# Comparative Toxicity Evaluation of Acephate and Chlorantraniliprole on *Drosophila melanogaster* (Meigen, 1830) Through Transcriptomics and Wing Geometric Morphometrics

Thesis submitted in
partial fulfilment of the requirements
for the award of the degree of

**DOCTOR OF PHILOSOPHY IN ZOOLOGY**

By

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**SEPTEMBER, 2024**

# DECLARATION

I hereby declare that the work presented in the thesis entitled **"Comparative toxicity evaluation of Acephate and Chlorantraniliprole on *Drosophila melanogaster* (Meigen, 1830) through transcriptomics and wing geometric morphometrics "** is based on the original work done by me under the guidance of **Dr. Y. Shibu Vardhanan**, Professor, Biochemistry & Toxicology Division, Department of Zoology, University of Calicut and has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis have undergone a plagiarism check using iThenticate software at the C.H.M.K. Library, University of Calicut, and the similarity index was found to be within the permissible limit. I also declare that the thesis is free from AI-generated content.

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# CERTIFICATE

This is to certify that this dissertation entitled **"Comparative toxicity evaluation of Acephate and Chlorantraniliprole on *Drosophila melanogaster* (Meigen, 1830) through transcriptomics and wing geometric morphometrics"** is an authentic record of independent work done by **Ms. Rahila. K.,** Department of Zoology, the University of Calicut under my guidance and supervision for the partial fulfillment of the requirements for the **Degree of Doctor of Philosophy in Zoology** and further no part of this dissertation has been presented before for any other Degree or Diploma.

I also certify that the corrections/suggestions from adjudicators have been incorporated in the revised thesis.

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# ACKNOWLEDGMENT

*First of all, I thank Almighty God for gifting me with life, motivation, patience, positivity and strength.*

*My PhD work will be incomplete without thanking some wonderful people who helped me a lot to achieve success in my research carrier.*

*It is a great pleasure for me to thank* ***Dr. Y. Shibu Vardhanan****, Professor, Department of Zoology, University of Calicut, who not only is my research supervisor, but this thesis is a product of his guidance, continuous support, inspiration and constructive criticism throughout my research work. His guidance and encouragement have always been instrumental in helping me throughout the research and writing of this thesis. Without his guidance, I would not have been able to complete my Ph.D. successfully.*

*I express my deep sense of gratitude to the present Head, Dr. C.D. Sebastian, and former Heads, Dr. E. M. Manogem, Dr. E. Pushpalatha, and Dr. Y. Shibu Vardhanan, of the Department of Zoology, University of Calicut, for providing me with all the basic infrastructural facilities necessary to carry out my research work.*

*I respectfully acknowledge my MSc. Teachers (Dr. M. Gogul Das, Dr. N. Ramani, Dr. M. Nasser, Dr. Y. Shibu Vardhanan, Dr. V. M. Kannan, Dr. E. M. Manogem, Dr. E. Pushpalatha, Dr C.D. Sebastian, Dr. K. C. Chithra and Dr. M. Zubair) for their teaching, motivation and inspiration during post-graduation days.*

*Words cannot express my sincere gratitude to Mr. Anand, P. P., my colleague and senior research scholar, whose unwavering support and vast knowledge greatly aided me in completing my Ph.D. work. Without him, it would not be possible for me to complete this work.*

*I would like to express my heartfelt thanks to all my senior lab mates, Dr. M.S. Siva Prasad, Dr. Shameema Kodasseri, Mrs. Jinsha Peter, and Mrs. Hima K.S., for their support and guidance throughout the completion of my work.*

*I feel blessed to have a group of co-workers without whose friendly help, support and cooperation; it would have been very hard to carry out my work. It's a*

*great pleasure for me to express heartfelt gratitude and love for my lab mates, Dr. Neethu, C.B., and Dr. Seena. S. Mrs. Gayathri. M., Mrs. Renu, V.V., for their unfailing cooperation and constant support during several steps of my research life. Moreover, express my sincere thanks to other members of our lab Ms. Mehabooba, B., Mr. Abhishek Buxi., Dr. Abhilash, P. L., Ms. Anjumol. P., for their help and support. Also, remember and express thanks to Anusree, A.S. (former M. Phil student), may your heart and soul find peace and comfort. I express my thanks to Ms. Jinu Jishad for her help during submission.*

*I want to extend my sincere gratitude to the CSIR-UGC for providing financial support for this research. I would like to express my heartfelt gratitude to the University of Calicut's CSIF (Central Sophisticated Instrument Facility) for providing the instrumentation facilities. I would also like to extend my thanks to the department projects (UGC-SAP and DST-FIST) for providing instrumental facilities.*

*I extend my heartfelt thanks to all my teachers and non-teaching staff in the Department of Zoology, University of Calicut, for their selfless support and assistance. Special thanks to former librarian Mr. Sandosh K. V., and present librarian (Mr. Abdul Latheef) for help and support.*

*My sincere heartfelt thanks go to all my friends in the Department of Zoology for their enthusiastic support and help during my research. Special thanks to M. Sc. friends of batch 2012-2014 for their support and care.*

*I am immensely indebted to my parents (Mr. Abdu koothumadan & Mrs. Fathima. K. M.), whose unconditional support and encouragement helped me to be strongly grounded and to strive for success in my academic and research life. The affectionate presence of my brothers (Mr. Sadique Ali & Mr. Muhammed Shameer. K. M.), and sisters (Mrs. Raziya. K. M., Dr. Rajna. K. M., & Ms. Raheema. K. M.) in my life, helped me to face all odds with patience. I would like to thank in law members of our family: Mrs. Nasreen Habeeb. P., Mr. Rafeeque M. T., Mr. Navas for their support. I thank full to niece and nephews of my home (Fathima Rinsha M.T., Rabeeh, M.T., Razan. M. T., Inara Mehak., Mryam Nasreen Ali, Rania Nasreen Ali).*

*As I turn to thank my husband's family, heartfelt thanks to my father in law (Mr. Muhammed musliyar) for your prayer and support. And also express thanks to my mother-in-law (Mrs. Kadeeja T.), I realise how truly indebted I am to her for all the love she has given me and the sacrifices she has made to help me reach here, especially for managing my little kids. Thanks for all the lovely in sister in laws (Mrs. Rahmathuneesa, Mrs. Najmathunneesa, Mrs. Fairoosunnisa & Mrs. Fathima muhsina) and brothers-in-law (Mr.Abdul Rasheed M. K., Mr.Mansoor. A., Mr. Rasheed. K., Mr. Naheem. A., & Mr. Shamsudheen K. T.), and children of our family (Shabeeb, shuhaib, Shaima, Mehafin, Nusaif, Rushda, Rusna, Rasa, Nuhman, Adnan, Faizan, and Hamdan).*

*I want to extend special thanks to Mr. Saidalavi Haji (Moothappa) and Abdul Kareem. P. (Kunchippa), and in law, grandmother (Kunippathumma), who, although no longer with us, continues to inspire me with their prayers and support. I extremely grateful to Mrs. Arifa. P., and all relatives and neighbours for their support.*

*Moreover, most all, I owe my deepest gratitude to my husband, Mr. Sharafudheen. K. T., for his affection, encouragement, understanding, and patience. He supported me without any complaint or regret, which enabled me to complete my thesis. Also, deep thanks to my lovely stars, Muhammed Minhaj K.T., and Ummul Khair K.T., for their kind cooperation, patience, and love. Moreover, heartfelt apologies for taking up your time with Mom.*

*Aside from those listed above, I would like to express my sincere gratitude to everyone who has assisted me, either directly or indirectly, in the completion of this thesis.*

***Rahila. K.***

Dedicated to Society

# ABSTRACT

Pesticides are a necessary component of contemporary farming methods and are often applied to manage pests to increase agricultural yield. Pesticides are regularly used for pest control in agriculture. Therefore, the hazards of using these chemicals outweigh their benefits, and residues of these substances are frequently detected in food products and the environment. The non-selective and ignorant usage of pesticides causes harmful effects on non-target organisms. The present study investigated the non-target effects of two pesticides, acephate and chlorantraniliprole, on the model organism *Drosophila melanogaster.* Acephate and chlorantraniliprole are two commonly used pesticides in organophosphate and anthranilic diamide groups, respectively. These two chemicals have different modes of action. Acephate has anti cholinesterase activity and chlorantraniliprole acts on insect ryanodine receptors.

Sub-lethal exposure to acephate and chlorantraniliprole elicited a wide range of adverse effects on lifecycle parameters like hatchability, pupation, emergence, and stabilised body weight of the adult flies. The hatchability percentage gives a clear picture regarding early sub-lethal toxic effects. Our study revealed a concentration-dependent decrease in hatchability in the treated group. Acephate and chlorantraniliprole significantly impaired the entire organismal growth and early development. The intoxicated organism's pupation, emergence, survival rate and weight showed sound differences. This is a promising toxicological index for evaluating the overall life process. Early embryonic studies revealed the effects of both pesticides qualitatively and quantitatively.

Our study used RNA-seq technology (transcriptomics) to investigate the toxic effects of sub-lethal concentrations of acephate and chlorantraniliprole in *D. melanogaster*. We focused on the unique effects of each pesticide, which were directly linked to their mode of action. Both pesticides significantly altered various pathways and genes crucial for pesticide resistance and immunological response. In the acephate-treated group, 2,031 genes were up-regulated, whereas 1812 genes were down-regulated. Compared to the control group, 1369 genes were up-regulated, and 773 were down-regulated in the chlorantraniliprole-treated group. Our findings suggest that sublethal acephate and chlorantraniliprole exposures can cause up- or down-regulation of P450s, ESTs, GSTs, UGTs, and ABC transporters, which are crucial for detoxification. These findings are particularly important for

understanding the whole range of detoxification-related gene activities in *D. melanogaster*, making a significant scientific contribution to the field.

The third chapter evaluated the acephate and chlorantraniliprole-induced morphological alterations in the wings of *D. melanogaster*and also transcriptomic validation of genes responsible for wing development. The geometric morphometric (GMM) tool was used to detect fine-scale changes in wing size and shape. Concentration-dependent changes in wing shape were exhibited in both the pesticide-treated group, and these changes were independent of the sex of the organisms and validated by different multivariate analyses. The degree of shape variations differed between acephate and chlorantraniliprole treatments, which is strictly due to the different modes of action of the two pesticides. Pesticide-induced stress leads to changes in the functional integrity of the wing, with the clear separation of proximal-distal wing modules observed in pesticide-treated groups. Transcriptome analysis reveals the differential expression of genes controlling wing vein development, wing development, and other significant genes involved in shaping the wing and regulating wing function in *D. melanogaster*.

Our study's key findings concluded that sub-lethal concentrations of both pesticides significantly altered the normal life cycle of *D. melanogaster* and inhibited the expression of immunological genes, heat shock proteins, and resistance genes in a non-targeted insect species. We recommend that the sub-lethal concentrations of acephate and chlorantraniliprole significantly affect the immune and resistance mechanisms of non-targeted organisms. We also highlighted the potential of our study's findings to enhance the pesticide safety assessment process. By combining Transcriptomics and GMM analysis with toxicity studies, researchers can identify potential toxicity liabilities early and move forward with only those chemicals that have both efficacy at the target and a low potential for toxicity in the non-target population. We also emphasised the importance of advanced studies, such as proteomics and metabolomics, in elucidating detoxification pathways, thereby facilitating the development of scientific strategies to protect non-target species from xenobiotics.

**Keywords**: Acephate, Chloranthraniprole, Pesticides, Non-trageted organism, Transcriptomics, Geometric morphonetrics, *Drosophila melanogaster*.

# സംഗ്രഹം

മനുഷ്യരാശിക്ക് ഭക്ഷ്യസുരക്ഷാമേഖലയിൽ എന്നും കീടങ്ങൾ ഒരു വെല്ലുവിളി തന്നെയാണ്. കീടനാശിനികൾ സമകാലിക കൃഷരീതികളുടെ അനിവാര്യ ഘടകമാണ്. കാർഷിക വിളവ് വർദ്ധിപ്പിക്കുന്നതിനും കീട നിയന്ത്രണത്തിനും കീടനാശിനികൾ പലപ്പോഴും ഉപയോഗിക്കാറുണ്ട്. അതിനാൽ ഇത്തരം രാസവസ്തുക്കൾ ഉപയോഗിക്കുന്ന തിന്റെ അപകട സാധ്യത അവയുടെ ഗുണങ്ങളേക്കാൾ കൂടുതലാണ്. കൂടാതെ ഈ പദാർത്ഥങ്ങളുടെ അവശിഷ്ടങ്ങൾ ഭക്ഷ്യഉൽപ്പന്നങ്ങളിലും പരിസ്ഥിതിയിലും പതിവായി കാണപ്പെടുന്നു. തുടർന്നുണ്ടാകുന്ന മലിനീകരണം പ്രകൃതിയിലെ ആവാസ വ്യവസ്ഥയെ അസന്തുലിതമാക്കുന്നു. കീടനാശിനികൾ ഉപയോഗിക്കുന്നതിലെ അജ്ഞതയും, കൈകാര്യം ചെയ്യുന്നതിലുള്ള വൈദഗ്ദ്ധ്യവും കീടങ്ങളല്ലാത്ത ജീവികളിൽ ദോഷഫലങ്ങൾക്ക് കാരണമാവുന്നു. ഈ പഠനത്തിന്റെ പ്രഥമ ഉദ്ദേശം സാധാരണയായി ഉപയോഗിക്കുന്ന രണ്ട് കീടനാശിനികളായ അസഫേറ്റിന്റെയും ക്ലോറാൻട്രനിലിപ്രോലിന്റെയും ലക്ഷ്യമല്ലാത്ത ജീവികളിലുള്ള മാറ്റങ്ങൾ, ഡ്രോസോഫില മെലനോഗാസ്റ്റർ (പഴയീച്ച) എന്ന പരീക്ഷണ മാതൃക ജീവികളിൽ ഉണ്ടാകുന്ന മാറ്റങ്ങളാണ്. ഓർഗനോ ഫോസ്ഫേറ്റ് വിഭാഗത്തിൽപ്പെടുന്ന അസഫേറ്റും ആൻത്രാനിലിക് ഡൈഅമൈഡ് വിഭാഗത്തിൽപ്പെടുന്ന ക്ലോറാൻട്രനിലി പ്രോലും സാധാരണയായി കൃഷിയിടങ്ങളിൽ ഉപയോഗിക്കുന്ന രണ്ട് കീടനാശിനികളാണ്. ഈ രണ്ട് കീടനാശിനികളുടെയും പ്രവർത്തനരീതി വ്യത്യസ്തതയുള്ളതാണ്. അസഫേറ്റിന് കോളിൻഎസ്റ്ററേസ് വിരുദ്ധ പ്രവർത്തനവും, ക്ലോറാൻട്രനിലിപ്രോൾ പ്രാണികളിലെ റയനോഡിൻ റെസപ്റ്ററുകളിലും പ്രവർത്തിക്കുന്നു.

അസഫേറ്റിൻറെയും, ക്ലോറാൻഡ്രനിലിപ്രോലിന്റെയും ഉപമാരകമായ എക്സ്പോഷൻ ഈച്ചകളുടെ മുട്ട വിരിയൽ, പ്യൂപ്പേഷൻ, പ്രാണികളുടെ ആവിർഭാവം, ഈച്ചകളുടെ ശരീരഭാരം എന്നിങ്ങനെയുള്ള ജീവിത ചക്ര സൂചികകളിൽ വൈവിധ്യമാർന്ന പ്രതികൂല ഫലങ്ങൾ ഉണ്ടാകുന്നു. മുട്ട വിരിയുന്നതിൻറെ ശതമാനത്തിലുള്ള കുറവ് ആദ്യകാല വിഷ ഫലങ്ങളെ കുറിച്ച് വ്യക്തമായ ചിത്രം നൽകുന്നു. അസഫേറ്റും ക്ലോറാൻട്രനിലിപ്രോലും മുഴുവൻ ഈച്ചകളുടെ വളർച്ചയേയും ആദ്യകാല ജീവിത വികാസത്തേയും ഗണ്യമായി തടസ്സപ്പെടുത്തി. കീടനാശിനി പ്രയോഗിച്ച് ജീവികളുടെ പ്യൂപ്പേഷൻ ആവിർഭാവം, അതിജീവിത നിരക്ക്, എന്നിവയിൽ സ്പഷ്ടമായ വിത്യാസങ്ങൾ കാണിച്ചു. ഭ്രൂണ പഠനങ്ങൾ രണ്ട് കീടനാശിനികളുടെയും ഗുണപരമായ അളവിലും ബാഹ്യഘടനയിലും മാറ്റങ്ങൾ കാണിച്ചു. പഴയീച്ചകളിലെ അസഫേറ്റിന്റെയും ക്ലോറാൻട്രനിലി പ്രോലിന്റെയും മാരകമായ തന്മാത്ര വിഷഫലങ്ങൾ പഠിക്കുന്നതിനുള്ള വിലപ്പെട്ട സാങ്കേതിക വിദ്യയാണ് RNA sequencing (Transcriptomics)ങ്ങളുടെ പഠനം ഉപമാരക അളവിലുള്ള കീടനാശിനികളുടെ തന്മാത്ര വിഷഫലങ്ങളിൽ ശ്രദ്ധകേന്ദ്രീകരിച്ചു. പഠനത്തിൽ നിന്ന് കീടനാശിനി ചികിത്സിച്ച രണ്ട് ഗ്രൂപ്പുകളുടെ വിവിധ ജീനുകളുടെ പ്രവർത്തനങ്ങൾ വ്യക്തമായി വേർതിരിച്ചറിയാൻ കഴിയും. ഓരോ കീടനാശിനിയും അവയുടെ പ്രവർത്തന രീതിയുമായി നേരിട്ട് ബന്ധിപ്പിച്ചിരിക്കുന്ന തനതായ ആവിഷ്കാരരീതി കാണിച്ചു. രണ്ട് കീടനാശിനികളും കീടനാശിനി പ്രതിരോധ പ്രതികരണത്തിനും നിർണ്ണായകമായ വിവിധ ജീനുകളുടെയും, ജീനുകളുടെ പാതകളെയും ഗണ്യമായി മാറ്റുന്നു. അസഫേറ്റ് ഉപയോഗിച്ച് ജീവികളിൽ ഏകദേശം 2031 ജീനുകളുടെ അതിക നിയന്ത്രണവും,

552 ജീനുകളുടെ ക്രമാതീതമായ കുറവും രേഖപ്പെടുത്തി. അതേസമയം ക്ലോറാൻഡ്രുനിലിപ്രോൾ പ്രയോഗിച്ചവയിൽ യഥാക്രമം 1369 ജീനുകളുടെ കൂടുതലും 773 ജീനുകളുടെ കുറവും കണ്ടെത്തി. ഞങ്ങളുടെ കണ്ടെത്തലുകൾ അനുസരിച്ച് ഉപകാരമായ അളവിലുള്ള അസഫേറ്റും ക്ലോറാൻഡ്രുനിലിപ്രോലും p450s, ESTകൾ, GSTകൾ, UGTകൾ എബിസി ട്രാൻസ്പോർട്ടറുകൾ എന്നിവയുടെ ജീനുകളുടെ പ്രവർത്തനത്തെ കൂട്ടുകയോ കുറക്കുകയോ ചെയ്യുന്നതിന് കാരണമാകുന്നു.

പഴയീച്ചയിലെ ചിറകുകളിൽ അസഫേറ്റും ക്ലോറാൻഡ്രുനിലിപ്രോലും മൂലമുണ്ടാകുന്ന രൂപമാറ്റങ്ങളും ചിറകുകളുടെ വികാസത്തിന് ഉത്തരവാദികളായ ജീനുകളുടെ ട്രാൻസ്ക്രിപ്റ്റോമിക് പഠനവും മൂന്നാം അധ്യായത്തിൽ ഉൾപ്പെടുത്തിയിരിക്കുന്നു. ജ്യോമട്രിക്ക് മോർഫോമെട്രിക് (GMM) ടൂൾ ഉപയോഗിച്ച് ചിറകിന്റെ വലിപ്പവും ആകൃതിയിലുള്ള മാറ്റങ്ങളും പഠന വിധേയമാക്കി. കീടനാശിനികൾ ഉപയോഗിച്ച് പ്രാണികളിലെ ചിറകിന്റെ ആകൃതി, അവയിലുപയോഗിച്ച കീടനാശിനിയുടെ അളവിനെ ആശ്രയിച്ച് മാറ്റങ്ങൾ ഉണ്ടായിട്ടുണ്ട്. ഈ മാറ്റങ്ങൾ ലിംഗ ഭേദമന്യേ കാണപ്പെടുന്നു. ഈ കാര്യങ്ങൾ വ്യത്യസ്ത മൾട്ടിവാരിയേറ്റ് വിശകലനം വഴി സാധൂകരിക്കപ്പെട്ടു. രണ്ട് കീടനാശിനികൾ പരസ്പരം താരതമ്യം ചെയ്യുമ്പോൾ കാണുന്ന ചിറകിന്റെ ആകൃതി വ്യത്യാസം തികച്ചും അതാത് കീടനാശികളുടെ പ്രവർത്തന രീതിയിലുണ്ടാവുന്ന വ്യത്യാസം കാരണമാണ്. ഈ ആകൃതി വ്യത്യാസം പ്രാണികളുടെ ചിറകിന്റെ സുഗമമായ പ്രവർത്തനത്തിന് തടസ്സം സൃഷ്ടിക്കുന്നു. കീടനാശിനികൾ ഉപയോഗിച്ച പ്രാണികളിൽ പ്രോക്സിമൽ (Proximal) ഡിസ്റ്റൽ (Distal) ഭാഗങ്ങളുടെ വേർതിരിവ് കാണാൻ സാധിച്ചു. ഇത്തരം വേർതിരിവുകൾ സാധാരണ ചിറകിന്റെ ധർമ്മത്തെ കാര്യമായി ബാധിക്കാനിടയുണ്ട്. ട്രാൻസ്ക്രിപ്റ്റോം വിശകലനത്തിലൂടെ ചിറകുകളുടെ വികസനത്തിനും, വികാസത്തിനും ആകൃതിക്കും കാരണമാകുന്ന പല ജീനുകളുടെയും അസ്വാഭാവികത പ്രകടമാക്കി.

രണ്ട് കീടനാശിനികളുടെയും ഉപമാരക അളവ് പഴയീച്ചയുടെ സാധാരണ ജീവിതചക്രത്തെ മാറ്റിമറിച്ചതായി കണ്ടെത്തി. ടാർജറ്റ ചെയ്യാത്ത പ്രാണികളുടെ പ്രതിരോധ ശേഷി ജീനുകൾ, ഹീറ്റ്ഷോക്ക് പ്രോട്ടീനുകൾ, പ്രതിരോധ ജീനുകൾ എന്നിവയിലും മാറ്റങ്ങൾ വരുത്തുന്നു. ഇതിലൂടെ അസഫേറ്റിന്റെയും ക്ലോറാൻ ഡ്രുനിലിപ്രോലിന്റെയും ഉപമാരക അളവ് ലക്ഷ്യം വെയ്ക്കാത്ത ജീവികളുടെ രോഗപ്രതിരോധ ശേഷിയെയും പ്രതിരോധത്തെയും സാരമായി ബാധിക്കുമെന്ന് ഞങ്ങൾ കണ്ടെത്തിയിരിക്കുന്നു. ട്രാൻസ്ക്രിപ്റ്റോമിക്സ്, GMM എന്നിവയുടെ കൂട്ടായ വിശകലനം കീടനാശിനി സുരക്ഷാ വിലയിരുത്തൽ പ്രക്രിയയെ സുഗമമാക്കാൻ ഗവേഷകരെ പ്രാപ്തരാക്കുന്നു. ഇതുവഴി ഗവേഷകർക്ക് കീടങ്ങൾക്ക് എതിരെ പ്രവർത്തനക്ഷമത കൂടിയതും എന്നാൽ ലക്ഷ്യം വെയ്ക്കാത്ത ജീവികളിൽ കുറഞ്ഞ പ്രവർത്തനമുള്ളതുമായ കീടനാശിനികൾ കണ്ടെത്താൻ സഹായിക്കുന്നു. ലക്ഷ്യം വെയ്ക്കാത്ത ജീവികളെ സംരക്ഷിക്കുന്നതിന് പ്രാപ്തമാക്കുന്ന തരത്തിലുള്ള ശാസ്ത്രീയ അറിവുകൾക്ക് പ്രോട്ടിയോമിക്സ്, മെറ്റബോളോമിക്സ് തുടങ്ങിയ വിപുലമായ പഠനങ്ങൾ ആവശ്യമാണ്.

**സൂചകപദങ്ങൾ:** അസിഫേറ്റ്, ക്ലോറന്ത്രാനിപ്രോൾ, കീടനാശിനികൾ, നോൺ-ടാഗേറ്റഡ് ഓർഗാനിസം, ട്രാൻസ്ക്രിപ്റ്റോമിക്സ്, ജ്യാമിതീയ മോർഫോണട്രിക്സ്, ഡ്രോസോഫില മെലനോഗാസ്റ്റർ.

# ABBREVIATIONS

| | | |
|---|---|---|
| LC50 | : | Letahal Concentration |
| AchE | : | Acetyl Cholinestrase |
| ANOVA | : | Analysis of Variance |
| CVA | : | Canonical Variate Analysis |
| DAPI | : | 4'6-Diaminido- 2-Phynylindole |
| DFA | : | Discriminant Function Analysis |
| EC50 | : | Effective Concentration |
| GO | : | Gene Ontology |
| GST | : | Glutathaione-S-Transferace |
| HSP | : | Heat Shock Protein |
| KEGG | : | Kyoto Encyclopedia of Genes and Gnomes |
| NGS | : | Next Generation Sequencing |
| PBT | : | Phosphate Buffered Saline |
| PCA | : | Pricipal Component Analysi |
| PLS | : | Partial Least Square |
| RyR | : | Rynodine Receptor |
| SNP | : | Single Nucleotide Polymorphism |
| SOD | : | Super Oxide Dismutase |

# CONTENTS

# General Introduction

# GENERAL INTRODUCTION

A drastic increase in the global population has put pressure on the agricultural system, leading to increased food production (Saravi & Shokrzadeh, 2011; Yadav et al., 2020). To meet the ever-growing demand for food, the agricultural sector largely depends on agrochemicals like herbicides, fungicides, and nematicides, and these chemicals control pest populations and increase food yields (Vandergragt et al., 2020). Tons of synthetic pesticides are applied to fields to increase yields. Approximately 2 million tons of pesticides are ingested globally every year for crop protection (Alavanja, 2009; De et al., 2014). This process has deterrent effects on non-target organisms because 98% of sprayed pesticides directly or indirectly affect them (Alavanja et al., 2001). Various studies have shown that 80% of the pollutants directly pollute the environment (Gill & Garg, 2014). Likewise, pesticide application on plants contaminates the soil, surface, and groundwater by leaching down pesticide residues from the soil (Damalas & Eleftherohorinos, 2011). Pesticides, heavy metals, and other toxic substances interfere with the normal functioning of various species, including humans and aquatic organisms.

Today, pesticides have entered food chains and are prone to bioaccumulate at higher trophic levels (Mostafalou & Abdollahi, 2013). Many pesticides pose a severe threat to non-target and non-pest organisms (Venter et al., 2006). Many of them are heavily toxic to exposed insects (Storkey et al., 2012), birds (Mitra et al., 2011), mammals (Chambers, 2019), amphibians (Brühl et al., 2013) and fish (Wu et al., 2013). Beneficial insects, such as pollinators, predators, and parasitoids, are often compromised by the sub-lethal effects of pesticides, which are frequently overlooked (Connolly, 2013). Pesticide contamination results in considerable declines in wild and managed pollinators, denoted depletion in crop productivity and honey yields (Berenbaum, 2016). Exposure to agrochemicals is a significant threat to human health. Pesticide toxicities are exhibited in a variety of ways, ranging from mild illness, like slight skin irritation or allergic conditions, to severe symptoms, like

solid headache, dizziness, or nausea (Alewu & Nosiri, 2011). Chronic exposures can cause human abnormalities, ranging from cancer to other significant diseases (Srivastava & Kesavachandran, 2019).

However, pesticides are a necessary component of contemporary farming methods and are often applied to manage pests to increase agricultural yield. Pesticides are introduced into the environment to prevent, control, discourage, and lower the number of insects, weeds, rodents, fungi, and other undesirable pests and toxic materials of biological or chemical origin. The term "pests" refers to species (plants, animals, and bacteria) that have a detrimental effect on food, human health, comfort, and the economy (Soliman et al., 2015). Pesticides are considered practical tools in agriculture for controlling pests, minimising harm to crop plants, and minimising losses during harvest (Rembiałkowska, 2007; De Castro et al., 2020). Pesticides can be classified as destructive, repellent, or mitigating agents and categorised based on the target organism (Brethour & Weersink, 2001). Depending on their physiological effects, they can also be classified based on their modes of action, such as targeting animals, plants, or microbes (Montesinos & Bardaji, 2008). The proper application of pesticides strikes a balance between minimising adverse effects on the environment, particularly on non-target creatures, and obtaining high economic and aesthetic returns on agricultural goods (Osteen & Fernandez-Cornejo, 2013; Sánchez-Bayo et al., 2002; Desneux et al., 2007). Pesticides are regularly used for pest control in agriculture. Therefore, the hazards of using these chemicals outweigh their benefits, and residues of these substances are frequently detected in food products and the environment. The non-selectivity of pesticides causes harmful effects on non-target organisms (Dudley et al., 2017).

Pesticides are grouped into several categories based on their chemical nature: organochlorines, organophosphates, carbamates, pyrethroids, and anthranilic diamides. Organophosphates form a wide range of pesticides, discovered in the 19$^{th}$ century but only used widely in the 1930s. German scientist Gerhard Schrader synthesised many organophosphates in the market, of which parathion is the most prominent and is still used as a common pesticide (Costa, 2006). During the Second

World War, organophosphate compounds like Sarin, Soman and tabun were used as nerve warfare agents. Today, organophosphorous compounds are generally used to shield crops, livestock, and human health. Sadly, due to global usage, many people are constantly exposed to low-dose organophosphates. As mentioned by the World Health Organisation, each year, 3 million people experience acute organophosphate poisoning through contaminated food, water, and by breathing polluted air (WHO, 2001). Chemically, Organophosphates are phosphoric acid derivatives and are considered one of the broad-spectrum pesticides, comprising a diverse group of chemicals (Gámiz-Gracia et al., 2005). These chemicals control a wide range of pests, weeds, and plant diseases due to their varied functions. They act as acetylcholinesterase (AChE) inhibitors, interfering with neurotransmitters across a synapse (Bora et al., 2012). Consequently, nervous impulses fail to pass across the synapse, causing a quick contraction of voluntary muscles and paralysis, which leads to death. Some widely used organophosphorus insecticides include parathion, malathion, dichlorvos, diazinon, acephate and glyphosate. The organophosphate compounds are characterised by a covalent bonding of carbon to phosphate bond (C-P) substituted with one of its four carbon to oxygen to phosphorus bonds of phosphate ester (Wanner & Metcalf, 1992). The C-P bond is defined as chemically and thermally inert, making these compounds resistant to extreme conditions such as chemical hydrolysis, photolysis, and thermal decomposition. Organophosphate pesticides have significantly enhanced agricultural productivity and increased fruitful crop yields (Bolognesi, 2003). Organophosphates are reaching to non-targets through contaminated air, water and soil. The discharge of organophosphates in agricultural fields results in the contamination of crops, fruits, and vegetables, which affects herbivores and humans throughout the food chain. Organophosphates pollute water broadly, influencing fish and other non-target mammals. By inhaling contaminated air, organophosphates can reach livestock, birds, and humans. Additionally, residues from the meat of herbivores and dairy products may also be ingested by humans (Manna, 2021).

Acephate is a major organophosphate pesticide commonly used in India, the United States, China, France, and Japan. It is generally used against insects with sucking and chewing mouthparts, such as aphids, thrips, sawflies, fire ants, and lepidopteran larvae (Das et al., 2008). It is generally applied in agricultural fields, such as those for potatoes, tomatoes, lettuce, carrots, and capsicum, to enrich yields and the economy. Acephate also inhibits AChE activity in synapses, leading to tremors, paralysis, and ultimately, the death of target organisms. Surprisingly, acephate may undergo hydrolysis and cleavage on its amide linkage, converting it into a more toxic compound, Methamidophos (Chang et al., 2009). Methamidophos is a class I toxic compound and is more toxic and harmful than acephate. Hence, Acephate and its hydrolysed products may be toxic for organisms other than target pests.

Acephate is a systemic pesticide, so it can readily be taken up from polluted water in the soil through the roots and its residues deposited in various parts, mainly the leaves of the plants (Roberts & Hutson, 1999). The residual proportions of acephate in plants have been reported in previous studies. In previous studies, acephate residues were reported in tomatoes (Trevizan et al., 2005), mango pulp (Mohapatra et al., 2011), and Chinese eggplants (Dasika et al., 2012). However, due to the persistence of acephate in vegetables and fruits, non-target users, including humans, are at high risk of susceptibility to this chemical. Even though acephate is a potent anti-cholinesterase action in insects and mammals (Spassova et al., 2000), other toxicity reports are also available. Acephate promotes DNA destruction in leucocytes of Swiss albino mice (Rahman et al., 2002). It has been reported to be a potent mutagen, as it was found to enhance chromosomal damage and micronuclei formation in human peripheral lymphocytes and bone marrow cells of chicks (Özkan et al., 2009). Moreover, acephate induces infertility in humans by badly affecting the motility, capacitation, vitality, and functional integrity of the plasma membrane and DNA of sperm (Dhanushka & Peiris, 2017). This also increases oxidative stress in

rats by affecting the functions of antioxidant enzymes such as SOD, Catalase and Glutathione peroxidase (Datta et al., 2010).

Anthranilic diamide insecticides have been discovered as one of the most promising new classes of insecticides due to their potent insecticidal efficacy and high margins of mammalian safety. Diamide insecticides are remarkably effective against a wide range of pests in insect orders of Lepidoptera, Diptera, Coleoptera, Hemiptera, and Isoptera. Diamide insecticides target the ryanodine receptors (RyR) by binding to this receptor, activating calcium channels existing on RyR and resulting in the extra release of calcium ions from muscle cells. This action eventually leads to uncontrolled muscle contraction, paralysis, and death (Cordova et al., 2006). Since the introduction of chlorantraniliprole as an insecticide in 2007, anthranilic diamides have rapidly garnered global attention due to their high efficacy, low mammalian toxicity, and unique mode of action. Anthranilic diamides substitute for neonicotinoid and pyrethroid pesticides in pest control for selected vegetable plants, due to their reduced risk of non-target effects and environmental concerns (Uesugi et al., 2021). With their broad application and inappropriate use, these insecticides have a considerable impact on plant development and non-target organisms. Various reports summarised that the diamide insecticides (flubendiamide) severely damaged the honey bees' antennal neurons by affecting calcium homeostasis (Kadala et al., 2020). Chlorantraniliprole and cyantraniliprole displayed decreased growth rate, weight, and reproduction of earthworms by increasing the level of reactive oxygen species (ROS), malondialdehyde (MDA) level (Liu et al., 2018). Increased levels of detoxification enzymes were reported in the acute and chronic toxicity of three diamides (flubendiamide, chlorantraniliprole, and cyantraniliprole) to *Daphnia magna* (Cui et al., 2017).

Chlorantraniliprole is a novel anthranilic diamide insecticide discovered by DuPont Ltd. It is generally used in rice, coffee, sugar cane, apple and peach crops and acts by activating the ryanodine receptor and damaging calcium balance in the cell (Lahm et al., 2007). Chlorantraniliprole terminates Lepidoptera insects (Temple

et al., 2009), as well as other pest orders like Coleoptera, Diptera, Isoptera and Hemiptera (Hannig et al., 2009). Previous studies discussed the effects of chlorantraniliprole in different insect species: *Choristoneura ro-saceana* (Sial et al., 2011), termites *Reticulitermes flavipes* (Spomer et al., 2009), honey bees *Apis mellifera* and bumble bees *Bombus terrestris* (Dinter et al., 2010) and *Leptinotarsa decemlineata* (Jiang, 2012). Another study demonstrated the efficacy of chlorantraniliprole against major stored-product insect species (Kavallieratos et al., 2013). Although chlorantraniliprole exhibits less toxicity to non-target organisms, some reports regarding its non-target effects are available. Chlorantraniliprole was acutely toxic to honey bees after exposure to 4- or 72-hour treatments at different concentrations (Williams et al., 2020).

The above information highlights the need for a study on the non-target effects of acephate and chlorantraniliprole on organisms. Using *D. melanogaster* as a model organism, we can examine the xenobiotic-induced effects of acephate and chlorantraniliprole. *D. melanogaster* is a holometabolous insect that ends its life cycle within two weeks at (25 ± 1) °C, starting from egg to adults (Ashburner & Thompson, 1978). Approximately 400 eggs are laid by a single female fly on fruits or decaying materials (Ashburner et al., 2005). *D. melanogaster* is commonly used as a model organism in genetics, biochemistry, cell biology, and developmental biology. Over the last few decades, it has been selected as a model to examine human diseases, primarily for toxicological studies (Rand, 2010; Singh et al., 2011; Sharma et al., 2012; Rand et al., 2012; Paula et al., 2012; Sudatti et al., 2013). The *D. melanogaster* provides excellent benefits as a model for biological studies. Their small size is suitable for mass culture in large numbers. Flies have fewer ethical concerns compared to vertebrate models and are easier to handle. The genome of *D. melanogaster* can be easily exploited to understand the functional features of genes. Moreover, they share prominent biochemical, behavioural, physiological and developmental similarities with the higher vertebrates, including humans. Approximately 75% of human disease-affecting genes have homologues in

*Drosophila* (Adams et al., 2000). The most common neurodegenerative diseases, like Alzheimer's disease and Parkinson's disease, have been successfully modelled in *Drosophila*. Due to the presence of insulin/insulin-like growth factor-I (IIS) signalling pathway in *Drosophila* (Brogiolo et al., 2001), Common human metabolic disorders such as diabetes and obesity are also studied in *Drosophila* (Ram & KarChowdhuri, 2014).

The toxicity of several xenobiotics and nanoparticles can be assessed in the embryonic, larval, pupal, and adult developmental stages of *D. melanogaster* (Hsu & Schulz, 2000). The behaviours of the larvae (foraging, chemotaxis, and phototaxis) and of adults (flight, chemotaxis, phototaxis, geotaxis, courtship and mating, aggression, and grooming) in response to chemicals can be examined in *D. melanogaster*. Numerous reports have documented the effects of pesticides and heavy metals on *D. melanogaster*. Researchers have observed a cadmium-induced decrease in egg fertility in Drosophila (Gelegen & Yesilada, 2000), and an organophosphate compound-activated stress gene activity has also been shown to inhibit acetylcholine esterase activity in the fruit fly model (Gupta et al., 2005). Tissue destruction and increased stress gene expression in the reproductive organs of fruit flies are caused by Cypermethrin (Karatas & Bahceci, 2008). Sub-lethal concentrations of endosulfan induced life-stage responses, such as delay in hatching, inhibition of pupation onset, and emergence failure, in *D. melanogaster* (Shameema et al., 2022). Hence, *Drosophila melanogaster* is a favourable model for toxicological examinations of chemicals and environmental pollutants. Therefore, the present study focuses on the comparative toxicity of acephate and chlorantraniliprole on the non-target model organism *D. melanogaster*. The study aims to demonstrate lifecycle alterations and wing morphological examinations, with a focus on whole-genome analysis.

**OBJECTIVES OF THE STUDY:**

- Evaluation of acute toxicity of cholrantraniliprole and acephate and dose-dependent effect on a model organism, *Drosophila melanogaster*.
- Differential expressions of genes in response to sublethal concentration of cholrantraniliprole and acephate using Transcriptomic analysis.
- Quantitative analysis of variation in wing shape using geometric morphometrics.

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# Chapter 1

# Comparative toxicity evaluation of Acephate and Chlorantraniliprole on *Drosophila melanogaster*

## 1.1 INTRODUCTION

Pesticides are chemical preparations used to prevent, destroy, repel, or mitigate pests. This includes insecticides, herbicides, fungicides, rodenticides, and more**.** While pesticides aim to manage or eradicate pests, their application may have unintended effects on non-target organisms, including plants, animals, humans, and beneficial insects. First, it is necessary to ascertain the impacts of pesticides on mortality to comprehend the hazardous effects of pesticides on any organism. One such metric, the median lethal concentration ($LC_{50}$), gives information on the concentration at which 50% of exposed or treated individuals die in a population.

Similarly, effective concentration ($EC_{50}$) represents the concentration of a substance required to produce a specific effect in 50% of the organisms or cells being studied. $EC_{50}$ is frequently used in toxicology to quantify a drug's potency or a substance's effectiveness in eliciting a biological response. It is often used in dose-response curves to determine the concentration at which a drug or compound produces half its maximum effect. These parameters are necessary for the toxicological evaluation of any chemical in animal models.

Acephate is a broad-spectrum organophosphate insecticide used in forestry and agriculture to eliminate insect pests. Acephate is a systemic insecticide that acts through both contact and stomach action (Tomlin, 1997). In soil, plants and insects convert acephate to methamidophos. It is another organophosphate insecticide that acts as an Acetylcholinesterase inhibitor (Rahman et al., 2002). Compared to acephate, methamidophos is more toxic to invertebrates and vertebrates. According to the US EPA (2011), acephate and methamidophos are highly toxic to bees and other beneficial insects, moderately toxic to birds, and non-toxic to Amphibians.

Reports are available that document the $LC_{50}$/ $LD_{50}$ of acephate and chlorantraniliprole on non-target species. The $LC_{50}$ of Acephate for bees is 1.2 µg/bee. Several fish species had 96-hour lower limit of detection ($LC_{50}$) values that were reported. These included rainbow trout (>1,000 mg/L), blue gill fish (2,050 mg/L), largemouth black bass (1,725 mg/L), channel catfish (2,230 mg/L), and goldfish (9550 mg/L) (Fisher et al, 1991). Technical grade acephate has an acute

oral $LD_{50}$ of 866 mg/kg in female rats, 945 mg/kg in male rats, 361 mg/kg in mice, 350 mg/kg in mallard ducks, 852 mg/kg in chickens, and 140 mg/kg body weight in ring neck pheasants (Worthing, 1987).

Chlorantraniliprole is an insecticide with a unique mechanism of action that targets ryanodine receptors (RyR) by binding to the receptor and activating the calcium channels associated with RyR, which releases a large amount of calcium ions from muscle cells. Diamide pesticides are highly effective against various pests from the Hemiptera, Isoptera, Diptera, Coleoptera, and Lepidoptera insect orders (Uesugi *et al.,* 2021). The reported $EC_{50}$ values for several species of larvae about chlorantraniliprole were 0.02 ppm for *Spodoptera frugiperda* (fall armyworm), 0.01 ppm for *Plutella xylostella*, and 0.05 ppm for *Heliothis virescens* (tobacco budworm) (Lahm et al*.,* 2007). Technical-grade chlorantraniliprole has an acute oral $LD_{50}$ of 0.119 mg/bee in *Apis mellifera,* 2250 mg/kg in *Colinus virginianus* (Northern Bobwhite), and 5,000 mg/kg in rats**.** Technical-grade Chlorantraniliprole with an $LD_{50}$ of 951 ug/L (95% CL = 741-1118 ug/L) is highly dangerous to crayfish, according to aquatic 96-hour median lethal toxicity ($LD_{50}$) data (Barbee et al., 2010).

*D. melanogaster* is a commonly used experimental animal due to its short generation period, ease of genetic manipulation, and ease of culture in laboratory settings. *D. melanogaster* is usually bred in a lab at 25°C on a maise meal yeast mixture. This creature goes through three larval stages, a pupal stage, and an adult stage, all of which happen in less than two weeks at a temperature of (25 ± 1°C) (Ashburner & Thompson, 1978). Fruit fly growth times vary according to changes in ambient temperature since they are sensitive to it. Females deposit 0.5 mm long eggs on over-ripe fruits in the wild, and the eggs hatch at 25°C after 20 to 22 hours. Within approximately 24 to 48 hours, the resultant larvae undergo two moults to develop into their second and third instars. Following its encapsulation, the larva goes through a four-day metamorphosis at 25°C in the puparium, where it remains immobile (Ashburner et al., 2005; Ram & Chowdhuri, 2014**).** Finally, a fly emerges from the puparium through a process known as eclosion. It is recognised that several outside variables affect the life cycle in different ways. At room

temperature (18°C), the life cycle takes 19 days; however**,** under heat stress (approximately 30°C), it is reduced to 11 days (Ashburner et al., 2005). It takes longer for development in crowded conditions (Chiang & Hodson, 1950). Similarly, other researchers have discovered a chemically induced delay in adult *Drosophila* emergence (Das et al., 2010; Rajak et al., 2013). Sublethal quantities of nitenpyram dramatically reduced *D. melanogaster's* longevity, pupation rate, eclosion rate, and egg output. Furthermore, following exposure, there was a noticeable increase in the mRNA expression of genes essential for metabolism and development (Ahmed et al*.,* 2022). Several variables, including diet, health, daily activities, and exposure to chemical or physical stress, determine the lifespan of both invertebrates and vertebrates. Pesticide exposure significantly alters the total life span of any organism (Wu et al*.,* 2011).

Early toxicity responses are evident from embryonic development and egg morphometrics. The egg is the first level of pesticide exposure, so measuring changes in the length and width of the egg provides a clear picture of quantitative morphometrics. In a subsequent investigation, those researchers reported that elevated chlorpyrifos levels significantly reduce the egg-hatching rate in *D. melanogaster***,** increasing embryonic mortality. At far lower quantities, no harmful effects were seen (Nazir et al., 2001). The results of yet another study showed a definite decline in egg fertility in those who had ever been exposed to cadmium (Gelegen & Yesilada**,** 2000).

To find the effective concentration that causes 50% eclosion inhibition and to investigate the effects of harmful chemical concentrations below the fatal threshold, toxicity assays were conducted in this work. The hatchability percentage, pupation rate, emergence pattern, body weight, and survival rate of the experimental organism *D. melanogaster* were all calculated to study developmental changes. We investigated how the hatchability and embryo development of *D. melanogaster* were affected by sublethal doses of acephate and chlorantraniliprole. Furthermore, we evaluated the alterations in gene expression linked to metabolism and development. With the help of transcriptome analysis, a potent tool in contemporary genomics, we can investigate how genes dynamically express themselves in response to pesticide

exposure. Understanding how pesticides modify gene expression at the transcriptome level will help identify essential pathways and processes of these toxic substances, as well as the genes involved in each life cycle event of *D. melanogaster*.

## 1.2 REVIEW OF LITERATURE

Acephate, also known as O, S-dimethyl acetyl phosphoramidothioate, is a commonly used insecticide for controlling pests in homes and crops (Debnath et al*.,* 2019). It is authorised in buildings, establishments, public health facilities, ornamental plants, food crops, and agricultural seeds (Debnath et al*.,* 2019; Kumar et al*.,* 2015). It is a popular insecticide for controlling chewing and sucking insects, and one of the most effective in managing aphid and thrips populations together (Lewis et al., 2016). Acephate is an insecticide that inhibits acetylcholinesterase, resulting in the accumulation of neurotransmitters in nervous system tissues and blood. This leads to muscle weakness, miosis, and fasciculation, which can potentially result in death (Colovic et al., 2013; Tsai & Lein, 2021). Acephate, a moderately hazardous class II pesticide, is restricted in several countries due to its tendency to degrade into the toxic metabolite methamidophos, which is classified as highly hazardous class IB (Chowdhary et al., 2014; Takayasu et al., 2019). The half-life of Acephate in soil, groundwater, and plants is three to six days, indicating average persistence. However, due to variations in soil qualities, this stability can occasionally be significantly improved (Pinjari et al., 2012; Kumar et al., 2015). Furthermore, acephate has a low rate of soil absorption, which raises the possibility of contaminating groundwater and aquatic environments (chai et al., 2010; Wang et al., 2019). Acephate poisoning is associated with bioactivation through metabolic conversion to methamidophos, which inhibits the nervous system's acetylcholinesterase enzyme (Yao et al*.,* 2018). Both target and non-target animals, such as microorganisms (Wu et al*.,* 2021), insects (Rajak et al., 2018), fish (Liu et al., 2018), birds (Vyas et al*.,* 1996), mammals (Jain & Vashishat, 2023), and aquatic invertebrates (Moulton et al., 1996), exhibit cytotoxic effects.

Different studies are conducted worldwide to test the toxicities of acephate in mammals, humans, invertebrates, and insects. Human exposure to acephate has detrimental consequences on metabolism, including hyperglycemia, elevated oxidative stress, dysfunctional lipid metabolism, DNA damage, and an increased risk of cancer (Du et al., 2014). Acephate is probably going to have cytotoxic and genotoxic effects on men's sperm, causing issues with sperm volume, motility, and cell membrane disorders. Respiratory depression, quadriplegia, or even death may result from prolonged exposure to Acephate (Beavers et al*.,* 2014). Humans exposed to Acephate over an extended period develop hyperglycemia, DNA damage, lipid metabolic disorders, an increased risk of cancer, and chromosomal abnormalities (Ozkan et al*.,* 2009).

Honeybees exposed to acephate experience weight loss and enzyme inhibition (Yao et al., 2018). When earthworms encounter acephate-containing soil, they also undergo oxidative stress, as indicated by changes in the state of antioxidant enzymes, lipid peroxidation, protein oxidation, and DNA damage (Phugare et al*.,* 2012). Acephate significantly reduces the antioxidant capacity of the liver and kidneys in birds, leading to increased lipid peroxidation, interleukin, and tumour necrosis factor levels in both organs, and affecting the immune system (Tripathi et al., 2012). In pregnant and nursing rats, acephate changes glucose metabolism and predisposes the offspring to adult-onset type 2 diabetes (Gill et al*.,* 2011). Overuse of methamidophos and Acephate can cause cancer, reproductive barriers, DNA damage, elevated blood sugar, and poor metabolism in rats (Maia et al., 2012; Araoud et al., 2016; Ribeiro et al*.,* 2016). In mice, exposure to Acephate may cause metabolic abnormalities. In contrast, in rats, alterations in specific endogenous metabolites lead to renal damage and disrupt normal metabolic processes, including glucose metabolism, nucleic acid metabolism, and protein metabolism (Hao et al*.,* 2012). Behera et al. (1989) evaluated the genotoxic potential of acephate *in vivo* in mice. Results showed a significant increase in chromosome aberrations, micronuclei, sperm shape abnormalities, and dominant lethal tests. The highest dose resulted in significant differences, suggesting that acetate may be a potential mutagen.

Rajak *et al.* (2014) investigated the impact of the organophosphate insecticide acephate on hemocyte abundance in Drosophila *melanogaster* larvae. Results showed a decrease in plasmatocyte and lamellocyte populations with increasing chemical concentrations**.** However, the number of crystal cells increased with concentration. Factors such as oxidative stress, induction of apoptosis, and mitotic failure may cause a reduced count. High melanin synthesis helps larvae combat chemical stress. Exposure to Acephate may cause ROS-mediated harmful reactions in addition to the conventional cholinesterase inhibition. According to research by Rajak et al. (2018), *D. melanogaster* can sustain damage from ROS-mediated oxidative stress that impairs its ability to move, maintain neuromuscular coordination, and engage in physical activity. In addition to lower brain and fat cell viability, the larval intestine displayed tissue damage. Increased cytochrome P450 (CYP450) and glutathione S-transferase (GST) activities were also observed in the study, suggesting that Drosophila may have hazardous reactions mediated by reactive oxygen species (ROS). At concentrations greater than 5 g/mL, Acephate contamination can damage DNA and kill fruit flies. It can also disrupt the equilibrium of antioxidant and oxidase enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), CYP450, acetylcholinesterase (AChE), and other enzymes (Rajak et al*.,* 2017). Another team examined the impact of chronic exposure to the pesticide Acephate on the male reproductive system of *D. melanogaster*. The results show altered testis structure, decreased germ cell viability, and increased activities of oxidative stress markers. The study also reveals altered expression of reproductive marker proteins, confirming the adverse effect of chronic Acephate exposure on male reproduction (Mandi et al., 2020).

In a lab environment, Jamal (2015) investigated the harmful effects of acephate on *Dysdercus cingulatus* fifth instar nymphs. Results showed that nymphal loss, survival duration, incomplete moulting, and number of malformed adults increased linearly with increasing concentrations. However, fecundity and fertility declined linearly with increasing concentrations. The ovaries of affected females showed anomalies, including reduced size, decreased mature oocytes, and abnormal

development and maturation of oocytes in the vitellarium. Rogers & Howell, in 1973, tested the toxicity of acephate and diazinon to harlequin bugs (*Murgantia histrionica*) on cabbage. In terms of lowering the adult population of *M. histrionica*, acephate was more successful than diazinon. Compared to 53–65% for diazinon, all acephate treatments resulted in reductions of >99.0% (Rogers & Howell, 1973).

A study found that acephate and methamidophos had a significant impact on dark-eyed juncos, with brain cholinesterase activity being depressed 80% and 60% after poisoning, respectively. The $LC_{50}$ for acephate was 1485 mg/kg, with early-dead birds less depressed. The amount of Acephate needed to cause cholinesterase depression is about one-fifth of the $LD_{50}$, but in some cases, cholinesterase activity may be near lethal levels (Zinkl, 1981). The roles of antioxidant stress and antioxidant protection in acephate-induced renal tubular cytotoxicity were examined in 1998. The study suggests that reactive oxygen species (ROS) may play a role in organophosphate-induced renal tubular injury. Renal tubular cells concentrate nephrotoxic chemicals, and renal injury from these compounds arises from excessive ROS production. The study found that acephate caused a concentration- and time-dependent increase in cell damage in renal tubular epithelial cells, which was suppressed by antioxidants 2-methylaminochroman and desferrioxamine. This suggests that oxidant stress may play a role in the pathogenesis of acephate-induced acute tubular necrosis and renal dysfunction observed in cases of acephate overdoses (Poovala et al., 1998).

Liu et al. (2018) investigated the developmental and neurotoxicity of synthetic organic insecticides, deltamethrin, acephate, and thiamethoxam, on zebrafish embryos and larvae. Results showed that exposure to deltamethrin and acephate caused embryo development delay, increased mortality, decreased chorion surface tension, and morphological deformities, including shorter body length, smaller eyes, and larger head-body angles. Thiamethoxam did not show significant developmental toxicity. Deltamethrin and acephate increased behaviour activity, while thiamethoxam altered locomotor activity.

Adult bees treated with acephate at residue concentrations did not exhibit a significant increase in mortality; however, esterase activity was significantly suppressed. Bees treated with binary mixtures of acephate with six pesticides showed lower esterase activity and body weight. The Stoneville bee population exhibited higher tolerance to acephate; however, chronic toxicity was evident in body weight loss and esterase suppression (Yao et al*.,* 2018). Ferreira et al. (2023) examined the effects of typical insecticides and herbicides on stingless bees in Brazil. Acephate-based insecticides reduced bee lifespan and flight ability, while glyphosate-based herbicides were safe for forager bees under realistic concentrations. Bee mortality increased with agrochemical concentrations.

Chlorantraniliprole is a member of the anthranilic diamide insecticide class, designed to control certain Dipteran, Coleopteran, and Lepidopteran pests on both annual and perennial crops in commercial agriculture. The most efficient entry mode is ingestion, which usually requires a lower dose for response. It has been reported that chlorantraniliprole is a persistent chemical in the environment and soil, with concentrations present in surface waters ranging from 0.1 to 9.7 μg/L. It activates the insect ryanodine receptor on the sarcoplasmic reticulum of muscles, the endoplasmic reticulum of neurons, or other cells. This results in the uncontrollably released release and depletion of internal $Ca^{2+}$ reserves upon specific binding. This causes the insect to stop eating and develop muscular dysfunction, which paralyses it (Bassi et al., 2009).

Worldwide, much research has been done on the toxicity of target-specific chlorantraniliprole. Chlorantraniliprole's insecticidal efficacy against common stored-product insect pests in various grain commodities was evaluated under laboratory conditions by Kavallieratos et al. (2013). The study found that mortality rates in multiple insects were similar after exposure to chlorantraniliprole. For *Liposcelisbos trychophila* adults, mortality was lower in maize and whole rice but increased with the dose. *Ephestia kuehniella* larvae exhibited significant differences, with a slight increase in mortality at the higher dose. *Rhyzopertha dominica* adults had higher mortality rates, while *Sitophilus oryzae* adults had lower

mortality rates. *Tribolium confusum* adults exhibited low mortality rates, but mortality rates increased proportionally for all commodities. Leaf-dip bioassays of chlorantraniliprole have high toxicity against *Plutella xylostella* larvae, with $LC_{50}$ values of 0.23 and 0.25 mg $L^{-1}$ for susceptible and field strains, respectively. Its sublethal effects include reduced pupation, pupal weight, adult emergence rates, female pre-oviposition period duration, decreased fecundity, egg hatch, and offspring survival rates (Han et al., 2012). Plata-Rueda et al. (2019) conducted a study on the effects of chlorantraniliprole on Hypothenemus hampei, the leading pest of coffee crops, and found it to be toxic. The insecticide reduced mobility and respiration rate, altering behavioural responses and locomotor activity, and reduced survivorship in adults exposed to the insecticide.

