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**Efekty działania nikotyny i acetamiprydu na  
*Drosophila melanogaster* i *Drosophila suzukii***

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Rozprawa doktorska wykonana pod kierunkiem  
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ADAM MICKIEWICZ UNIVERSITY IN POZNAN  
FACULTY OF BIOLOGII  
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DEPARTMENT OF GENETICS

PHD THESIS

**Effects of nicotine and acetamiprid on  
*Drosophila melanogaster* and *Drosophila suzukii***

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## Spis treści

1. Wykaz oryginalnych publikacji stanowiących rozprawę doktorską.....	7
2. Wykaz skrótów.....	8
3. Streszczenie .....	9
4. Abstract .....	11
5. Wstęp .....	13
5.1. Wprowadzenie.....	13
5.2. Główne cele rozprawy doktorskiej.....	18
5.3. Uzyskane rezultaty.....	20
5.4. Podsumowanie.....	24
5.5. Literatura.....	26
6. Publikacja I: <i>Genotoxic effects of sub-lethal doses of nicotine and acetamiprid in neuroblasts of Drosophila melanogaster and Drosophila suzukii</i> .....	32
7. Publikacja II: <i>Fluctuating asymmetry spotted wing Drosophila (Diptera: Drosophilidae) exposed to sublethal doses of acetamiprid and nicotine</i> .....	43
8. Oświadczenia współautorów o wkładzie w powstanie artykułów.....	61

## 1. Wykaz oryginalnych publikacji stanowiących rozprawę doktorską

Rozprawa doktorska składa się z dwóch oryginalnych publikacji naukowych opublikowanych w czasopismach z tzw. listy filadelfijskiej, o łącznym IF = 8.9:

1. **Lewandowska-Wosik A., Chudzińska E.M., Wojnicka-Półtorak A. 2024**, Genotoxic effects of sub-lethal doses of nicotine and acetamiprid in neuroblasts of *Drosophila melanogaster* and *Drosophila suzukii*, *Ecotoxicology and Environmental Safety*, 280(7):116585, doi:10.1016/j.ecoenv.2024.116585  
*Impact Factor = 6.2, Q1, 96 perc., 100 pkt. MNiSW*
2. **Lewandowska-Wosik A., Chudzińska E.M. 2024**, Fluctuating asymmetry spotted wing *Drosophila* (Diptera: Drosophilidae) exposed to sublethal doses of acetamiprid and nicotine, *Insects*, 15(9): 681, doi:10.3390/insects15090681  
*Impact Factor = 2.7, Q1, 85 perc., 100 pkt. MNiSW*

## 2. Wykaz skrótów

ACM – acetampiryd

DM – *Drosophila melanogaster*

DS – *Drosophila suzukii*

F1 – pierwsze pokolenie potomstwa

F5 – piąte pokolenie potomstwa

F10 – dziesiąte pokolenie potomstwa

FA – ang. *fluctuating asymmetry*; asymetria fluktuacyjna

LC50 – ang. *lethal concentration*; medialne stężenie śmiertelne

N – nikotyna

nAChRs – ang. *nicotinic Acetylcholine Receptors*; cholinergiczne receptory nikotynowe acetylocholiny

NEOs – ang. *neonicotinoid insecticides*; insektycydy neonicotynoidowe

OTM – ang. *Olive Tail Moment*

P – ang. *parental generation*; pokolenie rodzicielskie

SCGE – ang. *Single-Cell Gel Electrophoresis*; elektroforeza pojedynczych komórek w żelu

SP50 – ang. *50% survival percentage*; procent przeżycia (50%)

SWD – ang. *Spotted Wing Drosophila*; *Drosophila* plamoskrzydła

### 3. Streszczenie

Szybki rozwój rolnictwa wymusza produkcję szerokiej gamy różnych pestycydów, co rodzi wiele pytań dotyczących ich bezpieczeństwa i potencjalnych zagrożeń dla zdrowia i środowiska. Neonikotynoidy to klasa insektycydów - pochodnych nikotyny - które są szeroko stosowane w ochronie roślin. Silnie wpływają na cholinergiczne receptory nikotynowe acetylocholino w układzie nerwowym owadów, powodując nadmierną stymulację synaps nerwowych, paraliż i śmierć szkodników. Związki te są rozpuszczalne w wodzie, więc ich obecność nie ogranicza się do miejsc aplikacji, a zwiększone stężenia neonikotynoidów wykrywa się w całym środowisku. Oznacza to, że niezamierzenie stają się częścią pożywienia, co czyni je potencjalnie niebezpiecznymi dla innych organizmów, w tym także dla ludzi. Podobieństwo podstawowych mechanizmów komórkowych i rozwojowych między muchówkami i ludźmi uzasadnia centralną rolę modelu *Drosophila* w testach toksykologicznych. Niektóre gatunki należące do rodziny Drosophilidae to szkodniki, które niszczą duże części upraw i powodują poważne problemy ekonomiczne i środowiskowe. Kilkanaście lat temu do grupy intensywnie zwalczanych szkodników dołączyła muszka plamoskrzydła (DS) *Drosophila suzukii* (Mats.), nowy, inwazyjny gatunek (w Polsce zaobserwowana po raz pierwszy w 2014 r.). Jej żerowanie na dojrzewających owocach obniża plony i generuje znaczne straty finansowe. Obecnie jedynym preparatem z grupy neonikotynoidów zatwierdzonym do zwalczania muszki plamoskrzydłej jest acetamipryd. Pomimo wielu badań nad toksycznością nikotyny i acetamiprydu, eksperymenty, w których populacje są narażone na niskie dawki pestycydów przez wiele pokoleń, są nieliczne. Podstawę niniejszej rozprawy doktorskiej stanowią więc zagadnienia dotyczące tego problemu. Jej głównym celem było zbadanie skutków długotrwałego stresu wywołanego kontaktem z subletalnymi dawkami neurotoksycznych insektycydów na przykładzie dwóch gatunków owadów: *D. melanogaster* i *D. suzukii*. Aby prześledzić efekty działania toksyn, założone zostały wielopokoleniowe hodowle owadów na pożywkach z dodatkiem niskich dawek toksyn. Dla zminimalizowania różnic, reakcje stresowe były testowane w tych samych kontrolowanych warunkach. Oprócz acetamiprydu do analiz wykorzystano nikotyne, która jako substancja o dobrze zbadanych właściwościach spełniała rolę kontroli pozytywnej. Wielkość dawek subletalnych użytych do badań określono oceniając procent przeżycia (SP50). W celu

sprawdzenia wpływu insektycydów na organizm owadów wykonano analizę uszkodzeń DNA neuroblastów larwalnych z zastosowaniem metody kometkowej w warunkach alkalicznych, analizę asymetrii fluktuacyjnej skrzydeł informującą o zaburzeniach stabilności rozwojowej oraz testy wspinaczkowe (testy ujemnej grawitacji) oceniające aktywność lokomotoryczną owadów.

Otrzymane wyniki umożliwiły stwierdzenie, że subletalne stężenia testowanych pestycydów obniżają kondycję owadów i mają działanie neurotoksyczne. Przeprowadzenie wielopokoleniowych hodowli pozwoliło zaobserwować, że w kolejnych generacjach nasilenie negatywnych objawów jest zróżnicowane w zależności od zastosowanej substancji toksycznej oraz od badanego gatunku. Większą wrażliwością zarówno w przypadku acetamiprydu, jak i nikotyny, cechowała się *D. suzukii*. Jednak w przypadku acetamiprydu, przy subletalnych dawkach w dziesiątym pokoleniu zaobserwowano u tego szkodnika znaczną redukcję stopnia uszkodzenia DNA neuroblastów. Może to oznaczać, że owady docelowe przez kilka pokoleń rozwijają odporność na niskie stężenia tego pestycydu. Przeprowadzone badania pozwalają na lepsze zrozumienie działania testowanych insektycydów, a analiza ich skuteczności obserwowana przez kilka pokoleń owadów przynosi nowe informacje i pozwala szerzej spojrzeć na kształtowanie się adaptacji poprzez nabywanie oporności. Może to być wskazówką przy określeniu „złotego środka” między koniecznością ochrony zbiorów przed *D. suzukii* a koniecznością ochrony organizmów niebędących celem działania insektycydów, w tym także ludzi.

Słowa kluczowe:

*Drosophila melanogaster*, *Drosophila suzukii*, nikotyna, acetamipryd, neurotoksyczność, dawki subletalne, wielopokoleniowa hodowla

## 4. Abstract

The rapid development of agriculture has forced the production of a wide range of different pesticides, which raises many questions about their safety and potential health and environmental hazards. Neonicotinoids are a class of insecticides - nicotine derivatives - that are widely used in crop protection. They strongly affect the neuronal nicotinic acetylcholine receptors in the nervous system, causing excessive stimulation of neural synapses, paralysis and death of pests. These compounds are water-soluble, so their presence is not limited to the application sites, and increased concentrations of neonicotinoids are detected throughout the environment. This means that they unintentionally become part of the food, making them potentially dangerous to other organisms, including humans. The similarity of basic cellular and developmental mechanisms between flies and humans justifies the central role of the *Drosophila* model in toxicological tests. Some species belonging to the Drosophilidae family are pests that destroy large parts of crops and cause serious economic and environmental problems. Several years ago, the spotted winged fly (SWD) *Drosophila suzukii* (Mats.) joined the group of intensively controlled pests, a new, invasive species (in Poland first observed in 2014). Its feeding on ripening fruit reduces yields and generates significant financial losses. Currently, the only neonicotinoid product approved for the control of the spotted winged fly is acetamiprid. Despite many studies on the toxicity of nicotine and acetamiprid, experiments in which populations are exposed to low doses of pesticides for many generations are rare. Therefore, the basis of this doctoral dissertation is issues related to this problem. Its main goal was to investigate the effects of long-term stress induced by contact with sublethal doses of neurotoxic insecticides on the example of two insect species: *D. melanogaster* and *D. suzukii*. In order to investigate the effects of the toxins, multigenerational insect cultures were established on media with the addition of low doses of toxins. To minimize differences, stress reactions were tested in the same controlled conditions. In addition to acetamiprid, nicotine was used for the analysis, which as a well-studied substance played the role of positive control. The amount of sublethal doses for the studies was determined by assessing the percentage of survival (SP50). To examine the effect of insecticides on the insect organism, an analysis of DNA damage of larval neuroblasts was performed using the comet assay in alkaline conditions, an analysis of wing fluctuating asymmetry informing about disturbances in

developmental stability and climbing tests (negative gravity tests) assessing the locomotor activity of insects.

The results obtained allowed us to state that sublethal concentrations of the tested pesticides reduce the condition of insects and have a neurotoxic effect. Conducting multi-generational breeding allowed us to observe that in subsequent generations of insects the intensity of negative symptoms varies depending on the toxic substance used and the species tested. *D. suzukii* was known to have greater sensitivity to both acetamiprid and nicotine. However, in the case of acetamiprid, in the tenth generation a significant reduction in the degree of DNA damage of neuroblasts was observed in this pest at lower concentrations. This may mean that the target insects develop resistance to low concentrations of acetamiprid over several generations. The conducted studies allow for a better understanding of the action of the tested insecticides, and the analysis of their effectiveness observed over several generations of insects provides new information and allows for a broader look at the formation of adaptation through the acquisition of resistance. This may provide guidance in determining the “golden mean” between the need to protect crops from *D. suzukii* and the need to protect non-target organisms, including humans.

Keywords:

*Drosophila melanogaster*, *Drosophila suzukii*, nicotine, acetamiprid, neurotoxicity sublethal doses, multigenerational effects

## 5. Wstęp

### 5.1. Wprowadzenie

Rozwój rolnictwa wymusza wytwarzanie i wykorzystywanie szerokiej gamy różnych pestycydów, co rodzi wiele pytań dotyczących ich bezpieczeństwa i potencjalnych zagrożeń dla zdrowia i środowiska. Obecnie szeroko stosowane w ochronie roślin są neonikotynoidy (NEOs) - ogólnoustrojowe środki owadobójcze będące pochodnymi nikotyny. Związki te są rozpuszczalne w wodzie, więc ich obecność nie ogranicza się do miejsc aplikacji, a podwyższone stężenia neonikotynoidów wykrywane są nie tylko w glebie i wodzie, ale także w żywności (Chen i in. 2014, Simon-Delso i in. 2015). Oznacza to, że niezamierzenie mogą się one stać częścią diety, przez co stanowią potencjalne zagrożenie nie tylko dla owadów, ale także dla innych organizmów, w tym dla ludzi (Zhang i in. 2019).

W układzie nerwowym NEOs silnie wpływają na cholinergiczne receptory nikotynowe acetylocholin (nAChRs), powodując nadmierną stymulację synaps nerwowych, paraliż i śmierć szkodników. Neonikotynoidy zostały wprowadzone jako alternatywa dla wcześniej stosowanych insektycydów, które okazały się szkodliwe dla ludzi i środowiska. Ich niewątpliwą przewagą nad innymi grupami związków jest selektywność w stosunku do nAChRs owadów w porównaniu do receptorów kręgowców, co wynika z większej liczby podtypów nAChRs (konkretnie  $\alpha 4\beta 2$ ), wykazujących powinowactwo do neonikotynoidów u bezkręgowców (Tomizawa i Casida 2005, Sheeds i in. 2016)

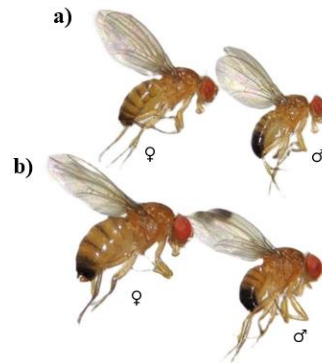
Zrozumienie mechanizmów odpowiedzialnych za funkcjonowanie układu nerwowego jest bardzo zaawansowane w dużej mierze dzięki badaniom przeprowadzonym na prostych modelach bezkręgowców, głównie na *Drosophila melanogaster* (Meigen, 1930). To dobrze poznany i najczęściej wykorzystywany w biologii organizm modelowy, od lat stosowany w badaniach dotyczących reakcji na stresy różnego typu, w tym także na stres związany z ekspozycją na pestycydy. Nie wszystkie gatunki z rodzaju *Drosophila* są tak użyteczne, niektóre należą do szkodników niszczących dużą część zbiorów i powodujących poważne problemy ekonomiczne i ekologiczne. Kilkanaście lat temu do grona intensywnie zwalczanych szkodników dołączył nowy, inwazyjny gatunek – muszka płamoskrzydła - *Drosophila suzukii*. Jego

gwałtowne rozprzestrzenianie się na terenie Europy (także w Polsce) wiąże się ze znacznymi stratami plonów owoców jagodowych i pestkowych np. borówki czy wiśni. Obecnie jedynym dopuszczonym do zwalczania tego szkodnika preparatem z grupy neonikotynoidów jest acetamipryd. Wiadomo, że w naturze owady mogą być narażone na różne dawki insektycydów, nie tylko śmiertelne, ale także subletalne. Może to wywołać zmiany sprzyjające rozwojowi odpornych lub przystosowanych owadów oraz wiele efektów, które mogą stanowić zagrożenie dla różnych, niebędących przedmiotem zwalczania organizmów. Mając to na uwadze, wykorzystałam wieloletnie doświadczenie w hodowli *D. melanogaster* prowadzonej w Zakładzie Genetyki Wydziału Biologii UAM do zbadania skutków długotrwałego stresu wywołanego kontaktem z subletalnymi dawkami neurotoksycznych insektycydów na przykładzie dwóch gatunków owadów z rodziny Drosophilidae: *D. melanogaster* i *D. suzukii*.

#### *Opis badanych gatunków*

Rodzina Drosophilidae obejmuje około 4400 gatunków, a sam rodzaj *Drosophila* liczy ich około 1600, z czego najbardziej znanym jest *Drosophila melanogaster* Meigen, 1830 (**Rys. 1a**) – owad będący uznanym i powszechnie wykorzystywanym organizmem modelowym w szeroko rozumianych badaniach biologicznych. *D. melanogaster* to kosmopolityczny gatunek wywodzący z Afryki Równikowej (Lachaise i in. 1988). Jest ludzkim komensalem, wykorzystującym jako źródło pokarmu głównie psujące się, gnijące owoce. Rozwijające się tam drożdże stanowią podstawowy składnik diety tych owadów, szczególnie niezbędny do prawidłowego rozwoju larw (Baumberger 1917). Za wysoką pozycję jaką *D. melanogaster* zajmuje w świecie nauki odpowiadają m.in. takie właściwości jak krótki cykl życiowy, produkcja liczego potomstwa, wyraźny dymorfizm płciowy czy wreszcie łatwość prowadzenia hodowli. Istotne znaczenie mają też: mała liczba chromosomów ( $2n = 8$ ; 3 pary autosomów i chromosomy płci XY), obecność chromosomów politenicznych, zsekwencjonowany genom (Adams i in. 2000), zawierający 125 milionów par zasad kodujących około 14 000 genów (Celnikier i in. 2003, Chen i in. 2008), dostępność żywotnych mutantów czy genetyczna podatność na kontrolę za pomocą systemu *GAL4/UAS* pozwalająca na uzyskanie ekspresji genów specyficznej dla konkretnego typu komórki lub tkanki (Zirin i in. 2024). *D. melanogaster* jest wykorzystywana w badaniach genetycznych (Nagarkar-Jaiswal S. i in. 2015, Ewen-Campen B. i in. 2017, Götze i in. 2022), badaniach dotyczących biologii rozwoju, chorób nowotworowych (Munnik i in. 2022, Sharpe i in. 2023), neurodegeneracyjnych (Yeates

i in. 2023, Deshpande i in. 2024), uzależnień (Kaun i in. 2012), rytmu okołodobowego i snu (Dubovy i Sehgal 2017), w nutrigenomice (Baenas i Wagner 2019), genotoksykologii (Naik i Mishra 2024) czy badaniu toksyczności insektycydów (Chmiel i in. 2019, Tasman i in. 2021).



**Rysunek 1.** Samica i samiec *Drosophila melanogaster* (a) oraz samica i samiec *Drosophila suzukii* (b) (zdj. A. Lewandowska-Wosik).

Drugim gatunkiem owadów stanowiącym obiekt podjętych w niniejszej rozprawie badań jest spokrewniony filogenetycznie z DM, należący do podgrupy „suzukii” grupy „melanogaster” - *Drosophila suzukii* Matsumura, 1931 (**Rys. 1b**). Muszka plamoskrzydła (ang. *Spotted Wing Drosophila*, SWD) to polifagiczny, inwazyjny gatunek wywodzący się z Azji Południowo-Wschodniej, który przez dekadę skolonizował ponad 20 krajów w Europie i obu Amerykach (Hauser 2011, Calabria i in. 2012, Asplen i in. 2015). Uważa się, że za ową szybką międzykontynentalną migrację odpowiada głównie globalny handel owocami (Lee i in. 2011), choć nie bez znaczenia jest też zdolność do aktywnego lotu (Tait i in. 2018, Vacas i in., 2019). Podobnie jak *D. melanogaster*, może szybko adaptować się do różnych środowisk i dlatego jest w stanie kolonizować różne nisze. Mimo, że europejskie populacje SWD są genetycznie mało zróżnicowane na skutek efektu przejścia w trakcie inwazji przez wąskie gardła demograficzne, gatunek ten wykształcił szereg mechanizmów pozwalających na ograniczenie szkodliwych konsekwencji niskiej zmienności genetycznej. Sukces ekspansji *D. suzukii* to również zasługa posiadania szeregu adaptacji do klimatu umiarkowanego, wysoka wydajność reprodukcyjna, czy wreszcie odpowiednia nisza i brak konkurencji (Rota-Stabelli i in. 2013). Muszka plamoskrzydła jest szkodnikiem upraw atakującym głównie owoce jagodowe jak wiśnie i borówki, ale i owoce dzikorosnące, w tym leśne (Bühlmann i Gossner 2022). W przeciwieństwie do *D.*

*melanogaster*, samica *D. suzukii* wykorzystując ostre, ząbkowane pokładełko przecina skórę i składa jaja, z których w owocach rozwijają się larwy. Uszkodzona skórka to otwarta droga dla patogenów, które powodują szybkie psucie i gnicie owoców, przez co stają się one nieatrakcyjne zarówno dla ludzi, jaki i dla innych kręgowców, np. ptaków roznoszących nasiona (Bühlmann i Gossner 2022). Niewielkie rozmiary jaj składanych wewnątrz owoców utrudniają wczesne wykrycie zagrożenia i sprawiają, że walka z tym szkodnikiem jest utrudniona.

