

**Sublethal Toxic Effects of Monocrotophos and Carbamazepine
on *Chrysomya megacephala* (Fabricius, 1794): A Multifaceted
Approach Employing Transcriptomics and Geometric
Morphometrics, with Forensic Implications**

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

DOCTOR OF PHILOSOPHY IN ZOOLOGY

By

RENU V V

Under the Guidance of

Dr. Y. SHIBU VARDHANAN



**BIOCHEMISTRY & TOXICOLOGY DIVISION
DEPARTMENT OF ZOOLOGY
UNIVERSITY OF CALICUT
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(ജന്തുശാസ്ത്ര പഠനവിഭാഗം)



Dr. Y. Shibu Vardhanan
Professor

UNIVERSITY OF CALICUT
Re-Accredited by NAAC with "A+" Grade

Calicut University (P.O.)
Malappuram (Dt.)
Kerala, India - 673 635
Tel : 0494 2407420 (Head)
0494 2407419 (Office)

No.

Date: 13th August 2025

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This is to certify that this dissertation entitled "**Sublethal Toxic Effects of Monocrotophos and Carbamazepine on *Chrysomya megacephala* (Fabricius, 1794): A Multifaceted Approach Employing Transcriptomics and Geometric Morphometrics, with Forensic Implications**" is an authentic record of independent work done by **Renu. V. V.**, 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.



Dr. Y. Shibu Vardhanan

Dr. Y. Shibu Vardhanan, Ph.D.
Professor
Department of Zoology
University of Calicut
Kerala, India, Pin: 673 635



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Dr. Y. Shibu Vardhanan

Professor

Dr. Y. Shibu Vardhanan, Ph.D.
Professor
Department of Zoology
University of Calicut
Kerala, India, Pin: 673 635

DECLARATION

I do hereby declare that thesis entitled “**Sublethal Toxic Effects of Monocrotophos and Carbamazepine on *Chrysomya megacephala* (Fabricius, 1794): A Multifaceted Approach Employing Transcriptomics and Geometric Morphometrics, with Forensic Implications**” is an authenticated record of the work carried out by me under the guidance and supervision of Dr. Y. Shibu Vardhanan, Professor, Biochemistry & Toxicology Division, Department of Zoology, University of Calicut and that no part of this has been submitted before for the award of any other Degree or Diploma. The contents of the thesis are undergone plagiarism check using **iThenticate** software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents.



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Prof. (Dr.) Y. Shibu Vardhanan
Biochemistry & Toxicology Division
Department of Zoology, University of Calicut



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Dr. Y. Shibu Vardhanan, Ph.D.

Professor

Department of Zoology

University of Calicut

Kerala, India, Pin: 673 635

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Department of Zoology

University of Calicut

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Renu V V.

DEDICATION

In recognition of the enduring spirit of Rationalism: to those who, driven by an unwavering commitment to truth, prioritize logic over dogma, evidence over assertion, and the relentless pursuit of knowledge above all else, demonstrating the integrity and courage to uphold their principles, even in solitude.

May Clarity Always Outrun Comfort.

ABSTRACT

Chrysomya megacephala, a blowfly species of the Calliphoridae family, plays a crucial role in forensic entomology by aiding in the estimation of post-mortem interval (PMI). This study aims to investigate the impact of sublethal doses of monocrotophos, an organophosphate insecticide, and carbamazepine, an anticonvulsant drug, on the developmental stages of *C. megacephala*, from embryogenesis to emergence. The life cycle of *C. megacephala* consists of four distinct stages: egg, larva, pupa, and adult, with the duration of each stage influenced by temperature and humidity. To assess the effects of these toxicants, the EC₅₀ values for monocrotophos and carbamazepine were estimated. Subsequently, the impact of sublethal doses on various developmental stages was examined. The molecular basis of these effects was further elucidated through the analysis of differentially expressed genes and transcriptomic data. The findings of this study contribute to the understanding of the influence of toxicants on the development of *C. megacephala*, which is essential for accurate PMI estimation in forensic entomology. Moreover, the results provide insights into the potential consequences of monocrotophos and carbamazepine exposure on the biology and development of this forensically important blowfly species.

Toxicological analysis is crucial for detecting harmful substances in organisms and assessing their impact on human health. This study investigated the chronic *in vivo* toxicity of monocrotophos and carbamazepine on *C. megacephala* using enzymatic biochemical assays and transcriptomic analysis. The activities of superoxide dismutase (SOD), acetylcholinesterase (AChE), glutathione reductase (GR), and glutathione S-transferase (GST) were evaluated in the third instar larvae of *C. megacephala* under control conditions and in the presence of sub-lethal concentrations of the toxicants. Biochemical assays, which are reliable and commonly employed techniques, were used to identify targets and understand biomolecular functions. The study aimed to elucidate the detoxification mechanisms involved in the response to monocrotophos and carbamazepine exposure. Additionally, differential gene expression (DGE) analysis was performed to investigate the molecular basis of the detoxification mechanism. The findings of this study contribute to the understanding of the toxic effects of pollutants on various molecular pathways and provide insights into the potential impacts of drugs on human physiology.

The integration of omics technologies, particularly transcriptomics, has significantly advanced the field of forensic entomology. This study presents a *de novo* transcriptomic profiling of the third instar larvae of *C. megacephala* to investigate the potential genes and pathways altered by sublethal concentrations of Carbamazepine

and Monocrotophos, and their effects on development and various biological activities. The larvae were selected at the point of transformation from the feeding to the non-feeding phase, assuming that transcripts present in both the larval and upcoming pupal stages could be detected. Due to the lack of a previously published genome for this species or any closely related species, de novo profiling was performed. Differentially expressed genes (DEGs) were matched against the EuKaryotic Orthologous Group (KOG) database to categorize them into molecular families, highlighting the most prevalent functional categories. Gene Ontology (GO) enrichment analysis was employed to interpret the high-throughput molecular data and formulate hypotheses about the underlying biological mechanisms. KEGG pathway enrichment analysis was used to functionally annotate DEGs, providing insights into their associated functions and pathways. The results of this study contribute to a better understanding of the altered genes and pathways due to the effects of Carbamazepine and Monocrotophos on the development and biological activities of *C. megacephala* larvae, ultimately enhancing the accuracy of postmortem interval estimation in forensic entomology.

Insect wings exhibit remarkable diversity in shape, size, and structure, reflecting their crucial role in insect evolution and radiation. The aerodynamic performance of insects is fundamentally tied to the specific attributes of their wings, and the interplay between shape and movement patterns determines both flying abilities and energy requirements. Geometric morphometric (GMM) analysis is a powerful tool for quantifying wing shape, offering efficient and statistically robust analyses that can connect abstract, multivariate outcomes to the physical structure of the original specimens. This study investigates the effects of two sublethal concentrations of monocrotophos, an organophosphate insecticide, and carbamazepine, an anticonvulsant, on wing development in *C. megacephala*, a forensically important dipteran species. Significant alterations in wing dimensions and morphology were observed using GMM analysis. Additionally, differential gene expression (DGE) associated with wing development was analysed, complementing and reinforcing the GMM results. The higher sublethal dose (1/3rd of EC₅₀) was used for DGE analysis, as it showed more promising results in basic entomotoxicological analyses. The study of the effects of these intoxicants on *C. megacephala* can also be critical from a forensic perspective, as the species is of significant forensic importance. The findings of this study contribute to our understanding of the impact of environmental toxicants on insect wing development and morphology, with potential implications for insect evolution, ecology, and forensic entomology.

Key words: Calliphoridae, Entomotoxicology, Monocrotophos, Carbamazepine, Embryotoxicity, Transcriptomics, GMM analysis

സംഗ്രഹം

കാലിഫോറിയ കടുംബത്തിലെ ഒരു ബ്ലോക്കിംഗ് (blowfly) സ്പീഷിസായ ക്രിസോമ്യ മെഗാസെഫല, മരണാനന്തര ഇടവേള (Post-Mortem Interval - PMI) കണക്കാക്കുന്നതിൽ സഹായിക്കുന്നതിനുള്ള ഫോറൻസിക് എൻറ്റോമോളജിയിൽ പ്രധാന പങ്ക് വഹിക്കുന്നു. ഓർഗാനോഫോസ്ഫേറ്റ് കീടനാശിനിയായ മോണോക്രോട്ടോഫോസിന്റെയും ആന്റികൺവൾസന്റ് മരുന്നായ കാർബമാസാപൈന്റെയും ഉപമാരക തോതുകൾ (sublethal doses), ഭ്രൂണജനനം മുതൽ ആവിർഭാവം വരെയുള്ള സി. മെഗാസെഫലയുടെ വികസന ഘട്ടങ്ങളിൽ ചെലുത്തുന്ന സ്വാധീനം അന്വേഷിക്കുക എന്നതാണ് ഈ പഠനത്തിന്റെ ലക്ഷ്യം. സി. മെഗാസെഫലയുടെ ജീവിതചക്രത്തിൽ നാല് വ്യത്യസ്ത ഘട്ടങ്ങൾ ഉൾക്കൊള്ളുന്നു: മുട്ട, ലാർവ, പ്യൂപ്പ, പ്രായപൂർത്തിയുള്ള ഫ്ലൈ. ഓരോ ഘട്ടത്തിന്റെയും ദൈർഘ്യം താപനിലയും ഈർപ്പവും ആശ്രയിച്ചിരിക്കുന്നു. ഈ വിഷവസ്തുക്കളുടെ ഫലങ്ങൾ വിലയിരുത്തുന്നതിന്, മോണോക്രോട്ടോഫോസിനും കാർബമാസാപൈനുമുള്ള EC₅₀ മൂല്യങ്ങൾ കണക്കാക്കി. തുടർന്ന്, വിവിധ വികസന ഘട്ടങ്ങളിൽ ഉപമാരക തോതുകളുടെ സ്വാധീനം പരിശോധിച്ചു. വ്യത്യസ്തമായ ജനപ്രകടനമുള്ള ജീനുകളുടെയും ട്രാൻസ്ക്രിപ്റ്റോമിക് ഡാറ്റയുടെയും വിശകലനത്തിലൂടെ ഈ ഫലങ്ങളുടെ തന്മാത്രാ അടിസ്ഥാനം (molecular basis) കൂടുതൽ വ്യക്തമാക്കി. ഈ പഠനത്തിന്റെ കണ്ടെത്തലുകൾ, സി. മെഗാസെഫലയുടെ വളർച്ചയിൽ വിഷപദാർത്ഥങ്ങൾ ഉണ്ടാക്കുന്ന സ്വാധീനത്തെ കുറിച്ചുള്ള അവബോധത്തിൽ വലിയ സംഭാവന നൽകുന്നു. ഫോറൻസിക് എൻറ്റോമോളജിയിൽ PMI കൃത്യമായി കണക്കാക്കുന്നതിന് അത്യന്താപേക്ഷിതമായ ഒരു ഘടകമാണിത്. മാത്രമല്ല, ഫോറൻസിക് പ്രാധാന്യമുള്ള ഈ ബ്ലോക്കിംഗ് സ്പീഷിസിന്റെ ജീവശാസ്ത്രത്തിലും വികാസത്തിലും മോണോക്രോട്ടോഫോസും കാർബമാസാപൈനുമായുള്ള സമ്പർക്കം ചെയ്യുന്നതിന് സാധ്യതയുള്ള പ്രത്യാഘാതങ്ങളെക്കുറിച്ചുള്ള ഉൾക്കാഴ്ചകളും ഫലങ്ങൾ നൽകുന്നു.

ജീവജാലങ്ങളിലെ ദോഷകരമായ വസ്തുക്കൾ കണ്ടെത്തുന്നതിനും മനുഷ്യന്റെ ആരോഗ്യത്തിലുണ്ടാകുന്ന സ്വാധീനം വിലയിരുത്തുന്നതിനും വിഷശാസ്ത്ര വിശകലനം (toxicological analysis) നിർണായകമാണ്. എൻസൈമാറ്റിക് ബയോകെമിക്കൽ അസേയകളും ട്രാൻസ്ക്രിപ്റ്റോമിക് അനാലിസിസും ഉപയോഗിച്ച് സി. മെഗാസെഫലയിൽ മോണോക്രോട്ടോഫോസിന്റെയും കാർബമാസാപൈന്റെയും ദീർഘകാല ഇൻ വിവോ വിഷബാധയെക്കുറിച്ച് ഈ പഠനം അന്വേഷിച്ചു. സുപ്പർഓക്സൈഡ് ഡിസ്ട്രേസ് (എസ്ഒഡി), അസെറ്റൽകോളിനെസ്റ്ററേസ് (എസിഎച്ച്ഇ), ഗ്ലൂട്ടത്തയോൺ റിഡക്ടേസ് (ജിആർ), ഗ്ലൂട്ടത്തയോൺ എസ്-ട്രാൻസ്ഫറേസ് (ജിഎസ്ടി) എന്നിവയുടെ പ്രവർത്തനങ്ങൾ സി. മെഗാസെഫലയുടെ മൂന്നാം ഇൻസ്റ്റാർ ലാർവകളിൽ നിയന്ത്രണ സാഹചര്യങ്ങളിലും വിഷവസ്തുക്കളുടെ മാർകമല്ലാത്ത സാന്ദ്രതയുടെ സാന്നിധ്യത്തിലും വിലയിരുത്തി. വിഷവസ്തുക്കളുടെ ലക്ഷ്യങ്ങൾ തിരിച്ചറിയുന്നതിനും ബയോമോളികുലാർ പ്രവർത്തനങ്ങൾ മനസ്സിലാക്കുന്നതിനും വിശ്വസനീയവും സാധാരണയായി ഉപയോഗിക്കുന്നതുമായ സാങ്കേതിക വിദ്യകളായ ബയോകെമിക്കൽ അസേയകൾ ഉപയോഗിച്ചു. മോണോക്രോട്ടോഫോസിനും കാർബമാസാപൈൻ എക്സ്പോഷറുമുള്ള പ്രതികരണത്തിൽ ഉൾപ്പെട്ടിരിക്കുന്ന വിഷവിമുക്തമാക്കൽ സംവിധാനങ്ങൾ (detoxification mechanisms) വ്യക്തമാക്കുക എന്നതാണ് പഠനം ലക്ഷ്യമിടുന്നത്. കൂടാതെ, വിഷവിമുക്തമാക്കൽ സംവിധാനത്തിന്റെ തന്മാത്രാ അടിസ്ഥാനം അന്വേഷിക്കുന്നതിനായി ഡിഫറൻഷ്യൽ ജീൻ എക്സ്പ്രഷൻ (DGE) വിശകലനം നടത്തി. ഈ പഠനത്തിന്റെ കണ്ടെത്തലുകൾ വിവിധ തന്മാത്രാ പാതകളിൽ മലിനീകരണത്തിന്റെ വിഷ ഫലങ്ങളെക്കുറിച്ചുള്ള മനസ്സിലാക്കലിനും മനുഷ്യ ശരീരശാസ്ത്രത്തിൽ മരുന്നുകളുടെ സാധ്യതയുള്ള പ്രത്യാഘാതങ്ങളെക്കുറിച്ചുള്ള ഉൾക്കാഴ്ചകൾക്കും സഹായിക്കുന്നു.

ഒമിക്സ് സാങ്കേതികവിദ്യകളുടെ, പ്രത്യേകിച്ച് ട്രാൻസ്ക്രിപ്റ്റോമിക്സിന്റെ സംയോജനം ഫോറൻസിക് എൻറ്റോമോളജി മേഖലയെ ഗണ്യമായി പുരോഗമിപ്പിച്ചു. കാർബമാസാപൈൻ, മോണോക്രോട്ടോഫോസ് എന്നിവയുടെ ഉപമാരക സാന്ദ്രതയാൽ മാറ്റം വരുത്തിയ സാധ്യതയുള്ള ജീനുകളും, പാതകളും, വികസനത്തിലും വിവിധ ജൈവ പ്രവർത്തനങ്ങളിലും അവയുടെ സ്വാധീനവും അന്വേഷിക്കുന്നതിനായി സി. മെഗാസെഫലയുടെ മൂന്നാം ഇൻസ്റ്റാർ ലാർവകളുടെ ഒരു ഡി നോവോ ട്രാൻസ്ക്രിപ്റ്റോമിക് പ്രൊഫൈലിംഗ് ഈ പഠനം അവതരിപ്പിക്കുന്നു. ലാർവ ഘട്ടത്തിലും വരാനിരിക്കുന്ന പ്യൂപ്പൽ ഘട്ടത്തിലും ഉള്ള ട്രാൻസ്ക്രിപ്റ്റുകൾ കണ്ടെത്താനാകുമെന്ന് അനുമാനിച്ചുകൊണ്ട്, ഭക്ഷണഘട്ടത്തിൽ നിന്ന് ഭക്ഷണം കഴിക്കാത്ത ഘട്ടത്തിലേക്ക് പരിവർത്തനം ചെയ്യുന്ന ലാർവകളെയാണ് തിരഞ്ഞെടുത്തത്. ഈ സ്പീഷിസിനോ അടുത്ത ബന്ധമുള്ള

സ്പീഷീസിനോ ജീനോം ഡാറ്റ പ്രസിദ്ധീകരിച്ചിട്ടില്ലാത്തതിനാൽ ഡി നോവോ ജീനോം പ്രൊഫൈലിംഗ് നടത്തേണ്ടിവന്നു. ഏറ്റവും പ്രചാരത്തിലുള്ള പ്രവർത്തന വിഭാഗങ്ങളെ എടുത്തുകാണിച്ചുകൊണ്ട്, അവയെ തന്മാത്രാ കട്ടാബങ്ങളായി തരംതിരിക്കുന്നതിന് വ്യത്യസ്തമായി പ്രകടിപ്പിക്കപ്പെട്ട ജീനുകൾ (DEG-കൾ) യുകാരിയോട്ടിക് ഓർത്തോളജസ് ഗ്രൂപ്പ് (KOG) ഡാറ്റാബേസുമായി പൊരുത്തപ്പെടുത്തി. ഉയർന്ന തൂപ്പ് (high-throughput) തന്മാത്രാ ഡാറ്റ വ്യാഖ്യാനിക്കുന്നതിനും അടിസ്ഥാന ജൈവ സംവിധാനങ്ങളെക്കുറിച്ചുള്ള അനുമാനങ്ങൾ രൂപപ്പെടുത്തുന്നതിനും ജീൻ ഓർ്റോളജി (GO) സമ്പൂഷ്ടീകരണ വിശകലനം ഉപയോഗിച്ച്, കെജുജിജി പാത്ത്വേ സമ്പൂഷ്ടീകരണ വിശകലനം ഉപയോഗിച്ച് ഡിഇജികളെ പ്രവർത്തനപരമായി വ്യാഖ്യാനിച്ചു, അതുവഴി അവയുടെ അനുബന്ധ പ്രവർത്തനങ്ങളെയും പാതകളെയും കുറിച്ചുള്ള ഉൾക്കാഴ്ചകൾ ലഭിച്ചു. കാർബമാസാപൈൻ, മോണോക്രോട്ടോഫോസ് എന്നിവയുടെ സ്വാധീനം മൂലം സി. മെഗാസെഫല ലാർവകളുടെ വികാസത്തിലും ജൈവിക പ്രവർത്തനങ്ങളിലും ഉണ്ടാകുന്ന മാറ്റങ്ങൾ മൂലം ഉണ്ടാകുന്ന ജീനുകളുടെയും പാതകളുടെയും മികച്ച ധാരണയ്ക്ക് ഈ പഠനത്തിന്റെ ഫലങ്ങൾ സംഭാവന നൽകുന്നു, ഇത് ആത്യന്തികമായി ഫോറൻസിക് എന്റമോളജിയിൽ പോസ്റ്റ്മോർട്ടം ഇടവേള എസ്റ്റിമേഷന്റെ കൃത്യത വർദ്ധിപ്പിക്കുന്നു.

പ്രാണികളുടെ ചിറകുകൾ ആകൃതിയിലും വലുപ്പത്തിലും ഘടനയിലും ശ്രദ്ധേയമായ വൈവിധ്യം പ്രകടിപ്പിക്കുന്നു, ഇത് പ്രാണികളുടെ പരിണാമത്തിലും വികിരണത്തിലും അവയുടെ നിർണായക പങ്ക് പ്രതിഫലിപ്പിക്കുന്നു. പ്രാണികളുടെ വായുസഞ്ചാര പ്രകടനം അടിസ്ഥാനപരമായി അവയുടെ ചിറകുകളുടെ പ്രത്യേക ഗുണങ്ങളുമായി ബന്ധപ്പെട്ടിരിക്കുന്നു, കൂടാതെ ആകൃതിയും ചലന രീതികളും തമ്മിലുള്ള പരസ്പരബന്ധം പരക്കൽ കഴിവുകളെയും ഊർജ്ജ ആവശ്യകതകളെയും നിർണ്ണയിക്കുന്നു. ജ്യാമിതീയ മോർഫോമെട്രിക് (GMM) വിശകലനം ചിറകിന്റെ ആകൃതി അളക്കുന്നതിനുള്ള ശക്തമായ ഒരു ഉപകരണമാണ്, ഇത് അമൂർത്തവും മൾട്ടിവേരിയേറ്റ് ഫലങ്ങളെ യഥാർത്ഥ മാതൃകകളുടെ ഭൗതിക ഘടനയുമായി ബന്ധിപ്പിക്കാൻ കഴിയുന്ന കാര്യക്ഷമവും സ്ഥിതിവിവരക്കണക്കനുസരിച്ച് ശക്തവുമായ വിശകലനങ്ങൾ വാഗ്ദാനം ചെയ്യുന്നു. ഫോറൻസിക് പ്രാധാന്യമുള്ള ഡിപ്റ്ററൻ ഇനമായ സി. മെഗാസെഫലയിൽ, ഓർഗാനോഫോസ്റ്റേറ്റ് കീടനാശിനിയായ മോണോക്രോട്ടോഫോസിന്റെയും ആന്റികൺവൾസന്റായ കാർബമാസാപൈനിന്റെയും രണ്ട് ഉപമാരക സാന്ദ്രതകൾ ചിറകിന്റെ വികാസത്തിൽ ചെലുത്തുന്ന സ്വാധീനം ഈ പഠനം അന്വേഷിക്കുന്നു. GMM വിശകലനം ഉപയോഗിച്ച് ചിറകിന്റെ അളവുകളിലും രൂപഘടനയിലും ഗണ്യമായ മാറ്റങ്ങൾ നിരീക്ഷിക്കപ്പെട്ടു. കൂടാതെ, ചിറകിന്റെ വികാസവുമായി ബന്ധപ്പെട്ട ഡിഫറൻഷ്യൽ ജീൻ എക്സ്പ്രഷൻ (DGE) വിശകലനം ചെയ്തു, ഇത് GMM ഫലങ്ങളെ പൂരകമാക്കുകയും ശക്തിപ്പെടുത്തുകയും ചെയ്തു. അടിസ്ഥാന എന്റോമോടോക്സിക്ക്കോളജിക്കൽ വിശകലനങ്ങളിൽ കൂടുതൽ വാഗ്ദാനകരമായ ഫലങ്ങൾ കാണിച്ചതിനാൽ, ഉയർന്ന ഉപമാരക തോത് ($1/3^{\text{rd}}$ of EC_{50}) DGE വിശകലനത്തിനായി ഉപയോഗിച്ചു. ഈ ലഹരിവസ്തുക്കൾ സി. മെഗാസെഫലയിൽ ചെലുത്തുന്ന സ്വാധീനത്തെക്കുറിച്ചുള്ള പഠനം ഫോറൻസിക് വീക്ഷണകോണിൽ നിന്ന് നിർണായകമാണ്, കാരണം ഈ ജീവിവർഗത്തിന് കാര്യമായ ഫോറൻസിക് പ്രാധാന്യമുണ്ട്. പ്രാണികളുടെ ചിറകുകളുടെ വികാസത്തിലും രൂപഘടനയിലും പാരിസ്ഥിതിക വിഷവസ്തുക്കൾ ചെലുത്തുന്ന സ്വാധീനത്തെക്കുറിച്ചുള്ള നമ്മുടെ ഗ്രാഹ്യത്തിന് ഈ പഠനത്തിന്റെ കണ്ടെത്തലുകൾ സംഭാവന നൽകുന്നു, കൂടാതെ പ്രാണികളുടെ പരിണാമം, പരിസ്ഥിതി, ഫോറൻസിക് എന്റമോളജി എന്നിവയിലും ഇവയുടെ സ്വാധീനം ചെലുത്താനുള്ള സാധ്യതയും കൂടുതലാണ്.

സൂചകപദങ്ങൾ കാലിഫോറിഡേ, എന്റോമോടോക്സിക്ക്കോളജി, മോണോക്രോട്ടോഫോസ്, കാർബമാസെപൈൻ, എത്രിയോടോക്സിസിറ്റി, ട്രാൻസ്ക്രിപ്റ്റോമിക്സ്, ജിഎംഎം അനാലിസിസ്.

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GENERAL INTRODUCTION

The Calliphoridae family consists of a diverse group of calyptrate flies found globally, encompassing species that hold ecological, veterinary, medical, and forensic significance. Known for their parasitic nature, the larvae of numerous blowflies, similar to some other dipteran larvae, are distinguished by their capacity to thrive in animal tissue (Nasser et al., 2021). It is believed that the name "blow flies" originates from Homer's renowned work, *The Iliad*, where he described the "blows of flies" that plagued the wounds of both injured and deceased soldiers (Merritt et al., 2009). Blowflies are often evaluated anthropocentrically (Heath, 1982). Although they can cause issues, they serve as important pollinators for horticultural plants, boosting seed production. If blowflies did not initially invade carrion, the decomposition of dead bodies would be hindered. The advantages of Calliphoridae include their consumption by humans, though other insects are more commonly eaten. Blowflies also hold religious significance or recreational purposes for some human groups. Another promising avenue is the utilization of Calliphoridae as producers of pharmacologically active substances, where compounds with antiviral and antitumor properties derived from blowfly maggots have been applied in treating viral infections (Yakovlev et al., 2019). Additionally, maggot enzymes and antimicrobial peptides represent another category of drug candidates that have garnered significant attention.

Chrysomya megacephala serve as a significant forensic indicator, as silent observers after a crime has occurred (Babu et al., 2022). These insects, belonging to the family Diptera: Calliphoridae, are among the earliest to colonize a corpse soon after death, making them highly valuable in forensic entomology (Hore et al., 2017). The development rates of blowflies (calliphorids) are used to determine the minimum time that has passed between death and discovery of the remains, a period known as the post-mortem interval (PMI) (Hans & Vanlaerhoven, 2021). Forensic entomology is essentially the examination of insects within the realm of forensic science (Bozdogan et al., 2023). It involves applying insect biology knowledge to legally investigate suspicious human and animal deaths. Insect evidence can also indicate if a body has

been relocated to a different location after death or if it has been disturbed at some point, either by animals or by the perpetrator revisiting the crime scene (Anderson, 1995). Nonetheless, the main goal of forensic entomology today is to establish the time elapsed since death. Most cases involving a forensic entomologist are at least 72 hours old, as other forensic techniques are just as accurate, if not more so, than insect evidence up to this point. However, beyond three days, insect evidence often becomes the most precise and sometimes the sole method for determining the time elapsed since death. Entomotoxicology is a developing field in forensic science that combines forensic entomology with toxicology. By incorporating toxicological analysis, it can provide essential information about the presence and effects of drugs or toxins in a biological system (Chakroborty & Sharma, 2023). This discipline shows great potential as a useful tool in forensic investigations, especially in cases involving deaths related to drugs or poisoning.

Monocrotophos (dimethyl [(E)-4-(methylamino)-4-oxobut-2-en-2-yl] phosphate) is a broad-spectrum organophosphate insecticide that has been widely used in many horticultural and agricultural crops. Monocrotophos is linked to the development of OP-induced intermediate syndrome, with a prevalence rate ranging from 10% to 40% among all OP poisoning cases (Bharali et al., 2023). The official ban on monocrotophos went into effect immediately upon publication on October 6, 2023. Despite the prohibition of monocrotophos, a pesticide known for its mutagenic properties, its presence in the market persisted due to inadequate enforcement measures. The ban did not effectively deter manufacturers from distributing the pesticide through online platforms (Das, 2024). Carbamazepine is commonly used to treat focal epilepsy and a range of non-epileptic conditions, such as neuropathic pain, schizophrenia, and bipolar disorder, in both children and adults. The first instance of an overdose was documented in 1967, with severe toxicity noted at levels above 40 mg/L, while the standard therapeutic range is between 4 and 12 mg/L (Al Khalili et al., 2023).

Enzyme assays are standardized experimental methods used to measure the activity or concentration of enzymes in biochemical or cell-based systems (Sarfraz et al.,

2022). These assays have two main objectives: (i) to detect a specific enzyme, confirming whether it is present or absent in a given sample, such as an organism or tissue, and (ii) to determine the amount of the enzyme in the sample (Bisswanger, 2014). Over the past couple of decades, "omics" technology has evolved from merely listing genes, proteins, and SNPs to conducting detailed, disease-specific analyses of meta-genetics, protein interactions, modifications, and pathway mapping (Ning & Lo, 2010). Large-scale genome-wide association studies and high-throughput methods have become more effective and productive in exploring largely uncharted areas. Numerous research fields fall under the category of omics, such as proteomics, transcriptomics, genomics, metabolomics, lipidomics, and epigenomics. These fields involve comprehensive studies of proteins, RNA, genes, metabolites, lipids, and either methylated DNA or modified histone proteins within chromosomes, respectively (Micheel et al., 2012).

Geometric morphometrics (GMM) is a sophisticated morphometric approach that facilitates the quantitative examination of shape and size differences in biological forms (Spani et al., 2025). This method employs Cartesian geometric coordinates to explore the relationships of scale and shape in structures, as opposed to relying on linear, areal, or volumetric measurements (Hadi, 2023). The integration of geometric morphometrics (GMM) with transcriptome analysis offers significant potential for elucidating the combined morphological and molecular foundations of variations induced by external factors in an organism.

This study investigates the effects of two distinct intoxicant classes, monocrotophos and carbamazepine, on the developmental stages of the forensically relevant dipteran species, *Chrysomya megacephala*. The research employs a combination of observational study, biochemical analysis, GMM analysis, and transcriptome analysis to achieve its objectives.

OBJECTIVES

1. Evaluation of chronic toxicity and dose-dependent effects of monocrotophos and carbamazepine on *Chrysomya megacephala*.
2. Evaluation of the biochemical parameters in third instar larvae exposed to the sub-lethal concentration of monocrotophos and carbamazepine.
3. De novo transcriptomic analysis and evaluation of differentially expressed genes in response to monocrotophos and carbamazepine exposure.
4. Geometric morphometric (GMM) analysis assessing the variation in wing morphology resulting from exposure to monocrotophos and carbamazepine.

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Chapter 1

Developmental Effects of Monocrotophos and Carbamazepine on *Chrysomya megacephala*: Implications for Forensic Entomology

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1

Developmental Effects of Monocrotophos and Carbamazepine on *Chrysomya megacephala*: Implications for Forensic Entomology

1.1 INTRODUCTION

Calliphoridae are usually the first family of insects to colonise on a cadaver, thereby playing a crucial role in estimating the post-mortem interval (PMI) in forensic entomology. Blowflies of the genus *Chrysomya* (Diptera: Calliphoridae) are of significant medical and sanitary importance due to their ability to cause myiasis in both animals and humans. These flies are also pivotal in forensic entomology as they assist in determining the PMI. The extensive distribution and global prevalence of issues associated with *Chrysomya megacephala* necessitate a comprehensive review of the species' biology. Such a review serves as a foundation for research into its management and applications, particularly within the context of forensic entomology (Bharti et al., 2007).

The study by Chen et al. (n.d.) provides a concise description of the life cycle of *Chrysomya megacephala*, delineating its four distinct developmental stages: egg, larva, pupa, and adult. Upon hatching, larvae experience rapid growth under warm conditions, typically completing development through three instars within a few days. The duration of these stages is influenced by temperature and humidity levels. The life cycle of this species, particularly the development time at varying temperatures, is well-documented due to its forensic significance. An adult female can deposit 200-325 eggs in faeces, carcasses, or decaying organic matter within a single day. While females are diurnally active, they prefer nocturnal oviposition when temperatures are elevated. Males generally emerge two to three hours before females.

Martín-Vega and Hall (2016) propose that a viable method for estimating the age of blowfly eggs involves utilizing embryonic morphological landmarks, which have also been employed for staging embryos of certain Diptera species used in pest control. For instance, developmental studies on the fruit fly *Drosophila melanogaster* (Diptera: Drosophilidae) have demonstrated a predictable sequence of morphological changes during embryogenesis (Campos-Ortega & Hartenstein, 1997). Notably, none of these studies have addressed the impact of intoxicants on the embryonic development of *Chrysomya megacephala* eggs. Therefore, it is imperative to develop a method to study development within the egg.

Monocrotophos [3-hydroxy-N-methyl-cis-crotonamide dimethylphosphate] is an organophosphate (OP) insecticide employed to combat a broad spectrum of insect pests, including piercing and sucking insects, leaf-eating beetles, bollworms, and caterpillars, across various horticultural and agricultural crops (Kumar et al., 2020). Organophosphate insecticides are generally recognised for their ability to inhibit acetylcholinesterase, an enzyme crucial for neurotransmission at cholinergic synapses, by facilitating the rapid hydrolysis of the neurotransmitter acetylcholine into choline and acetate (Kavitha & Venkateswara Rao, 2007). Organophosphorus (OP) poisoning remains the predominant cause of self-poisoning, particularly in developing regions. In the Indian subcontinent, it accounts for over 30% of suicides (Gunnell et al., 2007). A study by Acharya & Panda (2024) revealed that the poisoning due to monocrotophos constituted 2.95% of the poisoning cases and 5.26% of organophosphate poisoning during the study period (2012-21).

Carbamazepine, an anticonvulsant drug discovered by Walter Schindler in 1953, was initially employed to treat partial tonic-clonic seizures (Wang et al., 2023). Presently, it is extensively utilised for the management of epilepsy, peripheral neuralgia, partial and generalised seizures, bipolar disorder, and neuropathic pain (Nasif et al., 2022). Significant toxicity typically manifests at serum levels exceeding 40 mcg/mL in adult patients; however, toxicity may also occur at serum levels above 20 mcg/mL. The therapeutic dosage of carbamazepine is 15-25 mg/kg, with the required serum levels ranging from 4-12 µg/mL. In the context of suicide attempts, carbamazepine is a

relatively common clinical issue that can lead to coma, respiratory depression, arrhythmia, hemodynamic instability, and death (Yaylaci et al., 2012).

This chapter examines the rearing of the test organism and fundamental aspects of toxicological analysis, including the determination of the EC₅₀ values for monocrotophos and carbamazepine. It further examines the effect of sublethal doses of these toxicants on various critical developmental stages of *Chrysomya megacephala*, spanning from embryogenesis to emergence. Additionally, the molecular basis of these effects is elucidated through the analysis of differentially expressed genes and transcriptomic data.

1.2 REVIEW OF LITERATURE

Accurate estimation of the post-mortem interval (PMI) is critical for the effective investigation of suspicious deaths, and forensic indicators, such as blowflies, play a vital role in ensuring the accuracy of PMI estimation (Siddiki & SP, 2017). In forensic entomology, *Chrysomya megacephala* serves as a crucial indicator species due to its role as an early coloniser of vertebrate carrion. The application of knowledge regarding its developmental stages, from egg to adulthood, to specimens collected from remains is employed to estimate the minimum postmortem interval (minPMI), under the assumption that colonization occurred postmortem (Martínez-Sánchez et al., 2025). The life history and life table of the oriental latrine fly, *Chrysomya megacephala*, in laboratory conditions at 26°C were prepared by Gabre et al., (2005). Additionally, larvae of *Chrysomya megacephala* can be utilised as an alternative specimen for gunshot residue (GSR) detection when a bullet is not recovered at the crime scene, and the bullet wound can be traced even in highly decomposed bodies (Abd Rashid et al., 2013).

Many researchers have attempted to develop a potential larval diet for the rearing and culture maintenance of calliphorids, as the maggots have several medical, veterinary, and forensic applications, including maggot therapy, myiasis, and PMI estimation. Previous studies and expert opinions have indicated that artificial diets are the most appropriate dietary media for entomotoxicological studies (Mbatha, 2017). Daniels et al., (1991) devised and compared two simple artificial diets for *Lucilia sericata* with

agar and horse blood as the prime ingredients. Sherman & My-Tien TRAN (1995) attempted to develop a sterile tissue-based larval diet that could potentially be a convenient and inexpensive alternative growth medium, and Zheng et al., (2017) compared six sterilised diets: blood–yeast agar diet, decomposed beef liver diet, powdered beef liver diet, powdered fish diet, milk-based diet, and a chemically defined diet, for *Lucilia sericata*. Each of these dietary regimens exhibited certain limitations that rendered them unsuitable for use in toxicological studies with forensic applications.

Embryonic development initially relies on maternal mRNAs and proteins deposited in the egg before zygotic genome activation (Crofton et al., 2018). This maternal control is conserved across metazoans and establishes developmental trajectories. Barckmann & Simonelig (2013) studied the control of maternal mRNA stability in early embryos and observed that late oogenesis and early embryogenesis occur without transcription, relying on maternal mRNAs in oocytes. The research by Jackle (1979) focused on the processes of RNA and protein synthesis in the developing embryos of *Smittia* sp., utilising transcription inhibitors to explore these mechanisms. The study revealed that proteins synthesised before the formation of the blastoderm were directed by maternal mRNA. As the embryos progressed to the blastoderm stages, this maternal mRNA was replaced by embryonic mRNAs. Yoon et al. (2019) investigated the function of maternal mRNA in establishing embryo polarity through the localisation of mRNA transcripts in moth flies and mosquitoes. Their findings indicated that maternal mRNA serves as anterior determinants in both species. The earliest stages of animal development are governed by maternally deposited mRNA transcripts and proteins, and upon the zygote's ability to transcribe its own genome, maternal transcripts undergo degradation in a meticulously regulated process known as the maternal-to-zygotic transition (MZT) (Atallah & Lott, 2018).

Numerous incidents of mass poisoning by organophosphates (OPs) have been documented in developing countries, and organophosphorus (OP) poisoning represents the most prevalent form of poisoning in India. Among the various OP compounds, monocrotophos is associated with the highest mortality rate and

frequently necessitates mechanical ventilation (Bharali et al., 2023). Furthermore, monocrotophos is linked to the development of OP-induced intermediate syndrome, with a prevalence rate ranging from 10% to 40% among all OP poisoning cases. Another report by Patel et al., (2012) details an incident of mass monocrotophos poisoning resulting from the accidental consumption of millet flour contaminated with monocrotophos, affecting eight individuals who were severely poisoned. Human exposure to environmental chemicals has been a persistent issue throughout history. On July 16, 2013, a tragic incident occurred in Gandaman village, Bihar, where twenty-three children succumbed within a few hours after consuming a school meal contaminated with monocrotophos (Krause et al., 2013). On December 14, 2018, another incident of accidental monocrotophos consumption occurred in Karnataka due to the unintentional contamination of temple prasadam, resulting in the deaths of fifteen individuals and eighty birds (Gupta, 2018). Fatal poisoning due to accidental dermal absorption of monocrotophos is infrequently reported. However, there has been a documented case of fatal accidental monocrotophos poisoning in an adult female, resulting from dermal exposure while sleeping, with the pesticide detected in post-mortem blood and skin samples (Bodwal et al., 2019). Cases of deliberate self-poisoning with monocrotophos have been described, which exhibit a higher mortality rate compared to accidental exposure. In Maharashtra alone, pesticide poisoning had caused the death of 272 farmers in 4 years (2014-18). Monocrotophos, an unapproved compound reportedly responsible for the majority of these fatalities, remains included in the list of 66 pesticides utilised in India, despite being banned or subject to restricted use in other parts of the world (Mohan, 2018). The official ban on monocrotophos went into effect immediately upon publication on October 6, 2023. Although monocrotophos was banned, despite being among the mutagenic pesticides, it continued to be prevalent in the market due to limited enforcement. The ban did not prevent manufacturers from distributing the pesticide online (Das, 2024).

Swain & Mahananda (2014) investigated the impact of monocrotophos on the growth of *Limnonectis Limnocharis* through a 96-hour toxicity test to determine the LC_{50} , in conjunction with a growth experiment. The calculated LC_{50} was found to be lower than the recommended field dose; nevertheless, it resulted in a reduction in length and

body weight, as well as an extension in the duration of metamorphosis. This suggests that the application of monocrotophos, even at doses below the recommended level, can cause ecological disturbances and affect non-target organisms. A study conducted by Rao et al., (2005) examined the impact of monocrotophos on another non-target organism, subterranean termites, specifically *Odontotermes obesus*. The findings indicated that monocrotophos led to the inhibition of acetylcholinesterase activity and resulted in an 88% reduction in locomotor behavior. Similarly, monocrotophos caused an increase in the stadia period and a decrease in body weight, egg production, and longevity in *Rhynocoris longifrons* (George, 2024). Monocrotophos induced dose-dependent developmental abnormalities like pericardial edema, altered heart development, spinal and vertebral abnormalities in *Danio rerio* embryo (Pamanji et al., 2015; Raja Prabu & Murugan, 2021).

Carbamazepine is a pharmacological agent employed in the management of various neurological and psychiatric disorders, including seizure disorders, neuropathic pain syndromes, and bipolar disorder. However, its pharmacokinetic properties and side effect profile can result in significant toxicities, primarily due to its mechanism of action, which involves sodium channel blockade (Bridwell et al., 2022). In 2019, the US Poison Control Center documented 1,556 instances of carbamazepine toxicity, with 1,261 cases involving adults over the age of 20. Although fatalities due to carbamazepine are infrequent, with only three reported cases in 2019, moderate to severe symptoms are relatively common, as evidenced by 1,199 cases necessitating treatment at a healthcare facility. Carbamazepine intoxication is a relatively common occurrence and constitutes a significant proportion of poisoning cases among users of anticonvulsant medications (Nasif et al., 2022). In the absence of a specific antidote for carbamazepine overdose, management strategies are confined to gastric decontamination and supportive therapy. Given its high protein binding, the efficacy of extracorporeal elimination in cases of carbamazepine intoxication remains uncertain. Wang et al., (2023) conducted a review of data on 67 patients, which included 4 fatalities due to carbamazepine poisoning. Based on the summary of cases reported in the included articles, the cure rate of CBZ poisoning following symptomatic treatment was 82%, while the efficacy of hemoperfusion was 58.2%.

Carbamazepine intoxication associated with suicide attempts is a relatively prevalent clinical issue that may lead to coma, respiratory depression, arrhythmia, hemodynamic instability, and mortality (Yaylaci et al., 2012).

Jarvis et al., (2014) observed that carbamazepine can influence the development and behaviour, growth, and development of *Stenonema nymphs*. The study also noted alterations in the body dimensions of adult individuals. Chronic exposure to carbamazepine in *Ceriodaphnia dubia* resulted in a significant reduction in fecundity and neonate dry weight (Lamichhane et al., 2013). Additionally, it caused a notable delay in the median time to first brood, indicating slower maturation. Oetken et al. (2005) observed a concentration-dependent decrease in the emergence rate of *Chironomus riparius* in response to carbamazepine exposure. Oropesa et al., (2016) investigated the endocrine activity of carbamazepine using *Daphnia magna* as a model organism. Their study revealed that carbamazepine functions as an endocrine disruptor, as evidenced by its reduction of reproductive output, interference with sex determination, and induction of developmental abnormalities in offspring. Another study by Qiang et al., (2016) demonstrated that carbamazepine can induce accelerated embryonic development and behavioural changes in zebrafish. Similarly, in the marine copepod *Tigriopus fulvus*, carbamazepine increased the mean egg hatching time (Prato et al., 2023).

1.3 MATERIALS AND METHODS

1.3.1 Culture of Experimental Organism: *Chrysomya megacephala*

Chrysomya megacephala was chosen as the non-target organism for studies on acute toxicity and life cycle alteration. This species is a holometabolous dipteran with significant medical and forensic importance. Its life cycle comprises four distinct stages: egg, larva, pupa, and adult. Upon hatching, the larvae exhibit rapid growth in warm conditions, often completing development through three instars within a few days. The duration of these stages is dependent on temperature and humidity levels. The life cycle of this species, particularly its development time at varying temperatures, has been extensively documented due to its forensic significance.

Taxonomic position of *C. megacephala* is as follows:

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Family	:	Calliphoridae
Genus	:	<i>Chrysomya</i>
Species	:	<i>megacephala</i> (Fabricius, 1794)

The culture of the test organism, *Chrysomya megacephala* (strain DI212), was maintained in our laboratory, and primary identification of the species was performed using taxonomic keys (Old World Screw-Worm Fly: A Diagnostic Manual, 2017; Falk, 2016; Szpila, 2012; Whitworth, 2006, 2010), which was then confirmed by CO1 barcoding (Accession number: PQ136459).

1.3.2 Test chemicals

Two test chemicals from entirely different classes, monocrotophos (insecticide) and carbamazepine (pharmaceutical compound), were selected based on a comprehensive literature review. Toxicity studies were performed using analytical standards of Monocrotophos (Batch number: BCCC6539, CAS Number: 6923-22-4, Purity: 100%) and Carbamazepine (Lot number: MKCK3782, CAS Number: 298-46-4) purchased from Sigma-Aldrich.

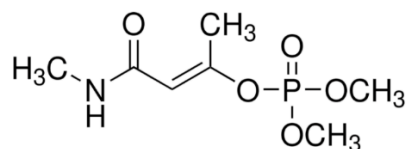
Monocrotophos

Monocrotophos, chemically known as dimethyl [(E)-4-(methyldamino)-4-oxobut-2-en-2-yl] phosphate, is a synthetic organophosphate that functions as an inhibitor of acetylcholinesterase and monoamine oxidase, and is utilized as a pesticide. It is a highly toxic, colorless or reddish-brown crystalline solid with a mild ester odor. Exposure to monocrotophos can occur via inhalation, ingestion, or dermal contact.

Chemical formula: $C_7H_{14}NO_5P$

Molecular weight: 223.16 g/mol

Chemical structure:



Carbamazepine

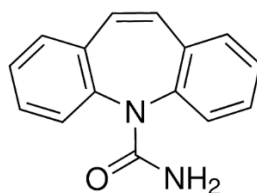
Carbamazepine is a dibenzazepine (5H-dibenzo[b,f]azepine) carrying a carbamoyl substituent at the azepine nitrogen, used as an anticonvulsant. It has a role as an anticonvulsant, an EC 3.5.1.98 (histone deacetylase) inhibitor, a mitogen, a glutamate transporter activator, an antimanic drug, an analgesic, a non-narcotic analgesic, an environmental contaminant, a xenobiotic, a drug allergen and a sodium channel blocker.

Carbamazepine is a dibenzazepine (5H-dibenzo[b,f]azepine), characterized by a carbamoyl group attached to the azepine nitrogen, and is used as an anticonvulsant. It functions as an anticonvulsant, EC 3.5.1.98 (histone deacetylase) inhibitor, mitogen, glutamate transporter activator, antimanic agent, analgesic, non-narcotic analgesic, environmental contaminant, xenobiotic, drug allergen, and sodium channel blocker.

Chemical formula: $C_{15}H_{12}N_2O$

Molecular weight: 236.27 g/mol

Chemical structure:



1.3.3 Range finding test and Definitive test

Separate range-finding tests were conducted for monocrotophos and carbamazepine. Thirty viable eggs were allocated to each experimental concentration, including the control, with the entire set replicated six times. The purpose of the range-finding test was to determine the highest concentration that resulted in 0% pupation inhibition and the lowest concentration that resulted in 100% pupation inhibition. Two effective concentrations ($50\% = a$ and $100\% = ar^2$) were identified from the range-finding test, and the geometric concentrations were calculated using formulas a/r^2 , a/r , a , ar , and ar^2 .

Five experimental concentrations were derived for both monocrotophos and carbamazepine for the definitive test. The concentrations determined for monocrotophos were 0.05, 0.1, 0.25, 0.559, and 1.25 $\mu\text{g/ml}$, whereas those for carbamazepine were 0.1, 0.22, 0.5, 1.118, and 2.5 mg/ml . Pupation inhibition was monitored and the 50% pupation inhibition (EC_{50}) was calculated using the Litchfield and Wilcoxon (1949) method as well as by probit analysis.

To ensure statistical rigor, 30 viable eggs were utilized across six independent replicates ($N=180$ per treatment group). This configuration satisfies prospective power requirements ($\text{power}=0.8$, $\alpha=0.05$), maximizing the sensitivity of downstream probit analyses and Dunnett's t-tests to precisely detect dose-dependent chemical impacts while strictly minimizing false positives.

1.3.4 Dose-dependent effects of monocrotophos and carbamazepine in the lifecycle parameters of *Chrysomya megacephala*

The effects of sublethal doses of both chemicals were assessed based on pupation time, pupal weight, and emergence time. Two concentration levels of monocrotophos, specifically $1/5^{\text{th}}$ of EC_{50} at 0.0464 $\mu\text{g/ml}$ and $1/3^{\text{rd}}$ of EC_{50} at 0.077 $\mu\text{g/ml}$, as well as carbamazepine, at $1/5^{\text{th}}$ of EC_{50} at 0.12 mg/ml and $1/3^{\text{rd}}$ of EC_{50} at 0.2 mg/ml , were selected. These concentrations are significantly lower than the calculated acute EC_{50} values. All life cycle parameter tests were conducted using these concentrations.

1.3.4a Hatching time

A single egg batch was extracted immediately after egg laying, and thirty eggs each from this batch were transferred to feed containing experimental concentrations of monocrotophos (0.0464 and 0.077 µg/ml) and carbamazepine (0.12 and 0.2 mg/ml), along with a control group. Six replicates of each experimental and control group were used in the study. The time taken for the eggs to hatch was recorded, and the variation in time between control and treatment groups were assessed. Statistical validation was done using the Dunnett's t-test.

1.3.4b Pupation time

Thirty eggs were transferred to feed containing experimental concentrations of monocrotophos (0.0464 and 0.077 µg/ml) and carbamazepine (0.12 and 0.2 mg/ml), along with a control group. Six replicates of each experimental and control group were used in the study. The number of pupae formed was recorded periodically and the differences between the control and treated groups were assessed. Dunnett's t-test was done for the comparison of control with the two different experimental concentrations of monocrotophos and carbamazepine.

1.3.4c Pupal weight

Thirty pupae from each experimental and control group were weighed on the second day of pupation. Six replicates of each experimental and control group were used in this experiment. An electronic balance (SHIMADZU, AP-224) with a sensitivity of 0.1 mg was used for weighing, and the weights were expressed in milligrams per 30 pupae. Statistical validation was done using Dunnett's t-test.

1.3.4d Emergence time

Thirty eggs were transferred to media containing experimental concentrations of monocrotophos (0.0464 and 0.077 µg/ml) and carbamazepine (0.12 and 0.2 mg/ml), along with a control group. Six replicates of each experimental and control group were

used in this study. The number of adults that emerged was recorded periodically and the differences between the control and treated groups were evaluated. Dunnett's t-test was done for the comparison of control with the two different experimental concentrations of monocrotophos and carbamazepine.

1.3.5 Embryotoxicity

The effects of sublethal concentration ($1/3^{\text{rd}}$ of EC_{50}) of monocrotophos and carbamazepine on the embryonic development of *Chrysomya megacephala* was analysed. Eggs of a single batch were collected within minutes of completion of egg-laying, divided into batches, and immediately transferred to the control and treatment feed sets. In order to image every stage of embryonic development, 10 eggs were periodically and randomly collected from each sample separately. The collected eggs were washed and treated with 0.3% sodium hypochlorite solution for a brief period. This process helps to remove the opaque chorion coating on the egg surface. Thus, the live embryos with the transparent vitelline coat were viewed under Zeiss Axio Scope A1 microscope and subsequently imaged using AxioCam 305.

1.3.6 Differential expression of genes involved in the development of *Chrysomya megacephala*

The effect of sublethal concentration ($1/3^{\text{rd}}$ of EC_{50}) of monocrotophos and carbamazepine on the expression of genes involved in embryonic development, pupation, and eclosion (emergence) was analyzed using *de novo* transcriptomics of the third instar larvae of *Chrysomya megacephala*. Sublethal concentration of $1/3^{\text{rd}}$ of EC_{50} of monocrotophos (0.077 $\mu\text{g/ml}$) and carbamazepine (0.2 mg/ml) with an exposure time of 96 hours (since the egg stage) was used for transcriptome analysis. The control and treatment sets had two independent replicates.

The DGE analysis is done on the following combinations:

- (a) Control vs Monocrotophos
- (b) Control vs Carbamazepine

Reads of each sample were mapped separately to CDS sequences obtained from the master assembly using BWA, and PCR duplicate reads were removed from the alignment file using Picard's MarkDuplicates.jar. Then, the reads mapping to each CDS were calculated for each sample using the idxstats program of Samtools. Finally, these mapped reads mentioned above were considered for differential gene expression (DGE) analysis. The read-mapped count from each sample was given, and finally, these mapped reads in the combination mentioned above were considered for differential gene expression (DGE) analysis. The read-mapped count from each sample was given as input for differential expression analysis using the edgeR Bioconductor package in R. For a comprehensive description of the procedures involved in transcriptomic analysis, please refer to the methodology section in Chapter 3.

1.4 RESULTS

1.4.1 Culture standardisation

Test organism

An initial survey was conducted on the Calicut University campus to identify the prevalent species of Calliphoridae in the area. *Chrysomya megacephala* was selected because of its adaptability and survival capability across a broad temperature range. This species is among the most abundant Calliphoridae in Kerala. Its life cycle involves complete metamorphosis, comprising egg, larval, pupal, and adult stages. Eggs were white, elongated, and laid in multiple batches. Hatching occurs within 8-12 hours, and the larval stage lasts approximately 3-5 days, contingent on the atmospheric temperature. The first and second instars are voracious feeders, whereas during the third instar stage, the larvae cease feeding and exhibit limited movement as they transition to the prepupal stage. Pupation results in the formation of a coarctate pupa, characterised by dark brown to black coloration. Emergence occurs within 2-5 days, depending on temperature and other external factors. Upon emergence, the adults were initially black with a metallic sheen developing a few hours later. Honey and water were provided for sustenance, while meat served as a protein source for egg maturation in adult females. Figure 1.1 illustrates the life cycle of *Chrysomya*

megacephala, and Figure 1.2 depicts the adult female (♀) and male (♂). In females, the eyes are spaced far apart. In contrast, males have eyes that meet at the front and top of the head, with the upper eye facets being noticeably larger than the lower ones.

Fresh chicken was used as an attractant to lure female flies to feed and oviposit. Newly laid eggs, along with females, were collected for rearing. Females were identified using taxonomic keys. The initial rearing experiment was successful, and from the emerged adults, a single female was introduced into a new cage to establish and maintain a culture of *Chrysomya megacephala*.

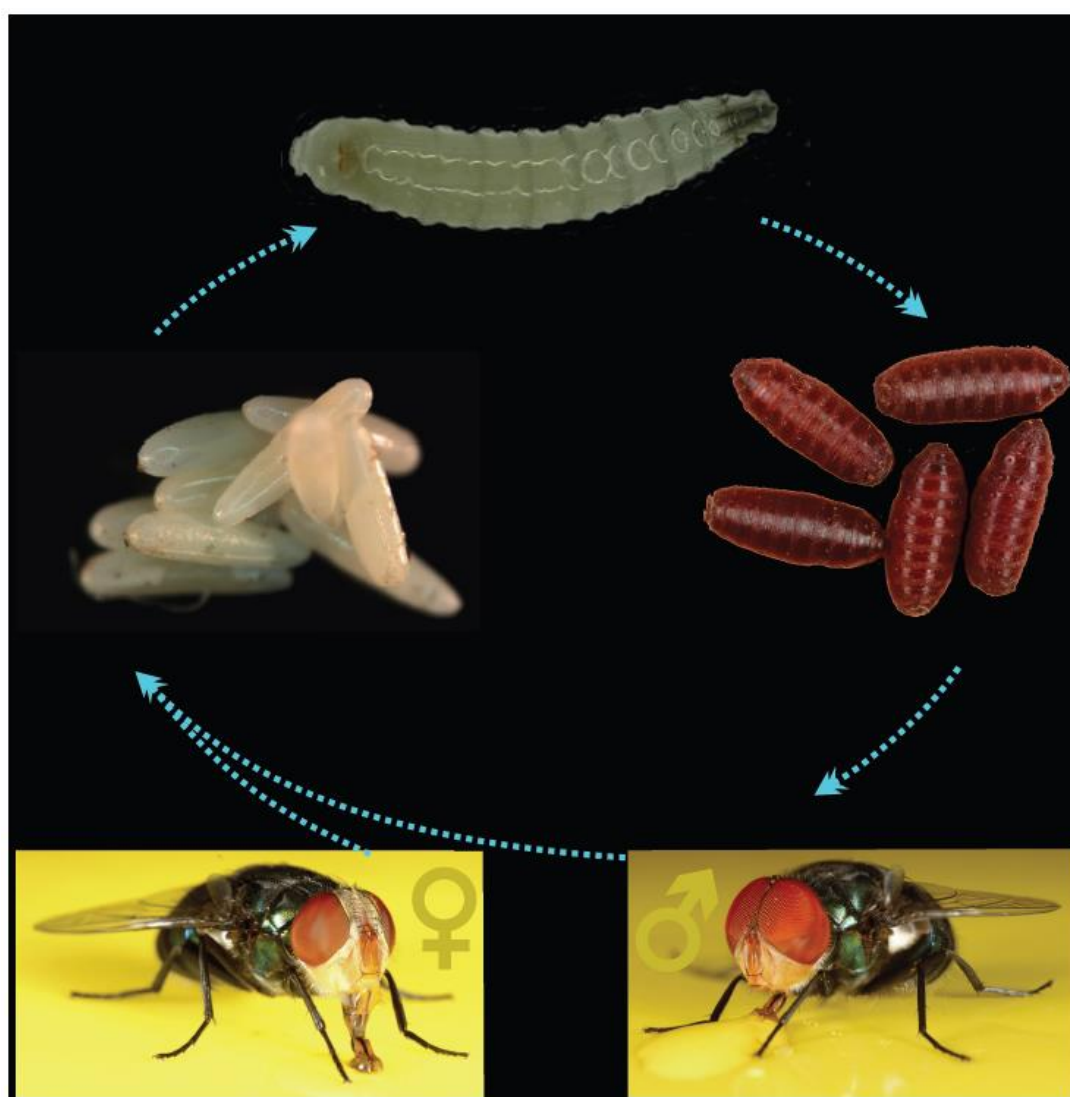


Fig 1.1: Life cycle of *Chrysomya megacephala*

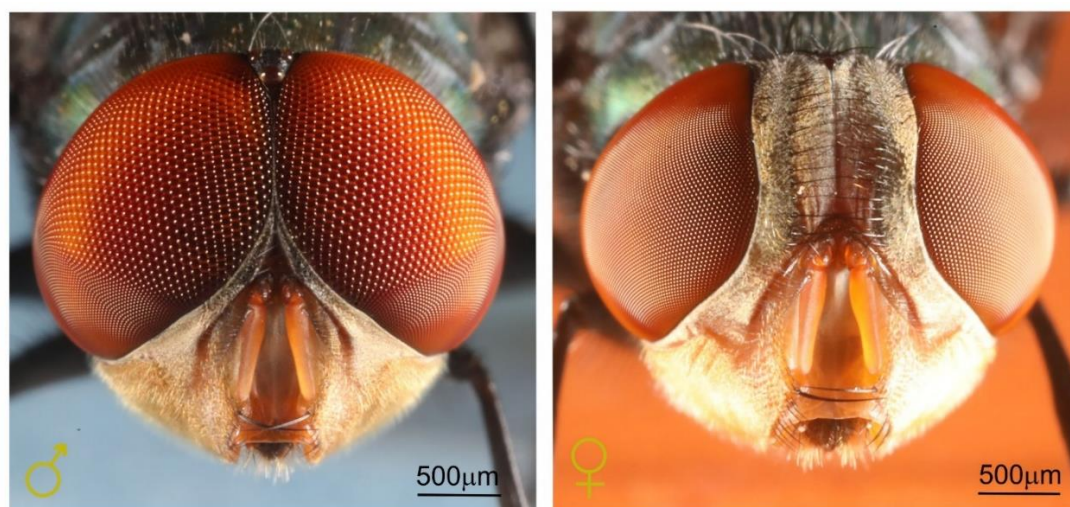


Fig 1.2: *Chrysomya megacephala* adult Female (♀) and Male (♂)

Feed standardization

A series of artificial feeds were evaluated to standardise the feed for culture maintenance and experimental set-up.