Lethal and sublethal effects of chlorantraniliprole on *Spodoptera cosmioides* were investigated by Lutz et al. (2018). The *S. cosmioides*is is an emerging soybean pest in Argentina. Results show that chlorantraniliprole is active against second-instar larvae and induces changes in the life cycle of exposed larvae, requiring them to spend more time completing all stages of development. Adult fecundity is also decreased. Bacca et al. (2021) evaluated the toxic effect of Chlorantraniliprole on the early immature stages of the potato tuber moth, *Tecia solanivora*. The research employed a concentration-mortality bioassay, exposing eggs to various concentrations of insecticides. The results showed that chlorantraniliprole had a high hatching success rate and 100% larval mortality, indicating its potential for controlling *T. solanivora* in stored potato tubers. The study also suggests chlorantraniliprole as a promising alternative for controlling *T. solanivora* populations resistant to neurotoxic insecticides, offering a hopeful solution to a challenging problem. A recent study investigated the effects of chlorantraniliprole and indoxacarb on the developmental stages, detoxification enzymes, reproductive activity, calling behavior, peripheral physiology, and pheromone titer of the cabbage moth *Mamestra brassicae*. Results showed that chlorantraniliprole is more susceptible than indoxacarb, with increased

developmental time, reduced egg viability, and weaker antennal responses. Both insecticides also reduced the enzymatic activity of glutathione S-transferases, mixed-function oxidases, and carboxyl esterases (Moustafa et al., 2023).

Although chlorantraniliprole is an insecticide that targets ryanodine receptors, research has suggested that it may have non-target effects. Wu et al. (2017) investigated how soil microbial populations and their activity were affected by chlorantraniliprole. The findings demonstrated notable temporal changes in soil microbial activity over the first 14 days of chlorantraniliprole treatment. Liu et al. (2018) evaluated the ecotoxicity of chlorantraniliprole on earthworms (*Eisenia fetida*) after a 42-day exposure. Results showed that chlorantraniliprole contents decreased to no more than 20% in the soil, and the accumulation of chlorantraniliprole in earthworms was significantly reduced. However, chlorantraniliprole induced excess production of reactive oxygen species, leading to oxidative damage to biomacromolecules. The study suggests that chlorantraniliprole poses a high risk to earthworms (Liu et al., 2018).

Abbas et al. (2018) found noticeably increased percentages of external abnormalities in the embryos treated with chlorantraniliprole. Chlorantraniliprole's teratogenic effects on chick embryos were evaluated at various commercial dosages (0.1, 0.5, 1.0, and 1.5 µl/1000 µl/egg). At the 12-day developmental stage, the treated embryos exhibit noticeably higher percentages of external abnormalities than the controls, suggesting that even a very low dosage of chlorantraniliprole may harm avian embryos. According to a study conducted by Nawaz et al. (2017), further evidence is needed to demonstrate that sublethal concentrations of chlorantraniliprole prevent *H. axyridis* from proliferating. This suggests that integrated pest management (IPM) tactics should take this into consideration.

Tuelher et al. (2017) studied the effects of chlorantraniliprole on non-targeted insect pests, such as the Neotropical brown stink bug, *Euschistus heros*. The study found that chlorantraniliprole caused low mortality regardless of

developmental stage, but sublethal exposure affected the sexual fitness of stink bug couples. Treated males and females showed higher mating numbers, while untreated males and treated females had longer survival times. The study also found that chlorantraniliprole-induced hormesis may have contributed to *E. heros* outbreaks in Brazilian soybean fields. Teixeira and co-workers experimented on three species of Rhagoletis fruit flies in 2008. A tarsal contact toxicity test revealed that when male and female flies of all species were exposed to a surface residual of 500 mg $L^{-1}$ of chlorantraniliprole, their mortality was considerably higher than that of the control. There was evidence of delayed egg laying in females who had consumed chlorantraniliprole, but there were no appreciable sub-lethal effects on the quantity or hatch of eggs deposited. Munhoz et al. (2013) examined the effects of chlorantraniliprole on silkworm (*Bombyx mori*), a commercial Brazilian hybrid. Results showed that *B. mori* is highly sensitive to chlorantraniliprole, with high mortality rates at 0.2 and 0.1 ppm. Symptoms of toxicity included cessation of feeding, regurgitation, delayed development, and incomplete ecdysis, which affected nutrient uptake and cocoon production. Chronic oral exposure of chlorantraniliprole to pollen can induce lethargic behavior in *Bombus terrestris* workers and their offspring. The study found that contact and pollen exposure did not affect worker survival; however, oral exposure via sugar water caused both acute and chronic toxicity. Severe sub-lethal effects on reproduction were recorded in nests orally exposed to pollen treated with chlorantraniliprole. This study emphasises the need for adequate risk assessment of potentially deleterious compounds with neurogenic targets (Smagghe et al*.,* 2013). Previous works clarified the issue that Acephate and Chlorantraniliprole are toxic to both target and non-target insects.

## 1.3 MATERIALS AND METHODS

### 1.3.1 Experimental Organism: *Drosophila melanogaster* Meigen

We selected *Drosophila melanogaster* as a non-target organism for an acute toxicity study and life cycle alterations. *D. melanogaster* (Figure 1.1) is a tiny Dipteran insect with egg, larva, and pupal stages before adulthood. *D.*

*melanogaster's* life span is approximately 40 days at 29 °C. The egg-to-adult development time is completed in 7 days at 28 °C. The average life span of *Drosophila* depends on environmental conditions. In laboratory conditions, females live for 26 days and males for 33 days.

The taxonomic position of *D. melanogaster* is as follows.

| | | |
|---|---|---|
| Kingdom | : | Animalia |
| Phylum | : | Arthropoda |
| Class | : | Insecta |
| Order | : | Diptera |
| Family | : | Drosophilidae |
| Genus | : | *Drosophila* |
| Species | : | *melanogaster* |


**Figure.1.1** *Drosophila melanogaster* Adults (A) Male, (B) Female.

*Drosophila* flies were collected from the *Drosophila* Stock Centre of Mysore University. The culture was reared in *Drosophila* medium and maintained at a constant temperature of (25±1 ºC) in the laboratory. The *Drosophila* culture medium was prepared according to the standard protocol formulated by the *Drosophila* Centre, Mysore (Table 1.1). *Drosophila* medium was prepared using wheat powder, molasses, agar-agar, yeast powder, and distilled water. Propionic acid was added to the medium as an antifungal agent.

**Table 1.1**: Compositions of *Drosophila* culture medium.

| Sl. No. | Items | Quantity |
|---|---|---|
| 1 | Wheat flour | 75gm |
| 2 | Molasses | 75gm |
| 3 | Baker'syeast | 15gm |
| 4 | Agaragar | 15gm |
| 5 | Propionicacid | 2.5ml |
| 6 | Water | 1125ml |

### 1.3.2 Test Chemicals:

Toxicity studies were performed using technical-grade acephate and chlorantraniliprole purchased from Sigma-Aldrich Co., USA. Stock solutions were prepared (200µg/ml). Due to chlorantraniliprole's low water solubility, acetone was used as the solvent. The IR spectra of both pesticides were analysed using a JASCO FTIR-4100 spectrometer, and the structures were compared with those in the literature.

**Acephate**

Acephate **(O, S-Dimethyl acetyl phosphoramide thioate2)** is a compound named Orthene and Ortran by Chevron Chemical Co. /Ortho Div.

Chemical formula: $\mathbf{C_4H_{10}NO_3PS}$

Molecular wieght: 181.6g/mol

Water solubility: 79 g/100 mL

Chemical structure:

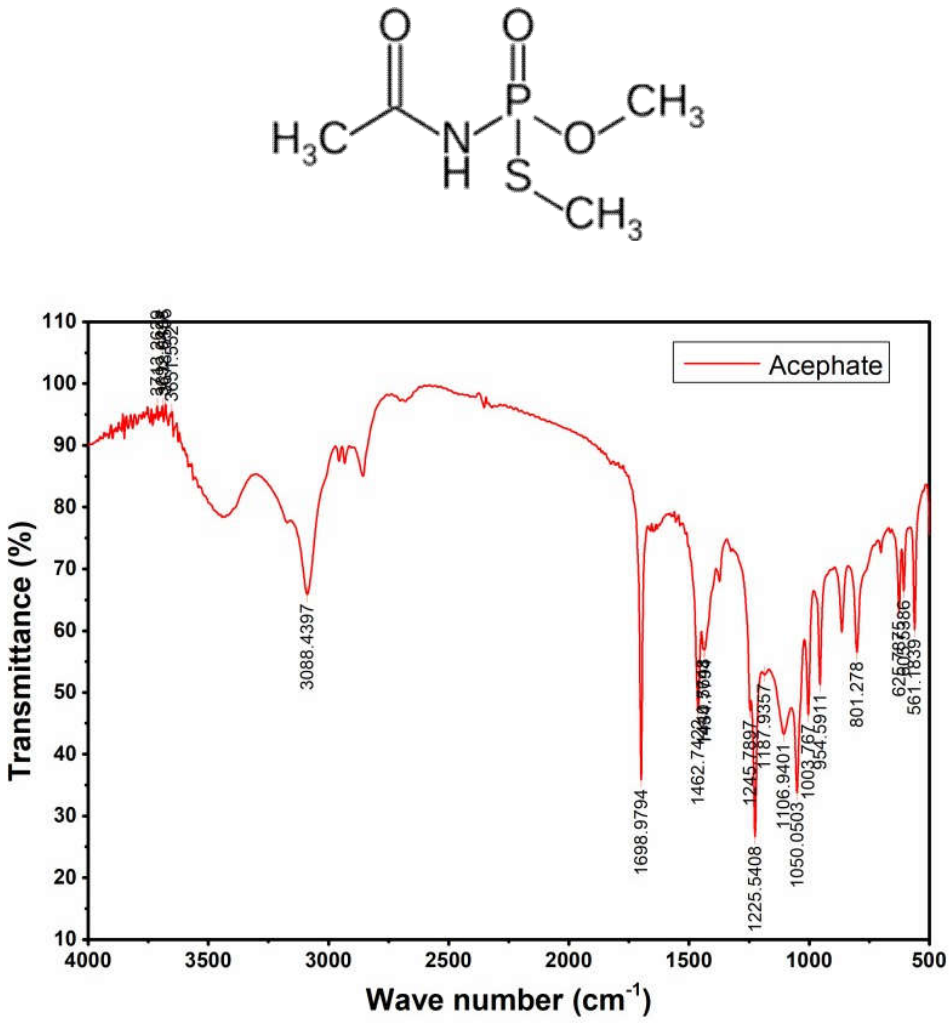



**Figure 1.2:** Chemical structure and IR spectra of Acephate

The IR spectra of acephate (Figure 1.2) were analysed and compared with the literature (Kumar et al., 2015), which showed a similar pattern. Acephate has a broadened peak in the range of 3000-3800cm$^{-1}$.

**Chlorantraniliprole**

Chlorantraniliprole **(3-Bromo-4′-chloro-1-(3-chloro-2-pyridyl)-2′-methyl-**

**6′ (methyl carbamoyl) pyrazole-5-carboxamide,** DPX-E2Y45, Rynaxypyr®, Coragen®) is a new compound by DuPont that belongs to a new class of selective insecticides called anthranilic diamides. It controls insect pests in various countries, including India, USA, Brazil, Canada, etc.

Molarmass: 483.15 g/mol

Molecular formula: $\mathbf{C_{18}H_{14}BrC_{l2}N_5O_2}$

Chemical structure:

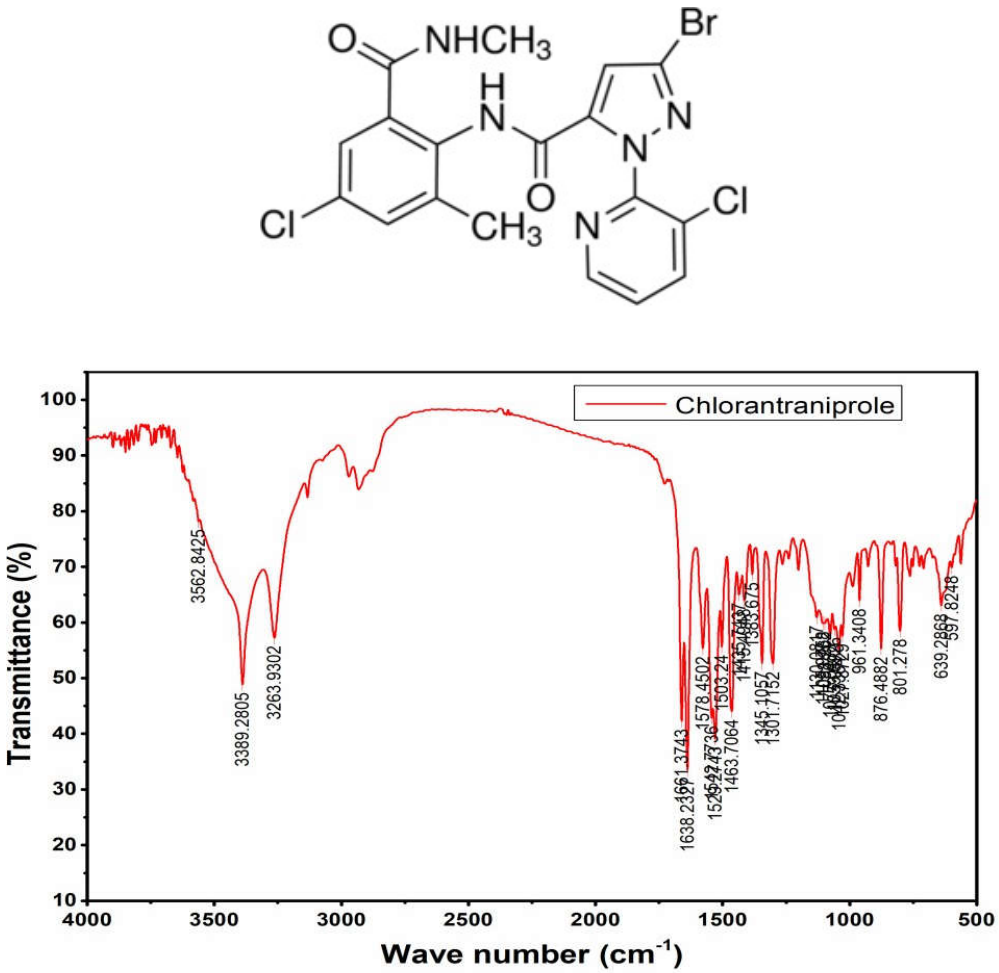



**Figure.1.3**: Chemical structure and IR spectrum of chlorantraniliprole

In the IR spectra of chlorantraniliprole (Figure 1.3), three peaks are seen in the region above 3200 $cm^{-1}$: one at 3563 $cm^{-1}$, another at 3389 $cm^{-1}$, and one at 3264 $cm^{-1}$. When we compare this with the spectrum of analytical grade, it suggests the

presence of a secondary amide group. As demonstrated in the literature (Liu et al., 2018), Chlorantraniliprole exhibits the same peak patterns for amide groups.

### 1.3.3 Range finding test

Separate range-finding tests were conducted for acephate and chlorantraniliprole. Six individual tests were arranged in parallel for acephate, and three different tests were conducted for chlorantraniliprole. Each test concentration had six replicates and thirty animals per replicate**.**

### 1.3.4 Definitive test

Two effective concentrations (50% = ***a*** and 100% = $ar^2$**)** were derived from the rigorous range-finding test, and the geometric concentrations were calculated using the formulas $a/r^2$, a/r, a, ar, and $ar^2$. Five experimental concentrations of acephate were obtained from the formulae for the definitive test. The arrived concentrations for Acephate were 0.7 µg/ml, 0.1 µg/ml, 1.4 µg/ml, 2.0 µg/ml, and 2.8 µg/ml and for chlorantraniliprole 0.0175 µg/ml, 0.025 µg/ml, 0.035 µg/ml, 0.05 µg/ml, and 0.07 µg/ml. The eclosion inhibitions were meticulously monitored and plotted on double logarithmic graph paper. The 50% eclosion inhibition ($EC_{50}$) was calculated using the Litchfield & Wilcoxon (1949) method.

### 1.3.5 Study of the changes in lifecycle parameters of *Drosophila*.

The sublethal toxicity response of chemicals was evaluated through the assessment of hatchability, pupation, emergence pattern, body weight, and survival rate of the organisms. Three concentration grades of acephate (0.19 µg/ml, 0.38 µg/ml and 0.63 µg/ml) and chlorantraniliprole (0.0029 µg/ml, 0.0058 µg/ml and 0.0096 µg/ml) were selected. The selected concentrations were far below the calculated acute $EC_{50}$ concentrations. All life cycle parameter tests were done with these concentrations. The chosen concentrations were in the $1/10^{th}$ of $EC_{50}$, $1/5^{th}$ of $EC_{50}$, and $1/3^{rd}$ of $EC_{50}$, respectively.

#### Hatchability of eggs

The healthy, uniform-sized eggs were transferred to culture medium

containing acephate (0.19 µg/ml, 0.38 µg/ml, and 0.63 µg/ml) and chlorantraniliprole (0.0029 µg/ml, 0.0058 µg/ml, and 0.0096 µg/ml), along with the control and vehicle control groups. After 24 hours, non-hatched eggs were counted, and the hatchability rate was recorded by subtracting non-hatched eggs from the total eggs exposed.

**Pupation rate**

Thirty *D. melanogaster* eggs were transferred to pesticide-treated media containing experimental concentrations of acephate (0.19 µg/ml, 0.38 µg/ml, and 0.63 µg/ml) and chlorantraniliprole (0.0029 µg/ml, 0.0058 µg/ml, and 0.0096 µg/ml) along with control and vehicle control (6 replicas). After four days of exposure, the number of pupae was recorded, and the difference between the control and treated groups was evaluated.

**Emergence pattern of *D. melanogaster***

Thirty-first instar larvae of *D. melanogaster* were transferred to a medium treated with experimental concentrations of acephate (0.19 µg/ml, 0.38 µg/ml, and 0.63 µg/ml) and chlorantraniliprole (0.0029 µg/ml, 0.0058 µg/ml and 0.0096 µg/ml) along with a control and vehicle control group (6 replicates) maintained in the standard laboratory conditions. Emergence patterns of *D. melanogaster* were evaluated using the method described by Gayathri and Krishnamurthy (1981). The number of flies emerging from each group was counted and recorded until the last fly emerged from the control. The emerging flies were removed from the vials at 24-hour intervals for further analysis.

**The adult body weight of *D. melanogaster***

Three-day-old flies from the respective experiments were taken for body weight measurement. Male flies were selected for body weight measurements. Eighty male flies (6 replicates of 30 flies in each vial) from each experiment were weighed using an electronic weighing balance (SHIMADZU, AP-224) with 0.1mg sensitivity, and weights were expressed in milligrams per 30 flies.

**Survival rate of adult *D. melanogaster***

The dose-dependent effect of Acephate on the life span of *D. melanogaster* flies was examined by recording the number of dead flies from the experimental groups. Six replicates of 30 flies from three distinct concentrations of acephate (0.19 µg/ml, 0.38 µg/ml and 0.63 µg/ml) and chlorantraniliprole (0.0029 µg/ml, 0.0058 µg/ml and 0.0096 µg/ml) along with a control and a vehicle control group were used in the experiment. The flies were transferred to the fresh vials every alternate day, and the number of dead flies was recorded until the end of the experimental period. The equation calculates survivorship probability.

$$\text{Survivors' probability} = \frac{\text{Number of flies survived from the group}}{\text{Total number of flies in the group}}$$

### 1.3.6 Embryotoxicity

Embryotoxicity is a method for measuring the early response of embryos to pesticides. Two parameters are used to analyse embryotoxicity, which studies quantitative embryological changes using microscopic imaging and qualitative morphological indexes. Embryological changes are determined using a neutral red stain and a DAPI stain. The changes were obtained by placing 15 eggs in plastic cups containing culture media. Two plastic cups, each containing 25g of media, a toxicant ($1/5^{th}$ of the $EC_{50}$), and a control and a vehicle control. The eggs were stained in the $1^{st}$ hour, early $3^{rd}$ hour, and late $3^{rd}$ hour and the embryological changes were observed. For length and width measurements, eggs were examined in the $1^{st}$ hour, $3^{rd}$ hour and $6^{th}$ hour.

**Neutral red staining of *Drosophila* embryo**:

The eggs are stained with neutral red, which consists of three steps: dechorionation, Fixation, and Staining.

Dechorionation: Eggs were collected from respective groups and subjected to dechorionation in 3% sodium hypochlorite for 5 minutes. Later, the eggs were washed with PBS buffer at pH 7.4 for a few minutes.

Fixation: The eggs were treated with Carnoy's fixative for 3 minutes. Composition of Carnoys fixative: Ethanol – 60 ml, Chloroform – 30 ml, and Acetic acid- 10 ml.

The eggs were then washed with PBS for a few minutes.

Staining: 5% neutral red was prepared and filtered before use. Neutral red was used to study egg damage to the vitelline membrane and egg apoptosis (LeMosy & Hashimoto, 2000).

Stained eggs were viewed under a darkfield microscope, ZEISS AxioScope A1, Germany, and a camera, Axiocam 305 colour. The software used was ZEN 3.0 Blue Edition. The embryos were stained and documented after three hours of exposure.

**DAPI staining of *Drosophila* embryo:**

The eggs were stained with DAPI, a process consisting of three steps: dechlorination, fixation, and staining.

Dechorionation and fixation: - The eggs were dechorionated with 1.5% sodium hypochlorite for 5 minutes. The eggs were washed in PBS for a few minutes to remove excess sodium hypochlorite**.** Fixation, and later, it was treated with Carnoy's fixative for 3 minutes. The eggs are washed with PBS for a few minutes**.**

Staining:- DAPI, chemically 4'6–diamidino-2-phenylindole, a blue fluorescent stain that binds to the adenine-thymine regions in double-stranded DNA, was prepared 5000 x using water as the solvent. DAPI counterstains were performed: fixed, methanol-dehydrated embryos were rehydrated in PBT and incubated with PBT/DAPI for 10 min. Embryos were washed three times in PBT for 1 minute, followed by longer washes in 3x PBT for 10 minutes. Stained embryos were mounted and stored in 70% glycerol/PBS till documentation. The eggs were viewed under a DAPI filter in UV light for fluorescent imaging in an Axioscope A1 Microscope and Zeiss Axiocam 305 colour camera. The software used was ZEN 3.0 Blue Edition for the first hour, the early third hour, and the late third hour.

Morphological changes: The length and width of the egg are observed by treating the eggs in media containing acephate and chlorantraniliprole ($1/5^{th}$ of $EC_{50}$), as well as control and vehicle control. The 15 eggs were selected for this, and the same was measured using ZEN 3.0 Blue Edition software on images acquired by the Zeiss Axiocam 305 Colour camera, located in the Axioscope A1 Microscope**.** The image was acquired during the $1^{st}$ hour, the early $3^{rd}$ hour, and the late $3^{rd}$ hour.

### 1.3.7 Differential gene expression study related to development

Differential expression of genes related to development was estimated using transcriptomic analysis. The 1/5$^{th}$ of the $EC_{50}$-treated third instar larvae were used for analysis. The transcriptome analysis involves four significant steps: 1-RNA extraction and quality control, 2-library preparation, 3-illumina sequencing, and 4-data analysis. Detailed methodologies were discussed in Chapter 2.

### 1.3.8 Statistical analysis of data

The $EC_{50}$ of pesticides was determined by probit analysis using the Litchfield and Wilcoxon method (1949). Non-parametric methods were used to analyse organismal responses, and Dunnett's t-test for multiple comparisons was used to compare toxicity responses. Kaplan-Meier tests were used to analyse survivorship with stratified log-rank tests, and all other data were analysed with one-way ANOVA using the SPSS software version 20.

## 1.4 RESULTS

### 1.4.1 Determination of acute median effective concentration ($EC_{50}$) of Acephate

Separate six range-finding tests for acephate were conducted. Each test concentration had six replicates and thirty animals per replicate. The results of the range-finding test indicated fluctuating values of percentage eclosion inhibition between 0.2 and 1.0 µg/ml.

The arrived concentrations for the median effective concentration of acephate were 0.7 µg/ml, 0.1 µg/ml, 1.4 µg/ml, 2.0 µg/ml, and 2.8 µg/ml. The mean eclosion inhibition percentage of selected concentrations of acephate (0.7, 1.0, 1.4, 2.0, and 2.8 µg/ml) was found to be 36.44±1.72%, 40.22±2.48%, 36.44±1.21%, 52.22±1.8%, and 72±2.09%, respectively.

The data were plotted on a double-logarithmic graph, and a 95% confidence interval was obtained by interpolating the method of Litchfield and Wilcoxon (1949). The eclosion inhibition percentage of different concentrations revealed that the exact $EC_{50}$ concentration was 1.9µg/ml, with a 95% confidence limit of 1.35-2.66 and a slope function of 3.1, with a confidence limit of 0.99-9.5 (Figure 1.4 and Table1.2).

**Table 1.2:** $EC_{50}$ and 95% confidence limits for eclosion inhibition of Acephate on *D. melanogaster*

| Pesticides | The number of organisms tested | Expected lethal concentration (ppm) | | | $EC_{50}$ and 95% confidence limits (µg/ml) | Slope and 95% confidence limits (µg/ml) |
|---|---|---|---|---|---|---|
| | | $EC_{16}$ | $EC_{50}$ | $EC_{84}$ | | |
| Acephate | 180 | 0.95 | 1.9 | 3.5 | **1.9** (1.35-2.66) | 3.1 (0.99-9.51) |

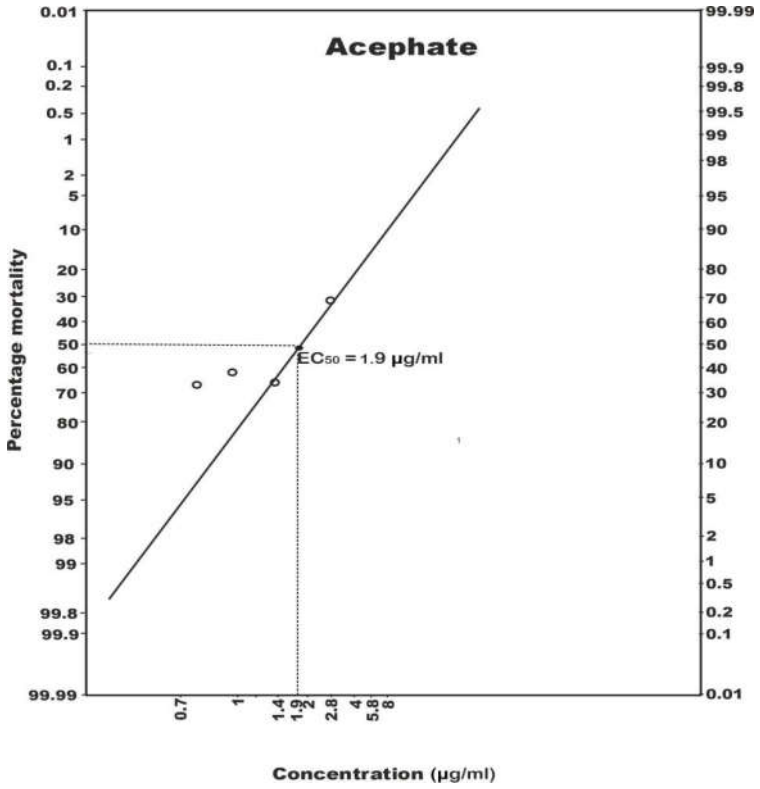



**Figure. 1.4:** $EC_{50}$ of Acephate on *D.melanogaster*

### 1.4.2 Dose-dependent (Acephate) changes in the lifecycle of *D.melanogaster*.

**Effects on hatchability of eggs.**

Sublethal concentrations of $1/10^{th}$, $1/5^{th}$ and $1/3^{rd}$, respectively, 0.19 µg/ml, 0.38 µg/ml, and 0.63 µg/ml, were uniformly mixed in media, and healthy eggs were transferred to intoxicated media for various experiments. After 24 hrs, the hatchability rate was recorded by subtracting non-hatched eggs from the total number of eggs exposed. After 24 hours of exposure, the hatchability percentages of different treatment groups were compared and presented in Figure 1.5. The results

(Table 1.3) showed a concentration-dependent decrease in hatchability compared with the control group. A significant reduction in hatchability (24.33±1.21, 20.83±1.722 $p<0.05$) was observed in the eggs treated with 0.38 µg/ml and 0.63 µg/ml concentrations compared with the control (27.6±0.81). The low hatchability rate observed in the 0.633 µg/ml treated groups indicated early toxicity responses. Compared to the control, a 22.78% reduction in hatchability was observed in the 0.633 µg/ml-treated groups.

**Table 1.3:** Effect of Acephate on *D. melanogaster* hatchability

| | **Experiment** | **Hatchability (mean ± stdev) *p<0.05** |
|---|---|---|
| 1 | Control | 27.6±0.81 |
| 3 | $1/10^{th}$ of $EC_{50}$ (0.19µg/ml) | 25.63±2.50 |
| 4 | $1/5^{th}$ of $EC_{50}$ (0.38µg/ml) | 24.33±1.21* |
| 5 | $1/3^{rd}$ of $EC_{50}$ (0.63µg/ml) | 20.83±1.722* |

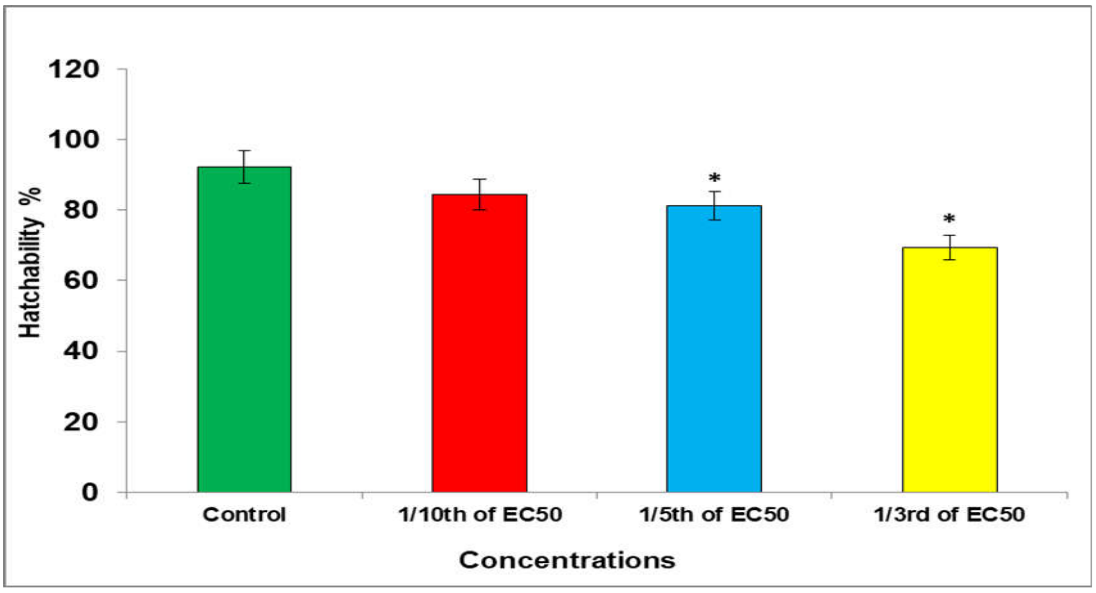



**Figure 1.5:** Effects of Acephate on hatchability of eggs of *D. melanogaster*, n=30 (mean±Stdev), significance ascribed as *p<0.05

**Effects of Acephate on Pupation Rate**

Pupation began on the $4^{th}$ day of exposure, in both the control and treated groups (Table 1.4). On $4^{th}$ day, a concentration-dependent increase in the pupation rate was observed. In treated groups, the pupation rate was higher (15.16±2.6 and

14.33±2.5 p<<0.05) compared to the control (9.3±1.5). The pupation ended on the 6th day in all groups except those treated with 0.63 µg/ml, in which it ended within two days. The pupation rate significantly differed among the three groups (F = 16.4; df = 3; P < 0.05) and decreased as the concentration of applied acephate increased (Figure 1.6). The incidences of decreased pupation in the 0.63 and 0.38 µg/ml groups were significantly different (24±1.095 and 25.33±1.21 p<0.05) when compared with the control (28.67±1.03).

**Table 1.4:** Effect of acephate on *D. melanogaster* day ways pupation

| Sl. No | Experiment | Pupation 4th day (mean ± stdev) *p<0.05 | Pupation 5th day (mean ± stdev) *p<0.05 | Pupation 6th day (mean ± stdev) *p<0.05 |
|---|---|---|---|---|
| 1 | Control | 9.3±1.5 | 27.3±0.81 | 28.67±1.03 |
| 2 | 1/10th of $EC_{50}$ (0.19 µg/ml) | 12.5±2.7 | 24±1.4 | 26.33±1.032 |
| 3 | 1/5th of $EC_{50}$ (0.38 µg/ml) | 14.33±2.5* | 24.33±1.03 | 25.33±1.21* |
| 4 | 1/3rd of $EC_{50}$(0.63 µg/ml) | 15.16±2.6* | 24±1.094 | 24±1.095* |

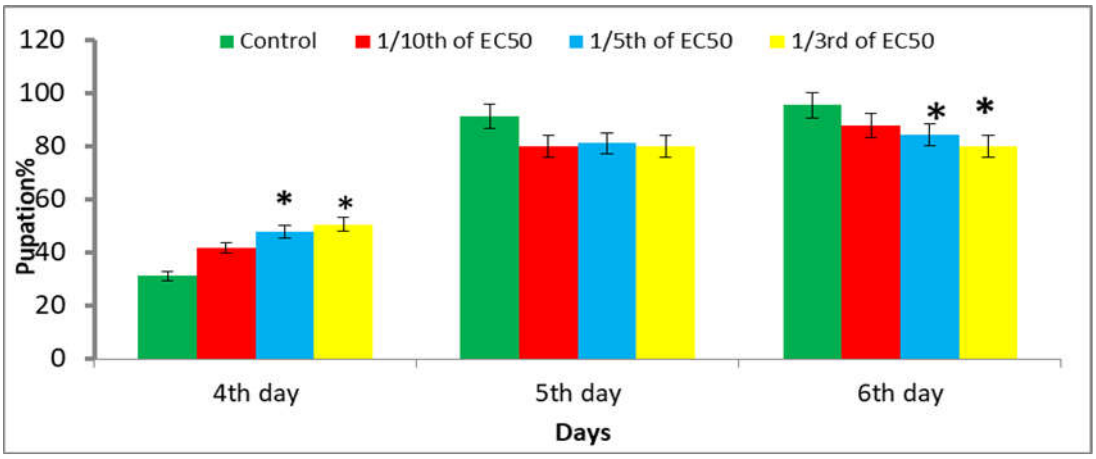



**Figure 1.6:** Effects of acephate on pupation of *D. melanogaster,* n=30 (mean± Stdev), significance ascribed as *p<0.05.

**Effects of Acephate on Emergence Pattern of *D. melanogaster***

The response to emergence in the control and treated groups differs significantly, and the results are presented in Figures 1.7, 1.8, and 1.9. In both the control and

experimental groups, emergence began on the 7th day and was almost complete by the 11$^{th}$ day (Figure 1.7). There was no significant variation between groups on the 7$^{th}$ and 8$^{th}$ day emergence (ANOVA variance test between groups showed df =3, F=1.02 and p >0.05 for the 7$^{th}$ day; df=3, F= 1.2 and p>0.05). An increased emergence was observed on the 9$^{th}$, 10$^{th}$, and 11$^{th}$ days. The emergence pattern on all three days (9th, 10th, and 11th) exhibited a significant concentration-dependent decrease (ANOVA test, $p < 0.05$) (Figure 1.8). The Dunnett t-test confirmed a significant difference ($p < 0.05$) between the control and treated groups. The total emergence (Table 1.5 & Figure 1.9) observed in the 0.63, 0.58, and 0.19 µg/ml treated groups was 22.67±0.8156, 25.33±1.2, and 26.33±1.032, respectively, compared to the control (28.67±1.03). Compared to the pupation rate, the emergence pattern of the 0.63 µg/ml-treated group showed a 4.5% difference. This indicates that not all pupae formed at a 0.63 µg/ml concentration complete their life cycle.

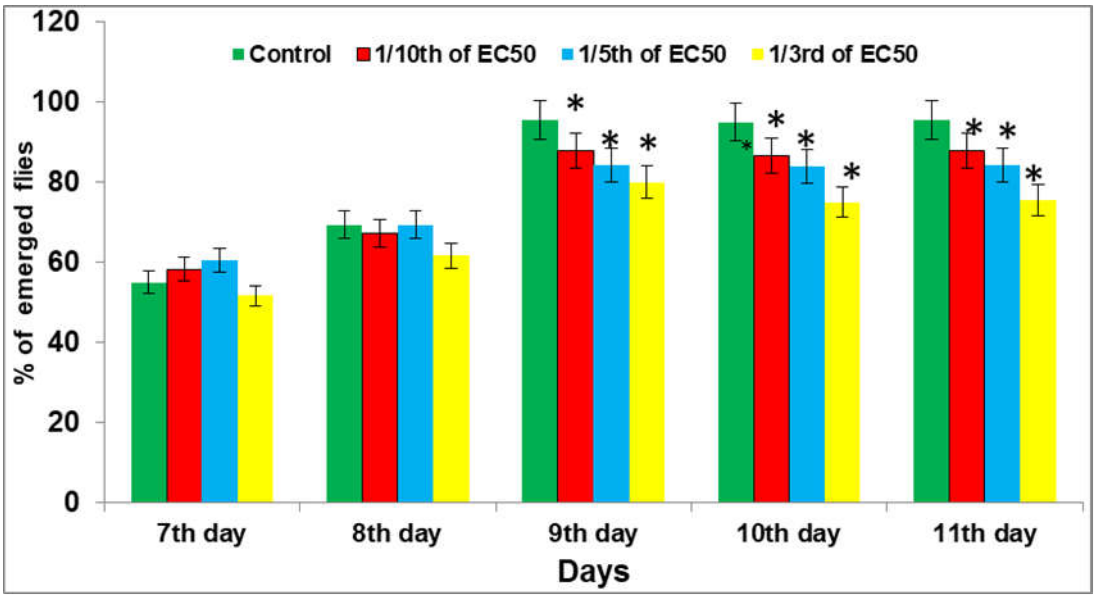



**Figure 1.7:** Effects of Acephate on the emergence pattern of *D. melanogaster,* n=30 (mean± Stdev), significance ascribed as *p<0.05.

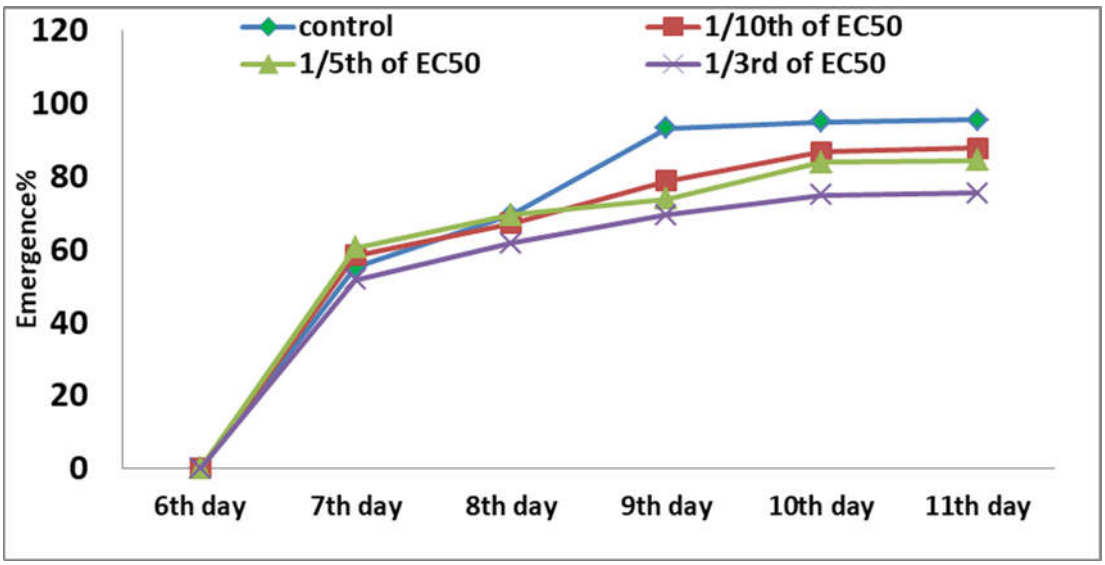



**Figure 1.8:** Effects of Acephate on the daily emergence pattern of *D. melanogaster.*

**Table1.5:** Effects of Acephate on total emergence of *D.melanogaster*

| Sl. No | Concentrations (µg/ml) | Emergence (mean ± stdev),*p<0.05 |
|---|---|---|
| 1 | Control | 28.67±1.032 |
| 2 | $1/10^{th}$ of $EC_{50}$ (0.19) | 26.33±1.03* |
| 3 | $1/5^{th}$ of $EC_{50}$ (0.38) | 25.33±1.21* |
| 4 | $1/3^{rd}$ of $EC_{50}$ (0.63) | 22.67±0.81* |

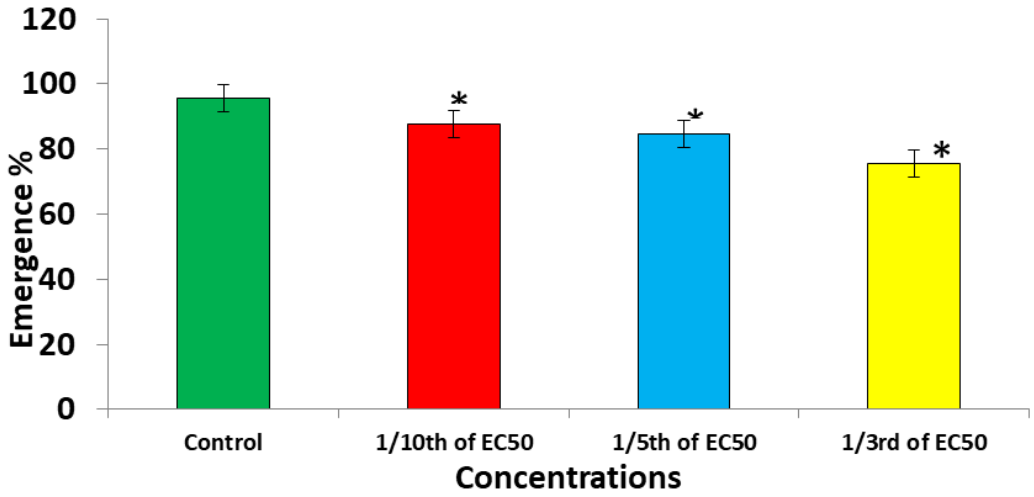



**Figure 1.9:** Percentage of mean emergence of *D. melanogaster,* n=30 (mean±Stdev), significance ascribed as *p<0.05.

**Effects of Acephate on the body weight of adult *D. melanogaster***

The stabilised weight of adult *D. melanogaster* flies that emerged from control and treatment groups was recorded after the 3rd day of emergence. Table 1.6 shows the concentration-dependent effects, and is graphically presented in Figure 1.10. A significant weight loss (14.96 ± 0.403, p<<0.05) was observed in higher concentration (0.63 µg/ml) treated flies when compared to control (16.48 ±.0.32).

**Table 1.6:** Effect of Acephate on body weight of *D.melanogaster*, significance ascribed as *p<0.05.

| Sl. No | Experiment | Adult body weight (mean ± stdev) *p<0.05 |
|---|---|---|
| 1 | Control | 16.48±0.32 |
| 3 | 1/10th of $EC_{50}$ (0.19µg/ml) | 16.28±0.18 |
| 4 | 1/5th of $EC_{50}$ (0.38µg/ml) | 16.10±0.223 |
| 5 | 1/3rd of $EC_{50}$ (0.63µg/ml) | 14.96±0.403* |

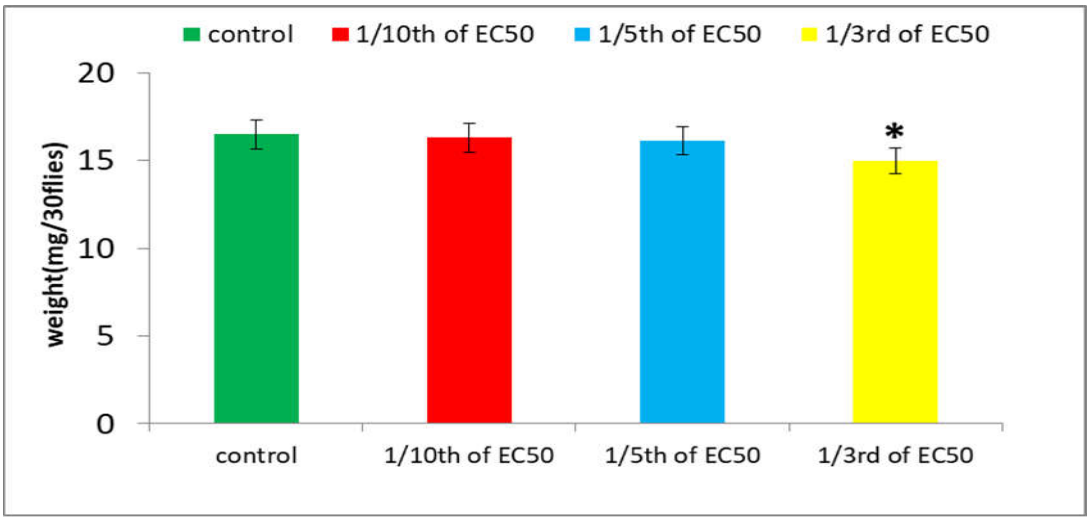


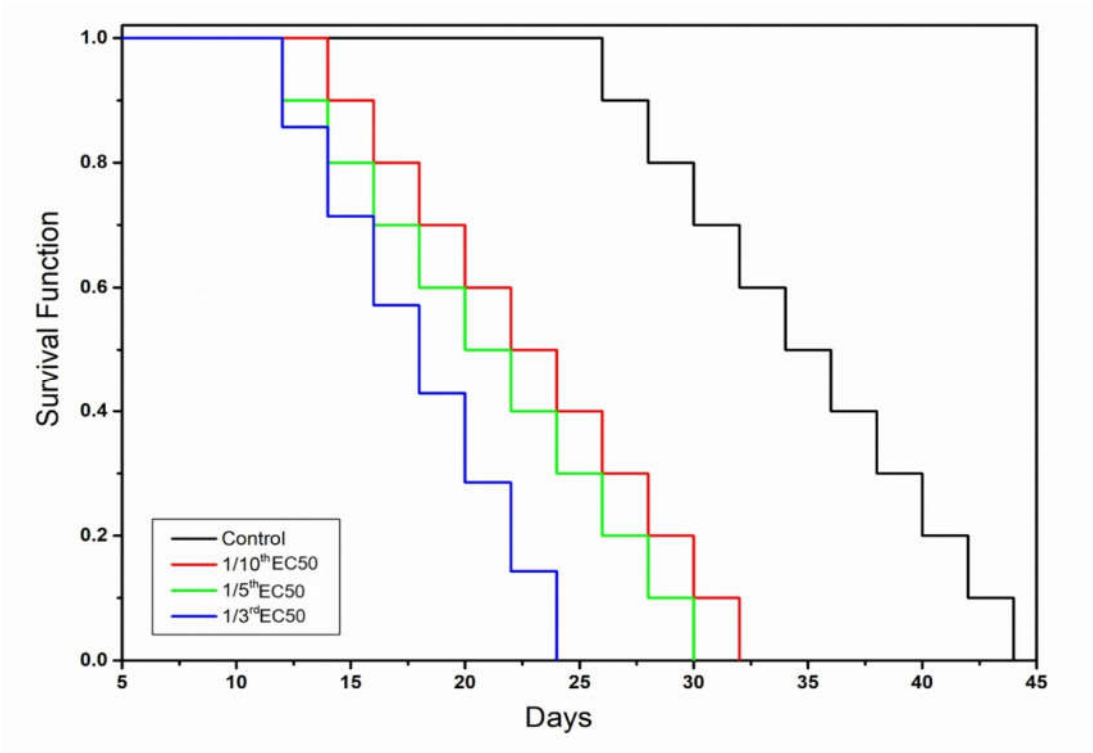

**Figure 1.10:** Effects of Acephate on body weight of *D. melanogaster*, n=30 (Mean ± stdev), significance ascribed as *p<0.05.

**Effects of Acephate on the survival rate of adult *D. melanogaster***

The life span of adult *Drosophila* was decreased upon Acephate treatment. Figure 1.11 shows the survival rate of adult flies exposed to chemicals and controls. The lifespan of adult Drosophila decreased upon exposure to Acephate and

was concentration-dependent. We used the Kaplan-Meier estimator, a non-parametric statistic, to compare the effects of Acephate on survival rate. The survival plot gives a clear picture of the difference in survival rate. The log-rank test detected a significant difference between the control and Acephate-treated groups. The log-rank test of significance showed a chi-square value of 102.69, with df, 3 and p values less than 0.0001. A concentration-dependent decrease in life span was observed**.** In control, death began on the 26th day and continued until the 44$^{th}$ day. Death occurred early in the treated groups (0.19 µg/ml, 0.38 µg/ml, and 0.63 µg/ml). For the flies treated with 0.19 µg/ml and 0.38 µg/ml, death occurred on the 14$^{th}$ day onwards. In contrast, the 0.63 µg/ml-treated flies showed signs of death starting on the 12$^{th}$ day. The total lifespan of the treated groups (0.19 µg/ml, 0.38 µg/ml, and 0.63 µg/ml) was reduced to 32, 30, and 24 days, respectively.

**Figure 1.11:** Effect of Acephate on survivorship of adult *D.melanogaster,* n=30 (mean ± Stdev)



### 1.4.3 Determination of acute median effective concentration ($EC_{50}$) of chlorantraniliprole

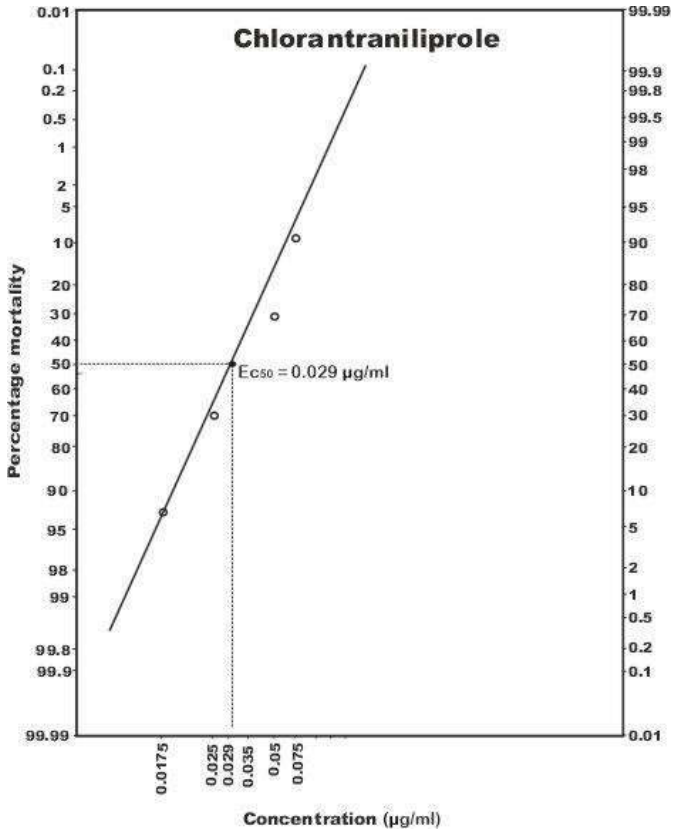
We tested different concentrations for the range-finding test of chlorantraniliprole. 0.3% v/v acetone control groups were also maintained for chlorantraniliprole at the experimental concentration. The experimental range was found to be between 0.02 and 0.04µg/ml.

From the range-finding test, we arrived at concentrations for the definitive test. The concentrations tested for chlorantraniliprole were 0.0175, 0.025, 0.035, 0.05, and 0.07µg/ml. The respective mean mortality percentages were found to be 30 ± 1.4%, 35.5 ± 1.6%, 57.2 ± 0.75%, 70 ± 0.89%, and 91.66 ±1.04%.

The $EC_{50}$ value of chlorantraniliprole was 0.029 µg/ml (Figure 1.12)**.** In addition, $EC_{16}$, $EC_{84}$, and slope function ratios for 95% probability were also calculated and presented (Table 1.7).

**Table 1.7:** $EC_{50}$ and 95% confidence limits for eclosion inhibition of Chlorantraniliprole on *D. melanogaster*

| Pesticides | Number of organisms tested | Expected lethal Concentration (µg/ml) | | | $EC_{50}$ and 95% confidence Limits (µg/ml) | Slope and 95% confidence Limits (µg/ml) |
|---|---|---|---|---|---|---|
| | | $EC_{16}$ | $EC_{50}$ | $EC_{84}$ | | |
| Chlorantraniliprole | 180 | 0.0185 | 0.029 | 0.049 | 0.029 (0.0245-0.0342) | 2.0 (1.42-2.82) |




**Fgure.1.12:** $EC_{50}$ of chlorantraniliprole

### 1.4.4 Dose-dependent (chlorantraniliprole) changes in the lifecycle of *D. melanogaster*.

**Effects of Chlorantraniliprole on Hatchability**

Sublethal concentrations of chlorantraniliprole**,** 1/3$^{rd}$ of the $EC_{50}$, 1/5$^{th}$, and 1/10$^{th}$, respectively, were 0.0096 µg/ml, 0.0058 µg/ml, and 0.0029 µg/ml, mixed uniformly in the media. Healthy eggs were transferred to the intoxicated media. Control and vehicle control were also maintained, as were the experiments. After 24 hours of exposure, the mean hatchability of the different treatment groups was recorded and found to be significant between groups at $p < 0.05$ (ANOVA tests, df = 4, F = 24.83). The result (Table 1.8) showed a concentration-dependent decrease in hatchability compared with the control group. It was noticed that there was no significant difference between the control and the vehicle control. Even though a reduction of mean hatchability was observed in all treated concentrations, a significant decrease was observed only in 1/3$^{rd}$ of $EC_{50}$ treated (19.6 ± 2.6, Dunnett t-test $p<<0.05$), compared to the control (27.16 ± 0.575). The low hatchability rate

observed in 1/3rd of $EC_{50}$-treated groups showed early toxicity responses (Figure 1.13).

**Table 1.8:** Effects of chlorantraniliprole on hatchability of eggs of *D. melanogaster*, n=30 (mean±Stdev).

| Sl.No. | Experiment | Hatchability (mean±stdev) *p<<0.05 |
|---|---|---|
| 1 | Control | 27.16±0.75 |
| 2 | Vehicle Control | 27.16±0.76 |
| 3 | 1/10th of $EC_{50}$ (0.0029µg/ml) | 26.6±1.50 |
| 4 | 1/5th of $EC_{50}$ (0.0058µg/ml) | 25.16±1.32 |
| 5 | 1/3rd of $EC_{50}$ (0.0096µg/ml) | 19.6±2.6* |

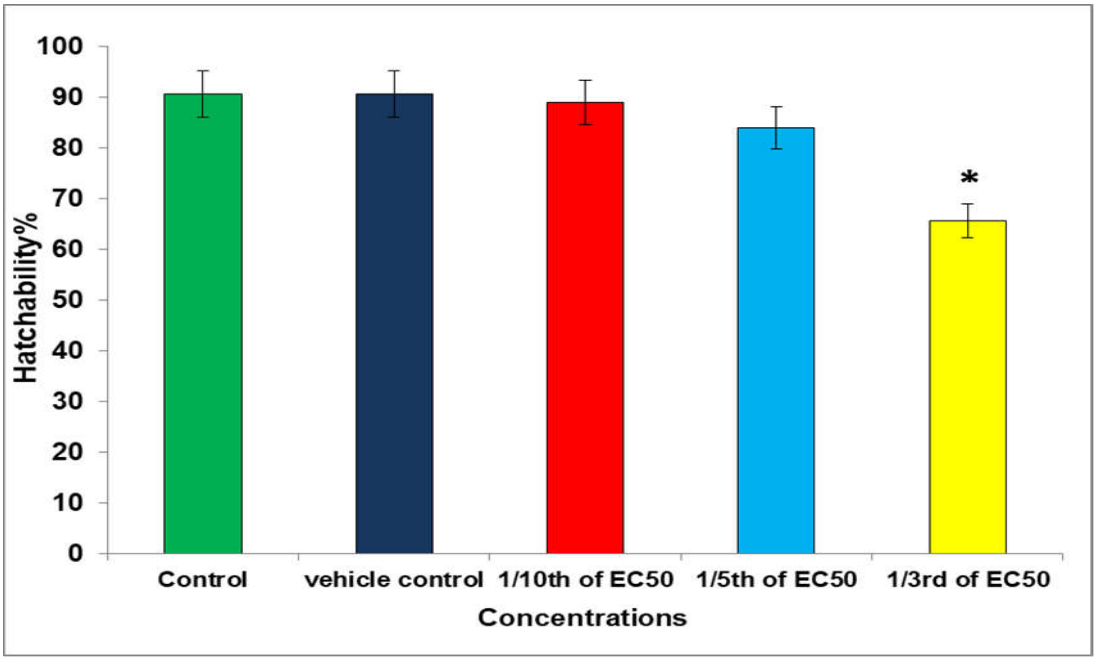



**Figure 1.13:** Effect of chlorantraniliprole on egg hatchability of *D. melanogaster,* n=30 (mean±Stdev), significance ascribed as *p<0.05

**Effects of Chlorantraniliprole on Pupation.**

Pupation began on the 4th day of exposure, in both the control and treated groups. The daily mean pupation pattern is given in Table 1.8. The pupation showed a significant difference between groups ($df = 4$, $F = 13.7$, $p < 0.05$). On the 4th day, a concentration-dependent increase in the pupation rate was observed. A significant

increase (7.83±1.72 and 5.0±1.41) in pupation rate was observed in treated (1/3rd of $EC_{50}$ and 1/5th of $EC_{50}$) groups. Compared to the control, the vehicle control did not show any significant difference. On the 5th day, compared to the treated groups, the pupation rate increased sharply in the control and vehicle control groups. The 5th-day pupation rate showed a concentration-dependent decrease in treated groups compared to the control (Figure 1.14). The mean pupation rate decreased with increasing concentrations on the 6th day (Table 1.9). In 1/10th of $EC_{50}$, 1/5th of $EC_{50}$, and 1/3rd of $EC_{50}$ treated groups, the mean pupation was noticed as (25.3±1.5, 24.67±1.03, and 22.5±4. $p<0.01$).

