Sustainable Crop Production: Novel Strategies for the Control of Dipteran Pests

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The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapter 3 "Myosuppressin signalling in the crop of *Drosophila melanogaster*" is based on experiments and sample preparations undertaken by the author of this thesis under the guidance of Prof. Isaac (Leeds University, UK) except for confirmation of deletions in knock-out mutant *DmsR-1*^{01} and *DmsR-2*^{01} flies that were kindly provided by Prof. Young Joon Kim, School of Life Sciences, Gwangju Institute of Science and technology, Gwangju, Republic of Korea.

Chapter 4 "Peptidergic control of the crop of the cabbage root fly, *Delia radicum*: a role for myosuppressin" contains experimental work, written part and analysis prepared by the author of this thesis under the supervision and guidance of Prof. Isaac (Leeds University, UK), Dr. Audsley, Dr. Down and Dr. Matthews (Fera Science Ltd., UK).

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Chapter 6 "Myosuppressin role and Neuropeptidome of medfly, *Ceratitis capitata*" is based on experiments and sample preparations undertaken by the author of this thesis under the guidance of Prof. Isaac (Leeds University, UK) and Dr. Audsley (Fera Science Ltd., UK) except for Orbitrap mass spectrometry analysis of *C.capitata* tissues that were examined by Dr. Ragionieri (Universtity of Cologne, Germany) and provided information were used to complement Table 6.3 of predicted masses of putative neuropeptides together with provided Figure 6.11 of mass spectra analysis of the brain and MS/MS fragmentation of myosuppressin peptide.

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Abstract

Neuropeptide signalling systems present promising avenues for the development of new pest control agents due to their integral roles in insect homeostasis, development, and behaviour. This project primarily examines the crop of the foregut, a muscular organ presents in almost all but not exclusively in the order Diptera, serving the dual purposes of food storage and regurgitation.

Myosuppressin and its receptors, which are well-conserved across insect orders, are known to inhibit muscle contractions in the gut, positioning them as prime candidates for disrupting gut function. A critical aspect of this study is understanding that only when ingested food efficiently passes through the foregut can the animal continue its feeding process, highlighting a potential target area for intervention.

Using mass spectrometry and immunohistochemistry, we confirmed the identity and spatial distribution of this peptide in the foreguts of two economically significant agricultural pests; cabbage root fly, *Delia radicum* and medfly, also known as Mediterranean fruit fly, *Ceratitis capitata*. Our physiological findings highlight myosuppressin capability to significantly inhibit spontaneous crop contractions, responding even to nano- and pico-molar peptide concentrations. Leveraging the adaptable genetics of *Drosophila melanogaster*, we delved into the potential roles of two myosuppressin G-protein-coupled receptors, DmsR-1 and DmsR-2, in regulating crop muscle contractions. Employing knock-out mutants for both receptors, we established that DmsR-1 plays a critical role in the robust inhibition of crop contractions. Myosuppressin was observed to degrade swiftly when exposed to peptidases in crop of *D. radicum*. Consequently, we tested a biostable myosuppressin receptor agonist, Benzethonium chloride (Bztc), for its potential to disrupt crop function. Microinjections of the native peptide led to complete oviposition inhibition, highlighting a potential new pathway for myosuppressin signalling that warrants further exploration. This finding emphasizes the potential of myosuppressin as an eco-friendly pest control agent. Given the substantial economic implications associated with the medfly, our neuropeptidome analysis offers a rich repository of prospective novel targets.

The discussion further delves into the advantages and challenges of harnessing peptidergic signalling for insect control. It particularly addresses the inherent biostability issues of peptides and underscores the necessity for metabolically stable analogues that can engage with peptide receptors while minimizing degradation.

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List of Abbreviations

µl	Microliter
µM	Micromolar
µg	Microgram
AMC	7-amino-4-methylcoumarin
BLAST	Basic Local Alignment Search Tool
BSA	Albumin bovine
Bztc	Benzethonium chloride
C	Caeca
CA	Corpora allata
CC	Corpora cardiaca
Cas9	CRISPR associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
cDNA	Complementary DNA
CNS	Central nervous system
CO_2	Carbon dioxide
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DMS	Dromyosuppressin
Dms-R1	Dromyosuppressin receptor 1
Dms-R2	Dromyosuppressin receptor 2
DNA	Deoxyribonucleic acid
E.D.	Ejaculatory duct
Gal4	Galactose-responsive transcriptional factor
GFP	Green fluorescent protein
GPRC	G-protein coupled receptor
GTX-PBS	Goat serum in Triton-X phosphate buffer saline
H_2O	Water
HCCA	α-cyano-4-hydroxycinnammic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	Hour

HG	Hindgut
HPLC	High-performance liquid chromatography
IC_{50}	Half maximum response of an inhibitor concentration
IgG	Immunoglobulin
IHC	Immunohistochemistry
KO	Knockout
M	Molar
MAG	male accessory glands
MALDI	Matrix-assisted laser desorption ionization
MALDI-TOF-MS	Matrix-assisted laser desorption ionization time of flight mass spectrometry
mg	Miligram
min	Minute
ml	Millilitre
MS	mass spectrometry
m/z	Mass/charge number of ions
NCS	Nerval central system
°C	Degrees Celsium
P	Proboscis
PBS	Phosphate buffer saline
pE	Pyroglutamic acid
PER	Proboscis Extension Reflex
RER	Rough Endoplasmic Reticulum
RFa	C-terminal Arg-Phe amide
RH	Relative humidity
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RP-HPLC	Reverse phase high performance liquid chromatography
Sec	Second
SNS	Stomatogastric nervous system
SP	Signal peptide
TFA	Trifluoroacetic acid

TX-PBS	Triton X-100 in phosphate buffer saline
U.K.	United Kingdom
U.S.A.	United States of America
UAS	Upstream activation sequence
v/v	Volume per volume
VDRC	Vienna Drosophila Resource Centre
w/v	weight per volume

Chapter 1 General Introduction

1.1 Dipterans

1.1.1 Background

Dipterans, derived from the Greek words 'di' and 'phera' meaning 'two wings,' represent the 'true flies' order. These insects, many of which are significant agricultural pests, inhabit both terrestrial and aquatic environments. Characterized by a single pair of wings and reduced hindwings transformed into structures called halters, the order encompasses approximately 158,000 species across over 180 families. Notable members include fruit flies, horse flies, flower flies, mosquitoes, and midges (Merritt et al., 2009; Wiegmann and Richards, 2018).

Their impact on agriculture is substantial, as they can cause extensive damage to cultivated crops and livestock, leading to significant economic losses. This damage is inflicted in various ways: direct attacks on plants by species like fruit flies and gall midges, and indirect harm through the spread of plant diseases by vectors such as leaf miners (Family: Agromyzidae) and fungus gnats (Families: Sciaridae and Mycetophilidae). Certain species also adversely affect livestock health (Merritt et al., 2009; Sarwar and Sarwar, 2020). Understanding and managing these pests is vital for food security and sustainable agricultural practices.

The holometabolic life cycle of flies (Fig 1.1) is a key factor in their agricultural impact. In the larval stage, they engage in continuous feeding, consuming plant tissues, roots, or organic matter. This intense feeding can lead to significant crop damage and yield loss. Following two larval molts, a critical surge in ecdysteroid hormone triggers metamorphosis. The larva then enters a 'wandering' phase, ceasing feeding and preparing for the pupal stage. This metamorphosis marks a radical transformation, culminating in the emergence of an adult fly, a stage with different feeding behaviors and ecological impacts (Yamanaka et al., 2005; Žitňan et al., 2007; Aghajanian et al., 2016).

As adults, their feeding habits change, but the impact remains considerable. The significant dietary difference between larval and adult stages is mirrored in the adaptation of their mouthparts. Larvae, hatching from eggs deposited by females in naturally protective environments, are equipped with mouth hooks for consuming a solid diet. Adults, conversely, rely on well-adapted mouthparts in the form of a proboscis to ingest liquid diets. Adult flies, such as fruit flies and gnats, may feed on plant nectar or other substances that do not possess any direct impact on crops. However, they can also act as vectors for various plant diseases. By transmitting pathogens and pesticide-degrading

microorganisms during feeding (Guo et al., 2017), they play a significant role in the spread of diseases across agricultural fields and play a significant role in increased resistance to diverse range of pesticides.

The lifespan of fly species varies greatly, influenced by environmental factors. A notable example is seen in common fruit flies of Drosophilidae family. These flies typically have an average lifespan of 70 days, which can extend up to ninety days in standard room temperatures of approximately 25°C (Piper and Partridge, 2018). Interestingly, when subjected to suboptimal conditions, their lifecycle can be substantially prolonged, in some cases even doubling in duration. This adaptability in different environments highlights Diptera's complexity and their ability to colonize diverse regions, from temperate to tropical zones, as seen in species like the Mediterranean fruit fly (Ricalde et al., 2012).

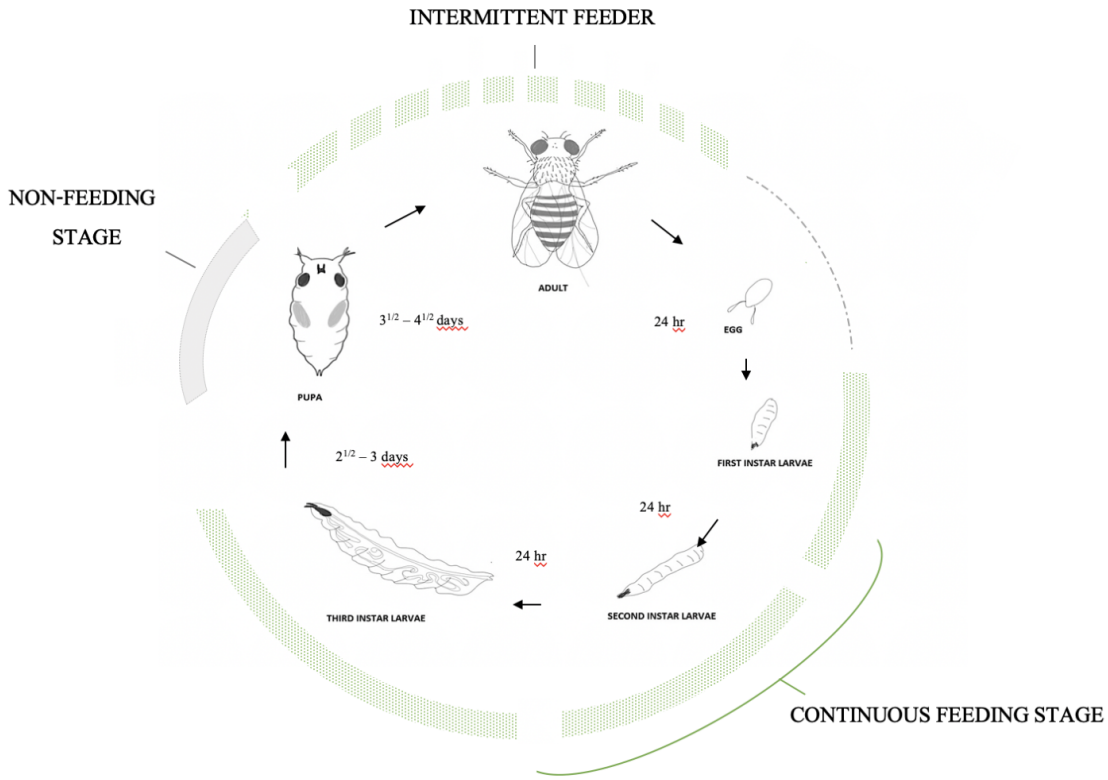


Figure 1.1 presents development and feeding stages of the fruit fly, *Drosophila melanogaster*, a common example of the Diptera order. This species follows a holometabolous life cycle, characterized by distinct feeding phases: the larval and the adult stages. In the larval stage, the fruit fly exhibits continuous feeding, a notable contrast to the intermittent feeding pattern observed in the adult stage. These two distinct feeding phases are separated by a non-feeding pupal stage, marking a major transition in the fly's development. The developmental cycle encompasses four stages: egg, larva, pupa, and adult. Larvae hatch from eggs approximately 24 hours after oviposition. Subsequently, they progress through three instar stages, each marked by a molting event. Upon completing the third instar, larvae enter a wandering phase, eventually transitioning into the non-feeding pupal stage. Within less than a week, the final molting phase takes place, forming a hard protective case around the immobile pupa. This casing is vital for the pupa's protection as it undergoes metamorphosis, emerging as a fully developed adult fly. (Drawing inspired by Miroschnikow et al., 2020).

1.1.2 Beneficial Diptera

Dipterans are renowned for their diverse influences on ecosystems, which include aiding seed dispersal, functioning as crucial pollinators, and playing an indispensable role in nutrient recycling and decomposition in both aquatic and soil environments (Gullan and Cranston, 2014). With the alarming decline of bee populations globally, there has been a surge in research focusing on Diptera as alternative pollinators, a trend particularly

noticeable in regions like Australia, where agricultural systems heavily depend on bee-driven pollination services (Cook et al., 2020). Their environmental impact varies, with some members being classified as 'keystone' species due to their disproportionate effects on ecosystem functioning and community structure (de Visser et al., 2012). For instance, in South Africa, the evolutionary biology of the long-tubed flowers and their key pollinators, the tangle-veined flies (Moegistorhynchus longirostris, Wiedemann, 1819) of the family Nemestrinidae, has been extensively documented (Barraclough and Slotow, 2010).

Other Dipterans are categorized as 'Ecosystem Engineers.' As defined by Jones et al., (1994), these are organisms that directly or indirectly modulate the availability of resources to other species, by causing physical state changes in biotic or abiotic materials. This activity can lead to the modification, maintenance, or creation of a favourable habitats. For example, in aquatic environments, the abundance of larval flies often represents a primary or substantial portion of the diet for other organisms (Adler and Courtney, 2019). Additionally, gall midges (Diptera: Cecidomyiidae) have been experimentally shown to uniquely drive diversification and structural changes in arthropod communities by altering their habitats (Crawford et al., 2007).

1.1.3 Diptera as pests

Dipterans are recognized for their dual roles in ecosystems: while some species contribute positively, they can simultaneously or independently be identified as pests in agriculture and horticulture, or as vectors of human and animal diseases. These diseases are transmitted through various means, including fecal matter, blood-feeding, and regurgitation (Sela et al., 2005). The classification of certain Dipterans as pests is based on the physical, medical, or economic harm they inflict on humans. Emameh et al., (2015) published a detailed list of the most harmful Dipterans, showcasing their impact in medicine, veterinary medicine, and agriculture.

An illustrative example of a problematic Dipteran pest is *Ceratitis capitata*, commonly known as the medfly, that is recognized as one of the most destructive agricultural pests, causing significant economic impacts (Szyniszewska and Tatem, 2014). Its resilience has imposed substantial financial stress on global agriculture, particularly affecting the fresh fruit and vegetable trade, which is valued at approximately €135 billion annually (Ministry of Foreign Affairs CBI, 2021). The invasion of medflies has led countries such as Iran to classify them as a primary pest shortly after their establishment (Rajabiyan et

al., 2015). In California alone, this species poses a major threat to the US$43 billion agricultural industry. The Department of Food and Agriculture estimates that a permanent medfly infestation could result in annual losses of up to US$1.8 billion (California Department of Food and Agriculture, 2012). These costs are attributed to direct production losses, increased monitoring and control expenses, and the impacts of embargoes or stricter quarantine regulations on exports. Siebert and Cooper (2008) have noted that such market losses could lead to significant job reductions.

The expansion of global trade and transportation in the late 19th century has also played a critical role in the spread of dipteran pest species to most fruit-producing countries in Europe (Diamantidis et al., 2011; Karsten et al., 2013). The climate change is also lately significantly influencing biodiversity by altering the distribution of native and non-native species, subsequently affecting crop yields. A study by Deutsch et al., (2018) predicts a 10 to 25% reduction in global yield for every degree increase in the global mean surface temperature. This impact is attributed to the rising populations and metabolic rates of insects, especially in temperate regions. For instance, the medfly, originally from sub-Saharan Africa, has spread worldwide, partly due to its superior adaptability to various weather conditions compared to other tropical fruit flies. In higher temperatures, adults exhibit bimodal activity, being active during the cooler hours of early morning and late afternoon, while seeking shade during midday heat (Hendrichs and Hendrichs, 1998). In more temperate conditions, usually below 30°C, they show a unimodal behavior pattern, focusing activity around midday (Hendrichs et al., 1991), making eradication attemps particularly challenging. When medflies face a cold temperature, research has shown that as an adult they can survive and even overwinter in various stages: as eggs, larvae in fruits, or as pupae in the ground (Martinez-Ferrer et al., 2010). Intriguingly, although primarily fruit feeders, larvae can occasionally develop on alternative hosts such as flowering plant blooms or stems, advancing their survival and colonization attempts (White and Elson-Harris, 1992). Furthermore, adult flies, naturally capable of migrating up to 20 km, can now travel further distances due to stronger winds and air currents associated with climate change (Copeland et al., 2002; OEPP/EPPO, 2011). The multifaceted nature of the medfly's life cycle and behavior, including its diverse dietary preferences, ability to thrive in various climatic conditions, and increased mobility due to climate change, significantly complicates eradication efforts. For farmers, these aspects present persistent challenges especially when these flies become classified as quarantine species, as it happened in the UK that follows The European and Mediterranean Plant

Protection Organization (EPPO) guidelines. Their classification of medfly as an A2 quarantine pest means strict regulatory controls, adding extra costs and layers of complexity to agricultural management, and essential continuous monitoring to prevent infestations.

The introduction to the Diptera order aimed to highlight their dual role in ecosystems as both pollinators and pests, revealing the challenges they pose in agricultural management. This intricate relationship becomes especially pertinent in the context of pesticide use. As we transition to discussing pesticides, we will explore the balance between controlling Diptera populations and addressing environmental concerns. This next section will delve into the development of sustainable pest control methods, reflecting on the lessons learned from Diptera to better appreciate the ecological impact of pesticides.

1.2 The historical and economical background of pesticides

From historical perspective, the third agricultural revolution or so-called ‘green revolution’ was initiated between 1950 and the late 1960s and marks the adaptation of innovative technologies to intensify agricultural production. Recognized as the “father of the green revolution”, the agricultural scientist Norman Borlaug was awarded a Nobel Peace Price in the 1970s as recognition for saving an estimated 1 billion people from starvation and reducing infant mortality. According to a review from (Pingali, 2012), the “Green Revolution" also contributed to a global reduction of poverty with the creation of numerous jobs, and reduction in greenhouse gas emissions by avoiding the conversion of thousands of hectares of land cultivated with heavy agricultural machinery (Pimentel and Pimentel, 1990). This worldwide spread of new techniques mainly consisted of using high yielding varieties of cereals combined with fertilizers, chemical pesticides and controlled irrigation. This resulted in an increase in all cultivated crop yields, with an estimated 160% increase in the case of world grain production (Kendall and Pimentel, 1994)Following these new agriculture practices, pesticides became a necessity to keep up with annual crop production with a current estimate of 45% loss due to pest infestation (Abhilash and Singh, 2008; Singh et al., 2017). Pesticide usage has seen a dramatic fiftyfold increase since 1950, with current applications amounting to over 3 million tons of pesticides used annually, with the distribution being 47.5% for herbicides, 29.5% for insecticides, 17.5% for fungicides, and 5.5% for other types, as reported by De et al., (2014) and Sharma et al., (2020). This substantial use of insecticides is particularly relevant in the context of managing Diptera populations, underscoring the scale of efforts required to control these pests. The market analysis by Björkman and Niemelä, (2015) estimated the global pesticide market at the producer level to be around US$56 billion in 2012. This included expenditures of US$25 billion for herbicides, US$16 billion for insecticides, US$14.5 billion for fungicides, and approximately US$0.5 billion for other types of pesticides. This significant investment in pesticides stresses their crucial role in modern agriculture, which is increasingly important in light of the World Health Organisation's projection. According to WHO (2020), an 80% increase in farming is needed in developing countries to sustain a growing global population, predicted to reach 9.7 billion by 2050. This projection highlights the escalating demand for agricultural productivity, where pesticides play a key role in ensuring crop yield and quality.

1.2.1 Classification of Pesticides

Pesticides, a common tool in households, gardens, and farms, are categorized into several classes based on their target pests and chemical structures. This classification is essential for understanding their distinct modes of action, effectiveness, and potential environmental impacts. Broadly, pesticides are divided into herbicides, aimed at controlling unwanted plants and weeds; insecticides, designed to combat various insect pests; fungicides, which prevent fungal infections in crops; and rodenticides, used for controlling rodent populations. Additionally, there are more specialized categories like algicides, targeting plant-like organisms, miticides managing mite's population and nematicides for nematode worms (Suiter et al., 2008; Tuzimski, 2015).

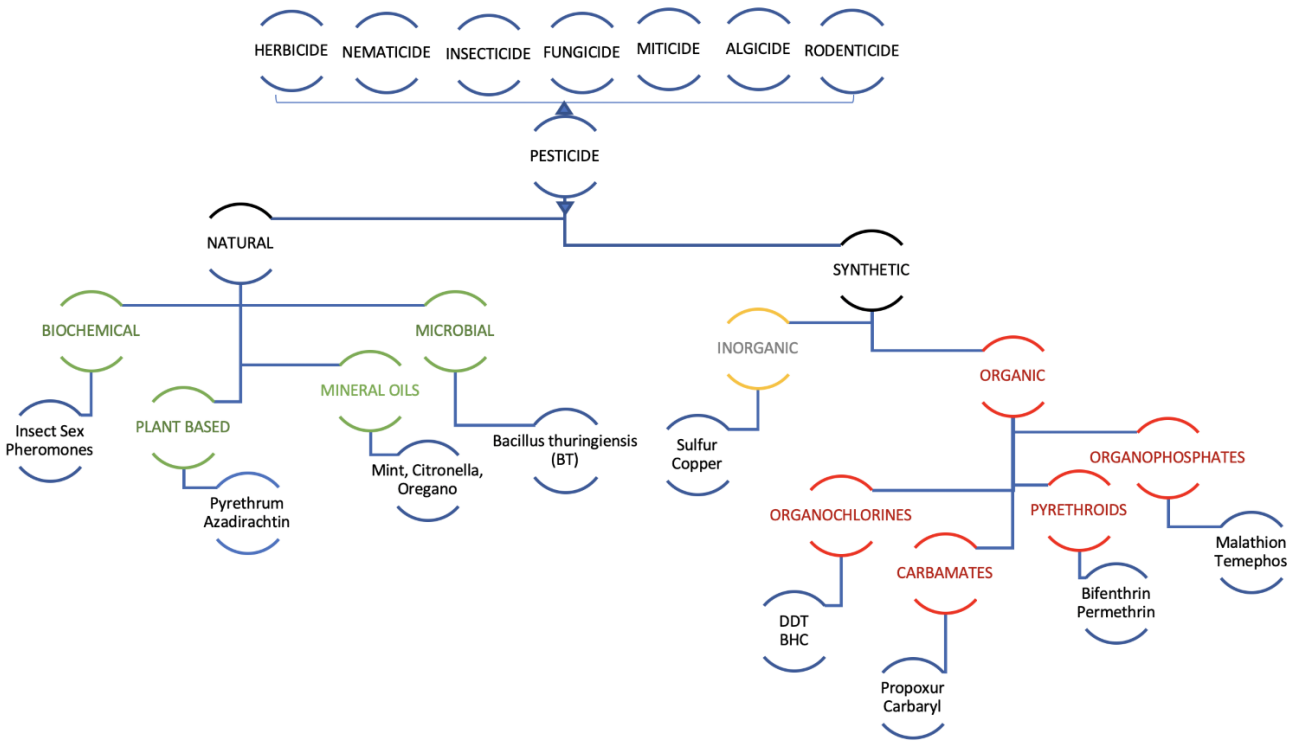


Figure 1.2 Classification of pesticides according to their chemical composition. Pesticides can be categorized based on their origin, being either natural or synthetic. Synthetic pesticides are further divided based on their carbon content, divided into two groups: inorganic formulations (such as sulfur or copper compounds) and organic pesticides. The latter form the majority of commercial pesticides and are further subdivided into four major groups: organochlorines, carbamates, pyrethroids, and organophosphates. Pesticides of natural origin, also referred to as biopesticides, mimic naturally occurring chemicals, such as insect growth regulators or chitin synthesis inhibitors (Kaur et al., 2019).

The schematic was modified from Pathak et al., (2022).

Each class of pesticides is formulated to address specific challenges in agriculture and public health, and their usage varies depending on the pest problem, crop type, and environmental considerations, as illustrated in Fig. 1.3 below. This figure highlights the range of insecticide types used to protect various crops grown in the United Kingdom, with data sourced from the FERA Pesticide Usage Survey Report (Ridley et al., 2020).

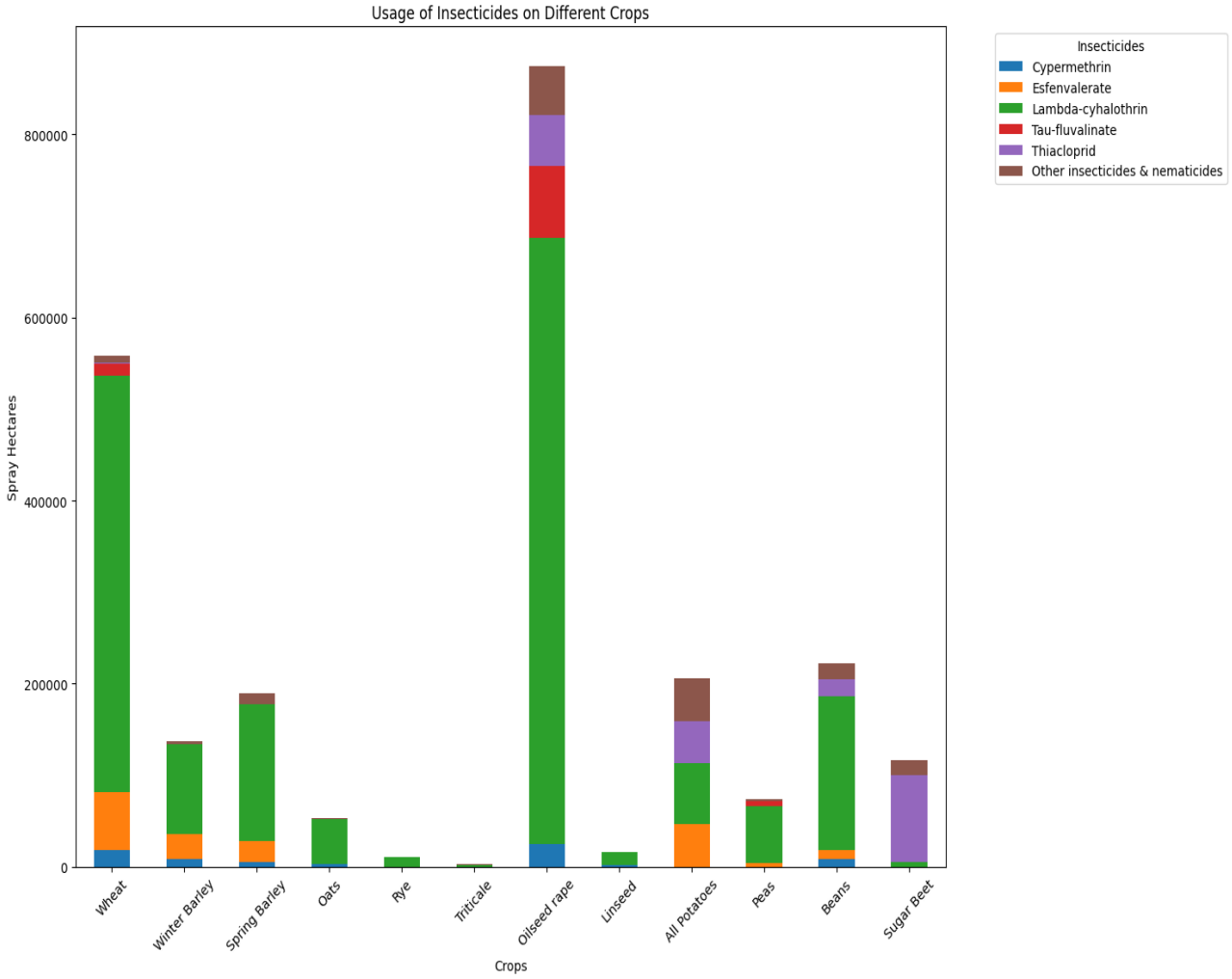


Figure 1.3 An illustrative overview of most common insecticides applied to crops in the United Kingdom, highlighting specific insecticide-crop pairings. (colour code: blue- Cypermethrin, orange- Esfenvalerate, green- Lambda-cyhalothrin, red- Tau-fluvalinate, violet- Thiacloprid, brown- Other insecticides and hematicides. The latter category includes acetamiprid, cypermethrin, cyfluthrin, chlorpyrifos, deltamethrin, fluopyram and pymetrozine). For more detailed usage of pesticides including herbicides, molluscidices, repellents and other control agents applied in the UK please refer to Pesticide Usage Survey Report 295, Arable Crops in the United Kingdom (https://pusstats.fera.co.uk/published-reports, accessed on 05/12/2023). Data were retrieved from Pesticide Usage Survey and graphical representation was generated by Google Colab with integrated matplotlib package (https://colab.research.google.com).

1.2.1 Sustainable Crop Agriculture through Integrated Pest Management

A comprehensive and environmentally conscious approach to pest control, aligning with current trends towards more sustainable and ecologically friendly farming methods is the philosophy of the concept of Integrated Pest Management (IPM) invented in 1950's. It is an approach that relies on the commitment to practically keep pest populations below an economic injury level, using effective and environmentally sensitive control methods (Damos et al., 2015). While there are numerous techniques to control pests, IPM takes into account a combination of three main factors to achieve its best practices; the first factor is a requirement for deep knowledge of the ecosystem where pests need to be controlled as it is highly focused on using natural methods to suppress a target pest. For example, the introduction, the increase or the protection of predators in an ecosystem could be favourable in controlling a specific pest. The second element is the inclusion of economic threshold levels (ETL), which consist in defining an ETL at which the harmfulness of the pest will require control measures. In practice, it consists of sampling all insects present in the ecosystem, measuring their benefits and potential harm against the economic damage level defined for the crop. This threshold gives the advantage of avoiding the unnecessary use of chemical control often applied on crops as prevention. In cases such as the mosquito *Aedes aegypti* (Diptera: Culicidae), a vector of yellow fever, the impact caused on human health may involve an ETL valued of zero.

The third IPM element to include is the full understanding of the biology and ecology of all species present in the agro-ecosystem. A detailed appreciation of the role of each species and their damage level is essential in the establishment of a control strategy that fits with IPM principles. For example, understanding the pest population dynamics require analysis on the lifecycle of pest in the crop, its biotic and abiotic mortality factor. Sequencing this knowledge at various stages of the crop cycle may reduce the application of pesticides to a specific growth stage (Morallo-Rejesus, B., Rejesus, 1992; Flint, M., 2012; Lefebvre et al., 2015).

1.2.2 Population Detection, Control and Eradication Methods

1.2.2.1 Trapping

A common practice to diminish the use of insecticide is to attract-and-kill pests in a device containing lures mixed with insecticides such as ammonium acetate, putrescine and trimethylamine. These devices can either attract and capture the targeted pest, in which case they are referred as “mass trapping” or attract and not retain the pest which is called “lure and kill”. The food-based lures are imitating the protein requirement needed by the insect to reach its reproductive maturity. The higher need of protein of female flies to develop their eggs often results in a significant decline of female population. While the use of food-based synthetic insecticides inside those devices can reduce the need of a wide application of chemical pesticides on the crops, the efficiency of this technique has shown to be limited to lower pest density populations. Additional techniques such foliage baiting or cover spraying are often required to obtain pest population under control, making mass trapping an efficient method for continuous monitoring purposes (Fao et al., 2005; El-Sayed et al., 2006; Rel and Akyol, 2017).

1.2.2.2 Foliage baiting

This makes chemical control the most common method used to eradicate medfly. Within this method, foliage baiting and cover spraying are the most widely used. While the first one is used to control the adult population only, cover spraying is applied to kill medflies at all stages of their lifecycle. Foliage baits consist of spraying a combination of a protein and an insecticide, usually during the early hours of the day to prevent the fruits or leaves from burning. While it is understood that females are highly dependent on protein source to develop their eggs, those baits are an attractive food source for both sexes (Umeh and Onukwu, 2011; Vargas et al., 2015).

1.2.2.3 Cover spraying

On the other hand, cover spraying consists of spraying a defined amount of insecticide directly on the fruits. The main advantage of this approach over other methods, is in the detrimental effect to all life stages of medflies; via contact for adult medfly or penetration for eggs and larvae (Broughton et al., 2004; Broughton and Rahman, 2017). However, the lack of specificity of the active component of the pesticide means that wide range of insects could also be exterminated, as mentioned in the case of banned malathion insecticide (Ben Jemaa et al., 2010).The other difficulty relies in the consistency of the

amount of spray to avoid local high concentration which could results in high harmful residues level left in the fruits (Grewal et al., 2017).

For example, the application of malathion, a potent organophosphate (OP) insecticide that targets acetylcholinesterase, was the predominant and preferred method for plant protection against medfly. Malathion is an inhibitor and its mode of action consists of preventing their nervous system from functioning properly in two main steps. Unprotected by external shelter (host's pulp, soil, egg or pupae casing), the targeted adult medfly is susceptible to the OP (Gupta et al., 2002; Colović et al., 2013). However, in 2006, the potential negative effects on human health as well as its side-effects on the environment and ecosystems, resulted in a ban under annex I EU directive 91/414/EEC (Ben Jemaa et al., 2010).

1.2.3 Chemical Pesticides: A Critical Look at Modern Agriculture's Double-Edged Sword

While the use of chemical pesticides has been beneficial in increasing crop yields, their excessive application has led to several adverse effects. This is particularly evident in the case of numerous chemicals used in industry and agriculture, which, when spilled into the environment, persist for exceptionally long periods. These chemicals not only accumulate but also move up through the trophic levels of the food chain. Reflecting this concern, the 2004 Stockholm Convention categorized certain pesticides as Persistent Organic Pollutants (POPs) to safeguard human health and the environment. POPs, including Dichloro-diphenyl-trichloroethane (DDT), Heptachlor, or Pentachlorobenzene, are known for their toxicity, resistance to degradation, and tendency to accumulate in biological systems. They also have the capacity to be transported across international boundaries via air and water, settling far from their original release points in both terrestrial and aquatic ecosystems, as illustrated in Figure 1.4. Using those multiple pathways, it has been proved that POPs can affect non-targeted organisms in the environment by disrupting endocrine and reproductive systems in living organisms (Sarmah et al., 2004; Asiamah et al., 2020). The majority of chemical insecticides were developed a couple of decades ago and as such do not necessarily abide to the regulations and health and safety requirements for nowadays farming. Retrospective surveying of those insecticides to abide current regulations are of remarkably prohibitive cost and agrochemical companies thus abandon insecticides that already made it to the shelves (Finch and Collier, 2000). Currently, the process of developing a new chemical pesticide

is both time-consuming and costly, taking approximately 15 years and an investment of around $20 million. On average, only one in every 10,000 compounds initially screened eventually makes it to final commercial production (Tuzimski, 2015).

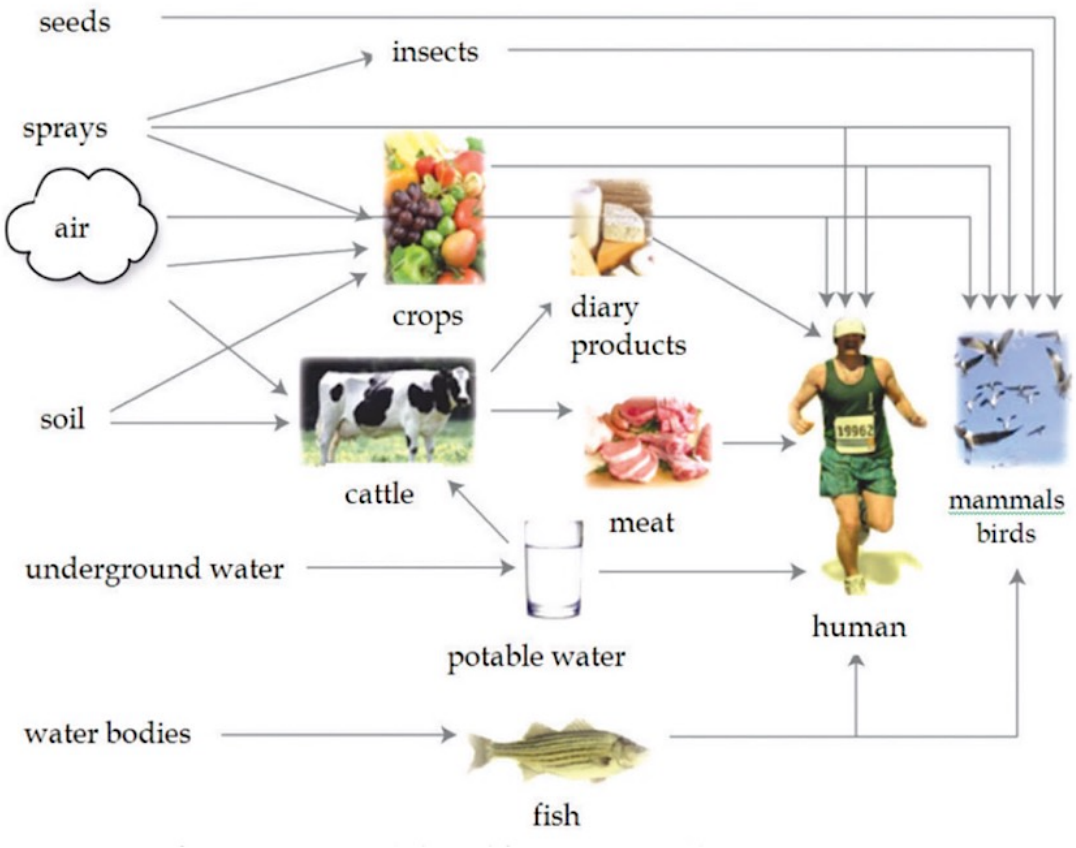


Figure 1.4 Main routes of organophosphate exposure in animals and humans. This illustration depicts the primary pathways through which animals and humans are exposed to organophosphate residues. Direct exposure routes are shown, including inhalation of contaminated air and dermal contact with contaminated soil and water. Additionally, indirect exposure routes are highlighted, encompassing the ingestion of contaminated water and food. The diagram provides a comprehensive overview of the environmental and dietary factors contributing to organophosphate exposure.

(reproduced from WHO, 2001 in Elersek, T. , Filipic, 2011)

1.2.3.1 Beyond the Crop Fields: The Far-Reaching Effects of Pesticides on Our Well-being

Pesticides such as dichlorodiphenyltrichloroethane (DDT), chlordane, aldrin, dieldrin, endrin, mirex, heptachlor, and hexachlorobenzene, even when present in trace amounts

in food or through environmental exposure, are associated with significant immediate and long-term health effects on humans. Studies have shown that chronic exposure to these substances, even at low concentrations, can lead to a weakened immune system, hormonal imbalances, diminished cognitive abilities, reproductive health issues, and an increased risk of developing cancers (Yadav et al., 2015). Notably, farmers, who are regularly exposed to chlorinated pesticides such as organochlorines, and methyl bromide, a halogenated fumigant pesticide, are particularly at risk. They have been observed to have an elevated likelihood of developing prostate cancer and both types of asthma – allergic and non-allergic (Alavanja et al., 2003; Hoppin et al., 2009).

1.2.3.2 Unravelling the Impact of Pesticides on the Environment

Pesticides are also considered as the major factor for unprecedent large-scale insect biomass decline (Hallmann et al., 2017). Numerous studies and models on pesticides impact reported the significant patterns of pollinator decline (Sponsler et al., 2019), resulting in tighten regulations and withdrawal of several commonly used pesticides across the globe (Maroni et al., 1999; Aktar et al., 2009; Damalas and Eleftherohorinos, 2011). Furtermore Wagner's 2020 review highlights a stark decline in insect populations and a loss of species diversity, with the use of insecticides being partially blamed for this worrying environmental trend (Wagner, 2019). For example, in the case of honeybees, which are recognized by a large public as symbol of the rapid decline of keystone pollinators, this threat has resulted in the controversial restriction of the use of neonicotinoids across Europe (Woodcock et al., 2016). The controversy over restricting neonicotinoids in Europe stems from balancing environmental concerns about honeybee decline with agricultural reliance on these highly effective, broad-acting pesticides, and the complexity of finding suitable alternatives. Its replacement by sulfoximines for example, as the alternative, also raised concerns as it has also shown a detrimental effect on bumblebee colonies (Siviter et al., 2018). This matter highlights conflict between various stakeholders, including farmers, scientists, environmentalists, and policymakers, each with differing priorities and opinions.

Beyond the noted effects on human and animal health above, the significant concern lies in the environmental accumulation of pesticides, primarily resulting from spillage and runoff, rather than just their presence in food, leading to unintentional exposure in both wildlife and humans with far-reaching ecological and health implications. Striking revelation in 2020 published data indicated that over one-fifth (22%) of monitoring sites in European rivers and lakes had pesticide levels exceeding thresholds of concern.

Furthermore, a 2019 study alarmingly found that 83% of tested agricultural soils in Europe were contaminated with pesticide residues, highlighting widespread soil pollution issues. According to the Land Use and Coverage Area frame (LUCAS) database, more than 80% of topsoil samples contain one or more pesticide residues, and over half of these show a mixture of two or more pesticides (European Environmental Agency, 2023). Yet, the synergistic effects of combined pesticides remain a largely unexplored area, only with recent reports on their effects on ecosystems and health (Siviter et al., 2021).
As a result of those findings, the European Commision has set a target for 50% reduction in both, the use and risk of chemical pesticides, and to dedicate at least third of agricultural land to organic farming, to foster fair, healthy, and sustainable food systems in Europe by 2030.

1.2.4 Innovative Solutions: Beyond Chemical Pesticides in Agriculture

In an effort to mitigate the detrimental impacts of chemical pesticides in agriculture, the adoption of Integrated Pest Management (IPM) strategies offers a range of innovative solutions. Biological control for example takes a significant part where natural enemies of insect pests including predators, pathogens, competitors and parasitoids are used to restrain their population while protecting crops systems. Additionally, the complexity to produce, distribute and apply in the fields, studies have shown variable success of this method and additional chemicals were needed for the complete collapse of medfly populations (Panizzi, 2012).
Building upon the foundation of biological control, another alternative within IPM strategies is the Sterile Insect Technique (SIT). This approach involves releasing sterilized males over a wide area to mate with females from wild population, thereby disrupting the breeding patterns of pest populations (Pérez-Staples et al., 2021). However, the eradication of pests, such as medflies, is complicated by their unique mating behavior known as lek polygyny. This pre-copulatory courthip behaviour means that females are the ones to pick the fitttest male from ‘mating arena’ known as ‘lek’. In those lekking areas males headbut competitors to defent their territory and display mating body postures, wing movements and release pheromones to attract observing females (Niyazi et al., 2008). The lek polygyny behavior implies that a successful Sterile Insect Technique (SIT) requires the release of a large number of sterilized males. For example, in Florida, the US Department of Agriculture maintains a minimum release ratio of 10 sterile males for every wild male. This approach, combined with quarantine restrictions and chemical

ground treatments, is essential to achieve a population collapse of fruit flies (medflies) (Ben Jemaa et al., 2010; OEPP/EPPO, 2011; El-Lissy, 2021). Adding to the complexity, behavioural resistance against sterile males were also documented (Panizzi, 2012; Pérez-Staples et al., 2021). While Deguine et al., (2021) have detailed various Integrated Pest Management (IPM) methods, these often require the repeated use of chemical controls, which lack specific efficiency against the targeted pests.

To address this issue, new methodologies such as RNA interference (RNAi) have been tested as biological insecticides that aim to specifically inhibit the function of genes in target pest, reducing its toxicity impact on non-target species (Price and Gatehouse, 2008; Huvenne and Smagghe, 2010; Taning et al., 2016). To enhance the stability of double-stranded RNA (dsRNA) against environmental factors before uptake target pest, one technique involves its encapsulation in polymeric nanoparticles. This method helps prevent dsRNA degradation, thereby facilitating its effective delivery and function. Following this process, dsRNA enters the cell and is cleaved into small interfering RNAs (siRNAs). The siRNA binds to the RNA-induced silencing complex (RISC) leading to the specific degradation of messenger RNA (mRNA) inside the target animal. While showing promising results when directly injected, the RNAi response is limited when inducted through feeding or topical application (Pugsley et al., 2021). The main obstacle lies in creating nanoparticles capable of protecting dsRNA from degradation, in the environment and during digestion to allowing full completion of its mode of action. Another burden for its use as a pest control is in the process and the cost of full-scale production for a high variety of pesticides formulation applied in range of weather conditions and type of crops. The encapsulation of dsRNA in polymers, crucial for the efficacy of RNAi, must also adhere to toxicity and environmental regulations (Bellés, 2010). This consideration is part of a broader shift in global pesticide usage, increasingly governed by new regulations aimed at phasing out conventional pesticides harmful to humans and non-target species. These efforts advocate for the adoption of environmentally friendly biocides, which could also mitigate insect resistance (reviewed by Tataridas et al., 2022). Initiatives like the European Citizen's 'Save Bees and Farmers' (https://www.savebeesandfarmers.eu/eng) and 'Towards a chemical pesticide-free agriculture' (https://www.era-pesticidefree.eu), supported by key policy propositions in the 'European Green Deal' by the European Commission, exemplify this shift.

The rising demand for biopesticides, driven by increasing public concern over chemical pesticide spills and residues, is reshaping the pest control industry. As the global market

for biopesticides is expected to exceed US$ 9.5 billion by 2025 (Davies et al., 2019), integrating these into Integrated Pest Management (IPM) systems and exploring new modes of action, such as targeting insect neuropeptides, are becoming increasingly important.

Central to the effectiveness of these approaches is the understanding that ingestion is a principal pathway for pests to absorb pesticides. Understanding the role of the digestive tract in how pests absorb pesticides is vital in this context. Therefore, the following subchapter will be dedicated to a detailed exploration of the digestive system and its function. This focus is essential not just for comprehending the action of current pesticides but also for paving the way for the development of new, more targeted pest control solutions.

1.3 The digestive tract, its structure and functioning

Regardless of feeding habits, all adult flies share a common anatomical organization of the digestive tract with each compartment having a specialised role in digestion, excretion, and water balance. Presumably reflecting the distinctive feeding habits, the organization of the larval and adult gut differs considerably. Figure 1.5 provides a detailed comparison of the digestive tract structures in both larval and adult stages, illustrating the developmental changes.

While larvae are equipped with mouth hooks allowing them to feed continuously on solid diet and two pairs of elongated tubules 'gastric ceca' formed behind the proventriculus, the adult foregut owns a diverticulated crop. The crop, emerging as a single duct from the oesophagus, extends into the abdomen where it forms an expandable sac, enveloped by highly contractile muscles (Stoffolano et al., 2010). This, in turn, allows a large amount of liquid diet to be ingested and stored. The contracting crop lobes initialise digestion by mixing the food with regurgitated enzymes, presumably of midgut origin (Lemaitre and Miguel-Aliaga, 2013). In the adult, the digestive tract begins with the pharynx and oesophagus, followed by the proventriculus and the already mentioned crop that all together form the foregut (stomodeum). The foregut leads to the midgut (mesenteron), where much of the digestion and nutrient uptake occurs, and ends with the hindgut (proctodeum) that is important for water balance and the control of defaecation. The intestine is covered with circular and longitudinal visceral muscles that generate peristaltic movements and assist with grinding and moving food along the gut. A layer of impermeable cuticle covers the apical epithelium of the foregut and hindgut, a protection against digested pathogens, abrasive particles and harmful substances. The midgut's semi-permeable barrier consists of the peritrophic matrix as shown in Figure 1.6. Although this extracellular structure is poorly defined, it is known to contain a network of chitin-binding proteins 'peritrophins' and chitin fibrils. In terms of function, it adds additional strength, protects against abrasive particles and pathogens, but primarily segregates gut into the endo- and ectoperitrophic space thanks to a range of pores present (commonly between 7-9 nm but some might be up to 36 nm) thus acts as a sieve (Terra, 2001). It is hypothesized that primary enzyme degradation occurs within the confined endoperitrophic space by polymer hydrolyses, followed by secondary digestion of passed-through particles now present in ectoperitrophic fluid. The digestion is completed via enzymes trapped in the glycocalyx, glycoproteins that residue within microvilli, an extension of epithelial cells present in the midgut.

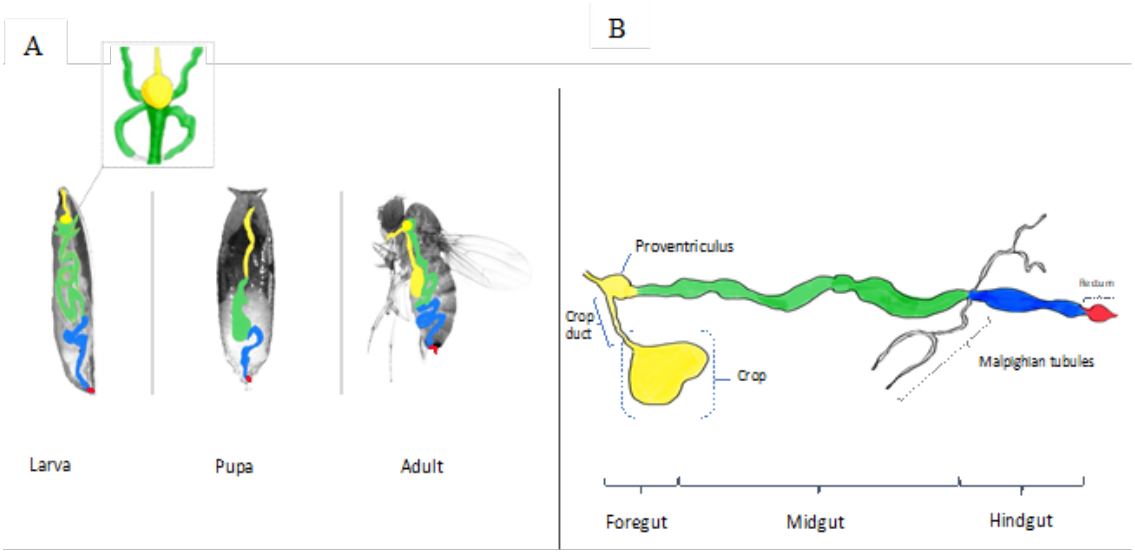


Figure 1.5 Organization of dipteran digestive tract depicting the organisational structure through (A) larval, pupal and adult stages separated by colour code; yellow, foregut; green, midgut; blue, hindgut, red, rectum. (B) Adult digestive tract differs considerably from larvae. The two pairs of ceca in larva (in square illustrating elongated finger-like structures attached to proventriculus, forming a part of midgut) are lost and rebuilt during pupal metamorphosis into an adult crop, forming a distinctive part of adult foregut.

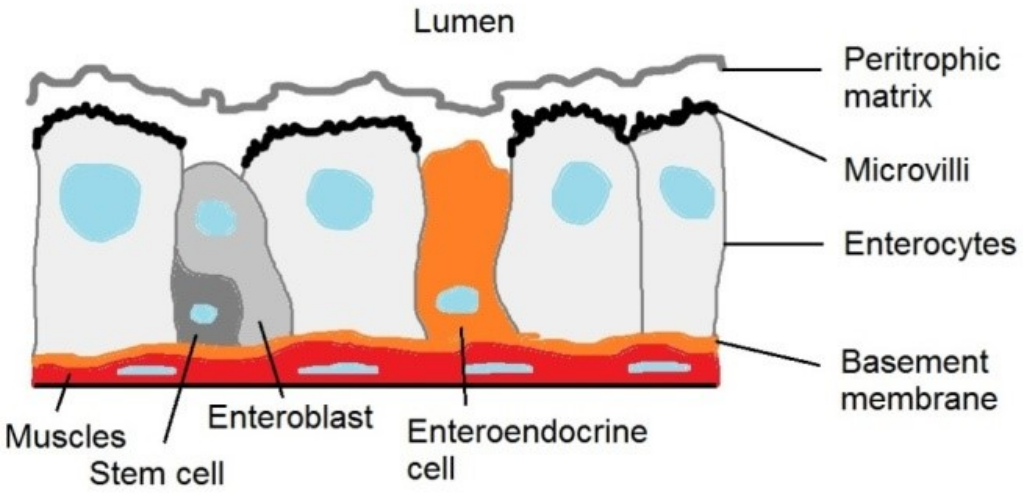


Figure 1.6 The structure of midgut epithelium in adult *D. melanogaster*. Predominant enterocytes are accompanied by enteroendocrine cells and progenitor stem cells, all attached to basement membrane.

(redrawn from (Lemaitre and Miguel-Aliaga, 2013)

The digestive tract is further subdivided along the midgut (Marianes and Spradling, 2013). In the structure of model insect *Drosophila*'s midgut, cells are attached basally to the basement membrane and separated from the lumen environment by the peritrophic membrane. A specialized copper-cell region present in the middle midgut facilitates uptake of metal and essential micronutrients. Four distinct groups of cells are present in the midgut; differentiated enterocytes, enteroendocrine cells, transient enteroblasts and intestinal stem cells (Kux and Pitsouli, 2014). The main function of enterocytes is to secrete digestive enzymes and absorption, however cell damage releases growth factors that stimulate enteroblast differentiation. Between enterocytes there are dispersed enteroendocrine cells that secrete peptide hormones and play a role in innate immunity and possibly release of digestive enzymes (Fig. 1.7). Progenitor cells, referred to as enteroblasts differentiate into enterocytes or enteroendocrine cells that in turn replace delaminated cells to maintain tissue integrity (Buchon et al., 2013; Buchon and Osman, 2015).

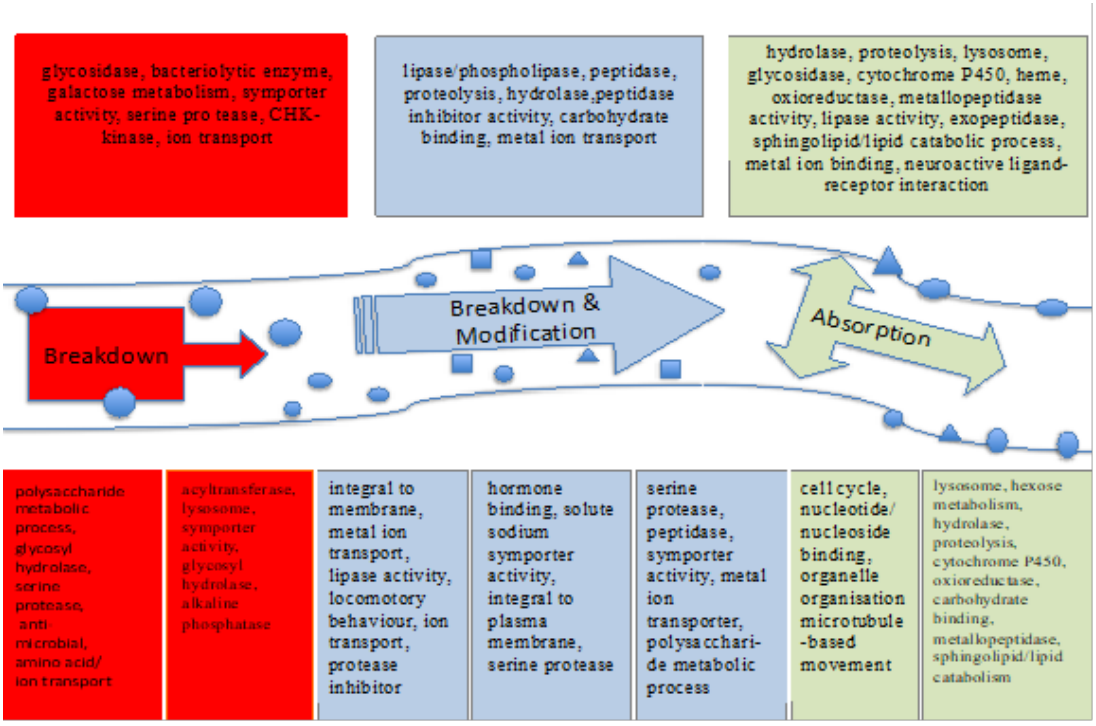


Figure 1.7 The spatial localisation of digestion occurring in adult *D. melanogaster* digestive tract based on RNAseq analysis that led to the identification of distinct features of subregions present in *D. melanogaster* digestive tract.

(redrawn from Marianes and Spradling, 2013)

For more detailed morphological structures, gene expression patterns and 3D models follow ‘Flygut: an atlas of the *Drosophila* adult midgut’ dabatase (http://flygut.epfl.ch).

Having explored the intricate anatomy of the gut, we now turn our attention to the complex interplay between the nervous system and feeding behavior, where neuronal control plays a pivotal role in regulating digestive processes and food intake. Neuropeptides that interfere with the passage of ingested food through the foregut are particularly intriguing as biopesticide candidates, as the ability of an animal to proceed with feeding critically depends on the smooth transition of food through this initial part of the digestive system.

1.4 Neural control of feeding behaviour

For every animal, humans included, the majority of their daily routine is to obtain nutrition and energy from a diet, to maintain and support needs of growth, development, and to sustain activities required for foraging and reproduction. Flies that are exposed to nutritional deficiencies during their larval stages of development, display reduction in size, limited ability to withstand starvation, inadequate immune response to pathogens together with low reproductive competence after reaching maturity.

In Dipterans, the mouthparts and gastrointestinal tract are both innervated by neural circuits originating from the CNS and the stomatogastric nervous system (SNS), the latter network containing peripheral ganglia that in turn regulate muscles of the pharynx and gut. Numerous studies have demonstrated the importance of the subesophageal ganglion, that is situated in the ventral region of the adult brain, believed to be a centre for the initiation of feeding and in regulation of food uptake (reviewed by Spit et al., 2012).

The brain functions as a master regulator, interpreting signals from endocrine cells distributed throughout the body, including those in the gut. These cells secrete various neuropeptides that engage in sophisticated bi-directional communication within the 'brain-gut endocrine axis'. They play a crucial role in signaling and interpreting nutritional states and environmental conditions, thereby maintaining energy balance and influencing feeding decisions (Chopra et al., 2022). The immunohistochemical localisation of 'brain-gut' peptides in CNS, SNS and enteroendocrine cells is further indication that implies a role in the regulation of feeding and digestion. Peptides briefly described below are believed to be directly involved in coordination of feeding that exerts their function primarily in two ways; directing mouthparts and muscle mobility and thus playing crucial role of initialisation and modulation of digestion via gut peristalsis, and/or influence the release of digestive enzymes from enteroendocrine cells present in the gut.

It should be also briefly mentioned that beyond the neuropeptides discussed below, additional neuropeptides/neurohormones are synthesized by neurosecretory cells in the brain that contribute to complex feeding circuits. Among these, eight *Drosophila* insulin-like peptides (DILPs) and Neuropeptide F (NPF), the long isoform of sNPF, are notable. Both are primarily produced in the brain but modulate feeding behavior through different mechanisms. DILPs, predominantly synthesized in the brain, particularly in the insulin-producing cells (IPCs) of the pars intercerebralis, regulate feeding behavior though systemic release and acting, for example, on abdominal fat body (Sudhakar et al., 2020). They are involved in regulating energy homeostasis and hunger signaling, responding to

low circulating nutrients and altering the activity of neurons involved in food seeking and consumption (Semaniuk et al., 2021). In contrast, the expression and secretion of NPF is a notable example of a peptide that promotes feeding behavior in *D. melanogaster* though acting within a brain (Shen and Cai, 2001). It acts within its site of synthesis, influencing feeding motivation and apetitive memory through NPF receptors in dopaminergic neurons that in turn regulate food intake (Krashes et al., 2009).

As the exploration of pesticides, gut anatomy, and the basics of neuronal regulation of feeding concludes, a shift in focus is necessary to understand the deeper mechanisms at play. This transition leads into the intriguing subject of neuropeptides, describing their nature, structure and synthesis, regulatory mechanisms, and specific examples that are crucial in controlling feeding behavior.

1.5 Neuropeptides

1.5.1 Background

Neuropeptides, ancient signaling molecules thought to have evolved even before the emergence of animal nervous systems 600 million years ago (Sachkova, 2022), present a promising avenue for pest control. Despite being highly conserved, neuropeptides represent some of the most structurally and functionally diverse compounds in the animal kingdom (Altstein, 2009). This diversity and specificity make them ideal candidates for developing selective biopesticides that align with the increasing market demand and regulatory requirements.

Essentially all living organisms respond to their external environment and modulate the internal state to maintain homeostasis, while adjusting to growth, aging and reproductive behaviour necessary for survival of the species (Vanden Broeck, 2001; Gäde and Goldsworthy, 2003; Altstein, 2009; Spit et al., 2012; Caers et al., 2015). Neuropeptides can act either locally within the central nervous system (CNS) as neuromodulators or as circulating neurohormones released into haemolymph, reaching multiple circuits reflecting their pleiotropic roles (Nässel, 2002). Neuropeptides are synthesised and released by both peptidergic neurons and endocrine tissues, as illustrated by the peptidome of *D. melanogaster* where 24 neuropeptides are expressed in both the CNS and the enteroendocrine cells of the midgut, thus acquiring the term ‘brain-gut’ peptides (Reiher et al., 2011). It is not unusual for both, central and peripheral neurons to co-express small non-peptide neurotransmitters, for instance acetylcholine, glutamate or serotonin, allowing for increased flexibility of neuronal signalling. Likewise, neurosecretory cells and enteroendocrine cells of gut are also capable of synthesis of more than one peptide. The co-localization of neuropeptides and neuronal transmission is discussed in detail by (Nässel and Winther, 2010) and further reviewed by Nässel (2018).