### *Zastosowane insektycydy*

W badaniu zostały wykorzystane dwie substancje toksyczne: nikotyna i acetamipryd. Nikotyna jest bardzo silną neurotoksyną alkaloidową występującą naturalnie w *Nicotiana tabacum* i *N. rustica*, której skuteczność jako insektycydu była znana od dawna (Tomizawa i Casida 2005). Obecnie jej stosowanie jako pestycydu jest zabronione w Europie i USA ze względu na wysokie ryzyko zatrucia, szczególnie dla zapylaczy, i duże ryzyko nabycia odporności na tę substancję (Trocza i in. 2021). W przypadku *D. melanogaster* wiadomo, że narażenie na nikotynę w trakcie rozwoju zmniejsza zarówno przeżywalność, jak i wagę dorosłych owadów, opóźnia rozwój (Chudzińska i in. 2016, Velazquez-Ulloa 2017) i wpływa na strukturę mózgu, np. poprzez zwiększenie powierzchni półkul mózgowych w mózgach larw (Morris i in. 2018). W przeprowadzonych przeze mnie badaniach nikotyna została wykorzystana jako kontrola pozytywna. Acetamipryd, czyli N-(6-Chloro-3-pyridylmethyl)-N'-cyano-N-(methyl-d<sub>3</sub>)acetamidine jest jednym z insektycydów dopuszczonych i zalecanych do stosowania w celu ograniczenia liczebności *D. suzukii*. Związek ten to insektycyd chloronikotynylowy nowej generacji o podobieństwie strukturalnym do nikotyny. Podobnie jak nikotyna jest agonistą nikotynowych receptorów cholinergicznym acetylocholino (nAChRs) u owadów. Jako insektycyd neonikotynoidowy z grupy cyjanoamidynowej nie jest klasyfikowany jako toksyczny dla pszczoł (Pruszyński 2011).

### *Materiał i metody*

W badaniach zostały wykorzystane owady należące do dwóch gatunków: *Drosophila melanogaster* (DM) – szczep dziki Oregon pochodzący z hodowli Zakładu Genetyki Uniwersytetu im. Adama Mickiewicza w Poznaniu oraz *D. suzukii* (DS) zebrany z sadów w okolicach Wrześni w województwie wielkopolskim i wprowadzony do hodowli ZG UAM. Owady były hodowane na podłożu składającym się z mąki

kukurydzianej, drożdży, agaru, cukru i kwasu propionowego, w następujących warunkach: temperatura  $23^{\circ}\text{C}\pm 2$ , wilgotność 60%, w cyklu 12 godzin światło:12 godzin ciemność.

W celu sprawdzenia, czy konsekwencją narażenia na insektycydy może być uszkodzenie DNA neuroblastów, przeprowadziłam test kometkowy *comet assay* metodą alkaliczną (elektroforeza pojedynczych komórek w żelu; SCGE) z barwieniem fluorescencyjnym bromkiem etydyny i analizą obrazów z wykorzystaniem mikroskopu fluorescencyjnego. *Comet assay* to czuła metoda pozwalająca na wykrywanie w pojedynczych komórkach uszkodzeń DNA w postaci pęknięć nici. Używana jest m.in. do badania genotoksyczności i mechanizmów działania różnych substancji chemicznych (Gaivão I. i Sierra L.M. 2014, Sierra L.M. i in. 2014, Rodriguez i in. 2023).

Aby ocenić wpływ ACM i N na motorykę owadów dorosłych zastosowałam test, który wykorzystuje naturalną tendencję *Drosophila* do wspinania się wbrew grawitacji (test ujemnej grawitacji, inaczej test wspinaczkowy). Jest on używany do ilościowego określenia wpływu mutacji genetycznych i/lub warunków środowiskowych na motorykę owadów, m.in. w badaniach chorób neurodegeneracyjnych, np. choroby Parkinsona, głównie przy użyciu modelu *D. melanogaster* (Madabattula i in. 2015).

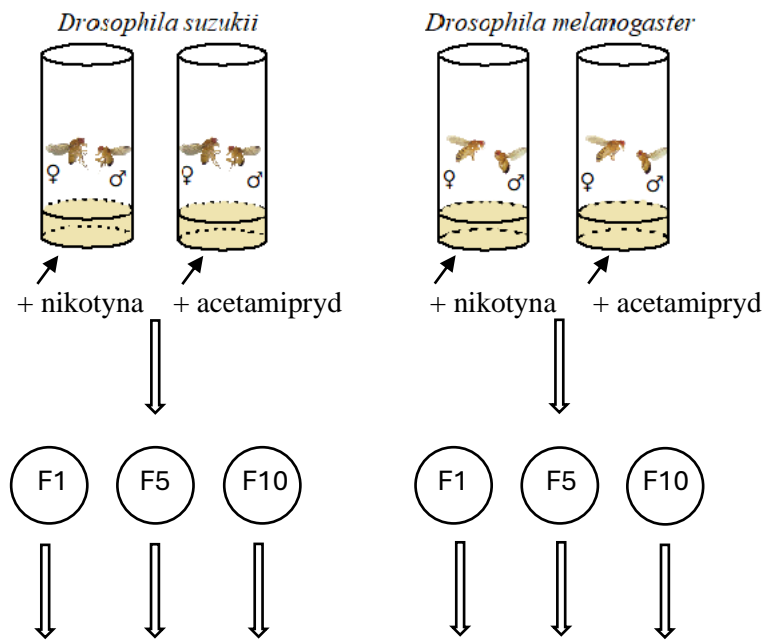
Jako biomarker stresu wywołanego przez insektycydy zastosowałam wskaźnik asymetrii fluktuacyjnej - FA (Graham i in. 2010, Büyükgüzel i in. 2020, Graham 2021). Mierzy on niewielkie, losowe odchylenia od idealnej symetrii wynikające z zaburzenia stabilności rozwojowej, za którą odpowiadają procesy zapewniające spójność fenotypową organizmu w ustalonych warunkach środowiskowych i genetycznych pomimo istnienia niewielkiej zmienności w trakcie rozwoju, czyli tzw. szumu rozwojowego (Debat i David 2001, Debat i Petronnet 2013). Zakładając, że organizm jest kontrolowany przez te same geny i jest poddany takim samym wpływom środowiskowym, pojawienie się asymetrii fluktuacyjnej i jej wzrost świadczy o spadku stabilności rozwojowej (Palmer i Strobeck 1986). Dwustronnie symetryczne struktury, które wpływają na funkcjonowanie i sprawność organizmu powinny cechować się jak najmniejszą asymetrią (Palmer i Strobeck 1986). Badania wykazują, że morfologia skrzydeł owadów jest w znacznym stopniu zależna od czynników stresowych (Hoffmann i in. 2005), dlatego zdecydowałam się na analizę użytkowania skrzydeł pod kątem FA. Realizując to zadanie wykonywałam pomiary (program CellSens Olympus) wybranych żyłek z prawego i lewego skrzydła.

## 5.2. Główne cele rozprawy doktorskiej

Pomimo licznych badań nad toksycznością insektycydów prowadzonych między innymi na *D. melanogaster*, eksperymenty, w których populacje byłyby narażone na subletalne dawki neurotoksyn przez wiele pokoleń są rzadkie i zaledwie kilka z nich dotyczy acetamiprydu (Lin i in. 2020, Sakaguchi i in. 2022, Zhang i in. 2022). Z jednej strony lepsze zrozumienie działania tych substancji może przyczynić się do skuteczniejszej walki z niebezpiecznymi szkodnikami, a analiza ich skuteczności testowana na kilku pokoleniach owadów może ułatwić szersze spojrzenie na kształtowanie się adaptacji poprzez nabywanie odporności na stosowane pestycydy. Z drugiej strony weryfikacja postawionych w rozprawie hipotez badawczych może dostarczyć ważnych danych dotyczących ryzyka spożycia insektycydów neonikotynoidowych w dawkach subletalnych, które mogą być niebezpieczne dla konsumentów „niedocelowych”, np. dla owadów pożytecznych czy ludzi. Dlatego **głównym celem niniejszej rozprawy było określenie skutków długotrwałego stresu wywołanego kontaktem z subletalnymi dawkami neurotoksycznych insektycydów (nikotyny i acetamiprydu) na przykładzie dwóch gatunków owadów z rodziny Drosophilidae (Rys. 2).**

Cele szczegółowe obejmowały:

- I. Zbadanie skutków działania acetamiprydu i nikotyny na owady z gatunku *Drosophila melanogaster* i *Drosophila suzukii* na poziomie behawioralnym, organizmalnym i komórkowym;
- II. Porównanie efektów długotrwałej ekspozycji na substancje toksyczne między dwoma blisko spokrewnionymi filogenetycznie gatunkami owadów;
- III. Stwierdzenie, czy wielopokoleniowa ekspozycja owadów na insektycydy spowoduje wykształcenie się odporności.



- I. testy przeżycia
- II. comet assay
- III. asymetria fluktuacyjna
- IV. testy behawioralne / wspinaczkowe

**Rysunek 2.** Schemat przeprowadzonych badań.

*Hipotezy badawcze*

**Założenie podstawowe:** testowane owady wykażą wielopoziomowe objawy stresu wywołanego działaniem acetamiprydu i nikotyny, ale w kolejnych pokoleniach nasilenie tych objawów będzie się zmniejszać ze względu na pojawienie się tolerancji w odniesieniu do zastosowanych pestycydów.

### *Hipotezy szczegółowe*

1. Subletalne stężenie testowanych pestycydów obniża kondycję owadów i ma działanie neurotoksyczne;
2. Odpowiedź na działanie insektycydów jest zbliżona u blisko spokrewnionych gatunków;
3. Wielopokoleniowa ekspozycja na insektycydy powoduje powstawanie odporności u szkodników.

### *Zadania badawcze*

Aby lepiej zrozumieć mechanizm działania toksyn, prowadziłam wielopokoleniową hodowlę owadów. Reakcje stresowe *D. melanogaster* - kosmopolitycznego gatunku modelowego i *D. suzukii* - inwazyjnego szkodnika, analizowano w tych samych kontrolowanych warunkach.

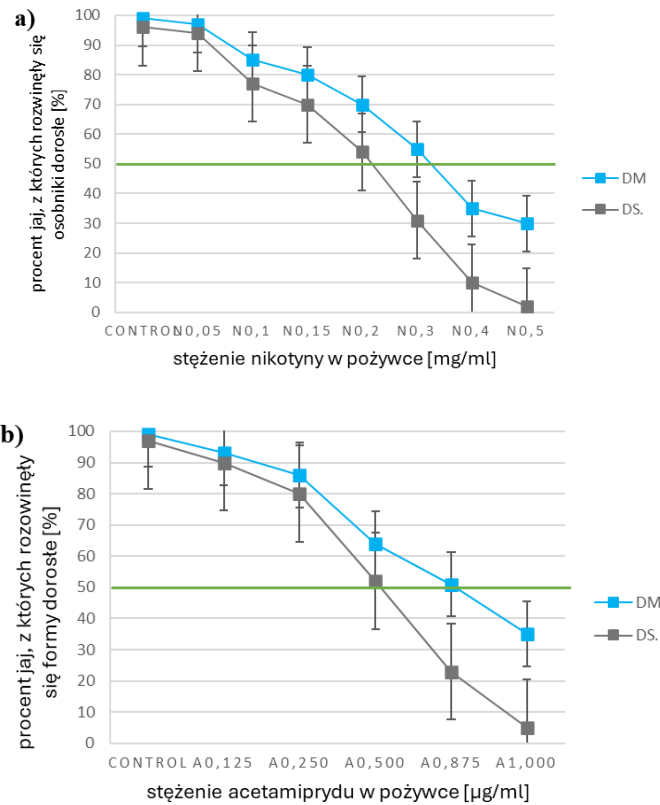
Zaplanowałam i wykonałam następujące **zadania badawcze**:

1. Standaryzacja warunków hodowli *Drosophila melanogaster* i *D. suzukii*;
2. Określenie wskaźnika przeżywalności 50% (SP50) dla badanych substancji i wybór do eksperymentów dawek subletalnych;
3. Prowadzenie hodowli ciągłej dziesięciu pokoleń *Drosophila melanogaster* i *D. suzukii* w warunkach ekspozycji na badane insektycydy;
4. Sprawdzenie z wykorzystaniem testu kometkowego, czy narażenie na acetamipryd i nikotynę skutkuje uszkodzeniem DNA neuroblastów larwalnych *Drosophila*;
5. Zbadanie czy wybrane toksyny zaburzają stabilność rozwojową, co może znaleźć odzwierciedlenie w postaci asymetrii fluktuacyjnej - biowskaźnika stresu;
6. Przeprowadzenie testów wspinaczkowych w celu stwierdzenia czy powyższe związki wpływają na lokomotorykę owadów;
7. Porównanie wyników uzyskanych dla *Drosophila melanogaster* i *D. suzukii*.

### **5.3. Uzyskane rezultaty**

Wskaźnik przeżywalności 50% (SP50) DM i DS określiłam na podstawie liczby jaj, z których po upływie 14 dni rozwinęły się osobniki dorosłe (**Rys. 3**). Testy przeprowadziłam na podłożach zawierających wyjściowo acetamipryd w stężeniach od 0,125 do 1  $\mu\text{g/ml}$  i nikotynę w stężeniach od 0,05 do 0,5  $\text{mg/ml}$ . Po analizie wyników wybrałam następujące subletalne stężenia, na które owady były narażane w sposób ciągły

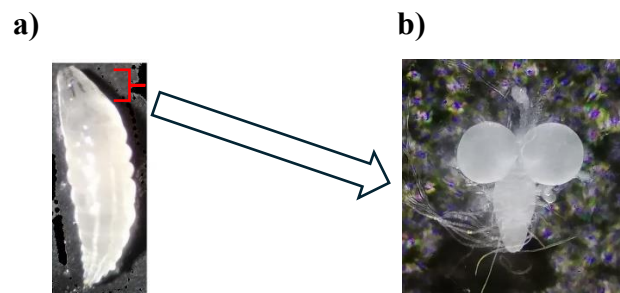
przez dziesięć kolejnych pokoleń: ACM: 0,125, 0,250, 0,500 i 0,875  $\mu\text{g/ml}$  dla obu gatunków oraz N: 0,05, 0,1, 0,15, 0,2, 0,3, i 0,5  $\text{mg/ml}$  dla DM, i N: 0,05, 0,1, 0,15, 0,2  $\text{mg/ml}$  dla DS.



**Rysunek 3.** Wykresy przeżycia przedstawiające procent jaj *D. melanogaster* i *D. suzukii*, z których na pożywkach z dodatkiem różnych stężeń nikotyny (a) i acetamiprydu (b) po 14 dniach rozwinęły się formy dorosłe. Linia zieloną oznaczono poziom 50%.

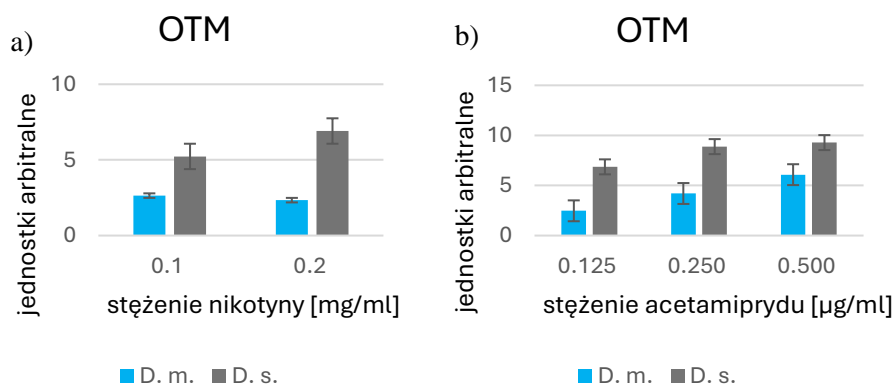
W przypadku DS wpływ N obniżał płodność i liczba osobników z pokolenia na pokolenie stopniowo i znacząco spadała, a przy najwyższym stężeniu N (0,2  $\text{mg/ml}$ ) nie było możliwe doprowadzenie hodowli do pokolenia F10. W przypadku DM niewielki spadek liczby owadów z pokolenia na pokolenie obserwowano tylko przy najwyższym stężeniu N (0,5  $\text{mg/ml}$ ); nie był on jednak na tyle duży, aby doprowadzić do całkowitego wyginięcia owadów. Pod wpływem ACM rozwój jaj został przyhamowany, a niektóre larwy pierwszego stadium obumarły. Wrażliwość gatunków celowego i niecelowego na ACM i N była wyraźnie różna.

Do oceny neurotoksycznych skutków zastosowanych insektycydów zgodnie z rekomendacjami wykorzystałam larwy trzeciego stadium (**Rys. 4a**), z których izolowałam mózgi będące źródłem neuroblastów (**Rys. 4b**).



**Rysunek 4.** Larwa *Drosophila* trzeciego stadium (**a**) i wyizolowany z niej mózg (**b**) (zdj. A. Lewandowska-Wosik).

Okazało się, że już małe dawki ACM i N powodują uszkodzenie DNA neuroblastów u owadów obu gatunków, przy czym większą wrażliwością charakteryzuje się gatunek *D. suzukii*. Porównanie otrzymanych wartości istotnego parametru OMT<sup>1</sup> dla DM i DS przy różnych stężeniach nikotyny i acetamiprydu w pierwszym pokoleniu owadów wykazało, że im wyższa zawartość insektycydów w pożywce tym większe uszkodzenie DNA jest obserwowane (**Rys. 5**).



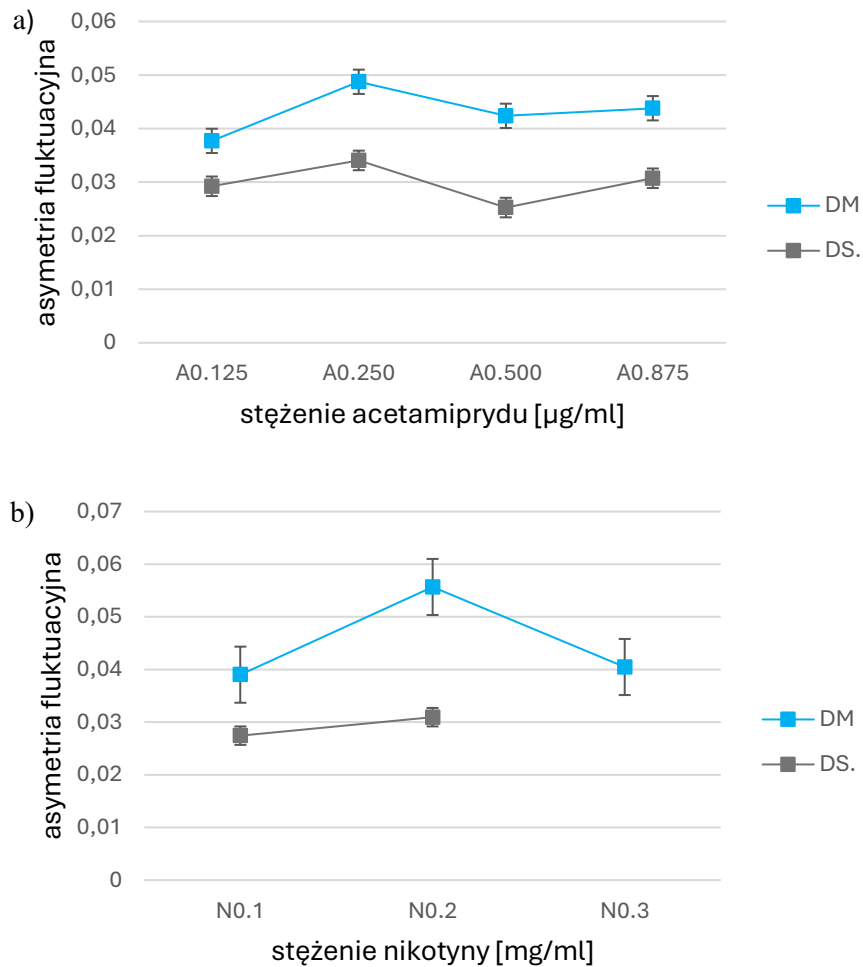
**Rysunek 5.** Porównanie wielkości uszkodzeń DNA neuroblastów na podstawie średnich wartości parametru OMT, przy narażeniu na różne dawki nikotyny (**a**) i acetamiprydu (**b**) dla gatunków *Drosophila melanogaster* i *D. suzukii*; zaznaczono błąd standardowy. Pierwsze pokolenie owadów.