**Table 1.9:** Effects of Chlorantraniliprole on pupation of *D. melanogaster*, n=30 (mean ± SD).

| Sl. No | Experiment | Pupation 4th day (mean ± stdev) *p<<0.05 | Pupation 5th day (mean ± stdev) *p<<0.05 | Pupation 6th day (mean ± stdev) *p<<0.05 |
|---|---|---|---|---|
| 1 | Control | 4.167±0.752 | 27.3±0.81 | 28.67±1.03 |
| 2 | Vehicle Control | 4.5±1.04 | 28±0.89 | 29±0.899 |
| 3 | 1/10th of $EC_{50}$ (0.0029µg/ml) | 5.0±1.41 | 10±1.67* | 25.3±1.50* |
| 4 | 1/5th of $EC_{50}$ (0.0058 µg/ml) | 5.0±1.41 | 12±1.414* | 24.67±1.03* |
| 5 | 1/3rd of $EC_{50}$ (0.0096 µg/ml) | 7.83±1.72* | 16.16±2.4* | 22.5±4.08* |

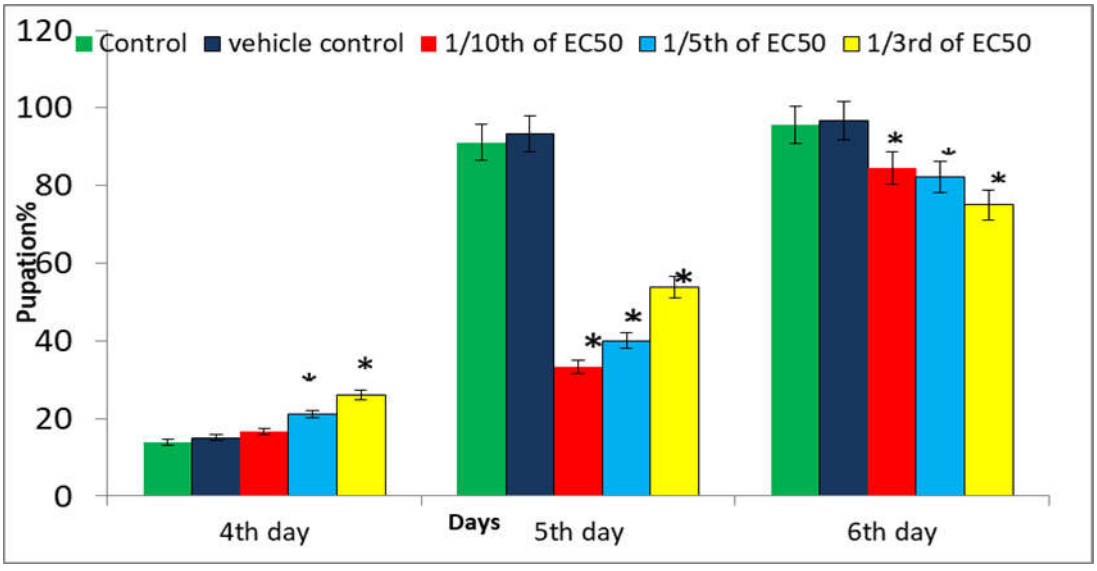



**Figure 1.14**: Effect of chlorantraniliprole on pupation of adult *D. melanogaster* , n=30 (mean ± Stdev) *p<<0.05

**Effects of Chlorantraniliprole on Emergence Pattern of *D. melanogaster***

The treatment of *D. melanogaster* with different concentrations of Chloranthraniliprole showed considerable variation in the emergence of adult flies. Treated flies observed a noticeable delay in emergence compared to the control and vehicle control. The effect was significant between groups ($p<0.001$) (Figures 1.15 & 1.16). Control and vehicle control do not elicit any considerable variation. The 1/3$^{rd}$ of the $EC_{50}$-treated groups showed a remarkable delay in emergence; in all other experiments, including control and vehicle control, emergence started on the 7$^{th}$ day or later. In control and vehicle control, emergence was completed on the 11th day of exposure, but in the treated groups, it lasted until the 15$^{th}$ day. Total emergence on the 15$^{th}$ day (Table 1.10 and Figure 1.17) revealed a concentration-dependent decrease (21±1.41, 23.16±1.69, and 24.67±1.21 $p<0.05$) in chlorantraniliprole-treated (0.0096, 0.0058, and 0.0029µg/ml) groups, respectively.

**Table 1.10:** Effects of Chlorantraniliprole on total emergence of *D. melanogaster*, n=30 (mean ± SD).

| Sl. No | Concentrations (µg/ml) | Emergence (mean ± stdev) *p<0.05 |
|---|---|---|
| 1 | Control | 28.67±1.032 |
| 2 | Vehicle control | 29.0±0.89 |
| 3 | 1/3$^{rd}$ of $EC_{50}$ | 24.67±1.2* |
| 4 | 1/5$^{th}$ of $EC_{50}$ | 23.16±1.16* |
| 5 | 1/10$^{th}$ of $EC_{50}$ | 21.0±1.4* |

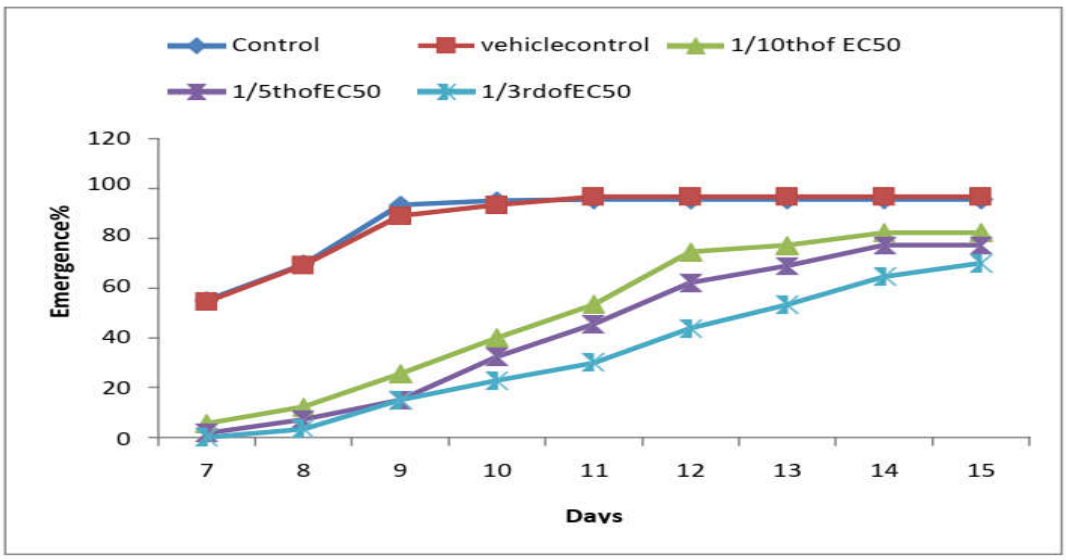



**Figure 1.15:** Effect of Chlorantraniliprole on th e emergence of adult *D. melanogaster*.

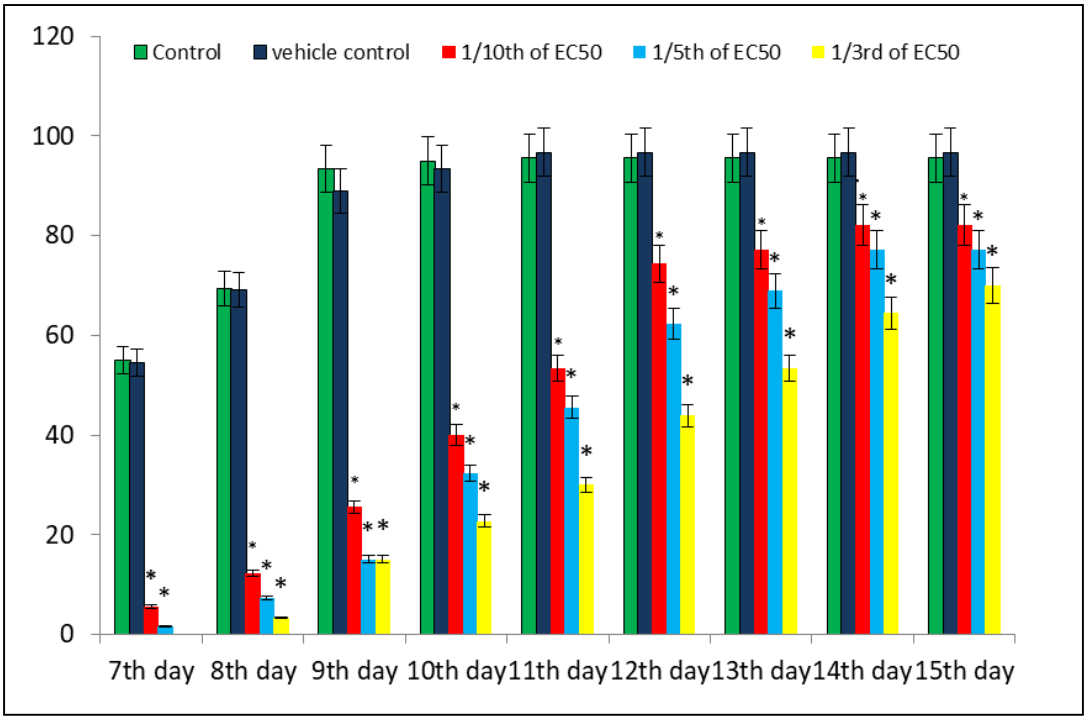



**Figure 1.16:** Effect of chlorantraniliprole on day ways emergence of adult *D. melanogaster,* n=30 (mean ±Stdev) *p<<0.05

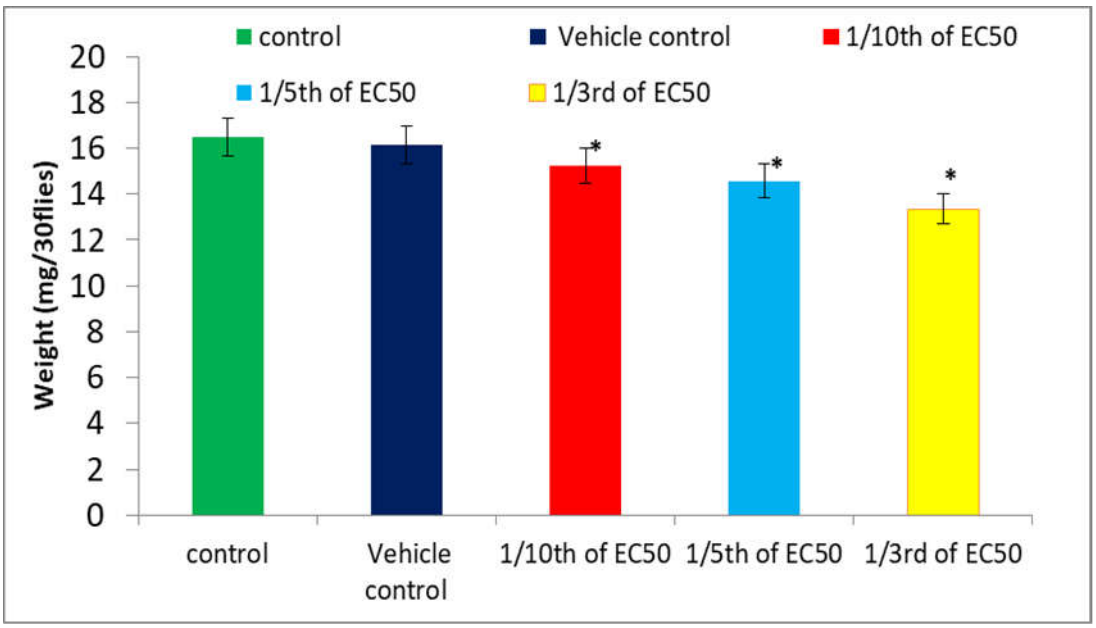



**Figure 1.17:** Effect of chlorantraniliprole on the emergence of adult *D. melanogaster,* n=30 (mean ± SD)*p<<0.05

**Effects of Chlorantraniliprole on Body Weight of Adult *D. melanogaster***

After treatment with chlorantraniliprole, *D. melanogaster* showed significant changes in the life cycle parameters like hatchability, pupation, and emergence. These changes are also reflected in the adult body weight of flies. The results are described in Table 1.11 and Figure 1.18. Compared to the control, vehicle control did not exhibit any significant variation. Flies that emerged in chlorantraniliprole treatment (1/3$^{rd}$ of $EC_{50}$, 1/5$^{th}$ of $EC_{50}$, and 1/10$^{th}$ of $EC_{50}$) had significant reductions in weights 13.36 ± 0.347, and 14.57 ± 0.33 and 15.23±0.13 mg per thirty male flies ($p < 0.01$) compared to the control (16.48 ± 0.32) and the effect is concentration dependent.

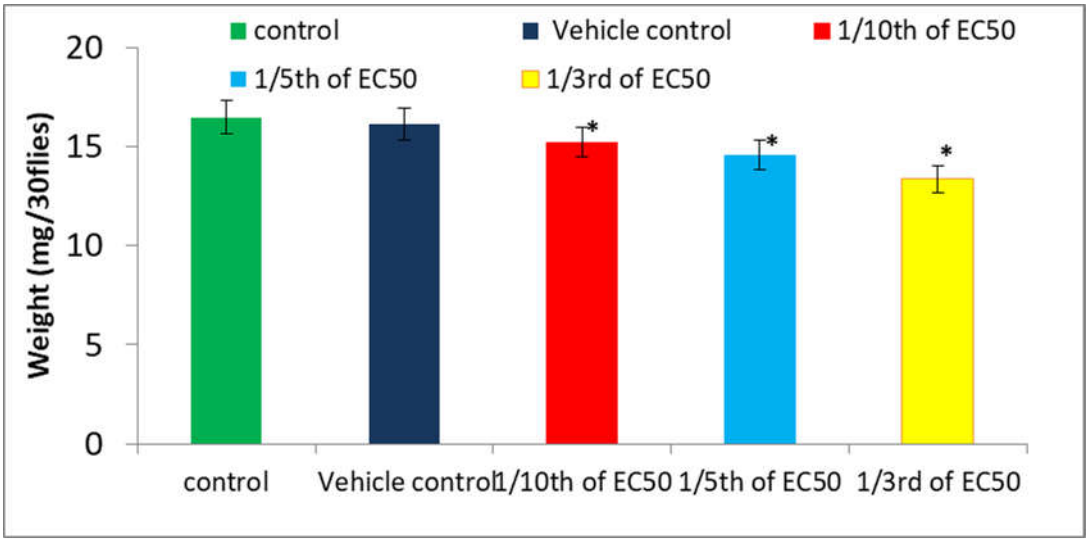



**Figure 1.18:** Effect of chlorantraniliprole on body weight of adult *D. melanogaster,* n=30 (mean ± SD)*p<<0.05

**Table 1.11:** Effects of chlorantraniliprole on adult body weight of *D. melanogaster*, n=30 (mean ± SD), significance ascribed as *p<0.05

| Sl. No | Experiment | Adult body weight (mean ± stdev) *p<<0.05 |
|---|---|---|
| 1 | Control | 16.48±0.32 |
| 2 | Vehicle Control | 16.14±0.21 |
| 3 | 1/10$^{th}$ of $EC_{50}$ (0.0029µg/ml) | 15.23±0.13* |
| 4 | 1/5$^{th}$ of $EC_{50}$ (0.0058µg/ml) | 14.5±0.33* |
| 5 | 1/3$^{rd}$ of $EC_{50}$ (0.0096µg/ml) | 13.36±0.34* |

**Effects of Chlorantraniliprole on the Survival Rate of Adult *D. melanogaster***

The analysis compares the impact of chlorantraniliprole treatment on the survival rate. We used the Kaplan-Meier estimator, a non-parametric statistic, to examine the effects of chlorantraniliprole on survival rate. A log-rank test detected a significant difference between the control and chlorantraniliprole-treated groups.

The lifespan of adult *Drosophila* decreased after treatment with chlorantraniliprole. The Control and vehicle control did not elicit variation; it is clear from the survival plots (Figure 1.19). The log-rank test of significance yielded a chi-square value of 195.14, with 4 degrees of freedom and a p-value of less than 0.0001. A concentration-dependent decrease in life span is observed. In control, death began on the 26th day and continued until the 44$^{th}$ day. Death started early in treated groups (1/3$^{rd}$ of $EC_{50}$, 1/5$^{th}$ of $EC_{50}$ and 1/10$^{th}$ of $EC_{50}$). In the flies treated with 1/3$^{rd}$ of the $EC_{50}$, death occurred on the 10th day onwards. In contrast, in 1/5$^{th}$ of the $EC_{50}$ µg/ml-treated flies, death started on the 12$^{th}$ day, and in 1/10$^{th}$ of the $EC_{50}$-treated groups, death began on the 14$^{th}$ day. The total lifespan of treated groups (0.0096µg/ml, 0.0058µg/ml and 0.0029µg/ml) was reduced to 32, 28, and 26 days, respectively.

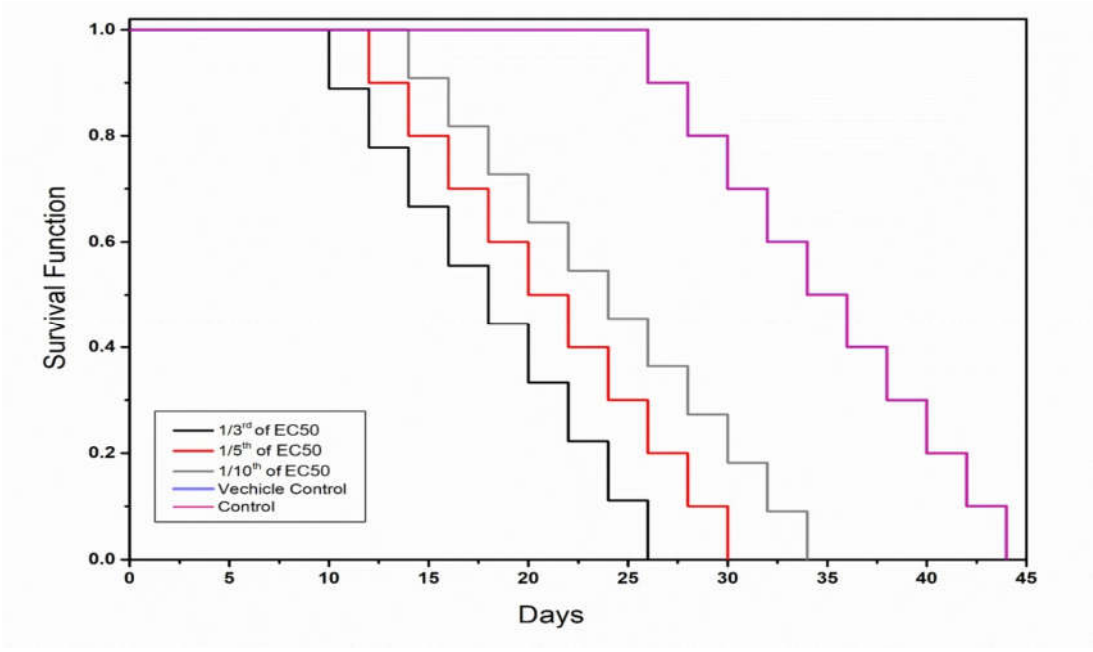



**Figure 1.19:** Effect of chlorantraniliprole on the survival rate of adult *D. melanogaster,* n=30 (mean ± Stdev) *p<<0.001

### 1.4.5 Embyotoxicology

Pesticide treatment results in physical deformities in the *Drosophila* embryo, characterised by reduced egg size. The $1/5^{th}$ of $EC_{50}$ treated embryos showed significant variations in length and width compared to the control. There was a reduction in the egg length from 619.19±7.8 to 592.8±5.3 during the first hour of exposure to acephate (Table 1.12). Similarly, during the third hour, there was a reduction in the length of the egg from 655±4.4to 583.4 ±4.9, and during the sixth hour, there was a decrease in the length of the egg from 652.8 ± 2.56 to 571.45 ± 2.16. There was also a reduction in the egg's width; during the first hour, it decreased from 260.13 ± 3.8 to 196.5 ± 6.8. Similarly, during the third hour, the decrease in the width of the egg is from 260.1 ± 1.7 to 191.9 ± 5.1, and during the sixth hour, there is a decrease in the width of the egg from 255.6 ± 2.3 to 191.54± 5.8. There is a reduction in both lengths as well as the width of the egg (Fig 1.20).

**Table 1.12:** Length and width variations of *Drosophila* on exposure to acephate during the first, third and sixth hour (mean ± SD).

| Time | Control | | Acephate | |
|---|---|---|---|---|
| | Length (µm) | Width (µm) | Length (µm) | Width (µm) |
| 1hr | 619.19±7.8 | 260.13±3.8 | 592.8±5.3 | 196.5±6.8 |
| 3 hr | 655±4.4 | 260.1±1.7 | 583.4±4.9 | 191.9±5.1 |
| 6 hr | 652.8±2.56 | 255.6±2.3 | 571.45±2.16 | 191.54±5.8 |

**Table 1.13:** Length and width variations of *Drosophila* on exposure to chlorantraniliprole during the first, third and sixth hour (mean ± Stdev).

| | Control | | Vehicle control | | Chlorantraniliprole | |
|---|---|---|---|---|---|---|
| | Length (µm) | Width (µm) | Length (µm) | Width (µm) | Length (µm) | Width (µm) |
| 1hr | 619.19±7.8 | 260.13±3.8 | 610.5±7.8 | 260.9±4.2 | 589.3±4.5 | 189±6.4 |
| 3hr | 655±4.4 | 260.1±1.7 | 650.8±3.3 | 259.3±5.3 | 572.4±5.9 | 184.4±2.03 |
| 6hr | 652.8±2.56 | 255.6±2.3 | 651.73±3.13 | 259.25±3.5 | 572.6±1.2 | 183.5±1.8 |

A similar trend was observed in chlorantraniliprole-treated eggs**.** In the first hour, there was a reduction in the size of eggs both in terms of length and width. The

length was reduced from 619.19±7.8 to 589.3±4.5 in chlorantraniliprole-treated eggs compared to the control. Vehicle control showed no remarkable variation in length and width compared to control (figure 1.13). The highest difference in length was observed in the third-hour eggs treated with chlorantraniliprole (figure 1.20). A tremendous difference in width was also observed; during the first hour, it was from 260.13±3.8 to 189 ± 6.4. Similarly, during the third hour, the reduction in the width of the egg is from 260.1 ± 1.7 to 184.4 ± 2.03, and during the sixth hour, there is a decrease in the width of the egg from 255.6 ± 2.3 to 183.5±1.8 (figure 1.21).

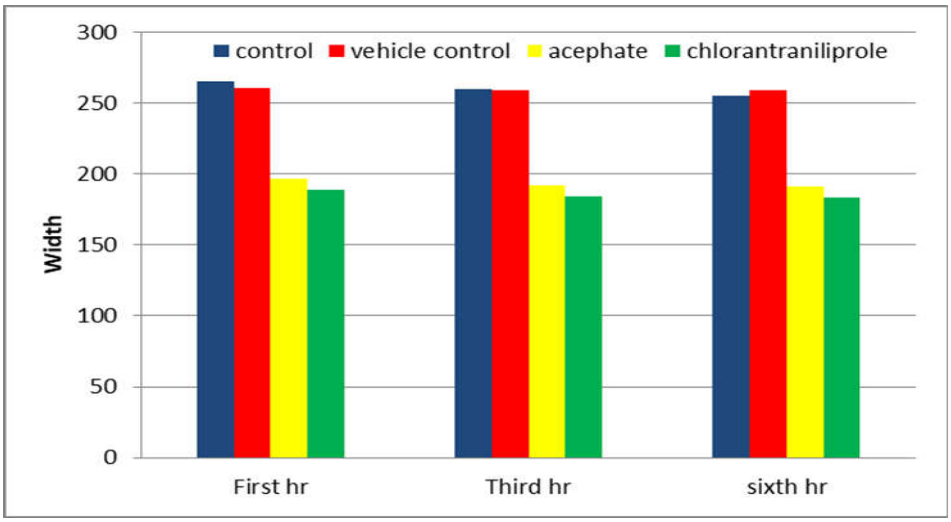



**Figure 1.20:** Variation in the width of the eggs of *D. melanogaster* in control and exposure to acephate and chlorantraniliprole (mean ± SD).

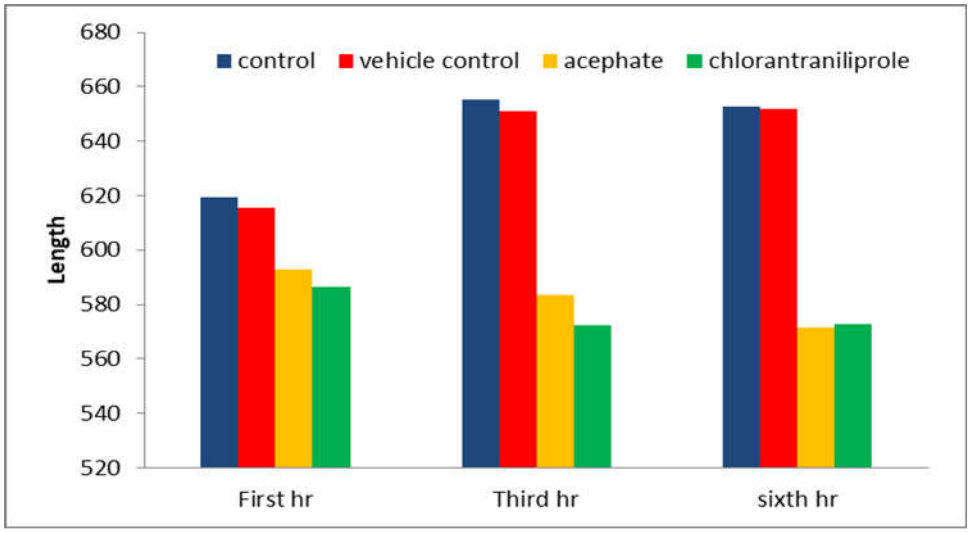



**Figure 1.21:** Variation in the length of the eggs of *D. melanogaster* in control and exposure to acephate and chlorantraniliprole (mean ± SD).

During the first hour of embryo development, egg cytoplasm can be seen retracting from the vitelline envelope at both poles, and the space at the posterior pole occurs at this time. No such retraction in the cytoplasm is seen compared to the control in pesticide-treated eggs. As development progresses at the third hour, early gastrulation events occur: the ventral and cephalic furrows form, and the pole cells continue to shift dorsally in both control and vehicle control. The pole cell plate is in a horizontal position at the later stages. The plate continues to tilt, forming a pocket (the amnioproctodeal invagination). The beginning of the cephalad (head wards) movement of this invagination also started in this stage. The dorsal folds and amnioproctodeal invagination were visible in control eggs; no more amnioproctodeal invaginations visible in pesticide-treated groups were observed (figure 1.22 and 1.23).

Neutral red uptake determines the lethal effect of acephate and chlorantraniliprole on *D. melanogaster*. The $1/5^{th}$ of $EC_{50}$ concentrations of the acephate and chlorantraniliprole were examined in this study. The neutral red staining helps to locate and determine the damage in the vitelline membrane. The neutral red uptake increases with time, indicating the toxins' lethal effect on the vitelline membrane. The egg is also stained to determine the gross cell morphology and nuclear movement. DAPI-stained eggs were viewed under a fluorescence microscope, and the morphology was determined under blue fluorescence, showing a considerable difference between the control and toxicant-treated eggs. Vehicle control also shows the difference in development in the third hour (Figure 1.23).

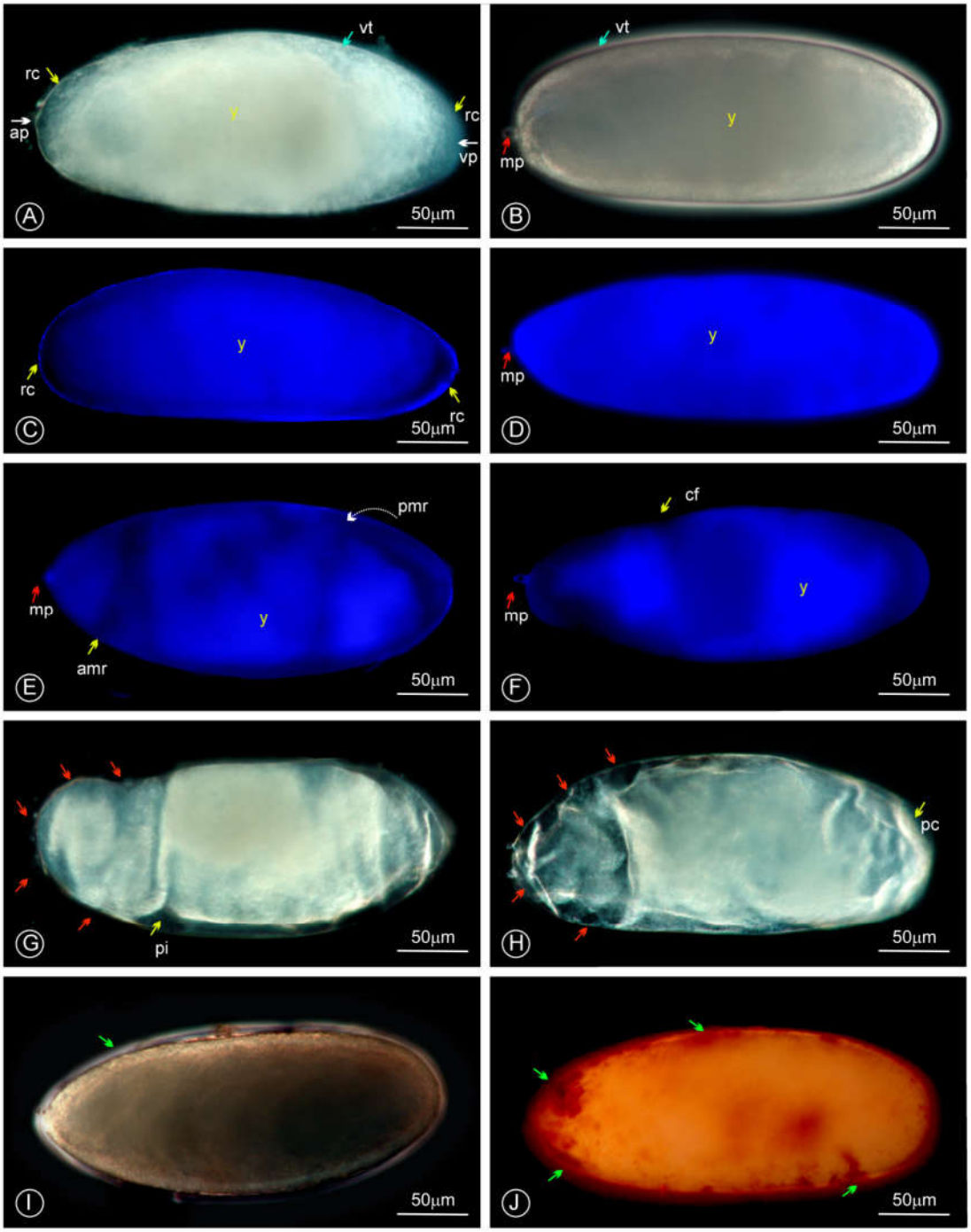



**Figure 1.22:** Demonstration of the impact of acephate on the earlier development of *D. melanogaster*. (A) 1st hour control (B) 1st-hour acephate (C) 1st hour DAPI control (D) 1st-hour DAPI acephate (E) Early third-hour DAPI control (F) Early third hour DAPI acephate (G) Late third-hour control (H) Late third-hour acephate (I)After third-hour neutral red control (J) After third-hour neutral red acephate. ap-animal pole, vp-vegetal pole, vt-vitelline membrane, y-yolk protein, rc- retracting cytoplasm, amr-anterior midgut rudiments, pmr-posteriormidgut rudiments, and cf-cephalic furrow.

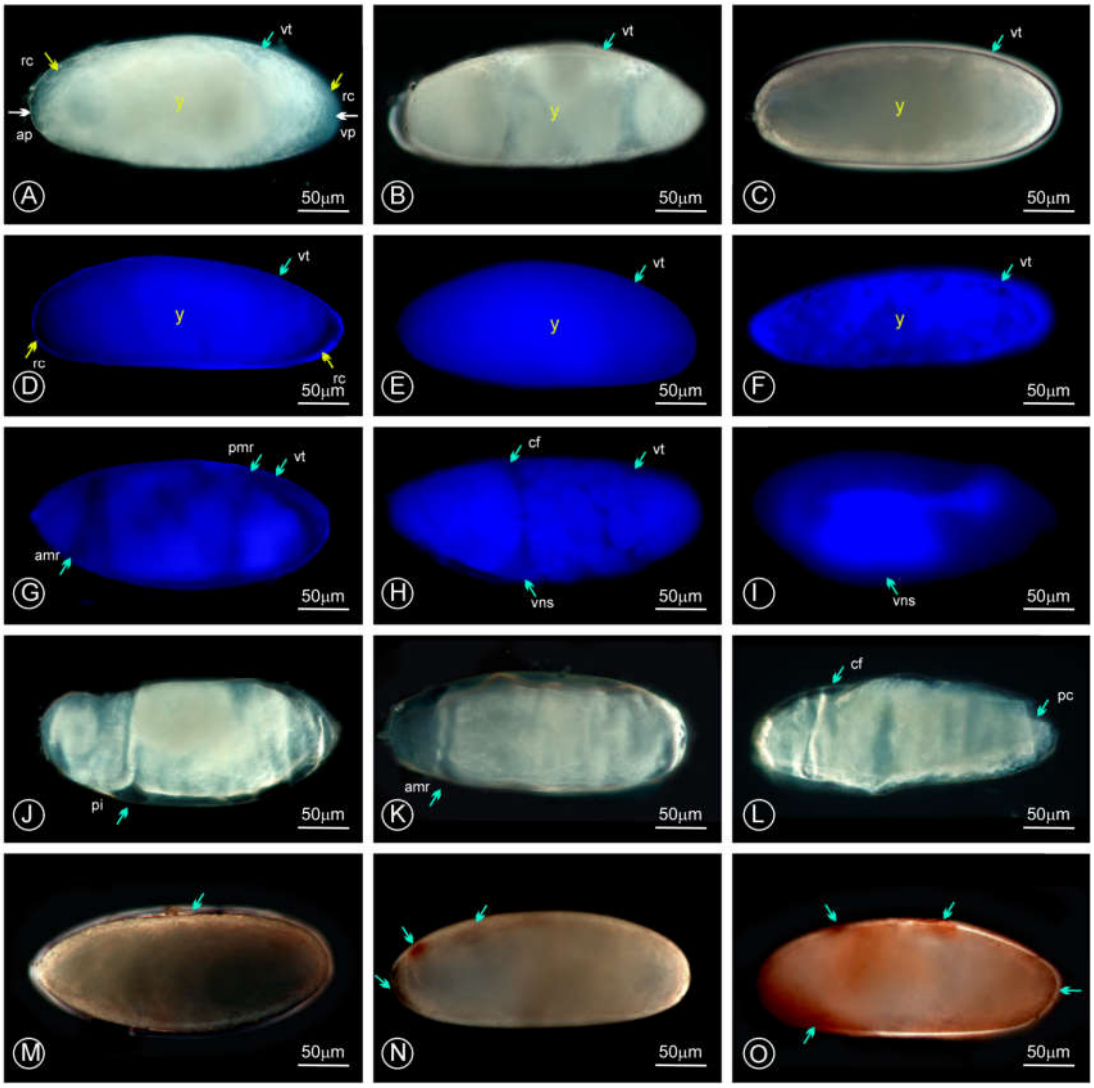



**Figure 1.23:** Impact of chlorantraniliprole in the earlier development of *D. melanogaster*. (A) 1st hour control (B) 1st hour vehicle control (C) 1st hour chlorantraniliprole (D) 1st hour DAPI control (E) 1st hour DAPI vehicle control (F) 1st hour DAPI chlorantraniliprole (G) Early third hour DAPI control (H) Early third hour DAPI vehicle control (I) Early third hour DAPI chlorantraniliprole (J) Late third hour control (K) Late third hour vehicle control (L) Late third hour chlorantraniliprole (M) After third hour neutral red control (N) After third hour neutral red vehicle control (O) After third hour neutral red chlorantraniliprole. ap-animal pole, vp-vegetal pole, vt-vitelline membrane, y-yolk protein, rc- retracting cytoplasm, amr-anterior midgut rudiments, pmr- posterior midgut rudiments, and cf-cefalic furrow, vns- ventral nervous system.

### 1.4.6 Transcriptome analysis and differential gene expression studies

Transcriptomic analysis was performed on the control group, and larvae were treated with 1/5th of the $EC_{50}$ of acephate and chlorantraniliprole for 48 hours. We

studied the differential expression of genes controlling life cycle parameters of *D. melanogaster* third instar larvae. The DEGs, which encode detoxification enzymes such as cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), carboxylesterases (COEs), UDP glucosyl transferases (UGTs), and ATP-binding cassette transporters (ABCs), were therefore examined in this work. The samples treated with acephate differed from controls in the differential expression of 29 genes encoding detoxification enzymes. Five of the nine downregulated P450s have logfc values greater than 2. Nearly -3.06 is the most excellent log2fold change value (log2fc value) for the *Cyp4e1* transcript. Out of the four GST genes, all four had lower transcription levels than the flies in the control group, with *GstD2* having the highest log2 fold change (log2fc) value of -2.42.

Fifteen genes were identified in samples treated with chlorantraniliprole: eight were down-regulated, and the other seven were up-regulated**.** These genes included four P450 genes, two GST genes, one COE gene, four UGT genes, and four ABC genes**.** UGTs with high expression values (*ugt36F1*-3.8) were identified among these detoxifying genes. The major GSTs up-regulated during chlorantraniliprole induction were identified as *GstE14* and *GstE5*.

It is very well known that the HSP family plays a vital role in pesticide stress. We have examined the expression of various families of heat shock proteins (HSPs). In acephate-treated groups, out of thirteen hsps, eight were significantly over-transcribed. The most over-transcribed HSP family is the *HSP 70* family with six genes (*Hsp70Aa, Hsp70Ab, Hsp70Ba, Hsp70Bb, Hsp70Bbb* and *Hsp70Bc*). The eight significantly up-regulated Hsps genes have log2fc values above two. Six Hsp70s (*Hsp70Aa, HspAb, HspBa, HspBb, HspBbb,* and *HspBc*) were significantly over-expressed with chlorantraniliprole treatment.

Compared to the control, the acephate- and chlorantraniliprole-treated groups showed differential expression of genes involved in embryo development, instar larval development, pupation, feeding behaviour, circadian rhythm, and insect hormone biosynthesis.

The overall changes in the gene expression related to the organismal responses of *D. melanogaster* to acephate and chlorantraniliprole. In the acephate-treated group, ouib and wech, genes involved in instar larval development showed up-regulation without a significant p-value. The *sas* genes involved in instar larval development showed considerable down-regulation**.** Genes involved in pupation also showed significant differential expression with two down-regulated genes (*cyp18a1* and *ude*) and one up-regulated gene (*Blimp-1*). Metamorphosis genes also showed variable expressions, with *Eig71Ec* and *Eig71Ea,* having the highest log2fc value above three. The *Yp3* gene *(vitellogenin)* showed significant up-regulation in the acephate-treated group. Unlike the control, insect hormone biosynthesis genes showed significant expression differences. *phm, sad* and *scro* genes with significant log2fc values (Table 1.14).

Detailed differential expressions of genes in chlorantraniliprole groups are given (Table 1.15). Acephate and chlorantraniliprole share some common genes, but there were differences in the log2fc values. The highly up-regulated genes reported are *Lk* genes in circadian rhythm, *ugt36f1* genes in detoxification, and *Takl1*genes in apoptosis. Out of four genes in insect hormone biosynthesis, three genes were down-regulated.

**Table 1.14:** Differential expressions of genes in acephate-treated *D. melanogaster.*

| Function | Gene Name | Regulation | Log2foldchange | P-value |
|---|---|---|---|---|
| Instar larval development | *ouib* | Up | 1.65 | 0.2312 |
| | *wech* | Up | 1 | 0.1348 |
| | *sas* | Down | -1.42 | 0.0225 |
| Pupation | *Blimp-1* | Up | 1.99 | 0.0041 |
| | *Cyp18a1* | Down | -1.45 | 0.01414 |
| | *Ude* | Down | -2.03 | 0.00135 |
| Pupariation | *Ilp8* | Up | 3.708 | $5.47\times10^{-5}$ |
| | *Snmp1* | Down | -1.951 | 0.01049 |
| metamorphosis | *Eig71Ec* | Up | 3.3812 | $8.74\times10^{-6}$ |

| | | | | |
|---|---|---|---|---|
| | *Eig71Ea* | Up | 3.152 | $7.77\times10^{-5}$ |
| | *EbpIII* | Down | -2.462 | 0.000107 |
| | Cyp18a1 | Down | -1.4502 | 0.014 |
| Embryo development ending in birth or egg hatching | *Yp3(vitellogenin)* | Up | 1.752 | 0.04279 |
| | *lnR* | Up | 1.199 | 0.0818 |
| | *elav* | Up | 1.87 | 0.0674 |
| | *arg* | Down | -1.506 | 0.011 |
| Chorion-containing eggshell formation | *llp8* | Up | 3.708 | $5.47\times10^{-5}$ |
| | *fs(1)N* | Up | 1.287 | 0.223 |
| Insect hormone biosynthesis | *Aldh* | Down | -1.896 | 0.0437 |
| | *CG11617* | Down | -1.769 | 0.0264 |
| | *Phm* | Up | 2.151 | 0.0223 |
| | *Sad* | Up | 2.081 | 0.0208 |
| | *Nfl* | Down | -1.415 | 0.01674 |
| | *Cyp18a1* | Down | -1.453 | 0.01414 |
| | *Jheh1* | Down | -1.516 | 0.01097 |
| | *scro* | Down | -2.885 | 0.0076 |
| Circadian rhythm | *Cry* | Up | 1.032 | 0.47 |
| | *SIFa* | Up | 2.28 | 0.198 |
| | *E23* | Up | 1.574 | 0.027 |
| | *Hll* | Up | 2.377 | 0.00128 |
| | *HK* | Down | -1.105 | 0.08 |
| | *bgm* | Down | -1.194 | 0.044 |
| | *Acer* | Down | -1.343 | 0.0268 |
| | *InR* | Up | 1.199 | 0.0818 |
| Larval feeding | *BM-40* | Down | -1.7013 | 0.0386 |
| | *LK* | Up | 2.907 | 0.0373 |
| | *5HT2A* | Down | -1.4075 | 0.138 |

| | | | | |
|---|---|---|---|---|
| Embryonic development | *phm* | Up | 2.215 | 0.0223 |
| | *exp* | Down | -1.675 | 0.0063 |
| | *mey* | Up | 1.6914 | 0.0325 |
| | *m* | Up | 3.129 | 0.0085 |
| | *kkv* | Down | -1.396 | 0.0477 |
| Heatshock protein | *hsp70Bb* | Up | 3.53 | $3.7\times10^{-5}$ |
| | *hsp70Ba* | Up | 3.495 | $2.3\times10^{-5}$ |
| | *hsp70Aa* | Up | 3.191 | $2.36\times10^{-5}$ |
| | *hsp70Bbb* | Up | 2.973 | $8.47\times10^{-5}$ |
| | *hsp70Ab* | Up | 3.208 | 0.000161 |
| | *hsp70Bc* | Up | 2.901 | 0.00226 |
| | *hsp67Bc* | Up | 2.66 | 0.00024 |
| | *hsp67Ba* | Up | 2.099 | 0.0048 |
| Detoxification mechanism | *cyp4ad1* | Up | 2.124 | 0.0084 |
| | *CG13594* | Up | 1.896 | 0.0376 |
| | *Cyp311a1* | Up | 1.8088 | 0.044 |
| | *Cyp304a1* | Up | 1.355 | 0.045 |
| | *Cyp4e1* | Down | -3.063 | $2.72\times10^{-5}$ |
| | *Cyp4c3* | Down | -2.714 | $5.15\times10^{-6}$ |
| | *Ugt37c1* | Down | -2.94 | $1.22\times10^{-5}$ |
| | *GstD2* | Down | -2.424 | 0.00149 |
| | *Cyp49a1* | Down | -1.976 | 0.00146 |
| | *Cyp6w1* | Down | -2.345 | 0.002 |
| | *Cyp6a17* | Down | -1.8 | 0.0024 |
| | *Cyp4aa1* | Down | -1.783 | 0.00422 |
| | *Cyp4d21* | Down | -2.27 | 0.00469 |
| | *Cyp6a18* | Down | -1.77 | 0.005 |
| | *GstD10* | Down | -1.7434 | 0.0045 |

| | | | | |
|---|---|---|---|---|
| | *GstD3* | Down | -1.7232 | 0.005 |
| | *Ugt37A3* | Up | 1.687 | 0.014 |
| | *Ugt303A1* | Up | 2.42 | 0.042 |
| Apoptosis | *Tak1* | Up | 3.285 | 0.00078 |
| | *Hid* | Up | 1.77 | 0.023 |
| | *Eip93F* | Up | 3.308 | $4.08\times10^{-5}$ |
| Chitin-based embryonic cuticle biosynthetic process | *kkv* | Down | -1.3961 | 0.04744 |
| | *dib* | Up | 2.093 | 0.2593 |
| | *mmy* | Down | -1.2484 | 0.0594 |
| | *pot* | Down | -1.0145 | 0.1344 |

**Table 1.15:** Differential expressions of genes in chlorantraniliprole treated *D. melanogater.*

| Gene function | Gene name | regulation | Log2foldchange | P value |
|---|---|---|---|---|
| Instar larval or pupal development | *Ac76E* | Down | -1.019 | 0.013 |
| Embryo development ending in birth or egg hatching | *Yp3* | Up | 1.0278 | 0.0683 |
| Pupariation | *CG30033* | Up | 1.7389 | 0.589 |
| Ecdysone | *Eip74EF* | Down | -1.0188 | 0.01308 |
| | *Kr-h1* | Down | -1.6206 | 0.00521 |
| | *ImpE1* | Down | -1.289 | 0.0034 |
| Metamorphosis | *Sox14* | Down | -1.238 | 0.00354 |
| | *Kr-h1* | Down | -1.6206 | 0.00521 |
| | *Ptth* | Up | 1.0077 | 0.1992 |
| | *CG30033* | Up | 1.738 | 0.5897 |
| Insect hormone biosynthesis | *CG10352* | Up | 2.192 | 0.0599 |
| | *CG8757* | Down | -1.298 | 0.8904 |
| | *CG40485* | Down | -1.11 | 0.194 |
| | *Antdh* | Down | -1.0435 | 0.405 |
| Circadian rhythm | *SIFa* | Up | 1.8234 | 0.1119 |
| | *Lk* | Up | 2.395 | 0.0024 |
| | *RYa* | Up | 1.701 | 0.5902 |
| Feeding behavior | *Hug* | Up | 1.218 | 0.0764 |
| | *Dsk* | Up | 1.333 | 0.2934 |
| | *pain* | Down | -1.167 | 0.007 |
| Embryo development | *exu* | Up | 1.101 | 0.00624 |

| | | | | |
|---|---|---|---|---|
| | *asRNA: CR45145* | Down | -1.447 | 0.1947 |
| | *foy* | Down | -1.004 | 0.0201 |
| | *smash* | Down | -1.109 | 0.00635 |
| | *CR40005* | Up | 1.312 | 0.8075 |
| Chorion containing eggshell formation | *Muc12Ea* | Up | 1.18 | 0.415 |
| | *Cp7Fa* | Up | 1.66 | 0.286 |
| Heat shock proteins | *Hsp70Bb* | Up | 1.004 | 0.0134 |
| | *Hsp70Aa* | Up | 1.217 | 0.0054 |
| | *Hsp70Bbb* | Up | 1.2422 | 0.00336 |
| | *Hsp70Ab* | Up | 1.009 | 0.0131 |
| | *Hsp70Bc* | Up | 1.097 | 0.0077 |
| Detoxification mechanism | *Ugt303A1* | Up | 1.941 | 0.00912 |
| | *Ugt36F1* | Up | 3.803 | 0.0176 |
| | *GstE5* | Up | 1.187 | 0.043 |
| | *Cyp4p1* | Down | -1.609 | 0.00226 |
| | *Cyp6a20* | Down | -1.3 | 0.03 |
| | *Cyp4ad1* | Up | 1.433 | 0.0038 |
| | *Cyp12d1-d* | Up | 1.616 | 0.0382 |
| Apoptosis | *Takl2* | Up | 1.5829 | 0.0151 |
| | *Takl1* | Up | 2.2615 | 0.0031 |
| | *Eip74EF* | Down | -1.018 | 0.0130 |
| | *grim* | Down | -1.30033 | 0.628 |
| Chitin-based embryonic cuticle biosynthetic process | *dpy* | Down | -1.04199 | 0.0102 |
| | *knk* | Down | -1.0254 | 0.018 |
| | *kkv* | Down | -1.10922 | 0.0061 |

## 1.5 DISCUSSION

The present study demonstrates that acephate intoxication significantly affects the lifecycle performance of *Drosophila melanogaster*. The $EC_{50}$ value (concentration causing 50% eclosion inhibition) has arrived from the range-finding tests, and definitive toxicity bioassays were conducted based on the range-finding analysis. Our findings show that the estimated $EC_{50}$ value of Acephate (98% purity) for *D. melanogaster* is 1.9µg/mL. Remarkably, 50% eclosion inhibition was observed after exposure to a 1.9 µg /mL acephate concentration. The conventional method of treating organophosphate toxicity has focused on receptor-specific effects on multi-cellular creatures' muscarinic, nicotinic, and central nervous systems (Peter

& Cherian, 2000; Wadia et al., 1974). This results in neurotoxic effects that include tremors, convulsions, and neuromuscular paralysis, characterised by the complete contraction of muscles throughout the body, as well as the organism's eventual death (Gupta, 2006). Therefore, the neurotoxic properties of acephate might have played a significant role in eclosion inhibition following acephate exposure. Previous studies have shown that the estimated $LC_{50}$ value of the market formulation of Acephate (75% purity) for *D. melanogaster* is only 14.45µg/ml (Rajak et al*.,* 2014). Our $EC_{50}$ values were much lower than those of this study, and the reason for this variation may be the differences in the purity of acephate used. Our results showed that the estimated chlorantraniliprole $EC_{50}$ value for *D. melanogaster* is 0.029µg/ml. According to earlier investigations, the estimated $EC_{50}$ value of chlorantraniliprole for adult *D. melanogaster* flies is 0.73µg/ml (Gao et al., 2021), and the $EC_{50}$ value of chlorantraniliprole for *Spodoptera exigua* (Lepidoptera: Noctuidae) is only 6.7 µg/L (Lai & Su, 2011). Huang et al. (2016) determined that the $EC_{50}$ value of chlorantraniliprole at 72 hours for *Chilo suppressalis* (Lepidoptera: Crambidae) is 210 µg/L.

Sub-lethal exposure to acephate and chlorantraniliprole elicited a wide range of adverse effects on lifecycle parameters like hatchability, pupation, emergence, and stabilised body weight of the adult flies. The hatchability percentage gives a clear picture regarding early sublethal toxic effects. Our study revealed a concentration-dependent decrease in hatchability in the treated group, which is significant at higher concentrations (0.63µg/ml). It was reported that Acephate's sub-lethal doses influenced the egg hatchability in *Dysderus cingulatus*, in a concentration-dependant manner (Jamal, 2015). It revealed the Acephate-induced developmental delay in *Drosophila*. Chlorantraniliprole-treated groups showed a concentration decrease in hatchability. A significant reduction in hatchability was observed only in the eggs treated with $1/3^{rd}$ of $EC_{50}$ concentrations (19.6±2.6 $p<<0.05$). Chlorantraniliprole is a contact and systemic insecticide, so the chance of absorption through the cuticle suggests significant ovicidal and larvicidal activity Bassi et al. (2007). A comparative study of three insecticides (thiamethoxam, acephate and deltamethrin) on zebrafish (Liu et al., 2018), acephate and deltamethrin

induced developmental delay, malformation and decreased embryonic surface tension. The dose-dependent toxicity of compound Agallol3 in the hatchability of *Drosophila* was reported by Gayathri and Krishnamurthy (1981). Ioriatti et al. (2008) previously reported that infield trials of chlorantraniliprole against *Lobesia botrana,* when applied at a field rate of 35 mg/L, caused more significant than 20% egg mortality. It indicated that chlorantraniliprole was a potent chemical that induced egg mortality.

The present investigation revealed a reduction in the hatching rate of eggs, which may have resulted from the insecticide accumulation within the eggs, ultimately leading to their direct demise. Inappropriate yolk integration may have prevented the embryo from completing its developmental stages when eggs are deposited but do not hatch (Kaur et al., 1993). It was validated by the transcriptome analysis in acephate and chlorantraniliprole-treated groups, which showed regulated levels of the yolk protein gene (*Yp3*). The over-transcribed vitellogenin protein may be involved in the inappropriate incorporation of yolk. The observed decrease in egg hatching may be due to the failure of the embryos to pierce the surrounding vitelline membrane, probably due to a weakened chitinous mouth hook assembly required for hatching, or may be due to extra thickening of the chorion layer (Wilson & Cryan, 1997). Genes encoding "structural constituent of chitin-based larval cuticle" showed down-regulation in acephate and chlorantraniliprole-treated larvae. More than ten genes were downregulated compared to the control, with the highest logfc value in both samples. Moreover, down-regulated chitin structural genes may be the reason for weakened mouth assembly.

In insects, pupation is metabolically an active period in metamorphosis, during which drastic developmental rearrangements occur. The initiation of pupation can be used as a developmental transition point to assess the growth pattern alterations in *Drosophila sp*. (Zinke et al., 1999). Our results illustrate a significant rise in early pupation rate in higher concentration treated groups, possibly due to concentration-induced reduction in larval duration. Transcriptome analysis validated that three genes involved in instar larval development are differentially expressed, one gene with significant down regulation. These differentially expressed larval

development genes may be one of the reasons for the early pupation in pesticide-treated groups. The process of pupation starts at the end of the third instar larval period; towards the end of the third larval instar, a pulse of ecdysone causes puparium formation, which starts the prepupal stage of development (Robertson, 1936, and Jiang et al., 1997). In the acephate-treated group, two ecdysone inducible genes are significantly up-regulated with high log2fold value (*Eig71Ec*-3.38, *Eig71Ec*-3.15). Chlorantraniliprole toxicity also induced differential expression of ecdysone-related genes. Likewise, pesticides may alter the micro-environment of the test organisms and subsequently modify the early developmental events in contrast with the control.

Pesticide-induced toxicity may act as stress on the medium; they reduce the larval period to avoid toxic stress. It was cleared from the findings of Ashburner et al., (2015) high temperature-induced early pupation in *Drosophila*. The results corroborate Podder and Roy (2014) ideas, which suggested the exposure-dependent alterations in the pupal formation of *D. melanogaster* to toxic chemical Cryolite. Even though the number of pupae that appeared on the fourth day was high in a higher concentrated pesticide medium, the total number of pupae on $6^{th}$ day is inversely proportional to the pesticide concentrations (in acephate and chlorantraniliprole). The current study illustrated a concentration-dependent decrease in pupation rate on the $6^{th}$ day of exposure. The decreased pupal number may be associated with the genes involved in the pupation. In acephate-treated larvae, three genes involved in pupation show variation; two genes were down-regulated out of three. Chlorantraniliprole-treated groups also showed remarkable changes in gene expression patterns.

The adverse effects of acephate on the development of the test organism were evident in a significant decrease in the number of adult flies. This observation coincides with previous studies on the effects of various environmental chemicals and industrial waste on different organisms (Gupta et al., 2007; Sidhique et al., 2009; Sharma et al., 2012). The emergence of the organism has been a robust endpoint for detecting lifecycle anomalies (Pauman et al., 2008). Our study reaffirms the lethal nature of acephate, causing the death of insects in their larval or

pupal stages, resulting in reduced emergence of adult flies. Acephate, by blocking AChE, interferes with the standard transmission of nerve impulses in synaptic regions (Wang et al., 2013).

Circadian clocks regulate pre-adult development and emergence in *D. melanogaster* fruit flies. Faster clocks accelerate development, while slower clocks slow it down (Kumar et al., 2007). *Drosophila* flies' adult emergence timing is influenced by their developmental state, developmental clock phase, and environmental conditions (Pittendrigh, 1974; Qiu & Hardin, 1996; Pittendrigh, 1954). The differential gene expression study of acephate and chlorantraniliprole confirms the alterations in the genes related to the circadian clock. In the treated group, the four genes regulating circadian rhythm were up-regulated. Out of four genes, the up-regulated *E23* gene (early genes at 23) showed significant up-regulation with a 1.5 log2fc value.