- a. Dried-Blood feed:** Fresh goat blood (200 ml) was collected from a slaughterhouse in two bottles (100 ml each) with two different anticoagulants, sodium citrate and potassium oxalate. The contents of each bottle were split into two equal parts, and one part from each bottle was subjected to sonication for 5 minutes in 5-second interval pulses. Both the sonicated and non-sonicated samples were subsequently frozen at -50°C using a lyophilizer. Following this process, the samples were obtained as a fine powder, intended to serve as the primary component of the artificial feed. However, the yield was significantly lower than that anticipated. Additionally, the lyophilisation process required approximately 7 days (11-12 hours per day) to complete, which was deemed excessively time-consuming. Owing to these issues, the dried blood meal was ultimately discarded. Figure 1.3 illustrates a flow chart of the steps involved in preparing the dried blood feed.

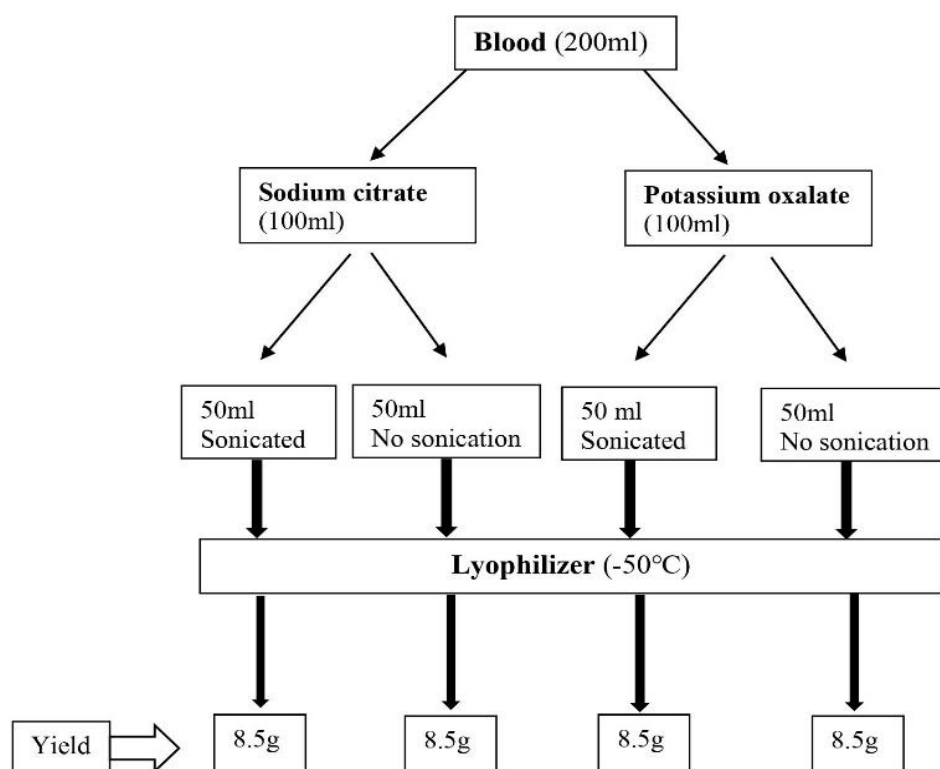


Fig 1.3: Dried blood feed

b. Cooked-blood feed:

Semolina	-	100g
Agar	-	20g
Propionic acid	-	3ml
Yeast	-	25g in 100ml sugar solution (25g sugar/100ml)
Goat blood	-	675ml + anticoagulant
Water	-	225ml

Pros

- No foul smell
- Can be stored in the fridge for later usage
- Inexpensive protein source

Cons

- High rate of larval death
- Larval development duration high
- Pupation unsuccessful

c. Cooked liver feed:

Semolina	-	100g
Agar	-	20g
Propionic acid	-	3ml
Yeast	-	25g in 100ml sugar solution (25g sugar/100ml)
Goat liver(ground)	-	675g
Water	-	225ml

Pros

- No foul smell
- Can be stored in the fridge for later usage

Cons

- High rate of larval death
- Larval development duration is high
- Pupation unsuccessful

d. Milk powder-based feed

Semolina	-	50g
Agar	-	10 g

Propionic acid	-	1.5 ml
Milk powder	-	50g
Water	-	700ml

Pros

- No foul smell
- Can be stored in the fridge for later usage
- Development completed and adults emerged successfully

Cons

- Extreme developmental delay
- Larval instars showed reduced size and mass
- Showed a delay of 7-10 days for emergence

e. Milk-based feed

Semolina	-	50g
Agar	-	10 g
Propionic acid	-	1.5 ml
Milk	-	100ml
Water	-	600ml

Pros

- No foul smell
- Can be stored in the fridge for later usage
- Development completed and adults emerged successfully
- Developmental delay was less than 5 days (for emergence)

Cons

- Developmental delay was observed

f. Milk and egg-based feed

Semolina	-	50g
Agar	-	10 g
Propionic acid	-	1.5 ml
Milk	-	100ml
Chicken egg	-	50g (1 no)
Water	-	600ml

Pros

- No foul smell
- Can be stored in the fridge for later usage
- Improved larval size and mass compared to the feed (e)
- Development completed and adults emerged successfully
- Developmental delay was less than 5 days (for emergence)

Cons

- Developmental delay was observed

Among the various feeds evaluated, both of the milk-based diets demonstrated promising outcomes. However, the observed delay in the lifecycle renders them suboptimal for experimental purposes. Nonetheless, these feeds are suitable for culture maintenance and mass rearing in maggot therapy. Consequently, the experimental setup employs a feed composed of ground chicken meat and semolina. The inclusion of semolina serves to maintain the feed's texture as the meat undergoes

putrefaction, thereby ensuring the drug concentration in the test medium remains unaffected by preserving a uniform texture. This approach offers an advantage over using whole animals as feed, which necessitates determining the dose across different body parts to assess the effects of varying doses accurately. In contrast, utilising an artificial feed comprising ground meat and a stabiliser such as semolina circumvents these issues and facilitates a normal rate of development due to the use of a natural protein source. Figure 1.4 presents a simple graphical depiction of the culture set up. Upon hatching directly atop the feed, the larvae remain there, consuming the feed until they reach the non-feeding migratory third-instar stage. At this point, they transition to the provided sawdust to initiate pupation. Following emergence, the flies are transferred to a fly cage.



Figure 1.4: *Chrysomya megacephala* culture set-up during egg-pupal stages

1.4.2 Selection of Test Chemicals

Two test chemicals from distinct categories, namely an insecticide (Monocrotophos) and a pharmaceutical compound (carbamazepine), were selected based on a comprehensive literature review. The objective was to identify commonly misused chemicals for homicidal purposes from credible sources. Monocrotophos is a highly toxic organophosphate that, despite being legally prohibited, is extensively utilised in India, where it is associated with a significant number of both accidental and intentional overdoses. In contrast, carbamazepine is a mood-stabilising medication employed in the management of various chronic conditions, including psychiatric disorders such as depression, mania, bipolar disorder, dyscontrol syndrome, schizophrenia, and alcohol withdrawal syndrome (Birkhimer et al., 1985).

1.4.3 Determination of median effective concentration (EC₅₀) of Monocrotophos

Six distinct range-finding tests were performed for monocrotophos, with each assay concentration comprising six replicates and 30 specimens per replicate. The results of these assays demonstrated variable pupation inhibition percentages, ranging from 0.1 to 1.0 µg/ml.

The determined concentrations for the median effective concentration (EC₅₀) of monocrotophos were 0.05 µg/ml, 0.1 µg/ml, 0.25 µg/ml, 0.559 µg/ml, and 1.25 µg/ml. The mean pupation inhibition percentages at selected concentrations were 8.88%, 41.11%, 47.77%, 76.66%, and 88.88%, respectively.

The data were represented on a double logarithmic graph, and a 95% confidence interval was derived using the interpolation method of Litchfield and Wilcoxon (1949). The percentages of pupation inhibition at various concentrations indicated that the precise EC₅₀ concentration was 0.23 µg/ml (Table 1.1).

Table 1.1: Toxicity of monocrotophos

Effective concentrations (EC) ($\mu\text{g/ml}$)			95% confidence limits of EC₅₀	Slope	95% confidence limits of slope
EC ₁₆	EC₅₀	EC ₈₄			
0.1	0.23	0.64	0.1-0.48	2.541	1.320-4.884

1.4.4 Dose-dependent effects of monocrotophos on the lifecycle parameters of *Chrysomya megacephala*

Chrysomya megacephala, exposed to the sub lethal concentrations ($1/5^{\text{th}}$ of EC₅₀- 0.0464 $\mu\text{g/ml}$ and $1/3^{\text{rd}}$ of EC₅₀- 0.077 $\mu\text{g/ml}$) of monocrotophos showed dose-dependent effects. The larvae subjected to the toxicant demonstrated an overall reduction in size in the second- and third-instar stages, compared to the control set. Eggs were allowed to develop, hatch and grow in the intoxicated feed to evaluate their effects on life cycle parameters, like hatching time, pupation time, pupal weight and emergence time.

1.4.4a Hatching time

Sublethal concentrations of $1/5^{\text{th}}$ and $1/3^{\text{rd}}$ of EC₅₀ were uniformly mixed in the feed, and healthy eggs were transferred to intoxicated media. The time taken by the eggs to hatch was recorded and the results showed a dose-dependent variation in hatching time. The treatments exhibited a decreased hatching time with the highest sublethal treatment showing a statistically significant reduction in the time taken for hatching/embryonic development. The hatching time of the control and treatment groups were compared and presented in Table 1.2 and Figure 1.5.

Table 1.2: Effect of monocrotophos on hatching time of *Chrysomya megacephala* eggs

Experiment	Hatching time (in minutes) (Mean $\pm$ Std Error)
Control	774.16$\pm$61
$1/5^{\text{th}}$ of EC ₅₀ (0.0464 $\mu\text{g/ml}$)	660.33$\pm$50.15
$1/3^{\text{rd}}$ of EC ₅₀ (0.077 $\mu\text{g/ml}$)	535.83$\pm$33.87

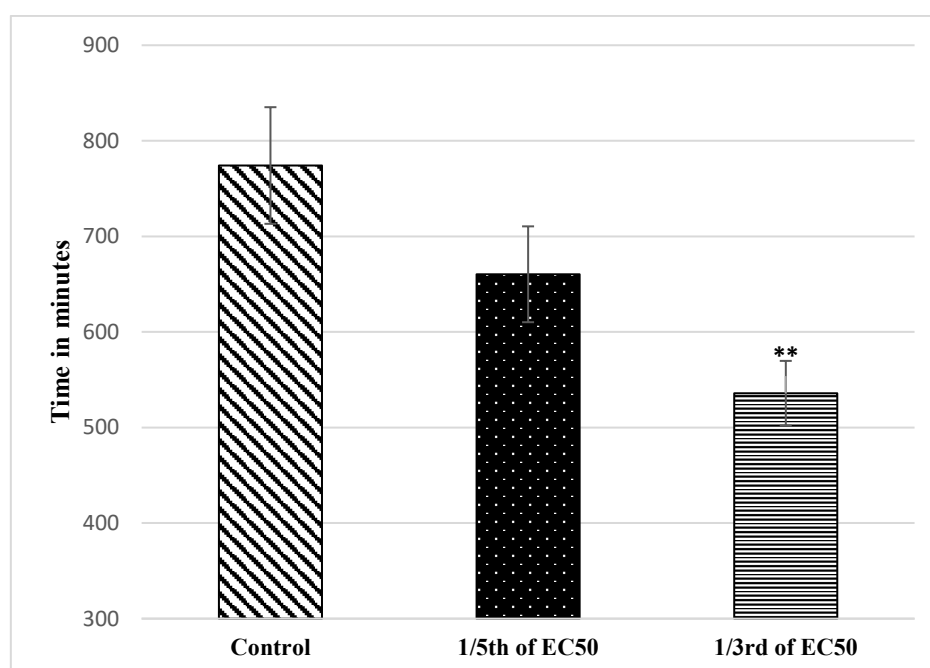


Fig 1.5: Effect of monocrotophos on hatching time of *Chrysomya megacephala* eggs (** $p < 0.01$, *** $p < 0.001$)

1.4.4b Pupation Time

The toxic effect of sub-lethal concentrations of monocrotophos on pupal formation is estimated by evaluating the pupation time. Pupation was commenced in all three sets (control and test groups) from the 90th hour post-egg laying. The average number of third-instar larvae that pupated at each time interval in the control and treatment groups was determined from six sets of replicas. Monocrotophos induced a dose-dependent acceleration in development rate, causing early pupation in *Chrysomya megacephala*. Larvae exposed to the lower sublethal concentration, 1/5th of EC₅₀, exhibited statistically significant acceleration in pupation rate in four out of nine-time intervals, whereas the larvae developing at 1/3rd of EC₅₀ showed statistically highly significant accelerated pupation in all intervals, but two (Table 1.3 and Figure 1.6).

Table 1.3: Effect of monocrotophos in pupation time of *Chrysomya megacephala*

Sl. No	Experiment	90 hours (mean $\pm$ Std Error)	96 hours (mean $\pm$ Std Error)	102 hours (mean $\pm$ Std Error)	108 hours (mean $\pm$ Std Error)	114 hours (mean $\pm$ Std Error)	120 hours (mean $\pm$ Std Error)	126 hours (mean $\pm$ Std Error)	132 hours (mean $\pm$ Std Error)	138 hours (mean $\pm$ Std Error)
1	Control	0.66 $\pm$ 0.33	1.33 $\pm$ 0.42	2.33 $\pm$ 0.21	5.33 $\pm$ 0.49	9.33 $\pm$ 0.42	15.33 $\pm$ 1.54	22 $\pm$ 1.39	24.33 $\pm$ 0.76	28.83 $\pm$ 0.3
2	1/5 th of EC ₅₀ (0.0464 μ g/ml)	1.66 $\pm$ 0.49	2.66 $\pm$ 0.66	5.33 $\pm$ 0.55	9 $\pm$ 0.85	12.5 $\pm$ 0.42	17.83 $\pm$ 1.1	21.66 $\pm$ 0.71	25.5 $\pm$ 1.05	25.66 $\pm$ 1.14
3	1/3 rd of EC ₅₀ (0.077 μ g/ml)	5.16 $\pm$ 0.47	6.83 $\pm$ 0.47	10.33 $\pm$ 0.49	12.83 $\pm$ 0.54	14.66 $\pm$ 0.55	17 $\pm$ 0.51	19.66 $\pm$ 0.42	20.16 $\pm$ 0.70	20.66 $\pm$ 0.55

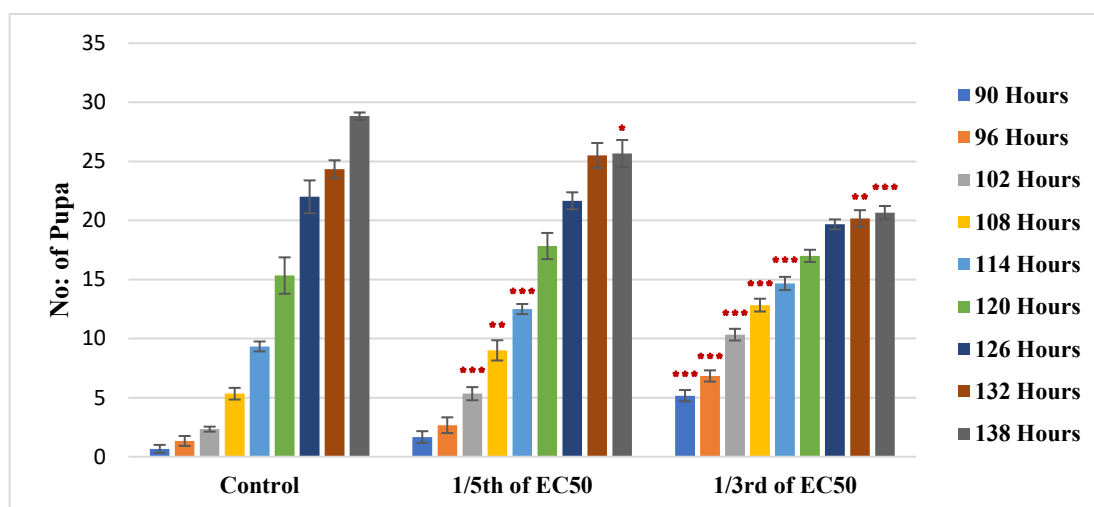


Fig 1.6: Effect of monocrotophos on pupation time of *Chrysomya megacephala* (** $p < 0.01$, *** $p < 0.001$)

1.4.4c Pupal weight

The pupal weight showed a dose-dependent reduction due to the exposure to monocrotophos. The pupal size was also smaller in the treatment sets. Table 1.4 and Figure 1.7 show the effect of monocrotophos on the pupal weight of *Chrysomya megacephala*. The highest sub-lethal concentration, 1/3rd of the EC₅₀, exhibited a statistically significant reduction in pupal weight.

Table 1.4: Effect of monocrotophos in pupal weight of *Chrysomya megacephala*

Experiment	Pupal Weight (mg) (Mean $\pm$ Std Error)
Control	65.56 $\pm$ 1.11
1/5 th of EC ₅₀ (0.0464 μ g/ml)	61.53 $\pm$ 1.95
1/3 rd of EC ₅₀ (0.077 μ g/ml)	58.28 $\pm$ 0.71

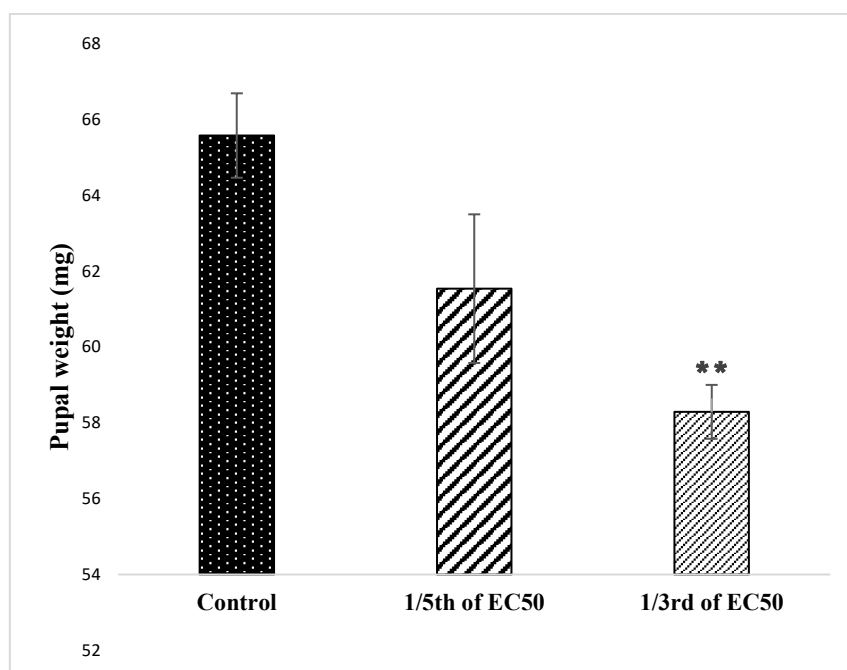


Fig 1.7: Effect of monocrotophos in pupal weight of *Chrysomya megacephala* (** $p < 0.01$, *** $p < 0.001$)

1.4.4d Emergence time

The effect of sub-lethal levels of monocrotophos on the overall development of *Chrysomya megacephala* was determined by analysing the emergence rate. The emergence of adults from their pupal stage began in control and treatments at 216 hours after egg laying. The highest sub-lethal concentration, 1/3rd of EC₅₀, caused a statistically highly significant accelerated emergence rate in three out of four time intervals chosen for observation (Table 1.5 and Figure 1.8).

Table 1.5: Effect of monocrotophos on emergence time of *Chrysomya megacephala*

Sl. No	Experiment	216 hours (mean ± Std Error)	222 hours (mean ± Std Error)	228 hours (mean ± Std Error)	234 hours (mean ± Std Error)
1	Control	2.16±0.3	4.83±0.9	19.16±3.04	27.66±0.61
2	1/5 th of EC ₅₀ (0.0464 µg/ml)	3.66±0.45	7.16±0.7	23.33±1.17	25.66±1.14
3	1/3 rd of EC ₅₀ (0.077 µg/ml)	6.16±0.79	15±1.29	19.66±0.55	20.66±0.55

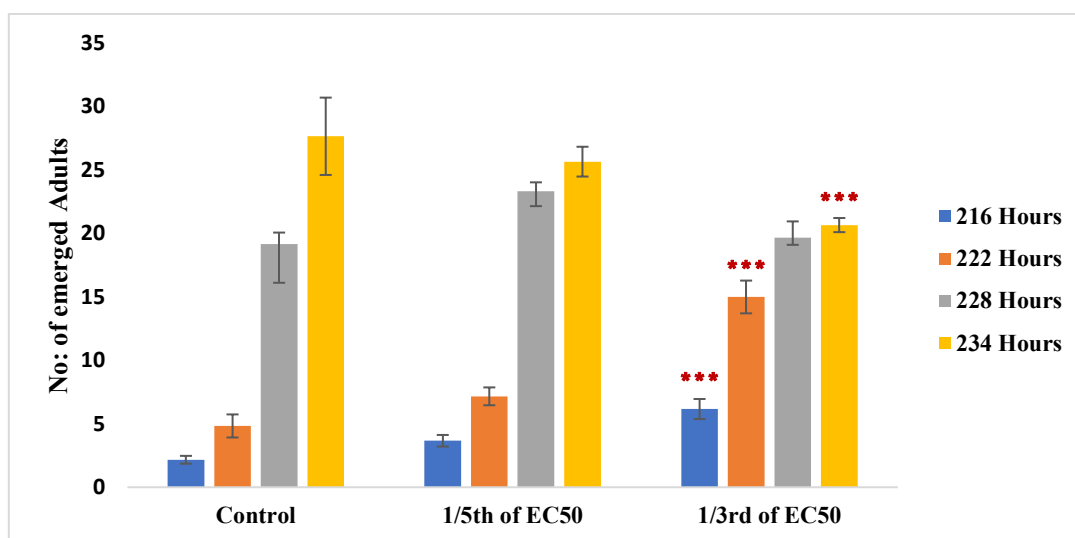


Fig 1.8: Effect of monocrotophos on emergence time of *Chrysomya megacephala* (** $p < 0.01$, *** $p < 0.001$)

1.4.5 Determination of median effective concentration (EC₅₀) of Carbamazepine

Six distinct range-finding tests were performed for carbamazepine, with each assay concentration comprising six replicates and 30 specimens per replicate. The results of these assays demonstrated variable pupation inhibition percentages, ranging from 0.2 to 1.0 mg/ml.

The determined concentrations for the median effective concentration (EC₅₀) of monocrotophos were 0.1 mg/ml, 0.22 mg/ml, 0.5 mg/ml, 1.118 mg/ml, and 2.5 mg/ml. The mean pupation inhibition percentages at selected concentrations were 5%, 25%, 54.55%, 76.11%, and 78.88%, respectively.

The data were represented on a double logarithmic graph and a 95% confidence interval was derived using the interpolation method of Litchfield and Wilcoxon (1949). The percentages of pupation inhibition at various concentrations indicated that the precise EC₅₀ concentration was 0.6 mg/ml (Table 1.6).

Table 1.6: Toxicity of Carbamazepine

Effective concentrations (EC) (mg/ml)			95% confidence limits of EC ₅₀	Slope	95% confidence limits of slope
EC ₁₆	EC ₅₀	EC ₈₄			
0.16	0.6	1.025	0.245-1.465	2.729	1.320-4.884

1.4.6 Dose-dependent effects of carbamazepine in the lifecycle parameters of *Chrysomya megacephala*

Chrysomya megacephala, exposed to the sub-lethal concentrations ($1/5^{\text{th}}$ of EC_{50} - 0.12 mg/ml and $1/3^{\text{rd}}$ of EC_{50} - 0.2 mg/ml) of carbamazepine showed dose-dependent effects. The larvae subjected to the toxicant demonstrated a slight overall reduction in size in the second- and third-instar stages, compared to the control set. Eggs were allowed to develop, hatch and grow in the intoxicated feed to evaluate their effects on life cycle parameters, like hatching time, pupation time, pupal weight and emergence time.

1.4.6a Hatching time

Sublethal concentrations of $1/5^{\text{th}}$ and $1/3^{\text{rd}}$ of EC_{50} were uniformly mixed in the feed and healthy eggs were transferred to intoxicated media. The time taken by the eggs to hatch was recorded and the results showed no variation in hatching time. Eggs were placed in the control, and both the treatment sets initiated and completed hatching at the same time.

1.4.6b Pupation Time

The toxic effect of sub-lethal concentrations of carbamazepine in pupal formation is estimated by evaluating the pupation time. Pupation was commenced in all three sets (control and test groups) from the 120th hour post-egg laying. The average number of third-instar larvae that pupated at each time interval in the control and treatment groups was determined from six sets of replicas. Carbamazepine induced a dose-dependent deceleration in development rate, causing delayed pupation in *Chrysomya megacephala*. In the control set, pupation was completed at 138 hours. In contrast, the larvae in the $1/5^{\text{th}}$ of EC_{50} set completed pupation by 150 hours, and the larvae in the $1/3^{\text{rd}}$ of EC_{50} required 162 hours to complete the pupation. Larvae exposed to the lower sublethal concentration, $1/5^{\text{th}}$ of EC_{50} , exhibited statistically significant deceleration in pupation rate at two out of eight time intervals, whereas the larvae developing at $1/3^{\text{rd}}$ of EC_{50} showed statistically highly significant delay in pupation in all intervals, but two (Table 1.7 and Figure 1.9).

Table 1.7: Effect of carbamazepine in pupation time of Chrysomya megacephala

Sl. No	Experiment	120 hours (mean $\pm$ Std Error)	126 hours (mean $\pm$ Std Error)	132 hours (mean $\pm$ Std Error)	138 hours (mean $\pm$ Std Error)	144 hours (mean $\pm$ Std Error)	150 hours (mean $\pm$ Std Error)	156 hours (mean $\pm$ Std Error)	162 hours (mean $\pm$ Std Error)
1	Control	15.33 $\pm$ 1.54	22 $\pm$ 1.39	27.16 $\pm$ 0.70	28.83 $\pm$ 0.30	28.83 $\pm$ 0.30	28.83 $\pm$ 0.30	28.83 $\pm$ 0.30	28.83 $\pm$ 0.30
2	1/5th of EC₅₀ (0.12 mg/ml)	12.5 $\pm$ 1.25	17.33 $\pm$ 1.28	20.66 $\pm$ 1.96	23.16 $\pm$ 2.40	27.66 $\pm$ 0.98	28.83 $\pm$ 0.47	28.83 $\pm$ 0.47	28.83 $\pm$ 0.47
3	1/3rd of EC₅₀ (0.2 mg/ml)	1.16 $\pm$ 0.98	2.33 $\pm$ 1.47	9.166 $\pm$ 2.05	13.16 $\pm$ 1.01	19.83 $\pm$ 1.53	23.33 $\pm$ 1.74	27.33 $\pm$ 0.55	27.66 $\pm$ 0.66

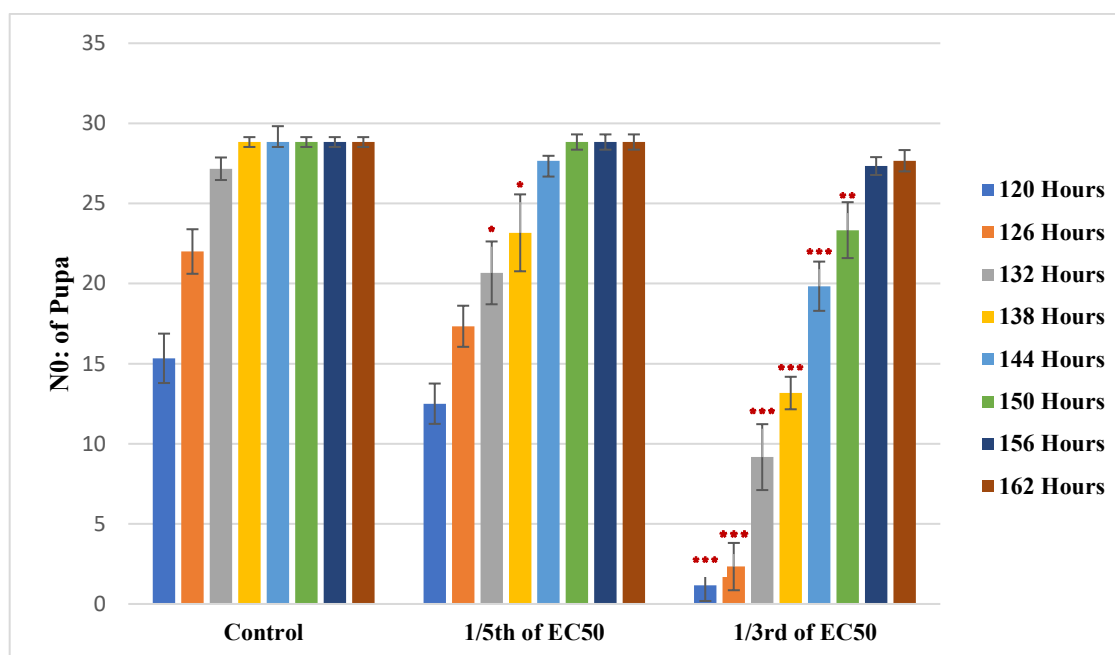


Fig 1.9: Effect of carbamazepine on pupation time of *Chrysomya megacephala* (** p < 0.01, *** p < 0.001)

1.4.6c Pupal weight

The pupal weight showed a dose-dependent reduction due to the exposure to carbamazepine. Table 1.8 and Figure 1.10 show the effect of carbamazepine in the pupal weight of *Chrysomya megacephala*. The highest sub-lethal concentration, 1/3rd of the EC₅₀, exhibited a statistically significant reduction in pupal weight.

Table 1.8: Effect of carbamazepine in pupal weight of *Chrysomya megacephala*

Experiment	Pupal Weight (mg) (Mean ± Std Error)
Control	58.38±1.25
1/5 th of EC ₅₀ (0.0464 µg/ml)	55.6±1.47
1/3 rd of EC ₅₀ (0.077 µg/ml)	50.77±1.33

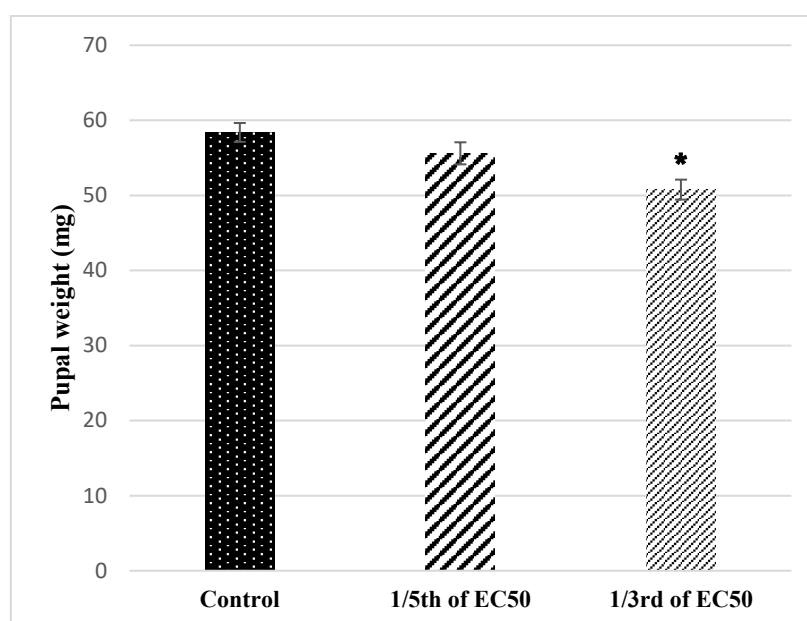


Fig 1.10: Effect of carbamazepine in pupal weight of *Chrysomya megacephala* (** $p < 0.01$, *** $p < 0.001$)

1.4.6d Emergence Time

The effect of sub-lethal levels of carbamazepine on the overall development of *Chrysomya megacephala* was determined by analysing the emergence time. The emergence of adults from their pupal stage began in control and treatments at 222 hours after egg laying. The highest sub lethal concentration, 1/3rd of EC₅₀, caused a statistically significant delay in emergence rate in all time intervals chosen for observation (Table 1.9 and Figure 1.11).

Table 1.9: Effect of carbamazepine in emergence time of *Chrysomya megacephala*

Sl. No	Experiment	222 hours (mean ± Std Error)	228 hours (mean ± Std Error)	234 hours (mean ± Std Error)
1	Control	4.83±0.9	19.16±3.04	27.66±0.61
2	1/5 th of EC ₅₀ (0.12 mg/ml)	4.33±0.84	12.83±0.94	27.5±0.61
3	1/3 rd of EC ₅₀ (0.2 mg/ml)	1.66±0.42	10.83±1.66	24±0.73

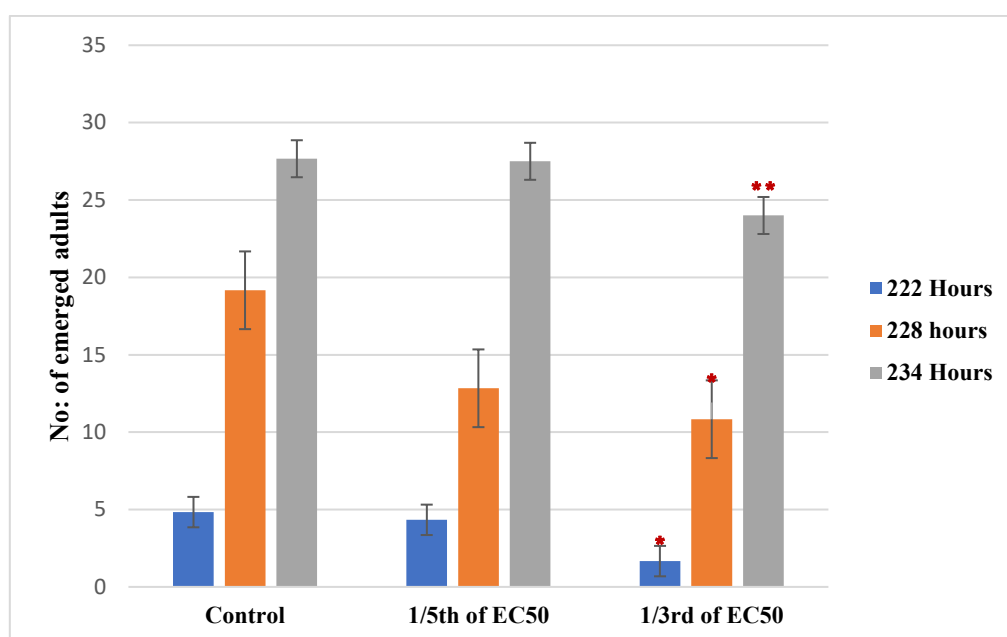


Fig 1.11: Effect of carbamazepine in emergence time of *Chrysomya megacephala* (** $p < 0.01$, *** $p < 0.001$)

1.4.7 Embryotoxicity

The study of dose-dependent effects of monocrotophos and carbamazepine revealed that the sublethal dosage of $1/3^{\text{rd}}$ of EC_{50} induced statistically significant variations in all the life cycle evaluations. Therefore, that dose was selected for the study on the effect of monocrotophos and carbamazepine in the embryonic development of *Chrysomya megacephala*. DGE analysis was performed to track the maternal mRNA involved on embryonic development using *de novo* transcriptomics. Third-instar larvae of *Chrysomya megacephala* were exposed to sublethal concentrations of $1/3^{\text{rd}}$ of EC_{50} of monocrotophos ($0.077 \mu\text{g/ml}$) and carbamazepine (0.2 mg/ml), since the egg stage with an exposure time of 96 hours was used for transcriptome analysis. Both the control and treatment sets had two independent replicates.

Visualisation of embryonic development

Visualisation of egg development is done in order to examine the effect of monocrotophos and carbamazepine on the embryo. The procedure aids in egg age estimation which is typically determined by measuring the time required for the eggs

to hatch. The visualisation method is always the easiest method for age estimation in any organism at any stage. In the Calliphorid egg, the presence of opaque chorion completely cuts off any visualisation without pre-treatment unless it reaches the IXth stage of development.

Removal of the chorion from the egg surface to expose the vitelline membrane is the major step in visualisation. Six different methods were tested for this purpose.

1. Sodium hypochlorite treatment followed by Hot Water Killing (HWK)

The eggs were washed and treated with 0.3% sodium hypochlorite solution for a few minutes, and then washed again and subjected to HWK by dipping in near-boiling water for 10 seconds. The eggs were then viewed under a Zeiss Axio Scope A1 microscope. The samples were then stored in 70% ethyl alcohol. The HWK caused severe damage to the early-stage eggs making it impossible to see the morphological landmarks of development. The latter stages which are close to hatching (after approximately 90% of development is complete), were the only ones that survived the HWK done after sodium hypochlorite treatment. The stored samples belonging to the last stages got damaged once it was taken out of the alcohol after a day.

2. HWK followed by sodium hypochlorite treatment

The eggs were washed and subjected to hot water killing (HWK) and then treated with 0.3% sodium hypochlorite solution. This method was tested to determine if the washing out of the chorion is the reason why the egg loses its structure in early stages in method 1. However, even this procedure was unsuccessful in obtaining intact morphological landmarks in the early stages. There were severe damage and the only successful stages obtained were after the completion of about 70-80% of development.

3. Sodium hypochlorite treatment followed by alcohol fixation

The eggs were washed and treated with 0.3% sodium hypochlorite solution for a few minutes and then washed again and transferred to 70% ethyl alcohol. The eggs were then viewed under a Zeiss Axio Scope A1 microscope. The sodium hypochlorite treatment helped in clearing the chorion and visualising the embryo. But most eggs in every stage of development were damaged by dehydration making it difficult to track every stage of development. Only a very small numbers of eggs were intact in most of the stages, indicating that we need a very large number of samples to make this work at every stage.

4. Alcohol fixation followed by sodium hypochlorite treatment

The result of method 4 is very similar to that of method 3. The eggs were initially fixed by placing them in 70% ethyl alcohol for 5-10 minutes and then treated with 0.3% sodium hypochlorite for a few minutes, and then transferred to 70% ethyl alcohol for storage after observing under a microscope. The issue of dehydration persisted, causing damage to most of the eggs at every stage.

5. Alcohol fixation and storage for 4-5 days

This is a method where no clearing agent was used to clear off the chorion. The eggs were washed and then directly transferred to 70% ethyl alcohol, where they were stored in the fridge to allow the alcohol to remove the chorion. The eggs were checked daily to maximise visibility of morphological landmarks. After 4-5 days, the chorion was cleared off, but the visualisation also failed as the eggs were shrunk within seconds once they were taken out of alcohol.

6. Live embryo visualisation by sodium hypochlorite treatment

This is so far the most successful method. The eggs were washed thoroughly and treated with 0.3% sodium hypochlorite solution for a few minutes. They were then visualised under a Zeiss Axio Scope A1 microscope and subsequently imaged using

Axiocam 305. In this method, the egg is not subjected to any fixation. Therefore, development continues after the treatment. It is one of the main advantages of this method, in that we can observe the development of the same egg for at least 3 to 4 hours, which means we can visualise all the stages of development even with a very low sample size. The treatment with hypochlorite solution for the removal of the chorion is a very simple visualisation method that can be used in earlier stages, and it does not require any tedious laboratory equipments. The only requirement is a light microscope. Therefore, this technique can easily be applied in the field, and the stage of development/age (and thereby the PMI) can be estimated within minutes.

The stages of embryonic development were determined based on the studies on the fruit fly *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) and on the studies on *Calliphora vicina* eggs which have both shown a predictable sequence of morphological changes occurring during embryogenesis (Campos-Ortega & Hartenstein, 1997; Martín-Vega & Hall, 2016). The key features of the major stages of development of *Chrysomya megacephala* eggs are as follows;

Stage I – Homogenous Yolk: In freshly laid eggs, the yolk is distributed uniformly throughout the egg. This homogeneous distribution of yolk granules results in the zygote filling the egg completely without leaving any empty space. The living egg in stage 1 is uniformly dark in the centre and slightly light at its periphery. This lasts for only a few minutes and soon change to a stage where there was a significant retraction of cytoplasm from the vitelline envelope, leading to the formation of an empty space in the anterior and posterior ends.

Stage II – Clear Cytoplasmic Rim: At 1 hour and 3 minutes, the egg acquires a clear cytoplasmic rim at the periphery of the embryo, which depicts Stage II of embryonic development. Now, the embryo acquires a dark centre in a relatively broad clear zone at the periphery, reflecting the presence of dividing blastoderm nuclei. During this stage, the space at the anterior pole of the embryo disappears.

Stage III – Cephalic Furrow: The next crucial developmental event is the cephalic furrow formation. This stage was observed at 2 hours and 10 minutes after egg laying. After gastrulation, endodermal and mesodermal invaginations occur, along with the shortening of lateral cells, forming dorsal folds and cephalic furrow.

Stage IV – Stomodeal Invagination: On the ventral side of the anterior pole, the embryo moves away from the vitelline envelope and forms a stomodeal cell plate from which the invagination of stomodeum (the primordia of the foregut) occurs. In this stage, the transient appearance of segmentation in the mesodermal layer is visible. Also, the germ band gets fully extended around the embryo. This stage is achieved in 3 hours and 25 minutes after egg laying.

Stage V – Clypeolabrum formation: The formation of clypeolabrum (the fused plate of the clypeus and labrum) by further invagination of stomodeum occurs at this stage. Clypeolabrum can be observed as an apparent protuberance at the anterior end. The germ band starts to shorten during this interval, along with the retraction of the posterior part of the embryo from the vitelline membrane. 3 hours and 48 minutes is the time taken post-egg laying to reach this stage of development.

Stage VI – Segmentation: By 5 hours and 4 minutes, thoracic and abdominal segmentation are completed, and the segments are visible. As the involution of the head segment begins, a dorsal fold covers the clypeolabrum. As a result, the germ band shortens on the dorsal surface, and the posterior gap between the embryo and the vitelline membrane is reduced.

Stage VII – Sack-shaped Gut: The dorsal fold completely covers the clypeolabrum making it invisible, after 6 hours and 33 minutes. Then, gut formation becomes visible as a J-shaped to a sack-shaped, yolk-filled tube.

Stage VIII – Coil-shaped Gut: As the gut formation continues, the sack-shaped tube gradually gets transformed into a coil-shaped structure. The coiling is visible from 7 hours and 18 minutes onwards. The coiling is very gradual, starting with 2-3 bud-like

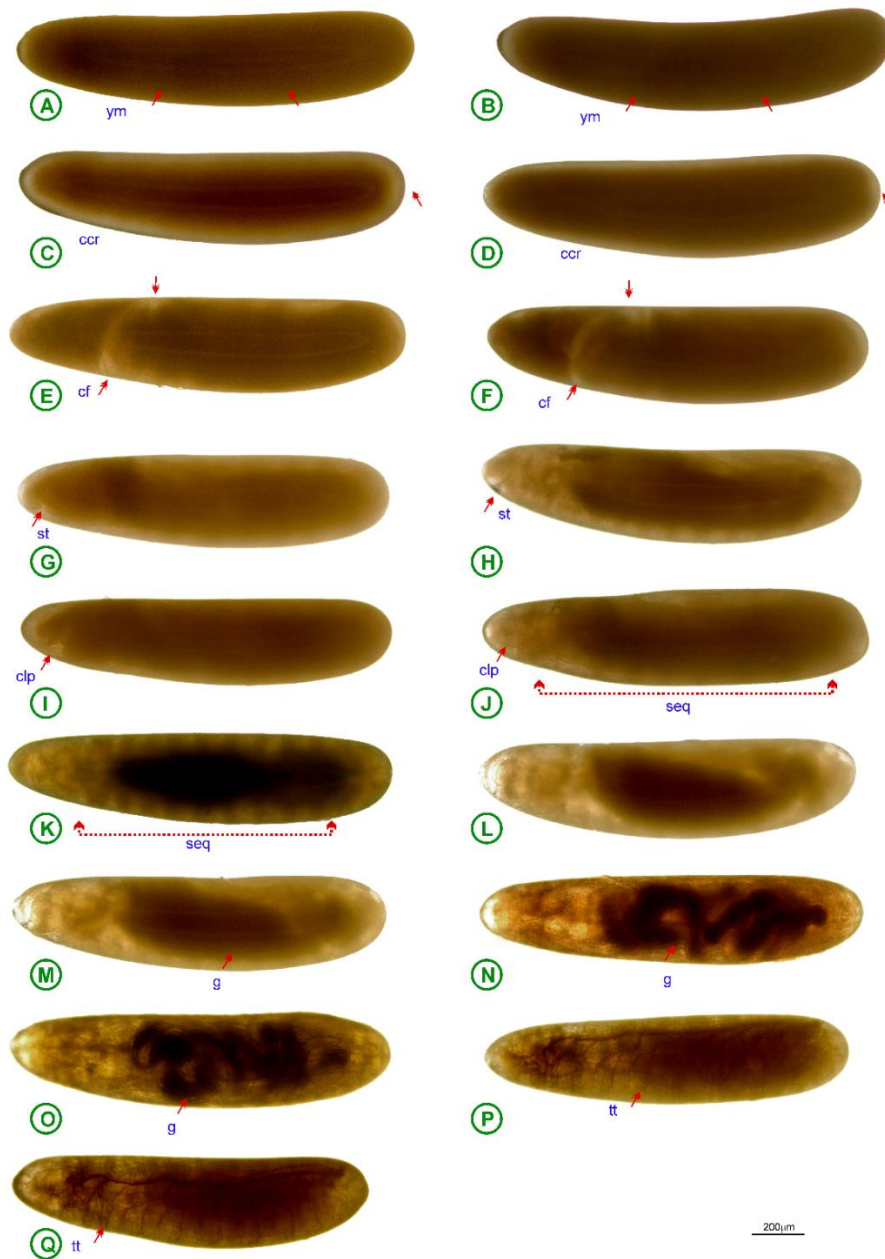
coils and ending with a heavily coiled structure. The yolk is progressively digested during this process. This coiled gut becomes thinner during further development, but it can still be observed until the end of embryonic development. The peristaltic movement of larvae in the gut region becomes visible at this stage, making imaging difficult from here onwards.

Stage IX – Spine Bands, Posterior Spiracle and Tracheal System: The sequence of development is so rapid after the coil-shaped gut formation. The sequence that begins within 8 hours and 56 minutes after egg laying includes the formation of spine bands, posterior spiracle, and a fully developed tracheal system essentially in this order.

Stage X – Hatching: The eggs hatched at 9 hours and 11 minutes.

1.4.7a Monocrotophos

Figure 1.12 is a visualisation of the stages of developmental significance in the control eggs of *Chrysomya megacephala* along with the major variations shown by the eggs due to the exposure to monocrotophos in the corresponding time intervals. The eggs developed in the monocrotophos-treated feed showed visually recognisable variations from developmental stage IV onwards. The eggs in the treatment set showed the accelerated rate of development from stage IV onwards. When the control set reached the stage V of development, the treatment set had already moved past stage V and was showing initial signs of structures formed during stage VI. From the next stage onwards, the monocrotophos treatment set was always one stage ahead in its development, until hatching. The hatching time of the eggs in the treatment sets was also accelerated to an average of 7 hours and 33 minutes instead of the 9 hours and 11 minutes in control set.



[ym- yolky middle, ccr- clear cytoplasmic rim, cf- cephalic furrow, st- stomodeal invagination, clp- clypeolabrum, seq- segmentation, g- gut, tt- tracheal trunk]

Fig 1.12: Impact of Monocrotophos on the embryonic development of *Chrysomya megacephala*. (A)- Control- Stage I, (B) Monocrotophos- Stage I, (C) Control- Stage II, (D) Monocrotophos- Stage II, (E) Control- Stage III, (F) Monocrotophos- Stage III, (G) Control- Stage IV, (H) Monocrotophos- Stage IV, (I) Control- Stage V, (J) Monocrotophos- Stage V & VI intermediate, (K) Control- Stage VI, (L) Monocrotophos- Stage VII, (M) Control- Stage VII, (N) Monocrotophos- Stage VIII, (O) Control- Stage VIII, (P) Monocrotophos- Stage IX, (Q) Control- Stage IX.

Transcriptome Analysis of Maternal mRNA Involved in Embryonic Development

Transcriptomic analysis and Differential Gene Expression (DGE) analysis revealed the variation in the expression of several maternally transcribed gene products, which exhibit gradient expression in the later stage of the lifecycle. Given its maximum exposure to the intoxicant via feed, the third instar stage is used for the DGE analysis, revealing several maternal mRNA involved in embryonic development that exhibit differential expression. Even though this is not a direct reflection of gene expression during embryogenesis, these genes indicate the effect of monocrotophos on maternal mRNA expression. Table 1.10 represents a list of maternal mRNAs with a pivotal involvement in embryonic development that are differentially expressed in response to monocrotophos exposure. There are certain maternal mRNAs that are later replaced by zygotic mRNAs which code for the same protein, showing differential expression are listed in Table 1.11. These zygotic genes also play vital roles beyond their involvement in embryonic development.

Table 1.10: Monocrotophos-induced differential expression of maternal mRNAs involved in embryonic development of *Chrysomya megacephala*

Gene	Function	Up-regulated ▲	Down-regulated ▼
<i>tudor</i>	Required during oogenesis for the formation of primordial germ cells and for normal abdominal segmentation.	1	3
<i>exuperantia</i>	Ensures the proper localization of the mRNA of the bicoid gene to the anterior regions of the oocyte thus playing a fundamental role in the establishment of the polarity of the oocyte.	0	1
<i>staufer</i>	Encodes a double-stranded RNA binding protein involved in mRNA localization. It contributes to anterior and posterior patterning, nervous system development and long-term memory.	0	1
<i>pumilio</i>	It is an essential factor in the translational regulation of hunchback mRNA in the early embryo.	0	2

Table 1.11: Monocrotophos-induced differential expression of zygotic genes involved in embryonic development of *Chrysomya megacephala*

Gene	Function	Up-regulated ▲	Down-regulated ▼
<i>Kinesin heavy chain</i>	Encodes the force generating subunit of kinesin-1, a microtubule motor protein. It functions in the long-distance transport of cytoplasmic "cargoes" such as mRNAs, protein complexes, and organelles.	0	1
<i>maelstrom</i>	As a component of the meiotic nuage, plays a central role during oogenesis by repressing transposable elements and preventing their mobilization, which is essential for the germline integrity.	0	1
<i>aubergine</i>	Required for dorsal-ventral as well as anterior-posterior patterning of the egg. Required during oogenesis for primordial germ cell formation and activation of RNA interference. During early oogenesis, required for <i>osk</i> mRNA silencing and polarization of the microtubule cytoskeleton. During mid-oogenesis, required for <i>osk</i> mRNA localization to the posterior pole and efficient translation of <i>osk</i> and <i>grk</i> . During embryogenesis, required for posterior localization of <i>nanos</i> (<i>nos</i>) mRNA, independently of <i>osk</i> , and pole cell formation. Forms a complex with <i>smg</i> , <i>twin</i> , <i>AGO3</i> and specific piRNAs that targets <i>nanos</i> mRNA (and probably other maternal mRNAs) for deadenylation promoting its decay during early embryogenesis.	0	3

<i>mini spindles</i>	Involved in oocyte microtubule cytoskeleton organization and bicoid mRNA localization.	0	3
<i>cup</i>	Adapter protein that plays a central role in localization of transcripts in the oocyte and in young embryos. Required for correct localization of eIF4E1 in the developing oocyte. Required for translational repression of oskar (osk) mRNA. Also required for the translational repression of nanos (nos) mRNA. Promotes the accumulation of the germ plasm components osk, vas and stau at the posterior pole of the oocyte and is required for germ cell development. Represses orb positive autoregulatory activity which prevents premature activation of orb and ensures its accumulation specifically in the developing oocyte. In 0-1 hour embryos, forms a complex with me31B, cup, tral and pAbp which binds to various mRNAs including maternal mRNAs, and down-regulates their expression during the maternal-to-zygotic transition.	0	1
<i>dystroglycan</i>	Encodes a major non-integrin extracellular matrix (ECM) receptor that connects the ECM to the actin cytoskeleton. It regulates animal survival and temperature preference, muscle integrity, myotendinous and neuromuscular junction formation and function, nervous system development, axon pathfinding, rhabdome differentiation, neuronal stem cell division and epithelial polarity.	0	1
<i>mago nashi</i>	It is involved in germline development, germplasm assembly and photoreceptor differentiation.	1	0

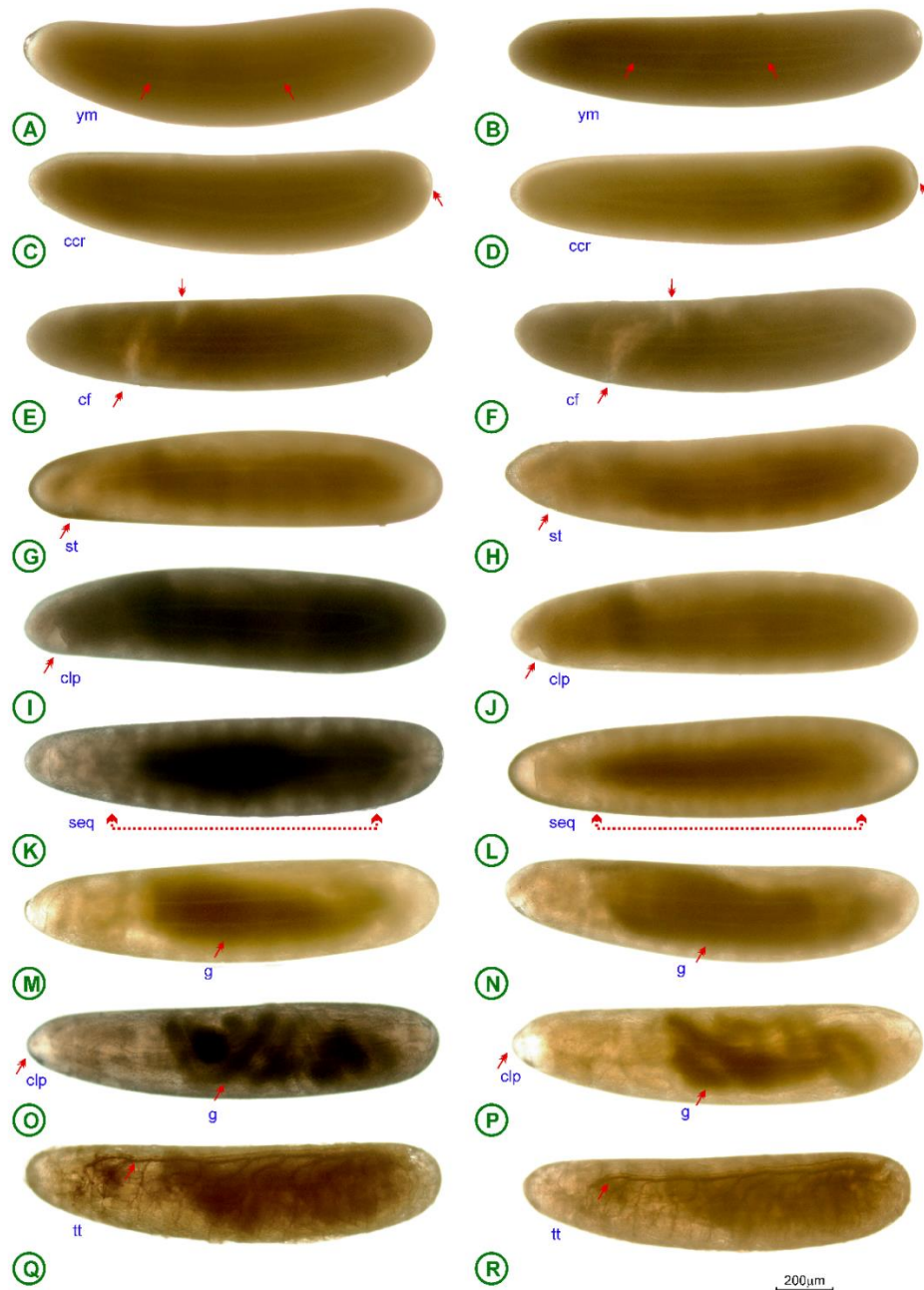
<i>Rabenosyn-5</i>	Encodes a Rab5 -binding effector that controls entry of endocytosed cargo into the early endosome.	1	0
<i>encore</i>	Encodes a protein involved in the regulation of germline mitoses. It promotes the upregulation of gurken mRNA translation in the dorsal anterior corner of the oocyte, allowing normal dorso-ventral pattern formation.	0	3
<i>Rab6</i>	Encodes a member of the Rab family, which are small Ras-like GTPases involved in vesicle trafficking. The Rab6 product localizes on late Golgi, and regulates vesicle trafficking within Golgi, post-Golgi, and endosome to Golgi.	0	1
<i>tudor</i>	Required during oogenesis for the formation of primordial germ cells and for normal abdominal segmentation.	0	8
<i>Laspl</i>	Encodes a member of the nebulin family. It plays subtle roles in modulating the actin cytoskeleton in ovaries, testes, and muscles, by serving as a scaffold protein binding multiple partners, especially actin and myosin.	0	5
<i>Heat shock protein 83</i>	Molecular chaperone that promotes the maturation, structural maintenance and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction.	0	1
<i>Moesin</i>	Encodes an Ezrin, Radixin, Moesin (ERM) protein involved in cortical cytoskeleton stability. It regulates the products of crb and Rho1. The product of Moe contributes to apical-basal polarity, mitotic spindle organisation and epithelial integrity.	0	4

<i>Clathrin heavy chain</i>	Encodes a protein that forms part of the clathrin complex, which is the major component of coated vesicles.	0	1
<i>torso-like</i>	Encodes a protein present in many organs. In particular it is produced and secreted by two cell clusters in the egg chamber, initially anchored at the vitelline membrane and then translocated to the oocyte plasma membrane where it is required for the activation of the receptor encoded by tor.	1	1
<i>spire</i>	Encodes a de novo WH2 domain-containing actin nucleation factor that polymerizes linear actin filaments, as well as bundles and/or crosslinks actin and microtubules. It is a downstream effector of the product of Rho1 and is involved in many cytoskeleton-related developmental processes, including ooplasmic streaming, nurse cell architecture, and endocytosis.	0	1
<i>bicaudal</i>	Encodes the β subunit of the nascent polypeptide-associated complex. It regulates osk mRNA localization, which in turn specifies the anterior/posterior axis of the egg.	0	6
<i>gustavus</i>	Encodes a cullin-RING ubiquitin E3 ligase specificity receptor. It interacts genetically and physically with the product of Cul5, and putatively regulates stability of target proteins including the one encoded by vas. It is involved in oogenesis and germ cell maintenance.	2	0
<i>valois</i>	The product of vls is required for proper localization of the product of tud to the posterior pole of the	0	1

	oocyte during germ line specification.		
<i>atypical protein kinase C</i>	Encodes a member of the conserved Par complex and is asymmetrically localized to the apical cortex, where it phosphorylates and thus excludes the basal determinants encoded by <i>mira</i> and <i>numb</i> . It is involved in neuroblast proliferation and self-renewal.	0	1
<i>Serendipity δ</i>	Transcriptional activator that controls <i>bicoid</i> gene expression during oogenesis.	2	1
<i>gurken</i>	The asymmetrical localization of <i>grk</i> mRNA is key for its function during oogenesis, to establish anterior-posterior and dorso-ventral axes in the egg and embryo.	0	2
<i>squid</i>	encodes a member of the hnRNP family of RNA binding proteins. It is involved in the localization and translational regulation of <i>grk</i> mRNA in oogenesis.	0	3

1.4.7b Carbamazepine

Figure 1.13 is the visualisation of the stages of developmental significance in the control/normal eggs of *Chrysomya megacephala* along with the carbamazepine treated eggs in corresponding time intervals. There are not many visually perceptible variations in embryonic development observed between the control and carbamazepine treatment set by means of live embryo visualisation by sodium hypochloride treatment. The temporal variations in development and hatching, in comparison to the control, was also absent in carbamazepine treated set.