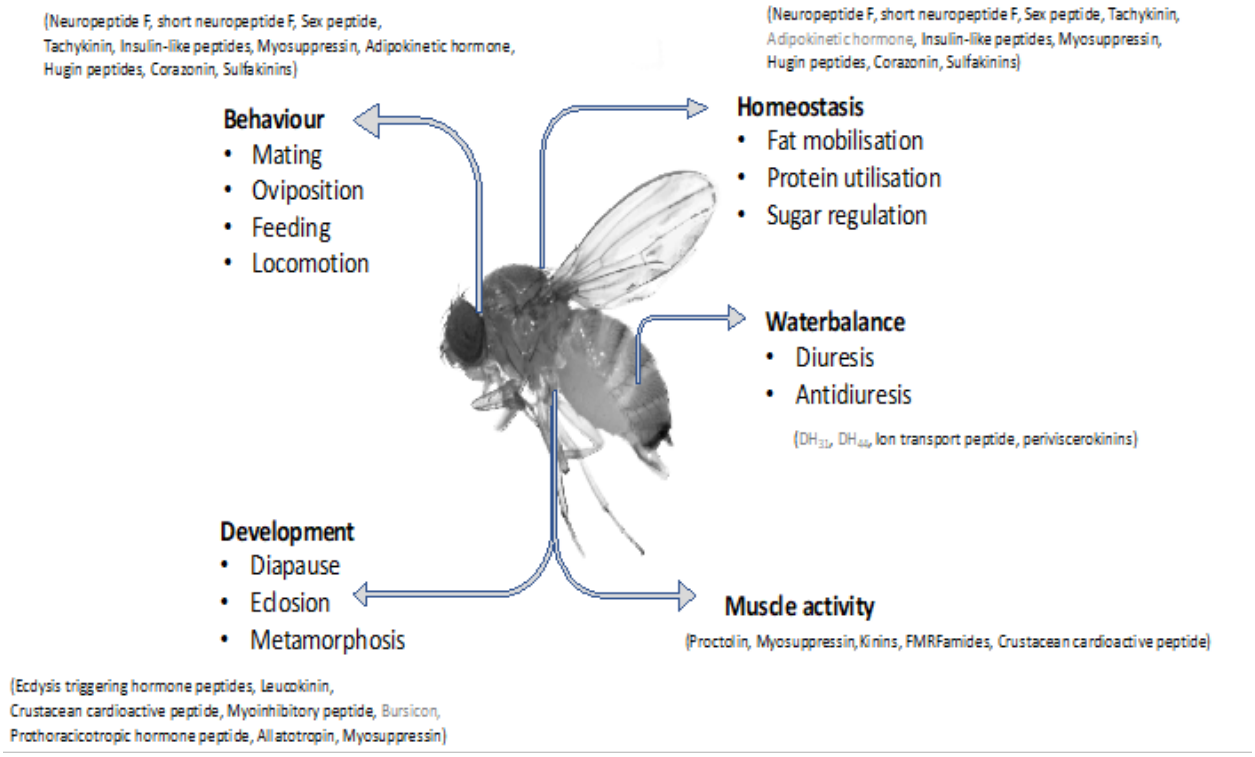


Figure 1.8 Illustration of diverse role of neuropeptides. Every aspect of fly behaviour and internal function is under a direct and/or indirect influence of neuropeptides. Most neuropeptides can be implicated in more than one function, for instance short Neuropeptide F (sNPF). Released both as a neurohormone and a local co-transmitter is involved in regulating taste sensing and feeding, walking, memory and odour perception in the fly (Nässel and Zandawala, 2019).

(Modified from Scherkenbeck and Zdobinsky, 2009).

The extraction of myotropic substances from proctodeal nerves located in hindgut of the American cockroach (*Periplaneta americana)* led to the first neuropeptide discoveries in insects dating back to 1970's, where isolation and Edman degradation sequencing identified the novel compound, called proctolin, and unleashed active exploration within insect neuroendocrinology (Wegener and Gorbashov, 2008). Since then, for the past two decades thanks to ongoing development of analytical and DNA sequencing techniques mentioned below, 50 neuropeptide families across 400 different insect species have been identified (Vanden Broeck, 2001; Yeoh et al., 2017). For *D. melanogaster* the estimation is 50 genes encoding neuropeptide precursors and equally 50 precursors for neuropeptide receptors (Nässel and Zandawala, 2019). Before the publication of the *D. melanogaster* genome (Adams et al., 2000), the pre-genome era meant that peptidomics relied on

analytical separation though liquid chromatography together with bio-assays and mass spectrometry techniques. It also meant laborious dissections of several hundreds to thousands of neurohaemal tissues, organs containing neurosecretory cells responsible for the storage and release of neuropeptides and/or neurohormones into haemolymp. Purified extracts were then bioassayed to determine either their function or pharmacological effect, as presented by strenuous dissections of over thousands of abdominal ganglions from crayfish, *Orconectes limosus* that led to the discovery of myotropic orcokinin peptide (Stangier et al., 1992). Before the era of mass spectrometry, Edman sequencing developed in 1950's was applied to sequentially cleave amino acids from the N-terminus of the peptide before identification by HPLC-based amino acid analysis. Compared to current techniques, the method is time consuming and importantly it is restricted to the peptides that do not have protected N-termini (protein termini and their modifications are reviewed by Marino et al., 2015). A change came in 1990's when the mass spectrometry was used to identify peptides directly from range of biological samples, but still mainly focusing on known neuropeptides or to study their degradome ie. the repertoire of peptidases responsible for the cleavage and regulation involved in neuropeptide activation and/or degradation (Predel, 2001; Schrader et al., 2014). The biologically active form of a neuropeptide can be validated either directly through single tissue profiling (Chapter 3, Figure 3.7 Mass spectrum from the direct analysis of a *D. melanogaster* crop nerve bundle) that provides direct evidence of the neuropeptide presence and form in a specific tissue sample or following peptide separation though reversed-phase liquid chromatography (RP-HPLC) analysis (Chapter 6, Fig. 6.11) that allows separation and identification of the neuropeptide after it has been isolated, further confirming its structure and active form.

The advance and the ease of "NextGen' (next generation) sequencing and widening taxonomic coverage has resulted in over 100 published Diptera genomes (http://1kite.org) is allowing the exploration of novel neuropeptides and detailed phylogenetic studies (Wegener and Gorbashov, 2008; Wiegmann and Richards, 2018; Bläser and Predel, 2020). Conserved structural features of precursors deduced from genome sequencing allow us to uncover the evolutionary relationship between neuropeptide signaling pathways across taxa/animal phyla. All those features are based on identity of structural motifs, a crucial factor for the development of species-specific bioactives (Khaldi, 2012; Romanova and Sweedler, 2015; Koehbach and Jackson, 2015). Despite significant structural diversity, the presence of conserved motifs, such as cysteines and hydrophobic

cores, aids in the identification and discovery of novel neuropeptides, as demonstrated by recent identification of putative insulin-like peptides (Wang et al., 2015). Assembled transcripts allow the identification of orthologous precursors and structural features of putative neuropeptides or their receptors, such as a signal peptide, open reading frames, basic cleavage (processing) sites (Wiegmann and Richards, 2018; Nässel and Zandawala, 2019; Bläser and Predel, 2020). A plethora of databases (NCBI, UniProtKB, MEROPS, DINeR, NeuroPep, NeuropPedia, NeuroPP) are now accessible to aid anyone to mine and compare transcripts leading to a discovery of novel insect peptides, for example RYamide (Hauser et al., 2010), trissin (Ida et al., 2011), natalisin (Jiang et al., 2013) or limostatin (Alfa et al., 2015).

The continual advancement in automatization for mining of putative neuropeptide precursors in the form of transcripts, detecting the presence of a signal peptide, cleavage sites and/or conserved hydrophobic regions accelerated the identification and annotation of neuropeptides within and outside of insect phyla. Not always does identification of a pre-pro-protein sequence lead to the mature peptide structure (Chapter 6, Table 6.3; Nässel and Zandawala, 2019). Thus, although several publications are based on *in silico* data only, particularly for species of economic or medical importance (Christie et al., 2008; Veenstra et al., 2012; Adamson et al., 2015), *in silico* analysis is often combined with mass spectrometry-based methods to confirm the predicted mature peptide as shown in Figure 1.9 that illustrates common workflow for identification of novel neuropeptides.

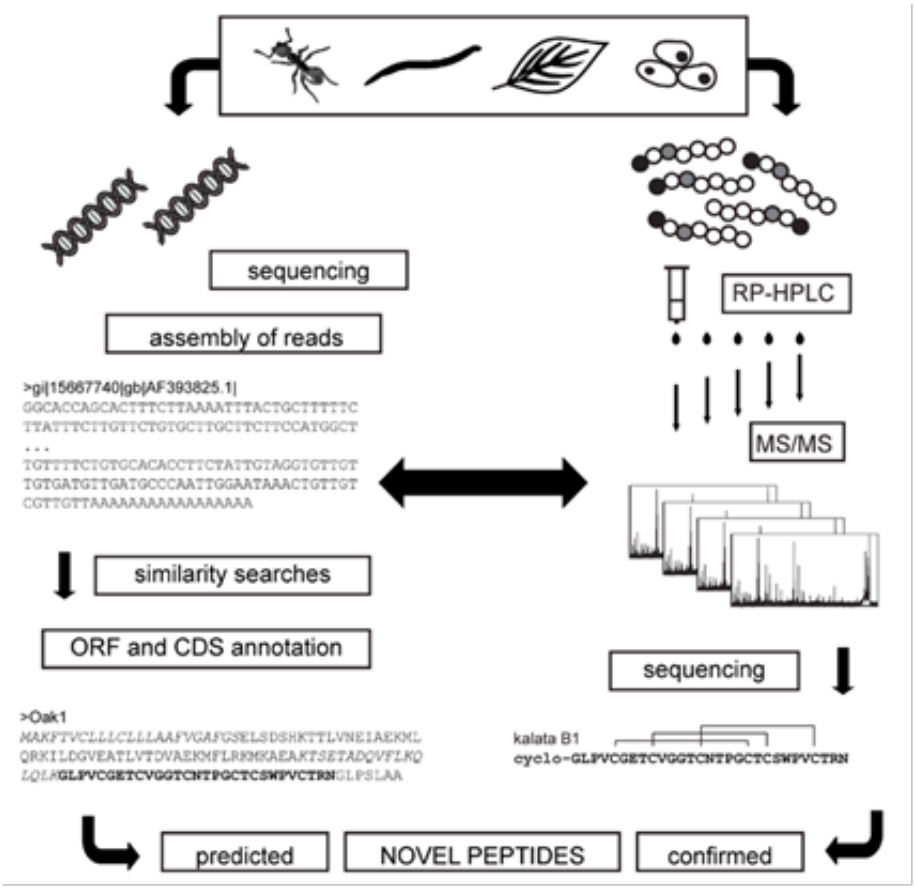


Figure 1.9 Common workflow for the discovery of novel neuropeptides. *In silico* mining (left side flow) predicts the neuropeptides from sequenced data obtained either through 'open access' databases or though sample sequencing. Predicted molecular weight of putative peptides can be matched to homologous peptides of known species. de novo sequencing (right side flow) pools peptide sequencies directly from tandem mass spectrometry (MS/MS) data that rely on initial peptide separation through RP-HLPC. Aligned expressed sequence tags (EST) of peptides are then matched to depositories of homologous species.

(Image credit Koehbach and Jackson, 2015)

1.5.2 Biosynthesis and peptide maturation

The origin of neuropeptides begins by the gene transcription in the nucleus that releases a protein-coding mRNA into the cytoplasm of neurosecretory cell. Once reaching the ribosome, the translation of pre-pro-protein mRNA begins and hydrophobic amino acids, termed as the 'signal sequence' at the N-terminus of pre-pro-protein directs the complex into the rough endoplasmic reticulum (RER) for translation and folding. Inside the lumen

of the RER, a signal peptidase converts the pre-pro-protein to the pro-protein which is further processed to generate smaller peptides. Often, many duplicates of identical or structurally similar peptides (isopeptides) can be found on a single pro-protein (Masler, 1994; Altstein and Nässel, 2011). For instance, *Drosophila* FMRFamide precursor encodes eight closely related propeptides that, although related vary in their functions (Randall S Hewes et al., 1998). Following translocation in the Golgi, pro-peptide/pro-peptides are packed into secretory granules, where they undergo limited cleavage by trypsin-like convertases that cleave at the C-terminus of basic amino acids, followed by carboxypeptidases that remove these basic recognition sequences (Rholam and Fahy, 2009). It should be noted that many insect neuropeptides have blocked termini (e.g. an N-terminal pyroglutamate and an amidated C-terminus) to protect against exo-peptidases. Also, some peptides form disulfide bridges providing additional structural protection against both exo- and endo-peptidase attack (Masler et al., 1991). The depolarization of neuronal axon terminals result in release of mature neuropeptide by exocytosis, whether it is at synaptic region to act in a restricted manner, as it happens in central nervous system, or as a non-targeted secretion via ‘varicosities’ leading to a long-distance effect (Zupanc, 1996; Ribeiro-da-Silva and Hökfelt, 2000). Consequently, neuropeptides have the ability to act equally as neuromodulators or neurohormones, depending on their mode and site of release. For instance, if neuropeptide is released into haemolymph, they can effectively modulate long-distance receptors on peripheral targets in endocrine fashion. Hence, it is anticipated that neuropeptides can be versatile, acting as a synaptically released neuromodulator, a local neurohormone and as circulating hormone, the latter with potency within a nanomolar concentration (Nässel, 2002).

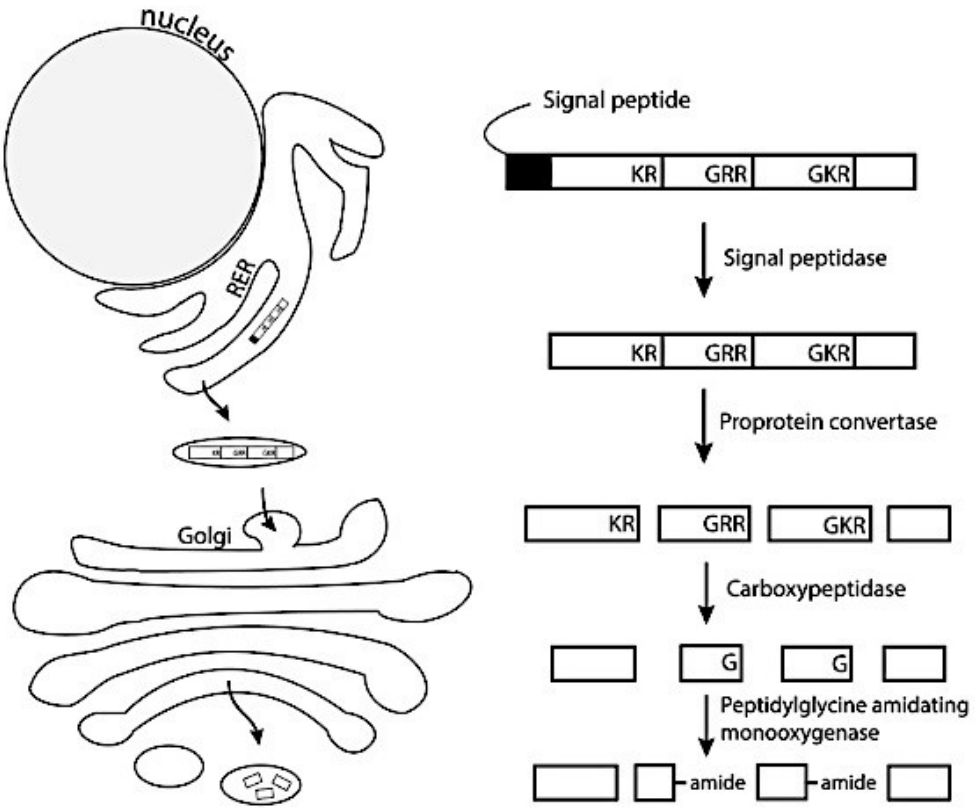


Figure 1.10 Overview of general neuropeptide processing and maturation. All neuropeptides are initially synthesized from corresponding mRNA that is transcribed from the nuclear gene. Following translation by ribosomes, the prepropeptide, upon translocation to Golgi, signal peptidase removes signal peptide (SP) to form prohormone that is later processed by specific proprotein convertases and carboxypeptidases into endogenous peptide. Before being packed into secretory vesicles, many forms of modifications follow; to protect and improve the bioavailability of mature peptide by post-translational modifications in the form of glycosylation, C-terminal amidation, N-terminal pyroglutamate, acetylation, or sulfation.

(Credit: Image taken from De Haes et al., 2015).

1.5.3 Neuropeptide signalling

In order to perform their biological functions neuropeptides and other signalling messengers require selective binding to cell surface receptors that transduce the signal from outside to inside the target cell. For many insect neuropeptides this is achieved through G-protein-coupled receptors (GPCRs) of which around 200 were predicted from the *D. melanogaster* genome(Hauser et al., 2006). So far, over 70 genes have been assigned for Class A (the rhodopsin-like family) receptors, 20 belonging to class B (the secretin-like family), 5 to Class C (the metabotropic glutamate-like family) and equally

5 for Class F (frizzled/smoothened family) based on well conserved structural motifs (Liu et al., 2021).

GPCRs constitute the largest superfamily of signal transducing proteins on the surface of target cells. Structurally, a GPCR receptor is built from a single polypeptide folded into spherical helices across a cell membrane, exposing seven hydrophobic transmembrane segments regions while containing an extracellular amino terminus and an intracellular carboxyl terminus. Each transmembrane region, consisting of 20-30 hydrophobic amino acids, is interconnected with interchanging intracellular and extracellular loops. Those are flanked on each end with N-terminal polypeptide chain located at the extracellular side or with C-terminus tail that resides on the intracellular side as illustrated in Fig. 1.11 (Verlinden et al., 2014). Residue variation within large transmembrane regions and loops positioning open to the extracellular space, form neuropeptide binding site that determine receptor specificity to its ligand and those unique patterns are responsible for exceptionally high affinity of receptor for neuropeptides in nanomolar concentration range (Scherkenbeck and Zdobinsky, 2009; Caers et al., 2012).

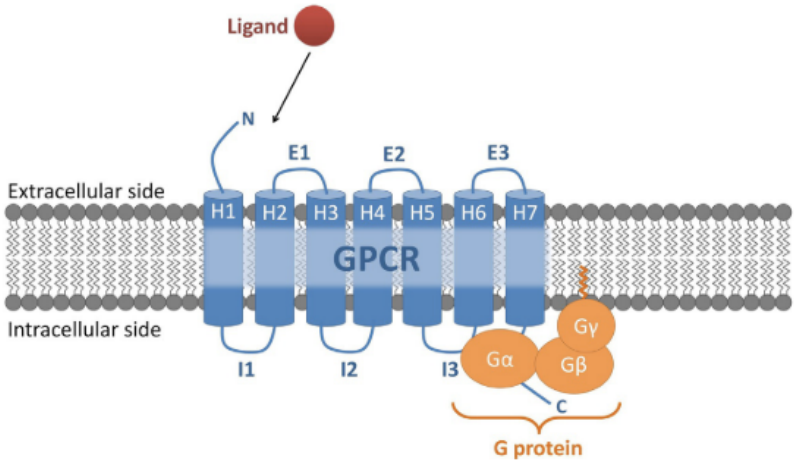
A single receptor can be promiscuous and respond to unrelated peptides as demonstrated by the myoinhibitory peptide (MIP) that activates the sex peptide GPCR (SPR) (Poels et al., 2010). A FMRFamide receptor can be activated not only by FMRFamide but also by other structurally-related peptides with a similar C-terminal sequence, albeit at higher concentrations, such as short neuropeptide F or dromyosuppressin (Egerod et al., 2003). Recently, it was shown how the co-sharing pigment dispersing factor (PDF) receptor with diuretic hormone 31 (DH31) is contributing towards locomotor coordination (Goda et al., 2019), illustrating overall pleiotrophy of neuropeptide signalling pathways across the organism.

1.5.4 Signal transduction, internalisation and desensitisation

The regulation of neuropeptide receptor response is tightly regulated and achieved either two ways. Receptor can either be desensitised in the presence of ligand by undergoing phosphorylation, palmitoylation and internalisation or overall downregulation of receptor expression on the cell surface. Receptor activation depends on imposing conformational changes within molecular switches that, upon binding, allows transmembrane region alteration and hydrogen bond proliferation; for example, FMRFa receptor contains an ionic lock present on TM3 linked to TM6 keeps the receptor in inactivate state while

preventing G protein binding (Rasmussen et al., 2015). Once a ligand binds to its receptor, a conformational change activates heterotrimeric guanine-nucleotide-binding regulatory G proteins. Once activated, G protein associate with guanosine triphosphate (GTP) that function as GTPases, that in turn activate an intracellular hydrolytic cascade initiating downstream signalling. The uncoupled G protein subunits then interact with variety of enzymes (adenylate cystate, kinases or phospholipase C) to generate secondary messengers, particularly by intracellular Ca^{2+} mobilization.

A set of enzymes 'G-protein- coupled receptor kinases, such as protein kinase A (PKA) and protein kinase C (PKC) then begin phosphorylation of the C-terminus of the activated receptor. that leads to the recruitment of β-arrestin. Those regulatory proteins bind with high affinity to kinase-phosphorylated receptor, uncoupling it from G-protein leading to a receptor desensitization. In some cases, arrestins can impose their effect even without receptor phosphorylation (Kelly et al., 2008; Hanlon and Andrew, 2015).



(Image credit Verlinden et al., 2014)

Figure 1.11 Illustration of characteristic organisation of G-protein-coupled receptor. The strict organisation of conserved 7 transmembrane regions (blue) and variable organisation of intra- (I1, I2, I3) and extra-cellular (E1, E2, E3) loops defines a ligand binding site specificity. G-proteins interacting with receptor are heterotrimetic; in inactive state G-protein alpha binds to guanosine diphosphate (GDP) and, as a complex unit, stay attached to the receptor. However, in the presence of signal, conformation change in the receptor leads to the G protein activation and guanosine treiphosphate (GTP) replaces bound GDP attached to the alpha unit. This response leads to the separation of G protein subunits into two; GTP-attached alpha monomer and beta-gamma dimer. This, in turn, dissociate both separate entities from the receptor allowing to interact them with other membrane proteins. G protein activity is functional only during alpha subunit association with GTP. Once GTP is hydrolysed to GDP, it "switch" back into inactive heterodimer, that reconnects back with the inactive receptor (Predescu et al., 2019).

It should be noted that ligand binding capacity of the receptor is also influenced by its neighbouring receptors, other ligands, signalling effectors and enzymes present in proximity, that are fully described in a review by Verlinden et al., (2014). There is another important aspect of neuropeptide signalling regulation that involves promiscuous peptidases, present either in the haemolymph or on the surface of cells that can efficiently terminate peptide signalling by degradation to inactive fragments. For a review of peptidases, their specificity and mode of action see Isaac et al., (2009).
The functionality of neuropeptides in the nervous system is a testament to their versatility and specificity in physiological processes, especially in regulating feeding behavior. This broad overview of neuropeptide functionality sets the step for a more focused examples of individual neuropeptides.

The following subchapter introduces specific examples, such as family of FMRFamide-like petides, known for its role in appetite modulation, or short Neuropeptide F, which is associated with satiety and energy balance. Each of these neuropeptides, along with others like Allatostatins and Kinins, represents a unique piece of the complex puzzle of feeding regulation, offering insights into the intricate interplay between the nervous system and the digestive processes.

1.5.5 FMRFamide-like peptides (FaRPs)

Initially discovered as molluscan peptide nearly 30 years ago, it was shown to be a cardioexcitatory peptide with a potent muscular excitatory activity (Greenberg and Price, 1979). It is now formed as a large superfamily of neuropeptides compromising of diverse set of peptides that share a common C-terminal FMRFamide consensus, that is further divided into major subgroups based on their structures and activities; FMRFamides, Myosuppressins, Sulfakinins and two neuropeptide F(NPF)-like groups (Merte and Nichols, 2002; Haselton et al., 2008). Diversity of receptor binding affinity between each group imply that they are not in fact closely related and are evolutionally distant (Cazzamali and Grimmelikhuijzen, 2002) nevertheless the majority of members share strong myoregulatory effects on visceral muscles of various kinds.

Table 1.1 FMRFamide-like "FaRPs" family of peptides in *D. melanogaster**

Family	Acronym	Peptide sequence
FMRFa	FMRFa-1	SVKQNDFMHFa
	FMRFa-2	DPKQDFMRFa
	FMRFa-3	TPAEDFMRFa
	FMRFa-4	SDNFMRFa
	FMRFa-5	SPKQDFMRFa
	FMRFa-6	PDNFMRFa
	FMRFa-7	SAPQDFVRSa
	FMRFa-8	MDSNFIRFa
Sulfakinin	SK-0	NQKTMSFa
	SK-1	FDDYGHMRFa
	SK-2	GGDDQFDDYGHMRFa
Myosuppressin	MS	TDVDHVFLRFa
Neuropeptide F	NPF	SNSRPPRKNDVNTMADAYKFL-QDLDTYYGDRARVRFa
Short NPF	sNPF-1	AQRSPSLRLRFa
	sNPF-1_{4-11}	SPSLRLRFa
	sNPF-2	WFGDVNQKPIRSPSLRLRFa
	sNPF-2_{12-19}	SPSLRLRFa
	sNPF-3	KPQRLRWa
	sNPF-4	KPMRLRWa

*Nomenclature for neuropeptides present in *D. melanogaster* usually hold a prefix of dro- but for the purpose of clarity it was omitted.

(Adapted from Nässel and Zandawala, 2019)

1.5.6 FMRFamide peptides

FMRFamides are group of abundant peptides exclusively found in dipterans and hemipteran species. In *D. melanogaster* there are 11 copies from which five are distinctive mature peptides, all encoded by a single preprohormone. In contrast to other peptides belonging to FaPRs family, not only do all FMRFamides bind to a single receptor, they are also capable to activate ligand-gated ion channels and thus elicit a rapid response (Cazzamali and Grimmelikhuijzen, 2002; Meeusen et al., 2002). In flies, the action of peptides was implicated in heartbeat, salivary glands secretion and wall muscle

contractions (Nässel, 2002). Immunoreactive material in *R. prolixus* was detected in CNS and associated ganglia (frontal ganglia and hypocerebral ganglion) and the oesophageal nerve from where neuronal processes extend the crop and gut muscles. Indeed, following application of peptide to foregut, led to the increase of spontaneous contractions (Tsang and Orchard, 1991). The feeding regime of this species was also found to correlate with increased amount of circulating peptides in the haemolymph (Elia et al., 1993). The FMRFamide expression pattern using antisera is very similar between dipterans with localisation in the CNS (brain lobes, optic lobes and ventral ganglion), gastrointestinal and reproductive tracts (Nichols, 2006). Detailed distribution map depicting RFamide signal observed within CNS of larvae, pupa and adult is well portrayed in review by Nässel, (2002).

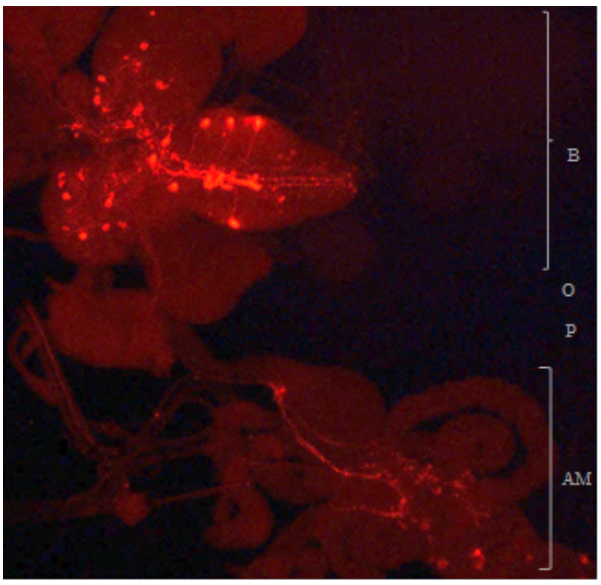


Figure 1.12 Whole mount of third instar larva of *D. melanogaster* depicting RFamide immunoreactivity across the brain, oesophagus, proventriculus and anterior part of midgut. (B, brain; O, oesophagus; P, proventriculus, AM, anterior midgut). Image obtained by the author using immunohistochemical staining method described in Ch 2. Materials and Methods, subchapter 2.2.2.2).

1.5.7 Myosuppressin

One of the most potent anorexic effects exerted on insects was seen when myosuppressin was incorporated into a diet (Schoofs et al., 2017).As the name suggests, it is a strong down regulator of muscular contractions. It was shown to affect the heart, foregut, and hindgut muscles, depending on species. In flies, while myosuppressin itself is not present in the gut, the presence within CNS, stomatogastric system and two cell bodies attached to the rectum and reproductive tract, McCormick and Nichols (1993) label it as a brain-gut peptide. Importantly, inhibitory effects of the peptide delivered to the crop muscles via crop nerve bundles from corpora-cardiaca/corpora allata complex is considered to be a key regulator of the crop, thus having a major effect on feeding regulation (Gough et al., 2017).

1.5.8 Sulfakinins

Since the first discovery of sulfakinins (SK) in cockroach *Leucophae amaderae*, they were implicated in the regulation of the activity of various visceral muscles, but not in every species studied (Duve et al., 1994; East et al., 1997; Wei et al., 2000; Maestro et al., 2001; Downer et al., 2007; Yu et al., 2012). They are structurally related to gastrin/cholecystokinin peptides that regulate food intake in vertebrates (Schoofs et al., 2013). Following immunolocalisation of these peptides in cockroaches (*P. americana* and *B. germanica*) that revealed staining of oesophageal nerves terminating on crop muscles (East et al., 1997), it was shown that SK peptides increase spontaneous contractions of the foregut and hindgut muscles and when injected SK elicits a potent antifeeding response (Maestro et al., 2001) as also observed in locust, *Schistocerca gregaria* (Wei et al., 2000). On the other hand, in tested flies (*Calliphora vomitoria* and *Phormia regina*) SK-like immunoreactivity is restricted to the CNS and exogenous peptide did not interfere with crop muscular contractions, but still managed to elicit a significant reduction in food intake (Duve et al., 1994; Downer et al., 2007). Likewise, the knockdown of SK receptors in kissing bug (*R. prolixus*) mediated an increase in volume of blood taken (Bloom et al., 2019), further supports the role of sulfakinins in feeding and satiety.

1.5.9 Neuropeptide F peptides

NPF peptides belong to the FaPRs family and NPF expression was mapped in *D. melanogaster* using the Gal4-UAS expression system and revealed a signal in both the CNS and midgut enteroendocrine cells of the adult (Veenstra et al., 2008). Based on those findings, the authors concluded that immunohistochemical staining observed in the enteroendocrine cells of the adult midgut using RFamide is most likely due to antibody cross-reactivity towards NPF peptide. *In situ* hybridisation and immunohistochemistry detected the expression of this peptide in the brain and midgut of adult fly (Brown et al., 1999). Functionally, the peptide was linked to the regulation of foraging and feeding, aggression and influencing learning, reproduction and stress responses (Li et al., 2018). Feeding regulation is also under the control of four shorter version of NPF, termed as short neuropeptides F (sNPFs) and encoded on a separate gene. These peptides are also localised in the *Drosophila* CNS, particularly in the brain and hypocerebral ganglion. sNPF-specific antibody also highlighted immunoreactive fibres extending from hypocerebral ganglion towards the anterior midgut area. No evidence was obtained for the presence of sNPF in enteroendocrine cells (Veenstra et al., 2008), however, overexpression of this peptide gene leads to increase in appetite, body size and weight (Lee et al., 2004).

1.5.10 Tachykinins

In *Drosophila* tachykinins exert myostimulatory effect on midgut contractions (Nässel, 1999), as it does on foregut and midgut contractions of locusts and cockroaches (Schoofs et al., 1990; Dick R Nässel, 2002). Both, tachykinin and sNPF encourage feeding behaviour in flies. From the detection of immunostaining in enteroendocrine cells of *L. maderae* midgut, Winther and Nassel proposed a hormonal role for those peptides since the peptide synthesis correlates with satiety in various insects (Winther and Nässel, 2001). Lange (2001) demonstrated the fluctuation of tachykinins in endocrine cells through starvation in midgut of malnourishment locusts (*L. migratoria*).

1.5.11 Proctolin

Proctolin is another example of cardioactive and myostimulating peptide present in motorneurons and CNS in *Drosophila* that affects a broad range of visceral and skeletal muscles in variety of insects (Starratt and Brown, 1975; Holman et al., 1991; Audsley

and Weaver, 2009). Besides of co-localisation in some neuronal bodies with tachykinin-related peptides and GABA co-transmitter effectively regulating rhythm-generating pattern, the presence of this peptide in haemolymph also led to the suggestion of neurohormonal role (Nässel, 2002; Spit et al., 2012).

1.5.12 Allatostatins

As in the case of FaPRs above, peptides are subdivided into families based on their structural characteristics; Alt-A, Alt-B that are more commonly recognised as myoinhibitory peptides (MIPs) and Alt-C. All are implicated in insect response to dietary intake, affecting gut motility in either excitatory or inhibitory way and regulation of enzyme release. Interestingly, as noted by Dick R. Nässel (2002), although all members are present in insects and capable of myotropic activity, this activity is species-specific. For instance, while members of the Allatostatin-A (Alt-A) family affect muscular contractions in cockroaches, they are ineffective in flies. Similarly, peptide secretion is inhibited by Allatostatin-B (Alt-B) members in crickets, but not in cockroaches (Nässel, 2002).

Focusing on Alt-A members, they were found to initiate and inhibit muscular rhythmicity in locust (Zilberstein et al., 2004) but, as already mentioned, while they are not capable of inhibiting muscle contractions in *Drosophila* they act as a potent inhibitors of starvation-induced feeding (Ji Chen et al., 2016) that corresponds to the immunoreactivity observed in the brain, thoracico-abdominal ganglion and enteroendocrine cells in this insect. Alt-B peptides, also known as myoinhibitory peptides or MIPs, were discovered and named according their potent inhibitory activity on visceral muscles of the hindgut and oviduct (Schoofs et al., 1991). While MIPs isolated from moth and crickets have both potent inhibitory action on foregut and hindgut muscles in cockroaches, only the latter resulted in significant feeding when delivered via injection into same species (Aguilar et al., 2004). Whether it is the case in *D. melanogaster* remains to be determined, however, in general terms it is recognised that MIP peptides affect feeding by interfering with the frequency and the amplitude of gut contractions. As with the previous allatostatins, peptides of Ast-C family were localised within the CNS and SNS together within anterior, mid- and posterior enteroendocrine cells of the *D. melanogaster* gut (Lemaitre and Miguel-Aliaga, 2013) where they have potent myoinhibitory activity (Price et al., 2002). In general, spatial localisation of allatostatins and their myotropic effects on muscles of

the foregut strongly suggest a potent regulatory role in food intake by regulating gut peristalsis.

1.5.13 Kinins

Kinins were initially discovered as myostimulatory peptides based on hindgut assay in the cockroach *L. maderae* and thus firstly referred to as myokin (Holman et al., 1986b). The number of peptides vary greatly across species; while eight 'leuco'kinins were identified in cockroach, (*L. maderae*), only a single 'lymno'kinin is present in mollusc, (*Lymnaea stagnalis*), six 'pev'kinins in whiteleg shrimp, (*Penaeus vannamei*) and only one in *D. melanogaster* (Torfs et al., 1999; Nässel and Winther, 2010). Following studies imply their functions in secretion of digestive enzymes, ecdysis, metabolism and locomotion, tracheal air-filling and promoting fluid excretion from Malpighian tubules, depending on species (Coast, 1998; Allan et al., 2005; Yamanaka et al., 2006; Kim et al., 2018; Zandawala et al., 2018). Delivery of 'helico'kinin peptide though microinjection into larvae of tobacco budworm, *Heliothis virescens* prevented weight gain and increased mortality (Seinsche et al., 2000). Kinins gained interest in the research aiming at has shown to possess a strong antifeeding effect when delivered in the form of stable analogue (Lange et al., 2016). In *D. melanogaster*, the expression of peptide is restricted to a small section of the brain and subesophageal ganglion, a region implicated in food choice and feeding behaviour, and thoracico-abdominal ganglion, where it co-localizes with a diuretic hormone to regulate diuresis and food intake (reviewed inNässel, 2021). Interfering with kinin signaling regulates meal size (Al-Anzi et al., 2010). In the blood-feeding *R. prolixus*, a correlation has been observed between the kinin levels in the midgut's endocrine cells and the feeding activity, as reported by Bhatt et al., (2014).

Outside of typical 'brain-gut peptides' mentioned above, several others, such as diuretic hormones (DH), regulator of fluid secretion by Malpighian tubules indirectly contribute to the inhibition of feeding as demonstrated in moth larvae (Keeley et al., 1992; Ma et al., 2000). Additionally, corazonin and adipokinetic hormone (AKH) peptides both, although primarily myotropic peptides, alter metabolism and feeding patterns in flies without being among others identified by peptidomic analysis in fly's enteroendocrine cells (Van Der Horst, 2003; Pauls et al., 2014; Gáliková et al., 2015; Kučerová et al., 2016; Kubrak et al., 2016).

The synchronized signalling pathways of neuropeptides regulating satiety, feeding and digestion are still poorly characterized due to pleiotropy of majority peptides and overall complexity of secondary effects on homeostasis, metabolism and general activity.

After providing a background on Diptera and an extensive introduction that discusses the effects of pesticides, the detailed structure of insect digestive systems, and the function of neuropeptides in insect biology, the discussion now transitions to the specific aims and objectives of this research.

1.6 Aims and Objectives

This research aims to explore the potential of neuropeptide signaling in agricultural pest control, focusing specifically on myosuppressin. Its role in insect homeostasis, development, and behavior is a primary area of study, targeting Dipteran pests for biocontrol strategies. Key objectives include the investigation of myosuppressin in the foreguts of *D. radicum* and *C. capitata*. This involves using mass spectrometry and immunohistochemistry to confirm its identity and distribution. Another objective is to study the myosuppressin G-protein coupled receptors, Dms-R1 and Dms-R2, in the model organism *D. melanogaster*. This research focuses on their role in crop muscle contractions, with knock-out mutants used for validation. The resistance of myosuppressin to crop peptidases in *D. radicum* is also assessed. Benzethonium chloride is evaluated as a potential biostable agonist for disrupting crop function. The effects of native myosuppressin peptide injections on oviposition are explored, suggesting new avenues in myosuppressin signaling. Additionally, a comprehensive neuropeptidomics library from *C. capitata* is compiled for future research.

The study concludes with a discussion on the feasibility and challenges of using peptidergic signaling for pest control. It addresses critical issues like peptide biostability and the need for metabolically stable analogs, contributing to sustainable agricultural practices.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Drosophila melanogaster and *Delia radicum* myosuppressin (TDVDHVFLRFamide), *Ceratitis capitata* myosuppressin (SDVDHVFLRFamide), and truncated short neuropeptide F (sNPF$^{4-11}$, SPSLRLRFamide) were custom synthesized by Biomatik (Cambridge, Ontario, Canada) of molecular biology grade (>90% purity by HPLC). For *ex vivo* bioassay experiments, the synthetic peptide was dissolved in fly saline (for composition see Table 2.1) and stock solutions of 10 mM were stored at -20 ºC. 7-Amino-4-methylcoumarin (AMC), Dulbecco's Phosphate Buffered Saline (PBS) and Benzethonium chloride (Bztc) were purchased from Sigma-Aldrich Company Ltd., (Gillingham, U.K.). AMC-RPPGFSAFK(DNP) and L-Threonine 7-amido-4-methylcoumarin (Thr-AMC) were purchased from Enzo Life Sciences Ltd., (Exeter, U.K.) and Insight Biotechnology Ltd., (Wembley, U.K.) respectively. Solvents required for samples analysis through high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) were purchased from Sigma-Aldrich (Dorset, UK). Rhodamine phalloidin was obtained from ThermoFisher Scientific (Loughborough, U.K.). Dyes to visualise and measure feeding, and excreta were bromophenol blue from Sigma-Aldrich (Dorset, U.K.) and Natural Blue Food Colouring, Ocado bought from a local supermarket. pH indicators were purchased from Sigma-Aldrich (Dorset, U.K.). The solid poly(methyl methacrylate) (PMMA) particles labelled with Sudan III and the pH-responsive poly(2-vinylpyridine) (P2VP) microcapsules containing FITC-dextran were synthesized by Dr Areej Al-Khalaf and Calum Ferguson, School of Process and Chemical Engineering, University of Leeds (Ferguson et al., 2018).

2.1.2 Insect salines

Drosophila saline was prepared from research grade chemicals listed in Table 2.1 and adjusted to pH 7.2 by the addition of NaOH as required. Recipe for Phormia saline (Table 2.2) was followed as described by Chen and Friedman (1975).

Table 2.1 Drosophila saline composition

Stock solution	Final concentration (mM)
2M NaCl (58 g/500 ml)	140
1M KCl (3.7 g/50 ml)	5
1M $MgCl_2$ (10 g/50 ml)	1
0.5M $CaCl_2$ (2.7 g/50 ml)	5
0.5M $NaHCO_3$ (2.1 g/50 ml)	4
HEPES (free acid)	5
H_2O	up to 1 L

*Adjusted with the addition of NaOH and HCl to reach pH 7.2

Table 2.2 Phormia saline composition

Solution A	
Stock solution	Final concentration (mM)
NaCl (0.993 g/l)	17
KCl (0.417 g/l)	5.6
MgCl2 (100 ml of 0.01 M)	1
CaCl2 (2.4 ml of 1M)	2.4
Glutamic acid (14.27 g/l)	97
Glutamine (6.43 g/l)	44
Proline (11.17 g/l)	97
Alanine (4.27 g/l)	48
Glycine (1.95 g/l)	26
NaOH (3.91 g/l)	97.8
HEPES (2.86 g/l)	12

Solution B	
Stock solution	Final concentration (mM)
Histidine- HCl	0.958 g
NaOH (1N, 4g/100 ml)	4.26 ml
add H2O to 100 ml	

Complete solution
50 ml solution A + 7 ml solution B
pH=7.0

* Final saline solution was adjusted to pH 7.0 with the addition of NaOH.

2.2 General Methods

2.2.1 Insect maintenance

2.2.1.1 Fruit fly, Drosophila melanogaster

All stocks listed in Table 3.2 were kept in the incubator (MIR-253, Sanyo, UK) at 26 °C with 12/12 light-dark photoperiod cycle and 65% R.H. on a standard diet (7.38% (w/v) fine oatmeal, 0.84% (w/v) fly agar, 0.84% (w/v) yeast, 5% (w/v) molasses, 3.4% (w/v) methyl 4-hydroxy benzoate), unless mentioned otherwise.

To ensure age uniformity, eggs were collected synchronously within a 2-4 hr window, allowing for controlled tracking of developmental stages. Standardised nutritional conditions were maintained, with consistent food composition and regulated larval density to minimize developmental variability. Pupae were segregated based on sex-specific traits prior to emergence, and their sex was further confirmed under a microscope as adults emerged, ensuring accurate and reliable collection of virgin flies for our experiments. Careful monitoring of pre-emergence conditions such as larval food quality and density was conducted to ensure optimal development and health of the flies, critical aspect for the reliability and consistency of the experimental methods listed below.

2.2.1.2 Cabbage root fly, *Delia radicum*

Insects were reared at 20 °C, a photoperiod of 16L: 8D and 65 % R.H and adults were maintained on a diet consisting of dry yeast powder, sugar, dried skimmed milk powder as previously described (Finch and Coaker, 1969).

2.2.1.3 Medfly, *Ceratitis capitata* (Wiedemann)

Insects were reared in cages (BugDorm-1, NHBS Ltd.) under operating conditions of 21 °C, 65 % R.H., 16:8 light-dark regime in quarantine facilities (FERA Science Ltd., York, UK). Larval rearing diet consisted of bran (24.2%), yeast (8.1%), sugar (16.2%), sodium benzoate (0.5%), citric acid (1.8%), hot water (49.2%). Adults were provided with sugar and enzymatic yeast hydrolysate (3:1) and water *ad libitum*.

2.2.2 Staining of the crop and crop nerve bundle of adult insects

2.2.2.1 Phalloidin staining

Phalloidin staining of actin filaments was used to observe the crop's muscle structure. Dissected tissues were fixed in 4% (wt/v) paraformaldehyde for 20 min at room temperature. After washing five times (10 min per wash) in PBS, permeabilized with 0.3% Triton X-100 in PBS (PBST) for 1 hr and washed again with PBS, as above. Rhodamine phalloidin solution (1:100 in PBST) was added to the samples and incubated for 20 minutes at room temperature before being extensively washed in PBS five times (10 min per wash) and mounted in DAPI-Fluoromount-G mounting media (2BScientific, UK). Slides were viewed using a confocal Zeiss LSM 700 inverted microscope.

2.2.2.2 Immunohistochemistry (IHC)

Third instar larvae and adult flies were anaesthetized with CO_2 and chilled on ice before being dissected in the appropriate saline (2.1.2, Table 1). Samples were fixed in 4% (wt/v) paraformaldehyde in PBS, overnight in a cold room. After five 10 min washes in 0.3% (v/v) Triton X-100 in PBS (PBST), samples were placed in a blocking solution of 10% (v/v) goat serum in PBST (GPBST) for 1 hr at room temperature. Samples were incubated with a primary antibody in 5% GPBST for 48 hr at 4 ^{o}C. After five washes in PBST, samples were further incubated with a secondary antibody (Table 2.3), diluted 1 in 500, with 10 % GPBST. After washing the tissues with PBST (5 10 min washes), samples were mounted in DAPI Fluoromount-G (2BScientific, Upper Heyford, U.K.) and covered with a cover slip, which was sealed with clear nail vanish. Slides were stored in the dark at 4 °C until viewed using either a Zeiss Axiophan fluorescent microscope, Leica M165 FC apochromatic stereo microscope with CoolLED's pE-300^{white} illumination or a confocal LSM700 inverted microscope.

IHC controls included omitting primary antibody from the procedure above and, in the case of the RFamide primary antibody, some tissues were incubated with antiserum that had been blocked by preincubation with peptide (1 mg/ml overnight at 4 ^{o}C). Antibodies used are listed in Table 2.3.

Table 2.3 Antibodies used in double staining IHC against GFP and RFa. Prior to use, both primary and secondary antibodies were dissolved in blocking solution (GTPBS).

Target	Antibody	Specificity	Host	Manufacturer	Concentration
Myosuppressin	1^0 GFP	IgG2a, monoclonal 3E6 (anti-GFP, MaB 3e6)	mouse	Life Technologies, Ltd., UK	1:1000
	1^0 RFamide	anti-H-Phe-Met-Arg-Phe-NH_2, polyclonal	rabbit	Peninsula, California	1:1000
	2^0 Alexa Fluor 488	goat anti-mouse IgG (H+L), 2 mg/mL	goat	Life Technologies Ltd., UK	1:500
	2^0 Alexa Fluor 594	goat anti-rabbit IgG [H+L], 2mg/ml	goat	Life Technologies Ltd., UK	1:500
Muscle assembly/ Myosin heavy chain gene	1^0 GFP	IgG2a, monoclonal 3E6 (anti-GFP, MaB 3e6)	mouse	Life Technologies, Ltd., UK	1:1000
	2^0 Alexa Fluor 488	goat anti-mouse IgG (H+L), 2 mg/mL	goat	Life Technologies Ltd., UK	1:500

2.2.3 *Ex vivo* crop bioassay

Three-day old adult females were collected from insect stock cultures (*D. melanogaster*, *D. radicum* and *C. capitata*) and deprived of food and water for 24 hr prior to use to ensure that the crop was devoid of food. The procedure described by Haselton et al. (2004) was followed. In brief, flies were separated into individual containers and allowed to acclimatise for 30 min. From a stock solution prepared by dissolving 28 micrograms of Natural Blue Food Colouring (from Ocado) into 1 mL of 1M sucrose to achieve a 0.5% concentration, a 4 µL droplet was carefully dispensed for feeding the flies. This method ensured consistent dye exposure and sucrose concentration in each sample.

After 10 min feeding, flies were anesthetized with CO_2 and the crop exposed under a drop of physiological saline (*Drosophila* saline for *D. melanogaster* and *C. capitata*; *Phormia* saline for *D. radicum*). The crop duct was cut from the proventriculus (cardia) and the crop transferred immediately into 40 µl of saline. After 1 min, the crop was viewed using a stereo microscope and muscle contractions were recorded to determine the basal rate. Saline was replaced by test solutions containing different concentrations of either myosuppressin peptide or benzethonium chloride (*D. melanogaster*, Dms 10^{-4} M – 10^{-10} M, Bztc 10^{-4} M – 10^{-6} M; *D. radicum*, Dms 10^{-4} M – 10^{-11} M, Bztc 10^{-4} M – 10^{-6} M; *C. capitata*, Ms 10^{-5} M – 10^{-12} M) and the rates of contractions were measured visually for a further 1 min using the 'two-pipette transfer system' which limits disturbance to the crop tissue (Stoffolano et al., 2013). After a 1 min adjustment period, contractions were counted for the following minute and compared to the basal contraction rate for each tissue. Test solutions were washed out with physiological saline to observe the recovery of muscle activity. A further procedural control was performed where saline was substituted with carrier saline only. Each crop was used only once. Dose-response plots were generated and analysed using Prism (version.7, Prism Software Corporation, U.S.A.) Graphs were generated using AviLine software (http://biolpc22.york.ac.uk/avianal/avi_line/) as described by Norville et al., (2010). Individual crop movements were video recorded using a Leica EZ4 W stereomicroscope with an integrated 5-megapixel camera and analysed using ImageJ and the AviLine macro set to monitor pixel changes across a line set in each frame (640 x 480 pixels, at 5 frames/sec). The mean squared differences between pixel intensity of crop muscle and background during contraction at 200 msec intervals were saved in a Microsoft® Excel (Office 365, Version 16.22) format. The output of intervals and mean of peaks height resulted in amplitude of the crop contractions shown as graph data in Figure 4.5, Chapter 4.

2.2.4 Direct tissue profiling of the crop nerve bundle by MALDI-TOF spectrometry

The analysis of the crop nerve bundle dissected from adult *D. melanogaster, D. radicum* and *C. capitata* flies followed the method described in Bell et al., (2018). Nerve bundles were directly transferred on to a MALDI-TOF MS plate into 1 µl of HPLC-grade water. Blotting with filter paper removed excess water and 0.5 µl matrix solution (α-Cyano-4-hydroxycinnammic acid (HCCA), Sigma-Aldrich; 10 mg/ml in 70% acetonitrile 0.1%

trifluoroacetic acid (TFA)) was added and allowed to dry at room temperature. Samples were analysed using a Voyager DE STR MALDI TOF MS (Applied Biosystems, Warrington, UK). Settings for laser intensity and the number of sub-spectra were adjusted to individual sample. The measured monoisotopic masses ($[M+H]^+$) were compared to the monoisotopic masses of reference peptides calculated using the Applied Biosystems Data Explorer software. A calibration of the Voyager was performed with an external calibration mixture containing des-Arg-bradykinin, angiotensin 1, Glu-fibrinopeptide B and neurotensin (Applied Biosystems) or angiotensin I, angiotensin II, substance P, bombesin, ACTH clip 1-17, ACTH clip 18-39, and somatostatin 28 (Bruker Daltronic) (Audsley et al., 2015).

2.3 Chapter-specific methods

2.3.1 Chapter 3. Myosuppressin signalling in the crop of *Drosophila melanogaster*

General methods relating to insect maintenance (subsection 2.2.1.2), staining of the crop and crop nerve bundle (subsection 2.2.2), Phalloidin staining (2.2.2.1), IHC (2.2.2.2), Crop bioassay (2.2.3) MALDI TOF MS (2.3.4) are all described in this Chapter 2. Materials and Methods, subsection 2.2 General Methods.

2.3.1.1 Fly strains

Experimental flies listed in Table 2.4 were maintained as described in the section of 2.2.1 Insect maintenance, subsection 2.2.1.1 Fruit fly, *Drosophila melanogaster.*

Table 2.4 Experimental *D. melanogaster* strains used in this study

Purpose	Fly strain	Resources	References
Control	wild-type Canton-S (CS)	Department of Genetics, University of Cambridge, UK	http://flybase.org/reports/FBsn0000274.html
	w^{1118}		http://flybase.org/reports/FBal0018186.html
Muscle-expressing GFP	$Mhc^{Wee\text{-}P26}$	A kind gift from Dr. C. J. H. Elliot, University of York	https://flybase.org/reports/FBgn0264695.html
GAL4 lines	pJFRC 81-10x UAS-IVS-Syn21-GFP-P10 (super high expression cytoplasmic GFP)	Bloomington Drosophila Stock Centre	Pfeiffer *et al.*, 2012 BDSC http://bdsc.indiana.edu
	Dms-Gal4 (39278, P[GMR61H01-GAL4]attP2)	Bloomington Drosophila Stock Centre	BDSC http://bdsc.indiana.edu
CRISPR knock-out null mutants for *DmsR-1*^{01} & *DmsR-2*^{01}	Dms-Receptor 1, *DmsR-1*^{01} [dmsr1 02/TM6BTb1 (♯1 by CRISPR) ID 2005; YJK_Vie_1980.05Oct15]	A kind gift received from Prof. Young-Joon Kim, School of Life Sciences, Gwangju Institute of Science and Technology (GIST), Gwangju, South Korea	
	Dms-Receptor 2, *DmsR-2*^{01} [dmsr2 02/TM6BTb1 (♯1 by CRISPR) ID 2009; YJK_Vie_1984; 05Oct15]		

2.3.1.2 GFP-labelling of the muscle fibres of the crop using *MhcWee-P26*

D. melanogaster ($Mhc^{Wee\text{-}P26}$), containing a small P element insert driving the expression of green fluorescent protein (GFP) in myosin heavy chain in muscle filaments, was kindly provided by Dr. C. J. H. Elliot, University of York. Flies were reared in a 26 °C incubator (MIR-253, Sanyo, UK) and maintained on diet as described in subsection 2.2.1.1 GFP fluorescence was observed and recorded using a Leica M165 FC apochromatic stereomicroscope. Tissues were also stained using anti-GFP antibodies as described in 3.2.3 (see below).

2.3.1.3 The UAS/Gal4 binary expression system for reporting *Dms* expression

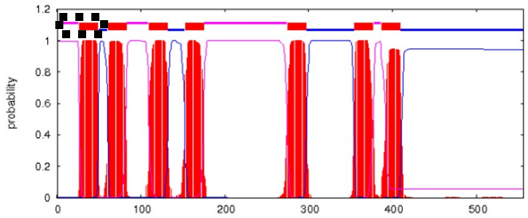
The UAS/Gal4 bipartite expression system was used to investigate the expression of *Dms* (CG6440) in the *D. melanogaster* crop and associated nerves. Pupae containing UAS regulatory sequence pJFRC 81-10x UAS-IVS-Syn21-GFP-P10 (super high expression cytoplasmic GFP) were collected and sex segregated. Virgin females were crossed with males carrying *Dms-Gal4* (39278, P[GMR61H01-GAL4]attP2), from The Bloomington Drosophila Stock Center (BDSC), Indiana University Bloomington, Indiana, United States. All flies were reared in 26 °C incubator (MIR-253, Sanyo, UK) and kept on standard fly diet as above. Tissues expressing *GFP* were stained with an anti-GFP antibody as described in the General methods section (2.2.1.1) to provide samples for confocal microscopy. Parental strains were screened to eliminate any background activity.

2.3.1.4 *In vivo* functional analysis of myosuppressin receptors

To determine the functional role of myosuppressin receptors, knockout mutant flies (*DmsR-1*^{01} and *DmsR-2*^{01}) carrying deletions were generated in the w^{1118} genetic background using CRISPR/Cas9 methodology by Ivana Daubnerova and Young-Joon Kim at Gwangju Institute of Science and Technology, Gwangju, Republic of Korea (Fig. 2.1). While the w^{1118} strain as background strain is readily used as a marker, mutation in white gene has also other genetic characteristics (please refer to 'Phenotypic Data for Allele': Dmel/w^{1118} http://flybase.org/reports/FBal0018186.html). To mitigate the potential influence of background traits associated with the w^{1118} strain, we incorporated a secondary control utilizing a wild-type Canton-S (CS) strain as a comparative baseline.

The deletion of 1040 bp and 3104 bp in *DmsR-1*^{01} and *DmsR-2*^{01} correspond to 22 amino acids, including start codon methionine and the first extracellular domain of Dms-R1 (Fig. 2.1 A) and 164 amino acids spanning four of the seven transmembrane helices of Dms-R2 (Fig. 2.1 B). *DmsR-2*^{01} flies were maintained with the TM6B balancer chromosome that carries the Tb1 dominant mutation, which results in squat larvae and pupae. DmsR-2-/ DmsR-2- displayed the non-tubby phenotype. *DmsR-1*^{01} flies were maintained as DmsR1-/DmsR-1- stocks. Validation of knock-out receptor mutants was confirmed by agarose gel electrophoresis of PCR products of decreased size, produced by primers flanking the deleted region in *DmsR-1*^{01} (Fig. 2.2 A, from 1595bp to 560bp) and for *DmsR-2*^{01} (Fig. 2.2 B, from 3874bp to 772bp). As a control, a secondary line of *DmsR-1*^{01} and *DmsR-2*^{01} mutants with deletion products of different size were used corresponding to deletion products of 762bp and 1091bp, respectively. Deletion validations were kindly provided on request by Prof. Young-Joon Kim, School of Life Sciences, Gwangju Institute of Science and Technology, Republic of Korea.

A



B

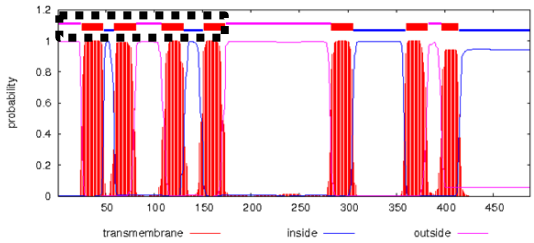


Figure 2.1 Prediction of the transmembrane domains and deleted protein regions for DmsR-1 and DmsR-2 in *D. melanogaster*.
The TMHMM Expasy software (www.cbs.dtu.dk/services/TMHMM-2.0) predicted 7 transmembrane helices based on (A) DmsR-1 receptor sequence highlighting deletion in first transmembrane protein region and (B) DmsR-2 receptor sequence indicating four affected transmembrane regions. Red color represents transmembrane region, blue lines intracellular segments while pink lines mark predicted extracellular sections. Black dash squares mark deleted protein regions generated by CRIPR/Cas9 method.

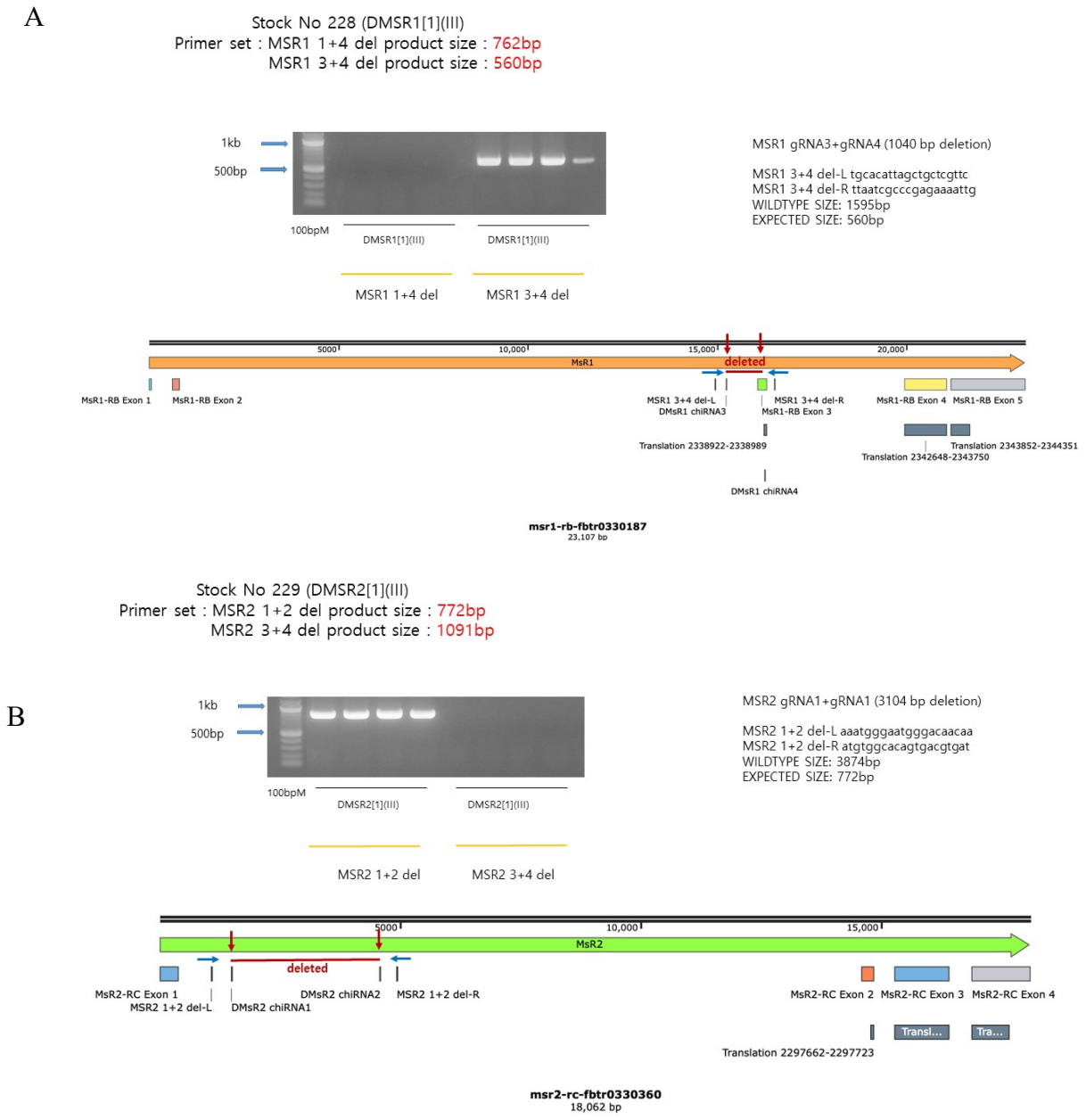


Figure 2.2 Confirmation of CRISPR/Cas9-generated KO mutants targeting *DmsR-1* (CG8985) and *DmsR-2* (CG13803) in *D. melanogaster* as a result of 1040 bp and 3104 bp deletions, respectively. Agarose gel electrophoresis of purified PCR products of two mutant lines per receptor A, *DmsR-1* No. 228 (DMSR1[1](III); and B, *DmsR-2* No. 229 (DMSR2[1](III) were provided by Korean Drosophila Stock Centre, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea. Since all experimental lines were kept as homozygous stock the confirmation of deletion was indeed observed as a single band. Figures courtesy of Prof. Young-Joon Kim, School of Life Sciences, Gwangju Institute of Science and Technology, Republic of Korea and used with permission).

Continued on following page

Figure 2.2 (continued from previous page)

A. The primer set for homozygous *DmsR-1*^{01} experimental stock flanking 560bp deletion product confirmed 1040bp deletion (MSR1 3+4 del). Primers designed against 833bp deletion confirmed the primer specificity by lacking band corresponding to 762bp deletion product size (MSR1 1+4 del).
B. The primer set for homozygous *DmsR-2*^{01} null mutants confirmed deletion of 3104bp producing a single band corresponding to expected deletion product of 772bp (MSR2 1+2 del).

2.3.1.5 *Drosophila* excretion assay

Five-day old *w*^{1118}, CS and *DmsR-1*^{01} flies were starved for 4 hr prior to feeding for 24 hr on either 5 mM Bztc in agar (2% wt/v) containing sucrose (5%, wt/v), bromophenol blue (0.5%, wt/v) or the same recipe without Bztc. Flies were then anaesthetised with CO_2, and five individuals transferred into a clean glass tube. Openings were covered with Parafilm punctured in several places. Through the opening, 10 µl of sucrose diet was pipetted on to the side of the tube. After 24 hr, flies were frozen for 3 hr before removal. Each tube was washed with 300 µl of distilled water using a vortex mixer and visually inspected to ensure that all excreta was dissolved. Samples of 200 µl were then transferred into the well of a 96-well plate and the absorbance measured at 595 nm (Spectramax 340PC Microplate Reader, Molecular Devices).