Furthermore, five genes involved in the "positive regulation of circadian cycle" were differentially expressed, with three genes being down-regulated, in the group treated with acephate. Likewise, the chlorantraniliprole-treated groups showed differential expression of *SIFa* genes that control the "positive regulation of circadian cycle." The differential expression of genes involved in the circadian cycle may contribute to the impaired emergence pattern in acephate and chlorantraniliprole-treated *D. melanogaster.*

The test organism's body weight served as a valuable indicator to show the impacts of additive toxicity. According to the current study, the organisms' body weight significantly decreased in high-concentration treatment groups. The weight loss observed at higher dosages might be attributed to oxidative stress-induced cell development retardation. Liu et al., 2019 demonstrated the effect of ciprofloxacin in adult *Drosophila*; at higher concentrations, ciprofloxacin produces oxidative stress, exhibits a shortened lifespan, and reduces body size and weight. Similar results were also reported (Hanning et al., 2009) in *Helicoverpa armigera* with reduced larval body mass, emergence ratio, adult longevity, and egg hatchability in both parent and offspring generation. Flies that emerged in chlorantraniliprole treatment (1/3$^{rd}$ and

$1/5^{th}$ µg/ml) had significantly lower weights (13.36 ± 0.347and 14.57 ± 0.33) compared to the control (16.48 ±0.32 mg per five larvae), and the effect is concentration-dependent. Adult body weight in dipterans is a fitness-related trait since it is directly connected to the flying ability, fecundity, and the number of eggs in females and sperm count in males (Sibley et al., 2001; Ponlawat& Harrington, 2007). Weight loss may be due to reduced feeding. Reduced feeding habits of *H. armigera* resulted in reduced body weight in chlorantraniliprole treatment. *H. armigera* stopped feeding after exposure for 20.3 minutes. It is speculated that long-time starvation may be the reason for the body weight decline. In some instances, oxidative stress may be the reason for reduced body weight. In ciprofloxacin-treated *Drosophila,* oxidative stress induced a shortened lifespan and reduced body size and weight (Liu. 2019). The chlorantraniliprole may act as a stressor to induce feeding cessation. From the previous reports, feeding and body weight are directly related. Genes in the feeding process also show differential expression in Acephate and chlorantraniliprole-treated groups. When we focused on pesticide-treated groups, gene *Lk* involved in the "negative regulation of feeding behaviour" showed significant up-regulation with a 2.39 log2fold value in chlorantraniliprole-treated groups and also significant down-regulation of a gene involved in "feeding behaviour". *Lk* genes showed significant up-regulation in the chlorantraniliprole-treated experiment with a 2.9 log2fc fold value.

Gene expression results confirmed that one acetylcholine-gated channel complex gene up-regulated in the acephate-treated group. This over-transcribed gene also contributes to unbalanced synaptic impulses. This acephate activity might produce nervous imbalance inside the body, resulting in the death of exposed flies, thereby accounting for variation in the emergence percentage of adult flies. Chlorantraniliprole acts on insect ryanodine receptors; the transcriptome study proved the down-regulated *ryr* genes in chlorantraniliprole treated larvae.

Embryotoxicology studies primarily examine early pesticide toxicity. From the hatchability experiment, a concentration-dependent decrease in egg hatchability was observed. The exposure of acephate and chlorantraniliprole to the eggs of *D. melanogaster* results in morphological changes, which include the change in the

length and width of the eggs. As a result of increasing exposure to toxicants throughout time, the eggs' length and width decreased. In a related study where the length and width of the eggs were measured after they were exposed to the toxicant mercuric nitrate, it was found that the exposure to the toxicant had no discernible impact on the length and width of the eggs when compared to the embryonic and maturation times. There was a complete impairment in foetal growth, and none of the larvae hatched after being exposed to 200 mg/l and 400 mg/l (Abnoos et al., 2013). The eggs in this study are stained with DAPI and examined under a fluorescence microscope using the DAPI filter. The purpose of the DAPI stain is to examine nuclear movement and cell morphology. The eggs treated with the toxicant showed irregular cell multiplication instead of control irregularities in cell movement. The genes involved in various embryonic processes show differential expression in acephate and chlorantraniliprole-treated groups. More genes are differentially expressed in the treated group than in the chlorantraniliprole group. In acephate-treated groups, genes participated in "embryonic development via the syncytial blastoderm", "post-embryonic development", "regulation of embryonic cell shape", "embryonic pattern specification", and "morphogenesis of embryonic epithelium" were differentially expressed. These genes may directly or indirectly contribute to the profound changes in embryo development. Chlorantraniliprole-treated larvae showed differential expression of the following gene groups; "embryonic development via the syncytial blastoderm", "morphogenesis of embryonic epithelium", and "regulation of embryonic cell shape".

In this investigation, the toxicant exposure causes the egg to absorb neutral red. When compared with control during the first hour, neutral red uptake is visible and increases during the third hour. Utilising neutral red uptake, one can assess changes in the vitelline membrane. In the head region, staining is more visible, indicating neuron degeneration. A related study found that the creation of the dorsoventral axis, which four serine protease family members regulate, is caused by a ventral signal from the perivitelline region. Eggshell biogenesis depends on Nudel protease, and mutations in the *Nudel* gene result in eggs with aberrantly permeable vitelline membranes to neutral red dye. In vitro membrane cross-linking, eggshell

biogenesis, and embryonic patterning, Nudel protease is critical (LeMosy, & Hashimoto, 2000). Even though the Nudel protein plays a significant role in embryonic development, our transcriptome result validated that *Nudel* genes were neutrally regulated in both experimental groups. Neutral red penetration to the embryonic cuticle is possible only when the embryonic chitin layer is weakened. The weakened embryonic cuticles may result from gene expression related to the "chitin-based embryonic cuticle biosynthetic process". This group of genes showed downregulation in acephate and chlorantraniliprole experimental groups.

Sublethal exposure to acephate and chlorantraniliprole elicited a wide range of adverse effects on lifecycle parameters like hatchability, pupation, emergence, and stabilised body weight of the adult flies. Toxicity-induced differences in gene regulation and expression may be the primary reason for the adverse effect on the cycle.

## 1.6 CONCLUSIONS

Acephate and chlorantraniliprole are widely used to control a broad spectrum of pests. Apart from toxic to-target pests, non-target organisms are also affected. The present study highlights areas like $EC_{50}$ values for acephate and chlorantraniliprole, assessing concentration-dependent effects on the early developmental stages, body weight, and survival rate. The investigation unequivocally demonstrates how acephate and chlorantraniliprole affect *D. melanogaster*. These parameters serve as functional indices for researching the mode of action and toxicity assessment. Transcriptome analysis revealed some of the genes responsible for the pesticide-induced effects.

The study concluded that

- The $EC_{50}$ values of acephate and chlorantraniliprole were 1.9 and 0.029 µg/ml) respectively.
- Acephate and chlorantraniliprole significantly impaired the entire organismal growth and early development.

- In the present study, the survival rate and weight of the intoxicated organism showed sound differences. This is a promising toxicological index for evaluating the overall life process.
- Early embryonic studies reveal the effects of both pesticides qualitatively and quantitatively.

# Chapter 2

# Transcriptomic profiling of sub-lethal concentration of Acephate and Chlorantraniliprole on *Drosophila melanogaster*

## 2.1 INTRODUCTION

In today's agriculture, pesticides are essential since they help shield crops from various pests and diseases. Pesticides are now necessary for modern agricultural operations due to the increased demand for food security worldwide (Popp et al., 2013). However, the widespread use of these compounds has given rise to severe worries about their possible negative impacts on the environment and public health. Particularly concerning is the issue of pesticide toxicity, which can seriously affect both human health and the ecological balance (Alengebawy et al., 2021). With the help of transcriptome analysis, a potent tool in contemporary genomics, we can investigate how genes dynamically express themselves in response to pesticide exposure. Understanding how pesticides modify gene expression at the transcriptome level will help us to identify essential pathways and processes that affect these toxic substances (Simões et al., 2018).

Attempts to study gene expression started in the 20$^{th}$ century. In the early 20$^{th}$ century, scientists began deciphering the genetic code, highlighting the function of RNA in protein production and DNA as the genetic material. Initially, researchers concentrated on individual genes and their expression patterns using northern blotting and quantitative PCR. Several sophisticated tools for genome-wide expression monitoring have been available and often employed since the introduction of microarray technology. It gave researchers a more complete picture of gene expression patterns, enabling them to simultaneously quantify hundreds of gene expressions in a single experiment. The technical and scientific possibilities for studying gene expression offer a snapshot of the entire genome with an accuracy that would have been unthinkable some years ago. A significant advancement occurred with the introduction of high-throughput RNA sequencing (RNA-Seq) in the mid-2000s. RNA-Seq supplanted microarrays by as the most popular technique for transcriptome study. It provided increased dynamic range, improved accuracy, and the capacity to identify new transcripts and alternative splicing. The transcriptome displays a comprehensive collection of messenger RNAs under specific conditions (Stahl et al., 2012). Whole transcriptome analysis measures gene expression heterogeneity in cells, tissues, organs, and even the entire body. It captures coding

and non-coding RNA (Jiang et al., 2015). The transcriptome covers the whole set of transcripts in a cell, together with their abundance, for a particular physiological state or developmental stage, which is known as the transcriptome. Comprehending the transcriptome is fundamental to unravelling the functional components of the genome, revealing the molecular components of cells and tissues, and understanding both growth and illness (Wang et al., 2009). This analysis is also significant because it offers the initial stages of functional evaluation and annotation of genes and genomes previously identified through DNA sequencing (Granjeaud et al.,1999), creates schematics for reconstructing genetic interaction networks to comprehend biological systems, growth, and development, and cellular processes (Altman & Raychaudhuri, 2001); develops molecular fingerprints of disease processes and prognoses to identify possible targets for diagnostics and medication development (Hsiao et al., 2000; Celis et al., 2000), and provides chances to investigate the interaction between the pathogen and the host to find novel solutions for both preventative and therapeutic intervention (Manger & Relman, 2000). Comprehensive transcriptome analysis offers a basis for investigating genetic networks and regulatory pathways that govern both qualitative and quantitative traits crucial to human health and agriculture (Jiang et al., 2015).

The transcriptome shows the entire set of messenger RNAs present under defined conditions. Since RNA synthesis is essential to transferring genetic information in prokaryotic and eukaryotic cells, extracted RNA has been the focus of numerous analytical and diagnostic methods. The availability of simple and efficient systems for generating synthetic RNA has likewise led to the development of methods for researching bio-molecule interactions with specific RNA sequences (Stahl et al., 2012). Various technologies analyse mRNA expression levels or differential mRNA expression, including Northern blots, RNase protection Assay (RPA), RT-PCR, macroarrays, microarrays, differential display RT-PCR, serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS), expressed sequence tag (EST). Sequence-based methods provide the most specificity, while hybridization-based methods are less expensive and can process more samples. None are considered the best (Fryer et al., 2002).

Genes can produce many RNA species, and transcription patterns of genes are frequently complex. One technique for analysing RNA in a lab setting is the northern blot. Separated pure RNA fragments from a biological sample (blood or tissue) are passed through a matrix or sieve-like gel with the help of an electric current, allowing the smaller fragments to pass through faster than the more significant pieces. After the RNA fragments are removed from the gel or matrix, they are exposed to a DNA probe tagged with a chemical, fluorescent, or radioactive substance on a solid membrane. The tag makes it possible to see RNA fragments in the northern blot that have complementary sequences to the DNA probe sequence (Sabelli & Shewry, 1995). One experiment can lead to multiple conclusions. Measuring the transcript's size and identifying transcripts with alternative splices is possible. RNA abundance in many samples can be directly compared based on their relative abundance using northern blotting. Currently, it is possible to determine the absolute amount of mRNA abundance by spiking RNA samples with varying quantities of an artificial sense-strand RNA target or exogenous standard to create a standard curve that can be used to compare the signal from experimental samples. However, these technologies have significantly advanced gene expression research without limitations. Relative to RT-PCR and nuclease protection assays, northern blotting is often less sensitive. For blot hybridisation to be used for detection, a sequence of DNA or RNA needs to be copied about 100,000 times (Streit et al., 2009).

On the other hand, single copies of DNA following reverse transcription (RNA) can be amplified by PCR to easily measurable quantities. Most researchers agree that the conventional Northern blot is the "gold standard" for measuring mRNA expression levels because it analyses one gene at a time. A northern blot's intrinsic drawback is that it can only typically analyse one gene at a time. Large-scale quantitative comparisons are further undermined by blot-to-blot variability. Numerous blots are needed to analyze more than ten to twenty RNA samples, and the procedures use a significant amount of RNA and other reagents, which adds to the expense and time of large-scale analysis (Fryer et al., 2002). Even though these technologies paved the way for milestones in the gene expression study field, there

are limits to these technologies. For instance, RNA extraction is necessary for PCR-based and northern blotting methods; however, RNA molecules may be destroyed. Furthermore, the location of gene expression inside cells or tissue cannot be ascertained by Northern blotting or PCR techniques (Wang et al., 2012 and Hittu et al., 2021).

Northern blotting techniques detect and size mRNA species, which are not typically a function of PCR or RPA. They involve denaturing and size-separating RNA fragments on agarose gels, then transferring the RNA ladder onto 1.nitrocellulose. This method can be used to isolate complete novel transcripts from mRNA pools, although it is time-consuming and cumbersome. RNAse protection assays (RPA) offer 20-50 times greater sensitivity than northern blots, allowing accurate identification and quantification of different mRNA species within gene families, even with high sequence homology. The RNase protection assay is a sensitive technique used to detect and measure the abundance of specific mRNAs in total cellular RNA samples. It uses 32P-labeled antisense RNA probes. These probes are hybridised in solution to their corresponding cellular mRNAs. After that, non-hybridising (single-stranded) RNA species are broken down by RNases, the RNases are eliminated by treating the RNA with proteinase K, the cRNA: mRNA complexes are extracted with phenol, and the hybridising cRNA fragments are isolated electrophoretically (Ma, 1996). This technique relies on forming an absolute specific cRNA: mRNA hybrid, making it a quantifiable method for multiple mRNA species detections. RNAse protection assays offer high throughput screening of numerous transcripts but require large amounts of radioactivity and high levels of mRNA purification. Despite these challenges, they are more easily executed, quantified, and adapted to large samples than northern blotting (Sanbrook et al., 1989). The primary drawback of RPA is the need for more details on transcript size (Qu & Boutjdir, 2007).

RT-PCR for mRNA expression analysis gained popularity in the early 1990s. The real-time, fluorescence-based RT-PCR is a genomic age-enabling technology for detecting mRNA (Bustin, 2000). This method has two steps: reverse transcription (R.T.), the initial step of the process, and the second stage, which is

necessary to maximise the sensitivity and specificity of the PCR. In this procedure stage, the RNA virus enzymes are used to transcribe the viral RNA genome into a template for host transcription systems *in vivo*. The avian myeloblastosis virus (AMV reverse transcriptase) and the Molony murine leukaemia virus (Mo-MLV reverse transcriptase) were the sources of the first RT enzymes ever employed. Genetically altered versions of these enzymes with higher activity have emerged as the preferred agents. Reactions involving mRNA and nucleotides are carried out at 42°C, and the enzymes are relatively heat-labile (especially when compared to Taq polymerase). Its homogeneous assay avoids post-PCR processing, offers a wide dynamic range, and realises the PCR's quantitative potential (Ginzinger, 2002). Quantitative real-time polymerase chain reaction (QRT-PCR) is a method for continuously monitoring amplification processes using fluorescent reporter dyes. It is based on PCR reaction kinetics and can detect sequence-specific PCR products as they accumulate in real-time, and it measures a small amount of DNA sample. It measures the increase in fluorescent signal, corresponding to DNA production during each PCR cycle. The process involves converting RNA to cDNA, PCR amplification, and detecting amplified products.

Quality control is recommended to inconsistencies and maximise reproducibility (Jozefczuk & Adjaye, 2011). Reverse transcription quantitative PCR (RT-PCR) is a highly accurate, fast, and sensitivity-driven method for gene expression analysis, making it the gold standard for medium-throughput analysis. However, it requires careful execution due to numerous quality issues affecting the results' accuracy and reliability (Derveaux, 2010). Quantitative PCR (RT-PCR) offers a wide dynamic range and higher reliability than conventional PCR due to its ability to identify individual reactions, which deviates in amplification efficiency and provides more precise quantitative results (Wilhelm & Pingoud, 2003). Real-time PCR offers numerous advantages for measuring gene expression compared to previous techniques. It doesn’t require post-amplification processing and can generate quantitative data with an accurate dynamic range of 7 to 8 log orders of magnitude (Morrison et al., 1998). The sensitivity of real-time PCR assays is 10,000–100,000 times higher than that of RNase protection assays (Wang & Brown,

1999), 1000 times higher than that of dot blot hybridisation (Malinen et al., 2003), and it can even identify a single transcript (Palmer et al., 2003). Furthermore, real-time PCR assays have lower coefficients of variation (SYBR® Green at 14.2% and TaqMan® at 24%) than endpoint assays like band densitometry (44.9%) and probe hybridisation (45.1%) (Schmittgen et al., 2000). They can also reliably detect gene expression differences as small as 23% between samples (Gentle et al., 2001). The primary disadvantage of real-time PCR is the high cost of the reagents and equipment needed. Furthermore, it has a significant drawback due to its high sensitivity, high experimental design, and need for a thorough understanding of normalisation techniques for accurate conclusions (Marisa & Juan, 2005).

The comparative EST sequence study was made accessible by developing automated, high-throughput sequencing technology. Comparative EST analysis, a large-scale gene expression analysis, was first used in the 1990s (Adams et al., 1993; Lee et al., 1995). It involves creating cDNA libraries representing all expressed mRNAs in a cell or tissue, sequencing thousands of cDNAs, and creating a database to identify and count expressed genes. This method is reliable and cost-effective but has faced criticism for its high sequencing costs and low abundance of transcripts. EST sequencing remains crucial for studying organisms without sequenced genomes and discovering novel transcripts, particularly splice variants (Adams et al., 1991; Jongeneel, 2000; Nagaraj et al., 2007).

While the expressed sequence tag (EST) methodology assesses a restricted set of genes at a time, methods such as complementary DNA subtraction and differential display offer partial information regarding differences in gene expression (Hedrick et al., 1984; Liang & Pardee, 1992 and Adams et al., 1992). SAGE, or serial gene expression analysis, uses a short nucleotide sequence tag and concatenates numerous tags inside a single clone to enable a quick, in-depth examination of thousands of transcripts. If these markers are separated from a specific point, they have sufficient information to identify a transcript individually. As in computer serial transmission, concatenating these tags enables practical serial analysis of transcripts (Fields et al., 1994; Velculescu et al., 1995). SAGE is a method for generating short nucleotide sequences (tags) from mRNA species. It

involves extracting poly ($A^+$) RNA, transcribing it into biotinylated double-stranded cDNA, and digesting it with a type II restriction enzyme. The fragments are isolated using streptavidin beads and ligated to two different linkers. The resulting sequence data is analysed to identify genes expressed in cells and their levels. This information forms a library that can be used to analyze differences in gene expression between cells. SAGE can be used to identify disease-related genes, analyse drug effects on tissues, and provide insights into disease pathways. Its most attractive feature is its ability to quantitatively evaluate the expression pattern of thousands of genes without prior sequence information. This feature has been used in three powerful applications: defining transcriptomes, analysing differences between cancer cell gene expression patterns, and identifying down-stream targets of oncogenes and tumour suppressor genes (Velculescu et al., 2000; Tuteja & Tuteja, 2004).

MPSS is a method that uses sequencing for gene identification and parallel processing to provide ultrahigh-throughput analysis. It involves creating a cDNA library on microbeads and imaging through complex, multistep sequencing reactions. This method provides sequence-level gene identification and can identify previously unknown transcripts. The parallel-processing system simultaneously interrogates hundreds of thousands of transcripts, providing unprecedented sensitivity. MPSS is most valuable when used with an existing genome or EST database (Brenner, 2000; Tyagi, 2000).

DNA microarrays, also known as 'gene chips,' are a popular global platform for gene expression analysis. They create a spotted array of thousands of different DNA molecules, such as oligonucleotides or cDNAs, corresponding to thousands of genes (Hsiao, 2001; Kurella, 2001). A biochemical reaction series generates a fluorescently labelled cRNA or ss-cDNA probe, which is hybridised into the microarray and scanned with a laser scanner. The expression levels are measured by the fluorescence intensity of the bound probe to each spot (Nagasawa, 2001; Moch et al., 1999). Multiple microarray platforms exist from various commercial entities, and many institutions have robotic equipment to create their custom microarrays. The predominant microarray platforms are affymetrix oligonucleotide microarrays

and glass slide cDNA or oligonucleotide microarrays (Lipshutz, 1999; Jain, 2000). However, they are relatively expensive, have uncertainty regarding binding specificity, and exhibit lower sensitivity compared to sequence-based methods. Microarray assays, a fundamental departure from porous membranes, enable parallelism, miniaturisation, multiplexing, and automation (Schena, 1996; Lockhart, 1996). These features provide performance specifications that earlier technologies could not achieve. Microarray assays enable the acquisition and analysis of massive parallel data, thereby accelerating experimental progress and facilitating meaningful comparisons between genes or gene products (DeRisi et al., 1997; Hsiao et al., 2000). They may eventually allow the analysis of the entire human genome in a single reaction. Miniaturisation of conventional assays is a trend in biomedical research, reducing reagent consumption, reaction volumes, and sample concentration. Multiplexing, the process of analysing multiple samples in a single assay, increases the accuracy of comparative analysis. Advances in manufacturing technologies and automation are increasing the proliferation of microarray assays. DNA microarrays can be used to identify genes, investigate the physiological impact of genetic variations, and/or screen for mutations and polymorphisms (Heller, 2002). Early disease identification is already facilitated by genomic profiling, which may also facilitate early intervention. Furthermore, genomic research should advance our understanding of the molecular characteristics of diseases, aid in the development of more accurate diagnostic tools, and ultimately lead to new targets for therapeutic intervention (McCarthy et al., 2013).

Microarrays are becoming increasingly popular in bioinformatics, but several challenges can arise when using them. These include technical issues with the chip, labelling, and scanning, as well as bioinformatics issues such as image analysis and gene identification (Fryer et al., 2002). The first step is to fabricate a microarray using a glass slide, a spotting robot, and DNA for spotting. The quality of the slide is essential, and it should be evenly planned to reduce optical noise. The source of DNA for spotting is a significant consideration, and there needs to be a clear consensus on the best material to use for spotting on microarrays (Knight, 2001). Many commercial arraying instruments are effective, but they vary in terms of cost,

throughput, spot density, and ease of use. The buffer used to spot a microarray determines the size and morphology of the spots, and the DNA needs to be covalently linked to the slide using high-energy ultraviolet rays or exposure to high temperatures (Romanov et al., 2014). Hybridising the labelled probe to the immobilised target is critically dependent on efficient mixing, which can significantly reduce hybridisation time and increase data reproducibility from chip to chip (Stears et al., 2000). Confocal laser scanners produce high-quality digital images of fluorescent signals from the chips, but they should be adjusted to maximise the dynamic range and reduce signal saturation. Image processing is the final step for transforming microarray scanned images into a database of expression values (Yang et al., 2001).

Hybridisation-based approaches have been developed to deduce and quantify the transcriptome using custom high-density oligo microarrays. These methods have high throughput and are relatively inexpensive, but they have limitations, including reliance on genome sequence knowledge, high background levels, and a limited detection dynamic range. Comparing expression levels across different experiments can be difficult and requires complex normalisation methods (Okoniewski et al., 2006; Royce et al., 2007). Sequencing-based methods determine cDNA sequences but are limited by their low throughput, high cost, and non-quantitative nature. Tag-based methods, such as SAGE, CAGE, and MPSS, offer high throughput and precise measurement of gene expression levels. However, these methods rely on expensive Sanger sequencing technology, cannot map short tags to reference genomes, and only analyze a portion of transcripts. RNA-Seq technology is an advanced approach to large-scale RNA analysis, combining the advantages of previous methods. It enables low-cost monitoring of the transcriptional landscape of a whole genome, without introducing biases associated with arrays (Wang et al., 2009). It also provides information on transcript structures (exon-exon boundaries) without cloning biases. RNA-Seq is sensitive enough to detect transcription for genes with low expression levels, which are usually missed by EST analysis. This technology offers a more accurate and efficient method for RNA analysis (Kukurba & Montgomery, 2015). With the advent of high-throughput next-generation

sequencing (NGS), RNA analysis using complementary DNA (cDNA) sequencing has become possible, significantly transforming the field of transcriptomics (Royce et al., 2007).

RNA-Seq has been used to quantify expression levels, identify differentially expressed genes (Wilhelm et al., 2008; Cloonan et al., 2008), and measure expression outside annotated loci. It has also been used to detect alternative splice forms (Nagalakshmi et al., 2008; Mortazavi et al., 2008), identify new transcriptional events, and refine annotated gene structures (Wilhelm et al., 2008; Mortazavi et al., 2008). RNA-seq is an experimental procedure that produces cDNA sequences from entire RNA molecules, enabling the quantification of gene expression under specific conditions. It has a dynamic detection range (>8,000 fold), low background signals, and single-base pair resolution (Wang et al., 2009). It reveals open reading frames containing "hypothetical" genes and, unlike hybridisation-based technologies, can be applied to species lacking a complete reference genome assembly (Kolker et al., 2004; Galperin et al., 2010). RNA-seq is a tool for quantitative analysis of RNA and investigative analysis of novel transcript species, including long non-coding RNA, miRNA, siRNA, and small RNA classes (Hangauer et al., 2013; Trapnell et al., 2010; and Robertson et al., 2010). It has been used to identify enhancer RNA, provide details regarding transcriptional start sites, and study biological problems (Liu et al., 2011; Arnold et al., 2013). Moreover, gene fusions, endogenous retrotransposons, disease-associated single-nucleotide polymorphisms, and allele-specific expression can all be detected (Erwin et al., 2014). In stem cell biology and neuroscience, single-cell RNA-seq analysis is frequently used to investigate cellular heterogeneity and diversity (Wilhelm et al., 2008; Saliba et al., 2014; Wilson et al., 2015).

Although preparation, fragmentation, and library creation can be biassed, RNA-seq technology is thought to be impartial (Wang et al., 2009). When oligo primers are used for poly (A) mRNA enrichment but canonical histones and non-coding RNA are absent, this can be undesirable or unfavourable (Marzluff et al., 2008; Yang et al., 2011). Interpreting RNA-Seq datasets needs sophisticated computational programs. Because RNA-seq data creation is a dynamic process, new

computational methods are required on a regular basis. While mature tools meet basic requirements, challenges like differential gene expression analysis and fusion gene detection require comprehensive solutions. A typical RNA-seq workflow includes three primary sections: experimental biology, computational biology, and systems biology. Experimental biology encompasses the methods of RNA collection, first-strand synthesis, and library building, which generate millions of short reads from the NGS sequencer and are included in the experimental section. RNA preparation methods vary depending on the sequencing platform and purpose, but sample quality is crucial for unbiased analysis. Poly-A-selection is effective for whole-transcriptome analysis, while the Illumina platform's labelled molecules achieve high mRNA capture efficiency (Turnbull et al., 2020).

Transcriptome analysis has been established as an enabling technique in the drug discovery process, providing a significant benefit of the genomics revolution in biomedical research. Pre-clinical toxicity is the one field in drug development where transcriptome analysis is expected to have the most considerable influence. Transcriptome analysis can significantly enhance the drug safety assessment process by enabling drug development teams to identify potential toxicity liabilities early and move forward with only those molecules that have both efficacy at the target and a low potential for toxicity in the human population. This is possible in conjunction with other new high-content data-generating technologies. In preclinical toxicology, transcriptome analysis is mainly used to 1) comprehend toxicity mechanisms, 2) forecast toxicity, 3) create *in vivo* and *in vitro* surrogate models and screens; and 4) create toxicity biomarkers (Storck et al., 2002; Yang et al., 2004; Searfoss et al., 2005; Qin et al., 2016; and Szabo & Devlin, 2019).

Transcriptomic strategies, such as RNA-Seq, have been widely used in biomedical research for disease diagnosis and profiling (Ozsolak & Milos, 2011). These methods identify transcriptional start sites, alternative promoter usage, and novel splicing alterations, which are crucial for understanding human disease (Costa et al., 2013). RNA-Seq can also identify disease-associated single-nucleotide polymorphisms (SNPs), allele-specific expression, and gene fusions, contributing to

disease-causing variants (Khurana et al., 2016). Its potential for understanding immune-related diseases is expanding rapidly (Proserpio & Mahata, 2016).

Human pathogen RNA-Seq has emerged as a reliable technique for measuring changes in gene expression, detecting new virulence factors, predicting drug resistance, and elucidating host-pathogen immunological interactions (Suzuki et al., 2014; Wu et al., 2008). Dual RNA-Seq profiles RNA expression in pathogens and hosts, enabling the study of dynamic response and interspecies gene regulatory networks. With the use of this method, it is possible to investigate interspecies gene regulatory networks and dynamic responses in both interaction partners, from the initial contact to invasion, pathogen persistence, and eventual host immune system clearance (Westermann et al., 2012; Durmuş et al., 2015).

Transcriptomics identifies genes and pathways that respond to environmental stresses, revealing novel transcriptional networks within complex systems. Comparative analysis of chickpea lines reveals distinct profiles associated with drought and salinity stresses (Garg et al., 2016). A co-regulated group of genes essential for producing and maintaining biofilms was identified by analysing gene expression during the biofilm formation process of the fungal pathogen Candida albicans (García-Sánchez, 2004). Additionally, transcriptome profiling offers vital insights into drug resistance mechanisms.

All transcriptomic approaches have been especially helpful in discovering the roles of genes and identifying those responsible for specific phenotypes. A correlation between the phenotype and genes linked to metal uptake, tolerance, and homeostasis was found in the transcriptomics of Arabidopsis ecotypes exhibiting hyperaccumulation of metals (Verbruggen et al., 2009). It has been possible to improve the annotation of gene activities in economically significant organisms (like cucumbers) (Li et al., 2011) or endangered species (eg, koalas) by integrating RNA-Seq datasets from various tissues (Hobbs et al., 2014). Since RNA-Seq read assembly does not rely on a reference genome, it is perfect for studying the gene expression of non-model animals with under-developed or nonexistent genomic resources. Additionally, RNA-Seq can be used to locate previously unidentified

protein-coding regions in genomes that have already been sequenced (Grabherr et al., 2011). Transcriptomics is most frequently used to analyse a cell's mRNA content. Nevertheless, non-coding RNAs with direct activities (such as those involved in protein translation, DNA replication, RNA splicing, and transcriptional control) that are not translated into proteins can also use the same methods (Christov et al., 2006; Kishore & Stamn, 2006; & Hüttenhofer et al., 2004). Numerous non-coding RNAs have an impact on disease states, such as neurological, cardiovascular, and cancer disorders (Esteller, 2011).

Molecular initiating processes and downstream transcriptional and proteomic events provide valuable insights into adverse outcome pathways, which can be used to predict the effects of chemicals on physiological systems (Liang et al., 2018). Toxicity studies aim to prevent adverse health effects from human exposure to xenobiotics. Detecting target organ toxicity before permanent damage is crucial. Understanding the mechanisms underlying xenobiotic-induced toxicity is essential for managing potential adverse health effects. The highly dynamic transcriptome responds sensitively to xenobiotic exposure. Expression profiling of the entire transcriptome can be a sensitive indicator of toxicity. Differentially expressed genes and products can be used as biomarkers for detecting exposure or toxicity. Global gene expression profiling is a sensitive and mechanistically relevant approach for detecting and understanding target organ toxicity associated with xenobiotic exposure. Past studies have demonstrated the application of transcriptome analysis in the early detection of toxicity and elucidating molecular mechanisms (Cui & Paules, 2010).

Several studies have tried to implement transcriptomics analysis to gain insight into the interactions of combined chemicals. To connect phenotypic and toxicant-driven changes in gene, protein, and metabolite expression, a new branch of toxicology has arisen called toxicogenomics, which links toxicology with information-rich genomic technologies. The goal is to produce more predictive data (David, 2020). Toxicogenomics can enhance the efficiency of toxicity testing by assessing gene regulation across the genome, enabling the rapid classification of compounds based on their expression profiles, and identifying potential mechanisms

of action and genes under similar regulatory control (Aardema & MacGregor, 2003). Transcriptomics can be utilised in genetic toxicology to follow up on positive genotoxicity results and elucidate the mechanism of action (Newton et al., 2004). Advances in DNA array technology allow gene expression microarrays to measure and characterise genome-wide gene expression changes, significantly impacting the field (Bumgarner, 2013). The main goal of toxicity studies is to prevent adverse health effects from human exposure to xenobiotics. Detecting target organ toxicity and understanding the underlying mechanisms is crucial. The highly dynamic transcriptome can respond sensitively to xenobiotic exposure. Expression profiling of the entire transcriptome and identifying genes affected by exposure can be sensitive indicators of toxicity. Differentially expressed genes can be used as biomarkers for exposure and toxicity detection (Waring et al., 2001; Hamadeh et al., 2002; Amin et al., 2004; Heinloth et al., 2004; Sellamuthu et al., 2012; Otava et al., 2015).

Compounds induce a specific pattern of gene expression changes based on their mode of action, and DNA damage triggers a stress response that leads to alterations in gene expression across various biological pathways (Fornace et al., 1992). These distinct expression profiles for various classes of chemicals may serve as "fingerprints," facilitating the classification of compounds and enabling the prediction of their mode of action, thereby improving the process of identifying hazards (Hamadeh et al., 2002). Previous research has demonstrated the utility of global transcriptomics in identifying target organ toxicity induced by pesticides in a timely manner and clarifying the molecular mechanisms underlying the toxicity. These are essential steps in preventing harmful health consequences that arise from human exposure to pesticides. This study will discuss pesticide-induced effects as specific examples to illustrate the application of transcriptome analysis in detecting toxicity and determining the molecular mechanisms underlying non-target organism toxicity. Using *D. melanogaster* as a non-target insect model, we conducted transcriptome research on two pesticides, namely acephate and chlorantraniliprole.

Acephate is a common organophosphate insecticide used to kill or repel insects with sucking or chewing mouthparts, such as aphids, thrips, sawflies, fire

ants, and lepidopteran larvae (Das et al., 2008). Acephate is recognised for its anti-cholinesterase activity in insects and mammals (Spassova et al., 2000), which has been linked to DNA damage in Swiss albino mice (Rahman et al., 2002). It is a potent mutagen, increasing chromosomal aberrations and micronuclei formation in human lymphocytes and chicks (Özkan et al., 2009). Acephate also promotes infertility in humans and induces oxidative stress in rats (Dhanushka & Peiris, 2017). In *D. melanogaster*, chronic exposure to acephate causes ROS-mediated damage at both the organismal and sub-organismal levels (Rajak et al., 2018).

Anthranilic diamide chlorantraniliprole belongs to anthranilic diamide insecticide and acts by altering ryanodine receptors (RyRs) in the sarcoplasmic reticulum membrane, resulting in acute muscular contraction brought on by an excess of $Ca^{2+}$ release in the cytosol, which stops insect feeding and kills the insect. This process causes permanent paralysis in insect bodies (Nauen, 2006). Due to their low risk, diamide insecticides control crop pests, but their broad application and indiscriminate use can significantly impact the ecological environment. Flubendiamide, chlorantraniliprole, and cyantraniliprole have been found to cause severe damage to honey bee antennal neurons (Kadala et al., 2020) and inhibit the growth rate, weight, and reproduction of earthworms (Liu et al., 2019). Acute and chronic exposure to these insecticides can cause developmental abnormalities in *Daphnia magna* embryos, and even low levels can cause ecological risks to aquatic ecosystems. These insecticides can also decrease antioxidant enzyme activities (Cui et al., 2017).

Therefore, using *D. melanogaster* as a model organism, we performed a transcriptome-based gene expression profiling and subsequent biochemical/organismal experiments to identify genes and the biological pathways significantly affected by chloranthraniliprole and acephate. We chose this organism because of its substantial genetic and genomic resources, fewer ethical challenges, and homology to humans. Moreover, using *Drosophila* complies with the guidelines provided by the European Centre for the Validation of Alternative Methods (ECVAM) (Festing et al., 1998). Due to its short life cycle, ease of handling, low maintenance expenses in the lab, and accessibility to molecular genetics with an

extensive range of publicly available resources, the *Drosophila* model has long been recognised as a versatile and potent model. The translatability of research findings from flies to humans is supported by the substantial conservation of genes encoding essential developmental and physiologic pathways across *Drosophila* and mammals (Rand et al., 2023). The *Drosophila* model helps research mechanistic and descriptive toxicology investigations. Under regulated genetic backgrounds and standardised laboratory conditions, it enables the exploration of gene-by-environment interactions. In terms of determining risk variables for hazardous exposure, this model excels. Mechanistic toxicology examines how toxins affect various biological systems, ranging from molecular to organismal levels. The fly model provides a comprehensive system for studying mechanistic toxicology at multiple levels due to its history of uncovering genetic and developmental biology (Huang & Lee, 2023).

Therefore, the primary goal of this study was to obtain high-quality transcriptome data for *D. melanogaster* using next-generation sequencing (NGS) techniques and identify genes implicated in target proteins and putative enzymes involved in the detoxification of pesticides. Moreover, identifying differentially expressed genes would offer valuable information regarding the molecular mechanisms underlying *D. melanogaster*'s responses to acephate and chlorantraniliprole treatment. The alterations in the global gene expression patterns of the control and treated groups were another goal of this investigation. Differential gene expression patterns also benefit each pesticide's specific detoxifying mechanism. The last, but not least, goal is to compare the toxicity of acephate and chlorantraniliprole at the gene expression level.

## 2.2 REVIEW OF LITERATURE

Pesticides are essential for achieving sustainable agricultural production. They are a cornerstone for pest control in a world where the population is constantly increasing, and over 2 billion people are expected to be fed in the next 40 years (Gianessi, 2013). A pest is any living thing that negatively affects human activity, whether in terms of appearance, cost, or health (Hassall, 1982). In agricultural and

urban settings where insects, fungi, and weeds must be carefully controlled, pesticides are defined as agrochemicals designed to combat the detrimental effects of pests (Cremlyn, 1991). Pesticides belong to the biocides class used in agriculture primarily to neutralise, eliminate, or severely limit pests. Pesticides can be categorised as chemical family members, such as carbamates, organochlorines, and organophosphorus compounds, or as agents targeting specific organisms, including fungi, herbicides, and insecticides (Jeyaratnam, 1990). Although using pesticides in agriculture has numerous advantages, it also poses immediate and long-term health risks. Both manufactured and naturally occurring pesticides benefit humanity, but can be toxic. They are found in air, water, and food, posing a significant threat to human health and the health of other organisms. Unintentional exposure to xenobiotics can lead to adverse health outcomes such as cardiovascular (Lippmann, 2014), renal (Prudente et al., 2018), pulmonary (Ratanachina et al., 2020), neurological (Vellingiri et al., 2022), reproductive (Sifakis et al., 2011), immune system (Lee& Choi, 2020), and cancer-related diseases (Varghese et al., 2021). The World Health Organisation estimated that 3 million individuals are hospitalised annually due to pesticide poisoning (Jeyaratnam, 1990).

Furthermore, pesticides have the potential to persist in the environment and contaminate air, water, soil, and food (Gilden et al., 2010). As a result, the toxicological research on pesticides has garnered significant interest, with over 300 publications annually (Wang & Wu, 2015). Organisms are exposed to pesticides daily and have been linked to adverse health effects. Effective strategies must be developed to prevent these effects. Early detection of toxicity and understanding the mechanisms become crucial. This includes identifying molecular targets, interacting with xenobiotics, disrupting cellular networks, and understanding the onset and progression of toxicity, as well as structural and functional impairment in target organs and systems. This prevents life-threatening adverse health effects. It requires powerful techniques to capture global changes in biological systems. Recent developments in molecular biology, computer science, mathematics, and bioinformatics have led to the emergence of "omics" techniques, including genomics, transcriptomics, proteomics, lipidomics, metabolomics, adductomics, and

epigenomics. These techniques enable simultaneous measurement of global or all definable entities of an "ome". Pesticides can affect global gene expression through genetic and epigenetic mechanisms. Genetic mechanisms involve direct interaction with DNA, potentially leading to DNA damage or mutations (Poirier, 2016).

On the other hand, epigenetic mechanisms, indirectly affect DNA through the epigenome, causing gene expression changes (Labib et al., 2016). Primary epigenetic mechanisms include DNA methylation (Bird, 2011), histone modification (Mudbhary & Sadler, 2011), and interaction with non-coding RNA (Costa, 2008). The epigenome plays a crucial role in regulating gene expression under various toxic conditions. *In vitro*, *in vivo*, and epidemiological studies have demonstrated that xenobiotics can induce changes in gene expression through their impact on the epigenome (Zhang et al., 2012). Transcriptomics has been widely employed in pesticide toxicity studies over the past few years. Toxicity studies aim to prevent adverse health effects from human exposure to xenobiotics. Detecting target organ toxicity before permanent damage is crucial. Understanding the mechanisms underlying xenobiotic-induced toxicity is essential for managing potential adverse health effects. The transcriptome, a highly dynamic biological system, responds sensitively to pesticide exposure. Expression profiling of the entire transcriptome can be a sensitive indicator of toxicity. Differentially expressed genes can be used as biomarkers to detect exposure or toxicity. Global gene expression profiling is a sensitive and relevant approach for detecting and understanding target organ toxicity associated with xenobiotic exposure. Past studies have demonstrated the application of transcriptome analysis in the early detection of toxicity and elucidation of molecular mechanisms (Waring et al., 2001; Otava et al., 2015).

The transcriptome is the entire collection of messenger RNA (mRNA) and non-coding RNA (ncRNA) transcripts generated by a specific type of cell, organism, or cell (Morozova et al., 2009). The phenomenon of multiple cell types arising from the same genetic makeup of cells, each with a distinct purpose in the operation of a multicellular organism, is a fascinating mystery in molecular biology. The fact that distinct cell types within an organism activate (or express) distinct gene sets (transcriptomes), resulting in different cell fates and roles, has been linked to this

phenotypic variety. Therefore, biologists have long been interested in the relationship between gene expression patterns and cellular fate and function. The initial attempts to comprehend cellular transcriptomes were counting and analysing total cellular RNA from various organisms, tissues, or disease states to find relevant transcripts. The northern blot technique was used in the initial candidate gene-based investigations (Alwine et al., 1977). Transcriptome analysis research has progressed from the detection of single mRNA molecules to the comprehensive profiling of gene expression and genome annotation using microarrays and EST sequencing, respectively (Schena et al., 1995; Boguski et al., 1995). Transcriptome analysis techniques based on sequencing have become increasingly widespread due to the development of a panel of next-generation sequencers with a substantially better sequencing throughput than the most advanced Sanger sequencer (Harismendy et al., 2009). Gene expression profiling, mutational profiling, non-coding RNA discovery and detection, aberrant transcriptional event identification, RNA editing site discovery, genome annotation, and alternative isoform finding have all been made possible by transcriptome sequencing data (Yin et al., 2008; Ng et al., 2006; Ruan et al., 2007; and Morozova et al., 2009).

Insecticide imparts toxicity in various ways, including cellular, physiological, and molecular levels. Further detrimental effects are also revealed by looking into the molecular pathways. Understanding the molecular alterations in agricultural pests and pests' responses to insecticides is crucial to managing insecticide resistance, which is integral to effective insect pest management. Environmental toxicologists were able to characterise the toxicant-mediated perturbations in gene transcription and to identify the molecular pathways involved in the toxic response by analysing changes in global gene expression that accompany chemical exposure. This analysis provided mechanistic insight into chemical-mediated cellular toxicity (Royland & Kodavanti, 2008). Various techniques are used to study gene expression profiles in biological samples in response to xenobiotic exposure. These include northern hybridisation (Kendall & Riley, 2000), quantitative real-time PCR (QRT-PCR) (Song & Freedman, 2005), subtractive hybridisation (Volz et al., 2005), serial analysis (Taulan et al., 2004),

differential display (Tomita et al., 2002), microarray analysis (Sellamuthu et al., 2011), and next-generation sequencing (Klaperet al., 2014). The most widely used gene expression profiling techniques are quantitative real-time PCR (QRT-PCR), microarray analysis, and next-generation sequencing. QRT-PCR has been used to determine the expression profiles of single or limited transcripts, but it has limitations from a systems toxicology perspective. High-throughput techniques, such as microarrays and next-generation sequencing, are necessary to study systems-level toxicity. The microarray technique is a high-throughput global transcriptome analysis technique that uses DNA molecules to bind or hybridise in a nucleotide sequence-specific manner. It enables the detection of almost all genes in a biological sample at a given time, allowing for the identification of differences in global gene expression profiles in response to xenobiotic exposure (DeRisi, 1997; Hsiao et al., 2000). The technique involves the isolation of high-quality RNA, reverse transcription of mRNA, cRNA synthesis and labelling, hybridisation of probes with targets, detection of signal intensity, and analysis of the resulting data. Microarray-based transcriptome analysis has applications in drug development, as well as in environmental and occupational toxicology (Kukurba & Montgomery, 2015). Next-generation sequencing (NGS) is a global technique for analysing the transcriptome, which determines gene expression profiles in biological samples. It involves RNA isolation, removal of abundant molecules, library preparation, PCR amplification, and sequencing. The cost of NGS has decreased, allowing for more widespread use in global transcriptome profiling (Finotello& Camillo, 2015). Transcriptome analysis is a component of toxicogenomics, which aims to help scientists understand the cellular and molecular effects of chemicals on biological systems. Toxicogenomics encompasses proteomic, metabonomic, and genomic research (Hamadeh et al., 2002). The application of gene expression to comprehend the impacts of chemical and other environmental stresses on biological systems has accelerated due to the rapid development and evolution of genomic, proteomic, and metabonomic-based technologies (Yu et al., 2016).

When exposed to toxicants, the body undergoes dynamic physiological changes that can be systematically detected through transcriptomics.

Transcriptomics can identify exposure to hazardous compounds at an earlier stage, as minor metabolic changes often precede the biochemical alterations induced by xenobiotics (Joseph, 2017). Changes in metabolism are linked to the control of specific genes and proteins. Safety screening, biomarker identification, and research into toxicity mechanisms are among the applications of transcriptomics in toxicological studies. Therefore, in this study, we used D. melanogaster as a non-target model to identify global gene expression changes in response to exposure to acephate and chlorantraniliprole. Over the past ten years, there has been a noticeable increase in the use of *D. melanogaster* in toxicological research (Ong et al., 2015). Due to its short life cycle, low maintenance costs, molecular genetic accessibility, abundance of publicly available strains, and extensive data resources, the *Drosophila* model has long been recognised as a flexible and potent model for developmental biology and genetics. The fly model is particularly pertinent and topical for developing novel approach methodologies and advances in precision toxicology due to these aspects and recent unique developments in genomics and metabolomics (Rand et al., 2023). Global gene expression research in *Drosophila* has previously shed light on changes in various biological systems in response to chemical treatment (King-Jones et al., 2006). Multiple reports have linked *D. melanogaster* and transcriptomic studies to extrapolate the xenobiotic effects of different pesticides. An early work reported a Genome-wide analysis of phenobarbital-inducible genes in *D. melanogaster* (Sun et al., 2006). Sharma et al. (2011) analysed the global gene expression changes and subsequent adverse effects of endosulfan using *D. melanogaster*. Gao et al. (2021) evaluated the transcriptomic responses of six insecticides to *D. melanogaster*.

Jin et al. (2016) conducted a transcriptome sequencing study of the widespread locust Epacromius coerulipes, which is targeted by the chemical pesticide butenafipril. A genomics database for *E. coerulipes* was created with the transcriptome sequencing results, offering a theoretical resource for mechanistic research on pesticide resistance and a molecular foundation for gene function studies. A study using two strains of *E. Coerulipes*, a pesticide-resistant (PR) strain and a pesticide-sensitive (PS) strain,, found 63,033 unigenes, of which 39.87% were recognised as known proteins. The study identified 2,568 differentially expressed genes, comprising 1,646 up-regulated and 922 down-regulated genes. The metabolic

process category was the largest group with a high frequency of differentially expressed genes. Six of the 50 metabolic pathways linked to pesticide metabolism involved differentially elevated genes.

Yang et al. (2016) studied the genes involved in xenobiotic detoxification and stress response following exposure to the new insecticide cycloxaprid in the white-backed planthopper, *Sogatella furcifera*. It was discovered that GSTd, CYP4, and CYP6 are involved in pesticide detoxification and play a significant role in the development of pesticide resistance. Furthermore, *Hsp70* and small *Hsp* genes exhibited up-regulation upon high-dose treatment and down-regulation upon low-dose treatment, respectively. These results contribute to understanding why rice plant hoppers react quickly to pesticide stress.

Diamondback moth pesticide resistance was studied by Hsu and colleagues (2016) in relation to differentially expressed genes. In the case of resistant and susceptible strains, differential expression data showed that 427 transcripts (150 with annotations) exhibited down-regulation, and 944 transcripts (803 with annotations) showed overexpression. It was discovered that resistance mechanisms such as penetration, detoxification, and behaviour response were linked to 6.8% of differential expression transcripts. The investigation additionally found that in trade-off scenarios, a few transcripts associated with smell perception were down-regulated.

Shi et al. (2017) examined the molecular impacts of neonicotinoid pesticides, particularly thiamethoxamon, on honey bees. In honey bees exposed to 10 ppb thiamethoxam over 10 days, the researchers identified 609 differentially expressed genes (DEGs), of which 225 were upregulated and 384 were downregulated. Thiamethoxam affects biological processes such as ribosome function, oxidative phosphorylation, tyrosine metabolism, and drug metabolism. The enriched differentially expressed genes (DEGs) were associated with metabolism, biosynthesis, and translation. The results shed light on the intricate relationships between neonicotinoid pesticides and honey bees.

The impact of mosquito chemical environments on insecticide-driven selection mechanisms is examined by Poupardin et al. (2012). They evaluated the effects of pesticide selection and xenobiotic exposure on larvae using *Aedes aegypt*i

as a model species. The results showed that three specific strains exhibited a minor but noteworthy rise in permethrin resistance. According to microarray research, the selection of insecticides altered gene transcription, influencing cell metabolism, transport, and detoxification. According to the study, long-term metabolic pathways that lead to pesticide resistance may be influenced by the chemical environment of insects.

The harmful effects of chlorantraniliprole on autumn armyworms (*Spodoptera frugiperda*), a significant pest of maize in Latin America, were examined by Xu et al. (2022). The study Utilises transcriptome, biochemical, and bioassay analysis to investigate gene expression changes in third-instar larvae. The findings indicate that 1,266 differentially expressed genes (DEGs) are caused by exposure to $LC_{10}$ chlorantraniliprole, of which 578 are upregulated and 688 are downregulated. They examined how chlorantraniliprole affected the transcription of genes linked to detoxification. Following exposure to chlorantraniliprole, $LC_{10}$, eleven P450S, three ESTs, one GST, and one ABC transporter were found; four P450S and three ESTs were down-regulated, and seven P450S, one GST, and one ABC transporter were induced. Conversely, in response to chlorantraniliprole, $LC_{30}$, twenty-seven P450s, six ESTs, one GST, one UGT, and four ABC transporters were detected. He and colleagues conducted another trial in 2023 using *S. frugiperda*. The group investigated how the diamide pesticide chlorantraniliprole affects autumn armyworms (*Spodoptera frugiperda*) in China. The study discovered that 3309 genes were expressed differently in FAW larvae under stress caused by chlorantraniliprole, resulting in growth inhibition and fatal effects. Chlorantraniliprole's target proteins, two RyR genes, have single nucleotide polymorphisms (SNPs) found in the study. The results indicate a high likelihood of suppressing FAW in China by applying chlorantraniliprole, maybe because of the RyR SNP frequency's stabilised 4743 M.

Li et al. (2022) investigated the toxicity bioassay and differentially expressed chlorantraniliprole (CAP) genes in *Mythimna separata* larvae. RNA-sequencing produced 43,055 unigenes with an average length of 1010 bp, and screening revealed 567 up-regulated and 692 down-regulated DEGs that responded to CAP therapy. The outcomes demonstrated that 24 hours after exposure, CAP was

effective against third-instar larvae and had increased GST activity. The investigation also found 35 GST genes.

Pesticide application kills the intended pest and negatively impacts beneficial species, such as biological control agents. Applications of sulfoxaflor, a pesticide that targets pests in agroecosystems, indirectly endanger the essential biological control agent *Harmonia axyridis* (Pallas). Nawaz et al. (2018) examined the transcriptome profile of second-instar *H. axyridis* larvae subjected to sulfoxaflor insecticide using high-throughput RNA-seq technology. 794 significant differentially expressed genes (DEGs) associated with several KEGG pathways, such as drug metabolism and xenobiotic metabolism, were found in the study. According to the findings, sulfoxaflor significantly altered the transcriptome profile and pathways of *H. axyridis*, opening the door for more in-depth molecular research. According to Qiong et al. (2022), the biological control efficacy of predatory arthropods, *Eocanthecona furcellata*, was observed to be diminished when insecticide exposure to λ-cyhalothrin was present. After the sublethal exposure of λ-cyhalothrin, they built a comparative transcriptome analysis and found 4364 upregulated and 1043 down regulated differentially expressed genes. Flupyradifurone (FPF), a parasitic natural enemy of aphids, has lethal and sublethal effects on *Binodoxys communis*. Gao et al. (2023) studied these effects. The sublethal concentrations of FPF's $LC_{10}$ and $LC_{25}$ were found to have detrimental effects on *B. communis*, lowering survival rates, adult lifespan, parasitism, and emergence.

Furthermore, FPF affects the following generation beyond generations. A total of 1429 differentially expressed genes (DEGs) that were substantially altered between the FPF-treated and control groups were found using RNA-Seq transcriptomic analysis. The metabolic pathways include peroxisomes, glutamate metabolism, carbon metabolism, fatty acid metabolism, and amino acid metabolism, where these DEGs are primarily abundant. According to a study by Wu et al. (2022), the ladybird beetle (*Coccinella septempunctata*), which is an excellent vector for biological control, is negatively affected by cycloxaprid in terms of survival, development, longevity, ability to reproduce, and ability to prey. Demographic growth indices, such as net reproduction rate, were considerably reduced by the sublethal dosage of cycloxaprid. Differentially expressed genes (DEGs) between the

treatment and control groups were identified by transcriptome expression, which also identified pathways linked to xenobiotic metabolism, P450 metabolism, retinol metabolism, carcinogenesis, and steroid hormone production. Wang et al. (2023) examined the transcriptome expression of the neonicotinoid insecticide Sulfoxaflor about *C. septempunctata's* demographic fitness. These findings showed that between the LR30 treatment and control groups, there were 544 up-regulated and 338 down-regulated significantly differentially expressed genes (DEGs) for transcriptome expression

The pesticide toxicity and global gene expression changes in biological samples were investigated using different organisms, including microorganisms to vertebrates. Unintentional spills and pesticide misuse can result in environmental pollution, either present or past, and raise questions about potential threats to higher eukaryotes and non-target microorganisms within ecosystems. In a eukaryotic model of *Saccharomyces cerevisiae*, Gil et al. (2018) examined transcriptome responses to sub-lethal concentrations of six pesticide-active ingredients. The transcriptional profiles of a standardised yeast population were investigated in the study following exposure to the pesticide carbofuran, the herbicides alachlor, S-metolachlor, diuron, and methyl (4-chloro-2-methylphenoxy) acetate, as well as the fungicide pyrimethanil. Venn and hierarchical clustering studies show that the active ingredients have different transcriptional patterns. Pesticide toxicity and stress response are predicted by functional enrichment analysis. The fungicide mancozeb, used in agriculture, has been linked to Parkinson's disease and cancer. Santos et al. (2009) used *S. cerevisiae* to analyze the molecular mechanisms of mancozeb toxicity and adaptation. Quantitative proteomics revealed target genes and transcription activators related to yeast response to stress, including oxidative stress, protein translation, and degradation.

In *Danio rerio* (Zebra fish), transcriptome-based identification of genes responsive to the organophosphate insecticide phosmet was investigated by Vasamsetti et al. in 2021. Two thousand one hundred ninety (2190) differentially expressed genes (DEGs) were found using whole transcriptome analysis, of which 822 and 1368 were highly up-and down-regulated genes, respectively. System processes, as well as sensory and visual perception, were among the primary biological pathways affected by phosmet poisoning. Significant enrichment of

metabolic pathways, calcium signalling routes, actin cytoskeleton regulation, cardiac muscle contraction, drug metabolism, phototransduction, and other enzymes was found by Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis. Wang et al. (2021) assessed the cardiotoxic consequences of exposing zebra fish larvae to environmental Mancozeb concentrations. The transcriptome study predicted that the activation of Notch and apoptosis-related signalling pathways, which cause pericardial oedema, myocardial fibrosis, and congestion, may be connected to Mancozeb-induced cardiac developmental toxicity in zebrafish.

Additionally, differential gene expression analysis revealed that after MZ therapy, cyp-related genes (*cyp1c2* and *cyp3c3*) were dramatically elevated, which was associated with myocardial cell death. Yang et al. (2022) investigated the potential toxicity of the imidazole fungicide Prochloraz to aquatic species. Prochloraz exposure at high concentrations resulted in 345 up-regulated and 76 down-regulated differential expression genes (DEGs) in larval zebrafish, influencing the metabolism of amino acids, glycolipids, and oxidative stress. Furthermore, there was a clear impact on biochemical markers associated with glycolipid metabolism, which was evident in entire larvae with higher TG and lower Glu levels. Additionally, Prochloraz was shown to cause oxidative stress in larval zebrafish by altering the activities of associated genes and upregulating their levels.