<sup>1</sup> OMT stanowi iloczyn procentowej zawartości całkowitego DNA w ogonie i odległości między środkami masy regionu głowy i ogona [OTM = (średnia ogona - średnia głowy) x % DNA w ogonie].

Szczegółowy opis metod i wyniki powyższych badań zostały zaprezentowane w publikacji: **Lewandowska-Wosik A., Chudzińska E.M., Wojnicka-Póltorak A. Genotoxic effects of sub-lethal doses of nicotine and acetamiprid in neuroblasts of *Drosophila melanogaster* and *Drosophila suzukii*, *Ecotoxicology and Environmental Safety*, 2024, 280(7):116585, doi:10.1016/j.ecoenv.2024.116585.**

W artykule zostały zawarte również wyniki testu wspinaczkowego przeprowadzonego w celu oceny aktywności lokomotorycznej owadów. Pierwszym wnioskiem wynikającym z powyższych badań była obserwacja, iż test ujemnej grawitacji nie jest odpowiedni do porównania sprawności lokomocji badanych gatunków bowiem *D. suzukii* nie wykazywała takiej tendencji do przemieszczania się „do góry” jak *D. melanogaster*. Ponadto owady te poruszały się wolniej i częściej zatrzymywały podczas wspinaczki. Ciekawy wynik otrzymano dla DM - nikotyna w najniższym stężeniu wpłynęła pobudzająco na owady, pozostałe dawki N i ACM prowadziły do zaburzeń lokomocji.

Rezultaty uzyskane na podstawie analiz asymetrii fluktuacyjnej wykazały przydatność tego wskaźnika jako biomarkera stresu wywołanego przez oba testowane insektycydy. Metodyka i wyniki powyższych badań przedstawione zostały w publikacji: **Lewandowska-Wosik A., Chudzińska E.M. Fluctuating asymmetry spotted wing *Drosophila* (Diptera: Drosophilidae) exposed to sublethal doses of acetamiprid and nicotine, 2024, *Insects*, 15(9): 681, doi:10.3390/insects15090681.** Zaobserwowałam pojawienie się asymetrii fluktuacyjnej w użyłkowaniu skrzydeł zarówno u owadów gatunku DM (wyniki nieopublikowane), jak i DS. Ogólny wskaźnik FA (dla wszystkich mierzonych żyłek razem) przy każdym z badanych stężeń nikotyny i acetamiprydu okazał się wyższy dla *D. melanogaster* niż dla *D. suzukii* (**Rys. 6**). Nie zaobserwowano stałej tendencji do wzrostu FA wraz ze wzrostem stężeń insektycydów.



**Rysunek 6.** Średnia wartość współczynnika asymetrii fluktuacyjnej (FA) obserwowana przy kolejnych stężeniach acetamiprydu (a) i nikotyny (b) dla *D. melanogaster* i *D. suzukii*. Pierwsze pokolenie owadów.

#### 5.4. Podsumowanie

**I. Pozytywnie zweryfikowano postawioną hipotezę badawczą, że subletalne stężenie testowanych pestycydów obniża kondycję owadów i ma działanie neurotoksyczne;**

1. Ekspozycja owadów obu gatunków na subletalne dawki acetamiprydu i nikotyny zmniejsza przeżywalność owadów i ogranicza ich płodność.
2. Badane insektycydy zaburzają stabilność rozwojową owadów, co przejawia się we wzroście poziomu asymetrii fluktuacyjnej w użyłkowaniu skrzydeł.
3. Acetamipryd i nikotyna nawet w niskich dawkach powodują uszkodzenia DNA neuroblastów larwalnych, co potwierdza ich działanie neurotoksyczne.

## **II. Odrzucono hipotezę zgodnie z którą odpowiedź na działanie insektycydów jest zbliżona u blisko spokrewnionych gatunków.**

1. Większą wrażliwością zarówno w przypadku ACM jak i N cechuje się *D. suzukii*. Z pokolenia na pokolenie liczebność populacji owadów narażonych na insektycydy znacząco się zmniejsza. W przypadku *D. melanogaster* przy zastosowaniu takich samych dawek chemikaliów efekty ich działania są słabsze.
2. Zaburzenia lokomotoryczne zaobserwowane u owadów dorosłych w przypadku DM są częściowo skorelowane ze wzrostem uszkodzeń DNA w neuroblastach larw. Nie ma tu jednak pełnej korelacji – najniższa dawka nikotyny okazała się być pobudzająca. W przypadku acetamiprydu najniższe stężenie powoduje najmniejsze uszkodzenia DNA, ale największe zaburzenia lokomocji.
3. Test ujemnej grawitacji będący jednym z powszechniej stosowanych testów do badania lokomocji u *D. melanogaster* okazał się mniej przydatny w przypadku *D. suzukii*. Znacznie mniejsza ruchliwość DS w porównaniu z DM i niechęć do wspinania się wbrew grawitacji może być konsekwencją reakcji na stres. Obserwacje obu gatunków autorstwa Hansena i in. (2019) wykazały bowiem niższą aktywność *D. suzukii* wyhodowanych w warunkach laboratoryjnych.

## **III. Wielopokoleniowa ekspozycja na insektycydy powoduje powstawanie odporności u szkodników.**

1. Przeprowadzenie wielopokoleniowych hodowli pozwoliło zaobserwować, że w kolejnych generacjach owadów nasilenie negatywnych objawów jest zróżnicowane w zależności od zastosowanej substancji toksycznej oraz od badanego gatunku.
2. W przypadku nikotyny - samice DM składają mniej jaj i wykluwa się z nich mniej larw. Tendencja ta nie wykazuje istotnych zmian między pokoleniami. W przypadku DS liczba osobników z pokolenia na pokolenie znacząco spada, a przy najwyższym badanym stężeniu N (0,15 mg/ml i 0,2 mg/ml) nie było możliwe doprowadzenie hodowli do 10. pokolenia owadów dorosłych.
3. Pod wpływem acetamiprydu następuje zahamowanie rozwoju jaj, a część larw obumiera już w pierwszym stadium. Wrażliwość gatunku docelowego i niecelowego na ACM i N jest tu wyraźnie różna i wyższa dla DS.
4. Negatywny wpływ nikotyny na DNA w neuroblastach larw systematycznie wzrasta wraz ze wzrostem dawek a brak istotnych różnic w uszkodzeniach DNA

w porównaniu z pokoleniem F1 sugeruje, że DM i DS pozostają wrażliwe na N w badanych pokoleniach.

5. W przypadku acetamiprydu, stopień uszkodzeń DNA neuroblastów u DS spowodowanych narażeniem na trzy różne dawki ACM, w pokoleniach F1 i F5 był bardzo podobny, jednak w F10 zaobserwowano znaczną redukcję tych zaburzeń przy niższych stężeniach niż w próbie kontrolnej. Owady docelowe wydają się rozwijać odporność na niskie stężenia ACM przez kilka pokoleń. Wyższa dawka (0,500 µg/ml) nadal powoduje uszkodzenia tak duże jak w pokoleniu F1. W przypadku DM w pokoleniach F5 i F10 zaobserwowano największe uszkodzenia DNA przy najniższej dawce, co sugeruje brak odporności.

Podsumowując należy stwierdzić, że nikotyna i acetamipryd w dawkach niższych niż stosowane w celu zwalczania szkodników powodować mogą wiele niekorzystnych efektów biologicznych u *Drosophila melanogaster* i *D. suzukii*. Owe zmiany, dotyczące ograniczenia przeżywalności, uszkodzenia DNA neuroblastów larwalnych i zaburzenia stabilności rozwojowej przejawiające się w postaci asymetrii fluktuacyjnej dotyczą obu badanych gatunków. Badania potwierdzają skuteczność działania acetamiprydu na szkodnika jakim jest DS, ale i niekorzystne działanie na organizm niecelowy jakim jest DM i to na wielu różnych poziomach. Wskazuje to na konieczność dalszego wnikliwego obserwowania skutków, które ten neonikotynoid może wywoływać u innych bezkręgowców, w tym u pożytecznych zapylaczy czy kręgowców, wliczając ludzi. Tym bardziej, że subletalne dawki które badano są znacznie niższe niż te docelowe stosowane przez rolników czy sadowników, a takie stężenia mogą znajdować się w glebie, wodzie, w roślinach i stanowić potencjalne, pośrednie zagrożenie.

Z jednej strony lepsze zrozumienie działania insektycydów może przyczynić się do skuteczniejszej walki z groźnymi szkodnikami, a analiza ich efektywności przetestowana na kilku pokoleniach owadów przynosi nowe informacje i pozwala na szersze spojrzenie na kształtowanie się adaptacji poprzez nabycie odporności na stosowane pestycydy. Z drugiej strony weryfikacja hipotez badawczych dostarcza ważnych danych dotyczących ryzyka spożywania subletalnych dawek acetamiprydu, które mogą być niebezpieczne dla przypadkowych konsumentów, w tym także dla ludzi. Ponieważ jednak wpływ acetamiprydu na różne stadia rozwojowe i różne tkanki jest złożony, a w dysertacji zamieszczono jedynie wybrane aspekty, nie różnicując chociażby efektów w zależności od płci, istnieje potrzeba dalszej oceny neurotoksyczności acetamiprydu i możliwości

rozszerzenia badań na inne metody ograniczania szkodników np. przez stosowanie substancji podstawowych czy naturalnych insektycydów.

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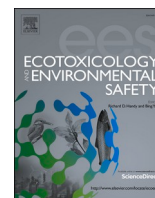
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## **6. Publikacja I:**

**Lewandowska-Wosik A., Chudzińska E.M., Wojnicka-Półtorak A. 2024,** Genotoxic effects of sub-lethal doses of nicotine and acetamiprid in neuroblasts of *Drosophila melanogaster* and *Drosophila suzukii*, *Ecotoxicology and Environmental Safety*, 280(7):116585, doi:10.1016/j.ecoenv.2024.116585

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## Genotoxic effects of sub-lethal doses of nicotine and acetamiprid in neuroblasts of *Drosophila melanogaster* and *Drosophila suzukii*

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### ABSTRACT

Neonicotinoids form a class of insecticides that are chemically related to nicotine and are widely used in crop protection. They have adverse effects on the neuronal nicotinic acetylcholine receptors (nAChRs). One of the neonicotinoids approved for control of the invasive pest *Drosophila suzukii* is acetamiprid. Despite concerns regarding its genotoxicity and data indicating the presence of small amounts of this substance in fruits intended for consumption, effects of its low doses on nerve cells are yet to be investigated. To determine whether the neurotoxic effects are species-specific and vary depending on the insecticide present in diet, multigenerational cultures of *Drosophila melanogaster* and *D. suzukii* were prepared, in this study, in media supplemented with different concentrations (below the LC50) of acetamiprid and nicotine. Acetamiprid, analogous to nicotine, caused damage to the DNA of neuroblasts in both species, at sublethal concentrations, along with a decrease in mobility, which remained at a similar level over subsequent generations. *D. suzukii* was found to be more sensitive to nicotine and acetamiprid, due to which the genotoxic effects were stronger even at lower doses of toxins. The results collectively indicated that even low concentrations of acetamiprid affect the stem cells of developing fly brain, and that long-term response to the tested insecticides is species-specific.

### 1. Introduction

The effects of insecticides used in agriculture and horticulture are not always limited to the target organisms. Harmful substances can directly or indirectly affect invertebrates and vertebrates by accumulating in animal or plant tissues, which are food sources for other organisms, including humans. Neonicotinoids (new nicotine-like insecticides) are a class of systemic insecticides that are widely used in crop protection. They affect the neuronal nicotinic acetylcholine receptors (nAChRs) in the nervous system strongly, causing overstimulation of nerve synapses, paralysis, and eventually death of the pests (Tomizawa and Casida, 2003). These compounds are water soluble, due to which their presence is not limited to their place of application. Increased concentrations of neonicotinoids have recently been detected not only in soil and water but also in food (Bonmatin et al., 2015). This makes it potentially hazardous not only to insects but also to humans. Cells of the developing nervous system are particularly sensitive to this type of insecticides, and exposure to neonicotinoids at an early age could cause, among other things, abnormal development of neurons and reduced neurogenesis

(Laubscher et al., 2022). Therefore, in-depth research on the effects of chronic acetamiprid exposure on developing brain cells is urgently required. One of the models used in this type of research is *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), which can be utilised to explain the mechanisms underlying the functioning of the nervous system in human neurodegenerative diseases and to assess the harmfulness of toxins widespread in the environment (Velazquez-Ulloa, 2017; Tasman et al., 2021). The significant role of fruit fly as a model in toxicological research is justified by the similarities in basic cellular and developmental mechanisms between flies and humans. Flies exhibit a wide range of responses relevant to understanding the effects of neurotoxins on humans, leading to the development of optimal endpoints in the study of nervous system-toxin interactions (Tomizawa and Casida, 2003). Some species of the genus *Drosophila* are pests that extensively destroy crops and cause serious economic and environmental problems. Several years ago, *Drosophila suzukii* (Matsumura, 1931), called spotted wing drosophila (SWD), joined the group of intensively combated pests.

Acetamiprid is one of the few plant protection products approved for control of *D. suzukii*. Like other neonicotinoids, it acts as a selective

**Abbreviations:** LC50, lethal concentration 50; SWD, spotted wing Drosophila; nAChR, neuronal nicotinic acetylcholine receptors; DNT, developmental neurotoxicity; ECHA, European Chemicals Agency; EFSA, European Food Safety Authority; SP, survival percentage; DsDNA, double-stranded DNA.

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agonist of nAChRs in the central nervous system of insects (Wallace, 2005). It is highly volatile, water-soluble, and is widely used against pests that destroy fruit plantations; therefore, its residues are often detected in food. N-Desmethyl-acetamiprid, the major metabolite of acetamiprid, was found in cerebrospinal fluid samples from 8-year-old children (Laubscher et al., 2022). Unequivocal neurotoxic effects of this insecticide was observed in bees, hens, and rats treated with a dose below the LD50 value (Hernandez-Jerez et al., 2022). Despite the doubts regarding its genotoxicity and neurotoxicity, including developmental neurotoxicity (DNT), the European Chemicals Agency (ECHA) and European Food Safety Authority (EFSA) have not changed their recommendations on the use of acetamiprid. Approval was in fact extended till 2033. Another effective insecticide known for many years is nicotine, a potent alkaloid neurotoxin naturally found in *Nicotiana tabacum* and *N. rustica*. Currently, its use as a pesticide is prohibited in Europe and the USA. In case of *D. melanogaster*, exposure to nicotine during

development is known to reduce both the survival and weight of adult insects, delay development (Chudzińska et al., 2016; Velazquez-Ulloa, 2017), and affect the structure of the brain by increasing the area of the cerebral hemispheres in larval brains (Morris et al., 2018).

To expand our knowledge of the toxic activity of the studied insecticides, we addressed the following questions in this study: (1) Does multigenerational exposure of insects to a diet containing sublethal doses of insecticides have neurotoxic effects on *D. melanogaster* and *D. suzukii* larvae? (2) Is the response to insecticides similar regardless of the species? and (3) Does multigenerational exposure to insecticides lead to the development of pest resistance? Clarification of these issues may facilitate the understanding of neurotoxicity associated with the responses of target and non-target organisms to acetamiprid and its environmental impact.

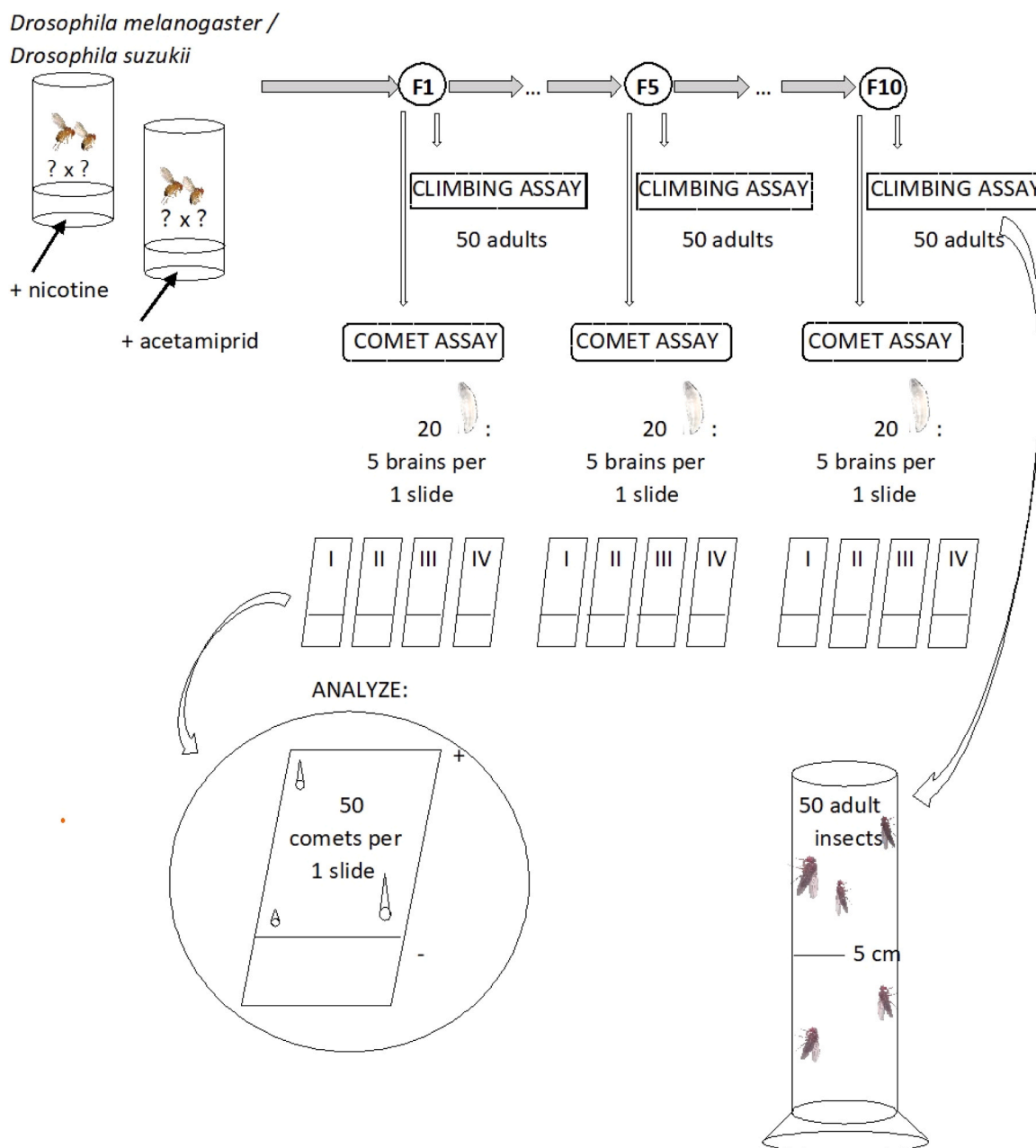


Fig. 1. Schematic representation of the experiments performed on the two species, namely *D. melanogaster* and *D. suzukii*, over 10 generations, with exposure to acetamiprid and nicotine. Comet assay was conducted on larva stage and climbing assay was on imagoes.