2.3.1.6 Feeding assay

Five-day old CS, *w*^{1118} and *DmsR-1*^{01} flies (Al-Anzi et al., 2010) were sex-separated and deprived of food overnight in an incubator (26°C, 12:12 hr light: dark cycle, 65% relative humidity) and only provided with moist tissue paper as a source of water. Prior to taking images, flies were provided with a mixture of sucrose (2 ml, 5% wt/v) containing Dr. Oetker food color dye (1.5 mg) in form of a 20 µl drop, and allowed to feed *ad libitum* for 60 min. At the same time, 10 starved females from each group were separated and allowed to feed for 10 min as described above but were dissected in fly saline to visualize food in the crop, midgut and hindgut. Images were captured using a stereomicroscope (Leica EZ4 W) with an integrated 5-megapixel camera.

2.3.2.7 Stress assay to measure longevity under starvation conditions

Adult (1-2 days old post-eclosion) flies (CS, *w*^{1118} and *DmsR-1*^{01}) were separated by sex and each individual was placed in a glass tube (100 x16 mm) that was either empty or contained agar (2%, wt/v) as a source of water. The open end of the tube was secured with a cotton wool plug and the tube was inserted into an activity monitor (DAM16 Locomotor Activity Monitor, Trikinetics Inc., USA) that was kept at 26°C, 12 hr:12 hr

light: dark cycle in an incubator (MIR-253, Sanyo, UK) set for 65% humidity. Fly movement was monitored by recording every 5 min the number of infrared beam breaks generated as the fly moved up and down the tube. The data collected was processed using DAMFileScan application (Trikinetics Inc.) and Excel (Microsoft Office) (Woods et al., 2014).

2.3.1.8 Statistical analysis

All collected data, where appropriate, were analyzed by GraphPad Prism version 6 software (San Diego, USA) for statistical analysis and to generate graphical output. A two-way ANOVA was employed to analyze both longevity and crop contractions recovery rate, whereas a one-way ANOVA was utilized for the excretion assay. All initial results were subsequently subjected to post-hoc Tukey's multiple comparison tests to further elucidate differences between groups.

2.3.2 Chapter 4. Peptidergic control of the crop of the cabbage root fly, *Delia radicum*: a role for myosuppressin

2.3.2.1 Survival and food intake assays

Adult females (3–5 days post eclosion) were transferred to 25 × 95 mm vials containing either 6 ml of 5% sucrose (wt/vol) and 2% agar (wt/vol) (control diet) or the same sucrose/agar mixture containing 5 mM Bztc (treatment diet). For both control and treatment groups, the diet was replenished weekly until all flies were dead. For measuring food intake, 0.5% bromophenol blue (wt/vol, Sigma-Aldrich) was included in both the control and the treatment (5 mM Bztc) sucrose/agar diet. Flies were allowed to feed for 24 h before pairs of females were transferred to 6 mm diameter glass tubes and fed from a drop (10 µl) of 5% (wt/vol) sucrose solution without added dye. After 24 h, the flies were removed, and the empty tubes were washed with 300 µl of distilled water. Pooled washes from three tubes, containing excreta from six flies, were measured at the absorbance of 595 nm wavelength using a SpectraMax 340PC Microplate Reader Spectrophotometer (n = 6).

2.3.2.2 Injection of adult *D. radicum* with myosuppressin

Females (2 days post-eclosion) were anaesthetized under CO_2 and injected with either 1 µl of PBS (controls) or 1 µl PBS containing 6.4 µg of myosuppressin. Flies were monitored twice daily until all flies had died.

2.3.2.3 Metabolism of myosuppressin by crop enzymes

Twenty crops were dissected under Phormia saline and disrupted using a glass homogenizer (0.1 ml glass Wheaton Micro Tissue Grinder, Fisher Scientific) in 100 µl of 0.1 M HEPES buffer (pH 7.5, 10 µM Zn) with 20 upward down strokes. The homogenate was stored in aliquots at 4 °C until required. To study the degradation of myosuppressin, 10 µl of the crop homogenate diluted 5-fold was added to 200 µl of 100 µM myosuppressin in 0.1 M HEPES buffer, pH 7.5 and incubated at 24 °C. At different time points 20 µl aliquots were removed and added to 5 µl 8% (v/v) TFA to terminate enzyme activity. Each reaction was performed in quadruplicate. Acidified samples were centrifuged (4 °C, 12,000g for 20 min) and the supernatant was diluted 10-fold with 0.1% TFA prior to reversed-phase high-performance liquid chromatography (Beckman gold chromatography system, Beckman Coulter, U.K. Ltd) using a 150 × 4.6 mm Kinetex reverse phase column (Phenomenex, Macclesfield, U.K.), eluted with a 10–60% acetonitrile 0.1% TFA gradient at a flow rate at 1 ml/min over 25 min. HPLC fractions (1 ml) of were collected and concentrated using a Savant Speed Vac concentrator (Thermo Electron, U.K.) to less than 10 µl. The mass of HPLC-purified metabolic breakdown products was determined by MALDI-TOF mass spectrometry using a Voyager DE STR MALDI TOF mass spectrometer. A single 0.5 µl droplet of sample from HPLC fractions was mixed with 0.5 µl of matrix (HCCA) and spotted onto a MALDI-TOF plate. The collected mass spectra fragmentation patterns were compared with those generated by Protein Prospector software (University of California, U.S.A.). The UV (214 nm) peak area (uVmin) of myosuppressin in samples were measured and the reduction in peak area after incubation with crop homogenate at different time periods was used to determine the half-life ($t_{1/2}$) of myosuppressin. Under the separation conditions described above, myosuppressin eluted at 9.6 min.

2.3.2.4 Detection of membrane and soluble crop peptidases

To prepare a high-speed membrane and supernatant preparation, 15 crops were homogenised in 0.5 ml of PBS,) using a glass homogeniser (Jencons, East Grinstead, U.K.) and 20 up and down strokes of the pestle. The resulting homogenate was centrifuged at 55,000g for 1 hr at 4 °C using a Beckman Optima™ MAX bench-top ultracentrifuge and TLA110 rotor (Beckman Instruments Inc, Palo Alto, Ca, U.S.A.). The pellet was re-suspended in 0.5 ml of PBS and both the pellet and supernatant were stored frozen until required. Endopeptidase assays were conducted by measuring the initial rate of increase in fluorescence from cleavage of the quenched substrate 1.6 µM AMC-

RPPGFSAFK(DNP) by 10 µl of enzyme in 100 µl of MES buffer, pH 6.5 in a 96-well black plastic plate (Corning Life Sciences, High Wycombe, U.K.) using a FLUOstar Omega (BMG LABTECH GmbH, Offenburg, Germany) with the excitation λ set at 330 nm and emission λ set at 410 nm. The same assay conditions were used for detecting aminopeptidase activity using 1.6 µM Thr-AMC, except that the initial rate of increase in fluorescence was determined with the excitation λ set at 355 nm and emission λ set at 460 nm. The amount released was calculated from a standard curve of AMC. Activities for both enzymes are expressed as pmol of substrate cleaved/h/crop equivalent.

2.3.2.5 Statistical analysis

All graphs and statistical analyses were performed using GraphPad Prism 7 for Windows. Survival curves were compared by the Kaplan-Meier log-rank survival analysis for each treatment group.

2.3.3 Chapter 5. Feeding dipteran pests with pH-responsive microcapsules

2.3.3.1 Feeding dipteran pests *C. capitata* and *D. radicum* with pH indicator dyes and pH-responsive microcapsules

To encourage feeding, female flies were starved overnight. The feeding solutions were prepared as phenol red 0.4% wt/vol and bromophenol blue 0.5% wt/vol. Those were then mixed with a 10% sucrose solution and presented to the flies as the sole food and water source for 1 hr. Flies were then anesthetized using carbon dioxide (CO_2) and following dissection, pictures were taken using a Leica stereomicroscope fitted with a Q-imaging camera.

Prior to feeding, nanoparticles (PMMA, P2VP 30% wt/vol) were divided into aliquots of 1ml, spun and excess liquid discarded. Samples were washed twice in 500 µl of 10% ethanol solution followed by wash of dH_20 only. Excess liquid was discarded, and pellets were resuspended in 10% (w/v) sucrose and kept at 4 °C. Overnight starved adults of *C. capitata* and *D. radicum* of both sexes were allowed to feed *ad libitum* while kept individually in petri dishes for 24 hr and were immediately dissected afterwards. Samples were analysed under a Leica M165 FC apochromatic stereo microscope.

2.3.4 Chapter 6. Neuropeptides of *Ceratitis capitata:* the neuropeptidome and the role of Myosuppressin

2.3.4.1 Chemicals

Medfly myosuppressin (SDVDHVFLRFamide) was synthesised by Biomatik, Canada (>95% purity). For *ex vivo* bioassay experiments, the synthetic peptide was dissolved in *Drosophila* saline (for composition see current Chapter 2, Method 2.1.2, Table 2.1) and stock solutions of 10 mM were stored at -20 ºC. Chemicals used are listed in Chapter 2, 2.1.1 Chemicals.

2.3.4.2 Fly culture

Refer to Chapter 2.2 General Methods, subsection 2.2.1.3 Medfly, *Ceratitis capitata*

2.3.4.3 Staining of the crop muscle and nerve

Refer to Chapter 2.2, subsection 2.2.2 Staining of the crop and crop nerve bundle of adult insects

2.3.4.4 Phalloidin staining

Refer to Chapter 2.2, subsection 2.2.2 Staining of the crop and crop nerve bundle of adult insects, 2.2.2.1 Phalloidin staining

2.3.4.5 Immunohistochemistry

The immunohistochemical protocol using the RFamide antibody is described in Chapter 2.2, subsection 2.2.2.2

2.3.4.6 *Ex vivo* assay of inhibitory activity of myosuppressin on the crop

Refer to Chapter 2.2 Materials and Methods, subsection 2.2.3 *Ex vivo* bioassay

2.3.4.7 Effect of injecting adults with myosuppressin

2.3.4.7.1 Adult injections

Adults at 5-7 days old (50 females and 50 males) were chilled on ice prior to the administration of 0.5 µl of 10 µM or 10 mM myosuppressin using a Hamilton syringe, effectively administering 6.25 ng and 6.26 µg of peptide, respectively. The injection was aimed towards either the softer cuticle under the wing or under the scutum of the dorsal thorax. Fly saline (described in Chapter 2, Method 2.3.1, Table 2.1) was injected into a separate batch of flies to provide control data. Every individual was checked for alertness and normal locomotor behaviour before transferring to the rearing cage. Cages were set

either for control or test flies, where both sexes were kept together. Flies were provided with a standard diet of sucrose, yeast and water *ad libitum* and survival was monitored over a 4-week period.

2.3.4.7.2 Excreta assay

Forty-five 3-5 day old females were injected with 0.5 µl solutions as described above (fly saline for control insects, and either 6.25 ng or 6.26 µg medfly myosuppressin peptide). All flies were alert and responded to touch before being placed into a glass tube covered with Parafilm (Sigma-Aldrich, Dorset, U.K.) that was punctured to provide air and an opening for a liquid diet. This was repeated for four replicates comprising 15 adults per treatment group. For 48 hr, adults received daily 30 µl of solution containing 1M sucrose and blue dye (Natural Blue Food Colouring, Ocado) per tube.

2.3.4.7.3 Statistical analysis

The effect of medfly myosuppressin on the crop peristalsis was analysed by non-linear regression analysis using Prism version 6.0e (GraphPad software, California USA). Mortality of individuals in control and test groups injected with myosuppressin across the period of 4 weeks was analysed using Mantel-Cox survival analysis. The data collected from absorbance readings from excreta assay were analysed using one-way analysis of variance (ANOVA), followed by Tukey (HSD) mean comparison. All graphs were the output of the Prism version 6.0e (GraphPad software, La Jolla California USA).

2.3.4.8 Neuropeptidomics

2.3.4.8.1 *In silico* identification of putative neuropeptides

2.3.4.8.1.1 Data mining

To identify preprohormone sequences and to predict mature forms of the neuropeptides for *C. capitata*, the amino acid sequences of *D. melanogaster* neuropeptides were retrieved from Neuropeptide Research DiNeR database (http://www.neurostresspep.eu/diner/insectneuropeptides) (Yeoh et al., 2017) and used as queries to search through protein databases using BLASTp suite (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Each retrieved amino acid sequence was subjected to analysis using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) to predict the location of signal peptide cleavage. In cases where SignalP failed to predict

signal peptide sequence, Phobius software was used (a signal peptide predictor accessible (http://phobius.cgb.ki.se) (Käll et al., 2007).

Predictions of cleavages within preprohormone sequences and post-translational modifications were manually sought to predict the structures of mature peptides and any medfly-specific processing of peptides. Sequence alignments were generated using ClustalW (Larkin et al., 2007) while pairwise alignment comparison of identity and similarity of individual putative propeptides were calculated by EMBOSS Needle (Rice et al., 2000).

2.3.4.8.1.2 *Ceratitis capitata* Myosuppressin receptor

Translated sequences for *D. melanogaster* myosuppressin receptors (Dms-R1, 478bp, CG8985, FBgn0035331; Dms-R2, 488bp, CG43745, FBgn0264002) from FlyBase (https://flybase.org) were used as queries against the *C. capitata* genome using the BLASTp suite. The identified protein sequence of the putative *C. capitata* myosuppressin G-protein coupled receptor protein 139 (XP_02071789.1) was then aligned with *D. melanogaster* myosuppressin receptors (Dms-R1; Q9W025, Dms-R2; Q9W027) retrieved from Uniprot (https://www.uniprot.org) and analysed using the sequence alignment program

Clustal Omega version 1.2.4 (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.3.4.8.1.3 Molecular weight prediction of mature neuropeptides

A mass list of mature peptides was created based on predicted cleavage sites and post-translational modifications of putative neuropeptides and calculated against predicted patterns retrieved from Protein Prospector sequence database (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct, University of California, USA).

2.3.4.9 Mass spectrometry analysis

2.3.4.9.1 Fractionation of neuropeptides by high-performance liquid chromatography (HPLC)

One hundred tissues of the adult CNS (brain, thoracico-abdominal ganglion or abdominal ganglion) and the *corpora cardiaca* were dissected from adult *C. capitata* in insect saline and were placed in acidic methanol (87% methanol, 5% glacial acetic acid) and kept on

ice for 30 min before being centrifuged (12 000 x g) at 4ºC for 20 min. The supernatant was removed and diluted 10-fold with 0.1% trifluoroacetic acid (TFA) and injected onto a chromatography system (Beckman System Coulter, UK) equipped with Jupiter C_{18} 5 µm 300 Å reversed-phase column (250 mm x 2.1 mm; Phenomenex, UK). The column was eluted with a linear gradient increasing from 10 to 60% acetonitrile/0.1% TFA (v/v) and the elution of each sample was monitored at 214 nm with the flow rate set at 0.2 ml/min, as previously described (Audsley et al., 2011). Fractions (0.2 ml) were collected and concentrated to less than 10 µl by Savant Speed Vac concentrator (Thermo Electron, UK) for mass analysis.

2.3.4.9.2 HPLC samples preparation for MALDI-TOF analysis

For MALDI-TOF mass spectrometry aliquots (0.3 µl) from the HPLC fractions were mixed (1:1) with matrix solution (α-Cyano-4-hudroxycinnamic acid reconstructed in 70% acetonitrile and 0.1% TFA) before applying to the MALDI sample plate and allowed to dry.

2.3.4.9.3 Orbitrap analysis

Samples for analysis using the Orbitrap (Q ExactivePlus Hybrid Quadrupol-Orbitrap, Thermo Fisher Scientific, Waltham, MA, USA) were prepared from 20 brains, corpora cardiaca, thoracico-abdominal ganglia and abdominal ganglia were collected into polypropylene tubes containing 20 µl of extraction buffer, methanol, double distilled water and trifluoroacetic acid either in the ratio 90:9:1 by volume or 50:49:1 by volume. An equal volume of de-ionized water was added to all samples which were then centrifuged for 1 min at 13 000 rpm. Following sonication and centrifugation for 15 min, supernatants were concentrated to less than 10 µl by Savant Speed Vac concentrator (Thermo Electron, UK) and stored at -20 ºC until analysis. The acquired spectra were obtained in a data dependent mode with full MS1 acquisition at 70,000 resolution with automatic gain control target (AGC target) at 3e6. The maximum injection time was set to 80 ms and scanning array limited to 400 and 6000 m/z. The 10 most intense peaks were selected for HCD fragmentation and measured in the Orbitrap mass analyser with a resolution of 35,000, AGC target at 1 e6, maximum IT at 120 ms and dynamic exclusion set at 25 s (Ragionieri et al., 2017).

2.3.4.9.4 Fragmentation analysis

Data acquired from Orbitrap analysis were analysed with MaxQuant software (1.5.3.30) and MS/MS spectra were matched against an internal database containing the transcriptome-derived precursor sequences. Settings were adjusted to include post-translational modifications of acetylation at the N-terminus, oxidation at methionine, amidation of C-terminal, pyroglutamate from glutamine, pyroglutamate from glutamic acid, and disulfide bridges.

Chapter 3 Myosuppressin signaling in the crop of *Drosophila melanogaster*

3.1 Introduction

3.1.1 The crop

The crop is a characteristic organ for almost all adult insect species. It is an expandable super-contractile muscular organ of the intestine that coordinates engagement of pumps and sphincters to control the movement of ingested food along the gut. Crop expansion, as illustrated in Figure 3.1, allows a substantial amount of liquid to be consumed and stored within a relatively short amount of time and thus reducing exposure to competition and predation. Particularly in diapausing species, crop serves as a critical storage site for carbohydrates, essential for energy reserves during periods of inactivity (Stoffolano and Haselton, 2013).

Immediately after the adult engages in feeding, the liquid diet is drawn into the opening of an extended proboscis that relies on the expansions of a cibarium chamber to generate a pumping frequency, thus permitting suction and translocation of diet though the oesophagus into the crop

Figure 3.1 Crop expansion in fruit fly, *Drosophila melanogaster*. Located in abdomen of fly, the crop is capable of impressive expansion to accommodate ingested diet. Starvation reduces the crop to a smaller, immobile sac of wrinkle appearance (left, dissected crop from overnight starved fly). In the fully fed fly (right, dissected crop from overnight starved fly allowed to feed prior dissection), the crop can expand impressively into a unilateral sac that protrudes into the abdominal cavity. (The author created a collage of *D. melanogaster* $^{MHCWee\text{-}P26}$>*GFP* images, captured using a Leica M165 stereomicroscope, before antibody staining. These images were subsequently arranged and managed in Microsoft PowerPoint).

Resting fully fed flies have developed a system of regurgitation and vomiting food onto their mouthparts to allow excess water to evaporate and nutrients to concentrate in the crop (Fig.3.2). In certain species this behavior can serve as a nuptial gift offering, in others as a form of nutrient sharing between co-habiting flies (Stoffolano and Haselton, 2013). This has been consequently linked to the spread of pathogenic bacteria responsible for secondary infections in fruits and for transmitting human pathogens, particularly *Escherichia coli* and *Salmonella enterica* (Sela et al., 2005; Olafson et al., 2014). Further theories propose that re-extrusion of oral droplets onto the fly's proboscis is a means of moistening and initiating food digestion through mixing food with digestive enzymes or as a form of thermoregulation to maximise muscle performance (May, 1985; Hendrichs and Cooley, 1992). Recent studies also revealed a critical role for the crop in the complex lekking behavior observed in fruit flies of the Tephritidae family (Walse et al., 2008; Guillén et al., 2019).

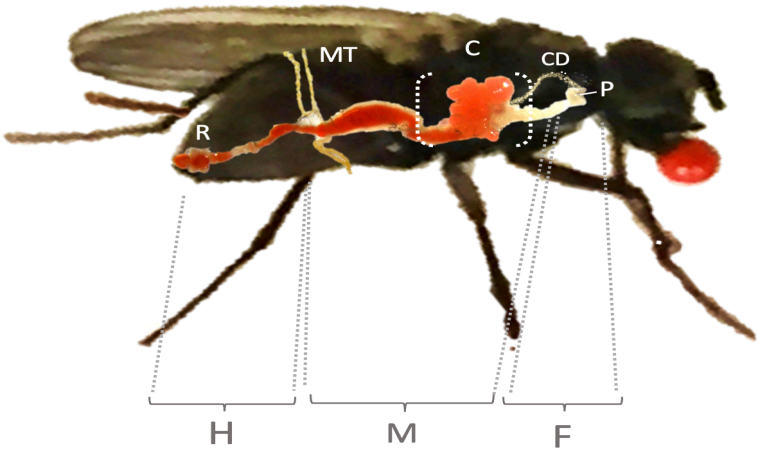


Figure 3.2 Regurgitation in cabbage root fly, *Delia radicum*. The crop (C) content in fully fed fly is vomited back on top of the proboscis. Prior dissection, fly was fed with sudan-red tinted diet to visualise regurgitation in this species. The crop sits in the upper part of abdomen and is connected to the oesophagus via the crop duct (CD) in front of proventriculus (P). Together they form part of the foregut of digestive tract. (C, crop; CD, crop duct; P, proventriculus, MT, Malpighian tubules; R, rectum; F, foregut; M, midgut; H, hindgut). (The author created a collage of *D. radicum*, captured using a Leica EZ4 W stereomicroscope, before and after dissection of digestive tract to evaluate sudan red labelled particles feeding. These images were subsequently arranged and managed in Microsoft PowerPoint).

Details of the anatomy and physiology of the fly crop are limited to a small number of species but is well described in a review by Stoffolano and Haselton (2013). It defines a crop as a diverticulated organ of the digestive tract, composed of three parts; an expandable sac located in the upper part of abdomen, a crop nerve bundles and a slender hollow tube, the crop duct, found in the thorax that facilitates transport of ingested bolus from the oesophagus to the crop. Anatomically, the crop lumen is formed by cuticular intima that provides support needed for epithelial cells to build cuticular lining. The crop duct is followed by a pair of crop nerve bundles originating from retrocerebral complex that extensively proliferate across the whole muscular surface of crop sac. The duct and crop itself are enveloped by contractile muscles that create a network of coordinated pumps and sphincters. The functionality of the whole system is dependent on hydrostatic pressure, where ingested food is pushed in or out by the major pumps (Thomson, 1975; Stoffolano et al., 2010). The course of movement is led by the threshold of hydrostatic pressure present in the crop lobes; a self-governing system free from neuronal control input. As a safe-guard mechanism, next to the mechanical interchanging function of pumps and sphincters, the neuropeptide innervation by inhibitory myosuppressin prevents unwanted contractions of the crop muscles (Thomson and Holling, 1976; Stoffolano and Haselton, 2013). The failure to regurgitate and control crop contractions has proven to be lethal in flies (Haselton et al., 2004; Blumenthal, 2008; Stoffolano et al., 2013; Gough et al., 2017).

3.1.2 Myosuppressin and its receptors

The myosuppressin neuropeptide (pEDVDHVFLRFamide) was first isolated from the head of the cockroach, *Leucophaea maderae, and* was the first insect peptide to be recognized as an effective peptidergic inhibitor of gut peristalsis (Holman et al., 1986a). Insect myosuppressin peptides have a highly conserved primary structure of $X_1DVX_2HX_3FLRFamide$, where X_1 represents pQ, T or P while X2 could be either D, G or V and X_3 replaced with V or S (Nässel, 2002). A decade after its discovery, the precursor from cockroach *Diploptera punctata* was characterized from the sequence of the cloned cDNA (Donly et al., 1996) with subsequent studies noting that other insect myosuppressin precursors have a similar structure (Audsley and Weaver, 2009). Table 3.1 below depicts the conservation of the structure of myosuppressin among insects from diverse orders. The identification and characterization of myosuppressin precursors in

various insects, along with the noted structural similarities across different species, calls for careful consideration in the development of control strategies, particularly in formulating protective coating with potential attractants. This approach is crucial to ensure specificity and minimize the risk of inadvertently affecting non-target species.

Table 3.1 Comparative Conservation of Myosuppressin Peptide Sequences Across Insect Orders

UniProt ID	Family	Organism	Common name	Sequence	Significance	Reference
P61849 NEMS_DROME	Diptera	*Drosophila melanogaster*	Fruit fly	TDVDHVFLRFa	Insect model organism	Nichols et al., 1992
Q7PUD4_ANOGA	Culicidae	*Anopheles gambiae*	African malarian mosquito	TDVDHVFLRFa	Disease vector (malaria, dengue fever)	Holt et al., 2002
P85816 · NEMS_RHOPR	Hemiptera	*Rhodnius prolixus*	Kissing bug	QDLDHVFMRFa	Disease vector (Chagas disease)	Ons et al., 2009
E2A3M7 · NEMS_CAMFO	Hexapoda	*Camponotus floridanus*	Carpenter ant	QDVDHVFLRFa	Decomposer	Schmitt et al., 2015
P85527 · NEMS_APIME	Hymenoptera	*Apis mellifera*	Honeybee	QDVDHVFLRFa	Pollinator	Hummon et al., 2006
C0HKU2 · NEMS_AGRIP	Lepidoptera	*Agrotis ipsilon*	Cutworm	QDVVHSFLRFa	Agricultural pest	Diesner et al., 2018
U3U9Y2_NILLU	Hemiptera	*Nilaparvata lugens*	Brown planthopper	QDVDHVFLRFa	Agricultural pest	Tanaka et al., 2014
D7EIL9_TRICA	Coleoptera	*Tribolium castaneum*	Red flour beetle	QDVDHVFLRFa	Agricultural pest	Richards et al., 2008
P84306 · FARP_LOCMI	Orthoptera	*Locusta migratoria*	Migratory locust	PDVDHVFLRFa	Agricultural pest	Schoofs et al., 1993

Source: The UniProt Knowledgebase (UniProtKB), https://www.uniprot.org/

Date accessed: 08.09.2022

Unlike other insect neuropeptides belonging to the superfamily of FMRFamide-like peptides (FLPs), only a single copy of the mature decapeptide, flanked with endoproteolytic cleavage sites at both termini, is present in the precursor (Vilaplana et al., 2004). In *D. melanogaster,* the myosuppressin gene (*Dms*, *CG6440*, FBgn0011581) is expressed in all stages of development. Firstly Dms-containing cells are presentin the developing embryonic nervous system (McCormick and Nichols, 1993). During larval

development, the numbers of dms-positive cells increase in the brain and also appear in the ventral ganglia. The number of dms cells increase further in the adult brain and staining is also seen in the corpora cardiaca, two rectal cells and in neuronal fibres in the crop. The physiological actions of this peptide in insects is predominantly as a potent myoinhibitor, reducing cardiac, visceral, skeletal and flight muscle contraction (Lange et al., 1995; Dickerson et al., 2012). Further detailed studies revealed the truly pleiotropic nature of myosuppressin, when the peptide was shown to be involved in stimulating release of several digestive enzymes in scallop *Pecten maximus*, cockroach *D. punctata*, or in beetle *Rhynchophorus ferrugineus* (Nachman et al., 1997; Fusé et al., 1999; Harshini et al., 2002), the release of hormone from the hypocerebral ganglion complex (Veelaert et al., 1998; Hendrichs and Hendrichs, 1998), regulating pupal diapause (Yamada et al., 2017), in regulation of the writhing of Malpighian tubules (Coast, 1998) and in affecting escape responses, locomotion and sleep behaviour (Klose et al., 2010; Iannacone et al., 2017).

Two Dms receptor genes, *Dms-R1* (CG8985) and *Dms-R2* (CG43745/CG13803), have been characterised by heterologous expression of the receptor cDNAs in cultured mammalian cells, confirmed by an inositol 1,4,5-triphosphate (IP3)/Ca2+- mediated bioluminescence response mediated by *Drosophila* myosuppressin and localised by Northern blots in adults and larvae, depending on receptor (Egerod et al., 2003). RNA-seq data indicate low expression of *Dms-R1* in the nervous system, heart and salivary gland, while the majority of *Dms-R1* expression was found in the crop of both sexes (Table 3.2, Chintapalli et al., 2007; Leader et al., 2018; Krause et al., 2022). *Dms-R2* is also expressed in the same adult tissues, but the levels in the crop appear to be 90% lower than that of *Dms-R1*. Protein sequence alignment of *Dms-R1* and *-R2* generates a similarity score of over 65% (Fig. 3.3). Both expressed receptors respond exclusively to myosuppressin and equally well to nanomolar concentrations in a dose-dependent manner (Egerod et al., 2003; Marygold et al., 2016).

Molecular modeling has investigated the interaction between myosuppressin and both receptors, highlighting unique features of switch motifs and binding pockets, particularly within TM3 and TM6 regions needed for receptor activation, reflecting their exclusive interactions to single ligand (Rasmussen et al., 2015).

Table 3.2 *D. melanogaster* myosuppressin receptors Dms-R1 (Gene ID: FBgn0035331) and Dms-R2 (Gene ID: FBgn0264002) expression in larvae and adult stage. Data pooled from FlyAtlas2 database based on RNA-Seq data. Abundance is reported in logarithmic scale and colour intensity from white, low abundance, to black, high abundance. Expression specific to the crop is highlighted in the red rectangle. (https://motif.mvls.gla.ac.uk/FlyAtlas2) (Krause et al., 2022).

Dromyosuppressin receptor 1 Dms-R1				Dromyosuppressin receptor 2 Dms-R2		
Male	Female	Larva	Tissue	Male	Female	Larva
3.5	3.5		Head	1.4	1.6	
2.1	1.8		Eye	1.4	1.0	
5.4	5.1	1.5	Brain / CNS	2.3	2.0	0.5
4.3	4.4		Thoracicoabdominal ganglion	3.5	3.6	
105	92		Crop	9.4	9.1	
0.0	0.0	0.0	Midgut	0.5	0.2	0.5
0.0	0.0	0.0	Hindgut	0.3	0.3	0.3
0.0	0.0	0.0	Malpighian Tubules	0.1	0.0	0.0
0.1	0.2	0.1	Fat body	0.7	0.5	0.0
1.7	0.8	0.2	Salivary gland	0.5	0.2	0.0
1.4	0.9		Heart	2.0	1.7	
		0.1	Trachea			0.0
	0.0		Ovary		0.0	
	0.0		Virgin Spermatheca		0.4	
	0.1		Mated Spermatheca		0.2	
0.1			Testis	0.2		
0.0			Accessory glands	1.3		
1.8	1.4	0.1	Carcass	1.6	1.7	0.0
0.0	0.0		Rectal pad	1.4	1.3	
		0.2	Garland cells			0.2

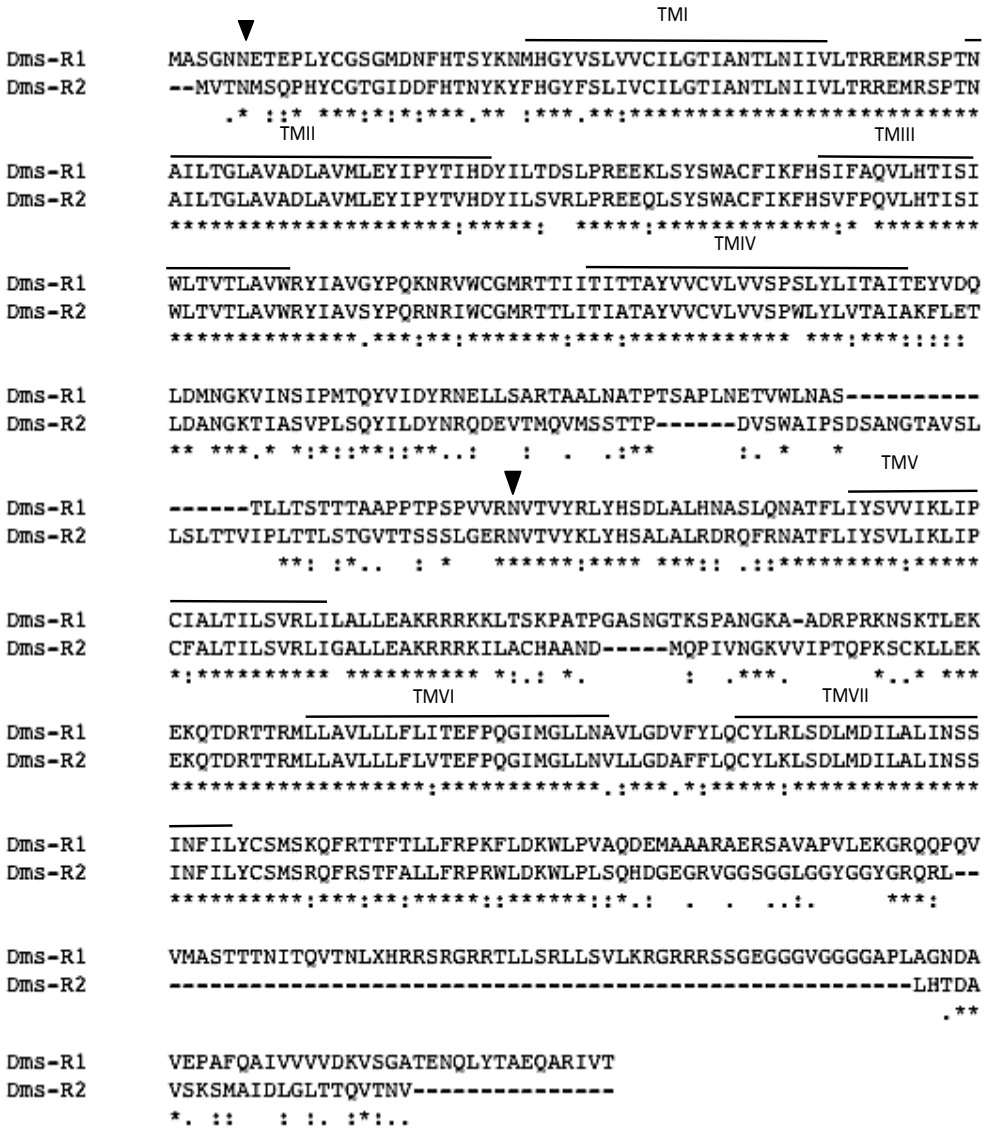
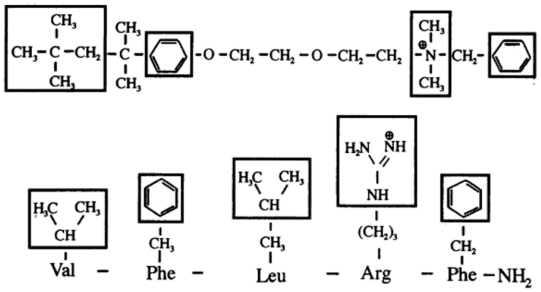


Figure 3.3 Alignment of two myosuppressin receptor proteins (Dms-R1, CG8985) and Dms-R2 (CG13803/CG43745), sharing over 65% similarity. Seven transmembrane helices TMI-TMVII are overlined and two glycosylation sites are indicated with full arrowhead. (Needle-EMBOSS-pairwise-alignment-following-Needleman
Wunsch Algorithm https://www.ebi.ac.uk/Tools/psa/,TMHMM2.0 predicted glycosylated s ites https://services.healthtech.dtu.dk/service.php?TMHMM-2.0).
(Symbols for the degree of conservation: "-", mismatch or gap; ". " (period), conservation of weakly similar properties; " : " (colon), conservation between strongly similar properties; " * " (asterisk), fully conserved residue).

3.1.3 Benzethonium chloride (Bztc), a non-peptide myosuppressin analogue

Bztc was first reported by Nachman and colleagues to be a non-peptide analogue of myosuppressin (Nachman et al., 1996). It shares chemical features with the C-terminal pentapeptide, VFLRFamide, which is critical for natural myosuppressin peptides to function as a receptor ligand. Nachman et al. (1996) highlighted common structural features between Bztc and the pentapeptide, namely two phenyl rings and a positively charged group with branch-chain hydrophobic tail (Fig. 3.4). Using the locust oviduct preparation Bztc mimicked the actions of the locust myosuppressin, also known as SchistoFLRFamide (PDVDHVFLRFamide), by reversibly inhibiting both proctolin and neutrally-evoked contractions of the oviduct. Furthermore, Bztc competitively displaced radiolabelled SchistoFLRFamide from both high and low affinity receptors on membranes prepared from the oviduct. Also, commercially known as hyamine, Bztc is a relatively stable synthetic quaternary ammonium salt that resists degradation in the presence of digestive enzymes and was thus used in proof-of-concept experiments to support the proposal that myosuppressin signaling is a potential new insecticidal target. Myosuppressin peptides are potent inhibitors of feeding when either ingested by or injected into the pest species, *Spodoptera littoralis, Lacanobia olercea* and *Acyrthosiphon pisum*, stimulating interest in the potential for Bztc to work as an anorexic agent inhibiting insect feeding(Vilaplana et al., 2008; Matthews et al., 2009; Down et al., 2011; Schoofs et al., 2017). The presence of myosuppressin innervation of the fly crop, the high potency of the peptide in inhibiting crop contractions *ex vivo* and the expression of *DmsR-1* in the crop of *D. melanogaster*, together suggest that myosuppressin control of the crop is an attractive target for a non-peptide analogue to disrupt food intake and gut physiology. Furthermore, Richer and colleagues showed that Bztc inhibited spontaneous muscle contractions when applied to the crop of *P. regina* (Richer et al., 2000). This was the first attempt to interfere with the function of the dipteran crop by feeding with Bztc in the diet. No study, however, has yet shown that Bztc can effectively reduce feeding.



(Image credit: Lange et al., 1995)

Figure 3.4 Illustration of Bztc structure (above) and the C-terminal pentapeptide fragment VFLRFamide of myosuppressin (below), highlighting structural similarities proposed by Nachman et al., (1996).

3.1.4 Experimental aim

This research was initiated to address the notable gap in published data regarding the role of myosuppressin in modulating the activity of the adult *D. melanogaster* crop, utilizing a myosuppressin-GAL4 line sourced from the Janelia FlyLight project. The method employed used a highly expressing UAS-GFP (pJFRC81-10XUAS-IVS-Syn21-GFP-P10) line and immunohistochemical (IHC) detection of expressed GFP in fixed isolated adult tissue. This part of the study focused on the nervous system and, in particular, the adult foregut. The inhibitory effect of myosuppressin on *D. melanogaster* crop muscle was also studied to determine its EC_{50}. Myosuppressin receptor null mutants, donated by the Korea Drosophila Resource Center, provided compelling evidence that Dms-R1 is the key receptor for the inhibitory response to nanomolar concentrations of the peptide. In addition, the role of myosuppressin signaling in feeding behavior was investigated.

3.2 Results

3.2.1 Single tissue profiling of the *D. melanogaster* crop nerve bundle

To fully understand the regulatory role of myosuppressin in the muscular contractions of the crop, it is imperative to revalidate the presence of its biologically active native form in the neuronal pathways innervating the crop. Additionally, confirming the specificity of RFamide staining is crucial, given that the C-terminal antibody used in our study is reactive to five different families of FaPR peptides. This step is essential to ensure the precision of our insights regarding the influence of myosuppressin on crop muscle activity.

Over the m/z range of 500-2500, a major single monoisotopic mass ion peak (*m/z*) of 1247.6 corresponding to Dms was present in the mass spectrum retrieved from single tissue of the crop nerve bundle (Fig. 3.5). Three minor signals, *m/z* 972.5, 1012.5 and 1118.6 were also present, however, their ion masses could not be matched to any known *D. melanogaster* neuropeptide.

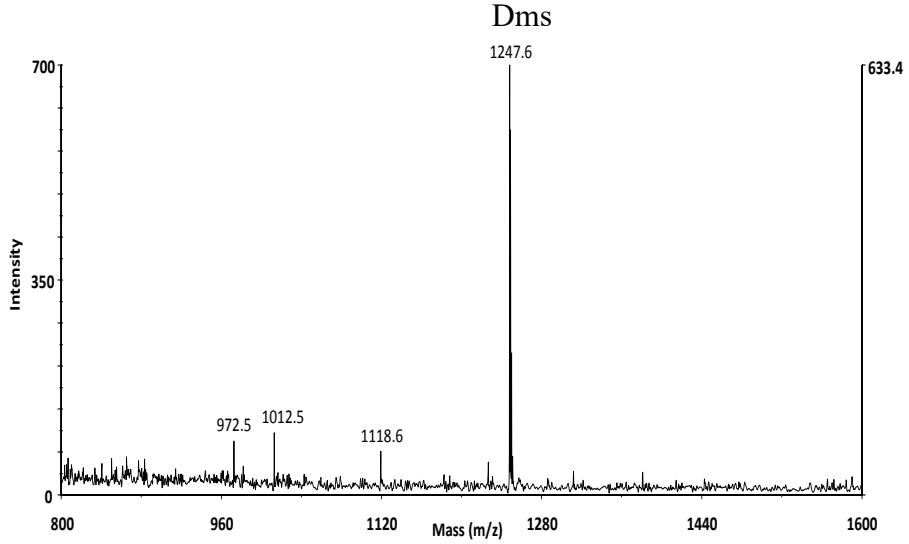


Figure 3.5 Mass spectrum from the direct analysis of a *D. melanogaster* crop nerve bundle. MALDI-TOF MS identified a major peak representing monoisotopic mass ($[M+H]^+$) of Dms (*m/z* 1247.6), and less intense unknown peaks of masses (*m/z* 972.5, 1012.5 and 1118.6).

3.2.2 The muscle structure of the *D. melanogaster* crop

Mhc$^{Wee\text{-}P26}$ driving the expression of GFP within myosin heavy gene (gift from C.J.H. Elliot, York;Clyne et al., 2003), illuminated the muscle fibre assembly of the crop (Fig. 3.6 A) and crop duct (Fig. 3.6 B). The crop duct, stemming from the oesophagus as an elongated slender tube, passes from the thorax into the abdomen, where it widens to form a conical region which connects to the anterior base of the diverculated crop sac. The anterior base of the crop displays a strong pattern of striated muscle fibres in an arrowhead formation (Fig. 3.6 B). Once merged, the anterior base of the crop contains closely stacked striated fibres in circular formation. Fibres radiate outwards, forming a structure described by Thomson (1975) as the main crop pump. The fibres appear to be present on top of longitudinal fibres that, align next to each other, spread towards the posterior region of the crop. Two patterns can be observed; a small number of fibres continue straight until they reach the top of the crop, while most fibres subdivide. Division occurs approximately halfway along the crop surface, where they cross a neighboring "branch" depending on the width of stretched crop lobe area.

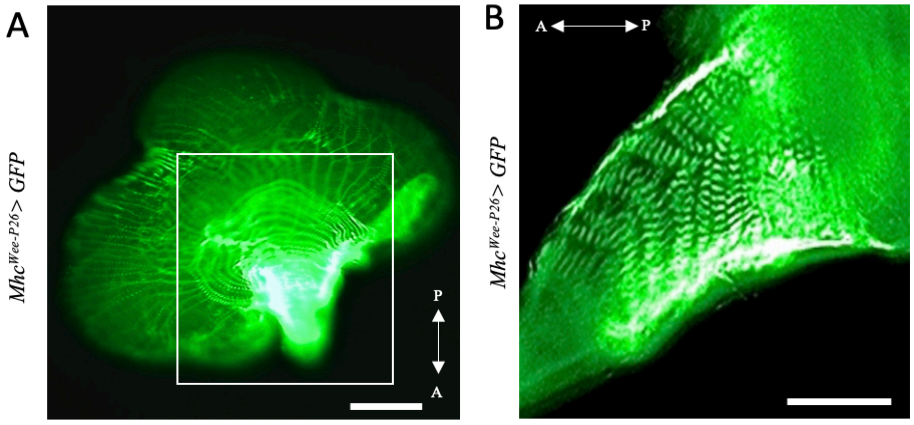


Figure 3.6 Expression of GFP within myosin heavy chain gene in muscle assemblies of fruit fly, *D. melanogaster* MHC$^{Wee\text{-}P26}$>GFP. Whole-mounted crop tissue dissected from adult expressing the GFP-tagged myosin was labelled with anti-GFP (green) mouse antibody and screened under Leica M165FC fluorescent stereo microscope. (A) Circular pattern of muscles surrounding the main crop pump (square) and linear muscle fibers running along the crop surface. The scale bar represents 100 µm. (B) Posterior conical region of crop duct region. The striated muscle pattern forms between interconnection of crop duct and crop base. The scale bar represents 50 µm.

3.2.3 *Dms* expression in the crop of adult *D. melanogaster*

Transgenic *Dms-GAL4* males (P[GMR61H01-GAL4]attP2) were crossed with females carrying 'super high' cytoplasmic GFP reporter pJFRC81 construct (10XUAS-IVS-Syn21-GFP-p10) to trace myosuppressin neuronal tracts associated with the fore gut. An antibody against GFP was used to localise expression of *Dms-GAL4*.

Several *Dms-GAL4* neuronal axons were seen to emanate from the hypocerebral ganglion/corpora cardiaca complex (HG-CC-CA) that reside in the posterior region of the oesophagus (Fig. 3.7 B). GFP labelled axons leave from this region in two directions; (i) towards the proventriculus (aka cardia) that functions as a valve between the oesophagus and the midgut; (ii) as bilateral nerve bundles reaching towards the crop.

A unilateral nerve passes from HG-CC-CA complex and runs over the surface of the proventricular bulb, where it splits into two continuing fibres (Fig. 3.7 B) that extend along one third of the length of the anterior midgut. Two crop nerve bundles run alongside a crop duct into abdominal area. Passing the surface of main crop pump and reaching the crop midline leads to the prominent expansion into a network of immunoreacted fibers covering area of crop lobes. (Fig. 3.7 A). Results were validated by screening: (i) samples where the primary antibody has been omitted and (ii) parental lines screened under the fluorescent microscope for background autofluorescence (Fig. 3.8).

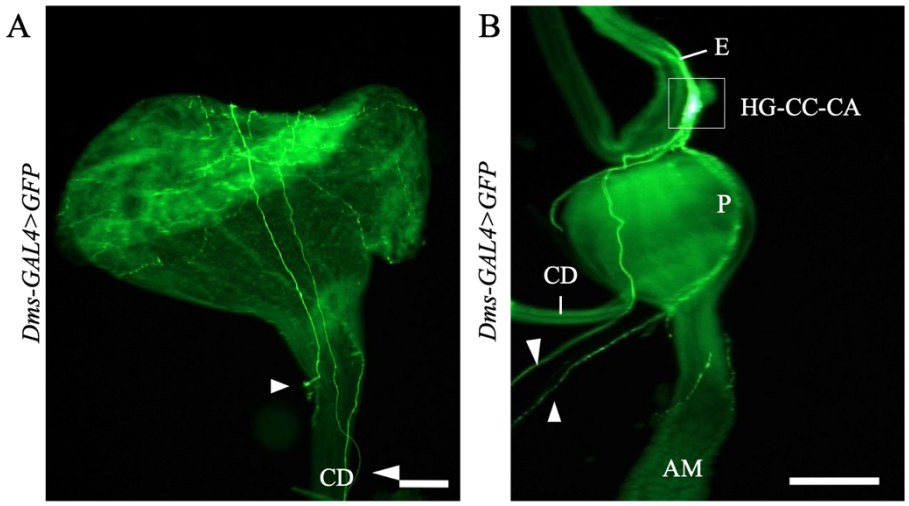


Figure 3.7 *Dms-GAL4* mediated GFP expression in foregut of adult *D. melanogaster*. Whole-mounted adult tissues expressing the GFP reporter under the control of *Dms*-GAL4 transgene were stained with anti-GFP antibody and pictures taken by Leica M165FC fluorescent stereo microscope. (A) Immunoreactive fibres run alongside the crop duct (CD) and branch across the crop surface. The scale bar represents 50 μm (B) Descending immunoreactive fibres from HG-CC-CA complex reach the proventriculus (P) and continue either towards anterior part of midgut (M) or in the form of bilateral nerves (white arrowheads) towards crop. The scale bar represents 100 μm. (E, esophagus; HG-CC-CA, hypocerebal ganglion-corpora cardiaca complex; CD, crop duct; P, proventriculus; M, midgut, F, foregut; AM, anterior midgut region).

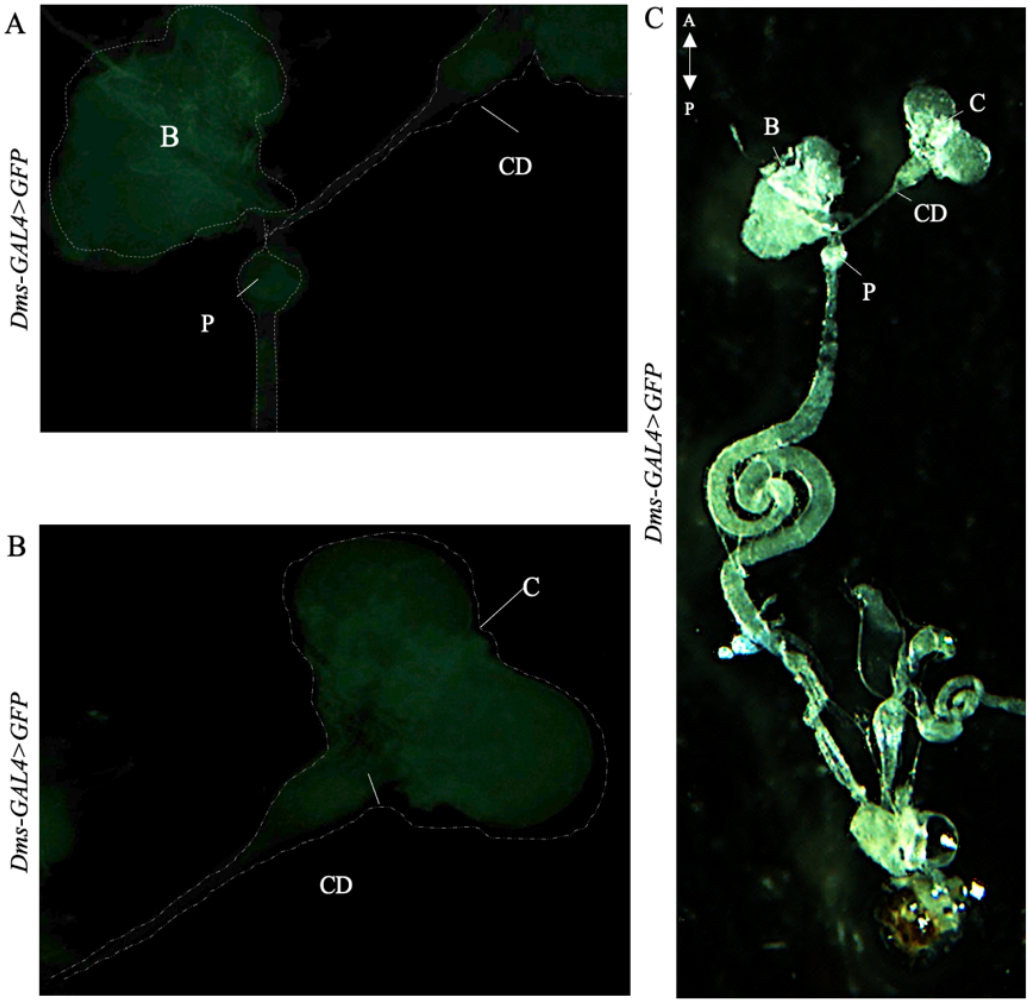


Figure 3.8 Control experiment for non-specific binding between secondary antibody (Alexa Fluor 488, green) and non-target epitopes in *Dms-GAL4>GFP* preparation. Working immunohistochemical solutions excluded primary (anti-GFP) antibody. Samples were screened using a Leica M165 FC apochromatic stereo microscope. (A) Detail of brain and proventriculus under 10x magnification. (B) Detail of the crop and crop duct under 10x magnification. (C) Bright field image of unfixed whole adult dissection exposing the alimentary tract under 2.5x magnification. (B, brain; P, proventriculus; C, crop; CD, crop duct).

3.2.4 *In vitro* crop motility bioassay to investigate the effect of Dms and the functionality of DmsR-1 and DmsR-2 receptors in crop physiology

Baseline data was first obtained for crop muscle contractions in order to establish the effect of Dms on muscle contractions of the semi-isolated crop tissue and particularly the

involvement of DmsR-1 and DmsR-2. For these physiological experiments spontaneous contractions were recorded from the same muscular lobe of the crop. On average, 48±11 contractions per min (s.e.m., n = 35) were recorded for the crop basal rate. The peristalsis of the crop musculature from control wild-type w^{1118} flies responded strongly to the application of Dms (IC_{50} = 4.11 x 10^{-9} M), as did crops from flies lacking a functional *DmsR-2*^{01} (IC_{50} = 8.23 x 10^{-9} M). In contrast, crops from *DmsR-1*^{01} flies showed a 1000-fold reduction in response to the peptide (IC_{50}= 2.16 x 10^{-6} M). The inhibitory action of Dms was reversed on washing out the peptide with saline, except in crops lacking functional DmsR-1, where contractions remained unaffected (Fig. 3.10). The non-peptide agonist Bztc also inhibited crop contractions but did not discriminate between w^{1118} and *DmsR-1*^{01} flies; the half maximal inhibitory effects of Bztc were 5.77 x 10^{-6} M and 8.22 x 10^{-6} M, respectively (Fig. 3.9).

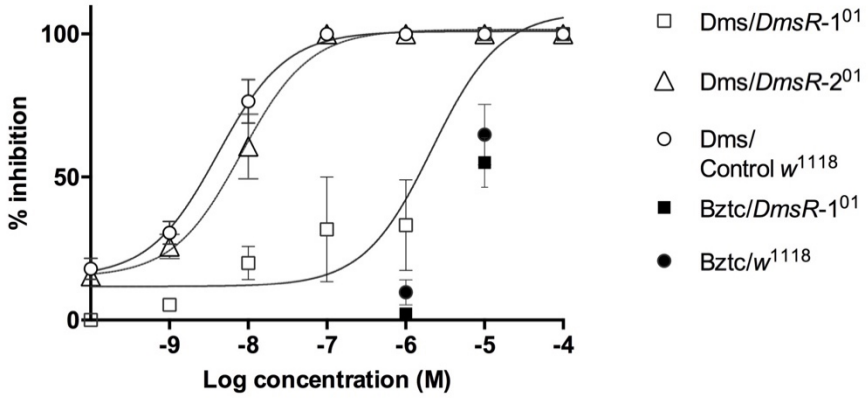


Figure 3.9 The effect of Dms and Benzethonium chloride (Bztc) on spontaneous crop contractions in *D. melanogaster.* Isolated crops from adult females were exposed to Dms and the non-peptide analog Bztc, and their crop contractions counted to determine IC_{50} values by employing the "two-pipette transfer system" described by Stoffolano et al., (2013). Data are expressed as the % inhibition of spontaneous contractions counted for a 1 min period after adding either Dms or Bztc solutions. GraphPad Prism 6.0 was used for the analysis and graphical representation. (w^{1118} ○, IC_{50}= 4.1x10^{-9} M; ●, IC_{50}= 5.8x10^{-6} M; *DmsR-1*^{01} □, IC_{50}= 2.2x10^{-6} M; ■, IC_{50}= 8.2x10^{-6} M; and *DmsR-2*^{01} Δ, IC_{50}= 8.2x10^{-9} M). Each point represents the mean ± s.e.m (n = 5).

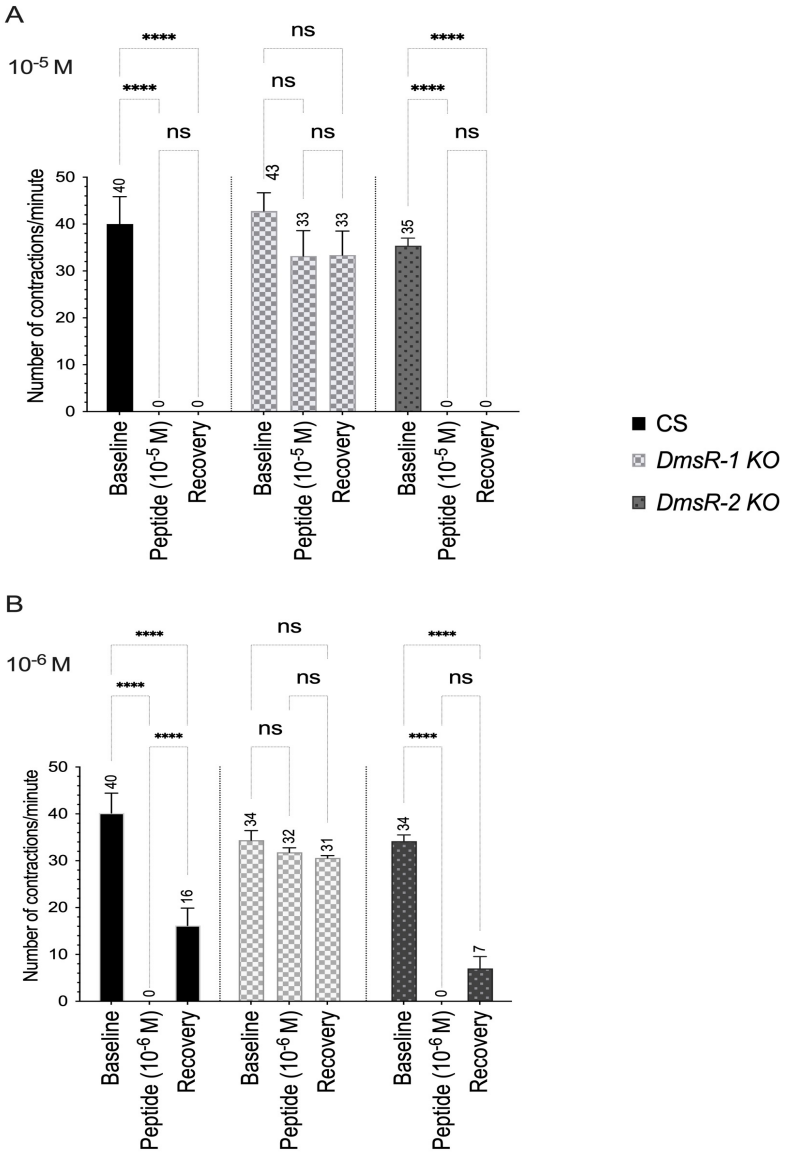


Figure 3.10 Crop contractions recovery rate in *D. melanogaster* after exposure to Dms. Semi-isolated crops were exposed to Dms of either (A) 10^{-5} M or (B) 10^{-6} M concentration and the effect on the spontaneous contractions counted for a minute in control (CS) and mutant flies *DmsR-1*^{01} and *DmsR-2*^{01}. Recovery of the spontaneous contractions in control flies was dose-dependent as crops recovered from inhibition when threated with 10^{-6} M peptide, but not when crops were exposed to 10^{-5} M peptide concentration. Two-way ANOVA analysis followed by a post-hoc Tukey tests were performed by using GraphPad Prism 6.0. Error bars represent the mean ± s.e.m (n = 5), number above the error bar shows the mean calculated for sum of contractions per treatment per fly strain.

3.2.5 Dms signaling and food intake

The results reported in 3.3.4 show that DmsR-1, and not DmsR-2, is necessary for the sensitive response of the crop muscle to applied Dms peptide, a result that is consistent with the transcript levels reported in FlyAtlas2 (Table 3.2). To further investigate the role of *DmsR-1*^{01} on food intake and feeding behavior, several standard feeding assays were conducted using CS, *w* ^{1118} and *DmsR-1*^{01} flies.

Adults of both sexes were starved overnight (17 hr) before being offered a blue sucrose solution. After a 1 hr feeding period, images of the abdomen with the crop distended with blue food were captured. All flies lacking a functional DmsR-1 showed a clear difference in crop expansion compared to both *CS* and *w*^{1118} individuals. In contrast, *DmsR-1*^{01} flies had partially expanded crops, that is only half of the abdomen appeared blue (Fig. 3.11). Further support for the importance of *DmsR-1*^{01} in food intake was sought by following the transit of labelled food along the alimentary tracts of females that were offered food for just 1 min (Fig. 3.12). As expected, only 20% of *DmsR-1*^{01} females had food in the crop and midgut after the short feeding regime.

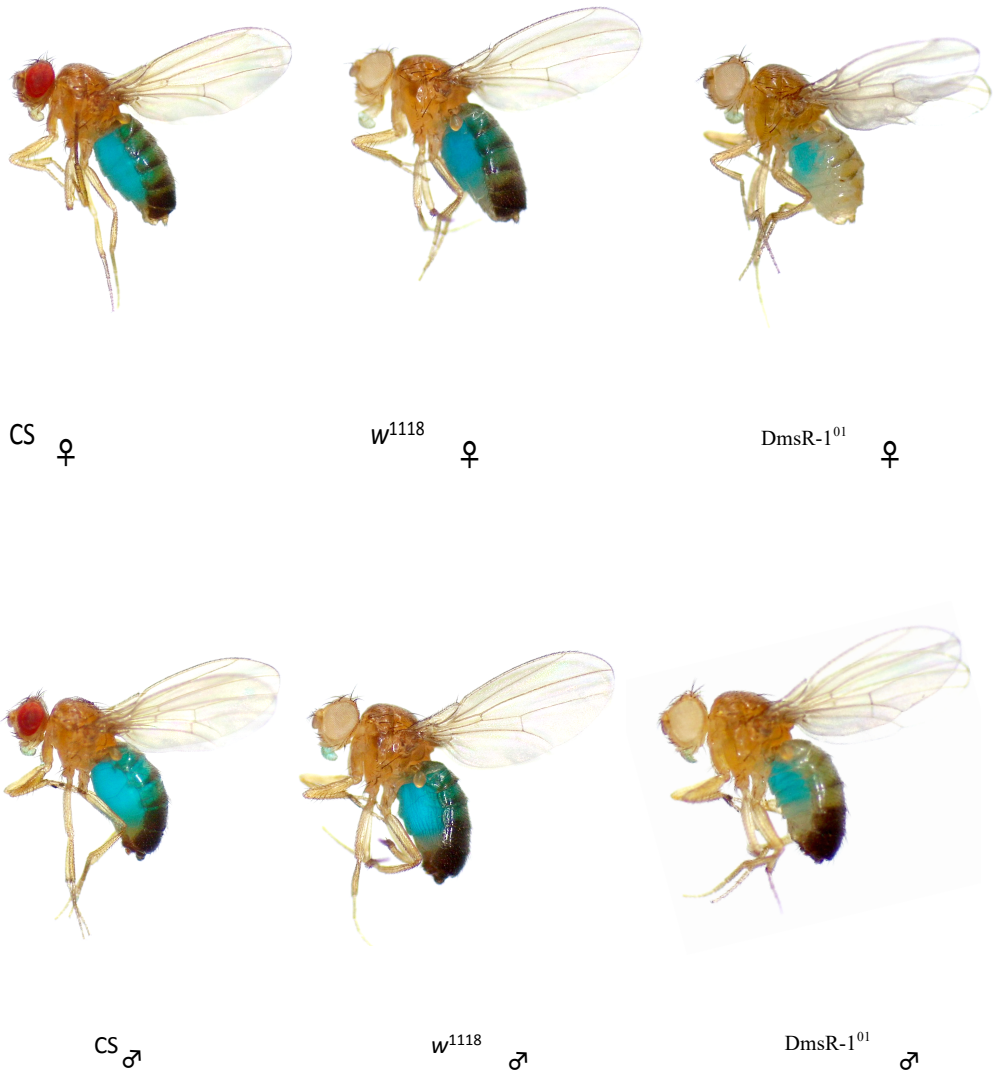


Figure 3.11 Representative images of *CS*, w^{1118} and *DmsR-1*^{01} flies after feeding on sucrose/blue dye for 1hr. Each group contained 10 male and female individuals.