A study by Sharma et al. (2011) using D. melanogaster found differential expression of 256 genes controlling development, stress, immune response, and metabolism in exposed larvae. The findings revealed diminished or delayed emergence in exposed organisms, as well as abnormalities in the hind legs. These results demonstrate the deleterious effects of endosulfan on living things and shed light on potential genetic alterations that may affect higher organisms. Phenobarbital administration caused altered gene expression in *D. melanogaster* larvae, according to a study by Sun et al. (2006). Four genes showed up-regulation, and eleven showed down-regulation, out of the seventeen stimulated to express more. In metabolically DDT-resistant *Drosophila* strains, several genes were found to be overexpressed, suggesting potential health hazards. Enhanced metabolism is one of the most significant mechanisms for insecticide resistance. It can be obtained by overexpressing or structurally changing detoxification genes like ATP-binding cassette transporters (ABCTs), glutathione S-transferases (GSTs), cytochrome P450s

(CYPs), and UDP-glucuronosyl transferases (UGTs) (James & Davey, 2009; Yoon et al., 2011; Gao et al., 2021). Identifying the critical enzymes involved in insecticide detoxification can be achieved by examining the induction profile of insect detoxification enzymes. While piperonyl butoxide and two recognised inducers, phenobarbital and caffeine, stimulated the transcription of many detoxification genes in *Drosophila*, including those involved in insecticide metabolism, exposure to high quantities of pesticides only slightly affected the transcription of other detoxification genes (Willoughby et al., 2006; Willoughby et al., 2007). Midgut genes of *D. melanogaster* larvae in their third instar were examined in a study conducted by Li et al. (2008). This allowed the number of GSTs and cytochrome P450s linked to the midgut to be calculated. Additionally, the putative DNA transcription factor binding motifs (TFBMs) of the third-instar larvae's midgut-expressed GSTs, carboxyl esterases, and cytochrome P450s were found. Since some of the putative TFBMs for the midgut-associated GSTs were linked to the oxidative stress response, they also investigated how hydrogen peroxide, an oxidative stressor, affected GST expression. Researchers inferred that serine proteases accounted for most of the proteolytic enzymes discovered. Using oligoarray analysis, they discovered that the midgut expressed approximately 40% of the cytochrome P450 genes and 74% of the glutathione S-transferases (GSTs) in the *D. melanogaster* genome.

Gao et al. (2021) analysed transcript profiles of *D. melanogaster* treated and untreated with six insecticides and compared them to identify inducible metabolic processes that may lead to pesticide tolerance/resistance. The study found that pretreatment with six insecticides significantly increased the tolerance of *D. melanogaster* to lethal concentrations. Commonly responding genes were identified, with 26 over-transcribed and 30 under-transcribed transcripts. Over-transcribed genes were related to olfactory behavior and immune-related genes, while under-transcribed genes were related to the mitochondrial respiratory chain and egg shell formation and motion. The roles of tolerance-related genes were not directly related to metabolic detoxification, but rather to the restoration of homeostasis. Previous research using a similar method found that specific cuticular protein genes, detoxification genes, and proteolysis-related genes may be involved in the initial tolerance induction in *P. xylostella* and *F. occidentalis* (Gao et al., 2020). Martelli et

al. (2023) evaluated the transcriptional fingerprints of imidacloprid and spinosad at low-dose exposures using RNA-sequencing in *D. melanogaster*. In contrast to decreased expression of immune-related genes in both tissues, both pesticides cause up-regulation cytochrome P450 and glutathione S-transferase genes in the brain and down-regulation in the fat body. Spinosad primarily affects lysosomal activity, protein folding, and genes related to reproduction. Co-expression analysis revealed a strong connection with glial cell markers, but minimal to no correlation between genes affected by spinosad and neurons expressing *nAChRα6*. Additionally, they identified and verified the expression of *nAChRα6* in male germline and fat body cells through experiments. Thiamethoxam is a commonly used pesticide that has been shown to delay insect growth and development, reduce fecundity, and alter the architecture of the molecule in *D. melanogaster* (Li et al., 2020). It also lowers the viability of hemocytes and fat body cells and damages DNA. RNA sequencing demonstrated that genes implicated in immunological responses (IM4), hemocyte proliferation (RyR), and stratum corneum proteins (Lcp65Ag3, Cpr65Ax1) were significantly increased. The expression of genes linked to lipid metabolism (*sxe2*), lifespan (*Atg7* and *NalZ*), pupa development (*IIp8*, *Blimp-1*), female fertility (*Ddc*), male mating behaviour (ple), and neural retina development (*Nnad*) was substantially lower.

Previous studies have shown that transcriptomics analysis is an effective tool for studying pesticide/ xenobiotic toxicity. There is extensive research on the toxicity of pesticides to non-targeted model organisms, as well as to beneficial species such as those used in biological control, and to pest species. So, our study "comparative transcriptome profiling of acephate and chlorantraniliprole in non-targeted insect model *D. melanogaster"* will pave the way for a milestone in toxicogenomics.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Chemicals

Acephate (CAS No: 30560-19-1) and Chlorantraniliprole (CAS No: 500008-45-7) were procured from Sigma-Aldrich (St. Louis, MO, USA). Acephate was prepared in double-distilled water, as chlorantraniliprole was diluted in acetone due to its low solubility in water. The sublethal concentration, $1/5^{th}$ of EC50, was used

for the RNA sequencing experiment. For acephate, it was 0.38µg/ml and 0.0058µg/ml for chlorantraniliprole.

### 2.3.2 **RNA Sequencing**

Whole *Drosophila* larvae grown in $1/5^{th}$ of $EC_{50}$ concentration of pesticides, vehicle control and control groups were collected and washed three times in TRIzol and RNA was extracted using the Qiagen RNeasy mini kit (Cat. No.74106). A single Tommy micro-smash homogenisation cycle was performed, and larvae in Trizol were moved to Tommy tubes using four stainless steel beads. 200 µl of chloroform was used for phase separation, and the centrifuge was run for 15 minutes at 4°C at 13,200 rpm. After thoroughly combining the aqueous phase with half of the absolute alcohol volume, the RNeasy spin column was loaded into a 2 ml collection tube. The tubes were passed through and disposed of after being centrifuged for one minute at 10,000 rpm. The DNase I (Cat No. 79254) treatment on the column and subsequent column washes were performed in accordance with the manufacturer's instructions. Nuclease-free water was used to elute the RNA from the column.

### 2.3.3 Library Preparation

Library preparations at Genotypic Technology Pvt. Ltd., Bangalore, India, involved generating RNA sequencing libraries using the Illumina-compatible NEBNext Ultra II Directional RNA Library Prep Kit (New England BioLabs, MA, USA). A total of 500 ng of RNA was extracted to isolate, fragment, and prime mRNA. First-strand and second-strand synthesis were applied to mRNA that had been primed and fragmented. NEBNext sample purification beads were used in purifying the double-stranded cDNA. Following the NEBNext® UltraTM II Directional RNA Library Prep methodology, purified cDNA was end-repaired, adenylated, and ligated to Illumina adapters. Second-strand excision was then performed using the USER enzyme at 37 ˚C for 15 minutes. Illumina Universal adapter used in this study were: 5'-AATGATACGGCGACCACCGAGATCTACA CTCTTTCCCTACACGACGCTC TTCCGATCT-3' and index adapter. 5'-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC [INDEX] ATCTCGTATG

CCGTCT TCTGCTTG-3'. After adapter-ligated cDNA was purified using NEBNext beads, it underwent 11 cycles of enrichment for adapter-ligated fragments and indexing (98˚C for 30 sec, cycling (98˚C for 10 sec, 65˚C for 75 sec, and 65˚C for 5 min). Before a library quality control inspection, NEBNext beads were used to purify the sequencing library's final PCR products. Thermo Fisher Scientific, MA, USA, used a Qubit fluorometer to quantify Illumina-compatible sequencing libraries, and an Agilent 2200 was used to analyse the fragment size distribution.

### 2.3.4 Illumina Sequencing

Illumine sequencing done by following the manufacturer's instructions; the libraries were paired-end sequenced for 150 cycles using an Illumina HiSeq X Ten sequencer (Illumina, San Diego, USA).

### 2.3.5 Examining Data

To under take transcriptome analysis, low-quality reads and adapter sequences were removed from the raw data by processing. Expression analysis was done, and the high-quality readings were considered for alignment with the reference genome using a spliced aligner. To learn more about the functional role of each expressed gene, down-stream analysis using GO annotation and pathway analysis was performed using the expression data.

### 2.3.6 Raw data processing

The raw reads were processed using FastQC (Andrews et al., 2010) for quality assessment and pre-processing, which includes removing adapter sequences and low-quality bases.

### 2.3.7 Mapping and Yield

Mapping and yielding using HISAT (Kim et al., 2015) with default parameters, the pre-processed high-quality data was aligned to the *Drosophila melanogaster* reference genome retrieved from the FlyBase database to determine

the alignment percentage. The reads were divided into two categories: aligned reads, which match the reference genome, and unaligned reads.

### 2.3.8 Expression Analysis

Gene abundance was estimated and calculated using the Featurecounts tool (Liao et al., 2014). Gene absolute counts were calculated and utilised in the investigation of differential expression.

### 2.3.9 Differential Expression Analysis

DESeq was used to calculate the differentially expressed genes. Genes were categorised as up, down, and neutrally regulated based on the log2fold change cut off of +/- i.e. ontology (GO) and pathway analysis Genes were functionally annotated based on homology using the BLAST tool against "*Drosophilidae*" family protein sequences available from the Uniprot database. If a match was found with an e-value less than e -5 and a minimum similarity greater than 30%, genes were assigned a homolog protein from other organisms. Pathway analysis was done on the KAAS (Moriya et al., 2007) server using *D. melanogaster* as the reference organism for pathway identification for all samples. Based on the best hit information, the KEGG database annotates genes in complete genomes with the KEGG orthology (KO) identifiers, or the K numbers. Compiled pathways per gene were mapped to the DE genes.

## 2.4 RESULTS

### 2.4.1 Primary Analysis

The primary step in the transcriptome analysis is the extraction of RNA from the samples. The samples include control, vehicle control, acephate and chlorantraniliprole treated samples, we include pesticide-treated samples in duplicate. The samples that passed the quality assessment with optimal yield and concentration were listed in Table 2.1.

Table 2.1: Estimated RNA Concentration and Purity

| | | Nano Drop QC | | | | | Qubit QC | | | SampleQualityControl | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sl. No. | Sample Name | ng/ul | 260/280 | 260/230 | Volume (µl) | Yield (ng) | Qubit Conc. (ng/ul) | Volume (µl) | Yield (ng) | ND Purity Ratios | Qubit Yield | Tape RNA Integrity | TAPE#RIN |
| 1 | Control | 884.2 | 2.25 | 1.91 | 60 | 53052 | 704 | 60 | 42240 | Optimal | Optimal | Optimal | 7.7 |
| 2 | Vehicle control | 801.8 | 2.28 | 2.13 | 60 | 48108 | 582 | 60 | 34920 | Optimal | Optimal | Optimal | 7.5 |
| 3 | Acephate treated | 994.6 | 2.29 | 2.03 | 60 | 59676 | 726 | 60 | 43560 | Optimal | Optimal | Admissible | 6.4 |
| 4 | Acephate treated | 903.9 | 2.29 | 2.4 | 50 | 45195 | 588 | 50 | 29400 | Optimal | Optimal | Optimal | 7 |
| 5 | Chlorantraniliprole | 916.5 | 2.25 | 2.24 | 80 | 73320 | 678 | 80 | 54240 | Optimal | Optimal | Optimal | 7.8 |
| 6 | Chlorantraniliprole | 839.8 | 2.25 | 1.89 | 60 | 50388 | 644 | 60 | 38640 | Optimal | Optimal | Optimal | 7.5 |

The Illumina-compatible sequencing libraries showed an average fragment length of 482 bp and sufficient concentration to obtain the desired amount of sequencing data. Table 2.2 lists the concentration of libraries obtained and the indices used.

Table 2.2: Description of Libraries

| Sl. No. | Sample ID | Qubit Conc. (ng/ul) | Vol(ul) | Yield (ng) | Barcode ID | Index Sequence 1 |
|---|---|---|---|---|---|---|
| 1 | Control | 12 | 10 | 120 | S762 | TTACCGAC |
| 2 | Vehicle control | 7.32 | 10 | 73.2 | S713 | TCGTCTGA |
| 3 | Acephate treated | 7.14 | 10 | 71.4 | S736 | TTCCAGGT |
| 4 | Acephate treated | 10.9 | 10 | 109 | S709 | TACGGTCT |
| 5 | Chlorantraniliprole | 8.1 | 10 | 81 | S732 | AAGACCGT |
| 6 | Chlorantraniliprole | 5.64 | 10 | 56.4 | S774 | CAGGTTCA |

The data obtained from the sequencing run was de-multiplexed using Bcl2 fastq software v 2.20, and FastQ files were generated based on the unique dual barcode sequences. This sequencing quality was assessed using FastQC v 0.11.8 software. The adapter sequences were trimmed, and bases above q30 were considered. Low-quality bases were filtered off during read pre-processing and used for downstream analysis.

The raw data sequencing quality was assessed. The adapter sequences were trimmed, and low-quality bases were filtered off during read pre-processing. The reads with a Ph redscore >q30 were used in downstream analysis.

We generated an average of 19.85 million paired-end raw data using Illumina sequencing technology, whereas an average of 19.74 million paired-end reads were retained as high-quality (>q30) data. An average of 99.46% (Table 2.3) of high-quality reads were retained for the down-stream analysis.

Table 2.3: Illumina paired-end read statistics

| Sample | Total Reads | Processed Reads | % high-quality data |
|---|---|---|---|
| Control | 17366953 | 17277326 | 99.48 |
| Vehicle control | 17150599 | 17051256 | 99.42 |
| Acephate treated | 19599199 | 19469685 | 99.34 |
| Acephate treated | 21499127 | 21390539 | 99.49 |
| Chlorantraniliprole | 19908345 | 19806868 | 99.49 |
| Chlorantraniliprole | 23609048 | 23491368 | 99.50 |

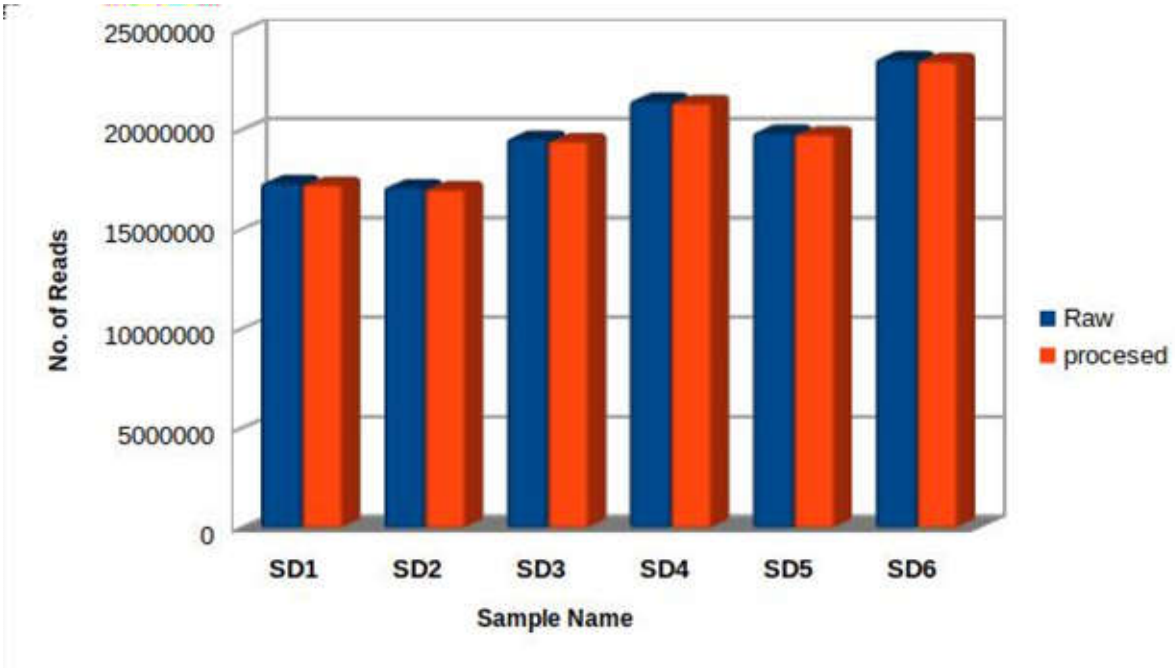



**Figure 2.1**: Read statistics (raw and processed reads) representing the total number of reads generated

The reference genome (*Drosophila melanogaster*) was downloaded from the FlyBase database. When all processed reads were aligned to the *D. melanogaster* reference, an average of 96.25% of the reads showed complete mappability to the reference (Table 2.4).

Table 2.4: Sample-wise alignment statistics to the reference genome

| Sample | Total Reads | Processed Reads | % Alignment [*D. melanogaster* Reference] |
|---|---|---|---|
| Control | 17366953 | 17277326 | 96.63% |
| Vehicle control | 17150599 | 17051256 | 96.33% |
| Acephate treated | 19599199 | 19469685 | 95.53% |
| Acephate treated | 21499127 | 21390539 | 95.85% |
| Chlorantraniliprole | 19908345 | 19806868 | 96.54% |
| Chlorantraniliprole | 23609048 | 23491368 | 96.59% |

An alignment percentage between 70% and 95% to the reference genome is typically achievable in a standard experiment. However, the number depends on multiple factors, such as sample quality, RNA quality, genome coverage, and the relatedness of the genome and alignment parameters.

Genes were identified and quantified based on the alignment of reads. The total number of aligned gene reads was calculated using features related to gene expression. The read counts for individual genes were converted into expression profiles at the gene level, represented in the expression matrix. The details of the total number of genes expressed in all the samples are provided in (Table 2.5).

Table 2.5: Number of expressed genes

| Sample Name | No. of expressed genes |
|---|---|
| Control | 13444 |
| Vehicle control | 13483 |
| Acephate treated | 13923 |
| Acephate treated | 13910 |
| Chlorantraniliprole | 13778 |
| Chlorantraniliprole | 13708 |

Compared to the control group, treated groups showed a high level of gene expression. In the control group, a total of 13444 genes were expressed, and in the in-vehicle control groups, 13483 genes were expressed. In acephate-treated groups, 13917 genes were expressed, and about 455 genes were overexpressed compared to the control group. In the chlorantraniliprole-treated groups (13743), a similar pattern was observed in several of the expressed genes; the number was also calculated to be higher than in the control and treated groups.

### 2.4.2 Differential expression

2.4.2a Control *vs.* Acephate

Relative to the control group, volcano plots were constructed (Figure 2.2A) by integrating each transcript's P-value and fold change to illustrate the general scattering and filter the differentially expressed genes in the acephate-treated group. In the pesticide-treated group, 15% of (2,031 genes) were up-regulated, whereas 13.64% (1812 genes) were down-regulated. 461 genes showed more than a 2-fold difference in expression (up-regulated) in the acephate-treated group compared to the control group. Similarly, 552 genes showed less than -2-fold difference in expression in down-regulated genes. Detoxification genes, heat shock protein genes, and chromosome-associated genes are the significant over-transcribed genes observed in the acephate-treated group. Furthermore, 1030 unigenes were identified as having differentially expressed genes in response to the stress-inducing effects of acephate (Figure 2.2B, Venn diagram). A heatmap was generated for the top 20 genes expressed (Figure 2.3). The impacted genes included detoxification enzyme-encoding genes, cuticle protein-encoding genes, and chitin metabolism-related enzymes, all of which are linked to metabolic resistance.

The significantly up-regulated genes include those governing transcription factors, G-protein-coupled receptors, chromosome-associated proteins, heat shock proteins, cytochrome P450, proteolysis, and carbohydrate metabolic processes, among others. Differential gene expression, which primarily occurs at the transcriptional level and is governed by intricate regulatory networks of transcription factors (TFs), is crucial for certain cellular fates and activities. Of forty-seven up-

regulated transcription factor genes, 16 genes were significantly over-transcribed ($p<0.05$). *slbo, danr, dan, fd59A, btd,* and *Lmx1a* genes were the highly up-regulated genes with log2fc value above three. Likewise, 39 chromosome-associated genes were upregulated in acephate-treated samples, but only six genes significantly contributed to the differentially expressed genes (DEGs). The genes with significant up-regulation were *rn, Clk, Klp59D*, Ald2, aura, and *CG15306*. We have identified 23 up-regulated genes of G-protein-coupled receptors, three of which (LOAF, Dop2R, and AstC-R1) are highly significant ($p < 0.05$) with a log2fc value above 2.

In acephate-treated larvae, detoxification genes, heat shock protein genes, and chromosome-associated genes are significantly over-transcribed. The KEGG pathway analyses revealed that acephate treatments have a significant impact on the "Metabolism of xenobiotics by cytochrome P450" and "Drug metabolism - cytochrome P450" pathways. The DEGs, which encode detoxification enzymes such as cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), carboxylesterases (COEs), UDP glucosyltransferases (UGTs), and ATP-binding cassette transporters (ABCs), were therefore examined in this work. As indicated in Table 2.6, samples treated with acephate differed from controls in the differential expression of 29 genes encoding detoxification enzymes. These DEGs are comprised of 14 P450s, 4 GSTs, 1 COEs, 4 UGTs, and 6 ABCs. Among them, 9 P450s, 4 GSTs, 1 COEs, 2 UGTs, and 2 ABCs were down-regulated.

**Table 2. 6:** Differential expression of detoxification enzymes in different treatment

| Gene group | Control vs. Acephate | | | Control vs. Chlorantraniliprole | | | Vehicle control vs. Chlorantraniliprole | | |
|---|---|---|---|---|---|---|---|---|---|
| Detoxification enzymes | Total | Up | Down | Total | Up | Down | Total | Up | Down |
| P450 | 15 | 5 | 9 | 4 | 2 | 2 | 3 | 1 | 2 |
| GST | 4 | 0 | 4 | 2 | 1 | 1 | 1 | 0 | 1 |
| COE | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 |
| UGT | 4 | 2 | 2 | 4 | 2 | 2 | 3 | 0 | 3 |
| ABC Transporter | 6 | 4 | 2 | 4 | 2 | 2 | 3 | 1 | 2 |

The significant genes with Log2fc values below 2 were *Cyp4e1, Cyp4c3, Ugt37c1, CG33178, GstD3, Cyp6w1, Cyp4d20*, and *Cyp4d21*. Of the 14 transcripts associated with P450s, 5 showed up-regulation compared to the control, whereas the remaining 9 transcripts showed down-regulation. Compared to the control group, the up-regulated transcripts *Cyp4ad1* and *phm* displayed the greatest expression difference, with an average fold change of more than two. Five of the nine down-regulated P450s have log2fc values greater than 2. Nearly -3.06 is the greatest log2fc value for the *Cyp4e1* transcript. Out of the four GST genes, all four had lower transcription levels than the flies in the control group, with *GstD2* having the highest logfc value of -2.42. There were two up-regulated and two down-regulated genes among the four differentially expressed genes (DEGs) that were UGTs.

It is well known that the HSP family plays an essential role in pesticide stress. We have examined the expression of different HSP families. Out of thirteen ships, eight were significantly overtranscribed. The most overtranscribed HSP family is the HSP 70 family, with six genes (Hsp70Aa, Hsp70Ab, Hsp70Ba, Hsp70Bb, Hsp70Bbb, and Hsp70Bc). The eight significantly up-regulated Hsps genes have Log2fc values above two.

Acephate-induced stress was evident in the upregulation of stress protein genes and exhibited high variations in immune response genes. One hundred nineteen immune-related genes were differentially expressed between control and acephate-treated samples, with 19 being up-regulated and 100 being down-regulated (Table 2.6). The DEGs are divided into 13 groups, which includeToll and Imd regulating pathway genes (12 DEGs), hemolymph lipopoly saccharide-binding proteins (LBPs, 1 DEGs), lectins (1 DEGs), scavenger receptors (SRs, 5 DEGs), integrins (4 DEGs), superoxide metabolism (Antioxidase, 1 DEGs), cuticle proteins (86 DEGs), rho GTPase-activating proteins (Rho-family proteins, 4 DEGs), down syndrome cell adhesion molecule-like proteins (DSCAMs, 2 DEGs), innate immune response genes (2 DEG) and antimicrobial peptide (2 DEG).

The major pathways involved in acephate treatment include various transcription factors, ubiquitin, G protein-coupled receptors, chromosome-associated

proteins, protein folding, and modification pathways. In addition to these significant pathways, other pathways are also affected by pesticide-induced stress. Some of these pathways include the TGF-beta signalling pathway, the hedgehog pathway, the Wnt pathway, and the longevity-regulating pathway. They all showed significantly higher expression levels than control-group samples, with an average Log2fc value greater than or equal to 1.5.

In the 47 transcripts related to transcription factors, out of which 16 genes were highly significant, all were up-regulated compared to the control, danr showed higher expression with a Log2fc value of 5.44. The other major transcription factors include *Dan, Slbo, Btd, Fd59A, and Lmx1A, all of which have logFC* values above 3.

The seventeen ubiquitin system-related genes were significantly overexpressed. The CG12402 gene showed a maximum Log2fc value of 2.4. The G protein-coupled receptor pathway was also overexpressed during acephate treatment. The 16 genes were upregulated, with Log2fc values ranging from 3.8 to 1.01. The astC-R1 gene showed the highest fold change, 3.8.

The xenobiotic-induced system also affects the signalling pathways of *D. melanogaster*. Compared to the control, the genes (*talk 1, Ror* and *CanB*) involved in the wnt signalling pathway were also over-transcribed in acephate-treated groups. The talk 1 and Ror genes have Log2fc values greater than 3, and the Can B gene has a 1.5 Log2fc value. The major genes over-expressed in the TGF-beta signalling and hedgehog pathway were Actbeta and CG14669, respectively.

While most of the DEGs were over-transcribed, the others were commonly under-expressed following treatment with a sublethal concentration of acephate. The top-down regulated DEGs with a log2foldchange of more than 5 should be focused on, such as cuticle protein and the chitin-based larval cuticle's structural constituent. The primary constituent of the insect epidermis, midgut peritrophic membrane, and tracheal system is chitin. The significantly under-transcribed genes involved in body morphogenesis and chitin-based larval cuticle development include *Twdl C, Twdl V, Twdl N, Twdl J, Twdl B, Twdl K, Twdl P, Twdl O, Twdl M,* and *Twdl Y.* Cuticle proteins are critical structural elements of insect tissues and affect how well pesticides penetrate an insect's body. Additionally, compared to the control group, the expressions of six genes "structural constituent of chitin-based cuticle" were

down-regulated fourfold. The effect of acephate on suppressing larval growth and reducing the pupation rate was consistent with the downregulation of genes involved in chitin production and those encoding cuticle proteins under stress-induced conditions.

Furthermore, 1030 unigenes were identified as having differentially expressed genes in response to the stress-inducing effects of acephate (Figure 2.2B). The impacted genes included detoxification enzyme-encoding genes, cuticle protein-encoding genes, and chitin metabolism-related enzymes, all of which are linked to metabolic resistance.

2.4.2b Control *vs.* Chlorantraniliprole

Transcriptomic analysis was performed on the control group and larvae treated with $1/5^{th}$ of the $EC_{50}$ of chlorantraniliprole for 48 h. It produced an average of 19.85 million paired-end raw data, whereas 19.74 million paired-end reads were retained as high-quality (>q30) data. Nearly 99.46% (Table 2.3 ) of total reads were retained as high-quality (>q30) data. Relative to the control group, volcano plots were constructed (Figure 2.4A) by integrating both the P-value and fold change of each transcript (P-value1) to illustrate the general scattering of the transcripts and to identify differentially expressed genes in the chlorantraniliprole-treated group.

Compared to the control group, 1369 genes were up-regulated, 773 were down-regulated, 521 unigenes were identified as DEGs in the treatment group, and also 295 unigenes were expressed only in the control (Figure 4B). Depending on the log2 fold change, moreover, half of the DEGs exhibit 1- to 2-fold variations in their expression levels, whether they are up- or down-regulated. A heatmap was generated for the top 20 expressed genes (Fig. 2.5). To examine the gene expression patterns of *D. melanogaster* larvae, pairwise comparisons were made between the control and treatment groups. The top-up-regulated DEGs include proteins involved in proteolysis, chromosome-associated proteins, G protein-coupled receptors, genes regulating carbohydrate metabolism pathways, ribosome biogenesis, detoxification, immunity-related genes, and heat shock proteins. The major down-regulated genes include chitin-based larval cuticle proteins, chitin-binding proteins, transcription factors, cytoskeleton proteins, calcium ion-binding proteins, ATP-binding proteins, and detoxification genes involved in drug metabolism.

Proteolysis is a part of cellular homeostasis. The DEGs implicated in proteolysis exhibit significant fluctuations compared to the control. A total of 96 DEGs related to proteolysis were examined; 65 had up-regulated expression, while the remaining DEGs had down-regulated expression. Of the 65 DEGs related to proteolysis, 17 genes had a logfc value greater than two, while CG17283 and *CG8329* had log2fc values more significant than five. Four of the 31 genes in the down-regulated proteolysis DEGs have a log2fc of less than two. The *LP17541p* was a highly under-expressed proteolysis gene.

Numerous DEGs were discovered to be involved in detoxification, including genes for ATP-binding cassette transporters (ABCs), glutathione S-transferases (GSTs), UDP glucosyltransferases (UGTs), and cytochrome monooxygenases (P450s). Fifteen genes were identified in samples treated with chlorantraniliprole: eight were down-regulated, and the other seven were up-regulated. These genes included four P450 genes, two GST genes, one COE gene, four UGT genes, and four ABC genes. Among these detoxifying genes UGTs with high expression value (*ugt36F1*-3.8). The major GSTs upregulated during chlorantraniliprole induction were identified as *GstE14* and*GstE5*.

Hsps are molecular chaperones that pesticides can induce. In the current study, six Hsp70s (*Hsp70Aa, HspAb, HspBa, HspBb, HspBbb, and HspBc)* and two *Hsp* protein binding genes *(CG32640 & CG30075*) were significantly overexpressed with chlorantraniliprole treatment.

In the chlortraniprole-treated groups, nine genes involved in the carbohydrate metabolism process were over-expressed, with three having log2fc values greater than three. *Amyrel, Amy-p,* and *Cht9* are among the genes that are highly expressed. The production of ribosomes (ribosome biogenesis) is a complex, tightly controlled process associated with cell development and proliferation. In our study, nine differentially expressed genes (DEGs) regulating ribosome biogenesis were overexpressed. Two genes (*Npc2d & Npc2f*) implicated in the intracellular cholesterol transport pathway were overexpressed, with *Npc2d* having a high expression value of 4.97.

KEGG enrichment analysis was performed on DEGs, and the transcriptome pathway map showed that chlorantraniliprole affects a variety of genes involved in

drug metabolism in third-instar larvae, with P450s serving as one of the key pathways for insecticide metabolism. The other major affected pathways include the G protein-coupled receptor pathway, peptidase inhibitor pathways, Toll and imd signalling pathway, insect hormone biosynthesis pathway, mitochondrial biogenesis pathway, etc.

RyR has been identified previously as the target protein for chlorantraniliprole; in our investigation, the ryr gene was underexpressed, which could suggest that chlorantraniliprole induces specific transcriptome toxicity. Along with the *ryr* gene, genes governing calcium ion binding, transport, and homeostasis were also under-transcribed.

The significant under-expressed genes in chlorantraniliprole-induced stress were "chitin-binding" and "structural constituent of chitin-based cuticle". Within this group, 36 DEGs were down-regulated. *TwdlF* displayed the lowest expression value of -4.76; five had log2fc values less than two. Gene-regulating transcription factors also reported down-regulation with chlorantraniliprole therapy. Nine of the 15 transcription factors that were down-regulated were noticeably under-expressed.

Due to the low water solubility of chlorantraniliprole, acetone was used as the solvent. In all our experiments, acetone was considered the vehicle control. In the group treated with chlorantraniliprole and the vehicle control, 13306 genes were expressed. In the treatment group, 890 unigenes were identified as DEGs, 1416 genes were up-regulated, 970 genes were down-regulated (Figure 2.6A), and 177 were expressed solely in the vehicle control group (Figure 2.6B). A heatmap was created for the top 20 genes expressed (Figure 2.7). The differential expression of 11 genes encoding detoxification enzymes was observed in samples treated with acephate compared to the controls, as shown in Table 2.6. Out of which, nine DEGs were down-regulated.

2.4.2c Acephate vs. Chlorantraniliprole

The transcriptome profiles of the *D. melanogaster* larvae exposed to one-fifth of the $EC_{50}$ of acephate and chlorantraniliprole. The 432 genes were exclusively upregulated in the acephate treatment, and 551 were over-transcribed in

chlorantraniliprole treatment (P value < 0.05) (Fig. 2.8A). Despite their differences in mode of action, these larvae exhibit distinct expression profiles after exposure to acephate and chlorantraniliprole, even when, in some cases, they share similar Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway terms. These pesticides affect several biological and metabolic processes, including proteolysis, xenobiotic P450 metabolism, immunological response, carbohydrate metabolism, transcription factors, protein modification, heat shock proteins, chromosome-associated proteins, G-protein coupled receptors, and apoptosis. Around 888 and 397 genes were down-regulated in acephate and chlorantraniliprole, respectively (P value < 0.05). Processes associated with chitin binding, body morphogenesis, chitin-based cuticle development, and metabolism of xenobiotics by cytochrome P450 were down-regulated in acephate and chlorantraniliprole-treated samples. In comparing the up-regulated genes in these pesticides' treatments, genes controlling proteolysis were the most abundant over-transcribed genes. In acephate and chlorantraniliprole-treated groups, 35 and 65 genes governing proteolysis were significantly transcribed, respectively. Compared to the control, the number of unique genes also differed between the two pesticide groups; 1030 genes were in the acephate group, and 521 genes were in the chlorantraniliprole-treated group. When we compared the overall gene expression patterns between the control, vehicle control, acephate-treated, and chlorantraniliprole-treated groups, it was clear that 12867 genes were expressed in all groups. The number of unique genes differed between groups. Unigenes in control and vehicle control, respectively, 54 and 64, and acephate and chlorantraniliprole, each containing 293 and 182 unique genes (Figure 2.8B). A heatmap was created for the top 20 genes expressed (Figure 2. 9).

Upon thorough examination of the differentially expressed genes (DEGs), we could discern a distinct difference between the groups treated with acephate and those treated with chlorantraniliprole. The acephate group showed 16 up-regulated transcription factors, whereas the chlorantraniliprole group only showed differential expression of two transcription factors. Similarly, the transcript of the chromosome-associated protein showed variation. Nine transcripts were up-regulated in chlorantraniliprole treatment and were only observed in acephate treatment.

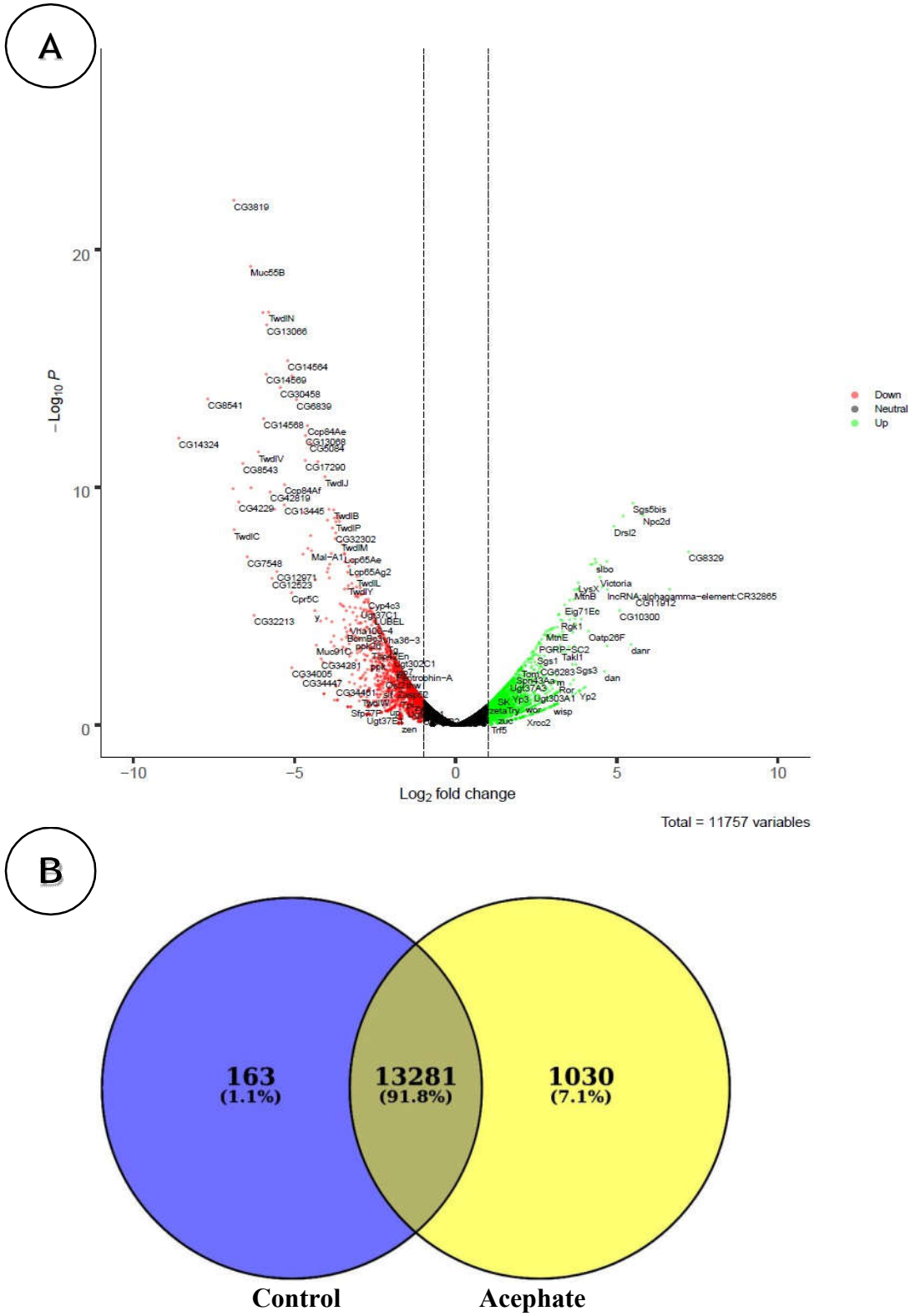



**Figure 2.2**: Comparative expression of acephate vs. control group in *D. melanogaster*. (A) Volcano plots of up-and down-regulated genes in 1/5$^{th}$ EC50 acephate treatment group compared with the control group; (B) Venn diagram of differential genes between the control and acephate treatment group.

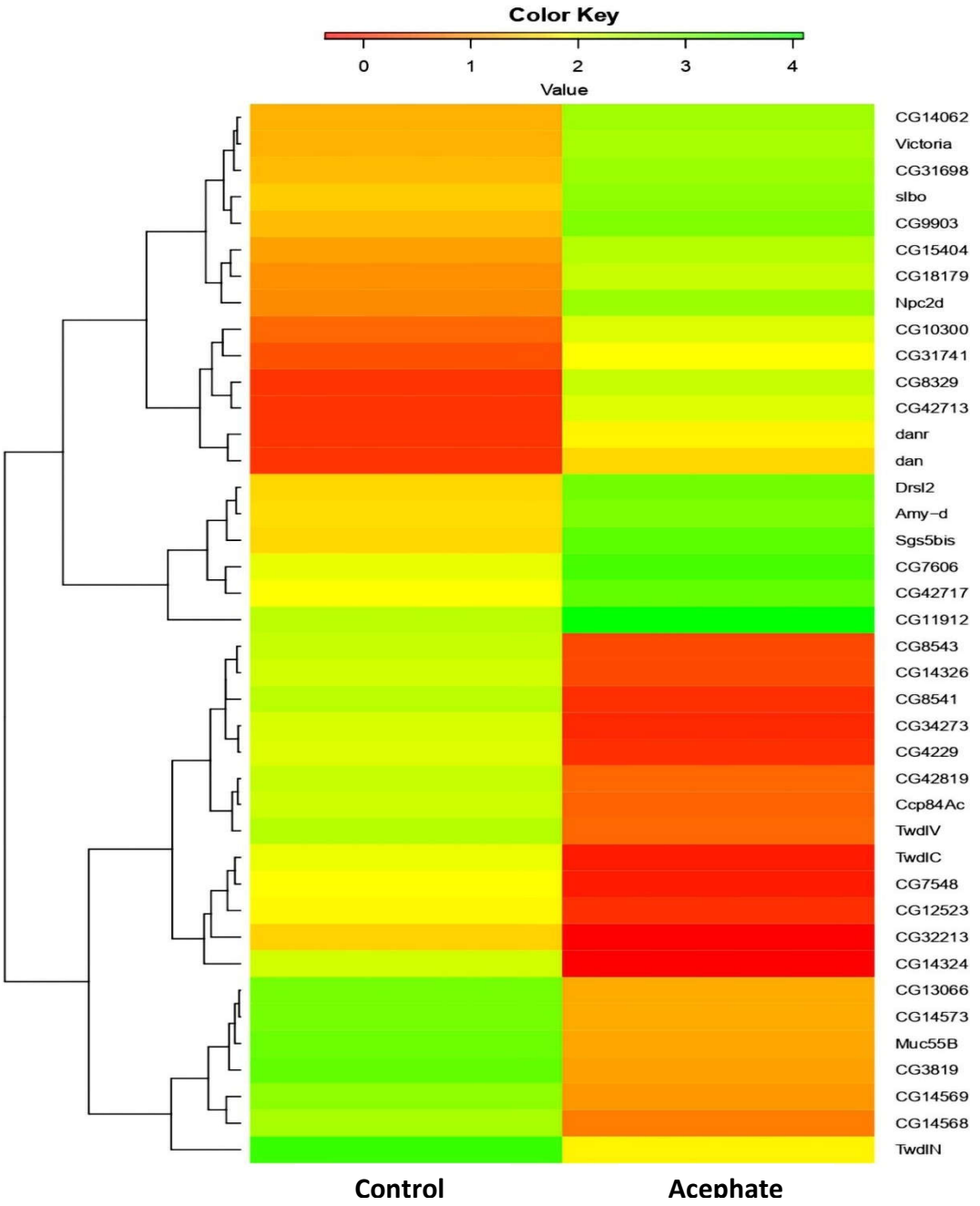



**Figure 2.3**: Heatmaps of DEGs in 1/5$^{th}$ $EC_{50}$ Acephate treatment groups compared with the control group.

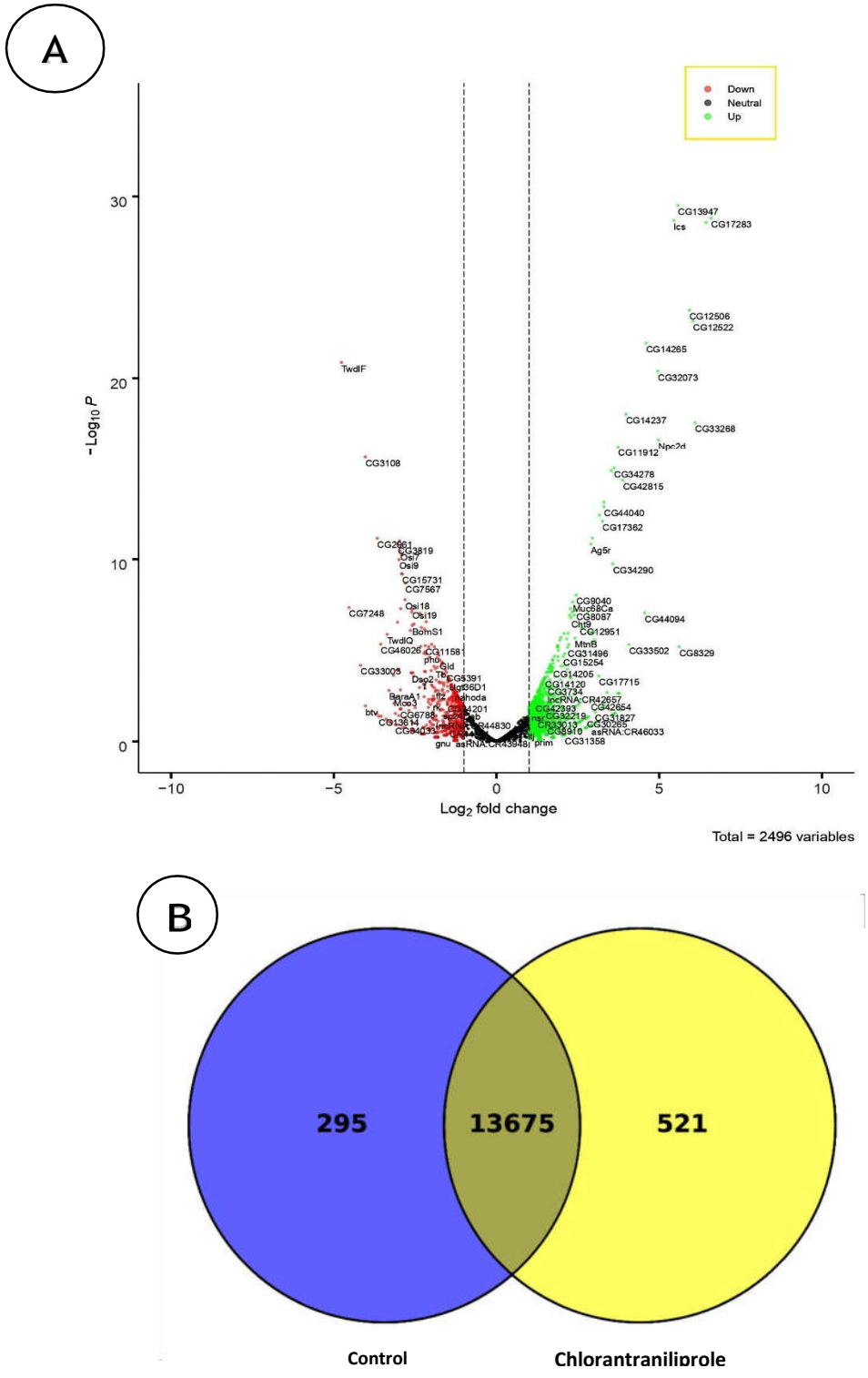



**Figure 2.4**: Comparative expression of chlorantraniliprole vs. control group in *D. melanogaster*. (A) Volcano plots of up- and down-regulated genes in the 1/5th $EC_{50}$ chlorantraniliprole treatment group compared with the control group; (B) Venn diagram of differential genes between the control and chlorantraniliprole treatment group.

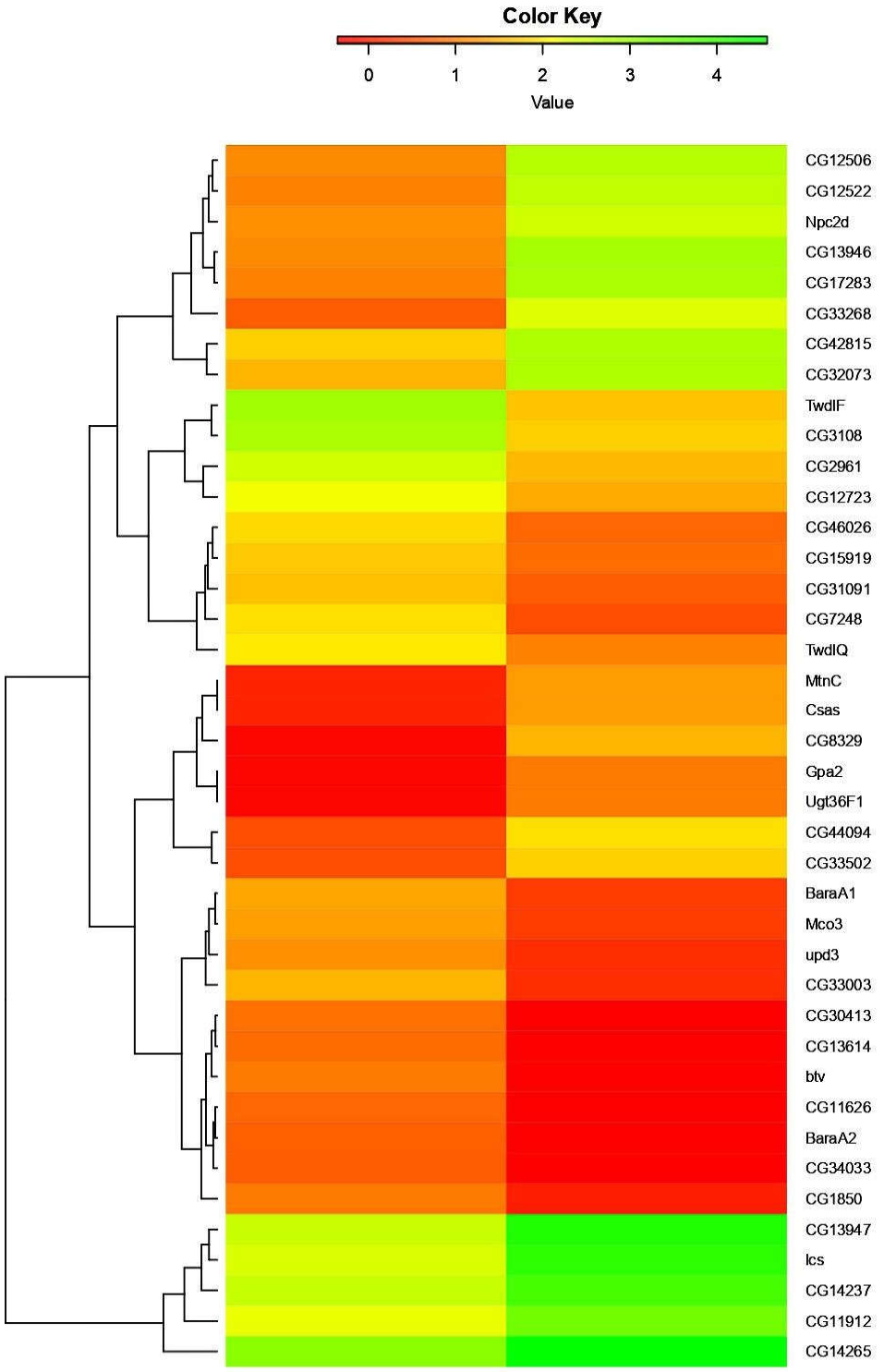



**Figure 2.5**: Heatmaps of DEGs in 1/5$^{th}$ $EC_{50}$ chlorantraniliprole treatment groups compared with the control group.

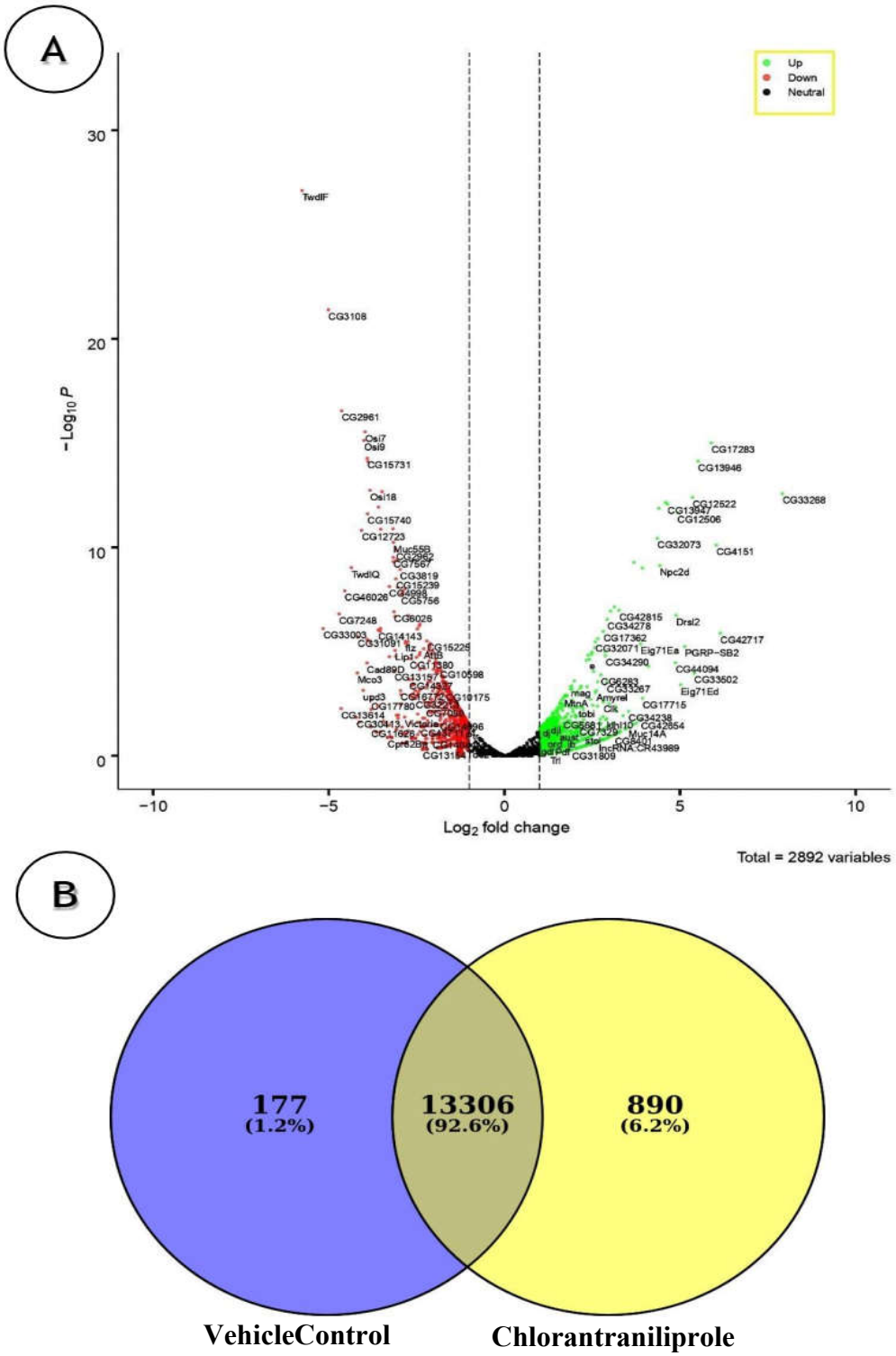



**Figure 2.6**: Comparative expression of chlorantraniliprole vs vehicle control group in *D. melanogaster*. (A)Volcano plots of up- and down-regulated genes in the 1/5$^{th}$ $EC_{50}$ chlorantraniliprole treatment group compared with the vehicle control group; (B) Venn diagram of differential genes between the vehicle control and the chlorantraniliprole treatment group.

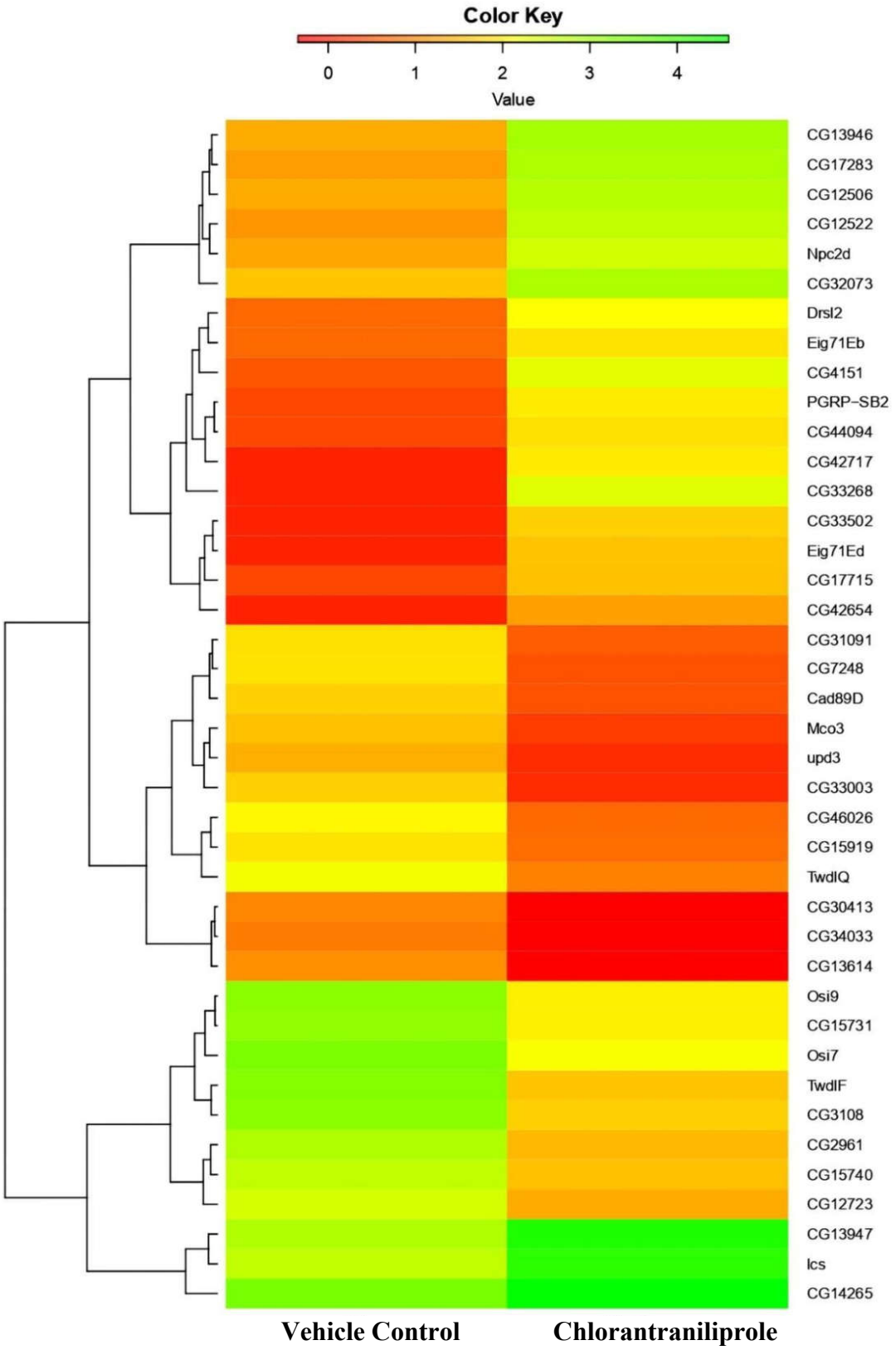



**Figure 2.7**: Heatmap of DEGs in 1/5$^{th}$ EC50 chlorantraniliprole treatment groups compared with the vehicle control group.