[ym- yolky middle, ccr- clear cytoplasmic rim, cf- cephalic furrow, st- stomodeal invagination, clp- clypeolabrum, seq- segmentation, g-gut, tt- tracheal trunk]

Fig 1.13: Impact of Carbamazepine on the embryonic development of *Chrysomya megacephala*. (A)- Control- Stage I, (B) Carbamazepine- Stage I, (C) Control- Stage II, (D) Carbamazepine- Stage II, (E) Control- Stage III, (F) Carbamazepine- Stage III, (G) Control- Stage IV, (H) Carbamazepine- Stage IV, (I) Control- Stage V, (J) Carbamazepine- Stage V, (K) Control- Stage VI, (L) Carbamazepine- Stage VI, (M) Control- Stage VII, (N) Carbamazepine- Stage VII, (O) Control- Stage VIII, (P) Carbamazepine- Stage VIII, (Q) Control- Stage IX, (R) Carbamazepine- Stage IX.

Transcriptome Analysis of maternal mRNA involved in embryonic development

Transcriptomic analysis and Differential Gene Expression (DGE) analysis revealed the variation in the expression of several maternally transcribed gene products which exhibits gradient expression in the later stage of lifecycle. Considering the fact that it is the stage with maximum exposure to the intoxicant via feed, the third instar stage is used for the DGE analysis and the result revealed a number of maternal mRNA involved in embryonic development that shows differential expression. Even though this is not a direct reflection of gene expression during embryogenesis, these genes give an indication on the effect of carbamazepine on maternal mRNA expression. Table 1.12 shows the list of maternal mRNAs with a pivotal involvement in embryonic development that are differentially expressed due to carbamazepine exposure. There are certain maternal mRNAs which are later replaced by zygotic mRNAs which codes for the same protein. Such genes that are involved in embryonic development showing differential expression are listed in Table 1.13. Most of these zygotic genes also have vital roles other than their involvement in embryonic development.

Table 1.12: Carbamazepine-induced differential expression of maternal mRNAs involved in embryonic development of *Chrysomya megacephala*

Gene	Function	Up-regulated ▲	Down-regulated ▼
<i>tudor</i>	Required during oogenesis for the formation of primordial germ cells and for normal abdominal segmentation.	1	3
<i>exuperantia</i>	Ensures the proper localization of the mRNA of the bicoid gene to the anterior regions of the oocyte thus playing a fundamental role in the establishment of the polarity of the oocyte.	1	0
<i>staufer</i>	Encodes a double-stranded RNA binding protein involved in mRNA localization. It contributes to anterior and posterior patterning, nervous system development and long-term memory.	0	1
<i>pumilio</i>	It is an essential factor in the translational regulation of hunchback mRNA in the early embryo.	0	2

Table 1.13: Carbamazepine-induced differential expression of zygotic genes involved in embryonic development of *Chrysomya megacephala*

Gene	Function	Up-regulated ▲	Down-regulated ▼
<i>Kinesin heavy chain</i>	Encodes the force generating subunit of kinesin-1, a microtubule motor protein. It functions in the long-distance transport of cytoplasmic "cargoes" such as mRNAs, protein complexes, and organelles.	0	1
<i>maelstrom</i>	As a component of the meiotic nuage, plays a central role during oogenesis by repressing transposable elements and preventing their mobilization, which is essential for the germline integrity.	0	1
<i>aubergine</i>	Required for dorsal-ventral as well as anterior-posterior patterning of the egg. Required during oogenesis for primordial germ cell formation and activation of RNA interference. During early oogenesis, required for <i>osk</i> mRNA silencing and polarization of the microtubule cytoskeleton. During mid-oogenesis, required for <i>osk</i> mRNA localization to the posterior pole and efficient translation of <i>osk</i> and <i>grk</i> . During embryogenesis, required for posterior localization of <i>nanos</i> (<i>nos</i>) mRNA, independently of <i>osk</i> , and pole cell formation. Forms a complex with <i>smg</i> , <i>twin</i> , <i>AGO3</i> and specific piRNAs that targets <i>nanos</i> mRNA (and probably other maternal mRNAs) for deadenylation promoting its decay during early embryogenesis.	0	3

<i>mini spindles</i>	Involved in oocyte microtubule cytoskeleton organization and bicoid mRNA localization.	0	3
<i>cup</i>	Adapter protein that plays a central role in localization of transcripts in the oocyte and in young embryos. Required for correct localization of eIF4E1 in the developing oocyte. Required for translational repression of oskar (osk) mRNA. Also required for the translational repression of nanos (nos) mRNA. Promotes the accumulation of the germ plasm components osk, vas and stau at the posterior pole of the oocyte and is required for germ cell development. Represses orb positive autoregulatory activity which prevents premature activation of orb and ensures its accumulation specifically in the developing oocyte. In 0-1 hour embryos, forms a complex with me31B, cup, tral and pAbp which binds to various mRNAs including maternal mRNAs, and down-regulates their expression during the maternal-to-zygotic transition.	0	1
<i>dystroglycan</i>	Encodes a major non-integrin extracellular matrix (ECM) receptor that connects the ECM to the actin cytoskeleton. It regulates animal survival and temperature preference, muscle integrity, myotendinous and neuromuscular junction formation and function, nervous system development, axon pathfinding, rhabdomere differentiation, neuronal stem cell division and epithelial polarity.	0	1
<i>mago nashi</i>	It is involved in germline development, germplasm assembly and photoreceptor differentiation.	1	0

<i>Rabenosyn-5</i>	Encodes a Rab5 -binding effector that controls entry of endocytosed cargo into the early endosome.	0	1
<i>encore</i>	Encodes a protein involved in the regulation of germline mitoses. It promotes the upregulation of gurken mRNA translation in the dorsal anterior corner of the oocyte, allowing normal dorso-ventral pattern formation.	0	3
<i>Rab6</i>	Encodes a member of the Rab family, which are small Ras-like GTPases involved in vesicle trafficking. The Rab6 product localizes on late Golgi, and regulates vesicle trafficking within Golgi, post-Golgi, and endosome to Golgi.	0	1
<i>Laspl</i>	Encodes a member of the nebulin family. It plays subtle roles in modulating the actin cytoskeleton in ovaries, testes, and muscles, by serving as a scaffold protein binding multiple partners, especially actin and myosin.	1	4
<i>Heat shock protein 83</i>	Molecular chaperone that promotes the maturation, structural maintenance and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction.	0	1
<i>Moesin</i>	Encodes an Ezrin, Radixin, Moesin (ERM) protein involved in cortical cytoskeleton stability. It regulates the products of crb and Rho1. The product of Moe contributes to apical-basal polarity, mitotic spindle organisation and epithelial integrity.	1	3
<i>Clathrin heavy chain</i>	Encodes a protein that forms part of the clathrin complex, which is the major component of coated vesicles.	0	1

<i>torso-like</i>	Encodes a protein present in many organs. In particular it is produced and secreted by two cell clusters in the egg chamber, initially anchored at the vitelline membrane and then translocated to the oocyte plasma membrane where it is required for the activation of the receptor encoded by <i>tor</i> .	1	1
<i>spire</i>	Encodes a de novo WH2 domain-containing actin nucleation factor that polymerizes linear actin filaments, as well as bundles and/or crosslinks actin and microtubules. It is a downstream effector of the product of <i>Rho1</i> and is involved in many cytoskeleton-related developmental processes, including ooplasmic streaming, nurse cell architecture, and endocytosis.	0	1
<i>bicaudal</i>	Encodes the β subunit of the nascent polypeptide-associated complex. It regulates <i>osk</i> mRNA localization, which in turn specifies the anterior/posterior axis of the egg.	0	6
<i>gustavus</i>	Encodes a cullin-RING ubiquitin E3 ligase specificity receptor. It interacts genetically and physically with the product of <i>Cul5</i> , and putatively regulates stability of target proteins including the one encoded by <i>vas</i> . It is involved in oogenesis and germ cell maintenance.	1	1
<i>valois</i>	The product of <i>vls</i> is required for proper localization of the product of <i>tud</i> to the posterior pole of the oocyte during germ line specification.	1	0
<i>atypical protein kinase C</i>	Encodes a member of the conserved Par complex and is	0	1

	asymmetrically localized to the apical cortex, where it phosphorylates and thus excludes the basal determinants encoded by <i>mira</i> and <i>numb</i> . It is involved in neuroblast proliferation and self-renewal.		
<i>Serendipity δ</i>	Transcriptional activator that controls <i>bicoid</i> gene expression during oogenesis.	0	3
<i>gurken</i>	The asymmetrical localization of <i>grk</i> mRNA is key for its function during oogenesis, to establish anterior-posterior and dorso-ventral axes in the egg and embryo.	0	2
<i>squid</i>	encodes a member of the hnRNP family of RNA binding proteins. It is involved in the localization and translational regulation of <i>grk</i> mRNA in oogenesis.	1	2

1.4.8 Differential expression of genes involved in the development of *Chrysomya megacephala*

To determine differences in gene expression levels in *Chrysomya megacephala* under the effect of monocrotophos and carbamazepine, the clean reads from control and treatments, containing two independent replicates, were assembled to generate de novo assembled transcriptome. A total of 17,865 genes showed differential expression in control *vs.* sub-lethal monocrotophos treatment of *Chrysomya megacephala*, among which 8,731 were downregulated and 9,134 were upregulated. A total of 17984 genes showed differential expression in control *vs.* sub-lethal carbamazepine treatment of *Chrysomya megacephala*, among which 9489 were downregulated and 8495 were upregulated. Among these, there were several genes present that are directly involved in many major processes in development including, pupation, molting and eclosion. The list of major gene ontology terms and respective genes that are differentially expressed and are involved in the process of pupation is given in Table 1.14 (control *vs.* monocrotophos) and Table 1.15 (control *vs.* carbamazepine).

Differentially expressed genes involved in molting and eclosion due to the exposure to monocrotophos and carbamazepine are listed in Table 1.16 and Table 1.17, respectively.

Table 1.14: Control vs. Monocrotophos GO terms and differentially expressed genes involved in pupation

GO terms	Gene	Up-regulated ▲	Down-regulated ▼
Pupal development (GO:0035209)	Cytochrome P450 18a1	0	1
	Glucose dehydrogenase	12	10
Pupal chitin-based cuticle development (GO:0008364)	Glucose dehydrogenase	12	10
Instar larval or pupal development (GO:0002165)	rhomboid	0	2
	anchor	7	7
	Strawberry notch	0	1
	ecdysone-induced protein 75B	0	3
	Glucose dehydrogenase	12	10
	Nedd4	0	1
	singed	1	1
	Star	0	1
	split ends	0	7
	pygopus	0	2
	patched	0	2
	Delta	2	2
	smoothened	0	1
	folded gastrulation	0	1
	Ultrabithorax	0	1
	Ecdysone receptor	0	4
	Stromal interaction molecule	0	3
	Cytochrome P450 301a1	1	0
	Torsin	2	0
	broad	0	7
	Elongator complex protein 3	1	1
	armadillo	4	3
	wntless	1	0
	ADP ribosylation factor 1	1	0

C15	1	0
held out wings	1	0
jing	0	2
atonal	1	1
Epidermal growth factor receptor	1	3
Notch	0	4
kinase suppressor of ras	0	1
LIM domain kinase 1	2	0
apterous	1	0
G protein-coupled receptor kinase 2	0	1
Son of sevenless	0	1
eyes absent	1	0
Serrate	0	4
gustavus	2	0
parvin	1	0
fizzy-related	0	1
hopscotch	0	1
misshapen	0	1
Larval cuticle protein 1	0	1
string	1	0
Yippee	1	3
shaggy	1	1
Hairless	0	1
division abnormally delayed	0	1
Rap1 GTPase	0	2
Death-associated inhibitor of apoptosis 1	3	2
Larval cuticle protein 9	1	0
penguin	1	0
drumstick	1	0
engrailed	0	1
cut	0	1
ecdysoneless	0	1
ebi	1	0
frataxin	1	0
Larval cuticle protein 2	6	7
Rabenosyn-5	1	0

Pupal cuticle protein	11	6
hemipterous	0	1
escargot	0	1
homothorax	1	3
tricornered	1	0
fritz	2	0
Topoisomerase 1	0	1
decapentaplegic	0	1
neuralized	1	3
spinster	0	4
Cyclin-dependent kinase 8	2	0
furry	0	2
Dual oxidase	0	2
halfway	0	1
Chromodomain-helicase-DNA-binding protein 1	0	3
Rac1	2	1
Meosin	0	4
winged eye	0	2
teashirt	1	0
spalt major	0	1
asteroid	0	1
gurken	0	2
capicua	0	4
headcase	1	2
Rab5	0	1
unkempt	1	2
Dystroglycan	0	1
Dystrophin	0	3
Ribosomal protein L7	2	1
CTD nuclear envelope phosphatase 1	1	1
Neuroglian	0	1
ultraspiracle	0	1
vestigial	0	1
brahma	1	0
frizzled	0	4
torso-like	0	1
quail	2	0
araucan	1	2

	mastermind	0	2
	pangolin	4	1
	fringe	0	1
	kayak	1	6
	Rho1	5	1
	Nostrin	0	1
	Death-associated protein kinase related	0	2
	prickle	1	1
	Suppressor of Hairless	0	1
	p115	0	1
	tolloid	0	2
	Coat Protein	1	0
	Transcription factor AP-2	1	0
	pointed	0	1
	scarface	0	1
	ovo	0	2
	hedgehog	0	1
	inturned	0	1
	spitz	0	1
	Chip	1	0
	Protein kinase N	0	3
	Mothers against dpp	1	6
	Cytochrome P450 18a1	0	1
	costa	0	1
Pupariation (GO:0035073)	torso-like	1	1
	ultraspiracle	0	1
	halfway	0	1
	Ribosomal protein L7	2	1
	Ecdysone receptor	0	4
Pupation (GO:0035074)	Cytochrome P450 18a1	0	1
Instar larval or pupal morphogenesis (GO:0048707)	rhomboid	0	2
	anchor	7	7
	ebi	1	0
	Rabenosyn-5	1	0
	Pupal cuticle protein	11	6
	strawberry notch	0	1
	hemipterous	0	1
	escargot	0	1

homothorax	1	3
Nedd4	0	1
fritz	2	0
tricornered	1	0
singed	1	1
Star	2	1
decapentaplegic	0	1
neuralized	1	3
split ends	0	7
pygopus	0	2
Cyclin-dependent kinase 8	1	0
furry	0	2
Dual oxidase	0	2
miniature	0	1
patched	0	2
Delta	2	2
smoothened	0	1
folded gastrulation	0	1
Chromodomain-helicase- DNA-binding protein 1	0	3
Rac1	2	1
Meosin	0	4
Ultrabithorax	0	1
winged eye	0	2
Ecdysone receptor	0	4
spalt major	0	1
teashirt	1	0
Stromal interaction molecule	0	3
Cytochrome P450 301a1	1	0
asteroid	0	1
gurken	0	2
capicua	0	4
broad	0	7
armadillo	4	3
wntless	1	0
Rab5	0	1
ADP ribosylation factor 1	1	0
C15	2	0
unkempt	1	2

held out wings	1	0
jing	0	2
atonal	1	1
Epidermal growth factor receptor	1	3
Dystroglycan	0	1
Dystrophin	0	3
Notch	0	4
kinase suppressor of ras	0	1
LIM domain kinase 1	2	0
apterous	1	0
G protein-coupled receptor kinase 2	0	1
Son of sevenless	0	1
eyes absent	1	0
frizzled	0	4
vestigial	0	1
brahma	1	0
quail	2	0
araucan	1	2
mastermind	0	4
Serrate	0	4
gustavus	2	0
pangolin	4	1
parvin	1	0
fringe	0	1
fizzy-related	0	1
hopscotch	0	1
kayak	1	6
Rho1	5	1
Nostrin	0	1
misshapen	0	1
Death-associated protein kinase related	0	2
prickle	2	1
Suppressor of Hairless	0	1
p115	0	1
tolloid	0	2
Transcription factor AP-2	1	0
string	1	0

	Yippee	1	3
	shaggy	1	1
	pointed	0	1
	scarface	0	1
	ovo	0	2
	Hairless	0	1
	hedgehog	0	1
	division abnormally delayed	0	1
	inturned	0	1
	Rap1 GTPase	0	2
	spitz	0	1
	Death-associated inhibitor of apoptosis 1	3	2
	penguin	1	0
	drumstick	1	0
	Chip	1	0
	sprouty	0	1
	Protein kinase N	0	3
	engrailed	0	1
	Mothers against dpp	1	7
	Cytochrome P450 18a1	0	1
	costa	0	1
	cut	0	1
Structural constituent of pupal chitin-based cuticle (GO:0008011)	Pupal cuticle protein	11	6
	obstructor-E	4	3

Table 1.15: Control vs. Carbamazepine GO terms and differentially expressed genes involved in pupation

GO terms	Gene	Up-regulated ▲	Down-regulated ▼
Pupal development (GO:0035209)	Cytochrome P450 18a1	0	1
	Glucose dehydrogenase	8	14
Pupal chitin-based cuticle development (GO:0008364)	Glucose dehydrogenase	8	14
	rhomboid	0	2

Instar larval or pupal development (GO:0002165)	anchor	5	7
	Strawberry notch	0	1
	ecdysone-induced protein 75B	0	3
	Glucose dehydrogenase	8	14
	Nedd4	0	1
	singed	1	1
	Star	0	1
	split ends	0	7
	pygopus	0	3
	patched	0	2
	Delta	0	4
	smoothened	0	1
	folded gastrulation	0	1
	Ultrabithorax	0	1
	Ecdysone receptor	0	4
	Stromal interaction molecule	1	2
	Cytochrome P450 301a1	1	0
	Torsin	1	1
	broad	0	7
	Elongator complex protein 3	2	0
	armadillo	4	3
	wntless	0	1
	ADP ribosylation factor 1	1	0
	C15	0	2
	held out wings	0	1
	jing	0	2
	atonal	1	1
	Epidermal growth factor receptor	1	3
	Notch	0	4
	kinase suppressor of ras	0	1
	LIM domain kinase 1	0	2
	apterous	1	0

G protein-coupled receptor kinase 2	0	1
Son of sevenless	0	1
eyes absent	1	0
Serrate	1	3
gustavus	1	1
Distal-less	0	2
parvin	1	0
fizzy-related	0	1
hopscotch	0	1
misshapen	0	1
Larval cuticle protein 1	0	1
string	0	1
Yippee	0	4
shaggy	1	1
Hairless	0	1
division abnormally delayed	0	1
Rap1 GTPase	1	1
Death-associated inhibitor of apoptosis 1	1	4
Larval cuticle protein 9	0	1
penguin	1	0
drumstick	1	0
engrailed	0	2
cut	0	1
ecdysoneless	0	1
ebi	1	0
frataxin	0	1
Larval cuticle protein 2	1	10
Rabenosyn-5	0	1
Pupal cuticle protein	10	8
hemipterous	1	2
escargot	0	1
homothorax	1	3
tricornered	0	1
fritz	2	0
Topoisomerase 1	0	1
decapentaplegic	0	1
neuralized	1	3

spinster	0	4
Cyclin-dependent kinase 8	1	1
furry	0	2
Dual oxidase	0	2
halfway	1	0
wingless	1	0
Chromodomain-helicase-DNA-binding protein 1	0	3
Rac1	1	2
Meosin	1	3
winged eye	0	3
teashirt	0	1
spalt major	2	0
asteroid	1	0
gurken	0	2
capicua	1	3
headcase	0	3
Rab5	1	0
unkempt	0	3
Dystroglycan	0	1
Dystrophin	0	3
Ribosomal protein L7	2	0
invected	1	0
CTD nuclear envelope phosphatase 1	2	0
Neuroglian	0	1
ultraspiracle	0	1
vestigial	0	1
brahma	1	0
frizzled	0	4
torso-like	1	1
quail	2	0
araucan	1	2
mastermind	0	4
pangolin	2	3
fringe	0	1
kayak	1	6
Rho1	5	1

	Nostrin	0	1
	Death-associated protein kinase related	0	2
	prickle	1	2
	Suppressor of Hairless	0	1
	p115	0	1
	tolloid	0	2
	Coat Protein	2	0
	Transcription factor AP-2	1	0
	pointed	0	1
	scarface	0	1
	ovo	0	1
	hedgehog	0	1
	inturned	0	1
	spitz	1	0
	Chip	1	0
	sprouty	0	1
	Protein kinase N	0	4
	Mothers against dpp	3	5
	Cytochrome P450 18a1	0	1
	costa	0	1
Pupariation (GO:0035073)	torso-like	1	1
	ultraspiracle	0	1
	halfway	1	0
	Ribosomal protein L7	2	0
	Ecdysone receptor	0	4
Pupation (GO:0035074)	Cytochrome P450 18a1	0	1
Instar larval or pupal morphogenesis (GO:0048707)	rhomboid	0	2
	anchor	5	7
	ebi	1	0
	Rabenosyn-5	0	1
	Pupal cuticle protein	10	8
	strawberry notch	0	1
	hemipterous	1	2
	escargot	0	1
	homothorax	1	3
	Nedd4	0	1
	fritz	2	0

tricornered	0	1
singed	1	1
Star	1	2
decapentaplegic	0	1
neuralized	1	3
forked	2	1
split ends	0	7
pygopus	0	3
Cyclin-dependent kinase 8	1	0
furry	0	2
Dual oxidase	0	2
miniature	0	1
patched	0	2
wingless	1	0
Delta	0	4
smoothened	0	1
folded gastrulation	0	1
Chromodomain-helicase-DNA-binding protein 1	0	3
Rac1	1	2
Meosin	1	3
Ultrabithorax	0	1
winged eye	0	2
Ecdysone receptor	0	4
spalt major	2	0
teashirt	0	1
Stromal interaction molecule	1	2
Cytochrome P450 301a1	1	0
asteroid	1	0
gurken	0	2
capicua	1	3
broad	0	7
armadillo	4	3
wntless	0	1
Rab5	1	0
ADP ribosylation factor 1	1	0

C15	0	2
unkempt	0	3
held out wings	0	1
jing	0	2
atonal	1	0
Epidermal growth factor receptor	1	3
Dystroglycan	0	1
Dystrophin	0	3
Notch	0	4
kinase suppressor of ras	0	1
LIM domain kinase 1	0	2
apterous	1	0
G protein-coupled receptor kinase 2	0	1
Son of sevenless	0	1
eyes absent	1	0
frizzled	0	4
vestigial	0	1
brahma	1	0
quail	2	0
araucan	1	2
mastermind	0	4
Serrate	1	3
gustavus	1	1
pangolin	2	3
Distal-less	0	2
parvin	1	0
fringe	0	1
fizzy-related	0	1
hopscotch	0	1
kayak	1	6
Rho1	5	1
Nostrin	0	1
misshapen	0	1
Death-associated protein kinase related	0	2
prickle	1	2
Suppressor of Hairless	0	1

	p115	0	1
	tolloid	0	2
	Transcription factor AP-2	1	0
	string	0	1
	Yippee	0	4
	shaggy	1	1
	pointed	0	1
	scarface	0	1
	ovo	0	1
	Hairless	0	1
	hedgehog	0	1
	division abnormally delayed	0	1
	inturned	0	1
	Rap1 GTPase	1	1
	spitz	1	0
	Death-associated inhibitor of apoptosis 1	1	4
	penguin	1	0
	drumstick	1	0
	Chip	1	0
	sprouty	0	1
	Protein kinase N	0	3
	engrailed	0	2
	Mothers against dpp	3	5
	Cytochrome P450 18a1	0	1
	costa	0	1
	cut	0	1
Structural constituent of pupal chitin-based cuticle (GO:0008011)	Pupal cuticle protein	10	8
	obstructor-E	3	5

Table 1.16: Control vs. *Monocrotophos* GO terms and differentially expressed genes involved in molting and eclosion

GO terms	Gene	Up-regulated ▲	Down-regulated ▼
Molting cycle (GO:0042303)	ecdysoneless	0	1
	Dual oxidase	0	2
	yippee	1	3
	shaggy	1	1
	Larval cuticle protein 2	6	7
	Ecdysone-induced protein 75B	0	3
	Glucose dehydrogenase	12	10
	frizzled 2	0	1
	pangolin	4	1
	Ecdysone receptor	0	4
	Larval cuticle protein 9	1	0
	rigor mortis	0	1
	yellow	5	2
	armadillo	4	3
	pygopus	0	2
	Larval cuticle protein 1	0	1
Molting cycle process (GO:0022404)	Dual oxidase	0	2
	yippee	1	3
	Ecdysone receptor	0	4
	Ecdysone-induced protein 75B	0	3
	yellow	5	2
Molting cycle, chitin-based cuticle (GO:0007591)	frizzled 2	0	1
	ecdysoneless	0	1
	Dual oxidase	0	2
	yippee	1	3
	shaggy	1	1

	Larval cuticle protein 2	6	7
	Ecdysone-induced protein 75B	0	3
	Glucose dehydrogenase	12	10
	pangolin	4	1
	Ecdysone receptor	0	4
	Larval cuticle protein 9	1	0
	rigor mortis	0	1
	yellow	5	2
	armadillo	4	3
	pygopus	0	2
	Larval cuticle protein 1	0	1
Cuticle development involved in chitin-based cuticle molting cycle (GO:0042337)	frizzled 2	0	1
	shaggy	1	1
	pangolin	4	1
	Larval cuticle protein 9	1	0
	Larval cuticle protein 2	6	7
	armadillo	4	3
	Glucose dehydrogenase	12	10
	pygopus	0	2
	Larval cuticle protein 1	0	1
Eclosion (GO:0007562)	Chip	1	0
	broad	0	7
	period	2	0
	lark	2	0
	timeless	2	2
Eclosion rhythm (GO:0008062)	period	2	0
	timeless	2	2
	lark	2	0

Negative regulation of eclosion (GO:0045804)	lark	2	0
Regulation of eclosion (GO:0007563)	Chip	1	0
	lark	2	0

Table 1.17: Control vs. Carbamazepine GO terms and differentially expressed genes involved in molting and eclosion

GO terms	Gene	Up-regulated ▲	Down-regulated ▼
Molting cycle (GO:0042303)	ecdysoneless	0	1
	Dual oxidase	0	2
	yippee	0	4
	shaggy	1	1
	Larval cuticle protein 2	1	10
	wingless	1	0
	Ecdysone-induced protein 75B	0	3
	Glucose dehydrogenase	8	14
	frizzled 2	0	1
	hugin	0	1
	pangolin	2	3
	Ecdysone receptor	0	4
	Larval cuticle protein 9	0	1
	rigor mortis	0	1
	yellow	2	4
	armadillo	4	3
	pygopus	0	3
	Larval cuticle protein 1	0	1
Molting cycle process (GO:0022404)	Dual oxidase	0	2
	yippee	0	4
	Ecdysone receptor	0	4

	Ecdysone-induced protein 75B	0	3
	yellow	2	4
Molting cycle, chitin-based cuticle (GO:0007591)	frizzled 2	0	1
	ecdysoneless	0	1
	Dual oxidase	0	2
	yippee	0	4
	shaggy	1	1
	Larval cuticle protein 2	1	10
	Ecdysone-induced protein 75B	0	3
	Glucose dehydrogenase	8	14
	pangolin	2	3
	Ecdysone receptor	0	4
	Larval cuticle protein 9	0	1
	rigor mortis	0	1
	yellow	2	4
	armadillo	4	3
	pygopus	0	3
	Larval cuticle protein 1	0	1
Cuticle development involved in chitin-based cuticle molting cycle (GO:0042337)	frizzled 2	0	1
	shaggy	1	1
	pangolin	2	3
	Larval cuticle protein 9	0	1
	Larval cuticle protein 2	1	10
	armadillo	4	3
	Glucose dehydrogenase	8	14
	pygopus	0	3
	Larval cuticle protein 1	0	1
Eclosion	Chip	1	0

(GO:0007562)	broad	0	7
	period	2	0
	lark	2	0
	timeless	1	3
Eclosion rhythm (GO:0008062)	period	2	0
	timeless	1	3
	lark	2	0
Negative regulation of eclosion (GO:0045804)	lark	2	0
Regulation of eclosion (GO:0007563)	Chip	1	0
	lark	2	0

1.5 DISCUSSION

The extensive distribution and global issues associated with *Chrysomya megacephala* necessitate an examination of its biology to support research on its control and potential uses, especially in the field of forensic entomology (Badenhorst & Villet, 2018). Therefore, detailed studies on the effects of intoxicants with forensic potential on various life stages of the species are very relevant. The present study demonstrates that the selected intoxicants, monocrotophos, and carbamazepine induced varied levels of disparities in various aspects such as embryonic development, pupation and emergence.

The EC₅₀ value (concentration causing 50% pupal inhibition) was determined using range-finding tests, and definitive toxicity bioassays were conducted based on the results of the range-finding analysis. Current findings show that the estimated EC₅₀ value of monocrotophos for *Chrysomya megacephala* is 0.23 µg/ml. A few previous studies have determined the LC₅₀ values of monocrotophos for various species. In *Drosophila melanogaster*, the concentrations were 0.68 µg/mL for 3rd instar larvae and 0.56 µg/mL for pupae (Das et al., 2024). For tadpoles of *Limnonectis limnocharis*, a Rice Field Frog, the LC₅₀ of monocrotophos was 0.59 mg/l (Swain & Mahananda, 2014), 0.148 µg cm⁻² for subterranean termites, *Odontotermes obesus* (Rao et al., 2005), and 20.42 mg/l for fish, *Oreochromis niloticus* (Vroumsia et al., 2014). In all

of these studies, commercial formulations of monocrotophos under various brand names were used. However, in the present study, the analytical standard of monocrotophos was used, which may have contributed to the variations in the lethal concentration values, as well as variations in the species. Similarly, the EC₅₀ value of carbamazepine on *Chrysomya megacephala* was determined to be 0.6 mg/ml. Unlike monocrotophos, most studies on carbamazepine were based on environmentally realistic concentrations or chronic exposure studies. The range of environmentally realistic concentrations were between 0.3–3.0 and 6.0–9.0 µg/L (Prato et al., 2023). The determination of the median acute toxicity level is relevant in the current study, as the application of the toxicity is targeted to have a forensic application that is usually based on accidental or intended overuse/abuse of drugs.

Previous research on zebra fish embryos indicated that monocrotophos can negatively affect embryonic development, leading to various developmental issues such as pericardial edema, altered heart development, and spinal and vertebral anomalies, all in a concentration-dependent manner (Pamanji et al., 2015; Murugan, 2021). A study by Shamapari & Nagaraj., (2023) investigated the impact of Anti-epileptic drugs (AEDs) on *Drosophila melanogaster*. Their research revealed that prenatal administration of AEDs to fruit flies resulted in significant teratogenic effects, leading to various malformations. The authors suggested that these observations in fruit flies may be linked to the diverse abnormalities observed in humans following AED use. The research by Qiang et al., (2016) demonstrated that exposure to environmental levels of carbamazepine accelerated embryonic development in zebra fish. Oropesa et al., (2016) evaluated the effects of carbamazepine on *Daphnia magna* embryos and found that, although the drug does not directly cause embryotoxicity, maternal exposure disrupts normal gastrulation and organogenesis.

The primary distinction in the effects of monocrotophos and carbamazepine in *Chrysomya megacephala* was observed in the hatching time of eggs. Monocrotophos induced a dose-dependent reduction in hatching time, with the highest concentration resulting in a statistically significant decrease. Conversely, carbamazepine did not affect hatching time at either dose when compared to the control group. To further

elucidate these effects, embryo visualisation and differential gene expression (DGE) analysis were conducted. The investigation into embryo visualisation provided insights into developmental stages that are either bypassed or rapidly advanced due to the influence of monocrotophos. In the case of carbamazepine exposure, although there was no change in hatching time, the DGE analysis indicated potential effects on the embryo of *Chrysomya megacephala*.

In various animal species, early embryos rely on maternal messenger RNA. The initial phases of embryonic development are directed by genetic material from the mother, including mRNAs and proteins, which are deposited into the egg (Crofton et al., 2018). Mothers play a pivotal role in controlling early developmental stages across metazoan species. The genetic material supplied by mothers is crucial for many initial developmental processes and lays the groundwork for later stages. This maternal genetic contribution is a significant yet often overlooked form of parental investment. The presence of maternal mRNA, along with the delayed start of embryonic mRNA production, suggests that the early development of insect embryos likely depends heavily, if not entirely, on maternal mRNA (Herbert, 1980). Studying the formation of the primary axis (head-to-tail) in fly (Diptera) embryos offers valuable insights into the evolution of essential gene functions during early development (Yoon et al., 2019). This developmental process relies on specific maternal mRNAs, known as ‘anterior determinants’, located at the egg's anterior pole. Interestingly, these determinants have evolved throughout the evolutionary history of flies. The initial stages of animal development, including late oogenesis and early embryogenesis, occur without transcription and rely on maternal mRNAs stored in oocytes (Barckmann & Simonelig, 2013). These maternal mRNAs then undergo a general decay in embryos, where the control of development shifts from the maternal to the zygotic genome in a carefully regulated process known as the maternal-to-zygotic transition (MZT) (Atallah & Lott, 2018). Upon examination of the transcriptomic analysis of third instar larvae of *Chrysomya megacephala*, differential expression of several maternal mRNAs with significant roles in embryonic development was observed. Although this does not directly reflect gene expression during embryogenesis, these genes provide insights into the effects of monocrotophos and carbamazepine on maternal mRNA

expression. Additionally, a subset of mRNAs initially present as of maternal origin and later become zygotic (MZT), which also play crucial roles in embryogenesis, were found to be differentially expressed. This finding further indicates the impact of monocrotophos and carbamazepine on embryonic development in the current generation of flies, both through direct exposure and via maternal mRNA produced by exposed individuals in subsequent generations.

The transcriptomic analysis of third-instar larvae of *Chrysomya megacephala* revealed differential expression of maternal mRNAs with a gradient expression pattern, which are directly involved in the major processes in embryonic development, including axis formation. This may indicate that monocrotophos and carbamazepine can potentially impact embryonic development in *Chrysomya megacephala*. The differential expression of zygotic mRNAs showing MZT and with direct involvement in embryonic development may also suggest an effect of intoxicants on maternal mRNA formation for the next generation. Further elaborate studies based on transcription analysis of the embryonic stages of *Chrysomya megacephala* are needed to confirm these findings. However, the current results suggest an effective egg visualisation method, as well as a possible impact of monocrotophos and carbamazepine on molecular expression in *Chrysomya megacephala*.

A study conducted by Mandi et al., (2024) on chronic monocrotophos exposure demonstrated an accelerated developmental rate in *Drosophila melanogaster*. The findings suggest that early pupation may result from the toxic food medium creating unfavourable conditions, thereby inhibiting the normal, extended larval duration. The current study also observed a similar response in *Chrysomya megacephala*, where exposed to monocrotophos led to an accelerated developmental rate, culminating a reduced pupal duration and early pupation, which holds significance in entomotoxicological analysis (Renu & Vardhanan, 2025).

The study on the effect of carbamazepine on *Ceriodaphnia dubia* revealed that carbamazepine can cause a significant decrease in fecundity and neonate dry weight (Lamichhane et al., 2013). It had also delayed the first brood release indicating a slower maturation. In the nonbiting midge *Chironomus riparius*, carbamazepine

caused a significant and concentration-dependent decrease in emergence (Oetken et al., 2005). In mayfly nymphs (*Stenonema* sp.), exposure to carbamazepine caused developmental and behavioural changes (Jarvis et al., 2014). The current study also shows similar effects in *Chrysomya megacephala*. The sublethal doses of carbamazepine caused a concentration-dependent delay in pupation time and emergence and induced a concentration-dependent decrease in pupal weight.

Chitin deacetylases (CDAs) catalyze the N-deacetylation of chitin, a critical process for chitin modification, and the knockdown of the CDA1 gene significantly prolonged the developmental period in the final instar larvae of *Leptinotarsa decemlineata* (Wu et al., 2019). In *Heortia vitessoides*, chitin deacetylase 1 and 2 genes play essential roles in the larval–pupal and pupal–adult transitions, exhibiting relatively high mRNA expression levels during the larval–pupal transition (Wang et al., 2019). Transcriptome analysis revealed that exposure to monocrotophos in the third instar larvae of *Chrysomya megacephala* resulted in the differential expression of chitin deacetylase 1 genes, with 4 out of 7 differentially expressed genes being up-regulated. In carbamazepine-exposed larvae, 5 genes of chitin deacetylase 1 genes were downregulated and 1 was upregulated, accounting to the delayed pupation and emergence time.

The inhibition of the Chitinase gene in *Spodoptera frugiperda* affected the degradation of chitin in the old epidermis and the formation of the new epidermis (X. Y. Liu et al., 2022). In our study, 21 out of 33 differentially expressed Chitinase genes (including Chitinase-like proteins and putative Chitinase genes) were up-regulated, indicating a faster degradation rate of the old epidermis in monocrotophos-treated individuals and 13 out of 31 genes were down-regulated in carbamazepine-treated individuals.

Juvenile hormone epoxide hydrolase (JHEH) is involved in the degradation of juvenile hormones and plays a role in insect development and molting processes Z. Liu et al., (2022). Juvenile hormone acid methyltransferase (JHAMT) is a rate-limiting enzyme that converts JH acids or inactive precursors of JHs to active JHs in the final step of JH biosynthesis in insects (Guo et al., 2021). In summary, JHEH

inhibits juvenile hormones, while JHAMT promotes their expression. Our transcriptome analysis revealed that 3 JHEH genes were up-regulated and 2 JHAMT genes were downregulated, suggesting an accelerated metamorphosis as a consequence of monocrotophos toxicity. In the carbamazepine treatment set, 1 JHEH gene was down regulated and 2 genes were up-regulated. In the case of juvenile hormone acid methyltransferase, 2 genes were down-regulated.

Analysis of the differentially expressed genes associated with the pupation process under the Gene Ontology (GO) terms ‘pupal development’, ‘pupal chitin-based cuticle development’, ‘instar larval or pupal development’, ‘pupariation’, ‘pupation’, ‘instar larval or pupal morphogenesis’, and ‘structural constituent of pupal chitin-based cuticle’ corroborated the observations regarding the impact of monocrotophos and carbamazepine on pupation time. Similarly, the genes categorised under the GO terms ‘molting cycle’, ‘molting cycle process’, ‘molting cycle, chitin-based cuticle’, and ‘cuticle development involved in chitin-based cuticle molting cycle’ which are implicated in various aspects of molting, and genes under the GO terms ‘eclosion’, ‘eclosion rhythm’, and ‘regulation of eclosion’ that are involved in eclosion process, were also differentially expressed following the exposure to monocrotophos and carbamazepine. This indicates the molecular basis of the initial observations. The expression of several key genes involved in pupation, molting, eclosion, and subsequent emergence was uniquely affected by monocrotophos and carbamazepine in *Chrysomya megacephala*.

Entomotoxicology represents a medicolegal application of forensic entomology, wherein toxins may induce alterations in the developmental rate of necrophagous insects, potentially leading to significant modifications in post-mortem interval (PMI) estimates. If these factors are overlooked during death investigations, particularly in cases of suicide and deliberate self-harm involving pesticides, which are major yet under-recognised public health issues in the developing world, errors may ensue (Sharma & Singh, 2016). The emerging field of entomotoxicology is currently concentrated on two primary research trajectories (Byrd & Castner, 2001). One trajectory examines the potential use of carrion-feeding arthropods as alternative

specimens in toxicological analyses. At the same time, the other investigates the impact of tissue-borne toxins and contaminants on the developmental processes of juvenile insects that consume these materials. The present study demonstrates that the developmental rate of *Chrysomya megacephala* is altered due to exposure to monocrotophos and carbamazepine, with transcriptomic analysis corroborating these findings.

1.6 KEY FINDINGS

- ❖ The EC₅₀ concentrations for Monocrotophos and Carbamazepine are 0.23 µg/mL and 0.6 mg/mL respectively.
- ❖ Sublethal concentrations of monocrotophos and carbamazepine induced dose-dependent effects in the life cycle parameters of *Chrysomya megacephala* causing significant developmental impairments.
- ❖ Monocrotophos induced significant acceleration in the rate of embryonic development causing an early hatching compared to control set.
- ❖ In the case of hatching time and embryonic development, carbamazepine caused no perceivable variations in the current study/method of study.
- ❖ Transcriptome analysis revealed the differential expression of numerous genes corroborating the observations.

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Chapter 2
Investigating Detoxification Mechanisms: Chronic Toxicity
Assessment of Monocrotophos and Carbamazepine Using
Biochemical and Molecular Techniques

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2

Investigating Detoxification Mechanisms: Chronic Toxicity Assessment of Monocrotophos and Carbamazepine Using Biochemical and Molecular Techniques

2.1 INTRODUCTION

A toxicological analysis is used to detect harmful substances in an organism. This is accomplished by analysing the sample extract through a series of bioassays, which act as a distinctive fingerprint for the sample's toxicity (Verma et al., 2024). This method aids in identifying and evaluating the effects of toxicants on various environmental or biological samples based on dose. Toxicity tests provide information about the adverse effects of a toxicant and assess the risk associated with the dose-response relationship. The concept of toxicity analysis involves evaluating the impact of a specific sample on laboratory animals, monitoring its effects when exposed to increasing doses, and ultimately assessing its potential harmful impact on human health (Arome & Chinedu, 2013).

The toxicity profile of a pharmacological or chemical agent is just as crucial as the details regarding its beneficial applications. Although there are several levels of toxicity testing, the most frequently evaluated are acute, sub-acute, sub-chronic, and chronic toxicities (Chinedu et al., 2015). Acute toxicity testing is a brief evaluation conducted to determine potential risks from a single exposure to a toxic substance. In contrast, sub-acute toxicity studies are carried out to understand the effects of different dosage levels, offering a more realistic assessment of toxic doses than acute evaluations (Verma et al., 2024). Sub-chronic toxicity testing aims to identify any harmful effects that might arise from repeated exposure over a moderate duration. Meanwhile, chronic toxicity analysis is typically conducted over extended periods and involves larger groups of model organisms (*Sub-Chronic and Chronic Toxicity*

Studies, n.d.). This type of testing aims to identify long-term, cumulative, and delayed adverse effects that may not become apparent until after prolonged exposure.

Due to the inability to systematically test the human system, research on the potential impacts of drugs on human physiology has largely relied on examining drug effects in model systems, such as living animal organisms (*in vivo*) or cultivated cell lines from animals and humans (*in vitro*). Additionally, the recent technological advancements in molecular biology and bioinformatics have enabled researchers to thoroughly investigate the toxic effects of pollutants on various molecular pathways. The broad spectrum of omics technologies, including genomics and proteomics, supports in-depth analysis of cellular processes within individuals or biodiversity, particularly in response to diverse environmental stimuli (Zhang et al., 2018). Additionally, methods such as transcriptomics and metabolomics are being employed and further assessed in the field of investigational toxicology (Ellinger-Ziegelbauer & Ahr, 2014).

A biochemical assay is a method used to analyse and measure cellular activities, such as apoptosis and cell signalling, or metabolic reactions (*Biochemical Assays*, n.d.). These assays are a dependable and commonly employed technique that aids in identifying targets and comprehending biomolecular functions. Biochemical assays are utilized to detect, measure, and examine the binding or activity of biological molecules, like enzymes (*Biochemical Assays Articles from across Nature Portfolio*, n.d.). They offer users additional insights into the possible mechanisms of action of a compound and how these compounds affect enzymatic activity in both canonical and non-canonical pathways (*Cell-Based Assay or Biochemical Assay? Now That's the Question...*, 2023). Biochemical assays are broadly categorised into several types based on the specific substances or interactions. The most common types are, enzyme assay, immunoassay, ligand binding assay, and cell-based assay (*What Is a Biochemical Assay? Types and Applications*, 2025). Enzyme assays are employed to assess the functionality of enzymes, which are proteins that catalyse biochemical reactions. These assays are crucial for understanding metabolic pathways and can aid in evaluating enzyme kinetics. Conversely, immunoassays utilize the unique

specificity of antibodies to detect and quantify proteins, hormones, or other molecules. Ligand binding assays are commonly used to explore the interaction between a protein and a potential drug candidate. Another category, cell-based assays, is dedicated to analysing the biochemical processes occurring within living cells.

This chapter investigates the chronic *in vivo* toxicity of monocrotophos and carbamazepine on *Chrysomya megacephala* using enzymatic biochemical assays and transcriptomic analysis. The activities of superoxide dismutase (SOD), acetylcholinesterase (AChE), glutathione reductase (GR), and glutathione S-transferase (GST) were assessed in the third instar larvae of *Chrysomya megacephala* under control conditions and in the presence of sub-lethal concentrations of monocrotophos and carbamazepine. This study aimed to elucidate the mechanisms involved in detoxification. Additionally, the molecular basis of detoxification mechanism was investigated through differential gene expression (DGE) analysis.

2.2 REVIEW OF LITERATURE

Insects have developed a range of detoxification strategies to withstand natural toxins (Cumming et al., 2008). These strategies can also enable insects to resist insecticides, with the extent and nature of these mechanisms varying significantly. The detoxification strategies are divided into three phases: phase I, phase II (which involves metabolizing enzymes), and phase III (which involves transporters) (Aioub & Ashour, 2023). The main enzymes responsible for the detoxification processes in phases I and II are cytochrome P450 monooxygenase (CYP450), glutathione S-transferase (GST), and carboxylesterase (CarE). On the other hand, phase III is mainly controlled by ATP-binding cassette (ABC) transporters. Phase I reactions encompass oxidation, hydrolysis, and reduction.

Numerous studies have investigated the impact of various intoxicants on the detoxification mechanisms in insects, with the majority employing biochemical assays. Experimental findings suggest that various pesticides have a common impact: they trigger oxidative stress in different cell types and animal models (Sule et al., 2022). This stress, induced by pesticides, arises from reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are linked to various diseases including

cancer, inflammation, and cardiovascular and neurodegenerative disorders. ROS and RNS are capable of activating at least five distinct signalling pathways, including mitochondrial-induced apoptosis. Some limited in vitro research also suggests that external antioxidants might mitigate or prevent the harmful effects of pesticides. A mini-review by Maslakova & Silivanova (2025) indicated that insecticides containing various active components can significantly impact antioxidant levels in different insect species. This effect is sometimes observed as an increase in enzyme activities, while in other instances, it results in a decrease. Consequently, the induction of oxidative stress and the reduction of insects' antioxidant capacity are the primary toxicity mechanisms of most insecticides.

Leelaja & Rajini (2013) investigated the biochemical and physiological responses of *Caenorhabditis elegans* to exposure to a sublethal dose of monocrotophos. Their findings indicated a reduction in the activity of acetylcholinesterase and carboxylesterase, along with an increase in reactive oxygen species (ROS) and a decrease in superoxide dismutase (SOD) activity, indicating that even low concentrations of monocrotophos can cause physiological and biochemical alterations, impacting the fitness of *Caenorhabditis elegans*. (Franeta et al., 2018) examined the activities of several enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferase (GST), as well as the total free SH groups in *Ostrinia nubilalis* when exposed to various insecticides such as indoxacarb, chlorantrilinipole, and a combination of chlorantraniliprole and lambda cyhalothrin. The findings indicated that these insecticides significantly impact the antioxidative defence mechanisms in the larvae of the European corn borer, with chlorantraniliprole having a particularly notable effect. Research conducted by Abou-Elghar et al. (2024) demonstrated that sublethal concentrations of the insecticides abamectin, emamectin benzoate, imidacloprid, indoxacarb, and spinosad caused oxidative stress in *Callosobruchus maculatus*. This stress resulted in an increase in glutathione reductase (GSH) and GPx levels, while SOD levels were decreased. In the model organism *Galleria mellonella*, exposure to the pesticide thiamethoxam led to oxidative stress and genotoxic effects, resulting in a reduction in SOD and CAT levels (Kayis et al.,

2019). (Staroň et al. (2017) investigated the toxicity of formetanate and its impact on the antioxidant enzyme system in *Apis mellifera*. The observed reduction in SOD specific activity, along with increased CAT and GST activities, indicates the presence of oxidative stress. By comparing the toxicity levels in larvae with those in adult bees, it can be inferred that larvae might be less sensitive to formetanate than adult bees.

Exposure to fluopyram led to oxidative stress in *Caenorhabditis elegans*, as evidenced by an increase in ROS production, lipofuscin and lipid buildup, and elevated MDA levels (Liu et al., 2022). Conversely, fluopyram exposure significantly reduced the activity of the target enzyme SDH and antioxidant enzymes, including SOD, CAT, and GST. Additionally, there was a change in the expression of genes related to oxidative stress (e.g., *gst-4*, *sod-3*, *fat-7*, *mev-1*, and *daf-16*), intestinal damage (e.g., *mtm-6*, *nhx-2*, *opt-2*, *pkc-3*, *par-6*, *act-5*, and *egl-8*), and cell apoptosis (e.g., *ced-13*, *ced-3*, *egl-38*, *efl-2*, *cep-1*, and *lgg-1*). Tu et al. (2018) conducted a molecular analysis of the mitochondrial manganese superoxide dismutase (mMnSOD) in *Chilo suppressalis*, revealing that mMnSOD plays a crucial role in defending against oxidative damage. Li et al. (2021) characterized the copper/zinc superoxide (prSOD1) gene from *Pieris rapae* and found that when exposed to sublethal levels of chlorantraniliprole, there was an increase in prSOD1 expression. The resulting protein demonstrated antioxidant properties and exhibited high stability under varying thermal and pH conditions, confirming that PrSOD1 encodes a functional enzyme with antioxidant capabilities. A study by Gong & Diao (2017) demonstrated that a comprehensive transcriptomic and proteomic approach has enabled the understanding that detoxification processes in honey bees involve numerous genes and pathways, along with alterations in energy metabolism and cellular stress responses. The P450 genes play a significant role in the direct detoxification of xenobiotics and insecticides in honey bees, and their expression can be influenced by honey and pollen components, leading to increased tolerance to other xenobiotics or insecticides. Additionally, P450s serve as crucial detoxification enzymes that facilitate synergistic interactions between acaricides/insecticides and fungicides by either inhibiting P450 activity through fungicides or creating competition for detoxification enzymes between acaricides.

Through genomic analysis of detoxification genes in *Aedes aegypti*, Strode et al. (2008) constructed an array with unique oligonucleotide probes for these genes and compared their expression levels in insecticide-resistant and susceptible strains. This study found that most detoxification genes belong to two gene families: the CYP9 P450s and the Epsilon GSTs. Another study by Pavlidi et al. (2013) focused on the transcriptome and phylogenetic classification of major detoxifying genes in *Bactrocera oleae*, identifying 55 P450, 43 GST-, 15 CCE-, and 18 ABC transporter genes. Some of these detoxification genes are potentially involved in the olive fruit fly's capacity to manage xenobiotics, including plant phytotoxins and insecticides.

2.3 MATERIALS AND METHODS

2.3.1 Protein estimation by Lowry's method

The protein estimation using Lowry's method was performed on the third instar larvae of *Chrysomya megacephala* to understand the effect of sublethal concentration (1/3rd of EC₅₀) of monocrotophos and carbamazepine. Sublethal concentration of 1/3rd of EC₅₀ of monocrotophos (0.077 µg/ml) and carbamazepine (0.2 mg/ml) with an exposure time of 96 hours (since the egg stage) was used for protein estimation. The control and treatment sets had six independent replicates. 0.1 mL of tissue homogenate was taken and made up to 1 mL. 5 mL of reagent C was added to each tube, including the blank (water), mixed well and incubated at 37°C for 3 minutes. Then, 0.5 ml of Folin-Ciocalteu reagent was added, mixed well and incubated at 37°C for 3 minutes (Lowry et al., 1951; Peterson, 1979). The blue colour was developed, and absorbance was measured at 660nm using UV-VIS spectrophotometer (SHIMADZU – UV 1900i). A standard graph was plotted and the amount of protein in the sample was calculated.

2.3.2 Biochemical Analyses

All biochemical assays utilized six independent biological replicates (N=6) per treatment group. This configuration satisfies prospective power requirements (power=0.8, α =0.05), maximizing parametric test sensitivity to precisely detect dose-dependent toxicological responses.

2.3.2a Estimation of Superoxide Dismutase (SOD)

50µl of tissue samples were added to the reaction mixture containing 50mM phosphate buffer (7.8), 45µM methionine, 5.3mM riboflavin and 84µM potassium ferric cyanide and 0.1M NBT. The tubes were then incubated at 25°C for 10 minutes and the absorbance was read on spectrophotometer at 600nm (Misra & Fridovich, 1977).

$$\text{Percentage of Inhibition} = ((\text{Control}-\text{Test})/\text{Control}) \times 100$$

$$\text{Enzyme Units} = \text{Percentage Inhibition} / 50$$

2.3.2b Estimation of Acetylcholinesterase (AChE) activity

DNTB was dissolved in this buffer containing 0.1M NaCl and 0.02 M MgCl₂. Acetylthiocholine iodide (ATCI) was dissolved in deionized water. 100µL of 3mM DNTB, 60µL of buffer (50 mM Tris pH 8.0), 20µL of each homogenized sample was added to 96-well plate. After mixing, the plate was incubated for 15 minutes (25°C) and then the absorbance was measured at 412nm. Then reaction was then initiated by the addition of 20µL of ATCI. After mixing, absorbance was measured at two time intervals (5minutes, 20 minutes) (Kuvvelkar et al., 2021).

$$\text{Enzyme activity} = \frac{\Delta \text{Absorbance} * \text{volume of reaction (mL)} * 1000 * \text{dilution factor}}{13.33 * \text{volume of sample}}$$

2.3.2c Glutathione Reductase (GR) Assay

The assay system was composed of 1 mL of phosphate buffer, 0.1 mL of EDTA, 0.1 mL of sodium azide, 0.1 mL of oxidized glutathione, and 0.1 mL of the enzyme source, with the total volume adjusted to 2 mL using distilled water. The tubes were incubated for 3 minutes, after which 0.1 mL of NADPH was added (Bergmeyer et al., 1983). Absorbance was measured at 340 nm using a UV-VIS spectrophotometer (SHIMADZU – UV 1900i) at 15-second intervals for a duration of 2 to 3 minutes. For each series of measurements, control samples were prepared by substituting water for oxidized glutathione. Enzyme activity was quantified as micromoles of NADPH oxidized per minute per milligram of protein.

$$\text{Units/mL} = \frac{\frac{OD}{\text{minute}} \times \text{Dilution factor} \times \text{Total volume}}{\text{sample volume} \times 6.22}$$

2.3.2d Glutathione S-Transferase (GST) Assay

0.1mL homogenized samples were taken into corresponding test tubes, and the following reagents were added. 0.02mL of 0.5mM CDNB, 0.05mL GSH, 0.68mL of distilled water and 0.2mL of 100mM potassium phosphate buffer were added (Habig et al., 1974). The absorbance was measured at 340nm using a UV-VIS spectrophotometer (SHIMADZU – UV 1900i).

$$\text{GST specific activity} = \frac{od / \text{Minute} \times V \times d \times 1}{V_{enz} \times emM}$$

d - dilution factor of the original sample

emM - extinction coefficient for CDNB conjugate at 340nm- 9.6M

V_{enz} - 0.1mL

V - total reaction volume

2.3.3 Transcriptomic analysis of genes involved in detoxification

The effect of sublethal concentration (1/3rd of EC₅₀) of monocrotophos and carbamazepine on the expression of genes involved in the detoxification process was analyzed using de novo transcriptomics of the third instar larvae of *Chrysomya megacephala*. Sublethal concentration of 1/3rd of EC₅₀ of monocrotophos (0.077 µg/ml) and carbamazepine (0.2 mg/ml) with an exposure time of 96 hours (since the egg stage) was used for transcriptome analysis. The control and treatment sets had two independent replicates.