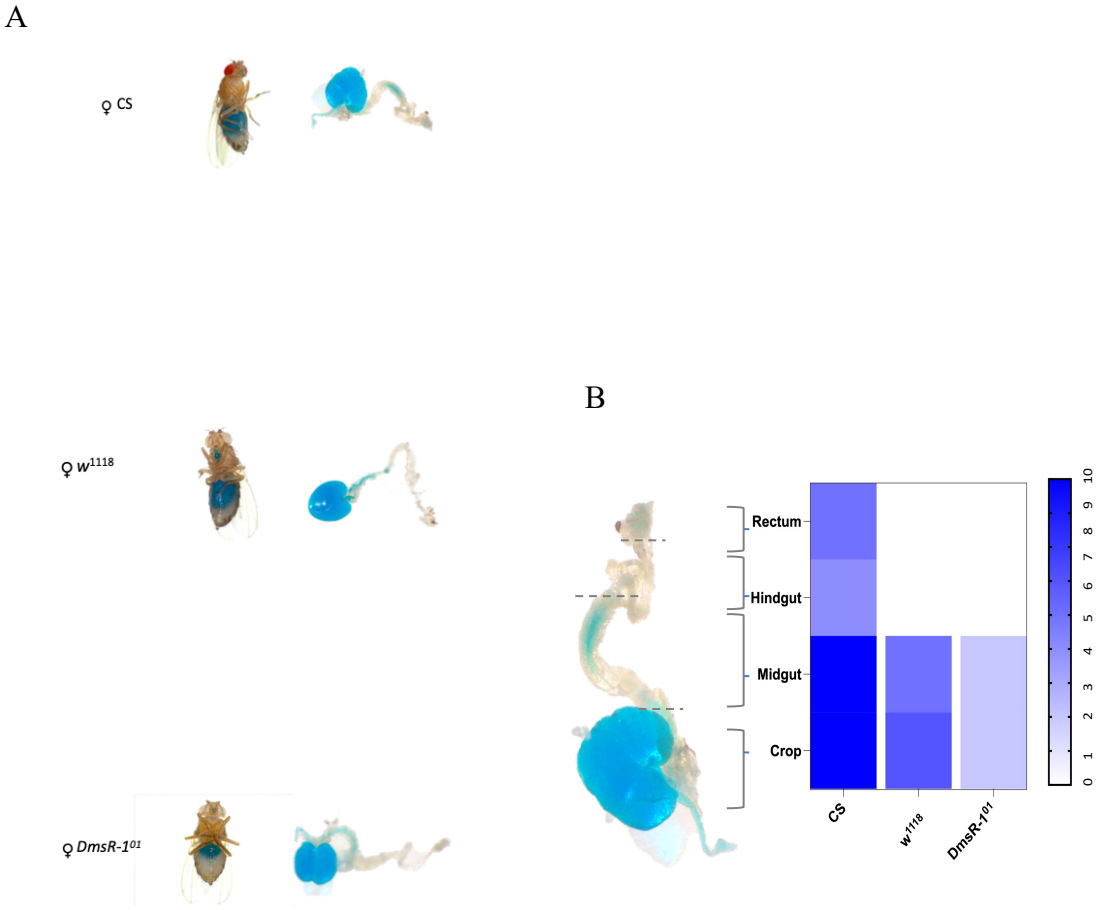


Figure 3.12 Transit of food along the intestine in *D. melanogaster* females. (A) This panel demonstrates the transit of blue dye-enhanced sucrose feed in females of three *D. melanogaster* strains (CS, w^{1118}, and *DmsR-1*^{01}). Flies were starved overnight and then allowed to feed on the dyed food for 1 minute before their alimentary tracts were dissected. The progression of the ingested food was tracked from the crop, through the midgut and hindgut, and into the rectum. (B) A heatmap illustrates the distribution of the ingested dye-enhanced feed as it transited the alimentary tract (n=10). Adjacent to the heatmap, a color scale is provided, representing the intensity of the dye detected in the samples. This scale correlates with the number of positive samples, offering a visual representation of dye concentration across different gut sections; darker colour on the scale signify higher frequencies of positive dye detection. This colour gradient allows for quick and intuitive assessment of dye distribution patterns in the alimentary tract across different strains. Statistical analysis using Tukey's post-hoc test, following a two-way ANOVA, revealed significant differences. Specifically, the crop of *DmsR-1*^{01} showed a significantly higher presence of dye compared to CS (p=0.0035), as well as in the midgut (*DmsR-1*^{01} vs. CS, p=0.0034). No significant differences were observed in the hindgut across groups. In the rectum, CS differed significantly from both w^{1118} (p=0.0361) and *DmsR-1*^{01} (p=0.0361) in dye accumulation.

3.2.6 Excretion assay

The amount of food ingested and passed along the alimentary canal was measured indirectly by quantifying the amount of blue dye excreted from previously starved female flies fed for 24 hr on sucrose containing blue food colouring. An analysis of variance (ANOVA) in wild type CS and w^{1118} adults did not significantly differ from each other in terms of the amount of dye in the recovered excreta, thus suggesting they ingest a similar amount of food in this time period ($p=0.8542$). There were, however, significant differences between wild type /control groups (CS and w^{1118}) and *DmsR-1*^{01} flies with the amount of dye collected in the faeces of *DmsR-1*^{01} flies being much lower (Fig. 3.13). Post hoc Tukey multiple comparisons test validated distinct reduction in fecal deposition in both, CS and w^{1118} flies exposed to 5 mM Bztc in diet (CS $p<0.0001$, w^{1118} $p<0.0001$). In contrast, flies exposed to diet containing Bztc that lacked functional Dms-R1 gave the matching pattern of amount of dye in collected excreta to those fed with sucrose only ($P=0.9943$). The lack of Bztc in diet given to *DmsR-1*^{01} flies resulted in vaguely noticeable decrease in defecation but not to a significantly relevant level.

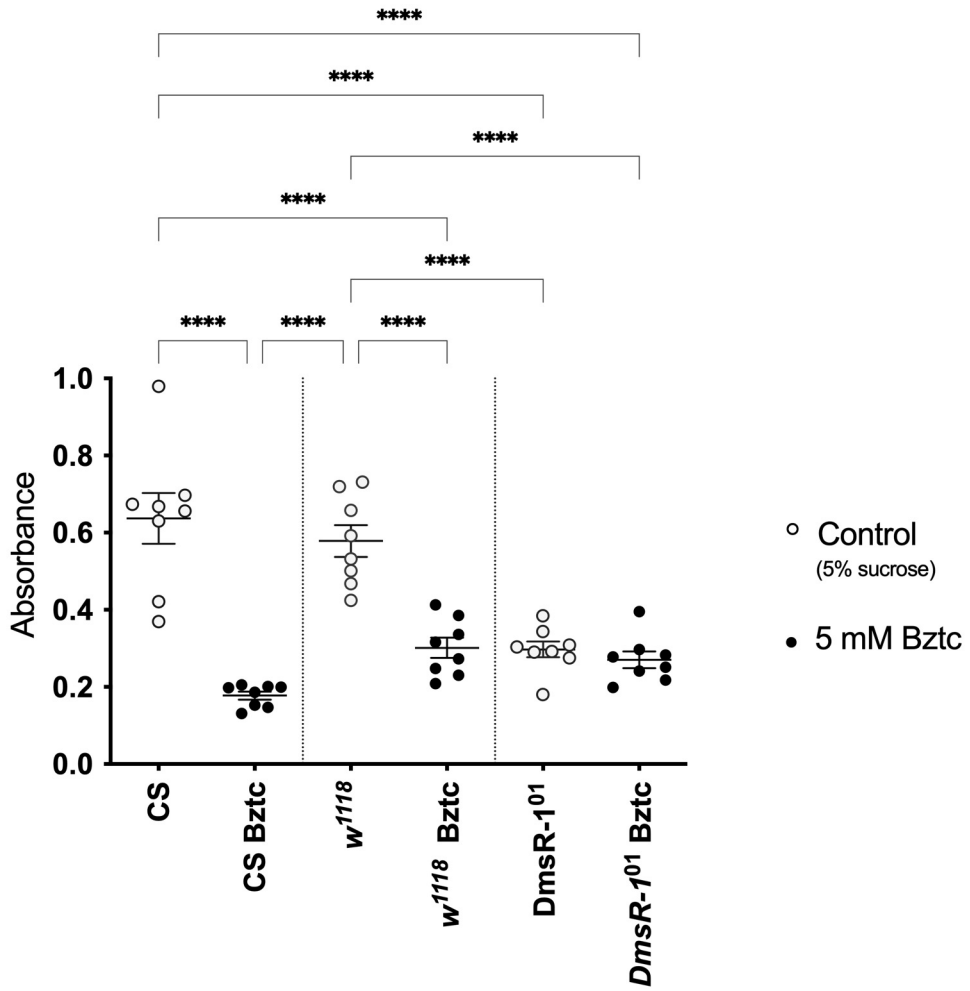


Figure 3.13 Measurement of the impact of Bztc on feeding through excretion assay. Comparison of excreta volumes collected from untreated groups, including controls (CS, w^{1118}) and flies lacking functional myosuppressin receptor (*DmsR*-1^{01}). An Ordinary one-way ANOVA revealed a significant difference only in the *DmsR-1*^{01} group compared to both controls (CS vs. w^{1118}, p=0.8542; CS vs. *DmsR-1*^{01}, p<0.0001; w^{1118} vs. *DmsR-1*^{01}, p<0.0001). Addition of Bztc to the diet resulted in a significant reduction in defecation in both control groups (CS, p<0.0001; w^{1118}, p<0.0001). In contrast, excretion in flies lacking the functional Dms-R1 receptor was not affected by Bztc (p=0.9943). Each point represents excreta collection from 5 individuals (n=8 biological replicates, 5 flies per replicate, mean ± s.e.m.). Data were analyzed using a one-way ANOVA with post-hoc adjustments made through Tukey's multiple comparisons test. Only significant differences are graphically represented in the form of brackets above the columns (**** p < 0.0001).

3.2.7 The effect of dietary stress on locomotor activity and longevity of control and *DmsR-1^{01}* flies

Following the observation of altered waste elimination in *D. melanogaster*, as evidenced by reduced excreta levels in flies without functional myosuppressin receptor (*DmsR-1^{01}*), it is crucial to explore the implications on overall physiological health. This leads us to investigate two key areas: locomotor activity and longevity. Locomotor assays will provide insights into the neuromuscular and metabolic health of these flies, while longevity assays might help understand how these metabolic changes affect lifespan.

Sex-separated *CS, w^{1118}* and *DmsR-1^{01}* flies were subjected to either desiccation conditions by depriving access to both food and water or starvation, where flies were provided with 2% (w/v) agar as a source of water without an energy source. Locomotor activity of individual flies under standard environmental conditions (see methods) was used as a surrogate method for measuring both well-being and survival with the time of the last recorded movement (beam break) in a 5 min period used as a surrogate for the time of death (Gough et al., 2017).

Both control groups, exposed to stressor, either in the form of desiccation or starvation, did not show significant impact on longevity between sexes (n=16, Fig. 3.17). CS strain, both males and females, on average had a median lifespan of 28 hr when provided with water (♀ 28 ± 3 hr and ♂ 28 ± 3 hr) and 16 hr without it (♀ 17 ± 3 hr and ♂16 ± 2 hr). *w*^{1118} flies lasted around 40 hr when provided with water (♀ 42 ± 3 hr and ♂38 ± 3 hr) and survived for around 18 hr without it (♀ 19± 2 hr and ♂17 ± 1 hr). In both cases, the impact of desiccation halved the lifespan of flies compared to starvation. Sex did not provide any advantage in dehydration tolerance for any group exposed to desiccation stress (CS ♀17 ± 3 hr vs. ♂16 ± 2 hr; *w*^{1118} ♀19 ± 2hr vs. ♂17 ± 1 hr; Dms-R1♀28 ± 3 hr vs. ♂27 ± 1 hr). On the other hand, *Dms-R1* KO flies, regardless of sex, outlived CS and *w*^{1118} flies, and showed outstanding resilience towards starvation conditions in females (*Dms-R1* KO, ♀ 72 ± 3 hr), on average three times longer than CS females (♀ 28 ± 3 hr) and survived nearly twice as long compared to control *w^{1118}* females (♀ 42 ± 2 hr).

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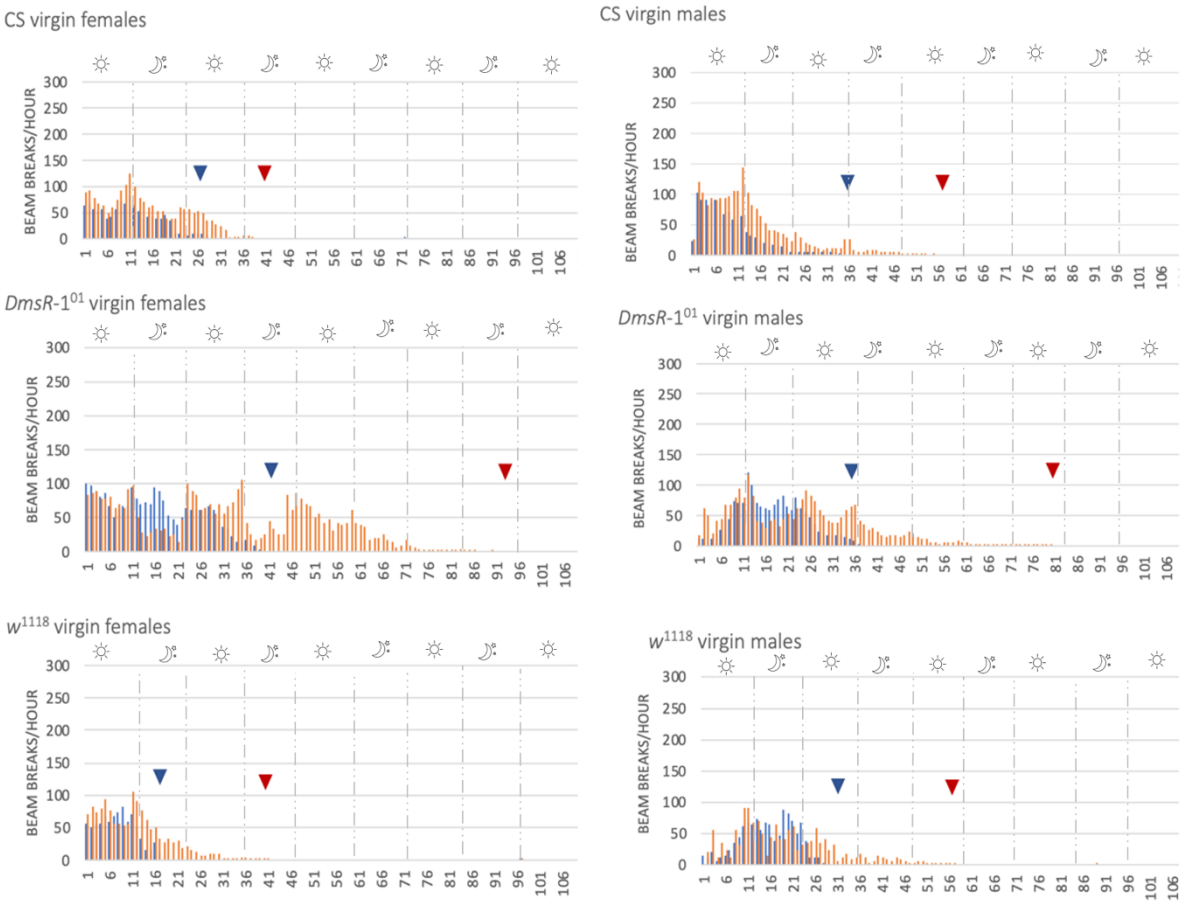


Figure 3.14 Locomotor activity of virgin *Drosophila* females CS, *DmsR-1*^{01} and w^{1118} (left) and virgin males CS, *DmsR-1*^{01} and w^{1118} (right) provided with access to water in the form of 2% (wt/v) agar (red) or exposed to desiccation stress, being deprived of food and water (blue). Flies were monitored for 6 days (144 hr). Movement activity was recorded using the Trikinetics activity monitors as described by Gough et al. (2017) and are expressed as the mean number of beam breaks per hr (n =16, mean ± s.e.m). Each 12/12-hour light/dark cycle is separated by dash line and indicated by graphical input above the graph in the form of sun ☼ – light or moon ☽- dark. Arrow above the graph indicates the time of death of last remaining individual (blue arrow-desiccation, red arrow- starvation).

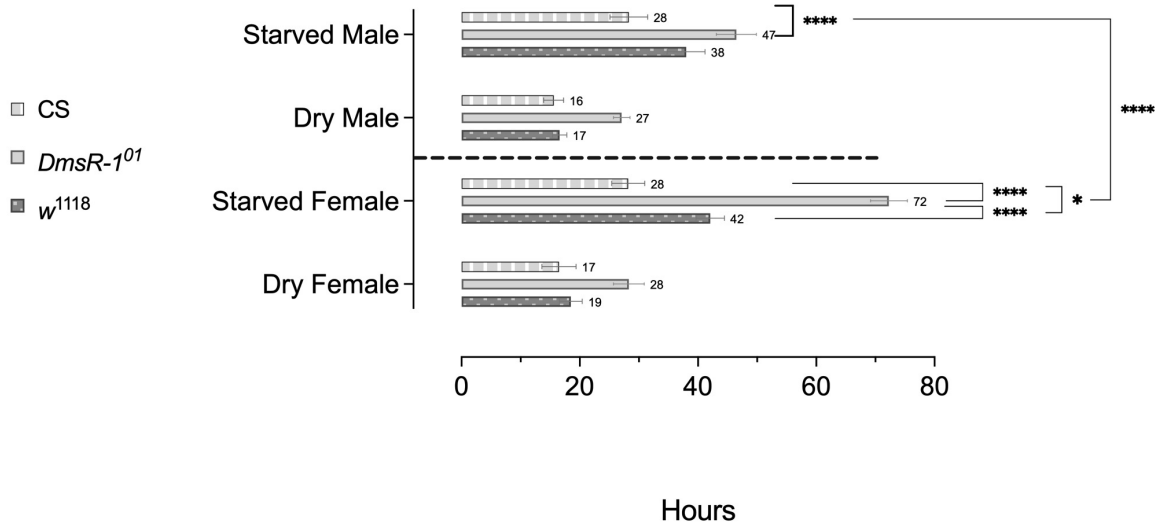


Figure 3.15 The longevity of *CS*, w^{1118} and *DmsR-1* KO virgin males (top) and virgin females (bottom) was tested under desiccation and starvation conditions. Flies were deprived of water (dry) or provided with 2% (w/v) agar as a source of water (starved). Within the male subgroups, analysis showed no significant differences in mean values among the male flies devoid of water (CS, $DmsR\text{-}1^{01}$, w^{1118}). Among the Starved Male groups, significant differences were identified; Starved Male CS group demonstrated a statistically significant difference compared to $DmsR\text{-}1^{01}$ flies (****, $p < 0.0001$), while comparisons involving w^{1118} (CS vs. w^{1118} and $DmsR\text{-}1^{01}$ vs. w^{1118}) showed no significant differences. Statistical analysis of the 'Dry' and 'Starved' female groups revealed no significant differences among Dry Female subgroups (CS, $DmsR\text{-}1^{01}$, w^{1118}). In contrast, significant differences were observed among Starved Female subgroups; Starved Female CS flies showed a significant difference compared to both $DmsR\text{-}1^{01}$ (****, $p < 0.0001$) *and* w^{1118} (*, $p = 0.0105$) group, while Starved Female $DmsR\text{-}1^{01}$ flies differed significantly from *w1118* (****, $p < 0.0001$). The time of death was determined by the lack of locomotion measured by Trikinetics activity monitor recording individual break beam in 5 min interval (Mean survivorship ± s.e.m, n=16, two-way ANOVA followed by post-hoc Tukey's multiple comparisons test).

3.3 Discussion

The crop, previously noted as a muscular organ serving both as a storage site and an initial digestion location, is not thoroughly described in fruit fly *D. melanogaster*. Most existing publications on this topic rely heavily on the detailed anatomical and physiological descriptions of black blowfly, *Phormia regina*'s crop provided by Stoffolano et al. (2010). The crop of *D. melanogaster* is likely to exhibit unique adaptations, reflecting the species' distinct dietary preferences and ecological roles. The fruit fly is considerably smaller than the blowfly, a distinction that manifests not only in physical structure but also in their respective feeding behaviors. *D. melanogaster* primarily consumes yeast and microorganisms found on decaying fruit, whereas *P. regina*, being larger, has a diet that includes a broader range of decaying organic materials, such as animal carcasses. It is, therefore, anticipated that the crop of *D. melanogaster* is specialized for the intake of finer food particles, a specialization that would influence its crop's capacity, the elasticity of its muscle fibers, and the rate at which it empties (Thomson, 1975; Liscia et al., 2012; Stoffolano and Haselton, 2013).

This chapter begins with an in-depth examination of the musculature in the crop of *Drosophila melanogaster*, employing GFP-driven visualization of myosin filaments using the MhcWee-P26 GAL4 line as detailed by Clyne et al. (2003). The observed myosin filaments, in conjunction with actin fibers, form sarcomere units that constitute a super-contractile muscle sheath enveloping the crop's cuticular lamina. This intricate arrangement highlights a sophisticated muscular system, potentially mirroring functional aspects seen in other dipteran species. Stoffolano and colleagues compared the musculature surrounding the crop to a very similar muscle layer covering *D. melanogaster* ovaries (Hudson et al., 2008; Stoffolano et al., 2010). Interestingly, myosuppressin-like fibres were observed across the whole peritoneal sheath of the *D. melanogaster* ovary and oviduct, a tube composed of single layer of tissue surrounded by muscles (P. Pribylova, data not shown).

The pattern of crop contractions can vary depending on species. Yet, most often the shape of fruit fly crop has two large lobes beating with powerful, but slower strokes in synchrony with small, lobes that contract erratically fast. Those are often located on the sides of the border between crop and crop duct. *D. melanogaster* crops that were observed in the present study contracted regularly, but their lobe strokes were erratic moving with irregular frequency and amplitude of beating. For instance, the whole crop could stop contracting, then appear to have small rhythmic patterns of contractions in the large lobe,

expand in size and then completely stop. Conversely, the whole structure would appear still except for a small lobe at the base, with fast powerful strokes that could stop and restart anytime. Occasional touches with a dissecting metal needle was enough to restart the whole contracting cascade of lobe strokes. This behavior is indicative of the presence of stretch-sensitive mechanism in crop muscle that can evoke contractions without any other neural input.

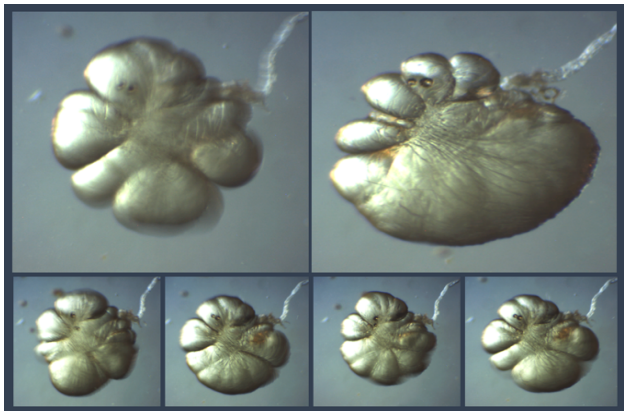


Figure 3.16 Lobe formation dynamics in the *D. melanogaster* crop. This figure presents a collage of screenshots extracted from a video recording (captured using a Leica EZ4 W stereomicroscope equipped with an integrated 5-megapixel camera) showcasing an isolated crop immersed in fly saline. The images vividly demonstrate the crop's remarkable flexibility and dynamic lobe formation. Notably, this particular sample was excluded from the crop assay due to its atypical behavior, characterized by unpredictable merging and formation of lobes on the surface.

Crop muscles appear to generate strong contractions that are occasionally powerful enough to move the tissue from a microscopes' field of view, turning or pushing the crop in any direction. How a fly regulates the strength of lobe strokes and their formation during feeding, and especially how regurgitation takes place remains unknown. Yet, there must be synchrony between different muscle groups to direct ingested food in, mix it and/or push it out either further into midgut or to regurgitate it back in the form of droplets. An in-depth physiological study of muscular peristalsis throughout the foregut, supplemented by video microscopy, would enhance our understanding of how the foregut manages the passage of food along the alimentary canal for digestion, and in some cases, the regurgitation of liquids.

This chapter also describes the use of a GFP reporter to map *Dms*-GAL4 neurons of the retrocerebral complex and the crop nerve bundle (CNB). The retrocerebral complex, located above the proventriculus, consists of paired corpora allata (CA) and corpora

cardiaca (CC). Together they are considered as a major neurohemal organ known to synthesize and store a variety of neuropeptides (full list in Predel et al., 2004). Confocal imaging suggested direct Dms innervation of the peptide-containing CC. Besides immunoreactive fibres extending towards the proventriculus, there are *Dms*-GAL4 axons in the CNB, the bilateral nerves emanating from the CC. In pairs, those nerves exit from CC to run alongside of the crop duct and before reaching the crop lobes, they branch out into a web-like structure covering the whole crop surface, making contact with the crop musculature. The GFP pattern is in agreement with a report by McCormick and Nichols (2012), who used antiserum recognising the N-terminus of Dms to immuno-label neuronal tracts of the *D. melanogaster* crop.

To learn more about the identity of the peptides delivered from the CC towards the crop, the CNB was subjected to single tissue profiling using MALDI-TOF (Matrix-assisted laser desorption/ionization-time of flight) mass spectrometry, which identified the presence of Dms ($[M+H]^+$ of *m/z,* 1247). This molecular ion, whilst dominating the spectrum, was accompanied by less intense ions (*m/z, 972.5*, 1118.6 and 1012.5). The 972.5 ion might correspond to the monoisotopic mass (*m/z,* 974.5) of $sNPF^{4-11}$ (SPSLRFamide), that was found in the CNB spectra of both *D. radicum* (Chapter 4; Bell et al., 2018) and *D. suzukii* (Audsley and Down, 2015). The significance of these findings is not clear since $sNPF^{4-11}$ had no inhibitory effect when applied to the crop of *D. radicum* even at high doses (Chapter 4, Fig. 4.6). Yet, it has been reported as one of the stored neuropeptides within neurosecretory cells of *D. melanogaster* CC/retrocerebral complex (Carlsson et al., 2013). In *Drosophila,* sNPF is associated with responses to food stimuli and implicated in the control of metabolism, energy homeostasis and response to stress (reviewed by Cui and Zhao, 2020).

Transcriptomics data in FlyAtlas2 shows that there is 'very high' expression of *DmsR-1* and 'moderate' expression of *DmsR-2* in the crop of *D. melanogaster,* suggesting that *DmsR-1* is the more important receptor on the crop muscle for transducing the inhibitory Dms signal (Krause et al., 2022). The availability of *Dms* receptor KO mutants allowed the testing of the relative importance of these two receptors in crop physiology. The *ex vivo* data reported in this chapter comparing the inhibition of crop muscle from wild type and both KO mutant genotypes, firmly supports the above conclusion that DmsR-1 is primarily responsible for mediating the potent inhibitory actions of Dms. The IC_{50} for wild type and $DmsR\text{-}1^{01}$ crops is in the low nM range and is comparable to the IC_{50} values (40 nM) obtained for both receptors when expressed in Chinese hamster ovary cells

(Egerod et al., 2003). The IC_{50} of 4.1 nM for the wild type (w^{1118}) crop indicates greater crop muscle sensitivity to the peptide compared with the crop of *D. radicum* (44 nM, Chapter 3; Bell et al., 2018) but is comparable to the value reported for *D. suzukii* (2.3 nM, (Gough et al., 2017). Even in the absence of a functional *DmsR-1*, Dms was still capable of inhibiting crop contractions, but at much higher peptide concentration. This weaker activity might be explained by either functional redundancy from DmsR-2 or activation of other G-protein coupled receptors e.g. myosuppressin is also capable to act on FMRFa receptor (above 10^{-8} M) (Cazzamali and Grimmelikhuijzen, 2002) that is also present within crop tissue but in low amount (CG13398,.FBgn0032042, https://www.flymine.org/flymine/report/Gene/1364026).

Bztc, a non-peptide myosuppressin receptor agonist, inhibited muscle contractions of both w^{1118} and $DmsR\text{-}1^{01}$ flies, but at relatively high concentrations compared to Dms with an IC_{50} of around 5.8 µM, which was close to the IC_{50} values recorded for *D. suzukii* (5.7 µM) and *D. radicum* (7.2 µM). This lower agonist potency was not unexpected for a non-peptide ligand and is consistent with previous reports that the IC_{50} for the inhibition of proctolin-induced contractions by Bztc is two orders of magnitude greater than for locust myosuppressin, SchistoFLRFamide (Sedra et al., 2014; Lange et al., 2016). Data in this chapter indicates that Bztc inhibition was not acting through *DmsR-1.* The work of Egerod et al. also could not elicit specific DmsR1/2 responses to Bztc in cell culture at concentrations below 100 µM, casting doubt on the specificity of this chemical in *D. melanogaster* (Egerod et al., 2003).

We might expect that $DmsR\text{-}1^{01}$ flies have a problem in regulating the function of the crop. How this problem might manifest is not easy to predict, not only because of the variable roles that the crop has in food storage, digestion and regurgitation, but also because some receptor redundancy is expected. In addition, the receptor KO is obviously not tissue specific and various compensatory actions might kick in. Nevertheless, several studies were conducted to investigate the impact of the loss of a functional DmsR-1 might have on food intake. Using blue-labelled sucrose as a food source, it was clear that $DmsR\text{-}1^{01}$ flies, feeding *ad libitum* after starvation, have smaller crops, reduced food intake and excretion compared to *CS* and the genetic control w^{1118} . Dms signaling in the crop probably functions to stop contractions and relax the muscle allowing for rapid crop expansion and uptake of food into the organ and eventual passage down the alimentary canal. Bztc also reduced the amount of excreta produced when fed to both wild type and $DmsR\text{-}1^{01}$, but this effect might be due to non-specific activity, such as phagodeterrence.

This concern was also very briefly mentioned by Haselton et al., (2004) when feeding Bztc to *M. domestica*.

DmsR-1^{01} KO males and females that were subjected to desiccation stress, showed resilience compared to control (CS and *w*^{1118}) flies with up to 2-fold increase in survival. It was observed that sex did not have an impact on survival/stress resistance when flies were subject to desiccation, specifically (CS ♀17 ± 3 hr vs. ♂16 ± 2 hr; *w*^{1118} ♀19 ± 2 hr vs. ♂17 ± 1 hr; Dms-R1♀28 ± 3 hr vs. ♂27 ± 1 hr). Again, there was no obvious disparity in lifespan between control flies exposed to desiccation. On average CS and w^{1118}, both males and females, survived 17 hr. Yet, flies lacking functional receptor shown higher resistance to desiccation, surviving twice longer with lifespan on average of 28 hr for both sexes. High tolerance to desiccation is presumed to be a result of gaining advantage in any of those three physiological traits; water content available within a body, water availability as result of metabolism and/or urine production and finally result of adjusted water loss (Nghiem et al., 2000). The exact role of Dms in stress responses is not fully understood, but an intriguing possibility is that the inability of flies to fully engorge their crop, particularly after periods of starvation, might activate stress responses. These responses could include reduced water loss and increased mobilization of energy reserves like glycogen, providing essential 'reserves' during challenging times.

Likewise, it is known that the dipteran crop can have a role in water balance by regurgitation of liquid crop contents (Guillén et al., 2019). The failure to fully expand the crop with liquid during feeding might help conserve water under desiccation conditions. Whether Dms and/or Dms-R1 signalling contribute to metabolic changes govern by fat body that serves as major energy reservoir (review on lipid metabolism in *Drosphila* by Heier and Kühnlein, (2018) and in turn, delay fly to succumb under stress conditions should be further investigated. In view of those points, assaying older flies and measuring their internal metabolic state via quantification of fat body and their metabolites in the haemolymph might be helpful to investigate whether Dms and/or Dms-R1 has an important role to the internal metabolic state.

The study hints at potential link between crop functionality and desiccation tolerance. However, the exact mechanisms and whether these are directly related to crop physiology remains to be fully explored. Also, the observation of crop contractions and their pattern were based on manual stimulation and video analysis. This approach might not fully capture the natural dynamics in the living organism in its natural environment. Multiple knock-out lines would help to determine if the observed effect of DmsR-1^{01} are specific

to the targeted gene. Also, limitation of our study arises from the fact that the DmsR-1 knockout line is derived from the w^{1118} line, potentially introducing background genetic variations (see Materials and Methods, that could influence our results and interpretations, especially when comparing with other genetic backgrounds or wild-type strains.

Chapter 4 Peptidergic control of the crop of the cabbage root fly, Delia radicum: a role for myosuppressin

4.1 Introduction

The cabbage root fly, *Delia radicum* (L.) (Diptera: Anthomyiidae), is a pest of brassicas in Europe and North America and poses a major and chronic threat to the commercial production of brassica crops (Blackshaw et al., 2012). The flies overwinter as pupae and in the spring the emerging females lay their eggs on the soil close to the base of cruciferous plants. Following egg hatch, the larvae feed on the host plant's root system and it is this life stage that is the most damaging (Biron et al., 1998). While cultural control methods like crop rotation and fencing can help mitigate damage, the reliance on chemical insecticides, particularly on organophosphates, remains the primary approach to manage cabbage fly's population. However, it's important to note that cabbage larvae have developed resistance to numerous active ingredients in pesticides, presenting an ongoing challenge in effective pest management (Myrand et al., 2015). Also, the absence of a fully sequenced genome or comprehensive transcriptome for the cabbage fly significantly hampers our understanding of its biology and resistance mechanisms.

Myosuppressin has been identified by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) in the brain and retrocerebral complex (corpus cardiacum) from a range of dipteran species (Wegener et al., 2006; Hauser et al., 2010; Predel et al., 2010; Rahman et al., 2013; Caers et al., 2015), including both larval and adult stages of *Delia radicum* (Audsley et al., 2011; Zoephel et al., 2012). Mass spectrometry has also identified myosuppressin in the larvae and crop nerve bundle of adult *D. radicum* (Zoephel et al., 2012; Audsley et al., 2015) but data on the function of myosuppressin in this species are lacking. As in other dipteran species, the crop in *Delia radicum* has a critical role in the transfer of food to the midgut (as demonstrated in fruit fly, *D. melanogaster,* Chapter 3 and medfly, *C. capitata* in Chapter 5). Dysfunctionality of the crop can result in profound reductions in survival of the adult fly (Peller et al., 2009; Ren et al., 2014) and therefore targeting the neuronal control of the crop of cabbage root fly is an attractive strategy in the search for a novel pest control strategy.

In this chapter we have investigated the role of myosuppressin in regulating crop motility of *D. radicum*. We confirm that myosuppressin is the dominant peptide in the crop nerve bundle and that this peptide is a potent inhibitor of crop muscle contractions. We have also undertaken experiments to assess the potential of myosuppressin and the myosuppresin receptor agonist benzethonium chloride (Bztc) to disrupt gut function in this important pest of brassica crops in our efforts to identify targets for the development of new insect control chemicals.

4.2 Results

4.2.1 Peptidergic innervation of the D. radicum crop

Immunostaining of a whole mount preparation of the crop of adult *D. radicum* using antibodies recognizing the RFamide epitope of myosuppressin revealed prominent innervation by a network of immunoreactive fibres extending over the central region of the crop sac with individual projections reaching towards the lobes (Fig. 4.1). These immuno-reactive fibres originate from two axons emanating from the corpora cardiaca (Fig. 4.2 A) that travel along the lateral sides of the crop duct (Fig. 4.2 B) towards the crop sac. Reaching the base of the crop, they undergo prominent division (Fig. 4.1 A). Figure 4.2 A shows 2 cells from which the immunoreactive material in the crop nerve originates. Other processes originating from the corpora cardiaca project over the proventriculus (Fig. 4.2 B) and terminate on the surface of the anterior midgut. Despite the size difference, with females being larger than males, no notable differences were observed in the crop preparations between the two sexes. The antibody specificity was confirmed when tissues were incubated either with secondary antibody alone or with antibody pre-absorbed with peptide, which abolished the immuno-reactivity. The staining in the midgut is the result of cross-reactivity to FaRPs present in the enteroendocrine cells.

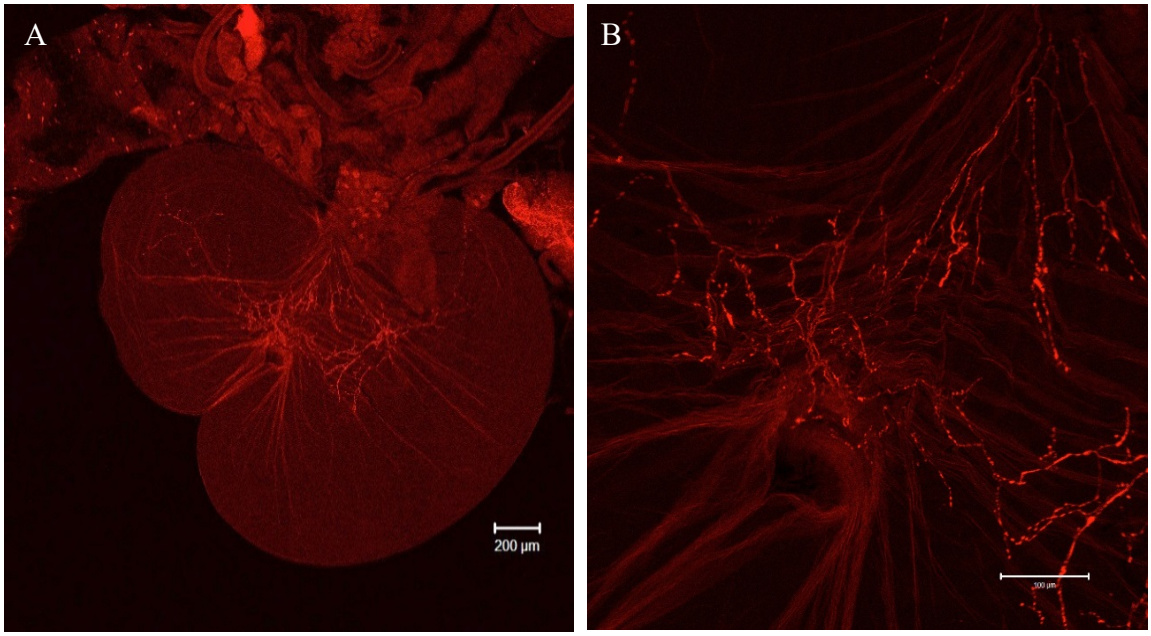


Figure 4.1 Whole-mount preparation of the crop of cabbage root fly *D. radicum*. (A) Confocal microscopy revealed prominent anti-RFamide immunoreactivity within a crop area. The scale bar represents 200 microns. (B) Close-up of *D. radicum* crop tissue innervated by immunoreactive fibers stained with antisera to RFamide. The bar in the lower right-hand corner represents 100 microns.

4.2.2 Phalloidin staining of the crop muscle

Confocal microscopy shown phalloidin-stained actin filaments of longitudinal muscle fibers in *D. radicum* crop and crop lobe (Fig.4.2) arranged as 'interconnecting bridges' between strong circular muscles. Falsely stained epithelium beneath the muscles layer had a folded appearance, presumably reflecting the expandable function needed during feeding and regurgitation. Clear difference between crop musculature can be observed between *D. radicum* and *C. capitata* phalloidin-stained myofibres reported in Chapter 6.

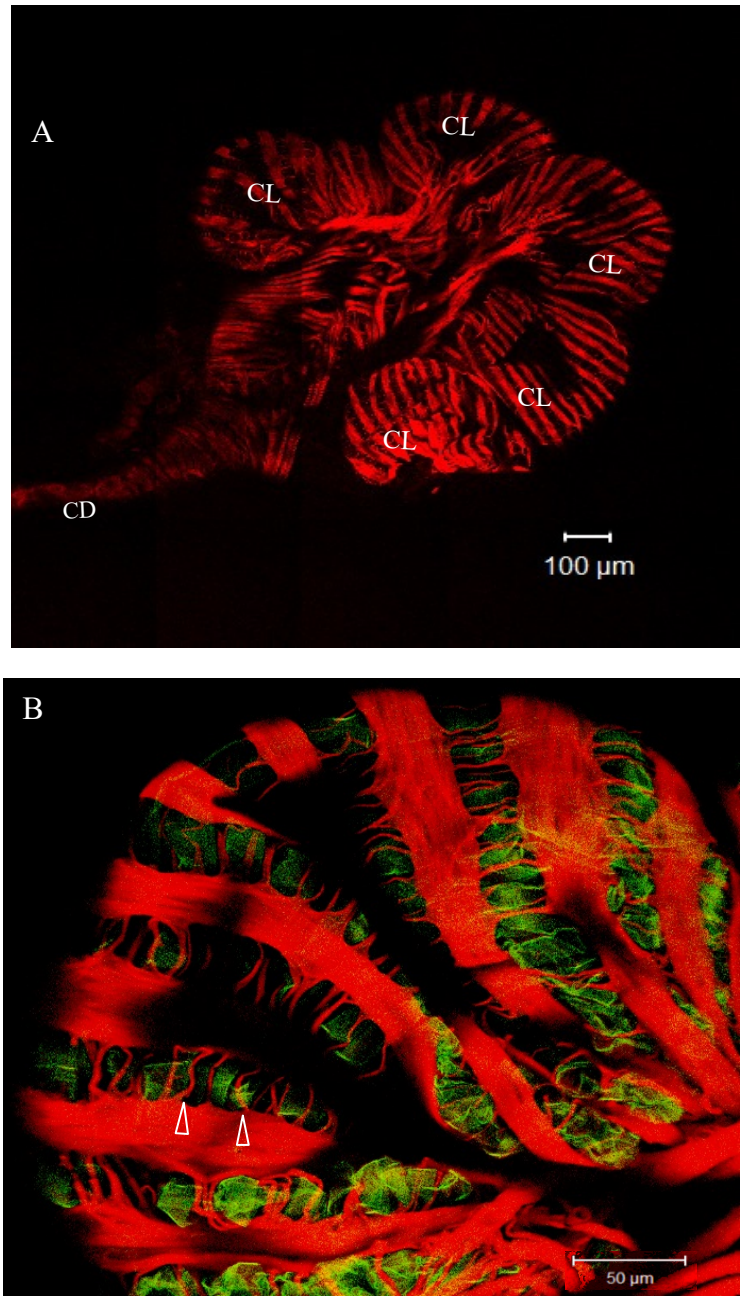


Figure 4.2 Phalloidin-stained crop of cabbage root fly, *Delia radicum*. The crop musculature of crop duct (CD), crop base (CB) and several crop lobes (CL) were visualised by treating semi-isolated crops with phalloidin staining (red). (A) A strong assembly of filamentous actin fibres were enveloping the whole crop, crop duct, and several crop lobes attached to the crop base. (B) A close-up image was taken to visualise the muscular organisation of a crop lobe, showing a regularly spaced (on average < 10 µm apart), fine strings of filaments, creating a network of interconnecting bridges (hollow arrowhead) across a strong (~ 40µm wide) muscle filaments. The higher magnification also revealed a cuticular lamina (tissue autofluorescence) protruding between interconnected filaments. The scale bar represents 50 microns. Images were taken by Zeiss 700 confocal microscopy.

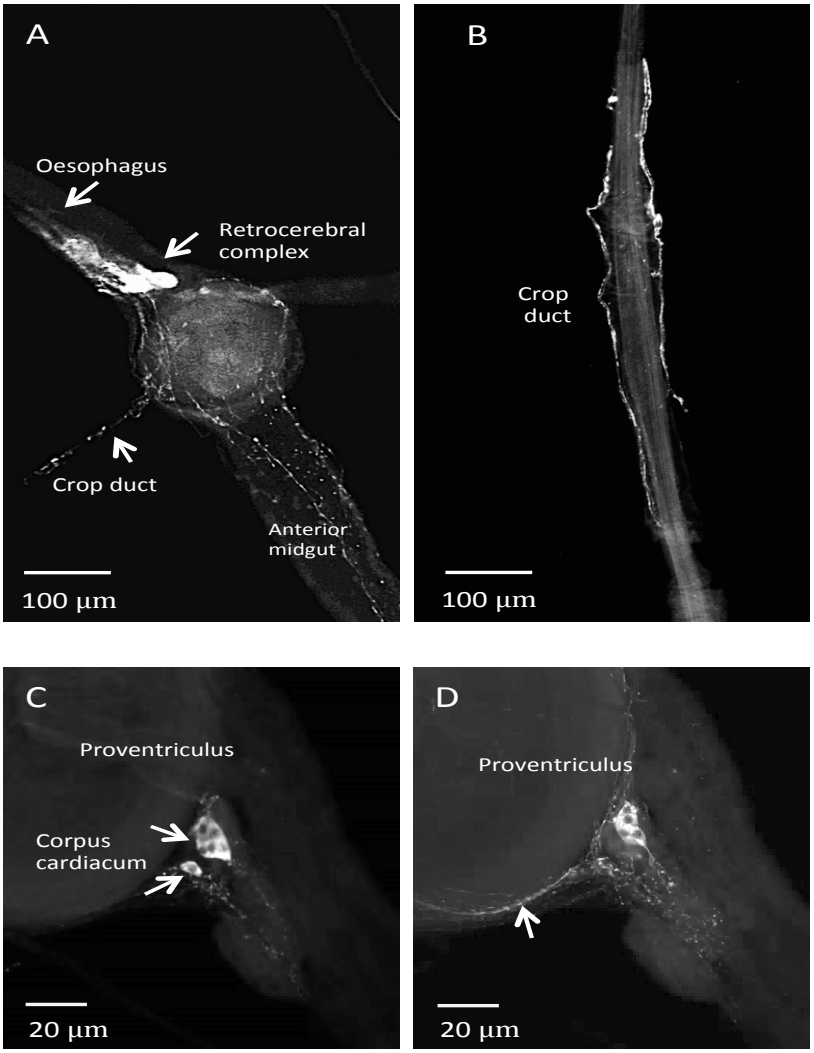


Figure 4.3 Immunohistochemical localization of myosuppressin-like peptide in whole mounts of the foregut and midgut of adult *D. radicum*. Axons on the surface of the oesophagus enter the retrocerebral complex (A). Axons leave the retrocerebral complex and continue to run across the proventiculus surface where they divide, passing over the anterior midgut (A) and along crop duct surface (B) to the lobes of the crop. Scale bar represents 100 µm. The confocal z-stack images of *D. radicum* retrocerebral complex stained with RFamide antibody to localise myosuppressin-like material. Two cells in the corpora cardiaca, identified by arrows in (C), were prominently stained together with immuno-labelled axons, which originate in the corpus cardiacum, covering the proventriculus (white arrow in (D)). The scale bar represents 20 µm.

4.2.3 Mass analysis of crop nerve bundle peptides

In the mass range of 500-2500 Da, two prominent monoisotopic mass ion peaks *m/z*, 974.7 and 1247.8 were present in the mass spectra obtained from single tissue extracts of the crop nerve bundle. These signals correspond to the monoisotopic masses of myosuppressin (TDVDHVFLRFamide) and sNPF$^{4-11}$ (SPSLRFamide), respectively. The sodium adduct of the myosuppressin ion (m/z, 1269.8) was also present (Fig. 4.4). The presence of both the peptide and its sodium adduct provides dual confirmation of the myosuppressin's identity as well as it does helps verifying the accuracy of the mass spectrometer calibration.

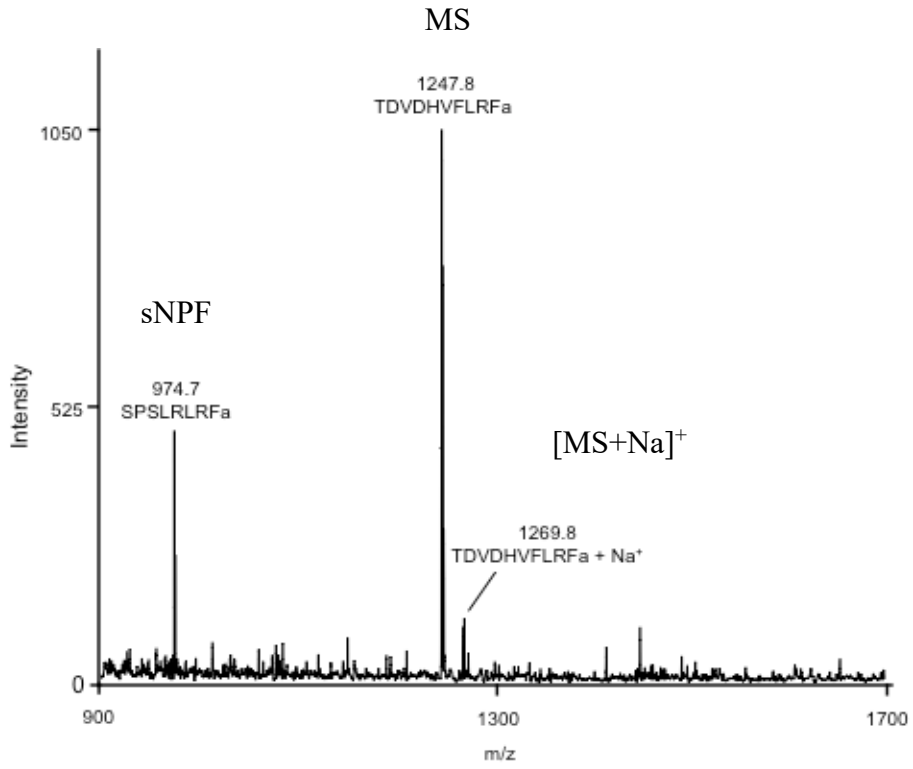


Figure 4.4 Mass spectrum of a single crop nerve bundle from adult *Delia radicum*. Numerical values represent the monoisotopic masses ($[M+H]^+$) or sodium adduct ($[M+Na]^+$).

4.2.4 Inhibition of crop muscle contractions by myosuppressin and Bztc

Myosuppressin inhibited spontaneous contractions of semi-isolated preparations of adult *D. radicum* crop in a dose-dependent manner (Fig. 4.6) with an apparent EC_{50} of 4.4 x10^{-8} M. Spontaneous contractions were recovered when the peptide solution was removed and washed from crop preparations with physiological saline (Fig. 4.5). The

application of the non-peptide agonist Bztc to the isolated crop tissue also reduced the frequency of spontaneous contractions but was less potent (EC_{50} 7.2 $x10^{-6}$ M). Importantly, the crop tissues recovered from inhibitory effect of 1 and 10 µM Bztc, but not 100 µM Bztc, when washed with fresh saline. $sNPF^{4-11}$ had no significant effect on spontaneous contractions of the crop even at high concentrations (10^{-4} M).

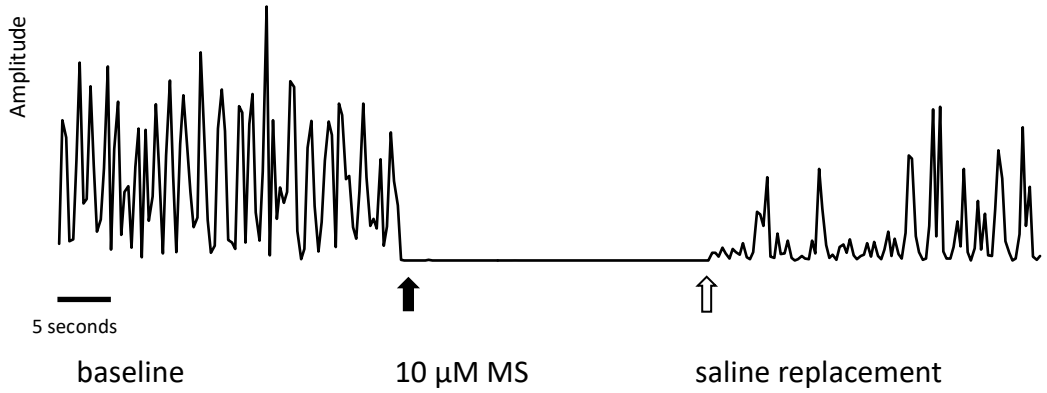


Figure 4.5 Inhibition of spontaneous contractions of the *D. radicum* crop. Spontaneous crop contractions were recorded before and after the application of 10µM myosuppressin peptide (black arrow). The open arrow indicates the removal and replacement of peptide solution with physiological saline. Peptide removal allowed the recovery of spontaneous contractions, as shown by pattern of recorded peaks occurring after hollow arrow. Each individual crop movements were output of intervals and mean of peaks height resulted in amplitude of the crop contractions shown as graph data by method published by Norville et al., (2010).

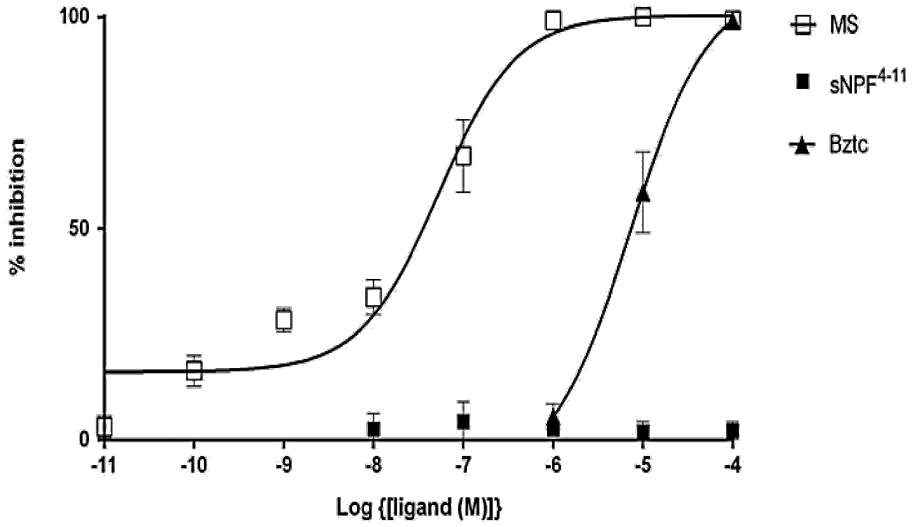


Figure 4.6 Dose-response curves for the inhibition of spontaneous contractions of the crop of adult *D. radicum* by myosuppressin, Benzethonium chloride (Bztc), and short neuropeptide F (sNPF). Data are the mean ± s.e.m., n = 5-10.

4.2.5 *In vivo* effect of peptides and Bztc on adult survival and food ingestion

Single injection of myosuppressin into adult female *D. radicum* had no effect on survival compared to control (saline injected) flies (Kaplan-Meier log-rank survival analysis, P=0.667, 0.416) (Fig. 4.7). In contrast, there was a significant difference in the survivorship between the controls and flies maintained on 5 mM Bztc/agar diet (Log-rank test, P<0.0001) (Fig. 4.8). The survival median was 5 days for Bztc-fed flies, whereas control flies lived for up to 12 days (Fig. 4.9). All the flies fed with Bztc died by day 10, whereas it took 27 days for all the control flies to die. When Bztc was included in the diet containing a food dye, the amount of coloured food passing into the faeces was much less than that occurring in the absence of the agonist, suggesting reduced consumption of food (Fig. 4.10).

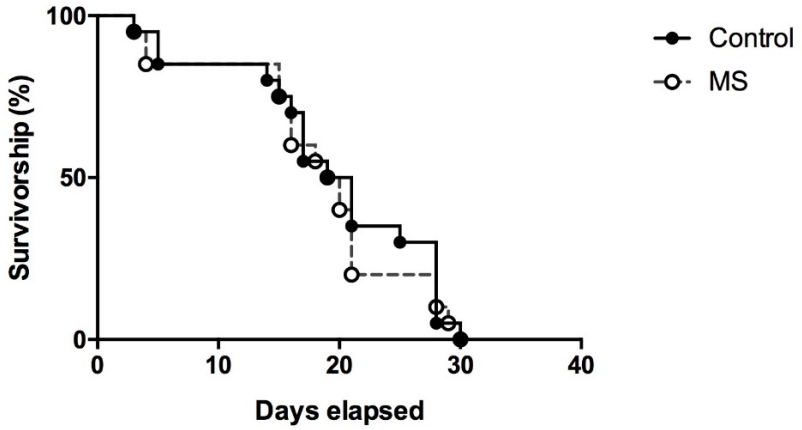


Figure 4.7 Survivorship curve of *D. radicum* females injected with either 1 µl of PBS (controls) or 1µl PBS containing 6.4 µg of myosuppressin (MS). Statistical analysis revealed no significant difference between two groups (Mantel-Cox test, p=0.6676, n=40). Flies were monitored twice daily.

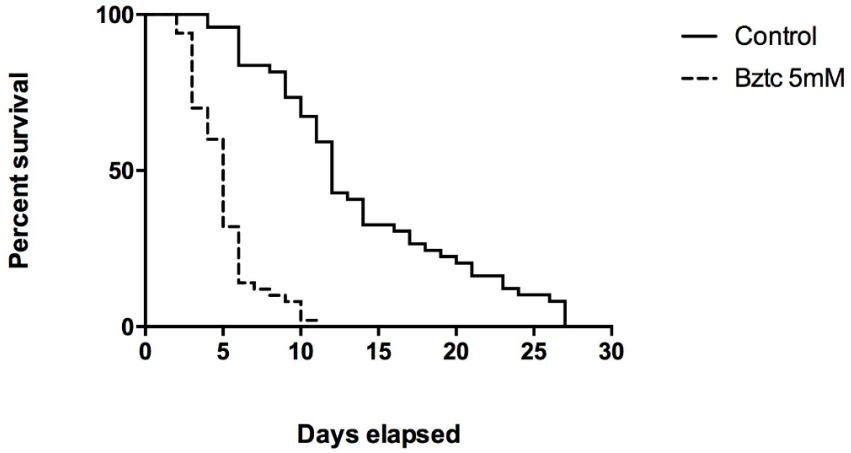


Figure 4.8 Bztc in the diet increases mortality rate. Survival curves of adult female *D. radicum* fed either on 5% sucrose agar (controls, solid line) or sucrose agar containing 5 mM Bztc (dashed line). The diet containing 5mM Bztc caused significant difference in the survival curve compared to the control flies (Kaplan-Meier log-rank survival analysis, P<0.0001, n=96). Flies were monitored daily.

	Days	
Survivorship (%)	Control	5 mM Bztc
75	9	3
50	12	5
25	19	6
0	27	9

Figure 4.9 Percentage survivorship based on the number of days that elapsed before the flies died fed on sucrose agar (control) or sucrose agar containing 5 mM Bztc. The median survival for the control and test group was day 12 and 5, respectively.

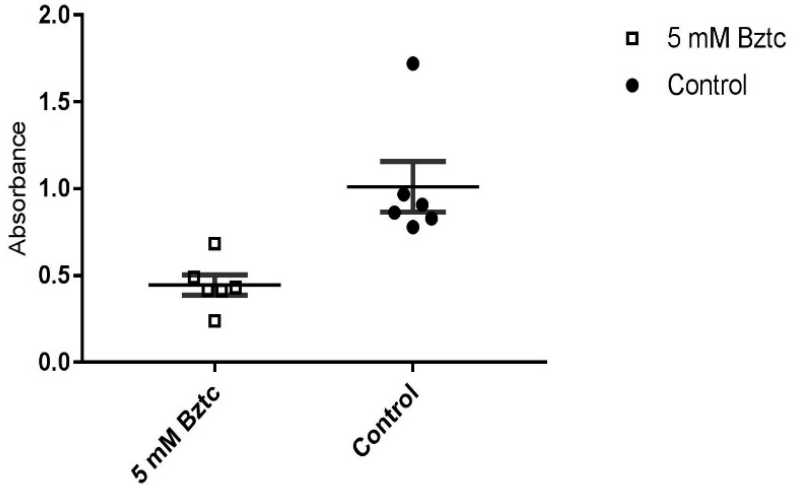


Figure 4.10 Benzethonium chloride (Bztc) reduces ingestion and excretion of sucrose/food dye. The amount of dye in the faeces of *Delia radicum* after 24 hr of feeding was determined spectrophotometrically (595 nm) and the results are expressed as the mean ± s.e.m. (n = 6). Differences in the means values are statistically significant (t-test, P <0.001).

4.2.6 Degradation of myosuppressin by crop peptidases

The degradation of myosuppressin by crop peptidases is vital in the context of developing effective pest control strategies. Understanding the dynamics of how this peptide is degraded by crop peptidases is crutial for determining its stability and effectiveness as a

bioinsecticide. It is essential to protect the peptide from premature breakdown but also understand how the peptide is degraded by crop peptidases aids the development of modified or synthetic analogues, enhancing longevity and effectiveness.

HPLC with uv detection was used to monitor the reduction in 2 nmoles of myosuppressin when incubated with crop homogenate. Myosuppressin degradation by crop peptidases was rapid with an estimated half-life of *c.* 2 min (R^2= 0.9398). MALDI-TOF mass analysis of HPLC fractions identified a number of degradation products (Table 4.1, Fig. 4.11), five of which retained the amino terminus (N-terminus) and six had Phe-amide at the carboxy terminus (C-terminus). One peptide fragment (DVDHVFLR) was truncated at both termini. The mass spectrometric data indicated the involvement of crop aminopeptidases as well as endopeptidase activity capable of cleaving the Arg-Phe peptide bond (Fig. 4.12). In support of this hypothesis, we used fluorogenic aminopeptidase and endopeptidase substrates to show that the crop possessed both peptidase activities (Fig. 4.13). These enzyme activities were measured in both a soluble and a membrane fraction separated from each other by high-speed centrifugation. Around 85% of the endopeptidase (85 ± 2 pmoles/hr) and 68% of the aminopeptidase activity (3.11 ± 0.08 pmole/hr) were located in the soluble fraction.