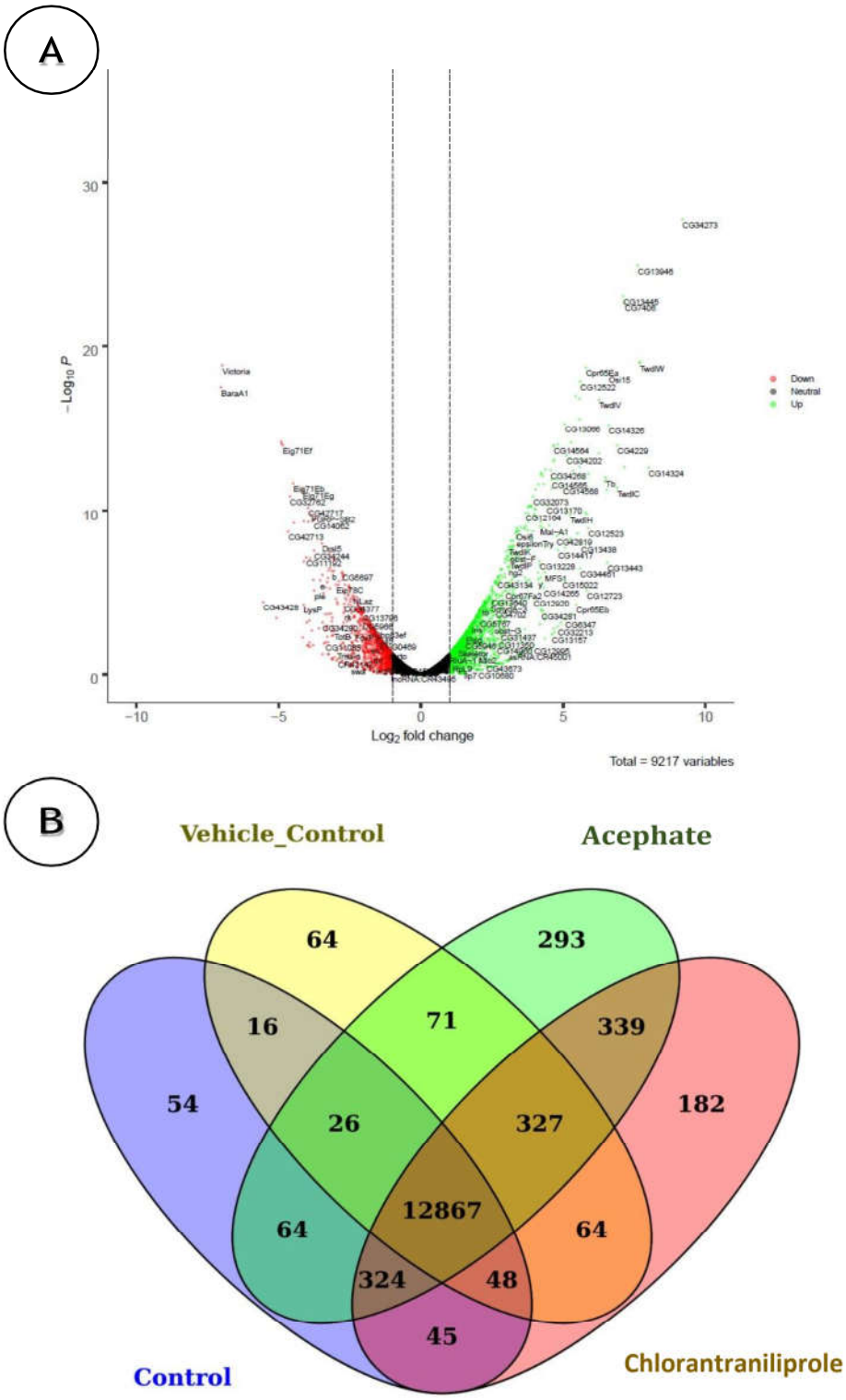
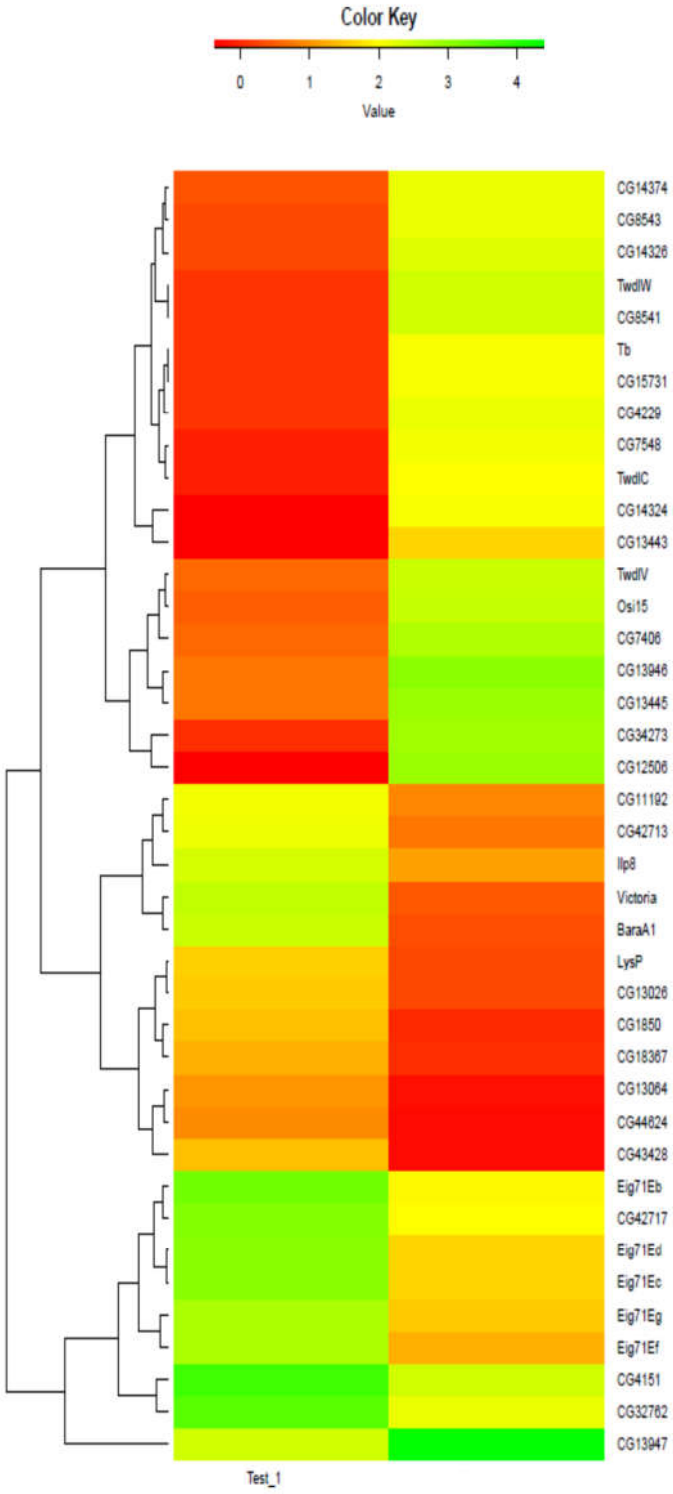



**Figure 2.8**:Comparative expression of acephate vs. chlorantraniliprole in *D. melanogaster*. (A) Comparative Volcano plots of up and down-regulated genes in 1/5$^{th}$ $EC_{50}$ of acephate and chlorantraniliprole treatment group; (B) Venn diagram of differential genes between chlorantraniliprole and acephate treatment group.




**Acephate** **Chlorantraniliprole**

**Figure 2.9**: Comparative heatmaps of DEGs in 1/5$^{th}$ $EC_{50}$ of acephate and chlorantraniliprole treatment groups.

## 2.6 DISCUSSION

It is widely accepted that chemicals and other toxins initially cause molecular alterations in biology. Xenobiotic-induced alterations in gene expression may ultimately lead to cellular-level biological changes. Pre-treatment with sub-lethal concentrations of different insecticides has been shown to change the gene expression profile of *D. melanogaster*. (Gao et al., 2020). Currently, RNA-seq analysis is a widely used technique for identifying insecticide-responsive genes (Shu et al., 2021). In pesticide toxicology, investigating gene expression regulation mechanisms may aid in pinpointing the early and mechanistically significant cellular events associated with a specific reaction (Rodrigues et al., 2019). Pesticide-induced toxicity is a complex phenomenon that can interfere with cellular homeostasis, particularly in protein synthesis (Lushchak et al., 2018). Proteolysis is a component of cellular homeostasis; it can sometimes be triggered by environmental stresses, such as heat or biological or chemical assaults (Pham et al., 2014). Our result revealed that the unbalanced proteolysis of these pesticides triggered various physiological responses in *D. melanogaster*. In the acephate and chlorantraniliprole samples, the proteolysis genes with the highest log2fc values were *CG8329, CG12951*, and *CG11912*. Additionally, unbalanced proteolysis may result in the under- or over-regulation of specific proteolysis genes. Our findings confirmed that several proteolysis genes were overexpressed in both pesticide-treated groups, especially in the chlorantraniliprole-treated groups, which showed 65 proteolysis differentially expressed genes (DEGs). When compared to vehicle control, 40 DEGs were over-expressed in chlorantraniliprole-treated groups. In larvae exposed to acephate, 13 genes were downregulated at a significant level, most of which were part of the trypsin enzyme family.

All insects contain a varied class of heme-thiolate enzymes encoded by the large multigene family known as cytochrome P450S (Feyereisen, 1999). The *Drosophila* P450 genes are only partially characterised, although several have been linked to xenobiotic detoxification (Chung et al., 2009). The GST, COE, UGTs, and ABC transporters are also involved in the metabolism of xenobiotics, as well as the

P450 genes. Generally, xenobiotic metabolism includes multiple biotransformation pathways, including Phase I (P450 and esterase), II (GST and UGT), and Phase III (ABC transporter) enzyme systems. Xenobiotics are classified into non-toxic forms through the Phase I and II enzyme systems. The byproducts of Phase I processes frequently serve as substrates for Phase II enzymes, allowing the elimination of substances from the body. In Phase III, for example, water-soluble chemicals are transported and eliminated by ABC transporters (Amezian et al., 2021). In this study, DEGs engaged in biosynthesis, transport, and catabolism of secondary metabolites were primarily categorised into P450 enzymes (significantly concentrated in the "metabolism of xenobiotics by cytochrome P450″ and "drug metabolism-cytochrome P450″ pathways) in both treatments. Insecticide and xenobiotic metabolism are known to be regulated by genes of the CYP6 family, which confer tolerance and resistance (Feyereisen, 2006). CYP6 groups contribute to pesticide resistance in various arthropod species. The *Cyp6g1, cyp6d5*, and *cyp4ad1* are most significantly over-transcribed genes in acephate-treated larvae. Likewise, *cyp4p1* and *cyp6a20* were up-regulated in chlorantraniliprole treatment, and this finding is consistent with a previously reported study on *F. Occidentalis* (Gao et al., 2020). Even though the up-regulation of pesticide resistance genes and detoxification genes are general phenomena, our study's promising results and most striking results were that most genes involved in xenobiotic metabolism were down-regulated. Out of 18 under-expressed detoxification genes, 10 genes (*cyp4e1, cyp4c3, cyp9a1, cyp6w1, cyp4d20,cyp4d21, GstD2, Ugt37C1, CG33178,* and *CG10226*) had a below minus two log2fc value; this result pointed to the inactivation of resistance genes. Genes that control drug metabolism were more negatively regulated in acephate-treated larvae than in chlorantraniliprole treatment. In acephate-treated samples 29, 18 genes were down-regulated significantly, and eight had log2fc values above two. Eight xenobiotic metabolic genes were significantly under-transcribed in chlorantraniliprole-treated larvae in response to pesticide stress. This is further supported by the results of our vehicle control versus chlorantraniliprole DEGs, which confirmed that nine detoxifying genes were downregulated in chlorantraniliprole samples. Our study

results rely more on the decreased resistance, even though some detoxifying genes are up-regulated.

The pesticide-exposed larvae exhibited significant differences in the expression of immune-related genes. The fruit fly *D. melanogaster* employs a variety of innate defence mechanisms, many of which are similar to those found in higher organisms. These mechanisms include physical barriers, as well as systemic and local immune responses (Lemaitre & Hoffmann, 2007).

As far as down-regulated immune genes are concerned, cuticular proteins are major under-expressed genes. In addition to metabolic defence, cuticular and target-protein resistance also play a role in insect resistance to insecticides. Insect cuticular resistance is strongly related to chitin, the main component of the insect epidermis, midgut peritrophic membrane, and tracheal system. The rice brown planthopper is affected by the biological insecticide validamycin, which has been demonstrated to disrupt the chitin production pathways, leading to aberrant morphologies or even mortality (Tang et al., 2017). Similarly, in *Spodoptera frugiperda*, chlorantraniliprole treatment resulted in the down-regulation of seven of the eight critical enzymes in the chitin biosynthesis pathway, along with two genes encoding enzymes related to the chitin catabolism pathways (He et al., 2023). Three immune-related gene groups were involved in the intricate process of the insect immune response. The first group, including Pattern recognition receptors (PRRs), such as PGRP, GRP, LBP, lectin, SR, hemolin, integrins, DSCAM, and tetraspanins, was responsible for recognising pathogens (Wang et al., 2020; Shu et al., 2021). The second set of genes controls and activates the expression of the third group of genes, which encode antimicrobial peptides. These immune signalling pathways include the Toll, immunodeficiency (IMD), Janus kinase/signal transduction, and activator of transcription (JAK/STAT) pathways (Teixeira, 2012). Antimicrobial peptides (AMPs) are produced when the innate immune system recognises microorganisms or chemicals that are not self. Although it was initially thought that chemical pesticides had no impact on the generation of antimicrobial peptides (James & Xu., 2012), several studies have

currently shown that insecticide exposure significantly affects the expression level of some antimicrobial peptides.

When insects were subjected to insecticides at sublethal doses, an over-transcription of AMPs was observed. In response to acetic acid, ethanol, and 2-phenylethanol exposure, numerous AMPs regulated by the Toll and IMD pathways were elevated in *D. melanogaster* (Seong et al., 2020). When *D. melanogaster* was exposed to six insecticides, six AMPs, and one PGRP regulated by the Toll and IMD pathways, these genes were overexpressed (Gao et al., 2021). In contrast to the previous study, our study speculated that there are more expressions of immune-related genes than overexpression. In acephate and chlorantraniliprole-treated samples, 100 and 61 genes were down-regulated, respectively. Even though effective immune responses are vital for survival, they require high energy input (Lazzaro & Galac, 2006) because they utilise resources from different physiological processes (Zerofsky et al., 2005 & DiAngelo et al., 2009). Intense or protracted immune responses can result in metabolic imbalance, which can cause wasting in mammals and flies, affecting metabolism, reproduction, and responses to environmental stress (Fitzpatrick & Young, 2013). Several examples of antimicrobial peptide under expression have also been observed when insects are exposed to sub-lethal pesticide stress. For instance, in *D. melanogaster*, a low dose of imidacloprid dramatically reduced the expression levels of *diptericin A* and *drosomysin-like 2* (Martelli et al., 2020). Lack of resources from other metabolisms may cause the down-regulation of immune response genes.

In addition to the expression of resistance genes, Hsps play an essential role in pesticide resistance, serving as molecular chaperones. According to Nazir et al. (2003), the larvae of *D. melanogaster* treated with captafol displayed significant Hsp70 expression. Additionally, six Hsps were down-regulated, while two Hsp70s were up-regulated in a population of *Plutella xylostella* that was resistant to chlorpyrifos. In *Sogatella furcifera* exposed to imidacloprid treatment, six Hsp70s were elevated, whereas one Hsp was down-regulated, according to Zhou et al., (2018). The Hsps are thus clear markers of pesticide stress. The acephate-treated *D.*

*melanogaster* larvae revealed 13 central genes encoding Hsp proteins, including *hsp70, hsp60, hsp27, hsp67, hsp23, hsp68*, and *hsp26*. However, in the case of chlorantraniliprole-treated larvae, only 6 Hsp genes were over-expressed, and all of them belong to the *Hsp70* family of genes. When we compared the genes involved in the detoxification mechanisms of two pesticides, more detoxification genes were differentially expressed in the acephate-treated groups. Clear-cut data that detoxification genes were under-expressed in chlorantraniliprole and acephate-treated groups, indicating reduced resistance. In support of detoxification genes, immunity response genes also showed under-expression in both pesticides, especially in the acephate-treated group. The increased expression of HSP70 genes may protect against pesticide-induced immunotoxicity. D'Souza et al. (2022) study explored the protective role of Heat Shock Protein 70 (Hsp70) against benzene-induced immunotoxicity and its effects on development. DEGs responding to proteolysis and chromosome-associated proteins validated that more DEGs are expressed in the chlorantraniliprole group. Although a low EC50 value was recorded in the chlorantraniliprole-treated groups, the acephate-treated groups showed the highest number of DEGs related to detoxification, immune response, and stress response. The decreased response to these genes in chlorantraniliprole-treated groups may be due to the insect-specific mode of action of chlorantraniliprole.

The principal energy and substrate source for an insect's everyday activities is its metabolism of carbohydrates and energy. Curiously, in the acephate and chlorantraniliprole, essential participants in the metabolism of carbohydrates, including *Amy-d, Amy-p,* and *Amyrel* were up-regulated along with these three genes; in the chlorantraniliprole groups, six other genes involved in carbohydrate metabolism were up-regulated (*tobi, cht9, mal-A2, CG14740, Gpdh2*, and *Tk*). The outcomes align with the findings of studies conducted on *Frankliniella occidentalis* treated with sublethal concentrations of three different insecticides (Gao et al., 2020). They reported the up-regulation of alcohol dehydrogenase, GDH, 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase, and phosphoenolpyruvate carboxykinase. In Seven genes that regulate starch and glucose metabolism showed down-regulation in acephate-treated groups. However, different studies (Meng et al., 2019 & Xu et al.,

2022) reported gene down-regulation in carbohydrate metabolism. These differences might indicate that various pesticide treatments affect insects' glucose metabolism differently. According to our research, carbohydrates may be essential for protecting the body against stress caused by acephate and chlorantraniliprole. Up-regulation of carbohydrate metabolism genes in both pesticides is linked to *D. melanogaster's* resistance. However, the down-regulated genes also reported may be connected to other detoxification genes. One study, for example, hypothesised that the resistant olive fly's higher energy consumption would be required to activate their detoxifying mechanism, suggesting a role in insecticide resistance (Sagri et al., 2014).

## 2.7 CONCLUSION

When combined, RNA-seq technology (transcriptomics) is a valuable tool for studying the molecular mechanisms underlying the toxic effects of sub-lethal concentrations of acephate and chlorantraniliprole in *D. melanogaster* larvae. Most studies have evaluated the lethal dose of drugs. Nonetheless, our study focused on sub-lethal concentrations of pesticides. From the study, we can clearly distinguish various expression levels of transcripts in the two pesticide-treated groups. Each pesticide showed a unique way of expression directly linked to its mode of action. Both pesticides significantly alter the various pathways and genes crucial for pesticide resistance and immunological response. According to our findings, sublethal acephate and chlorantraniliprole exposures can cause up- or down-regulation of P450s, ESTs, GSTs, UGTs, and ABC transporters. These findings are significant for understanding the whole range of detoxification-related gene activities in *D. melanogaster* larvae. Key findings of the study concluded that the sub-lethal concentration inhibits the immunological genes, heat shock proteins, and resistance genes of a non-targeted insect species. Based on the study, we recommend that sub-lethal concentrations or residues of acephate and chlorantraniliprole significantly affect the immune and resistance mechanisms of non-targeted organisms. Our data will aid in the hazard and risk assessment for the environmental management of this insecticidal product by highlighting systematic toxicity processes in response to acephate and chlorantraniliprole.

Advanced studies, such as proteomics and metabolomics, are necessary to elucidate detoxification pathways, which facilitate the development of scientific strategies for protecting non-target species from xenobiotics.

# Chapter 3

# Effects of sub-lethal concentrations of Acephate and Chlorantraniliprole on wings of *Drosophila melanogaster*: Geometric morphometric analysis

## 3.1 INTRODUCTION

For some time, acephate insecticides have been widely used to control insects, particularly those that bite and chew, by directly affecting their nervous systems (Oyugi et al., 2024). These insecticides are effective, reasonably priced, and highly selective against pests like leaf miners, aphids, thrips, and infected wasps. Research on specific plants has revealed the presence of these pesticide residues, raising concerns about the safety of humans, animals, and other invertebrates (Datta et al., 2010). Despite being widely used in domestic and international agriculture, acephate has been subject to rigorous controls and partial prohibitions in several countries due to its potential for environmental and human toxicity (Sampaio et al., 2020; Morato et al., 2021). Long-term exposure to acephate and its metabolite methamidophos causes an accumulation of the substance in non-target organisms, increasing the risk of both direct and indirect toxicity. Thus, because methamidophos is exceptionally poisonous to non-target creatures, misusing acephate and methamidophos can result in severe environmental contamination and potentially fatal health issues (Ortega-Olvera et al., 2018; Lin et al., 2020). Regular intake of contaminated food causes these residues to gradually accumulate in humans, which can have detrimental health impacts like cancer, increased oxidative stress, hypertension, and disruption of reproductive health, all of which can result in high medical costs (Lin et al., 2022).

Recently, the Insecticide Resistance Activity Committee (IRAC) class 28 ryanodine receptor modifying diamides have been registered as the most effective form of insecticidal activity. They account for 8% of the agrochemical market and are becoming more and more popular every year increasingly popular yearly (Sparks & Nauen, 2015). The binding of chlorantraniliprole to the ryanodine receptor is thought to be specific for a variety of problem insects that feed on crops, especially lepidopteran species, compared to other insects, such as dipteran and hymenopteran species (Qi & Casida, 2013). Because of their minimal risk, diamide insecticides are employed in many applications (foliar, seed, and soil treatments) to control various crop pests (Oliveira et al., 2019). The ecological environment (plant development and non-target creatures) is significantly impacted by these insecticides' widespread

application and incorrect usage (Ma et al., 2021). Chlorantraniliprole and cyantraniliprole, the first insecticides of diamide, inhibit earthworm growth and reproduction by increasing reactive oxygen species (ROS) and malondialdehyde content (Liu et al., 2018 and Qiao et al., 2019). Cui et al. found that acute exposure to three diamide insecticides increased reactive oxygen species (ROS) and catalase activity, and decreased antioxidant enzyme activities. Chronic exposure could cause lethal and sub-lethal effects on aquatic ecosystems, indicating the potential ecological risks of even low-diamide insecticides (Cui et al., 2017).

Acephate and chlorantraniliprole affected the developmental, biochemical and molecular levels of *Drosophila melanogaster*. Previous chapters discussed the potential impact of acephate and chlorantraniliprole in *D. melanogaster*. However, relatively few studies have investigated the sublethal effects of pesticide exposure on non-target wing morphology.

In this chapter, we discussed the morphological variations resulting from the toxicity of acephate and chlorantraniliprole. Morphological variation is primarily measured in terms of size and shape. Exploring average shape and size variation can be accomplished with an efficient and sensitive tool known as geometric morphometric analysis. It can identify minute morphological changes in biology as a quantitative tool. In toxicology, quantitative analysis enables toxicologists to find answers to numerous morphological issues and identify any subtle changes induced by toxicity. Shape analysis is one approach to deciphering the reasons for variation and the morphological changes in animals resulting from evolution, age, disease, and selective breeding (Zelditch et al., 2004).

Wing shape is a candidate metric that may serve as a warning indicator of insect developmental stress (Hoffmann et al., 2002). The alterations in wing shape may be susceptible to a range of environmental stressors because the development of shape requires a series of genes that act throughout development (Garcia-Bellido, 1977 and GarciaBellido & de Celis, 1992), and slight modifications throughout development can result in considerable changes in shape (Emlen & Nijhout, 2000). Although the shape of the wings of *Drosophila* veins can vary, there is a slight

variation within the typical temperature range used for *Drosophila* cultures (Birdsall et al., 2000). There is so much evidence that shape changes occur in response to stress. Stress may include pesticide stress, physical stress or biological stress. Exposure to $CO_2$ modified the wing asymmetry and shape of the bumblebee *Bombus impatiens* (Klingenberg et al., 2001). In *D. melanogaster*, the wing aspect ratio is a measurement of the wings' width to their length and has been linked to environmental factors, especially temperature extremes (Azevedo et al., 1998). Pesticide and temperature-stress-exposed *Helicoverpa punctigera* moths exhibit distinct wing morphological changes, which may indicate an increased susceptibility to alterations in wing asymmetry and size (Hoffmann et al., 2002).

The wing of *Drosophila* is a well-suited model for examining the evolution and development of quantitative morphological diversity. It is one of the most extensively researched systems in developmental biology and quantitative genetics. In this study, the *Drosophila* wing was divided into two compartments: proximal and distal. Both modules have been considered promising candidates for independent developmental modules because they originate from different cell lineages representing different gene expression domains. Nowadays, geometric morphometric methods are frequently employed to investigate morphological integration and have been utilised to address issues regarding the developmental foundation of morphological alterations, such as the evolution of insect wings (Klingenberg et al., 2001; Klingenberg, 2004, 2008, 2009, 2013; Mitteroecker & Bookstein, 2009). Various analyses have been conducted to investigate and illustrate the integration patterns in flying insects (Klingenberg & Zaklan, 2000; Klingenberg et al., 2001; Klingenberg, 2009). Due to our increased understanding of the genetics and developmental processes of *D. melanogaster*, wing shape has been utilised extensively as a model feature (Palsson & Gibson, 2000; Bai et al., 2012). Although a growing body of research indicates that distinct regions along the proximo-distal (PD) axis of the *Drosophila* wing may have some degree of autonomy, modularity along this axis has not been thoroughly evaluated (Klingenberg, 2009). Following gene expression patterning, the adult *Drosophila* wing is split into two primary sections along the PD axis (Rodríguez et al., 2002; Perea et al., 2009): the wing base often

referred to as the proximal wing (Whitworth & Russell, 2003) or wing hinge, and the wing blade (Matamoro-Vidal, 2015). Although the *Drosophila* wing is a fully integrated structure, proximal-distal module separation is evident. Therefore, the primary purpose of this study is to investigate whether pesticide treatments are associated with proximal-distal module separation.

Although the wing is an adult structure, its development process begins in the embryonic stage. The wing is formed from a precursor group of 30 cells invading the embryonic ectoderm in the anterior/mid-part of the embryo (Bate & Martinez-Arias, 1991). These cells form the wing imaginal disc, a mono-layered sac of epithelial cells. The disc differentiates into a columnar cell layer, which develops into the adult wing, thorax, and peripodial membrane. The wing disc proper consists of a circular patch of cells (Alexis et al., 2015). In the first larval instar, the wing disc grows moderately without cell division (Madhavan & Schneiderman, 1977). It becomes exponential during the second and early third instar, resulting in 10-11 rounds of cell division (Garcia-Bellido & Merriam, 1971). The disc patterning establishes three body axes and five longitudinal proveins. During the growth phase, the wing disc shape changes from a nearly circular to an ellipsoid shape due to anisotropic growth. By the end of the third instar, the wing disc is an organised structure (Bittig et al., 2009). During metamorphosis, the wing imaginal disc folds to form a two-layered epithelium, with ventral and dorsal compartments (Fristrom & Fristrom, 1993). This process, known as evagination, involves cell rearrangements, shape changes, and division (Taylor & Adler, 2008; Kanca et al., 2014). Other key events include the larval epidermis perforated, the wing disc protruding, and the anterior-cross vein visualised. Proliferation is arrested from 6 hours before pupariation to 18 hours after pupariation, but the blade is considerably elongated. Between 18 and 35 hours after pupariation, the wing shape undergoes a profound alteration, becoming similar to that of the adult wing. This process is crucial to morphogenesis, as cell intercalations and divisions modify the wing's shape. The process is characterised by a significant rate of change and fragile tissue (Aigouy et al., 2010; Sugimura & Ishihara, 2013). Over that time, there is roughly one round of cell division, but the wing blade's area does change (Aigouy et al., 2010), most likely due to a decrease in the cell area. The final stages,

from the 35-hour apical phase to eclosion, are less well understood. During this period, the wing epithelium is embedded in the adult cuticle, which expands due to cell size and folds within the puparium (Fristrom & Fristrom, 1993). After eclosion, epithelial cells undergo an epithelial-mesenchymal transition, delaminate, and migrate into the thorax, leaving only a few living nerve cells and the folded adult cuticle (Kiger et al., 2007).

The multitude of genes influencing wing shape variation, in addition to environmental factors (Bitner-Mathe & Klaczko, 1999). The size and shape of the Drosophila wing are primarily determined by four major morphogenetic processes: spatial regulation of mitotic density, cell division orientation, biased cell rearrangements and intercalation, and differential cell death (Lecuit & Le Goff, 2007). Cells can change relative positions by remodelling their contacts with neighbouring cells, leading to tissue elongation (Bertet et al., 2004). Differential cell death can result in dramatic remodelling of tissue shape (Montero & Hurlé, 2010). Understanding these processes during development is crucial for explaining the variation in wing shape. According to Weber et al. (1999), natural variations in wing shape in Drosophila are controlled by numerous genes that likely act throughout their development. We can measure a wide range of wing shape parameters during pesticide treatments because all processes are interrelated due to the connections among the cells that make up the wing during development and in the adult. Any developmental alteration that impacts a single wing feature, like the vein's length, must also impact nearby wing areas; any one wing measurement incompletely captures wing variation (Mezey & Houle, 2005). To identify differentially expressed wing development genes following sub-lethal exposure to acephate and chlorantraniliprole, we employed transcriptome analysis in our study. Combining geometric morphometrics with transcriptome analysis will pave the way for significant milestones in the analysis of pesticide-induced wing shape variation.

## 3.2 REVIEW OF LITERATURE

Since people have relied on cultivating crops as their primary food source, insects have posed a persistent danger to the food supply. When synthetic pesticides first came into use over 50 years ago, there was much hope that they could permanently address the world's issues with food and agricultural output (Oberemok, 2015). Pesticide use is predicted to increase food production by 2.7 times between 2000 and 2050, endangering humans and the environment (Sexton et al., 2007). Even though pesticides are designed to affect target organisms, they sometimes affect non-target organisms, which compromises the ecosystem's ability to remain sustainable. Pesticides deteriorate in the environment over time at sub-lethal concentrations, which might impact many characteristics of pests and beneficial organisms (Biondi et al., 2012; Xu et al., 2017; He et al., 2020).

Pesticides at sublethal concentrations have the potential to alter the physiology of several systems, including the nervous (De Castro et al., 2020), muscular (Castro et al., 2021), integumentary (Zanuncio et al., 2016), respiratory (Plata-Rueda et al., 2019a), digestive (Fiaz et al., 2019), excretory (Catae et al., 2014), reproductive (Amaral et al., 2018), circulatory (Papaefthimiou &Theophilidis, 2001), and exocrine (Santos-Junior et al., 2019). Additionally, behaviour-altering systems include those that involve mobility (Plata-Rueda et al., 2019b), orientation (da Silva Rolim et al., 2020), feeding (Plata-Rueda et al., 2020b), and reproduction (Xu et al., 2017; Martínez et al., 2019a, b; 2021). There were significantly few studies available on the morphological variations caused by sublethal exposure to pesticides.

Numerous biological investigations in systematics, taxonomy, and evolution begin with analyses of the morphology of living things. Formally speaking, shape is defined as an object's geometry when its position, orientation, and size are removed (Kendall, 1977); thus, it is well-suited for quantitative study. The fundamental "tool" for analysing variations in morphological units is morphometry, which studies the size and form of morphological objects and the interactions between morphology and internal and external variables. Data from measurements are analysed statistically (Rohlf, 1990). These investigations can establish the presence and strength of an

external stressor's impact on the phenotypic or the size and shape of morphological structures and quantify that impact. Geometric morphometry (GMM) involves the mathematical definition of objects by arranging specific delineated points that describe their geometry. These objects are then subjected to statistical methods that examine shape differences, and direct graphical representation techniques are used to visualise the differences between the objects (Rohlf, 1990). Although geometric morphometry was created to meet the demands of taxonomic research, it has much potential for use in ecotoxicology since computer software may be used to track minute and subtle changes in structures and is a precise tool for measuring the morphological variation of a structure (Arambourou et al., 2012; Cvetković et al., 2020; Stanković et al., 2020; Savić-Zdravković et al., 2018; Rohlf& Slice, 1990; Bookstein, 1991; Goodall, 1991). This method enables the analysis of variations in the shape of organisms through a configuration developed from landmarks of the taxa (Kendall, 1977), which are extracted in geographic coordinates and facilitate the comparison of the shape of the structures within the studied population. The wing size and shape are the first morphological traits to alter due to environmental and genetic influences (Levine et al., 1996; Bouyer et al., 2007). Various authors suggest that morphological variations in aquatic individuals can indicate pollution and, therefore, be considered a potential tool for evaluating the quality of water sources (Arambourou et al., 2012; Adams et al., 2004; Odume et al., 2016).

Insects exposed to hazardous agents may have adverse effects that negatively affect their fitness or progeny. For instance, applying 1 mL of the insect growth regulator lufenuron topically to fifth instar nymphs on two strains of *Cimex lectularius* (Hemiptera: Cimicidae) at varying sub-lethal dosages caused anomalies in the morphology of the late instars and adults' legs (Campbell et al., 2017). Previous researchers have examined many morphological criteria to gain an understanding of the sublethal toxicities of pesticides. Morphological changes refer to structural deviations resulting from alterations in size and shape. Recent research assessed the insecticidal efficacy of Malathion and diatomaceous earth (DE) against *Tribolium castaneum*, a damaging pest that contaminates cereals. The findings showed changes in the exterior morphology of the larvae, including particles adhering to the parts

mouthparts and the abdominal end, a larger abdominal end, and smaller, more petite mouth and abdominal segments (Elmadawy et al., 2024).

The wing of *Drosophila* is a suitable model to examine the evolution and development of quantitative morphological diversity. It is one of the most extensively researched systems in developmental biology and quantitative genetics. *D. melanogaster* is extensively studied to explore morphological variations resulting from toxic stress. When *D. melanogaster* larvae were fed the pesticide cryolite along with their regular diet, the treatment caused aberrant changes to the morphology of their compound eyes (Podder et al., 2012). Flubendiamide exposure in eye third-instar larvae of *D. melanogaster* increased stress protein hsp70 levels in larvae, leading to severe morphological deformities in the adult eyes (Sarkar et al., 2014). Cvetković et al. (2020) investigated the effects of exposure to low concentrations of human food-grade titanium dioxide nanoparticles ($TiO_2$ E171) on *D. melanogaster* wing morphology over multiple generations using geometric morphometrics. Results showed a diminishment in sexual dimorphism in wings, with females separated from males. The treatment caused significant differences in wing morphology but did not alter the general wing pattern, indicating the change occurred within the usually allowed wing variation.

Morphometry is a sensitive quantitative tool which can be of great value in assessing morphological changes in organisms caused by toxic substances. Cerisier et al. (2019) investigated the suitability of geometric morphometry in determining the degree of malformations in several diatom species to measure the deviation from the standard shape. In the study, conventional and geometric morphometrics were employed to investigate the effects of lead and 4-nonylphenol (4-NP) on the morphological variation of the mouthparts in larvae of *Chironomus riparius*. Four factors were used to evaluate the mentum phenotypic response to pollutants: frequency of malformations, fluctuating mentum length asymmetry, fluctuating mentum shape asymmetry, and mentum mean shape alterations. Significant mean shape alterations were found for lead and 4-NP exposure, respectively, resulting in increased tooth size and teeth closure. However, those variances were insignificant

compared to mentum shape alterations by hereditary influences (Arambourou et al., 2012).

The previous findings suggested that organophosphorus insecticides caused sub-lethal effects on stored-product insects. The study investigated the sublethal impact of pirimiphos-methyl on the offspring of Tenebrio molitor, Prostephanus truncatus, and Rhyzopertha dominica, noxious insect pests of stored grains. The results show variability among species, with *T. molitor* being the most sensitive, causing significant deformations in the elytra and hindwings (Boukouvala et al., 2023). Other research, however, has demonstrated the detrimental effects of insecticides on wing size and shape. For example, *Trichogramma embryophagum* and *T. evanescens* exposed to *Ferulaassafoetida L.* (Umbelliferae) oil exhibited wing abnormalities compared to the control group.

Silva et al. (2023) investigated the effect of essential oil from the aerial part of *Croton tetradenius* on larval and pupal viability and wing geometric morphometrics of *Aedes aegypti* mosquitoes, with notable changes between the treatments and the control.

Previous studies have examined the impact of long-term exposure to cerium dioxide nanoparticles (CeO2 NPs) on the morphological characteristics of the aquatic midge *Chironomus riparius*. The study found significant sublethal changes in chironomids exposed to $CeO_2$ NPs, potentially leading to further changes in metabolism, diet, and behaviour. Two methods were used to analyse morphological variation: (1) Deformity rate analysis, which looked at visible morphological malformations, and (2) Geometric morphometric analysis (GM), which looked at variations in the size and shape of the adult male and female wings, as well as the larval mandibles and mentum. Geometric morphometric analysis was employed to detect changes in all morphological features analysed at both low and high $CeO_2$ NP concentrations (Savić-Zdravković et al., 2021).

A study investigated the forewing morphologies of 294 CM populations using geometric morphometric procedures and Finite Element Method (FEM). Results showed forewing shape differences among the three treatment populations, with the

movement of landmarks driving these differences (Pajač Živković et al., 2019). Morphological integration and modularity are another set of analyses that can be carried out using GM techniques to determine the developmental structure of morphology (Schumacher et al., 1997) and to provide insights into the non-target effects of pesticides. In these studies, the *Drosophila* wing was divided into two compartments: proximal and distal. Although the *Drosophila* wing is typically cited as an example of a single, completely integrated structure, the separation of proximal-distal modules has been reported in previous studies. The *Drosophila* wing's developmental PD axis is determined by genes expressed in circular domains during larval (Terriente et al., 2008). The adult wing is divided into two central regions: the wing blade and the wing base, also known as the proximal wing or wing hinge (Rodr´ıguez et al., 2002; Perea et al., 2009). Later, during pupal development, the wing disc folds and extends, elongating the wing along the PD axis. After 15 hours, the wing is evenly divided into the proximal hinge and distal blade (Matamoro-Vidal et al., 2015; Aigouy et al., 2010; Ray et al., 2015). The specific functions of the proximal and distal modules during flight differ, and their unique architectures reflect this distinction. While the wing base is the region that transmits the forces the flight muscles generate, the wing blade generates the aerodynamic forces that will produce the lift (Dudley, 2002). At the base of the wing, where wing bending moments during flapping are greatest, vein mass and density are highest (Dudley, 2002). As a result, flexural stiffness is greatest near the base of the wing and decreases towards the tip (Combes & Daniel, 2005). Numerous investigations have demonstrated that wing modifications peculiar to each continent along the PD axis cause the latitudinal variations in wing size seen in the populations of *Drosophila subobscura* in Europe and North America. In the New World, the size of the cline was caused by modifications in the distal half of the wing, not the proximal part (Gilchrist et al., 2001), as was the case in Europe (Huey et al., 2000). Therefore, the theory that the adult *Drosophila* wing might consist of a proximal and distal module is supported by genetic, developmental, functional, and evolutionary evidence (Muñoz-Muñoz et al., 2016). According to earlier research, the *Drosophila* wing exhibits a modular structure rather than a fully integrated one. Thus, we can better comprehend how insect wings have evolved to function in various contexts, such as stress and toxins, and perform tasks like flying, sound generation, visual communication, crypsis, or mechanical protection (Wooton, 1992).

This study employs transcriptome analysis and geometric morphometric methods to assess genomic and phenotypic variations in *D. melanogaster* exposed to sublethal concentrations of acephate and chlorantraniliprole. Both genetic and environmental disturbances can result in qualitative (Waddington, 1939; Garcia-Bellido &Santamaria, 1972; Lawrence &Morata, 1976; Held, 2002; and Blair, 2007) and quantitative (Palsson & Gibson, 2000; Mezey et al., 2005; Weber et al., 2005; and Debat et al., 2006) morphological effects. Specifically, wing shape is the perfect example of a "complex" characteristic. It displays a firmly integrated multivariate phenotype (Klingenberg & Zaklan, 2000; Dworkin & Gibson, 2006; Klingenberg, 2009); information from multiple signalling pathways must be included during wing development. The analysis of shape modifications at different spatial scales can be studied using different developmental processes, including wing patterning. Wing patterning, a developmental process, can be studied using geometric morphometrics techniques to analyse changes in vein disposition (Dworkin & Gibson, 2006). These morphological differences may be caused by genetic or environmental alterations, which can be identified using quantitative genetics approaches. QTL and linkage disequilibrium mapping, as well as P-element insertion investigations, have been the primary tools employed in previous quantitative and evolutionary genetic analyses (Lukacsovich et al., 2001; Bellen et al., 2004; and Carreira et al., 2011). Whole-genome expression profiling (transcriptome) reveals how local perturbations of gene function propagate throughout the complex network of genetic pathways operating in cells, tissues, and organisms (Dworkin et al., 2011). In this study, we use quantitative RNA-seq to compare gene expression patterns between control and 1/5th of the EC50 concentrations of acephate and chlorantraniliprole-treated *D. melanogaster* larvae to identify genes involved in wing development, wing growth, and wing differentiation in this dimorphic species.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Geometric Morphometric Analysis of Wings of *D. melanogaster*

Sub-lethal concentrations of acephate and chlorantraniliprole induced changes in wing morphometry of *D. melanogaster* were evaluated through Landmark-based geometric morphometric analysis. Landmark-based geometric morphometrics is a powerful approach to quantifying biological size and shape variation.

#### 3.3.1a Image acquisition and landmark digitisation

The wings of 2-day-old adult males and females emerged from the respective experimental groups (described in Chapter 3) and were selected for geometric morphometric analysis. The flies were anesthetised using diethyl ether for wing extraction. Each fly's right and left wings were dissected and mounted on clean microscopic slides using DPX and photographed in the trinocular microscope, Zeiss Axio Scope A.1 Fluorescence microscopes with Axiocam 305 multi-chrome CCD camera. The captured images were transformed using tps Util version 1.68 x 64 - software (2016). Fifteen anatomical points were chosen for landmarks to minimise bias in digitising landmark locations (Figure 3.1). Digitisation and scale factors were performed using tps Dig2 version 2.26 (2016). The landmarks are the homologous points on the wing surface of *D. melanogaster*. Around 16,048 wings were included from the various groups in the present study. The concentration-dependent changes in wing shape were studied by grouping the right- and left-wing shapes. They left wings in different groups (Table 3.1). The position description of landmarks and abbreviations is presented in Table 3.2.

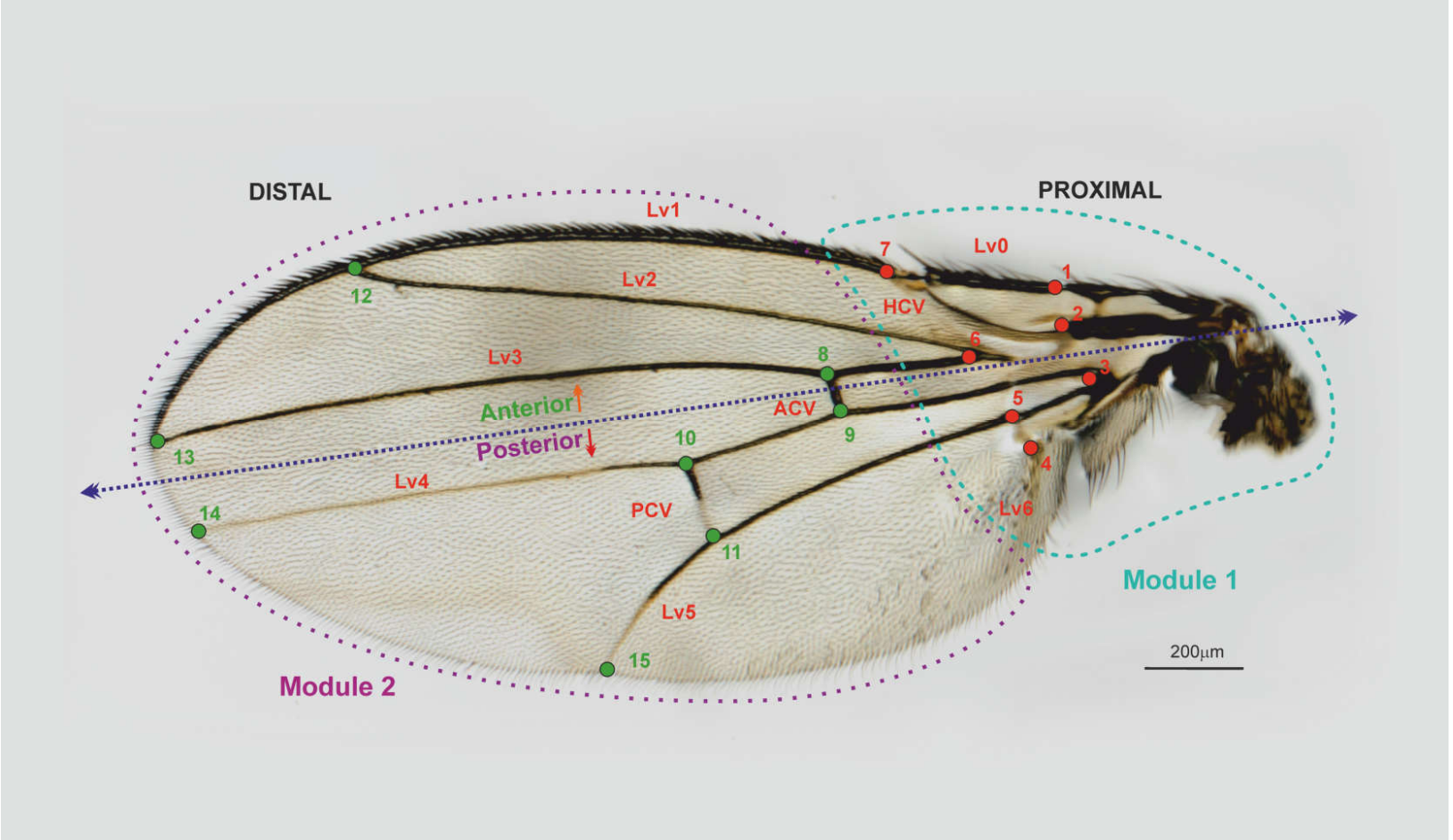



**Figure 3.1** Wing of *D. melanogaster* with landmarks and proximal-distal module separation.

Table 3.1: Experimental groups studied.

| Sl. No | Denotation | Experimental group | Total No. of wings |
|---|---|---|---|
| 1 | **CO** | Control | 3686 |
| 2 | **VC** | Vehicle control | 1791 |
| 3 | **AA** | 1/3$^{rd}$ of $EC_{50}$Acephate treated | 1680 |
| 4 | **AB** | 1/5$^{th}$ of $EC_{50}$ Acephate treated | 1724 |
| 5 | **AC** | 1/10$^{th}$ of $EC_{50}$Acephate treated | 1677 |
| 6 | **CA** | 1/3$^{rd}$ of $EC_{50}$ Chlorantraniliprole treated | 1766 |
| 7 | **CB** | 1/5$^{th}$ of $EC_{50}$ Chlorantraniliprole treated | 2256 |
| 8 | **CC** | 1/10$^{th}$ of $EC_{50}$ Chlorantraniliprole treated | 1468 |

Table 3.2: Landmark description for *D. melanogaster* wing

| Landmark | Location | Description of Landmark |
|---|---|---|
| 1 | **HB** | Humeral Break |
| 2 | **$Rs_1$** | Proximal end of Radial Sector |
| 3 | **$CuA_1$** | Proximal end of Cubitus Anterior 1 |
| 4 | **An** | Alula Notch |
| 5 | **Cu-$A_1$** | Anterior end of Cubito-Anal Cross Vein |
| 6 | **$Rs_2$** | The distal end of the Radial Sector |
| 7 | **$DR_1$** | The distal end of Radian 1 |
| 8 | **R-$M_1$** | The anterior end of Radio-median Cross Vein |
| 9 | **R-$M_2$** | Posterior end of Radio-median Cross Vein |
| 10 | **M-$Cu_1$** | Anterior end of Medio-Cubital Cross Vein |
| 11 | **M-$Cu_2$** | Posterior end of Medio-Cubital Cross Vein |
| 12 | **$DR_{2+3}$** | The distal end of Radian 2+3 |
| 13 | **$DR_{4+5}$** | The distal end of Radian 4+5 |
| 14 | **$DM_1$** | The distal end of Median 1 |
| 15 | **$CuA_2$** | Distal end of Cubitus Anterior 1 |

Measurement error has become an essential factor for analysing shape variation, particularly for studies of fluctuating asymmetry (Palmer & Strobeck, 1986), particularly for studies of fluctuating asymmetry (Palmer & Strobeck, 1986;

Palmer, 1994). Hence, replicate measurements were taken on everyone. Each wing was photographed twice to evaluate the variation in the image acquisition process. The landmarks were digitised twice on all images to assess the precision of the digitising step.

### 3.3.1b Data Analysis

The geometric morphometric analysis was performed using MorphoJ software, Version 1.06d, Java Version: 1.8.0-101, designed to analyse actual biological data (Klingenberg, 2011).

### 3.3.1c Generalised Procrustes Analysis (GPA)

After the landmark digitisation, the data of *Drosophila* wings were imported into MorphoJ, and a complete Procrustes superimposition was conducted by orthogonal projection to correct size and orientation. In the Procrustes analyses, the shape is characterised mathematically by removing the effects of location, scale, and rotation through transformations (Klingenberg & McIntyre, 1998; Debat & David 2000; Klingenberg et al., 2002).

### 3.3.1d Shape Variation and Visualisation of *Drosophila* Wings

Various methods visualised the shape variations between and among groups.

### 3.3.1e Discriminant Function Analysis (DFA)

A multivariate technique separates two or more observations based on variables measured for each experimental unit and identifies the contribution of each variable to the separation (Solberg, 1978).

### 3.3.1f Partial Least Squares (PLS)

To examine the patterns of covariation between two or more sets of variables, one or more of which contain shape data. PLS may relate shape data to other types of data (e.g., ecological information, experimental conditions) or other shape variables (Mateos-Aparicio, 2011).

### 3.3.1g Principal Component Analysis (PCA)

The principal component analysis is a way to determine the patterns in the data and express the data to highlight their similarities and differences. PCA converts a set of observations of possibly correlated variables into a set of observations of possibly correlated variables into values of linearly uncorrelated variables called principal components. PCA reduces the number of dimensions after finding the pattern; thus, it compresses the data without significant information loss. Principal components (PCs) are visualised directly as patterns of simultaneous displacements of landmarks related to one another (Berner, 2011).

### 3.3.1h Canonical variate analysis (CVA)

CVA is a method used to find the shape features that best distinguish among multiple groups of specimens (Gumiel et al., 2003; Villemant et al., 2007). This study compares the acephate and chlorantraniliprole-treated group to the control and vehicle-control groups. Discriminant analysis, scatter plot graphs, etc., were used to visualise the shape variation between two sets of variables using CVA (Adams et al., 1994).

### 3.3.2 Morphological integration and modularity

Morphological integration refers to the coordination among different traits within an organism, forming a cohesive system composed of coordinated parts. Thus, organisms are composed of several hierarchical and partially autonomous units called modules, which are sets of tightly integrated traits loosely related to traits of other such assemblies. Integration can deflect the response to a selection direction of maximum genetic variation by concentrating variation on some specific directions and limiting it in other directions. In this study, the *Drosophila* wing has been divided into proximal and distal compartments, representing the wing base and blade, respectively.

We tested the hypothesis that the adult *D. melanogaster* wing may be composed of a proximal and distal module. To test this hypothesis, the digitised landmarks were subdivided into two subsets of seven and eight landmarks, respectively (wing base: landmarks 1–7; wing blade: landmarks 8–15 (Figure 3.1).

The magnitude of integration between the subsets of landmarks was quantified as the RV coefficient. When the RV coefficient for the two tested subsets of landmarks was lower than 95% of the distributional values, it was considered statistically significant ($P < 0.05$), and the modularity hypothesis was confirmed.

### 3.3.3 Differential Expression of Genes Related to Wing Development

#### RNA Extraction and Quality Control

RNA was extracted from *Drosophila* larvae using a Qiagen RNeasy mini kit. The larvae were homogenised in a Tommy microsmash, separated with chloroform, and loaded into an RNeasy spin column. DNase I treatment and column washes were performed. RNA was eluted using nuclease-free water, and its concentration and purity were quantified using a Nanodrop Spectrophotometer. Sample integrity was assessed using Tapestation. RNA concentration was quantified using a Qubit RNA HS assay kit. The concentration and purity of RNA were quantified using a Nanodrop Spectrophotometer (Thermo Scientific, 2000).

#### Library Preparation

RNA sequencing libraries were prepared with Illumina-compatible NEBNext® Ultra™ II Directional RNA Library Prep Kit (New England BioLabs, MA, USA) at Genotypic Technology Pvt. Ltd., Bangalore, India. Approximately 500 ng of total RNA was taken for mRNA isolation, fragmentation and priming. Fragmented and primed mRNA was further subjected to first-strand synthesis followed by second-strand synthesis. The double-stranded cDNA was purified using NEBNext sample purification beads. Purified cDNA was end-repaired, adenylated and ligated to Illumina adapters as per NEBNext® Ultra™ II Directional RNA Library Prep protocol, followed by second-strand excision using the USER enzyme at 37 ˚C for 15 minutes.

#### Illumina Sequencing

The libraries were paired-end sequenced on an Illumina HiSeq X Ten sequencer for 150 cycles (Illumina, San Diego, USA) following the manufacturer's instructions.

**Data Analysis**

Transcriptome analysis was performed by processing the raw data to remove low-quality reads and adapter sequences. The high-quality reads were considered for alignment with the reference genome using a spliced aligner, and expression analysis was performed.

## 3.4 RESULTS

### 3.4.1 Morphological variations

In the previous chapter, we focused on and explained embryotoxicity, life cycle changes and molecular responses of two pesticides on *D. melanogaster*. In this chapter, we debated wing morphological changes caused by chlorantraniliprole and acephate. To test morphological variations, we used Geometric Morphometrics (GMM) tools. Geometric morphometrics is an effective technique for visualising minute morphological changes and illustrating how organisms respond to varying concentrations of toxicants or environmental changes. The current study aims to characterise the variations in size and shape of the wings of *D. melanogaster* intoxicated with three different concentrations of acephate and chlorantraniliprole when compared with control and vehicle control groups. One-way ANOVA results confirmed that mean squares for each variation were more significant than the error components, indicating that the measurement error was minimal. The degree of measurement error did not significantly affect the results. So, any deviations or changes expressed in the further analysis are strictly due to the toxicity effects of the two pesticides, not due to the landmarking errors.

#### *3.4.1a Control and Vehicle* Control

The control wing shape of the first six PCs explained 62.47% of the total population, and 18.95% of wing shape variation was explained by the first PC (Figure 3.2). PC1 shape variation is explained along with anterior-posterior landmark displacement. Anterior–posterior landmarks 1, 6, and 7 displaced to the posterior direction. More displacement was seen in distal landmarks 12, 13, 14, and 15 than in proximal landmarks. Compared to the two intersectional landmarks, 8 and 11,

intersectional landmarks 9 and 10 are more displaced (Figure 3.3). These variations are considered natural variations that exist in *D. melanogaster*.

