tree *Azadirachta indica A.Juss.* (*Meliaceae*) is probably the biorational insecticide with greatest agriculture use nowadays due to its broad insecticide activity. The current study, evaluated the lethal and sublethal effects of azadirachtin on larval avoidance, food intake and digestive enzymes of *Drosophila melanogaster* larvae as biological model. Azadirachtin was applied topically at two doses LD_{25} (0.28 µg) and LD_{50} (0.67 µg) on early third instars larvae.

Results evaluated 24h after treatment showed that larvae exhibited significant repellence to azadirachtin and prefer keeping in untreated arenas rather than moving to treated one. In addition, azadirachtin avoidance was more marked in larvae previously treated with this compound as compared with naïf larvae (controls). Moreover, azadirachtin treatment decreased significantly the amount of larval food intake. Finally, azadirachtin reduced significantly the activity of larval α-amylase, chitinase and protease and increased the activity of lipase. This finding showed that azadirachtin induced behavioral and physiological disruption affecting the ability of the insect to digest food. This rapid installation of avoidance and long term antifeedancy might reinforce the action of azadirachtin and provide a new behavioral strategy for integrated pest management programs.

Keywords: Azadirachtin, *Drosophila melanogaster*, Larval avoidance, Food intake, Digestive enzymes.

Introduction

Pesticides have played a crucial role in increasing agricultural productivity [1]. However, increasing awareness regarding environmental and human health impacts of conventional pesticides reduced their availability and promoted the search for alternative control strategies and reduced risk pesticides [2]. Consequently, a drastic re-emergence of interest in the use of natural pesticides, or biopesticides, especially plant-derived compounds was noted [3, 4, 5, 6]. The prominent botanical pesticides that have received the best of attention are those issued from the neem tree, *Azadirachta indica* A. Juss. (Meliaceae) [3, 7, 8]. Azadirachtin, produced as secondary metabolite, is the principal active constituent in neem extracts [3]. Due to its chemical complexity, azadirachtin has many anti-insect properties but no resistance problem [3]. In addition, this compound has also been reported as safer for non-target organisms; however, this earlier perception has been recently challenged specially in relation with natural enemies and pollinators [9, 10, 11, 12]. Despite these problems, azadirachtin still a recognizable alternative to conventional insecticides in integrates pest management programs. Azadirachtin has multiple modes of action including antifeedancy, fecundity suppression and sterilization, oviposition repellence, growth inhibition, detrimental effects on morphology and changes in biological fitness [13, 14, 3, 15, 16, 17]. However, at the moment, if the broad areas of its mode of action were established, the details were still not fully understood.

The major action of azadirachtin was its ability to modify or suppress hemolymph ecdysteroid and juvenile hormone titers by inhibiting the release of morphogenetic peptide hormone (PTTH) and allatotropins from the brain-*corpus cardiacum* complex leading to its well documented IGR effects [18, 19, 20]. The behavioral effects of azadirachtin are more controversial and seem to vary between insect orders and even species [21, 22, 23, 24].

The ability to discriminate between edible and toxic food is crucial to survive and reproduce, and all animals must locate and selects the diet that maximises fitness [25]. Feeding behavior is both mediated by the detection through contact chemoreceptor on the insect's chemosensillas and sensory code integration by the central nervous system [26]. This primary antifeedancy effects of azadirachtin were reported in various orders of insect's including Lepidoptera, Coleoptera, Hemiptera, Isoptera, Diptera and Orthoptera [3]. An internal feedback mechanisms called secondary antifeedancy are initiated following the onset of physiological toxic effects arising from azadirachtin uptake or ingestion, but the reports are not consistent [27, 28]. Moreover, decrease in food intake after azadirachtin treatment has been reported to dependent upon the insect species and concentration used [3, 28]. Recently, we have reported that the preimaginal exposure to azadirachtin affects food selection and digestive enzymes in adults of *Drosophila melanogaster* suggesting a memorability of a learned avoidance and a long term antifeedancy of this compound [29]. In order to estimate the precocity of installation of these effects and their evolution through the insect development; the present study, investigated the acute lethal and sublethal effects of azadirachtin on larval avoidance, food intake and digestive enzymes 24h after azadiarchtin application on third instar larvae of *Drosophila melanogaster*, one of the most promising animal models used for research on mechanisms of nutrient selection and regulation.

2. Material and methods

2.1. Flies

Experimental stocks of *D. melanogaster* (wild-type Canton-S) were reared in glass vials containing a yeast/cornmeal/agar laboratory medium containing an anti-fungal agent and kept at 25 ± 2°C, 70% relative humidity under a 12 h light/dark photoperiod. Flies were transferred

every three days to avoid larval competition and regularly provide abundant progeny for testing.

2.2. Insecticide and treatment

A Commercial formulation of azadirachtin, the Neem Azal-TS (1 % EC; Trifolio-M GmbH, Lahnau, Germany) was dissolved in acetone and topically applied (1 µl per insect) on early third-instar larvae of *D. melanogaster* at two doses, 0.67µg and 0.28µg corresponding respectively to LD_{50} and LD_{25} (doses that caused respectively a mortality of 25% and 50% of immature stages) [16]. Controls were exposed only to the solvent (1µl acetone) and all flies were kept under the same conditions as cited above. All experiments were done 24 h after treatment.

2.3. Larval preference assay

Groups of 20 larvae from controls and treated series (LD_{25} and LD_{50}) were collected and transferred to a petri dishes (Nunc ; diameter = 66 mm, height = 15 mm) containing filter paper (Whatmann, Cat 42; diameter = 55 mm) divided into two concentric circles (28 mm for the inner circle). The outer circle was soaked with 10 ml of azadirachtin (0.25 µg/ml) whereas the inner circle with 10 ml of acetone. After five minutes for solvents evaporation, the filter paper was humidified with 1 ml of distilled water in order to facilitate the displacement of the larvae. The larvae were placed in the inner circle, after five minutes the number of larvae present on each zone was determined and the larval preference index (LPI) was calculated as follows: (number of larvae on azadirachtin zone – number of larvae on control zone) / (number of larvae on azadirachtin zone + number of larvae on control zone). In theory, azadirachtin preference index can vary between -1 (strong aversion against azadirachtin) and +1 (strong attraction to azadirachtin).

2.4. Food intake estimation

The quantification of food intake was performed as previously described by Wong et al. [30]. For each experiment five larvae (Controls, LD_{25} and LD_{50}) were provided fresh food medium containing 2.5% (w/v) blue food dye (FD &C Blue no 1). After 30 minutes of feeding, only the blue larvae were selected.

Larvae were homogenised in 200 µl of distilled water. A further 800 µl of distilled water was added and the suspension cleared by centrifugation at 13,000 g for 15 min. The absorbance of the supernatant was read at 629 nm. Age-matched larvae exposed to non-dyed food were used as the baseline during spectrophotometry. The absolute amount of food intake was calculated from a standard curve made by serial dilution in water of a sample of blue food.

2.5. Digestive enzymes assay

- *Sample preparation*

For enzyme extracts individual's midgut of larvae from controls and treated series (LD_{25} and LD_{50}) were dissected out under a strereomicroscope and rinsed in ice cold saline buffer (0.15 M NaCl). A pool of ten midguts were homogenized in 1 ml of universal buffer (pH 7) and centrifuged at 13,000 g for 15 min. The supernatant was used as the enzyme source. Six replicates were used for each dose. The protein concentrations in each sample were determined in parallel according to Bradford [31] and used to calculate the specific activity.

- *Enzyme assay*

α-amylase activity was assayed using the dinitrosalicylic acid (DNS) as the reagent and 1% soluble starch as substrate [32]. Twenty microliters of the enzyme were incubated for 30 min at 35°C with 100 µl universal buffer (pH 7) and 40 µl soluble starch. The reaction was stopped by adding 100 µl DNS and heating in boiling water for 10 min prior to reading the absorbance

at 540 nm. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35°C.

Chitinase activity was determined using colloidal chitin [33] as the substrate according to Miller [34]. Chitinase activity was assayed, based on dinitrosalicylic acid (DNS) method [34]. 250 µl of enzyme solution was added to 250 µl of 1% colloidal chitin in citrate phosphate buffer (0.05 M, pH 6.6), after incubation for 30 min at 50°C with continuous shaking (120 rpm), the mixture was heated in a boiling-water bath for 5 min, cooled at room temperature and then centrifuged at 5000 g for 5 min. 150 µl of the supernatant were mixed with 300 µl of universal buffer and 200 µl of DNS; the mixture was incubated for 10 min in boiling water bath and then cooled at 4°C. The absorbance was measured at 540 nm. The activity was calculated from a standard curve of known concentration of N-acetylglucosamine. One enzyme unit was defined as the amount of enzyme that produces 1 µmol of reducing sugar per hour under reaction conditions.

The lipase assays were carried out as described by Tsujita et al. [35]. Ten microliters of the enzyme and 18 µl of p-nitrophenyl butyrate (50 mM) as a substrate were added to 172 µl of universal buffer (pH 7) and incubated at 37°C for 30 min. The absorbance was read at 405 nm and one unit of lipase activity was defined as the amount of enzyme necessary to liberate 1 µmol of *p*-nitrophenyl per minute under assay conditions.

General protease activity was assayed using casein (1%) as substrate [36]. 200 µl of 1% casein solution was added to 100 µl enzyme and 100 µl universal buffer (pH 7), the mixture was incubated at 37°C for 60 min. Proteolysis was stopped by the addition of 800 µl of 5% trichloroacetic acid (TCA). The mixture was centrifuged at 8000 g for 15 min and the absorbance was recorded at 280 nm. The activity was calculated from a standard curve using tyrosine with a known concentration.

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 of larval preference was subjected to Kruskal-Wallis test and pairwaise multiple comparison using Dunn's method. Index preference was assessed using Mann-Whitney test. Data of quantification of food intake and activity of digestive enzymes were subjected to one-way analysis of variance (ANOVA) followed by a post-hoc HSD Tukey test. 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 larval preference

Azadirachtin treatment (LD_{25} and LD_{50}) significantly affect larval preference for untreated and azadirachtin-treated filter paper arenas ($P<0.001$) (Fig. 1). Larvae previously exposed to azadirachtin showed a highest aversion to this substance compared to naïve larvae ($P<0.01$). Larvae exhibited significant repellence to azadirachtin and prefers keeping in untreated arenas and avoid moving to the treated one ($P<0.01$) (Fig. 1).

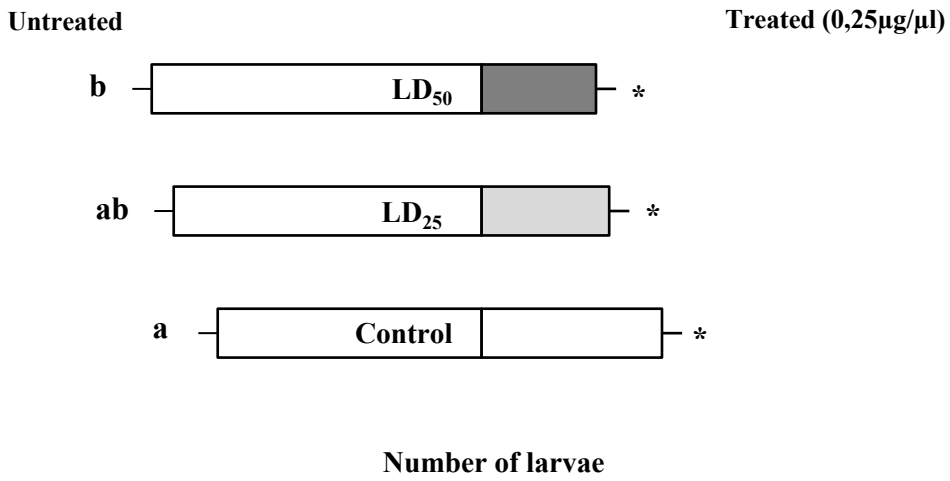

Figure.1. Effect of Azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on larval preference subjected to two-choice test on azadirachtin-treated surface (0.25 µg/µl). Different small letters indicate a significant difference between control and treated individuals in the same surface. The asterisks indicate a significant difference between individuals of the same dose in the different surface (untreated and treated).

The larval preference index (LPI) of *D. melanogaster* (Controls, LD_{25} and LD_{50}) is shown in Fig. 2. The means values of LPI recorded in azadirachtin-treated zone (0.25µg/µl) were -0.18, -0.42 and -0.44 respectively for controls, LD_{25} and LD_{50}. Indeed, Mann-Whitney test showed significant differences between the control and LD_{25} (Mann-Whitney test U = 1.00; P = 0.023), control and LD_{50} (Mann-Whitney test U = 1.00; P = 0.023) and no significant effects between the two tested doses (Mann-Whitney test U = 11.50; P>0.05) (Fig. 2).

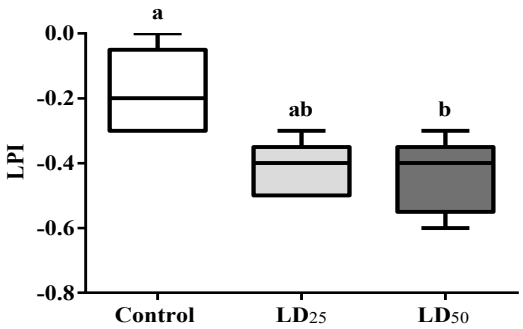

Figure.2. Larval preference index (LPI) of third instars larvae of *D. melanogaster* for azadirachtin-treated surface (0.25 µg/µl). Different small letters indicate a significant difference between control and treated individuals.

3.2. Effect of azadirachtin on food intake

Measurements of food intake and potential antifeedant effects caused by azadirachtin treatment were evaluated using spectrophotometry and shown in Fig. 3. Azadirachtin treatment of early third instars larvae caused a significant reduction of food intake, 24h after treatment, with a dose dependent relationship ($F_{2, 14} = 25.80$; $P<0.001$). The means values recorded were 2.40 ± 0.03 µg/larva for the controls, 1.96 ± 0.13 µg/larva for the LD_{25} and 1.36 ± 0.11 for the LD_{50} (Fig. 4). The food ingested rate in azadirachtin-treated larvae decreased by about 30% at 45% respectively for LD_{25} and LD_{50} compared with controls larvae (Fig. 3).

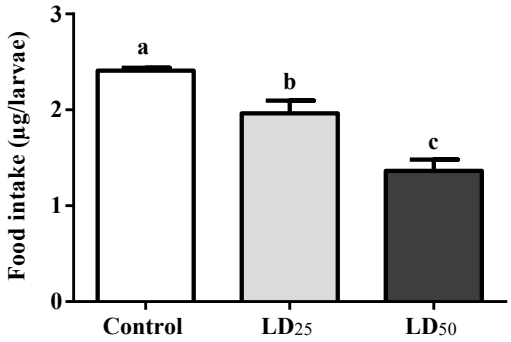

Figure.3. Effect of Azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on food intake (µg/larva), (m ± SE; n = 5 replicates of 5 insects). Different small letters indicate a significant difference between control and treated series ($P<0.05$).

3.3. Effect of azadirachtin on digestive enzyme activities

Midguts from azadirachtin-treated (LD_{25} and LD_{50}) larvae showed significantly ($F_{2, 15} = 45.01$; $P<0.001$) lower α-amylase activity in comparison to midguts from controls larvae (Fig. 4). The mean values recorded were 4.86 ± 0.15 µmol/min/mg protein for controls, 3.43 ± 0.13 µmol/min/mg protein for the LD_{25} and 3.14 ± 0.11 µmol/min/mg protein for the LD_{50}. Statistical analysis revealed no significant difference between the two tested doses ($P>0.05$) (Fig. 4).

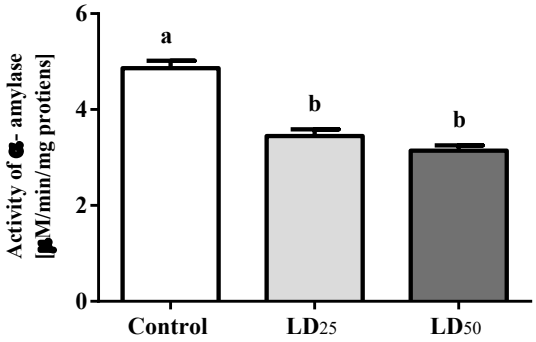

Figure.4. Effect of Azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of α-amylase, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series (P<0.05).

As a consequence of treatment, chitinase activity decreased in treated larvae when compared with control larvae ($F_{2, 15} = 202.4$; P<0.001) (Fig. 5). The means values recorded were 0.64 ± 0.009 mmol/min/mg proteins for controls, 0.51 ± 0.005 mmol/min/mg protein for the LD_{25} and 0.44 ± 0.006 mmol/min/mg protein for the LD_{50}. Statistical analysis revealed significant difference between the two tested doses (P<0.05) (Fig. 5).

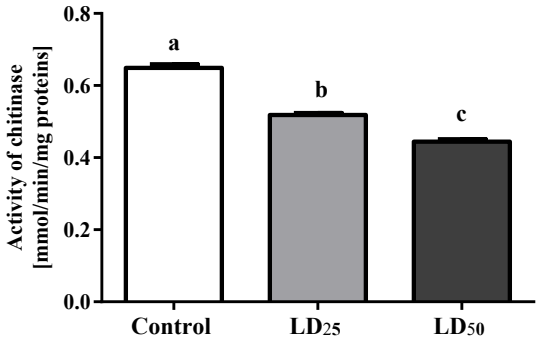

Figure.5. Effect of Azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of chitinase, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series (P<0.05).

Lipase activity increased in treated larvae as compared with control larvae ($F_{2, 15} = 80.90$; $P<0.001$) and the highest activity was observed in larvae treated by LD_{50} (Fig. 6). The means values recorded were 0.18 ± 0.002 µmol/min/mg protein for controls, 0.25 ± 0.007 µmol/min/mg protein for the LD_{25} and 0.27 ± 0.004 µmol/min/mg protein for the LD_{50}. Statistical analysis revealed no significant difference between the two tested doses ($P>0.05$) (Fig. 6).

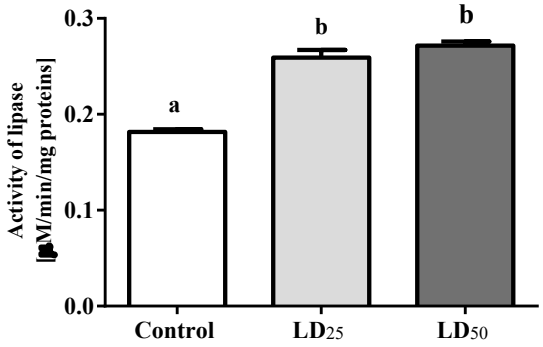

Figure.6. Effect of Azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of lipase, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series ($P<0.05$).

General protease activity was lower in azadirachtin-treated-larvae (LD_{25} and LD_{50}) as compared with the controls larvae ($F_{2, 15} = 10.08$; $P = 0.0017$) (Fig. 7). In control series the mean values recorded were 0.80 ± 0.034 mmol/min/mg protein. In treated series the mean values recorded were 0.59 ± 0.044 µmol/min/mg protein for the lowest dose LD_{25} and 0.57 ± 0.038 µmol/min/mg protein for the highest dose LD_{50}. Statistical analysis revealed no significantdifference between the two tested doses ($P>0.05$) (Fig. 7).

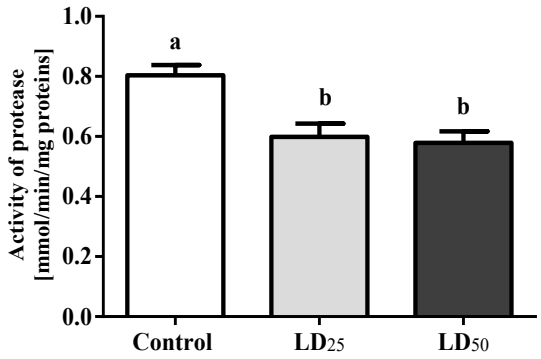

Figure.7. Effect of Azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of protease, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series (P<0.05).

4. Discussion

In this study, we investigated the effects of azadirachtin on larval avoidance, food intake and digestive enzymes in larvae of *D. melanogaster*. Results show that larvae preferentially staying in untreated filter paper arenas rather than moving in azadirachtin-treated one. In addition, azadirachtin avoidance was more marked in larvae previously treated (24 h before experiments) with this compound as compared with naïf larvae (Controls).

Azadirachtin avoidance has also been reported in other insect species, which are apparently able to detect the insecticide on the treated surface [37, 38, 23, 24]. In addition, the impact of previous exposure on insect behaviour was reported in different insect's species [39, 40, 41]. Recently, Kilani-Morakchi et al. [29] demonstrated that larval experience with azadirachtin can alter the physiological and behavioral responses of adults flies and that the aversion (primary and secondary antifeedancy) to this compound can be conserved through the metamorphosis suggesting a memorability and avoidance learning memory. In the current experiments, the behavioral effects sparked by azadirachtin seem to appear very early after treatment (24 h) and the first experience (topical application) reinforce the avoidance effect of this compound. Results demonstrate the precocity of this learning memory which may contribute to reinforce the insecticidal activity of this biopesticide

and provide a new behavioural strategy for controlling agricultural pests.

In *Drosophila,* a complex olfactory system allows animals to avoid noxious substances [42]. Moreover, the response of larvae to toxic or bitter compounds is mediated by gustatory receptors and can lead to aversive behavior resulting in food intake cessation [43, 44]. This primary antifeedancy is mediated by the projection of neurons to the protocerebrum which acts as gustatory interneuron's relaying avoidance information to higher brain centres in *Drosophila* larvae [26, 44].

A secondary antifeedant effect including a long-term reduction in food intake, and direct detrimental effects on different insect tissues such as muscles, fat body and gut epithelial cells is also reported [3].

In the current experiments, azadirachtin-treatment of early third instar larvae reduced significantly larval food-intake at the two tested doses (LD_{25}, LD_{50}) as compared to the controls. Previously, azadirachtin is reported to reduce food consumption in different insect's species [20, 45, 28]. The antifeedant activity of azadirachtin varied significantly based on the concentration used and the insect species [3, 28].

Inhibition of feeding behaviour results from stimulation of deterrent receptors by azadirachtin often coupled with an inhibition of sugar receptors [46], these effects results to bad nutrition, starvation, or restriction of food intake [3]. Moreover, neuronal circuits that control feeding must integrate information on both the palatability of food source ant its nutritional content [47]; indeed, *D. melanogaster* seems to be able to discriminate food texture and integrate the chemical and physical qualities of food [48] which may contribute to avoiding azadirachtin-treated food.