The DGE analysis is done on the following combinations:

(a) Control vs Monocrotophos

(b) Control vs Carbamazepine

Reads of each sample were mapped separately to CDS sequences obtained from the master assembly using BWA, and PCR duplicate reads were removed from the alignment file using Picard's MarkDuplicates.jar. Then, the reads mapping to each CDS were calculated for each sample using the idxstats program of Samtools. Finally, these mapped reads mentioned above were considered for differential gene expression (DGE) analysis. The read-mapped count from each sample was given, and finally, these mapped reads in the combination mentioned above were considered for differential gene expression (DGE) analysis. The read-mapped count from each sample was given as input for differential expression analysis using the edgeR Bioconductor package in R. For a comprehensive description of the procedures involved in transcriptomic analysis, please refer to the methodology section in Chapter 3.

2.4 RESULTS

2.4.1 Protein estimation by Lowry's method

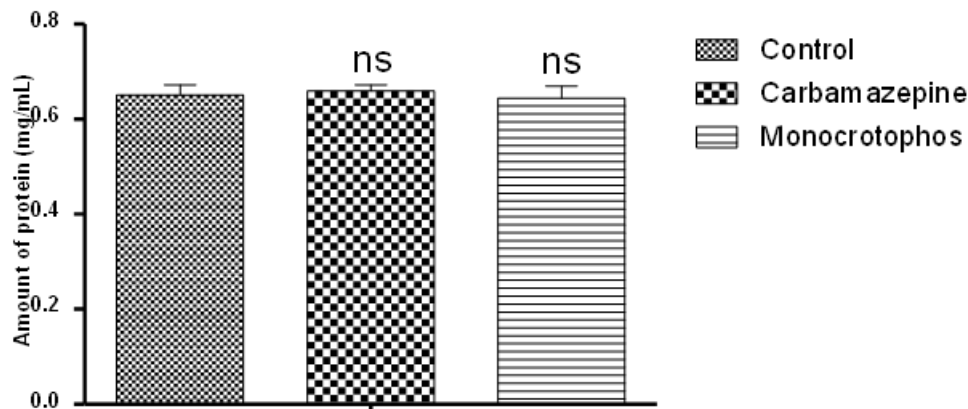
Protein quantification is essential for determining the total protein content within a sample or a formulated product. Accurate quantification is critical, as numerous other assays depend on precise protein content measurements to generate reliable data. Prior to conducting biochemical analyses, protein estimation is vital as it establishes a baseline measurement of total protein content, thereby ensuring the accuracy and reliability of subsequent analyses. This preliminary step facilitates the normalization of various biochemical assays, which require precise protein concentrations for accurate data interpretation.

The Folin-Lowry method is a prevalent technique for protein quantification, predicated on the principle of colour development through the reduction of phosphomolybdic-phosphotungstic components in the Folin-Ciocalteu reagent by

amino acids, notably tyrosine and tryptophan, present in proteins. In this method, proteins in the sample undergo a series of reactions involving cupric ions in an alkaline environment, resulting in the formation of a coloured complex that is further intensified by the Folin-Ciocalteu reagent. The intensity of the resultant blue colouration is directly proportional to the protein concentration within the sample, thereby enabling the quantitative assessment of protein content. Protein estimation revealed no significant variation between the control and test sets. Table 2.1 and Figure 2.1 illustrates the protein estimation of samples, with the Y-axis representing the amount of protein and the X-axis representing the treated samples.

Table 2.1: Protein estimation by Lowry's method

Experiment	Amount of Protein (mg/mL) (Mean $\pm$ Stdev (std error))
Control	0.6512 $\pm$ 0.036038 (0.012013)
Monocrotophos	0.64448 $\pm$ 0.043436 (0.014479)
Carbamazepine	0.65953 $\pm$ 0.02191 (0.007303)



(ns- not significant)

Figure 2.1: Graphical representation depicting the protein estimation of monocrotophos- and carbamazepine-treatment against control

2.4.2 Biochemical Analyses

Biochemical analyses, encompassing the estimation of superoxide dismutase (SOD), acetylcholinesterase (AChE) activity, glutathione reductase (GR) assay, and glutathione S-transferase (GST) assay, were performed to elucidate the effects of monocrotophos and carbamazepine on the biochemical processes of *Chrysomya*

megacephala. Additionally, these analyses aimed to elucidate the detoxification mechanisms activated in response to exposure to monocrotophos and carbamazepine.

2.4.2a Estimation of Superoxide Dismutase (SOD)

The enzyme superoxide dismutase (SOD) facilitates the dismutation of superoxide into oxygen and hydrogen peroxide (H_2O_2). As such, it functions as a vital antioxidant defence mechanism in nearly all cells exposed to oxygen. The results indicate a statistically significant reduction in SOD concentration in both monocrotophos and carbamazepine treatment groups compared to the control, suggesting an increase in oxidative stress within the test samples. Table 2.2 and Figure 2.2 illustrates the estimation of SOD in the samples, with the Y-axis representing enzyme units per milligram of protein and the X-axis denoting the treated samples.

Table 2.2: Estimation of SOD

Experiment	Enzyme Units/mg of protein (Mean $\pm$ Stdev (Std Error))
Control	0.49903 $\pm$ 0.021799 (0.007266)
Monocrotophos	0.22037 $\pm$ 0.001129 (0.000376)
Carbamazepine	0.36786 $\pm$ 0.013257 (0.004419)

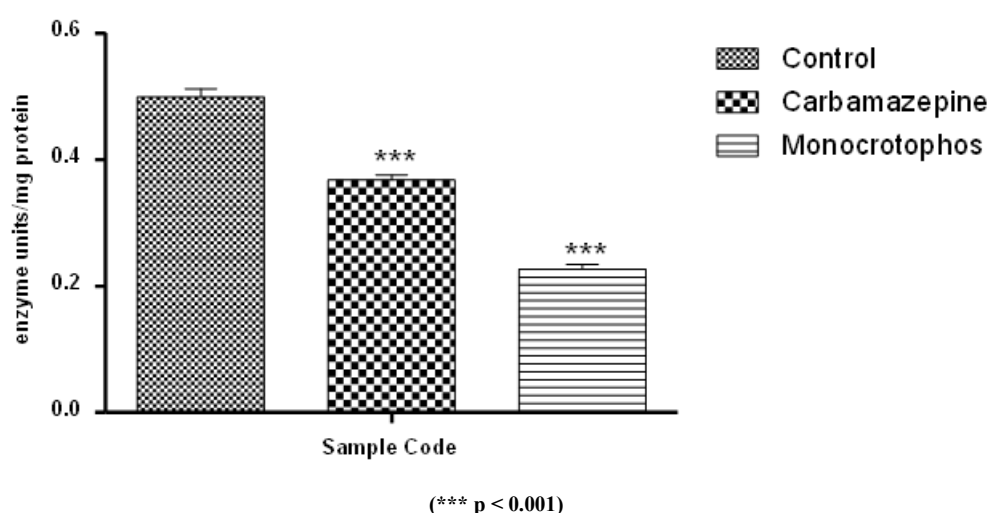


Figure 2.2: Graphical representation depicting the superoxide dismutase assay of monocrotophos- and carbamazepine-treated against control

2.4.2b Estimation of Acetylcholinesterase (AChE) activity

Acetylcholinesterase (AChE) activity was evaluated using a modified 96-well microplate assay based on Ellman's method. This enzyme facilitates the hydrolysis of the substrate acetylthiocholine, producing thiocholine, which subsequently reacts with Ellman's reagent (DTNB) to form 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate, detectable at 412 nm. The analysis indicated a reduction in enzyme activity in the groups treated with carbamazepine and monocrotophos. Although the decrease in activity was statistically insignificant in the carbamazepine-treated group, it was highly significant in the monocrotophos-treated group. The reduction in AChE activity can be an indication of the presence of a nerve agent or pesticide. Table 2.3 and Figure 2.3 illustrates the estimation of AChE activity in the samples, with the Y-axis representing enzyme activity per milligram of protein and the X-axis denoting the treated samples.

Table 2.3: Estimation of AChE

Experiment	Enzyme Activity/mg protein (Mean $\pm$ Stdev (Std Error))
Control	0.26559 $\pm$ 0.027396 (0.009132)
Monocrotophos	0.12391 $\pm$ 0.010494 (0.003498)
Carbamazepine	0.23351 $\pm$ 0.016935 (0.005645)

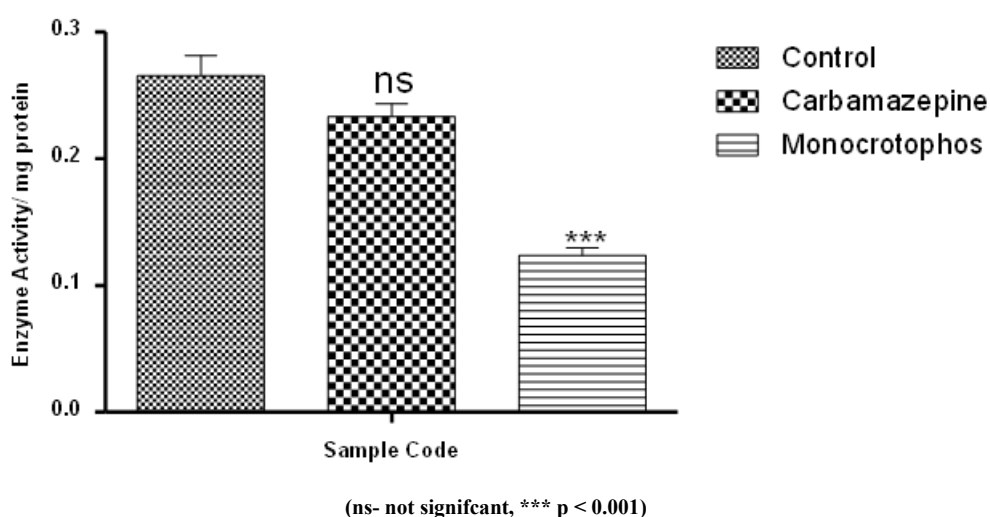


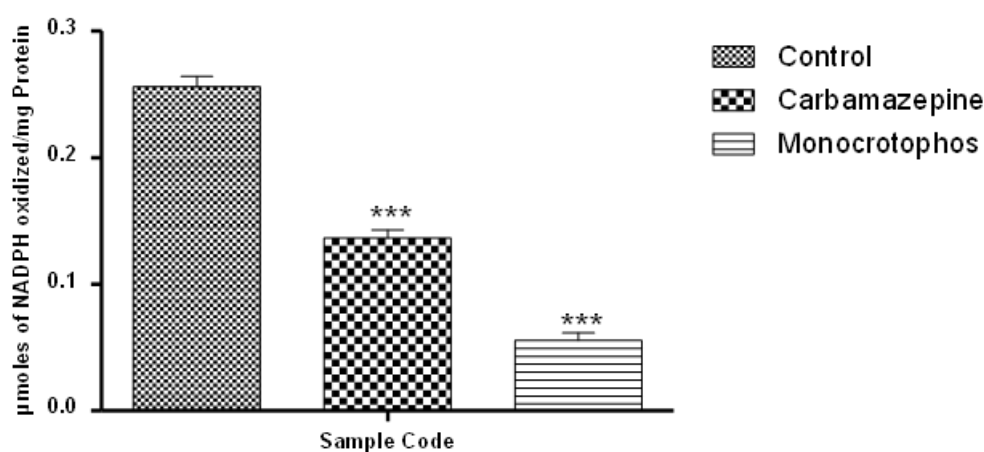
Figure 2.3: Graphical representation depicting the acetylcholinesterase activity of monocrotophos- and carbamazepine-treatment against control

2.4.2c Glutathione Reductase (GR) Assay

Glutathione reductase (GR) facilitates the conversion of oxidized glutathione to its reduced form, utilizing NADPH as a substrate. The quantity of NADPH consumed serves as a direct indicator of the enzyme's activity. The results indicate a statistically significant reduction in oxidised NADPH concentration in both monocrotophos and carbamazepine treatment groups compared to the control, suggesting an impaired oxidative stress defence and potential cellular damage in the test organism. Table 2.4 and Figure 2.4 shows the estimation of GR activity of samples, with the Y axis showing the micromoles of NADPH oxidised per milligram of protein, and the X axis showing the samples treated.

Table 2.4: Estimation of GR

Experiment	$\mu\text{moles of NADPH oxidized/mg Protein}$ (Mean $\pm$ Stdev (Std Error))
Control	0.25583 $\pm$ 0.014632 (0.004877)
Monocrotophos	0.0555 $\pm$ 0.010643 (0.003548)
Carbamazepine	0.1366 $\pm$ 0.011219 (0.00374)



(*** p < 0.001)

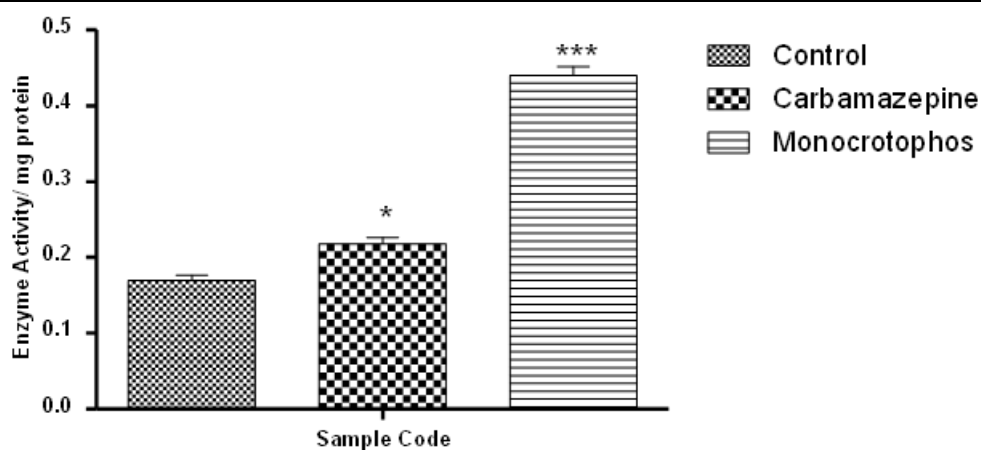
Figure 2.4: Graphical representation depicting the glutathione reductase activity of monocrotophos- and carbamazepine-treatment against control

2.4.2d Glutathione S-Transferase (GST) Assay

In the Glutathione S-Transferase (GST) assay, the reaction is quantified by observing the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH), as indicated by an increase in absorbance at 340 nm. One unit of enzyme activity is defined as the amount that catalyses the conjugation of 10.0 nmol of CDNB with reduced glutathione per minute at 25°C. The results demonstrate a statistically significant increase in enzyme activity in both the monocrotophos and carbamazepine treatment groups compared to the control group, suggesting an elevation in oxidative stress and enhanced activation of detoxification mechanisms within the test samples. Table 2.5 and Figure 2.5 illustrates the estimation of GST activity in the samples, with the Y-axis representing enzyme activity per milligram of protein and the X-axis representing the treated samples.

Table 2.5: Estimation of GST

Experiment	Enzyme Activity/mg Protein (Mean $\pm$ Stdev (Std Error))
Control	0.16935 $\pm$ 0.013368 (0.004456)
Monocrotophos	0.44041 $\pm$ 0.019889 (0.00663)
Carbamazepine	0.21805 $\pm$ 0.014326 (0.004775)



(*p < 0.05, *** p < 0.001)

Figure 2.5: Graphical representation depicting the glutathione s-transferase activity of monocrotophos- and carbamazepine-treatment against control

2.4.3 Transcriptome analysis of genes involved in biochemical process and detoxification mechanism

Transcriptomic analysis has identified the differential expression of several genes associated with biochemical processes and detoxification mechanisms as a result of exposure to both monocrotophos and carbamazepine. These intoxicants have led to the altered expression of genes involved in the synthesis of SOD, AChE, and GSH. The DGE analysis further revealed the differential expression of numerous genes implicated in detoxification mechanisms. Table 2.6a and Table 2.6b present the differentially expressed genes related to the expression of SOD, AChE, and GSH for monocrotophos and carbamazepine, respectively. While the expression of particular genes cannot be directly equated with actual protein expression, the comparison of specific gene expression in monocrotophos- and carbamazepine-treated sets yields relative expression data that can be considered analogous to the actual outcomes of biochemical assays.

Tables 2.7a and 2.7b enumerate the major gene families exhibiting differential expression involved in the detoxification mechanisms of monocrotophos and carbamazepine, respectively. Additionally, a comprehensive list of detoxification and stress signalling genes showing differential expression due to monocrotophos and carbamazepine exposure, along with their respective functions and Gene Ontology, is provided in Table 2.8a and Table 2.8b, respectively. In both scenarios, it has been observed that nearly all genes associated with detoxification are upregulated following exposure to monocrotophos and carbamazepine. This suggests a direct impact on the homeostasis of *Chrysomya megacephala*. Figure 2.6a and Figure 2.6b present heatmaps illustrating the differentially expressed genes, identified based on higher statistical significance, that are implicated in the detoxification process because of exposure to monocrotophos and carbamazepine, respectively.

Table 2.6a: Differentially expressed genes associated with the expression of enzymes in biochemical assays conducted on *Monocrotophos*-exposed *Chrysomya megacephala*

Assay/Enzyme	Genes	Significantly Down-regulated ▼	All Down-regulated ▼	Significantly Up-regulated ▲	All Up-regulated ▲
Superoxide Dismutase (SOD)	1 extracellular superoxide dismutase 1 [Cu-Zn]	0	2	0	3
	2 superoxide dismutase 2 [Mn]	0	0	0	1
	3 superoxide dismutase 1 [Cu-Zn]	0	0	0	1
	4 copper chaperone for superoxide dismutase	0	1	0	2
Acetylcholinesterase (AChE)	1 acetylcholinesterase	0	3	0	1
Glutathione S-transferase (GST)	1 glutathione S-transferase 1	0	0	1	8
	2 microsomal glutathione S-transferase 1	0	2	0	3
	3 glutathione S-transferase theta-1	0	2	0	5
	4 Glutathione S-transferase E14	0	0	0	1
	5 glutathione S-transferase C-terminal domain-containing protein	0	0	0	1

Table 2.6b: Differentially expressed genes associated with the expression of enzymes in biochemical assays conducted on Carbamazepine-exposed *Chrysomya megacephala*

Assay/Enzyme	Genes	Significantly Down-regulated ▼	All Down-regulated ▼	Significantly Up-regulated ▲	All Up-regulated ▲
Superoxide Dismutase (SOD)	1 extracellular superoxide dismutase 1 [Cu-Zn]	0	2	0	3
	2 superoxide dismutase 2 [Mn]	0	0	0	1
	3 superoxide dismutase 1 [Cu-Zn]	0	0	0	1
	4 copper chaperone for superoxide dismutase	0	0	0	3
Acetylcholinesterase (AChE)	1 acetylcholinesterase	0	1	0	3
Glutathione S-transferase (GST)	1 glutathione S-transferase 1	0	2	1	11
	2 microsomal glutathione S-transferase 1	0	2	0	3
	3 glutathione S-transferase theta-1	0	2	0	5
	4 Glutathione S-transferase E14	0	0	0	1
	5 glutathione S-transferase C-terminal domain-containing protein	0	0	0	1

Table 2.7a: Gene families demonstrating varied expression patterns associated with the detoxification mechanisms in *Chrysomya megacephala* subjected to monocrotophos exposure

Gene Family	Significantly down-regulated	All Down-regulated	Significantly up-regulated	All Up-regulated
Acetylcholinesterase	0	3	0	1
Heat shock protein 70	0	0	0	1
Esterase	0	4	0	22
Cytochrome P450	1	51	2	68
Metallothionein-like protein	0	2	0	1
Hydroxyacylglutathione hydrolase	0	0	0	2
UDP-glucuronosyltransferase	0	2	0	2
Glutathione S-transferase	0	4	1	19
Phospholipid hydroperoxide glutathione peroxidase	0	0	0	2
Multidrug resistance-associated protein	1	19	0	3
Dual oxidase	0	2	0	0
Cytochrome b5	1	7	0	13
Ubiquinol-cytochrome-c reductase	0	1	0	1
Oxidation resistance protein	0	2	0	0
Nose resistant to fluoxetine protein	0	3	0	4
Superoxide dismutase	0	3	0	7
Peroxidasin	0	0	0	1
Sur	0	1	0	0
Alkyl hydroperoxide reductase	0	0	0	1
Catalase	0	0	0	2
Peroxioredoxin	0	1	0	6
Thioredoxin reductase	0	3	0	2
Myeloid differentiation primary response protein	0	1	0	4

Juvenile hormone epoxide hydrolase	0	0	0	3
Sulfiredoxin	0	0	0	2
Tetracycline resistance protein	0	1	0	1
Catecholamines up	0	0	0	1
UV radiation resistance-associated gene protein	0	0	0	2
Mitochondrial amidoxime reducing component	0	0	0	2
Kelch-like ECH-associated protein	0	0	0	1
Zinc/cadmium resistance protein	0	0	0	1
Apoptosis-resistant E3 ubiquitin protein ligase	0	1	0	0
Cytochrome b560	0	0	0	1
Cytochrome b561	0	0	0	5

Table 2.7b: Gene families demonstrating varied expression patterns associated with the detoxification mechanisms in *Chrysomya megacephala* subjected to carbamazepine exposure

Gene Family	Significantly down-regulated	All Down-regulated	Significantly up-regulated	All Up-regulated
Acetylcholinesterase	0	1	0	3
Heat shock protein 70	0	1	0	0
Esterase	0	9	0	18
Cytochrome P450	0	39	2	82
Metallothionein-like protein	0	2	0	1
Hydroxyacylglutathione hydrolase	0	0	0	2
UDP-glucuronosyltransferase	0	1	0	3
Glutathione S-transferase	0	4	0	20
Phospholipid hydroperoxide glutathione peroxidase	0	0	0	2

Multidrug resistance-associated protein	0	7	0	1
Dual oxidase	0	2	0	0
Cytochrome b5	0	6	0	13
Ubiquinol-cytochrome-c reductase	0	1	0	1
Oxidation resistance protein	0	2	0	0
Nose resistant to fluoxetine protein	0	6	0	0
Superoxide dismutase	0	2	0	8
Peroxidase	0	1	0	0
Sur	0	1	0	0
Catalase	0	0	0	3
Peroxiredoxin	0	2	0	5
Thioredoxin reductase	0	0	0	5
Myeloid differentiation primary response protein	0	4	0	1
Juvenile hormone epoxide hydrolase	0	1	0	2
Sulfiredoxin	0	1	0	1
Tetracycline resistance protein	0	0	0	2
Catecholamines up	0	0	0	1
UV radiation resistance-associated gene protein	0	2	0	0
Mitochondrial amidoxime reducing component	0	1	0	2
Kelch-like ECH-associated protein	0	0	0	1
Zinc/cadmium resistance protein	0	0	0	1
Apoptosis-resistant E3 ubiquitin protein ligase	0	1	0	0
Cytochrome b560	0	0	0	1
Cytochrome b561	1	3	0	2

Table 2.8a: Differentially Expressed Genes related to the detoxification mechanism and stress signalling in *Chrysomya megacephala* exposed to monocrotophos

Function	Genes		Significantly Down-regulated ▼	All Down-regulated ▼	Significantly Up-regulated ▲	All Up-regulated ▲
Detoxification GO:0098754	1	Peroxisredoxin 1	0	0	0	1
	2	cytochrome P450 6v1	0	0	0	1
	3	cytochrome P450 6g1	0	1	0	3
	4	Copper chaperone for superoxide dismutase	0	1	0	2
	5	Sulfiredoxin	0	0	0	2
	6	Catecholamines up	0	0	0	1
	7	Mitochondrial amidoxime reducing component	0	0	0	2
	8	Superoxide dismutase 2 (Mn)	0	0	0	1
	9	Superoxide dismutase 1	0	2	0	4
Detoxification of inorganic compound GO:0061687	1	Catecholamines up	0	0	0	1
Detoxification of nitrogen compound GO:0051410	1	Mitochondrial amidoxime reducing component	0	0	0	2
Cellular detoxification GO:1990748	1	Peroxisredoxin 1	0	0	0	1
	2	Sulfiredoxin	0	0	0	2
	3	Peroxisredoxin 2	0	0	0	1
	4	Superoxide dismutase 1	0	2	0	4

	5	Mitochondrial amidoxime reducing component	0	0	0	2
	6	Copper chaperone for superoxide dismutase	0	1	0	2
	7	Superoxide dismutase 2 (Mn)	0	0	0	1
Cellular oxidant detoxification GO:0098869	1	Peroxiredoxin 1	0	0	0	1
	2	Sulfiredoxin	0	0	0	2
	3	Peroxiredoxin 2	0	0	0	1
	4	Superoxide dismutase 1	0	2	0	4
	5	Copper chaperone for superoxide dismutase	0	1	0	2
	6	Superoxide dismutase 2 (Mn)	0	0	0	1
Removal of superoxide radicals GO:0019430	1	Peroxiredoxin 1	0	0	0	1
	2	Copper chaperone for superoxide dismutase	0	1	0	2
	3	Peroxiredoxin 2	0	0	0	1
	4	Superoxide dismutase 2 (Mn)	0	0	0	1
	5	Superoxide dismutase 1	0	2	0	4
Hydrogen peroxide catabolic process GO:0042744	1	Peroxiredoxin 1	0	0	0	1
	2	Peroxiredoxin 2	0	0	0	1
	3	Peroxiredoxin 5	0	1	0	2
	4	Catalase	0	0	0	2
Response to toxic substance GO:0009636	1	Peroxiredoxin 1	0	0	0	1
	2	Peroxiredoxin 2	0	0	0	1
	3	Superoxide dismutase 1	0	2	0	4
	4	cytochrome P450 6v1	0	0	0	1
	5	cytochrome P450 6g1	0	1	0	3
	6	Myd88	0	1	0	4

	7	Sulfiredoxin	0	0	0	2
	8	Mitochondrial amidoxime reducing component	0	0	0	2
	9	Copper chaperone for superoxide dismutase	0	1	0	2
	10	Cytochrome P450 6a2	0	0	1	3
	11	Catecholamines up	0	0	0	1
	12	Cytochrome P450 12d1 proximal	0	0	0	1
	13	Superoxide dismutase 2 (Mn)	0	0	0	1
JNK cascade GO:0007254	1	ebi	0	0	0	1
	2	eiger	0	0	0	3
	3	parkin	0	1	0	1
	4	Ubiquitin-related modifier 1	0	1	0	0
	5	Rac2	0	0	0	1
	6	hemipterous	0	1	0	0
	7	Rac1	0	1	0	2
	8	Death-associated inhibitor of apoptosis 1	0	2	0	3
	9	Tankyrase	0	1	0	0
	10	grindelwald	0	0	0	2
	11	roadkill	0	3	0	1
	12	misshapen	0	1	0	0

Table 2.8b: Differentially Expressed Genes related to the detoxification mechanism and stress signalling in *Chrysomya megacephala* exposed to carbamazepine

Function		Genes	Significantly Down-regulated ▼	All Down- regulated ▼	Significantly Up- regulated ▲	All Up- regulated ▲
Detoxification GO:0098754	1	Peroxiredoxin 1	0	0	0	1
	2	cytochrome P450 6v1	0	0	0	1
	3	cytochrome P450 6g1	0	1	1	3
	4	Copper chaperone for superoxide dismutase	0	1	0	2
	5	Sulfiredoxin	0	0	0	2
	6	Catecholamines up	0	0	0	1
	7	Mitochondrial amidoxime reducing component	0	0	0	2
	8	Superoxide dismutase 2 (Mn)	0	0	0	1
	9	Superoxide dismutase 1	0	2	0	4
Detoxification of inorganic compound GO:0061687	1	Catecholamines up	0	0	0	1
Detoxification of nitrogen compound GO:0051410	1	Mitochondrial amidoxime reducing component	0	0	0	2
Cellular detoxification	1	Peroxiredoxin 1	0	0	0	1

GO:1990748	2	Sulfiredoxin	0	0	0	2
	3	Peroxiredoxin 2	0	0	0	1
	4	Superoxide dismutase 1	0	2	0	4
	5	Mitochondrial amidoxime reducing component	0	0	0	2
	6	Copper chaperone for superoxide dismutase	0	1	0	2
	7	Superoxide dismutase 2 (Mn)	0	0	0	1
Cellular oxidant detoxification GO:0098869	1	Peroxiredoxin 1	0	0	0	1
	2	Sulfiredoxin	0	0	0	2
	3	Peroxiredoxin 2	0	0	0	1
	4	Superoxide dismutase 1	0	2	0	4
	5	Copper chaperone for superoxide dismutase	0	1	0	2
	6	Superoxide dismutase 2 (Mn)	0	0	0	1
Removal of superoxide radicals GO:0019430	1	Peroxiredoxin 1	0	0	0	1
	2	Copper chaperone for superoxide dismutase	0	1	0	2
	3	Peroxiredoxin 2	0	0	0	1
	4	Superoxide dismutase 2 (Mn)	0	0	0	1
	5	Superoxide dismutase 1	0	2	0	4
	1	Peroxiredoxin 1	0	0	0	1

Hydrogen peroxide catabolic process GO:0042744	2	Peroxiredoxin 2	0	0	0	1
	3	Peroxiredoxin 5	0	1	0	2
	4	Catalase	0	0	0	2
Response to toxic substance GO:0009636	1	Peroxiredoxin 1	0	0	0	1
	2	Peroxiredoxin 2	0	0	0	1
	3	Superoxide dismutase 1	0	2	0	4
	4	cytochrome P450 6v1	0	0	0	1
	5	cytochrome P450 6g1	0	1	1	3
	6	Myd88	0	1	0	4
	7	Sulfiredoxin	0	0	0	2
	8	Mitochondrial amidoxime reducing component	0	0	0	2
	9	Copper chaperone for superoxide dismutase	0	1	0	2
	10	Cytochrome P450 6a2	0	0	1	3
	11	Catecholamines up	0	0	0	1
	12	Cytochrome P450 12d1 proximal	0	0	0	1
	13	Superoxide dismutase 2 (Mn)	0	0	0	1
JNK cascade GO:0007254	1	ebi	0	0	0	1
	2	eiger	0	0	0	3
	3	parkin	0	1	0	1

	4	Ubiquitin-related modifier 1	0	0	0	1
	5	Rac2	0	0	0	1
	6	hemipterous	0	2	0	1
	7	Rac1	0	2	0	1
	8	Death-associated inhibitor of apoptosis 1	0	4	0	1
	9	Tankyrase	0	1	0	0
	10	grindelwald	0	0	0	2
	11	roadkill	0	4	0	0
	12	misshapen	0	1	0	0

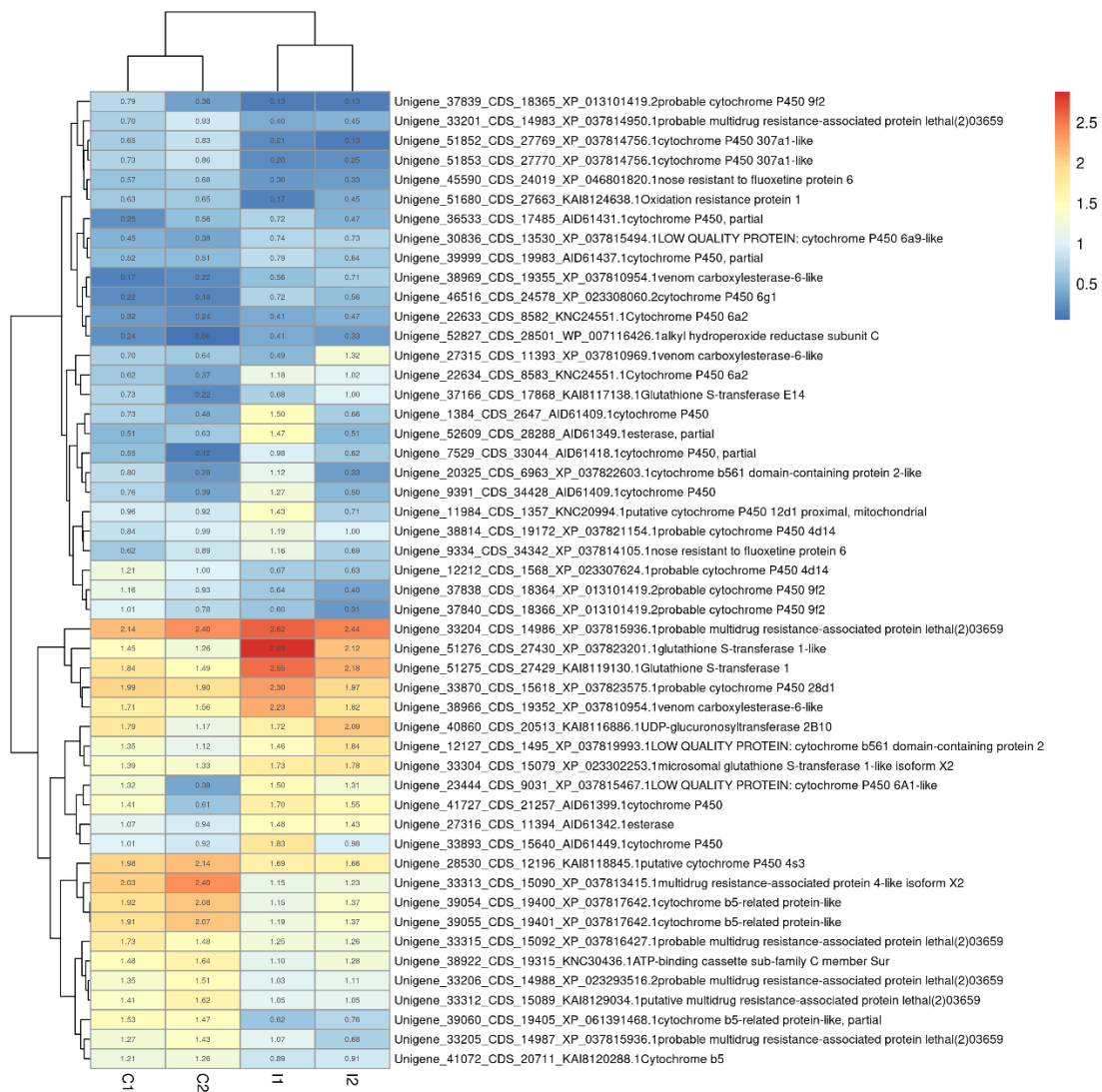


Figure 2.6a: Heatmap representing differentially expressed genes involved in detoxification mechanism due to monocrotophos exposure, where shades of blue represent downregulated genes and shades of red represent highly expressed genes (C- Control, I- Monocrotophos)

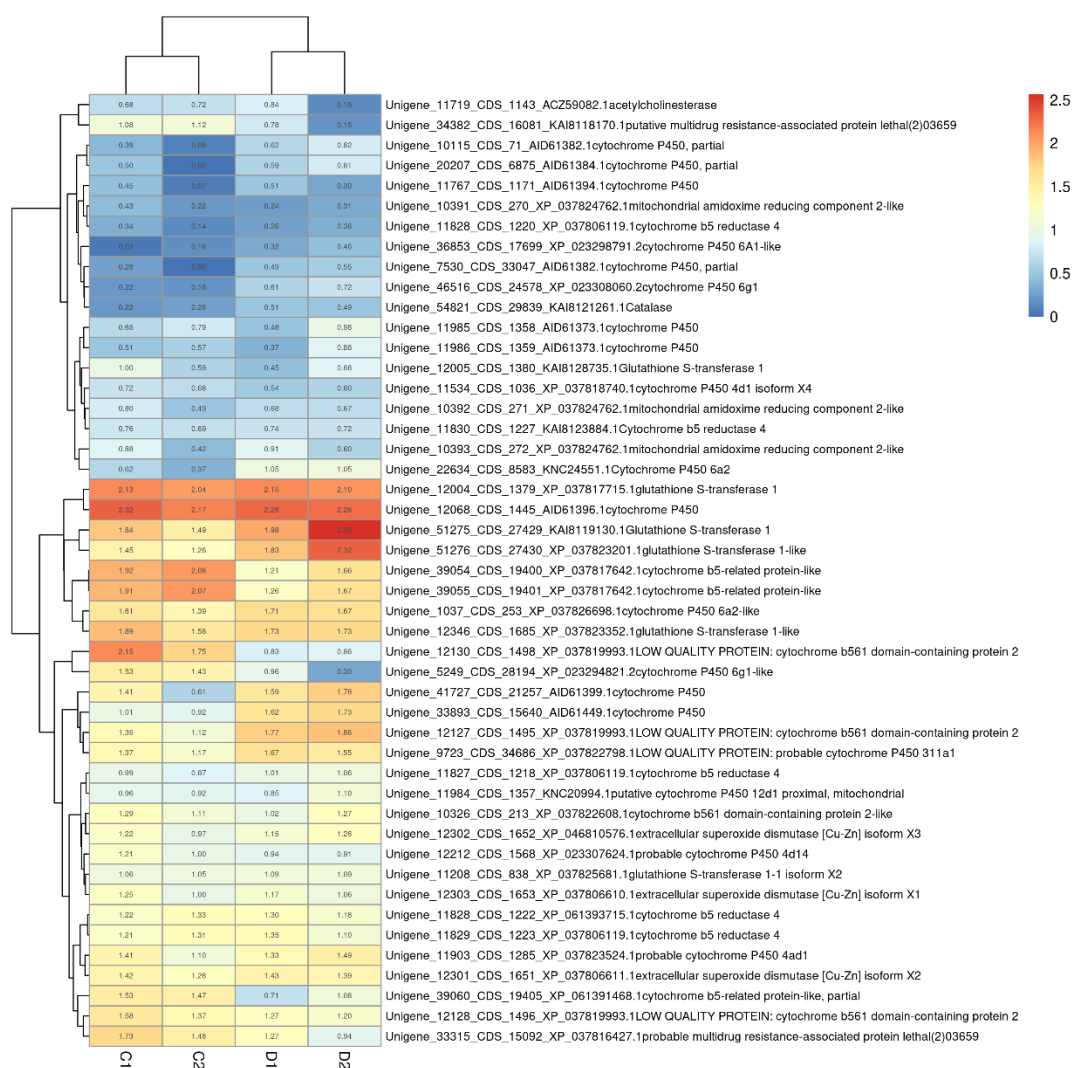


Figure 2.6b: Heatmap representing differentially expressed genes involved in detoxification mechanism due to carbamazepine exposure, where shades of blue represent downregulated genes and shades of red represent highly expressed genes (C- Control, D- Carbamazepine)

2.5 DISCUSSION

Insects encounter a variety of toxins, known as xenobiotics, throughout their lifecycle. These toxins are either naturally produced by plants, often referred to as allelochemicals, or synthetically produced by humans in the form of insecticides. To withstand these natural toxins, insects have developed various detoxification mechanisms (Aioub & Ashour, 2023). Upon exposure to toxins, insects initiate a detoxification program, which is activated across multiple organs and cells to enhance their tolerance to the toxins. The molecular mechanisms underlying this program within the insect body have been extensively studied and are well understood (Yang et al., 2024). In mammals, detoxification processes are typically divided into three phases: Phase I (functionalization), Phase II (conjugation), and Phase III (excretion). This classification is equally applicable to insects (Kshatriya & Gershenzon, 2024). Detoxification is thus categorized into three phases: Phase I, Phase II (involving metabolizing enzymes), and Phase III (involving transporters). Insect herbivores commonly possess detoxification enzymes from several major families. These enzymes play a crucial role in transforming toxins by attaching polar substituents, which diminishes their toxicity, enhances their solubility in water, and promotes their elimination from the organism.

This chapter presents an examination of the biochemical assays of enzymes pertinent to Phase I, specifically superoxide dismutase (SOD) and acetylcholinesterase (AChE), as well as Phase II, including glutathione reductase (GR) and glutathione S-transferase (GST) in the non-target organism *Chrysomya megacephala*. The chapter further explores the differential gene expression associated with detoxification processes in response to exposure to monocrotophos and carbamazepine.

The induction of excessive reactive oxygen species (ROS) in insects is attributed to their exposure to various stress factors throughout their lifespan. In response, insects have evolved a complex network of enzymatic antioxidant systems to mitigate the effects of ROS (Felton & Summers, 1995). In insects, enzymes such as superoxide dismutase (SOD) and glutathione S-transferases (GSTs) function as antioxidants. SOD is regarded as a primary defence mechanism against oxidative damage, while

GST serves as an early indicator of the induction of the detoxification system. Superoxide dismutase (SOD) is an enzyme involved in detoxification, facilitating the conversion of superoxide anions, which are reactive oxygen species, into oxygen and hydrogen peroxide (Yamamoto & Yamaguchi, 2022).

SOD activity in all insecticide-treated groups increases with higher concentrations. However, capacity of SOD to scavenge O_2^- is limited, and when O_2^- generation surpasses scavenging capacity of SOD, its activity decreases at higher concentrations of plant extracts and chemicals (Kolawole et al., 2014). Reduced SOD activity leads to accumulation of ROS, causing oxidative damage in insecticide-stressed organisms and promoting the accumulation of O_2^- and H_2O_2 . The current study presents analogous findings, wherein the levels of superoxide dismutase (SOD) and glutathione reductase (GR) are markedly diminished, and the levels of glutathione S-transferase (GST) are significantly elevated in the biochemical assays of *Chrysomya megacephala* larvae subjected to monocrotophos and carbamazepine treatments. This suggests an increase in oxidative stress within the organism because of intoxication. Notably, the elevation in GST levels was more pronounced in the group exposed to monocrotophos compared to the group exposed to carbamazepine. Transcriptome analysis of the same treatment groups indicated an overall upregulation of genes encoding both SOD and GST. In the case of SOD, this contradiction may be attributed to post-transcriptional regulation. Beyond transcriptional control, epigenetic regulation and post-transcriptional modifications significantly influence the modulation of functional SOD levels (Miao & St. Clair, 2009). These processes involve the stabilization of SOD mRNAs and the regulation of their translation. Formetanate (insecticide) exposure caused a decrease in SOD and an increase in GST in *Apis mellifera* (Staroň et al., 2017) and the activity of superoxide dismutase (SOD) was significantly decreased by abamectin, indoxacarb and spinosad in *Callosobruchus maculatus* (Kolawole et al., 2014). GST activities were significantly increased and the SOD activity was decreased in *Ostrinia nubilalis* treated with chlorantraniliprole insecticide (Franeta et al., 2018).

The impact of sublethal concentrations of monocrotophos on the nematode *Caenorhabditis elegans* resulted in the inhibition of acetylcholinesterase activities (75–86%) and carboxylesterase activities (76–81%) (Leelaja & Rajini, 2013). These alterations were accompanied by notable oxidative impairments, as evidenced by an increase in reactive oxygen species, a decline in glutathione content, and a decrease in superoxide dismutase activity. Exposure to monocrotophos in the rat brain, even at low doses, resulted in an increase in reactive oxygen species and a decrease in the activities of glutathione, superoxide dismutase (SOD), catalase, and cholinesterase (Karumuri et al., 2019). Sublethal doses of dimethoate in *Bombyx mori* also resulted in a similar decrease in the levels of SOD and glutathione reductase (GR). This suggests that dimethoate may have disrupted the reactive oxygen species (ROS) equilibrium, favouring the generation of free radicals, which consequently resulted in extensive damage to the insect organism (Maslakova & Silivanova, 2025). Severe diquat poisoning induces an increase in reactive oxygen species (ROS) production, thereby overwhelming the cellular protective mechanisms (Magalhães et al., 2018). These mechanisms include nonenzymatic components, as well as antioxidant enzymes including superoxide dismutase (SOD). The overwhelming of these defences results in oxidative stress and, consequently, apoptosis (Sule et al., 2022).

The chronic exposure to carbamazepine caused the reduction in the activities of superoxide dismutase (SOD) and glutathione reductase (GR) in *Corbicula fluminea* (Chen et al., 2014). The concurrent presence of carbamazepine and Cu in *Danio rerio* intensified the impact on the antioxidant system, as evidenced by the inhibition of hydroxyl radicals (IHR), superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) (Jia et al., 2020). However, this combination did not affect the nervous system, specifically Acetylcholinesterase (AChE).

Biochemical assays in the current study also revealed a reduction in acetylcholinesterase (AChE) levels in both treatment groups compared to the control group. Notably, a statistically significant decrease was observed in the monocrotophos treatment group. This finding aligns with the toxicologically relevant mode of action of monocrotophos, which involves the inhibition of cholinesterase, an enzyme critical

for proper nerve function in insects (Skripsky & Loosli, 1994). Additionally, transcriptome analysis corroborated these findings with supporting data on gene expression. In *Danio rerio*, high concentrations of carbamazepine have been shown to inhibit acetylcholinesterase activity, resulting in increased acetylcholine levels (Siebel et al., 2010). Chronic exposure to carbamazepine in rats yielded similar results (Sudha et al., 1995). Exposure of the stonefly, *Claassenia sp.*, to the organophosphate insecticide chlorpyrifos resulted in a significant reduction in acetylcholinesterase (AChE) levels, ranging from 30.7% to 45.1%, at concentrations nearing lethality (Day & Scott, 1990).

Transcriptomic analysis has identified the differential expression of several genes associated with the detoxification of monocrotophos and carbamazepine in *Chrysomya megacephala*. Although both upregulation and downregulation of detoxifying genes were observed, most genes involved in detoxification were found to be upregulated. This suggests the activation of various detoxification mechanisms in response to monocrotophos and carbamazepine, corroborating the findings of biochemical assays.

2.6 KEY FINDINGS

- ❖ The enzymatic biochemical assays showed an increased oxidative stress, potential cellular damage and enhanced activation of detoxification mechanisms within the test samples.
- ❖ Transcriptomic analysis identified multiple gene copies associated with stress signalling and detoxification mechanisms as being differentially expressed.
- ❖ Although both upregulation and downregulation of detoxifying genes were observed, most genes were upregulated suggesting the activation of various detoxification mechanisms in response to monocrotophos and carbamazepine, corroborating the findings of biochemical assays.

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Chapter 3

***De Novo* Transcriptomic Analysis of *Chrysomya megacephala*: Investigating the Effects of Sublethal Exposure to Monocrotophos and Carbamazepine**

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3

***De Novo* Transcriptomic Analysis of *Chrysomya megacephala*: Investigating the Effects of Sublethal Exposure to Monocrotophos and Carbamazepine**

3.1 INTRODUCTION

Advances in biology, particularly molecular biology, computer science, mathematics, and bioinformatics, have significantly fostered the development of various “omics” techniques like genomics (genome or DNA study), transcriptomics (transcriptome or mRNA study), proteomics (proteome or proteins study), lipidomics (lipidome or lipids study), metabolomics/metabonomics (metabolome or metabolites/small molecules study), adductomics (DNA adducts from xenobiotic exposure study), and epigenomics (epigenome study) which are vital in systems toxicology (Joseph, 2017). Transcriptomics is the study of the 'transcriptome,' a term commonly recognised as encompassing the full array of ribonucleic acid (RNA) molecules, also known as transcripts, that are expressed within a particular biological unit, such as a cell, tissue, or organism (Skerrett-Byrne Anthony et al., 2022). Transcriptome analysis employs high-throughput methods to investigate the complete set of RNA transcripts produced by the genome under specific conditions or within a particular cell, which are primarily employed to examine specific sample pairs and often involve varying external conditions, such as hormone effects or toxin exposure (Blumenberg, 2019).

The field of forensic entomology has seen significant advancements through the integration of omics technology (Shao et al., 2023). This cutting-edge approach has enhanced research in various areas, including species identification, phylogenetic analysis, developmental biology, and behavioural studies of insects. As a result, new methods for estimating the postmortem interval (PMI) have emerged, leading to

improved accuracy in determining the time of death. These developments have played a crucial role in accelerating the progress of forensic entomology as a discipline.

Kinaret et al., (2020) stated that unlike conventional toxicity assessments which depend on extensive *in vivo* phenotypic observations and supportive *in vitro* models, the continuous development of new materials and chemicals requires a more profound understanding of molecular changes in exposed biological systems. Therefore, by scrutinising molecular alterations, transcriptomics enables the study of organisms' responses to environmental, chemical, and physical agents. The discussion of hepatotoxicity and pulmonary toxicity induced by xenobiotic exposure serves as a specific example to illustrate the potential application of transcriptomics, or global gene expression analysis, in preventing adverse health effects (Joseph, 2017). Toxicogenomics research conducted in various laboratories worldwide demonstrates that the crucial steps and sequence of molecular events can be examined to provide comprehensive insights into toxic mechanisms (Heijne et al., 2005). Thus, it has enabled the more precise and earlier identification of adverse effects animal toxicity studies. Recent advances in DNA sequencing technology, and the relative affordability of these technologies, have dramatically increased the generation of sequence data of non-model organisms, offering significant potential for species of medical, veterinary, and economic importance (Carvalho et al., 2010).

Molecular biologists often focus on identifying which genes are expressed or suppressed in cells under different environmental conditions or following xenobiotic exposure, and the method of 'differential gene expression' (DGE/DEG) provides a methodical approach to generate libraries and microchip arrays of 'genetic signatures' that are unique to known enzyme activators and toxic xenobiotics (Rockett et al., 1999.). These signatures can subsequently be used to detect and analyse xenobiotics with unknown biological properties. The Eukaryotic Orthologous Groups (KOGs) constitute a eukaryote-specific adaptation of the Clusters of Orthologous Groups (COGs) framework, which is designed to identify orthologous and paralogous proteins (The KOG Browser, n.d.). Differentially expressed genes (DEGs) can be matched against the KOG database to categorise them into molecular families. The

KOG functional annotation process can highlight the most prevalent functional categories. Additionally, unigenes can be annotated using the KOG database through a BLAST search (De Alvarenga et al., 2005).

Ontology, within a specific domain of knowledge, functions as a structured representation of information. Typically, ontologies comprise a collection of concepts, terms, or classes along with the relationships that connect them. These frameworks offer a standardised vocabulary for expressing knowledge within a particular domain. Gene Ontology (GO) provides a comprehensive framework for understanding the biological domain in terms of molecular functions, cellular components, and biological processes associated with genes (Ashburner et al., 2000). As described by Tomczak et al., (2018), GO consists of two primary components: ontology structure and GO annotations. The ontology is organised as a hierarchical tree-like arrangement of concepts known as GO terms, and their interrelationships. GO annotations comprise a comprehensive list of all genes linked to specific ontology terms describing their characteristics. They also stated that GO enrichment analysis is widely employed to interpret high-throughput molecular data and formulate hypotheses about the underlying biological mechanisms in experimental studies.

The KEGG (Kyoto Encyclopedia of Genes and Genomes) database, initiated in 1995 by the Kanehisa Laboratory at Kyoto University's Bioinformatics Center, is an important bioinformatics resource that enables the prediction of protein interaction networks in various cellular processes (KEGG Pathway Enrichment Analysis, n.d.). It consolidates the latest insights into molecular interaction networks, which include pathways and complexes, with genomic information on genes and proteins sourced from genome projects (GENES / SSDB / KO database), alongside data on biochemical compounds and reactions. Consequently, KEGG pathway enrichment analysis is frequently employed in bioinformatics to functionally annotate differentially expressed genes, providing insights into their associated functions and pathways.

This chapter deals with the de novo transcriptomic profiling of the third instar larvae of *Chrysomya megacephala* to assess the potential genes and pathways altered by the sublethal concentrations of Carbamazepine and Monocrotophos and their effects on

development and various biological activities. The third instar larval stage comprises of 2 distinct phases: rapidly feeding phase, and a non-feeding migratory phase. The larvae for the analysis were selected at the point of transformation from the feeding to the non-feeding phase. At this stage of development, it is assumed that transcripts present in both the larval and upcoming pupal stages could be detected. The de novo profiling was performed because of the lack of any previously published genome of this species or any closely related species. Eukaryotic Orthologous Group (KOG), Gene Ontology (GO), and KEGG enrichment pathways of differentially expressed genes (DEGs) were analysed to get a better understanding on the altered genes and pathways due to the effects of carbamazepine and monocrotophos.

3.2 REVIEW OF LITERATURE

Integrating traditional toxicology with comprehensive analysis of extensive molecular and functional alterations across various biological levels offers a promising systems toxicology approach for examining the complete range of xenobiotic toxicity (Sturla et al., 2014). This methodology involves investigating and understanding all possible reactions within a biological system when exposed to xenobiotics. By combining classical toxicological methods with quantitative assessments of large-scale network changes, researchers can better understand how xenobiotics affect living organisms at multiple organisational levels (Joseph, 2017). Searfoss et al., (2005) stated that the major contribution of the genomics revolution in biomedical research is the establishment of transcriptome analysis as an essential technology in drug development and in the field of pre-clinical toxicology.

Omics techniques are increasingly being utilised in forensic entomology studies, offering valuable insights for species identification, phylogenetic analysis, detection of developmentally significant differentially expressed genes, and genetic-level interpretation of behavioral traits in forensically relevant species (Shao et al., 2023). These applications provide crucial data for estimating the postmortem interval (PMI). Nonetheless, the application of omics methodologies in forensic entomology remains constrained, particularly in the domains of transcriptomics, proteomics, and metabolomics.

A study conducted by Pi et al., (2024), on intrapuparial stage aging and postmortem interval (PMI) based on the developmental transcriptomes of forensically important *Aldrichina grahami* (Diptera: Calliphoridae) gene expression revealed that stage-specific DEGs and those with consistent expression trends could enhance the accuracy of age estimation for intrapuparial Calliphoridae, thereby improving PMI prediction. Furthermore, genes exhibiting similar expression patterns could be utilised as candidate variables to refine and enhance statistical models for PMI estimation. Using a multi-marker gene pool offers additional reference points for validating the PMI estimation results, potentially increasing the reliability of the process.

Another transcriptomic study on the cold tolerance of *Aldrichina grahami* by Liu et al., (2020) identified functional genes and revealed variations in their expression patterns across different temperatures. This study analyzed differentially expressed gene (DEG) series clusters with upward or downward trends, which may be crucial for cold tolerance. Furthermore, LCP, CUP, and HSP indicate additional potential adaptations to cold environments. This research will provide valuable insights for future investigations into the molecular mechanisms underlying cold tolerance in *A. grahami*.

The de novo transcriptome analysis of *Calliphora vicina* pupae by Zajac et al., (2015) across 15 distinct developmental stages, using a digital gene expression profiling method called Massive Analysis of cDNA Ends (MACE), serves as the basis for molecular age determination. This study provides detailed gene expression profiles for all the detected transcripts, enabling the identification of differentially expressed genetic markers as potential candidates for estimating the molecular age of *C. vicina* pupae. Furthermore, this analysis offers insights into gene activity during pupal development and elucidates the relationships between various genes relevant to insect development in general.

The molecular basis of organophosphate insecticide resistance in *Cochliomyia hominivorax* was studied by Tandonnet et al., (2020), who revealed altered and allele-specific expression of genes involved in many biological processes, including proteolysis, olfactory perception, and cuticle metabolism. Transcriptomic analysis has

verified that resistant specimens predominantly manifest the Trp251Ser mutation in esterase E3, which imparts resistance to organophosphate (OP) and pyrethroid insecticides. Genes related to metabolic and detoxification processes, especially cytochrome P450s, were notably downregulated in resistant individuals, except for esterases, which were upregulated. The findings imply that the resistant specimens may employ additional mechanisms, such as reduced insecticide penetration and the avoidance of dimethyl-OP contaminated food. The identified genes and processes lay a crucial foundation for future functional studies and should be considered to enhance the current pest control methods.

Carbamazepine is a pharmaceutical agent used in the management of various neurological and psychiatric disorders, including epilepsy, neuropathic pain, and manic-depressive illness (Maan et al., 2023). The clinical manifestations of toxicity include a spectrum of neurological and cardiovascular symptoms, ranging from mild effects such as cognitive impairment, localised weakness, and increased heart rate to severe complications including convulsions, unconsciousness, low blood pressure, and abnormal heart rhythms (Bridwell et al., 2022).

Research by Silva et al., (2024) on the genetic analysis of zebrafish in their early developmental stages revealed that Carbamazepine (CBZ) affects gene expression in proportion to its concentration. Although only 25 genes were affected, exposure to CBZ interfered with genes related to developmental processes (including *crygmx11*, *org*, *klf2a*, *otos*, *stx16* and *tob2*) and the nervous system (such as *Rtn3*, *Gdf10*, and *Rtn3*). In contrast, it activates genes linked to behavioural responses (e.g., *prlbr* and *taar*). These results indicate that the ecological effects of CBZ should be considered seriously, especially in real-world situations where aquatic ecosystems are continuously exposed to domestic wastewater discharge.