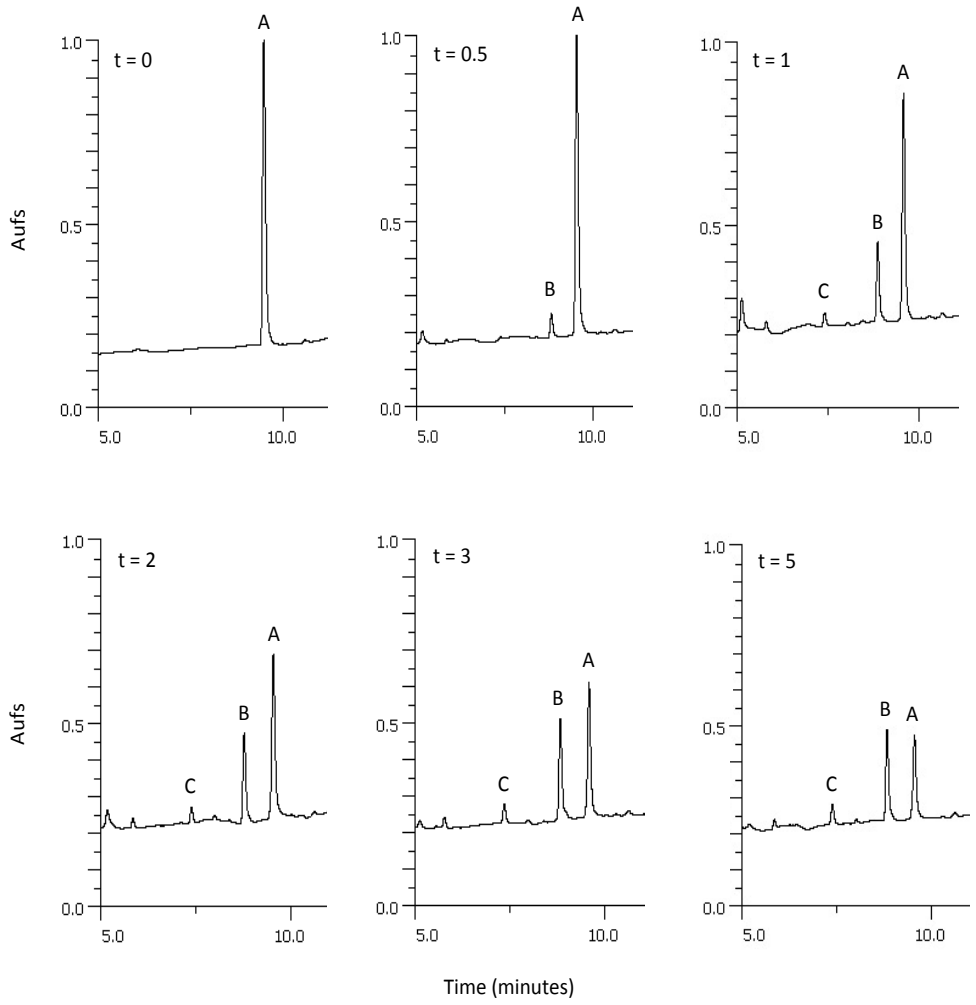


Figure 4.11 HPLC profiles of myosuppressin incubated with crop extract at times t = 0 min, t = 0.5 min, t = 1 min, t = 2 min, t = 3 min, and t = 5 min, showing the degradation of myossupressin (A) and the appearance of degradation products (B and C).

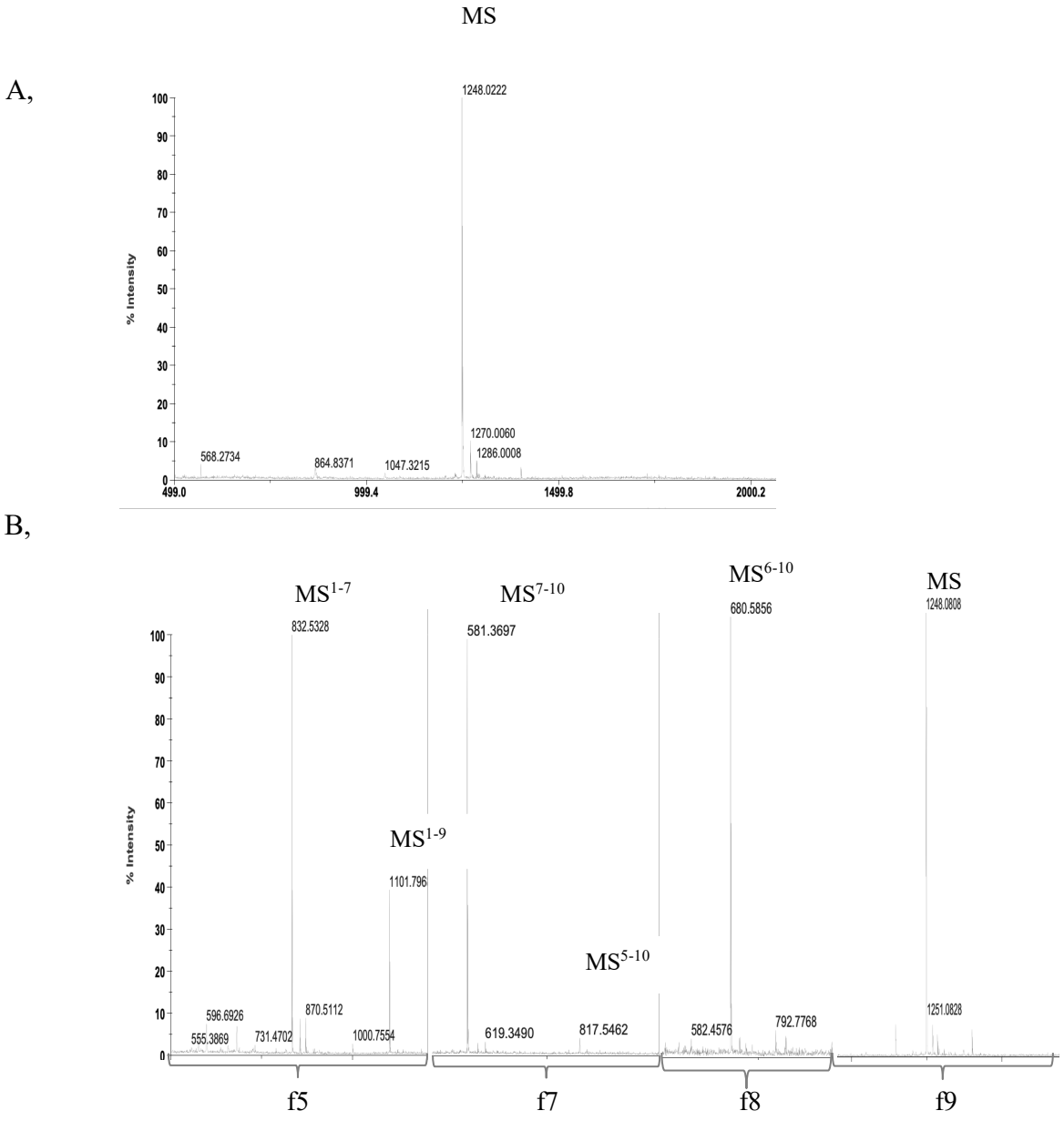


Figure 4.12 Mass spectra of the myosuppressin degradome. HPLC collected fractions of peptide incubation with *Delia radicum* crop homogenate were subject to MALDI-TOF analysis, resulting in spectra of (A) fraction 10 at time 0 (MS peak), (B) fractions 5 (MS^{1-7} ,MS^{1-9}), 7 (MS^{7-10} , MS^{5-10}), 8 (MS^{1-7}) and 9 (MS peak) after 5 min incubation. Numerical values represent the monoisotopic masses ($[M+H]^+$) that were subsequently matched to corresponding values calculated for hydrolysis products in Table 4.1.

Table 4.1 Monoisotopic masses ($[M+H]^+$) and sequences of myosuppressin hydrolysis products calculated by Protein Prospector software (University of California, U.S.A.)

Myosuppressin	Amino acid sequences	$[M+H]^+$
1-10 (intact)	TDVDHVFLRF-NH_2	1247.6
1-9	TDVDHVFLR	1101.5
1-8	TDVDHVFL	945.4
1-7	TDVDHVF	832.3
1-6	TDVDHV	685.3
1-5	TDVDH	586.2
2-10	DVDHVFLRF-NH_2	1146.6
3-10	VDHVFLRF-NH_2	1031.5
4-10	DHVFLRF-NH_2	932.5
5-10	HVFLRF-NH_2	817.4
6-10	VFLRF-NH_2	680.4
7-10	FLRF-NH_2	581.3
2-9	DVDHVFLR	1000.5

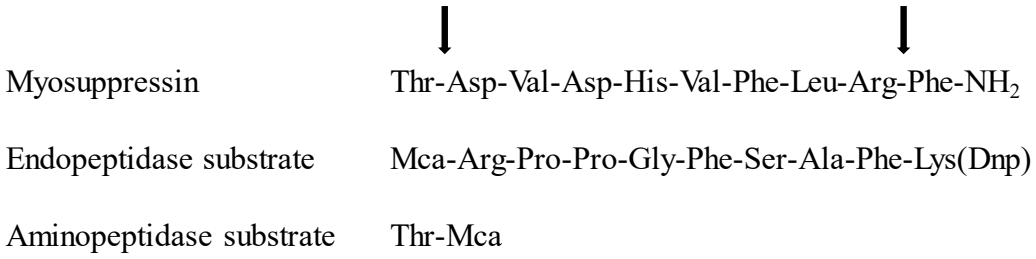


Figure 4.13 Predicted scissile peptide bonds (arrows) of myosuppressin and the structures of substrates used to measure endopeptidase and aminopeptidase activities of the crop.

4.3 Discussion

The release of regulatory peptides in response to external and internal cues is well known to have direct impact on feeding activity from the control of levels of digestive enzymes in response to food stimuli to effectively manoeuvring a food bolus through the gut via coordinated muscle contractions (reviewed by Audsley and Weaver, 2009; Spit et al., 2012). Previous studies have demonstrated that myosuppressins have a role in the regulation of feeding in several insect species. Leucomyosuppressin decreased food intake in the cockroach *Blattella germanica* (Aguilar et al., 2004), while injection of myosuppressin into *Spodoptera littoralis* resulted in anti-feeding behaviour (Vilaplana et al., 2008). In the pea aphid, *Acyrthosiphon pisum,* myosuppressin suppressed feeding resulting in mortality, most likely due to the inhibition of gut motility preventing the movement of food (Down et al., 2011). Feeding the non-peptide myosuppressin agonist Bztc to adult *M. domestica* and *D. suzukii* resulted in early mortality suggesting that myosuppressin signalling has potential as an insecticide target (Haselton et al., 2004; Gough et al., 2017).

The present results established the presence of myosuppressin and sNPF$^{4-11}$ within the crop nerve bundle (CNB) of *D. radicum* and provide several pieces of evidence in support of myosuppressin, but not sNPF$^{4-11}$, as an important regulator of crop function in this pest species. Myosuppressin was demonstrated in the CNB of *D. radicum* by direct peptide profiling using the same approach used by Audsley and colleagues (2015) to show the presence of myosuppressin in the CNB of *D. suzukii*. The current findings however differ from the previous study by the co-occurrence of myosuppressin and sNPF$^{4-11}$. Our identification of these two peptides were based on monoisotopic peaks $(M+H]^{+}$) that are in accordance with the peptide sequences and masses reported by Audsley et al., (2011) and Zoephel et al., (2012) in their peptidomics studies of the larval and adult central nervous system of *D. radicum,* respectively. Commercially available antiserum recognizing the C-terminus of FMRFamide was used to support the claim that myosuppressin/ sNPF$^{4-11}$ neurons extend to the crop muscle of flies (Gough et al. 2017). Myosuppressin and sNPF$^{4-11}$, as well as other insect FaRPs, share the Arg-Phe-amide sequence with FMRFamide and are expected to cross-react with FMRFamide antibodies. Consistent with this expectation, pre-incubation of the antiserum with synthetic myosuppressin blocked the staining of the *D. radicum* nervous system. The immunostaining of the axons in the crop nerve that project from the retrocerebral complex

to the crop muscle and spread over the surface of the crop is consistent with previous reports on the spatial distribution of FaRPs in other dipteran species including the housefly *M. domestica* (Haselton et al., 2004), the fruit fly *D. melanogaste*r (Dickerson et al., 2012b), blowfly *P. regina* (Richer et al., 2000), horn fly *H. irritans* and stable fly *Stomoxys calcitrans* (Meola et al., 1996). The widespread occurrence of FMRFamide-like immunoreactive material in the central and stomatogastric nervous system and enteroendocrine cells of dipterans suggests a general regulatory role for FaRPs in regulating feeding and digestion in this group of insects.

Consistent with an important role for myosuppressin in regulating crop function, the synthetic peptide powerfully inhibited the spontaneous contractions of the *D. radicum* crop musculature. Myoinhibition was observed with nM doses of peptide (EC_{50}, 44 nM) and the effect was immediate and long lasting, but reversible. Such potency is typical of insect peptide receptors and compares well with the potency (EC_{50}, 40 nM) of myosuppressin at activating two cloned G protein-coupled receptor genes (*DmsR-1* and *DmsR-2*) from *D. melanogaster* expressed in mammalian cell lines. Both *DmsR-1* and *DmsR-2* are expressed in the crop of *D. melanogaster,* but *DmsR-1* appears to be more important for myosuppressin signal transduction in the crop of this fruit fly (Chapter 3, unpublished data). In contrast, $sNPF^{4-11}$ failed to elicit a myoinhibitory response when applied to the crop at concentrations even as high as 0.1 mM leading us to conclude that myosuppressin probably works alone to inhibit *D. radicum* crop contractions.

When injected into adult *D. radicum*, myosuppressin had no measurable effect on feeding behaviour or mortality. This lack of a response could have resulted from a failure to reach target gut tissues and/or rapid inactivation. Myosuppressin was rapidly degraded ($t_{1/2}<$ 2min) when incubated with a homogenate of the *D. radicum* crop. MALDI-MS revealed a complex mixture of myosuppressin fragments that suggested multiple initial attacks by aminopeptidase and endopeptidase enzymes. The gradual reduction of native MS gave rise to appearance of UV peaks corresponding to MS^{1-7} and MS^{1-9}, in less than minute. Those were also the most abundant metabolites, indication that the major enzyme activity could be the result of carboxypeptidase(s) and endopeptidases present in homogenate. The latter, such as trypsin-like protease, cleaves arginine from the C-terminal (MS^{1-9}) whereas truncated fragments with hydrolyzed peptide bonds on carboxyl side (MS^{1-7}) are most probably metabolites from the action of carboxypeptidase-like enzymes. Additional emergence of small metabolites (MS^{5-10}, MS^{6-10} and MS^{7-10}) further indicates the activity of aminopeptidases by sequentially truncating fragments from N-termini. In

conclusion, MS appear to be susceptible to a proteolytic cleavage by endopeptidases, carboxypeptidases and aminopeptidases present in the crop, capable of generating biologically inactive metabolites in relatively short amount of time. Indeed, we confirmed the presence of both aminopeptidase and endopeptidase activities predominantly in a soluble fraction of the crop homogenate. A very similar pattern of rapid degradation was reported for leucomyosuppressin (pEDVDHVFLRF-NH_2) by gut extracts of two moths, *Lacanobia oleracea* and *Spodoptera littoralis* (Matthews et al., 2009; Down et al., 2011). A structure-activity study of the inhibitory activity of N-terminally truncated myosuppressin peptides on adult *D. melanogaster* crop contractions showed that removal of the N-terminal tripeptide resulted in loss of activity. When tested the same peptides were tested on larval gut, only the parent 10-mer peptide gave a full inhibitory response. A similar study of leucomyosuppressin inhibition of the cockroach (*Leucophaea maderae*) hindgut identified VFLRFamide as the core fragment, although this activity was at least two orders of magnitude below that of the intact peptide. In conclusion, myosuppressin is susceptible to rapid breakdown by peptidases present in the crop. Many of the fragments generated will probably have weak or no agonist activity on the *D. radicum* crop as shown by diminished inhibition activity of alanyl-substituted and N-terminal truncated analogues in *D. melanogaster* (Dickerson et al., 2012). These studies emphasise the need for myosuppressin analogues that are resistant to degradation by gut peptidases when testing as oral activity to have a long-lasting effect.

Benzethonium chloride (Bztc), a quaternary ammonium salt, is recognised as a myosuppressin agonist, sharing structural features (Chapter 3, Fig.3.4), with ability of a competitive displacement of radioactively labelled myosuppressin from both high- and low-affinity myosuppressin receptors in locust oviduct membranes (Lange et al., 1995). In the present study, the inhibitory effect of Bztc was 100-fold less potent compared to myosuppressin and the recovery of spontaneous contractions after the Bztc was replaced with saline was noticeably slower compared to the peptide. Our results are in accordance with findings reported by Stoffolano et al., (2013) and Lange et al., (1995), where in both instances Bztc reversibly inhibited muscle contractions. Furthermore, Richer (et al., 2000) described Bztc action in mM range to be equivalent to myosuppressin peptide, terminating spontaneous crop contractions in the blowfly *P. regina*. However, it remains unclear how Bztc mimics the effect of myosuppressin on muscle contractions. Egerod et al., (2003) could not demonstrate that heterologously expressed *Dms-R1* and *Dms-R2* of *D. melanogaster* were activated by Bztc in a specific manner and it is possible that some

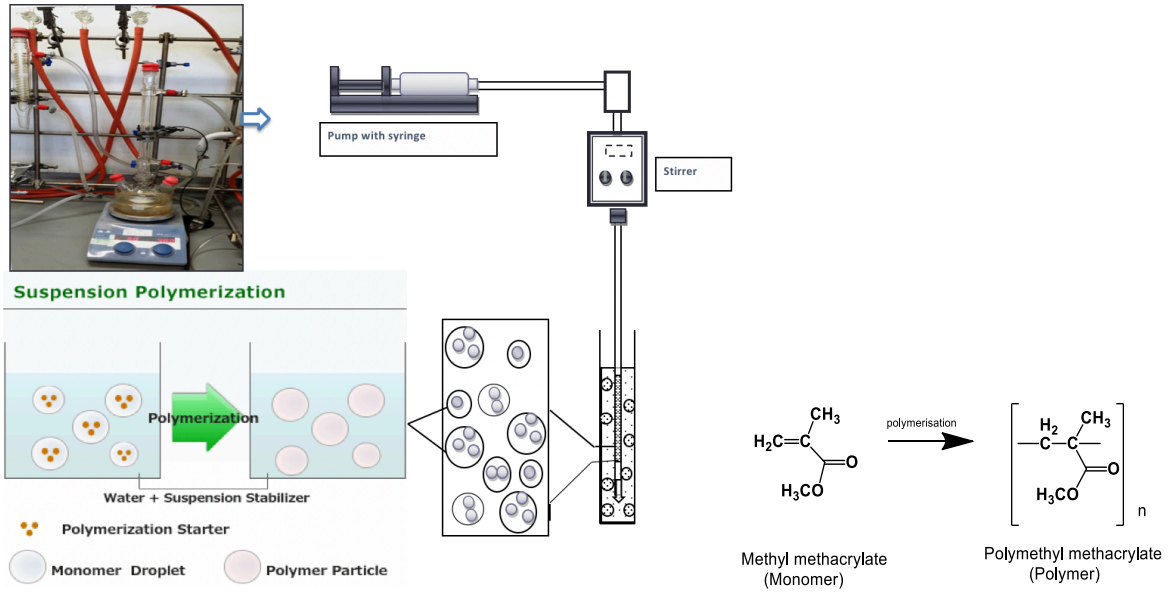
of the physiological effects of this quaternary ammonium salt results from its weak surfactant properties. Further investigations into Bztc's impact on food intake were conducted by testing it as part of the diet. Using dye-labelled food to follow diet ingestion and excretion in adult *D. radicum* showed that Bztc significantly affected food intake, likely contributing to the observed chemical toxicity, as evident by a marked reduction in lifespan. Given these findings, a deeper insight into the mechanisms underlying this toxicity is required before conclusively state that myosuppressin signaling is the pathway being targeted by Bztc.

Chapter 5 Feeding dipteran pests with pH-responsive particles

5.1 Introduction

The strategic use of encapsulation technologies has emerged as a transformative approach in the field of biological insect control, particularly for the delivery of water-soluble peptides. Such encapsulation serves as a defensive barrier, safeguarding the active compounds from environmental degradation and the harsh conditions within the gut digestive system (Rai and Posten, 2013). In collaboration with the Institute of Particle Science and Engineering, University of Leeds, this project's objective is to establish a proof-of-principle for the targeted delivery of novel insecticidal neuropeptides via smart microcapsules, engineered to release their contents selectively within the alkaline environment of the insect gut, thereby offering a safe and specific pest control solution. Based on observations and experiments detailed in previous chapter, which highlight the degradation of myosuppressin peptide by crop peptidases, there is a clear need to engineer protective delivery systems that can safeguard the peptide's integrity, ensuring the efficiency of its biopesticide action.

Advances in chemical engineering can drive the development of innovative methods to be applied in the agriculture sector. For example, nano-materials are being utilised in a variety of ways, as fertilizers, for soil improvement or for water purification (Dimetry and Hussein, 2016). Microcapsules have potential for the protection and delivery of bioinsecticides that are vulnerable to environmental and metabolic degradation. Bioinsecticides are often viewed as ‘greener’ alternatives offering improved selectivity and lower environmental impact, but their exploitation can be limited by poorly developed formulation and delivery systems. For example, encapsulation offers a possible solution to some of the major drawbacks of exploiting neuropeptides, such as neuropeptides and neuropeptide mimetics as insecticides- susceptibility to proteases and poor bioavailability.



(image credit: Dr.Areej Al-khalaf; Manga et al., 2012)

(image credit: Stickler, M., Rhein, 1992)

Figure 5.1 The synthesis of smart particles. The encapsulation via multiple emulsion suspension polymerisation could be very briefly described as a process of the incorporation of water phase into oil phase containing pH responsive monomer and emulsified using an ultratorax mixer (shown above). The water/oil/water emulsion is emulsified again and during heating oil phase it polymerises to form a pH-responsive shell. Interfacial polymerisation allows final separation and encapsulation of smaller particles through steering but this time in the presence of stabiliser. The emulsion is then allowed to polymerise by continuous stirring for several hours.

Recently, Ferguson et al., (2018) developed a novel delivery system for insect control agents using smart pH-sensitive microcapsules. The loading and release of the biomolecules from the microcapsules is controlled by changes in pH. The pH in the lumen of many insects is strongly alkaline, but in some dipteran insects there is an acid 'stomach' region in the midgut (Shanbhag and Tripathi, 2009). Microcapsules, made by polymerisation of 2-vinylpyridine monomers (P2VP) using a multiple emulsion template, responded to an alkali to acid pH change by dissolution. These microcapsules were loaded

with FITC-labelled dextran and fed to adult *Drosophila suzukii*. On reaching the acid region of the fly's midgut the FITC-labelled dextran was released and taken up across the gut wall.

To aid the design of pH-smart materials suitable for delivery of bioinsecticides to two of our target pests (medfly, *C. capitata* and the cabbage root fly, *D. radicum*), the pH profile of the gut of these economically important pest species was investigated using pH-sensitive dyes presented in the food. There was a particular focus on the changes that might occur along the length of the midgut (anterior, middle and posterior) to identify differential pH conditions that might be exploited in a similar manner to that described for *D. suzukii*.

In developing targeted delivery systems for neuropeptide-based biopesticides, understanding the ingestion capabilities of the target insects is critical. This chapter will detail our investigations into the size-selective feeding behaviors of different insect species, utilizing a spectrum of polydisperse solid poly(methyl methacrylate) (PMMA) particles. By marking these particles with the lipophilic dye, Sudan III, we assessed the upper size limit for ingestion. Our methodology reveals that the adult medfly, with its specialized feeding apparatus composed of fine tubes and micropores approximately 0.5 μm in diameter, inherently restricts food intake to liquids or particles smaller than 0.5μm. This study upholds the selective feeding mechanism of the medfly, providing a foundational understanding of its dietary limitations. In stark contrast, cabbage root fly demonstrates the ability to ingest and subsequently excrete PMMA particles exceeding 0.5μm. The implications of these distinct physiological differences are profound, prompting us to proceed with experiments involving pH-responsive P2VP microcapsules exclusively with *D. radicum*, as its ingestion mechanism aligns with the delivery specifications of our proposed biopesticide system.

5.2 Results

5.2.1 pH profiles of the intestines of *Ceratitis capitata* and *Delia radicum*

In order to assess the potential of pH-responsive polymer microcapsules for oral delivery of bioinsecticides, the pH environment of the intestine of medfly, *C. capitata* and the cabbage root fly, *D. radicum* was investigated by feeding pH-sensitive dyes incorporated into the diet.

Feeding phenol red to *C. capitata* resulted in intense red or pink colour (pH, ≥8) in the crop and in the anterior and posterior regions of the midgut (Fig. 5.1). A band of bright yellow was positioned around half-way along the midgut in between the two alkali sections, indicating a sharp change in pH from ≥8 to 6.8 or below.

Bromophenol blue is a pH indicator that turns yellow below pH 3 and is blue above pH 4.6. When the indicator was included in the medfly diet a strong blue colour was seen in the crop and anterior midgut with fainter blue in the posterior midgut (Fig. 5.2). Noticeably, no yellow staining of the midgut lumen was observed (10 samples), suggesting that the yellow region of Fig. 5.2 does not reach pH 3.

Phenol red ingested into the crop of *D. radicum* turned into a bright yellow colour (≤6.8). The colour changed from yellow to red after the cardiac valve that separates the foregut from the midgut indicating a rise in pH to ≥8 (Fig. 5.3). The contents of the hindgut appeared intermediate between red and yellow indicating another change in pH. Feeding bromophenol blue to the cabbage root fly stained the gut contents uniformly blue from the crop to the hindgut, indicating that intestinal pH does not drop to pH 3 .

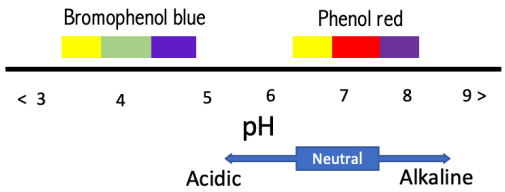


Figure 5.2 The scale of water-soluble pH indicators used to stain *Delia radicum* and *Ceratitis capitata* alimentary tract. In the presence of acid (pH <6.2) the Phenol red turns yellow. Increasing pH transits the colour to deep pink, indicative of alkaline environment. Bromophenol blue changes its colour from blue to shades of green to bright yellow at very low pH ≤3.

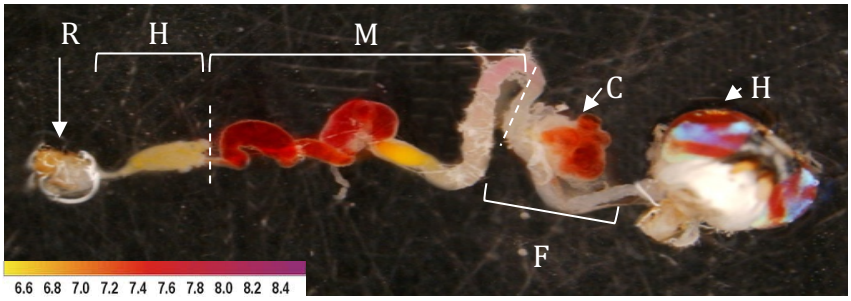


Figure 5.3 The gut of an adult medfly fed with the phenol red. Yellow stain of ingested indicator indicates an acid region of the midgut (<6.4), whereas red is alkaline (pH ≥8). (H-head capsule, C-crop, F-foregut, M-midgut, H-hindgut, R-rectum, arrow indicates anterior to posterior orientation; 7.3X magnification).

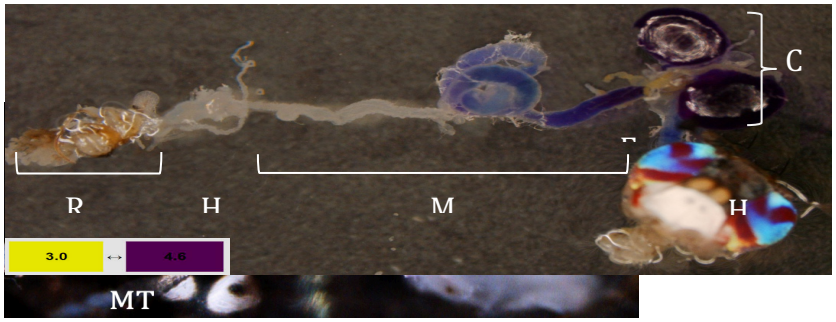


Figure 5.4 The gut of an adult medfly fed with bromophenol blue. The blue staining indicates pH >4.6 and the absence of yellow in (A) the whole gut regions indicates no pH ≤3. (C-crop, M-midgut, H-hindgut, R-rectum, MT-Malphigian tubules, arrow indicates anterior to posterior orientation; 7.3X magnification).

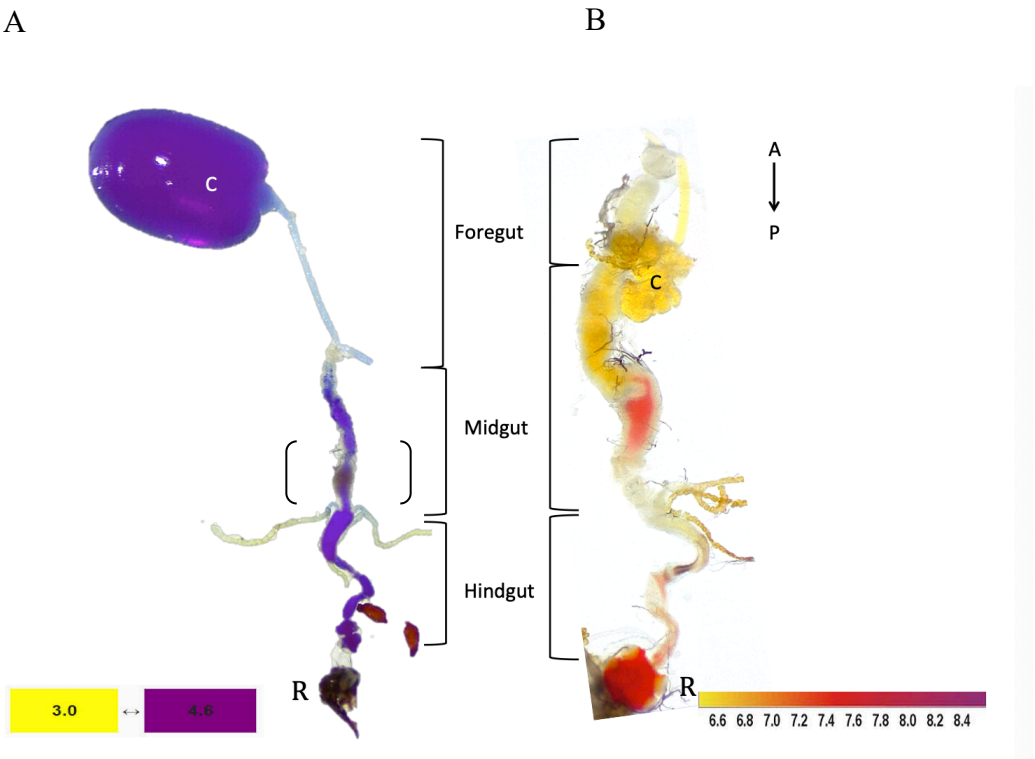


Figure 5.5 The gut of an adult *D. radicum* fed with (A) bromophenol blue and (B) phenol red. Diet of bromophenol blue stained the whole digestive tract in relatively uniform colour, except for midgut region highlighted in brackets. Phenol red -stained regions of yellow indicate a pH <6.4, whereas red marks alkaline (pH ≥8). Open brackets indicate the middle midgut region, a section of the digestive tract where acidic region is expected. (C, crop; the arrow indicates anterior to posterior orientation).

5.2.2 Feeding solid poly(methyl methacrylate) particles to Ceratitis capitata and Delia radicum to assess any size limitation by the mouth parts

To determine the size limit of particles that can be ingested and passed into the intestinal tract of adult *C. capitata* and *D. radicum,* we added solid poly(methyl methacrylate) particles (2 - 200 µm diameter, PMMA) labelled red with Sudan III to the sucrose diet. Flies were allowed to feed on the particles for 24 hr before the intestines were dissected and analysed for particles.

For *C. capitata,* careful screening of the crop, midgut, hindgut and rectum failed to detect any PMMA particles. The same experiment was conducted with adult *D. radicum* and for these flies ingested PMMA particles were observed throughout the digestive tract from the crop to the rectum (Fig. 5.6). Furthermore, in freshly dissected intestines, sudan red-

labelled PMMA could be seen being transported along the gut lumen by peristalsis. Noticeably, in several individuals 'bubbling' behavior typical of *D. radicum* was observed, where regurgitated crop contents contained PMMA particles. These observations suggest that that the presence of PMMA in the diet does not cause any serious obstruction or disturbance to feed (Fig.5.4).

5.2.3 Feeding pH-responsive poly(2-vinylpyridine) containing FITC dextran (P2VP/FITC) microcapsules to *D. radicum*

P2VP/FITC microcapsules fed to adult *D. radicum* were found concentrated in the crop and were present throughout the midgut, in the hindgut and in the rectum. It was therefore not unexpected to find intact fluorescent microcapsules in faeces produced by these flies. There appeared to be some non-particulate fluorescence, particularly in the crop, indicating some release of FITC-dextran. The presence of intact particles in the hindgut, rectum and in the faeces indicated a failure of the microcapsules to fully unload their cargo of FITC-dextran.

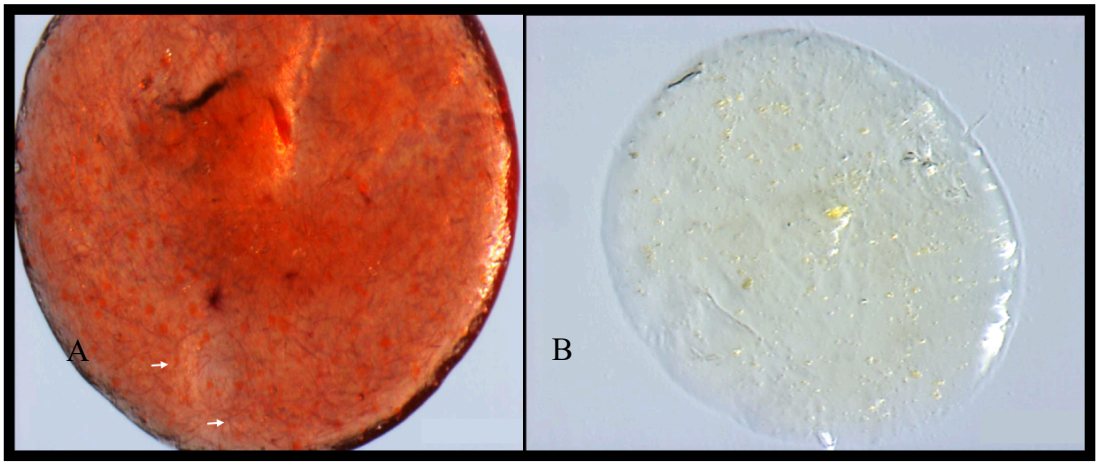


Figure 5.6 Regurgitated crop content and excretion containing stable PMMA particles in *D. radicum*. A, Image of regurgitated content from "bubbling" behaviour containing sudan red-labeled PMMA particles (white arrows; 40x magnification). B, Sample of excreta containing undigested particles (25x magnification).

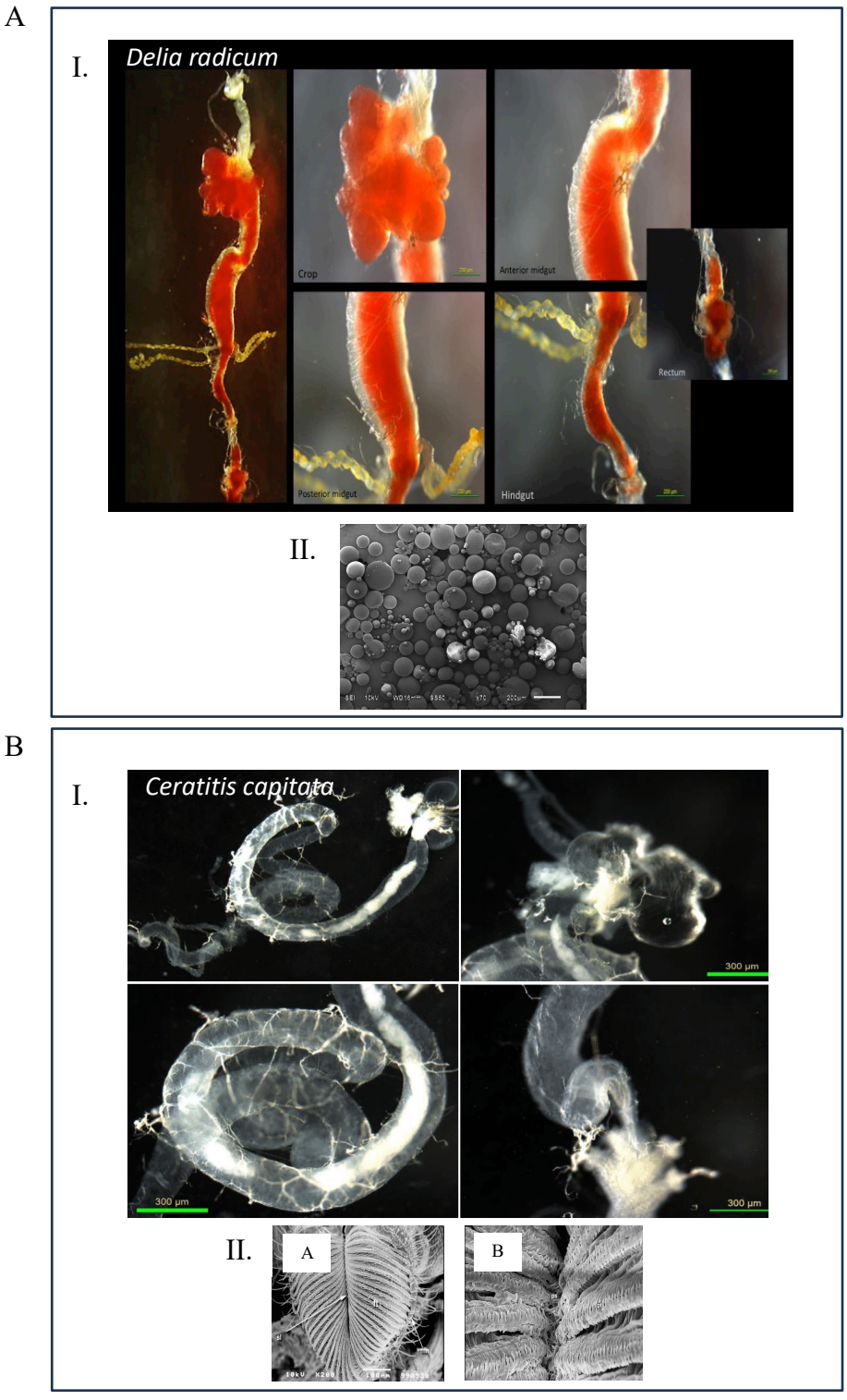


Figure 5.7 Collage of dissected adult guts of *D. radicum* (top) and *C. capitata* (bottom) fed with PMMA particles labelled with sudan red. A I. left; entire alimentary canal, left top; crop, left bottom; posterior midgut, right top; anterior midgut, right bottom; hindgut, far right; anus. A II. Scanning electron microscope image of PMMA particles emulsion after polymerization (scale 200 µm, credit: Dr. Al-khalaf, Academic visitor at Leeds University). B, Collage of medfly's alimentary tract fed with PMMA particles labelled with sudan red. Top left; entire alimentary canal, bottom left; midgut, right top; crop, right bottom; hindgut and rectum. B II. Electron micrographs of the mouth parts of adult *C. capitata* showing (A) labellar pseudotracheae structure that forms (B) interlocking tubular structures functioning effectively as 'sieve' (Credit images: Coronado-Gonzalez et al., 2008).

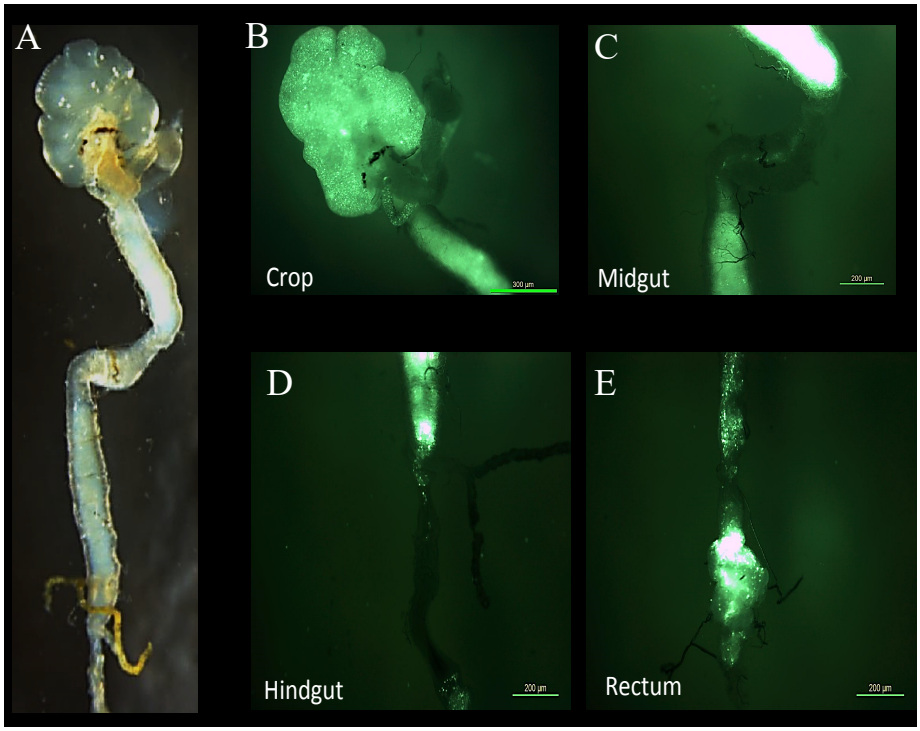


Figure 5.8 The gut of *D. radicum* fed P2VP/FITC-dextran microcapsules. (A) Bright field image. (B-E) fluorescent micrographs of the crop, midgut, hindgut, rectum.

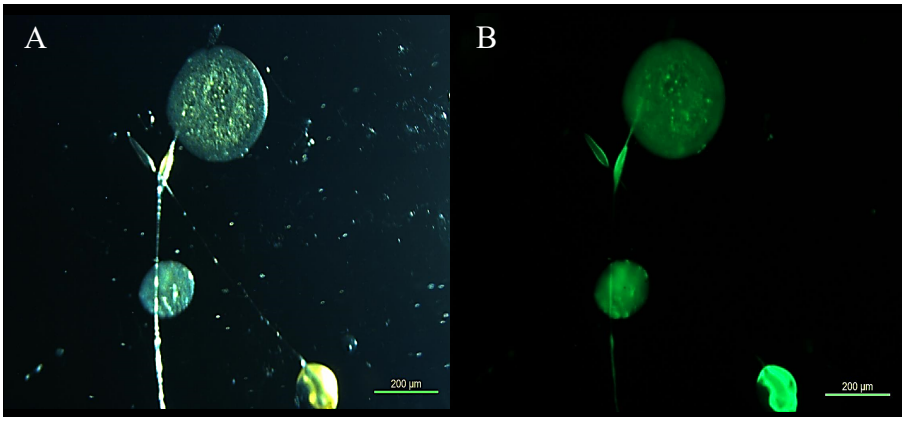


Figure 5.9 Image of excreta of *D. radicum* fed with fluorescent containing P2VP/FITC-dextran particles under (A) bright field and (B) fluorescent microscope.

5.3 Discussion

Encapsulation of water soluble biological insect control chemicals, such as peptides, can offer protection against degradation in the natural environment and when exposed to gut digestive enzymes. Ferguson et al. designed microcapsules suitable for sequestering hydrophilic molecules for oral delivery to the midgut of the fruit fly *D. suzukii* (Ferguson et al., 2018). They exploited sharp changes in the midgut pH of this insect to design smart pH-responsive microcapsules for the rapid release of a hydrophilic cargo. The capsules were made using a pH responsive monomer that polymerized to a polymer with a pKa of 4.2. At a pH below 4.2, these microcapsules swell due to protonation of the tertiary amine of 2-Vinylpyridine monomer, before dissolving and releasing their contents as a result of diffusion and osmotic pressure.

Microcapsules packed with FITC-dextran (10,000 Da) are stable at pH 6, but once the pH dropped to 3.8, full dissolution of capsules occurred rapidly. *In vivo* studies showed that the microcapsules released FITC-dextran once they reached the acid zone (pH<3) of the adult midgut of *D. suzukii*.

For this strategy to work in other insects, the capsules need to be small enough to be ingested intact into the digestive tract with ingested food. In the present study, solid PMMA polymers labelled with Sudan red with a size range from 64 to 200 µm, with mean lenghth of 124 µm (Fig. 5.7 A II.) were fed to both *C. capitata* and *D. radicum* to find any size restrictions. Unlike *D. suzukii* and *D. radicum*, adult *C. capitata* failed to ingest any of the PMMA particles (Fig. 5.7 B). Detailed observation of the crop, the whole length of the gut and rectum did not contain any particles except of bolus of ingested glucose. It reflects the intriguing differences between *C. capitata* and other dipteran flies in their peculiar adaptation of mouthpart structures resulting in selective food intake. This insect has many pseudotracheae tubules containing spikes that interlocks together, effectively creating a sieve in the medfly food canal cavity (Coronado-Gonzalez et al., 2008). This labellar filtering prevents anything above 0.5 µm entering the trachea. The food canal cavity that extends to the pharynx, has also restricted opening allowing only particles smaller than 40 µm to enter. Their anatomy allows them to filter anything above 0.5 µm in size from their diet.

The pH profile of the intestine of both *D. radicum* and *C. capitata* was investigated using pH indicator solutions. The pH of the cabbage root fly intestine changed from <6.4 in the crop, foregut and anterior midgut to >8 in the middle/posterior midgut. For adult medfly a sharp drop in pH was seen in the central region of the midgut, equivalent to the acid zone of *D. melanogaster* and *D. suzukii*, however, the pH of this zone was not as low as the acidic midgut region (pH < 3.0) of the Drosophila species (Shanbhag and Tripathi, 2009; Ferguson et al., 2018). The pH profile of the insect gut is not only a determinant for targeted pesticide release but also a factor in the stability of such compounds. For *D. radicum* and *C. capitata*, where the crop and anterior midgut exhibit a pH of less than 6.4, similar to the more alkaline midgut regions, insecticidal agents must be shielded from both the pH conditions and the peptidases prevalent in these digestive sections. This dual challenge necessitates enhanced protective measures for any bioactive agents administered, ensuring that they remain intact and effective until they reach their intended site of action within the insect gut. The creation of delivery systems that can withstand varying pH levels and enzymatic degradation is thus a critical step in the development of efficient and durable pest control solutions. The pH within an insect's digestive tract is often reflective of its dietary habits. For instance, in the German cockroach (*Blattella germanica*), the pH of the crop is not fixed but varies according to the type of food consumed, demonstrating the adaptability of the insect's digestive environment to its diet (Day and Powning, 1949).

P2VP/FITC-dextran microcapsules were ingested by adult *D. radicum* and passed into the gut uninterrupted. Solid microcapsules were present in the midgut, hindgut and rectal region and in excreta deposited on to the surface of a petri dish, showing that, unlike with *D. suzukii,* the P2VP did not dissolve in the intestine to release FITC-dextran. This difference between the two species probably reflects the observed weaker acidic environment found in the *D. radicum* midgut.

In conclusion, although pH-responsive microcapsules worked well to release water soluble cargo in adult *D. suzukii*, the same polymer was not suitable as a delivery system for *D. radicum* and *C. capitata* for two different reasons. Encapsulation is not appropriate for medfly because of the specialized mouthparts that filter out particles > 0.5 μm and for *D. radicum*, the chemistry of the polymer needs to be taken into consideration to respond and release its cargo in less acidic regions of digestive tract.

Alternatively, to enhance the stability and delivery of neuropeptides for biopesticide use, various strategies beyond traditional particle systems are employed. Liposomes offer a

biocompatible shield, encapsulating the active molecules for targeted release, as reported for dsRNA delivery in *D. suzukii* (Taning et al., 2016). Biodegradable polymer microcapsules provide a similar protective function with the added benefit of controlled degradation, while protein fusion techniques improve molecular stability, as demonstrated with diapause hormone (see Zhou et al., 2015). Additionally, bioconjugation techniques enable precise delivery, and chemical modifications of the neuropeptide itself can directly increase resistance to proteolysis (Kesharwani et al., 2012). Lastly, the use of silk fibroin particles is emerging as a promising method (Carissimi et al., 2021), leveraging the natural bioprotective properties of silk to safeguard these delicate molecules below the required range of 500 nm.

Chapter 6 Neuropeptides of *Ceratitis capitata*: the neuropeptidome and the role of Myosuppressin

6.1 Acknowledgment of contribution

All experiments and sample preparations were undertaken by the author of this thesis under the guidance of Prof. Isaac (Leeds University, UK) and Dr. Audsley (Fera Science Ltd., UK), except for mass spectrometry analysis of *C. capitata* tissues that were examined by Dr. L. Ragionieri (University of Cologne, Germany) and provided information were used to complement Table 5.3 of predicted masses of putative neuropeptides and provided the mass spectra analysis of the brain and MS/MS fragmentation of myosuppressin displayed in Fig. 6.10.

6.2 Introduction

6.2.1 The Mediterranean Fruit Fly, Ceratitis capitata background

One of the most destructive insect pests of agriculture is the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) that belong to the *Tephritidae* family, also referred to as "true fruit flies". It is recognized as a highly invasive species, is an EPPO A2 listed pest and subject to surveillance programmes and quarantine regulations, including import restrictions, across the world (Malacrida et al., 1998; OEPP/EPPO, 2011). It is extremely polyphagous being attracted to over 300 fruits, flowers, vegetables and nut trees (Liquido et al., 1991). Its origin can be traced to the eastern sub-Saharan region of Africa. Increased global trade, however, and ease of adaptation to ecologically diverse habitats has led to the successful invasion and establishment in many fruit-growing areas around the world (Fig. 6.1), (Gasperi et al., 1991; Diamantidis et al., 2011).

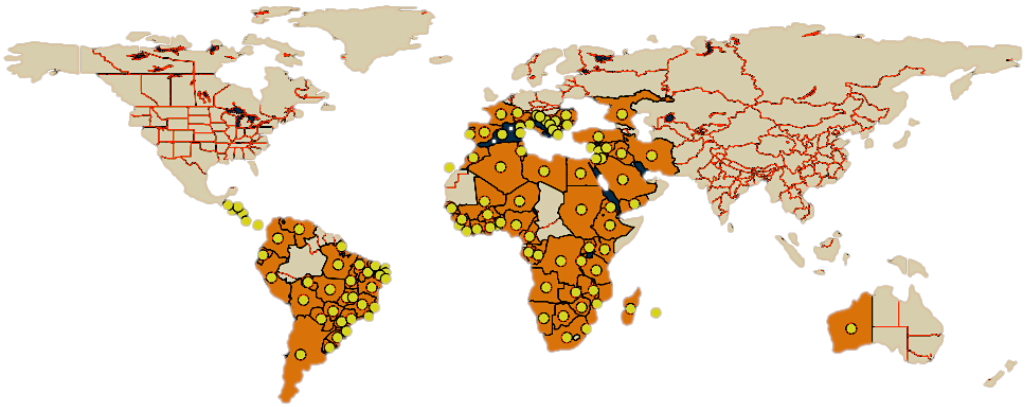


Figure 6.1 Distribution of *Ceratitis capitata* (yellow dot) across the world based on reports and surveillances deposited in European and Mediterranean Plant Protection Organization database (EPPO, 2019 Global Database, **https://gd.eppo.int**).

The agricultural nuisance is exacerbated by the characteristic oviposition behaviour of *C. capitata* females. They display plasticity in seeking an oviposition site, depositing eggs into immature, still ripening fruit and even attack crops that do not support the growth of larvae (Prokopy et al., 1989; Carey et al., 2006). The damaged flesh of those fruits often become subject to secondary bacterial and fungal infections resulting in the fruit decay and loss of market value. This consequentially leads to greater harvest loss with

devastating economic implications. It is estimated that the medfly causes annual losses of more than $US 2 billion to commercially important fruit (Malavasi, 2014). Protected from insecticides and parasites during maturation inside the fruit, the last larval instar has adopted a unique feature of escaping from its host plant to pupate in the soil. This is achieved by impressive leaps, whereby mouth hooks become firmly attached to the cuticle of the posterior end effectively forming a pressurized loop (Maitland, 1992), providing a rapid escape mechanism from potential danger. Additionally, their early morning synchronized emergence from the fruit host further reduces the risk of predation from generalist predators and pupal parasitoids (Cayol et al., 1994; Warburg and Yuval, 1997; Yuval and Hendrichs, 1999). The success of the medfly's migration and subsequent colonization can be attributed to many factors, such as global trade, increased crop production and associated transportation, tourism and smuggling of prohibited food (Malacrida et al., 2007). It has been proposed that invasive insect species, such as the medfly, are of significant financial burden to European countries with an estimate of over €12 billion a year (Szyniszewska and Tatem, 2014). The attempt to eradicate the medfy from the U.S.A., particularly in Florida and California, have hit the taxpayers heavily: a bill for California's effort to eradicate the medfly reached $500 million and Florida set aside $25 million to prevent the establishment of this pest. Moreover, once the presence of the medfly is confirmed, the impact of embargo on exports is estimated to be cost more than half million dollars together with losses of thousands of jobs (Hendrichs et al., 2002; Suckling et al., 2014; Siebert and Cooper, 2008).

6.2.2 Medfly's crop

The adult crop is common to all dipteran insects and primarily serves as storage organ for excess nutrients. The crop has been studied in some detail in *C. capitata* with circular muscle fibres lying on top of longitudinal muscle fibres (Caetano et al., 2006). An important function of the medfly crop is the regurgitation of liquid, which is thought to provide an efficient way of eliminating excess liquid from the diet. Some theories suggest that bubbling might be linked to the spread of symbiotic bacteria with the droplets providing a place for their proliferation and as a means of spreading the bacteria between flies by inoculating the plant surface with intestinal microbes (Drew and Lloyd, 1987; Hendrichs and Cooley, 1992). In contrast to other dipteran species that salivate for the purpose of digestion of ingested solid food, the short hypopharynx in medfly passes

liquids directly from the crop onto solid food and if the flow from the crop is interrupted, medfly cannot feed (Coronado-Gonzalez et al., 2008). Owing to the fact that the function of the crop is totally dependent on peristaltic movements of muscular lobes and sphincters that translocate the liquid/food to and from the crop, any disturbance of the regulation of crop muscle activity is likely to have deleterious effects and therefore be effective for pest control.

6.2.3 Medfly neuropeptidome

Over the last two decades, mass-spectrometry based neuropeptidomics approaches have proven successful in the structural characterization of signalling peptides in several insect pest species (Verleyen et al., 2004; Liu et al., 2006a; Hauser et al., 2006; Wegener et al., 2006; Predel et al., 2012; Zoephel et al., 2012; Rahman et al., 2013; Audsley et al., 2015). Such approaches in conjunction with the mining of peptide precursor genes from genome sequencing projects can provide valuable information about the post-translational processing and modification of neuropeptides in cells of the nervous and endocrine systems. Only recently the genome of *C. capitata* was published as a part of the i5k initiative (Evans et al., 2013; Papanicolaou et al., 2016) that allowed us to bioinformatically identify putative sequences for neuropeptides based on conserved features shared between the *Ceratitis* and *Drosophila* genomes.

6.3 Experimental aims

We advanced our investigation by employing phalloidin staining to define the crop musculature, complemented by immunohistochemistry to verify the presence of myosuppressin, building on the groundwork laid in *D. melanogaster* (Chapter 3) and *D. radicum* (Chapter 4). To validate the immunostaining approach in the crop, we analyzed the crop nerve bundle via mass spectrometry and evaluated the influence of the endogenous peptide through *ex vivo* assays on isolated crop tissues. Additionally, we assessed the *in vivo* impact of myosuppressin through abdominal injections, monitoring its effects on adult feeding behavior and longevity through survivorship and fecal output measurements. Finally, we explored publicly available genomic datasets to annotate and predict neuropeptide-encoding transcripts within the *C. capitata* genome based on *in silico* analyses.

6.4 Results

6.4.1 Localisation and functionality of medfly myosuppressin

6.4.1.1 Phalloidin staining

Phalloidin F-actin staining of crop revealed the prominent myofibres of the crop sac that were, approximately 15 µm wide. In the region where the crop duct attaches to the base of the sac there are circular muscles. All circular muscles originate from the base and radiate across the whole crop uniformly as a single unit. Each individual fibre is interconnected across the whole length with neighbouring myofibers with intercellular bridge build of thin longitudinal muscles. The whole musculature is underneath the cuticular lamina that on several places creases and folds, particularly in lobe areas (Fig. 6.2.

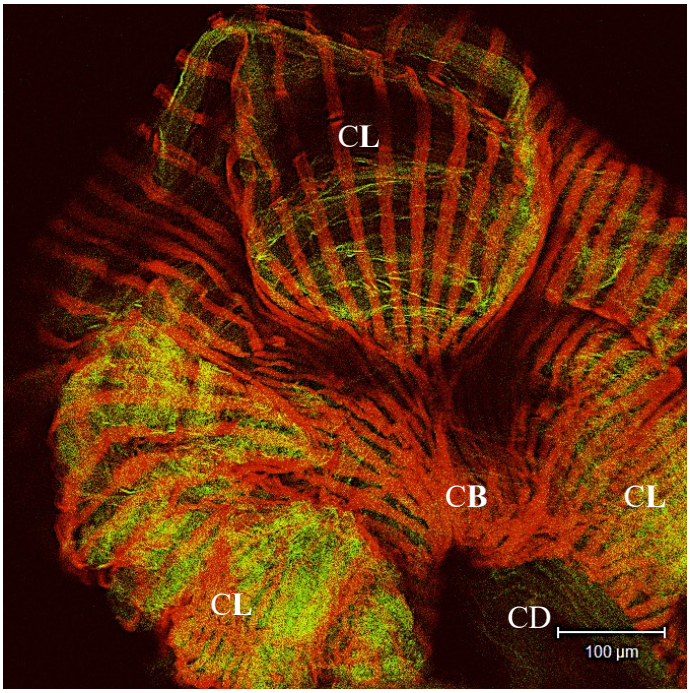


Figure 6.2 Confocal image of phallodin-stained crop musculature of *Ceratitis capitata*. myofibres (red) radiate from the base of the crop and spread across the whole crop sac and while shaping crop lobes, the also constrict cuticular lamina observed underneath (autofluorescent green). (CD-crop duct; CB-crop base; CL- crop lobe)

6.4.1.2 Immunolocalisation of myosuppressin-like material in the crop

Antibodies recognising the C-terminal RFamide epitope of myosuppressin were used to label two nerves that emanate from the hypocerebral complex and run alongside the crop duct on opposite sides. They extend over the whole crop sac reaching towards the crop lobes (Fig. 6.3 A). Higher magnification revealed a pattern of multiple immunoreactive fibres that, bunched together, covering on the crop surface while enclosing the crop lobe (Fig. 6.3 B).

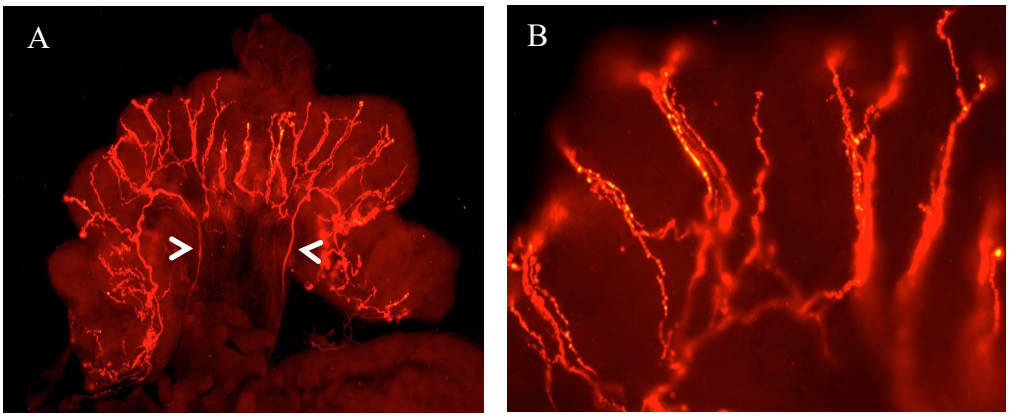


Figure 6.3 The whole mount of *C. capitata* stained with RFamide antibody. (A) Robust staining of immunoreactive fibres was observed across the whole crop surface. Immunoreactive material was also present within two crop nerves (white arrowhead), each running alongside of crop duct (10x magnification). (B) Close-up of the crop lobe presenting a very strong (40x magnification). Images were taken with Zeiss Axioplan fluorescence microscope.

6.4.1.3 Single tissue profiling of crop nerve bundle

The direct analysis of crop nerve bundle by MALDI-TOF identified a major prominent peak with a monoisotopic *m/z* of 1323.59, which matches the mass $[M+H]^+$ of corazonin neuropeptide. Of the less intense ions is one that matches the $[M+H]^+$ of 1234.64 for myosuppressin. The identity of mass ion 1296.72 is unknown.