## 2. Materials and methods

We investigated whether *Drosophila* larvae that survive exposure to the tested insecticides result in adult insects with lower neuronal efficiency, and hence, reduced locomotion abilities, and whether subsequent generations maintain this state. Since the concentrations of harmful substances polluting the environment can often be much lower than those used originally, we aimed to explore the consequences in neuroblasts that may have had exposure to doses below the LC50 over a long time. To check the same, insects chronically exposed to pesticides were farmed for many generations. Two toxic substances, namely nicotine and acetamiprid, were used in this study.

### 2.1. *Drosophila* husbandry and insecticide treatment

Schematic representation of the experiments is presented below (Fig. 1).

#### 2.1.1. *D. melanogaster* and *D. suzukii* strain

Experiments were conducted on two species belonging to the Drosophilidae family. *D. melanogaster* wild-type Oregon R<sup>+</sup>, obtained from a culture maintained at the Department of Genetics, Adam Mickiewicz University in Poznań, a well-studied model species was used in the experiment as a reference point. *D. suzukii* was established from wild individuals collected in 2019 from a location near Września (central Poland). *D. suzukii* was caught from the wild so that the experimental results obtained would best reflect the reaction of individuals from the wild European population. Both species were grown on modified sugar/yeast culture medium, as described previously (Chowański et al., 2018). All cultures were maintained at 23 ± 2 °C in a 12-h:12-h light:dark cycle at 60 % humidity.

#### 2.1.2. Survival analysis and exposure to toxic substances

Dose-response assays were performed for acetamiprid and nicotine. Nicotine was used as a positive control. In order to select doses of insecticides for further analysis, acetamiprid was added to the medium at concentrations of 0.125, 0.25, 0.50, 0.875, and 1.0 µl/ml and nicotine was added at concentrations of 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 mg/ml. Both reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). For survival analysis, newly eclosed adults (*D. melanogaster* and *D. suzukii*) were transferred from the stock populations to fresh vials containing standard culture medium. After 48 h, the imagoes were discarded from the bottles and the eggs were collected using a stereomicroscope; 100 eggs of each species were placed on a medium comprised of nicotine, acetamiprid, and standard medium. To assess survival, the number of hatched flies was counted two weeks after the eggs were laid. The collected data were used to calculate survival percentage (SP) and sublethal doses for transgenerational studies. The survival percentage of 50 % (SP50) was calculated as 50 % of the total number of eggs that developed into adult flies by day 14 after egg-laying.

In the next step, five reproductively viable mature males and females (*D. melanogaster* and *D. suzukii*) were transferred into vials containing a standard substrate with solutions of the tested compounds and the control. After 72 h, the parent insects (P) were removed. Each subsequent generation of adults (F1–F10) was transferred to a fresh medium containing pesticides at sub-lethal concentrations. Five replicates were performed for each concentration in eight independent experiments for both the species. Control cultures were prepared in parallel without the addition of nicotine or acetamiprid.

### 2.2. Bioassays

#### 2.2.1. Alkaline comet assay

Single-cell gel electrophoresis, known as the comet assay (OECD, 2016), was used to assess the degree of DNA damage in neuroblasts.

Alkaline modifications were used to detect single- and double-stranded DNA (dsDNA) damage. The comet assay, which is a valuable tool for genotoxicity studies (Langie et al., 2015), allows the detection of single- and double-stranded DNA breaks in the alkaline – modified form. Numerous studies using comet assays have demonstrated the genotoxic effects of various substances in the form of DNA damage in *Drosophila* brain cells (García-Sar et al., 2012), haemocytes (Sabella et al., 2011), midgut cells (Siddique et al., 2013), and S2 transfected cell lines (Guanggang et al., 2013).

Third-instar larvae (20 per generation) from the F1, F5, and F10 generations in the control and the cultures with analysed concentrations of nicotine and acetamiprid were collected (Fig. 1). According to the methodology refined for *Drosophila* (Sierra et al., 2014), the larvae were washed in 0.5 % sodium hypochlorite and deionised water, and gently dried thereafter. The larval brains were isolated, under a Zeiss stereomicroscope (5 per slide; 20 larval brains from one concentration) in a drop of Ringer's solution (130 mM NaCl, 35 mM KCl, and 2 mM CaCl<sub>2</sub>). Brain tissues were mechanically shredded with a tungsten wire and transferred to test tubes; 1 % low-melting point agarose (LMP agarose) dissolved in phosphate buffered saline (PBS) was added to the cell suspension. The whole preparation was transferred to slides coated with 1 % normal-melting point agarose (NMP agarose) dissolved in PBS, covered with a coverslip, and placed in a refrigerator at 4 °C. After 10 min, coverslips were removed, slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris 0.25 M NaOH, 77 % N-Laurylsarcosine sodium salt; 1 % Triton X-100 added before lysis) for 2 h at 4 °C in the dark. After lysis, the preparations were placed in an electrophoresis tank in fresh alkaline buffer (1 mM Na<sub>2</sub>EDTA and 300 mM NaOH, pH > 13) for 30 min, at 4 °C, in the dark. Electrophoresis was performed under the following conditions: 0.7 V/cm, 300 mA, (25 V/300 mA), 20 min, at 4 °C, in the dark. After electrophoresis, the slides were washed in a neutralising buffer (0.4 M Tris, pH 7.5) and dried. The slides were flooded with ethyl alcohol for 3 min and dried overnight in the dark. On the following day, they were stained using ethidium bromide (0.4 µg/ml).

#### 2.2.2. Climbing assay

Twenty-five pairs of *D. melanogaster* and *D. suzukii* adults from the F1, F5, and F10 generations, reared on medium containing the four tested concentrations of nicotine and acetamiprid, and a control were used to test negative geotaxis. Insects of one age, collected within 3 days after eclosion, were placed in a vertical transparent glass (length 25 cm; diameter 1.5 cm). The flies were set aside and allowed to acclimate for 30 min. During the whole experiment, identical conditions prevailed, i. e., temperature around 22 °C, natural light, the lamp being directed against the cylinders. The flies were gently tapped onto the bottom of the column, and after 8 s, photographs were taken with a digital camera. The flies that reached a diameter of 5 cm were counted. Five repetitions were performed for each experiment at 10-min intervals.

### 2.3. Image analysis

The comet assay slides were analysed under a fluorescence microscope (Olympus BX40) at 400 × magnification. Images of 50 comets on one slide were captured using the cellSens Entry 2.2 programme (Olympus Co.). Comets were analysed using the CaspLab software (Kořica et al., 2003). Three measures of DNA migration distance were used to assess DNA damage, namely tail length (TL), tail DNA% (i.e., tail intensity, % of tail DNA, that is, percentage of DNA in the tail), and tail moment. A parameter described by Olive et al. (1990), called the olive tail moment (OTM), was considered to be statistically significant (Kumaravel and Jha, 2006), and was calculated as the product of the percentage of DNA in the tail and the distance between the intensity centroids (centres of gravity) of the head and tail along the x-axis of the comet (Kumaravel et al., 2009). In the climbing assay, positions of the flies 8 s after knockdown were manually measured using an Olympus

camera. Photos of five replicates for each experiment were analysed using ImageJ software (Abramoff et al., 2004) and the number of individuals that covered the indicated distance was counted.

## 2.4. Statistical analysis

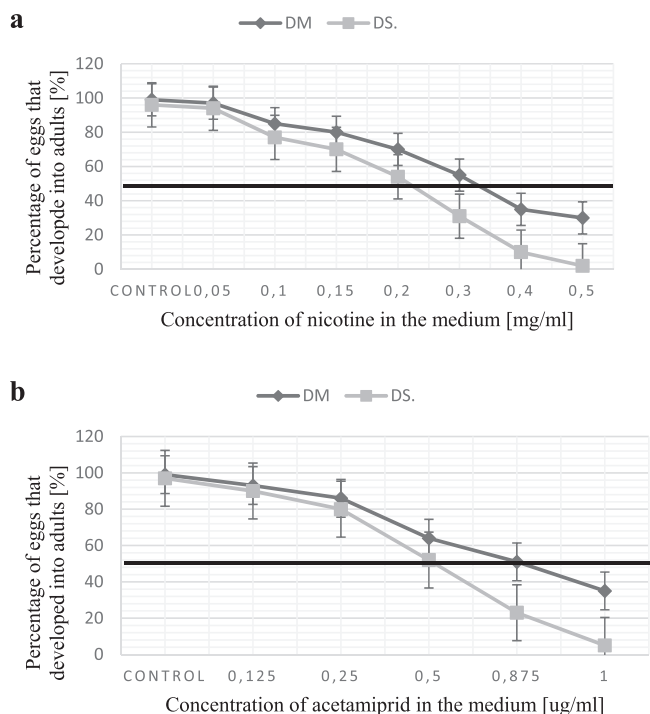
Data analysis was performed using STATISTICA StatSoft version 13 software. Results are expressed as the means  $\pm$  SE of five replicates for each series of experiments. The Shapiro-Wilk normality test, non-parametric Kruskal-Wallis test, median test, and multiple comparisons of mean ranks for all trials were performed. Values at  $p < 0.001$  were considered significantly different from those of the control ( $p < 0.05$ ). The median, maximum, minimum, and 25–75 % percentile for OTM, tail DNA%, and TL are shown as box and whisker plots. All data obtained from the analysis of comet images using CaspLab software, concerning the tail DNA content (tail DNA%), tail length (TL), and olive tail moment (OTM), were initially subjected to the Shapiro-Wilk test, which showed that the data distributions were not normal. In the next stage, non-parametric tests were used, including the Kruskal-Wallis test, which is a non-parametric equivalent of one-way ANOVA, median test, and multiple comparisons of mean ranks for all trials. The climbing assay results were analysed using a three-way ANOVA to reveal the effects of species, nicotine, and acetamidrid concentrations.

## 3. Results

### 3.1. Survival rate of insects

The survival rates of *D. melanogaster* and *D. suzukii*, calculated as the percentage of eggs that developed into adults, decreased after exposure to acetamidrid (A) and nicotine (N) (Fig. 2).

The highest concentration of N in the medium at which at least 50 %



**Fig. 2.** The percentage of eggs of *D. melanogaster* (DM) and *D. suzukii* (DS) that developed into adult forms after 14 days of exposure to (a) 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 mg/ml nicotine (N) and (b) 0.125, 0.250, 0.500, 0.875, and 1.0 µg/ml acetamidrid (A). Horizontal black line marks the concentrations of N and A at which the number of eggs that developed into imagoes exceeded the 50 % limit. Values are the average of five independent biological replicates; mean  $\pm$  SE (standard error),  $n = 5$ .

of *D. melanogaster* eggs developed into imagoes was 0.3 mg/ml, whereas that for *D. suzukii* was 0.2 mg/ml (Fig. 2a). The highest concentration of A in the medium at which at least 50 % of eggs developed into the imagoes was 0.875 µg/ml for *D. melanogaster* and 0.5 µg/ml for *D. suzukii* (Fig. 2b). The following sub-lethal concentrations were used for further analyses: for N, 0.05, 0.1, 0.15, 0.2, 0.3, and 0.5 mg/ml (the last two concentrations were for *D. melanogaster* only) and for A, 0.125, 0.250, 0.500, and 0.875 µg/ml (the last concentration was for *D. melanogaster* only).

The number of hatched eggs in case of N was significantly different between DM (*Drosophila melanogaster*) and DS (*Drosophila suzukii*) at all concentrations, except for the lowest (0.05 mg/ml). In case of A, statistically significant differences between DM and DS were observed at concentrations of 0.5 and 0.875 µg/ml.

### 3.2. Comet assay results

The single- and double-stranded DNA damage in third-instar larval neuroblasts of DM and DS, cultured in a medium with the addition of N (nicotine) for the OTM parameter, is shown in Fig. 3. The OTM values of F1, F5, and F10 generations of DM were significantly higher than those of the control after exposure to nicotine 0.1, 0.15, 0.2, 0.3 and 0.5 mg/ml (Fig. 3a). At the lowest concentration of N (0.05 mg/ml), a significantly increased OTM value was observed only in the F10 generation (see median). In case of DS, the OTM values increased significantly, compared to those of C, at concentrations of 0.05 and 0.1 mg/ml for all tested generations, being the lowest in F1 and the highest in F10 generation. Exposure to 0.15 and 0.2 mg/ml was lethal to DS F10 (Fig. 3b).

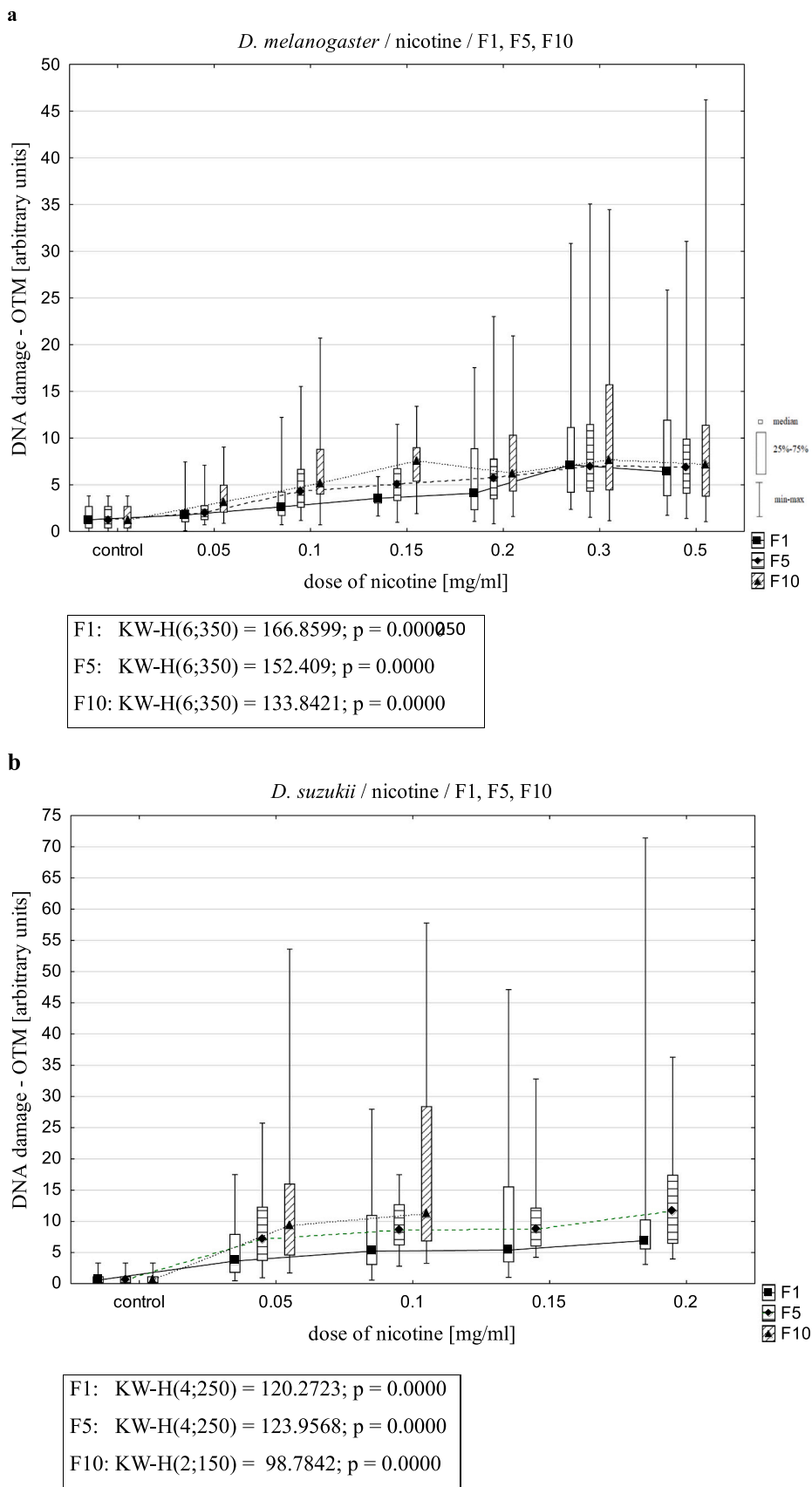
Results obtained from the analysis of insects cultured in the medium with added A (acetamidrid) for the OTM parameters are shown in Fig. 4.

The OTM value in the F1 generation of DM increased with higher concentrations of A in the medium. The highest DNA damage in F5 and F10 generations was caused by the concentration of 0.125 µg/ml. In the F10 generation, a slight decrease in the OTM parameter was observed at the highest concentration of 0.875 µg/ml, although it was not statistically significant (Fig. 4a). For DS, OTM was significantly increased in F1 and F5 generations, compared to that in control, after exposure to acetamidrid at 0.125, 0.250, and 0.500 µg/ml. In the F1 and F5 generations, the OTM levels were similar for all A concentrations, and in F10 the highest was at A 0.500 µg/ml (Fig. 4b).

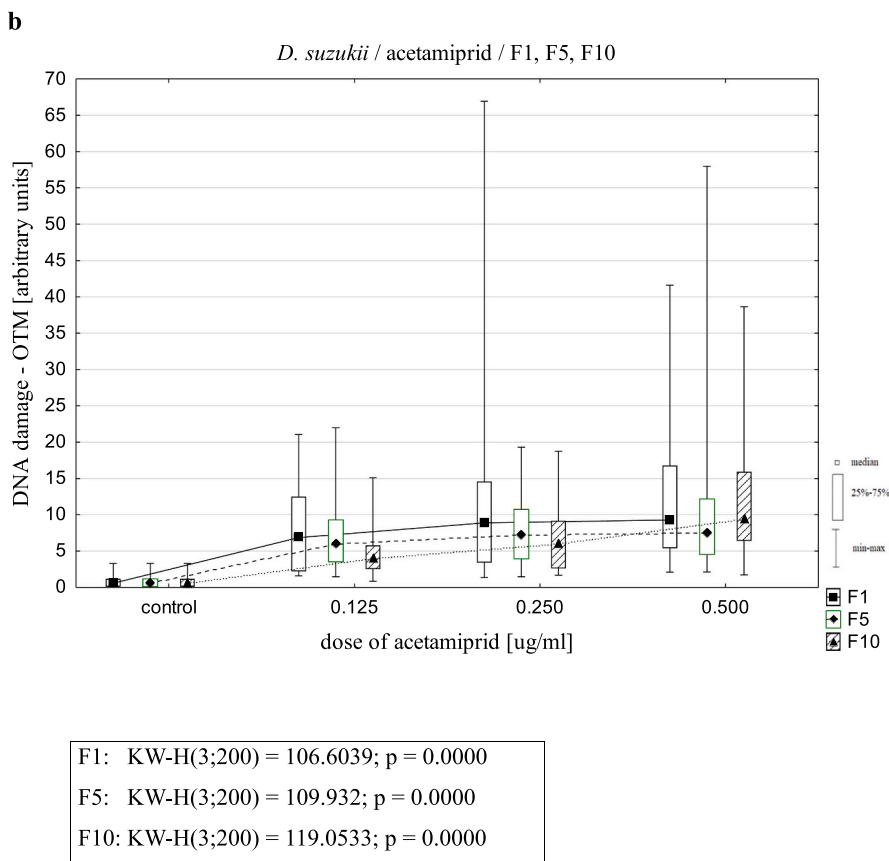
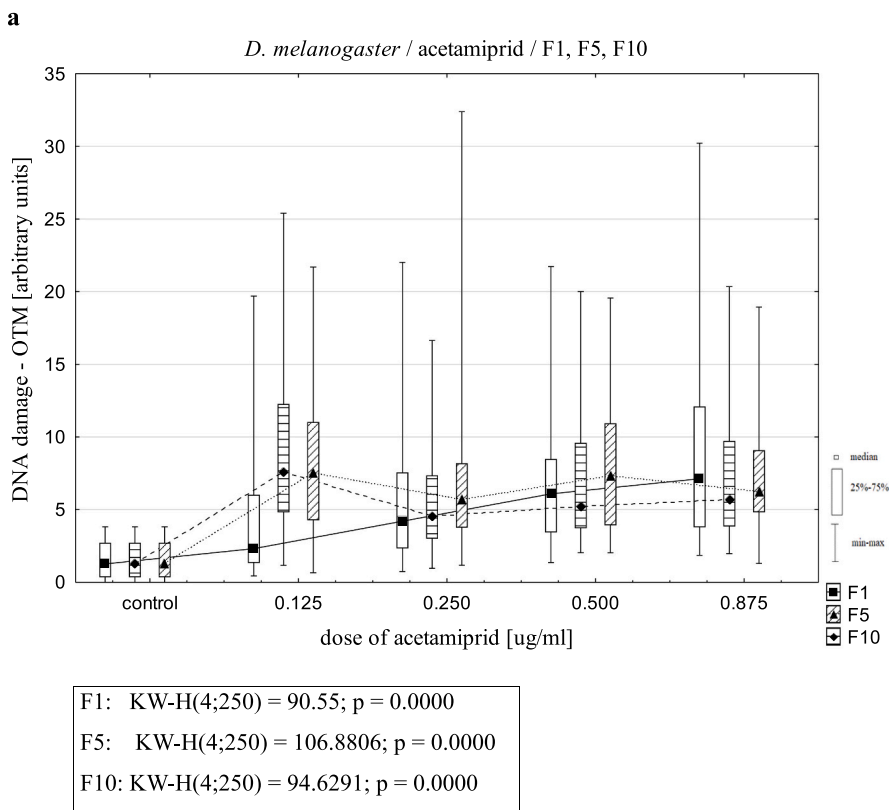
The tail DNA% parameter, obtained during the entire insect breeding period under the influence of toxins, was analyzed. The increase in median tail DNA% parameter between the control and the highest dose of N used for DM was 3.6-fold in F1, 3.6-fold in F5, and 4.1-fold in F10. For DS, it was 10.3-fold in F1, 14.1-fold in F5, and 15.2-fold in F10. For A DM tail DNA% 4-fold in F1, 4.3-fold in F5, and 4.1-fold in F10, whereas that for DS was 14-fold in F1, 13.5-fold in F5, and 14.5-fold in F10.