In addition, a reduction of insect weight under azadirachtin treatment was reported in *Periplaneta Americana* [49], *Helicoverpa armigera* [50] *Spodoptera eridania* [28] and *D. melanogaster* [51, 17]. Growth and nutrient intake are function-ally linked processes in development and growth and body mass are directly affected by nutrient uptake. Indeed, nutrient sensing systems play an important role in the physiological adjustment process which is governed by an evolutionary conserved systemic

nutrient sensor, the insulin/insulin-like growth factor signalling (IIS) pathway during insect growth [52]. In fact, Lai et al. [51] reported that the inhibition of growth and development caused by azadirachtin in *D. melangaster,* was consistent with those caused by disruption of the insulin/insulin-like growth factor signalling IIS pathway. Moreover, Qiao et al. [53] reported the neurotoxic effect of azadirachtin which might interfere with different endocrinological and physiological actions in insects.

Recent studies indicate that regulation of feeding is not only governed by gustatory information but also by internal sensors [54, 55]. Indeed, cells located in internal organs, including the gut, fat body and the brain, express receptors that detect nutrients or their metabolically processed derivates to regulate energy homeostasis and feeding behaviors [56].

Azadirachtin was reported to cause disruption in insect physiology and its ability to digest food [28]. The results obtained in the present study showed a clear disruption of digestive enzymes activities in *D. melanogaster* larvae after azadirachtin treatment as compared to controls. In intestine, the complex mix of dietary components ingested by flies is broken down by digestive enzymes before it is absorbed by the intestinal epithelium [57]. Azadirachtin was reported to affect digestive enzymes in several insect species [58, 27, 59]. In the current study, azadirachtin treatment of early third instar larvae of *D. melanogaster* reduced significantly gut α-amylase, chitinase and protease activities and increased lipasic activity.

α-amylase is an enzyme that degrades starch by hydrolyzing α-1,4-glucan bonds [60]. The reduction of α-amylase activity under azadirachtin treatment was reported in *Periplaneta Americana* [49], *Plodia interpunctella* [61] and *Glyphodes pyloalis* [27]. Lai et al. [51] demonstrate that azadirachtin reduced the activity of α-amylase in midgut of *D. melanogaster* by repressing expression of α-amylase gene.

Insect chitinases which belong to family of 18 glycosylhydrolases, detected in moulting fluid and gut tissues, are predicted to mediate the digestion of chitin present in exoskeleton and peritrophic membrane (PM) in the gut to chitooligosaccharides [62].

Azadirachtin reduced significantly the chitinase activity in *D. melanogaster* larvae which is in accordance with the results obtained in adults of the same species [29]. The peritrophic membrane protects the midgut from mechanical damage and toxic compounds; azadirachtin was reported to affect this PM leading to damage of cellular surfaces of the midgut [59]. In addition, in insects the expression of chitinase gene was regulated by 20E (20-hydroxyecdysone) [63], which may explain the effect observed in our results, since azadirachtin act as an antagonist of the 20E.

Lipases play a major role in storage and lipid mobilization. These enzymes are also the basic components in many of physiological process like, reproduction, growth, and defence against pathogens [64]. Our results showed that azadirachtin increased significantly the activity of lipase in the larvae midgut of *D. melanogaster*. Reduction in lipasic activity is mostly reported in literature after azadirachtin treatment [27, 64], but this effect differ with azadirachtin formulation and concentration. However, the increase in lipase activity observed in our study may be a result of an increasing of esterase activity needed for detoxification [64, 29]. In addition, according to the physiological and developmental needs of the insect, the acquired nutrients can be differentially allocated to different organs, processes and/or metabolic pathways which may explain the increase in lipase activity noted in third instars larvae of *D. melanogaster* to meet the demand for energy needed for metamorphosis.

Proteases are groups of enzymes that hydrolyze proteins to amino acids based on their catalytic mechanism [65]. In the present study, compared with the controls, protease activity was significantly reduced in larvae of *D. melanogaster* after azadirachtin treatment. The reduction of protease activity under azadirachtin treatment was reported in several insects' species [49, 27]. Azadirachtin prevents the secretion of Proteolytic enzymes and thus significantly impair ability of insects to digest and absorb nitrogenous food. Timmins and Reynolds [66] showed that azadirachtin significantly influenced the trypsine activity in the midgut of *Spodoptera litura.* Recently, several studies reported that azadirachtin interfere with activities of different enzymes such as α- amylase, lipase, α - and β - glucosidases, proteases, lactate dehydrogenase and glutathione S-transferase [27, 59, 15]. These

results may reflect interference of azadirachtin with regulation of feeding and metabolism which clearly supports the secondary antifeedancy action of azadirachtin included reduction in food consumption and digestive efficiency reducing access of nutrients for biological requirements [67, 68]. Furthermore, this reduction in efficiency of conversion of ingested and digested food may be a result of an energy conversion from biomass production into induction enzymes involved in detoxification of azadirachtin [28].

In conclusion, it is evident that this study has shown that azadirachtin affects larval avoidance, food consumption and digestion in *D. melanogaster.* This rapid installation of avoidance and antifeedancy reinforce the action of azadirachtin and is useful element for integrated pest management programs that involve behavioral manipulation of insect pests, including the harmful *D. suzukii,* like push-pull strategies for protected plant resource as safer alternative to conventional synthetic insecticides.

Acknowledgements

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Discussion

4 Discussion

4.1 Effets sur la fécondité et le choix d'oviposition

La ponte est une activité complexe et critique dans le cycle de vie d'un insecte, avec une variété de facteurs qui influencent à la fois sa physiologie et son comportement. Le choix du site de ponte est un facteur important pour la survie et la sécurité de la progéniture, dès lors que celle-ci exploite, au moins transitoirement, les ressources disponibles sur ce site ou son voisinage immédiat (Yang *et al.,* 2008).

Plusieurs facteurs peuvent influencer la préférence d'oviposition des femelles : la qualité et l'abondance de la nourriture, la densité des œufs, l'âge (Minkenberg *et al.,* 1992), la présence de prédateurs, la végétation environnante, les indices chimiques des congénères (Bentley & Day, 1989 ; Mokany & Shine, 2003), l'apprentissage social (Sarin & Dukas, 2009), ou encore l'expérience antérieure (Papaj & Prokopy, 1989).

Si la ponte représente un indicateur des conditions favorables au développement de la descendance, elle est également utilisée pour détecter l'aversion envers les composés toxiques pour les larves et les adultes (Possidente *et al.,* 1999). De plus, de nombreuses études ont utilisé des modèles de ponte pour distinguer des différences subtiles dans les préférences des plantes hôtes, ce qui a permis de mieux comprendre les comportements écologiques et physiologiques de différentes espèces de drosophiles (Ryan *et al.,* 2009 ; Olazcuaga *et al*., 2019).

Chez *Drosophila melanogaster*, la préférence des femelles pour la ponte peut être influencée par l'interaction sociale, la température, la texture ou la couleur du milieu de ponte (Battesti *et al.,* 2015). De même, une exposition préalable à des aliments ou composés particuliers peut également influencer le choix des femelles (Abed-Vieillard *et al.,* 2014 ; Flaven-Pouchon *et al.,* 2014).

Cette capacité des femelles de *D. melanogaster* à évaluer le meilleur site de ponte dépend à la fois de circuits neuronaux régulant ce choix comportemental, mais aussi des expériences sensorielles de l'insecte (Sarin & Dukas, 2009 ; Anagnostou *et al.,* 2010 ; Miller *et al.,* 2011).

Notre étude a démontré qu'une seule application topique d'azadirachtine (DL_{25} et DL_{50}) sur des larves L3 de *D. melanogaster* réduit le nombre d'œufs pondus chez les adultes ayant

survécu au traitement. Cet effet négatif sur la fécondité des femelles a été noté, non seulement chez les individus exposés de la génération parentale, mais aussi par des effets transgénérationnels sur les individus de la première génération F1 n'ayant jamais été directement exposés à l'insecticide.

L'impact de l'azadirachtine sur la reproduction a été observé chez différentes espèces d'insectes. Une réduction de la fécondité et de la fertilité après un traitement à l'azadirachtine a été signalée chez de nombreux insectes, notamment *Spodoptera littoralis* (Pineda *et al.,*2009), *Dysdercus cingulatus* (Pandey & Tiwari, 2011), *Blatta orientalis* (Tine *et al.,* 2012), *Helicoverpa armigera* (Ahmad *et al*., 2013), *Tuta absoluta* (Tomé *et al.,* 2013), *Galleria mellonella* (Er *et al.,* 2017) et *D. melanogaster* (Boulehbal *et al.,* 2015 ; Bezzar-Bendjazia *et al.,* 2016 ; Oulhaci *et al.,* 2018). Cette réduction a été corrélée à l'action négative de l'azadirachtine sur la synthèse des vitellogénines et/ou leur incorporation par les ovocytes (Boulehbal *et al.,* 2015).

Chez les insectes, les étapes du développement (mues, métamorphoses) et la reproduction sont contrôlées et coordonnées par le système neuroendocrinien qui sécrète les hormones nécessaires dont les ecdystéroïdes et l'hormone juvénile (Gilbert *et al*., 2002 ; Lafont *et al*., 2005 ; Gruntenko *et al*., 2009 ; Hiruma & Kaneko, 2013).

Chez les larves, l'hormone juvénile (JH) joue un rôle de « *status quo* » qui implique la suppression de la métamorphose prématurée et la détermination de la mue induite par les ecdystéroïdes en tant que larve (Truman & Riddiford, 2007 ; Jindra *et al.,* 2013 ; Dubrovsky & Bernardo, 2014 ; Rauschenbach *et al.,* 2017).

Chez les adultes, l'hormone juvénile joue un rôle gonadotropique et contrôle la reproduction en association avec l'hormone de mue, dont la forme active est la 20-hydroxyecdysone (20E). L'JH produite dans les *corpus allatum* stimule la biosynthèse des ecdystéroïdes dans l'ovaire. Les ecdystéroïdes produits dans le follicule ovarien stimulent la biosynthèse de la vitellogénine dans le corps adipeux ; la vitellogénine est ensuite captée par les ovaires à partir de l'hémolymphe (Raikhel *et al.,* 2004 ; Toivonen & Partridge, 2009). L'équilibre entre JH et 20E est d'une importance capitale pour un bon déroulement des événements de la reproduction.

Chez les insectes, y compris la drosophile, l'JH est la 20E sont partie intégrante des mécanismes neuroendocrinien liés à la réponse au stress (Rauschenbach *et al.,*1995 ;

Cymborowski, 1991) et jouent un rôle clé dans l'adaptation des insectes aux facteurs de stress en influençant le processus de reproduction (Gruntenko & Rauschenbach, 2008). En effet, et en plus des neuropeptides spécifiques à action prothoracicotrope, allatotrope et allatostatique, le métabolisme de l'JH et de la 20E peut être régulé par des amines biogènes tels que l'octopamine (OA) et la dopamine (DA) (Gruntenko & Rauschenbach, 2008).

Chez *D. melanogaster,* en plus des ecdystéroïdes et de l'hormone juvénile, les facteurs de signalisation de type insuline/insuline-like growth factor sont essentiels à la reproduction (Toivonen & Partridge, 2009). La vitellogénèse chez les femelles est stimulée sous l'action de l'JH et conduit au développement des ovocytes. L'action synergique de l'JH, de la 20-hydroxyecdysone (20E) et des voies de signalisation insuline/insuline-like growth factor contrôle l'homéostasie énergétique nécessaire à la formation des ovocytes (Toivonen & Partridge, 2009). Par conséquent, la réduction de la fécondité enregistrée au cours de nos expérimentations, peut être liée à l'action antagoniste de l'azadirachtine sur les principales hormones contrôlant le processus de reproduction (JH/ecdystéroïdes) (Navarro-Roldán & Gemeno, 2017).

Chez *Anopheles stephensi*, le traitement à l'azadirachtine a entraîné une anomalie de la structure des ovaires avec un arrêt complet de l'ovogenèse, une altération de la vitellogénèse et de la formation de l'enveloppe vitelline ainsi qu'une dégénérescence des cellules folliculaires (Lucantoni *et al.,* 2006). De plus, le traitement à l'azadirachtine a induit chez *Heteracris littoralis* un rétrécissement complet des ovaires avec suppression de la croissance des ovocytes, ainsi qu'une désintégration et une destruction des cellules folliculaires et des mitochondries (Ghazawi *et al.,* 2007).

L'azadirachtine réduit le succès de l'accouplement chez *D. melanogaster* et a un effet négatif sur le nombre et la taille des cystes et des ovocytes (Oulhaci *et al.,* 2018). Le traitement à l'azadirachtine affecte également la quantité de nourriture ingérée par cette espèce et l'activité des enzymes digestives dans l'intestin moyen, ce qui peut affecter l'ovogenèse et la vitellogénèse, puisque les taux d'ecdysone et d'JH sont affectés par la disponibilité des nutriments qui agissent comme régulateur positif sur la voie de l'insuline pour conférer aux ovaires la signalisation nécessaire à une ovogenèse normale (Badisco *et al.,* 2013 ; Jeong *et al.,* 2019).

L'azadirachtine altère également le cytosquelette en prévenant la formation de nouveaux filaments d'actine conduisant à une perturbation de la division cellulaire et à un blocage des processus de transport. Ce phénomène pourrait interférer avec le passage des nutriments des cellules nourricières vers l'ovocyte (Mordue *et al.,* 2010).

Nos résultats révèlent que le traitement des larves (L3 du début de stade) de *D. melanogaster* à l'azadirachtine (DL_{25}, DL_{50}) influence le choix du site de ponte des femelles survivantes au traitement et ce sur deux générations successives. Le traitement larvaire a augmenté l'effet répulsif de l'azadirachtine. En effet, les femelles testées de *D. melanogaster* ont préféré le milieu témoin pour leur ponte en évitant celui traité à l'azadirachtine et ce pour les deux doses testées. Le faible taux de ponte des mouches non exposées "naïves" sur le milieu traité à l'azadirachtine peut être expliqué par l'effet répulsif, bien connu, de l'azadirachtine ainsi que d'autres insecticides à base de Neem, tel qu'il a été rapporté par Silva *et al.,* (2012) chez la mouche commune *Ceratitis capitata.* Valencia-Botín *et al.,* (2004) suggèrent également que la propriété répulsive des extraits de Neem est le principal facteur responsable de la réduction du nombre d'œufs chez la mouche mexicaine *Anastrepha ludens*.

Les mouches ayant déjà été traitées à l'azadirachtine ont une aversion accrue à ce biopesticide par rapport aux mouches "naïves". De nombreuse études ont révélé qu'un traitement des sites de ponte à l'azadirachtine, entrainé un effet répulsif, dissuasif ou inhibiteur de la ponte chez plusieurs espèces d'insectes qui peuvent détecter le bioinsecticide sur une surface traitée (Cordeiro *et al.,* 2010 ; Tomé *et al.,* 2013 ; Bezzar-Bendjazia *et al.,* 2016). Cette inhibition du comportement d'oviposition peut avoir un impact précieux dans la lutte contre les insectes nuisibles.

De nombreuses études récentes ont démontré que le choix du site de ponte peut être influencé par l'expérience sensorielle individuelle et modifiée en fonction d'une expérience larvaire ou adulte (Dukas, 2008 ; Sarin & Dukas, 2009 ; Abed-Vieillard *et al.,*2014 ; Anderson *et al*., 2013 ; Abed-Vieillard & Cortot, 2016). La capacité des insectes à conserver la mémoire d'une exposition précoce affectant la réponse des adultes a été signalée (Dukas, 2008 ; Gerber *et al.,* 2009 ; Flaven-Pouchon*et al.,* 2014 ; Bezzar-Bendjazia *et al*., 2016).

Nos résultats ont également démontré que les effets négatifs d'une seule exposition larvaire à l'azadirachtine peuvent être transmis à la génération F1 *via* un effet transgénérationnel. Les substances toxiques pour l'environnement telles que les insecticides

sont capables de provoquer des altérations épigénétiques qui peuvent être transmises aux générations suivantes (Vandegehuchte *et al.,* 2011). Cela peut expliquer la fécondité réduite et l'évitement de la ponte chez notre génération non exposée (F1).

4.2 Effets sur le développement et la survie des adultes

Notre étude a démontré que l'azadirachtine (DL_{25} et DL_{50}) appliqué au début du troisième stade larvaire de la génération parentale peut affecter négativement divers traits de vie de *D. melanogaster*, de manière dose-dépendante, en réduisant significativement le nombre de larves, de pupes et d'adultes chez la génération parentale (exposée).

Nos résultats montrent également que l'exposition préimaginale à l'azadirachtine induit un sex-ratio biaisé en faveur des mâles de *D. melanogaster* et ceci uniquement pour la génération parentale, avec une relation dose-dépendante.

En effet, l'azadirachtine est connue pour réduire les taux de pupaison et d'émergence des adultes de nombreux insectes comme *Aphis glycines* (Kraiss *et al.,* 2008), *Plodia interpunctella* (Lynn *et al.,* 2012), *Aedes aegypti* (Koodalingam *et al.,* 2014), *H. arnigera* (Ahmad *et al.,* 2015) et *D. melanogaster* (Anjum *et al.,* 2010) affectant ainsi leur cycle de vie.

L'impact négatif de l'azadirachtine sur les stades immatures était attendu compte tenu de son action comme perturbateur de croissance des insectes en supprimant les pics de JH et 20E hémolymphatique (Gäde & Hoffmann, 2005). De plus, l'azadirachtine est connu pour provoquer une dégénérescence des noyaux des différentes glandes endocrines (glande prothoracique, *corpus allatum* et *corpus cardiacum*) qui contrôlent la mue et la métamorphose des insectes ce qui peut conduire à une perturbation généralisée du système neuroendocrinien (Quinn *et al.,* 2012).

Nos résultats montrent que le biopesticide prolonge également de manière significative la durée du développement des larves et des pupes, ce qui entraîne un retard important dans le développement des stades immatures. Ce retard considérable dans les transitions larve-pupe et pupe-adulte a été également rapporté chez *Culex pipiens* traité à l'azadirachtine (Alouani *et al.,* 2009). Un retard dans la formation des pupes a été rapporté par Lai *et al.* (2014) après traitement des larves du troisième stade de *D. melanogaster* à l'azadirachtine. Ce retard serait corrélé à une modification de l'expression des gènes contrôlant la régulation hormonale (Lai

et al., 2014) ce qui pourrait expliquer les perturbations du développement observées au cours de nos expérimentations.

De plus, le traitement induit des aberrations morphologiques chez les différents stades de développement (larves, pupes et adultes) et ce uniquement chez la génération exposée (génération P). Plusieurs anomalies ont été enregistrées pour les différents stades de développement dont : larves brûlées, larves et pupes non complètes, ailes déformées, taille corporelle plus petite et abdomen déformé. Les malformations enregistrées conduisent à la mort de l'insecte.

Chez les insectes, la succession des événements (mues, métamorphoses) au cours du développement est contrôlée et coordonnée par le système neuroendocrinien qui sécrète les hormones nécessaires dont les ecdystéroïdes et l'hormone juvénile (Gilbert *et al.,* 2002 ; Lafont *et al.,* 2005 ; Gruntenko *et al.,* 2009 ; Hiruma *et al.,* 2013).

La biosynthèse et la sécrétion des ecdystéroïdes sont assurées par les glandes prothoraciques, au cours des stades post-embryonnaires, sous l'effet d'une neurohormone prothoracicotrope (PPTH) (Gäde & Hoffmann, 2005). L'ecdysone, libéré dans l'hémolymphe, est rapidement converti en 20-hydroxyecdysone (20E) dans les différents organes périphériques (Marchal *et al.,* 2010). La 20E contrôle l'embryogénèse mais aussi le développement post-embryonnaire en initiant les différentes mues, et ce en agissant sur l'expression de gènes spécifiques *via* sa liaison aux récepteurs nucléaires des ecdystéroïdes (Kozlova & Thummel, 2003).

Chez la Drosophile, des pics de sécrétion de la 20 E sont observés 12h après le début de l'embryogénèse, avant chaque mue larvaire, avant le passage au stade pupal et durant la phase terminale de la différentiation des structures adultes. La PPTH est exprimée au cours du troisième stade larvaire de manière cyclique toutes les 8 heures ; avec un pic important environ 12 h avant la fin du 3ème stade larvaire qui déclenche la formation du puparium et le début de la phase pré-pupal. 12 heures plus tard, un pic d'hormone plus modeste met fin au stade pré-pupal et déclenche la pupaison (Fig. 35). Le stade pupal se caractérise par le pic d'hormone le plus important, corrélé au développement des structures adultes (Quinn *et al.,* 2012).

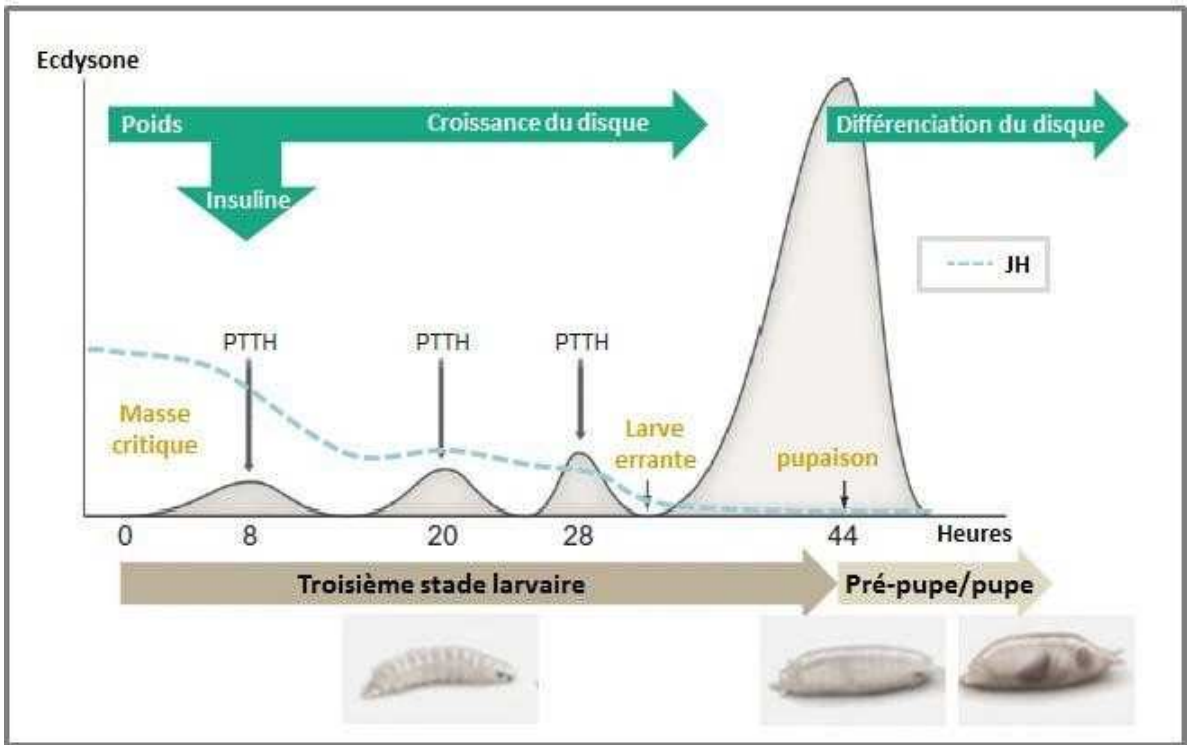

Figure 35. *Évolution des quantités d'hormone juvénile, d'ecdysone et de PTTH au cours des stades de développement de D.melanogaster (Rewitz et al., 2013).*

Les corps allates (*corpora allatum*) sont à l'origine de la biosynthèse de l'hormone juvénile dont la sécrétion est régulée par des neuropeptides activateurs, allatotropines ou inhibiteurs, allatostatines (Bellés & Maestro, 2005). L'JH libérée dans l'hémolymphe est transportée vers les tissus cibles grâce à des protéines vectrices (JH-binding proteins) qui la protègent contre les actions des hydrolases (Dubrovsky, 2005).