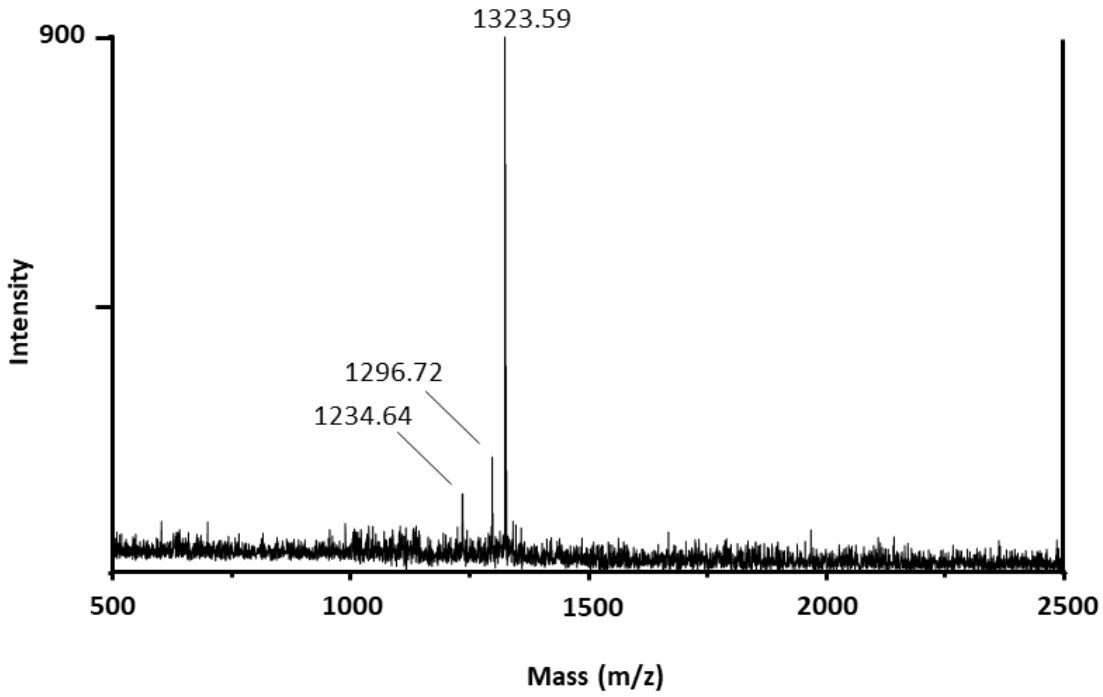


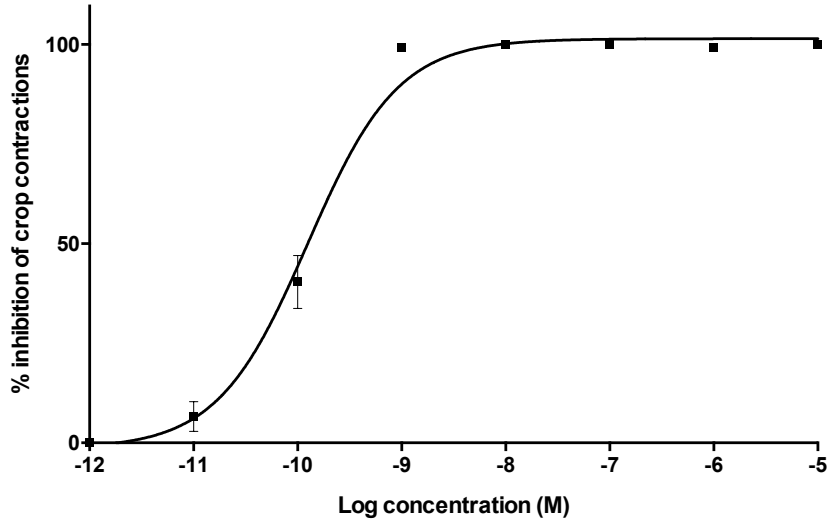
Figure 6.4 Direct analysis of single tissue of crop nerve bundle. A representative mass spectrum of direct analysis highlights a major peak (*m/z*) of 1323.59 matching the predicted mass for corazonin peptide. Amongst the minor peaks is the ion at *m/z* 1234.64 corresponding to medfly myosuppressin (SDVDHVFLRFa).

6.4.1.4 Crop bioassay

The spontaneous contractions of the medfly crop were strongly inhibited by myosuppressin in a dose-dependent manner with an IC_{50} of 1.3×10^{-10} M (Fig. 6.5). After the peptide solution was washed away with physiological saline, crop contractions

recovered demonstrating that the inhibitory effect of the peptide is reversible and non-toxic to the muscle apparatus.

A



B

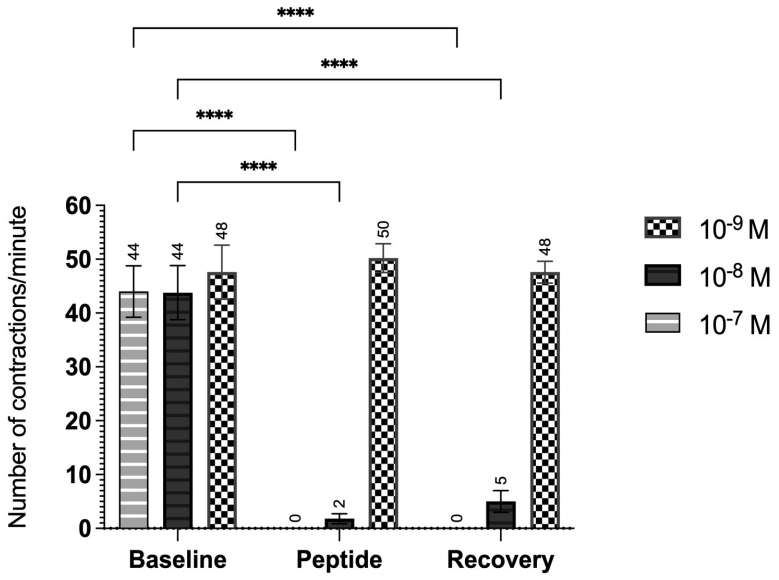


Figure 6.5 Dose response inhibition of crop peristalsis of *C. capitata* by myosuppressin. A, The neuropeptide is a potent inhibitor of contractions (IC_{50} =1.3 x 10^{-10} M). Data are expressed as the mean ± s.e.m. (n=40). B, The baseline is the number of crop contractions in fly saline counted for a 1 min period before the replacement of saline with peptide. Recovery of the spontaneous contractions was dose-dependent as crops recovered from inhibition by 10^{-9}M and 10-^{10}M but not at 10^{-8}M, Two-way Anova analysis followed by a post-hoc Tukey test performed by GraphPad Prism 6.0. The mean is presented above the error bar calculated as the sum of contractions per treatment. Only significant differences are graphically represented in the form of brackets above the columns (**** $p < 0.0001$).

6.4.1.5 The effect of injected myosuppressin on adult *C. capitata* survival, food intake and fecundity

To evaluate the effect of myosuppressin on food intake and fecundity, 50 male and 50 female adult flies were injected with 0.5µl saline (control) or 0.5µl saline containing either 6.25 ng of peptide, or 6.25 µg of peptide. After 24 hrs, mortality of flies injected with 6.25 ng myosuppressin (4 males and 4 females) was similar to that of the control group where (3 males and 3 females) showing that the injections did not cause significant harm to individuals. In both groups, females continued to lay eggs (24 hrs post-injection; control group, 159 eggs and test group, 138 eggs; 48 hours post-injection, control group, 148 eggs, test group, 135 eggs). When the dose was increased to 6.25 µg of peptide per fly the myosuppressin-injected adult females did not lay any eggs in the first 24 hrs post-injection, in contrast to the control group (159 eggs). This inhibition of egg laying was however only observed for the first day since the next day eggs were laid by both groups (test group, 148 eggs; control group, 132 eggs) (Fig.6.6).

Eggs collection time	*Treatment*†		
	Control (saline)	*6.25 ng*	*6.25 µg*
	Eggs number		
24 hr	159	138	0
48 hr	148	135	132

† Injection of 0.5 µl dose containing saline, 0.5 µl of 6.25 ng medfly myosuppressin or 0.5 µl 6.25 µg medfly myosuppressin peptide.

Figure 6.6 Effect of myosuppressin on female fecundity. The injection of 6.25 ng of peptide into adult females had no effect on fecundity at 24 and 48 hr compared to controls. However, females receiving a single dose of 6.25 µg peptide delayed ovipositing for 24 hr.

Daily observation of medfly behaviour or phenotypic response to a single dose of medfly myosuppressin peptide injection has not been noticed during a four-week observation. The survivorship did not differ in tested group to that observed in control group (Fig.6.7).

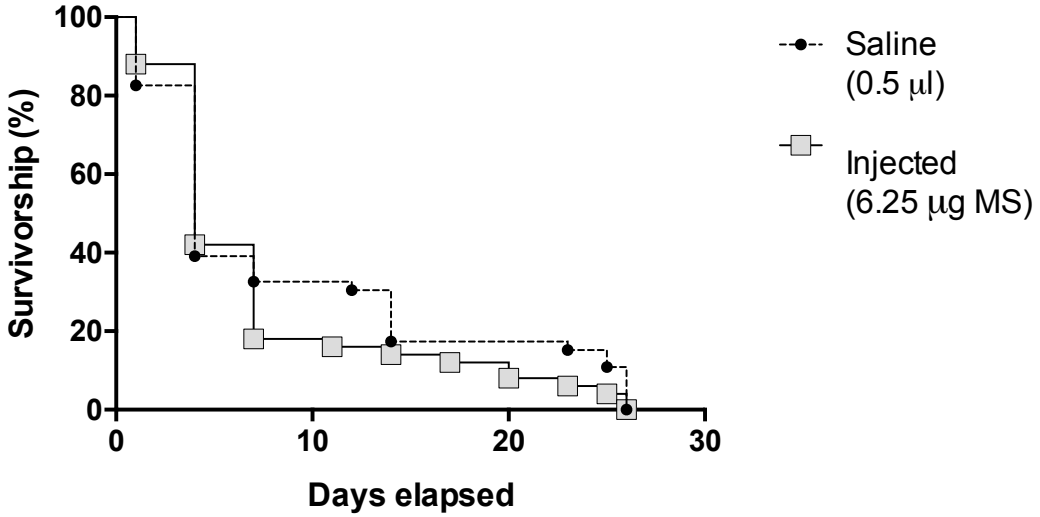


Figure 6.7 The effect of medfly myosuppressin on survivorship of *C. capitata* adults. Flies were injected with 0.5 μl single dose of either saline or 6.25 μg myosuppressin. Administered peptide had no significant effect compared to controls (P=0.3589, Log-rank Mantel-Cox test).

6.4.1.6 Excreta assay

After three-days feeding on sucrose-only solution, some individuals still retained blue dye in their crop. There was not a significant difference between individual columns (Fig. 6.8, P=0.3136), indicating that a single dose of administered peptide in nano- and microgram concentrations did not have any effect on feeding behaviour.

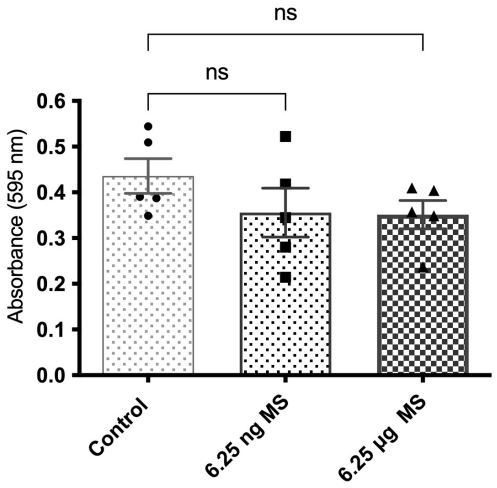


Figure 6.8 *In vivo* effect of medfly myosuppressin peptide on defecation rate. Delivery of peptide did not have any significant effect on the concentration of dye-evaluated defecation rate quantified by absorbance from collected faeces over 48 hr period (graph representation based on results in Table 6.1 below).

Table 6.1 Effect of myosuppressin *in vivo* on defecation rate measured over 48 hr period. Blue dye in the excrement was quantified by spectrophotometry at 595 nm.

In vivo *peptide effect on defecation*†	*Mean*	*Mean*	*Significance*	*p value*
Control vs. 6.25 ng	0.4358	0.3558	ns	1.905
Control vs. 6.25 µg	0.4358	0.3514	ns	2.009
6.25 ng vs. 6.25 µg	0.3558	0.3514	ns	1.1047

† One-way Anova comparison statistical analysis (Tukey's multiple comparison test)

ns- not significant

6.4.2 Neuropeptidome of medfly

6.4.2.1 *In silico* analysis

Using *Drosophila melanogaster* neuropeptide sequences as queries to search the predicted protein database of *C. capitata*, 85 putative mature neuropeptides were identified, some with alternatively spliced variants, from 42 precursor genes. Several precursor proteins were identified for 20 putative neuropeptides from unannotated transcripts (Table 6.2). Results of identity and similarity analysis of peptides between *Drosophila* and *Ceratitis* are listed in Table 6.3.

Due to the pleiotropic nature of almost all neuropeptides and peptide hormones identified, to describe in detail all associated functions and properties is beyond the scope of this chapter, however characteristics and unique features of medfly putative neuropeptides are briefly described in the Discussion (section 6.6.2.).

6.4.2.1.1 *Ceratitis capitata* myosuppressin receptor

Protein sequences for *Drosophila* myosuppressin receptors, Dms-R1 and Dms-R2 retrieved from the Uniprot database, labelled as Q9W025 and Q9W027 respectively, identified through BlastP analysis, the probable G-protein coupled receptor 139 (XP_02071789.1) is almost certainly the single myosuppressin receptor of *C. capitata*. (E-value = 0.0). The sequence alignment analysis by Clustal O ver. 1.2.4 has scored 71% and 68.3% identity against Dms-R1 and Dms-R2 (Fig.6.9). Following resubmission of the probable G-protein coupled receptor protein 139 amino acid sequence to TMHMM Expasy software (www.cbs.dtu.dk/services/TMHMM-2.0) highlighted seven transmembrane regions (Fig.6.10).

Figure 6.9 Alignment of putative *Ceratitis capitata* myosuppressin receptor, the probable G-protein coupled receptor 139 (XP_020717891.1) against protein sequences for *Drosophila melanogaster* myosuppressin receptors (Dms-R1, Q9W025 and Dms-R2, Q9W027) sharing 71% and 68.3% similarity. The 517 amino acid long sequence was delivered from NW_019376245.1 genome with predicted molecular mass of 57571 Da, recognised as a member of the class A family of seven-transmembrane G protein-coupled receptors. Seven transmembrane helices TMI-TMVII are overlined and two glycosylation sites are indicated with full arrowhead.
(Symbols for the degree of conservation: "-", mismatch or gap; ". " (period), conservation of weakly similar properties; " : " (colon), conservation between strongly similar properties; " * " (asterisk), fully conserved residue).

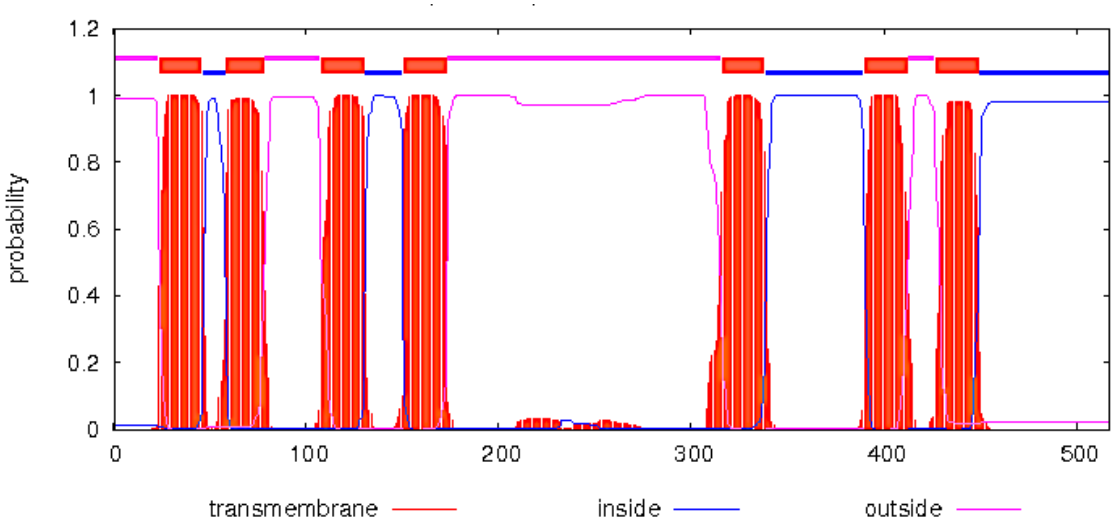


Figure 6.10 The prediction of the transmembrane regions in putative Myosuppressin receptor for *Ceratitis capitata*. Seven transmembrane regions (structural motifs TMI 26-51, TMII 57-82, TMIII 103-133, TMIV 145-165, TMV 310-335, TMVI 382-414, TMVII 425-450 identified in 517 amino acids long sequence) were located by TMHMM Server v. 2.0 of the protein sequence for the predicted *C. capitata* G-protein coupled receptor 139 (XP_020717891.1). Red color represents transmembrane region, blue lines intracellular segments while pink lines mark predicted extracellular sections.

Table 6.2 *C. capitata* neuropeptide sequences predicted by BLASTp analysis using *D. melanogaster* peptides as queries to interrogate the medfly predicted protein database.

	Peptide	Name	Predicted peptides in medfly (*Ceratitis capitata*)	*Drosophila* putative neuropeptides	Identity %	Similarity %
1	Adipokinec hormone (AKH)	PREDICTED: adipokinetic hormone [Ceratitis capitata]	QLTFSPDWG	QLTFSPDWG	100	100
2	Allatostatin-A/FGL	PREDICTED: allatostatin-A [Ceratitis capitata]	MERYAFGLG AYTYTNGGNGM LPVYNFGLG ARPYSFGLG NRPYSFGLG SLQEPPPHRYGFGLG	VERYAFGLG AYMYTNGGPMGM LPVYNFGLG SRPYSFGLG ----- TTRPQPFNFGLG	89 75 100 89 -- 33	100 75 100 100 -- 40
3	Allatostatin-B/MIPs	PREDICTED: allatostatins MIP [Ceratitis capitata]	NWQALQGPWG AWNKINAAWG NAPTWNKFRGAWG EPGWNNLKGMWG SSKDWAKLHGGWG	AWQSLQSSWG AWKSMNVAWG QAQGWNKFRGAWG EPTWNNLKGMWG DQWQKLHGGWG	60 60 77 92 62	70 70 77 92 62
4	Allatostatin-C/PISCF	PREDICTED: uncharacterized protein LOC101460921 [Ceratitis capitata]	QVRYRQCYFNPISCF	QVRYRQCYFNPISCF	100	100
5	Allatostatin CC	uncharacterized protein LOC101462522 isoform X1 [Ceratitis capitata]	AALLLDRLMVALHHALEQE DLNSINRATGETLMAKSFQ NGIGNGGRMYWRCYFNAVSCF	AALLLDRLMVALHHALEQE DLNQINRATGETLMPKSFQ IQPSGSGGGRAYWRCYFNAVSCF	100 90 71	100 90 75
6	Bursicon	bursicon [Ceratitis capitata]	QAEEAITAVDNDVSHIGDDCQVTPVIHVL QYPGCVPKPIPSFACVGRCASYIQVSGSKI WQMERSCMCCQESGEREAAVSLFCPKAK HGERKFKKVLTKAPLECMCRPCTSIEESGIIP QEIAGYSDEGPLNNHFRRIALQ	QPDSSVAATDNDITHLGDDCQVTPVIHVL QYPGCVPKPIPSFACVGRCASYIQVSGSKI WQMERSCMCCQESGEREAAVSLFCPKVK PGERKFKKVLTKAPLECMCRPCTSIEESGIIP QEIAGYSDEGPLNNHFRRIALQ	92	96
7	CAPA peptide	PREDICTED: cardio acceleratory peptide 2b [Ceratitis capitata]	CAP_{2b}1-TAGGPSGLIAFPRVG CAP_{2b}2-ASLIAFPRVG Pyrokinin-GAGPSATSGLWFGPRLG	GANMGLYAFPRVG ASGLVAFPRVG TGPSASSGLWFGPRLG	60 64 83	60 82 89
8	PK-beta & Hugin/PK-γ	PREDICTED: uncharacterized protein LOC105664747 [Ceratitis capitata]	SQKYDNFIEQPSAVMDLAPRLSSFDRIPFN DYNRIVSALKEMLRVNEEKSVMLRPRPG Hugin/PK-γ SAPFKPRLG	LRQLQSNGEPAYRVRTPRLG SVPFKPRLG	12 89	17 89
9	CCAP	PREDICTED: cardioactive peptide [Ceratitis capitata]	PFCNAFTGCG SYLPPYPLW	PFCNAFTGCG TYPSYPPFSLF	100 42	100 58
10	CCHa- 1	PREDICTED: uncharacterized protein LOC105665375 [Ceratitis capitata]	SCLEYGHSCWGAHG	SCLEYGHSCWGAHG	100	100
11	CCHa-2	PREDICTED: uncharacterized protein LOC101460931 [Ceratitis capitata]	GCNAYGHACYGGHG	GCQAYGHVCYGGHG	86	86
12	CNMa	PREDICTED: uncharacterized protein LOC101457297 [Ceratitis capitata]	QYMSPCHFKICNMG	QYMSPCHFKICNMG	100	100
13	Corazonin	PREDICTED: pro-corazonin [Ceratitis capitata]	QTFQYSHGWTSG	QTFQYSRGWTNG	84	92
14	DH31	PREDICTED: diuretic hormone class 2 isoform X1 [Ceratitis capitata]	TVDFGLARGYSGTQEAKHRMGLAAANFP GGPG	TVDFGLARGYSGTQEAKHRMGLAAANFA GGPG	97	97
		PREDICTED: diuretic hormone class 2 isoform X2 [Ceratitis capitata]	TVDFGLARGYSGTQEAKHRMGLAAANFP GGPG	TVDFGLARGYSGTQEAKHRMGLAAANFA GGPG	97	97

15	DH44	PREDICTED: uncharacterized protein LOC101456921 [Ceratitis capitata]	NKPSLSIVNPLDVLRQRLLLEIARRQMKENTRQVELNRAILKNVG	NKPSLSIVNPLDVLRQRLLLEIARRQMKENSRQVELNRAILKNVG	98	100
	EH	XP_004536647.1 eclosion hormone [Ceratitis capitata]	LPSIGHYGKRFDTMSGIDFIQICLNNCAQCKKMFGDYFQGQTCAESCLKFKGKAIPDCEDIGSIAPFLNALE	LPAISHYTHKRFDSMGGIDFVQVCLNNCVQCKTMLGDYFQGQTCALSCLKFKGKAIPDCEDIASIAPFLNALE	78	85
16	ETH-1	PREDICTED: uncharacterized protein LOC101457772 [Ceratitis capitata]	NDSPGFFLKITKNVPRLG	DDSSPGFFLKITKNVPRLG	84	90
17	ETH-2	PREDICTED: uncharacterized protein LOC101457772 [Ceratitis capitata]	SDSYFLKNMKAIPRIG	GENFAIKNLKTIPRIG	50	82
18	FMRFa-like peptides	PREDICTED: FMRFG-related peptides [Ceratitis capitata]	KSFNENFMRFG ASSDFMRFG GAEDFMRFG SANDFMRLG DARGDNFMRFG AANDFMRFG ASGDFMRFG ---------- NPSDFMRFG GGSNDFMRFG PQTDFMRFG ---------- AANQDFMRFG PDNFMRFG SPLAQPTISPNFMRFG SDTNFMRFG	SVQDNFMHFG ---------- ---------- ---------- DPKQDFMRFG DPKQDFMRFG DPKQDFMRFG DPKQDFMRFG DPKQDFMRFG TPAEDFMRFG TPAEDFMRFG SDNFMRFG SPKQDFMRFG PDNFMRFG SAPQDFVRSG MDSNFIRFG	55 ---- ---- ---- 60 60 60 ----- 70 60 70 ----- 70 100 38 67	64 ---- ---- ---- 70 60 60 ---- 80 70 70 ------ 80 100 38 89
19		FMRFG-related peptides isoform X1 [Ceratitis capitata]	KSFNENFMRFG ASSDFMRFG GAEDFMRFG SANDFMRLG DARGDNFMRFG AANDFMRFG ASGDFMRFG ---------- NPSDFMRFG GGSNDFMRFG PQTDFMRFG ---------- AANQDFMRFG PDNFMRFG SPLAQPTISPNFMRFG SDTNFMRFG	SVQDNFMHFG ---------- ---------- ---------- DPKQDFMRFG DPKQDFMRFG DPKQDFMRFG DPKQDFMRFG DPKQDFMRFG TPAEDFMRFG TPAEDFMRFG SDNFMRFG SPKQDFMRFG PDNFMRFG SAPQDFVRSG MDSNFIRFG	55 -- -- -- 60 60 60 -------- 70 60 70 ----- 70 100 38 67	64 -- -- -- 70 60 60 ------- 80 70 70 ------ 80 100 38 89
20	GPA2	PREDICTED: uncharacterized protein LOC101454664 [Ceratitis capitata]	QSWLKPGCHKVGNTRIISIPECVEFRITTNACRGFCESYAVPSIPFGQAIPGIFKPVKPVVSVGQCCNIMAAEEVQKRVLCMGGMRNITFKSAVSCSCYHCKKD	NSMGKDAWLRPGCHKVGNTRKITIPDCVEFTITTNACRGFCESFSVPSIPMMGSSLSVLFKPPKPVVSVGQCCNMMKSEEIQRRVLCIEGIRNVTFNSALCSCYHCKKD	99	99
21	GPB5	PREDICTED: glycoprotein hormone beta-5 [Ceratitis capitata]	SLSQLLEAQPIDSSPTTAPLGCHQRLYTYRITQADEQGRECWDYVSVRSCWGRCDSSEISDWKFPYKRSFHPVCVHATRQPAVAVLRNCHPEASEEIRRYEYMEAGSCHCHTCSTLDTSCEAPVNNIVDEKSSVKVLALTGSDSDALDY	SSLSEIKPMNNGHIVTPLGCHRRVYTYKVTQSDLQGHECWDYVSVWSCWGRCDSSEISDWKFPYKRSFHPVCVHAQRQLVVAILKNCHPKAEDSVSKYQYMEAVNCHCQTCSTQDTSCEAPANNEMAGGSRAIMVGADTKNLDY	62	76

22	ITP	PREDICTED: ion transport peptide isoform X3 [Ceratitis capitata]	SNFFDLECKGIFNKTMFFRLDRICEDCYQLF RETSIHRLCKQDCFGSQFFAACVEALQLHE EMDKYNEWRFTLG	SNFFDLECKGIFNKTMFFRLDRICEDCYQLF RETSIHRLCKQECFGSPFFNACIEALQLHEE MDKYNEWRDTLG	93	96
23	ITP-L1	PREDICTED: ion transport peptide-like isoform X2 [Ceratitis capitata]	SNFFDLECKGIFNKTMFFRLDRICEDCYQLF RETSIHRLCKQNCFTHETFGECLKVLMIPEE EVTQLQYFVKVINGSPMPFANLQ	SNFFDLECKGIFNKTMFFRLDRICEDCYQLF RETSIHRLCKANCFVHETFGDCLKVLLIDDE EISQLQHYLKVINGSPYPFHKPIYH	79	89
24	ITP-L2	PREDICTED: ion transport peptide-like isoform X4 [Ceratitis capitata]	SNFFDLECKGIFNKTMFFRLDRICEDCYQLF RETSIHRLCKKNCFDSKWFGECLKVLLIPSE EISKLQHFIKVINGSPISFPQS	SNFFDLECKGIFNKTMFFRLDRICEDCYQLF RETSIHRLCKKDCFDSKWFGECLKVLLIPEE EISNLQHFLRVVNGSPISFNMGPQT	89	94
26	Limostatin	PREDICTED: uncharacterized protein LOC101455320 [Ceratitis capitata]	AIVFRPLFVYKQQQI	AIVFRPLFVYKQQEI	93	100
27	Myosuppressin	PREDICTED: dromyosuppressin [Ceratitis capitata]	SDVDHVFLRFG	TDVDHVFLRFG	91	100
28	Neuropeptide F-1	PREDICTED: neuropeptide F-like [Ceratitis capitata]	SNSRPPRNNDISNMADALKYLQDLDTYYA DRARARFa	SNSRPPRKNDVNTMADAYKFLQDLDTYYG DRARVRFamide	78	87
29	Neuropeptide F-2	PREDICTED: neuropeptide F-like [Ceratitis capitata]	GNSRPPRNSDISNMADALKYLQDLDTYYG DRARARFa	SNSRPPRKNDVNTMADAYKFLQDLDTYYG DRARVRFamide	76	87
30	sNPF-check	PREDICTED: short neuropeptide F [Ceratitis capitata]	AQRSPSLRLRFG WFGDVNQKPIRSPSLRLRFG KPQRLRFG KPASLRLRFG	AQRSPSLRLRFG SPSLRLRFG KPQRLRWG KPMRLRWG	100 100 88 60	100 100 100 80
31	Natalisin 1/Natalisin 2/Natalisin 3	PREDICTED: uncharacterized protein LOC105664782 [Ceratitis capitata]	EQLDEDLTSGYLTEDPKPLRKGLHFQNIFNF NHAP-QYKDDPFIPPRG HNLPDLDALLNRYETFVPNRG DKIKDIFKYDDLFFPNRG	EKLFDGYQFGEDMSKENDPFIPPRG HSGSLDLDALMNRYEPFVPNRG DKVKDLFKYDDLFYPHRG HRNLFQVDDPFFATRG LQLRDLYNADDPFVPNG	28 68 78 --- ---	33 73 100 --- ---
32	Orcokinin A	PREDICTED: uncharacterized protein LOC101451285 isoform X1 [Ceratitis capitata]	NFDEIDKTSASFSTLNQLI	NFDEIDKASASFSILNQLV	84	90
33	Orcokinin B	PREDICTED: uncharacterized protein LOC101451285 isoform X2 [Ceratitis capitata]	TLDSIGGGHLI	GLDSIGGGHLI	91	91
34	PDF	PREDICTED: protein PDF [Ceratitis capitata]	NSLLSLPKSMNEAG	NSELINSLLSLPKNMNDAG	90	100
35	Proctolin	PREDICTED: uncharacterized protein LOC101462442 [Ceratitis capitata]	RYLPT	RYLPT	100	100
36	RYa/RYa2	PREDICTED: uncharacterized protein LOC101452386 [Ceratitis capitata]	PSFFVGSRYG NDRFFLGSRYG	PVFFVASRYG NEHFFLGSRYG	80 82	80 91
37	SIFa	PREDICTED: FMRFG-related neuropeptides [Ceratitis capitata]	AYRKPPFNGSIFG	AYRKPPFNGSIFG	100	100
38	SK-1/SK-2	PREDICTED: callisulfakinin [Ceratitis capitata]	FDDYGHMRFG GGEDQFDDYGHMRFG	FDDYGHMRFG GGDDQFDDYGHMRFG	100 93	100 100
39	Kinin	PREDICTED: leucokinin [Ceratitis capitata]	NSVVLGKKQRFHSWGG	NSVVLGKKQRFHSWGG	100	100
40	Trissin	PREDICTED: uncharacterized protein LOC101453784	SMTCDSCGNECTNACGTKNFRTCCFNYL	IKCDTCGKECASACGTKHFRTCCFNYL	71	86

41	NPLP1/APK/MTYa/IPNa	PREDICTED: neuropeptide-like 1 [Ceratitis capitata]	SVAALAAQGMLHP	SVAALAAQGLLNAPK	67	80
			SLATLAKNGQLPSLPDPEDVEPTAPNED	SLATLAKNGQLPTAEPGEDYGDADSGEPSEQ	52	61
			YVGSLARSGGFATYG	YIGSLARAGGLMTYG	73	87
			NIGTLARDYQLPQNG	NVGTLARDFQLPIPNG	75	88
42	NPLP1-4	neuropeptide-like 4 [Ceratitis capitata]	pQFYYGGYASPYSYAYSGYPY	pQYYYGASPYAYSGGYYDSPYSY	52	64
43	TRP	PREDICTED: tachykinins isoform X1 [Ceratitis capitata]	TPSGFIGMRG	APTSSFIGMRG	64	64
			APSTFYGVRG	APLAFVGLRG	60	70
			APTGFTGMRG	APTGFTGMRG	100	100
			GPVNGFVGLRG	APVNSFVGMRG	73	82
			SKSQRFVDFSNKFVAVRG (or pQRFVDFSNKFVAVRa?)	pQRFADFNSKFVAVRG	67	78
			MPLLALHGMRG	APNGFLGMRG	55	55
44		PREDICTED: tachykinins isoform X2 [Ceratitis capitata]	TPSGFIGMRa	APTSSFIGMRG	64	64
			APSTFYGVRa	APLAFVGLRG	60	70
			APTGFTGMRa	APTGFTGMRG	100	100
			GPVNGFVGLRa	APVNSFVGMRG	73	82
			SKSQRFVDFSNKFVAVRa	pQRFADFNSKFVAVRG	67	78
			MPLLALHGMRG	APNGFLGMRG	55	55
45		PREDICTED: tachykinins isoform X3 [Ceratitis capitata]	TPSGFIGMRa	APTSSFIGMRG	64	64
			APSTFYGVRa	APLAFVGLRG	60	70
			APTGFTGMRa	APTGFTGMRG	100	100
			GPVNGFVGLRa	APVNSFVGMRG	73	82

Table 6.3 List of *C. capitata* neuropeptides identified by genome and mass spectrometry

Peptide family	Sequence	Monoisotopic mass $[M+H]^+$	Genome	Mass (MS)*	Sequence (MS/MS)*
Adipokinetic hormone (AKH)	QLTFSPDWa	991.5	✓	✓	✓
	pQLTFSPDW	997.4 $[Na^+]$	✓	✓	✓
		1013.4 $[K^+]$		✓	
	QLTFSPDWGK	1161.5	✓	✓	
Allatostatin-A/FGL	MERYAFGLa	985.4	✓	✓	
	LPVYNFGLa	921.5	✓	✓	
	ARPYSFGLa	909.4	✓	✓	✓
	NRPYSFGLa	952.4	✓	✓	
	SLQEPPPHRYGFGLa	1596.8	✓	✓	✓
	AYTYTNGGNGM	1905.8	✓	✓	

Allatostatin-B/MIPs	NWQALQGPWa	1098.5	✓		
	AWNKINAAWa	1072.5	✓	✓	
	NAPTWNKFRGAWa	1446.7	✓	✓	
	EPGWNNLKGMWa	1330.8	✓	✓	✓
	DWAKLHGGWa	1067.4	✓	✓	
Allatostatin-C/PISCF	pQVRYRQCYFNPISCF	1905.9	✓	✓	✓
Allatostatin CC	AALLLDRLMVALHHALEQE	2143.8	✓		
	DLNSINRATGETLMAKSFQ	2096.6	✓		
	NGIGNGGRMYWRCYFNAVSCF	2416	✓		
Bursicon	QPDSSVAATDNDITHLGDDCQVTPVI-HVLQYPGCVPKPIPSFACVGRCASYIQ-VSGSKIWQMERSCMCCQESGEREAA-VSLFCPKVKPGERKFKKVLTKAPLECM-CRPCTSIEESGIIPQEIAGYSDEGPLNN-HFRRIALQ	15473.8	✓		
CAPA peptides					
CAP $_{2b}$ 1	TAGGPSGLIAFPRVa	1341.7	✓	✓	✓
CAP $_{2b}$ 2	ASLIAFPRVa	972.5	✓	✓	✓
Pyrokinin	GAGPSATSGLWFGPRVa	1559.0	✓	✓	✓
CCAP	PFCNAFTGCa	956.3	✓		
CCHamides					
CCH-1	SCLEYGHSCWGAHa	1446.5	✓		
CCH-2	GCNAYGHACYGGHa	1363.4	✓		
CNM	pQYMSPCHFKICNMa	1542.0	✓		
Corazonin	pQTFQYSHGWTSa	1323.6	✓	✓	
Calcitonin (CT)-like diuretic hormone (DH31)	TVDFGLARGYSGTQEAKHRMGLAAANF PGGPa	3176.5	✓	✓	
Corticotropin releasing factor (CRF)-like (DH44)	NKPSLSIVNPLDVLRQRLLLEIARRQMKE NTRQVELNRAILKNVa	5180.1	✓	✓	✓
Ecdysis triggering hormone (ETH)					
ETH-1	SPGFFLKITKNVPRLa	1716.0	✓		
ETH-2	KNMKAIPRIa	1069.3	✓	✓	

FMRFamide-like peptides	KSFNENFMRFa	1318.6	✓	✓	
	ASSDFMRFa	959.4	✓	✓	✓
	GAEDFMRFa	971.4	✓		
	SANDFMRLa	952.4	✓	✓	
	GDNFMRFa	885.4	✓		
	AANDFMRFa	970.4	✓		
	ASGDFMRFa	929.4	✓	✓	
	NPSDFMRFa	1012.4	✓	✓	
	GGSNDFMRFa	1029.4	✓		
	PQTDFMRFa	1040.4	✓	✓	
	AANQDFMRFa	1098.5	✓		
	PDNFMRFa	925.4	✓		
	SPLAQPTISPNFMRFa	1704.8	✓		
	SDTNFMRFa	1016.4	✓	✓	✓
Thyrostimulin/GPA2	SWLKPGCHKVGNTRIISIPECVEFRITTNACRGFCESYAVPSIPFGQAIPGIFKPVKPVVSVGQCCNIMAAEEVQKRVLCMGGMRNITFKSAVSCSCYHCKKD	11273.6	✓		
Thyrostimulin/GPB5	SLSQLLEAQPIDSSPTTAPLGCHQRLYTYRITQADEQGRECWDYVSVRSCWGRCDSSEISDWKFPYKRSFHPVCVHATRQPAVAVLRNCHPEASEEIRRYEYMEAGSCHCHTCSTLDTSCEAPVNNIVDEKSSVKVLALTGSDSDALDY	16765.9	✓		
Ion Transport Peptide (ITP)	SNFFDLECKGIFNKTMFFRLDRICEDCYQLFRETSIHRLCKQDCFGSQFFAACVEALQLHEEMDKYNEWRFTLa	8907.4	✓		
ITP-L1	SNFFDLECKGIFNKTMFFRLDRICEDCYQLFRETSIHRLCKQNCFTHETFGECLKVLMIPEEEVTQLQYFVKVINGSPMPFANLQ	10126.0	✓		
ITP-L2	SNFFDLECKGIFNKTMFFRLDRICEDCYQLFRETSIHRLCKKNCFDSKWFGECLKVLLIPSEEISKLQHFIKVINGSPISFPQS	9959.9	✓		
Kinin	NSVVLGKKQRFHSWGa	1742.2/916.2	✓		
	NSVVLa	529.9	✓		
Limostatin	AIVFRPLFVYKQQQI	1850.5	✓		

Ion Transport Peptide (ITP)	SNFFDLECKGIFNKTMFFRLDRICEDCYQ LFRETSIHRLCKQDCFGSQFFAACVEALQ LHEEMDKYNEWRFTLa	8907.4	✓		
ITP-L1	SNFFDLECKGIFNKTMFFRLDRICEDCYQ LFRETSIHRLCKQNCFTHETFGECLKVLMI PEEEVTQLQYFVKVINGSPMPFANLQ	10126.0	✓		
ITP-L2	SNFFDLECKGIFNKTMFFRLDRICEDCYQ LFRETSIHRLCKKNCFDSKWFGECLKVLLI PSEEISKLQHFIKVINGSPISFPQS	9959.9	✓		
Limostatin	AIVFRPLFVYKQQQI	1850.5	✓		
Myosuppressin	SDVDHVFLRFa	1233.6	✓	✓	✓
Neuropeptide F (NPF)	SNSRPPRNNDISNMADALKYLQDLDTYY ADRARARFa	4218.9/ 3424.0	✓	✓	✓
NPF-2	GNSRPPRNSDISNMADALKYLQDLDTYY GDRARARFa	4147.8/3382. 9	✓		
Short neuropeptide S (sNPF)	AQRSPSLRLRFa	1329.7	✓	✓	✓
	WFGDVNQKPIRSPSLRLRFa	2315.9	✓		
	PIRSPSLRLRFa	1340.8	✓	✓	
	PQRLRFa	815.4	✓	✓	✓
	PASLRLRFa	958.5	✓		
Natalisin	LNLSGHLEPPLSVEEPQYVIV	2333.9	✓		
	EQLDEDLTSGDYQKQYDPFIPPRa	2625.1 or 2608.1	✓		
	HNLPDLDALLNRYETFVPNRa	2396.9 or 2145.7	✓		
	DKIKDIFKYDDLFFPNRa	2173.7	✓		
Orcokinin	NFDEIDKTSASFSTLNQLI	2143.6	✓		
Orcokinin	TLDSIGGGHLI	1082.5	✓		

Pigment dispersing factor (PDF)	NSELINSLLSLPKSMNEAa	1959.5	✓		
Proctolin	RYLPT	649.0	✓		
RY amide	PSFFVGSRYa	1058.4	✓		
RY amide	NDRFFLGSRYa	1273.6	✓		
SIF amide	AYRKPPFNGSIFa/ PPFNGSIFa	1395.7/	✓	✓	✓
		877.5	✓	✓	
Sulfakinin (SK)					
SK-1	FDDYGHMRFa	1186.5	✓		
SK-2	GGEDQFDDYGHMRFa	1186.5	✓	✓	
Trissin	SMTCDSCGNECTNACGTKNFRTCCFNYL	3087.7	✓		
Neuropeptide-like 1 (NPLP1)/APK	SVAALAAQGMLHP	1265.7	✓		
NPLP1-2/MTYamide	YVGSLARSGGFATYa	1447.8	✓	✓	✓
NPLP1-3/IPNamide	NIGTLARDYQLPQNa	1602.0	✓		
NPLP1-4	pQFYYGGYASPYSYAYSGYPYYa	2512.9	✓		
Tachykinin-related peptides (TRP)	MPLLALHGMRa	1137.7	✓		
	TPSGFIGMRa	964.4	✓	✓	
	APSTFYGVRa	996.4	✓	✓	
	APTGFTGMRa	936.3	✓		
	GPVNGFVGLRa	1014.4	✓	✓	
	pQRFVDFSNKFVAVRa	1695.2	✓		

* Preliminary results from Orbitrap analysis and subsequent MS/MS fragmentation were provided by Dr. Ragionieri (University of Cologne, Germany).

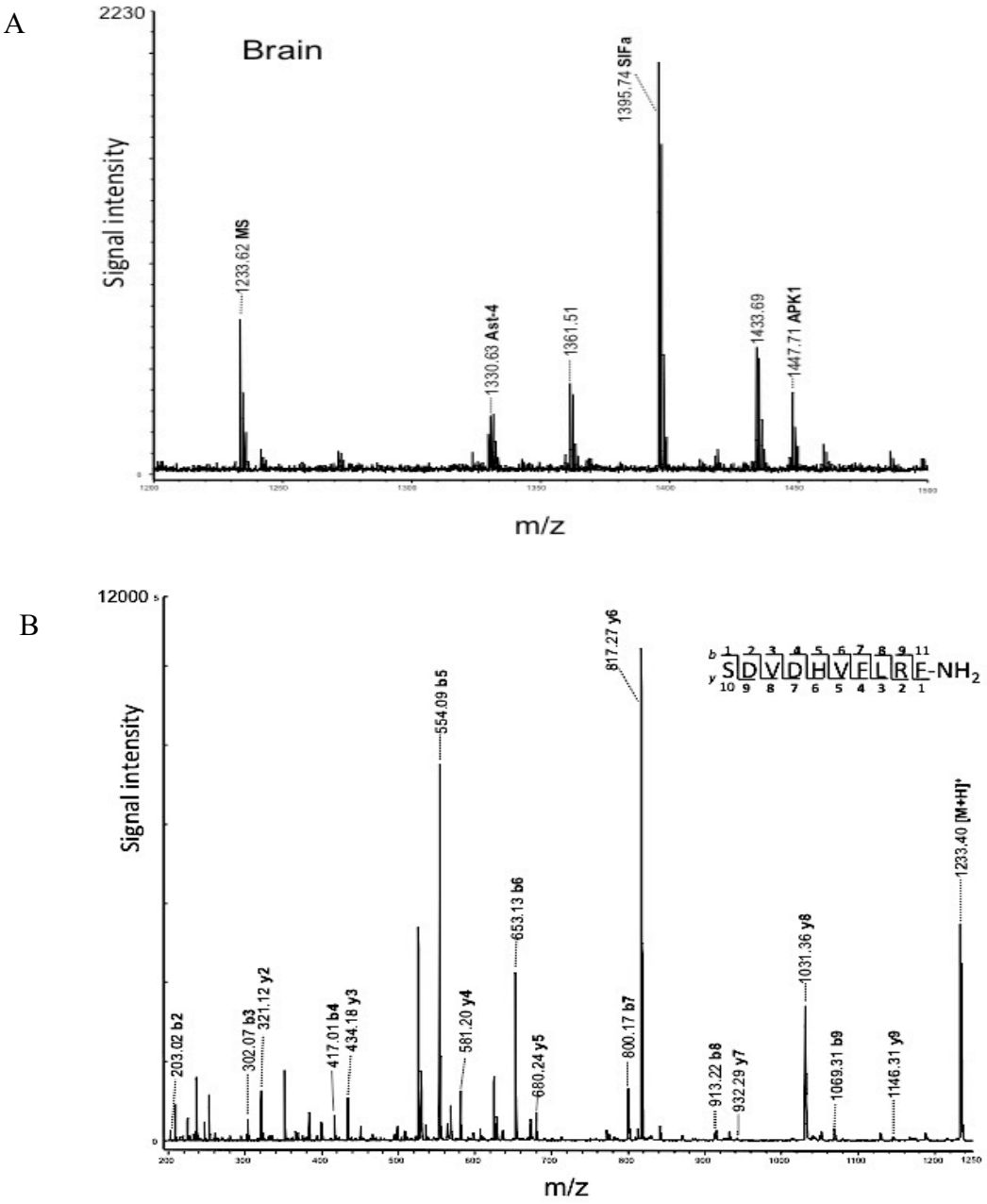


Figure 6.11 Mass analysis of HPLC fraction (A) derived from 100 *C. capitata* brains with ion masses ($[M+H]^+$) corresponding to myosuppressin (MS, 1233.62), Allatostatin-A (Ast-A, 1330.6), SIFamide (SIFa, 1395.7), and Neuropeptide-like precursor 1 (MTYa, 1447.7). (B) MS/MS fragmentation spectrum of the parent ion 1233.6. Mass analysis of collected HPLC fractions of brain and thoracico-abdominal ganglion are further as Supplementary data listed in Appendix.

6.5 Discussion

6.5.1 Myosuppressin signalling in medfly

The staining of the *C. capitata* crop with phalloidin revealed the anatomy of the muscle fibres that generate crop contractions. Noticeably, the isolated crop never appeared as a rounded, fully inflated sac as seen previously in other flies. The overall appearance of the crop in this 'relaxed' state was a horseshoe shape due to elongated lobes. The majority of the crop myofibres in *D. melanogaster* and *D. radicum* were concentrated in the centre of the crop, with fewer fibres reaching the actual lobes. In contrast, the medfly's whole crop surface including the lobes are equally covered with a network of fibres. Perhaps, the strong muscular organisation of the crop revealed by phalloidin explains the inhibitory potency of exogenously applied myosuppressin peptide (IC_{50}= 1.3 x 10^{-10} M) on crop peristalsis when compared to *D. melanogaster* (IC_{50}= 4.11 x 10^{-9} M) and the even weaker response for *D. radicum* (IC_{50}= 4.4 x 10^{-8} M). The robust muscular architecture of the crop is not only vital for digestive processes but also recently identified as key to male courtship displays, where males inflate their abdomens via air-filled crops, enhancing their size and thus attractiveness to potential female mates (Guillén et al., 2019).

Although the crop was very sensitive to myosuppressin (pM concentrations), muscles sorounding the midgut remained unaffected and continued to display peristalsis even in the presence of a high concentration (100 µM) of peptide (this study, but not presented) . Crop contractions were recovered within 1-3 min when the peptide (< 100 nM) was replaced with fresh saline. However, only in the presence of 1 nM myosuppressin was the tissue capable of regaining the initial frequency and strength of contractions. To validate the inhibitory effectiveness of myosuppressin and rule out potential bias from poor dissecting practice additional control studies were undertaken where several crops were repeatedly exposed to 10 nM peptide follwed by saline wash confirming the reversible nature of the inhibition. The observation that 1- 3 min is required for the crop to re-start spontaneous contractions might be explained by a slow desensitization of the activated myosuppressin receptor. Interestingly, Egerod et al. (2003) observed that both *D. melanogaster* cloned myosuppressin receptors take around 20 sec to desensitise, a much longer period compared to other insect neuropeptide receptors that desensitise in less than 5 sec. An *in silico* search performed through the basic local alignment search tool (BLAST) identified a single XP_020717891.1 transcript, coding for a myosuppressin-like G-protein coupled receptor (GPCR 139). GPCR 139 shares 71 and

68 % identity with Dms-R1 and Dms-R2, respectively. These GPCRs share significant structural similarities with FMRFamide receptors (E-value 6.25e-66). However, the true identity of GPCR 139 requires experimental confirmation by assaying the response of the receptor to myosuppressin and related peptides when expressed in a heterologous expression system for GPCRs (Egerod et al., 2003).

The direct tissue profiling of crop nerve bundle revealed a major molecular ion matching the predicted mass of *C. capitata* corazonin and many smaller ions, one of which matched myosuppressin. It has been shown for several dipterans that corazonin is present in both storage and glandular sections of corpora cardiaca that forms a major part of hypocerebral complex, the origin of the crop nerves (Predel, 2001; Predel et al., 2004; Hauser et al., 2010; Rahman et al., 2013).

A single dose of myosuppressin peptide injected into adult females did not intervene with the feeding behaviour based on defecation rate. Males were excluded from the experiment for their territorial aggression and competitive 'lekking' behaviour that might interfere with the experiment (Arita, L. H. and Kaneshiro, 1989; Warburg and Yuval, 1997; Papanastasiou et al., 2011). An anorexic effect of exogenous administration of myosuppressin was based on the reported outcome of studies on adults of different insect species (German cockroach *Blattella germanica* (Blattodea:Ectobiidae); cotton leafworm *Spodoptera littoralis* (Lepidoptera:Noctuidae); bright-line brown-eye moth *Lacanobia oleracea* (Lepidoptera:Noctuidae); pea aphid *Acyrthosiphon pisum* (Hemiphera:Aphididae) that were exposed to insect myosuppressin (Aguilar et al., 2004; Vilaplana et al., 2008; Matthews et al., 2009; Down et al., 2011). For example, in adult cockroaches myosuppressin delivered either in the diet or by injection exerted detrimental effects on individuals by limiting weight increase and lower food consumption as a result of inhibition of gut peristalsis (Vilaplana et al., 2008). It could only be speculated why the injection of myosuppressin (6.25 ng and 6.25 µg) into adult medflies did not result in any significant effect on food intake as measured by the excretion of food dye. It might have been due to rapid metabolic degradation of myosuppressin by proteolytic enzymes present in circulating haemolymph (Matthews et al., 2009). In most studies in other insects mentioned above, myosuppressin is protected at the N-terminus by pyroglutamic acid, providing protection against enzymatic degradation by aminopeptidases. however, at the myosuppressin of *C. capitata* is not protected at the N-terminus.

Injection of 6.25 µg (but not 6.25 ng) of myosuppressin did have the temporary effect of inhibiting egg laying suggesting that female reproduction is more sensitive to the peptide.

This suggests that targeting female reproductive processes could be an effective strategy in pest control. By day 2, however, both control and test groups were depositing comparable numbers of eggs. The mechanism behind the day 1 response is unclear, however, a recent study has found myosuppressin nerve termini in the uterus, common oviduct and lateral oviducts of female *D. melanogaster* and that the levels and distribution of immunoreactive myosuppressin changes after mating, suggesting a modulatory role for the peptide in regulating oviduct muscle contractions in this fruit fly (Heifetz et al., 2014). Whether myosuppressin plays a similar role in the female reproductive tract of *C. capitata* deserves investigation. Approaches through various experimental strategies, such as quantitative PCR to analyse the expression level of both, myosuppressin and its putative receptor gene in female reproductive tissues, functional bioassays as those applied to crops in *Drosophila,* and specific staining techniques to detect the presence and localization of myopsuppressin will help together provide a comprehensive understanding of myosuppressin in female reproductive physiology.

6.5.2 Medfly neuropeptidome

Throughout this study, both MALDI-TOF and Orbitrap mass spectrometry techniques were employed, utilizing MALDI-TOF for direct tissue profiling and Orbitrap for in-depth neuropeptidomic analysis of the medfly. In neuropeptidomics, both Orbitrap and MALDI-TOF mass spectrometry are powerful techniques, however they differ significantly in their operation and applications. Orbitrap mass spectrometry is known for its high resolution and accuracy, making it suitable for identifying and quantifying low-abundance neuropeptides in complex biological samples (Hu et al., 2005). It operates by trapping ions in an electrostatic field and measuring their frequencies, which provides precise mass determination. On the other hand, MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time Of Flight) mass spectrometry is commonly used for rapid and sensitive analysis of peptides (Walch et al., 2008; Salisbury et al., 2013). It employs a laser to ionize peptide samples that are co-crystallized with a matrix on a plate, and then measures the time it takes, also known as ‘time of flight’, for these ions to travel a fixed distance. While MALDI-TOF is less accurate in mass resolution compared to Orbitrap, it excels in its simplicity, speed, and the ability to analyze large peptides and proteins (Saidi et al., 2019). Each technique offers unique advantages in neuropeptidomics, with Orbitrap

providing in-depth, high-accuracy analysis and MALDI-TOF offering a faster, more accessible approach.

Genome sequencing projects have revealed that within and across insect orders there is high conservation of neuropeptide families and thus, even in species where the genome has not yet being fully annotated, it is possible to predict putative prohormones based on their homology to species with well characterised peptidomes (De Haes et al., 2015). After the publication of the *Drosophila* genome, a model organism for studying animal development and behaviour, *in silico* genome mining identified hypothetical 118 putative secretory neuropeptides (Adams et al., 2000; Liu et al., 2006b). To date, 42 are annotated as neuropeptides and neurohormones (Hewes et al., 1998; Yeoh et al., 2017). In the present study, translated transcripts of *D. melanogaster* peptide precursors were used as the query to mine the *C. capitata* genome with the aim of identifying neuropeptide precursors, referred to as prepropeptides, based on homology, exploiting conserved sequence motifs. Outside of the sequence for putative mature neuropeptide itself, characteristics of a signal peptide, mono- and dibasic proteolytic cleavage sites and post-translational modifications based on rules applied to insect peptide precursors (Veenstra, 2000) were identified allowing predicted mature peptide sequences to be aligned against *Drosophila* mature peptides. Resulting unique and prominent features are now briefly described and discussed below with complete identified transcripts from *C. capitata* listed in Appendix Fig.6.25.

Allatostatin peptides were named after their inhibitory role in the biosynthesis of juvenile hormone that is responsible for regulating development and reproduction in insects. Based on immunolocalisation studies, allatostatins belong to the group of so-called 'brain-gut' peptides with numerous physiological roles. Three types of allatostatins were isolated; FGLamides, $W(X)_6$Wamides and PISCFs. The first members of this family, Allatostatin-A (AST-A) peptides, were the first isolated/discovered in cockroaches. Their number varies between and within species, from over 40 putative peptides identified in the giant tiger prawn *Penaeus monodon* down to five isoforms in mosquitoes (Veenstra et al., 1997; Duve et al., 2002; Veenstra, 2009). In *D. melanogaster*, the function of AST-A is associated with foraging in larvae as well as in adults, while recently the peptide was associated with the regulation of both sleep and satiety (Jiangtian Chen et al., 2016). The medfly *allatostatin-a* precursor gene (XP_004531006.1) encodes six AST-A isoforms (Fig. 6.11). All putative peptides in the precursor, except AYTYTNGGNGM are followed by a glycine residue for C-terminal amidation and which is essential for

biological activity. Following a long signal peptide of 34 amino acids, the *C. capitata* precursor has four predicted peptides that are homologous to *D. melanogaster* drostatins1-4, sharing 100% similarity except for the non-amidated peptide AYTYTNGGNGM. An additional peptide *C. capitata* peptide (NRPYSFGLa) was predicted that was missing from the *D. melanogaster* precursor but matches to alike Ast-A peptide sequence in other pest species such as cabbage root fly, *D. radicum* (Audsley et al., 2011); sheep blowfly, *Lucilia cuprina* (Rahman et al., 2013) or the stable fly *Stomoxys calcitrans* (Liu et al., 2011). Finally, the last predicted sequence (SLQEPPPHRYGFGLa) shares a low level (33%) of common amino acid identity with that of *D. melanogaster*, however the same peptide sequence was found in five family members of Tephritidae fruit flies; *Rhagoletis zephyria, Zeugodacus cucurbitae, Bactrocera oleae, Bactrocera dorsalis and Bactrocera latifrons*. This leads to the assumption that the Ast-A peptide SLQEPPPHRYGFGLa is specific to this family of flies only. Intriguingly, SignalP 4.1 software failed to predict signal peptide cleavage and was missed in all prepropeptide sequences except for *Rhagoletis zephyria.* A combined transmembrane topology and signal peptide predictor Phobius however identified a cleavage site after 34th amino acid in the beginning of *C. capitata* prepropeptide and in the rest of the Tephritidae submitted prepropeptide sequences (Fig. 6.12, 6.13). The identification of this cleavage site provides valuable information for understanding protein processing, transition from an inactive precursor to its active form so the mature peptide can function correctly in Tephritidae. This have implications for both biological research and the development of novel pest management strategies.

The presence of all six Alt-A peptides in *C. capitata* was confirmed by preliminary results obtained from mass spectrometry analysis (Table 6.3).

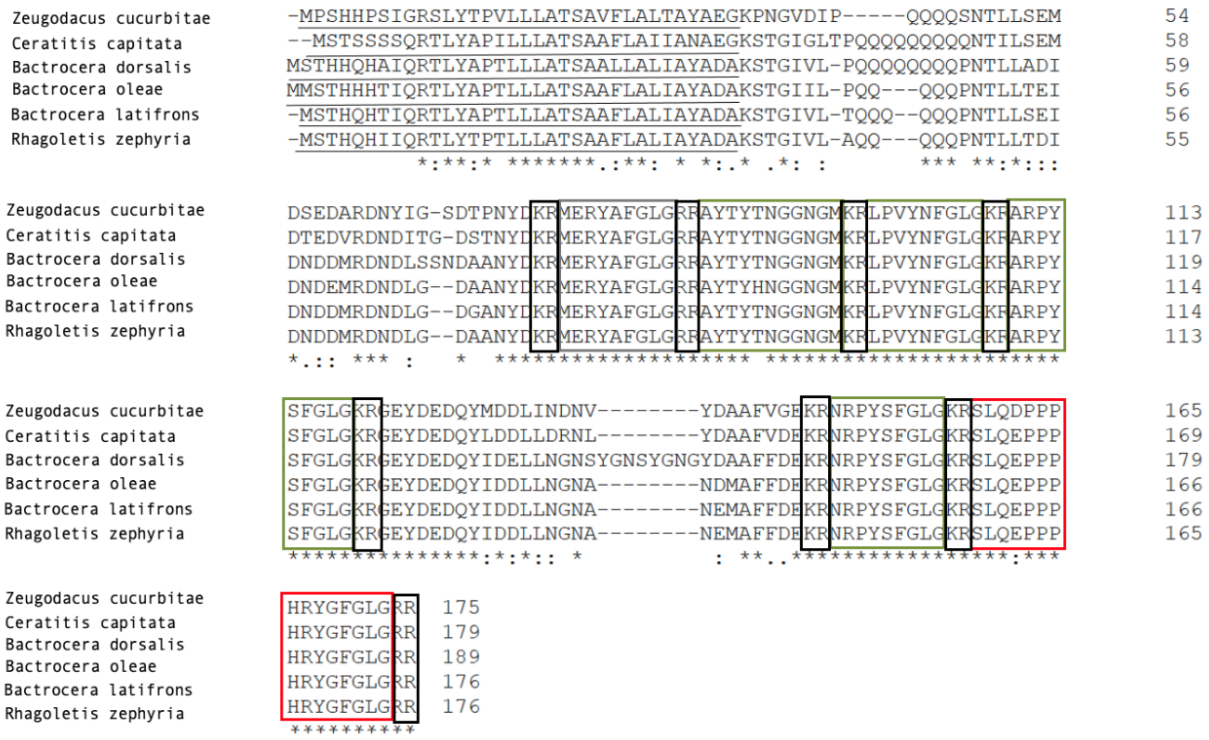
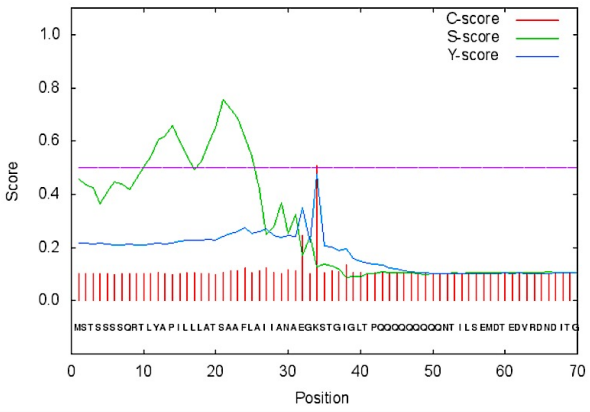


Figure 6.12 Alignment of Allatostatin-A prepropeptide sequences (green square) retrieved from *Zeugodacus cucurbitae* XP_011177714.1, *Ceratitis capitata* XP_004531006.1, *Bactrocera dorsalis* XP_011199722.2, *Bactrocera oleae* XP_ 014092355.1, *Bactrocera latifrons* XP_018791269.1 and *Rhagoletis zephyria* XP_017490250.1, the latter being the only one species having been identified with a putative signal peptide by SignalP 4.1. Yet, subsequent evaluation by Phobius predictor software identified signal peptide in all sequences analyzed (underlined by solid black line). The query for Allatostatin A putative peptide SLQEPPPHRYGFGLa (red square) has identified as a uniquely conserved peptide across six members of Tephritidae family, the true fruit flies.

A




```
# Measure  Position  Value    Cutoff   signal peptide?
  max. C    34        0.507
  max. Y    34        0.476
  max. S    21        0.755
  mean S     1-33     0.478
       D     1-33     0.477   0.500   NO
Name=XP_004531006.1     SP='NO' D=0.477 D-cutoff=0.500 Networks=SignalP-TM
```

B

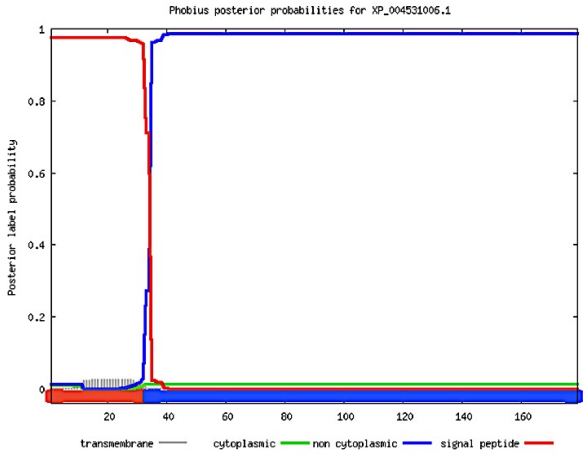


Figure 6.13 Signal peptide prediction for the Allatostatin A prepropeptide sequence of *Ceratitis capitata* (XP_004531006.1). (A) A cleavage site for signal peptide was not identified by SignalP 4.1 software. (B) A resubmitted query to Phobius predictor identified a 33-amino acid long putative signal peptide.