The Kruskal-Wallis test, median test, and multiple rank comparisons were performed for all trials. For each analysed concentration of N and A, the obtained results were statistically significant,  $p = 0.0000$ , i.e.,  $p > \alpha$ , where  $\alpha > 0.001$  (Fig. 3, Fig. 4), and confirmed the relationship between the doses of neurotoxins and DNA damage. The median test showed that in each of the analysed cases, the differences between control and test samples were statistically significant. In DM F1, an increase in DNA damage was observed with increasing doses of N and A, which was seen as an increase in the percentage of DNA in the tail and OTM. All the values obtained for the various experimental variants were significantly different from those obtained for the control samples.

In case of DS, an increase in DNA damage was visible in DNA content of the tail and in the OTM parameter, with an increase in the concentration of N and A in F1, F5, and F10. All values obtained for the various experimental variants were significantly different from those obtained in the control experiment. For DS, DNA damage in neuroblasts was higher in each successive generation exposed to N. The concentrations of N 0.15 mg/ml and 0.2 mg/ml proved to be lethal over time, to such an extent that the insects became completely extinct. In F10, response to



**Fig. 3.** Median, minimum, maximum, and percentile 25 % and 75 % obtained for the parameter OTM for insect neuroblasts grown on a medium containing nicotine (N). Results of the Kruskal-Wallis ANOVA and median test are shown in the table; a, *D. melanogaster*; b, *D. sukuzii*; OTM, olive tail moment; KW, Kruskal-Wallis's test; F1, F5, and F10, generations 1, 5, and 10.



**Fig. 4.** Median, minimum, maximum, and percentile 25 % and 75 % obtained for the parameter OTM for insect neuroblasts grown on a medium containing acetamiprid (A). Results of the Kruskal-Wallis ANOVA and median test are shown in the table; a, *D. melanogaster*; b, *D. sukuzii*; OTM, olive tail moment; F, generation; KW, Kruskal-Wallis's test; F1, F5, and F10, generations 1, 5, and 10.

the lowest concentration of A was lower than that in the previous generations; however, subsequent doses caused similarly high DNA damage, as observed in F5 and F10.

### 3.3. Climbing assay results

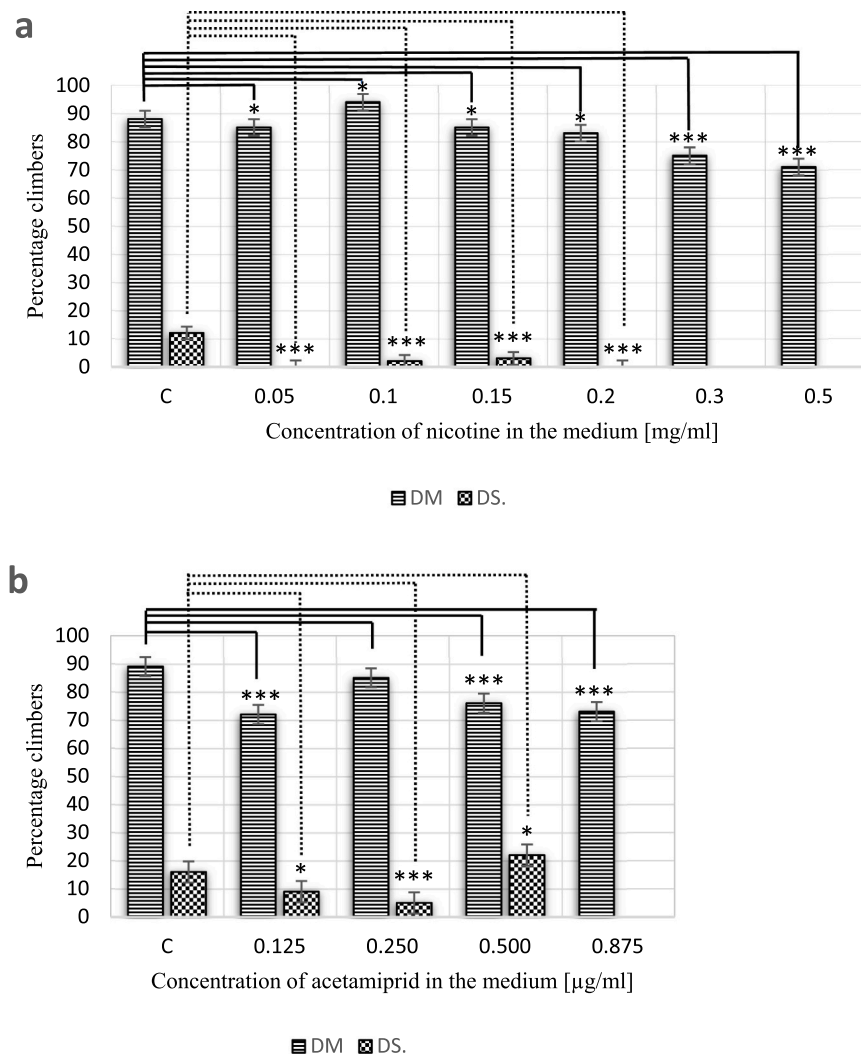
For DM, differences in the number of insects that reached 5 cm in 8 s were statistically significant in the F1 generation for all N concentrations compared to that in the control. At concentrations of N 0.3 and 0.5 mg/ml, reduction in the number of flies that passed 5 cm was the most significant ( $p < 0.001$ ). N at a concentration of 0.1 mg/ml caused greater mobility than C ( $p < 0.05$ ) (Fig. 5a). By extending the migration time to 16 s, the number of flies that crossed the 5-cm line was significantly lower ( $p < 0.05$ ) only at concentrations of 0.3 and 0.5 mg/ml. In case of A, the lowest number of insects that travelled 5 cm was observed at a concentration of 0.125  $\mu\text{g}/\text{ml}$ , compared to that in the control ( $p < 0.001$ ), both at 8 and 16 s. For doses of 0.500 and 0.875  $\mu\text{g}/\text{ml}$ , statistically significant differences were found at  $p < 0.001$  (Fig. 5b).

In F5 and F10 generations, 0.1 mg/ml N and 0.125  $\mu\text{g}/\text{ml}$  A did not have a significant effect on the mobility of insects. A low N dose did not increase DM mobility. Moreover, the reduction in flies climbing against gravity at all concentrations of both toxins, compared to the control, was

significantly lower than that in F1. DS showed much lower mobility than DM. Only a dozen percent of the control group covered 5 cm in 8 s. Each applied concentration of nicotine had a negative effect on the mobility of *D. suzukii*; differences with respect to the control sample were statistically significant ( $p < 0.001$ ) (Fig. 5a). In turn, the highest concentration of A caused an increase in the activity of *D. suzukii* compared to that in the control sample ( $p < 0.05$ ) (Fig. 5b). No significant difference was observed between F1 and subsequent generations.

## 4. Discussion

Our experiments showed that as the concentrations of N and A in the medium increased, the number of insects hatching from eggs decreased. This confirmed the effectiveness of the tested insecticides at sublethal doses and their ovicidal potential in both the species of *Drosophila*. Studies on the influence of N on DM fertility had shown its significant negative effects, such as delayed development, transformation, and increased mortality (Velazquez-Ulloa, 2017). As in earlier experiments, in case of N, all eggs that progressed to the larval stage developed into imagoes, whereas the N-exposed females laid significantly fewer and smaller eggs that hatched fewer larvae. Similar reductions in development and fertility have also been observed in mammals (Oyeyipo et al.,



**Fig. 5.** The percentage of flies *D. melanogaster* and *D. suzukii* that travelled the distance of 5 cm in 8 s. a) Insects bred on a medium containing N (nicotine) at a concentration of 0.1 mg/ml–0.5 mg/ml and on a C (control) medium; b) insects bred on a medium containing A (acetamiprid) at a concentration of 0.125  $\mu\text{g}/\text{ml}$ –0.875  $\mu\text{g}/\text{ml}$ . Values are the average of five independent biological replicates; means  $\pm$  SE (standard error),  $n = 5$ . Asterisks (\*) indicate the level of statistical significance of differences; \*  $p < 0.05$ ; \*\*\*  $p < 0.001$  (Tukey's post-hoc test) between the C sample and N or A.

2011 (non-male), Condorelli et al., 2017). We noted that in case of DS, N was so detrimental to fertility that the number of individuals from one generation to the next gradually and significantly decreased, and at the highest concentration of N (0.15 mg/ml or 0.2 mg/ml), it was not possible to bring the culture to the 10th generation even though the concentrations used did not cause such large losses in F1. For DM, a slight decrease in the number of insects from one generation to the next was observed only at the highest concentration of N (0.5 mg/ml); this was not large enough to lead to the complete extinction of insects in F10. Acetamiprid is chemically related to nicotine, and can reduce fertility of insects (Haramboure, 2017), nematodes (Kruk and Dziegielewska, 2020), birds (Humann-Guillemot et al., 2019), and mammals (Arican et al., 2020), including humans (Neghab et al., 2014). Other neonicotinoids may also be responsible for a decline in the number of pollinating insects (Wood and Goulson, 2017; Wagner, 2020). We observed that under the influence of A, egg development was inhibited and some of the first-instar larvae died. Sensitivities of the target and non-target species to A and N were quite obviously different.

Results of the comet assay showed that long-term exposure to low doses of N and A led to DNA damage in the brain neuroblasts of third-stage larvae. We focused on the effect of neurotoxins on the DNA of the developing brain cells, specifically on neuroblasts and stem cells of the larval brain, which produce many different neurones responsible for the complex behaviours of adults (Song et al., 2007; Homem and Knoblich, 2012). In the first generation of both species, the negative effect of A and N on DNA increased systematically with increasing doses, which was observed as DNA appearing like so-called comets with longer tails (higher percentage of DNA in the tail). This tendency to increase DNA damage caused by N had previously been observed in *Caenorhabditis elegans* (Sobkowiak and Lesicki, 2009) and human lymphocytes (Sobkowiak et al., 2014). In the following generations (F5 and F10), the trends were similar; each dose of the substance had a negative effect on the DNA of neuroblasts, and a significant increase in damage compared to that in the control sample was observed. The lack of significant differences in DNA damage compared to that in the F1 generation suggested that DM and DS remained sensitive to N over the tested generations.

The extent of damage to the DNA of DS neuroblasts, caused by exposure to three different doses of A, in both F1 and F5 generations was very similar; however, in F10, we observed a significant reduction of these disorders at lower concentrations than in the control sample. Target insects appeared to have developed resistance to the amounts of A, which was in agreement with research showing pests becoming resistant to neonicotinoids when exposed to low concentrations of A for several generations. The higher dose (0.500 µg/ml) was so large that it still caused damage as much as in the first generation. In case of DM, a different situation was observed in the F5 and F10 generations; neonicotinoids caused the greatest DNA damage at the lowest dose, the damage being greater than that caused by the highest concentration of A.

Comparing the results of the climbing and comet assays for *D. melanogaster*, we noticed that, in general, the increase in DNA damage in larval neuroblasts was accompanied by an increase in disturbance of adult insect locomotion. The fact that there was no such correlation in the two cases was interesting; the lowest concentration of nicotine (0.1 mg/ml) was the only one that led to an increase in fly mobility. This could be because the exposure of insects to nicotine during their entire development, especially during the larval stage, led to a decrease in sensitivity to the weakening effects of this substance at a certain, small dose, which is manifested by stimulation, not a decrease in locomotor activity (Velazquez-Ulloa 2017) but also an increase in DNA damage. In contrast, the lowest dose of acetamiprid caused the least DNA damage and most disturbed locomotion. Climbing tests, such as the negative gravity test, are good research methods to analyse motor abnormalities in neurodegenerative diseases, such as Parkinson's or Alzheimer's disease (Sekiya and Iijima, 2021; Madabattula et al., 2015), as well as to

study the negative effects of various substances (Moulin et al., 2021).

We decided to administer nicotine (acetamiprid) to insects via food in the nutrient medium since it is known to be effectively absorbed in this way. Moreover, with long-term exposure to this compound, *D. melanogaster* did not limit nicotine consumption, which is aversive under normal conditions (Sellier et al., 2011). Using the climbing test to study the locomotion of *D. melanogaster*, which was chronically exposed to various doses of nicotine, from egg to the imago stage, we found that a low concentration of toxin (0.1 mg/ml) caused the flies to become hyperactive, whereas higher doses interfered with the negative gravity. This was similar to the effect of volatile nicotine on insects (Velazquez-Ulloa, 2017). This could be because the exposure of insects to nicotine during the entire development, especially during the larval stage, led to a decrease in sensitivity to the weakening effects of this substance at a certain small dose, which is manifested by stimulation, not a decrease in locomotor activity (Velazquez-Ulloa 2017). Dworkin et al. (1993) had shown that a similar effect of nicotine was observed in rats owing to the similarity in certain mechanisms between *D. melanogaster* and mammals. *D. melanogaster*'s response to acetamiprid, however, differed. In this case, the lowest dose disturbed the negative gravity of the flies. A gradual increase in neonicotinoid concentration resulted in a gradual decrease in upward locomotion, though it was not as pronounced compared to that in the control sample as at the lowest dose.

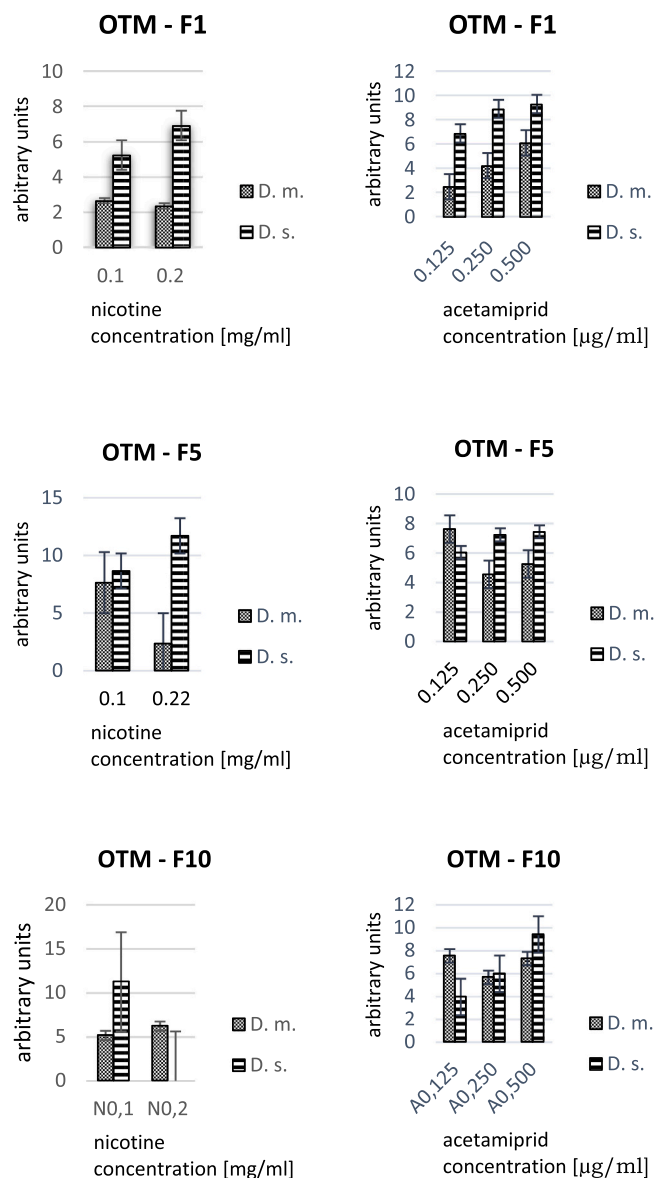
Chronic multigenerational exposure of insects to the tested toxins did not cause significant changes in the locomotion of *D. melanogaster* compared to that in the first generation of insects. However, in the fifth and tenth generations, the stimulatory effect of the lowest dose of nicotine was no longer observed. Our results showed that, after long-term exposure to nicotine and acetamiprid in food, the harmful effects on insects were slightly reduced. Although locomotion disturbances still occurred with increasing doses, it was not significant, and the sensitivity of *D. melanogaster* to toxins decreased.

Unfortunately, the negative-gravity test is not suitable for testing *D. suzukii*. Despite their phylogenetic similarity to *D. melanogaster*, there are some significant differences between them, including the much lower mobility of *D. suzukii* compared to *D. melanogaster*, and their reluctance to climb against gravity. Observations and comparisons of the circadian rhythms of both species by Hansen et al. (2019) had shown that lower activity of the *D. suzukii* bred under laboratory conditions may be a consequence of the stress response.

DM and DS are phylogenetically closely related. At first glance, their morphologies appear to be similar. However, one of the differences in structure, namely the presence of an enlarged ovipositor in the female DS, leads to significant differences in preferences regarding the substrate on which the eggs are laid. DS chooses fruits with much harder skin than DM. Sensory changes cause the smell of ripe fruits to cause egg-laying SWD, which is not observed in DM (Karageorgi et al., 2017) and CO<sub>2</sub> repellent for DM is ignored by DS (Krause Pham and Ray, 2015). *D. suzukii* is also much more susceptible to oxidative stress and is characterised by a lower activity of glutathione transferase (Ngueyn et al., 2016). We showed that *D. suzukii* is much more sensitive to nicotine and acetamiprid than *D. melanogaster*. This was noticeable in the reduced survival and fertility of this species when exposed to the toxins throughout their development. It was especially true for nicotine, where eggs and 1–2-day-old larvae were the most sensitive stages. The eggs and 1–2-day-old larvae of *D. melanogaster* were also sensitive, but not to the same extent as those of *D. suzukii*. In the former, at the same toxin concentration, many cells survived and developed into the imago stage.

We found that nicotine and neonicotinoids caused more DNA damage in the neurocytes of *D. suzukii* larvae than in those of *D. melanogaster*. Comparing the interspecific medians of the olive tail moment parameters, clear differences were observed when the insects were exposed to the same doses of neurotoxins (Fig. 6.).

Since the effect of acetamiprid on neurobehavior is complex, and we examined motor skills only in the adult generation of insects, without



**Fig. 6.** Comparison of the magnitude of DNA damage (median values) in *D. melanogaster* and *D. suzukii* neuroblasts owing to their exposure to the same doses of nicotine and acetamiprid (N and A) in F1, F5 and F10 generations. Values are the average of five independent biological replicates; means  $\pm$  SE (standard error),  $n = 5$ .

differentiating the effects depending on gender, there is a need to further assess the neurotoxicity of acetamiprid and the possibility of extending the research to other, for example natural insecticides.