L'hormone juvénile joue un rôle important dans le développement où elle maintient les caractères larvaires et inhibe la métamorphose (Dubrovsky, 2005). Chez la drosophile, les taux d'JH sont élevés au cours des premiers stades de développement, décroissent au dernier stade larvaire pour atteindre des quantités infimes lors du stade pupal ; les taux d'JH enregistrent un pic à l'émergence adulte nécessaire au démarrage de la vitellogenèse (Kozlova & Thummel, 2003 ; Dubrovsky, 2005).

L'équilibre entre ces deux hormones est donc essentiel au bon déroulement du développement des insectes, car toute interférence de ces hormones avec des sources exogènes d'agonistes ou antagonistes, peut conduire à une perturbation du développement de l'insecte (Dhadialla *et al.,* 2010 ; Smagghe *et al.,* 2012).

L'azadirachtine, en altérant la croissance et le processus de mue de plusieurs insectes, compromet leur survie. Selon Lai *et al.* (2014), l'azadirachtine réprime l'expression de différents gènes liés à la régulation hormonale, ce qui pourrait expliquer les aberrations du développement observées au cours de nos expérimentations. Il affecte également l'apport en nutriments et le métabolisme de la drosophile, compromettant les signaux nutritionnels qui entraînent une diminution du poids des insectes et de leur taux de croissance, ce qui se traduit par une réduction de la taille du corps et a un impact sur la survie. Le sex-ratio biaisé en faveur des mâles sous l'effet de l'azadirachtine a également été rapporté dans la littérature (Arnason *et al.,* 1985 ; Schneider *et al.,* 2004)

De plus, ce biopesticide est connu pour provoquer des changements structurels dégénératifs des noyaux dans toutes les glandes endocrines responsables du contrôle de la mue chez les insectes, ce qui contribuerait à une perturbation généralisée de la fonction neuroendocrine (Mordue *et al.,* 2010).

Plusieurs études ont rapporté l'inhibition de la synthèse des ecdystéroïdes après traitement par l'azadirachtine (Min-Li & Shin-Foon 1987 ; Feder *et al.,* 1988). Selon Anuradha *et al.* (2007), l'azadirachtine induit chez *D. melanogaster*, une dépolymérisation de l'actine conduisant à l'arrêt de l'activité cellulaire et à l'apoptose.

Les résultats obtenus pour la génération parentale (exposée) après traitement par application topique à l'azadirachtine, des larves L3 du début de stade de *D. melanogaster*, montrent une augmentation de la mortalité des stades immatures avec une relation dose dépendante. La transition larve-pupe est affectée par le traitement et le stade pupal semble être le stade le plus sensible conduisant à une inhibition de la mue adulte.

La génération F1 (non exposée) présente des effets moins marqués comparativement à la génération P. Toutefois, le nombre d'individus des séries témoins reste très élevé comparativement aux traitées. Ces résultats peuvent être expliqués par la possibilité d'une sélection directionnelle prenant place durant l'exposition à l'azadirachtine. Les individus les plus faibles ne pouvant survivre ou sont moins robustes et seul les individus les plus forts peuvent se reproduire.

Les résultats obtenus au cours de notre étude démontrent aussi qu'une seule application d'azadirachtine peut réduire de manière significative la survie des mouches sur deux générations successives (P : exposée et F1 : non exposée) alors que les insectes ont montré un

net rétablissement des taux de survie chez la deuxième génération (F2). Ces résultats reflètent des effets à long terme et différés à travers les stades de développement et les générations. Cet effet constant sur les deux premières générations peut être considéré comme un avantage pour la lutte, en compensant la photo-dégradation rapide bien connue et la faible persistance de l'azadirachtine dans l'environnement. L'action négative de l'azadirachtine sur le développement et la survie a été rapportée chez différentes espèces d'insectes (Hossain & poehling, 2006 ; Das *et al.,* 2006 ; Hasan & Ansari, 2011 ; Ahmad *et al.,* 2013 ; Tomé *et al.,* 2013 ; Ahmad *et al.,* 2015 ; Bezzar-Bendjazia *et al.,* 2016).

4.3 Effets sur la perception olfactive et gustative et le comportement locomoteur

La chimio-sensation est considérée comme l'un des sens les plus primaires. Comme tous les organismes vivants, les insectes utilisent les systèmes sensoriels pour percevoir les stimuli environnementaux et transmettre les réponses comportementales (Chen *et al.,* 2019 ; Xu *et al.,* 2019). Cette perception des substances chimiques présente dans l'environnement contribue à des comportements de survie cruciaux dont l'accouplant, la ponte des œufs, l'évitement des substances toxiques et entraîne une préférence de consommation alimentaires appropriées (Vosshall & Stocker, 2007 ; Bantel & Tessier, 2016).

Le système chimio-sensoriel comprend le système olfactif, qui est utilisé pour la détection des substances chimiques volatiles (odeurs), et le système gustatif (goût), qui permet la détection des composés solubles (Ebbs & Amrein, 2007 ; Bantel &Tessier, 2016 ; Chin *et al.,* 2018). Les deux modalités sensorielles stimulent les réponses neurologiques qui sont traitées et décodées par le cerveau pour produire le comportement attractif ou répulsif approprié (Vosshall & Stocker, 2007).

La capacité des insectes à modifier leur comportement en fonction d'une expérience antérieure est essentielle à leur survie (Chia & Scott, 2020). Des preuves de plus en plus nombreuses ont souligné un rôle essentiel de l'expérience préimaginale dans le comportement des insectes à l'âge adulte (Caubet *et al,*1992 ; Bezzar-Bendjazia *et al.,* 2016)

De plus, l'exposition à des facteurs de stress tels que les pesticides peut entraîner toute une série d'effets comportementaux qui peuvent être transmis à la génération suivante (Lu *et al.,* 2020). Notre équipe a récemment démontré que *D. melanogaster* peut moduler son comportement sur la base d'expériences antérieures avec l'azadirachtine (larves du troisième stade) induisant une modification de la ponte, de la préférence du site d'oviposition, et de la

sélection des aliments en augmentant l'aversion des adultes envers ce biopesticide (Bezzar-bendjazia *et al.,* 2016 ; Kilani-Morakchi *et al.,* 2017).

En effet, ces décisions sont souvent influencées par l'expérience sensorielle individuelle qui peut laisser une empreinte dans la vie adulte (Dukas, 2008 ; Abed-Vieillard *et al.,* 2014) modifiant ainsi la réponse comportementale en fonction d'un apprentissage induit par l'expérience par des modifications neurophysiologiques chez les insectes (Dukas, 2008 ; Little *et al.,* 2019).

Nos résultats montrent qu'une exposition larvaire unique à l'azadirachtine a un impact négatif sur l'olfaction, la gustation et la locomotion des adultes de *D. melanogaster*. Dans nos expériences d'olfactométrie, les mouches de la génération parentale des deux sexes, traitées précédemment au stade larvaire, ont montré une aversion au biopesticide en évitant l'odeur de l'azadirachtine. Néanmoins, aucun effet n'est noté sur les mouches témoins et sur la génération F1.

Ces résultats corroborent largement les conclusions d'études antérieures, qui montrent que l'exposition à l'azadirachtine affecte la réponse olfactive des mouches et a donc des effets potentiels sur la préférence alimentaire (Kilani-Morakchi *et al.,* 2017). Nos résultats suggèrent que les mouches adultes pourraient générer une mémoire d'aversion olfactive pour éviter l'azadirachtine.

En effet, l'apprentissage et la mémoire olfactif sont principalement contrôlés par les corps pédonculés (CP) (Tanaka *et al.,* 2008 ; Zhu *et al.,* 2020 ; Silva *et al.,* 2020). L'information olfactive est reçue par les CP *via* les neurones de projections provenant des lobes antennaires (De Belle & Heisenberg, 1994 ; Wolf *et al.,*1998). Chez *D. melanogaster*, un circuit neuronal centré au niveau des CP est crucial pour la formation et le stockage de la mémoire des odeurs (Aso *et al.,* 2012). Les principaux neurones des CP sont représentés par des cellules de Kenyon qui reçoivent un signal dopaminergique médiateur d'un renforcement aversif pour la formation de la mémoire olfactive (Qin *et al.,* 2012).

Chez la drosophile, malgré un remaniement drastique du système chimio-sensoriel périphérique et central lors de la métamorphose, certaines structures centrales, comme le lobe gamma des CP sont relativement conservées du stade larvaire au stade adulte (Armstrong *et al.,* 1998 ; Blackiston *et al.,* 2008 ; Aso *et al.,* 2014). Il est donc possible que le lien entre l'expérience larvaire et le comportement adulte puisse être lié au maintien de structures

neuronales des CP après la métamorphose, permettant la persistance dans le cerveau adulte d'une mémoire formée au stade larvaire (Tully *et al.,* 1994 ; Lee *et al.,* 1999). En effet, différentes études récentes soutiennent l'hypothèse selon laquelle l'expérience larvaire peut affecter le comportement des adultes et générer une mémoire d'apprentissage à travers les étapes du développement (Gerber *et al.,* 2009 ; Flaven-Pouchon*et al.,* 2014 ; Abed-Vieillard *et al.,* 2014 ; Bezzar-Bendjazia *et al.,* 2016 ; Ramírez *et al.,* 2016).

De plus, selon Pham & Ray 2015, l'aversion olfactive semble être conservée chez les différentes espèces de drosophiles, ce qui permet de généraliser l'action de l'azadirachtine à d'autres espèces nuisibles de drosophiles telles que *Drosophila suzukii*.

Nos données révèlent également que l'azadirachtine peut réduire de manière significative la réponse gustative, en inhibant le réflexe d'extension du proboscis des mouches et que l'expérience préimaginale peut renforcer cette inhibition.

Nos résultats d'électrophysiologie montrent que l'azadirachtine est détecté uniquement par les sensilles de type (S) et ces résultats sont en accord avec Weiss *et al.,* 2011. Nos résultats révélent étagement que l'intensité de réponse dépend de la concentration de la molécule. Les sensilles gustatives répondent à des doses plus faibles d'azadirachtine et l'intensité des réponses est très faible à la dose la plus élevée. La spécificité de réponse des mouches traités a été confirmé en comparant l'intensité des réponses des mouches témoins et traités. Les mouches traitées répondent plus à l'azadirachtine contrairement à la caféine qui ne révèle aucune différence par rapport aux témoins

Une étude récente a démontré que l'azadirachtine A pourrait inhiber de manière significative le réflexe d'extension du proboscis chez *D. melanogaster* (yan *et al.,* 2017). L'azadirachtine est perçue par les mouches comme une substance amère et son action anti-appétante primaire est médiée par des chimiorécepteurs de contact responsables du comportement d'aversion au niveau des pièces buccales et des pattes de l'insecte, bloquant les sites des récepteurs glucidiques responsables de la stimulation de l'alimentation, ce qui conduit à une inhibition de l'extension du proboscis.

Chez les Drosophiles, les circuits de signalisation des CP influencent les comportements innés, et certaines composantes des CP sont nécessaires à la formation de la mémoire gustative tandis que les PER fournissent une lecture comportementale claire à la perception gustative (Chia & Scott, 2020). Par conséquent, les CP semblent représenter le

centre de la mémoire dans le cerveau de la mouche et sont considérés comme un site central pour la mémoire chimiosensorielle (Masek & Keene, 2016). Cependant, la manière avec laquelle les circuits neuronaux influencent le comportement inné d'extension du proboscis reste peu connue (Chia & Scott, 2020).

Les amines biogènes, l'octopamine (OA), la sérotonine (5-HT) et la dopamine (DA) sont nécessaires à la transmission des signaux de renforcement de nombreux types de mémoire associative chez la drosophile (Masek & Keene, 2016). Les apports d'octopamine aux CP contribuent au conditionnement olfactif, aversif ou appétitif (Waddell, 2013) ; la sérotonine est nécessaire à la régulation de la locomotion et du comportement alimentaire ainsi que pour l'apprentissage et la mémoire (Bacqué-Cazenave *et al.,* 2020) ; alors que la dopamine est impliquée dans plusieurs rôles dans les fonctions neuronales, de la modulation des comportements locomoteurs, à l'apprentissage et à la mémoire appétitive ou aversive (Barron *et al.,*2010 ; Waddell, 2013 ; Masek *et al.,* 2015 ; Kaun & Rothenfluh, 2017 ; Pavlowsky *et al.,*2018).

Une réduction des taux d'OA, de 5-HT et de DA au niveau cérébral et hémolymphatique après traitement à l'azadirachtine a été rapportée chez *Acherontia styx* (Awad *et al.,* 1997). De plus, l'azadirachtine interfère avec la quantité de 5-HT dans les organes endocriniens et principalement dans le cerveau des criquets (Banerjee & Rembold, 1992).

Moulin *et al.,* (2020) ont rapporté qu'un dysfonctionnement transitoire de la signalisation dopaminergique, peut entrainer des perturbations du comportement des adultes de *D. melanogaster* et générer une mémoire aversive gustative qui peut être transmise à la descendance. En effet, l'azadirachtine pourrait exciter différents groupes de neurones dopaminergiques tels que les PPL1 et augmenter ainsi la libération de dopamine responsable d'une mémorisation de l'aversion (Yan *et al.,* 2017).

L'inhibition du réflexe d'extension du proboscis, chez les mouches adultes, observées dans nos résultats peut être liée à l'action agoniste de l'azadirachtine sur les neurones dopaminergiques tels que les PPL1 et PAM qui régulent la mémoire gustative aversive chez la drosophile.

En effet, Aso *et al.,* (2012) ont rapporté que les substances toxiques induisaient chez *D. melanogaster* la formation d'une mémoire dépendante de la sécrétion de dopamine au niveau cérébral. Récemment, il a été démontré que l'activation de neurones spécifiques des CP

et des neurones dopaminergiques inhibait l'extension du proboscis provoquée par le saccharose, démontrant que de nombreux compartiments des CP inhibent ce comportement inné (Chia & Scott, 2020).

De plus, l'azadirachtine est connu pour son action neurotoxique par blocage partiel des canaux calciques voltage-dépendants au niveau du système nerveux centrale de la drosophile (Qiao *et al.,* 2014). Cela peut également contribuer à l'évitement de l'azadirachtine puisque la formation de la mémoire associative s'accompagne de changements dans l'activité de Ca^{2+} au sein des lobes des CP (Davis, 2011).

Chez les insectes tels que la drosophile, comme chez d'autres organismes, l'activité locomotrice est directement ou indirectement impliquée dans presque toutes les activités comportementales (Martin, 2003).

Notre étude a démontré que l'azadirachtine appliqué topiquement chez les larves du troisième stade affecte négativement la réactivité locomotrice des adultes de *D. melanogatser*. Les performances des adultes traités au stade larvaire étaient moins bonnes que celles des témoins. L'altération de la réactivité locomotrice des mouches sous l'effet de l'azadirachtine pourrait affecter plusieurs aspects de la biologie de *D. melanogaster*, tels que la recherche de nourriture, l'alimentation, la reproduction et l'oviposition. Le déclin de la géotaxie négative est lié, chez de nombreuses espèces de drosophiles, à des troubles neurologiques connus chez l'homme ; en effet, le comportement de géotaxie négative requiert un réseau de neurones sensoriels intacts (Zhu *et al.,* 2020).

L'impact négatif de l'azadirachtine rapporté sur les amines biogènes peut expliquer le déficit locomoteur observé au cours de nos expérimentations. En effet, la dopamine est un neurotransmetteur clé dans le système nerveux de la drosophile pour un contrôle adéquat des activités locomotrices (Zhu *et al.,* 2020). En agissant comme un agoniste sur les récepteurs de la dopamine (Yan *el al.,* 2017), l'azadirachtine pourrait conduire au dysfonctionnement de la géotaxie observé dans nos résultats. De plus, l'effet neurotoxique de l'azadirachtine sur le système nerveux central (SNC) des mouches *via* l'inhibition de la transmission cholinergique excitatrice et le blocage partiel des canaux calciques (Qiao *et al.,* 2014) peut également avoir un impact négatif sur le comportement locomoteur.

L'azadirachtine est généralement associé à une activité anti-appétante très marquée et même à un comportement d'évitement chez un grand nombre d'espèces d'insectes, notamment

les hémiptères (Kumar & Poehling, 2007), les lépidoptères (Charleston *et al.,* 2006 ; Shannag *et al.,* 2015), les orthoptères (Capinera & Froeba, 2007), les coléoptères (Baumler & Potter, 2007) et les diptères (Kilani-Morakchi *et al.,* 2017).

Cet effet anti-appétant primaire, est médié par les chimiorécepteurs de contact et est impliqué dans la sélection et la discrimination des aliments (Lee *et al.,* 2010). Un signal envoyé au système nerveux central (SNC) permet à l'insecte d'éviter de s'approcher ou de se nourrir d'une substance potentiellement dangereuse.

L'effet anti-appétant primaire de l'azadirachtine semble être médié par les sensilles gustatives et est lié à un effet inhibiteur sur la vitesse de déclenchement des cellules sensibles au sucre en activant les cellules gustatives sensibles à l'amertume. Plusieurs espèces d'insectes sont extrêmement sensibles à l'anti-appétance primaire de l'azadirachtine, et préfèrent mourir de faim plutôt que d'ingérer ce biopesticide (Mordue & Nisbet, 2010).

Un effet anti-appétant secondaire, comprenant une réduction à long terme de la consommation alimentaire et des effets néfastes directs sur différents tissus d'insectes tels que les muscles, le corps gras et les cellules épithéliales de l'intestin a également été rapporté après traitement à l'azadirachtine (Koul & Isman, 1991 ; Timmins & Reynolds, 1992 ; Trumm & Dorn, 2000). Ces effets dépendent de l'espèce et de la concentration utilisée (Mordue *et al.,* 2010 ; Khosravi & Sendi 2013 ; Shannag *et al.,* 2015).

L'azadirachtine administré par application topique chez les larves de troisième stade réduit la consommation alimentaire et perturbe le comportement alimentaire chez *S. littoralis* (Martinez & van Emden, 1999). Chez les larves de deuxième stade de *Spodoptera eridania*, la consommation à court terme (2 jours) d'aliments traités à l'azatrol, une formulation commerciale d'azadirachtine, réduit le taux de consommation relatif, la quantité d'aliments ingérés, le taux de croissance, la digestibilité et le taux d'assimilation des aliments pendant toute la période de développement larvaire (Shannag *et al.,* 2015).

Chez *D. melanogaster*, une seule application topique d'azadirachtine sur les larves du début du troisième stade réduit de manière significative la quantité de nourriture ingérée par les larves et perturbe la capacité de l'insecte à digérer la nourriture en interférant avec les activités des enzymes digestives (Bezzar-Bendjazia *et al.,* 2017). Cet effet est également observé chez les adultes qui survivent à l'exposition pré-imaginale, procurant un effet

anti-appétant à long terme à travers les stades de développement pouvant renforcer l'activité insecticide de l'azadirachtine (Kilani-Morakchi *et al.,* 2017).

La réduction de la prise de nourriture et la capacité à convertir les nutriments en biomasse peuvent expliquer en partie les effets observés au cours de notre étude sur la reproduction et le développement de *D. melanogaster*. En effet, les mécanismes hormonaux contrôlant le développement, le métabolisme et la reproduction sont partiellement régulés par la prise de nourriture (Bernard & Lagadic, 1993 ; Klepsatel *et al.,* 2020).

Conclusion

5 Conclusion et perspectives

Les insectes peuvent moduler leur comportement sur la base d'expériences antérieures en début de vie et les divers changements induits par les insecticides chez la génération directement exposée peuvent persister chez les générations suivantes. Nos expérimentations ont été menées dans le but d'évaluer les effets d'une exposition unique d'une formulation commerciale d'azadirachtine, sur la reproduction, la nutrition et les propriétés aversives d'un insecte modèle, *Drosophila melanogaster*.

Le bio-insecticide a été administré par application topique (1µl/larve), sur des larves du début du troisième stade larvaire (L3) de *D. melanogaster* à deux doses 0,28 et 0,67 µg correspondants respectivement à la dose létale 25% et 50% (DL_{25} et DL_{50}) des stades immatures. Les travaux antérieurs de notre équipe ont révélé que l'exposition pré-imaginale à cette molécule affecte la reproduction et la survie des adultes survivants de la même génération. Ce travail de thèse en continuité avec les précédent travaux tend à évaluer les effets physiologiques et comportementaux de l'azadirachtine sur les générations successives jusqu'à restauration complète des paramètres étudiés.

L'azadirachtine affecte la fécondité des femelles en réduisant significativement le nombre d'œufs pondus chez les deux générations testées (P : exposées et F1 : non-exposées) avec un effet dose-réponse et une réduction plus marquée chez la génération P. Une restauration complète de la fécondité des insectes est observée chez la génération F2. De plus une aversion plus marquée à l'azadirachtine a été enregistrée chez les mouches ayant préalablement été exposées à l'insecticide au stade pré-imaginale.

L'exposition préimaginale à l'azadirachtine affecte le développement de *D. melanogaster* en réduisant significativement le nombre d'individu pour chaque stade de développement ainsi que la survie des adultes de la génération parentale (exposée). Un effet moins marqué sur la survie des adultes est observé chez la génération F1 (non exposée). Aucun effet n'est noté chez la génération F2. L'azadirachtine prolonge la durée du développement des stades larvaire et pupal, et affecte également la morphologie des mouches en induisant plusieurs malformations.

L'azadirachtine appliquée topiquement chez les larves du troisième stade, affecte négativement la réactivité locomotrice des adultes de *D. melanogaster* de la génération P avec une baisse des performances des adultes traités comparativement aux témoins.