Members of the allatostatin B family are also known as the myoinhibitory peptides (MIPs) and as in the *D. melanogaster* preprohormone sequence, all putative MIP-like peptides are flanked with dibasic cleavage sites of lysine (K) and arginine (R) that are predicted to release five putative MIP peptides all showing the hallmark of this peptide family, the conserved W(X)6Wamide motif. Even though each peptide differs by 1 and 3 amino acid substitutions, they still share over 70% similarity. Except for NWQALQGPWa, the rest of the medfly Alt-B/MIPs (AWNKINAAWa, NAPTWNKFRGAWa, EPGWNNLKGMWa and DWAKLHGGWa) were confirmed as mature peptides through mass spectrometry (Table 6.3)

The *Drosophila* Allatostatin C/PISCF peptide was matched with an uncharacterized protein (LOC101460921) with two isoforms (XP_020714951.1, XP_004526686.1), corresponding to all 15 amino acids of the long Ast-C peptide of *D. melanogaster* and an N-terminal pyroglutamic acid. Its presence was also confirmed by mass spectrometry.

The uncharacterized protein LOC101462522 with two isoforms (XP_012157921.1, XP_012157922.1), were identified as the allatostatin CC precursor. Each predicts 21-amino acids long ALT-CC peptide alongside single copies of two other peptides. While

in *Drosophila* two ALT-CC isoforms predict identical, non-amidated 23-amino acid peptides, in medfly there is a 2 amino-acid shorter version of Ast-CC, (NGIGNGGRMYWRCYFNAVSCF) with a conserved NAVT/SCF sequence common for Alt-CC peptides in insects. Both, medfly and *D. melanogaster* have two isoforms that appear without a signal peptide. However, it can be detected if the second methionine is used as the translation start site. Interestingly, another two single copies of peptides can be retrieved from the precursor; the sequence AALLLDRLMVALHHALEQE is present in both isoforms in both *D. melanogaster* and medfly. The next predicted mature peptide (DLNSINRATGETLMAKSFQ) is only partially conserved in the alternative isoforms XP_012157922.1 and NP_609483.2 (Fig.6.14).

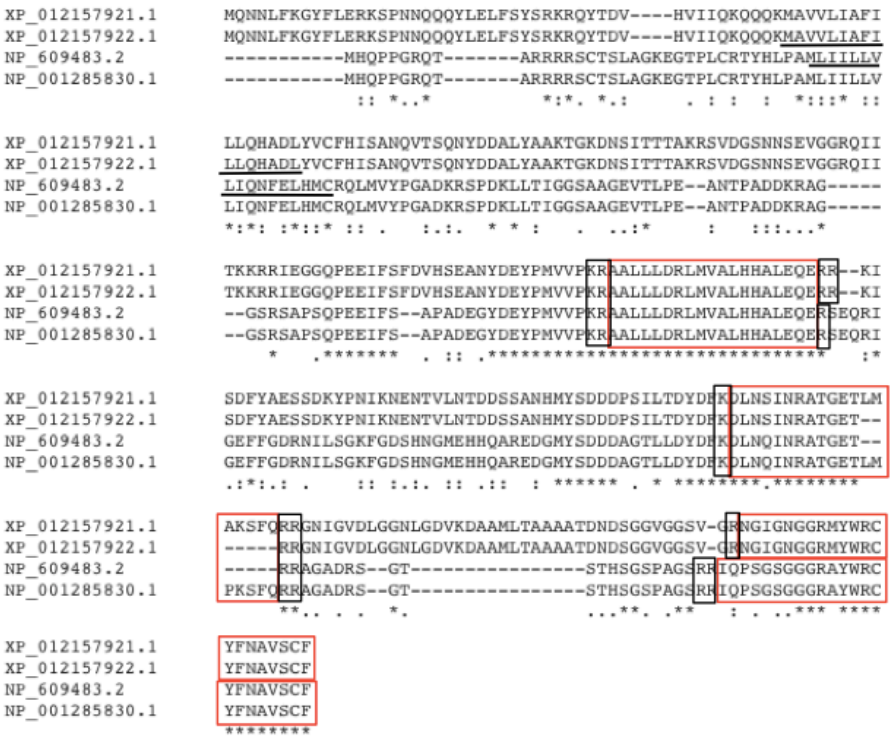


Figure 6.14 Alignment of two isoforms *Ceratitis capitata* for Allatostatin CC prepropeptide (XP_012157921.1, XP_012157922.1) followed by two sequences retrieved from *Drosophila melanogaster* genome (NP_609483.2, NP_001285830.1). Putative signal peptide is underlined by black solid line, which is only detected once the second methionine is exposed for translation. Red squares contain putative propeptide sequences while black squares indicate either single or diphasic cleavage site.

Adipokinetic hormones (AKH) are homologs of vertebrate glucagon and sometimes referred to as hyperlipaemic hormones (O'Shea and Rayne, 1992; Gáliková et al., 2015). They are metabolic peptides consisting of 8 to 10 amino acids that originate in glandular cells of the corpora cardiaca, a pair of neurohemal organs known to store and release numerous peptides originating from brain. Once AKH is secreted, it is released into the circulation to activate the release of lipid and glycogen from fat bodies that function as an equivalent to mammalian liver (Nässel, 2002). In adult flies AKH functions as a potent metabolic regulator of carbohydrates and lipid release from fat body cells in several insects while also implicated/coordinate in foraging behavior of starved individuals. A processing of single *akh* gene is giving rise to a one polypeptide that is either further modified or undergo altered cleavage. The putative AKH propeptides identified in medfly are also predicted to undergo post-translational modification of their N-terminus, where glutamine residue of the peptide cyclizes into pyroglutamic acid together with amidated carboxy terminus.

Mass spectra confirmed the presence of AKH (pQLTFSPDW) ions in both sodiated and potassiated forms. The detection of these multiple ionized forms not only provides additional verification of the peptide's identity and post-translational modifications but also confirms the robustness of our analytical approach, from *in silico* prediction through to proteolytic activation and extraction. Crucially, this finding indicates that the peptide has undergone processing and is present in its biologically active form. It was expected to find AKH in the CC. It was reported elsewhere that nerves originating from CC deliver AKH to the crop tissue via crop nerves that run alongside the crop duct (Lee and Park, 2004). In the present study mass analysis indicated the presence of corazonin, a peptide ancestrally related to AKH with with pyroglutamic acid at the N-terminus and C-terminal amidation. Previous published spectra of crop nerve bundle of other dipteran pest species *Drosophila suzukii* (Audsley et al., 2015) and *Delia radicum* (Audsley et al., 2011) did not find corazonin, but as already mentioned, it has been shown for several dipterans that corazonin is present in both, storage and glandular sections of corpora cardiaca that forms a major part of hypocerebral complex, the origin of crop nerves (Predel, 2001; Predel et al., 2004; Hauser et al., 2010; Rahman et al., 2013).

Another peptide hormone secreted by CC cells, which also co-localize with AKH, is limostatin, a suppressor of insulin signaling (Alfa et al., 2015). A single medfly precursor encodes for a *Drosophila* homolog with a putative non-amidated peptide sharing 100% identity.

The first FMRFamide peptide was isolated in mollusk for its potent cardioacceleratory effect (Orchard et al., 2001), and followed by the recognition of being part of broad family of neuropeptides that share FMRFa moiety, with occasional substitution within their C-termini. We have retrieved a two isoforms of prohormone precursor for FMRFamide-related peptides. Those sequences have shown to be identical except variation of missing 17 amino acids after methionine within second isoform (X2) that is also the only sequence present with a predicted signal peptide (Fig.6.15). A total of fourteen different peptides were deduced from the FMRFamide precursor sequence and half of those were confirmed as mature endogenous peptides by mass spectrometry, with two having their sequence confirmed.

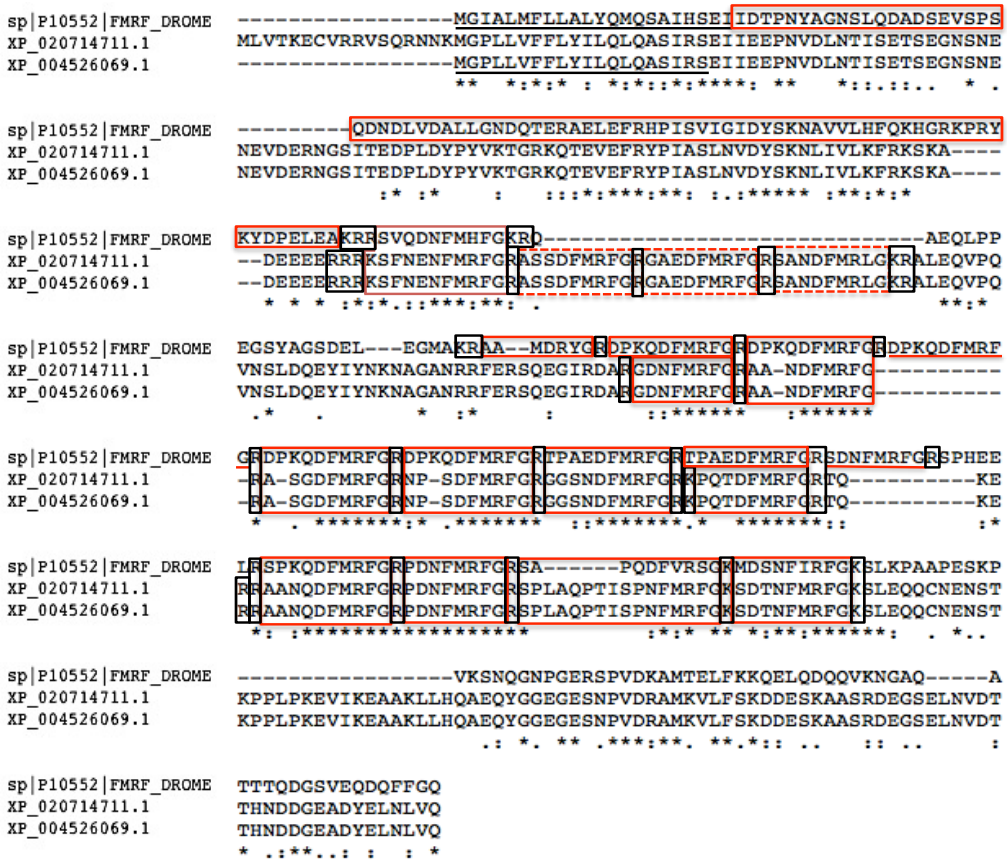


Figure 6.15 Single FMRFamide-like precursor (sp|P10552|FMRF_DROME) for *Drosophila melanogaster* was aligned against two *Ceratitis capitata* FMRFamide-like two isoforms (XP_020714711.1, XP_004526069.1). Putative signal peptide is underlined by black solid line. Red square contains shared putative propeptide sequences, red underline represents sequences unique to *D. melanogaster* and dotted red square indicates propeptide sequences only found in *C. capitata*. Black square indicates either single or diphasic cleavage site.

Myosuppressin (MS), a neuropeptide with characteristic FLRFa moiety shares a high degree of homology across species and is recognized for its potent myoinhibitory action on several muscle groups, particularly of those responsible for gut peristalsis (see Chapters 3 and 4). Medfly myosuppressin was found in a single precursor flanked with KR cleavage sites on both sides. The *C. capitata* decapeptide contains a substitution of Thr→Ser within the first N-terminal position, reducing its predicted mass to 1233.6 (m/z). Subsequently the structure was confirmed through fragmentation of the molecular ion by MS/MS (Fig. 6.11 B).

Physiological function of insect sulfakinins to the date is predominantly known of myotropic activity on various muscle groups (Schoofs et al., 2013). Only *C. capitata* sulfakinin 1 and 2 were found, both present on a single precursor protein separated from each other by KR dibasic cleavage sites. A third unusual *Drosophila* sulfakinin (SK-0, NQKTMSFGa) that does not share a common -GHMRa carboxy terminus moiety was not found in medfly (Fig.5.16 below).

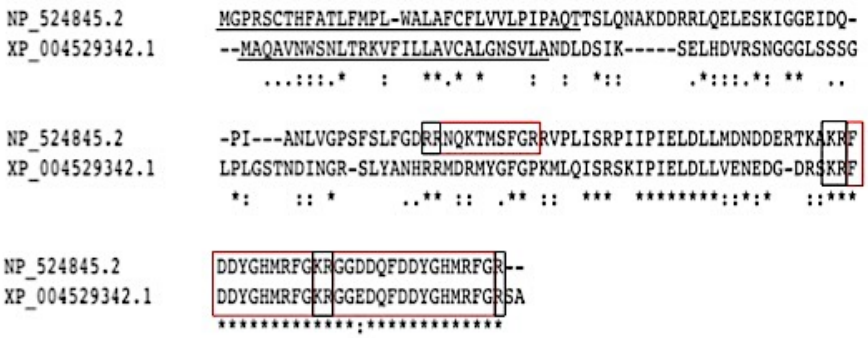


Figure 6.16 Sequence alignment analysis of sulfakinin *Drosophila melanogaster* prepropeptide (NP_524845.2) containing three putative propeptides compared to *Ceratitis capitata* (XP_004529342.1) that is missing a putative sequence identified as SK-0. Black underlines indicate putative signal peptides and red squares highlight propeptide sequences.

Neuropeptide F family is divided into short and long versions. A single prepropeptide encoded by the medfly short NPF gene contains four peptides, each ending with

glutamine and flanked with cleavage sites to be released as amidated mature peptides sharing RLRF C-terminal. One of the most prominent roles of sNPF in flies is to promote food intake and control of body size (Lee et al., 2004). Similarly, the long version of NPF influences feeding behaviour, but is also implicated in male courtship performance (Van Wielendaele et al., 2013). Two variations of transcripts encoding for a single, nearly identical 39 amino-acid long putative peptides exist in medfly (NPF-1 and NPF-2), differing only by 3 substitutions by S→G, N→S, A→G on position 1,9 and 29 respectively (Fig. 6.17), a unique feature within dipterans.

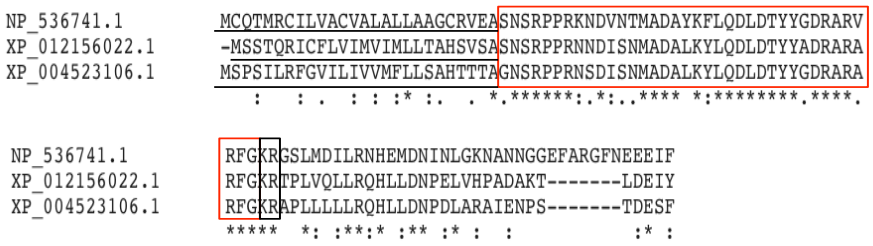


Figure 6.17 *Drosophila melanogaster* single sequence (NP_536741.1) aligned against two NPF *Ceratitis capitata* precursors (XP_012156022.1, XP_004523106.1). Black underlines indicate putative signal peptides while red squares mark propeptide sequence.

CCHa-1 and -2 hormones were identified on two separate preprohormones, LOC105665375 and LOC101460931, sharing 100% identity with those found in *D. melanogaster*. While the CCHa-1 sequence is identical to CCHa from *D. melanogaster*, the CCHa-2 of predicted peptide, differ in position 3 (Q→N) and 8 (V→A). CCHa peptides are 'brain-gut' neuropeptides/enteroendocrine peptides. CCHa-1 is found together with allatostatin A in enteroendocrine cells of the posterior region of midgut, whereas CCHa-2 is located in anterior enteroendocrine cells and co-localises with allatostatin C.

The neuropeptide CNMamide is associated with sperm rejection in female *D. melanogaster* (Lee et al., 2015). The identical sequence was identified within single transcript coding for an uncharacterized medfly protein (LOC101457297), flanked with

cleavage sites (K-RK). The presence of glutamine and glycine residues suggests subsequent post-translational modifications at both termini, to form N-terminal pyroglutamic acid and an amidated C-terminus.

Two peptides sharing a conserved amidated FPRV C-terminal motif, characteristic for CAPA peptides (or periviscerokinins) known for myotropic and osmoregulatory properties (Davies et al., 2012), were also identified. Based on alignment analysis, it is most likely that first peptide (TAGGPSGLIAFPRVa) might be further processed by aminopeptidase to shorter mature peptide, lacking first three amino acids from its N-terminus. The third peptide sequence identified corresponds to *D. melanogaster* pyrokinin-related peptide (PK-1) that is part of the pheromone-biosynthesis activating neuropeptide/diapause (PBAN) hormone family. The well-conserved sequence, sharing 88% similarity with *Drosophila* PK-1, contains additional glycine at the N-terminus together with a substitution of T→A at 2nd position and on 7th S →T.

An uncharacterised protein LOC105664747 contains sequences for putative mature pyrokinins, PK- β and hugin (PK-γ) peptides. While PK-γ is identical to that of *D. melanogaster* except for a substitution on the 2nd position where V→A, the PK-β appears unique to medfly (SQKYDNFIEQPSAVMDLAPRLSSFDRIPFNDYNRIVSALEMLRVNEEKSVMLRPRPG). The C-terminal residue, although amidated in both flies does not share a characteristic PRLa motif. Instead, it has a substitution of L→P. Also, the length of predicted mature peptide differs significantly. Alignment analysis has also shown that PRL motif sequence is found in the middle of putative propeptide but lacks cleavage sites and amino acids requited for posttranslational C-terminal amidation. Since the signal peptide is missing for the medfly precursor, it most likely represents the hugin precursor flanked with cleavage basic sites (K, RR) that would be processed at position 110 and 128 to produce a unique neuropeptide (EMLRVNEEKSVMLRPRPa) (Fig. 6.18). Across this chapter Cleavage sites are formed by dibasic motifs (combination of arginine or lysine) as showed by black boxes in sequence figures across this chapter however single basic residues (blue box below) are also known to be cleaved in precursors (Amare et al., 2006).

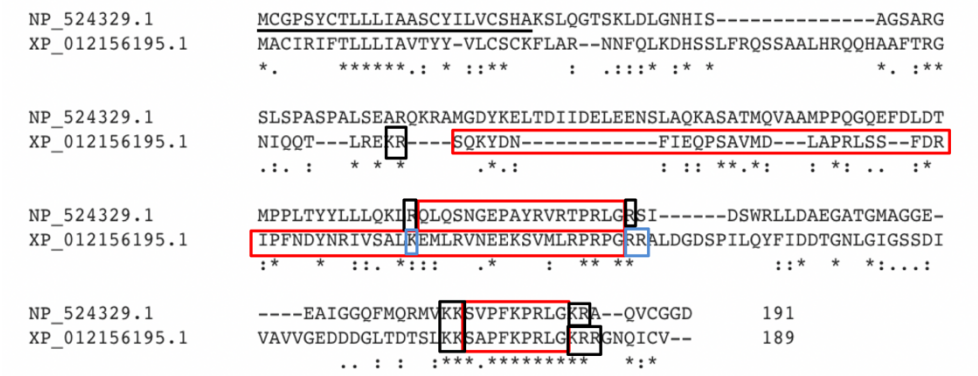


Figure 6.18 Alignment of *Drosophila melanogaster* precursor encoding for Pyrokinin (PK- β) and hugin (PK-γ) (NP_524329.1) and unannotated *Ceratitis capitata* sequence (XP_012156195.1). A signal peptide sequence was only found for *D. melanogaster* transcript (black underline). A hypothetical neuropeptide sequence could be processed from the medfly sequence if cleavage occurs at basic K and RR motifs (blue boxes).

Two isoforms of diuretic hormone (DH-31) prehormone sequences encoding for identical mature peptides flanked with cleavage sites KR and RR, were retrieved from the BLASt analysis. The mature peptides differed by a single substitution at the N-terminus (A→P). The *D. melanogaster* corticotropin-releasing factor (CRF)-like diuretic hormone (DH-44) matched an uncharacterised protein LOC101456921, predicted to encode an identical CRF-like peptide apart from a single amino acid substitution of S → T.

Ecdysis, a process of shedding old cuticle during the growth and development of insects is a very complex and precisely-regulated behaviour believed to be initiated and under the control of eclosion hormone (for comprehensive review refer to Park et al., 2002). In medfly, the eclosion hormone sequence of 79 amino-acid long prohormone is well conserved, with 78% common identity with the *D. melanogaster* precursor (Fig.6.19). Once the cascade is initiated, ecdysis is further regulated by the release of ecdysis triggering hormones (ETH) produced by specialised endocrine epitracheal or 'Inka' cells (Zitnan et al., 1996). Both ETH-1 and ETH-2 were identified on a single medfly preprohormone sequence - uncharacterized protein LOC101457772, with both sharing over 80% similarity. (ETH-1, 90% similarity and 84% identity; ETH-2, 50% identity and shared 81% similarity). Ecdysis is further coordinated and regulated by the release of additional factors, such as Crustacean Cardioactive peptide (CCAP) and bursicon hormone. Sequence alignment analysis revealed an identical CCAP sequence in both

medfly and *D. melanogaster*. Bursicon was also highly conserved (92% amino acid identity). In this study, we were unable to detect the prothoracicotropic hormone (PTTH) in *C. capitata*, a hormone known to be secreted from the corpora allata and crucial for initiating the release of ecdysone, which is necessary for ecdysis in *Drosophila* (Truman and Riddiford, 2002). This absence is unexpected yet not entirely surprising, given that a similar loss of PTTH and its associated receptors has been noted in several Hymenopteran genomes (Skelly et al., 2019). Particularly intriguing is the potential reasoning behind the loss of PTTH, which appears more frequently in social insects like bees, suggesting a shift towards reliance on external or abiotic cues for early development rather than internal cues. For further information on PTTH loss and its relation to diapause, please refer to Costa et al., (2022). Another hypothesis considers the medfly larvae's underground development (pupation), which could diminish the dependence on abiotic cues like light, known to regulate PTTH-producing brain neurosecretory cells, thereby implying the presence of alternative regulatory mechanisms.

Figure 6.19 Alignment of eclosion hormone transcripts of *Drosophila melanogaster* (NP_524386.1) and *Ceratitis capitata* (XP_004536647.1). Black underline highlights signal peptide sequence, red square indicates predicted mature peptide.

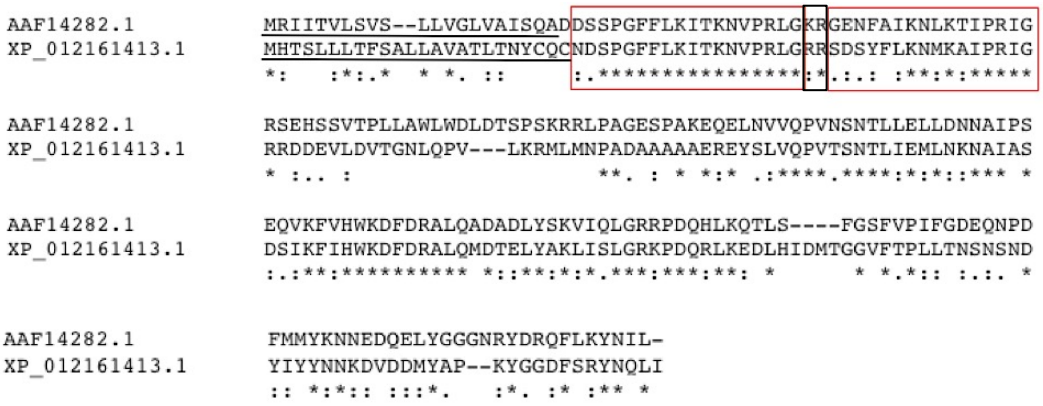


Figure 6.20 Alignment of transcripts encoding for ecdysis triggering hormone 1 (ETH-1) followed by ecdysis triggering hormone 2 (ETH-2) of *Drosophila melanogaster* (AAF14282.1) and unannotated transcript (XP_012161413.1) from *Ceratitis capitata* separated with diphasic proteolytic cleavage site (black square). Both sequences contain a putative signal peptide (black underline).

A precursor (uncharacterised protein LOC105664782) encoding three putative natalisin peptides, is each flanked with diphasic proteolytic cleavage site (RR, RK, KR and KK) with a glycine for post-translationally amidation. An alignment of each individual peptide highlighted particular difference within first peptide (Natalisin-1) sharing only 33% similarity and half the size of the *Drosophila* peptide. NTL-2 and NTL-3 on the other hand share a high degree of conservation, 73% and 100% identity respectively. However, no putative signal peptide was found.

Orcokinin peptides, another classical brain-gut peptide family with myotropic activity (Pascual et al., 2004), were identified in medfly as uncharacterised protein LOC101451285, encoding a single isoform (A & B) for each peptide. Sequence alignment revealed they are well conserved, sharing 90 and 91% similarity between species.

The pigment-dispersing factor 'PDF' is involved in the circadian system to regulate biological rhythmicity (Lear et al., 2009). A *C. capitata* PDF homologue was found that only differed from that of *D. melanogaster* by two substitutions (N→S) and (D→E).

The myoregulatory pentapeptide, proctolin (RYLPT) was identified within transcript for an uncharacterised protein LOC101462442. Proctolin was the second insect neuropeptide to be characterised and is widely distributed in arthropods, but surprisingly missing from several insect species (Orchard et al., 2011). The signal peptide cleavage site is directly followed by the proctolin sequence RYLPT tailed by arginine indicating proteolytic cleavage site, as it can be seen in other species. A search for a proctolin GPCR using the *D. melanogaster* transcript as the query identified the uncharacterised protein LOC101460750 (XP_023158717.1) with 1e-82 E value and sharing 75% protein sequence identity, indicating that both ligand and receptor are present in *C. capitata* (Fig. 6.21).

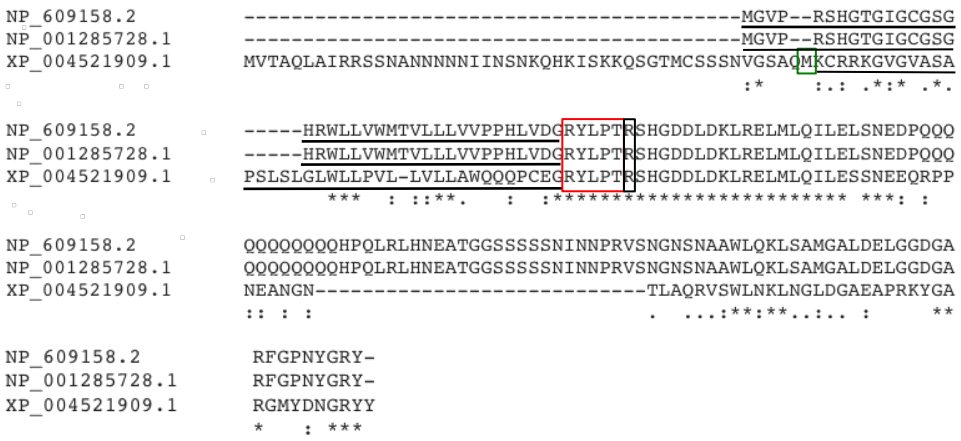


Figure 6.21 Sequence alignment of two proctolin *Drosophila melanogaster* isoforms (NP_609158.2, NP_001285728.1) and unannonated *Ceratitis capitata* transcript (XP_004521909.1). Putative signal peptide sequence is only detected after third methionine in *C. capitata* transcript is exposed (green square), directly followed by proctolin pentapeptide sequence and tailed with proteolytic single cleavage site (black square).

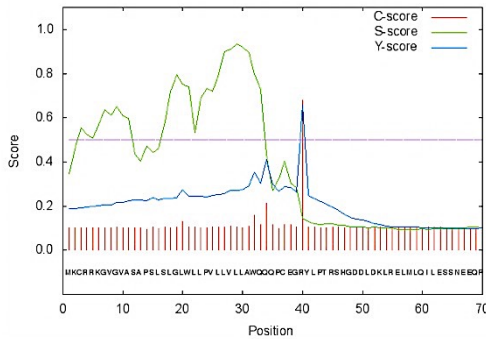


Figure 6.22 Unannotated transcript XP_004521909.1 of *Ceratitis capitata* was identified as proctolin precursor and analysed by SignalP 4.0 software to predicts the presence and cleavage site of 39-amino acid long putative signal sequence.

RYamide neuropeptides have only recently been implicated in feeding control and water homeostasis (Veenstra and Khammassi, 2017). The predicted medfly and *D. melanogaster* peptides are highly similar, sharing over 80% identity. As in *Drosophila*, both putative mature peptide sequences are present on a single preprohormone precursor and flanked with KR-R and RR-KR cleavage sites (Fig. 6.23). RYamide-specific antibody in *Drosophila* identified the presence of peptide within midgut enteroendocrine cells as well as in a pair of abdominal neurons innervating the rectal papillae.

```
NP_001104382.3      MNECV-------------------N-------------KLLHLKFLFYFILGIQKRPVFF      28
XP_004535985.1      MTQAVRQRGIIFLLTIIGLLNFLIEPVLSSSGSRDEQLSGDAVDKNGYEQLTLHKRPSFF      60
                    *.:.*                   :              .   :.   *  * ::*** **

NP_001104382.3      VASRYGRSTTYD--ESLKSRRKIFIVPRNEHFFLGSRYGKRSGKYLCLSREINKLIVRKRL      86
XP_004535985.1      VGSRYGRSSDGTAFSSSKTRRKLSVVPRNDRFFLGSRYGKRAEEGSVFEPYSPYE-QQNQ-      118
                    *.******:     .* *:**: :****::**********: :   :.        :::

NP_001104382.3      RNNDKERTPTLSFITKHFLMRNT-----------------------------------      109
XP_004535985.1      -QADNDAANNLNDMTVRHIGGNKQLYGELNNMDKPNQIQSPTMMSCMHTGFRNYYRCRNI      177
                     : *:: : .*. :* :.:  *.

NP_001104382.3      --------------------        109
XP_004535985.1      DEINNVINQLTVAHSVEERK        197
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Figure 6.23 RYamides (RYa1/RYa2) precursors alignment of *Drosophila melanogaster* (NP_001104382.3) and *Ceratitis capitata* (XP_004535985.1) transcripts, with only the latter containing a putative signal peptide (black underline). Both propeptides, highlighted in red box, are flanked with proteolytic cleavage sites on each side of putative peptide (black square).

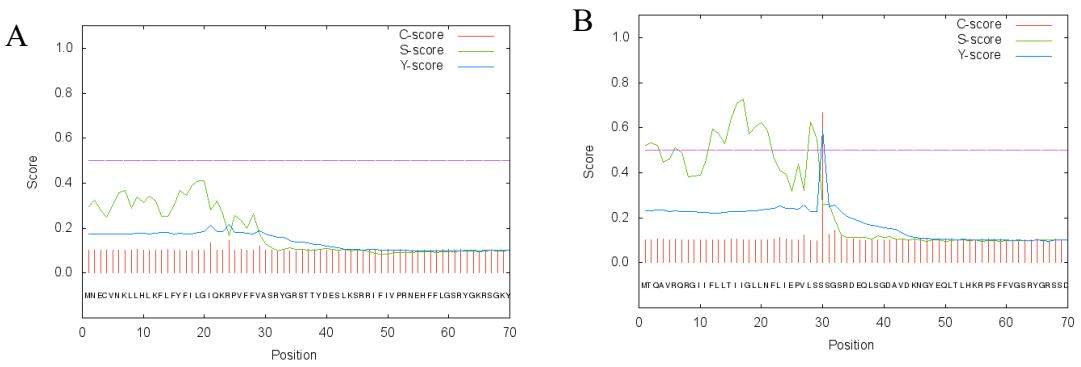

Figure 6.24 Quest for the presence of signal peptide sequence in RYamides (RYa1/RYa2) precursor. The analysis of (A) *Drosophila melanogaster* NP_001104382.3 transcript lacks potential cleavage site indicating the absence of signal peptide. Conversely, screening of (B) *Ceratitis capitata* RYa propeptides coding sequence (XP_004535985.1) identified the presence of 29-amino acid long signal peptide.

The dodecapeptide sequence of SIFamide peptide is preceded with a putative signal peptide followed by diphasic proteolytic sequence (KR) that allows post-translational amidation of its carboxy terminus. The propeptide sequence is conserved (100% identity) among many insect species including medfly. Although initially identified as a myotropic peptide, it is also proposed in paying role in sleep pattern or sexual behaviour (Park et al., 2014).

Another 'brain-gut' neuropeptide shown to be involved in regulation of feeding, together with myotropic and diuretic activity, is the insect kinin. The mature putative peptide is liberated from its single precursor by cleavage KR sites and is post-translationally amidated. The mature form of *D. melanogaster* kinin shares 100% identity with the predicted medfly peptide (Terhzaz et al., 2007; Zandawala et al., 2018).

The *C. capitata* transcript XP_012157482.1 coding for an uncharacterised protein LOC101453784 contains a putative Trissin propeptide, sharing over 86% peptide similarity. It is another recently identified, cysteine-rich neuropeptide that it is believed to have a role in antimicrobial defence (six cysteines in medfly and in *D. melanogaster*) (Ida et al., 2011).

A single transcript for ‘neuropeptide-like 1’ protein in medfly contains four putative sequences, each divided from each other by KR proteolytic sites. All are highly similar, on average sharing over 80% similarity except for APK peptide (*D. melanogaster* SVAALAAQGLLNAPK) sequence, with half of amino acids scoring 61% for similarity of predicted mature peptide. The only other neuropeptide-like sequence belonging to the NPLP family was found to be related to NPL-4 with post-translational modifications in the form of cyclization of N-terminal glutamine and carboxy terminal amidation. A single transcript for putative NPL-4 peptide, also known as YSY peptide retains uncharacterised function to this date. Based on FlyAtlas data, it is likely that it will escape the mass spectrometry detection since in adults it is predicted to be found in eye, heart and hindgut but not in the CNS.

Three transcripts in medfly are predicted to translate into tachykinin-related peptides (TRP), functionally assigned as olfactory-modulating peptides (Winther et al., 2006). Sequence alignment analysis revealed that first two transcripts encode 6 putative propeptides each, while third transcript bears a deletion of the last two prepropeptide amino acid sequences.

As this exploration of medfly neuropeptidomics draws to a close, the field's complexity and potential for new discoveries become increasingly apparent. The absence of certain anticipated neuropeptide genes in our findings underscores the nuanced nature of genomic research and emphasizes the importance of comprehensive methodologies and a readiness to embrace unforeseen results. Such findings prompt us to consider hitherto undiscovered genetic pathways or sequences that might significantly alter our understanding of medfly neurobiology. Therefore, moving forward requires not only thorough genomic analysis but also the integration of various scientific techniques to form a complete view. When addressing the supposed absence of genes, a cautious approach is paramount. This includes resequencing the genome to rule out any human errors in initial searches, followed by an extensive search across a wide range of related species or families. Employing laboratory methods like PCR, RT-PCR, or *in situ hybridization* (Li et al., 2006; Pfeiffer et al., 2008; Scolari et al., 2012) is crucial for confirming the presence of mRNA, which in turn validates the existence of these elusive 'ghost' genes. These techniques are instrumental in detecting and localizing specific genes for neuropeptides that may not be readily apparent in genomic searches. Likewise, the

application of mass spectrometry for tissue screening and antibody-based assays can provide additional insights into post-translational modifications and offer indirect evidence of these genes at the protein level (for more indepth review of techniques commonly applied in neuropeptidomics please refer to DeLaney et al., (2018)). Complementing these methods would shed light on the regulatory mechanisms of neuropeptide synthesis and function in the medfly. This complex approach will not only enhance our understanding of specific gene activities but also enriches our overall comprehension of the medfly's biology.

Chapter 7 General Discussion

As climate change is altering the distribution of pest's species (Björkman and Niemelä, 2015), it is likely to lead to a rise in the pesticide use. This increase in frequency of pesticide application would have negative effect on natural pest control and cause targeted pests to become more resilient towards current treatments, creating a vicious cycle where even more pesticides are needed. Over the past few decades, the widespread use of chemical insecticides has been met with increasing concern due to their ecological toxicity and the related development of resistance towards them (Siegwart et al., 2015). Many of those chemical insecticides, once hailed for their efficacy, have demonstrated detrimental effects on non-target species, including beneficial insects, aquatic life, and even terrestrial vertebrates (Sharma et al., 2020). Importantly, indiscriminate use of these chemicals has accelerated the evolution of field resistance in many insect populations, rendering them less potent or entirely ineffective (Magaña et al., 2008; Couso-Ferrer et al., 2011; Arouri et al., 2015; Hawkins et al., 2019; Guillem-Amat et al., 2020). Consequently, the dual challenge of ecological harm and rising resistance has compelled regulatory bodies and researchers to reconsider, and in many cases withdraw, the approval of many chemical insecticides, emphasizing the need for more sustainable and eco-friendly pest management solutions (Chandler et al., 2011). There's a pressing and undeniable need for innovative insecticidal compounds that brings new modes of action but also align harmoniously with ecological well-being, a crucial step in safeguarding our food supply.

This thesis focuses on examining the complex role of neuropeptides, specifically myosuppressin, and the role it has in the regulation of insect gut physiology, positioning it as a promising candidate for disrupting insect feeding behaviors. Our investigations targeted two key pest species, *D. radicum* and *C. capitata*, alongside the genetically versatile model organism, *D. melanogaster*. The use of *D. melanogaster* is particularly advantageous due to its comprehensive array of genetic tools and rich repository of biological insights, which are instrumental in defining and validating potential insecticidal targets. Neuropeptides and their receptors are increasingly recognized as key targets in insect pest control research due to their integral roles in the central nervous system, influencing functions such as mood, pain perception, appetite, sleep, learning, and memory (General Introduction, subsection 1.4; Halberg et al., 2015; Audsley and Down, 2015). The potential of neuropeptide signaling dysfunctions as insecticidal targets is particularly promising, given the presence of over 50 signaling peptide genes in insects. A prime example is myosuppressin, a myoinhibitory peptide originally discovered in the

cockroach *Leucophaea maderae* (Holman et al., 1986), known for its wide-ranging effects across different insect tissues including the nervous system, gut, reproductive organs, and heart. The myosuppressin system, with its myoinhibitory and anorexic activities, has been identified by numerous studies as a potent insecticidal target, paralleling the neurotoxic effects of many commercial insecticides (McCormick and Nichols, 1993; Vilaplana et al., 2008a; Matthews et al., 2009; Scherkenbeck and Zdobinsky, 2009; Down et al., 2011; Schoofs et al., 2017).

In the first experimental chapter of this research, mass spectrometry and the GAL4/UAS expression system were employed to expand on previous work of McCormick and Nichols (1993) showing Dms neuron innervation in the crop of *D. melanogaster*. Synthetic Dms applied in *ex vivo* assays significantly inhibited spontaneous crop contractions. The availability of DmsR-1 and DmsR-2 knockout (KO) mutant flies during this Ph.D. project has been instrumental in advancing our understanding of these pathways. They helped unveil the signaling mechanism that underpins the crop's control. It was evident that DmsR-1 played a pivotal role in this potent inhibition. Dms inhibit crops in both mutant lines, however in the case of flies lacking functional Dms-R1 the requirement is of substantially higher peptide concentration than for those observed in wild-type and DmsR-2 KO flies. For this reason, it is suggested that Dms-R2 has a potentially minor and redundant role in inhibiting Dms signal transduction within the crop. This finding was further supported by feeding experiments, where flies lacking DmsR-1 exhibit altered excretion behaviour compared to wild-type, while Dms-R2 KO flies had not shown significant difference. Perthaps, to strengthen the validity of those findings, and to enrich understanding of the complex molecular mechanisms governing crop activity in response to Dms signalling, gene expression analysis techniques such as quantitative PCR (qPCR) or RNA sequencing could be applied to quantify and compare the expression levels of each Dms receptor, across different genetic backgrounds and under various physiological conditions. Assessing gene expression in crops under different nutritional states (fed vs. starved) can also provide insights into the physiological relevance of individual receptor in metabolic regulation. It could also reveal how the absence of each receptor influences the expression of the other, and how they collectively respond to Dms. Finally, a double knockout mutant, where both DmsR-1 and DmsR-2 receptors are nonfuctional, present a unique opportunity to unravel the distinct contributions of these receptors to the inhibition of crop contractions by Dms. By comparing the response of the double KO mutants to that of the single KO strains we

used, together with wild-type controls, it could be determined the individual and combined roles of DmsR-1 and DmsR-2. If DmsR-1 indeed plays a key role in inhibiting contractions, one would expect to observe a marked reduction in Dms sensitivity in the DmsR-1 KO and double KO mutants compared to wild-type and DmsR-2 KO flies. The double KO mutants would be particularly informative, as any residual response to Dms in the absence of both receptors would point towards the existence of additional, unidentified pathways involved in mediating this effect. Conversely, a complete loss of response in the double KO mutants would solidify the understanding that DmsR-1 and DmsR-2 collectively account for the entirety of Dms-mediated inhibition, with DmsR-1 being the predominant player. This comprehensive analysis would enable to scrutinise the signaling mechanisms at play and emphasizes the invaluable role of genetic tools in elucidating complex neuropeptide signaling.

Notably the confirmation of the role of Dms-R1 in crop physiology recently appeared in a ground-breaking paper that demonstrated a role for Dms neurons and the crop in increasing food intake in post-mated females. Using flies that are allowed to feed *ad libitum,* Hadjieconomou et al., (2021) showed that the crop of mated females was larger than the crops of virgin females and males, concluding that Dms signaling is involved in regulating food intake, reproductive hyperphagia and reproductive fitness. Genetically activated Dms neurons resulted in greatly enlarged crops of flies fed *ad libitum,* whereas crop enlargement was not seen when Dms neurons were silenced in starved-refed insects. This result is similar to that reported in Chapter 3 for starved- refed DmsR-1 mutant flies. Hadjieconomou et al. (2021) propose that the *in vivo* role of Dms in the adult gut is to relax the crop muscle, allowing expansion and increased food intake. This role is mediated by DmsR-1 only, as DmsR-2 is not involved as suggested by the data presented in Chapter 3. The mechanism by which gut physiology is modulated by mating was attributed to elevated Dms release from neurosecretory neurons of the pars intercerebalis (PI) of the brain that directly innervate the crop via the crop nerve bundle. The authors conclude that flies feeding *ad libitum* relax and expand the crop to generate suction of ingested food, a mechanism regulated by peptide release from Dms neurons. The greater expansion and consequent increase in feeding seen in mated females is, in turn, coordinated by steroid hormones and peptides released from gut endocrine cells. It is highly likely that a similar integrated control of the crop is conserved in all adult flies. Thus, the crop can be considered as an important reproductive organ, at least in female dipterans, that respond to changes in mating status to increase feeding rates and provide

resources for egg production. Our findings in Chapter 6, reports on myosuppressin peptide being involved in fecundity when delivered via microinjection. The elicited response resulted in cessation of egg lying in females, that was recently supported by Hadjieconomou *et al.* (2020) linking crop functionality directly to the egg development in *D. melanogaster* females, labelling a crop as vital reproductive organ. Since this has only occurred at high concentration of peptide delivered through microinjection, to avoid fast degradation via digestive route, the design and stability of any myosuppressin analogue is paramount in further development to achieve potent insecticide effect.

Outside of digestive tract, staining of myosuppressin was also observed in reproductive organs. Interesting point was made by Stoffolano et al., (2010) who compared musculature surrounding the crop to those similar of those covering *D. melanogaster* ovaries (Hudson et al., 2008). Myosuppressin has been found in the female oviduct and uterus of *D. melanogaster* using specific antibodies, but its role is not known (Heifetz et al., 2014). Observation of myosuppressin-like staining was noted across the whole peritoneal sheath covering ovary and oviduct, a tube composed of single layer of tissue surrounded by muscles (Pribylova, unpublished data), and without further studies one could only speculate whether myosuppressin signalling has any effect on ovarian muscles contractility and thus regulate egg movement. Our lab has previously shown myoinhibitory role of MS in *Drosophila* male's reproductive organs (Dr. Abu-Hasan, PhD Leeds University) and oviductal motility was inhibited in locust (Lange et al., 1995). To make a conclusive role for myosuppressin in fecundity, one would need a further investigation into egg laying, ovarian physiology between mutants lacking individual receptor, together with the application of the peptide *in vitro* to observe any effect on the musculature sheet contractions surrounding female ovaries. To explore the role of myosuppressin in ovarian function, a comprehensive strategy encompassing molecular, genetic, and physiological methods is proposed. Gene expression analyses like qPCR and *in situ* hybridization will assess myosuppressin mRNA levels in the ovaries, while immunohistochemistry and confocal microscopy will visualize its protein distribution. The functional impact of the peptide on ovarian muscle contractility and egg movement should be examined through *ex vivo* ovary contraction studies following by calcium imaging, and egg-laying behavioral assays. Genetic tools such as RNAi and CRISPR/Cas9 gene editing are required to manipulate MS expression and observe the resulting phenotypic changes in dipteran families beyond *Drosophila* species, especially considering the presence of a single myosuppressin receptor, as identified in Chapter 6

for the medfly. These combined approaches will offer deep insights into the specific roles and mechanisms of myosuppressin in the ovaries of dipteran pests.

The high degree of structural and functional conservation of the chosen target across different dipteran pest species is a critical aspect of this research. *D. melanogaster* served as a template to retrieve and compare *D. radicum* and *C. capitata* MS precursors to fulfil the requirement for neuropeptide-analog to be effective in both species. Acquired data for cabbage root fly were based on mass spectrometry experimental data of the larval and adult central nervous system (Audsley et al. 2011; Zoephel et al. 2012) while medfly MS prepropeptide was retrieved in form of hypothetical protein based on unassembled WGS (whole genome shotgun, WGS ID CAJHJT010000000) as a part of i5K arthropod project (Papanicolaou et al., 2016). The alignment revealed a conservation of peptide sequence except for a single amino acid substitution of threonine with serine in the first position of medfly's N-terminus. They are fairly commonly interchanged due to their structural similarities. Both amino acids are small in size and possess a hydroxyl group on the side chain. Further studies are required to test whether this change at the N-terminus will alter signal transduction. However, this is unlikely since leucomyosuppressin, which is structurally homologous to myosuppressin, retains all its myoinhibitory properties across insect species (Meola et al., 1996). Moreover, alanine-substituted analogs tested by Dickerson et al. (2012) reported inhibitory activity of 95% and 91% in *in vitro* assays. The regulation of myosuppressin (MS) signaling in D. melanogaster, C. capitata, and D. radicum appears to be highly specific and tightly controlled. In these species, excess MS released into the haemolymph is likely to be rapidly degraded, as none of them exhibit the post-translational modification of cyclized N-terminal pyroglutamic acid, which confers structural stability and resistance to enzymatic degradation. This structural alteration, commonly seen in neuropeptides, enhances their bioavailability and durability in the haemolymph, allowing them to function effectively as circulating hormones. In contrast, species like the honeybee (*Apis mellifera*), tobacco moth (*Manduca sexta*), and cockroach (*Leucophaea maderae*) have shown resistance to aminopeptidases and enzymatic degradation due to such modifications, as outlined in Table 3.1 in Chapter 3, which illustrates MS sequence conservation across different species. Specifically, the effect of improved stability that myosuppressin peptide or its analogue can impose on individuals was illustrated by Vilaplana et al., (2008) studying leafworm, *Spodoptera littoralis,* where the highest food consumption was correlated with the lowest level of MS in the brain and haemolymph. To replicate the anorexic effect *in vivo*, the only alternative

to an unstable myosuppressin (degradation in crop is addressed in Chapter 4) is Benzethonium chloride (Bztc). It was a first neuropeptide analog discovered to mimic C-terminal pentapeptide of myosuppressin, and to this date is the only commercially available peptide mimetic to MS (Chapter 3, Figure 3.4 Illustration of Bztc structure and the C-terminal pentapeptide fragment VFLRFamide, Lange et al., 1995; Nachman et al., 1997). In the feeding experiments, myosuppressin had a significant impact on defecation rates and adversely affected longevity, as detailed in Chapters 3 and 4. However, *ex vivo* crop bioassays conducted on tissues lacking a functional myosuppressin receptor indicated a non-specific inhibitory action. Bztc, a non-peptide agonist, demonstrated a much lower potency, with an average EC_{50} in the range of 10^{-6} M. This potency aligns with the EC_{50} values recorded for both *D. suzukii* and *D. radicum*. Such reduced efficacy of a non-peptide ligand aligns with previous findings, where Bztc's EC_{50} for inhibiting proctolin-induced contractions was reported to be two orders of magnitude greater than that of locust myosuppressin, SchistoFLRFamide (Lange et al., 1995; Sedra et al., 2014). The loss, however, of a functional Dms-R1 had little effect on the inhibitory potency of the ligand (EC_{50}, 10^{-6} M) suggesting that Bztc does not act through the Dms-R1 receptor and exerts its effect via a different mechanism. If Bztc would act through MS receptor, as proposed by Lange et al. (1995), we would expect to see EC_{50} variation within and between species tested. While *Drosophila* crop was sensitive to the presence of MS in nanomolar concentraction, in *D. radicum*, the same response requires 10-fold concentrated solution of peptide to achieve equal inhibitory power. Yet, both crops were inhibited by matching concentration of Bztc (EC_{50}=$8x10^{-6}$ M and $7x10^{-6}$ M respectively) supporting generic inhibitory action of Bztc. This result is also consistent with the observation that Bztc did not activate Dms receptors in CHO cells at concentrations up to 10^{-4} M (Egerod et al., 2003). Possible explanation could be the presence of stretch-activated channels within a crop tissue that Stoffolano and colleagues (2013) reported for its ability to inhibit half of spontaneous crop contractions. It was demonstrated by testing an amiloride, a peptide toxin of spider origin known to acts on mechanosensitive channels within, matching Bztc, 10^{-6} M concentration range. This might also be explanation for myosuppressin inhibitory effect in crops devoid of DmsR-1. As mentioned above, the inhibitory effect was in the same 10^{-6} M concentration range.

Detailed structural anatomy and physiology of *D. melanogaster, D. radicum and C. capitata* crop musculature in literature is lacking and mainly refers to detailed description

of *Phormia*'s crop structure by Stoffolano et al. (2010). By some, the crop is referred to as an equivalent of mammalian stomach, since supercontractile muscles in the wall of the crop allows it to expand and collapse, and peristaltic waves of contractions passing through crop and crop duct allows ingested food to be thoroughly mixed, regurgitated or passed into midgut (Chapter 3, Subsection 3.1.1 The crop; Apidianakis and Rahme, 2011; Subramanian et al., 2013; Mistry et al., 2016).

In comparison, the anatomical structure of muscle fibres, that provide the structural support for underlying lamina and is responsible for the mixing and moving of ingested food, has shown a slight difference. Perhaps, the varying feeding habits of adult flies, such as the highly phytophagous medfly and the cabbage fly, which primarily feeds on flower dew, may be reflected in the noticeable differences in the strength of their crop muscles. This is observed despite the differences in their body sizes, with *D. melanogaster* being the smallest, followed by *C. capitata* and then *D. radicum*. Interestingly, when examining semi-isolated crop muscles, *D. melanogaster* and *D. radicum* exhibited similar contraction patterns, suggesting that factors beyond mere body size influence crop muscle functionality. In both fly species, the arrangement of relaxed and contracted muscle fibers facilitated the formation of crop lobes. These lobes, once relaxed, smoothed out the surface, giving the appearance of a uniform sac. In contrast, the medfly's crop consistently appeared in a constricted state, with several lobes visible on the surface. The grooves formed by these contracting lobes were more pronounced, and the overall shape of the crop resembled a horseshoe, as depicted in Figure 7.1 below.

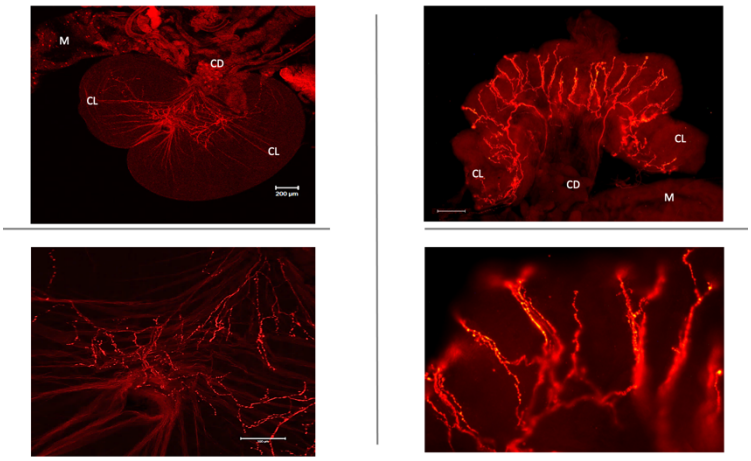

Figure 7.1 Myosuppressin-like staining of semi-isolated *Delia radicum* and *Ceratitis capitata* crops. In both species the immunoreactive fibres originate from crop nerve bundles present alongside the crop duct and extensively ramify across the crop surface. However, while bi-lobed sac of cabbage root fly is covered with singular immureactive fibres, that eventually divide into a fine network across the lobes, in medfly extensive branches spread across the shoe-like crop sac, forming a bundle of immunoreactive fibres.

To understand the differences in crop musculature, it may be necessary to examine other flies within the same family. Intriguingly, it has been noted that Caribbean fruit flies (*Anastrepha suspense*), which belong to the same Tephritidae family as medflies, protrude and deposit oral secretions from their crops in the form of droplets on leaves as lekking markers (Walse et al., 2008). These secretions contain sexual pheromones (specifically epianastrephin and anastrephin) and are attractive to both males and virgin females, playing a critical role in lekking- a mating behavior also observed in medflies. This function serves as a communicative strategy to gather competing males and an attractant during courtship. Furthermore, it has been proposed that the crop also participates in male courtship displays, where males 'inflate' their crops by inhaling air to appear larger and thus to become more attractive to females (Guillén et al., 2019). The crop's role in behaviors such as lekking, where it is used for depositing pheromones that attract mates, and in courtship displays, where its inflation makes males appear more attractive, highlights its significance in the reproductive and social behaviors of these species. Interventions that disrupt the crop's ability to produce or release these pheromones could effectively reduce mating success and, consequently, population growth in these pests. Additionally, the unique courtship displays involving the crop

suggest potential avenues for behavioral or pheromonal intervention. By targeting these specific aspects of crop functionality, pest control strategies can be refined to be more species-specific and environmentally friendly, reducing reliance on broad-spectrum chemical insecticides and contributing to more sustainable agricultural practices.

The pattern of myosuppressin-like staining achieved through commercially available RFamide antibody was quite uniform. It was previously used to demonstrate myosuppresin-like pattern in other dipterans (Meola et al., 1996; Haselton et al., 2004; Moffett and Moffett, 2005; Haselton et al., 2008; Gough et al., 2017). The issue of non-specific antibody that recognises the epitope of C-terminal RFamide, common to peptides of five families of FaPR peptides, such as Dms, SK, NPF, NPFs and FMRFa (Orchard et al., 2001), was addressed by MALDI-TOF single tissue analysis of crop nerve bundles from all three species, confirming the identity of myosuppressin. As expected, the presence of RFamide immunoreactivity was widely distributed across the CNS and SNS in all three species, with prominent immunoreactivity within subesophageal ganglion. This ventral region of the brain is considered as primary center of gustatory and feeding behavior (Wang et al., 2004). Previous studies identified the key role of stomatogastric nervous system in regulating feeding-related behavior in several insect species; ganglionectomy in the locust *S.gregaria* or moth *M.sexta* resulted in loss of appetite together with accumulation of food in the foregut and dysregulation of the muscle contractions, and notably interfered with crop emptying (Griss et al., 1991; Zilberstein et al., 2004; Rand and Ayali, 2010). Importantly, a prominent arborization over the crop surface was observed in each fly. While the spatial distribution of myosuppressin in *Drosophila* was already published by McCormick and Nichols (1993), we have only recently reported on localisation of RFa staining and myosuppressin detrimental effect in *Delia* (Bell et al., 2018; Chapter 4) and have manuscript in preparation to report on *Ceratitis* together with neuropeptidome (Chapter 6).

It is highly unlikely that the application of MS peptide in the form of insecticide would cause imminent lethality. Instead, the lack of nutrients, as a result of malfunctioned crop, would hamper reproductive success of individual; in males it would lower the competency to compete against other males (medfly males display lekking behaviour of aggression to defend its mating territory and “headbutting” its competitors (Briceño et al., 1999), while in females the lack of adequate nutrients would result in the cessation of ovarian development, particularly as a result of protein deficiency (Mirth et al., 2019), since each macronutrient in the form of carbohydrate, protein, lipid, vitamin or metal ion

is required during various stages of development to deliver viable batch of eggs. Concentrations of dietary nutrients was also shown to be a regulatory factor for hormones production, particularly of insulin-like peptides, juvenile hormone and ecdysone, that were implied in regulation of egg production (Mirth et al., 2019). Overall, malnutrition would thus lead to diminished reproductive success in individual, increased susceptibility to diseases and exposure to predation as result of reduced fitness. It would be expected to observe decline in environmental stress tolerance, overall survival and growth deterioration. Insecticidal potential of myosuppressin in the stable form of biocontrolling agent is summarised in Figure 7.2.

The practical aspect of utilizing neuropeptides as biopesticides is also the question of developing resistance against it. It is given that insects, particularly those with high reproductive rate and short life span, evolve resistance to any pesticide over time . The major advantage to focus on MS is the low selective pressure to develop resistant phenotype due to indispensable role of MS for the fly. As a novel target, there is less chance to encounter resistant individual. If selected target is already under pressure imposed by other pesticides, the weight of the pressure will select resistant individuals that passes resistant traits to the offspring, allowing rapid spread of resistant phenotype across the following populations. The pleiotropic effect of MS on feeding, locomotion and fecundity minimise the chance of developing resistance in established population. Lastly, reduced caloric intake leading to “weak individuals” lower migration/dispersal ability and effectively minimize the spread of induced resistant phenotypes as already reported for biopesticide resistance traits due to mutations or loss of receptors for aminopeptidase N, alkaline phosphatase or cadherins (Siegwart et al., 2015; Hawkins et al., 2019).

Those aspects are particularly important to take into account if considering future investments. The low persistence of neuropeptides due to quick degradation would inevitably lead to the increased number of treatments and consequently adding pressure for resistant traits selection within fly population. This and the overall fragility of neuropeptides in the form of biopesticide was a force behind the decision to collaborate with the Institute of Particle Science & Engineering at the University of Leeds to develop a protective pH smart particles. Evaluation of protective particles though feeding experiment (Chapter 5) however revealed a peculiar mouthpart adaptation in medfly that effectively filters particles from sucrose solution. contrary to whole dissection of cabbage fly, *D. radicum* where particles were present across the whole digestive tract, embedded

in tissues from crop to rectum. Interestingly, in both species crop appears to be on the alkaline spectrum, in contrast to midgut regions. Putting particles aside, those findings are indicative of digestive enzymes present in the crop, rather than being regurgitated from the midgut as is commonly believed. We might expect the same or similar pH to occur within both regions, crop and midgut, if regurgitation is to transfer digestive enzymes from midgut back to the crop. Other source of digestive enzymes, as shown by MS degradation in Chapter 4, should be considered.

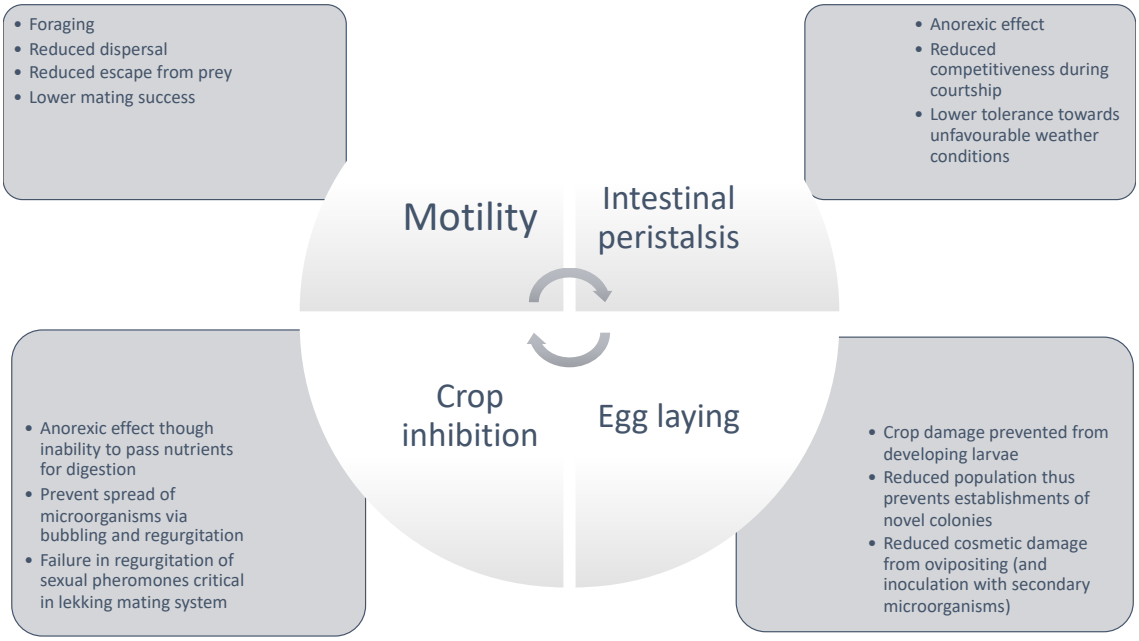

Figure 7.2 Proposed insecticidal effects of Myosuppressin (MS) on pest species. The pleiotropic nature of MS as an insecticidal agent could potentially impact pests in four significant ways: firstly, reduced motility may prevent escape, making pests more vulnerable to predators. Secondly, diminished foraging ability could lead to a decreased caloric intake, thereby reducing competitiveness during courtship. Thirdly, impaired movement could limit colonization and expansion into new areas. Fourthly, directly impacting crop and intestinal motility could result in less food intake, potentially leading to starvation. In the context of lekking mating behavior, the crop plays a vital role in depositing sexual pheromones within the lek area, which are essential for competitor signaling and female attraction. While the direct mechanism influencing egg laying is not fully understood, the ingestion of MS might inhibit this process, thus reducing the damage caused by larval feeding on crops. This could also mitigate secondary harm from oviposition, which can introduce microorganisms, and from the development of larvae. Grey arrows signify the interconnectedness between the various effectors. Several resultant effects could stem from more than one effector; for example, lower mating success could be due to a combination of reduced motility, crop inhibition, and/or impaired intestinal peristalsis. Additionally, inadequate nutrient uptake could arrest egg development, thereby preventing oviposition.

Particle development for insecticide delivery would have to overcome a few hurdles before to get commercialised. In our case, the composition of PMMA material is prone to photooxidation and photodegradation, interfering with particle stability (Babo et al., 2020). Even if short shelf-life can be accepted by customers, particles exposed to environmental pressure in the form of direct sunlight, acid rain, variation of soil pH, and fertilisers might hinder their protective shell due to pH variation. On the other hand, they could be finely "tuned" to provide a potential for attractants/repulsive coating increasing their selectivity to eliminate the risk of environmental contamination/pollution and residual contaminants in non-target organisms.

The final experimental chapter 'Chapter 6' was dedicated to the emerging pest in the U.K., *Ceratitis capitata*. The highly polyphagous medfly is considered as one of the most destructive pests in agriculture due to its unrestricted appetite. Chapter consists of two distinct parts. First follows the exploration of myosuppressin role in the medfly. As in previous chapters, detailed knowledge about morphological structure/physiology of crop musculature in literature is lacking. Phalloidin staining revealed a robust muscular organisation, that has shown extreme responsiveness to myosuppressin peptide. While half of spontaneous contractions of *Drosophila* and *Delia* crops were inhibited by myosuppressin at IC_{50} 10^{-9} and 10^{-8} M respectively, the direct effect of the peptide on *Capitata*'s crop muscular peristalsis was even more dramatic. While the inhibition in *D. melanogaster* and *D. radicum* occurred in dose-depended manner as reflected by curve in resulting graphs, *C. capitata* crop shown an excellent sensitivity to its native myosuppressin demonstrated by a curve of response 'all or nothing' (Chapter 6, Fig. 6.5).

The unique myosuppressin-like arborization across the crop surface showed a different pattern than those observed for *Delia* or *Drosophila*. In all cases, they originate from two crop nerve bundles, however in medfly, the crop is extensively covered with multiple bundles of fibres. That would also support different morphology. *D. melanogaster* or *D. radicum* the crop lobes formation appeared unique to medfly. The variation was particularly noticeable between individual lobes; *Drosophila* crop lobes, most of the time, appeared to be less contracted, shaping the whole organ into bilobed sac in contrast to *Capitata,* where crop lobes always appeared in a more contracted state making an appearance of more robust muscular organization that is addressed in Chapter 5. Furthermore, recent studies have suggested that in Tephritid flies exhibiting lekking behavior, the crop plays a multifaceted role. During lekking, males not only deposit

droplets enriched with pheromones from their crop within their territories but also use their crop to inflate their abdomens (Guillén et al., 2019). This abdominal inflation is believed to enhance their apparent size, a trait that increases attractiveness to females. Such diverse functionalities imply the necessity of a stronger and more robust muscular structure in the crop to accommodate these behaviors.

Dissections of medfly revealed a separate abdominal ganglion that is directly located on the top of the crop. A large nerve emanating from the posterior region of abdominal ganglion splits into two thinner nerves that divide across the top of the crop surface. Those then ramify into less noticeable fibres covering the crop lobes. This "Y" shape nerve assembly was strong-enough to withhold a pull with dissecting needle and still remained intact. It was unexpected, since in both *D. melanogaster* and *D. radicum* thoracico-abdominal ganglion is a fused three-lobed assembly that only differ in size, adequately to their body size. In medfly the abdominal ganglion is round in shape, set deeper within abdomen. The only reference found was of related tephritid fly, Caribbean fruit fly, *Anastrepha suspensa*, for which Fritz (2002)reported nerve fibres originating from a single abdominal ganglion to innervate female reproductive tract. Only recently Hadjieconomou and colleagues (2020) titled a crop as 'a key reproductive organ', that directly influence fecundity in females and interfering with crop function reduced the number of eggs to be laid. In direct relation to the crop, it is hypothesized that a nerve plexus emanating from a posterior region of thoracico-abdominal ganglion in blowfly, *P.regina* Gelperin (1971) respond to expanded surface of crop by signaling via stretch-responsive neurons, located within plexus of abdominal nerves, back to the centre nervous system (CNS). This system maintains constant negative feedback that is part of regulatory circuit required to terminate feeding and maintain satiety in flies. Interfering with signaling, experimentally demonstrated by severing connections between the crop and ganglion, resulted in hyperphagia i.e. uncontrolled feeding that in some cases let inflated crops to rupture (Gelperin, 1971; Hartenstein, 1997; Stoffolano and Haselton, 2013). Engagement in feeding and thus expansion of the crop lobes has correlated with increased firing frequency, suggesting a role in monitoring crop volume. Is it therefore possible that those nerves, directly associated with crop could respond to crop expansion accordingly in a form of negative feedback? It needs further evaluation whether those nerves emanating from abdominal ganglion do indeed interfere with crop filling and if so, could they form part of circuit that link the feedback from crop and reproduction?

Physiological studies in *D. melanogaster* determined pathway of most motor and sensory nerves emanating from this ganglion. While most nerves innervate body wall muscles, anterior 'prothoracic' segment is giving rise prothoracic leg nerves, from middle 'mesothoracic' segment exit nerves innervating flight muscles and projections towards gut, where they branch over to penetrate longitudinal muscles, and posterior metathoracic nerves terminate in muscles controlling fly halters (Power, 1948). It might then not be unexpected that MS in other species elicit muscular contractions of skeletal muscles and indirect fly muscles, as observed in cockroach, *Periplaneta americana* (Elia et al., 1993) or promoting a force of major flight muscles thus suspected of promoting a flight behaviour in *Manduca sexta* (Kingan et al., 1990). Moreover, early study done by Weeks and Truman, 1984) demonstrated that thoracicoabdominal nerves govern coordinated peristalsis of body-wall muscles that is required to escape from pupal case during eclosion. Indeed, phenotype of systemic RNAi interfering with Dms signaling was manifested by failure of wing expansion and unable to enlarge abdominal muscles in freshly eclosed flies (Dr. Iffah, personal communication). Lengthy immunoreactive fibres seen under thorax (most likely to be thoracic muscles) and abdominal cuticle (identified as abdominal muscles), might provide a basis for speculation whether the neuronal network caused a temporal paralysis that occurred in cabbage root fly, *Delia radicum* after delivering MS through microinjection into thorax area (Chapter 4). It raises an intriguing question whether the hormonally-released myosuppressin in *M. sexta* (pEDVVHSFLRFamide), which features a protective pyroglutamate residue at its N-terminus to guard against rapid degradation, could exert any detrimental effects in flies.

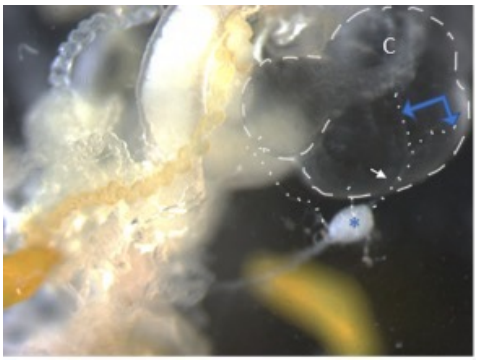

Figure 7.2 Live dissection of *Ceratits capitata* demonstrating an abdominal ganglion (blue star) located in crop proximity with extended fibres (highlighted with dotty lines) attached to the crop surface (outlined with dash line).

The second part of the experimental Chapter 6 is dedicated to medfly in an effort to 'fill the gap' in the literature for one of the most damaging pests in agriculture. It was therefore taken as a challenge to search through unannotated genome sequences to identify motifs of conserved and novel neuropeptides. Industrial collaboration has allowed access to quarantine facilities in National-Agrifood Innovation campus (FERA, York) and under the lead of Dr. Audsley allowed collection of samples from hundreds of dissections to be prepped and sent to the University of Cologne, Germany for Orbitrap-based mass spectrometry analysis. Unfortunately, full results were not received before the submission of this thesis. However, *in silico* identification of potential targets widens the opportunity to find an optimal candidate, either in the form of peptidomimetic or as RNAi target that holds great potential in the novel emerging field of pest control. The identification of a single MS receptor in medfly put this as an excellent RNAi target. Targeting neuropeptide receptor is an outstanding target since it is highly unlikely to develop resistance as the outcome of any mutation in a neuropeptide receptor gene is typically harmful to the individual (Hawkins et al., 2019). The chance of related receptor to take on the role is also less likely than in case of *Drosophila* where DmsR-1 and DmsR-2 share a high degree of similarity.

The results presented here extend our knowledge of the peptidergic control of crop contractions to two pest fly species, *D. radicum* and *C. capitata*. Myosuppressin was shown to be a powerful inhibitor of the spontaneous crop contractions in both species, establishing this signalling pathway as a potential and attractive target for disruptive chemicals, such as metabolically stable myosuppressin analogues. The indispensable and pleiotropic nature of neuropeptides such as the myosuppressins suggest that resistance might be less of a threat compared to synthetic chemicals. The issue of poor bioavailability, chemical and metabolic instability can be addressed with non-peptide analogues and new delivery systems, such as encapsulation or emultion enhanced with species-specific attractants to limit exposure to benefitial insects, pollinators and natural predators. However, the widespread application of peptide-based chemicals is likely to be challenged by high production costs as well as limited spectrum of insecticidal activity compared to broad-acting synthetic pesticides (Rosell et al., 2008). Still, as noted by Scherkenbeck and Zdobinsky (2009) the potency of neuropeptides is comparable to that of currently used chemical insecticides, which should make them attractive for

commercial development as a new generation of biopesticides (Scherkenbeck and Zdobinsky, 2009)

In a short summary, the quest to unveil an effective insecticidal agent for medfly control has been a meticulous journey through distinct yet interconnected stages. Beginning with the identification and characterization of promising neuropeptide in *D. melanogaster*, this research delved into the molecular intricacies that might hold the key to novel pest management strategies, laying the groundwork for further research. Rigorous assessment followed, scrutinizing the insecticidal efficacy and stability of myosuppressin, setting the benchmark for its potential use. Subsequent investigations unraveled the mode-of-action, shedding light on its physiological impacts on the target pests, *D. radicum* and *C. capitata*. The culmination of this effort was the evaluation of innovative delivery mechanisms, such as pH-responsive microcapsules, to establish a proof-of-concept for wider application. This not only paves the way for medfly control but also marks a significant step toward extending these findings to other dipteran pests, thereby broadening the horizon for sustainable pest management solutions.

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Appendix

Chapter 6

Drosophila melanogaster Dms-R1

Upper case letters indicates exons
Lower case letters marks introns
Blue letters highlight coding region
Underlines letters marks deletion

>3L:2322693..2345799
ACAGTCTTCATCGCCTCTCGGTGGCGTTCGCATCGTCGGCGCTTCGGTACATC
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DmsR1 (1040 bp deletion)

5'3' Frame 1

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5'3' Frame 1

MASGNNETEPLYCGSGMDNFHT 5 UTR and 22 amino acids lost

Drosophila melanogaster Dms-R2

Upper case letters indicates exons

Lower case letters marks introns

Blue letters highlight coding region

Underlines letters marks deletion

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DmsR2 (2617 bp deletion)
ATGGTCACGAACATGTCGCAGCCGCATTATTGCGGCACCGGCATCGATGATTT
CCACACAAA
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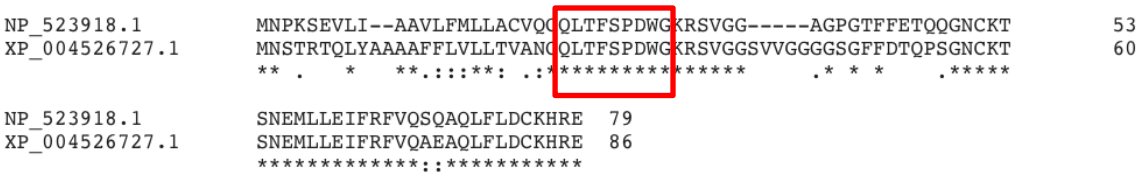
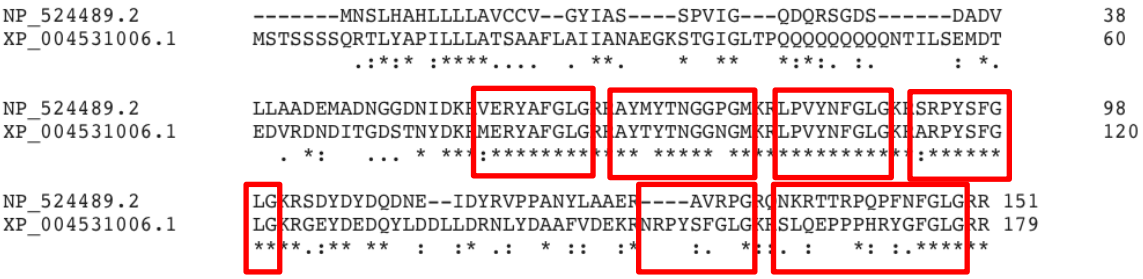
AGGAGCAGCTCAGCTACAGCTGGGCGTGCTTCATCAAGTTTCACTCGGTATT
TCCCCAGGTGCTGCACACCATCTCCATTTGGCTAACGGTGACGCTGGCAGTT
TGGCGGTACATAGCGGTAAGCTATCCGCAAAGGAATCGCATCTGGTGTGGAA
TGCGAACTACTCTGATCACCATAGCCACGGCCTATGTTGTCTGTGTCCTGGTA
GTGTCACCTT

Deletion includes following amino acid sequence:
5'3' Frame 1
MVTNMSQPHYCGTGIDDFHTNYKYFHGYFSLIVCILGTIANTLNIIVLTRREMRS
PTNAI
LTGLAVADLAVMLEYIPYTVHDYILSVRLPREEQLSYSWACFIKFHSVFPQVLH
TISIWL
TVTLAVWRYIAVSYPQRNRIWCGMRTTLITIATAYVVCVLVVSP
-164 amino acids (first 4 TM regions) and 5’ UTR lost

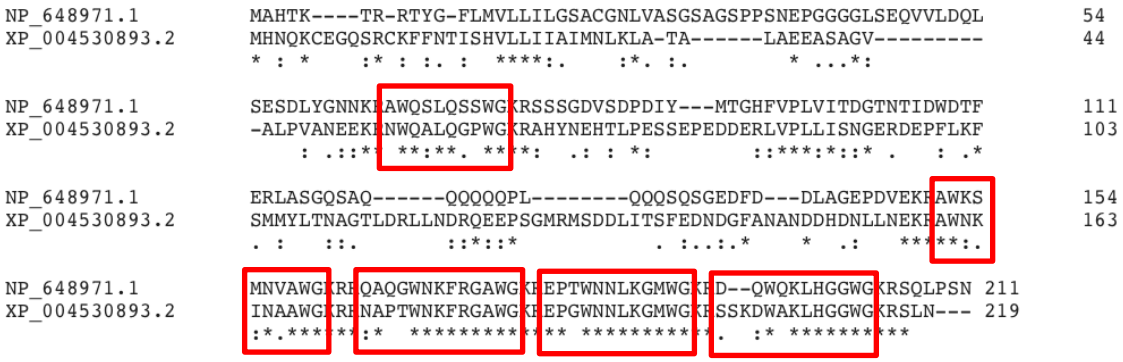
1- Adipokinec hormone (AKH)



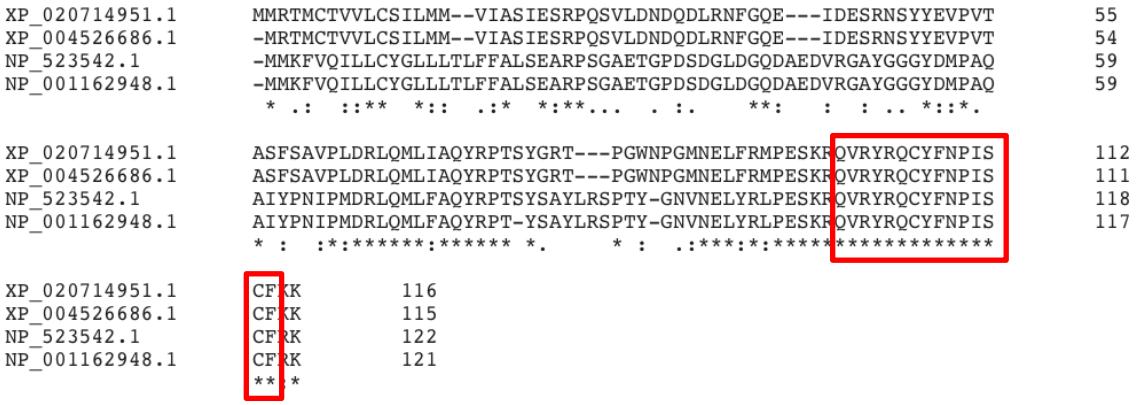
2- Allatostatin-A/FGL



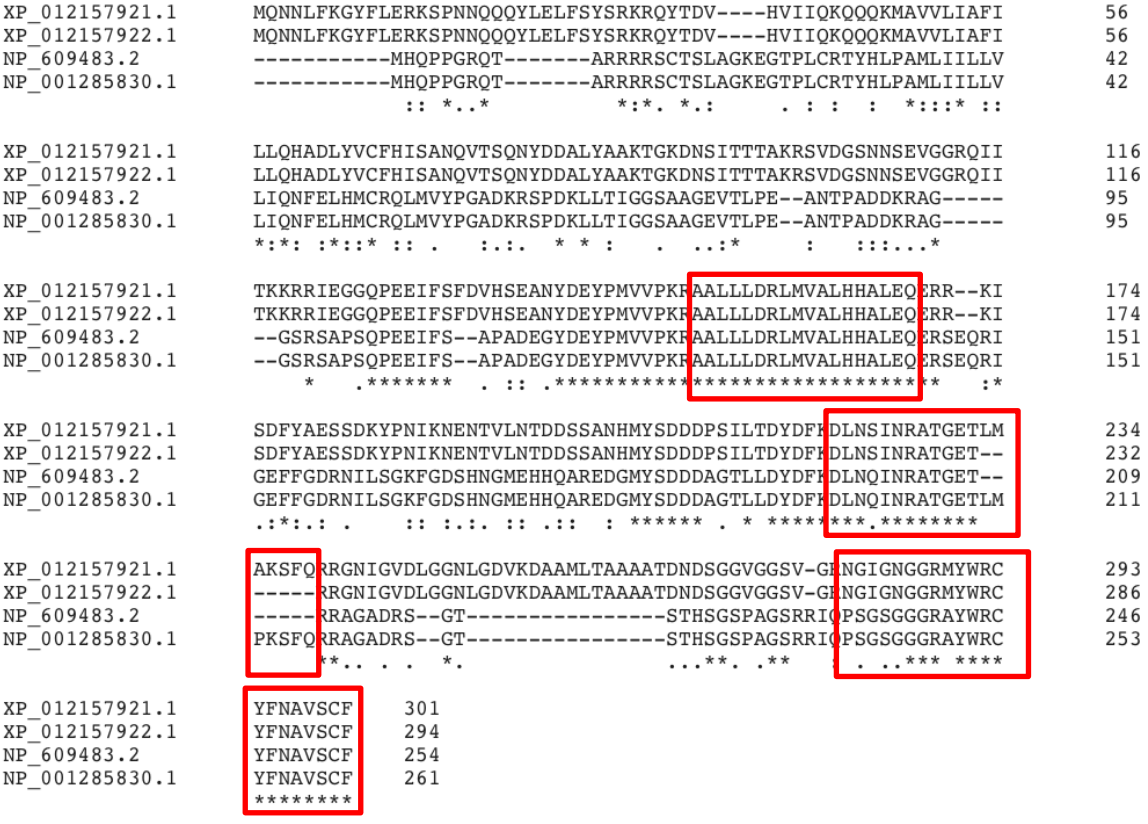
3- Allatostatin-B/MIPs



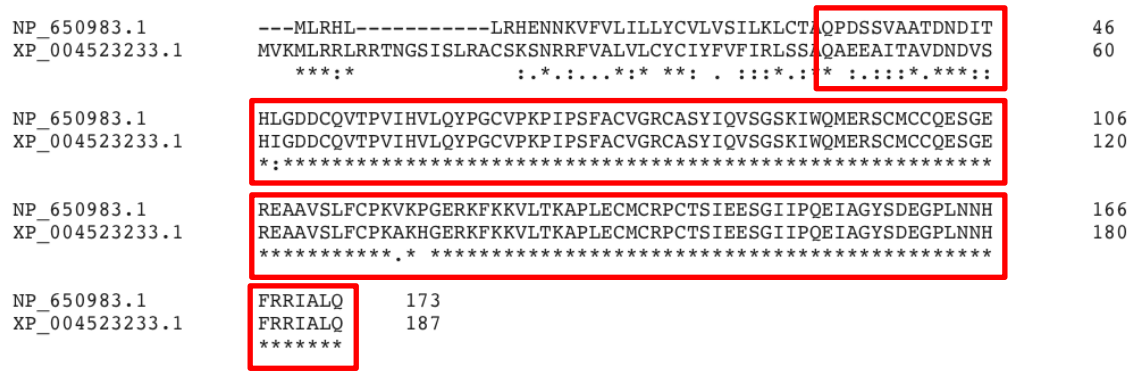
4- Allatostatin-C/PISCF



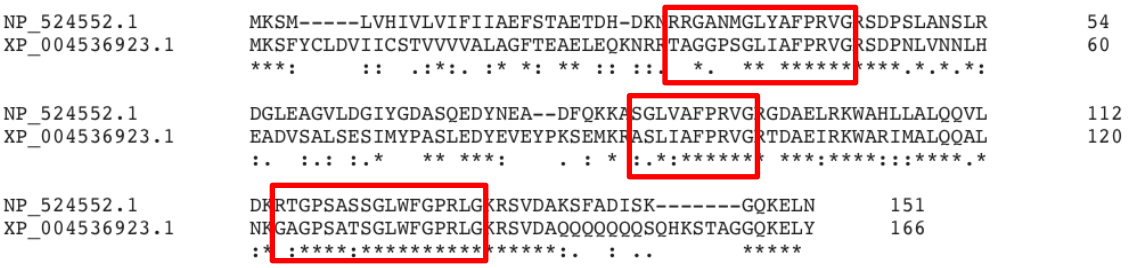
5- Allatostatin CC



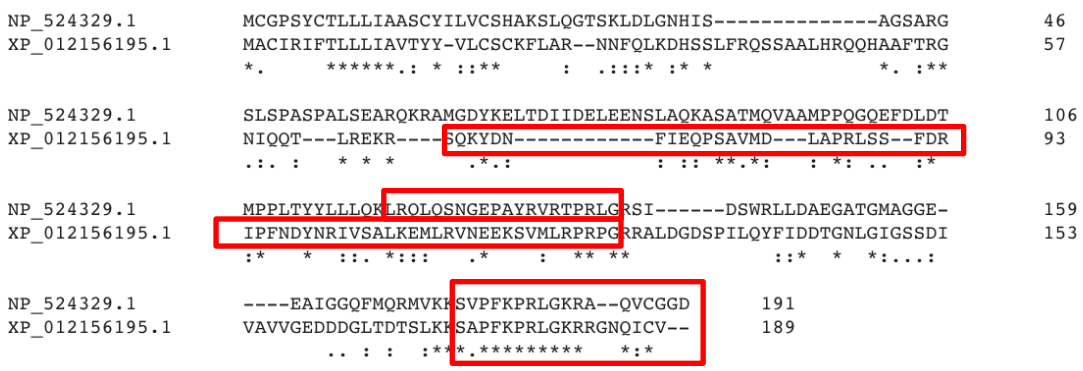
6- Bursicon



7- CAPA Peptides



8- Hugin/PK-γ



9- CCAP