## 5. Conclusions

In the present study, we evaluated the genotoxic effects of sublethal doses of nicotine and acetamiprid in neuroblasts of *Drosophila melanogaster* and *D. suzukii*. Sublethal doses of these toxins negatively affected the individual development and fertility of both the *Drosophila* species tested. The climbing test demonstrated the neurotoxic effects of these two substances, which caused disorders in locomotion abilities of the flies. We demonstrated that permanent long-term exposure to low doses of nicotine and acetamiprid led to DNA damage. It turned out that *D. suzukii* is much more sensitive to nicotine and acetamiprid, under constant exposure, than *D. melanogaster*. Furthermore, the insects developed resistance to neonicotinoids.

## Ethics declarations

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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## CRedit authorship contribution statement

**Anetta Lewandowska-Wosik:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Data will be made available on request.

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## **7. Publikacja II:**

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## Article

# Fluctuating Asymmetry Spotted Wing *Drosophila* (Diptera: Drosophilidae) Exposed to Sublethal Doses of Acetamiprid and Nicotine

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**Simple Summary:** Long-term exposure to low concentrations of insecticides can cause several adverse effects, both on target insects and on other organisms. Low doses can also generate tolerance in surviving offspring. One simple method to detect the consequences of long-term exposure to residual toxins is to measure deviations from ideal body symmetry. Fluctuating asymmetry (FA) is evidence that insects were stressed during development. This study aimed to verify FA in the wing veins of *Drosophila suzukii*, a fruit pest controlled by the insecticide acetamiprid. To determine whether low doses of insecticide in the diet induce the asymmetry effect, multigenerational insect breeding was carried out on media supplemented with different concentrations of two insecticides. Nicotine was used as a positive control. Even in the first generation, low doses reduced fertility and caused vein asymmetry. This effect persisted in subsequent generations, indicating a lack of tolerance that led to complete insect death after 10 generations. *D. suzukii* proved extremely sensitive to acetamiprid, and FA is a good index of this sensitivity.

**Abstract:** Long-term exposure to low concentrations of toxic substances can cause several adverse consequences ranging from molecular to morphological. Sublethal doses may also lead to increased tolerance in the offspring of surviving individuals. One of the consequences of such stress is deviations from the ideal body symmetry during development, reflected by increased levels of fluctuating asymmetry (FA). This research aimed to verify FA in the wing veins of insects belonging to the Drosophilidae family—*Drosophila suzukii*, a fruit pest controlled by the insecticide acetamiprid, a neonicotinoid. To determine whether FA varied depending on insecticides present in the diet, multigenerational cultures of *D. suzukii* were carried out on media supplemented with different concentrations (below the LC50) of two insecticides. Nicotine was used as a positive control. Fecundity decreased, the number of insects decreased, and breeding did not continue beyond the tenth generation. However, the FA level at different concentrations was similar, and high FA values were observed even at lower acetamiprid concentrations. We did not see significant changes in FA levels in subsequent generations. *D. suzukii* proved extremely sensitive to acetamiprid, and FA is a good index of this sensitivity.

**Keywords:** *Drosophila suzukii*; fluctuating asymmetry; acetamiprid; nicotine; sublethal doses; multigenerational cultures



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## 1. Introduction

All organisms are constantly exposed to harmful substances present in the environment. The rapid development of agriculture obliges the production of a wide variety of pesticides, raising many questions regarding their safety and potential health and environmental risks. Often, pesticide residues that have been partially decomposed or diluted accumulate in soil, water, and plants in low doses [1–3]. In this way, they can directly or

indirectly affect vertebrates and invertebrates. The best-known example of the negative impact of such substances on insects is pollinating insects. Bees can absorb pesticides from various sources, including food, direct contact with contaminated surfaces, inhalation, and exposure to water containing dissolved toxins. In most of these cases, insecticide concentrations are low, and their effects range from negligible to lethal [4–10].

One of the insects exposed to contact with insecticides at low concentrations is the vinegar fly, *Drosophila suzukii* (Matsumura, 1931), Diptera: Drosophilidae, also called the spotted wing Drosophila (SWD). It is a polyphagous fruit pest from Southeast Asia [11]. This species spread to Europe, Africa and Oceania [12]. This invasive species lays its eggs in intact fruits using its sclerotized ovipositor. Direct damage consists of larvae feeding on the pericarp, and secondary damage is caused by pathogens that penetrate the fruit, causing faster rotting and economic depreciation [13].

This pest is difficult to control, and many factors can expose its populations to sublethal doses of insecticides. One of these insecticides is acetamiprid (ACE (N-(6-chloropyridin-3-yl) methyl)-N'-cyano-N-methylethanimidamide), a neonicotinoid. Neonicotinoids are a class of insecticides that have a strong affinity for insect receptors, so it is believed that they are safe for other organisms [14]. Unfortunately, research shows that they can negatively affect other vertebrates, including mammals [14–16]. Neonicotinoids have a structure similar to nicotine; they are highly effective pesticides but are currently banned in Europe and the USA. (In this study, we used nicotine as a positive sample.) Like nicotine, neonicotinoids are agonists of nicotinic acetylcholine receptors in the nervous system. There is considerable concern about the bioavailability of neonicotinoids in the environment and the possible exposure of non-target organisms to insecticide residues, which have been detected at low concentrations in, e.g., pollen, nectar, soil, and water [17,18].

Sublethal effects are important for *D. suzukii* because its small size, feeding method, short development cycle, and use of different plant species as hosts mean that insecticides have a limited effect. Low doses of harmful substances can negatively affect the organism, especially in cases of long-term exposure [19,20]. This concerns various factors, such as survival, development, reproduction, learning ability, and behavior [4,21–23]. One method of determining whether chronic exposure to low concentrations of acetamiprid in food has negative effects is describing biomarkers of environmental stress such as fluctuating asymmetry (FA), a measure of developmental instability. The FA index reflects the degree of deviations between sides regarding the size of a given feature. FA is present when external stressors disrupt developmental processes that normally promote symmetrical growth. This is a non-directional, random, small deviation from perfect symmetry [24–26]. Among other insects, the emergence of FA under the influence of various factors has been demonstrated in *D. melanogaster* exposed to the antibiotic neomycin [27]; in *D. ananassae* as a result of nutritional stress [28]; and in *D. buzzatii* and *D. koepferae* raised on alternative breeding substrates [29]. Thus, we decided to determine to what extent it can be used to study *D. suzukii*. We aimed to test [i] whether exposing insects to sublethal doses of acetamiprid and nicotine in the diet causes deviations from wing symmetry; [ii] whether FA correlates with increasing concentrations of toxic substances; and [iii] whether there are FA changes in subsequent generations of insects compared with the F1 generation; in other words, can adaptation to these insecticides be observed?

## 2. Materials and Methods

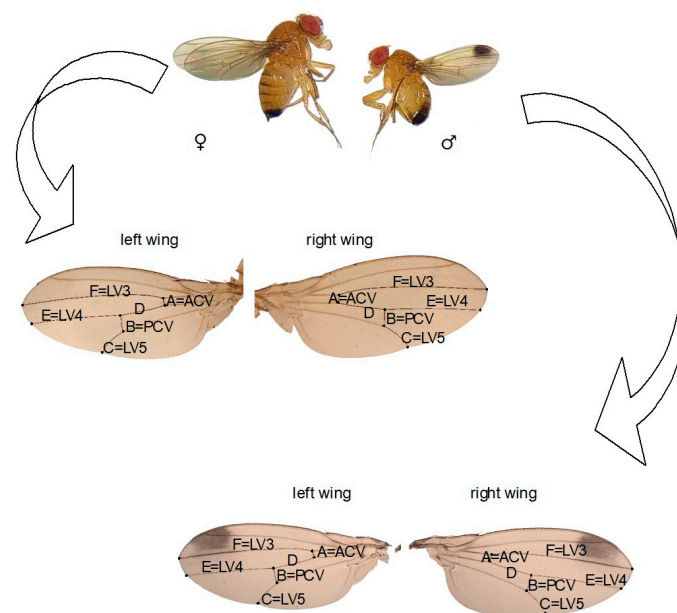
### 2.1. SWD Colony and Experimental Design

A *D. suzukii* colony was established from wild individuals collected in 2019 from infested fruit growing (sweet cherries) near Wrzesnia in central Poland (N 52°18'56.931" E 17°27'47.136"). The insects were reared on a modified sugar/yeast diet as described previously [30], in standard conditions: 23 ± 2 °C, under a 12:12 light-dark cycle, at 60% humidity. After optimizing rearing conditions, 5 virgin females and males were transferred to vials containing diet supplemented with sublethal toxin doses. After 72 h, the parent insects (P) were removed. The new, first generation of insects (definitely over

100 individuals in each bottle) was partly intended for analysis (30 individuals), and the rest was poured into new bottles with media so that the females would lay eggs and after 3 days poured out and disposed of. The new, second generation (three-day imago) was poured onto fresh diet with the tested substances, and the procedure was analogous until the tenth generation. Five replicates were performed for each concentration in seven independent experiments. Control diets without nicotine (N) and acetamiprid (A) were conducted in parallel. N and A were supplied by Sigma-Aldrich (St. Louis, MO, USA). To assess survival, emerged flies were counted on day 14 after oviposition. To calculate the percentage survival and sublethal doses in transgenerational studies, 50% survival percentage (SP50) was calculated as 50% of the total eggs that developed into adult flies. Dose-response tests were performed for acetamiprid and nicotine. To select insecticide doses for further analysis, acetamiprid was added to the medium at concentrations of 0.125, 0.25, 0.50, 0.875, and 1.0  $\mu\text{L}/\text{mL}$ , and nicotine was added at concentrations of 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5  $\text{mg}/\text{mL}$ . From the populations reared on standard medium, 100 *Drosophila suzukii* eggs were collected using a stereomicroscope and placed on a medium consisting of nicotine, acetamiprid, and standard medium. To assess survival, the number of hatched flies was determined. Three sublethal concentrations of acetamiprid were selected: 0.125, 0.250, and 0.500  $\mu\text{g}/\text{mL}$ . We also used 0.875, which is above SP50 doses. Two concentrations of nicotine were used as positive controls (sublethal, 0.1  $\text{mg}/\text{mL}$ ; above SP50 doses, 0.2).

## 2.2. FA Measurement in the Wing Veins

We randomly selected 15 female and 15 male *D. suzukii* flies (3 days old) reared on a diet with different concentrations of nicotine and acetamiprid from the first (F1), fifth (F5), and tenth (F10) generations. The insects were anesthetized using ether, and their wings were removed, placed on a slide, mounted in Euparal, and covered with a coverslip. Measurements of the veins of the left and right wings were taken under a microscope at  $4\times$  magnification using the CellSens software (Olympus Co., Tokyo, Japan). Six veins on the wing, marked A-F (Figure 1), were measured three times to reduce the risk of errors. FA was defined as the absolute difference between the veins of the right and left wings, standardized by the mean.



**Figure 1.** *Drosophila suzukii* adult. Right and left wings of a male and a female; from A to F, the measured veins are marked (LV 3,4,5 = longitudinal vein 3,4,5; PCV = posterior cross vein; ACV = anterior cross vein).

### 2.3. Statistical Analysis

Data were statistically analyzed using StatSoft Statistica (StatSoft Inc., Tulsa, OK, USA). The following statistics were calculated for vein length: arithmetic mean, minimum, maximum, standard deviation, and coefficient of variation. To determine the coefficient of FA, basic statistics, analysis of variance (ANOVA), the Shapiro–Wilk test, the Tukey test to check statistical significance, principal component analysis, and a dendrogram were calculated. Triplicate measurements of vein lengths were performed (90 measurements for each vein, A to F, from each of the six test concentrations plus the control, for generations F1, F5, and F10; F5—without A0.875 µg/mL, F10—without acetamiprid). The Kruskal–Wallis test determined the difference between triplicate wing measurements.

### 3. Results

The first results indicated that *D. sukuzii* was so sensitive to acetamiprid that it could not be continued to the tenth generation, although this attempt was made three times. In generation F5, no adults survived at a concentration of 0.875. In generation F10, at concentrations of 0.125, 0.250, and 0.500, only two to five adults were recorded, so proper analyses could not be performed.

#### 3.1. Vein Length

The absolute difference in the lengths of both veins yielded similar results, indicating high precision. Since there was no significant difference between replicates ( $p > 0.05$ ), the data were combined. Acetamiprid and nicotine affected the average length of *D. sukuzii* wing veins in F1. All veins except anterior cross-vein A were the longest in the control samples. In the remaining experiments, adding insecticides reduced vein length. No correlation was observed between the reduced length of the veins and the concentration of the tested toxic substances (Table 1).

**Table 1.** Descriptive statistics for five studied veins in *Drosophila sukuzii* wings. Yellow indicates the highest average vein length, and green indicates the lowest.

Vein	Concentration: A = Acetamiprid [µg/mL] N = Nicotine [mg/mL]	N	Mean [mm]	Min [mm]	Max [mm]	SD	V
A	C	270	64.41	54.42	75.14	5.624	8.724
	A0.125	180	57.62	46.71	81.34	8.005	13.89
	A0.250	180	67.81	52.97	75.88	5.597	8.817
	A0.500	180	69.92	60.66	82.705	5.508	7.877
	A0.875	90	66.02	56.13	76.47	6.653	10.077
	N0.1	270	60.23	49.53	68.67	5.21	8.64
	N0.2	270	62.35	50.03	70.56	4.53	7.27
B	C	270	195.07	166.13	225.26	17.038	8.734
	A0.125	180	183.48	151.61	232.45	23.92	13.032
	A0.250	180	189.47	170.64	207.69	11.771	6.032
	A0.500	180	194.72	148.17	218.5	15.391	7.904
	A0.875	90	187.81	160.29	217.05	14.741	7.85
	N0.1	270	170.32	146.49	195.92	12.83	7.53
	N0.2	270	173.44	153.87	192.72	12.62	7.28

Table 1. Cont.

Vein	Concentration:		Mean [mm]	Min [mm]	Max [mm]	SD	V
	A = Acetamiprid [ $\mu\text{g}/\text{mL}$ ]	N = Nicotine [ $\text{mg}/\text{mL}$ ]					
C	C	270	357.88	328.29	389.47	15.687	4.383
	A0.125	180	340.27	297.4	392.24	27.21	8.00
	A0.250	180	340.63	295.05	373.29	24.116	7.12
	A0.500	180	351.12	304.88	382.38	21.01	5.98
	A0.875	90	329.92	285.17	368.57	18.63	5.65
	N0.1	270	315.81	285.03	354.42	19.59	6.20
	N0.2	270	310.302	281.32	343.97	18.17	5.85
D	C	270	539.19	480.89	590.16	33.81	6.27
	A0.125	180	499.42	436.04	586.77	39.76	7.96
	A0.250	180	513.65	457.61	557.28	31.27	6.09
	A0.500	180	519.2	433.63	570.6	32.7	6.30
	A0.875	90	506.02	435.86	545.57	27.97	5.53
	N0.1	270	501.28	446.47	560.87	34.42	6.87
	N0.2	270	495.16	440.85	549.49	34.94	7.06
E	C	270	1145.43	1059.64	1244.9	65.01	5.68
	A0.125	180	1098.38	974.57	1264.04	73.99	6.74
	A0.250	180	1073.35	930.79	2272.98	65.58	6.11
	A0.500	180	1118.23	925.57	1231.3	755.5	6.75
	A0.875	90	1067.16	999.2	1210.04	66.31	6.21
	N0.1	270	1005.16	890.91	1114.17	64.98	6.46
	N0.2	270	1009.78	930.77	1106.28	60.45	5.99
F	C	270	1754.62	1609.57	1893.18	98.64	5.62
	A0.125	180	1676.69	1500.75	1936.74	120.99	7.22
	A0.250	180	1663.93	1523.23	1783.37	91.16	5.48
	A0.500	180	1718.81	1428.33	1886.32	108.53	6.31
	A0.875	90	1653.04	1487.69	1823.82	95.06	5.75
	N0.1	270	1580.19	1387.73	1753.63	102.91	6.51
	N0.2	270	1579.43	1447.36	1714.62	98.12	6.21

N = number of measurements; Min = minimum value; Max = maximum value; SD = standard deviation; V = coefficient of variation;   = highest average vein length;   = lowest average vein length.

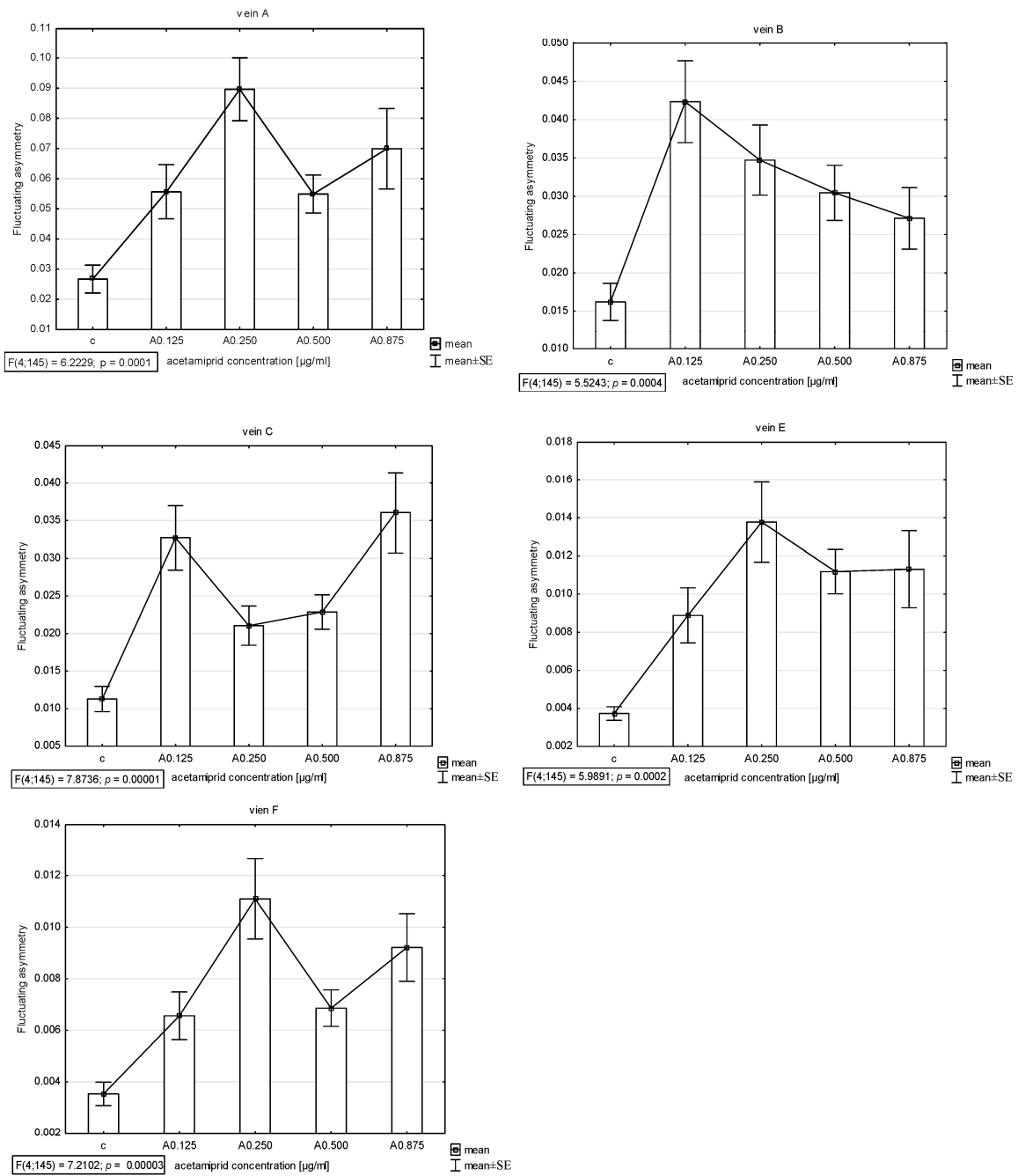
### 3.2. Fluctuating Asymmetry

The presence of asymmetry was confirmed via an ANOVA examining the differences in length between longer and shorter veins in pairs in all veins (A, B, C, E, and F) except D, which was excluded from further analyses. The Shapiro–Wilk test showed that the FA index distribution was normal. Thus, the descriptive statistics were calculated. The highest FA for vein A was noted for acetamiprid at a concentration of 0.250  $\mu\text{g}/\text{mL}$  and nicotine at a concentration of 0.2  $\text{mg}/\text{mL}$ ; for vein B, the highest FA was for acetamiprid at 0.125  $\mu\text{g}/\text{mL}$  and nicotine at 0.2  $\text{mg}/\text{mL}$ ; for vein C, the highest was for acetamiprid at 0.875  $\mu\text{g}/\text{mL}$  and nicotine at 0.2  $\text{mg}/\text{mL}$ ; for vein E, the highest was for acetamiprid at 0.250  $\mu\text{g}/\text{mL}$  and nicotine at 0.2  $\text{mg}/\text{mL}$ ; for vein F, the highest was for acetamiprid at 0.250  $\mu\text{g}/\text{mL}$  and nicotine at 0.1  $\text{mg}/\text{mL}$  (Table 2; Figures 2 and 3).