Le biopesticide affecte également le choix olfactif et gustatif des adultes de *D. melanogaster.* En effet, les résultats obtenus indiquent une aversion marquée pour l'azadirachtine. Une augmentation de la sensibilisation des sensilles gustatives chez les insectes ayant préalablement été exposés au traitement est notée au cours d'expériences d'électrophysiologie traduisant un comportement d'évitement à cette molécule.

L'azadirachtine affecte également la quantité de nourriture ingérée chez les adultes avec un effet plus maquée chez les insectes ayant survécu au traitement larvaire.

L'unique exposition pré-imaginale (L3) à l'azadirachtine interfère donc avec la fécondité, le développement, la survie, la locomotion, le choix du site de ponte ainsi que le système chimiosensoriel et la consommation alimentaire de *D. melanogaster*. Le traitement augmente l'effet aversif induit par l'azadirachtine sur deux générations successives, Cet effet à long terme d'une seule exposition larvaire à l'azadirachtine, à travers les stades de développement et des générations (effet transgénérationnel) représente un avantage certain à exploiter dans les programmes de lutte intégrée suggérant une possibilité de réduction des fréquences d'application.

A l'avenir il serait intéressant de compléter le présent travail par :

- Une étude de l'impact de l'azadirachtine sur la neurotransmission et les amines biogènes.
- Une analyse épigénétique pour comprendre la transmission trans-générationnelle.
- L'analyse des résidus de l'azadirachtine par le biais de l'HPLC.

Résumés

6 Résumés

6.1 Résumé

Si les effets de toxicité aigüe des pesticides sont assez bien connus, les effets à court et long terme d'une exposition non létale à un stade juvénile sont beaucoup moins étudiés. Dans ce travail, nous avons examiné les effets à long terme du Neem-Azal, qui contient 1% d'azadirachtine A, sur un insecte modèle, *Drosophila melanogaster*. Deux doses (DL_{25} : 0,28 µg, D_{50} : 0,67 µg correspondant respectivement à 25% et 50% de mortalité cumulée des stades immatures) ont été utilisées par application topique sur des larves du début du stade larvaire (L3). Les effets de cette unique exposition à l'azadirachtine ont été testés sur plusieurs générations successives jusqu'à restauration des paramètres étudiés.

L'exposition unique, en période pré-imaginale, à l'azadirachtine affecte la fécondité des femelles en réduisant significativement le nombre d'œufs pondus chez deux générations successives (P : exposée, F1 : non exposée). Une restauration complète de la fécondité est notée chez la génération F2. Le traitement larvaire affecte également le choix de ponte des femelles qui ont survécu au traitement avec une claire préférence de ponte sur un milieu témoins par rapport au milieu traité à l'azadirachtine et ce pour toutes les générations testées. Les mouches traitées à l'azadirachtine (début de stade L3) montrent une aversion plus marquée au biopesticide comparativement aux mouches "naïves".

Chez la génération parentale, l'exposition précoce à l'azadirachtine affecte le développement des adultes en réduisant le nombre de descendants, en induisant un retard du développement larvaire et pupal ; en biaisant le sex-ratio en faveur des mâles et en provoquant plusieurs anomalies morphologiques. L'azadirachtine réduit de manière significative la survie des adultes chez deux générations successives et ce comparativement aux témoins. De plus, l'azadirachtine affecte la réactivité locomotrice des adultes en réduisant significativement les scores géotaxiques obtenus chez les adultes des deux sexes en comparaison aux témoins.

L'azadirachtine affecte également la quantité de nourriture consommée par les mouches et perturbe le système chimiosensoriel des adultes en affectant leurs choix olfactifs et gustatifs. Les mouches préalablement traitées (début de stade L3) à l'azadirachtine ont une aversion plus marqué à cette molécule et évitent son odeur. Le biopesticide induit également une inhibition de l'extension du proboscis des adultes des deux sexes avec un effet plus marqué chez les

adultes préalablement traités au stade larvaire. Les réponses électrophysiologiques des sensilles gustatives montrent une augmentation de la sensibilité des sensilles gustatives à l'azadirachtine chez les mouches traitées au stade larvaire confirmant l'aversion plus marquée à cette molécule.

Mots clés : Azadirachtine, *D. melanogaster*, fécondité, développement, gustation, olfaction, locomotion.

6.2 Abstract

While the acute toxicity of xenobiotics on organisms is well known, long term effects of intermittent exposures are less studied. Here we examined the effects of a single larval exposure to a commercial antifeedant and insecticide Neem-Azal (containing 1% azadirachtin A), on *Drosophila melanogaster*. Two doses (LD_{25} : 0.28 µg, LD_{50} : 0.67 µg corresponding respectively to 25% and 50% cumulative mortality of immature stages) were used by topical application on early third instar larvae (L3). The possible adverse effects of this prior single exposure to azadirachtin experienced by the preceding generations were evaluated over three successive generations until full restoration of the studied parameters.

The single pre-imaginal exposure of azadirachtin affects female fecundity by significantly reducing the number of eggs laid over two successive generations (P and F1). Full restoration of affected fecundity was noted in the second generation (F2). Larval treatment also affects oviposition choices, the surviving flies avoiding treated substrates more than control flies for all tested generations.

Early exposure to azadirachtin also affects adult's development by reducing the number of organisms, delay larval and pupal development ; male biased sex ratio and induced morphological alterations. This exposure significantly reduces adult's survival during two successive generations. Moreover, azadirachtin was found to affect *Drosophila* adult's locomotor reactivity by significantly decreased the geotactic positioning scores of adults of both sexes as compared to controls.

Azadirachtin affects the amount of food intake by the flies and disrupts the chemosensory system of the adults by affecting their olfactory and gustatory choices. Results showed that previously treated flies (3rd instar larvae) showed an aversion to the biopesticide avoiding azadirachtin odor. The biopesticide also inhibited the proboscis extension response in the adults of both sexes with more marked effects in treated flies. The electrophysiological studies of gustatory response showed an increase of sensibility of gustatory sensilla to azadirachtin previously exposed flies confirming the more marked avoidance to this molecule.

Keywords : Azadirachtin, *D. melanogaster*, fecundity, development, tasting, olfaction, locomotion.

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Annexes

8 Annexes

8.1 Annexe 1. Production scientifique

- **Publications Internationales (4) :**

Maroua Ferdenache, Radia Bezzar-Bendjazia, Frédéric Marion-poll, Kilani-Morakchi Samira. **2019.** Transgenerational effects from single larval exposure to azadirachtin on life history and behavior traits of *Drosophila melanogaster*. *Sci Rep.* 9(1):17015.

Bilel Boulahbel, **Maroua Ferdenache**, Karima Sifi, Kilani-Morakchi Samira. **2021.** Larval exposure to azadirachtin induced locomotor deficits, and impairs olfactory and gustatory preference in adults of *D. melanogaster* (Diptera : Drosophilidae). Submitted to : *J asia-pac entomol*.

Kilani-Morakchi Samira, Radia Bezzar-Bendjazia, **Maroua Ferdenache**, Nadia Aribi. **2017.** Preimaginal exposure to azadirachtin affects food selection and digestive enzymes in adults of *Drosophila melanogaster* (Diptera : Drosophilidae). *Pestic Biochem Physiol.* **140**, 58–64.

Radia Bezzar-Bendjazia, Kilani-Morakchi Samira, **Maroua Ferdenache**, Nadia Aribi. **2017.** Azadirachtin induced larval avoidance and antifeeding by disruption of food intake and digestive enzymes in *Drosophila melanogaster* (Diptera : Drosophilidae). *Pestic Biochem Physiol.* **143**, 135–140.

- **Communications Internationales (7) :**

Maroua Ferdenache, Frédéric Marion-poll, Kilani-Morakchi Samira. **2019.** Sensitization of adult gustatory responses to azadirachtin following larval exposure in *Drosophila melanogaster* (Diptera : Drosophilidae). Eighth International Symposium on Molecular Insect Science 7-10 July 2019, Sitges, Spain. **Communication Poster.**

Maroua Ferdenache, Kilani-Morakchi Samira. Frédéric Marion-poll. **2019.** Pre-imaginal exposure to azadirachtin affect feeding in *Drosophila melanogaster*. 26th European Drosophila Research Conference 5-8 September 2019 Lausanne, Switzerland. **Communication Poster.**

Maroua Ferdenache, Kilani-Morakchi Samira, Radia Bezzar-Bendjazia, Frédéric Marion-poll. **2019.** Single larval exposure of azadirachtin affects development and

survivorship of Drosophila melanogaster (Diptera : Drosophilidae). 19ème Colloque Biologie de l'Insecte du 26 au 28 Juin 2019, Albi, France. **Communication orale**.

Maroua Ferdenache, Radia Bezzar-Bendjazia, Frédéric Marion-poll, Kilani-Morakchi Samira. **2018.** Impact de l'exposition pré-imaginale à l'azadirachtine sur la fécondité et la survie de deux générations successives d'adultes de *Drosophila melanogaster* (Diptera : Drosophilidae). 6ème Congrès Franco-Tunisien de Zoologie, au 6ème Congrès Franco-Maghrébin de Zoologie et aux 119èmes Journées Annuelles de la SZF - Paris 2018. 21 au 23 Novembre 2018. **Communication orale**.

Maroua Ferdenache, Kilani-Morakchi Samira, Radia Bezzar-Bendjazia, Frédéric Marion-poll. **2018.** Preimaginal exposure to azadirachtin affects digestive enzymes in adults of *Drosophila melanogaster* (Diptera : Drosophilidae). European Food Chemistry Congress" on July 26-27, 2018 at Amsterdam, Netherlands. **Communication orale.**

Maroua Ferdenache, Radia Bezzar-Bendjazia, Frédéric Marion-poll, Kilani-Morakchi Samira. **2018.** Impact de l'expérience larvaire sur la perception olfactive et gustative d'un biopesticide, l'azadirachtine chez *Drosophila melanogaster*. Conférence Internationale Francophone d'Entomologie (CIFE), SupAgro à Montpellier du 2 au 6 Juillet 2018. **Communication poster**

Kilani-Morakchi Samira, Radia Bezzar-Bendjazia, **Maroua Ferdenache. 2018.** Preimaginal exposure to azadirachtin affects digestive enzymes in adults of *Drosophila melanogaster* (Diptera : Drosophilidae). World Congress on Enzymology and Molecular Biology ", August 13-14, 2018 at Paris, France. **Communication orale.**

8.2 Annexe 2. Les molécules testées

Tableau 4 : *Liste des molécules utilisées*

Molécule	**Source**	**Pureté**	**Formule brute**
NeemAzal®-T/S	Trifolio	1% azadirachtine A	$C_{35}H_{44}O_{16}$
D-Saccharose	Sigma	≥99.5%	$C_{12}H_{22}O_{11}$
Caféine	Sigma	-	$C_8H_{10}N_4O_2$
Tricholine citrate	Sigma-Aldrich	≥65 % dans H_2O	$C_6H_5O_7$
Brilliant Blue R	Sigma-Aldrich	Pure	$C_{45}H_{44}N_3NaO_7S_2$

8.3 Annexe 3. Composition du milieu axénique utilisé pour l'élevage de *Drosophila melanogaster.*

Pour préparer 10 litres de milieu nutritif

120g d'Agar-Agar

830g de levure

830g de gaude

96g de nipagine

500mL d'alcool

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

M. Ferdenache[1,2,4], R. Bezzar-Bendjazia[1], F. Marion-Poll[2,3] & S. Kilani-Morakchi[1,4*]

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

The effect of insecticides and other toxicants on insects have been traditionally assessed using measures of the acute mortality as a single endpoint and have relied on the determination of the acute lethal dose/concentration[1]. However, in addition to the direct effect on lethality these compounds may also impair various key biological traits of the individuals that survive exposure through physiological and behavioral effects[1,2]. Among physiological effects, developmental success, morphological deformities, adult longevity, sex ratio, fertility and fecundity are commonly estimated[3,4]. Behavioral effects may be manifested as impairment in insect mobility, learning ability, host finding, sexual communication as well as feeding and oviposition behavior[5–10]. An accurate assessment of these effects is crucial to acquire knowledge on the overall insecticide efficacy for long-term management of pest insect populations, as well as on their selectivity toward non-target species[11]. Indeed, when studying susceptibility of organisms towards insecticides, and beside the short term influences on the directly exposed individuals, it is important to take into account the entire life-history as a comprehensive method for evaluating the total effect on insect population, including the impacts on the next generation which have important implications for the success of an Integrated Pest Management (IPM) program[2,4].

Among nowadays the insecticides used in sublethal effect studies, the botanical insecticides have been the subject of an increasing number of academic research as a potential option for an environment friendly pest management tools[12,13] due to their rapid degradation in the environment, low mammalian toxicity, low risk of resistance development in target pest populations and good selectivity to non-target arthropods[14–18]. Azadirachtin (AZA), a

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natural tetranortriterpenoid compound extracted from the neem tree, *Azadirachta indica*[19], is considered as one of the most promising plant compounds for pest control in organic agriculture[14,20]. AZA shows variable effects on insects including the model insect *Drosophila melanogaster*[21,22]. This triterpenoid acts as sterilant, insect growth regulators by disruption of the endocrine system, repellent, oviposition and feeding deterrent by activating bitter sensitive gustatory cells[23,24]. Larval exposure of *D. melanogaster* to sublethal doses of azadirachtin was found to affects various aspects of their physiology including digestive enzymes[25] and this effect is also further observed in the adults[10]. This pre-imaginal exposure affects not only the physiology and the fitness of flies but also adults oviposition and feeding preference[7,10].

Most studies on sublethal effects of insecticides are related to continuously or repeated exposure. This exposure provokes a generalized stress and activating a detoxification response such as up-regulated of cytochrome P450 genes which might lead to the detoxification of insecticide and even the development of resistance[26]. Moreover, the up-regulation is thought to provide versatility in environmental adaptation[27]. In botanical insecticide the potential fast desensitization to a feeding deterrent was reported[28,29]. Individual insects initially deterred by feeding inhibitor become increasingly tolerant due to repeated or continuous exposure[29]. Bomford and Isman[15] reported an habitation to pure azadirachtin in the tobacco cutworms which become less sensitive to the antifeedant properties of azadirachtin, but not to a neem containing a same absolute amount of azadirachtin. This might have an important implication to avoid desensitization to commercial neem-based insecticides which contains additional non AZA-compounds[15]. Larval exposure to Neem Azal, a commercial Azadirachtin-rich based formulation, was found to enhance avoidances of this compound in adults of *D. melanogaster* surviving from previously treated larvae[10,25]. This long-lasting avoidance is related to conditioned aversion and may be related to another mechanism such as sensitization[30,31] which also generally occurs after long term or repeated exposure and may increase avoidance to noxious stimulus[32]. Moreover, increasing evidence has highlighted the critical role of early life experience in adult physiology and behavior in insect[33]. Recent studies have revealed that insect can modulate their behavior on the basis of previous experiences early life and that various insecticide-mediated changes in the directly exposed generation can persist into the subsequent non-exposed generations[34,35]. Previously, we have focused on the impact of larval exposure to azadirachtin on adult's fitness (fecundity, survival) and oviposition site preference of the parental generation of *D. melanogaster* as a model organism for testing insecticide activity[7]. Current study aimed to evaluate, the possible adverse effects of this prior single exposure to azadirachtin experienced by the preceding generations on life table and oviposition site preference of the filial generations. We monitored the oviposition site preference, fecundity, development, sex ratio, survival and morphological abnormalities of exposed and non-exposed generations. All these parameters were investigated over generations until their restoration to predict the outcome of azadirachtin use on pest management practices.

Results

Fecundity and oviposition site preference. Azadirachtin, topically applied on the 3rd instar larvae (LD_{25} and LD_{50} of immature stages) affect fecundity of females by a significant reduction of the number of eggs laid as compared to controls ($KW = 24.73$; $p < 0.001$). This reduction was observed over two successive generations (parental and F1), however, the total eggs laid was higher in the unexposed generation (F1) than in parental (P) ones ($KW = 50.89$; $p < 0.001$) (Fig. 1). Full restoration of affected fecundity was noted in the second generations (F2).

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

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

The results concerning the dual choice experiments (Fig. 2) revealed an oviposition preference in control medium than in treated medium for all tested generations (P, F1 and F2). Furthermore, flies previously exposed to azadirachtin (early 3rd instar larvae) showed a highest aversion to this substance compared to naïve flies and led fewer eggs for the two first generations (P and F1) with a more marked effects for parental generation ($P < 0.001$).

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

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

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

Analyses of development. Results from development analysis of *D. melanogaster* are given in Tables 1 and 2, respectively for parental (exposed) and F1 (non-exposed) generation. Treatment of early third instar larvae at two tested doses (LD_{25} and LD_{50}) decreased the number of larvae, pupae and the final number of organisms

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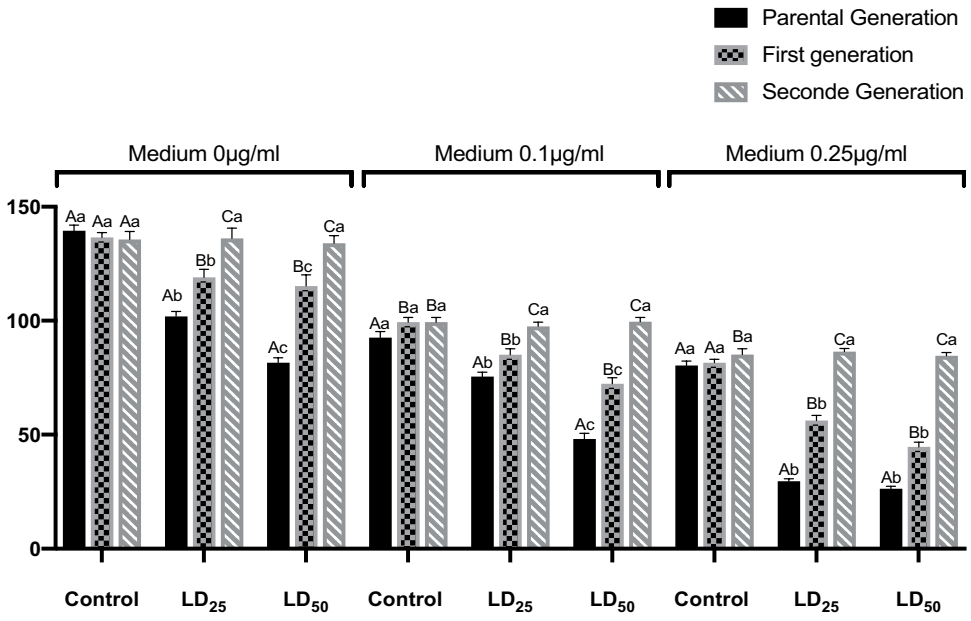

Figure 1. Effect of azadirachtin (LD_{25} and LD_{50}), topically applied on early third instars larvae of *D. melanogaster* on fecundity of females (number of eggs laid) subjected to non-choice experiments ($m \pm SE$; $n = 12$ replicates of 3 flies). Different small letters indicate a significant difference between control and treated individuals of the same medium ($P < 0.05$). Different capital letters indicate a significant difference between generations of the same medium ($P < 0.05$).

of parental generation with a dose-dependent relationship as expressed by the FNO which is always negative for the treated series. The development of F_1 *D. melanogaster* doesn't seem to be affecting by the early treatment of the parental generation. However, the FNO of tested flies (LD_{25}, LD_{50} and control) in treated medium was significantly lower than in the control medium for both generations. There is no difference between the number of organisms reached the pupae stage and the final number of organism in both generations. In addition, treatment of early third instar larvae increased significantly ($p < 0.001$) the duration of larval and pupal development as expressed by T_{50}, with dose-dependent manner only for the Parental generation (exposed) as compared to controls. There is no difference between the T_{50} of the tested flies in both treated and untreated medium.

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

Pre-imaginal exposure of azadirachtin induced a male-biased sex ratio only for the parental generation with a dose-dependent relationship (Fig. 5). Kruskal-Wallis test revealed significant effects between the different tested insect (Control, LD_{25} and LD_{50}) in untreated medium ($KW = 9.30$; $p = 0.0095$), medium 0.1 µg/ml ($KW = 8.02$; $p < 0.0181$) and medium 0.25 µg/ml ($KW = 18.85$; $p < 0.0001$) for the parental generation.

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

For the control series no mortality was recorded for both tested generations. For the treated series, the lowest dose (LD_{25}) decline the adult's survival to 49% for males and 36% for females of the P generation versus 94% for males and 84% for females of the F1 generation. The highest dose (LD_{50}) induced more marked effects on adult's survival with 27% for males and 16% for females of the P generation and 81% for males and 64% in females for the F1 generation. Survival of 100% was noted for males and females of the F2 generation.

Discussion

Azadirachtin's impact on reproduction have been reported on different insect species[21,41–45]. Our study has demonstrated that a single azadirachtin treatment (LD_{25}/LD_{50}) of *D. melanogaster* larvae reduced eggs number affecting negatively the fecundity of surviving females, not only through direct sublethal effects in exposed individuals, but also through transgenerational effects on F1individuals that were never directly exposed to the insecticide.