Monocrotophos (MCP), an organophosphate insecticide commonly applied to various crops, including rice, maize, sugarcane, cotton, soybeans, groundnuts, and vegetables, is considered an emerging contaminant that threatens terrestrial and aquatic ecosystems (Karthick et al., 2024). The effects of MCP on mammals and aquatic

organisms range from mild to severe, causing symptoms, such as confusion, anxiety, excessive salivation, seizures, and breathing difficulties.

A comprehensive transcriptome study conducted by Guo et al., (2017) on three species of aphids (*Acyrtosiphon pisum*, *Aphis gossypii*, and *Aphis glycines*) following MCP treatment revealed that across all three aphid species, there was an upregulation of genes associated with various functions. The activities encompass hydrolase activity, transmembrane transporter activity—primarily ion transmembrane transporter activity—oxidoreductase activity involving paired donors with the incorporation or reduction of molecular oxygen, protein dimerisation activity, and zinc ion binding.

3.3 MATERIALS AND METHODS

The transcriptomic workflow utilized independent biological replicates per group. This design satisfies standard high-dimensional sequence power criteria (power=0.8, $\alpha=0.05$), maximizing edgeR algorithmic sensitivity to precisely identify treatment-specific differential gene expression (DGE) patterns.

3.3.1 Chemicals

Analytic standards of Monocrotophos (Batch number: BCCC6539) and Carbamazepine (Lot number: MKCK3782) were purchased from Sigma-Aldrich. The sublethal concentrations of $1/3^{\text{rd}}$ of EC_{50} of Monocrotophos-I (0.077 $\mu\text{g/ml}$) and Carbamazepine- D (0.2 mg/ml) were used for transcriptome analysis.

3.3.2 RNA isolation & Library preparation

The culture of the test organism *Chrysomya megacephala* (strain DI212) was maintained in our laboratory, and the initial identification of the species was done using taxonomic keys (Old World Screw-Worm Fly: A Diagnostic Manual, 2017; Falk, 2016; Szpila, 2012; Whitworth, 2006, 2010) which was then confirmed by CO1 barcoding (Accession number: PQ136459).

The third-instar larvae of *Chrysomya megacephala* from both Control- C and Treatments;

- (i) Monocrotophos- I (96 hours of exposure via feed)
- (ii) Carbamazepine- D (96 hours of exposure via feed), were washed, immersed in Trizol reagent, and kept at -80°C.

RNA was isolated from the samples using the Trizol RNA isolation method. RNA quantity was measured using a Qubit® 4.0 fluorometer, and quality was analysed using 1% agarose gel. The paired-end sequencing library was prepared using the KAPA mRNA HyperPrep Kit for Illumina (CAT #KK8581).

The library preparation process was initiated with 500-1000 ng input. mRNA enrichment was performed as per the user manual, and mRNA was subjected to fragmentation, first & second-strand cDNA synthesis, end-repair, 3' adenylating, adapter ligation and low-quality data removal (Trim Galore version 0.6.4), selective enrichment of adapter-ligated DNA fragments through PCR amplification, followed by the validation of the library using the Agilent 4150 TapeStation (Agilent Technologies) with HSD100 ScreenTape, in accordance with the manufacturer's instructions. The final library was pooled with other samples, denatured, and loaded onto the flow cell. On the flowcell, cluster generation and sequencing were performed using the Illumina Novaseq 6000 platform to generate ~5-6 GB of 2×150bp paired-end (PE) clean reads from each library.

3.3.3 *De novo* assembly

As there is no published genome of this species or any closely related species, de novo assembly using all clean reads was done. Master/Combine assembly was performed taking high quality adapter trimmed and algal mapped reads of all samples together using Trinity (version 2.14.0) to generate common assembly for annotation as well as sample differential expression analysis.

3.3.4 Unigene prediction from the master assembly

Transcripts were further processed using the CD-HIT package for unigenes prediction. CD-HIT-EST executable was used to remove the shorter redundant transcripts when they were 100% covered by other transcripts with more than 90% identity (default parameter). The non-redundant clustered transcripts were then named as unigenes.

3.3.5 CDS prediction from unigenes and functional annotation of predicted CDS

CDS were predicted from the unigenes sequences using TransDecoder (version 5.6.0) at default parameters with the encoded protein length set to a minimum of 60 amino acids. The protein sequences corresponding to the predicted coding regions within the unigenes were subjected to a similarity search against NCBI's non-redundant (nr) database using the BLASTP algorithm with an e-value threshold of 1e-5. Simultaneously, all protein sequences were searched for similarity against Uniprot, KOG, and Pfam using BLASTP with an e-value threshold of 1e-5.

3.3.6 Differential expression analysis

The DGE analysis is done on the following combinations:

- (a) Control vs. Monocrotophos
- (b) Control vs. Carbamazepine

Reads of each sample were mapped separately to CDS sequences obtained from the master assembly using BWA, and PCR duplicate reads were removed from the alignment file using Picard's MarkDuplicates.jar. Subsequently, the reads aligning to each coding sequence (CDS) were computed for each sample utilizing the idxstats program of Samtools. These aligned reads were then employed for differential gene expression (DGE) analysis. The read-mapped count from each sample was provided, and ultimately, these mapped reads, as previously described, were utilized for DGE analysis. The read-mapped count from each sample was given as an input for differential expression analysis using the edgeR Bioconductor package in R.

3.3.7 Gene Ontology (GO) Sequence Distribution

The Gene Ontology (GO) project provides controlled vocabularies of defined terms representing gene product properties. These cover three domains: Cellular Component, the parts of a cell or its extracellular environment; Molecular Function, the elemental activities of a gene product at the molecular level, such as binding or catalysis; and Biological Process, operations or sets of molecular events with a defined beginning and end, pertinent to the functioning of integrated living units: cells, tissues, organs, and organisms. GO mapping was done to retrieve GO terms for all the BLASTP functionally annotated proteins against the NR database using OmicsBox.

GO sequence data for Differentially Expressed Genes (DEGs) were also obtained separately.

3.3.8 Pathway analysis

Ortholog assignment and mapping of the CDS and DEGs to the biological pathways were performed using the **KEGG Automatic Annotation Server (KAAS)**. All the CDS and DEGs were compared against the KEGG database using BLASTP with a threshold bit-score value of 60 (default). Results represented metabolic pathways of significant biomolecules such as carbohydrates, lipids, nucleotides, amino acids, glycans, cofactors, vitamins, terpenoids, polyketides, etc. The transcripts also represented the genes involved in metabolism, genetic information processing, environmental information processing and cellular processes.

3.4 RESULTS

3.4.1 RNA isolation & Library preparation

The initial step of transcriptome analysis is RNA isolation, followed by library preparation. For this, two independent replicates of all three samples, Control (C1, C2), Carbamazepine (D1, D2) and Monocrotophos (I1, I2), were used. The next-generation sequencing of the samples was performed on the Illumina platform. Statistics of raw data is shown in Table 3.1:

Table 3.1: Data statistics of next-generation sequencing

Sample	Total Reads in R1	Total Reads in R2	Total Reads (R1+R2)	Total Bases (R1+R2)	Total Data (GB)
Control 1 (C1)	2,00,88,885	2,00,88,885	4,01,77,770	6,38,82,65,430	6.3
Control 2 (C2)	1,88,79,399	1,88,79,399	3,77,58,798	6,00,36,48,882	6.0
Carbamazepine 1 (D1)	1,97,00,847	1,97,00,847	3,94,01,694	6,26,48,69,346	6.2
Carbamazepine 2 (D2)	1,52,82,476	1,52,82,476	3,05,64,952	4,85,98,27,368	4.8
Monocrotophos 1 (I1)	1,81,07,300	1,81,07,300	3,62,14,600	5,75,81,21,400	5.7
Monocrotophos 2 (I2)	1,74,88,687	1,74,88,687	3,49,77,374	5,56,14,02,466	5.5

3.4.2 *De novo* assembly

The clean reads from all samples were assembled to generate de novo assembled transcriptome. Using this reference, 84,444 transcript fragments ranging in length from 300 to 30,206 bp, with an average length of 1,283 bp and 2,014 bp long N50 (the length of the contig at 50% of the assembly arranged in descending order according to contig length) were identified. The statistical elements of the assembly are listed in Table 3.2

Table 3.2: Master assembly transcript statistics

Description	Statistics
Total no of transcripts	84,444
Total transcriptome size (bp)	10,83,42,937
Average transcript length (bp)	1,283
Maximum transcript length (bp)	30,206
Minimum transcript length (bp)	300
Transcript N50 (bp)	2014

3.4.3 Unigene prediction from the master assembly and CDS prediction from unigenes

Further processing of the master assembly revealed 58,073 unigenes ranging from 300 to 30,206 bp, with an average length of 1,109 bp and 1,751 bp long N50. Basic statistics for CDS prediction from unigene are given in Table 3.3

Table 3.3: CDS Statistics

Description	CDS Nucleotide	CDS Protein
Total no of CDS	34,922	34,922
Total CDS size (bp/aa)	3,25,02,474	1,08,34,158
Average CDS length (bp)	930	310
Maximum CDS length (bp)	27,804	9,268
Minimum CDS length (bp)	156	52

3.4.4 Functional Annotation of Predicted CDS

The top-hit species distribution revealed that *Lucilia cuprina* had the most hits, followed by *Lucilia sericata*. All protein sequences were searched for the similarity against NR, Uniprot, KOG and Pfam using BLASTP with an e-value threshold of $1e-5$. Results for similarity search against all 4 databases is depicted in Figure 3.1.

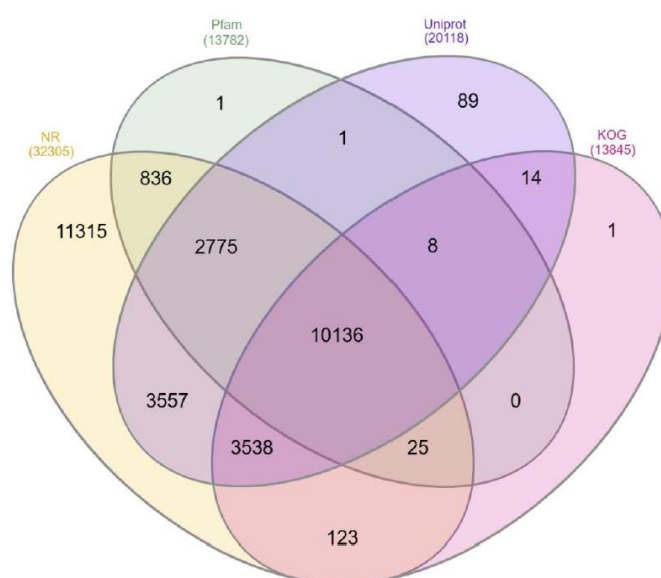


Fig 3.1: Venn diagram for annotated proteins in different databases

3.4.5 Differential Gene Expression

3.4.5a Control vs. Monocrotophos

A total of 17,865 genes showed differential expression in Control (C) vs sub-lethal Monocrotophos Treatment (I) of *Chrysomya megacephala*, among which 8,731 were downregulated and 9,134 were upregulated. This includes 30 significantly downregulated genes and 122 significantly upregulated genes, making it a total of 152 highly significant differentially expressed genes (DEGs).

Volcano plot (Figure 3.2a) and MA plot (Figure 3.2b) represent the DEGs of Control vs. Monocrotophos graphically. The volcano plot arranges the expressed genes along dimensions of biological as well as statistical significance where the red dots correspond to transcripts with q value < 0.05 . On the other hand, the MA Plot for DEGs visualises the differences by transforming the data onto M (log ratio) and A

(mean average) scales, then plotting these values. On the X-axis, the normalised counts for all the samples are plotted and, on the Y-axis, $\log_2$ foldchange is plotted. Red points represent q value less than 0.05, and black represents the q value is greater than 0.05.

The expression patterns of DEGs were shown as a Heatmap in Figure 3.3.

A list of transcripts was selected for heatmap generation based on the following criteria:

- ✓ Selection of highly significant 25 down and 25 upregulated transcripts based on q-value
- ✓ Transcripts must have proper annotations.

Heatmap package from R software was used for producing heatmap. The colour coding ranges from red to blue, where shades of red represent high transcripts expression and shades of blue represents low transcript expression.

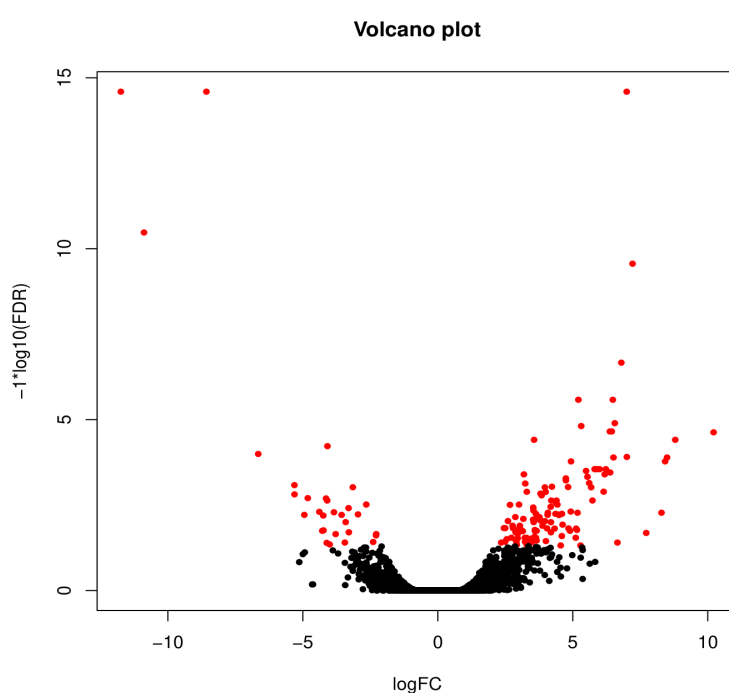


Figure 3.2a: Volcano plot of the distribution of expressed transcripts for Control vs. Monocrotophos. Red dots correspond to transcripts with q value < 0.05.

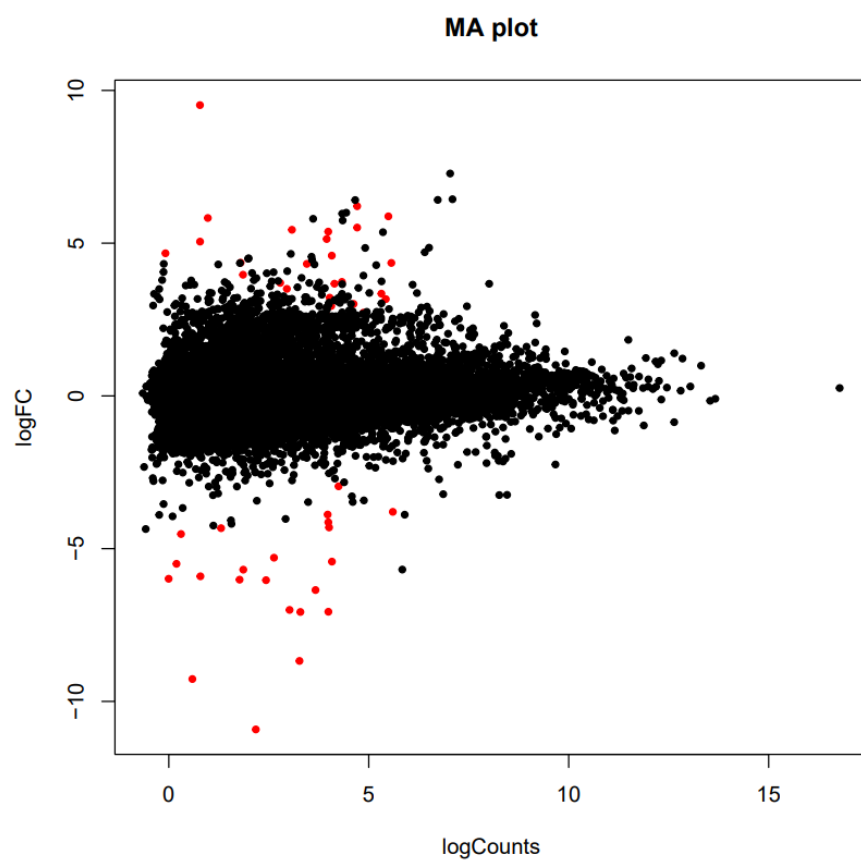


Figure 3.2b: MA plot showing differentially expressed transcripts for Control vs. *Monocrotophos*. On the X-axis, normalized counts for all the samples, and on Y-axis, $\log_2$ foldchange are plotted. Points are coloured with red if the q value is less than 0.05 and black if the q value is greater than 0.05.

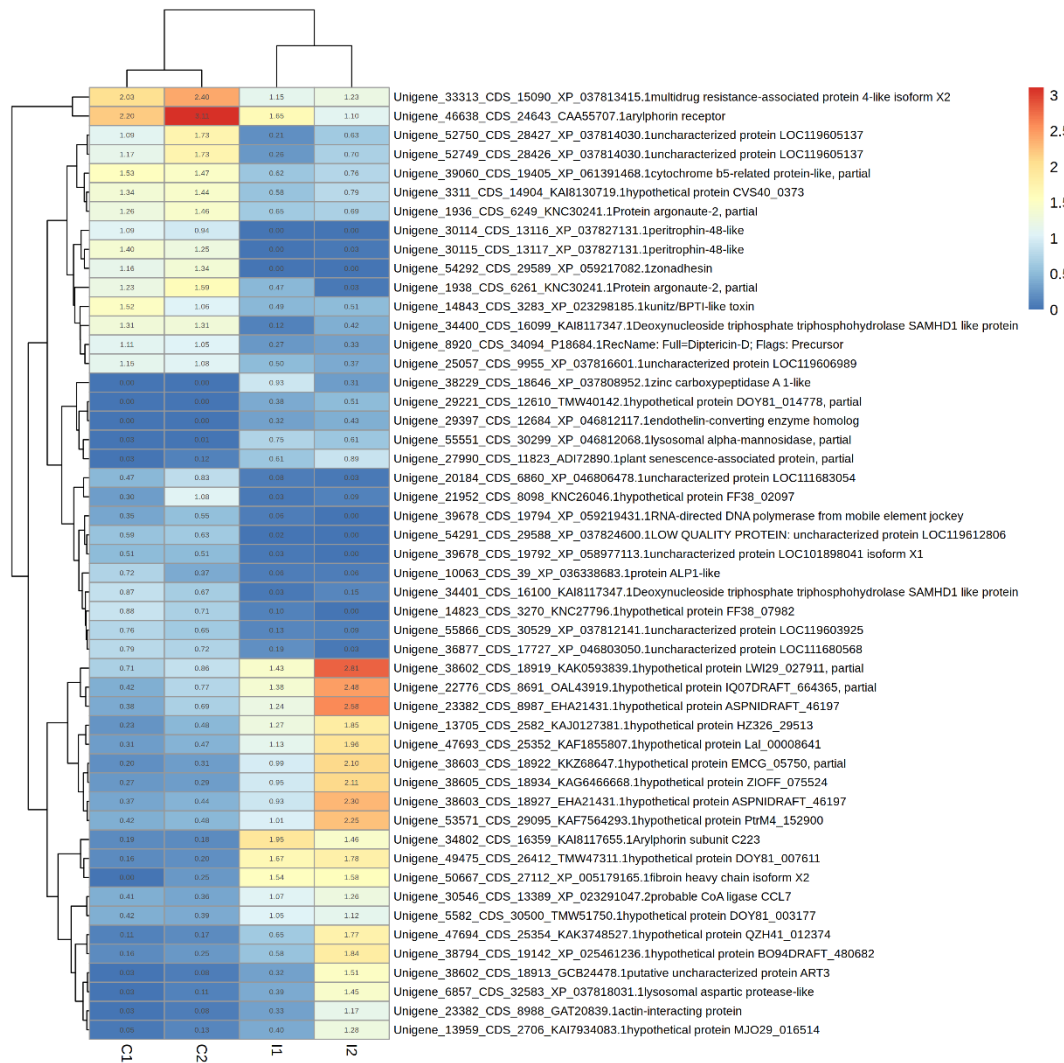


Figure 3.3: The Heatmap representing the most significant CDS expressed in both the samples was plotted using log10 of normalised read count values (CPM) for Control (C) vs. Monocrotophos (I), where shades of blue represent downregulated CDS and shades of red represent highly expressed CDS

3.4.5.b Control vs. Carbamazepine

A total of 17984 genes showed differential expression in Control (C) vs sub-lethal Carbamazepine Treatment (D) of *Chrysomya megacephala*, among which 9489 were downregulated and 8495 were upregulated. This includes 22 significantly downregulated genes and 26 significantly upregulated genes, making it a total of 152 highly significant differentially expressed genes (DEGs).

Volcano plot (Figure 3.4a) and MA plot (Figure 3.4b) are used to graphically represent the DEGs of Control vs. Monocrotophos. The volcano plot arranges the expressed

genes along dimensions of biological as well as statistical significance where the red dots correspond to transcripts with q value < 0.05 . On the other hand, MA Plot for DEGs visualises the differences by transforming the data onto M (log ratio) and A (mean average) scales, then plotting these values. On the X-axis, the normalized counts for all the samples are plotted, and on Y-axis, log2foldchange is plotted. Red points represent the q value less than 0.05, and black represents the q value is greater than 0.05.

The expression patterns of DEGs were shown as a Heatmap in Figure 3.5.

A list of transcripts was selected for heatmap generation based on the following criteria:

- ✓ Selection of highly significant 25 down and 25 upregulated transcripts based on q-value
- ✓ Transcripts must have proper annotations.

Heatmap package from R software was used for producing heatmap. The color coding ranges from red to blue where shades of red represent high transcripts expression and shades of blue represents low transcript expression.

The overall comparative expression of Control, Monocrotophos and Carbamazepine is shown in Figure 3.6.

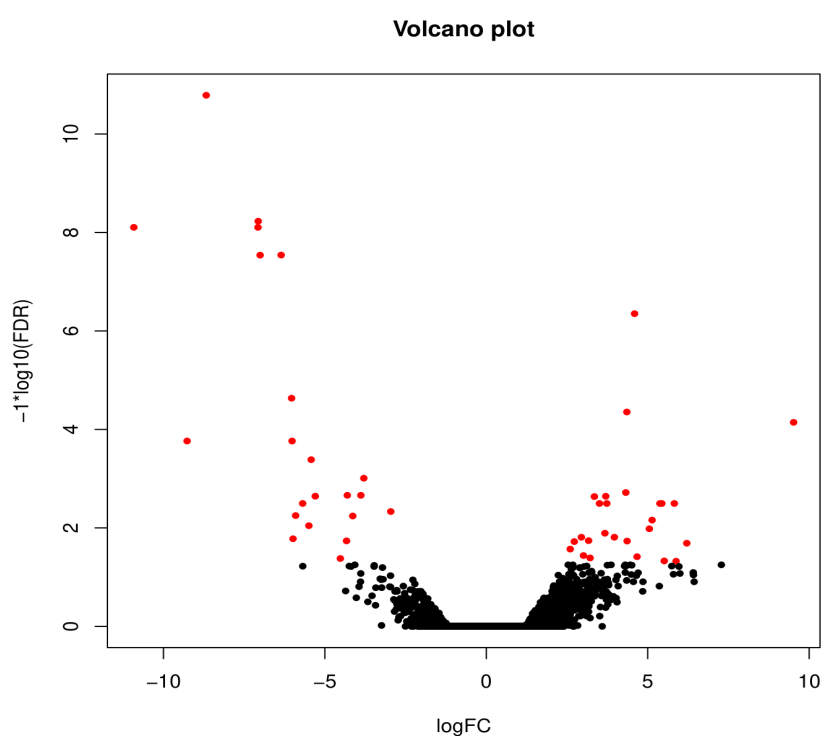


Figure 3.4a: Volcano plot of the distribution of expressed transcripts for Control vs. Carbamazepine. Red dots correspond to transcripts with q value < 0.05 .

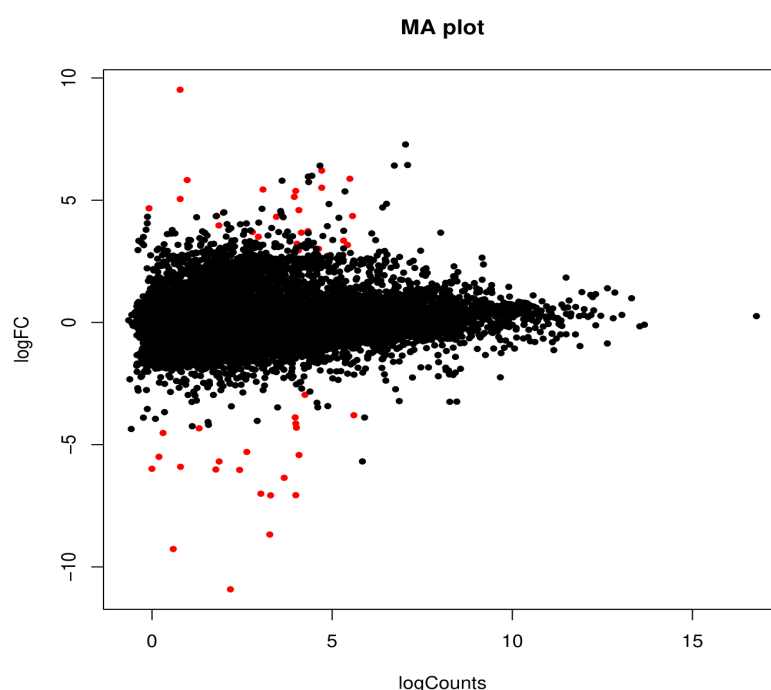


Figure 3.4b: MA plot showing differentially expressed transcripts for Control vs. Monocrotophos. On X-axis, normalized counts for all the samples, and on Y-axis, $\log_2$ foldchange are plotted. Points are coloured with red if the q value is less than 0.05 and black if the q value is greater than 0.05.

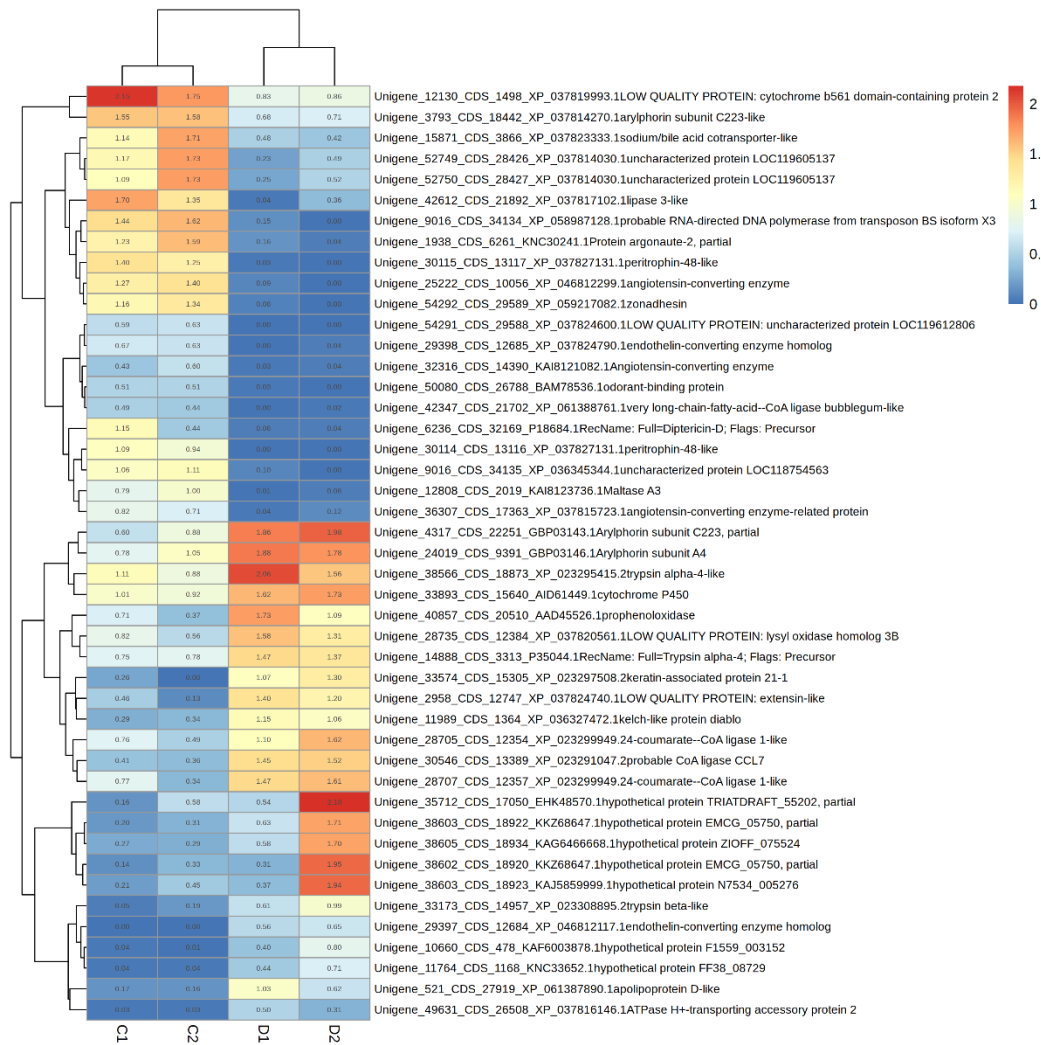


Figure 3.5: Heatmap representing the most significant CDS expressed in both the samples was plotted using $\log_{10}$ of normalised read count values (CPM) for Control (C) vs. Carbamazepine (D), where shades of blue represent downregulated CDS and shades of red represent highly expressed CDS

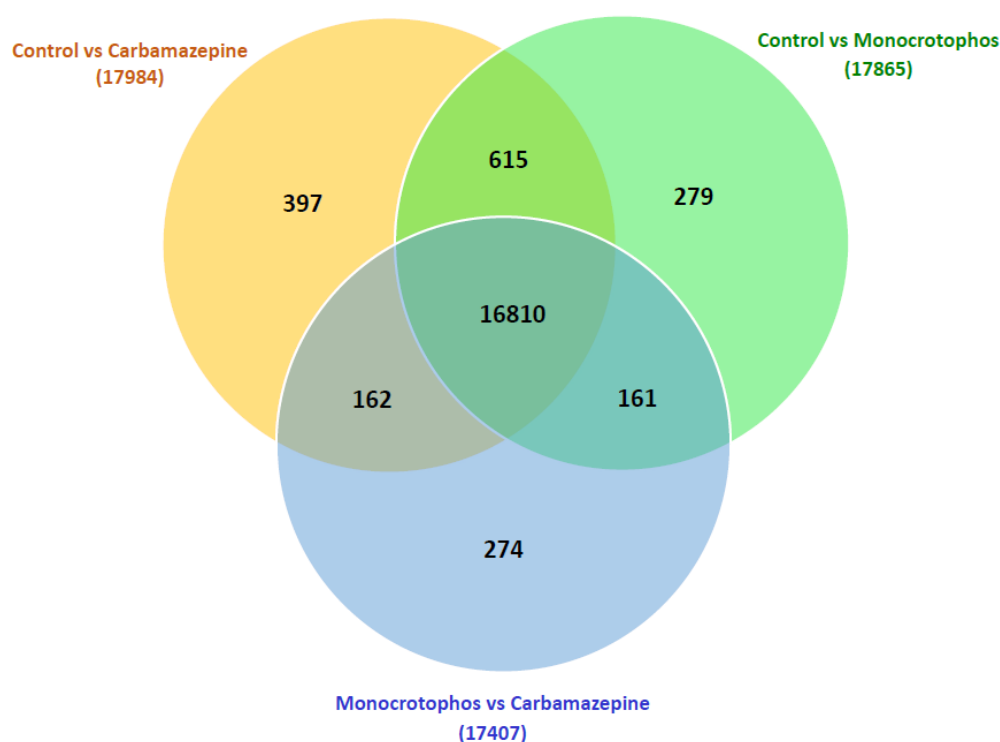


Figure 3.6: Comparative venn diagram of differential gene expressions of Control, Monocrotophos and Carbamazepine

3.4.6 KOG, GO and KEGG pathway analysis of DEGs

The general KOG classification of CDS is done initially with 25 classes, and respective counts are depicted in Figure 3.7.

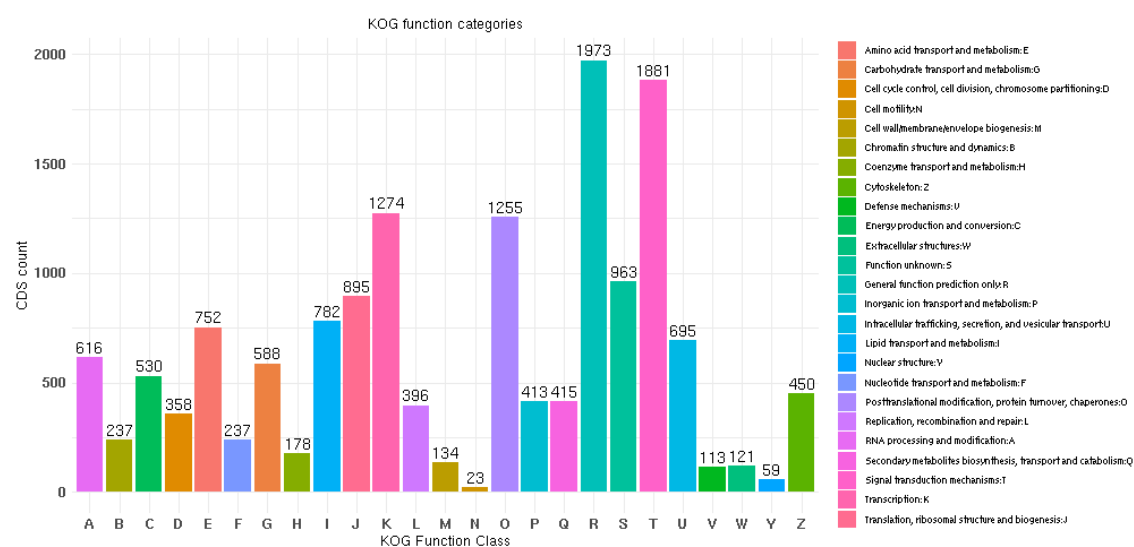


Figure 3.7: KOG classification for CDS

3.4.6a Control vs. Monocrotophos

The Eukaryotic Ortholog Groups (KOG) function categories of highly significant DEGs between Monocrotophos treated and Control group of *Chrysomya megacephala* includes several genes belonging to 10 gene clusters, and the largest cluster was of the genes involved in amino acid transport and metabolism (Figure 3.8). The other affected gene clusters due to monocrotophos exposure include genes involved in carbohydrate transportation and metabolism, energy production and conversion, lipid transport and metabolism, post-translational modification, protein turnover, chaperones, secondary metabolites biosynthesis, transport and catabolism, signal transduction mechanisms, and translation, ribosomal structure & biogenesis.

The most enriched Gene ontology (GO) terms are represented as a scatter plot, and out of 24 clusters, the most enriched clusters are proteolysis (GO:0006508) and serine-type endopeptidase activity (GO:0004252) (Figure 3.9a). Figure 3.9b shows the GO of all genes that are differentially expressed in Control vs. Monocrotophos-treated *Chrysomya megacephala*. All major gene groups that showed highly significant differential expression, and the number of genes involved are given in Table 3.4

The KEGG pathway analysis was performed to elucidate the biological pathways correlated with Monocrotophos exposure, and the highly significant 13 KEGG pathways are presented in Figure 3.10a, and highly significant differentially expressed gene groups involved in major pathways are listed in Table 3.5. The pathways most enriched and significantly impacted in the comparison between the Control and Monocrotophos groups were those related to transport and catabolism, lipid metabolism, and signal transduction. Figure 3.10b shows the KEGG pathways correspond to all differentially expressed genes (DEGs).

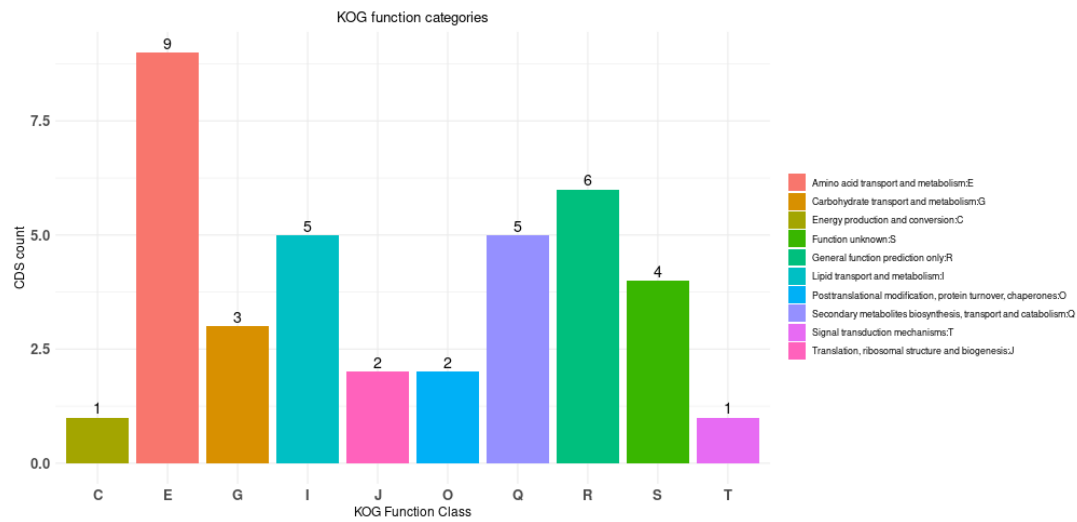


Figure 3.8: KOG function classification graph of highly significant DEGs of Control vs. *Monocrotophos*

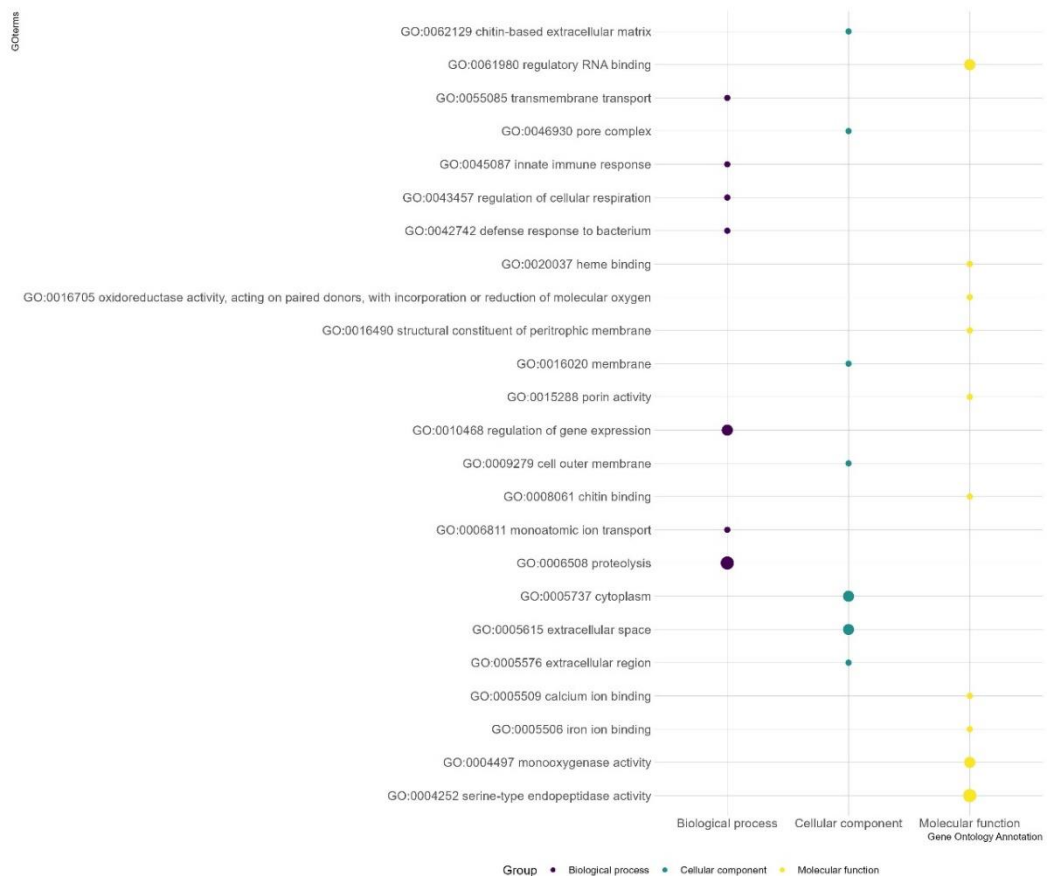


Figure 3.9a: Gene ontology scatter plot for highly significant genes of Control vs. *Monocrotophos*

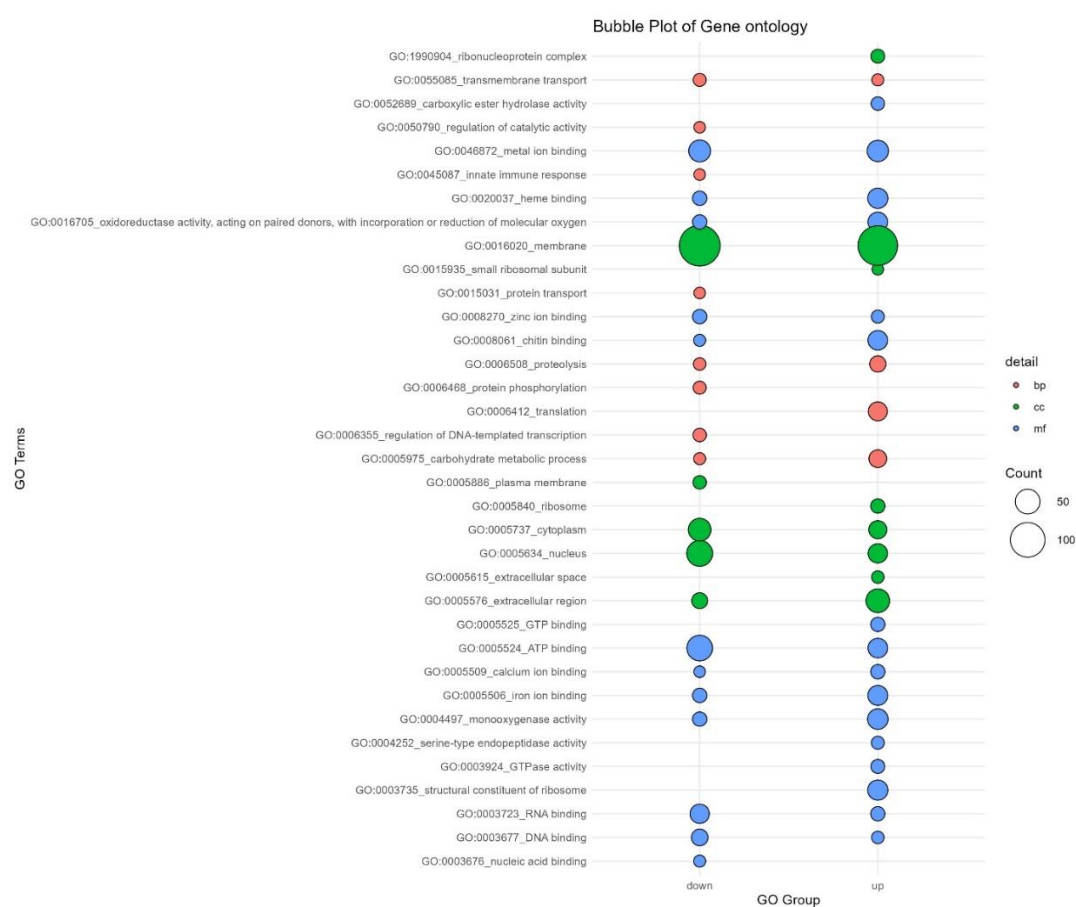


Figure 3.9b: Gene ontology scatter plot for all DEGs of Control vs. *Monocrotophos*

Table 3.4: Control (C) vs. *Monocrotophos* (I)- major gene groups that show highly significant differential expression, and the number of genes involved

Gene group	Significantly down-regulated	All Down-regulated	Significantly up-regulated	All Up-regulated
Amino acid transport and metabolism	0	156	9	311
Lipid transport and metabolism	1	256	4	300
Carbohydrate transport and metabolism	0	149	3	234
Energy production and conversion	0	80	1	245

Posttranslational modification, protein turnover, chaperones	0	384	2	539
Secondary metabolites biosynthesis, transport and catabolism	2	125	3	133
Signal transduction mechanisms	0	848	1	525
Translation, ribosomal structure and biogenesis	2	533	0	843
Chitin-based extracellular matrix	0	0	1	2
Chitin binding	0	11	1	31
Regulatory RNA binding	1	3	1	1
Transmembrane transport	1	13	0	11
Pore complex	0	0	1	1
Innate immune response	1	10	0	3
Regulation of cellular respiration	0	1	1	1
Oxidoreductase activity, acting on paired donors	1	16	0	31
Structural constituent of peritrophic membrane	0	0	1	2
Membrane	1	141	1	133
Porin activity	1	1	0	0
Cell outer membrane	1	1	0	0
Monoatomic ion transport	1	1	0	0
Proteolysis	2	12	1	21
Cytoplasm	1	42	1	26
Extracellular space	1	4	1	12

Extracellular region	0	20	1	46
Calcium ion binding	1	10	0	16
Iron ion binding	1	16	0	32
Monooxygenase activity	1	16	1	34
Serine-type endopeptidase activity	2	3	1	13

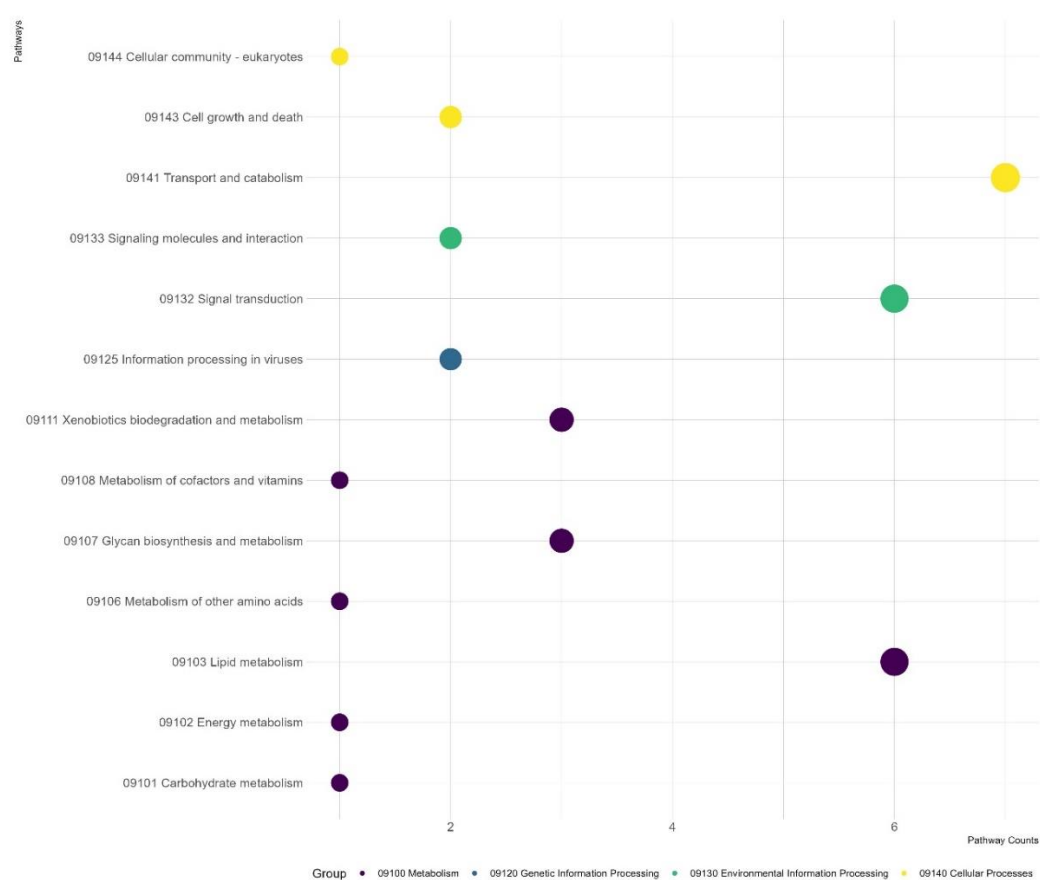


Figure 3.10a: KEGG enrichment scatter plot for highly significant genes of Control vs. *Monocrotophos*

Table 3.5: Control (C) vs. Monocrotophos (I)- highly significant differentially expressed gene groups involved in major pathways

Gene group	Sub group	Significantly down-regulated	Significantly up-regulated
Cellular community	Focal adhesion	0	1
Cell growth and death	Apoptosis	0	2
Transport and catabolism	Lysosome	3	2
	Autophagy	0	2
Signaling molecules and interaction	Neuroactive ligand-receptor interaction	0	1
	ECM receptor interaction	0	1
Signal transduction	Ras signaling pathway	2	0
	Hedgehog signaling pathway	0	1
	Sphingolipid signaling pathway	0	2
	P13-Akt signaling pathway	0	1
	Metabolism of xenobiotics by cytochrome P450	0	1
Xenobiotics biodegradation and metabolism	Drug metabolism-cytochrome P450	0	1
	Drug metabolism- other enzymes	0	1
Metabolism of cofactors and vitamins	Retinol metabolism	0	1
Glycan biosynthesis and metabolism	Other glycan degradation	3	0
Metabolism of other amino acids	Glutathione metabolism	0	1
	Other lipid metabolism	2	0
Lipid metabolism	Glycerophospholipid metabolism	1	1
	Arachidonic acid metabolism	1	0
	Linoleic acid metabolism	1	0
	Alpha-linoleic acid metabolism	1	0
	Pyruvate metabolism	0	1
Carbohydrate metabolism			

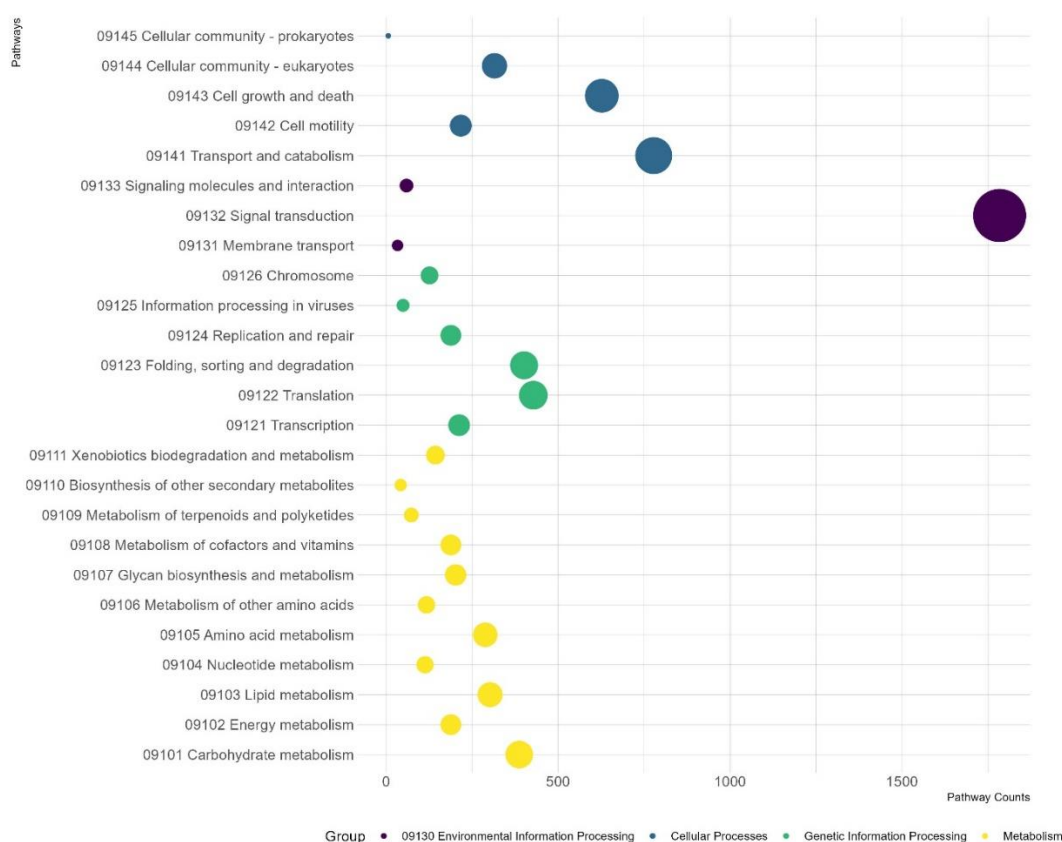


Figure 3.10b: KEGG enrichment scatter plot for all DEGs of Control vs. *Monocrotophos*

3.4.6b Control vs. Carbamazepine

The Eukaryotic Ortholog Groups (KOG) function categories of highly significant DEGs between Carbamazepine treated and Control group of *Chrysomya megacephala* include several genes belonging to 9 gene clusters, and the largest cluster was of the genes involved in amino acid transport and metabolism (Figure 3.11). The other affected gene clusters due to Carbamazepine exposure include genes involved in lipid transport and metabolism, and biosynthesis of secondary metabolites, transport and catabolism.

The most enriched Gene ontology (GO) terms are represented as a scatter plot, and out of 15 clusters, the largest is monooxygenase activity (GO:0004497), followed by iron ion binding (GO:0005506), oxidoreductase activity (GO:0016705) and heme binding (GO:0020037) (Figure 3.12a). Figure 3.12b shows the GO of all differentially

expressed genes in Control vs. Carbamazepine-treated *Chrysomya megacephala*. All major gene groups that showed highly significant differential expression, and the number of genes involved are given in Table 3.6.

The KEGG pathway analysis was performed to elucidate the biological pathways correlated with Carbamazepine exposure, and the highly significant 4 KEGG pathways are presented in Figure 3.13a, and highly significant differentially expressed gene groups involved in major pathways are listed in Table 3.7. The most enriched and significantly affected pathway of the Control vs. Carbamazepine was lipid metabolism. Figure 3.13b shows the KEGG pathways corresponds to all differentially expressed genes (DEGs).