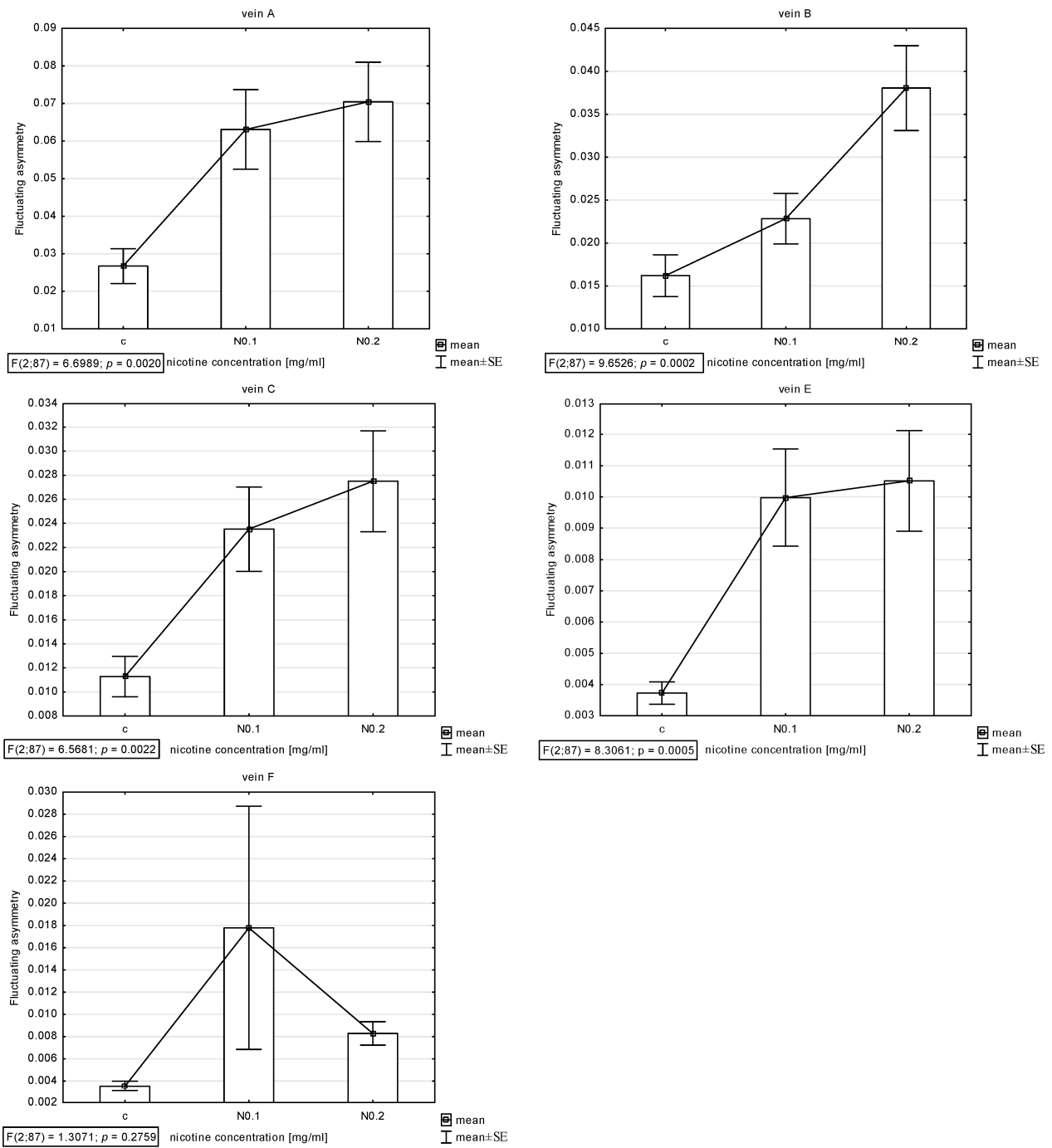
**Table 2.** Descriptive statistics for fluctuating asymmetry in five examined F1 *Drosophila suzukii* wing veins. Blue indicates the highest mean FA observed for acetamiprid, and green indicates the highest mean FA observed for nicotine.

Vein	Concentration: A = Acetamiprid [µg/mL] N = Nicotine [mg/mL]	N	Mean [mm]	Min [mm]	Max [mm]	SD	V
A	C	30	0.026709	0.001671	0.086230	0.025	94.83
	A0.125	30	0.055673	0.004383	0.182970	0.049	88.16
	A0.250	30	0.089643	0.014400	0.225400	0.057	63.65
	A0.500	30	0.054924	0.003820	0.117420	0.035	63.10
	A0.875	30	0.069924	0.004559	0.271510	0.073	104.27
	N0.1	30	0.063087	0.001311	0.300860	0.058	92.16
	N0.2	30	0.070425	0.001700	0.206100	0.058	82.07
B	C	30	0.016205	0.001827	0.050997	0.013	82.03
	A0.125	30	0.042344	0.011310	0.095230	0.029	69.14
	A0.250	30	0.034723	0.005800	0.134000	0.025	72.19
	A0.500	30	0.030442	0.003770	0.090200	0.020	64.65
	A0.875	30	0.027121	0.001325	0.090630	0.022	81.17
	N0.1	30	0.022847	0.001152	0.059800	0.016	70.68
	N0.2	30	0.038047	0.000950	0.105520	0.027	71.08
C	C	30	0.011279	0.001560	0.048240	0.009	81.71
	A0.125	30	0.032707	0.002972	0.092920	0.023	71.51
	A0.250	30	0.021047	0.001500	0.049600	0.014	68.11
	A0.500	30	0.022863	0.006325	0.049450	0.012	55.04
	A0.875	30	0.036076	0.001990	0.102430	0.029	81.45
	N0.1	30	0.023521	0.001485	0.060170	0.019	81.48
	N0.2	30	0.027501	0.000580	0.097310	0.023	83.49
E	C	30	0.003732	0.001014	0.007978	0.002	52.22
	A0.125	30	0.008884	0.001504	0.034520	0.008	89.21
	A0.250	30	0.013779	0.001190	0.059710	0.011	83.41
	A0.500	30	0.011182	0.001870	0.031630	0.006	56.72
	A0.875	30	0.011309	0.001350	0.049930	0.011	97.76
	N0.1	30	0.009978	0.001190	0.044610	0.008	85.28
	N0.2	30	0.010514	0.000380	0.026540	0.009	83.92
F	C	30	0.003535	0.001005	0.009060	0.002	70.37
	A0.125	30	0.006564	0.001660	0.023770	0.005	77.08
	A0.250	30	0.011096	0.001425	0.032600	0.008	76.78
	A0.500	30	0.006859	0.001775	0.017720	0.004	56.61
	A0.875	30	0.009213	0.001373	0.037380	0.007	77.88
	N0.1	30	0.017789	0.000030	0.333850	0.060	336.69
	N0.2	30	0.008290	0.001303	0.026690	0.006	69.59

N = number of measurements; Min = minimum value; Max = maximum value; SD = standard deviation; V = coefficient of variation;  = highest mean FA observed for acetamiprid;  = highest mean FA observed for nicotine.

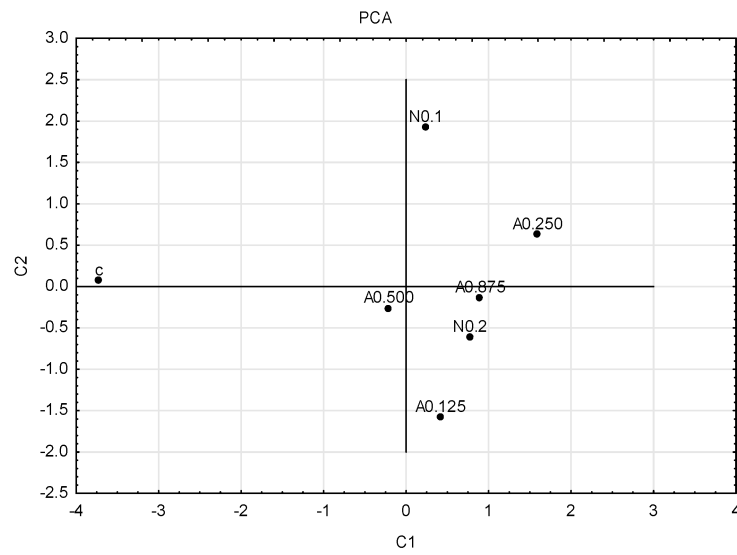


**Figure 2.** Graph of mean and standard error (SE) of fluctuating asymmetry for five studied veins (A, B, C, E, F) for four different concentrations of acetaminiprid (0.125, 0.250, 0.500, and 0.875 µg/mL) and control. ANOVA (F) and *p*-value calculated.

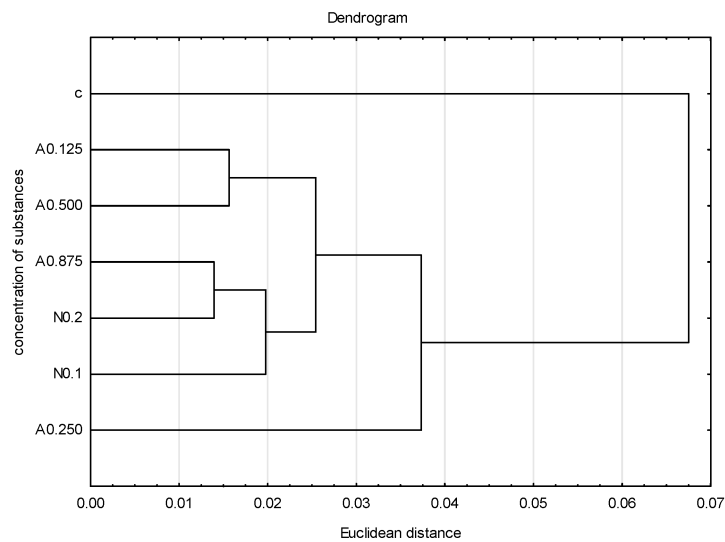


**Figure 3.** Graph of mean and standard error (SE) of fluctuating asymmetry for five studied veins (A, B, C, E, F) for two concentrations of nicotine (N: 0.1 and 0.2 mg/mL). ANOVA (F) and  $p$ -value calculated.

To graphically illustrate the obtained results, principal component analysis (PCA) (Figure 4) and cluster analysis (dendrogram) (Figure 5) were performed. Sublethal concentrations of acetamiprid at 0.125 and 0.500 formed one group, and concentrations of acetamiprid at 0.875 and nicotine at 0.2 (which are above SP50 doses) formed the other. The concentrations of acetamiprid at 0.250 and the sample without toxic substances were the most distinct.

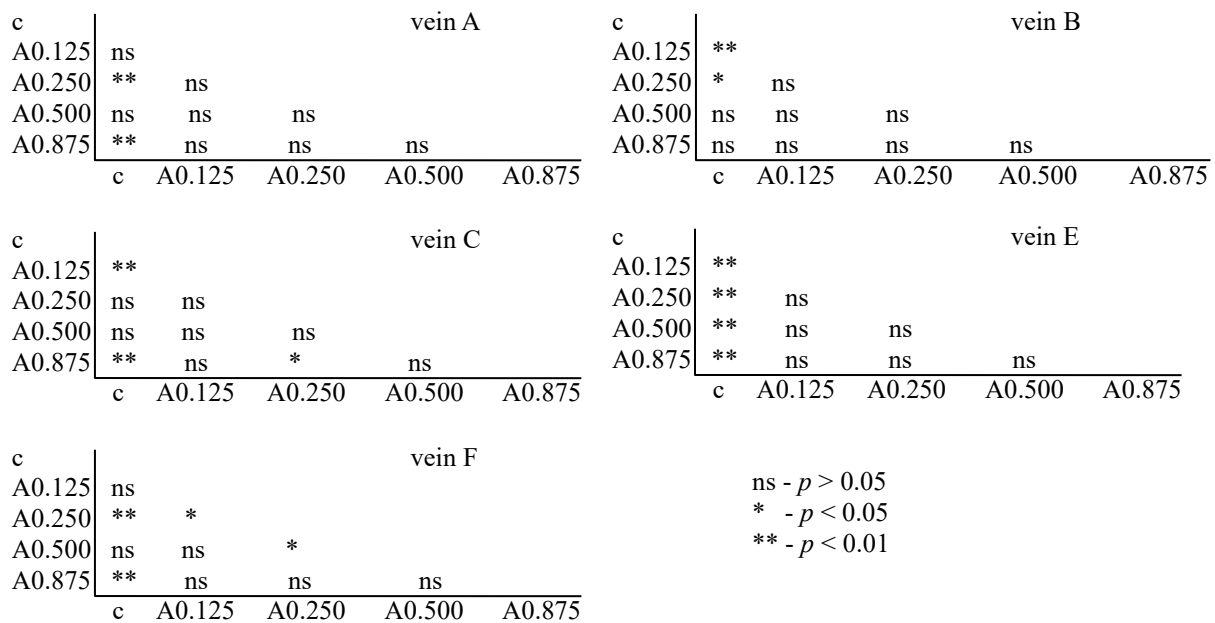


**Figure 4.** Graph of principal component analysis (PCA). First generation of insects; for four concentrations of acetamiprid (A = acetamiprid: 0.125, 0.250, 0.500, 0.875  $\mu\text{g}/\text{mL}$ ), for two concentrations of nicotine (N = nicotine: 0.1 and 0.2  $\text{mg}/\text{mL}$ ), and for the control (c).



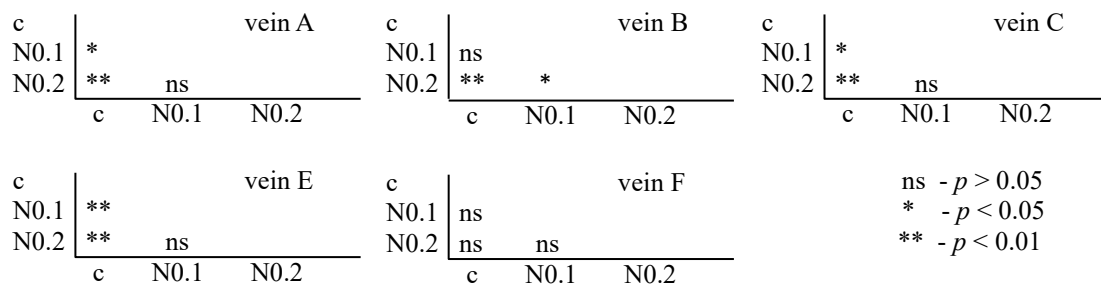
**Figure 5.** Dendrogram based on five tested veins (A, B, C, E, F) showing the hierarchical relationships between groups. Groups represent different concentrations of acetamiprid (A = acetamiprid: 0.125, 0.250, 0.500, and 0.875  $\mu\text{g}/\text{mL}$ ), of nicotine (N = nicotine: 0.1 and 0.2  $\text{mg}/\text{mL}$ ), and the control group (c).

The Tukey test was performed. The test showed that for acetamiprid, significant differences between the control and FA veins were observed: for vein A at concentrations of 0.250 and 0.875  $\mu\text{g}/\text{mL}$ ; for vein B at concentrations of 0.125 and 0.250  $\mu\text{g}/\text{mL}$ ; for vein C at concentrations of 0.125 and 0.875  $\mu\text{g}/\text{mL}$ ; for vein E at all tested concentrations; and for vein F at concentrations of 0.250 and 0.875  $\mu\text{g}/\text{mL}$ . In veins C and F, significant differences were also found between the 0.250 and 0.875  $\mu\text{g}/\text{mL}$  concentrations and 0.500 and 0.875  $\mu\text{g}/\text{mL}$  concentrations, respectively (Figure 6).



**Figure 6.** Tukey test for fluctuating asymmetry between tested acetamiprid concentrations (A = acetamiprid: 0.125, 0.250, 0.500, and 0.875 µg/mL) and control (c) for veins A, B, C, E, and F;  $p < 0.01$  = statistically significant differences,  $p < 0.005$  = statistically significant differences,  $p > 0.05$  = differences statistically insignificant (ns).

For nicotine, the Tukey test showed significant differences between control and FA veins: vein A at concentrations of 0.1 and 0.2 mg/mL; vein B at a concentration of 0.2 mg/mL; and veins C and E at concentrations of 0.1 and 0.2 mg/mL. In vein B, significant differences were also found between the 0.1 and 0.2 mg/mL concentrations (Figure 7).



**Figure 7.** Tukey test for fluctuating asymmetry between tested nicotine concentrations (N = nicotine: 0.1 and 0.2 mg/mL) and control (c) for veins A, B, C, E, and F;  $p < 0.01$  = statistically significant differences,  $p < 0.005$  = statistically significant differences,  $p > 0.05$  = differences statistically insignificant (ns).