Oviposition is a complex and critical activity in the life cycle of an insect with a variety of factors that influence both physiology and subsequent behavior, that lead to egg deposition by an insect which tries to ensure

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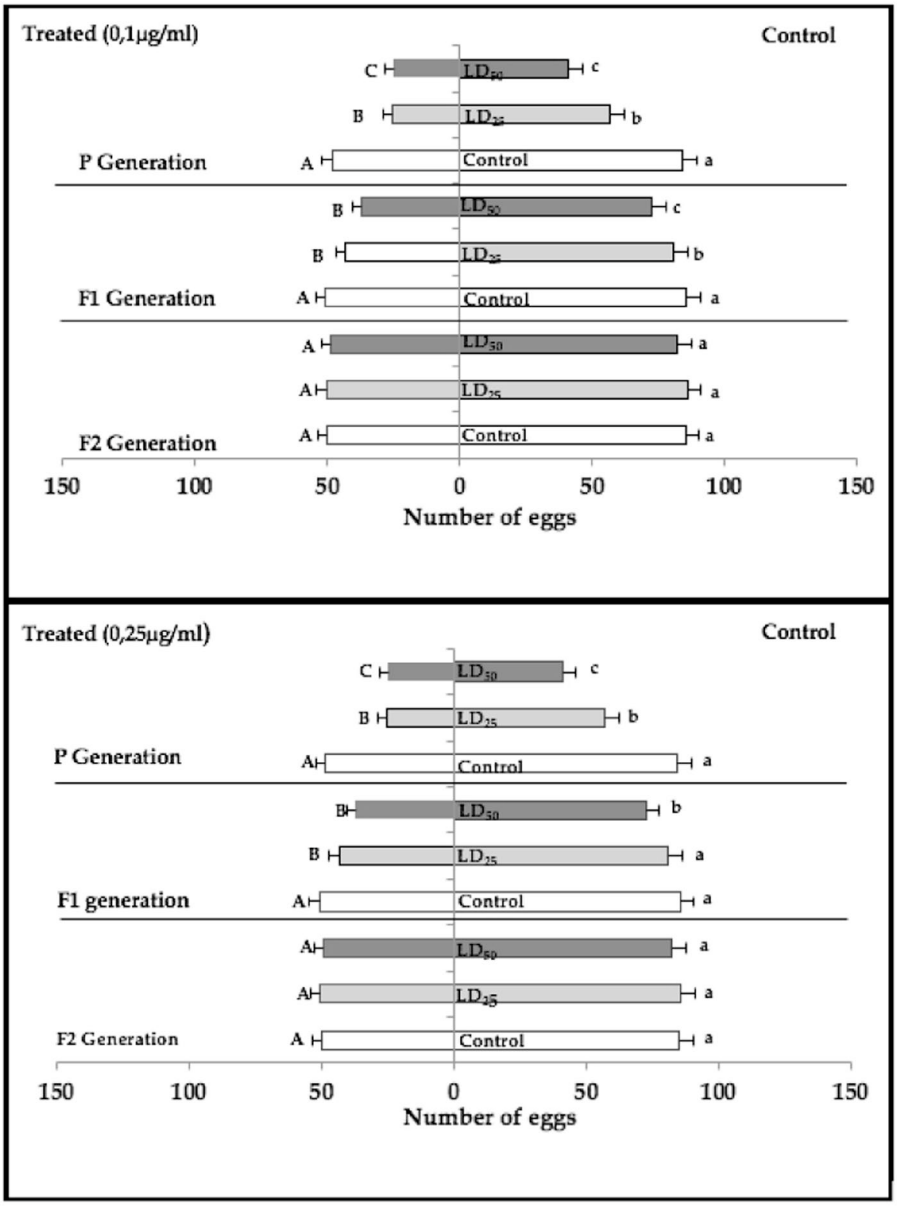

Figure 2. Egg-laying preference (m ± SE; n = 12 replicates) of female adults of *D. melanogaster* subjected to a free-choice test on food treated with azadirachtin at two doses (0.1 µg/ml and 0, 25 µg/ml). Different small letters indicate a significant difference between control and treated individuals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

safety to their progeny. Reduced fecundity and fertility after azadirachtin treatment has been reported in many insects including *Spodoptera littoralis*, *D. melanogaster, Galleria mellonella, Dysdercus cingulatus, Tuta absoluta* and *Helicoverpa armigera*[17,41,43–46] and could be correlated to the negative action of azadirachtin on yolk protein synthesis and/or its uptake into oocytes[21].

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

In *Anopheles stephensi*, azadirachtin treatment has led to abnormal ovaries structure with a complete arrest of oogenesis, vitellogenesis and vitelline envelope formation impairment, as well as follicle cells degeneration[48]. Ovaries of azadirachtin-treated females of *Heteracris littoralis* also showed complete shrinkage with suppressed oocyte growth[49], in addition to mitochondria disintegration and follicular cells destruction[49]. Moreover, Azadirachtin reduced mating success in *D. melanogaster* flies and negatively affected cyst and oocyte number and size[45]. Its treatment also affected food intake and digestive enzyme activity in the midgut, in these species[10].

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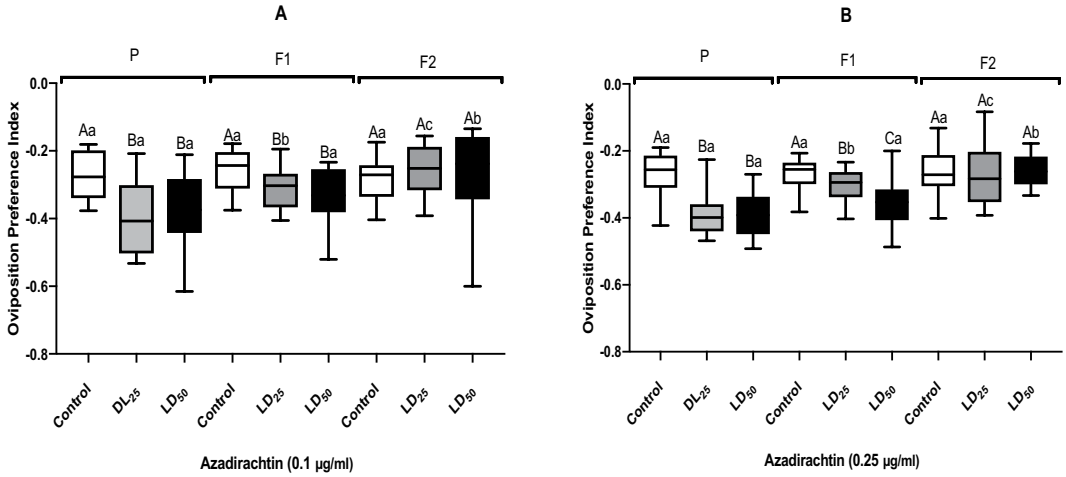

Figure 3. Oviposition preference index (m ± SE; n = 12 replicates) of female adults of *D. melanogaster* subjected to a free-choice test on food treated with azadirachtin at two doses (**A**: 0, 1 µg/ml; **B**: 0, 25 µg/ml). Different small letters indicate a significant difference between the same dose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

This may disturb oogenesis and vitellogenesis since ecdysone and JH rates are affected by nutrient availability, which acts as positive regulator on insulin pathway conferring ovaries the necessary signalling for a normal oogenesis[50,51].

In addition, tested flies of all generations preferred the control medium for oviposition avoiding the azadirachtin ones for the two tested doses and conditions (no-choice and free choice). A low oviposition rate of non-exposed (naïve) flies in azadirachtin-treated areas could be due to the known repellent, deterrent, and locomotor stimulation effects of azadirachtin and other neem-based insecticides, which were reported by Silva *et al.*[52] for medflies *Ceratitis capitata*. Valencia-Botín *et al.*[53] also suggest that the repellent property of neem extracts is the major factor responsible for the reduction of eggs number in *Anastrepha ludens* (Loew)[53]. The ovipository behavior inhibition may have a valuable impact in pest control.

In addition, flies who have already been treated (third instar larvae of P generation) showed an increased aversion to azadirachtin in comparison to the naïf flies. This continued for two successive generations (P and F1). When oviposition sites were treated with azadirachtin or other neem-based compounds, oviposition repellency, deterrency, or inhibition occurred in several insect species that are able to detect the bioinsecticide on the treated surface[7,14,43,54,55]. The capacity of insects to retain memory from early life exposure affecting the adult response was reported[38,56–58]. In *D. melanogaster*, females avoided oviposition on sites containing azadirachtin after larval exposure to the bio-insecticide[7].

Here, we have reported for the first time that the negative effects of a single larval exposure to azadirachtin can also be passed on to the F1 generation (transgenerational effects). Environmental toxicants such as insecticide are able to provoke epigenetic alterations, which can be inherited in the next generations[59]. This may explain the reduced fecundity and oviposition avoidance in the non-exposed generation (F1).

Our study has also demonstrated that azadirachtin applied during the third larval instar of parental generation (LD_{25} and LD_{50}) negatively affected various life traits of *D. melanogaster*, in a dose-dependent manner, as it significantly reduced larval, pupation, and emergence rate of the exposed generation. The biopesticide also significantly prolonged the larval and pupation period of development inducing important delays in immature stages development and affect sex ratios (with fewer females in the offspring) of the same generation. Additionally, the treatment induced morphological alterations of larvae, pupae and adults only in the exposed generation (P generation). The most prominent abnormalities were burned larvae, larva-pupa intermediate, pupa-adult intermediate, deformed wings, smaller body size and deformed abdomen. The recorded malformations finally resulted in insect dead. Similar results were noted in *D. melanogaster*[37], *Hyalomma anatolicum excavatum*[60] and *Spodoptera litura*[22]. Finally, a decline in adult's survival was noted for the two successive generations with more marked effects among the P generation.

Azadirachtin is known to reduce pupation and eclosion rates of many insects like *Aphis glycines*[61], *Plodia interpunctella*[62], *Aedes aegypti*[63] and *D. melanogaster*[21]. A negative impact of azadirachtin on the immature stages was expected due to its insect's growth disruptor (IGD) action by suppressing haemolymph ecdysteroid and JH peaks[25,64]. Furthermore, azadirachtin is known to cause nucleus degeneration in the different endocrine glands (prothoracic gland, *corpus allatum* and *corpus cardiacum*) controlling insects moulting and ecdysis, which could act as generalised disruptor of neuroendocrine system[24]. Azadirachtin alters the growth and molting process of several insects by compromising their survival[7,20,43,65,66]. Lai *et al.*[67] reported that azadirachtin down regulated the expression of different genes that are linked to hormonal regulation. This could explain the developmental aberrations observed in our results. Azadirachtin is also known to affect *Drosophila* nutrient intake and metabolism compromising the nutritional signals, which result in a decrease in insect weights and growth rates, and thus

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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 0.1 µg/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 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

Table 1. Effect of larval exposure to azadirachtin on development of parental generation (exposed) of *D. melanogaster*. Letters indicate a significant difference between the different tested doses of the same medium for each stage of development ($P < 0.05$). Different capital letters indicate a significant difference same doses tested of different medium ($P < 0.05$). (m ± 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
DL_{25}	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
DL_{50}	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 0.1 µg/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
DL_{25}	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
DL_{50}	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
DL_{25}	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
$DL_{50'}$	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

Table 2. Effect of larval exposure to azadirachtin on development of first generation (non-exposed) of *D. melanogaster*. Letters indicate a significant difference between the different tested doses of the same medium for each stage of development ($P < 0.05$). Different capital letters indicate a significant difference same doses tested of different medium ($P < 0.05$). (m ± SE; n = 15 replicates).

resulting in smaller body size impacting survival[10,25,37,66]. The male biased sex ratio under azadirachtin treatment was reported in literature[67,68].

In conclusion, the present study indicated that pre-imaginal exposure to sublethal doses of azadirachtin affects the fecundity, oviposition preference, and the survival of *D. melanogaster* of parent generation as well as the non exposed F1 generation. The treatment triggered life history traits variation in the P generation.

Results demonstrated that a single azadirachtin application significantly reduced the survival of flies over two successive generations (P: exposed and F1: unexposed) while insects showed clear recovery in the survival rates in the second generation (F2). These findings reflect a long term effects through developmental stage and generations. This effect is consistent in the two first generations could be considered as advantage for pest control by compensating the well-known fast degradation by sunlight and low persistence of azadirachtin in environment (half-life DT_{50}: 1.7–25d)[23,69] and suggest a possible reduction both in application frequency and total amount of pesticide used.

Furthermore, the decreased fecundity and survival in P and F1 generations indicated an absence of resurgence induction in offspring, even after full restoration in F2, when parental flies were treated. This translated an absence of hermetic effect, which is considered as a serious problem of exposure to sublethal doses in agriculture.

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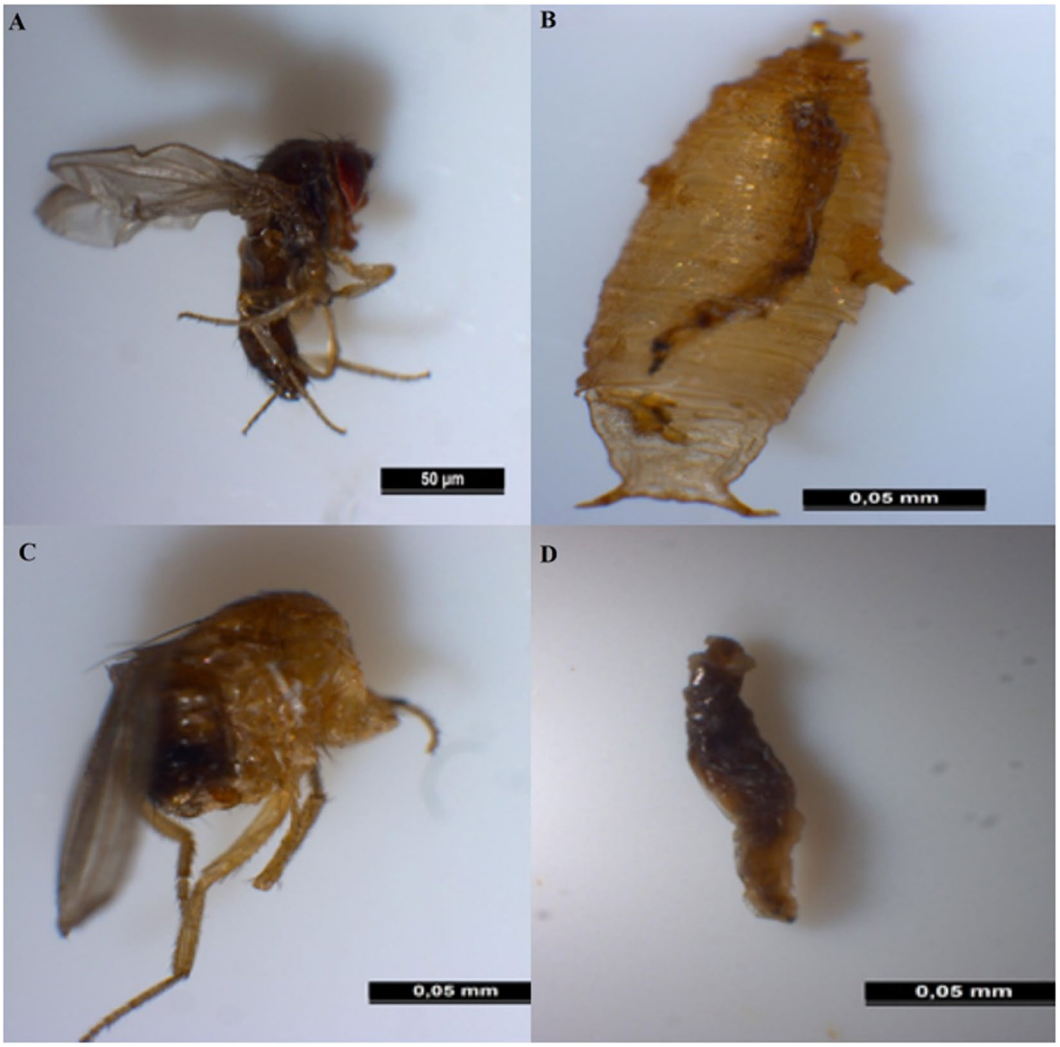

Figure 4. Examples of the most frequent malformations of *D. melanogaster* (n = 50). (**A**) Malformed abdomen and wings curved and smaller body shape; (**B**) dead adults inside pupae; (**C**) malformed adult; (**D**) burned larvae.

In addition, the treatment extended the aversive effect induced by azadirachtin to over two successive generations. This could contribute as push-pull strategies that increase its insecticidal effects in integrated pest management programs.

Material and Methods

Flies. Wild-type Canton-S strain of *D. melanogaster* flies were reared on artificial fly food (cornmeal/agar/yeast) at 25 °C, 70% humidity and 12D-12 L cycle[10].

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

Fecundity and oviposition site preference. We assessed the egg-laying performances of the females of *D. melanogaster* using a no-choice test. Three mated females (3 days old) that were pre-exposed to azadirachtin at the larval stage (LD_{25} and LD_{50}) were tested for 24 h in a petri dish (Ø = 65 mm) filled with 3 ml medium containing azadirachtin at two concentrations 0.1 and 0.25 µg/ml according to Bezzar-Bendjazia *et al.*[37] in addition to acetone as control medium. These concentrations were not lethal with the short exposure time (24 h) used. At the end of the test, flies were removed, and the number of eggs laid on each medium was counted. The control medium was used to test the possible effect of azadirachtin on female fecundity. The experiment was repeated 12

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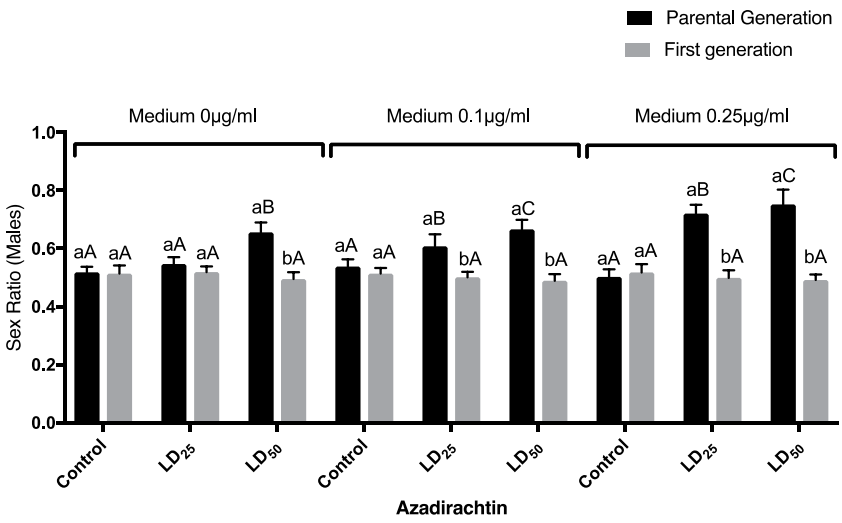

Figure 5. Effect of azadirachtin (LD_{25} and LD_{50}), topically applied on early third instars larvae of *D. melanogaster* on sex ratio of adults emerged. Different small letters indicate a significant difference between generations of the same medium ($P < 0.05$). Capital letters indicate a significant difference between control and treated individuals of the same medium ($P < 0.05$). ($m \pm SE$; $n = 15$ replicates).

times for each medium and each generation. Oviposition site preference was measured by means of dual choice experiments. Three fertilized females (3 days old) from controls and treated series (LD_{25} and LD_{50}) were allowed to oviposit for 24 h in a free choice egg-laying device. This device consisted of a two petri dishes either filled with control medium (acetone) or treated medium (azadirachtin: 0.1, 0.25 µg/ml). After 24 h, the egg-laying preference was assessed by counting the number of eggs laid in each medium. The test was performed for two successive generations with 12 replicates for each medium and generation.

Oviposition preference index (OPI) defined as (number of eggs on azadirachtin medium – number of eggs on control medium)/total number of eggs was calculated[38].

Development assays. Ten controls or pre-exposed (LD_{25} and LD_{50}) mated females (3 days old), named parental generation, were released into an oviposition box containing petri dishes filled with control (acetone) or treated medium (azadirachtin: 0.1 or 0.25 µg/ml) and left to lay eggs for 8 hours. At the end of the test, the flies were removed and a pool of 100 eggs for each experiment was transferred to a new petri dish containing the same medium. For all groups, we monitored the time course of larval development from egg to adult emergence by counting the number of third instar larvae, pupae, imagoes and their sex ratio, expressed as the number of males divided by the total number of emerged insects.

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

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

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

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

T = final number of organisms counted in treated medium.

C = final number of organisms counted in control medium.

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

Survival analysis of adults. Survival analysis was performed according to Linford *et al.*[40]. For each generation (P: exposed (LD_{25} and LD_{50}), F1: non-exposed) newly emerged adults were sexed and housed separately into a plastic vials (15 flies per vial) containing fresh food. Insects were transferred to new vial every 2 days. The flies were kept under observation for 15 days during which mortality was assessed every 24 h. Ten replicates were done for each dose and generation.

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

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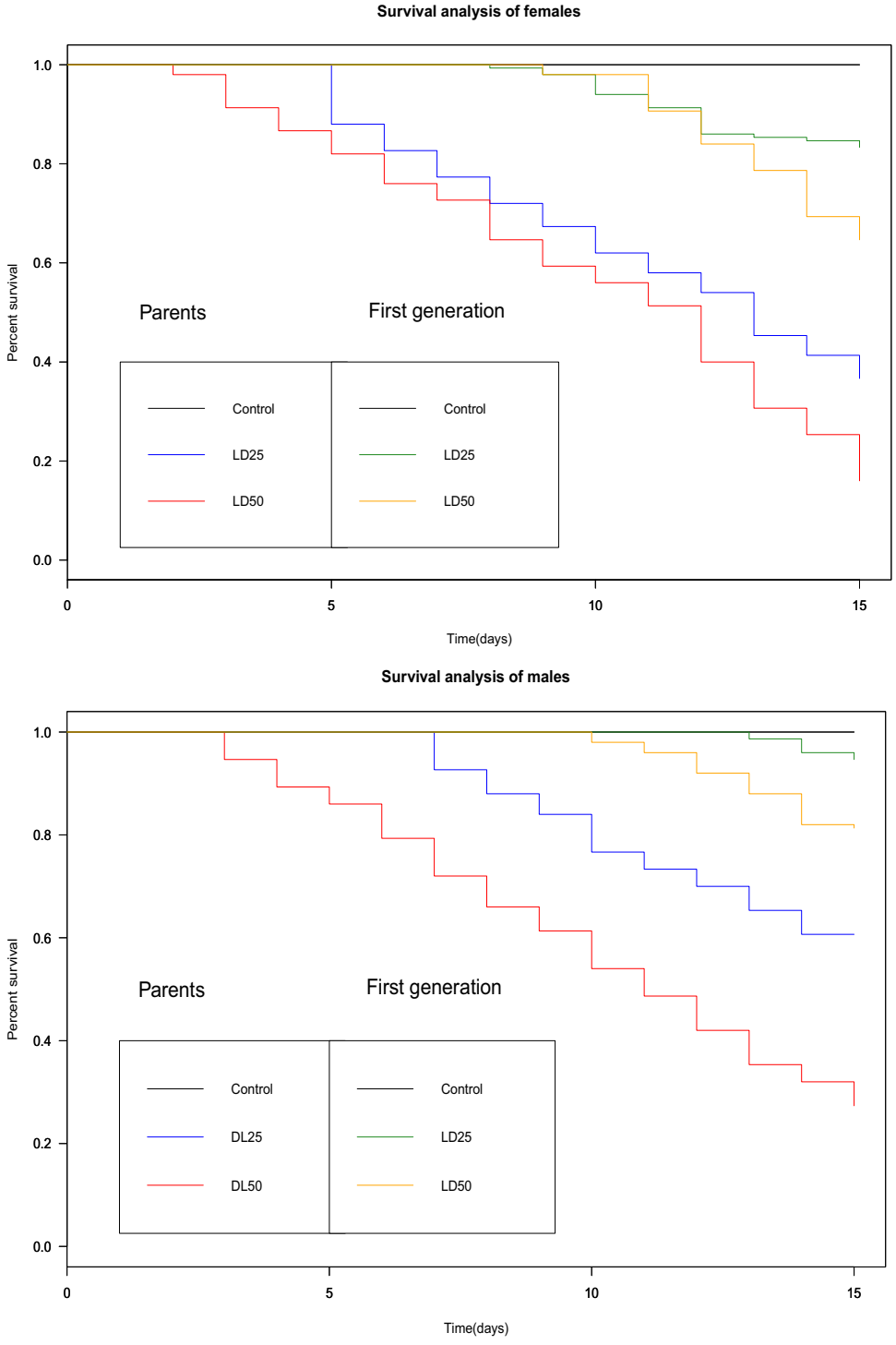

Figure 6. Effect of azadirachtin (LD_{25} and LD_{50}), topically applied on early third-instar larvae of *D. melanogaster* on the adult's survival (male and female) of two generations tested ($p < 0.05$).