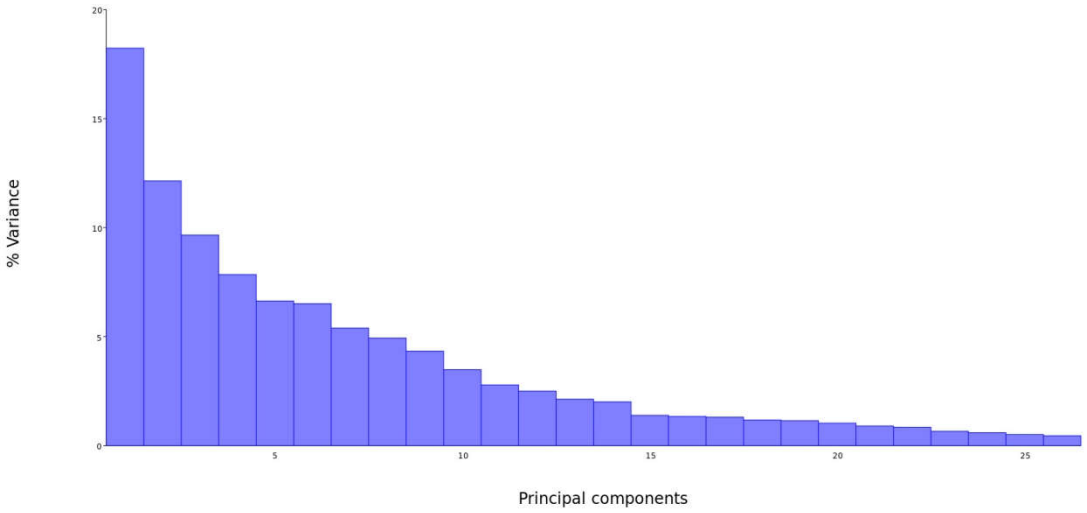



**Figure 3.2:** Percentage variance of principle component analysis in the control group

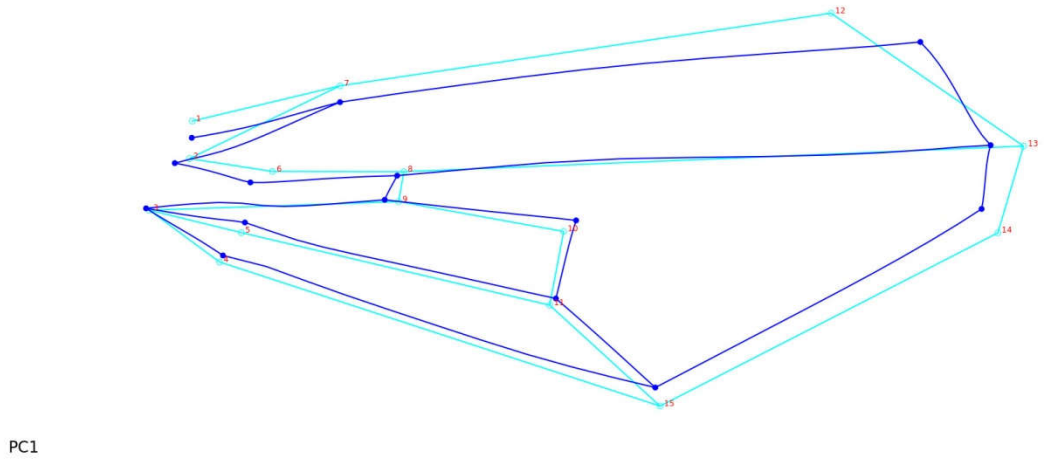



**Figure 3.2:** PC1 Shape morphology of the control group

In vehicle control-treated groups, the first five principal components explained 60% of the variance in the total vehicle control population (Figure 3.4). PC1 explained 26.2% of the variance, and wireframes showed shape changes related to extremes of variation along the PC1 axes. Shape variation at the base of the wings was attributed to the anterior proximal markers, which are 1, 2, 6, and 7. The wing blade shape variations were caused mainly by distal landmarks 12, 13, 14, and 15.

These crucial landmarks' displacement caused the wing blade compression (Figure 3.5).

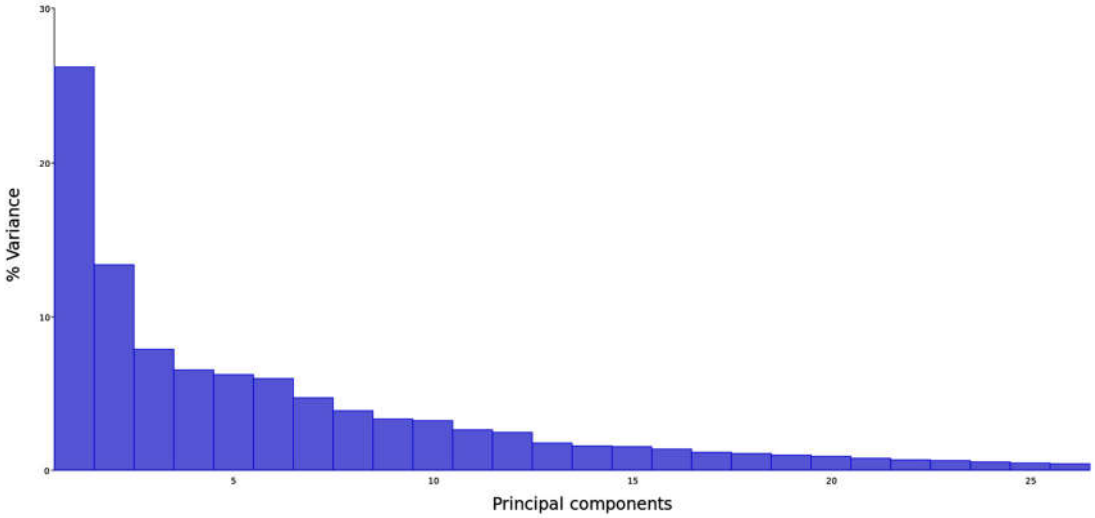



***Figure 3.4:*** Percentage variance of principle component analysis in vehicle control group

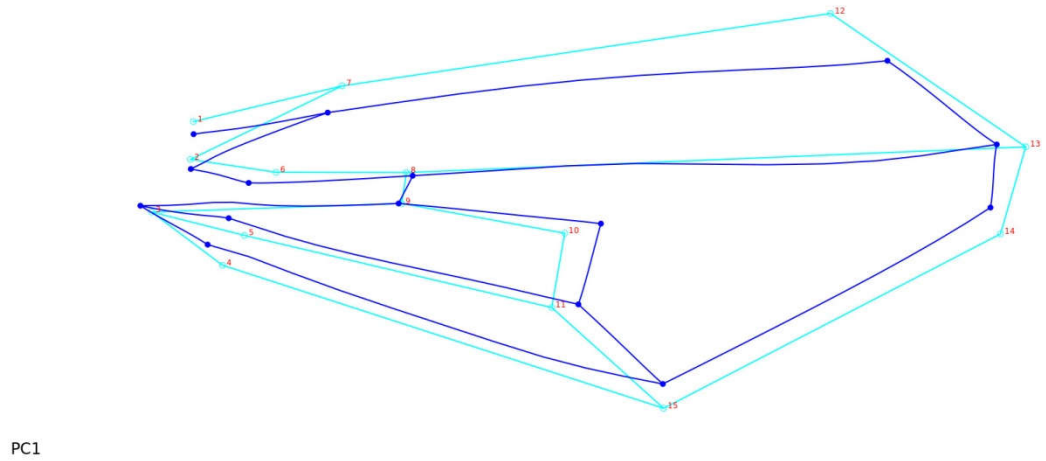



**Figure 3.5:** PC1 Shape morphology of vehicle control group

In control, flies' wings also showed hypothetical module separation with a significant RV value of 0.190. Wings of the vehicle control fly likewise displayed a considerable RV value of 0.343 (Figure 3.6).

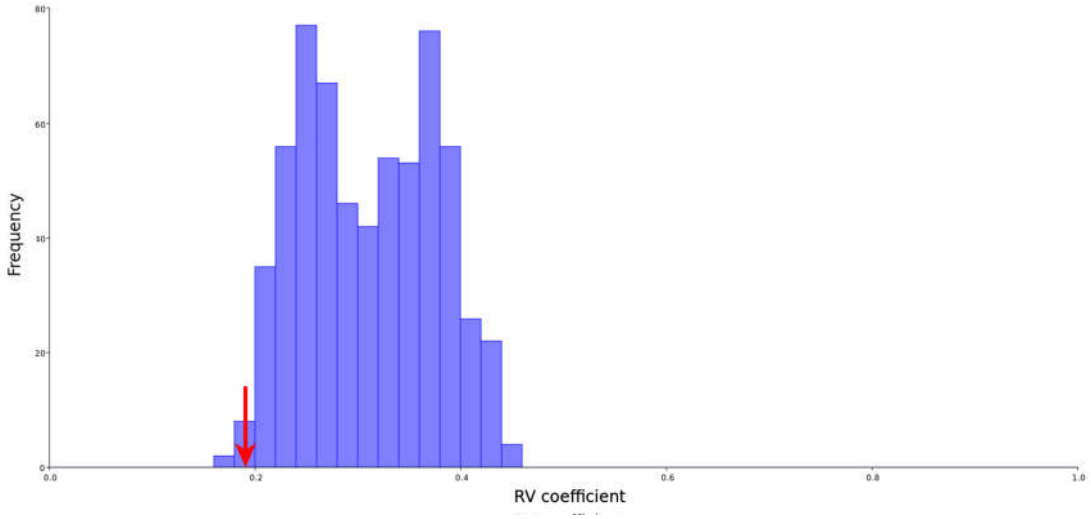



**Figure 3.6**: RV coefficient value for control wings.

### 3.4.1b Impact of Acephate on wing morphology of *D. melanogaster*

The three concentrations of acephate (A—0.63 µg/ml, B—0.38 µg/ml, and C—0.19 µg/ml) treated *Drosophila* wings exhibited wing size and shape variations. This suggests that the organophosphate pesticide significantly impacts *D. melanogaster* wing size and shape. Male and female wings also exhibit significant differences in shape and size.

***Morphological shape and size variations***: The concentration-dependent phenotypic variations in *D. melanogaster* were visualised and validated by PCA analysis. A total of 26 principal components (PCs) were cumulatively explained in 100% of the variations (Figure 3.7). The first two PCs explained the 37.3% variance (PC1=24.3% and PC2=13%). Significant shape changes were seen with most landmark displacement in the high-concentration treatment ($1/3^{rd}$ of $EC_{50}$). The landmarks in the proximal region (1, 2, 4, 5, 6, 8 and 9) were with small displacement when compared to distal landmarks, and only landmark 3 showed negligible displacement. Landmarks ending in longitudinal veins (12, 13, 14 and 15) showed more significant displacement. The landmark displacement in the anterior 7 and 12 resulted in the broadening of the leaf blade interiorly; likewise, in the posterior side, 11, 13, 14 and 15 displacements contributed to posterior wing width induction. Due to displacement

of 11 and 15, the longitudinal vein (LV5) length also increased. The 1/3$^{rd}$ of $EC_{50}$ treated wings showed overall broadening from standard wing shape (Figure 3.8).

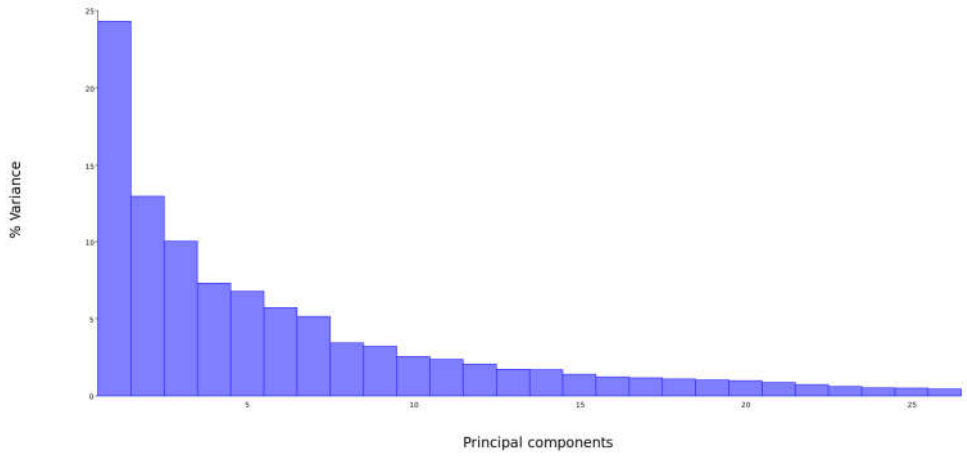



**Figure 3.7** Percentage variance of principle component analysis in acephate 1/3$^{rd}$ of $EC_{50}$ group

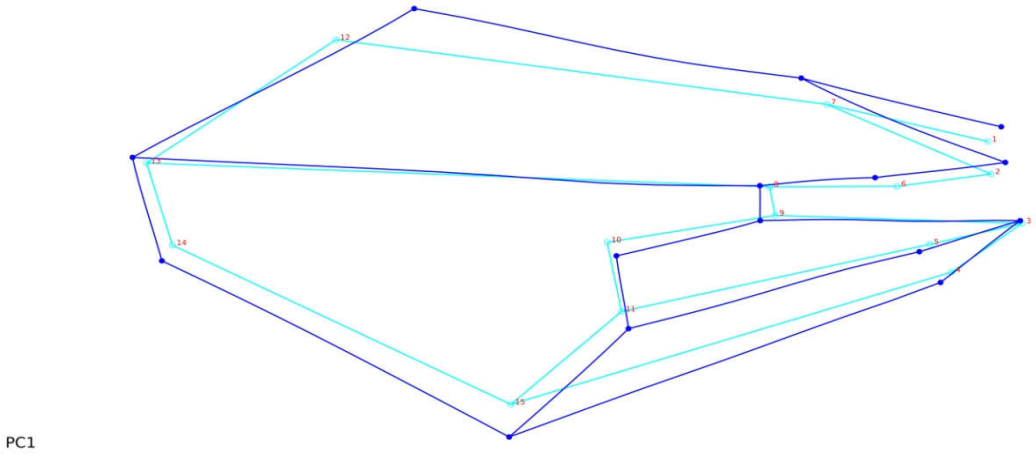



**Figure 3.8** PC1 Shape morphology of acephate 1/3$^{rd}$ of $EC_{50}$ group

In the 1/5$^{th}$ of $EC_{50}$, PC1 explained 16.48% of the total variance and PC2 and PC3, respectively, 13.28% and 9.7% of the variance (Figure 3.9). The principal wing shape deformations were observed with twelve or more landmark displacements. Anterior–proximal landmarks (1, 2, 6, and 7) displaced more to the posterior direction, so the wing margin was positioned more to the wing blade region. Similarly, the anterior-distal landmark (12) shifted more to the distal posterior side.

The results imply that the intersection between longitudinal vein 3 (LV3) and anterior cross vein and the intersection landmark between longitudinal vein 4 (LV4) and anterior cross vein moved to the more distal position. Similarly, the other two intersecting landmarks, 10 and 11, also shifted to the distal region. The posterior distal marginal landmark displaced more to the anterior than the control. The overall narrowing of the leaf blade area was observed with disposition in intersection landmarks (Figure 3.10).

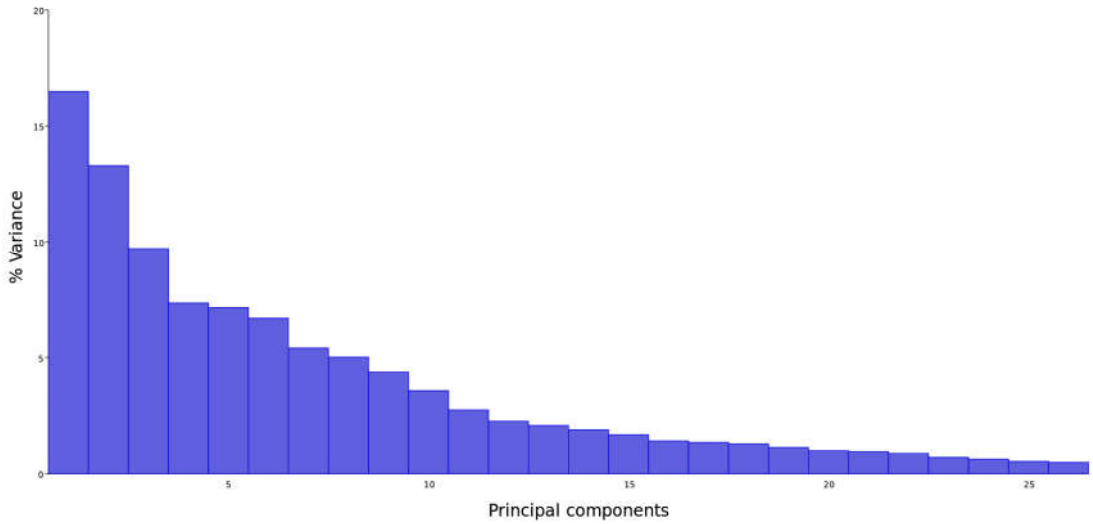



**Figure 3.9:** Percentage variance of principle component analysis in acephate 1/5$^{th}$ of $EC_{50}$ group

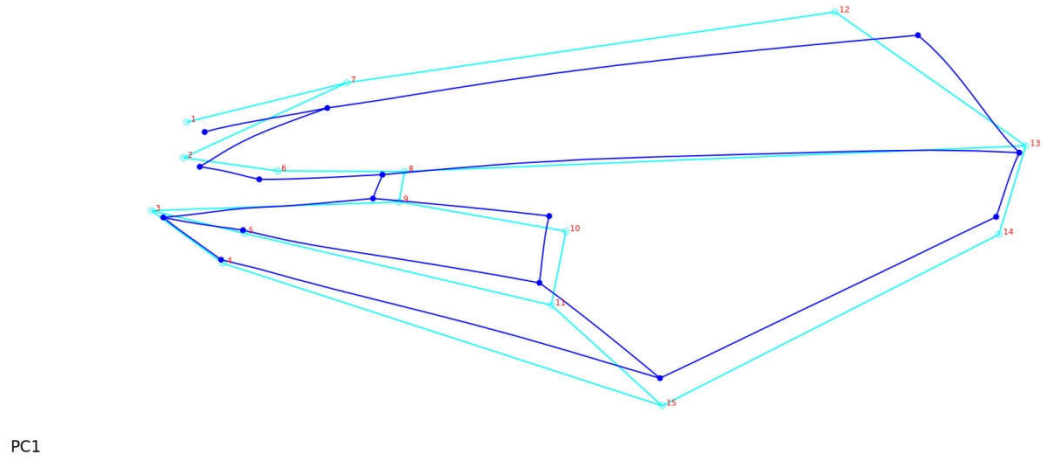



**Figure 3.10** PC1 Shape morphology of acephate 1/5$^{th}$ of EC $_{50}$ group

Regarding 1/10$^{th}$ of $EC_{50}$ acephate-treated wings, the variance percentages for PC1 and PC2, respectively, were 19.79% and 11.14% (Figure 3.11). In lower concentrations, treated wings were also observed to have wing shape changes. The anterior proximal landmark showed significant displacement from the original position. Landmarks 1 and 2 moved in the posterior direction. The intersection of longitudinal vein 0 and humeral cross vein also moved downwardly to the posterior direction. Posterior proximal landmarks with minimal displacement were observed from the results. Landmark 3 had negligible displacement, and the other two (4 and 5) showed minimal displacement. Anterior-distal marginal landmark displaced out of wing margin to posterior direction. Posterior distal landmarks located on the wing margin displaced more to the inner side of the wing blade. The prominent displacement of the LV4 and PCV intersection and LV5 and PCV were observed in 1/10$^{th}$ of $EC_{50}$ treated flies' wings (Figure 3.12).

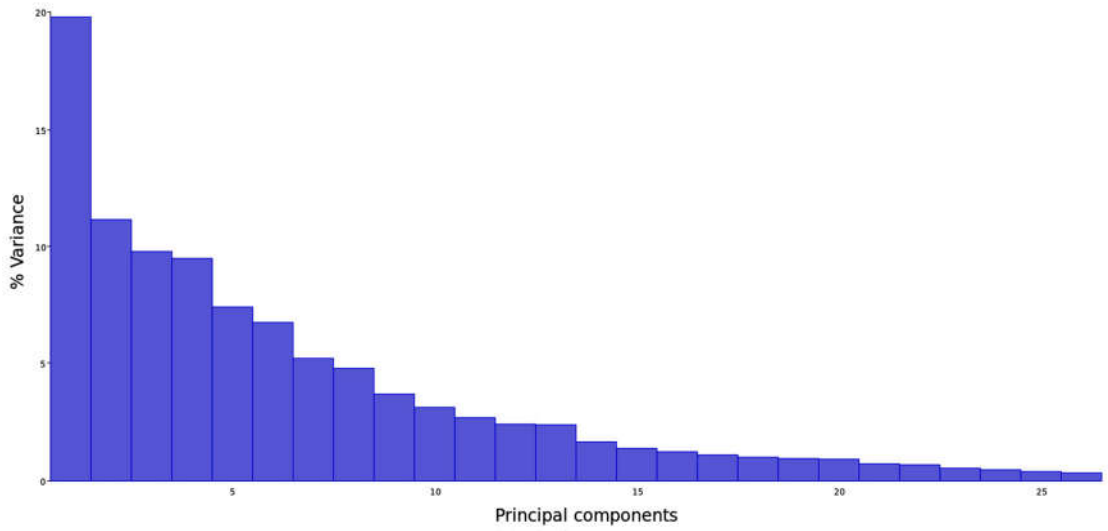



**Figure 3.11** Percentage variance of principle component analysis in acephate 1/10$^{th}$ of $EC_{50}$ group

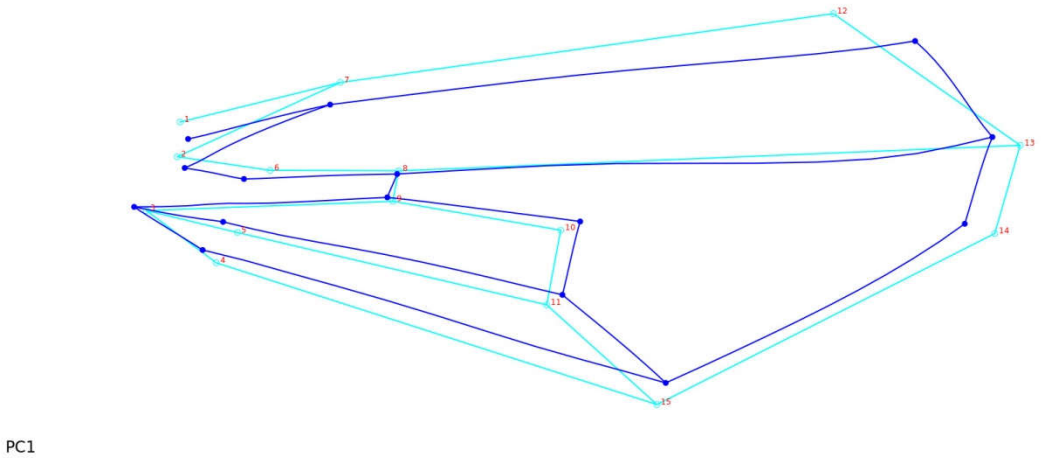



**Figure 3.12** PC1 Shape morphology of acephate 1/10$^{th}$ of $EC_{50}$ group

The organophosphate acephate-treated wings showed variation in wing size and shape. In higher-concentration treated wings, the landmarks were shifted away from the wing margin, resulting in the broadening of the wing area. In the case of lower-concentration treated groups, landmark displacement occurs more towards the inner side of the wing margin, resulting in the narrowing of the wing blade area.

***Multivariate analysis of concentration-dependent variations:***

The shape changes among the three acephate concentrations and control were analysed using 2B-Partial Least Square analysis (PLS). Block 1 PLS represents size covariation, and block 2PLS represents the shape covariations. Block 1 PLS showed a widened area; widening represents the heterogeneity between groups and size variations were prominently affected compared to control, the size differences in other groups diverged or altered. In Block 2 PLS data showed shape covariation between treated concentrations and control group. Compared to size, shape is more constructive (Figure 3.13).

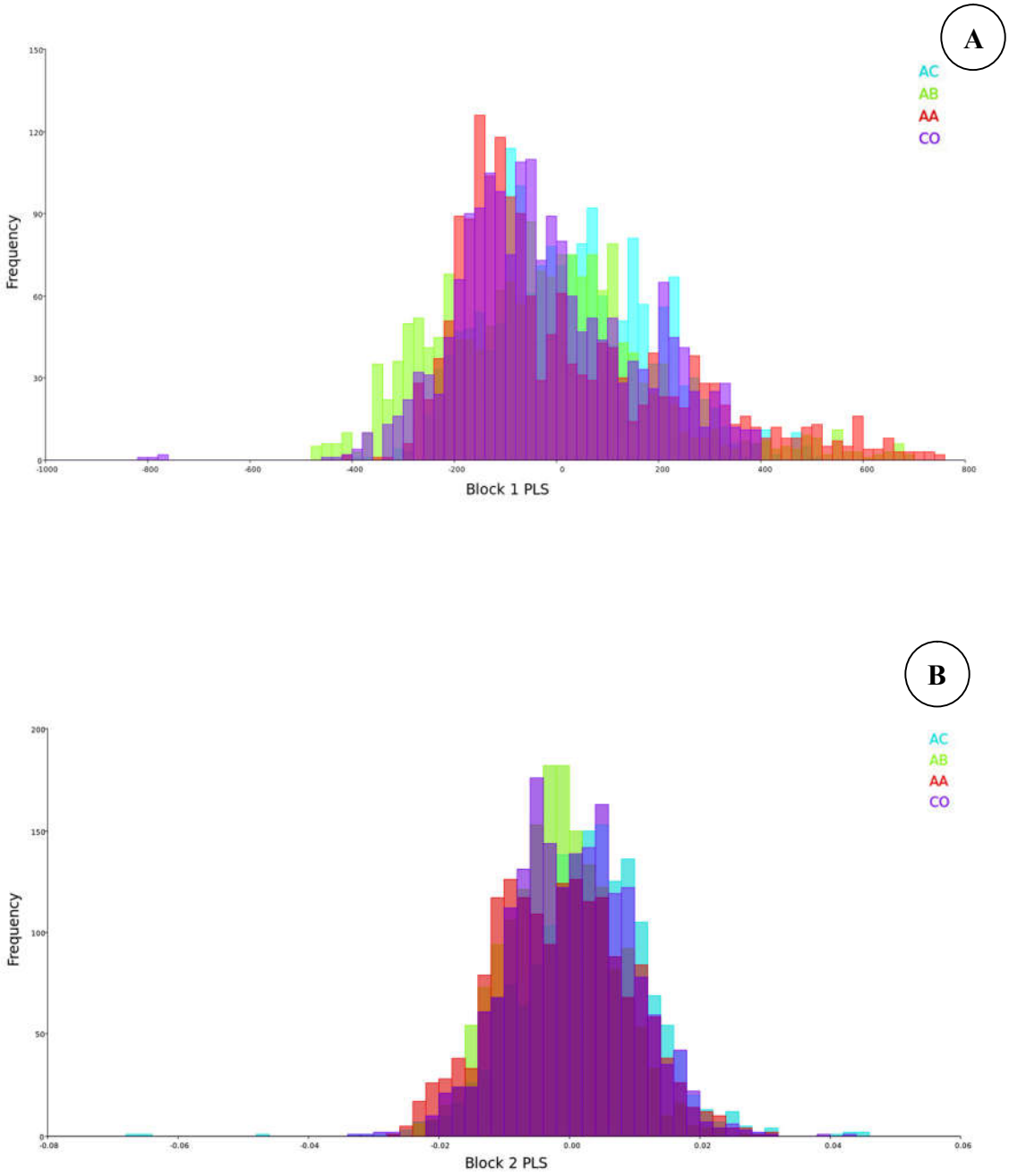



**Figure 3.13:** PLS analysis (A) Block 1 PLS represents size covariation between groups. (B)Block 2 PLS represents shape covariation between groups AA- 1/3$^{rd}$ of acephate-treated wings, AB- 1/5$^{th}$ of acephate-treated wings, AC- 1/10$^{th}$ of acephate-treated wings, CO-control group.

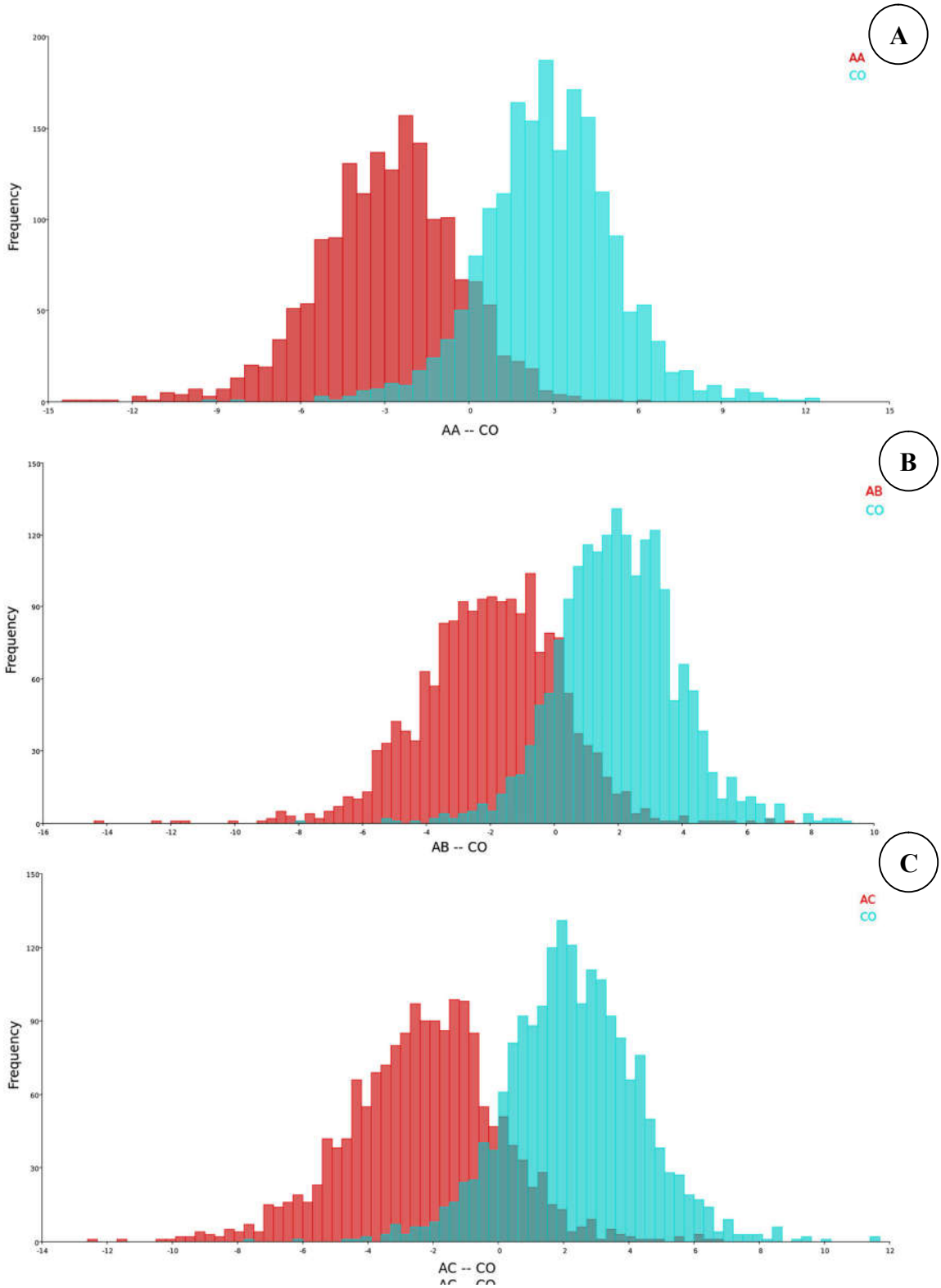



**Figure.3.14** Discriminant Function Analysis (DFA) of acephate treated groups. **A)** AA *vs.*CO, **B)** AB *vs.* CO, and **C)** AC *vs.* CO. CO- Control group, AA- 1/3$^{rd}$ of $EC_{50}$ of acephate-treated group, AB- 1/5$^{th}$ of $EC_{50}$ acephate-treated group, AC- 1/10$^{th}$ of $EC_{50}$ acephate-treated group

Discriminant Function Analysis (DFA) analysed concentration-dependent changes in wing shape. Two groups exist almost separately during 1/3$^{rd}$ of the $EC_{50}$ treated group compared with the control (Figure 3.14 A). Similar trends were observed in 1/5$^{th}$ and 1/10$^{th}$ of $EC_{50}$ treated groups compared with the control (Figure 3.14 B & C). The DFA of AA–CO represents 1/3$^{rd}$ of $EC_{50}$ and control, the Mahalanobis distance was 2.416, and the Procrustes distance was 0.012; the P-value for the permutation test revealed <.0001 for both Procrustes distance and Mahalanobis distance. In AB-CO (1/5$^{th}$ of $EC_{50}$ and control), the Procrustes distance and Mahalanobis distance respectively 0.011 and 2.003, respectively. Procrustes distance and Mahalanobis distance, respectively, were recorded as 0.011 and 2.155 in AC-and CO discriminant analysis (1/10$^{th}$ of the $EC_{50}$ treated group-control).

The morphospace analysis assessed by principal component analysis (PCA) and canonical variate analysis (CVA) represented the variation of morphological data in the largest variation coordinates (figure 3.15 A & B). PCA of pooled data of wings of control, 0.63 µg/ml, 0.38 µg/ml and 0.19 µg/ml treated groups confirmed the slightest variation between the three groups. However, few populations appear to be leaving the centroid. The 90% ellipse score was used to analyse and plot the data. The axis of CV1 and CV2 presented the first two large shape variances of all variance and points with different colours indicating the different groups of animals. With a specified probability level of 90%, the ellipse is displayed as an equal frequency ellipse. All acephate-treated groups tend to deviate from the control group. The treated groups are more overlapped and clustered with the slightest variation. CVA of males and females of acephate treatment also validated that the control male and female group deviated to the positive side and all treated males and females to the other side (Figure 3.16). A distinct variation of shape was observed even when the control male and female deviated to the same side.

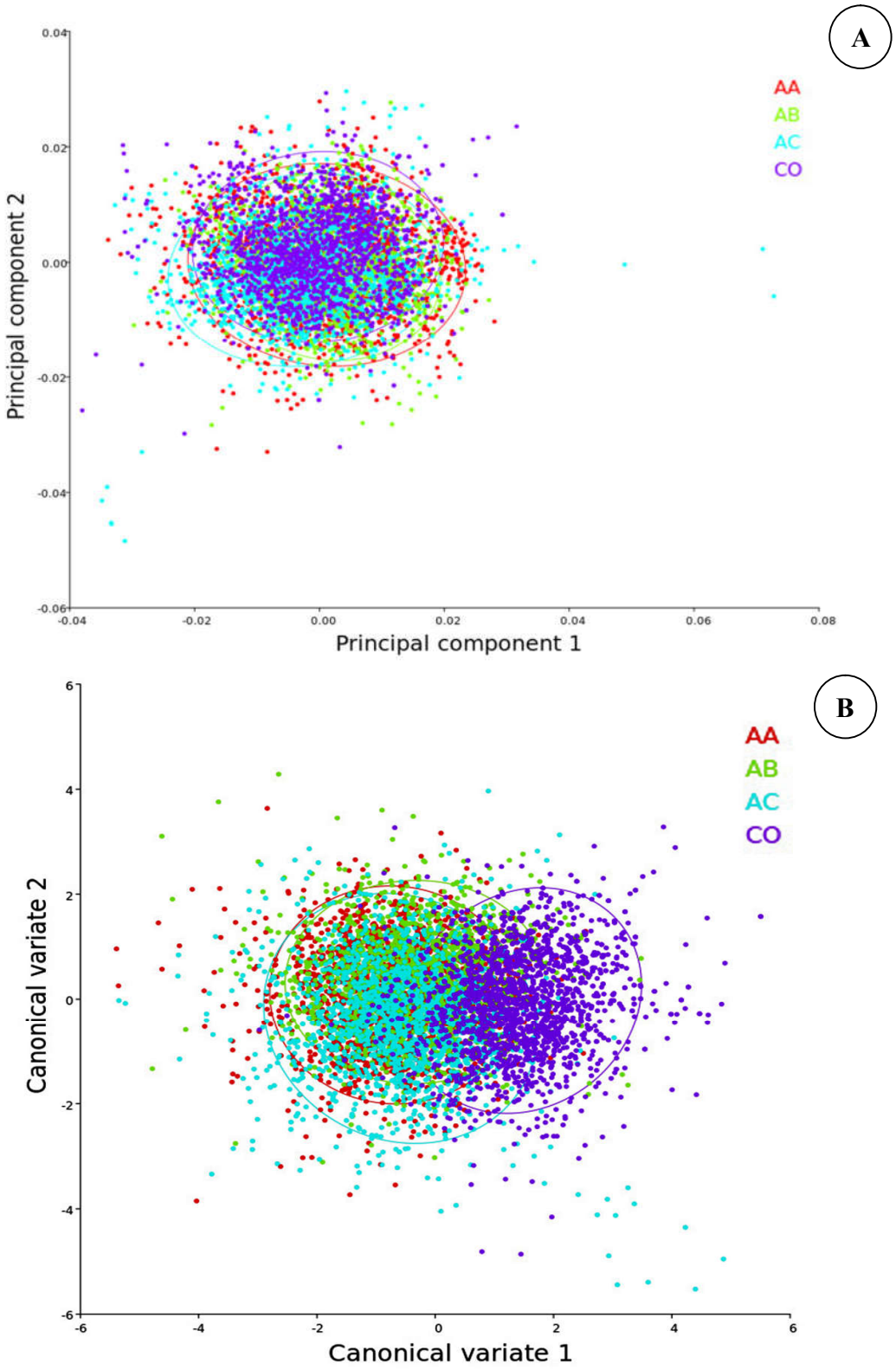



**Figure 3.15:** Morphospace analysis of acephate (A) PCA morphospace analysis; (B) CVA morphospace analysis, CO-Control group, AA- 1/3$^{rd}$ of $EC_{50}$ of acephate-treated group, AB- 1/5$^{th}$ of $EC_{50}$ acephate-treated group, AC- 1/10$^{th}$ of $EC_{50}$ acephate-treated group

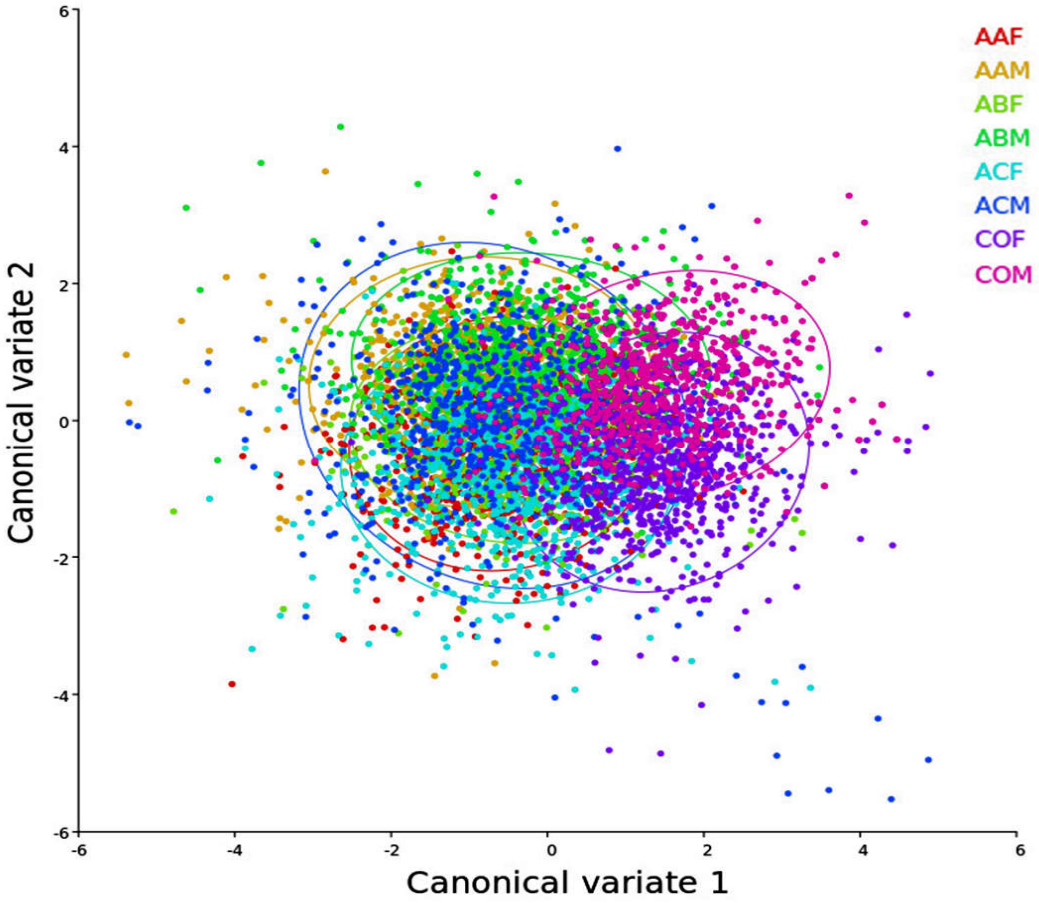



**Figure 3.16:** CVA morphospace analysis of males and females of acephate and control. COM- Control male, COF- Control female, AAM- 1/3$^{rd}$ of $EC_{50}$ of acephate-treated male, AAF- 1/3$^{rd}$ of $EC_{50}$ of acephate-treated female, ABM- 1/5$^{th}$ of $EC_{50}$ acephate-treated male, ABF- 1/5$^{th}$ of $EC_{50}$ acephate-treated female, ACM- 1/10$^{th}$ of of $EC_{50}$ acephate-treated male, ACF- 1/10$^{th}$ of $EC_{50}$ acephate-treated female.

***Modularity hypothesis analysis:*** Due to changes in wing shape, the proximal-distal coordination was further checked using modularity. The modularity hypothesis in this study states that the wing base and wing blade of a *Drosophila* are arranged in two modules along the proximal-distal (PD) axis. In all the cases, the RV value is less than 0.5, suggesting the existence of P-D modularity. When compared to control in each concentration (1/3$^{rd}$, 1/5$^{th}$and 1/10$^{th}$ of $EC_{50}$), the RV value changed; the altered RV value indicated the impaired coordination between proximal-distal modules (Figure 3.17). This impaired coordination is responsible for defective flight performance.

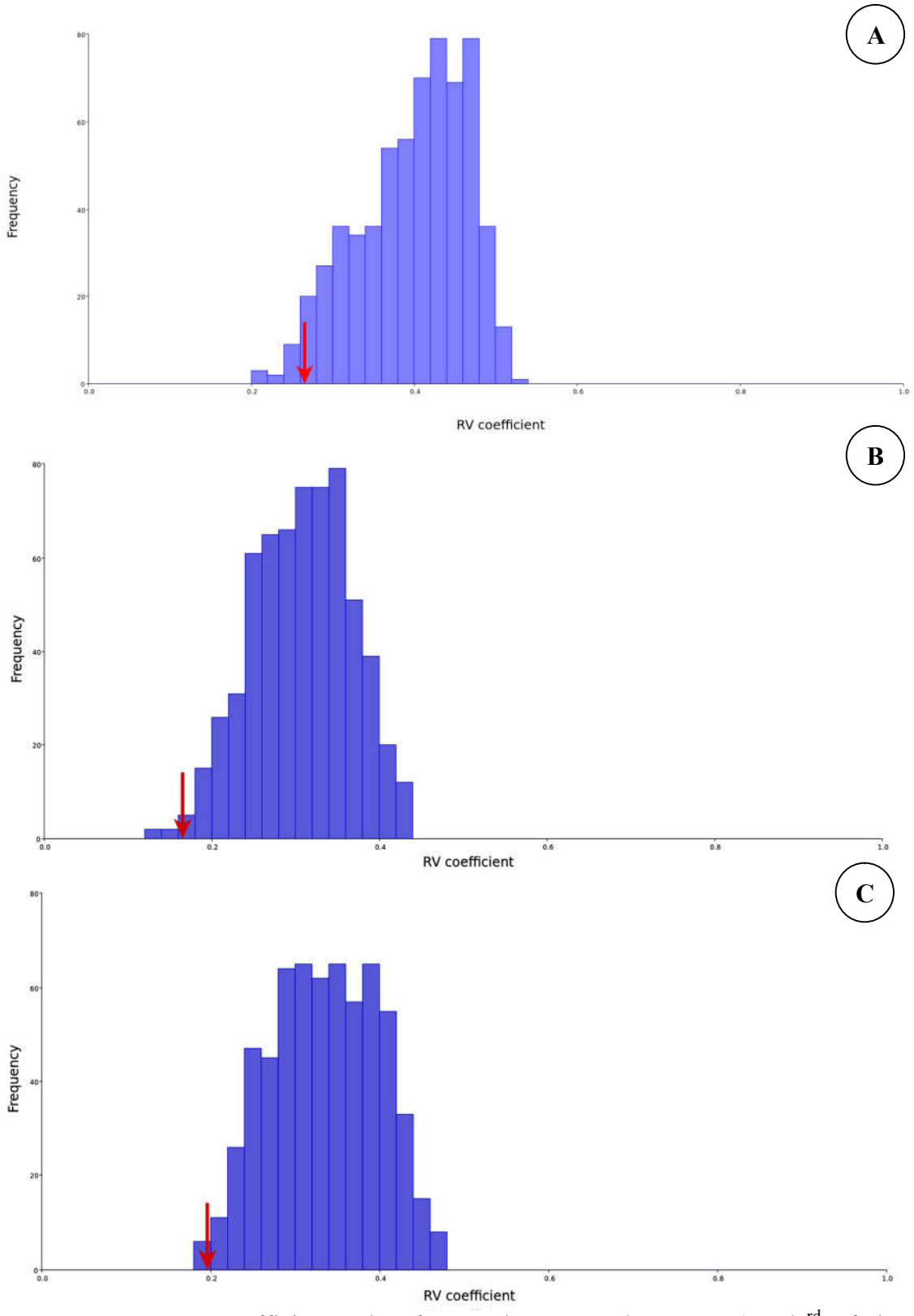



**Figure 3.17:** RV coefficient value for acephate treated group. A)- 1/3$^{rd}$ of the acephate-treated group, B)- 1/5$^{th}$ of the acephate-treated group, C)- 1/10$^{th}$ of the acephate-treated group.

### 3.4.1c Impact of Chlorantraniliprole on Wing Morphology of *D. melanogaster*

***Morphological shape and size variations***: 26 PCs were used to explain the variance within the A-1/3rd of chlorantraniliprole-treated wings (Figure 3.18). The first six PCs accounted for 70% of wing shape changes (PC1 21.45%, PC2% 17.28, PC3 10.59%, PC4 8.41%, PC5 7%, and PC6 5.28%). The proximal and distal landmarks displaced along PC1 followed an anterior-posterior compression. Distal landmarks12, 13 and 14 displaced to the proximal direction resulted in compression of the wing blade. Wing base shape changes due to proximal region landmarks 1, 2, and 3 displacements. The position of the transverse flexion line changed due to the posterior distal landmark 13. The four intersectional landmarks 8, 9, 10 and 11 displaced to the wing base direction (Figure 3.19 A). The 1/3rd of chlorantraniliprole has slender and narrowed wings.

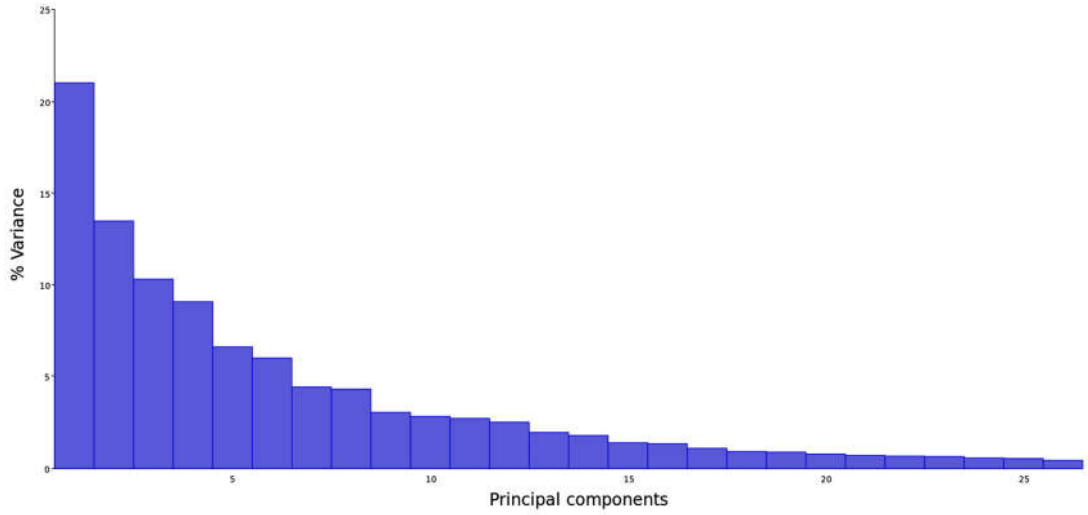



**Figure 3.18** Percentage variance of principle component analysis in chlorantraniliprole 1/3rd of EC $_{50}$ group

The distal portion of the wing blade showed a significant difference in the PC1 shape variance of the 1/5th of chlorantraniliprole-treated wings. Wing blade compression resulted from displacement of distal landmarks 12, 13, 14, and 15. The landmarks at the intersection of LV3-ACV and LV5-ACV have been repositioned to be more apparent. The posterior wing blade's landmarks 10 and 11 also shifted more noticeably. Proximal landmarks 1, 2 and 3 are displaced outside the wing base (Figure 3. 19B).

In 1/10th of chlorantraniliprole treated, wings displayed a compressed shape. Displacement of anterior marginal landmarks 7 and 12 to posterior resulted in

compression of the wing blade on the anterior side. Distal landmarks 13, 14 and 15 also contributed to the compression of the wind blade area. Intersectional landmarks 8, 9, 10, and 11 displaced more prominently than other landmarks (Figure 3.19C).

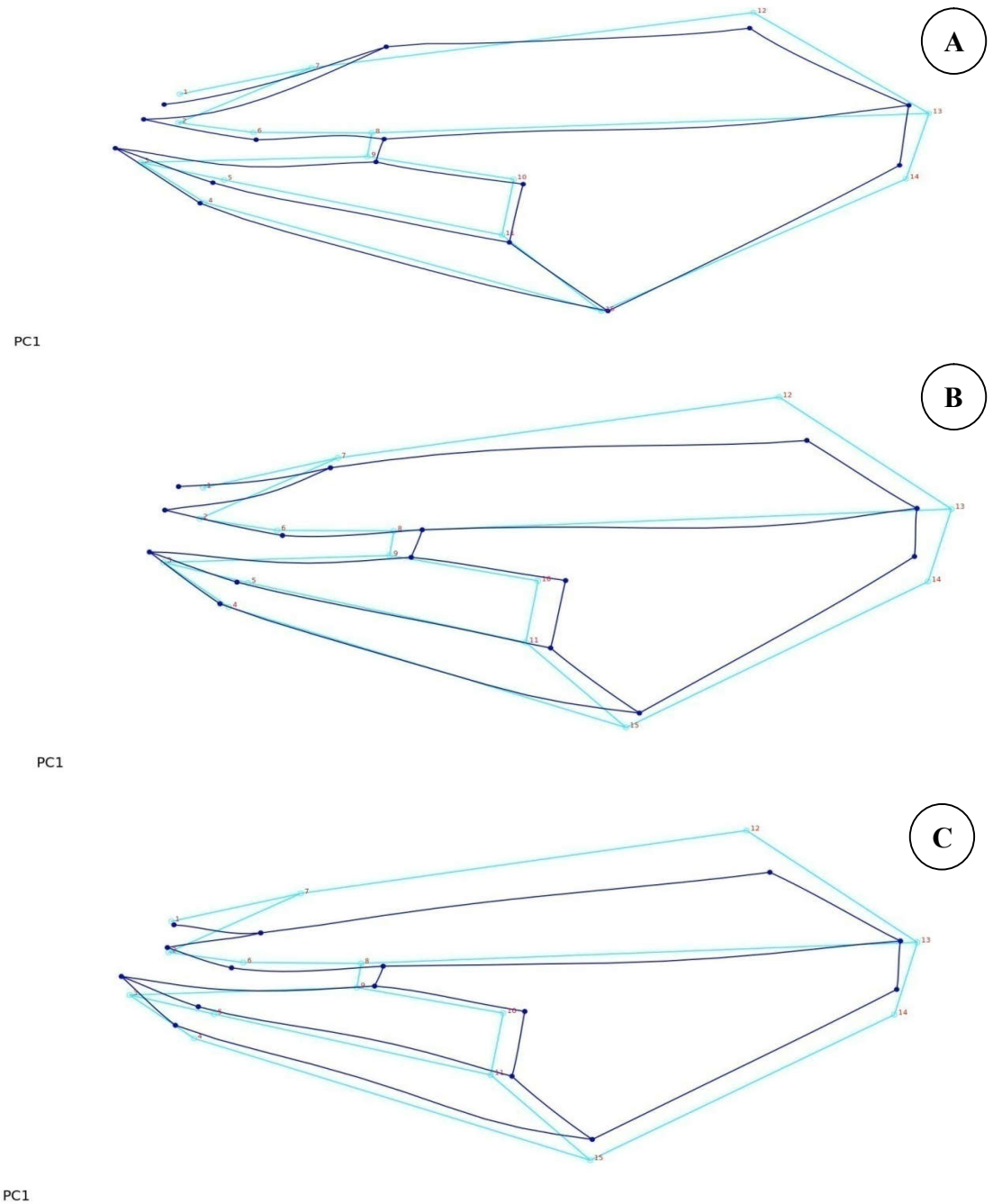



**Figure 3.19** A) PC1 Shape morphology of Chlorantraniliprole 1/3$^{rd}$ of $EC_{50}$ group (B) PC1 Shape morphology of chlorantraniliprole 1/5$^{th}$ of $EC_{50}$ group (C) PC1 Shape morphology of chlorantraniliprole 1/10$^{th}$ of $EC_{50}$ group

***Multivariate analysis of concentration-dependent variations:***

***DFA of wing shape revealed significant differences***

DFA of wing shape revealed significant differences between control, vehicle control and treatment groups. The DFA of CA-CO ($1/3^{rd}$ of $EC_{50}$-control) was reflected by the Mahalanobis distance of 0.8387 and the Procrustes distance of 0.00461 (Figure 3.20A). The P-value for the permutation test showed that the Mahalanobis and Procrustes distances were less than 0001. Similarly, in DA of CA-VC ($1/3^{rd}$ of EC50 treated-vehicle control), the P-value for permutation test revealed <0.0001 for both Procrustes distance and Mahalanobis distance. The Procrustes distance and ahalanobis distance were 0.00345 and 0.6792, respectively (Figure 3.21A).

Discriminant analysis of control and $1/5^{th}$ of $EC_{50}$ chlorantraniliprole treated wings (CB- CO) showed Procrustes distance of 0.00375 and Mahalanobis distance of 0.7830 (figure 3.20 B). The shape differences between $1/5^{th}$ of $EC_{50}$ chlorantraniliprole treated wings and vehicle control (CB-VC) by DA analysis with 0.00475 and 0.9146 Procrustes distance and Mahalanobis distance, respectively (Figure 3. 21 B).

The Procrustes distance of 0.00552 and Mahalanobis distance of 1.0616 were found in the discriminant functional analysis of the $1/10^{th}$ of $EC_{50}$ treatment and control groups (CC-CO) (Figure 3.20 C). $1/10^{th}$ of $EC_{50}$ µg/ml treatments with vehicle control discriminate wing shape with Procrustes distance of 0.00825 and Mahalanobis distance of 1.2754 (Figure 3.21 C). Additionally, permutation tests were conducted with 1,000 random runs to determine the significant P-value for the Procrustes and Mahalanobis distances of all experimental groups, the control, and the vehicle control.

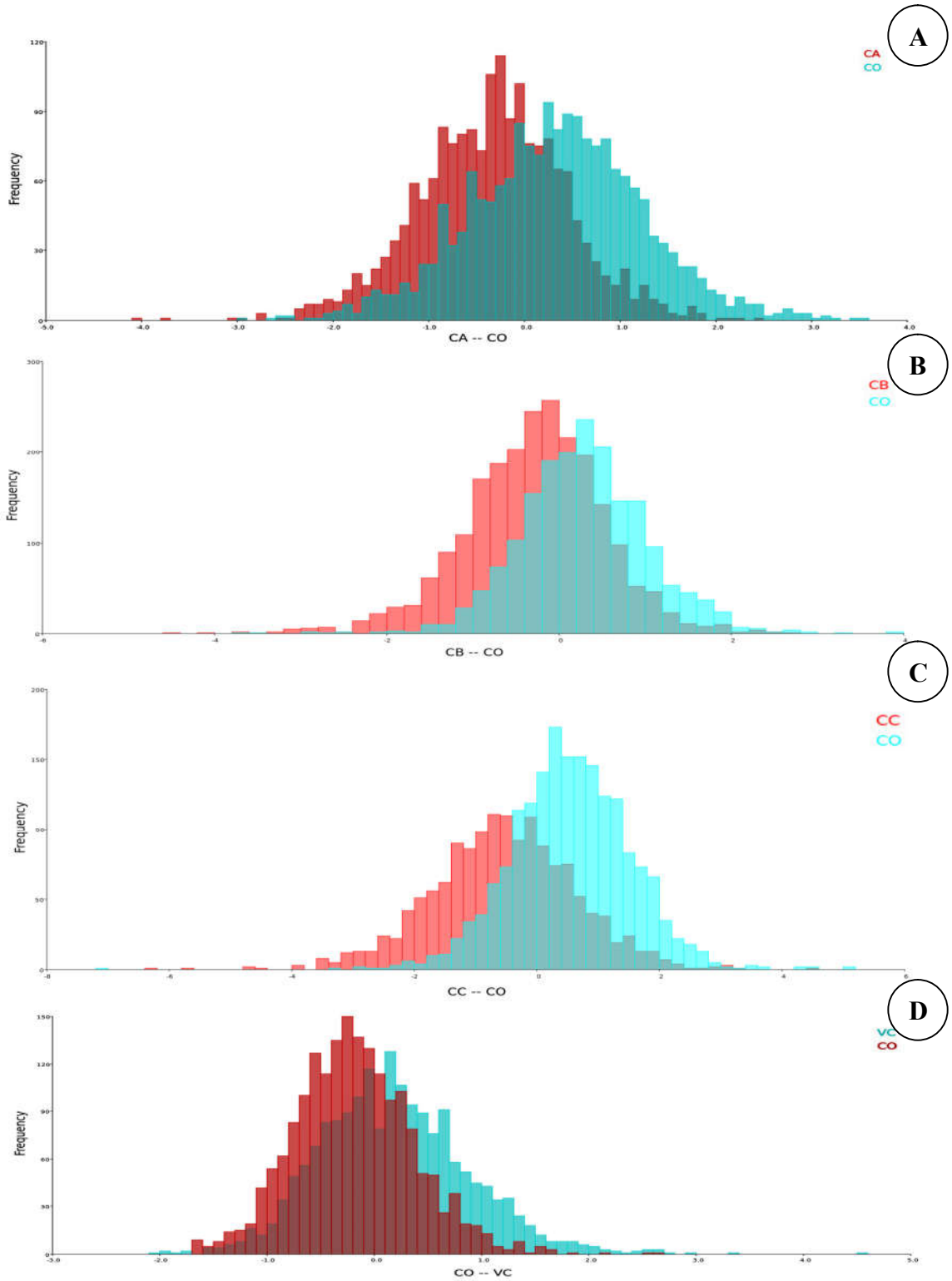



**Figure 3.20:** Discriminant Function Analysis(DFA) of chlorantraniliprole-treated group *vs.* control group **(A)** CA *vs.* CO, **(B)** CB *vs.* CO, **(C)** CC *vs.* CO, and **(D)** CO *vs.* VC. CO- Control group, CA- 1/3$^{rd}$ of $EC_{50}$ of the chlorantraniliprole-treated group, CB- 1/5$^{th}$ of $EC_{50}$ of the chlorantraniliprole-treated group, CC- 1/10$^{th}$ of $EC_{50}$ of the chlorantraniliprole-treated group and VC- vehicle control group.