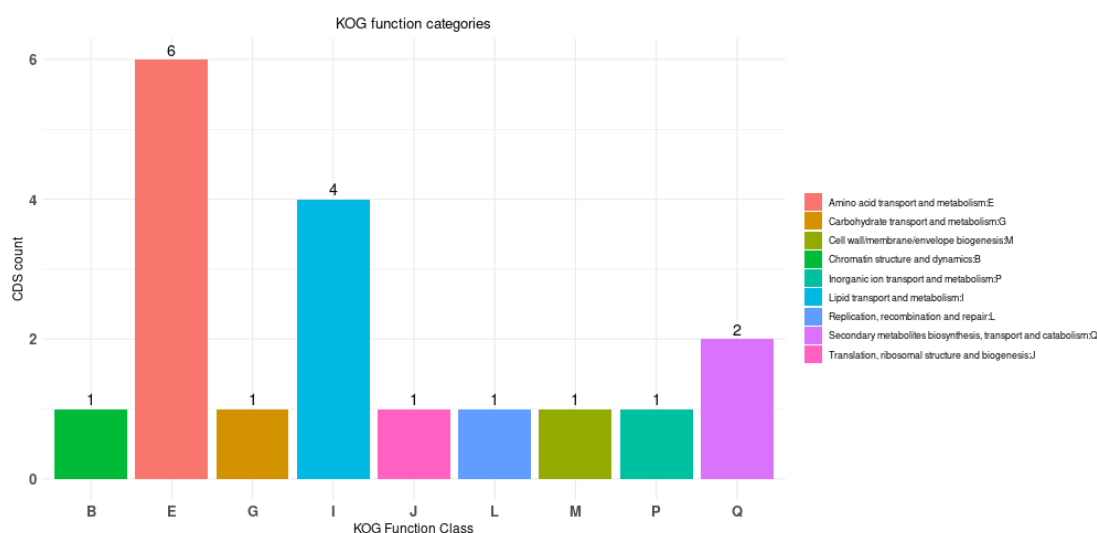


Figure 3.11: KOG function classification graph of highly significant DEGs for Control vs. Carbamazepine

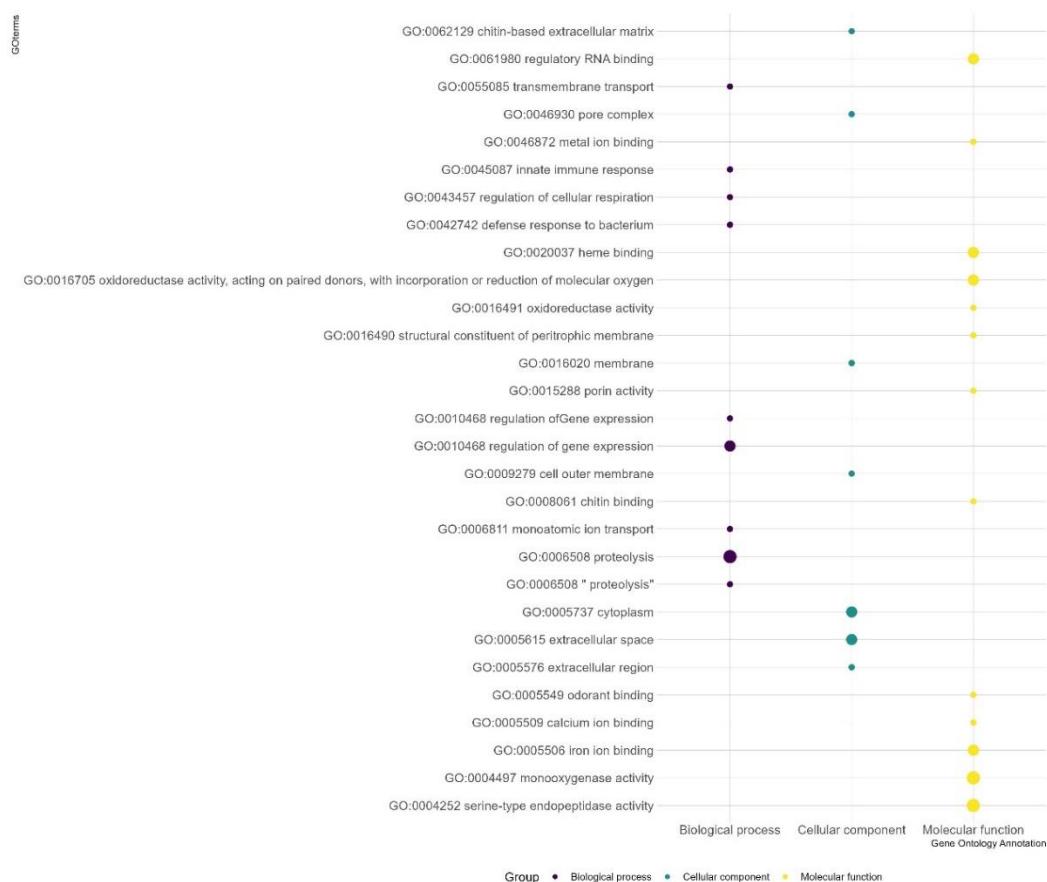


Figure 3.12a: Gene ontology scatter plot of highly significant DEGs of Control vs. Carbamazepine

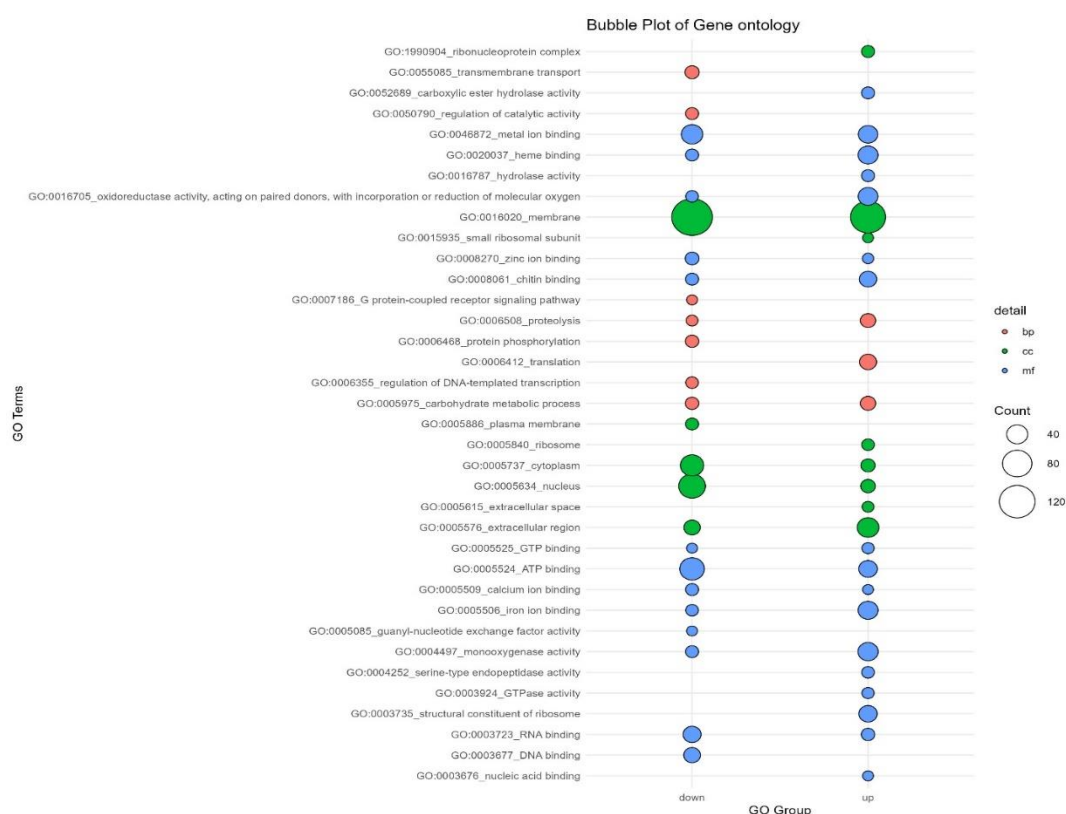


Figure 3.12b: Gene ontology scatter plot of all DEGs of Control vs. Carbamazepine

Table 3.6: Control (C) vs. Carbamazepine (D)- major gene groups that show highly significant differential expression, and the number of genes involved

Gene group	Significantly down-regulated	All Down-regulated	Significantly up-regulated	All Up-regulated
Regulatory RNA binding	1	3	1	0
Heme binding	0	13	2	36
Oxidoreductase activity, acting on paired donors	1	16	0	31
Membrane	0	134	1	116
Proteolysis	0	8	1	21
Cytoplasm	1	47	0	18
Extracellular space	0	4	1	12
Extracellular region	0	24	1	43
Odorant binding	1	4	0	3
Iron ion binding	0	12	2	36
Monooxygenase activity	0	13	3	37
Serine-type endopeptidase activity	0	1	1	14

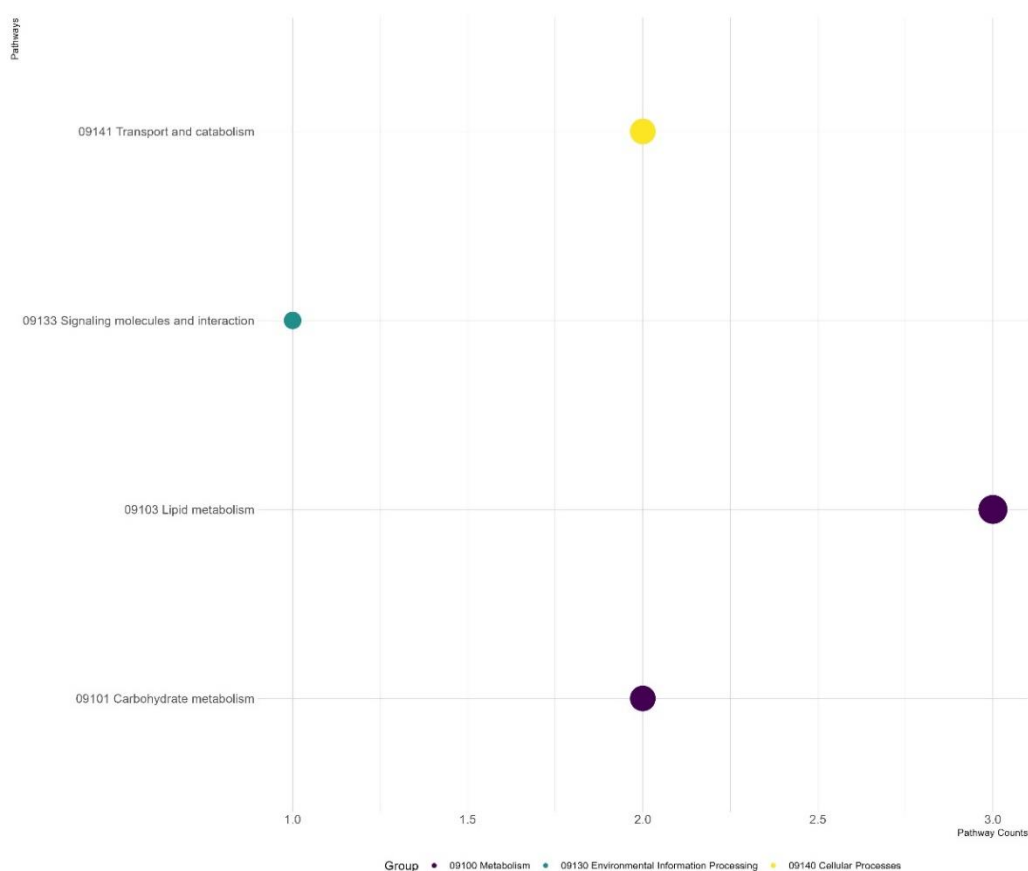


Figure 3.13a: KEGG enrichment scatter plot of highly significant DEGs of Control vs. Carbamazepine

Table 3.7: Control (C) vs. Carbamazepine (D)- highly significant differentially expressed gene groups involved in major pathways

Gene group	Sub group	Significantly down-regulated	Significantly up-regulated
Transport and catabolism	Lysosome	1	0
	Efferocytosis	1	0
Signaling molecules and interaction	Neuroactive ligand-receptor interaction	0	1
Lipid metabolism	Fatty acid biosynthesis	1	0
	Fatty acid degradation	1	0
	Steroid biosynthesis	1	0
Carbohydrate metabolism	Galactose metabolism	1	0
	Starch and sucrose metabolism	1	0

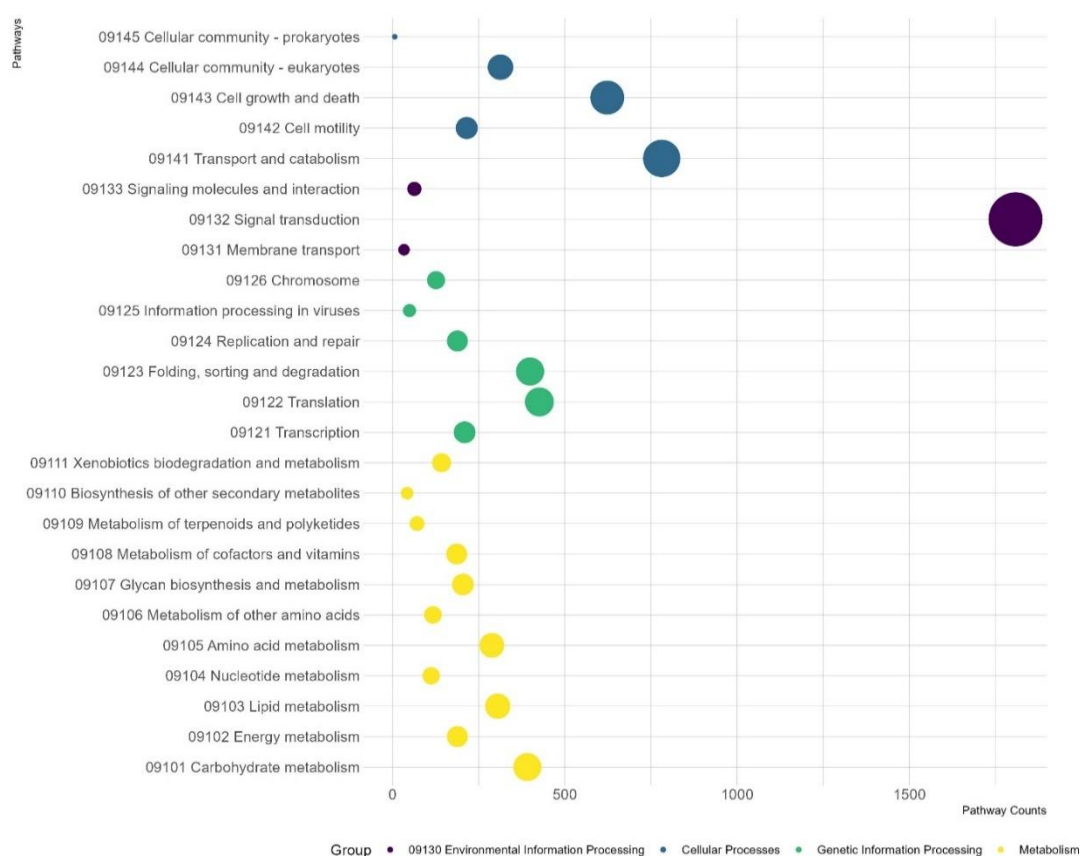


Figure 3.13b: KEGG enrichment scatter plot of all DEGs of Control vs. Carbamazepine

3.5 DISCUSSION

Insect transcriptomic studies have emerged as a valuable method for elucidating mechanisms associated with various aspects of insect biology. This approach, which effectively and thoroughly interprets genomic information from insects, enables researchers to explore gene function, physiological and morphological alterations, behavioural characteristics, growth and developmental processes, adaptations to environmental conditions, evolutionary relationships, and interactions between species. The comprehensive nature of transcriptomic analysis makes it an essential tool in advancing our understanding of insect biology across multiple domains (Shao et al., 2023; Wang et al., 2021). By investigating the expression patterns and associated metabolic pathways of these genes, researchers can gain a deeper understanding of insect growth, development, and reproductive processes. Additionally, integrating morphological features with an in-depth examination of the connection between

candidate genes and developmental timing can offer a conceptual foundation for molecularly defining insect growth stages (Meng et al., 2022).

This chapter deals with the gene expression profiling of *Chrysomya megacephala* in response to the sub-lethal concentrations of Monocrotophos and Carbamazepine. Monocrotophos [3hydroxy-N-methyl-cis-crotonamide dimethylphosphate] is a fast-acting organophosphate insecticide with both systemic and contact action, used in a wide variety of crops against a wide range of pests on cotton, sugar cane, tobacco, potatoes, peanuts, tomatoes, and ornamentals (*Monocrotophos*, n.d.). Carbamazepine, in contrast, is an anticonvulsant and analgesic medication employed to manage seizures and alleviate pain associated with trigeminal neuralgia (Carbamazepine, n.d.).

Chronic exposure to small amounts of organophosphates has deleterious effects on carbohydrate metabolism (Karami-Mohajeri & Abdollahi, 2011). The acute effects of acephate and methamidophos, both organophosphates, in rats demonstrated a notable reduction in amino acid levels and an elevation in blood glucose levels within the treatment groups, suggesting a decline in amino acid metabolism (Spasova et al., 2000). The chronic effects of pesticide toxicity in broiler chickens resulted in an increase in blood glucose levels and ATPase activity, suggesting that pesticide toxicity adversely impacts glycolysis (Garg et al., 2004). A differential protein expression study by Games et al. (2016) suggested a reduction in amino acid and lipid metabolism in the midgut of *Culex quinquefasciatus* larvae exposed to the insecticide temephos. The current study, based on the molecular responses of *Chrysomya megacephala* to sublethal monocrotophos exposure, fortifies these findings. There was a highly significant variation (log2FC significantly higher than zero with q-value <0.05) in the expression of genes involved in amino acid (9 genes), carbohydrate (3 genes), and lipid (5 genes) transportation and metabolism of the test group to the control group due to monocrotophos exposure.

In *Cochliomyia hominivorax*, as a response to dimethyl-OP, genes involved in proteolysis (mostly serine-type endopeptidases) were found to be up-regulated in the resistant larvae (Tandonnet et al., 2020). Similar results were found in our work,

where the GO terms ‘proteolysis’ (GO:0006508) and ‘serine-type endopeptidase activity’ (GO:0004252) were found to be up-regulated in the GO enrichment analysis of *Chrysomya megacephala* in response to Monocrotophos exposure. Out of 33 differentially expressed genes under ‘proteolysis’, 12 were down-regulated, 21 were up-regulated, and out of 16 genes under ‘serine-type endopeptidase activity’, 3 were down-regulated, and 13 genes were up-regulated. The other major genes found to be showing a significant variation in expression in the GO enrichment analysis are genes involved in monooxygenase activity (GO:0004497), regulatory RNA binding (GO:0061980), and regulation of gene expression (GO:0010468). Table 3.4 shows a detailed report on all the significant differentially expressed gene groups and their expression variations.

The KEGG pathway analysis revealed significant differential expression of several genes involved in various pathways in response to monocrotophos exposure. These pathways include Transport and catabolism, Lipid metabolism, Signal transduction, Xenobiotics biodegradation and metabolism, and Glycan biosynthesis and metabolism. Each of these pathways consists of several gene groups involved in significant functions that are either up-regulated or down-regulated, affecting the organism's normal functioning. A detailed gene report of significantly affected pathways is listed in Table 3.5.

The sublethal dose of Carbamazepine in *Chrysomya megacephala* also induced a highly significant variation (log2FC significantly higher than zero with q-value <0.05) in the expression of many major gene groups, including amino acid transport and metabolism (6 genes), and lipid transport and metabolism (4 genes). Although only one gene showed significant downregulation in carbohydrate transport and metabolism, 379 genes were differentially expressed in this cluster, with 180 genes downregulated and 199 genes upregulated. Table 3.6 provides a comprehensive report on all significantly differentially expressed genes and their expression changes.

The KEGG pathway analysis of Control vs. Carbamazepine also showed significant differential expressions in 4 major gene clusters, which are transport and catabolism, signalling molecules and interaction, lipid metabolism and carbohydrate metabolism.

All four of these pathways consist of several gene groups involved in significant functions that are either up-regulated or down-regulated, affecting the organism's normal functioning. A detailed gene report of significantly affected pathways is listed in Table 3.7. The gene ontology enrichment analysis revealed 12 highly enriched GO terms with genes/gene groups showing significantly high differential expression. The GO term with the highest gene count in terms of highly significant DEGs was monooxygenase activity (GO:0004497), with 3 genes showing significantly high upregulation, and the GO term 'membrane' (GO:0016020) shows 134 down-regulated genes and 116 up-regulated (1 highly significant) genes.

There are a few previous studies on the effects of antiepileptic drugs which showed similar results. The chronic effects of pentylenetetrazol, a convulsant drug, on *Drosophila melanogaster* showed down-regulation of genes enriched in several processes including transcription, cell differentiation, cell communication, neurogenesis, axonogenesis, axon guidance, glutamine family amino acid biosynthetic process and glutamate metabolism (Singh et al., 2009; Singh et al., 2011). Conversely, up-regulated transcripts were linked to axonogenesis, metabolism (particularly carbohydrate metabolism), and signalling. In this investigation, *Drosophila melanogaster* was subjected to a 7-day pentylenetetrazole regimen, followed by the administration of antiepileptic drugs (AED) after discontinuing PTZ treatment. The aim was to examine the impact of AED through molecular expression analysis. Post-AED transcriptome analysis indicated the down-regulation of genes involved in carbohydrate and lipid metabolism. In *Drosophila* culture cells, valproate, an anticonvulsant for epilepsy treatment, led to downregulation of genes associated with stress and immune responses, as well as carbohydrate metabolism (Herteleer et al., 2016). The transcriptome analysis of *Sparus aurata* brain, post Carbamazepine exposure showed significantly enriched genes involved in the glycoprotein biosynthetic process, glycoprotein metabolic process, glycosylation, lipid biosynthetic process and various lipase-associated genes (Hampel et al., 2017). This study also elicited enrichment of GO term 'membrane' namely, 'organelle membrane' and 'mitochondrial membrane' (16 and 8 DEGs).

The de novo transcriptome analysis of *Chrysomya megacephala* offered extensive insights into the full range of RNA transcripts produced by the entire genome. Both intoxicants uniquely modified the typical expression patterns of numerous major gene clusters, reflecting their distinct modes of action. Certain clusters exhibited differential expression in response to both Monocrotophos and Carbamazepine, including those involved in amino acid, lipid, and carbohydrate transport and metabolism, secondary metabolite biosynthesis, transport and catabolism, as well as translation, ribosomal structure, and biogenesis. Many of these genes are components of metabolic pathways, suggesting that this might be part of the insect's primary response to any xenobiotic exposure. Previous chapters have already addressed detailed reviews on developmental effects and detoxification mechanisms, other major gene clusters affected by the intoxicants. In summary, this transcriptomic study indicates that even at sublethal doses, both monocrotophos and carbamazepine impact the normal development and biological functions of *Chrysomya megacephala*. This also suggests that gene expression studies can provide a more comprehensive understanding of the effects of xenobiotics on an organism, even when there is no visible phenotypic or biochemical evidence. Such studies can reveal impacts that might otherwise go unnoticed.

From a forensic perspective, a more thorough understanding of gene expression levels throughout all developmental stages of forensically relevant insects can provide valuable insights and lead to more precise results in areas such as postmortem interval (PMI) determination. Furthermore, the complete transcriptomic data could be incorporated into a specialised database for entomotoxicology research, potentially making significant contributions to the field in the future.

3.6 KEY FINDINGS

- ❖ Out of 17,865 and 17,984 DEGs in Monocrotophos and Carbamazepine treated groups, 17,425 genes were common in both groups.
- ❖ 440 genes were exclusively expressed in Monocrotophos and 559 genes were exclusive to Carbamazepine treatment group.

- ❖ There was a highly significant variation in the expression of genes involved in amino acid (9 genes), carbohydrate (3 genes), and lipid (5 genes) transportation and metabolism of the test group to the control group due to monocrotophos exposure.
- ❖ The sublethal dose of Carbamazepine in *Chrysomya megacephala* also induced a highly significant variation in the expression of many major gene groups including amino acid transport and metabolism (6 genes), and lipid transport and metabolism (4 genes).

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Chapter 4

**Geometric Morphometric Analysis and Gene Expression
Dynamics in Wing development of *Chrysomya megacephala***

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4

Geometric Morphometric Analysis and Gene Expression Dynamics in Wing development of *Chrysomya megacephala*

4.1 INTRODUCTION

Insects comprise over one million documented species, with the majority possessing the ability to fly. The development of wings and their adaptive benefits have enabled them to become one of the most successful groups in evolutionary terms on the planet. The remarkable variety in wing shape, size, and colour patterns directly reflects the crucial role wings have played in insect evolution and in the radiation of insects (Parchem et al., 2007). Insect wings exhibit a diverse range of complexities in their structure. The aerodynamic performance of insects is fundamentally tied to the specific attributes of their wings, including their size, shape and structure (Salcedo et al., 2019). The diversity of wing shapes is remarkable, and the interplay between shape and movement patterns determines both flying abilities and energy requirements (Ray et al., 2016). Thus, the flight performance of insects is a direct result of how their wings interact with the surrounding air, which is influenced by a combination of kinematics and morphology. While the primary purpose of most wings is to create aerodynamic forces, sophisticated flight patterns require more than just maximum force generation. The structure of insect wings may reflect compromises or adaptations for various aspects of flight performance, such as efficiency, adaptability, agility, or equilibrium (Combes, 2010). Furthermore, insect wings must operate over an exceptionally long functional lifespan, often beating millions of times or more, and must withstand impacts and tears without experiencing a structural breakdown.

Shape analysis plays a crucial role in numerous biological studies. As statistical methods have evolved, so too has the complexity of analysing these types of data. The

advancement of statistical techniques has led to more sophisticated approaches in examining shape-related information in biological research. Quantifying the shape of wings is challenging, and simply using the aspect ratio to describe insect wings has significant limitations (Betts & Wootton, 1988). This issue can be remedied by using Geometric morphometric (GMM) analysis to quantify wing shape. Geometric morphometrics analyses Cartesian geometric coordinates of morphological structures rather than linear, areal or volumetric variables (Lawing & Polly, 2010). This approach emphasises maintaining geometric information throughout a study, offering efficient and statistically robust analyses that can easily connect abstract, multivariate outcomes to the physical structure of the original specimens (Slice, 2007). All geometric morphometric techniques utilise two-dimensional or three-dimensional landmark coordinates, which represent biologically or geometrically corresponding points on the measured objects (Mitteroecker & Schaefer, 2022). These methods vary in how they parameterize shape (geometric information independent of location, scale, and orientation) and form (geometric information independent of location and orientation, but not scale) of the landmark configurations. A key advantage of geometric morphometrics is that it preserves the geometry of landmark configurations throughout the analysis, allowing statistical results to be represented as actual shapes or forms (Mitteroecker & Gunz, 2009). After isolating shape from size, position, and orientation of landmark configurations, the resulting Procrustes shape coordinates can be used for statistical analysis. Techniques like principal component analysis, multivariate regression, or partial least squares analysis, which maintain these properties, can visualise results as shapes or deformations. Geometric morphometrics' robust visualisation tools and numerous shape variables enable an exploratory analysis style, identifying and quantifying previously unknown shape features.

Numerous toxicological and molecular investigations have examined the effects of major insecticides on wing development and malformation in insects. These studies have found that crucial stages of adult wing formation occur during the larval phases in various insect groups, including those belonging to the class Diptera (Carreira et al., 2011; De Celist & Garcia-Bellido, 1994; Ou et al., 2014; Sobala & Adler, 2016; Waddington, 1940). Klein (2001) studied the early stages of wing disc development

in the fly using *Drosophila* model. The study stated that the wing originates from imaginal discs, which are single-cell epithelial sheets of undifferentiated, multiplying cells. During embryonic growth, the wing imaginal disc begins with approximately 20 cells and expands through three larval phases, reaching around 75,000 cells by the late third instar (about 96 hours post-hatching). This disc forms a single-layered epithelium and the wing primordium is arranged in concentric circles, with central structures (wing blade and margin) surrounded by peripheral structures (hinge). By the late third instar, the wing primordium's major components—hinge, blade, and margin—can be identified using molecular markers. The formation processes commence at the third larval instar (roughly 48 hours after hatching) and are controlled by patterning centers located at the dorsoventral (D-V) and anteroposterior (A-P) compartment boundaries. Therefore, a transcriptome profiling of the final instar larvae can give a comprehensive prediction of the major shape, size, and axis variations expected in the wings of adult fly.

Monocrotophos is an organophosphate insecticide used to control a wide range of pests. Carbamazepine, on the other hand, is an anticonvulsant used to treat a wide range of neurological diseases such as epilepsy, neuropathic pain, and schizophrenia. There are few to no studies on the effect of both of these intoxicants on the wing development of insects. The study of the effects of these intoxicants on *Chrysomya megacephala*, a forensically important Diptera, can also be critical from a forensic perspective. This chapter investigates the effects of two sublethal concentrations of monocrotophos and carbamazepine on wing development in *Chrysomya megacephala*. Significant alterations in wing dimensions and morphology were observed using geometric morphometric analysis (GMM). Furthermore, this chapter delves into the differential gene expression (DGE) associated with wing development, complementing and reinforcing the GMM results. For DGE analysis, the higher sublethal dose (1/3rd of EC₅₀) was used as it showed more promising results in basic entomotoxicological analyses, as seen in the first chapters.

4.2 REVIEW OF LITERATURE

Geometric morphometrics involves the statistical examination of shape using Cartesian coordinate data from landmarks (Mitteroecker & Gunz, 2009). The process begins by isolating shape from factors such as overall size, position, and orientation of landmark configurations. Subsequently, the resulting Procrustes shape coordinates can be employed for statistical analysis. When examining landmark data specifically, we can explore the notions of size and shape (Richtsmeier et al., 2002). It is important to note that because there is no single, universally accepted definition of size and shape. They can only be identified in relation to a chosen size proxy.

A comprehensive study by Salcedo et al., (2019) examined 789 insect wings from 25 families, employing extensive computational analysis to investigate their morphology. The research focused on three key aspects: (1) wing dimensions and outline shape, (2) venation patterns, and (3) the configuration and arrangement of wing membrane areas. These morphological analyses supplement quantitative techniques for describing wing structure and provide valuable insights for investigating wing functionality and evolutionary development. Research conducted by Betts & Wootton, (1988) on the natural flight patterns of six butterfly species revealed potential links between wing shape and flight performance. Their findings suggested that factors such as aspect ratio, wing loading, and wing inertia may influence flight capabilities in these insects.

Sublethal concentrations of neonicotinoid pesticides, such as imidacloprid and dinotefuran, were found to substantially increase the production of long-winged adults in both macropterous and brachypterous populations of *Nilaparvata lugens*, commonly known as the brown planthopper (Bao et al., 2009). When *Trichogramma ostriniae*-parasitized host eggs were exposed to herbicides like atrazine and difenoconazole, and fungicides such as propiconazole and topramezone, the incidence of wing abnormalities increased significantly compared to the untreated control group. The genotoxic potential of different concentrations of organophosphate pesticides (methyl parathion, azamethiphos, dichlorvos, and diazinon) was evaluated using the wing somatic mutation and recombination test (SMART) in *Drosophila melanogaster*

(Çakir & Sarikaya, 2005). The study revealed a positive relationship between the total number of mutations and the quantity of wings exhibiting mutations. Additionally, mutations were categorized based on their size and type per wing. The insecticides were ordered according to their decreasing genotoxic effects as follows: diazinon, dichlorvos, methyl parathion, and azamethiphos.

Research conducted by Sarikaya & Yüksel (2008) using the wing spot test in *Drosophila* larvae demonstrated that oxcarbazepine and carbamazepine, both antiepileptic medications, exhibit genotoxic properties in this species. The study found that the *Drosophila* used in the experiments had significantly reduced survival rates compared to the control group, indicating that both drugs have toxic effects on *Drosophila melanogaster* larvae.

Žikić et al., (2024) investigated the impact of two insecticides, the pyrethroid α -cypermethrin and the organophosphate pirimiphos-methyl, on the wing structure of *Tribolium castaneum* and *Tribolium confusum*. They employed geometric morphometric (GMM) analysis to examine the elytra and hindwings. The research revealed that *T. castaneum*'s hindwings underwent alterations in response to both insecticides. The α -cypermethrin treatment induced more pronounced changes in wing shape compared to pirimiphos-methyl. Notably, for *T. castaneum*, even brief exposure to the insecticides (as short as 5 minutes) was sufficient to trigger morphological changes in the hindwings. Another research by Boukouvala et al., (2023) employed geometric morphometrics to investigate the potential impact of brief *pirimiphos-methyl* exposure on parental females on the wing structures of first-generation *Rhyzopertha dominica*, *Prostephanus truncatus*, and *Tenebrio molitor*. The research revealed that *T. molitor* exhibited the most significant alterations, particularly in the elytra and hindwings, with males showing more pronounced morphological changes compared to females. Additionally, the hindwings of *P. truncatus* displayed structural modifications following 36 hours of *pirimiphos-methyl* exposure.

A study by Montaña-Campaz et al., (2019) used geometric morphometric (GMM) techniques to examine deformities and shape variations in the mentum and wing of

Chironomus columbiensis exposed to water affected by mining, agricultural, and cattle-raising activities. The research identified four types of mentum deformities in *C. columbiensis*: tooth absence, increased tooth count, tooth fusion, and gaps between teeth. These abnormalities were not present in the control group. Additionally, significant differences in mentum and wing morphometry were observed between the control and treated groups. The results indicate that anthropogenic activities lead to changes in the larval mouthparts and adult wing morphology (broader wings with diminished basal area) of *C. columbiensis*. These structural alterations may be attributed to various stress factors, including xenobiotics processed under conditions of environmental pollution.

Through targeted RNA interference, Ray et al., (2016) successfully altered *Drosophila* wing shape beyond the species' natural variation, directly impacting flight performance as evidenced by physical modelling of the modified wing geometry. Their research demonstrated that adjusting the expression of a single gene could substantially enhance aerial agility, suggesting that the natural *Drosophila* wing shape is suboptimal for certain crucial flight performance characteristics. Research conducted by Carreira et al., (2011) reveals that wing shape has a complex genetic foundation, with multiple genes contributing to this trait in a sex-specific manner. This multifaceted genetic basis, which is largely independent of wing size, encompasses genes associated with developmental and metabolic functions, particularly those that regulate cellular processes such as motility, adhesion, communication, and signal transduction. The research suggests that to comprehend the genetic underpinnings of wing shape, it is necessary to combine the understanding of vein patterning regulation by signalling pathways with cellular-level processes occurring during wing development. (Baonza et al., 2000) investigated the role of epidermal growth factor receptor (DER) signalling in *Drosophila* wing development. Inhibiting DER signalling during the second larval instar results in notum-to-wing transformations and wing duplications. DER signalling confines the wing subregion in the developing wing disc and specifies posterior identity by restricting Wingless expression to the dorsal-posterior quadrant, suppressing wing-organizing activities, and maintaining Engrailed expression in posterior compartment cells.

In a study by Combes & Daniel, (2003), the connection between wing venation patterns and flexibility was investigated. The researchers examined the flexural stiffness in both spanwise and chordwise directions and analysed wing venation across 16 insect species from six different orders. Their findings revealed that spanwise flexural stiffness increases with the cube of wing span, while chordwise flexural stiffness increases with the square of chord length. Wing size accounted for over 95% of the variation in flexural stiffness. The study found minimal residuals, which showed no correlation with standardized independent contrasts of wing venation characteristics. Notably, spanwise flexural stiffness was 10 to 100 times greater than chordwise stiffness in all species studied. A finite element model demonstrated that veins along the leading edge play a crucial role in creating this spanwise-chordwise anisotropy.

4.3 MATERIALS AND METHODS

4.3.1 Geometric Morphometric analysis of wings of *Chrysomya megacephala*

Sub-lethal concentrations ($1/5^{\text{th}}$ of EC_{50} and $1/3^{\text{rd}}$ of EC_{50}) of Monocrotophos and Carbamazepine induced changes in wing morphometry of *Chrysomya megacephala* were evaluated through Landmark-based geometric morphometric analysis, which is a powerful approach to quantify biological size and shape variation.

All geometric morphometric shape analyses and size variations utilized structured sample allocations per group. This configuration satisfies prospective power requirements (power=0.8, $\alpha=0.05$), maximizing the sensitivity of Procrustes superimpositions and principal component analyses (PCA) to precisely detect dose-dependent structural anomalies.

4.3.1a Image acquisition and landmark digitisation

The wings of 2 days-old adult males and females emerged from the respective experimental groups (Table 4.1) were selected for geometric morphometric analysis. The flies were anesthetised using diethyl ether, and the wings were dissected and mounted on clean microscopic slides using DPX. Individual wings were photographed using Zeiss Stereo Discovery V20, a modular motorised stereo zoom microscope with

Zeiss Axiocam 506 colour camera 19-bit full resolution CCD Sensor, and converted to mathematical coordinates using tps Util version 1.68 x 64- software (2016). Twenty-one anatomical points were chosen for landmarks to minimise bias in digitising landmark locations (Figure 4.1). Landmarking and scale factors were performed using tps Dig2 version 2.26 (2016). A total of 12,500 wings from all the groups were used in the present study and the concentration-dependent changes in wing shape were studied by grouping the right- and left-wing shapes. The experimental groups studied and their denotations are given in Table 4.1. The position description of landmarks and abbreviations are presented in Table 4.2.

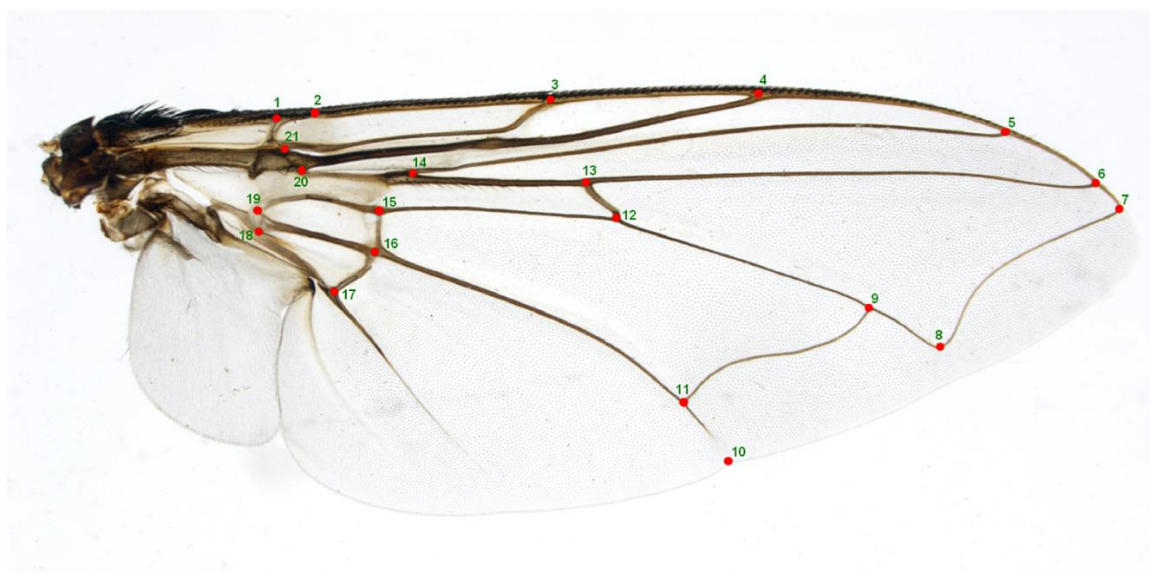


Figure 4.1: Wing of *Chrysomya megacephala* with landmarks

Table 4.1: Experimental groups studied

Sl. No	Denotation	Experimental group
1	MO	Monocrotophos- Control
2	MA	Monocrotophos- 1/5 th of EC ₅₀
3	MB	Monocrotophos- 1/3 rd of EC ₅₀
4	CO	Carbamazepine- Control
5	CA	Carbamazepine- 1/5 th of EC ₅₀
6	CB	Carbamazepine- 1/3 rd of EC ₅₀

Table 4.2: Landmark description for *Chrysomya megacephala* wing

Landmark	Location	Description of Landmark
1	HC	Costal-Humeral junction
2	H	Humeral break
3	CS	Intersection of Costa and Subcosta
4	CR ₁	Intersection of Costa and Radial vein 1
5	DR ₂₊₃	Distal end of the Radial vein 2+3
6	DR ₄₊₅	Distal end of the Radial vein 4+5
7	DM ₁	Distal end of Median 1
8	DM ₂	Distal end of Median 2
9	DMCu	Distal intersection of Medio-cubital cross veins
10	DCu	Distal end of Cubitus
11	PMCu	Proximal intersection of Medio-cubital cross veins
12	MR ₄₊₅	Midpoint branch of Radial vein 4+5
13	MM ₂	Midpoint branch of Medial vein 2
14	OR2	Origin of Radial vein 2+3 and Radial vein 4+5
15	MCuM	Intersection of Medio-cubital cross vein to Medial vein
16	MCuCu	Intersection of Medio-cubital cross vein to Cubital vein
17	CuAA	Intersection of Cubito-anal cross vein to Anal vein
18	CuA	Bifurcation between the Cubital and Anal vein
19	MCu	Bifurcation between the Median and Cubital vein
20	OR1	Bifurcation of Radial vein 2+3 from Radial vein 1
21	OS	Origin of Subcosta and Radial vein 1

As measurement error is essential for analysing shape variation, replicate measurements should be taken on each individual. For that, each wing was photographed twice to evaluate the variation in the image acquisition process. The landmarks were also digitised twice on all images to assess the precision of the digitising step.

4.3.1b Data Analysis

After the landmark digitisation, the data of *Chrysomya megacephala* wings were imported into MorphoJ (Version 1.06d, Java Version: 1.8.0-101), and a complete Procrustes superimposition was conducted by orthogonal projection to correct size and orientation, to perform the geometric morphometric (GMM) analysis. In the Procrustes analyses, the shape is characterised mathematically by removing the effects of location, scale, and rotation through transformations. Multivariate analyses like Principal Component Analysis (PCA), Generalised Procrustes Analysis (GPA), Discriminant Function Analysis (DFA), Partial Least Squares (PLS) and Canonical variate analysis (CVA) were done using MorphoJ, to quantify the biological size and shape variations.

4.3.2 Transcriptomic analysis of wing-related genes

The DGE analysis was done to determine the alterations in genes related to wing structure and formation using *de novo* transcriptomics. The sublethal concentrations of $1/3^{\text{rd}}$ of EC_{50} of Monocrotophos-I (0.077 $\mu\text{g/ml}$) and Carbamazepine- D (0.1975mg/ml) were used for transcriptome analysis. The culture of the test organism *Chrysomya megacephala* (strain DI212) was maintained in our lab, and the primary identification of the species was done using taxonomic keys (Old World Screw-Worm Fly: A Diagnostic Manual, 2017; Falk, 2016; Szpila, 2012; Whitworth, 2006, 2010) which was then confirmed by CO1 barcoding (Accession number: PQ136459).

The third-instar larvae of *Chrysomya megacephala* from both Control- C and Treatments;

- Monocrotophos- I (96 hours of exposure via feed (I)
- Carbamazepine- D (96 hours of exposure via feed) (D), were washed, immersed in Trizol reagent, and kept at -80°C .

4.3.2a RNA isolation & Library preparation

RNA was isolated from the samples using the Trizol RNA isolation method. RNA quantity was measured using a Qubit® 4.0 fluorometer, and quality was analysed by using 1% agarose gel. The paired-end sequencing library was prepared using the KAPA mRNA HyperPrep Kit for Illumina (CAT #KK8581).

The library preparation process was initiated with 500-1000 ng input. mRNA enrichment was performed as per the user manual, and mRNA was subjected to fragmentation, first & second-strand cDNA synthesis, end-repair, 3' adenylating, adapter ligation and low-quality data removal (Trim Galore version 0.6.4), selective enrichment of adapter-ligated DNA fragments through PCR amplification, followed by validation of Library on Agilent 4150 tape station (Agilent Technologies) using HSD100 ScreenTape® as per manufacturer's instructions. The final library was pooled with other samples, denatured, and loaded onto the flow cell. On the flowcell, cluster generation and sequencing were performed using the Illumina Novaseq 6000 platform to generate ~5-6 GB of 2×150bp paired-end (PE) clean reads from each library.

4.3.2b *De novo* assembly

As there is no published genome of this species or any closely related species, de novo assembly using all clean reads was done. Master/Combine assembly was performed, taking high quality adapter-trimmed and algal-mapped reads of all samples together using Trinity (version 2.14.0) to generate common assembly for annotation and sample differential expression analysis.

4.3.2c Differential expression analysis

The DGE analysis is done on the following combinations:

- (a) Control vs. Monocrotophos
- (b) Control vs. Carbamazepine

Reads of each sample were mapped separately to CDS sequences obtained from the master assembly using BWA, and PCR duplicate reads were removed from the alignment file using Picard's MarkDuplicates.jar. Then, the reads mapping to each CDS were calculated for each sample using the idxstats program of Samtools. Finally, these mapped reads mentioned above were considered for differential gene expression (DGE) analysis. The read-mapped count from each sample was given, and finally, these mapped reads in the combination mentioned above were considered for differential gene expression (DGE) analysis. The read-mapped count from each sample was given as an input for differential expression analysis using the edgeR Bioconductor package in R.

4.4 RESULTS

4.4.1 Geometric morphometric analysis of the wings of *Chrysomya megacephala*

The geometric morphometric analysis involved the identification of 21 essential landmarks on the wing surface, selected for their anatomical and taxonomic relevance. This analysis was conducted across all experimental sets to evaluate the morphological alterations caused by the intoxicants. Each experiment set included approximately 500 wing specimens (250 male and 250 female), with every specimen photographed twice to minimise potential sampling errors.

One way Procrustes ANOVA was employed to assess landmarking error in the datasets, revealing that the mean squares for each variation exceeded the error components significantly. This indicated minimal measurement error and confirmed that deviations from the control set were solely attributable to intoxicant effects. To investigate the effects of sublethal monocrotophos and carbamazepine doses on wing morphology, several analytical techniques like Principal Component Analysis (PCA), Discriminant Function Analysis (DFA), Partial Least Squares (PLS), and Canonical variate analysis (CVA) were done.

4.4.1a Effect of Monocrotophos on wing morphology of *Chrysomya megacephala*

The sublethal concentrations of Monocrotophos selected for the study are $1/5^{\text{th}}$ of EC_{50} (MA- 0.0464 $\mu\text{g/ml}$) and $1/3^{\text{rd}}$ of EC_{50} (MB- 0.077 $\mu\text{g/ml}$). These are then compared to the Control (MO) to understand the size and shape variations.

% variance and Principal Component Analysis

In the Control group (MO) of *Chrysomya megacephala*, 38 principal components (PCs) accounted for the variation in 100% population (Figure 4.2). PC1 explains the variation in 53.27% of the control population, and the most significant shifts were observed in distal landmarks 8, 9, 10, and 11, which moved towards the posterior area (Figure 4.3a). Landmarks 9 and 11 represent the intersections of medio-cubital cross veins, whereas landmarks 8 and 10 mark the distal ends of the median and cubitus, respectively. Compared to the distal shifts, the proximal regions of the wing exhibited minimal displacement. PC2 covers the variations of 10% of the population, and the major shifts are seen in the medio-cubital cross vein and distal end of cubitus, which are shifted posteriorly, and the radio-medial cross vein, which showed proximal displacement (Figure 4.3b). 8.6% of the population represented in PC3 showed major displacements that resulted in a proximo-distal elongation as well as a slight broadening of wings towards the posterior part (Figure 4.3c). These variations are considered typical within the natural population of *Chrysomya megacephala*.

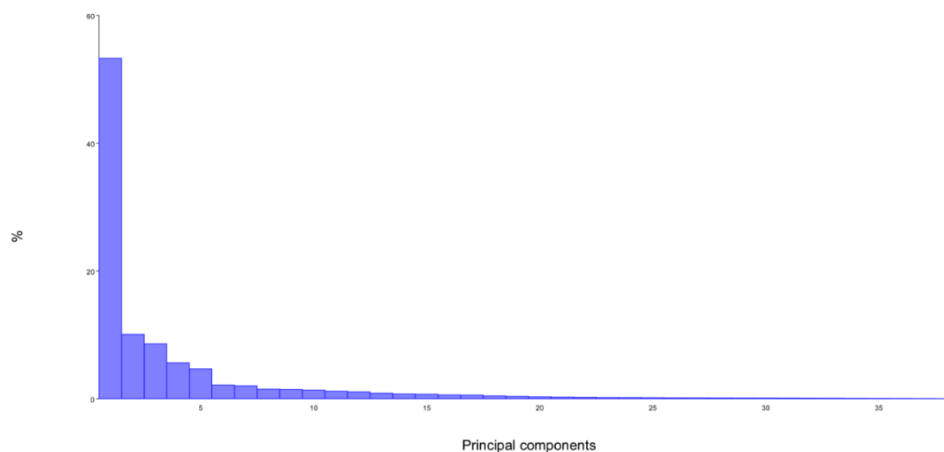


Figure 4.2: Percentage variance of Principal component analysis in the *Monocrotophos* Control (MO) set

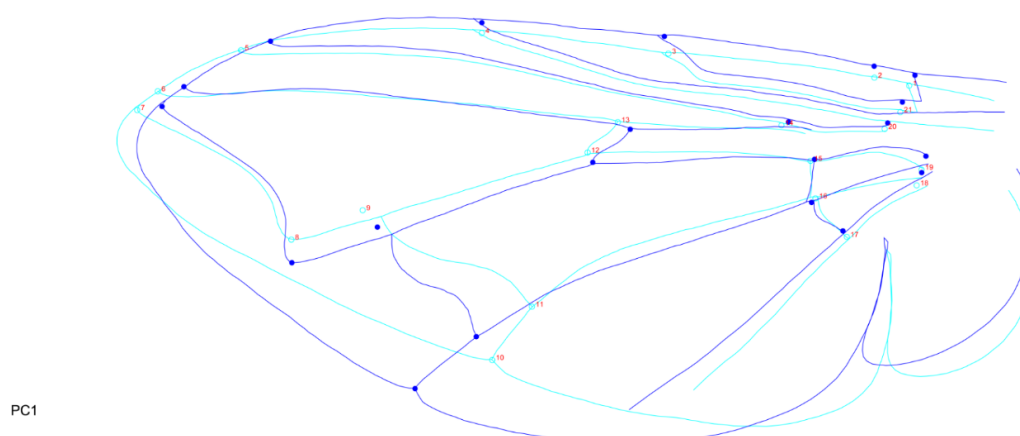


Figure 4.3a: PC1 Shape morphology of the Control (MO) set

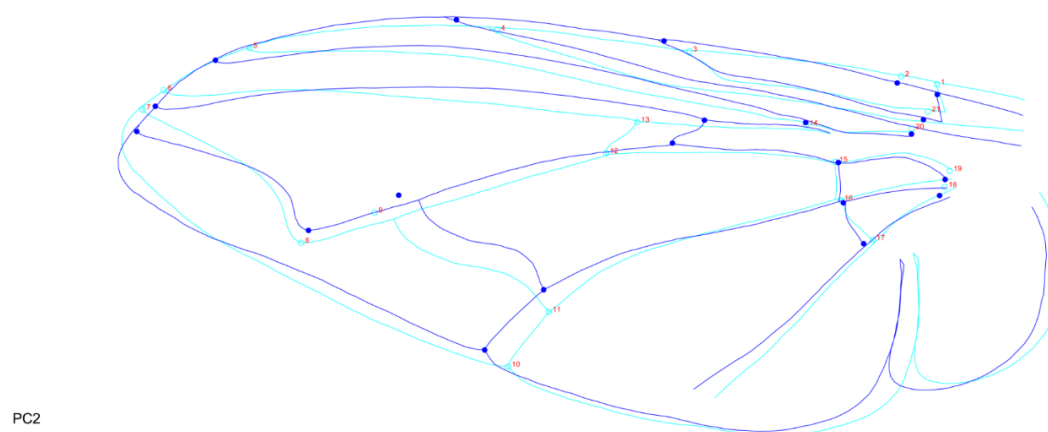


Figure 4.3b: PC2 Shape morphology of the Control (MO) set

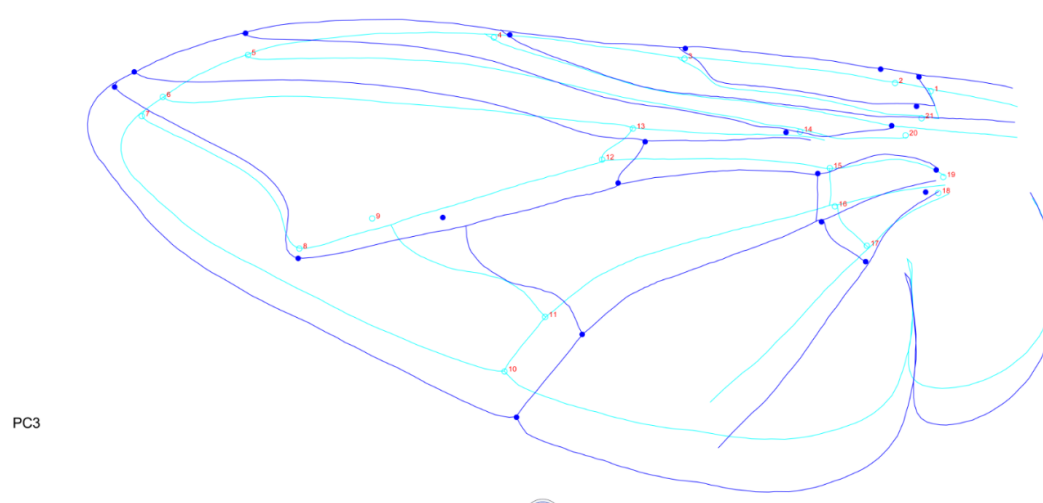


Figure 4.3c: PC3 Shape morphology of the Control (MO) set

For the lower concentration Monocrotophos treatment group (1/5th of EC₅₀- MA), variations in 100% of the population were explained by 38 PCs. The first three PCs explained the wing morphology variations in 45.78% of the population as shown in Figure 4.4. Among these, PC1 covers 16.84% of variations. The landmarks in PC1 that were attributed to the highest variations were landmarks 3, 4, 6, 7, 10, 11, 12, and 13 (Figure 4.5a). The costa-subcosta and costa-radial 1 intersections were displaced further anteriorly, while the medio-cubital cross vein and the distal end of the cubitus shifted posteriorly. The distal ends of the radial vein 4+5 and median were shifted proximally, whereas the radio-medial cross vein was displaced distally. Variations showed by 15.54% of the population is covered by PC2, and the major displacements resulted in the anteroposterior narrowing of the wing blade (Figure 4.5b). This was caused due to the posterior shifting of anterior points like costa-humeral junction, humeral break, bifurcation of radial vein 2+3 from radial vein 1, and the origin of subcosta and radial vein 1, the anteroproximal shifting of posterior areas including distal end of cubitus, medio-cubital cross vein, radio-medial cross vein, medio-cubital cross vein, and cubito-anal cross vein. Other major displacements in PC2 included the posterior shifting of distal ends of radial vein 4+5 and median, and the shifting of radio-medial cross vein towards the distal region. PC3 covering 13.38% of the population depicted the variation in the proximal intersection of the medio-cubital cross vein and distal end of cubitus that showed anteroproximal shifting, and the medio-radial cross vein that shifted towards the distal region (Figure 4.5c).

In summary, even though the PC1 population exhibited a slight anteroposterior broadening of the wing blade, both PC2 and PC3, which cover a combined 28.92% of the population, showed significant levels of anteroposterior narrowing of the wing blade. Therefore, we can conclude that Monocrotophos at this particular dose appeared to induce an anteroposterior compression in the wing blade of *Chrysomya megacephala*.

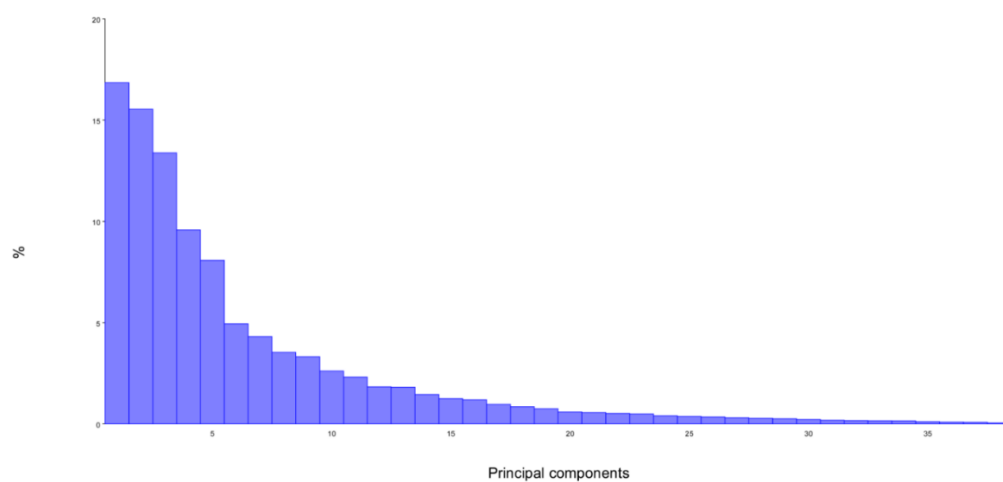


Figure 4.4: Percentage variance of Principal component analysis in Monocrotophos 1/5th of EC₅₀ (MA) set

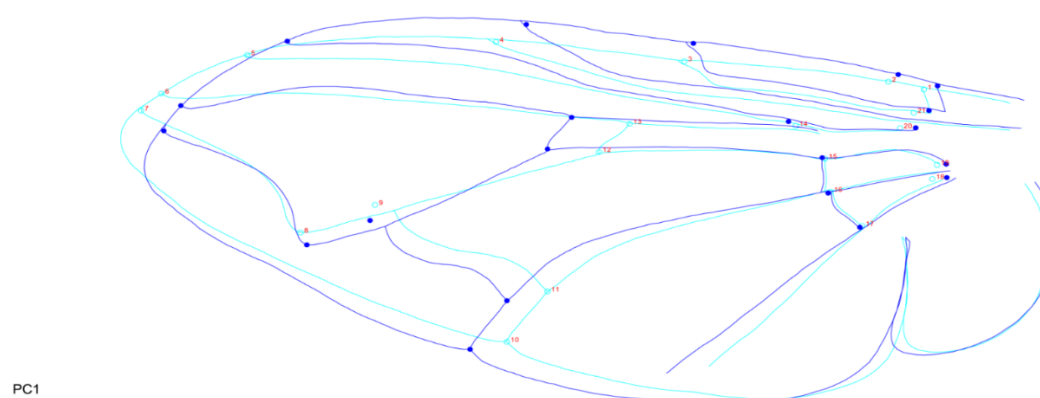


Figure 4.5a: PC1 Shape morphology of Monocrotophos 1/5th of EC₅₀ (MA) set

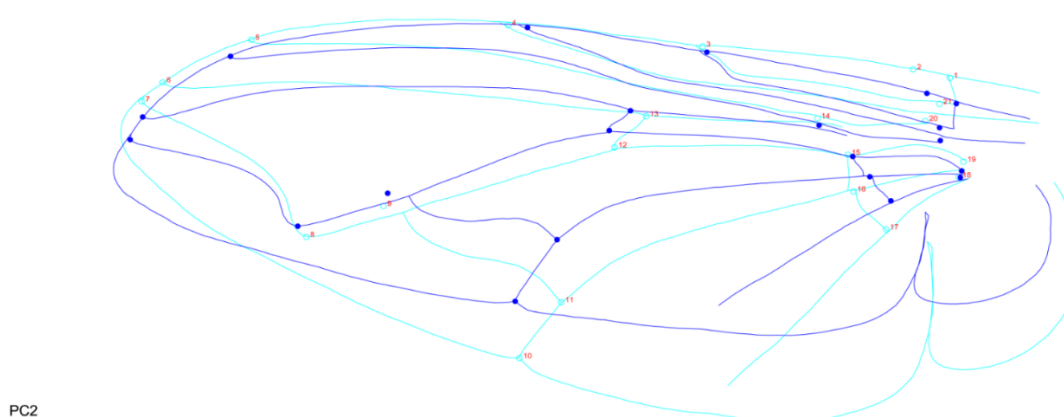


Figure 4.5b: PC2 Shape morphology of Monocrotophos 1/5th of EC₅₀ (MA) set

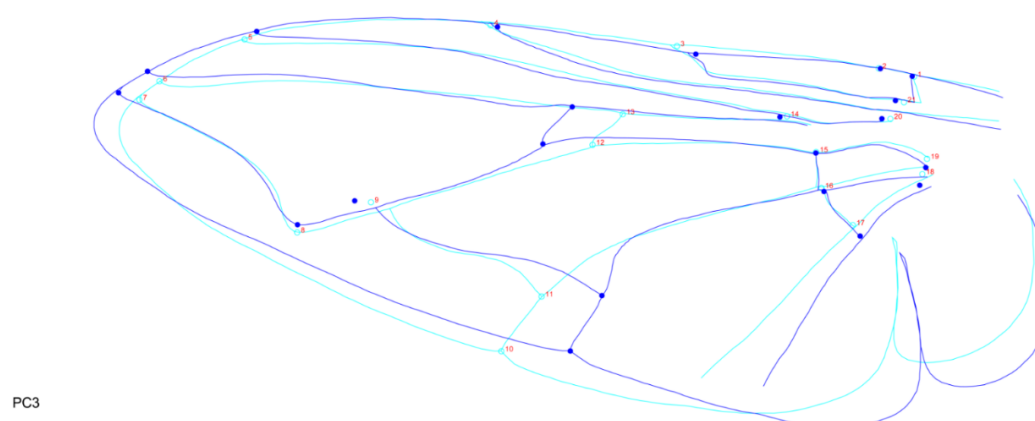


Figure 4.5c: PC3 Shape morphology of *Monocrotaphos* 1/5th of EC₅₀ (MA) set

Chrysomya megacephala wings treated with 1/3rd of EC₅₀ also had a total of 38 PCs that cumulatively explained 100% of the variation. PC1 explained 24.31% of the population variation, while PC2 and PC3 accounted for 13.44% and 10.92%, respectively (Figure 4.6). The figure 4.7a illustrates the PC1 shape morphology, depicting the major displacements at landmarks 3, 4, 6, 7, 9, 10, 11, 12, and 13. The anteriorly located morphologically important areas, such as the costa-subcosta and costa-radial 1 intersections, were displaced posteriorly. The distal ends of the radial veins (R2+3 and R4+5) and median vein moved distally. In contrast, posteriorly located areas, including the intersections of the medio-cubital cross veins and the distal end of the cubitus, shifted anteriorly. The radio-medial cross vein was the sole region that exhibited proximal displacement. PC2 exhibited anterior shifting of the intersection of costa and radial 1, the distal end of radial vein 2+3 towards the anterior side, distal shifting of the radio-medial cross vein, and anteroproximal displacement of the distal end of cubitus and medio-cubital cross vein (Figure 4.7b). PC3 depicted the variation in areas such as the costal-humeral junction, humeral break, distal end of radial vein 4+5, distal end of median, bifurcation of radial vein 2+3 from radial vein 1, and the origin of subcostal and radial vein 1 that exhibited anterior shifting (Figure 4.7c). Posterior shifting was also observed in areas such as the medio-cubital and radio-medial cross veins.