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

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Author contributions

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

Competing interests

The authors declare no competing interests.

Additional information

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Azadirachtin induced larval avoidance and antifeeding by disruption of food intake and digestive enzymes in *Drosophila melanogaster* (Diptera: Drosophilidae)

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ABSTRACT

Botanical insecticides are a promising alternative to reduce the harmful effects of synthetic chemicals. Among the botanical biopesticides, azadirachtin obtained from the Indian neem tree *Azadirachta indica* A. Juss. (*Meliaceae*) is probably the biorational insecticide with greatest agriculture use nowadays due to its broad insecticide activity. The current study, evaluated the lethal and sublethal effects of azadirachtin on larval avoidance, food intake and digestive enzymes of *Drosophila melanogaster* larvae as biological model. Azadirachtin was applied topically at two doses LD_{25} (0.28 μg) and LD_{50} (0.67 μg) on early third instars larvae.

Results evaluated 24 h after treatment showed that larvae exhibited significant repellence to azadirachtin and prefer keeping in untreated arenas rather than moving to treated one. In addition, azadirachtin avoidance was more marked in larvae previously treated with this compound as compared with naïf larvae (controls). Moreover, azadirachtin treatment decreased significantly the amount of larval food intake. Finally, azadirachtin reduced significantly the activity of larval α-amylase, chitinase and protease and increased the activity of lipase. This finding showed that azadirachtin induced behavioral and physiological disruption affecting the ability of the insect to digest food. This rapid installation of avoidance and long term antifeedancy might reinforce the action of azadirachtin and provide a new behavioral strategy for integrated pest management programs.

1. Introduction

Pesticides have played a crucial role in increasing agricultural productivity [1]. However, increasing awareness regarding environmental and human health impacts of conventional pesticides reduced their availability and promoted the search for alternative control strategies and reduced risk pesticides [2]. Consequently, a drastic re-emergence of interest in the use of natural pesticides, or biopesticides, especially plant-derived compounds was noted [3,4,5,6]. The prominent botanical pesticides that have received the best of attention are those issued from the neem tree, *Azadirachta indica* A. Juss. (Meliaceae) [3,7,8]. Azadirachtin, produced as secondary metabolite, is the principal active constituent in neem extracts [3]. Due to its chemical complexity, azadirachtin has many anti-insect properties but no resistance problem [3]. In addition, this compound has also been reported as safer for non-target organisms; however, this earlier perception has been recently challenged specially in relation with natural enemies and pollinators [9,10,11,12]. Despite these problems, azadirachtin still a recognizable alternative to conventional insecticides in integrates pest management programs.

Azadirachtin has multiple modes of action including antifeedancy, fecundity suppression and sterilization, oviposition repellence, growth inhibition, detrimental effects on morphology and changes in biological fitness [13,14,3,15,16,17]. However, at the moment, if the broad areas of its mode of action were established, the details were still not fully understood.

The major action of azadirachtin was its ability to modify or suppress hemolymph ecdysteroid and juvenile hormone titers by inhibiting the release of morphogenetic peptide hormone (PTTH) and allatotropins from the brain-*corpus cardiacum* complex leading to its well documented IGR effects [18,19,20]. The behavioral effects of azadirachtin are more controversial and seem to vary between insect orders and even species [21,22,23,24].

The ability to discriminate between edible and toxic food is crucial to survive and reproduce, and all animals must locate and selects the diet that maximises fitness [25]. Feeding behavior is both mediated by the detection through contact chemoreceptor on the insect's chemosensillas and sensory code integration by the central nervous system [26]. This primary antifeedancy effects of azadirachtin were reported in

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various orders of insect's including Lepidoptera, Coleoptera, Hemiptera, Isoptera, Diptera and Orthoptera [3]. An internal feedback mechanisms called secondary antifeedancy are initiated following the onset of physiological toxic effects arising from azadirachtin uptake or ingestion, but the reports are not consistent [27,28]. Moreover, decrease in food intake after azadirachtin treatment has been reported to dependent upon the insect species and concentration used [3,28]. Recently, we have reported that the preimaginal exposure to azadirachtin affects food selection and digestive enzymes in adults of *Drosophila melanogaster* suggesting a memorability of a learned avoidance and a long term antifeedancy of this compound [29]. In order to estimate the precocity of installation of these effects and their evolution through the insect development; the present study, investigated the acute lethal and sublethal effects of azadirachtin on larval avoidance, food intake and digestive enzymes 24 h after azadirachtin application on third instar larvae of *Drosophila melanogaster*, one of the most promising animal models used for research on mechanisms of nutrient selection and regulation.

2. Material and methods

2.1. Flies

Experimental stocks of *D. melanogaster* (wild-type Canton-S) were reared in glass vials containing a yeast/cornmeal/agar laboratory medium containing an anti-fungal agent and kept at 25 ± 2 °C, 70% relative humidity under a 12 h light/dark photoperiod. Flies were transferred every three days to avoid larval competition and regularly provide abundant progeny for testing.

2.2. Insecticide and treatment

A Commercial formulation of azadirachtin, the Neem Azal-TS (1% EC; Trifolio-M GmbH, Lahnau, Germany) was dissolved in acetone and topically applied (1 µl per insect) on early third-instar larvae of *D. melanogaster* 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 25% and 50% of immature stages) [16]. Controls were exposed only to the solvent (1 µl acetone) and all flies were kept under the same conditions as cited above. All experiments were done 24 h after treatment.

2.3. Larval preference assay

Groups of 20 larvae from controls and treated series (LD_{25} and LD_{50}) were collected and transferred to a petri dishes (Nunc; diameter = 66 mm, height = 15 mm) containing filter paper (Whatman, Cat 42; diameter = 55 mm) divided into two concentric circles (28 mm for the inner circle). The outer circle was soaked with 10 ml of azadirachtin (0.25 µg/ml) whereas the inner circle with 10 ml of acetone. After five minutes for solvents evaporation, the filter paper was humidified with 1 ml of distilled water in order to facilitate the displacement of the larvae. The larvae were placed in the inner circle, after five minutes the number of larvae present on each zone was determined and the larval preference index (LPI) was calculated as follows: (number of larvae on azadirachtin zone − number of larvae on control zone) / (number of larvae on azadirachtin zone + number of larvae on control zone). In theory, azadirachtin preference index can vary between − 1 (strong aversion against azadirachtin) and + 1 (strong attraction to azadirachtin).

2.4. Food intake estimation

The quantification of food intake was performed as previously described by Wong et al. [30]. For each experiment five larvae (Controls, LD_{25} and LD_{50}) were provided fresh food medium containing 2.5% (w/v) blue food dye (FD & C Blue no 1). After 30 min of feeding, only the blue larvae were selected.

Larvae were homogenized in 200 µl of distilled water. A further 800 µl of distilled water was added and the suspension cleared by centrifugation at 13,000*g* for 15 min. The absorbance of the supernatant was read at 629 nm. Age-matched larvae exposed to non-dyed food were used as the baseline during spectrophotometry. The absolute amount of food intake was calculated from a standard curve made by serial dilution in water of a sample of blue food.

2.5. Digestive enzymes assay

2.5.1. Sample preparation

For enzyme extracts individual's midgut of larvae from controls and treated series (LD_{25} and LD_{50}) were dissected out under a stereomicroscope and rinsed in ice cold saline buffer (0.15 M NaCl). A pool of ten midguts were homogenized in 1 ml of universal buffer (pH 7) and centrifuged at 13,000*g* for 15 min. The supernatant was used as the enzyme source. Six replicates were used for each dose. The protein concentrations in each sample were determined in parallel according to Bradford [31] and used to calculate the specific activity.

2.5.2. Enzyme assay

α-Amylase activity was assayed using the dinitrosalicylic acid (DNS) as the reagent and 1% soluble starch as substrate [32]. Twenty microliters of the enzyme were incubated for 30 min at 35 °C with 100 µl universal buffer (pH 7) and 40 µl soluble starch. The reaction was stopped by adding 100 µl DNS and heating in boiling water for 10 min prior to reading the absorbance at 540 nm. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35 °C.

Chitinase activity was determined using colloidal chitin [33] as the substrate according to Miller [34]. Chitinase activity was assayed, based on dinitrosalicylic acid (DNS) method [34]. 250 µl of enzyme solution was added to 250 µl of 1% colloidal chitin in citrate phosphate buffer (0.05 M, pH 6.6), after incubation for 30 min at 50 °C with continuous shaking (120 rpm), the mixture was heated in a boiling-water bath for 5 min, cooled at room temperature and then centrifuged at 5000*g* for 5 min. 150 µl of the supernatant were mixed with 300 µl of universal buffer and 200 µl of DNS; the mixture was incubated for 10 min in boiling water bath and then cooled at 4 °C. The absorbance was measured at 540 nm. The activity was calculated from a standard curve of known concentration of *N*-acetylglucosamine. One enzyme unit was defined as the amount of enzyme that produces 1 µmol of reducing sugar per hour under reaction conditions.

The lipase assays were carried out as described by Tsujita et al. [35]. Ten microliters of the enzyme and 18 µl of *p*-nitrophenyl butyrate (50 mM) as a substrate were added to 172 µl of universal buffer (pH 7) and incubated at 37 °C for 30 min. The absorbance was read at 405 nm and one unit of lipase activity was defined as the amount of enzyme necessary to liberate 1 µmol of *p*-nitrophenyl per minute under assay conditions.

General protease activity was assayed using casein (1%) as substrate [36]. 200 µl of 1% casein solution was added to 100 µl enzyme and 100 µl universal buffer (pH 7), the mixture was incubated at 37 °C for 60 min. Proteolysis was stopped by the addition of 800 µl of 5% trichloroacetic acid (TCA). The mixture was centrifuged at 8000*g* for 15 min and the absorbance was recorded at 280 nm. The activity was calculated from a standard curve using tyrosine with a known concentration.

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 of larval preference was subjected to Kruskal-Wallis test and pairwise multiple comparison using Dunn's method. Index preference was assessed using Mann-Whitney test. Data of quantification of food

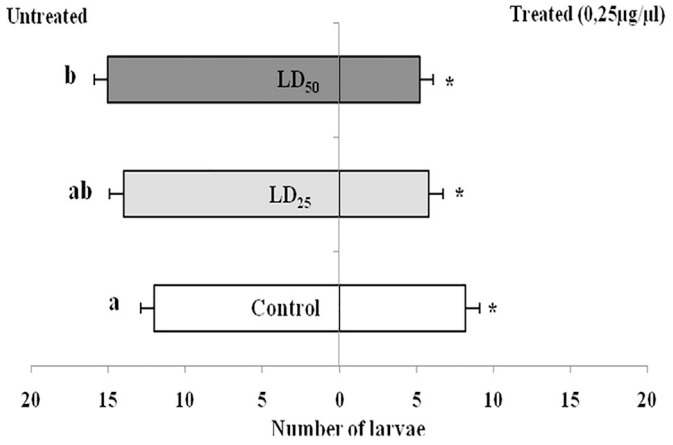

Fig. 1. Effect of azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on larval preference subjected to two-choice test on azadirachtin-treated surface (0.25 µg/µl). Different small letters indicate a significant difference between control and treated individuals in the same surface. The asterisks indicate a significant difference between individuals of the same dose in the different surface (untreated and treated).

intake and activity of digestive enzymes were subjected to one-way analysis of variance (ANOVA) followed by a post hoc HSD Tukey test. 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 larval preference

Azadirachtin treatment (LD_{25} and LD_{50}) significantly affect larval preference for untreated and azadirachtin-treated filter paper arenas ($P < 0.001$) (Fig. 1). Larvae previously exposed to azadirachtin showed a highest aversion to this substance compared to naïve larvae ($P < 0.01$). Larvae exhibited significant repellence to azadirachtin and prefers keeping in untreated arenas and avoid moving to the treated one ($P < 0.01$) (Fig. 1).

The larval preference index (LPI) of *D. melanogaster* (Controls, LD_{25} and LD_{50}) is shown in Fig. 2. The means values of LPI recorded in azadirachtin-treated zone (0.25 µg/µl) were − 0.18, − 0.42 and − 0.44 respectively for controls, LD_{25} and LD_{50}. Indeed, Mann-Whitney test showed significant differences between the control and LD_{25} (Mann-Whitney test U = 1.00; P = 0.023), control and LD_{50} (Mann-Whitney test U = 1.00; P = 0.023) and no significant effects between the two tested doses (Mann-Whitney test U = 11.50; $P > 0.05$) (Fig. 2).

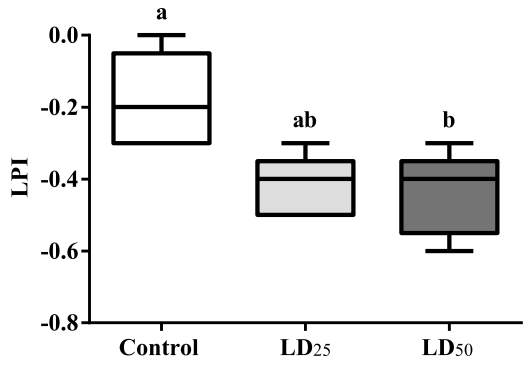

Fig. 2. Larval preference index (LPI) of third instars larvae of *D. melanogaster* for azadirachtin-treated surface (0.25 µg/µl). Different small letters indicate a significant difference between control and treated individuals.

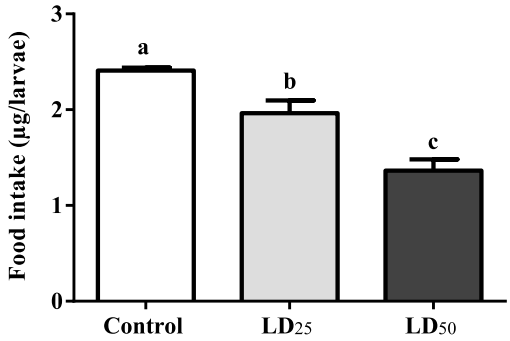

Fig. 3. Effect of azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on food intake (µg/larva), (m ± SE; n = 5 replicates of 5 insects). Different small letters indicate a significant difference between control and treated series ($P < 0.05$).

3.2. Effect of azadirachtin on food intake

Measurements of food intake and potential antifeedant effects caused by azadirachtin treatment were evaluated using spectrophotometry and shown in Fig. 3. Azadirachtin treatment of early third instars larvae caused a significant reduction of food intake, 24 h after treatment, with a dose dependent relationship ($F_{2, 14} = 25.80$; $P < 0.001$). The means values recorded were 2.40 ± 0.03 µg/larva for the controls, 1.96 ± 0.13 µg/larva for the LD_{25} and 1.36 ± 0.11 for the LD_{50} (Fig. 4). The food ingested rate in azadirachtin-treated larvae decreased by about 30% at 45% respectively for LD_{25} and LD_{50} compared with controls larvae (Fig. 3).

3.3. Effect of azadirachtin on digestive enzyme activities

Midguts from azadirachtin-treated (LD_{25} and LD_{50}) larvae showed significantly ($F_{2, 15} = 45.01$; $P < 0.001$) lower α-amylase activity in comparison to midguts from controls larvae (Fig. 4). The mean values recorded were 4.86 ± 0.15 µmol/min/mg protein for controls, 3.43 ± 0.13 µmol/min/mg protein for the LD_{25} and 3.14 ± 0.11 µmol/min/mg protein for the LD_{50}. Statistic analysis revealed no significant difference between the two tested doses ($P > 0.05$) (Fig. 4).

As a consequence of treatment, chitinase activity decreased in treated larvae when compared with control larvae ($F_{2, 15} = 202.4$; $P < 0.001$) (Fig. 5). The means values recorded were 0.64 ± 0.009 mmol/min/mg proteins for controls, 0.51 ± 0.005 mmol/min/mg protein for the LD_{25} and 0.44 ± 0.006 mmol/min/mg protein for the LD_{50}. Statistic analysis revealed significant difference between the two tested doses ($P < 0.05$) (Fig. 5).

Lipase activity increased in treated larvae as compared with control

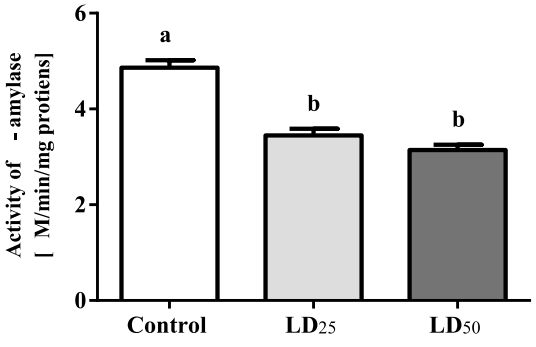

Fig. 4. Effect of azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of α-amylase, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series ($P < 0.05$).

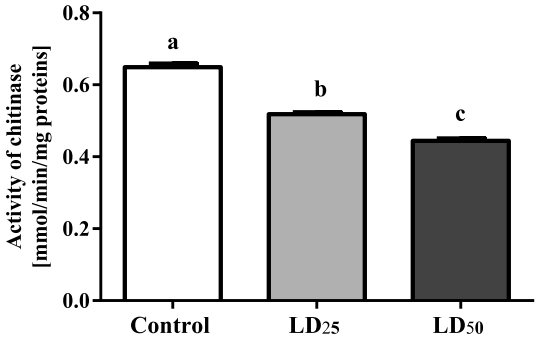

Fig. 5. Effect of azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of chitinase, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series (P < 0.05).

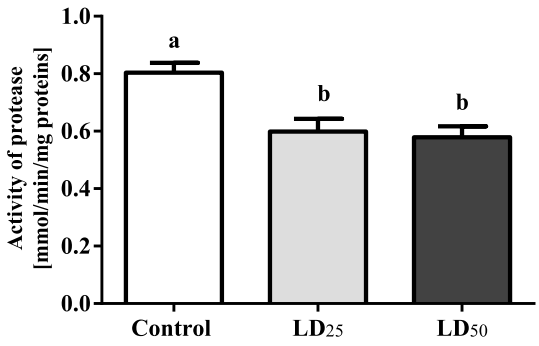

Fig. 7. Effect of azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of protease, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series (P < 0.05).

larvae ($F_{2, 15}$ = 80.90; P < 0.001) and the highest activity was observed in larvae treated by LD_{50} (Fig. 6). The means values recorded were 0.18 ± 0.002 µmol/min/mg protein for controls, 0.25 ± 0.007 µmol/min/mg protein for the LD_{25} and 0.27 ± 0.004 µmol/min/mg protein for the LD_{50}. Statistic analysis revealed no significant difference between the two tested doses (P > 0.05) (Fig. 6).

General protease activity was lower in azadirachtin-treated-larvae (LD_{25} and LD_{50}) as compared with the controls larvae ($F_{2, 15}$ = 10.08; P = 0.0017) (Fig. 7). In control series the mean values recorded were 0.80 ± 0.034 mmol/min/mg protein. In treated series the mean values recorded were 0.59 ± 0.044 µmol/min/mg protein for the lowest dose LD_{25} and 0.57 ± 0.038 µmol/min/mg protein for the highest dose LD_{50}. Statistic analysis revealed no significant difference between the two tested doses (P > 0.05) (Fig. 7).

4. Discussion

In this study, we investigated the effects of azadirachtin on larval avoidance, food intake and digestive enzymes in larvae of *D. melanogaster*. Results show that larvae preferentially staying in untreated filter paper arenas rather than moving in azadirachtin-treated one. In addition, azadirachtin avoidance was more marked in larvae previously treated (24 h before experiments) with this compound as compared with naïf larvae (Controls).

Azadirachtin avoidance has also been reported in other insect species, which are apparently able to detect the insecticide on the treated surface [37,38,23,24]. In addition, the impact of previous exposure on insect behavior was reported in different insect's species [39,40,41]. Recently, Kilani-Morakchi et al. [29] demonstrated that larval experience with azadirachtin can alter the physiological and behavioral responses of adults flies and that the aversion (primary and secondary antifeedancy) to this compound can be conserved through the

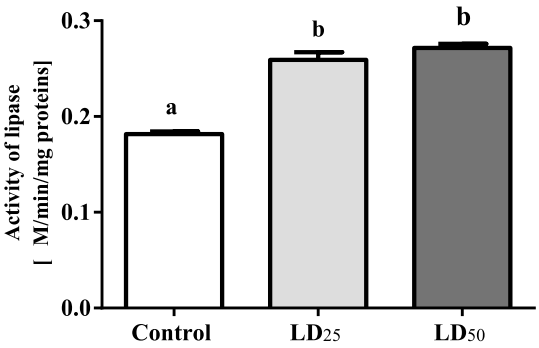

Fig. 6. Effect of azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of lipase, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series (P < 0.05).

metamorphosis suggesting a memorability and avoidance learning memory. In the current experiments, the behavioral effects sparked by azadirachtin seem to appear very early after treatment (24 h) and the first experience (topical application) reinforce the avoidance effect of this compound. Results demonstrate the precocity of this learning memory which may contribute to reinforce the insecticidal activity of this biopesticide and provide a new behavioral strategy for controlling agricultural pests.

In *Drosophila*, a complex olfactory system allows animals to avoid noxious substances [42]. Moreover, the response of larvae to toxic or bitter compounds is mediated by gustatory receptors and can lead to aversive behavior resulting in food intake cessation [43,44]. This primary antifeedancy is mediated by the projection of neurons to the protocerebrum which acts as gustatory interneuron's relaying avoidance information to higher brain centres in *Drosophila* larvae [26,44].

A secondary antifeedant effect including a long-term reduction in food intake, and direct detrimental effects on different insect tissues such as muscles, fat body and gut epithelial cells is also reported [3].

In the current experiments, azadirachtin-treatment of early third instar larvae reduced significantly larval food-intake at the two tested doses (LD_{25}, LD_{50}) as compared to the controls. Previously, azadirachtin is reported to reduce food consumption in different insect's species [20,45,28]. The antifeedant activity of azadirachtin varied significantly based on the concentration used and the insect species [3,28].