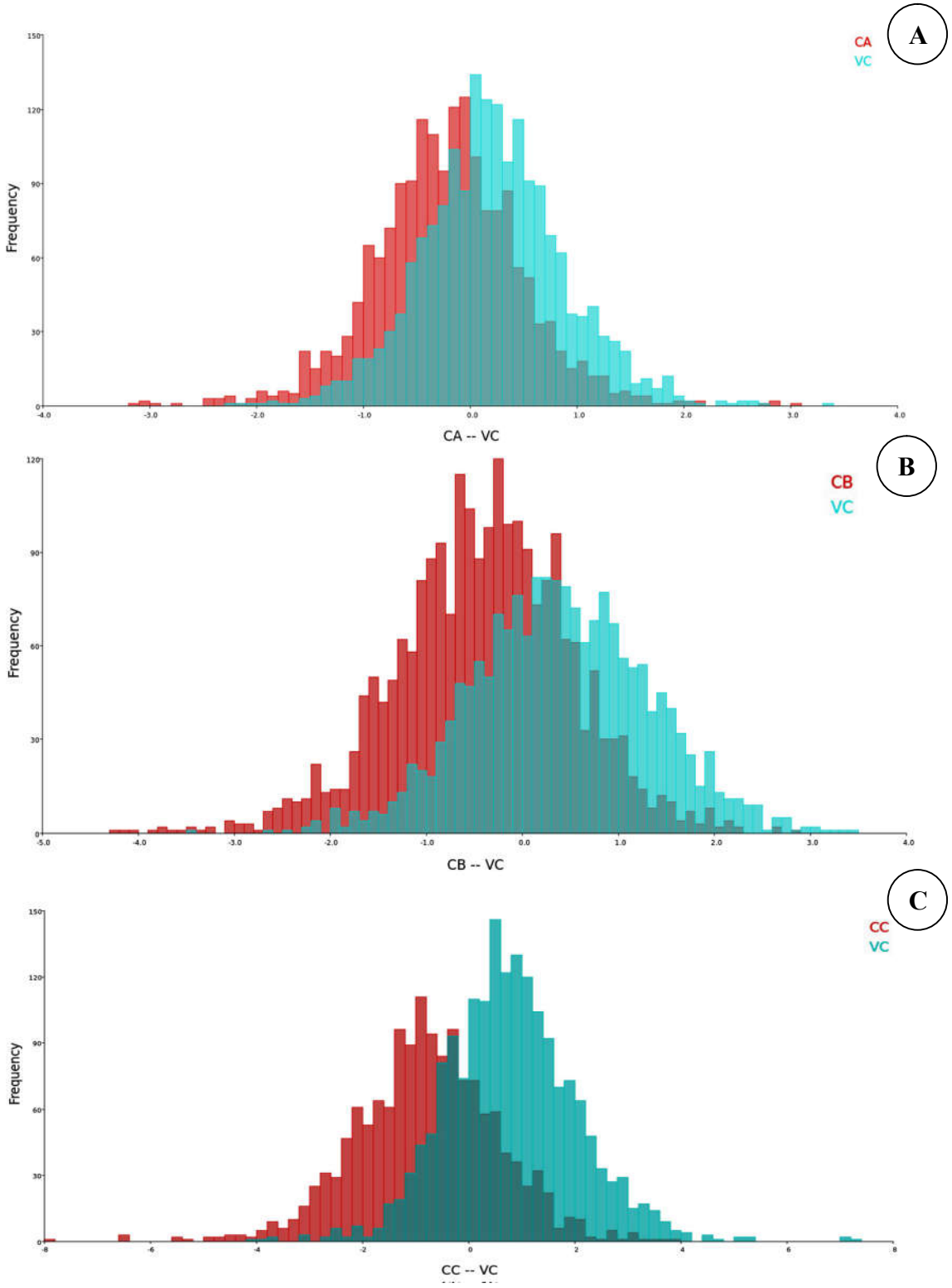



**Figure 3.21:** Discriminant Function Analysis (DFA) of chlorantraniliprole-treated group *vs.* Vehicle control group **(A)** CA *vs*. VC, **(B)** CB *vs.* VC, **(C)** CC *vs*. VC. CA-1/3$^{rd}$ of $EC_{50}$ of chlorantraniliprole-treated group, CB-1/5$^{th}$ of $EC_{50}$ of chlorantraniliprole-treated group, CC- 1/10$^{th}$ of $EC_{50}$ of chlorantraniliprole-treated group and VC- vehicle control group.

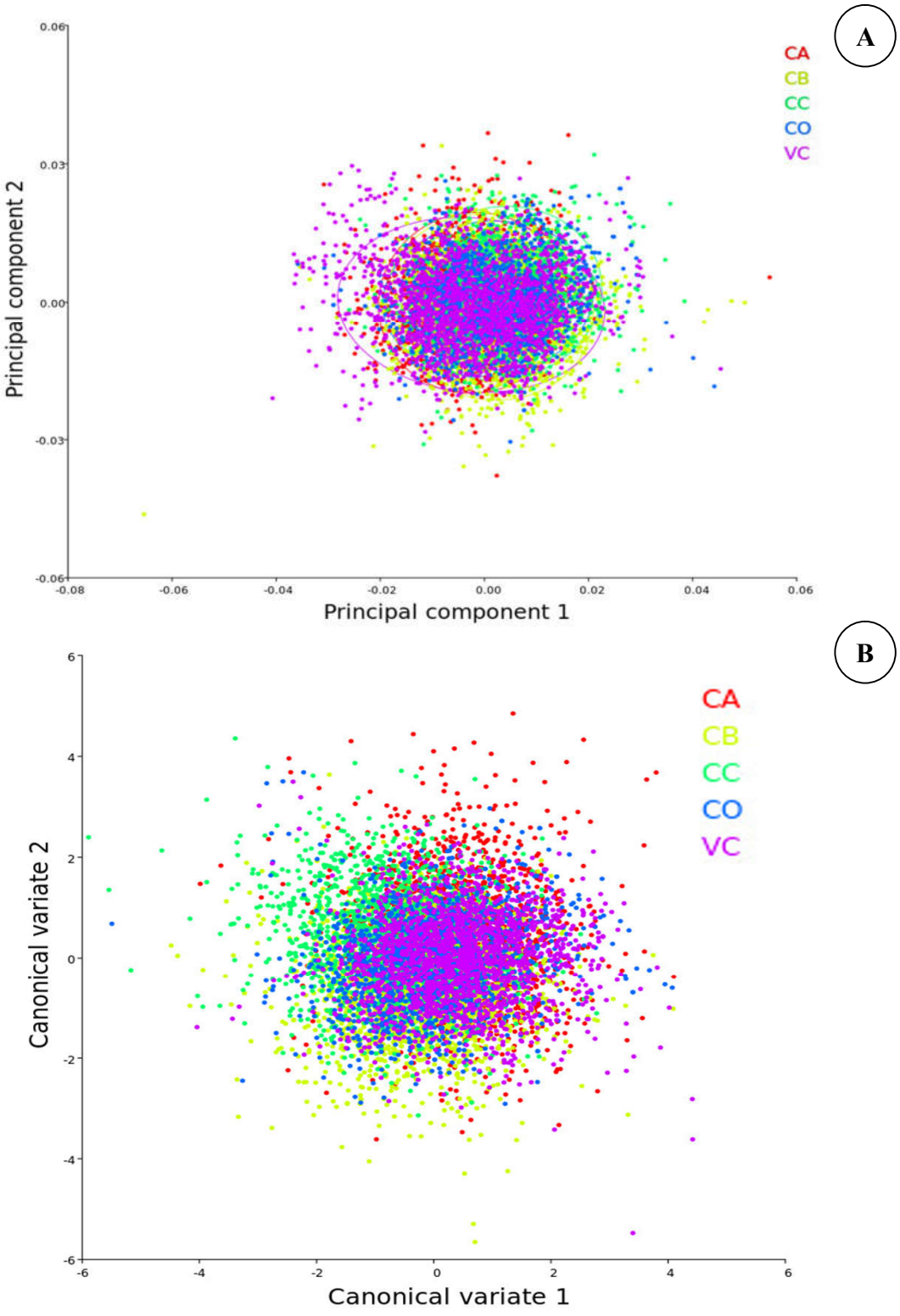



**Figure 3.22:** Morphospace analysis of chlorantraniliprole **(A)** PCA morphospace analysis; **(B)** CVA morphospace analysis, CO-Control group, CA- 1/3$^{rd}$ of $EC_{50}$ of chlorantraniliprole-treated group, CB- 1/5$^{th}$ of $EC_{50}$ of chlorantraniliprole-treated group, CC- 1/10$^{th}$ of $EC_{50}$ of chlorantraniliprole-treated group.

Wing shape discrimination between control and vehicle control was also tested to observe changes in the shape variation (Figure 3.20D). DFA analysis revealed significant discrimination, as indicated by Procrustes distance (0.0043) and Mahalanobis distance (0.6311), respectively.

The variations are further expressed in PCA and CVA morphospace analysis (Figure 3.22). The highly diverged data sets are described in morphospace. This divergence indicated the heterogeneity and plasticity between groups. The principal component analysis (PCA) of the pooled data from the wings of control, vehicle control, 0.0096 µg/ml, 0.0058 µg/ml, and 0.0029 µg/ml treated groups confirmed the slightest variation between the three groups (Figure 3.22A).

***Modularity analysis:*** Wings treated with chlorantraniliprole at $1/3^{rd}$ of $EC_{50}$ showed an RV coefficient value of 0.163, below 0.5 and noteworthy (Figure 3.23A).

The module partitioning from proximal to distal with the lowest RV value (0.157), module separation with minimal RV value displayed different landmarks in proximal and distal modules (proximal-1, 2, 3, 6, 7, 8, 9; distal-4, 5, 10, 11, 12, 13, 14, 15). In $1/5^{th}$ of $EC_{50}$ chlorantraniliprole-treated wings, the RV value of hypothetical proximal-distal modules was 0.217 (Figure 3.23B). The hypothesised proximal-distal modules with an RV value of 0.2192 were observed in the wings treated with $1/10^{th}$ of the $EC_{50}$ (Figure 3.23C).

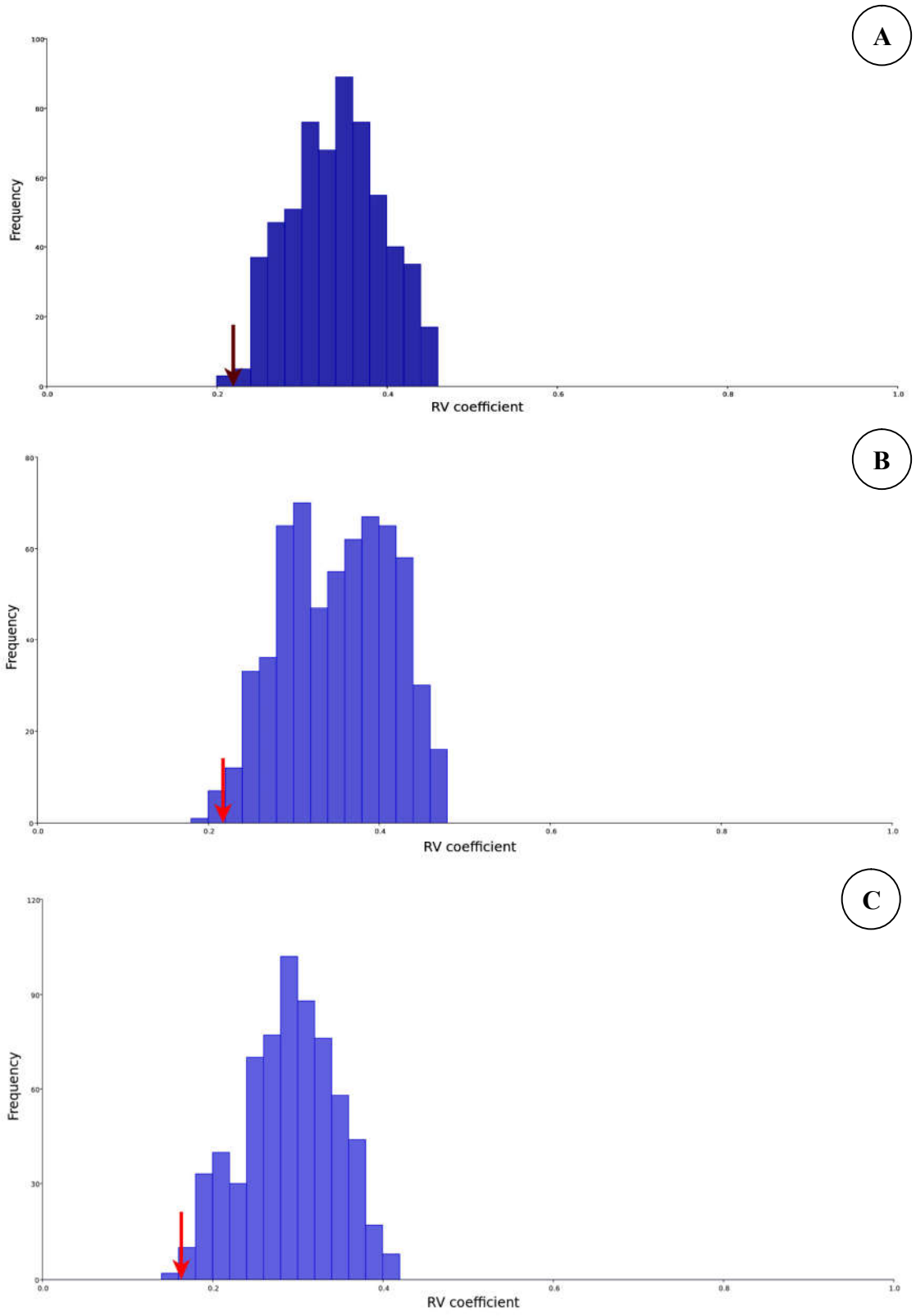



**Figure 3.23:** RV coefficient value for chlorantraniliprole-treated wings. A) 1/3$^{rd}$ of chlorantraniliprole-treated wings, B) 1/5$^{th}$ of chlorantraniliprole-treated wings, C) 1/10$^{th}$ of chlorantraniliprole-treated wings.

### 3.4.1d Comparative analysis –Acephate *vs.* Chlorantraniliprole

A discriminant analysis was conducted between different treatment groups (AA-CA, AB-CB and AC-CC). Procrustes distance and Mahalanobis distance showed significant discrimination between acephate and chlorantraniliprole treatment groups. DFA plots (Figure 3.24) also enhanced the result by minimising the overlapping area. The permutation test for Procrustes distance and Mahalanobis distance revealed that the p-value for all combinations was <.0001.

In the DFA figure 3.24A, there was little overlap between the $1/3^{rd}$ of $EC_{50}$-treated acephate and the $1/3^{rd}$ of $EC_{50}$-treated chlorantraniliprole (AA-CA). The Procrustes distance is 0.014, and the Mahalanobis distance is 2.402. The acephate treated at $1/5^{th}$ of $EC_{50}$ and the chlorantraniliprole treated at $1/5^{th}$ of $EC_{50}$ (AB-CB) had low overlap and demonstrated discriminant function (Figure 3.24 B).

The CV1 and CV2 explained 87.44% of the total variance between the different concentrations of acephate and chlorantraniliprole. All acephate-treated groups tend to deviate from the control group, with the major deviation observed to the positive side of the graph. The acephate-treated groups showed minimal overlap compared to the chlorantraniliprole-treated groups (Figure 3.25A). Even though the chlorantraniliprole group showed more overlap with the control and vehicle control groups, some variation was also observed. The CVA of males and females treated with acephate and chlorantraniliprole also validated that the acephate-treated male and female groups deviated to the positive side. In contrast, all chlorantraniliprole-treated males and females deviated to the other side, along with the control and vehicle control groups. A distinct variation in shape was observed, even when the males and females of each experiment deviated to the same side (Figure 3.25 B).

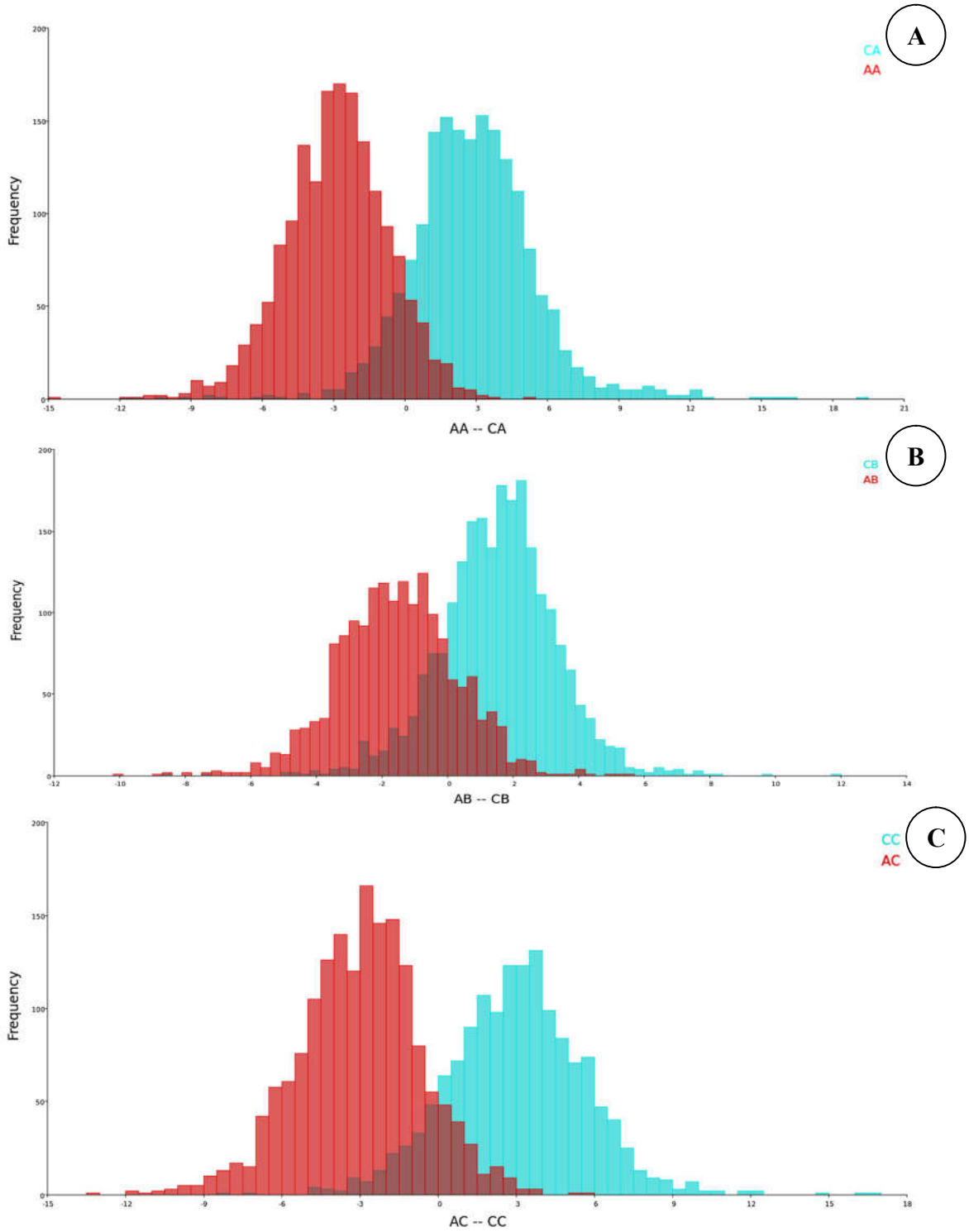



**Figure 3.24:** Discriminant function analysis of acephate vs. chlorantraniliprole. (A) AA *vs.* CA, (B) AB *vs.* CB and (C) AC *vs.* CC. AA- 1/3$^{rd}$ of $EC_{50}$ of acephate-treated group, AB- 1/5$^{th}$ of $EC_{50}$ of acephate-treated group, AC- 1/10$^{th}$ of $EC_{50}$ of acephate-treated group, CA- 1/3$^{rd}$ of $EC_{50}$ of chlorantraniliprole-treated group, CB- 1/5$^{th}$ of $EC_{50}$ of chlorantraniliprole-treated group, CC- 1/10$^{th}$ of $EC_{50}$ of chlorantraniliprole-treated group.

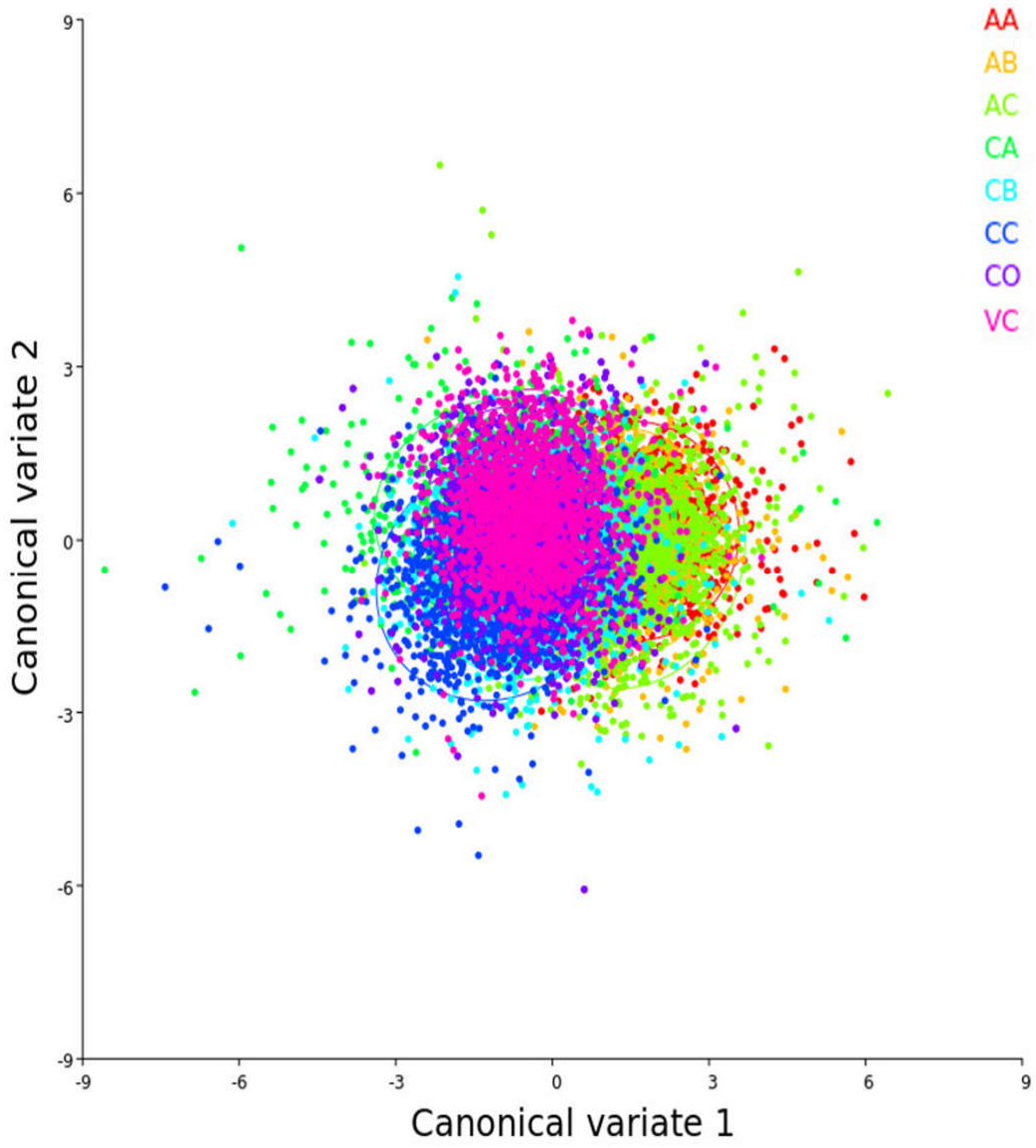



**Figure 3.25A**: CVA morphospace analysis, representing 87.44% of the variance. The total variance of the acephate and chlorantraniliprole-treated groups. CO- control group, VC- vehicle control group, AA- 1/3$^{rd}$ of $EC_{50}$ of the acephate-treated group, AB- 1/5$^{th}$ of $EC_{50}$ acephate-treated group, AC- 1/10$^{th}$ of $EC_{50}$ acephate-treated group, CA- 1/3$^{rd}$ of $EC_{50}$ of chlorantraniliprole -treated group, CB- 1/5$^{th}$ of $EC_{50}$ chlorantraniliprole -treated group, CC- 1/10$^{th}$ of $EC_{50}$ chlorantraniliprole -treated group.

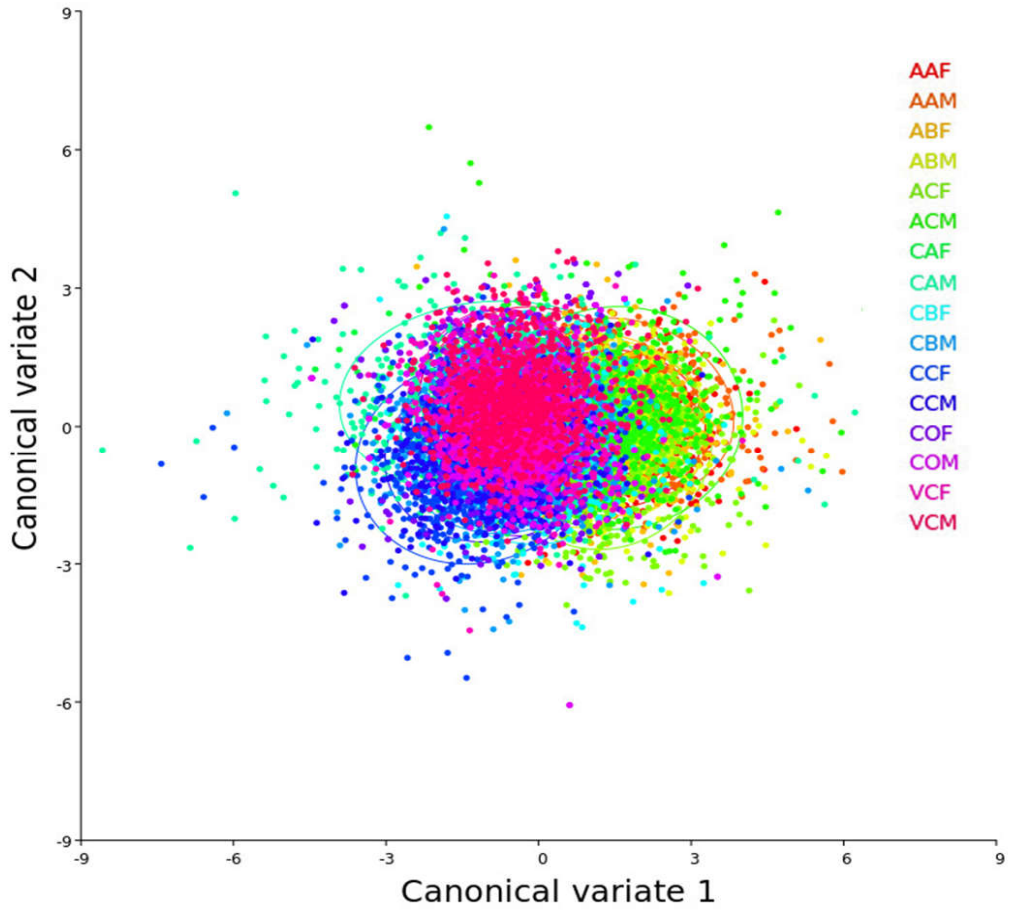



**Figure 3.25B**: CVA morphospace analysis, representing 87.44% of the variance. Male and female group variance in acephate and chlorantraniliprole-treated groups. COM-Control male, COF-Control female, AAM- $1/3^{rd}$ of $EC_{50}$ of acephate-treated male, AAF- $1/3^{rd}$ of $EC_{50}$ of acephate-treated female, ABM- $1/5^{th}$ of $EC_{50}$ of acephate-treated male, ABF- $1/5^{th}$ of $EC_{50}$ of acephate-treated female, ACM- $1/10^{th}$ of $EC_{50}$ of acephate-treated male, ACF- $1/10^{th}$ of $EC_{50}$ of acephate-treated female. CAM- $1/3^{rd}$ of $EC_{50}$ of chlorantraniliprole -treated male, CAF- $1/3^{rd}$ of $EC_{50}$ of chlorantraniliprole -treated female, CBM- $1/5^{th}$ of $EC_{50}$ chlorantraniliprole -treated male, CBF- $1/5^{th}$ of $EC_{50}$ chlorantraniliprole-treated female, CCM- $1/10^{th}$ of $EC_{50}$ chlorantraniliprole -treated male, CCF- $1/10^{th}$ of $EC_{50}$ chlorantraniliprole -treated female.

### 3.4.2 Transcriptomic validation of genes involved in wing development

RNA sequencing and transcriptome analysis confirmed that the genes responsible for wing development were primarily affected by exposure to acephate and chlorantraniliprole. The transcriptome study was conducted with 0.38 µg/ml of acephate and 0.0058 µg/ml ($1/5^{th}$ of $EC_{50}$) of chlorantraniliprole, along with these two experimental concentrations. Control groups were maintained for acephate; the

control and vehicle groups were maintained for chlorantraniliprole. The differential gene expression (DGE) analysis revealed that in the acephate-treated group, 15% of genes (2,031 genes) were upregulated, whereas 13.64% of genes (1,812 genes) were downregulated. Furthermore, 1030 unigenes were identified as having differentially expressed genes in response to the stress-inducing effects of acephate. Compared to the control in the chlorantraniliprole-treated group, 1369 genes were up-regulated, 773 were down-regulated, and 521 unigenes were identified as DEGs in the treatment group. Additionally, 295 unigenes were expressed only in the control group.

**3.4.2a Genes involved in wing development in acephate-treated *D. melanogaster*.**

We studied the differential expression of genes controlling wing vein development, wing development and other significant genes involved in the wing shape and wing function of *D. melanogaster*. Detailed differential expressions of genes in the acephate group are given in Table 3.3. Compared to control in acephate-treated groups, five genes involved in the imaginal disc-derived wing vein specification were downregulated. The *CG17600* and *grk* genes displayed significant downregulation. Wing disc development-governing genes also showed differential expression, with four upregulated genes and two downregulated genes. The *Ple* genes showed significant expression with a log2fc value of 2.43. The *smp-30* genes controlling negative regulation of imaginal disc-derived wing size showed significant downregulation with a log2fc value (-2.087). Ten genes involved in imaginal disc-derived wing morphogenesis are also differentially expressed in acephate-treated *D. melanogaster*. Out of ten, half were up-regulated, and the others were down-regulated. The Hedgehogs, hippos and notch pathways have essential roles in wing development. Our study confirmed the differential expression of *Dl* genes (log2fc value-1.61) involved in the notch signalling pathway. Seven genes involved in the negative regulation of the notch pathway were transcribed, and gene *Obst-A*, involved in the positive regulation of notch signalling, was under-transcribed. The Hedgehog pathway, which governs five genes, was upregulated. Four genes involved in the hippo pathway are also differentially expressed. The *MRP* and *Dpp* genes, which play a crucial role in wing development, exhibited differential expression, with the *MRP* genes showing significant downregulation.

**Table 3.3:** Differentially expressed genes related to wing development in acephate treatment

| Function | Gene name | Regulation | Log2fc value | p-value |
|---|---|---|---|---|
| Imaginal disc-derived wing vein specification | *tx* | DOWN | -1.25205473 | 0.053461 |
| | *CG17600* | DOWN | -1.224173175 | 0.038114 |
| | *Atx-1* | DOWN | -1.01312149 | 0.517918 |
| | *grk* | DOWN | -1.385211983 | 0.029408 |
| | *msk* | DOWN | -1.005936117 | 0.119059 |
| Wing disc development | *Sox15* | UP | 1.269904213 | 0.155389 |
| | *ple* | UP | 2.43460256 | 0.03907 |
| | *vg* | UP | 1.440060141 | 0.101573 |
| | *RpS28a* | UP | 2.099716052 | 0.451702 |
| | *Irk1* | DOWN | -1.45901044 | 0.103649 |
| | *Vajk3* | DOWN | -2.365190905 | 0.026383 |
| Negative regulation of imaginal disc-derived wing size | *smp-30* | DOWN | -2.087191945 | 0.000433 |
| Imaginal disc-derived wing morphogenesis | *fw* | DOWN | -1.10314114 | 0.062646 |
| | *If* | DOWN | -1.706708698 | 0.010753 |
| | *Msk* | DOWN | -1.005936117 | 0.119059 |
| | *By* | DOWN | -1.320628157 | 0.033451 |
| | *Shot* | DOWN | -1.035396458 | 0.207928 |
| | *m* | UP | 3.129193596 | 0.008567 |
| | *dy* | UP | 1.948695101 | 0.224079 |
| | *tnc* | UP | 1.243527226 | 0.095065 |
| | *Septin5* | UP | 1.383312226 | 0.1262 |
| | *asRNA:CR31429* | UP | 1.032666074 | 0.321174 |
| Apposition of dorsal and ventral imaginal disc-derived wing surfaces | *Dl* | UP | 1.610288654 | 0.019842 |
| | *if* | DOWN | -1.706708698 | 0.010753 |
| | *pot* | DOWN | -1.014516626 | 0.134425 |
| | *by* | DOWN | -1.320628157 | 0.033451 |
| | *stck* | DOWN | -1.047688574 | 0.098595 |
| | *Ilk* | DOWN | -1.068006737 | 0.077517 |
| Hedgehog pathway | *CG7094* | UP | 0.136933658 | 0.792145 |
| | *Pka-C2* | UP | 0.195372587 | 0.866315 |
| | *SkpE* | UP | 0.476535686 | 1 |
| | *CG14669* | UP | 0.267100943 | 0.95604 |
| | *gskt* | UP | 0.077880957 | 0.624515 |
| Notch signalling | *Dl* | UP | 1.610288654 | 0.019842 |

| pathway | | | | |
|---|---|---|---|---|
| Negative regulation of notch signalling | *Tom* | UP | 2.041540098 | 0.003863 |
| | *insb* | UP | 1.53338289 | 0.197953 |
| | *phyl* | UP | 1.192965266 | 0.251629 |
| | *E(spl)malpha-BFM* | UP | 1.448240051 | 0.035216 |
| | *E(spl)m6-BFM* | UP | 1.523825701 | 0.16716 |
| | *Ocho* | UP | 2.089387886 | 0.061174 |
| | *Brd* | UP | 1.883051524 | 0.047042 |
| Positive regulation of notch signalling | *obst-A* | DOWN | -1.735079073 | 0.016231 |
| Hippo pathway | *asRNA:CR44984* | DOWN | -1.668480193 | 0.824407 |
| | *upd1* | UP | 1.765233398 | 0.12674 |
| | *CG10508* | UP | 1.186907617 | 0.169092 |
| | *upd3* | UP | 1.612303111 | 0.45454 |
| Others | *MRP* | DOWN | -1.482015883 | 0.028073 |
| | *Dpp10* | UP | 1.511823124 | 0.103955 |

### 3.4.2b Genes involved in wing development in chloarntraniliprole-treated *D. melanogaster.*

We studied the differential expression of genes responsible for wing development in *D. melanogaster* treated with 0.0058 µg/ml ($1/5^{th}$ of the $EC_{50}$) of chlorantraniliprole. The genes involved in wing disc development, imaginal disc-derived wing morphogenesis, imaginal disc-derived wing vein specification, the hedgehog pathway, the hippo pathway, and regulators of the notch pathway showed variable expression compared to the control. Five genes involved in wing disc development (*RpS28a, Sdb, Duox, Ddc* and *Irk1*) showed differential expression. The *RpS28a* genes were found to be in regulation, with a 2.55 log2fc value. The *Sdb, Duox, Ddc* and *Irk1* genes were under-transcribed during chlorantraniliprole exposure. Overtranscription of the imaginal disc-derived wing vein specification *lncRNA: CR46500* gene contributed to the changes in wing vein development. Seven genes that participated in imaginal disc-derived wing morphogenesis showed up- and down-regulation. Out of seven, six genes were down-regulated (*shot, Duox, fog, if, tnc and Sdb)* and the *lncRNA:CR44221* gene was up-regulated. A remarkable expression of genes involved in the hedgehog pathway was also observed in the chloratraniliprole-treated groups. The up-regulated (*Pka-C2* and *SkpE)* and down-

regulated genes (*ihog* and *sona*) showed significant log2fc values. Five downregulated hippo signalling pathway genes were also observed in the DGE analysis. Out of five, four genes (*crb, Magi, Dlg5* and *upd3*) have significant log2fc values. The *LanA* gene plays a significant role in wing shape formation, and our study reports the regulation of LanA. Similarly, the *MRP* gene also showed significant under-regulation. The major findings were consolidated in Table 3.4.

**Table 3.4:** Differentially expressed genes related to wing development in chlorantraniliprole treatment.

| Function | Gene name | Regulation | Log2fc value | p value |
|---|---|---|---|---|
| Imaginal disc-derived wing vein specification | lncRNA: CR46500 | UP | 2.33882462 | 0.3196797 |
| Wing disc development | RpS28a | UP | 2.55285093 | 0.08461128 |
| | Sdb | DOWN | -1.27802806 | 0.00166758 |
| | Duox | DOWN | -1.1019345 | 0.01152966 |
| | Ddc | DOWN | -1.66630037 | 4.9568E-05 |
| | Irk1 | DOWN | -1.10261712 | 0.11289104 |
| Imaginal disc-derived wing morphogenesis | lncRNA: CR44221 | UP | 2.03950018 | 0.43350003 |
| | shot | DOWN | -1.06081572 | 0.00886546 |
| | Duox | DOWN | -1.1019345 | 0.01152966 |
| | fog | DOWN | -1.00451014 | 0.02017317 |
| | if | DOWN | -1.06349738 | 0.00841082 |
| | tnc | DOWN | -1.36928258 | 0.00103799 |
| | Sdb | DOWN | -1.27802806 | 0.00166758 |
| Hedgehog pathway | ihog | DOWN | -1.09195164 | 0.01351515 |
| | sona | DOWN | -1.80002881 | 1.2598E-05 |
| | Pka-C2 | UP | 1.4374392 | 0.01133072 |
| | SkpE | UP | 1.0928315 | 0.17142905 |
| Negative regulation of notch pathway | Brd | UP | 1.16259527 | 0.07032826 |
| | insb | UP | 1.08350397 | 0.18362035 |
| | CG31139 | UP | 1.02107109 | 0.11007241 |
| | upd2 | DOWN | -1.64622902 | 0.18602072 |
| | crb | DOWN | -1.33507202 | 0.00173542 |
| | Dlg5 | DOWN | -1.24992091 | 0.00417306 |
| | upd3 | DOWN | -3.14662298 | 0.00804561 |
| | Magi | DOWN | -1.08949916 | 0.01918162 |
| Others | MRP | DOWN | -1.06773418 | 0.00815717 |
| | LanA | DOWN | -1.01239904 | 0.01258268 |

## 3.5 DISCUSSION

Geometric morphometric analysis is a reliable and efficient tool for exploring average shape and size variation. It can identify minute morphological changes in biology as a quantitative tool. Previous chapters have demonstrated that acephate and chlorantraniliprole can induce developmental and molecular abnormalities. In the current study, wing shape abnormalities of *D. melanogaster* were investigated after exposure to sub-lethal concentrations of acephate and chlorantraniliprole. As far as we know, no prior research has been done on the sub-lethal effects of acephate and chlorantraniliprole on *D. melanogaster* wing shape. Interestingly, the two selected pesticides responded differently to the wing morphology of *D. melanogaster*, exhibiting variability in wing deformations. Based on the centroid size ANOVA and Procrustes shape ANOVA, the current results verified that, when compared to control and vehicle control, substantial size and shape variations were present in the wings of flies treated with different concentrations of acephate and chlorantraniliprole.

Previous studies have shown the direct impact of pesticides on the deformations of insects during their larval stages. Earlier research has shown that insecticidal treatments may cause morphological changes in exposed insects or their progeny. For instance, Han et al. (2012) reported that the weight of pupae was decreased when diamond back *Plutella xylostella* larvae were exposed to $LC_{10}$ or $LC_{25}$ doses of the anthranilic diamide chlorantraniliprole. The tobacco cutworm, *Spodoptera litura*, produces morphologically aberrant pupae and adults from third-instar larvae treated with Insect Growth Regulator (IGR) isoxazoline fluralane, as demonstrated by Liu et al. (2018). Applying the anthranilic diamide cyantraniliprole at an $LC_{30}$ concentration to the progenitor 3$^{rd}$ instar larvae of the oriental tobacco budworm, *Helicoverpa assulta*, increased the proportion of malformed adults in the same generation and increased the production of progeny (Dong et al., 2017).

In the present study, in order to find out the concentration dependant wing shape variations, different multivariate statistical techniques such as partial least square analysis (PLS), Discriminant function analysis (DFA), principal component analysis (PCA) and canonical variate analysis (CVA) were used to evaluate the

pattern of shape variations of different experimental groups. All the multivariate analyses consistently demonstrated wing shape deformation induced by acephate and chlorantraniliprole in *D. melanogaster*. Both acephate and chlorantraniliprole exhibited wing size co-variation between treatment groups and controls. Significant sexual dimorphism also existed between treatment groups and control groups. Previous researchers have pointed out the pesticide-induced variation in wing size and shape in both male and female flies. Pesticide-induced wing size variations have recently been studied. Male and female *Chironomus columbiensis* wings were differently affected by acute and chronic sublethal imidacloprid exposures. Male fly wings were more enlarged when exposed acutely and exhibited more wildly asymmetrical patterns when exposed chronically. The researchers also observed female flies with highly asymmetric wings during chronic and acute exposure (Montaño-Campaz et al., 2023). Shatha et al. (2023) examined the impact of Egy Pride, an insecticide, on the genetic variation and wing morphology of the Cucurbita fruit fly, *Dacus ciliatus*. Results showed a 67% match rate between treated and untreated specimens, with notable differences in wing shape and size. The wing size of the treated specimens varied, with a central size measurement of 868.6 microns in the treated group and 850.8 microns in the control group.

According to our findings, *D. melanogaster* eggs exposed to sublethal concentrations of acephate and chlorantraniliprole showed changed wing shapes. In acephate-treated concentrations, $1/3^{rd}$ of $EC_{50}$-treated wings showed broadening in the anterior side of the wing. In lower acephate concentration, the treated group was displayed with a narrowed wing blade. Likewise, chlorantraniliprole sub-lethal concentration treated wings showed with narrowed wing base and blade. For *Triatoma infestans*, there have also been reports of wing shape changes brought on by juvenile sublethal exposures to deltamethrin (Natterro et al. 2019 and Natterro et al., 2021). *T. infestans* adults subjected to sublethal deltamethrin concentrations showed bigger, less symmetrical, and less canalised (i.e., reduced individual variation within genotypes) wings. These observations may collectively compromise genetic and environmental canalisation and developmental stability (Natterro et al. 2021). The concentration-dependent trans-generational effects of deltamethrin on the wing shape

of *Chironomus columbiensis* were more stable when individuals were exposed to higher concentrations (Montaño-Campaz et al., 2022). Our study pointed to the concentration-dependent variations in wing shape, represented in discriminate function histograms. When comparing each concentration with the control, the overlapping region was minimal, and in higher concentrations, treated acephate and chlorantraniliprole displayed higher heterogeneity. The comparison between two different concentrations cleared the concentration-dependent variation in wing shape. Recent studies by Siva et al. (2023) revealed the impact of *Croton tetradenius* essential oil on *Aedes aegypti* wing geometric morphometrics, pupal viability, and larval viability. They compared different essential oils concentrations against the control.

The proximo-distal (PD) axis of the *Drosophila* wing is not fully assessed, but some degree of autonomy may exist between different regions along this axis (Klingenberg, 2009). During the larval stage, the PD axis is determined by genes expressed in circular domains (Terriente et al., 2008). The adult wing is divided into two central regions: the wing blade and the wing base, also known as the proximal wing or wing hinge. Later in pupal development, the wing disc folds and extends, elongating along the PD axis. Due to varying genetic regulation and mechanical stresses, these two distinct cell populations undergo hinge contraction and blade elongation along the PD axis to achieve mature wing shapes (Aigouy et al., 2010; Ray et al., 2015). In the present study, the PD separation was present. Compared to the control, the treated wing displayed variable RV values, indicating decreased integration between the two modules in sub-lethal concentrations of acephate and chlorantraniliprole. Decreased coordination between modules will result in improper flight mechanisms. Although flight is the primary function of the entire wing, the specific roles that the wing base and wing blade play during flight differ, and their unique structures reflect this. The wing blade generates the aerodynamic forces that produce lift, while the wing base transmits the forces generated by the flight muscles (Dudley, 2002). The flight mechanism occurs due to the coordination with the proximal-distal module. Even though the module separation is present in the control group, the treated group displayed greater changes than the control group. These

changes indicated the sublethal toxicities of acephate and chlorantraniliprole. Improper module separation, defective wing shape and size changes lead to weakened flight mechanisms. Disabled flight mechanisms in non-target insects, particularly in pollinators, can negatively impact their ecological services. Previous studies conducted on a wild insect pollinator, the bumble bee *Bombus ter□restris*, found that exposure to an accurate dose of imidacloprid, a widely used pesticide, affected the flight performance of a bumblebee, *B. ter-restris*. The exposed workers flew at a higher velocity over the first ¾ km of flight, which may lead to reduced flight distance and duration, potentially affecting their potential forage area and pollination service capabilities (Kenna et al., 2019). Furthermore, flight is essential for successful foraging because gathering nectar and pollen may require several hours of flying and heavy lifting daily (Schneider et al., 2012; Brodschneider et al., 2009). Pesticides (acephate and chlorantraniliprole) induced wing shape alterations that directly and indirectly affect non-targeted organisms, especially in their ecological functions. An insect's flight distance can reveal its capability for pollination and its foraging range (Schulke et al., 2001; Osborne et al., 2008). Reduced flight capacity may hinder honeybees' ability to collect food and their coping mechanisms during colony stress (Blanken et al., 2015).

Imaginal wing discs of the *D. melanogaster* are prototypes of the adult wings and are a classical tissue for studying wing growth and development. Numerous inter- and intra-organ variables are necessary for the growth of the discs, including morphogens, mechanical stresses, food levels, and hormones that affect gene expression and cell proliferation (Gou et al., 2020). So, applying sub-lethal concentrations of acephate and chlorantraniliprole may damage normal wing development and growth. The transcriptome analysis confirmed the differential expression of different genes involved in wing growth, development, size and shape. The induction of vestigial (*vg*) gene expression at the wing disc is the earliest requirement in wing development, and it acts as a transcriptional regulator of other genes in wing development (Couso et al., 1995; Cohen et al., 1992). In the treated group, the *vg* gene showed up-regulation along with other wing development genes. The *Ple* genes involved in pigmentation and sclerotisation of the wing (Neckameyer

& Quinn, 1985) also showed increased expression in acephate-treated larvae. Compared to the control 2.4 times increase in *Ple* gene expression in acephate control, 2.4 times increase in *Ple* gene expression was noticed in the treated group. Previous studies have demonstrated that phenotypes such as held-out wings, thicker veins, venation pattern abnormalities, undersized wings, and ablation of nearly the entire wing are produced when any *Irk* sub-unit in the wing is rendered inactive (Adachi-Yamada et al., 1999; Chen et al., 1998; Dahal et al., 2012; de Celis, 1997; Ralston & Blair, 2005; Shimmi et al., 2005; Spencer et al., 1982; Zecca et al., 1995 and Zeng et al., 2007) and in our study regulation of the *Irk1* gene was noticed in both acephate and chlorantraniliprole treated groups. The cell fate and wing disc formation process in *Drosophila* is controlled by morphogens such as the concentration-dependent short-range signal molecule Hedgehog (*Hh*), remote morphogenetic element Decapentaplegic (*Dpp*), and Wingless (*wg*). Notch plays a crucial role in the development of the imaginal wing disc. The wing is divided into anterior, posterior, dorsal, and ventral compartments. The dorsal-ventral (dyv) boundary is formed through the localised activation of Notch signalling, which is required for the expression of genes involved in forming the dyv boundary and wing margin patterning. Notch activation at the dyv boundary is also required for the localised expression of (*wg*), vestigial (*vg*), Distal-less (*Dll*), and cut (*ct*) (García-Bellido et al., 1973; Blair, 1995; Couso et al., 1995; Diaz-Benjumea & Cohen, 1995; Rulifson & Blair, 1995). In our study, many genes involved in the notch signalling pathway were differentially affected by the sublethal effects of acephate and chlorantraniliprole. Wing-patterning molecules like *Dpp* and *Notch* influence wing growth through Hippo signaling through the Fat-Dachsous pathway (Rogulja & Irvine, 2005). Three genes involved in the hippo pathway were up-regulated in the treated group.

The geometric morphometrics analysis, combined with transcriptome analysis, paved the way for understanding the impact of pesticides on the wing shape of the non-target insect model. Wing shape is a crucial factor in enabling various functions in insects, including flight, protection, visual communication, heat retention, and orientation. Sub-lethal concentrations of acephate and chlorantraniliprole lead to differential expression of wing development genes. Sub-lethal concentrations of

acephate and chlorantraniliprole lead to the differential expression of wing development genes, and these gene expression changes contribute more or less to the changed wing shape in *D. melanogaster*.

## 3.6 CONCLUSIONS

This chapter aims to evaluate the morphological alterations in the wings of D. melanogaster induced by acephate and chlorantraniliprole, as well as the transcriptomic validation of genes responsible for wing development. The geometric morphometric (GMM) tool was used to detect fine-scale changes in wing size and shape.

The study results included the following:

- Geometric morphometrics emerges as an unparalleled tool in this study, enabling the quantification and visualisation of subtle size and shape variations between control and pesticide-treated groups.
- Notably, concentration-dependent changes in wing shape were observed in both the pesticide-treated group, independent of the sex of the organisms, and were validated by different multivariate analyses.
- The degree of shape variations was different in the acephate and chlorantraniliprole treatments purely due to the various modes of action of the two pesticides.
- Pesticide-induced stress leads to changes in the functional integrity of the wing, as evidenced by the clear separation of wing modules observed in the pesticide-treated groups.
- Transcriptome analysis illuminates the differential expression of genes controlling wing vein development, wing development, and other significant genes involved in the wing shape and function of *D. melanogaster.*

# General Conclusion

# GENERAL CONCLUSIONS

The present study attempted to evaluate the toxicity effects of acephate and chlorantraniliprole against a non-target insect model, *Drosophila melanogaster* Meigen. The present study concluded as follows:

- Acephate and Chlorantraniliprole significantly impaired the entire life cycle and early development. Embryological studies reveal both pesticides' qualitative and quantitative effects, which agree with the egg hatchability results. The transcriptomic analysis enlightens the genes responsible for the toxic effects of both pesticides.
- Overall, the results provide a comprehensive understanding of sublethal toxicities of acephate and chlorantraniliprole on life cycle traits of *D. melanogaster,* developing new strategies in integrated pest management (IPM).
- Transcriptomic analysis correlates all other experimental results to a common point; hence, sublethal concentrations of both pesticides induced changes at the molecular level of the organism, and each pesticide showed a unique way of expression directly linked to its mode of action.

# Future Scope & Recommendations

# RECOMMENDATIONS

Since pesticides were first released onto the market, pesticide poisoning has become a global concern. Although pesticides are used to destroy hazardous species, they can also kill non-target organisms, whether intentionally or unintentionally. The present study evaluated the non-target effects of two pesticides, acephate and chlorantraniliprole, on the model organism *Drosophila melanogaster.* Acephate and chlorantraniliprole are commonly used pesticides in organophosphate and anthranilic diamide groups, respectively. These two chemicals have different modes of action. Acephate has anti-cholinesterase activity, and chlorantraniliprole acts on insect ryanodine receptors. Acephate and chlorantraniliprole are used primarily against a wide range of insect pests. Even though insect pests are the target organisms, non-target organisms, such as pollinators, natural enemies, and predators, are also affected. The non-targeted effects of both pesticides were examined using different methods using the model organism *Drosophila melanogaster*. *Drosophila* shares prominent biochemical, behavioural, physiological, and developmental similarities with higher vertebrates, including humans.

The present study encompassed various aspects of transcriptomics and geometric morphometrics. Toxicity effects on different lifecycle events were analysed, and the molecular toxicity of both pesticides was evaluated using transcriptomics. Transcriptomic analysis identified differentially expressed genes in response to sub-lethal concentrations of acephate and chlorantraniliprole. Morphological changes in wing architecture were analysed using Geometric Morphometric tools. The results concluded that the pesticides exerted size and shape variations, as well as variations in the functional integrity of the wing structure. Defective wing shape and size changes, along with improper module separation, lead to disabled wing function, especially flight mechanisms. Disabled flight mechanisms in non-target insects, especially pollinators, could negatively affect their ecological services. The study concluded the sublethal toxicity responses of acephate and chlorantraniliprole in *D. melanogaster.*

From this perspective, the present study suggests the following recommendations.

The present study highlights the significance and need for highly targeted pesticides to minimise effects on non-targeted organisms. Therefore, the results obtained on the model organism (*D. melanogaster*) can be extrapolated to human toxicity studies, helping in the development of predictive models for human health.

In the case of *D. melanogaster*, nearly all genes have been identified and studied. Nearly 75% of the *Drosophila* genome is homologous with higher vertebrates. Hence, this study can be used as a basis for monitoring gene-level changes in higher organisms, including humans.

A detailed field study is essential to assess the environmental impact of these pesticides and the alterations to ecological services provided by non-target species, which are crucial for maintaining ecological balance.

# FUTURE SCOPE

- Further investigations at the proteomic and metabolomic levels can deepen the understanding of the molecular mechanisms triggered by acephate and chlorantraniliprole, validating transcriptomic data and uncovering potential biomarkers of toxicity.

- Comparative Studies with Other Non-Target Species: Expanding similar studies to other beneficial or ecologically significant non-target organisms could provide broader ecological insights into the sublethal risks associated with these pesticides.

- Long-term and Multi-generational Effects**:** Future research should focus on the chronic, cumulative, and transgenerational effects of sublethal pesticide exposure, which may reveal delayed impacts on fitness and gene expression.

- Integration into IPM Strategies: Insights from this study can inform the development of more targeted and sustainable pest management practices. Exploring pesticide combinations, application timing, and dosage optimisation may help reduce unintended harm to non-target species.

- Environmental Monitoring and Risk Assessment: The toxicological markers identified in *D. melanogaster* could be used for environmental biomonitoring and in developing more refined ecological risk assessments.

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# Publications and Presentations

# Publications and Presentations

**Publications**

- **Rahila, K.,** & Vardhanan, Y. S. (2024). Comparative transcriptome profiling of two pesticides, Acephate and Chlorantraniliprole in non-targeted insect model, *Drosophila melanogaster*. *Pesticide Biochemistry and Physiology*, *203*, 106023.

**Presentations**

- **Rahila, K.,** & Vardhanan, Y. S. (2023). Trascriptome based identification of genes responding to the organophosphate pesticide Acephate in *Drosophila melanogaster* (Meigen, 1830). *2nd international conference on Advance Interdisciplinary Research (ICAIR-2023).* Digvijay Nath PostGraduate College, Gorakhpur, UP, India. April 07-09, 2023.

- **Rahila, K.,** & Vardhanan, Y. S. (2023). Unveiling the molecular resposes:Transcriptome analysis of *Drosophila melanogaster* (Meigen, 1830) exposed to anthranilic diamide pesticide chlorantraniliprole. *The 2nd international Conference on Research Methodology (ICRM-2023)*.Dr. Shakuntala Misra National Rehabilitation University, Lucknow, UP, India. October 28-30, 2023.