These findings suggest a slight proximodistal expansion of the overall wing blade. Major displacements were predominantly observed in the distal and posterior areas, whereas the proximal regions displayed relatively minor variations.

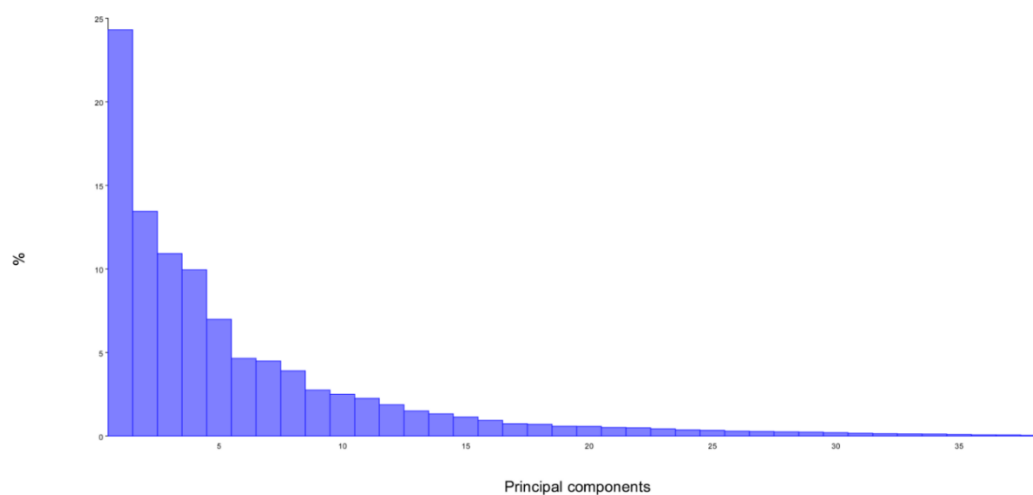


Figure 4.6: Percentage variance of Principal component analysis in *Monocrotophos* 1/3rd of EC_{50} (MB) set

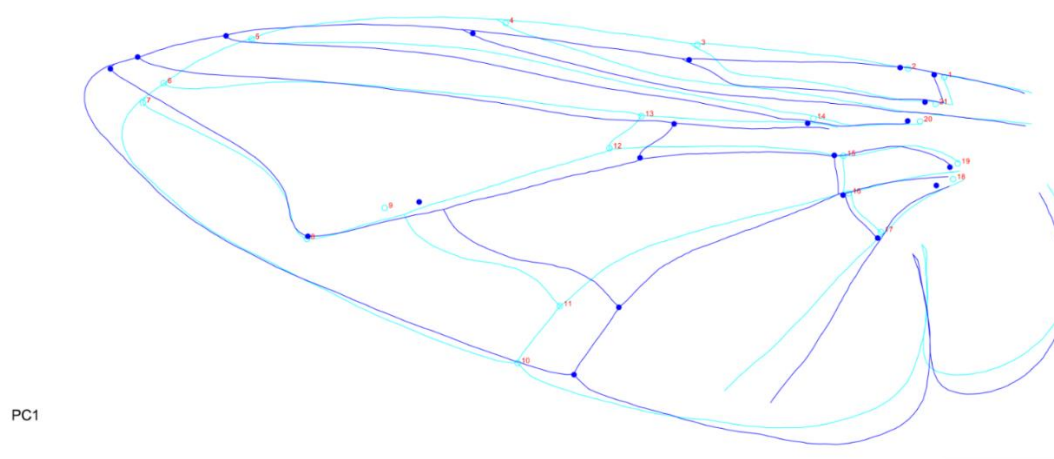


Figure 4.7a: PC1 Shape morphology of *Monocrotophos* 1/3rd of EC_{50} (MB) set



Figure 4.7b: PC2 Shape morphology of Monocrotophos 1/3rd of EC₅₀ (MB) set

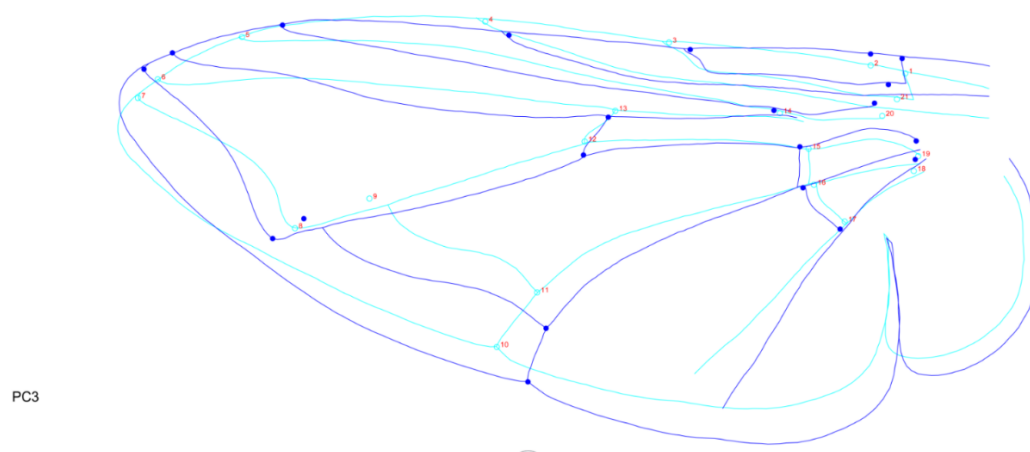


Figure 4.7c: PC3 Shape morphology of Monocrotophos 1/3rd of EC₅₀ (MB) set

Partial Least Square analysis (PLS)

The size and shape variations between the Control (MO) and the two concentrations of monocrotophos (MA and MB) were explained using 2B-PLS analysis. Block 1 PLS represents the size variation (Figure 4.8a), and Block 2 PLS explains the shape variation (Figure 4.8b). In comparison, size variation was more prominent than shape variation. In block 1, the frequency of the control set peaked at the points where the frequency of the higher dose of monocrotophos (MB) seemed to be lower and vice versa. The lower dose (MA) had a median/average frequency at these points. The shape variation explained by Block 2 PLS, on the other hand, showed fewer fluctuations in frequency. However, the frequencies of the treatment sets (MA and

MB) peaked significantly at certain points and the Control set (MO) also exhibited increased frequency in other areas. The results clearly point to the concentration-dependent variations of wing morphology of *Chrysomya megacephala*

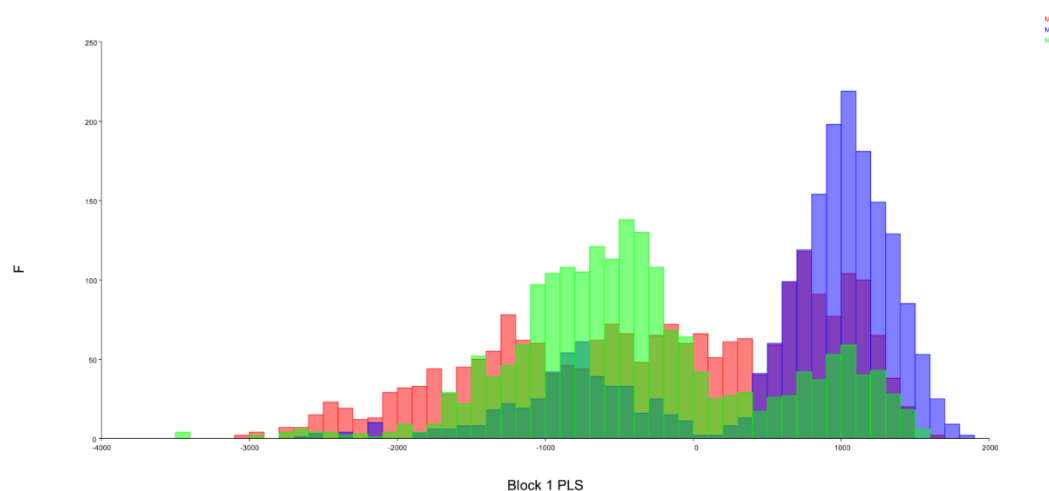


Figure 4.8a: Block 1 PLS representing the size variation in *Chrysomya megacephala* wings due to *Monocrotophos* exposure (MO- Control, MA- 1/5th of EC₅₀, MB- 1/3rd of EC₅₀)

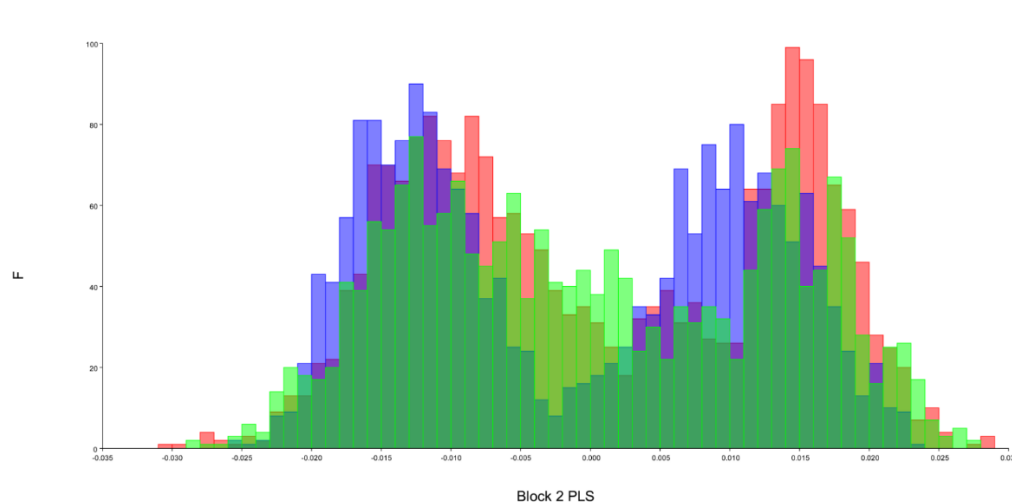


Figure 4.8b: Block 2 PLS representing the shape variation in *Chrysomya megacephala* wings due to *Monocrotophos* exposure (MO- Control, MA- 1/5th of EC₅₀, MB- 1/3rd of EC₅₀)

Discriminant Function Analysis (DFA)

Discriminant Function Analysis (DFA) revealed the concentration-dependent changes in wing shape. The wing shape of 1/5th of the EC₅₀ treatment set (MA) and the wing shape of 1/3rd of the EC₅₀ treatment set (MB) were compared with the wing shape of the control set (MO) (Figure 4.9a and Figure 4.9b). Both treatment sets (MA and MB) showed similar trends and were separated from the control set. Although there was some overlap, it was minimal, and the overall frequencies were also slightly higher in both treatment sets than in the control set.

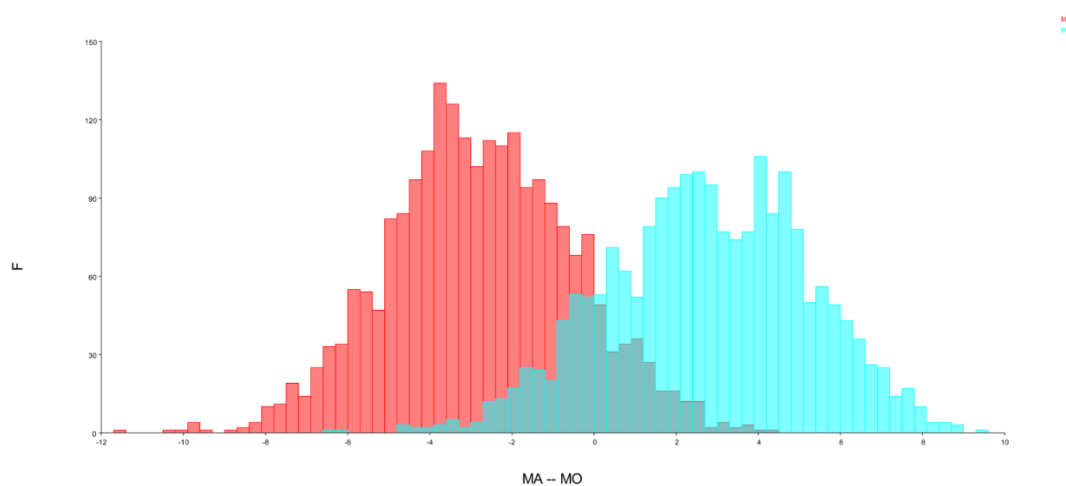


Figure 4.9a: Discriminant Function Analysis of Control (MO) vs. 1/5th of EC₅₀ (MA)

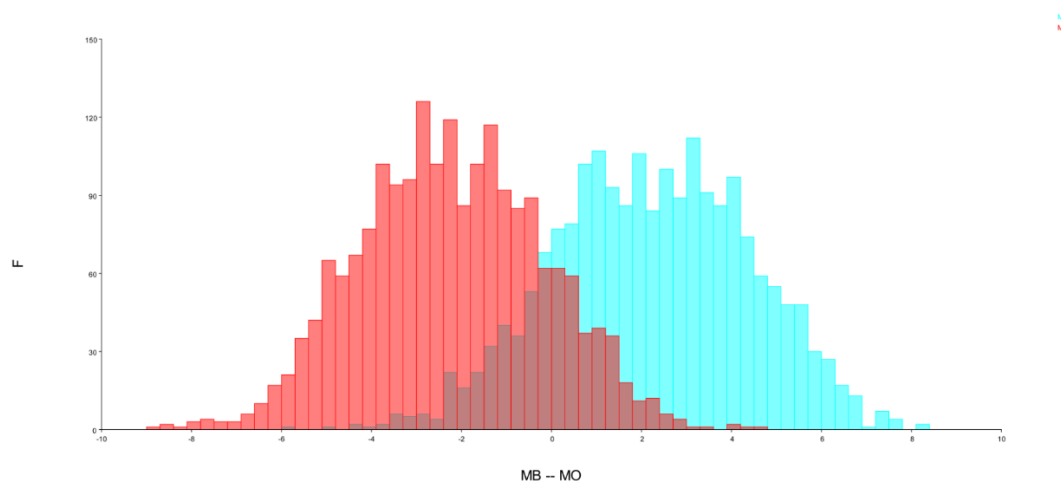


Figure 4.9b: Discriminant Function Analysis of Control (MO) vs. 1/3rd of EC₅₀ (MB)

Morphospace analysis by Principal Component Analysis (PCA) and Canonical Variate Analysis (CVA)

The PCA and CVA validated the variation in morphological data in the largest variation coordinates (Figure 4.10a and 4.10b). In PCA, the data of every individual wing in all three sets (MO, MA, and MB) were represented in the morphospace to understand the variations between the groups. A total of 38 PCs were present in the analysis and the first PC covered 53% variation. The analysis showed slight variation among the three groups and a small percentage of individuals in all three sets were located outside the centroid. CV1 covered 82.42% variation and the CVA analysis compared the average of each dataset to represent and understand the displacements in the morphospace owing to the effect of monocrotophos. CVA analysis revealed clustering and overlapping of all three sets, with the treatment sets (MA and MB) overlapping more than the Control cluster.

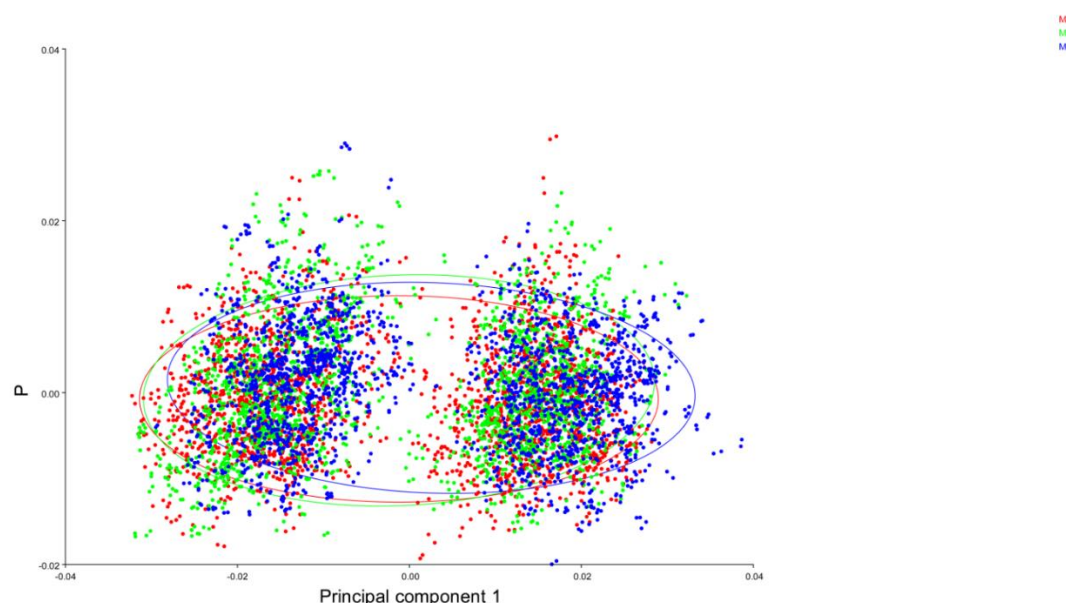


Figure 4.10a: PCA morphospace analysis of *Chrysomya megacephala* wings due to Monocrotophos exposure (MO- Control, MA- 1/5th of EC₅₀, MB- 1/3rd of EC₅₀)

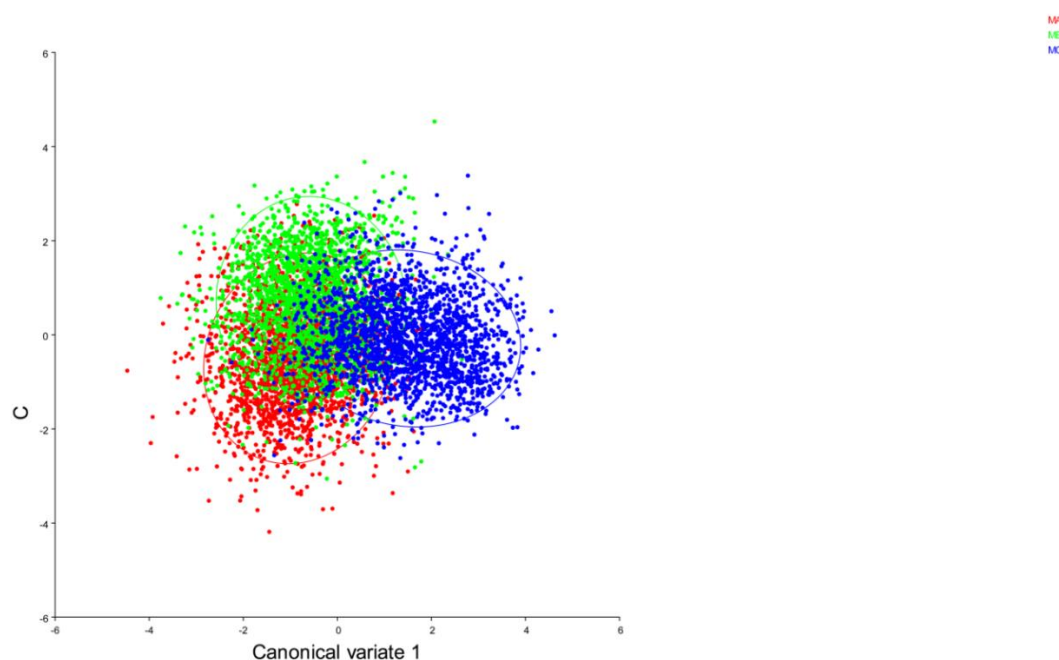


Figure 4.10b: CVA morphospace analysis of *Chrysomya megacephala* wings due to *Monocrotophos* exposure (MO- Control, MA- 1/5th of EC₅₀, MB- 1/3rd of EC₅₀)

4.4.1b Effect of Carbamazepine on wing morphology of *Chrysomya megacephala*

The sublethal concentrations of Carbamazepine selected for the study are 1/5th of EC₅₀ (CA- 0.118 mg/ml) and 1/3rd of EC₅₀ (CB- 0.1975 mg/ml). These are then compared to the Control (CO) to understand the size and shape variations.

% variance and Principal Component Analysis

In the Control set (CO) of *Chrysomya megacephala*, 38 principal components (PCs) cumulatively explained 100% of the variation. Among these, PC1 explained 20.32% of the population variation, while PC2 and PC3 accounted for 19.17% and 12.89%, respectively (Figure 4.11). The highest levels of variation in PC1 were the proximal shifts of the distal ends of the radial, median and cubitus veins, posterior displacement of the medio-cubital cross vein, and distal displacement of the radio-medial cross vein (Figure 4.12a). In the case of PC2, the major displacements were the distal displacement of the distal end of cubitus, distal intersection of medio-cubital cross vein, intersections of costa-subcosta and costa-radial vein 1, proximal shifts of radio-medial cross vein, and posterior shifting of distal ends of radius (2+3 and 4+5) and

median (Figure 4.12b). PC3 exhibited major variations in the distal ends of radial vein 4+5 and median that shifted anteriorly, medio-cubital cross vein, and the distal end of cubitus that shifted posteriorly, and radio-medial cross vein that showed proximal shifting (Figure 4.12c).

These variations are considered natural variations that occur within the normal population of *Chrysomya megacephala*.

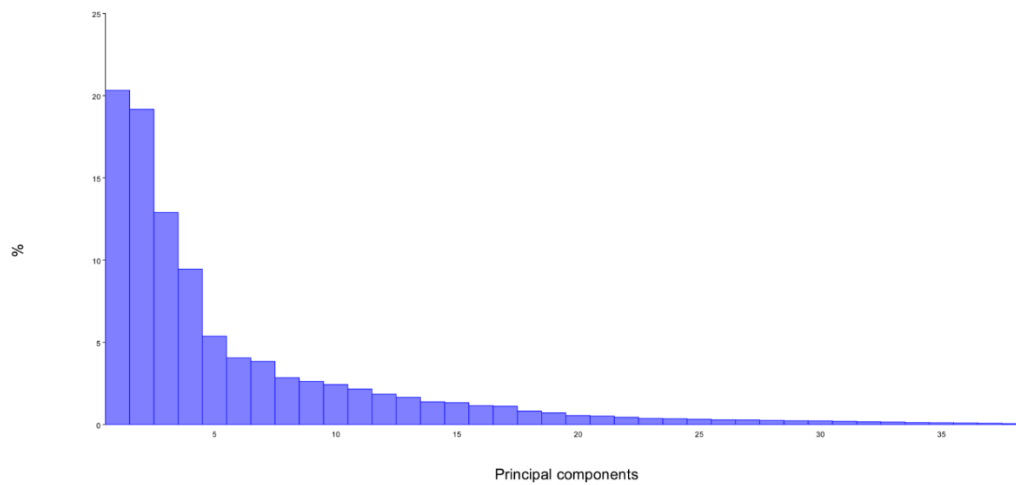


Figure 4.11: Percentage variance of Principal component analysis in the Carbamazepine Control (CO) set

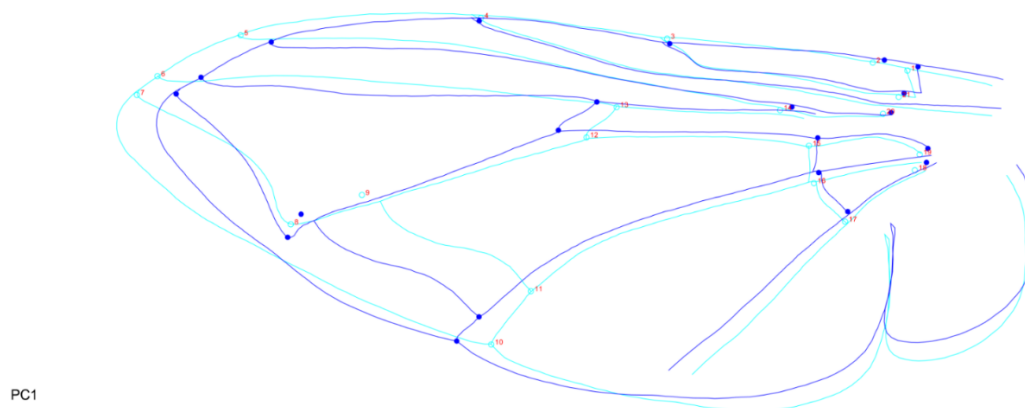


Figure 4.12a: PC1 Shape morphology of the Control (CO) set

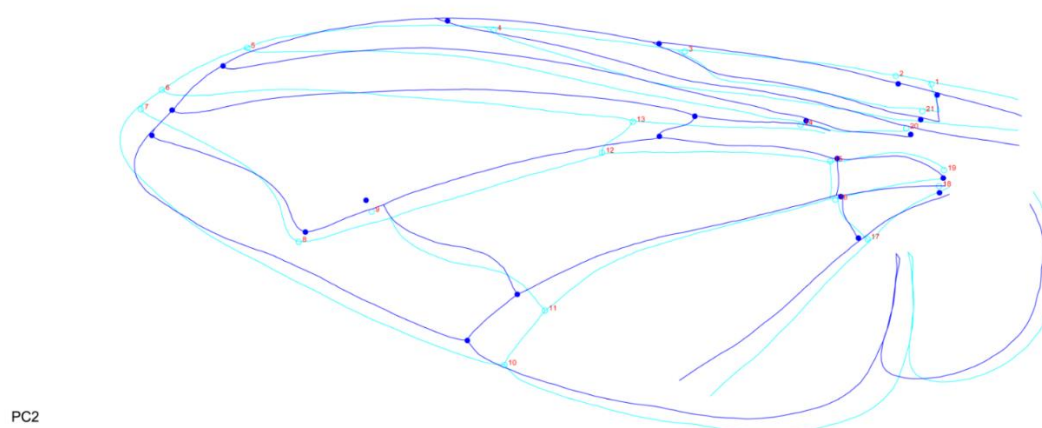


Figure 4.12b: PC2 Shape morphology of the Control (CO) set

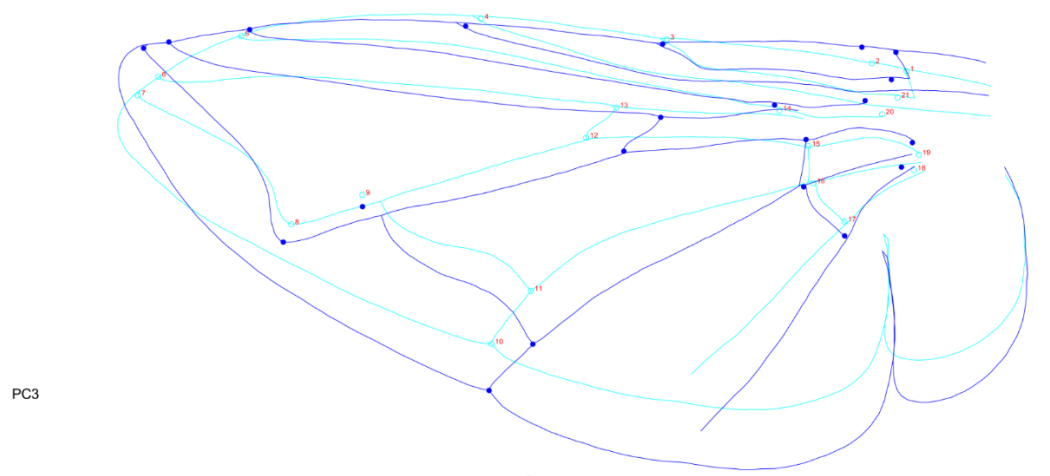


Figure 4.12c: PC3 Shape morphology of the Control (CO) set

In the lower dose Carbamazepine treatment set ($1/5^{\text{th}}$ of EC_{50} - CA), 100% of the variation was explained by 38 PCs. The first three PCs explained 54.29% of the population variation (PC1: 27.67%, PC2: 14.17%, PC3: 12.44%) in wing shape and size (Figure 4.13). The landmarks in PC1 that were attributed to the highest variations were landmarks 9, 10, 11, 12, and 13 (Figure 4.14a). The Radio-medial cross vein was displaced proximally, and the distal ends of the Cubitus and the Medio-cubital cross vein also showed displacement. The major displacements in PC2 were the posterior shifting of anterior regions, such as the intersections of costa-subcosta and costa-radial 1, distal ends of radial vein 2+3, anteroproximal displacement of radial vein 4+5 and the distal end of median, and anterior shifting of the radio-medial cross vein (Figure

4.14b). The distal end of the cubitus and medio-cubital cross vein also showed shifting. These displacements combined caused anteroposterior compression of the wing blade, making it narrower than the average. The major displacements in PC3 were the posterior shifting of the anteriorly located points such as the costal-humeral junction, humeral break, bifurcation of radial vein 2+3 from radial vein 1, origin of subcostal and radial vein 1, anterior shifting of posterior areas such as the distal end of cubitus and medio-cubital cross vein, and posterior shifting of the distal ends of the radius (2+3 and 4+5) and median (Figure 4.14c). PC3 also showed a slight overall narrowing of the wing in the anteroposterior plane.

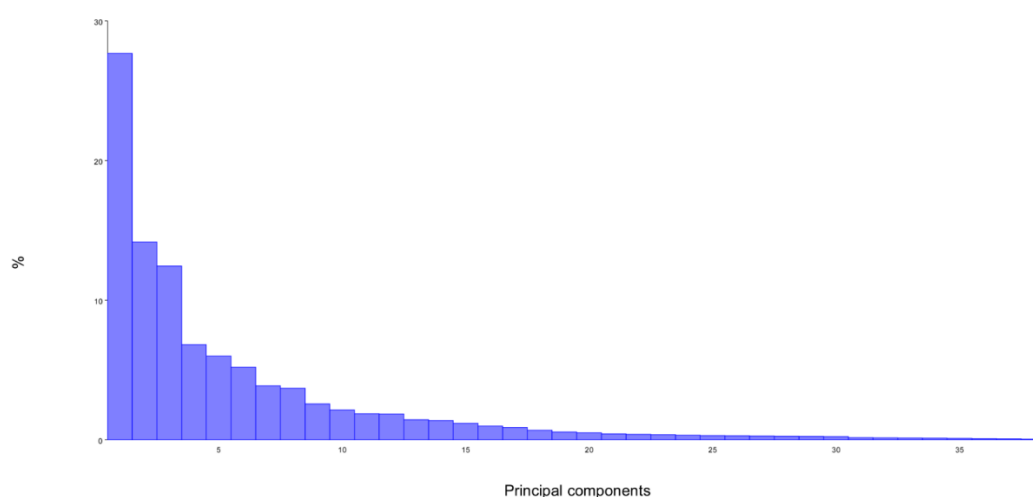


Figure 4.13: Percentage variance of Principal component analysis in Carbamazepine 1/5th of EC₅₀ (CA) set

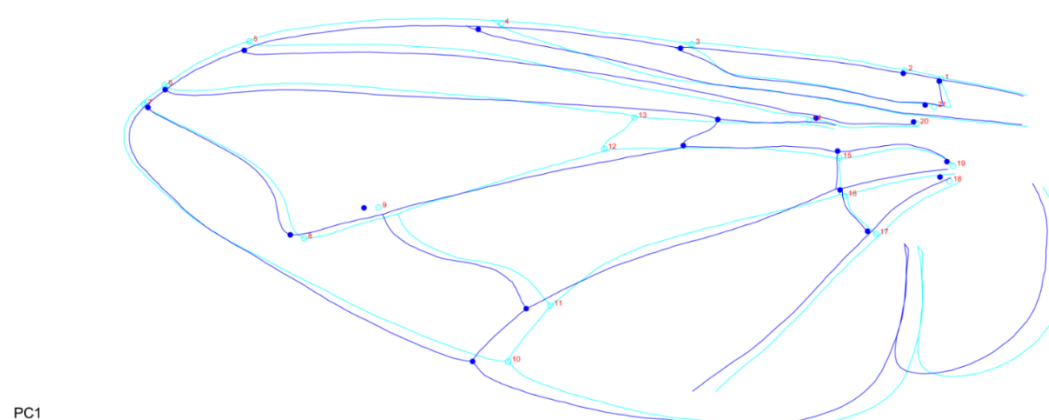


Figure 4.14a: PC1 Shape morphology of Carbamazepine 1/5th of EC₅₀ (CA) set

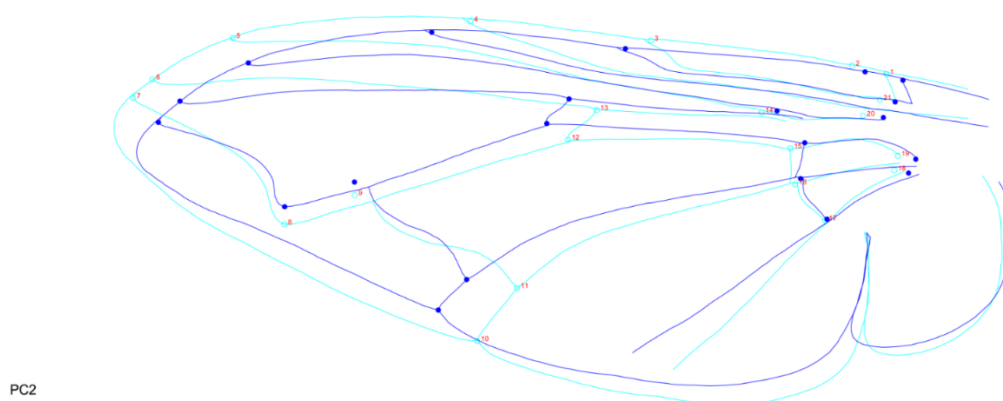


Figure 4.14b: PC2 Shape morphology of Carbamazepine 1/5th of EC₅₀ (CA) set

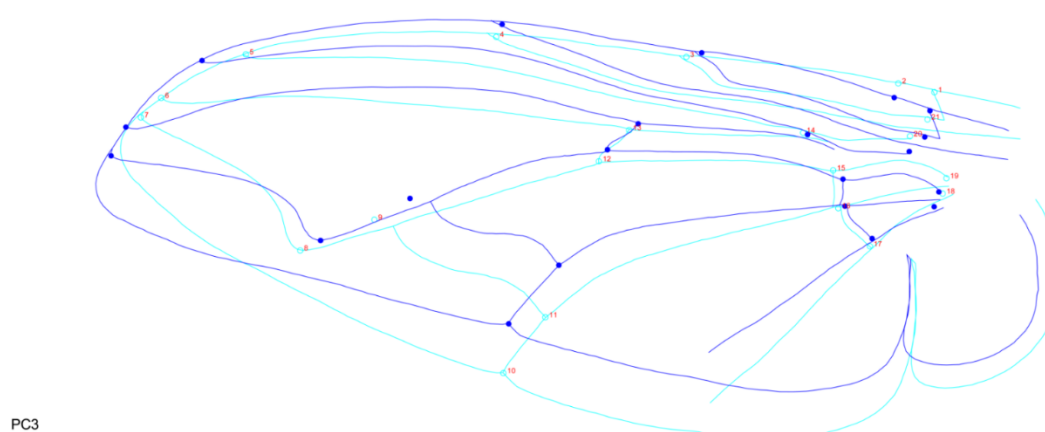


Figure 4.14c: PC3 Shape morphology of Carbamazepine 1/5th of EC₅₀ (CA) set

The 1/3rd of EC₅₀ treated wings of *Chrysomya megacephala* also had a total of 38 PCs that cumulatively explained 100% of the variation. PC1 explained 22.14% of the total population variation, whereas PC2 and PC3 explained 15.22% and 13.22% of the total variation, respectively (Figure 4.15). The figure 4.16a is the PC1 shape morphology depicting major displacements at landmarks 4, 10, 11, 12, and 13. The anteriorly located costa-radial vein, posteriorly located distal end of cubitus, and the proximal intersection of the medio-cubital cross vein were proximally displaced. The radio-median cross vein is the only region that has been distally displaced. Major displacements were observed mostly towards the distal areas. The proximal regions showed comparatively low displacements. PC2 exhibited major displacements such as posterior shifting of the costa-humeral junction, humeral break, bifurcation point

of radial veins, and the origin of subcostal and radius 1, anterior shifting of intersections of costa-subcosta and costa-radius, medio-cubital cross vein, and distal end of cubitus, and distal displacement of points such as the distal ends of radial veins and cubitus (Figure 4.16b). PC3 also showed major displacements, which resulted in the anteroposterior narrowing of the wing blade (Figure 4.16c). The major displacements were the posterior shifting of all the anterior-most wing border points, such as the costa-humeral junction, humeral break, intersections of costa-subcosta and costa-radius1, distal ends of radial veins and median, and the anterior shifting of medio-cubital cross vein, distal end of cubitus, and radio-medial cross vein.

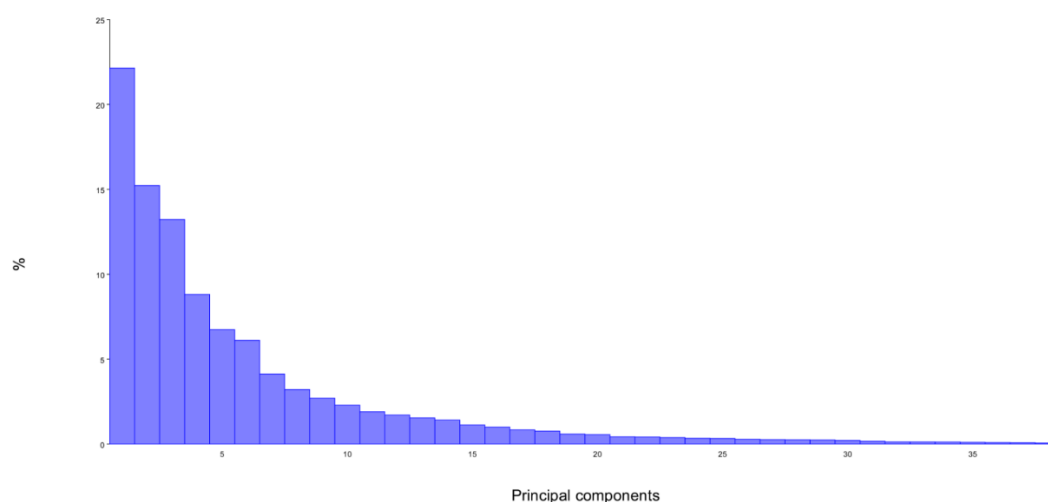


Figure 4.15: Percentage variance of Principal component analysis in Carbamazepine 1/3rd of EC₅₀ (CB) set

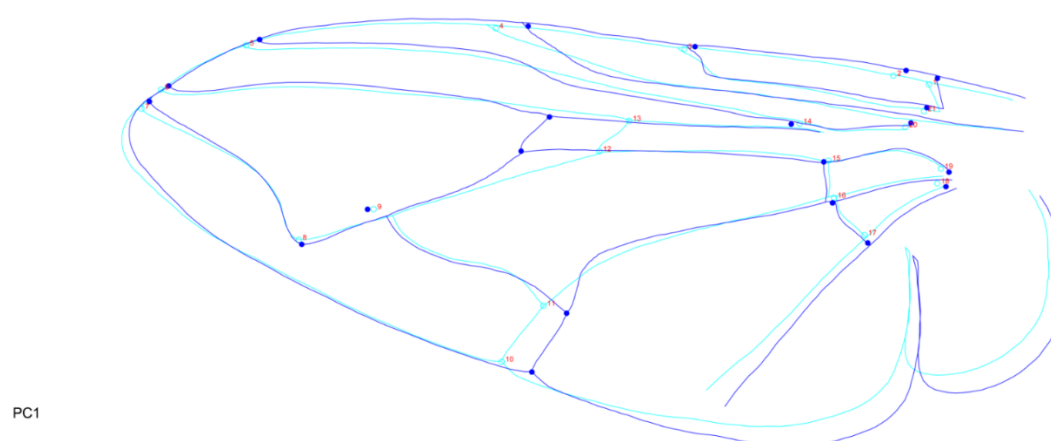


Figure 4.16a: PC1 Shape morphology of Carbamazepine 1/3rd of EC₅₀ (CB) set

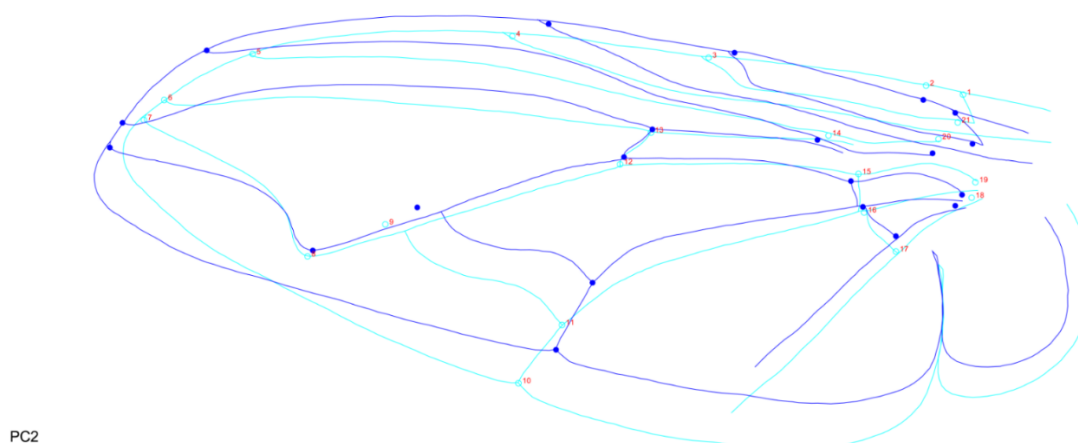


Figure 4.16b: PC2 Shape morphology of Carbamazepine 1/3rd of EC_{50} (CB) set

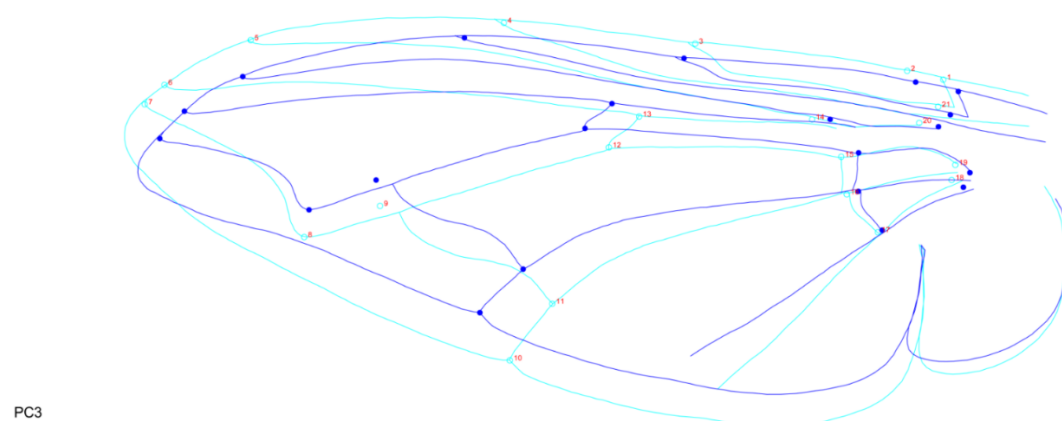


Figure 4.16c: PC3 Shape morphology of Carbamazepine 1/3rd of EC_{50} (CB) set

Partial Least Square analysis (PLS)

The size and shape variations between the Control (CO) and the two concentrations of carbamazepine (CA and CB) were explained using 2B-PLS analysis. Block 1 and Block 2 PLS represent the size and shape variation, respectively (Figure 4.17a and 4.17b) and the exposure to carbamazepine appeared to cause both size and shape variation. In block 1, the frequency of the control set showed an incredible peaking at the points where the frequency of the treated sets (CA and CB) was very low with CB being the lowest. At the same time, certain bars showed the highest frequencies for the treated groups where the frequency of the Control was the lowest. The shape variation explained by Block 2 PLS, on the other hand, showed lower levels of

frequency fluctuations. However, the frequencies of the treatment sets (CA and CB) peaked significantly at certain points where the Control set exhibited low frequency and vice versa, indicating a clear concentration-dependent influence of intoxicant in wing morphology.

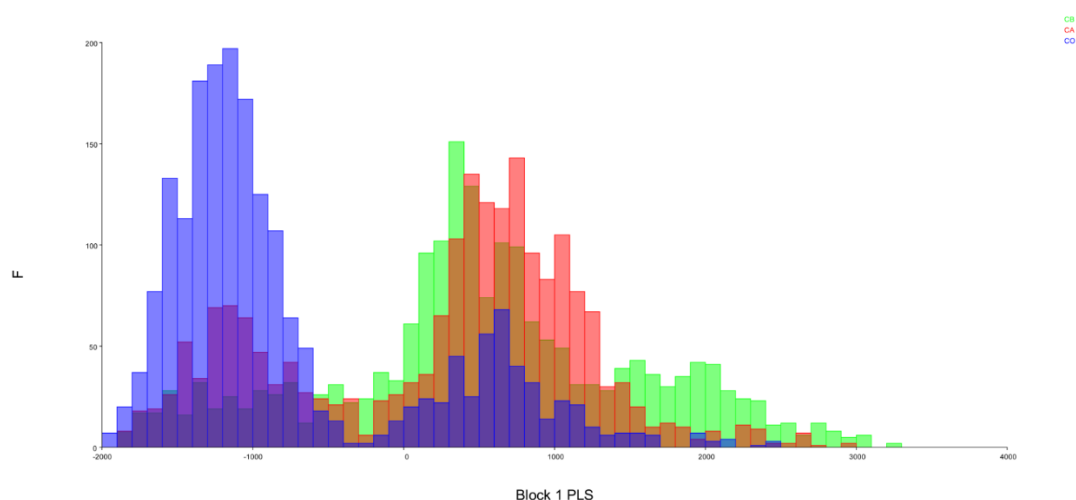


Figure 4.17a: Block 1 PLS representing the size variation in *Chrysomya megacephala* wings due to Carbamazepine exposure (CO- Control, CA- 1/5th of EC₅₀, CB- 1/3rd of EC₅₀)

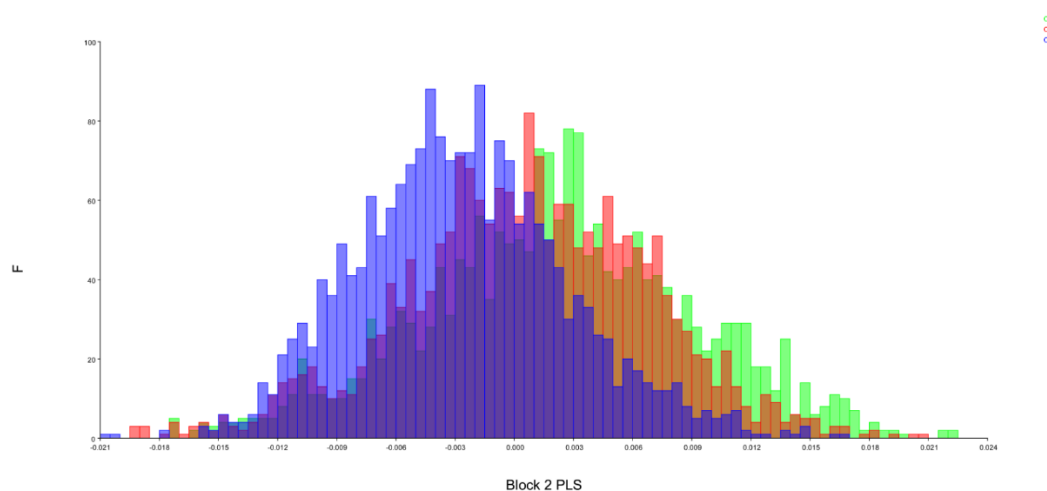


Figure 4.17b: Block 2 PLS representing the shape variation in *Chrysomya megacephala* wings due to Carbamazepine exposure (CO- Control, CA- 1/5th of EC₅₀, CB- 1/3rd of EC₅₀)

Discriminant Function Analysis (DFA)

Discriminant Function Analysis (DFA) revealed the concentration-dependent changes in wing shape. The wing shape of 1/5th of the EC₅₀ treatment set (CA) and the wing shape of 1/3rd of the EC₅₀ treatment set (CB) were compared with the wing shape of

the Control set (C) (Figure 4.18a and Figure 4.18b). Both treatment sets (CA and CB) showed similar trends, where they mostly existed separately from the Control set. However, there was some overlap between CO vs. CA and CO vs. CB. The overall frequencies were slightly lower in both treatment groups than in the control group.

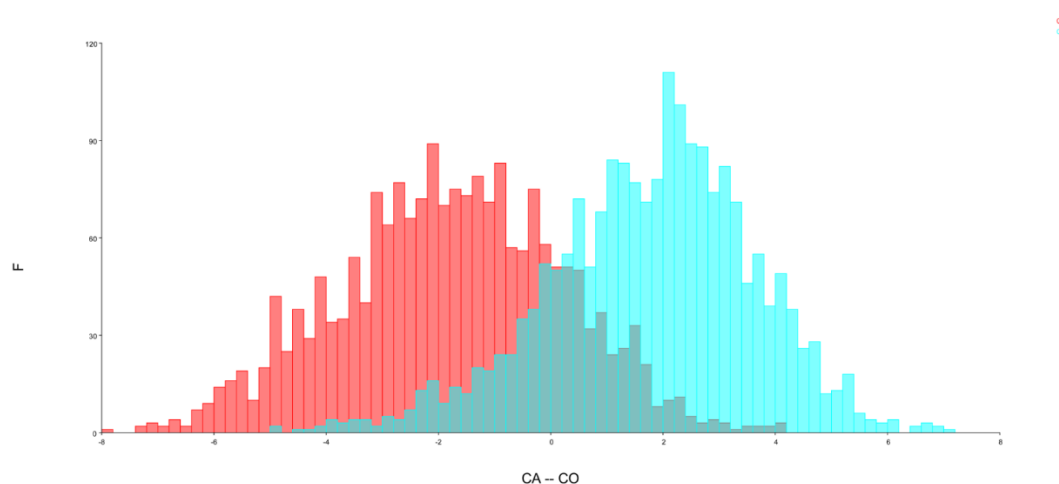


Figure 4.18a: Discriminant Function Analysis of Control (CO) vs $1/5^{\text{th}}$ of EC_{50} (CA)

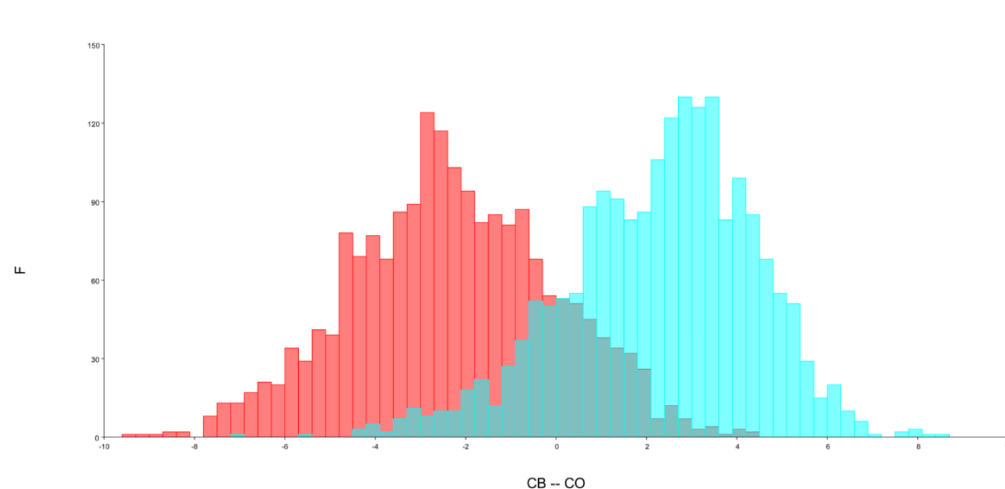


Figure 4.18b: Discriminant Function Analysis of Control (CO) vs $1/3^{\text{rd}}$ of EC_{50} (CB)

Morphospace analysis by Principal Component Analysis (PCA) and Canonical Variate Analysis (CVA)

The PCA and CVA validated the variation in morphological data in the largest variation coordinates (Figure 4.19a and 4.19b). In PCA, the data of every individual

wing in all three sets (CO, CA, and CB) were represented in the morphospace to understand the variations between the groups. A total of 38 PCs were present, and the first three PCs combinedly covered about 50.28% of the variation. The analysis showed slight variation among the three groups and a small percentage of individuals in all three sets were located outside the centroid. CV1 covered 91.85% of the variations and the CVA analysis compared the average of each dataset to represent and understand the displacements in the morphospace owing to the effect of monocrotophos. CVA analysis revealed clustering and overlapping of all three sets, with the treatment sets (CA and CB) overlapping more than the control cluster.

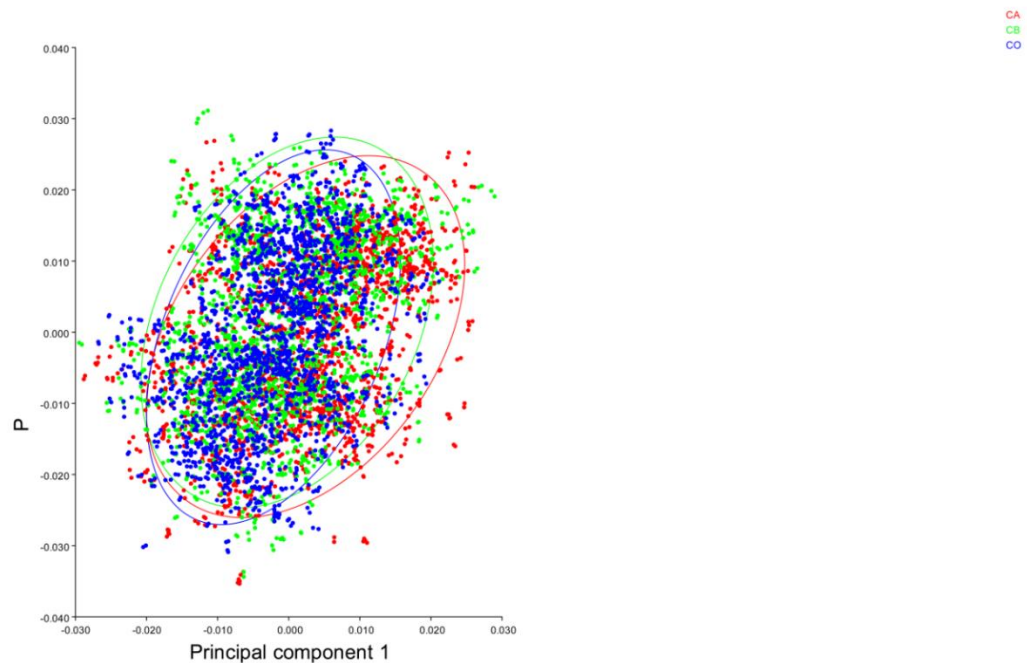


Figure 4.19a: PCA morphospace analysis of *Chrysomya megacephala* wings due to Carbamazepine exposure (CO- Control, CA- 1/5th of EC₅₀, CB- 1/3rd of EC₅₀)

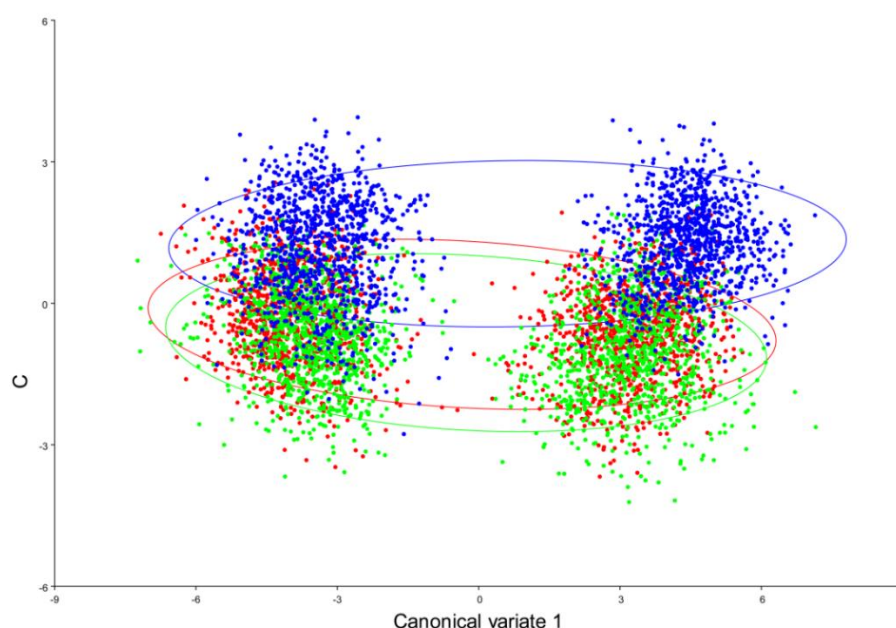


Figure 4.19b: CVA morphospace analysis of *Chrysomya megacephala* wings due to Carbamazepine exposure (CO- Control, CA- 1/5th of EC₅₀, CB- 1/3rd of EC₅₀)

4.4.1c Comparative analysis – Monocrotophos vs. Carbamazepine

Discriminant Function Analysis between the Controls (MO vs. CO), 1/5th of EC₅₀ (MA vs. CA), and 1/3rd of EC₅₀ (MB vs. CB) was performed to obtain an overall view of the analysis process. Apart from the Control sets, which showed the highest levels of overlap, the treatment sets were separated from each other with very little overlap (Figure 4.20a, 4.20b, and 4.20c).