Inhibition of feeding behavior results from stimulation of deterrent receptors by azadirachtin often coupled with an inhibition of sugar receptors [46], these effects results to bad nutrition, starvation, or restriction of food intake [3]. Moreover, neuronal circuits that control feeding must integrate information on both the palatability of food source ant its nutritional content [47]; indeed, *D. melanogaster* seems to be able to discriminate food texture and integrate the chemical and physical qualities of food [48] which may contribute to avoiding azadirachtin-treated food.

In addition, a reduction of insect weight under azadirachtin treatment was reported in *Periplaneta americana* [49], *Helicoverpa armigera* [50] *Spodoptera eridania* [28] and *D. melanogaster* [51,17].

Growth and nutrient intake are function-ally linked processes in development and growth and body mass are directly affected by nutrient uptake. Indeed, nutrient sensing systems play an important role in the physiological adjustment process which is governed by an evolutionary conserved systemic nutrient sensor, the insulin/insulin-like growth factor signalling (IIS) pathway during insect growth [52]. In fact, Lai et al. [51] reported that the inhibition of growth and development caused by azadirachtin in *D. melangaster*, was consistent with those caused by disruption of the insulin/insulin-like growth factor signalling IIS pathway. Moreover, Qiao et al. [53] reported the neurotoxic effect of azadirachtin which might interfere with different endocrinological and physiological actions in insects.

Recent studies indicate that regulation of feeding is not only

governed by gustatory information but also by internal sensors [54,55]. Indeed, cells located in internal organs, including the gut, fat body and the brain, express receptors that detect nutrients or their metabolically processed derivates to regulate energy homeostasis and feeding behaviors [56].

Azadirachtin was reported to cause disruption in insect physiology and its ability to digest food [28]. The results obtained in the present study showed a clear disruption of digestive enzymes activities in *D. melanogaster* larvae after azadirachtin treatment as compared to controls. In intestine, the complex mix of dietary components ingested by flies is broken down by digestive enzymes before it is absorbed by the intestinal epithelium [57]. Azadirachtin was reported to affect digestive enzymes in several insect species [58,27,59]. In the current study, azadirachtin treatment of early third instar larvae of *D. melanogaster* reduced significantly gut α-amylase, chitinase and protease activities and increased lipasic activity.

α-Amylase is an enzyme that degrades starch by hydrolyzing α-1,4-glucan bonds [60]. The reduction of α-amylase activity under azadirachtin treatment was reported in *Periplaneta Americana* [49], *Plodia interpunctella* [61] and *Glyphodes pyloalis* [27]. Lai et al. [51] demonstrate that azadirachtin reduced the activity of α-amylase in midgut of *D. melanogaster* by repressing expression of α-amylase gene.

Insect chitinases which belong to family of 18 glycosylhydrolases, detected in moulting fluid and gut tissues, are predicted to mediate the digestion of chitin present in exoskeleton and peritrophic membrane (PM) in the gut to chitooligosaccharides [62].

Azadirachtin reduced significantly the chitinase activity in *D. melanogaster* larvae which is in accordance with the results obtained in adults of the same species [29]. The peritrophic membrane protects the midgut from mechanical damage and toxic compounds; azadirachtin was reported to affect this PM leading to damage of cellular surfaces of the midgut [59]. In addition, in insects the expression of chitinase gene was regulated by 20E (20-hydroxyecdysone) [63], which may explain the effect observed in our results, since azadirachtin act as an antagonist of the 20E.

Lipases play a major role in storage and lipid mobilization. These enzymes are also the basic components in many of physiological process like, reproduction, growth, and defence against pathogens [64]. Our results showed that azadirachtin increased significantly the activity of lipase in the larvae midgut of *D. melanogaster*. Reduction in lipasic activity is mostly reported in literature after azadirachtin treatment [27,64], but this effect differ with azadirachtin formulation and concentration. However, the increase in lipase activity observed in our study may be a result of an increasing of esterase activity needed for detoxification [64,29]. In addition, according to the physiological and developmental needs of the insect, the acquired nutrients can be differentially allocated to different organs, processes and/or metabolic pathways which may explain the increase in lipase activity noted in third instars larvae of *D. melanogaster* to meet the demand for energy needed for metamorphosis.

Proteases are groups of enzymes that hydrolyze proteins to amino acids based on their catalytic mechanism [65]. In the present study, compared with the controls, protease activity was significantly reduced in larvae of *D. melanogaster* after azadirachtin treatment. The reduction of protease activity under azadirachtin treatment was reported in several insects' species [49,27]. Azadirachtin prevents the secretion of Proteolytic enzymes and thus significantly impair ability of insects to digest and absorb nitrogenous food. Timmins and Reynolds [66] showed that azadirachtin significantly influenced the trypsine activity in the midgut of *Spodoptera litura*. Recently, several studies reported that azadirachtin interfere with activities of different enzymes such as α-amylase, lipase, α- and β-glucosidases, proteases, lactate dehydrogenase and glutathione S-transferase [27,59,15]. These results may reflect interference of azadirachtin with regulation of feeding and metabolism which clearly supports the secondary antifeedancy action of azadirachtin included reduction in food consumption and digestive efficiency reducing access of nutrients for biological requirements [67,68]. Furthermore, this reduction in efficiency of conversion of ingested and digested food may be a result of an energy conversion from biomass production into induction enzymes involved in detoxification of azadirachtin [28].

In conclusion, it is evident that this study has shown that azadirachtin affects larval avoidance, food consumption and digestion in *D. melanogaster*. This rapid installation of avoidance and antifeedancy reinforce the action of azadirachtin and is useful element for integrated pest management programs that involve behavioral manipulation of insect pests, including the harmful *D. suzukii*, like push-pull strategies for protected plant resource as safer alternative to conventional synthetic insecticides.

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Preimaginal exposure to azadirachtin affects food selection and digestive enzymes in adults of *Drosophila melanogaster* (Diptera: Drosophilidae)

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ABSTRACT

Among the plant derived product, azadirachtin, a neem-based insecticide, is exceptional in having a broad range of bioactivity including toxicity, growth, development and reproduction effects, repellency and antifeedancy. If considerable progress on the physiological and biological activities and agricultural application of azadirachtin has been achieved, its exact mechanism of action remains uncertain. In this study, we aimed at assessing the lethal and sublethal behavioral and physiological effects of azadirachtin on *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) as biological model. Azadirachtin was applied topically at two doses LD_{25} (0.28 μg) and LD_{50} (0.67 μg) on early third instar larvae. Results showed that flies preferentially ingested control medium rather than azadirachtin-treated medium. Pre-imaginal exposure (L3) to azadirachtin increased aversion to this substance suggesting a memorability of the learned avoidance. In addition, all tested flies revealed a clear preference for solvent odour rather than azadirachtin odour. Moreover, azadirachtin treatment decreased significantly the amount of food intake in the adults of both sexes. Finally, azadirachtin was found to affect digestive enzyme activities in the midgut of flies. Indeed, an inhibition of α-amylase, chitinase, and protease activities and an increase of lipasic activity were noted. These results may reflect interference of azadirachtin with regulation of feeding and metabolism, and provide some evidence of a long term antifeedancy and delayed effects through developmental stage which may reinforce the insecticidal activity of this bioinsecticide.

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1. Introduction

Plants with pesticidal properties have been investigated for decades as alternatives to synthetics for developing sustainable agricultural practices and promote human and environmental safety [1,2]. The prominent botanical pesticides that have received the best of attention are those issued from the neem tree, *Azadirachta indica* A. Juss. (Meliaceae) [3]. Azadirachtin, a tetranortriterpenoid, is the most effective compound produced as a secondary metabolite of the neem tree [1]. This compound acts as repellent, oviposition and feeding deterrence, sterilant and as insect growth regulator (IGR) by interfering with both juvenile hormone and ecdysone titers, the two major hormones responsible for insect growth, development and reproduction [1,4,5].

Due to its short persistence and low mammalian toxicity, azadirachtin has been tested for various pests and is currently exploited in agriculture for crop protection against different phytophagous insects [6,4,7]. The compound has also been reported as safer for non-target organisms; however, this earlier perception has been recently challenged specially in relation with natural enemies and pollinators [8,9,10,11]. Despite these problems neem derived insecticides still valuable components in integrated pest management and its use is recommended for insecticide resistance control and organic production pest control [12,13].

The impairment of insect development, defined as reduced pupation, malformation and failure of adult emergence remain the most frequently reported effects of azadirachtin [14,15,16,17]. However, its behavioral effects still more controversial being somewhat dependent upon the insect species, time and/or method of application, and concentration used [14,18,7,17].

Insect use olfaction system to search and locate potential food [19] and thereafter contacting chemoreception, called primary antifeedancy, could confirm its quality and provide a basis for food selection and discrimination [20,21].

The antifeedant effect of azadirachtin was reported on large number of pest insects, including hemipterans [22], lepidopterans [23,24], orthopterans [25] and coleopterans [26]. A secondary antifeedant of azadirachtin consisting on a disruption in the physiology of the insect and its ability to digest food was also reported [27,28,24].

In insects such as the fruit fly *Drosophila melanogaster*, the ability to discriminate chemicals by taste and avoid toxins in food is critical to the survival and reproduction. Indeed, gustatory information were used to assess the suitability of potential food sources and calorie sensing to make

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critical decisions of feeding choices that maximizes fitness [29,30]. If the aversive and/or antifeedancy effects of azadirachtin was largely reported in literature [1,28], the possible conservation of this effects through metamorphosis was not investigation. Recently, we have demonstrated that azadirachtin, applied topically to third instars larvae, can affect fitness and eggs laying preference in adults of *D. melanogaster* suggesting an avoidance learning memory which may reinforce its insecticidal activity [17]. Here, we investigated the influence of azadirachtin previous experience (larval exposure) on predigestive and postdigestive effects on adults of *D. melanogaster* as indicated by behavioral responses of adults, food consumption and digestive enzymes.

2. Material and methods

2.1. Flies

The wild-type *D. melanogaster* Meigen flies (Diptera: Drosophilidae) were raised on standard fly medium made of cornmeal, yeast and agar at 25 °C and 70% relative humidity under a 12-h light:12-h dark cycle.

2.2. Insecticide and treatment

A commercial azadirachtin-bases insecticide formulation was used for the experiments (Neem Azal-TS, 1% EC; Trifolio-M GmbH, Lahnau, Germany) and dissolved in acetone for topical application (1 μl per insect) [17]. Azadirachtin was applied on early third-instar larvae of *D. melanogaster* at two doses, 0.28 μg and 0.67 μg corresponding respectively to LD_{25} and LD_{50} (doses that caused respectively a mortality of 25% and 50% of immature stages) [17]. Controls were exposed only to the solvent (1 μl acetone) and all flies were kept under the same conditions as cited above.

2.3. Two-choice food preference test

The food preference of adult males and females three-day-old were tested in binary choice using a 2-dye marker method blue-red [31] in 96-well microplates (Fig. 1A). The blue food-dye was FD&C Blue Dye no.1, used at a concentration of 0.5 mg/ml. The red food-dye was acid red, used at a concentration of 2 mg/ml. These food-dyes, at the above concentrations, were ascertained to be neither toxic nor were they metabolized [31]. The wells in the micro test plate were alternately filled with 10 μl azadirachtin treated food (0.25 μg/ml) marked with blue or red food-dye or control food containing the alternate food dye.

In each experiment, flies of both sexes (10 males and 10 females, tested in single sex groups) emerged from previously treated early 3rd instar larvae (LD_{25} and LD_{50}) were starved for 24 h and placed in plate. After being left to feed for 1 h at 25C° in the dark, the flies were conserved in −10C° during 24 h. A control series was done in parallel. Flies were classified into four groups according to abdominal coloring, i.e. blue (b), red (r), mixed (m) and uncoloured (Fig. 1). The fraction of the uncoloured flies was usually below 5%. Five replicates were used for each test. A preference index (PI) was calculated, according to the formula, where N represents de number of flies:

$$PI = \left[\left(N^b + N^m/2\right)/\left(N^b + N^m + N^r\right)\right] * 100$$

Paired control plates were prepared similarly, using standard food containing the alternate food dye, and used to test the possible effect of dyes on flies preference.

2.4. Olfactory response

Groups of adults three day-old of both sexes surviving from previously treated larvae (LD_{25} and LD_{50}) were tested in Y-maze assay according to Simonnet et al. [32] with slight modifications. The flies were starved for 17 h at 25 °C in glass tubes containing wet paper towel before testing. An Y-shape connector was used to join to two glass vials and a third vial (loading vial). A micro-pipette tips was passed through the foam stoppers to link the connector to the three vials to obtain a tightly sealed Y-maze. The narrow end of two pipette tips were cuts, to avoid any return of the fly once it has made its decision, and also a large end of the pipette tip forming the loading tube. Before connecting the “trap” vials, a filter paper (~ 5 mm) was placed in each

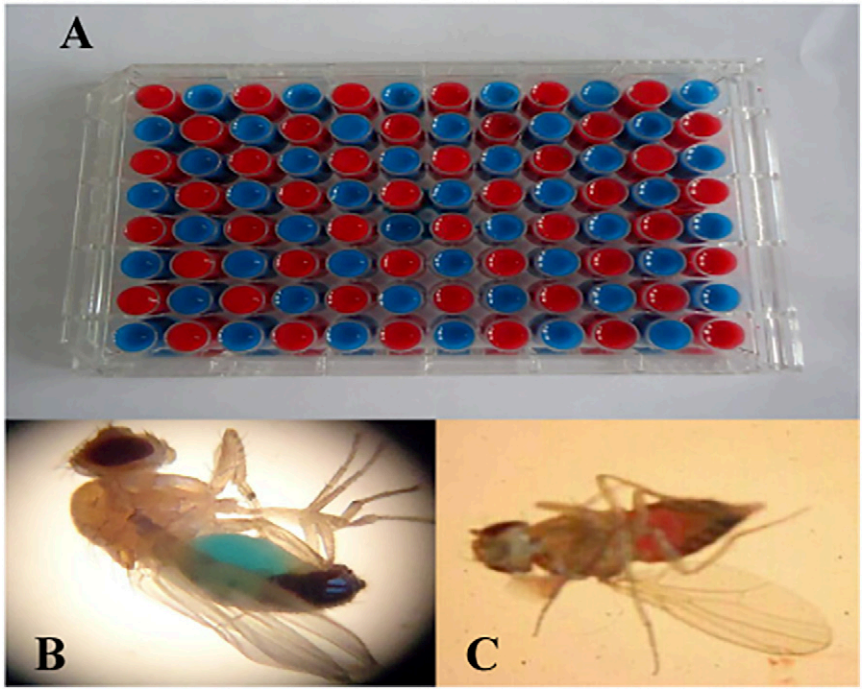

Fig. 1. Feeding preference test: (A) 96-well test plate filled with food marked with blue or red food-dye. (B) Abdomen colored in blue. (C) Abdomen colored in red.

vial. 50 µl of azadirachtin (0.25 µg/ml) were deposited on one filter paper, and 50 µl of acetone on the second filter paper. After a brief cooling on ice, flies were tested individually, to avoid group effects, and placed into the third vial. Forty replicates were used for each sex and each dose. Percentage of attraction was calculated of each group.

2.5. Quantification of food intake

Quantification of food intake was performed as previously described by Wong et al. [33]. For each experiment five 3-day-old adults males or females surviving from previously treated larvae (LD_{25} and LD_{50}) were provided fresh food medium containing 2.5% (w/v) blue food dye (FD &C Blue no 1) as food intake coloring marker. This Dye is not affected by gut pH and digestive enzymes [31]. After 30 min of feeding, flies were decapitated, to prevent eye pigment from interfering with the absorbance spectrum of the dye. Only the flies with blue abdomen were selected.

Flies were homogenised in 200 µl of distilled water inside an eppendorf tube, with the aid of a pestle for eppendorf (eppi-pistill). A further 800 µl of distilled water was added and the suspension cleared by centrifugation at 13,000*g* for 15 min. The absorbance of the supernatant was measured at 629 nm at room temperature. Age-matched flies exposed to non-dyed food were used as the baseline during spectrophotometry. The absolute amount of food intake was calculated from a standard curve ($y = 0.06x - 0.02$) made by serial dilution (0–15 µg/ml) in water of a sample of blue food.

2.6. Enzymatic determination

2.6.1. Preparation of enzyme extract

3-days old adult males or females surviving from previously treated larvae (LD_{25} and LD_{50}) were used to quantify the activity of digestive enzyme. Individual's midgut were dissected out under a stereomicroscope and rinsed in ice cold saline buffer (0.15 M NaCl). A pool of ten midguts were homogenised in 1 ml of universal buffer (pH 7) and centrifuged at 13,000*g* for 15 min. The supernatant was used as the enzyme source. Six replicates were used for each dose. The protein concentrations in each sample were determined, according to Bradford [34], and used to calculate the specific activity.

2.6.2. Assay of α- amylase activity

α-amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure [35], using 1% soluble starch as substrate. 20 µl of the enzyme were incubated for 30 min at 35 °C with 100 µl universal buffer (pH 7) and 40 µl soluble starch. The reaction was stopped by adding 100 µl DNS and heating in boiling water for 10 min. After cooling at room temperature, 100 µl of distilled water were added prior to reading the absorbance at 540 nm against a blank prepared in the same manner but with 20 µl of distilled water in place of the enzyme source. Maltose was used as standard solution in the concentration as (180 mg/ml). A calibration curve ($y = 0.86x - 0.048$) established with maltose (0–1 µM/ml) is used to convert the colorimetric readings into µM of maltose. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35 °C.

2.6.3. Assay of lipase activity

The activity of lipase was determined by the method of Tsujita et al. [36]. Ten µl of the enzyme was mixed with 18 µl *p*-nitrophenyl butyrate (50 mM) as a substrate, and 172 µl of universal buffer solution (pH 7). After incubation at 37 °C for 30 min, the absorbance was read at 405 nm against a blank prepared in the same manner but with 18 µl of distilled water in place of the *p*-nitrophenyl butyrate. One unit of lipase activity was defined as the amount of enzyme necessary to liberate 1 µmol of *p*-nitrophenyl per minute under assay conditions. A standard curve ($y = 57x + 0.10$) of *p*-Nitrophenol (0–0,025 µM/ml) was made from a solution of 0.05 µM/ml of *p*-Nitrophenol.

2.6.4. Assay of protease activity

Protease activity was measured determined using casein as the substrate [37]. The reaction mixture consisting of 200 µl of casein (casein dissolved in PBS to obtain a solution at 1%), 100 µl enzymes and 100 µl universal buffer (pH 7) was incubated at 37 °C for 60 min. Proteolysis was stopped by the addition of 800 µl of 5% trichloroacetic acid (TCA). The mixture was centrifuged at 8000*g* for 15 min and the absorbance was recorded at 280 nm against a blank prepared in the same condition but without enzyme source. The activity was calculated from a standard curve ($y = 0.51 \times - 0.06$) using tyrosine with a known concentration (0–25 mM/ml) prepared from a solution of 10 mM of tyrosine.

2.6.5. Assay of chitinase

2.6.5.1. Preparation of colloidal chitin. Colloidal chitin was prepared according to Shen et al. [38]. Fore grams of chitin powder (Crab shells, Sigma) was added slowly to 40 ml of concentrated HCl and kept at 4 °C for 1 h under vigorous stirring. The mixture was added to 1 l of ice-cold distilled water with rapid stirring and kept overnight at 35 °C. After filtration, the substrate was washed several times with sterile distilled water until the colloidal chitin became neutral (pH 7). One gram of the colloidal chitin (1% w/v) was dissolved in citrate phosphate buffer (0.05 M, pH 6.6).

2.6.5.2. Enzyme assay. Chitinase activity was determined with colloidal chitin as the substrate according to Miller [39]. 250 µl of enzyme solution was added to 250 µl of 1% colloidal chitin, after incubation for 30 min at 50 °C with continuous shaking (120 rpm), the mixture was heated in a boiling-water bath for 5 min, cooled at room temperature and then centrifuged at 5000*g* for 5 min. 150 µl of the supernatant were mixed with 300 µl of universal buffer and 200 µl of DNS; the mixture was incubated for 10 min in boiling water bath and the cooled at 4 °C. The reducing sugar released was measured by the DNS method at 540 nm using *N*-acetyl-D-glucosamine (GlcNAc) as standard. A blank was prepared in the same condition with 250 µl of distilled water in place of enzyme source. Enzymatic activity was estimated using a standard curve ($y = 4.88x - 0.13$) of *N*-acetylglucosamine (0–0,144 mg/ml) prepared from a solution of 0.5 mg/ml of *N*-acetylglucosamine. One unit of chitinase activity was defined as the amount of enzymes that produces 1 µmol of reducing sugar per hour under reaction conditions.

2.7. Statistical analyses

All statistical analyses were performed using Prism v 6.01 for Windows (GraphPad Software Inc., www.graphpad.com). Results are given as means ± standard errors (SE). The homogeneity of variances was checked using Bartlett's and Brown-Forsythe tests. Data on food preference index was assessed using Mann-Whitney test. Olfactory response was analyzed using the Chi-square (χ^2) test. Data of Quantification of food intake and activity of digestive enzymes were subjected to one-way analysis of variance (ANOVA) followed by a post-hoc HSD Tukey test.

3. Results

3.1. Two-choice food preference test

The sensitivity of adults of *D. melanogaster*, surviving from previously treated larvae (LD_{25} and LD_{50}), to azadirachtin was tested using 2-dye marker to discriminate the preferential ingestion of flies. Results show that flies preferentially ingested control medium rather than azadirachtin treated medium ($p < 0.01$). The marked food dyes also affected the choice of flies. Indeed, flies preferentially ingested the medium marked with the blue food-dye (53.76%) than the medium marked with the red food-dye (39%).

The preference index (PI) of adult males and females of *D. melanogaster* (Controls, LD_{25} and LD_{50}) are shown in Fig. 2. For males, the means values of PI recorded in azadirachtin-treated medium (0.25 μg/μl) marked with the blue food-dye were 33.21%, 15.00% and 13.00% respectively for controls, LD_{25} and LD_{50}. For females, the PI was 34.83%, 22.91% and 22.31% respectively for controls, LD_{25} and LD_{50}. Furthermore, the values of PI recorded in azadirachtin-treated medium (0.25 μg/μl) marked with the red food-dye were always null for both sexes from controls and treated (LD_{25} and LD_{50}) series. Mann-Whitney test showed significant differences (Mann-Whitney test $U = 0.00$; $p < 0.01$) between PI of previously treated flies and controls flies and no significant effects between the two tested doses (Mann-Whitney test $U = 10.00$; $p > 0.05$).

3.2. Effect on olfactory responses

Effects of azadirachtin on fly olfactory response were evaluated using Y-maze. The behavioral test showed a clear preference of fly to enter trap vial containing the solvent rather than azadirachtin. There was no difference between sexes and so data were pooled. Proportion of flies choosing the solvent was 90% for controls, 83.75% for LD_{25} and 77.75% for LD_{50}. Statistic analysis revealed no significant difference between the tested groups ($\chi^2 = 4.59$, $p > 0.05$).