No significant differences were observed when analyzing the F5 and F10 generations in relation to the first generation in terms of the mean FA for 0.125, 0.250, 0.500, and 0.875 µg/mL acetamiprid concentrations (Figure 8a) and 0.1 and 0.2 mg/mL nicotine concentrations (Figure 8b). Fluctuating asymmetry did not increase in subsequent generations and was smaller than in F1 in some cases.

a)

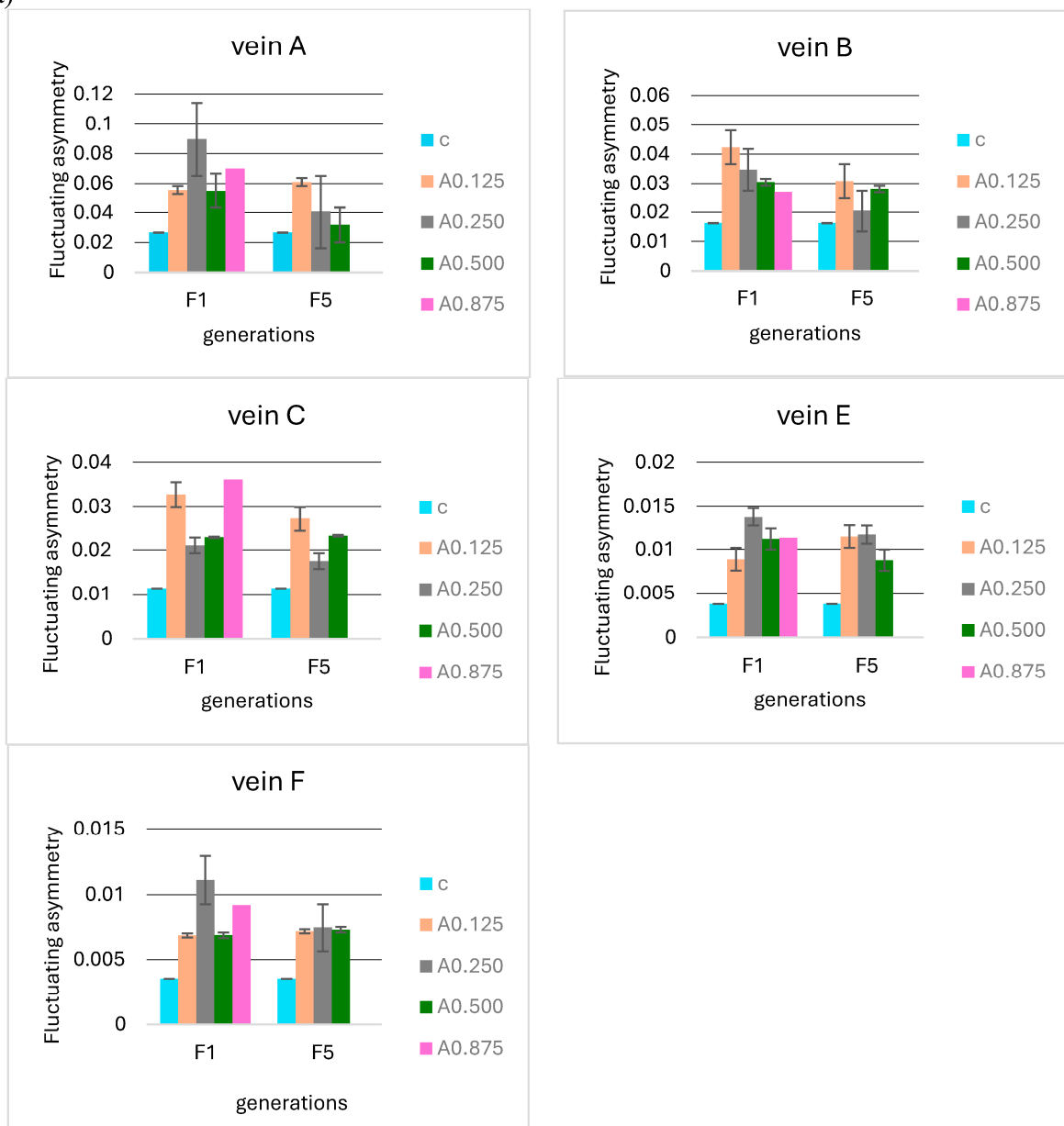
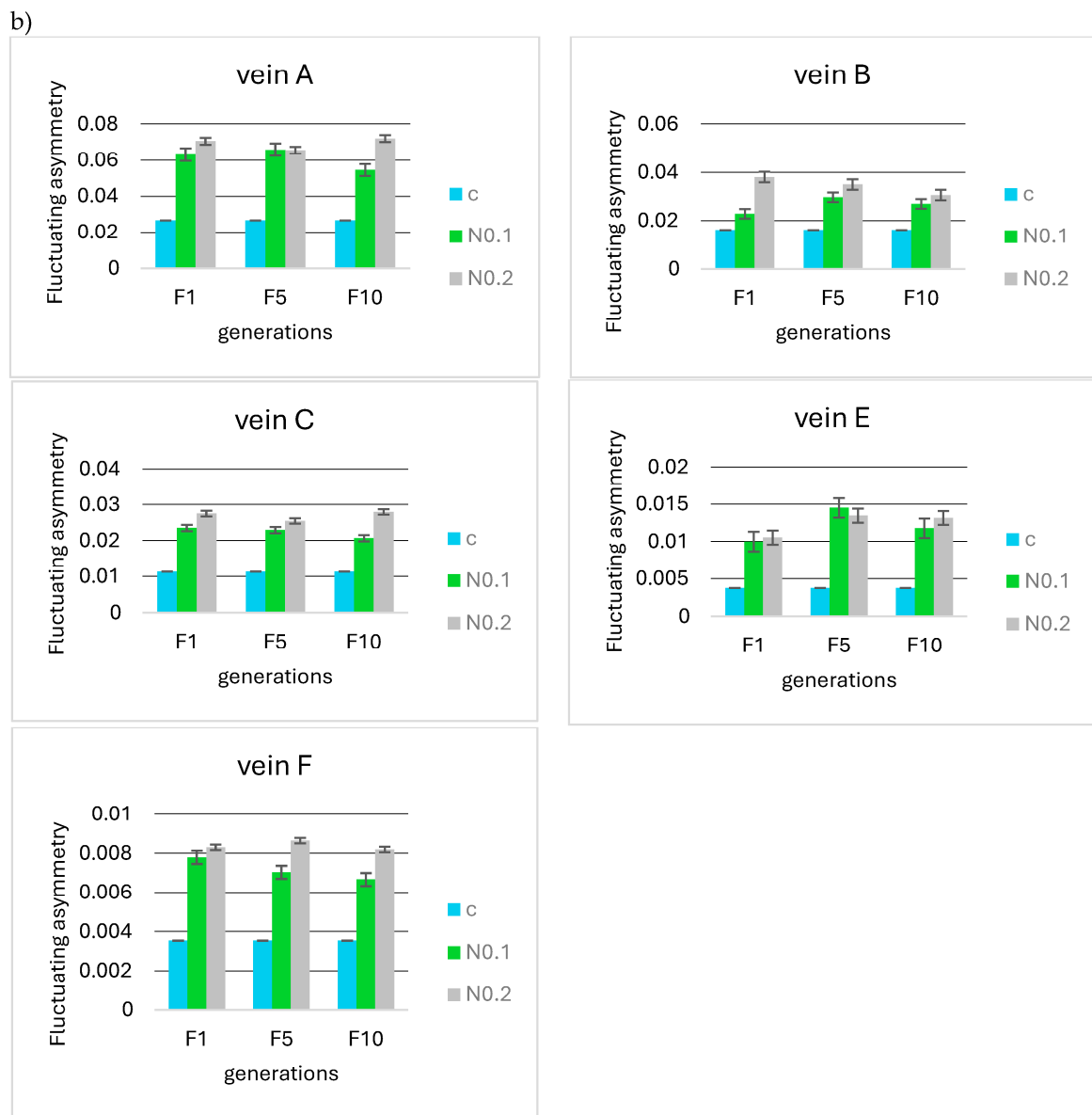
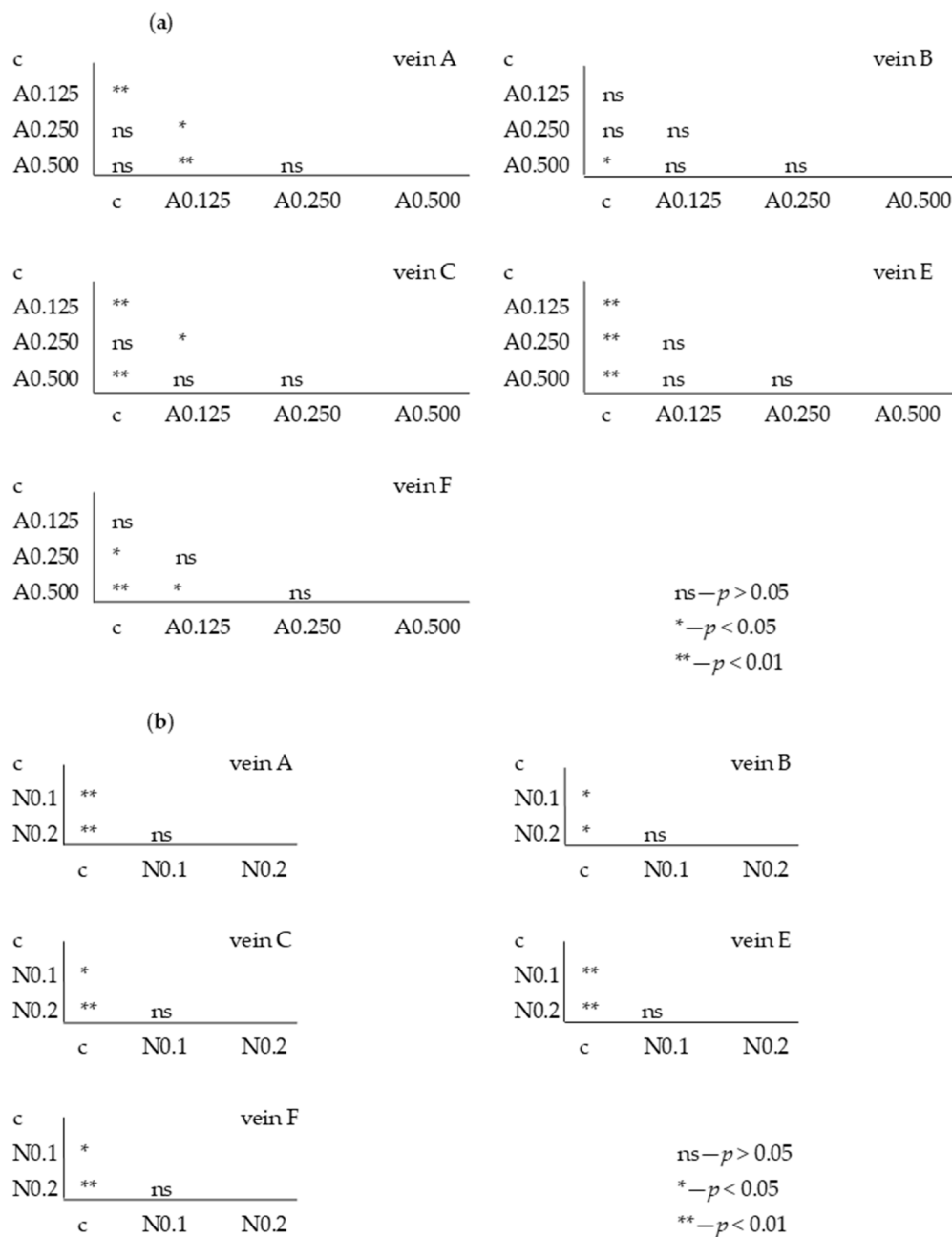


Figure 8. Cont.



**Figure 8.** Mean fluctuating asymmetry of veins A, B, C, E, and F observed in successive insect generations (F1 = first generation, F5 = fifth generation, F10 = tenth generation) for the control (c) and (a) different concentrations of acetamiprid (A0.125, 0.250, 0.500, 0.875  $\mu\text{g}/\text{mL}$ ) and (b) different concentrations of nicotine (N0.1 and 0.2  $\text{mg}/\text{mL}$ ).

For the fifth generation, the Tukey test was also performed, showing statistically significant differences for individual veins between the control sample in some acetamiprid and nicotine concentrations (Figure 9a); however, these were not significant between F1 and F5.



**Figure 9.** Tukey test for fluctuating asymmetry between control (c) and tested acetamiprid concentrations (A = acetamiprid: 0.125, 0.250, 0.500, 0.875  $\mu\text{g}/\text{mL}$ ) (a) and between control (c) and tested nicotine concentrations (N = nicotine: 0.1 and 0.2  $\text{mg}/\text{mL}$ ) (b) for veins A, B, C, E, and F;  $p < 0.01$  = statistically significant differences,  $p < 0.005$  = statistically significant differences,  $p > 0.05$  = differences statistically insignificant (ns); fifth generation.

For nicotine, the Tukey test showed significant differences between the FA of the control and all tested concentrations (Figure 9b), but these were not significant at the intergenerational level.

#### 4. Discussion

Despite the undoubted benefits of reasonable insecticide use, these chemicals can be serious risks to humans or the environment [31]. Neonicotinoids, a new group of insecticides, have become popular in recent years; initially, they were considered a safe alternative to other substances that control pests, but many of them are dangerous and have

already been banned. One of the few neonicotinoids currently approved is acetamiprid, a neurotoxin that affects the nervous system of insects and is used to control SWD, an invasive fruit pest. Despite renewed approval from the Commission Implementing Regulation, this substance has been detected in residues in many products, possibly posing previously unrecognized risks [32]. The EFSA recommends updating its residue limits based on new information on the risks involved and continuing research. We decided to use SWD to study the effects of sublethal doses of acetamiprid. Acetamiprid is classified as harmful to SWD parasitoids because it causes rapid mortality and reduced survival time in offspring and does not show parasitism [33]. As a positive control with a documented harmful effect on *Drosophila*, we used nicotine [23,34]. In earlier reports, we confirmed that in low doses, acetamiprid adversely affects *D. suzukii* development and reproduction, and we were interested in whether it similarly affects another bioindicator of stress, fluctuating asymmetry.

This small, random deviation from ideal symmetry measures developmental instability, a sensitive biomarker of environmental stress [24–26,35]. Higher FA has been proved to be a measure of susceptibility to pesticides in, for example, lizards [36], frogs [37], birds [38], fish [39,40], and invertebrates: Odonata [41], Apis [42], *Triatoma* [43,44], carabid beetles [45], and *Drosophila* [46,47].

FA in locomotor features may be particularly important in the fitness context; therefore, we decided to study wings—or more precisely, their veins—to determine whether long-term exposure to various sublethal acetamiprid and nicotine concentrations throughout the developmental period would cause asymmetry. Studies conducted on *D. melanogaster* have shown FA in wings under the influence of the antibiotic neomycin [27], but not all veins have shown FA, and it has not always been precisely correlated with increased antibiotic concentrations. In our studies, five of the six analyzed veins showed differences between the right and left wings under the influence of sublethal doses of insecticides, classified as FA after statistical analysis. Compared with the control, the differences were statistically significant and correlated with increased insecticide concentrations in most cases. The average wing vein length was smaller in the insects exposed to toxins than in the controls. Similar results have been reported for *D. melanogaster* by [27,30,48]. Their studies showed that both acetamiprid and nicotine, even in exceedingly small doses, are a strong controlling factor for SWD, disturbing developmental stability. Importantly, we can relate the results of our analyses to other studies on the effects of the same concentrations of acetamiprid and nicotine [23] and link the appearance of FA to locomotor dysfunction occurring under the influence of the tested toxins. This is a significant result. In subsequent generations of SWD, we did not observe major differences in the FA index. The results were similar, possibly indicating that insects do not acquire resistance to this pesticide. The reduced number of flies in subsequent generations indicates high acetamiprid activity even at low doses; with each subsequent generation, the number of insects exposed to insecticides decreased compared with the control culture. Acetamiprid had a greater effect on the decreasing numbers than nicotine used in higher concentrations. This also shows the validity of using FA as an environmental stress indicator. Exposure to low doses of insecticide and their influence on insects have been observed in many different experiments. Among sublethal effects, one can mention modification of antioxidant enzymes, altered neurotransmission, especially inhibited acetylcholinesterase activity, or structural aberrations [49]. Research on nicotine showed that sublethal effects, like reduced vitality or fertility, due to physiological malfunctions and structural malformations, may significantly decrease the fitness of flies [30]. A major problem with the widespread use of insecticides is the emergence of resistance. This enlarges the toxic effects of pesticides on the environment and leads to an increase in the amount of insecticides required to effectively control resistant insects. In the case of acetamiprid, we did not observe the acquisition of resistance expressed in fluctuating asymmetry. On the one hand, this confirms the effectiveness of this pesticide in controlling SWD even when used at low doses over several generations. On the other hand, attention should be paid to the risk of harmful effects of low doses in case of interactions

with non-target organisms, including humans. This is especially true since small amounts of residues of this compound can be found not only in the environment but also in food.

## 5. Conclusions

The conclusions from our research are as follows:

- Sublethal doses of acetamiprid and nicotine disrupt the developmental stability of *Drosophila suzukii*, which manifests as fluctuating asymmetry in wing veins.
- Exposing insects to these insecticides throughout development reduces wing vein length, consequently reducing wing size.
- It is likely that persistent FA in subsequent SWD generations affects increased mortality—assuming that more disturbed individuals do not develop from eggs or larvae.
- FA can be used to analyze the effects of acetamiprid even at low, sublethal concentrations.

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## **8. Oświadczenia współautorów o wkładzie w powstanie artykułów**


## OŚWIADCZENIA WSPÓLAUTORA O WKŁADZIE W POWSTANIE ARTYKUŁÓW


Niniejszym oświadczam, że mój wkład w powstanie artykułu:

Lewandowska-Wosik A., Chudzińska E.M., Wojnicka-Półtorak A. **Genotoxic effects of sub-lethal doses of nicotine and acetamiprid in neuroblasts of *Drosophila melanogaster* and *Drosophila suzukii***, *Ecotoxicology and Environmental Safety*, 2024, 280(7):116585, doi:10.1016/j.ecoenv.2024.116585

włączonego do mojej rozprawy doktorskiej polegał na:

sformułowaniu hipotezy badawczej, prowadzeniu hodowli eksperymentalnej owadów *D. melanogaster* i *D. suzukii*, wykonaniu analiz laboratoryjnych z zastosowaniem metody *comet assay*, wykonaniu analiz mikroskopowych, przeprowadzeniu testów przeżycia i testów wspinaczkowych owadów, statystycznym opracowaniu wyników i ich interpretacji, przygotowaniu artykułu do publikacji (autor korespondencyjny).

  
.....  
Mgr Anetta Lewandowska-Wosik

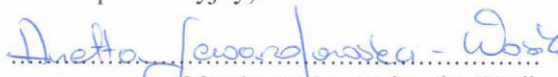
  
.....  
Prof. UAM dr hab. Ewa Chudzińska – Promotor


Niniejszym oświadczam, że mój wkład w powstanie artykułu:

Lewandowska-Wosik A., Chudzińska E.M. **Fluctuating asymmetry spotted wing *Drosophila* (Diptera: Drosophilidae) exposed to sublethal doses of acetamiprid and nicotine**, 2024, *Insects*, 15(9): 681, doi:10.3390/insects15090681

włączonego do mojej rozprawy doktorskiej polegał na:

sformułowaniu hipotezy badawczej, prowadzeniu hodowli eksperymentalnej owadów *D. suzukii*, przygotowanie preparatów, wykonanie analiz mikroskopowych, analizy statystyczne, interpretację wyników oraz przygotowanie artykułu do publikacji (autor korespondencyjny).

  
.....  
Mgr Anetta Lewandowska-Wosik

  
.....  
Prof. UAM dr hab. Ewa Chudzińska – Promotor

## OŚWIADCZENIE WSPÓLAUTORA O WKŁADZIE W POWSTANIE ARTYKUŁU

Niniejszym oświadczam, że mój wkład w powstanie artykułu:

Lewandowska-Wosik A., Chudzińska E.M., Wojnicka-Półtorak A. **Genotoxic effects of sub-lethal doses of nicotine and acetamiprid in neuroblasts of *Drosophila melanogaster* and *Drosophila suzukii***, *Ecotoxicology and Environmental Safety*, 2024, 280(7):116585, doi:10.1016/j.ecoenv.2024.116585

którego wiodącą i korespondencyjną autorką jest Anetta Lewandowska-Wosik polegał na:

współtworzeniu projektu badań, nadzorze nad badaniami i interpretacją wyników, krytycznej rewizji przygotowywanego artykułu.

  
.....

prof. UAM dr hab. Ewa Chudzińska

29.10.2024

## OŚWIADCZENIE WSPÓLAUTORA O WKŁADZIE W POWSTANIE ARTYKUŁU

Niniejszym oświadczam, że mój wkład w powstanie artykułu:

Lewandowska-Wosik A., Chudzińska E.M. **Fluctuating asymmetry spotted wing *Drosophila* (Diptera: Drosophilidae) exposed to sublethal doses of acetamiprid and nicotine**, 2024, *Insects*, 15(9): 681, doi:10.3390/insects15090681

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prof. UAM dr hab. Ewa Chudzińska

29.10.2024

## OŚWIADCZENIE WSPÓLAUTORA O WKŁADZIE W POWSTANIE ARTYKUŁU

Niniejszym oświadczam, że mój wkład w powstanie artykułu:

Lewandowska-Wosik A., Chudzińska E.M., Wojnicka-Półtorak A.  
**Genotoxic effects of sub-lethal doses of nicotine and acetamiprid in neuroblasts of *Drosophila melanogaster* and *Drosophila suzukii*, *Ecotoxicology and Environmental Safety*, 2024, 280(7):116585, doi:10.1016/j.ecoenv.2024.116585**

którego wiodącą i korespondencyjną autorką jest Anetta Lewandowska-Wosik polegał na:

współuczestniczeniu w pisaniu artykułu – przegląd, edycja, korekta.



prof. UAM dr hab. Aleksandra Wojnicka-Półtorak

29.10.2024