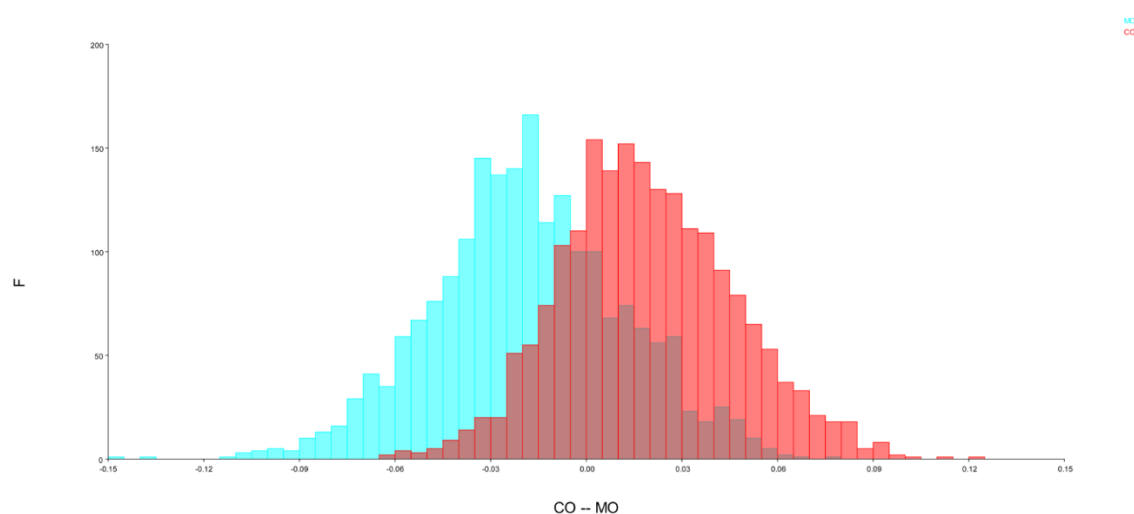


Figure 4.20a: Discriminant Function Analysis of Control sets (CO vs. MO)

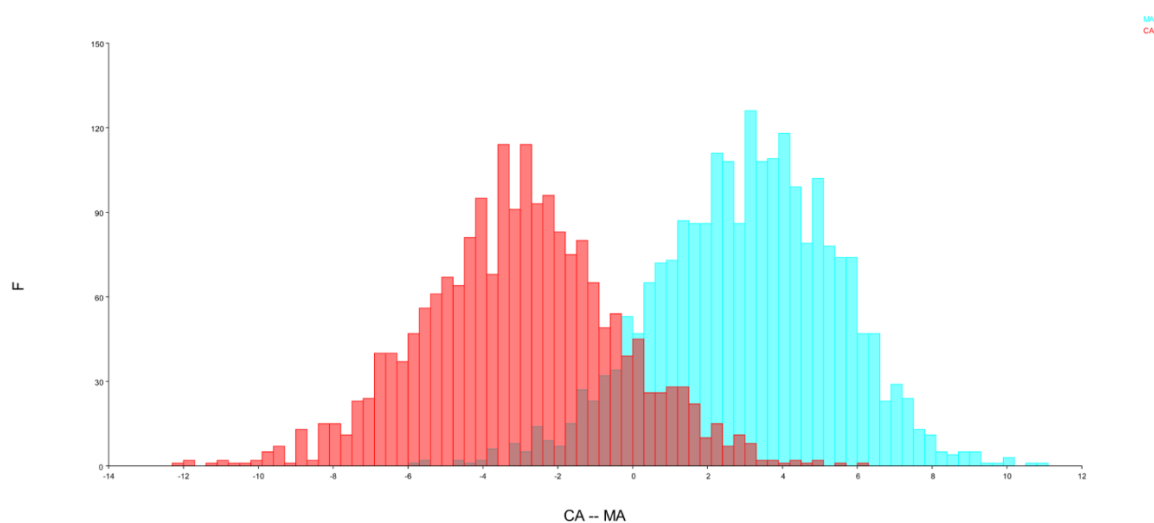


Figure 4.20b: Discriminant Function Analysis of 1/5th of EC₅₀ sets (CA vs. MA)

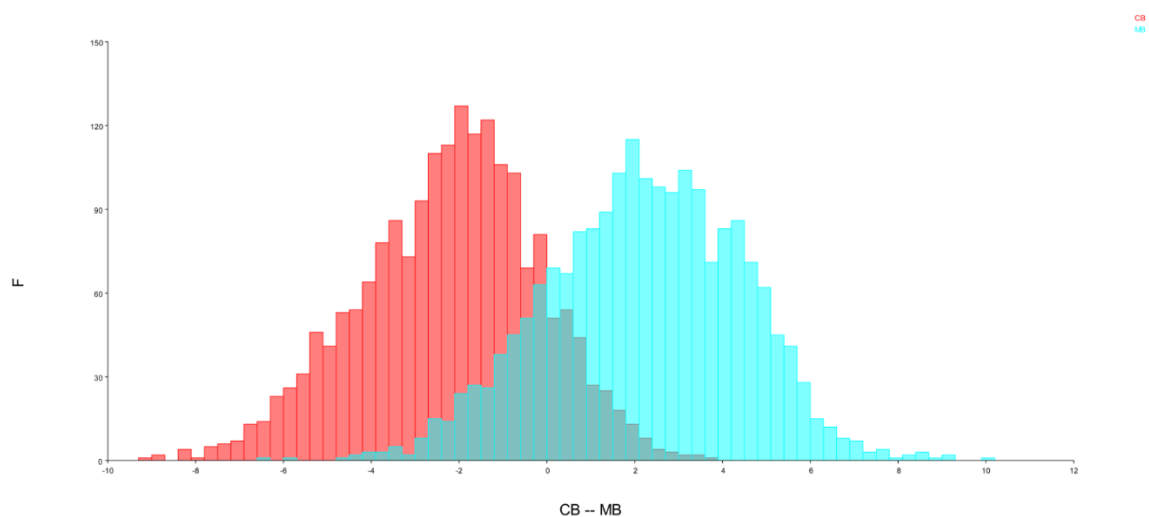


Figure 4.20c: Discriminant Function Analysis of 1/3rd of EC₅₀ sets (CB vs. MB)

The CVA morphospace analysis with CV1 covering 47.66% of the variation revealed certain levels of overlap (Figure 4.21). However, each of the intoxicants had its own separation in the morphospace, indicating variation from each other. The Control also appeared as another distinct cluster in the morphospace.

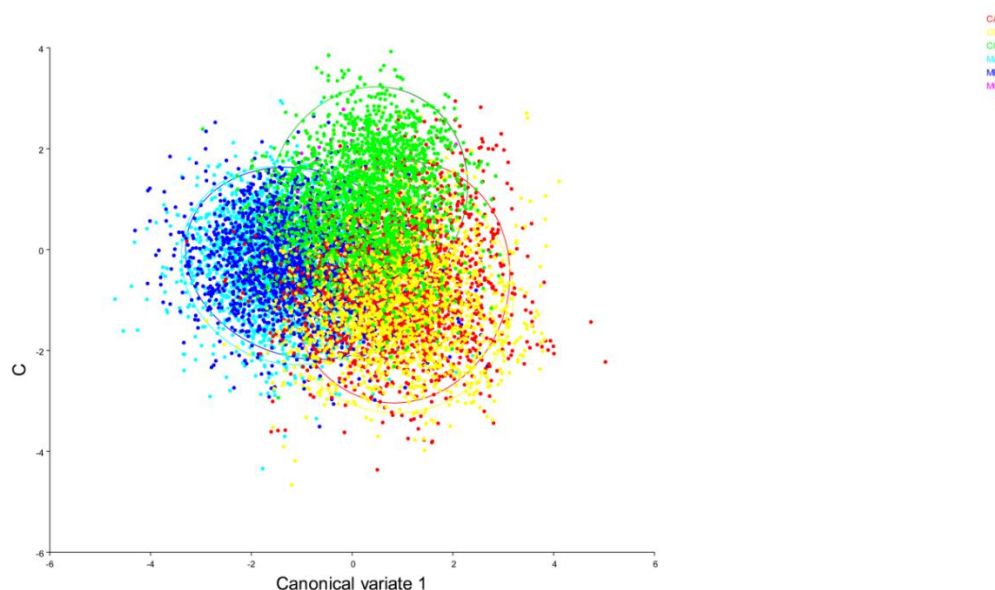


Figure 4.21: CVA morphospace analysis of *Chrysomya megacephala* wings (Carbamazepine: CO- Control, CA- 1/5th of EC₅₀, CB- 1/3rd of EC₅₀; Monocrotophos: MO- Control, MA- 1/5th of EC₅₀, MB- 1/3rd of EC₅₀)

4.4.2 Transcriptome analysis of genes involved in wing development

Transcriptomic analysis revealed the differential expression of several genes involved in wing development owing to the effects of both monocrotophos and carbamazepine. Both intoxicants have caused the differential expression of several genes involved in, wing and notum subfield formation, wing cell fate specification, wing disc development, wing disc dorsal/ventral pattern formation, wing disc morphogenesis, cell elongation involved in imaginal disc-derived wing morphogenesis, imaginal disc-derived wing margin morphogenesis, imaginal disc-derived wing morphogenesis, imaginal disc-derived wing vein morphogenesis, wing disc pattern formation, wing disc proximal/distal pattern formation, apposition of dorsal and ventral imaginal disc-derived wing surfaces, the establishment of imaginal disc-derived wing hair orientation, imaginal disc-derived wing expansion, negative regulation of imaginal disc-derived wing vein specification, regulation of imaginal disc-derived wing size, regulation of imaginal disc-derived wing vein specification, and cell hair.

Most genes affected by the intoxicants were the same, even though their expression patterns were unique. Four genes, invected, wingless, distal-less, and enhancer of split

my, were exclusive to carbamazepine exposure. Most of the differentially expressed genes were found to be down-regulated compared to the control set, due to the exposure to monocrotophos as well as carbamazepine. A detailed list of genes showing differential expression due to monocrotophos and carbamazepine exposure is given in Table 4.3 and Table 4.4, respectively.

Table 4.3: Differentially expressed genes involved in the wing development of *Monocrotophos*-exposed *Chrysomya megacephala*

Function	Gene		Up-regulated ▲	Down-regulated ▼
Wing and notum subfield formation (GO:0035309)	1	spalt major	0	1
	2	decapentaplegic	0	1
	3	rhomboid	0	2
	4	araucan	1	2
	5	Shaggy	1	1
	6	Epidermal growth factor receptor	1	3
Wing cell fate specification (GO:0035311)	1	rhomboid	0	2
Wing disc anterior/posterior pattern formation (GO:0048100)	1	smoothened	0	1
	2	interference hedgehog	0	2
	3	hedgehog	0	1
	4	serrate	0	4
	5	division abnormally delayed	0	1
		engrailed	0	1
	6	fringe	0	1
	7	patched	0	2
	8	mothers against decapentaplegic	1	7
	9	rasp	1	0
Wing disc development (GO:0035220)	10	cubitus interruptus	0	1
	1	frizzled	0	4
	2	rhomboid	0	1
	3	Anchor	7	7
	4	ebi	1	0
	5	rabenosyn	1	0
	6	strawberry notch	0	1
	7	interference hedgehog	0	2
	8	escargot	0	1
	9	homothorax	1	3

10	Nedd4	0	1
11	osa	0	2
12	fritz	2	0
13	tricornered	1	0
14	singed	1	1
15	decapentaplegic	0	1
16	neuralized	1	3
17	rasp	1	0
18	pygopus	0	2
19	dual oxidase	0	2
20	draper	0	5
21	split ends	0	5
22	presenilin	3	0
23	patched	0	2
24	smoothened	0	1
25	Delta	2	2
26	sin3a	0	2
27	dispatched	0	1
28	folded gastrulation	0	1
29	Chromodomain- helicase-DNA- binding protein 1	0	3
30	Rac-1	2	1
31	Ecdysone receptor	0	4
32	teashirt	1	0
33	abrupt	0	1
34	asteroid	0	1
35	Rab-7	1	0
36	gurken	0	2
37	deltex	1	2
38	capicua	0	4
39	armadillo	4	3
40	wntless	1	0
41	Rab5	0	1
42	ADP ribosylation factor 1	1	0
43	unkempt	1	2
44	held out wings	1	0
45	jing	0	2
46	Epidermal growth factor receptor	1	3
47	annexin B9	2	0
48	dystroglycan	0	1
49	dystrophin	0	3
50	Notch	0	4

51	crumbs	0	3
52	kinase suppressor of ras	0	1
53	LIM domain kinase 1	2	0
54	apterous	1	0
55	G protein-coupled receptor kinase 2	0	1
56	vestigial	0	1
57	frizzled	0	4
58	brahma	1	0
59	quail	2	0
60	araucan	1	2
61	mastermind	0	4
62	serrate	0	4
63	gustavus	2	0
64	pangolin	4	1
65	Spalt major	0	1
66	parvin	1	0
67	fringe	0	1
68	hopscotch	0	1
69	Rho1	5	1
70	Nostrin	0	1
71	Death-associated protein kinase related	0	2
72	prickle	2	1
73	Suppressor of Hairless	0	1
74	p115	0	1
75	tolloid	0	2
76	spenito	0	1
77	yippee	1	3
78	shaggy	1	1
79	cubitus interruptus	0	1
80	stubble	1	1
81	hairless	0	1
82	hedgehog	0	1
83	division abnormally delayed	0	1
84	inturned	0	1
85	Rap1 GTPase	0	2
86	pointed	0	1
87	scarface	0	1
88	spitz	0	1
89	penguin	1	0
90	chip	1	0
91	sprouty	0	1

	92	Protein kinase N	0	3
	93	engrailed	0	1
	94	mothers against decapentaplegic	1	7
	95	costa	0	1
Wing disc dorsal/ventral pattern formation (GO:0048190)	1	vestigial	0	1
	2	annexin B9	2	0
	3	draper	0	5
	4	osa	0	2
	5	pointed	0	1
	6	presenilin	3	0
	7	fringe	0	1
	8	Notch	0	4
	9	crumbs	0	3
	10	Rab7	1	0
	11	strawberry notch	0	1
	12	Delta	2	2
	13	hairless	0	1
	14	apterous	1	0
	15	division abnormally delayed	0	1
	16	Suppressor of Hairless	0	1
Wing disc morphogenesis (GO:0007472)	1	rhomboid	0	2
	2	anchor	7	7
	3	ebi	1	0
	4	osa	0	2
	5	Rabenosyn	1	0
	6	strawberry notch	0	1
	7	homothorax	1	3
	8	Nedd4	0	1
	9	fritz	1	0
	10	tricornered	1	0
	11	singed	1	1
	12	Star	0	1
	13	decapentaplegic	0	1
	14	neuralized	1	3
	15	Split ends	0	5
	16	pygopus	0	2
	17	furry	0	2
	18	Dual oxidase	0	2
	19	patched	0	2
	20	Delta	2	2
	21	smoothened	0	1
	22	folded gastrulation	0	1

23	Chromodomain-helicase-DNA-binding protein 1	0	2
24	Rac1	2	1
25	Ecdysone receptor	0	4
26	teashirt	1	0
27	Stromal interaction molecule	0	3
28	asteroid	0	1
29	gurken	0	2
30	capicua	0	4
31	armadillo	4	3
32	wntless	1	0
33	ADP ribosylation factor 1	1	0
34	unkempt	1	2
35	held out wings	1	0
36	jing	2	0
37	Epidermal growth factor receptor	1	3
38	dystroglycan	0	1
39	dystrophin	0	3
40	Notch	0	4
41	kinase suppressor of ras	0	1
42	LIM domain kinase 1	1	0
43	apterous	1	0
44	G protein-coupled receptor kinase 2	0	1
45	CTD nuclear envelope phosphatase 1	1	1
46	vestigial	0	1
47	frizzled	0	4
48	quail	2	0
49	araucan	1	2
50	mastermind	0	4
51	serrate	0	4
52	gustavus	2	0
53	pangolin	4	1
54	parvin	1	0
55	fringe	0	1
56	hopscotch	0	1
57	Rho1	5	1
58	Nostrin	0	1
59	Death-associated protein kinase related	0	2

	60	prickle	2	1
	61	Suppressor of Hairless	0	1
	62	p115	0	1
	63	tolloid	0	2
	64	yippee	1	3
	65	shaggy	1	1
	66	pointed	0	1
	67	scarface	0	1
	68	hairless	0	1
	69	hedgehog	0	1
	70	division abnormally delayed	0	1
	71	inturned	0	1
	72	Rap1 GTPase	0	2
	73	pointed	0	1
	74	scarface	0	1
	75	spitz	0	1
	76	penguin	1	0
	77	chip	1	0
	78	sprouty	0	1
	79	Protein kinase N	0	3
	80	engrailed	0	1
	81	mothers against decapentaplegic	1	7
Cell elongation involved in imaginal disc-derived wing morphogenesis (GO:0090254)	1	vestigial	0	1
	2	Rho1	5	1
	3	armadillo	4	3
	4	pangolin	4	1
Imaginal disc-derived wing margin morphogenesis (GO:0008587)	1	held out wings	1	0
	2	shaggy	1	1
	3	ebi	1	0
	4	osa	0	2
	5	Notch	0	4
	6	strawberry notch	0	1
	7	Delta	2	2
	8	hairless	0	1
	9	brahma	1	0
	10	frizzled	0	4
	11	mastermind	0	4
	12	serrate	0	4
	13	parvin	1	0
	14	fringe	0	1
	15	penguin	1	0
	1	rhomboid	0	2
	2	anchor	7	7

Imaginal disc-derived wing morphogenesis (GO:0007476)	3	ebi	1	0
	4	osa	0	2
	5	Rabenosyn	1	0
	6	strawberry notch	0	1
	7	homothorax	1	3
	8	Nedd4	0	1
	9	fritz	1	0
	10	tricornered	1	0
	11	singed	1	1
	12	Star	0	1
	13	decapentaplegic	0	1
	14	neuralized	1	3
	15	Split ends	0	5
	16	pygopus	0	2
	17	furry	0	2
	18	Dual oxidase	0	2
	19	patched	0	2
	20	Delta	2	2
	21	smoothened	0	1
	22	folded gastrulation	0	1
	23	Chromodomain-helicase-DNA-binding protein 1	0	2
	24	Rac1	2	1
	25	Ecdysone receptor	0	4
	26	teashirt	1	0
	27	abrupt	0	1
	28	asteroid	0	1
	29	Rab-7	1	0
	30	gurken	0	2
	31	deltex	1	2
	32	capicua	0	4
	33	armadillo	4	3
	34	wntless	1	0
	35	ADP ribosylation factor 1	1	0
	36	unkempt	1	2
	37	held out wings	1	0
	38	jing	0	2
	39	Epidermal growth factor receptor	1	3
	40	dystroglycan	0	1
	41	dystrophin	0	3
	42	Notch	0	4
	43	crumbs	0	3

	44	kinase suppressor of ras	0	1
	45	LIM domain kinase 1	2	0
	46	apterous	1	0
	47	G protein-coupled receptor kinase 2	0	1
	48	vestigial	0	1
	49	frizzled	0	4
	50	brahma	1	0
	51	quail	2	0
	52	araucan	1	2
	53	mastermind	0	4
	54	serrate	0	4
	55	pangolin	4	1
	56	parvin	1	0
	57	fringe	0	1
	58	Rho1	5	1
	59	Nostrin	0	1
	60	Death-associated protein kinase related	0	2
	61	prickle	2	1
	62	Suppressor of Hairless	0	1
	63	p115	0	1
	64	tolloid	0	2
	65	yippee	1	3
	66	shaggy	1	1
	67	pointed	0	1
	68	hairless	0	1
	69	hedgehog	0	1
	70	division abnormally delayed	0	1
	71	inturned	0	1
	72	Rap1 GTPase	0	2
	73	scarface	0	1
	74	spitz	0	1
	75	penguin	1	0
	76	chip	1	0
	77	sprouty	0	1
	78	engrailed	0	1
	79	mothers against decapentaplegic	1	7
	80	costa	0	1
Imaginal disc-derived wing vein morphogenesis (GO:0008586)	1	Nedd4	0	1
	2	tolloid	0	2
	3	brahma	1	0

	4	rhomboid	0	1
	5	Anchor	7	7
	6	Epidermal growth factor receptor	1	3
	7	decapentaplegic	0	1
	8	spalt major	0	1
	9	osa	0	2
	10	dystrophin	0	3
	11	asteroid	0	1
	12	hairless	0	1
	13	mothers against decapentaplegic	1	7
Wing disc pattern formation (GO:0035222)	1	spenito	0	1
	2	annexin B9	2	0
	3	draper	0	5
	4	osa	0	2
	5	pointed	0	1
	6	presenilin	3	0
	7	Notch	0	4
	8	patched	0	2
	9	crumbs	0	3
	10	cubitus interruptus	0	1
	11	strawberry notch	0	1
	12	Delta	2	2
	13	smoothened	0	1
	14	interference hedgehog	0	2
	15	hairless	0	1
	16	apterous	1	0
	17	hedgehog	0	1
	18	division abnormally delayed	0	1
	19	dispatched	0	1
	20	protein kinase	0	1
	21	vestigial	0	1
	22	serrate	0	4
	23	decapentaplegic	0	1
	24	teashirt	1	0
	25	fringe	0	1
	26	Rab-7	1	0
	27	split ends	0	5
	28	capicua	0	4
	29	engrailed	0	1
	30	Suppressor of Hairless	0	1
	31	mothers against decapentaplegic	1	7

	32	rasp	1	0
Wing disc proximal/distal pattern formation (GO:0007473)	1	hedgehog	0	1
	2	vestigial	0	1
	3	teashirt	1	0
Apposition of dorsal and ventral imaginal disc-derived wing surfaces (GO:0007475)	1	held out wings	1	0
	2	mastermind	0	4
	3	parvin	1	0
	4	penguin	1	0
	5	Delta	2	2
Establishment of imaginal disc-derived wing hair orientation (GO:0001737)	1	fritz	1	0
	2	frizzled	0	4
	3	LIM domain kinase 1	2	0
	4	prickle	2	1
	5	Rac1	2	1
	6	Rabenosyn	1	0
	7	inturned	0	1
Imaginal disc-derived wing expansion (GO:0048526)	1	armadillo	4	3
	2	pygopus	0	2
Negative regulation of imaginal disc-derived wing vein specification (GO:0110109)	1	sprouty	0	1
Regulation of imaginal disc-derived wing size (GO:0044719)	1	p115	0	1
Regulation of imaginal disc-derived wing vein specification (GO:0110107)	1	sprouty	0	1
Cell hair (GO:0070451)	1	Rho1	5	1
	2	tricornered	1	0
	3	yellow	6	2

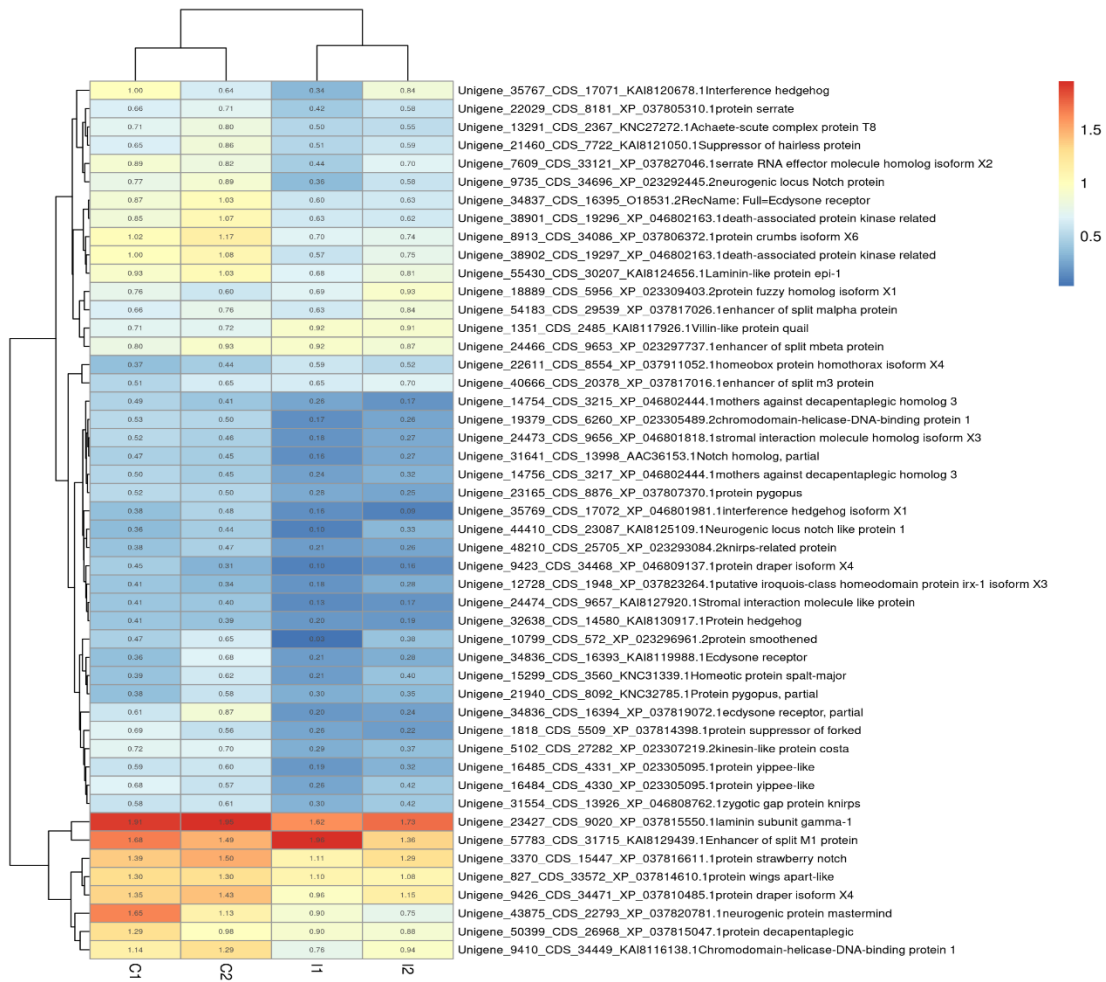


Figure 4.22: Heatmap representing differentially expressed genes involved in wing development due to monocrotophos exposure, where shades of blue represent downregulated genes and shades of red represent highly expressed genes (C- Control, I- Monocrotophos).

Table 4.4: Differentially expressed genes involved in the wing development of Carbamazepine-exposed *Chrysomya megacephala*

Function	Gene		Up-regulated ▲	Down-regulated ▼
Wing and notum subfield formation (GO:0035309)	1	spalt major	2	0
	2	decapentaplegic	0	1
	3	rhomboid	0	2
	4	araucan	2	1
	5	wingless	1	0
	6	shaggy	1	1
	7	Epidermal growth factor receptor	1	3
	1	wingless	1	0

Wing cell fate specification (GO:0035311)	2	rhomboid	0	2
Wing disc anterior/posterior pattern formation (GO:0048100)	1	smoothened	0	1
	2	interference hedgehog	0	2
	3	hedgehog	0	1
	4	serrate	1	3
	5	division abnormally delayed	0	1
	6	engrailed	0	2
	7	fringe	0	1
	8	patched	0	2
	9	mothers against decapentaplegic	3	6
	10	rasp	1	0
	11	cubitus interruptus	0	1
	12	invected	1	0
Wing disc development (GO:0035220)	1	frizzled	0	4
	2	rhomboid	0	2
	3	Anchor	5	7
	4	ebi	1	0
	5	rabenosyn	0	1
	6	strawberry notch	0	1
	7	interference hedgehog	0	2
	8	escargot	0	1
	9	homothorax	4	0
	10	Nedd4	0	1
	11	osa	0	2
	12	fritz	2	0
	13	tricornered	0	1
	14	singed	1	1
	15	decapentaplegic	0	1
	16	neuralized	1	3
	17	rasp	1	0
	18	pygopus	0	3
	19	dual oxidase	0	2
	20	draper	0	5
	21	split ends	0	7
	22	presenilin	3	0
	23	patched	0	2
	24	smoothened	0	1
	25	Delta	0	4
	26	sin3a	0	2
	27	dispatched	0	1
	28	folded gastrulation	0	1

29	Chromodomain-helicase-DNA-binding protein 1	0	3
30	Rac-1	1	2
31	Ecdysone receptor	0	4
32	teashirt	0	1
33	abrupt	0	1
34	asteroid	1	0
35	Rab-7	1	0
36	gurken	0	2
37	deltex	0	3
38	capicua	1	3
39	armadillo	4	3
40	wntless	0	1
41	Rab5	1	0
42	ADP ribosylation factor 1	1	0
43	unkempt	0	3
44	held out wings	0	1
45	jing	0	2
46	Epidermal growth factor receptor	1	3
47	annexin B9	2	0
48	dystroglycan	0	1
49	dystrophin	0	3
50	Notch	0	4
51	crumbs	0	3
52	kinase suppressor of ras	0	1
53	LIM domain kinase 1	0	1
54	apterous	1	0
55	G protein-coupled receptor kinase 2	0	1
56	vestigial	0	1
57	frizzled	0	4
58	brahma	1	0
59	quail	2	0
60	araucan	1	2
61	mastermind	0	4
62	serrate	1	3
63	gustavus	1	1
64	pangolin	2	3
65	spalt major	2	0
66	Distal-less	0	2
67	parvin	1	0
68	fringe	0	1

	69	hopscotch	0	1
	70	Rho1	5	1
	71	Nostrin	1	0
	72	Death-associated protein kinase related	0	2
	73	prickle	1	2
	74	Suppressor of Hairless	0	1
	75	p115	0	1
	76	tolloid	0	2
	77	spenito	0	1
	78	yippee	0	4
	79	shaggy	1	1
	80	cubitus interruptus	0	1
	81	stubble	1	1
	82	hairless	0	1
	83	hedgehog	0	1
	84	division abnormally delayed	0	1
	85	inturned	0	1
	86	Rap1 GTPase	1	1
	87	pointed	0	1
	88	scarface	0	1
	89	spitz	1	0
	90	penguin	1	0
	91	chip	1	0
	92	sprouty	0	1
	93	Protein kinase N	0	3
	94	engrailed	0	2
	95	mothers against decapentaplegic	1	8
	96	Enhancer of split my	1	0
	97	costa	0	1
Wing disc dorsal/ventral pattern formation (GO:0048190)	1	vestigial	0	1
	2	annexin B9	2	0
	3	draper	0	5
	4	osa	0	2
	5	pointed	0	1
	6	presenilin	3	0
	7	fringe	0	1
	8	Notch	0	4
	9	crumbs	0	3
	10	Rab7	1	0
	11	strawberry notch	0	1
	12	Delta	0	4
	13	hairless	0	1

Wing disc morphogenesis (GO:0007472)	14	apterous	1	0
	15	division abnormally delayed	0	1
	16	Suppressor of Hairless	0	1
	1	rhomboid	0	2
	2	anchor	5	7
	3	ebi	1	0
	4	osa	0	2
	5	Rabenosyn	0	1
	6	strawberry notch	0	1
	7	homothorax	1	3
	8	Nedd4	0	1
	9	fritz	1	0
	10	tricornered	0	1
	11	singed	1	1
	12	Star	0	1
	13	decapentaplegic	0	1
	14	neuralized	1	3
	15	Split ends	0	7
	16	pygopus	0	3
	17	furry	0	2
	18	Dual oxidase	0	2
	19	patched	0	2
	20	Delta	0	4
	21	smoothened	0	1
	22	folded gastrulation	0	1
	23	Chromodomain-helicase-DNA-binding protein 1	0	3
	24	Rac1	1	2
	25	Ecdysone receptor	0	4
	26	teashirt	0	1
	27	Stromal interaction molecule	1	2
	28	asteroid	1	0
	29	gurken	0	2
	30	capicua	1	3
	31	armadillo	4	3
	32	wntless	0	1
	33	ADP ribosylation factor 1	1	0
	34	unkempt	0	3
	35	held out wings	0	1
	36	jing	0	2

37	Epidermal growth factor receptor	1	3
38	dystroglycan	0	1
39	dystrophin	0	3
40	Notch	0	4
41	kinase suppressor of ras	0	1
42	LIM domain kinase 1	0	2
43	apterous	1	0
44	G protein-coupled receptor kinase 2	0	1
45	CTD nuclear envelope phosphatase 1	2	0
46	vestigial	0	1
47	frizzled	0	4
48	quail	2	0
49	araucan	1	2
50	mastermind	0	4
51	serrate	1	3
52	gustavus	1	1
53	pangolin	2	3
54	Distal-less	0	2
55	parvin	1	0
56	fringe	0	1
57	hopscotch	0	1
58	Rho1	5	1
59	Nostrin	0	1
60	Death-associated protein kinase related	0	2
61	prickle	1	2
62	Suppressor of Hairless	0	1
63	p115	0	1
64	tolloid	0	2
65	yippee	0	4
66	shaggy	1	1
67	pointed	0	1
68	scarface	0	1
69	hairless	0	1
70	hedgehog	0	1
71	division abnormally delayed	0	1
72	inturned	0	1
73	Rap1 GTPase	1	1
74	pointed	0	1
75	scarface	0	1
76	spitz	1	0

	77	penguin	1	0
	78	chip	1	0
	79	sprouty	0	1
	80	Protein kinase N	0	3
	81	engrailed	0	2
	82	mothers against decapentaplegic	3	6
Cell elongation involved in imaginal disc-derived wing morphogenesis (GO:0090254)	1	vestigial	0	1
	2	Rho1	5	1
	3	armadillo	4	3
	4	wingless	1	0
	5	pangolin	2	3
Imaginal disc-derived wing margin morphogenesis (GO:0008587)	1	held out wings	0	1
	2	shaggy	1	1
	3	ebi	1	0
	4	osa	0	2
	5	Notch	0	4
	6	strawberry notch	0	1
	7	Delta	0	4
	8	hairless	0	1
	9	brahma	1	0
	10	frizzled	0	4
	11	mastermind	0	4
	12	serrate	1	3
	13	parvin	1	0
	14	fringe	0	1
	15	penguin	1	0
Imaginal disc-derived wing morphogenesis (GO:0007476)	1	rhomboid	0	2
	2	anchor	5	7
	3	ebi	1	0
	4	osa	0	2
	5	Rabenosyn	0	1
	6	strawberry notch	0	1
	7	homothorax	1	3
	8	Nedd4	0	1
	9	fritz	1	0
	10	tricornered	0	1
	11	singed	1	1
	12	Star	0	1
	13	decapentaplegic	0	1
	14	neuralized	1	3
	15	Split ends	0	7
	16	pygopus	0	3
	17	furry	0	2
	18	Dual oxidase	0	2

19	patched	0	2
20	wingless	1	0
21	Delta	0	4
22	smoothened	0	1
23	folded gastrulation	0	1
24	Chromodomain-helicase-DNA-binding protein 1	0	3
25	Rac1	1	2
26	Ecdysone receptor	0	4
27	teashirt	0	1
28	abrupt	0	1
29	asteroid	1	0
30	Rab-7	1	0
31	gurken	0	2
32	deltex	0	3
33	capicua	1	3
34	armadillo	4	3
35	wntless	0	1
36	ADP ribosylation factor 1	1	0
37	unkempt	0	3
38	held out wings	0	1
39	jing	0	2
40	Epidermal growth factor receptor	1	3
41	dystroglycan	0	1
42	dystrophin	0	3
43	Notch	0	4
44	crumbs	0	3
45	kinase suppressor of ras	0	1
46	LIM domain kinase 1	0	2
47	apterous	1	0
48	G protein-coupled receptor kinase 2	0	1
49	vestigial	0	1
50	frizzled	0	4
51	brahma	1	0
52	quail	2	0
53	araucan	1	2
54	mastermind	0	4
55	serrate	1	3
56	pangolin	2	3
57	Distal-less	0	2
58	parvin	1	0

	59	fringe	0	1
	60	Rho1	5	1
	61	Nostrin	0	1
	62	Death-associated protein kinase related	0	2
	63	prickle	1	2
	64	Suppressor of Hairless	0	1
	65	p115	0	1
	66	tolloid	0	2
	67	yippee	0	4
	68	shaggy	1	1
	69	pointed	0	1
	70	hairless	0	1
	71	hedgehog	0	1
	72	division abnormally delayed	0	1
	73	inturned	0	1
	74	Rap1 GTPase	1	1
	75	scarface	0	1
	76	spitz	1	0
	77	penguin	1	0
	78	chip	1	0
	79	sprouty	0	1
	80	engrailed	0	2
	81	mothers against decapentaplegic	3	6
	82	costa	0	1
Imaginal disc-derived wing vein morphogenesis (GO:0008586)	1	Nedd4	0	1
	2	tolloid	0	2
	3	brahma	1	0
	4	rhomboid	0	2
	5	Anchor	5	7
	6	Epidermal growth factor receptor	1	3
	7	decapentaplegic	0	1
	8	spalt major	2	0
	9	osa	0	1
	10	dystrophin	0	3
	11	asteroid	1	0
	12	hairless	0	1
	13	split ends	0	7
	14	mothers against decapentaplegic	3	6
Wing disc pattern formation	1	spenito	0	1
	2	annexin B9	2	0

(GO:0035222)	3	draper	0	5
	4	osa	0	2
	5	pointed	0	1
	6	presenilin	3	0
	7	Notch	0	4
	8	patched	0	2
	9	crumbs	0	3
	10	cubitus interruptus	0	1
	11	invected	1	0
	12	strawberry notch	0	1
	13	Delta	0	4
	14	smoothened	0	1
	15	interference hedgehog	0	2
	16	hairless	0	1
	17	apterous	1	0
	18	hedgehog	0	1
	19	division abnormally delayed	0	1
	20	dispatched	0	1
	21	protein kinase	1	0
	22	vestigial	0	1
	23	serrate	1	3
	24	decapentaplegic	0	1
	25	teashirt	0	1
	26	fringe	0	1
	27	Rab-7	1	0
	28	split ends	0	7
	29	capicua	1	3
	30	engrailed	0	2
	31	Suppressor of Hairless	0	1
	32	mothers against decapentaplegic	3	6
	33	rasp	1	0
	34	Enhancer of split my	1	0
Wing disc proximal/distal pattern formation (GO:0007473)	1	hedgehog	0	1
	2	vestigial	0	1
	3	teashirt	0	1
Apposition of dorsal and ventral imaginal disc-derived wing surfaces (GO:0007475)	1	held out wings	0	1
	2	mastermind	0	4
	3	parvin	1	0
	4	penguin	1	0
	5	Delta	0	4
	1	fritz	2	0
	2	frizzled	0	4

Establishment of imaginal disc-derived wing hair orientation (GO:0001737)	3	LIM domain kinase 1	0	2
	4	prickle	1	2
	5	Rac1	1	2
	6	Rabenosyn	0	1
	7	inturned	0	1
Imaginal disc-derived wing expansion (GO:0048526)	1	armadillo	4	3
	2	pygopus	0	3
Negative regulation of imaginal disc-derived wing vein specification (GO:0110109)	1	sprouty	0	1
Regulation of imaginal disc-derived wing size (GO:0044719)	1	p115	0	1
Regulation of imaginal disc-derived wing vein specification (GO:0110107)	1	sprouty	0	1
Cell hair (GO:0070451)	1	Rho1	5	1
	2	tricornered	0	1
	3	yellow	3	4

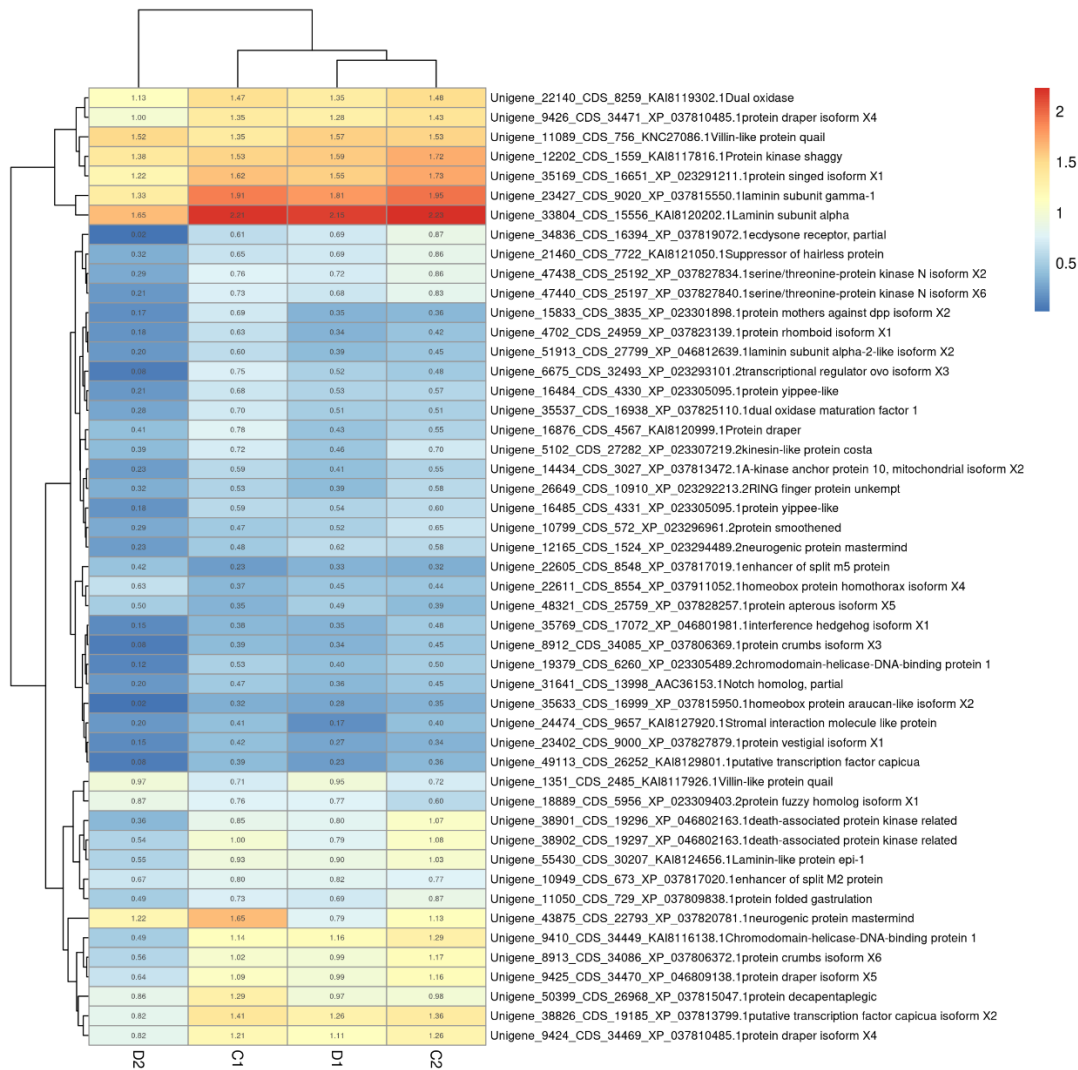


Figure 4.23: Heatmap representing differentially expressed genes involved in wing development due to carbamazepine exposure, where shades of blue represent downregulated genes and shades of red represent highly expressed genes (C- Control, D- Carbamazepine).

4.5 DISCUSSION

Geometric morphometrics, a discipline combining geometry and biology, explores form in two- or three-dimensional space (Richtsmeier et al., 2002). In this chapter, the effect of intoxicants on the wing structure of *Chrysomya megacephala* is studied using Geometric morphometric analysis (GMM). The findings were further confirmed using the differential gene expression study of genes involved in wing development. This chapter explores how intoxicants affect the wing structure of *Chrysomya megacephala* using Geometric morphometric (GMM) analysis and transcriptomic

analysis. GMM analysis revealed that monocrotophos and carbamazepine produced unique structural alterations in wings, with notable dose-related variations observed in both instances.

From a forensic perspective, GMM analysis has demonstrated its utility as a method for taxonomic identification. A study by (Nuñez et al., 2019) employed wing geometric morphometrics to distinguish between two closely related fly species (Muscidae) relevant to forensic investigations. Their approach utilised generalised Procrustes analysis and Discriminant Function Analysis. This research highlighted GMM as an economic, swift, and easily accessible quantitative tool for species identification. Furthermore, it established GMM as a straightforward and readily available tool in the field of forensic science.

A GMM investigation into phenotypic plasticity and morphological shape variation in *Danaus Plexippus* demonstrated that wing shape was influenced by gender, life history, and larval diet (Berns, 2014). GMM-based studies on seasonal variations conducted on three *Mansonia* species by Laojun et al., (2024) and on *Anopheles coluzzii* by Hidalgo et al., (2015) uncovered intraspecific wing shape and size fluctuations across different seasons. Research by Phanitchat et al., (2019) and Debat et al., (2003) on *Aedes albopictus* and *Drosophila simulans*, respectively, using geometric morphometric analysis confirmed that temperature fluctuations can indeed cause phenotypic plasticity in wing structure. These investigations also showed that, despite environmentally-induced changes, the wing architecture continues to provide a robust taxonomic indicator. The present investigation yields comparable findings when examining the control groups in both the Monocrotophos and Carbamazepine experimental sets. Although both groups were untreated, external influences such as temporal and seasonal factors resulted in some degree of difference between these two control populations.

The effect of organophosphate insecticide pirimiphos-methyl on the wing morphology of *Rhyzopertha dominica*, *Prostephanus truncatus*, and *Tenebrio molitor* (Coleoptera) was studied by Boukouvala et al., (2023). This GMM study results revealed that the organophosphorus insecticides may cause sex-specific variabilities on

wing morphology, and that it can also cause variable sub-lethal effects to stored-product insects. The effect of the same insecticide on the wing morphology of *Tribolium castaneum* and *Tribolium confusum* (Coleoptera) also revealed similar results where even the shortest exposure to insecticides (5 min) is enough to provoke shape changes in the hindwings.

Research conducted by (Sarıkaya & Yüksel, 2008) on the genotoxicity of oxcarbazepine and carbamazepine using the wing spot test demonstrated that both medications exhibited toxic effects in *Drosophila melanogaster*. Additionally, the study found that the flies used in the experiments had significantly reduced survival rates compared to the control group.

The plasticity of wing size adequately explains flight differences, with the swiftest flies exhibiting wings that have a narrower proximal section near the thorax and an extended shift towards the distal end (Fraimout et al., 2018). This distal distribution of wing area has been demonstrated to produce increased stroke force (Ellington, 1984). In this study, monocrotophos exposure resulted in a comparable effect, causing the wings of treated groups (1/5th and 1/3rd of EC₅₀) to narrow and expand distally. Transcriptome analysis also revealed several differentially expressed genes that can influence the antero-posterior pattern formation (11 genes) and proximo-distal pattern formation of wing disc (3 genes). Sublethal carbamazepine concentrations (1/5th and 1/3rd of EC₅₀) also led to slightly narrower wings. 12 genes involved in antero-posterior pattern formation, and 3 genes involved in proximo-distal pattern formation were differentially expressed in carbamazepine-exposed *Chrysomya megacephala*, fortifying these findings. However, the overall outcome for 1/5th of EC₅₀ also showed a wing morphology that was shortened proximodistally. In theory, longer and narrower wings are aerodynamically more efficient than shorter, wider ones due to reduced induced drag and associated power expenditure (DeVries et al., 2010). Wing morphology alterations impact insect flight aerodynamics, with long, slender wings being optimal for extended flight durations, and short, broad wings being ideal for slow, agile flight. Additionally, wide wing bases allow for a broader range of speeds, while narrow wing tips enable less expensive, extensive flight (Baleba et al., 2019).

Wing veins play a crucial role in enhancing wing flexibility, which reduces the moment of inertia (Wootton, 1992; Combes & Daniel, 2003). This, in turn, decreases the energy requirements and the stresses generated during oscillation. In addition, these veins increase the flexibility of the wing tip, allowing it to deflect more easily when subjected to excessive inertial forces or unexpected impacts. Therefore, minor alterations in wing vein location during development could modify wing flexibility, potentially affecting aerodynamic properties indirectly (Fraimout et al., 2018). Transcriptome analysis revealed the differential expression of 14 genes in the monocrotophos exposed group and 15 genes in the carbamazepine exposed group involved in wing vein development, which proves the potential roles of both intoxicants in the wing development of *Chrysomya megacephala*.

Comparative analysis of monocrotophos and carbamazepine using Discriminant Function Analysis and CVA morphospace analysis showed the least overlap and a very distinct morphospace between the two intoxicant sets. This indicates the potential of GMM analysis to clearly distinguish different intoxicants based on their effects on later stages of development, in this case, wing development.

During the early stages of insect development, the wing imaginal disk undergoes division into the wing and notum subfields. This subdivision process is critically dependent on the activity of the Wingless protein, which appears to be the initial component in the sequence of regulatory genes that drive wing formation in *Drosophila* (Baonza et al., 2000). The study also stated that the epidermal growth factor receptor (EGFR) pathway is crucial in defining the wing subregion within the developing wing disk and determining posterior identity. This is achieved through two main mechanisms: EGFR signalling limits Wingless expression to the dorsal-posterior quarter of wing discs, thereby inhibiting wing-organizing activities, and it also contributes to sustaining the expression of Engrailed in cells of the posterior compartment. Based on the current study, in the case of carbamazepine exposure, the differentially expressed genes under ‘wing and notum subfield formation’ included upregulation of wingless protein. This may indicate that carbamazepine affects wing development from the initial stages of differentiation. The epidermal growth factor

receptor (EGFR) was also found to be mostly down-regulated in both treatment sets. There are also other major genes involved in wing formation in this gene function group that were differentially expressed due to both monocrotophos and carbamazepine exposure namely, decapentaplegic, rhomboid, araucan, wingless and shaggy.

In the current study, geometric morphometric (GMM) analysis revealed monocrotophos and carbamazepine-induced effects on wing structure and development in *Chrysomya megacephala*. Differential Gene Expression (DGE) analysis detailed genes involved in wing development that had been altered by the effect of both intoxicants.

Principal component analysis and percentage variance visualised the displacements of major landmarks owing to the shape variations of the wings. These displacements were unique at different concentrations, in both intoxicants. Partial Least Square analysis (PLS), Discriminant Function Analysis (DFA), and Canonical Variate Analysis (CVA) were used to compare the different concentrations of same intoxicant to its control set. PLS analysis revealed that the size variation was more prominent than the shape variation in both intoxicants. There was a significant dose-dependent variation when comparing the two concentrations of the same intoxicant. DFA of both intoxicants showed a distinct separation of the two doses from the control set, while possessing similar trends. CVA analysis revealed clustering and overlapping of all three sets, with the treatment sets overlapping more than the control cluster in both monocrotophos and carbamazepine treatments.

These results were reinforced by the transcriptomic profiling results of the 1/3rd of EC₅₀ of both monocrotophos and carbamazepine with the control. DGE analysis revealed several genes owing to the major shape and size variation in the wing structure during the various stages of wing development. Although the genes involved in wing development that were differentially expressed were mostly the same, their expression patterns were unique for both intoxicants.

From the current results, it became evident that different classes of intoxicants, an insecticide and a drug, elicited their unique expression patterns in the later stage of

development (wing formation). The unique effects of both classes of intoxicants were visualised effectively using GMM analysis, and the results were successfully backed up by molecular data acquired by means of DGE analysis. Therefore, from a forensic viewpoint, GMM can be potentially used as a tool for identifying the class of intoxicants exposed to dipterans like *Chrysomya megacephala*, using morphological structures such as wings. Even though this area needs further detailed study and development of dedicated databases such as Home Of Forensic Entomology database (HOFE) for precise species-specific genetic information, it has the potential to become an important tool in entomotoxicology, in the future.

4.6 KEY FINDINGS

- ❖ GMM analysis revealed that monocrotophos and carbamazepine each produced unique structural alterations in wings, with notable dose-dependent variations observed in both instances.
- ❖ From the current results, it became evident that different classes of intoxicants, an insecticide and a drug, elicited their own unique expression patterns in wing formation.
- ❖ The unique effects of both classes of intoxicants were visualised effectively using GMM analysis, and the results were successfully backed up by molecular data acquired by means of DGE analysis.
- ❖ From a forensic viewpoint, GMM can be potentially used as a tool for identifying the class of intoxicants exposed to dipterans like *Chrysomya megacephala*, using the morphological structures such as wings.
- ❖ Even though this area needs further detailed study and development of dedicated databases such as Home Of Forensic Database (HOFE) for precise species-specific genetic information, it has the potential to become an important tool in entomotoxicology, in the future.

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Entomotoxicology represents a medicolegal application of forensic entomology, wherein toxins may induce alterations in the developmental rate of necrophagous insects, potentially leading to significant modifications in post-mortem interval (PMI) estimates. If these factors are overlooked during death investigations, particularly in cases of suicide and deliberate self-harm involving toxicants, which are major yet under-recognized public health issues in the developing world, errors may ensue. The emerging field of entomotoxicology is currently concentrated on two primary research trajectories. One trajectory examines the potential use of carrion-feeding arthropods as alternative specimens in toxicological analyses, while the other investigates the impact of tissue-borne toxins and contaminants on the developmental processes of juvenile insects that consume these materials. The present study demonstrates that the developmental rate of *Chrysomya megacephala* is altered due to exposure to monocrotophos and carbamazepine, with transcriptomic analysis corroborating these findings.

The enzymatic biochemical assays showed an increased oxidative stress, potential cellular damage and enhanced activation of detoxification mechanisms within the test samples. Transcriptomic analysis identified multiple gene copies associated with stress signalling and detoxification mechanisms as being differentially expressed. Although both upregulation and downregulation of detoxifying genes were observed, most genes were upregulated suggesting the activation of various detoxification mechanisms in response to monocrotophos and carbamazepine, corroborating the findings of biochemical assays.

The de novo transcriptome analysis of *Chrysomya megacephala* offered extensive insights into the full range of RNA transcripts produced by the entire genome. Both intoxicants uniquely modified the typical expression patterns of numerous major gene clusters, reflecting their distinct modes of action. Certain clusters exhibited differential expression in response to both monocrotophos and carbamazepine,

including those involved in amino acid, lipid, and carbohydrate transport and metabolism, secondary metabolite biosynthesis, transport and catabolism, as well as translation, ribosomal structure, and biogenesis. Many of these genes are components of metabolic pathways, suggesting that this might be part of the insect's primary response to any xenobiotic exposure. First two chapters addressed detailed reviews on developmental effects and detoxification mechanisms, which are other major gene clusters affected by the intoxicants. In summary, this transcriptomic study indicates that even at sublethal doses, both monocrotophos and carbamazepine impact the normal development and biological functions of *Chrysomya megacephala*. This also suggests that gene expression studies can provide a more comprehensive understanding of the effects of xenobiotics on an organism, even when there is no visible phenotypic or biochemical evidence. Such studies can reveal impacts that might otherwise go unnoticed. From a forensic perspective, a more thorough understanding of gene expression levels throughout all developmental stages of forensically relevant insects can provide valuable insights and lead to more precise results in areas such as postmortem interval (PMI) determination. Furthermore, the complete transcriptomic data could be incorporated into a specialized database for entomotoxicology research, potentially making significant contributions to the field in the future.

In the current study, geometric morphometric (GMM) analysis revealed monocrotophos and carbamazepine-induced effects on wing structure and development in *Chrysomya megacephala*. Differential Gene Expression (DGE) analysis showed a detailed report of genes involved in wing development that had been altered by the effect of both intoxicants. Principal component analysis and percentage variance visualised the displacements of major landmarks owing to the shape variations of the wings. These displacements were unique at different concentrations, in both intoxicants. Partial Least Square analysis (PLS), Discriminant Function Analysis (DFA), and Canonical Variate Analysis (CVA) were used to compare the different concentrations of same intoxicant to its control set. PLS analysis revealed that the size variation was more prominent than the shape variation in both intoxicants and that there was a significant dose-dependent variation when comparing

the two concentrations of the same intoxicant. DFA of both intoxicants showed distinct separation of the two doses from the control set, while possessing similar trends. CVA analysis revealed clustering and overlapping of all three sets, with the treatment sets overlapping more than the control cluster in both monocrotophos and carbamazepine treatments. These results were reinforced by the transcriptomic profiling results of the 1/3rd of EC₅₀ of both monocrotophos and carbamazepine with the control. DGE analysis revealed a number of genes owing to the major shape and size variation in the wing structure during the various stages of wing development. Although the genes involved in wing development that were differentially expressed were mostly the same, their expression patterns were unique for both intoxicants. From the current results, it became evident that different classes of intoxicants, an insecticide and a drug, elicited their own unique expression patterns in the later stage of development (wing formation). The unique effects of both classes of intoxicants were visualised effectively using GMM analysis, and the results were successfully backed up by molecular data acquired by means of DGE analysis. Therefore, from a forensic viewpoint, GMM can be potentially used as a tool for identifying the class of intoxicants exposed to dipterans like *Chrysomya megacephala*, using the morphological structures such as wings. Even though this area needs further detailed study and development of dedicated databases such as Home Of Forensic Entomology database (HOFE) for precise species-specific genetic information, it has the potential to become an important tool in entomotoxicology, in the future.

RECOMMENDATIONS

1. Improved Postmortem Interval (PMI) Estimation:

An in-depth analysis of gene expression profiles across all developmental stages of forensically-relevant insects can enhance the accuracy of PMI determination.

2. Development of Comprehensive Transcriptomic Databases:

Creating specialised, user-friendly databases for entomotoxicology research to store and facilitate access to transcriptomic data.

Integration of data into platforms like HOFE (Home of Forensic Entomology database) to support species identification and toxin detection.

3. Advances in Entomotoxicology and Toxicology:

Utilising gene expression data and morphological features for detecting and classifying toxicant-specific changes in insects.

Employing tools like Geometric Morphometrics (GMM) to identify chemical exposure through morphological changes in various structures of insects.

4. Broader Forensic and Scientific Applications:

Extending research to include a broader range of insect species relevant to different geographical regions.

Collaborating with other disciplines to refine forensic methods and integrate genomic, morphological, and chemical data.

5. Further Research Needs:

Conducting detailed studies to validate gene expression markers for forensic purposes. Furthermore, comprehensive field-validation trials tracking real-carrier breakdown against laboratory artificial control diets must be expanded to confirm the natural environmental accuracy of these molecular and morphological biomarkers.