3.3. Effect of azadirachtin on food intake

Amounts of ingested food in controls and treated series (DL_{25} and DL_{50}) were quantified using spectrophotometry and shown in Fig. 3. In control series the amount of ingested food was 1.910 ± 0.100 μg/fly for males and 3.100 ± 0.095 μg/fly for females. Azadirachtin treatments of early third instars larvae caused a significant reduction of food intake with a dose dependent relationship (Fig. 3). Indeed, the mean values recorded in treated series were 1.320 ± 0.029 μg/fly for males and 2.360 ± 1.150 μg/fly for females at the lowest dose (LD_{25}) and 1.080 ± 0.083 μg/fly for males and 2.360 ± 0.017 μg/fly for females at the highest dose (LD_{50}). ANOVA indicated significant effects of doses ($F_{2, 24} = 23.99$; $p < 0.001$), sex ($F_{1, 24} = 175.5$; $p < 0.001$) but no significant ($F_{2, 0.15} = 23.99$; $p > 0.05$) in doses-sex interaction.

3.4. Effect of azadirachtin on digestive enzyme

3.4.1. Effect on α- amylase activity

Results showed that azadirachtin treatment of early third instars larvae of *D. melanogaster* affected α-amylase activity of adult of both sex as compared to controls (Fig. 4). In males the mean values recorded were 5.470 ± 0.160 μmol/min/mg proteins for the controls, 3.430 ± 0.080 μmol/min/mg proteins for the LD_{25} and 2.260 ± 0.020 μmol/min/mg proteins for the LD_{50}. Similar results was observed for female with mean values of 4.47 ± 0.080 μmol/min/mg proteins for the controls, 3.510 ± 0.090 μmol/min/mg proteins for the LD_{25} and 2.440 ± 0.080 μmol/min/mg proteins for the LD_{50}. ANOVA indicated significant effects of doses ($F_{2, 30} = 396.2$; $p < 0.001$), and doses-sex interaction ($F_{2, 30} = 12.69$; $p < 0.001$) but no significant of sex ($F_{1, 30} = 3.77$; $p > 0.05$).

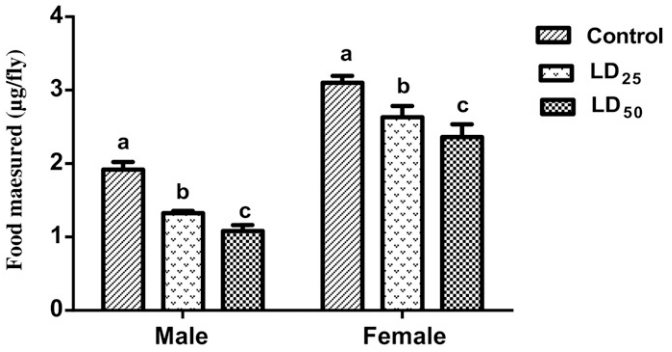

Fig. 3. Effect of azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on feeding rates (μg/fly) of adults male and female, (m ± SE; n = 5 replicates of 5 insects). Different small letters indicate a significant difference between control and treated series ($p < 0.05$).

3.4.2. Effect on lipase activity

The activity of lipase increased in adult's flies surviving from previously treated early third instars larvae. The highest activities were observed in females treated with LD_{50} (Fig. 5). The mean values recorded in the males were 0.054 ± 0.001 μmol/min/mg proteins for the control, 0.060 ± 0.003 μmol/min/mg proteins for the LD_{25} and 0.071 ± 0.003 μmol/min/mg proteins for the LD_{50}. In the female the mean values were 0.063 ± 0.002 μmol/min/mg proteins for the control, 0.077 ± 0.004 μmol/min/mg proteins for the LD_{25} and up to 0.101 ± 0.005 μmol/min/mg proteins for the LD_{50} (Fig. 6). ANOVA indicated significant effects of doses ($F_{2, 30} = 34.09$; $p < 0.001$), sex ($F1_{, 30} = 44.27$; $p < 0.001$) and doses-sex interaction ($F_{2, 30} = 4.72$; $p < 0.05$).

3.4.3. Effect on protease activity

Results showed that azadirachtin treatment of early 3rd instar larva (DL_{25} and DL_{50}) affected protease activity of adults of both sexes by a significant reduction compared to controls ($p < 0.001$) (Fig. 6). In control series the mean values recorded were 0.780 ± 0.014 mmol/min/mg proteins for males and 0.750 ± 0.012 mmol/min/mg proteins for females. The activity of protease for the lowest dose (LD_{25}) was 0.560 ± 0.022 mmol/min/mg proteins for males and 0.560 ± 0.011 mmol/min/mg proteins for females. At the highest dose (LD_{50}) the value recorded dropped to 0.410 ± 0.021 mmol/min/mg proteins for males and 0.450 ± 0.017 mmol/min/mg proteins for females (Fig. 7). ANOVA indicated significant effects of doses ($F_{2, 30} = 212.7$; $p < 0.001$), but no significant effects of sex ($F_{1, 30} = 0.0016$; $p > 0.05$) and doses-sex interaction ($F_{2, 30} = 2.34$; $p > 0.05$).

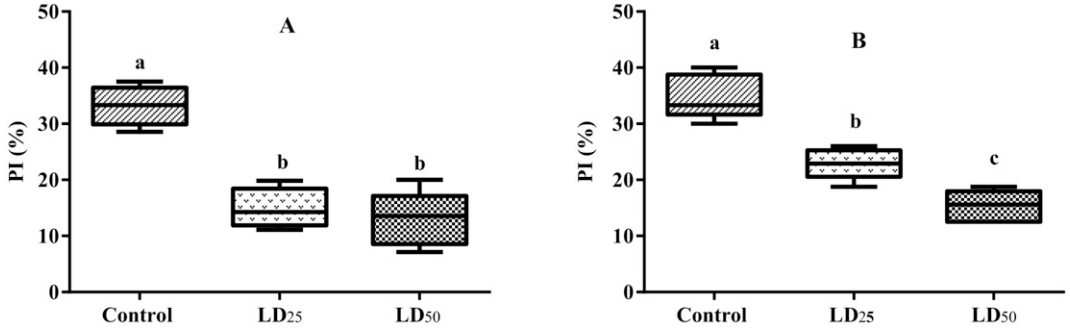

Fig. 2. Preference index (PI) of adults of *D. melanogaster* subjected to two-choice test on azadirachtin-treated food (0.25 μg/μl) marked with the blue food-dye and control medium marked with red food-dye. A: Male, B: female. Different small letters indicate a significant difference between control and treated individuals.

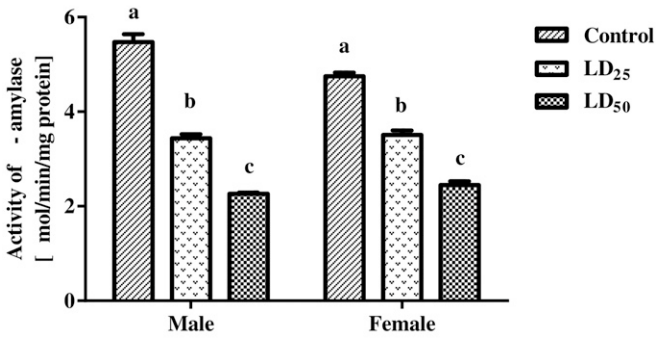

Fig. 4. Effect of azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of α-amylase of adults male and female, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series ($p < 0.05$).

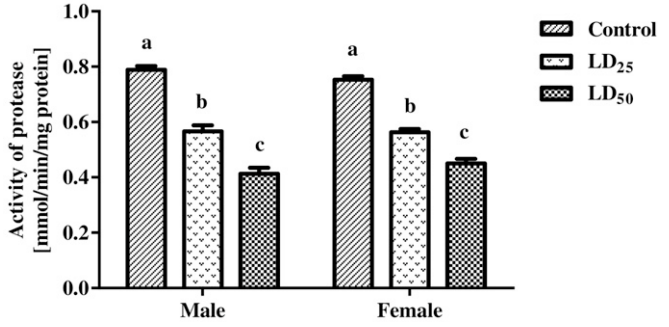

Fig. 6. Effect of azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of protease of adults male and female, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series ($p < 0.05$).

3.4.4. Effect on chitinase activity

As a consequence of larval treatment, the chitinolytic activity of adult of both sex of *D. melanogaster* decreased significantly as compared to controls ($p < 0.001$) (Fig. 7). ANOVA revealed significant effects of dose ($F_{2, 30} = 48.81$; $p < 0.001$) ($p < 0.001$), sex ($F_{1, 30} = 44.94$; $p < 0.001$) and dose-sex interaction ($F_{2, 30} = 8.67$; $p < 0.001$). The mean values for controls series were 0.440 ± 0.010 mmol/min/mg proteins and 0.540 ± 0.012 mmol/min/mg proteins respectively for males and females. In treated series the mean values recorded were 0.420 ± 0.009 mmol/min/mg proteins for the LD_{25} and 0.370 ± 0.005 mmol/min/mg proteins for the LD_{50} for males and 0.430 ± 0.012 mmol/min/mg proteins for the LD_{25} and 0.420 ± 0.010 mmol/min/mg proteins for the LD_{50} for females (Fig. 7).

4. Discussion

In this study, we evaluated the effects of azadirachtin treatment of early third instar larvae on feeding behavior and digestive enzymes of adults of *D. melanogaster*. In our results, flies preferentially ingested dyed control food rather than azadirachtin-treated food. Dyes appear to affect the choice of flies, with a clear preference for blue food-dye than the red food-dye. This preference for blue food-dye was reported by Fougeron [40]. Recently, Bantel & Tessier, [41] reported the impact of these dyes concentration on food preference in *D. melanogaster*. In addition, our results revealed that the antifeeding effect of azadirachtin was more marked in previously treated flies (third instar larvae) as compared with naïf flies suggesting that previous experience plays a major role in the feeding preference of adults of *D. melanogaster*.

The effects of azadirachtin on olfactory response were also evaluated on adults of *D. melanogaster*. All flies tested in dual choice revealed a clear preference for solvent odour rather than azadirachtin odour. Pre-imaginal exposure of flies to the bioinsecticide had no significant effects on insect olfactory orientation.

In insect, feeding preingestive inhibitors affect insect orientation, searching, and food selection by interfering with olfactory or gustatory chemosensillas [42]. A signal to the Central Nervous System (CNS) causes avoidance from further approach or feeding [42]. In *Drosophila* species attractive odourants have been studied extensively, but little is known about avoidance pathways [43]. However, the conservation of olfactory avoidance was reported in *Drosophila* species [43] which may be exploited as new behavioral strategies for controlling pest's species.

Previous studies have demonstrated that azadirachtin caused a strong antifeeding effects in several insects species [44,24,45]. This primary antifeeding effect of azadirachtin seems to be mediated by contact chemoreceptors and linked to an inhibitory effect on the rate of firing of sugar sensitive cells of the gustatory chemoreceptors [46]. Indeed, several species have been reported to be extremely sensitive to primary antifeedancy of azadirachtin, which prefer to starving to death rather than ingesting the biopesticide [47]. In addition, *D. melanogaster* seems to be able to discriminate food texture and integrate the chemical and physical qualities of food [48] which may contribute to avoiding azadirachtin-treated food.

Different recent studies support the hypothesis that a pre-imaginal experience can change the adult response and generate learning memory through developmental stages [49,50,51,17]. This learning avoidance of azadirachtin was reported in *D. melanogaster* oviposition sites selection after larval exposure (third instar larvae) to the bioinsecticide [17].

The primary antifeedancy is mediated by gustation and involved in discriminating between edible and toxic substance. However, a secondary antifeedant effect including a long-term reduction in food intake,

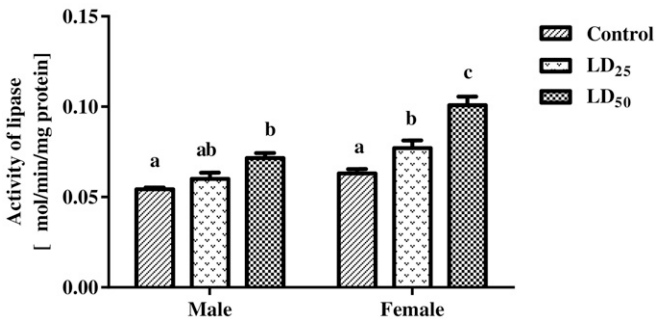

Fig. 5. Effect of azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of lipase of adults male and female, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series ($p < 0.05$).

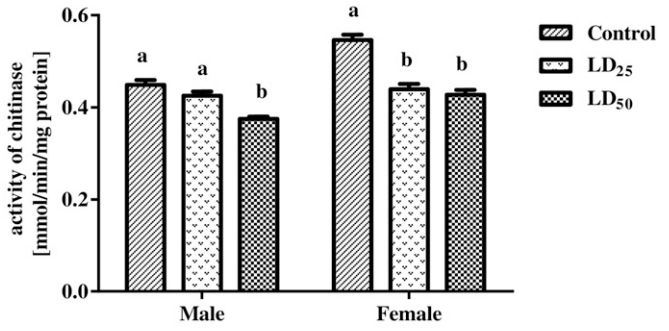

Fig. 7. Effect of azadirachtin, topically applied on early third instars larvae of *D. melanogaster* on activity of chitinase of adults male and female, (m ± SE; n = 6 replicates of 10 insects). Different small letters indicate a significant difference between control and treated series ($p < 0.05$).

and direct detrimental effects on different insect tissues such as muscles, fat body and gut epithelial cells [47].

In the current experiments, azadirachtin reduced significantly food intake of flies surviving to azadirachtin-treated third instar larvae (LD_{25}, LD_{50}) as compared to the controls. This long-term feeding reduction was reported in several insects' species and the level of food consumption differs upon species and was correlated to the concentration used [28,24]. In addition, azadirachtin was reported to affect negatively weight gain in *Spodoptera eridania* [24], *Plodia interpunctella* [52] and adults surviving from larval exposure of *D. melanogaster* [53]. 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 [54]. Lai et al. [55] reported that the inhibition of growth and development caused by azadirachtin in *D. melanogaster*, was consistent with those caused by disruption of the IIS pathway.

Azadirachtin was reported to cause disruption in insect physiology and its ability to digest food [24]. Our results showed a clear disruption of digestive enzymes activities responsible of the broken down of dietary components before its absorption by the intestinal epithelium. Similar results were reported in several insects species [56,28,57]. In the current study, azadirachtin reduced significantly the activity of α-amylase and chitinase of adults of *D. melanogaster* surviving to azadirachtin-treated third instar larvae (LD_{25}, LD_{50}).

Midgut α-amylases are involved in starch and other carbohydrate metabolism and its activities depends on feeding diet [58]. Insects Chitinases hydrolyze the β-(1–4) bonds of the residues of *N*-acetyl-β-D-glucosamine and are essential for moulting process as well as digestive enzymes compartmentalization because they are involved in the turn-over of the peritrophic matrices lining the gut epithelium [59]. The reduction of α-amylase activity under azadirachtin treatment was reported in *Periplaneta Americana* [44], *Glyphodes pyloalis* [28]. Moreover, Lai et al. [55], reported that azadirachtin reduced the activity of α-amylase in midgut of *D. melanogaster* by repressing expression of α-amylase gene. Azadirachtin was also reported to affect the peritrophic membrane, leading to damage of cellular surfaces of the midgut [28]. In addition, in insects the expression of chitinase gene was regulated by 20E [60], which may explain the effect observed in our results, since azadirachtin act as an antagonist of the 20E.

Lipases are responsible for hydrolysis the outer links of fat molecules. These enzymes play an important role in different physiological process such as storage and lipid mobilization, reproduction, growth and defence against pathogens [61]. In this study, lipase activity was significantly increased in azadirachtin-treated insects as compared to controls and the females showed the highest activity. This result is different from other reports where reduction in lipase is mostly reported after azadirachtin treatment; however, the observed effect was dependent on the chemical formulation and concentration of azadirachtin [28]. The increase in lipase activity noted in our experiments could be attributed to its esterase activity needed for detoxification [61]. However, the highest activities observed in female may be a result of a increasing of energy demand for reproduction [62].

Proteases have crucial role in food digestion by broken down proteins into amino acids [61]. Our results showed that azadirachtin caused a significant reduction of the protease activity in *D. melanogaster*. Low protease activity in the midgut of insects treated with azadirachtin was reported in *Periplaneta americana* [44] and *Glyphodes pyloalis* [28]. Moreover, Timmins & Reynolds [63] reported that azadirachtin reduces trypsin activity in the midgut of *Spodoptera litura.*

Our result supports the secondary antifeedancy action of azadirachtin which included a long-term reduction in food consumption and digestive efficiency. The precise effects of azadirachtin on food metabolism are not well known. However, different recent studies reported that azadirachtin interfere with activities of different enzymes such as α- amylase, lipase, α - and β -glucosidases, proteases, lactate dehydrogenase and glutathione S-transferase [58,28,57,16]. Jbilou et al. [64] and Almeida et al. [65] found that azadirachtin affect the midgut epithelial cells, resulting in inhibition of digestive enzymes, efficiency of digestion and nutrient absorption which reduce access of nutrients for biological requirements. Furthermore, this reduction in efficiency of conversion of ingested and digested food may be a result of an energy conversion from biomass production into induction enzymes involved in detoxification of azadirachtin [24].

In conclusion, preimaginal exposure to azadirachtin affects the behavioral and physiological processes that underlie food selection and digestion in *D. melanogaster*. This finding suggest a long term antifeedancy action which may reinforce behavioral avoidance of azadirachtin and contribute as push-pull strategies in integrated pest management programs using antifeedants as a safer alternative crop protectants.

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ÉCOLE DOCTORALE
Agriculture, alimentation, biologie, environnement, santé (ABIES)

Titre : Impacts d'un biopesticide, l'azadirachtine, sur la physiologie et le comportement de *Drosophila melanogaster*: Reproduction, nutrition et propriétés aversives.

Mots clés : Azadirachtine, *D. melanogaster*, fécondité, développement, gustation, olfaction, locomotion.

Résumé : Si les effets de toxicité aigüe des pesticides sont assez bien connus, les effets à court et long terme d'une exposition non létale à un stade juvénile sont beaucoup moins étudiés. Dans ce travail, nous avons examiné les effets à long terme du Neem-Azal, qui contient 1% d'azadirachtine A, sur un insecte modèle, *Drosophila melanogaster*. Deux doses (DL_{25} : 0,28 µg, DL_{50} : 0,67 µg correspondant respectivement à 25% et 50% de mortalité cumulée des stades immatures) ont été utilisées par application topique sur des larves du début du stade larvaire (L3). Les effets de cette unique exposition à l'azadirachtine ont été testés sur plusieurs générations successives jusqu'à restauration des paramètres étudiés.

L'exposition unique, en période pré-imaginale, à l'azadirachtine affecte la fécondité des femelles en réduisant significativement le nombre d'œufs pondus chez deux générations successives (P : exposée, F1 : non exposée). Une restauration complète de la fécondité est notée chezla génération F2. Le traitement larvaire affecte également le choix de ponte des femelles qui ont survécu au trainement avec une claire préférence de ponte sur un milieu témoins par rapport au milieu traité à l'azadirachtine et ce pour toutes les générations testées. Les mouches traitées à l'azadirachtine (début de stade L3) montrent une aversion plus marquée au biopesticide comparativement aux mouches "naïves".

Chez la génération parentale, l'exposition précoce à l'azadirachtine affecte le développement des adultes en réduisant le nombre de descendants, en induisant un retard du développement larvaire et pupal ; en biaisant le sex-ratio en faveur des mâles et en provoquant plusieurs anomalies morphologiques. L'azadirachtine réduit de manière significative la survie des adultes chez deux générations successives et ce comparativement aux témoins. De plus, l'azadirachtine affecte la réactivité locomotrice des adultes en réduisant significativement les scores géotaxiques obtenus chez les adultes des deux sexes en comparaison aux témoins. L'azadirachtine affecte également la quantité de nourriture consommée par les mouches et perturbe le système chimiosensoriel des adultes en affectant leurs choix olfactifs et gustatifs. Les mouches préalablement traitées (début de stade L3) à l'azadirachtine ont une aversion plus marqué à cette molécule et évitent son odeur. Le biopesticide induit également une inhibition de l'extension du proboscis des adultes des deux sexes avec un effet plus marqué chez les adultes préalablement traités au stade larvaire. Les réponses électrophysiologiques des sensilles gustatives montrent une augmentation de la sensibilité des sensilles gustatives à l'azadirachtine chez les mouches traitées au stade larvaire confirmant l'aversion plus marquée à cette molécule.

Title : Impacts of a biopesticide, azadirachtin, on physiology and behavior of *Drosophila melanogaster :* Reproduction, nutrition and aversive properties.

Keywords : Azadirachtin, *D. melanogaster*, fecundity, development, tasting, olfaction, locomotion.

Abstract : While the acute toxicity of xenobiotics on organisms is well known, long term effects of intermittent exposures are less studied. Here we examined the effects of a single larval exposure to a commercial antifeedant and insecticide Neem-Azal (containing 1% azadirachtin A), on *Drosophila melanogaster*. Two doses (LD_{25} : 0.28 µg, LD_{50} : 0.67 µg corresponding respectively to 25% and 50% cumulative mortality of immature stages) were used by topical application on early third instar larvae (L3). The possible adverse effects of this prior single exposure to azadirachtin experienced by the preceding generations were evaluated over three successive generations until full restoration of the studied parameters.

The single pre-imaginal exposure of azadirachtin affects female fecundity by significantly reducing the number of eggs laid over two successive generations (P and F1). Full restoration of affected fecundity was noted in the second generation (F2). Larval treatment also affects oviposition choices, the surviving flies avoiding treated substrates more than control flies for all tested generations.

Early exposure to azadirachtin also affects adult's development by reducing the number of organisms, delay larval and pupal development ; male biased sex ratio and induced morphological alterations. This exposure significantly reduces adult's survival during wo successive generations. Moreover, azadirachtin was found to affect Drosophila adult's locomotor reactivity by significantly decreased the geotactic positioning scores of adults of both sexes as compared to controls.

Azadirachtin affects the amount of food intake by the flies and disrupts the chemosensory system of the adults by affecting their olfactory and gustatory choices. Results showed that previously treated flies (3rd instar larvae) showed an aversion to the biopesticide avoiding azadirachtin odor. The biopesticide also inhibited the proboscis extension response in the adults of both sexes with more marked effects in treated flies. The electrophysiological studies of gustatory response showed an increase of sensibility of gustatory sensilla to azadirachtin previously exposed flies confirming the more marked avoidance to this molecule.

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