```
NP_651083.2         MRTSMRISLRLLALLACAICSQASLERENNEGTNMANHKLSGVIQWKYEKRPFCNAFTGC        60
XP_020717581.1      MKMSA-WKVWSMILLLCIHCTLQ---M--ETVSDELNHKLNGIIKWRYEKRPFCNAFTGC        54
                    *: *   .:  : ** *  *:       :  ::  ****.*:*:*:**********

NP_651083.2         GRKRTYPSYPPFSLFKRNEVEE-KPYNNEYLSEGLSDLIDINAEPAVENVQKQIMSQAKI        119
XP_020717581.1      GKKRSY--LPPYPLWKRAEIDEPKIYNDVDLSEGLSDLIDINAEPAVENVQKQIMSQAKI        112
                    *.**:*   **: *:** *::* * **:  ****************************

NP_651083.2         FEAIKEASKEIFRQKNKQKMLQNEKEMQQLEERESK        155
XP_020717581.1      FEAIKEASKEIFRQKSMKKLRQREENQDAVEGV---        145
                    ***************. :*: *.*:: : :*
```

10- CCHa- 1

```
NP_001097784.1      MWYSKCSWTLVVLVALFALVTGSCLEYGHSCWGAHGKRSGGKAVIDAKQHPLPNSYGLDS        60
XP_012161494.1      MWRSFVLATIFICLIFSTSVKGSCLEYGHSCWGAHGKRSSGPAIASGKAQRQLNALDEDN        60
                    ** *    *:.: : : : *.*****************.* *: ..* :   *: . *.

NP_001097784.1      VVEQLYNNNNNNQNNQDDDNNDDDSNRNTNANSANNIPLAAPAIISRRESEDRRIGGLKW        120
XP_012161494.1      S--NRFGTNK---FDFDETLTSAE-FSPHIPASAEDLPIELNDSTKLRKNNNIEQRHLRF        114
                       : :..*:    : *:  .. :        **:::*:      . *:.:: .   *::

NP_001097784.1      AQLM-----------RQHRYQLRQL--QDQQQQGRGRGGQGQYDAAAESWRKLQQALQAQ        167
XP_012161494.1      PLQLGTTDNNNSPPMEKGNRQSEPIDSPEQRQHSLQIQQQRKYPQHIERWHKFPLLGLRR        174
                       :           .: . * . :   :*:*:.     * :*    * *:*:      :

NP_001097784.1      IDAD-------NENYSGYELTK-------------------------    182
XP_012161494.1      IFGKSPLALSDAAAAAALELSTRNTENDLLQQLAGGRFDDVGVYYDFQ    222
                    * ..           :. **:.
```

11- CCHa-2

```
NP_650285.1         MKSTISLLLVVICTVVLAAQQSQAKRGCQAYGHVCYGGHGKRSLSPGSGSGTGVGGGMGE        60
XP_004530021.1      MNSSVSFLLVMICAVVLAAQQGQAKRGCNAYGHACYGGHGKRSLGSLTDE-------MLA        53
                    *:*::*:***:**:*******.******:****.*********.  :..       *

NP_650285.1         AASGGQEPDYVRPNGLLPMMAPNEQ-VPLEGDFNDYPARQVLYKIMKSWF-NRPRRPASR        118
XP_004530021.1      NNAYRNERDELRPNELLQAMPNDATGLLAEGEIETLPRYRL-IKFMRSLLGGHLKRPVER        112
                      :  :* * :*** **  *  :   :  **:::  *  ::  *:*:* : .: :**..*

NP_650285.1         LGELDYPLANSAELNGVN----      136
XP_004530021.1      PNEIDYPISAEGFSRDASNIFN      134
                     .*:***:: ..  ....
```

12- CNMa

```
XP_004519169.2      MTQRRTPAASAAINSHQIIFAFVASIVWLASDACALPAINTLLGLPTYNSHPQVDMQQAL        60
NP_647669.2         MSALSAPTTCGCSPVHWAIVIVLLSVAIGPGDAMARPARN----------------TQLL        44
NP_001261294.1      MSALSAPTTCGCSPVHWAIVIVLLSVAIGPGDAMARPARN----------------TQLL        44
                    *:   :*::...   *  *. .: *:.   .** * ** *                 * *

XP_004519169.2      LNELFDNVN-EDAFFSELKKY-----------QHQQPTYLSKWAGLRDILPTFDYGEEHA       108
NP_647669.2         FSELLGGGNDDNNYYGDQLKYQQQQQQQQEQKQQRVPAFARKWPSLRDLLLTVDYDDFGV       104
NP_001261294.1      FSELLGGGNDDNNYYGDQLKYQQQQQQQQEQKQQRVPAFARKWPSLRDLLLTVDYDDFGV       104
                    :.**:.. * :: ::.:  **           *:: *::  ** .***:* *.**.:  .

XP_004519169.2      DSGDTDDSYETSNTRLVDHLQQLATNSVDGIGVGDVRADNPAVIQPVMPFRKKPQSGPVA       168
NP_647669.2         TQESEEQ--VAPSSRLLARLHRLGDNG-----GGEELRY--NVVNELTNMPSKKVMPGHP       155
NP_001261294.1      TQESEEQ--VAPSSRLLARLHRLGDNG-----GGEELRY--NVVNELTNMPSKKVMPGHP       155
                     . . ::   : .:**: :*::*. *.      *:       *:: :  : .*

XP_004519169.2      FKDHGIKKNIQLHKQYMSPCHFKICNMGRKRNARYLDD      206
NP_647669.2         LKDHNTKKNVQFRKQYMSPCHFKICNMGRKRNAGFNSY      193
NP_001261294.1      LKDHNTKKNV----QYMSPCHFKICNMGRKRNAGFNSY      189
                    :***. ***:    ********************* : .
```

13- Corazonin

```
NP_524350.1         MLRLLLLPLFLFTLSMCMGQTFQYSRGWTNGKRSFNAASPLLANGHLHRASELGLTDLYD        60
XP_004533703.1      MFKLFISLAVLCLVATCFGQTFQYSHGWTSGKRAHRDSE---------------APEIYS        45
                    *::*::   .*  :: *:*******:***.***:.. :.                ::*.

NP_524350.1         LQDWSSDRRLERCLSQLQRSLIARNCVPGSDFNANRVDPDPENSAHPRLSNSNGENVLYS        120
XP_004533703.1      LQ-QDGDRKLERCLMQLQYLLRN--PLPIRA-------------AVQ--------TIAPA        81
                    **  ..**:***** ***  *     :*               *          .:  :

NP_524350.1         SANIPNRHRQSNELLEELSAAGGASAEPNVFGKH  154
XP_004533703.1      NSNLFGNHHQSNELYEELNAAEAN-----DYSKH  110
                    .:*: ..*:***** ***.** .       :.**
```

14- DH31

```
XP_012158294.1      MNTYSFNPSTNTKQVQQKTNAMMSNKVFCCSLVFVAVCLAFCLTSTEAAPFPRYQNSGY-        59
XP_020715207.1      MNTYSFNPSTNTKQVQQKTNAMMSNKVFCCSLVFVAVCLAFCLTSTEAAPFPS-QNSGY-        58
NP_523514.1         ----------------------MTNRCACFALAFLLFCLL-AISSIEAAPMPS-QSNGGY        36
NP_723401.1         ----------------------MTNRCACFALAFLLFCLL-AISSIEAAPMPRYQSNGGY        37
                                        *:*:  * :*.*: .**  .::* ****:*  *..*

XP_012158294.1      ----YNELEEVPDDILMELMARFGQTIMRARNDLENSKRTVDFGLARGYSGTQEAKHRMG        115
XP_020715207.1      ----YNELEEVPDDILMELMARFGQTIMRARNDLENSKRTVDFGLARGYSGTQEAKHRMG        114
NP_523514.1         GGAGYNELEEVPDDLLMELMTRFGRTIIRARNDLENSKRTVDFGLARGYSGTQEAKHRMG        96
NP_723401.1         GGAGYNELEEVPDDLLMELMTRFGRTIIRARNDLENSKRTVDFGLARGYSGTQEAKHRMG        97
                        **********:*****:***:**:**********************************

XP_012158294.1      LAAANFPGGPGRRRRSETEA        135
XP_020715207.1      LAAANFPGGPGRRRRSETEA        134
NP_523514.1         LAAANFAGGPGRRRRSETDV        116
NP_723401.1         LAAANFAGGPGRRRRSETDV        117
                    ****** ***********:.
```

15- DH44

```
NP_649922.2         MMKATAWFCPVLLTLLCATRLVCTAQRGAVGAGGAAGGSGAAAGGAEVGGSGRTNGYPLD        60
XP_004537274.1      -MKATTRLCPIVL-LICAARLVCTAQQQRYNH------------------NNGVAGYPLD        40
                     ****: :**::* *:**:*******:   .                  .. . *****

NP_649922.2         YPDGTRNSQDDFLLAKRNKPSLSIVNPLDVLRQRLLLEIARRQMKENSRQVELNRAILKN        120
XP_004537274.1      YADAK-LIQEDYLLEKRNKPSLSIVNPLDVLRQRLLLEIARRQMKENTRQVELNRAILKN        99
                    * *..   *:*:** ********************************:************

NP_649922.2         VGKRVVLRGGGGGGGSGAGGLAPKVSRRYRQQWPVERELERERQRERERERDAVREEQLD        180
XP_004537274.1      VGKRMLSTGAGDFNY--------KLPAHTQEQL------EY------------LLTHIPY        133
                    ****::  *.*. .         *:  : ::*       *            :  .

NP_649922.2         RQQLLPWKH-----FPSQLWSYGWA----------LSPYKESSQLQFADSQ-QSASTGPQ        224
XP_004537274.1      RQQLYSYYHGAQHPQLQQLWQPQYTNQLEYAPDVSVSEYLQRPAYTYEATLGATALAGSE        193
                    ****  : *       .***.  ::          :* * :     :  :   :* :* :

NP_649922.2         SQAL-PKQLQLLSYAKKPLDVAGMS-LARHRVSGNEANETNHENDDGNGASKNPARYVDD        282
XP_004537274.1      SKAEVDSQTPL--YLNALESTAGEGEVNIYKANGNDMTADN----------KAPESH---        238
                    *:*   .*  *  * :   ..** . :  ::..**: .  *          * *  :

NP_649922.2         GDNEGEDSYNDVGTEGVGLGLGMGVGLGLERFEVLEDKPNWANEEPNELVVV------NA        336
XP_004537274.1      -----------SQRAGVSSDAGALANNGLVAKNLS-GNEQPAVKDANQLVDGEGVDGNDG        286
                              **. . *  .. **   ::  .: : * :: *:**        :.

NP_649922.2         NDRVPWSFPYRFHKSQHNVN-       356
XP_004537274.1      NADYRLRYFYGLRKKHHSMRK       307
                    *      : * ::*.:*.:.
```

16- Eclosion hormone

```
NP_524386.1         MNC--KPLILCT--FVAVAMCLVHFGNALPAISHYTHKRFDSMGGIDFVQVCLNNCVQCK        56
AGB96048.1          MNC--KPLILCT--FVAVAMCLVHFGNALPAISHYTHKRFDSMGGIDFVQVCLNNCVQCK        56
XP_004536647.1      MHATKKFTIFAAAIAVALLCVLLPAADALPSIGHYG-KRFDTMSGIDFIQICLNNCAQCK        59
                    *:.  *  *:.:   **:   *:  .:***:*.**  ****:*.****:*:*****.***

NP_524386.1         TMLGDYFQGQTCALSCLKFKGKAIPDCEDIASIAPFLNALE   97
AGB96048.1          TMLGDYFQGQTCALSCLKFKGKAIPDCEDIASIAPFLNALE   97
XP_004536647.1      KMFGDYFQGQTCAESCLKFKGKAIPDCEDIGSIAPFLNALE   100
                    .*:********** ***************.**********
```

17- ETH-1

```
NP_524699.1         MRIITVLSVS--LLVGLVAISQADDSSPGFFLKITKNVPRLGKRGENFAIKNLKTIPRIG        58
XP_012161413.1      MHTSLLLTFSALLAVATLTNYCQCNDSPGFFLKITKNVPRLGRRSDSYFLKNMKAIPRIG        60
                    *:   :*:.*  * *. ::     :.*****************:*.:.: :**:*:*****

NP_524699.1         RSEHSSVTPLLAWLWDLETSPSKRRLPAGESPAKEQELNVVQPVNSNTLLELLDNNAIPS        118
XP_012161413.1      RRDDEVLDVTGNLQPV---LKRMLMNPADAAAAAAEREYSLVQPVTSNTLIEMLNKNAIAS        117
                    * :.. :                  **. : * *:* .:****.****:*:*::*** *

NP_524699.1         EQVKFVHWKDFDRALQADADLYSKVIQLGRRPDQHLKQTLS----FGSFVPIFGDEQNPD        174
XP_012161413.1      DSIKFIHWKDFDRALQMDTELYAKLISLGRKPDQRLKEDLHIDMTGGVFTPLLTNSNSND        177
                    :.:**:********** *::**:*:*.***:***:**: *      * *.*:: :.:. *

NP_524699.1         FMMYKNNEDQELYGGGNRYDRQFLKYNIL-      203
XP_012161413.1      YIYYNNKDVDDMYAP--KYGGDFSRYNQLI      205
                    :: *:*:: :::*.   :*. :* :** *
```

18- ETH-2

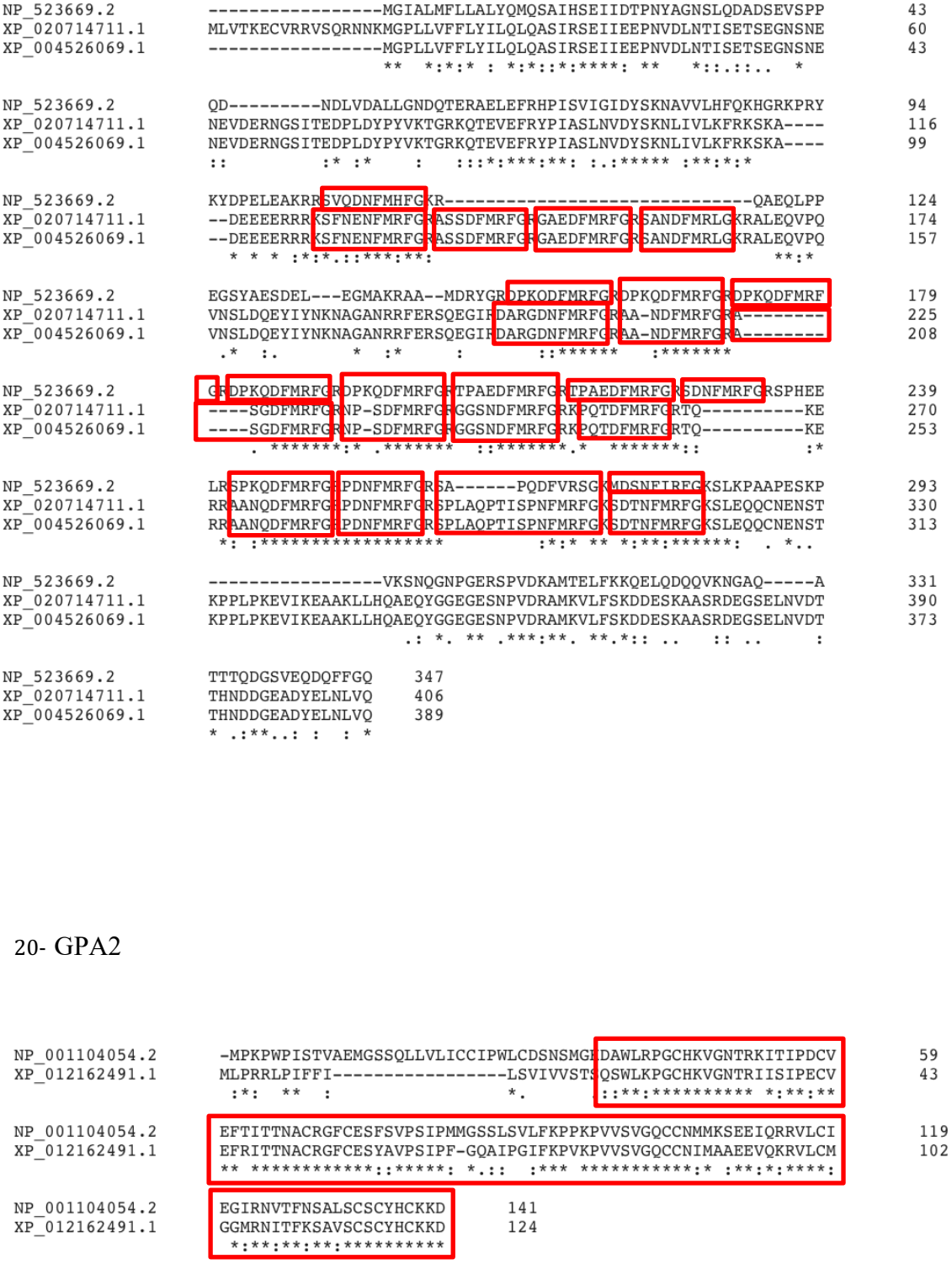
```
NP_524699.1         MRIITVLSVS--LLVGLVAISQADDSSPGFFLKITKNVPRLGKRGENFAIKNLKTIPRIG        58
XP_012161413.1      MHTSLLLTFSALLAVATLTNYCQCNDSPGFFLKITKNVPRLGRRSDSYFLKNMKAIPRIG        60
                    *:   :*:.*  * *. ::     :.*****************:*.:.: :**:*:*****

NP_524699.1         RSEHSSVTPLLAWLWDLETSPSKRRLPAGESPAKEQELNVVQPVNSNTLLELLDNNAIPS        118
XP_012161413.1      RRDDEVLDVTGNLQPV---LKRMLMNPADAAAAAAEREYSLVQPVTSNTLIEMLNKNAIAS        117
                    * :.. :                  **. : * *:* .:****.****:*:*::*** *

NP_524699.1         EQVKFVHWKDFDRALQADADLYSKVIQLGRRPDQHLKQTLS----FGSFVPIFGDEQNPD        174
XP_012161413.1      DSIKFIHWKDFDRALQMDTELYAKLISLGRKPDQRLKEDLHIDMTGGVFTPLLTNSNSND        177
                    :.:**:********** *::**:*:*.***:***:**: *      * *.*:: :.:. *

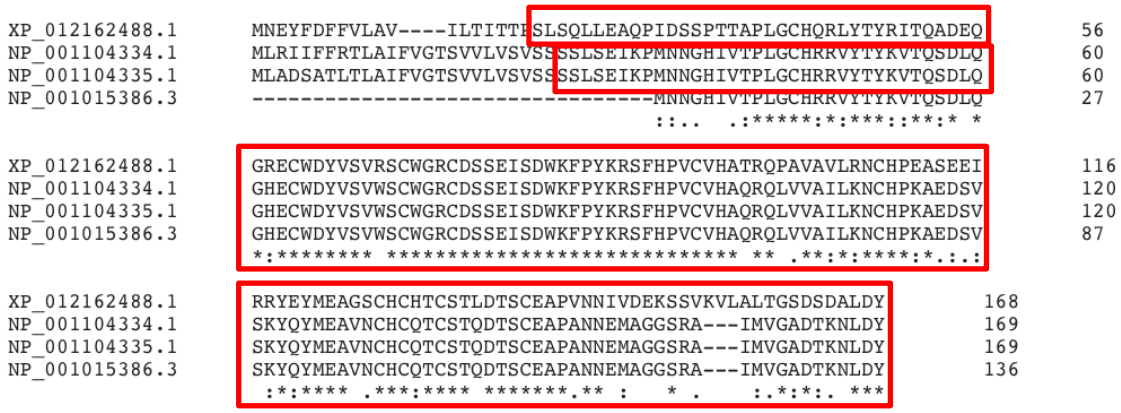
NP_524699.1         FMMYKNNEDQELYGGGNRYDRQFLKYNIL-      203
XP_012161413.1      YIYYNNKDVDDMYAP--KYGGDFSRYNQLI      205
                    :: *:*:: :::*.   :*. :* :** *
```

19- FMRFa-like peptides

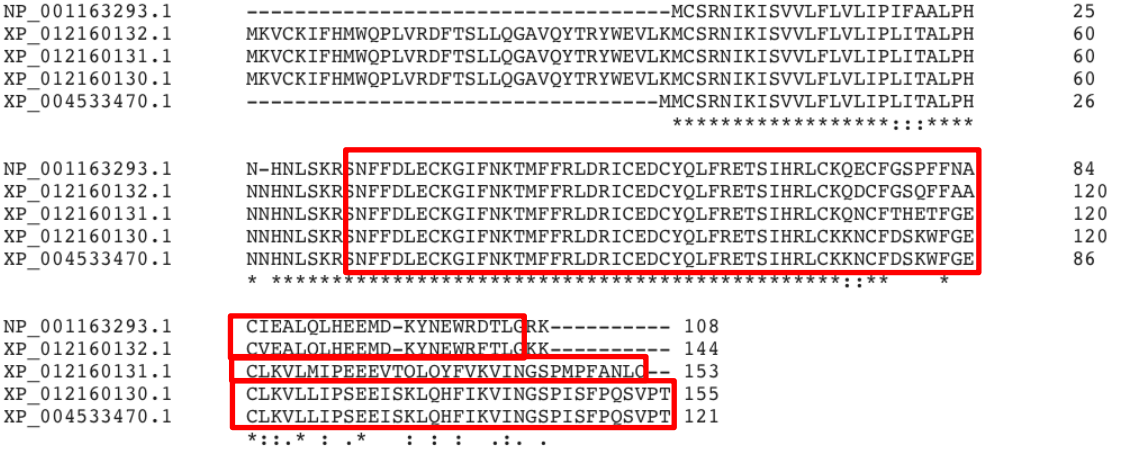


20- GPA2

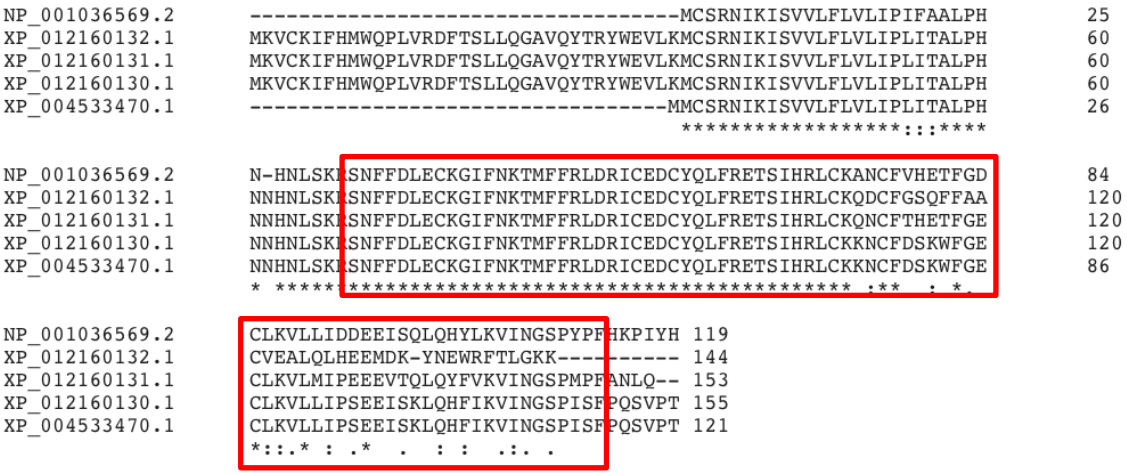
21- GPB5



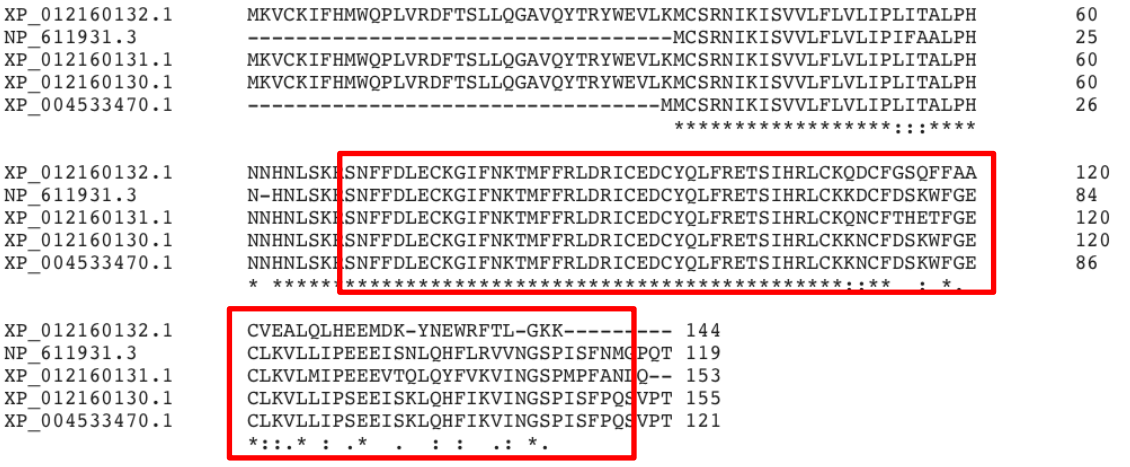
22- ITP



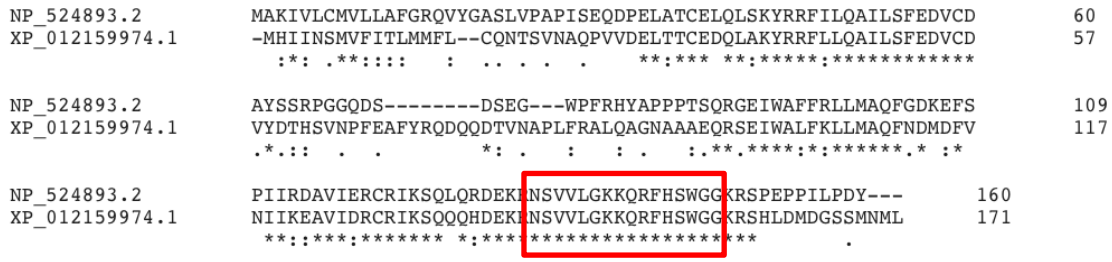
23- ITP-L1



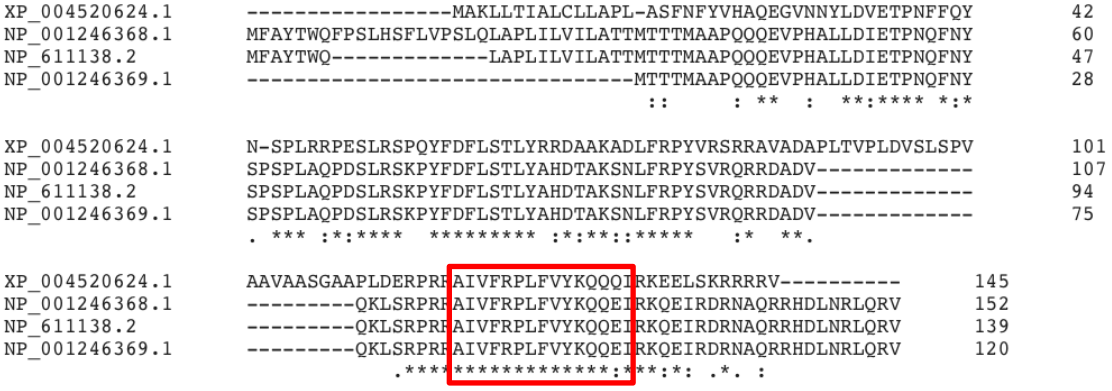
24- ITP-L2



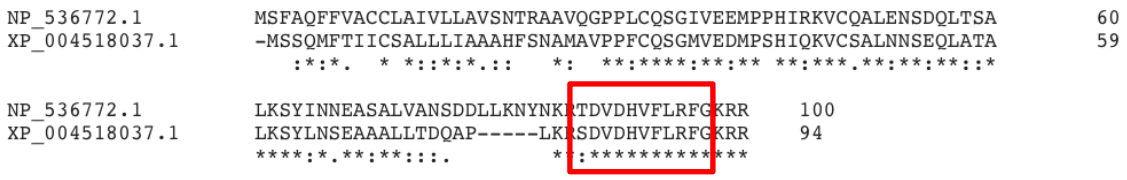
25- Leucokinin



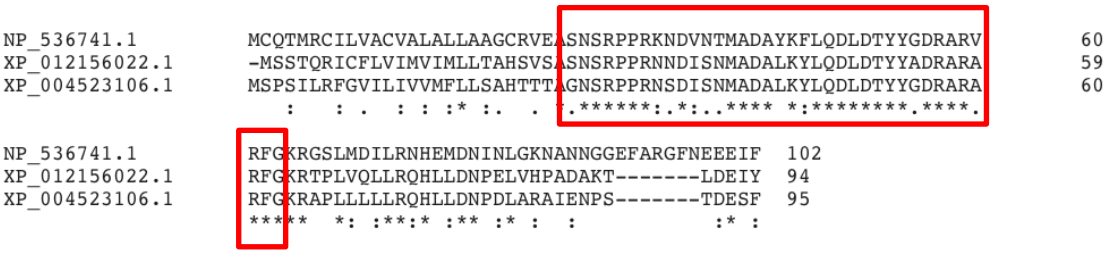
26- Limostatin



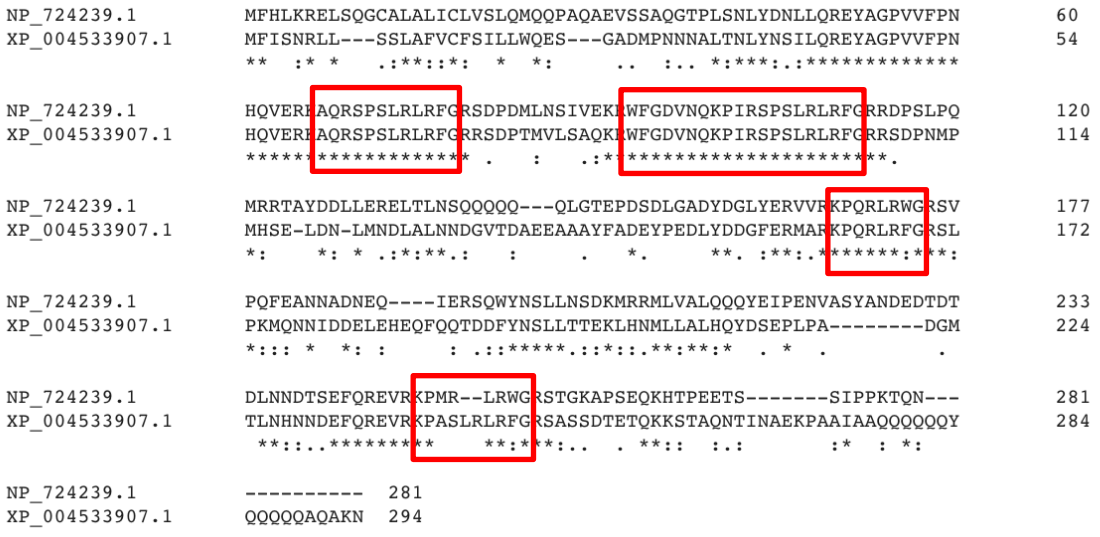
27- Myosuppressin



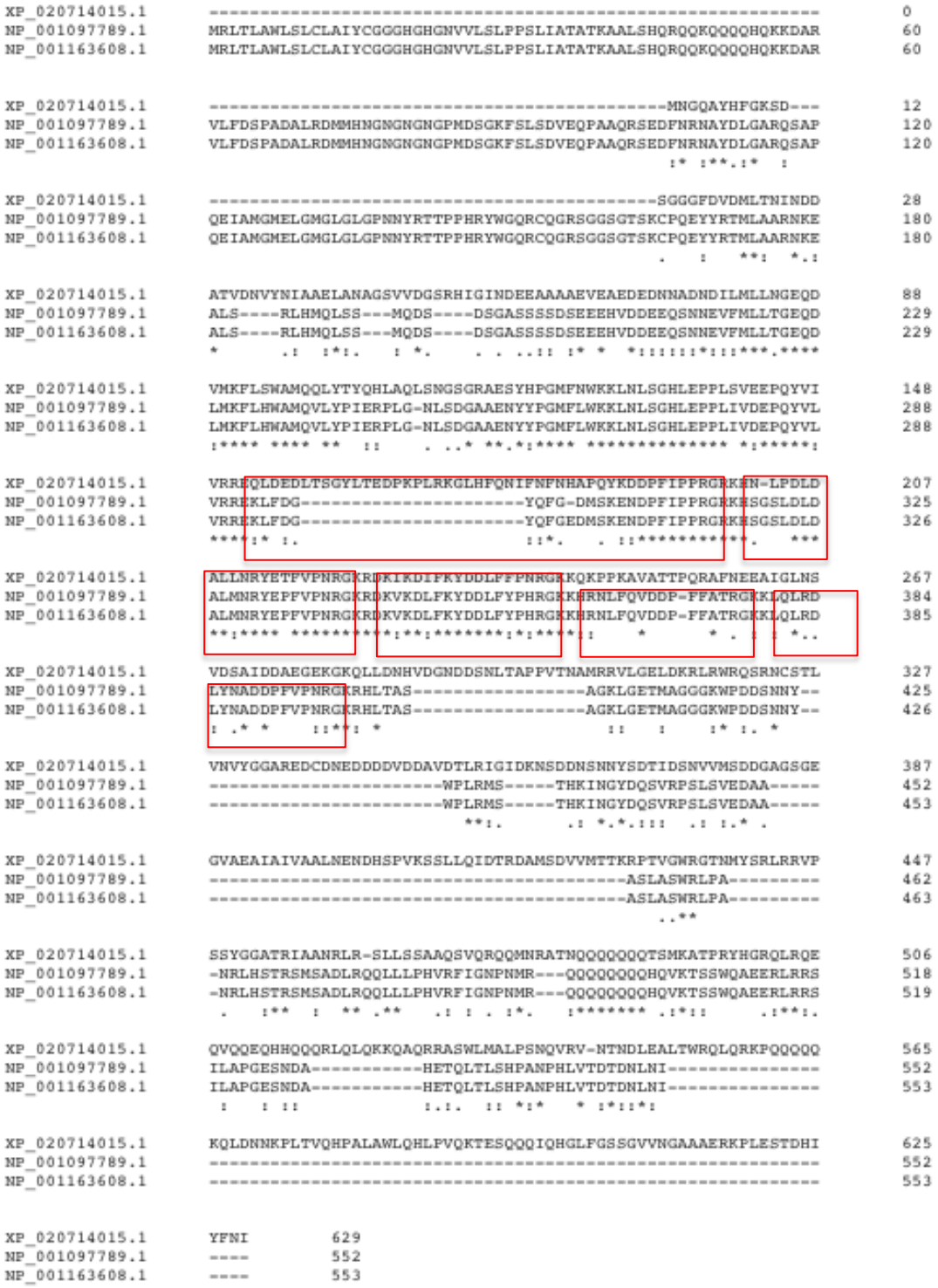
28- Neuropeptide F-1 & Neuropetide F-2



29- sNPF



30- Natalisin 1/Natalisin 2/Natalisin 3



31- Orcokinin A

```
NP_611852.2         MNLYVLLAVVSVFLNFIHAAPGVDISNDELLDGKYL---CEAGSKKYDGPFIVRLISAAN        57
XP_012160768.1      MKVIFPLSVV-VCVAYAMALPSKVS---TTYSDNYPAFKADFTNLNPDNIYI-RILMDLD        55
                    *:: . *:** * : :  * *.         ..:*    .:  . : *. :* *::   :

NP_611852.2         GQTVVCYECSQSEFKTKYSVKQCAAGKIGSGHHRDLVPYLVRMDP-------LYKDTWS-        109
XP_012160768.1      NRIRRCIYPNSIENILTILQNTCVPPK------CDFKKLLWYMDTMYNNAATMADDSWNN        109
                    .:   *   .. *   .   : *.  *       *:   *  **        : .*:*.

NP_611852.2         SKLKFNFDEIDKASASFSILNQLV    133
XP_012160768.1      NKVKFNFDEIDKTSASFSTLNQLI    133
                    .*:*:*******:***** ****:
```

32- Orcokinin B

```
NP_001261160.1        MNLYVLLAVVSVFLNFIHAAPGVDISNDELLDGKYLCEDLPAL-RELCNGNSVSL-----
XP_004534761.1        MKVIFPLSVV-VCVAYAMALPSK-------VSTTY-SDNYPAFKDELCNDYDYRTDNYNL
                      *:: . *:** * : :  * *.        :. .* .:: **:  ****. .

NP_001261160.1        ------RQTGLPNDDLSTSPLFNEFYKLFQRNVNIFLSKNKSPESLQERFSRTVSKRGLD
XP_004534761.1        FGQLVYILLTLPNEENNLETNCNKYMSISKRAFAVLLRLKNINRPKNEK-FESYPKRTLD
                            ***:: . .   *:: .: :* . ::*  ::  .  :*:  .:  ** **

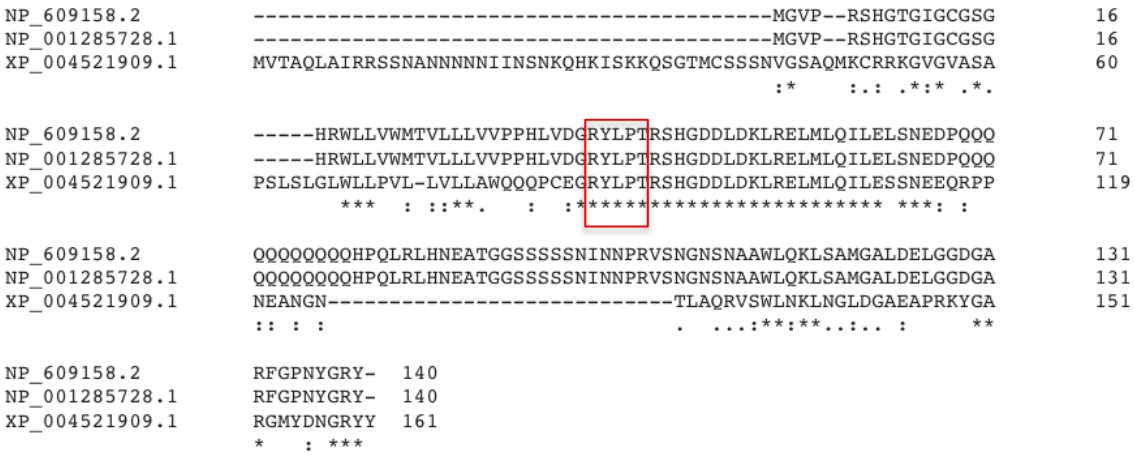
NP_001261160.1        SIGGGHLIKRTQSRQFLSD 127
XP_004534761.1        SIGGGHLIKRSDKIDF--- 126
                      **********::. :*
```

33- PDF

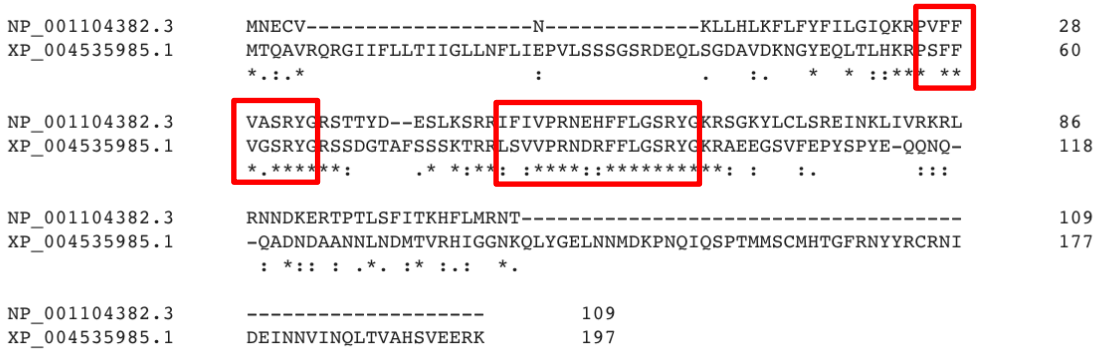
```
NP_524517.1         MARYTYLVALVLLAICCQWGYCGAMAMPDEERYVRKEYNRDLLDWFNNVGVGQFSPGQVA        60
XP_004530808.1      MVK--LLCIALALTLSSIFISSQALPTPDEDRFMEKEYNRELLNWLNSMQYPQ----PYA        54
                    *.:   *   : *::.. :  . *:  ***:*::.*****:**:*:*.:   *     *

NP_524517.1         TLCRYPLILENSLGPSVPIRKRNSELINSLLSLPKNMNDAGK  102
XP_004530808.1      VPCKYYPIN----SLTGPVPKRNSELINSLLSLPKSMNEAGK  92
                    . *:*  *     . : *: ***************.**:***
```

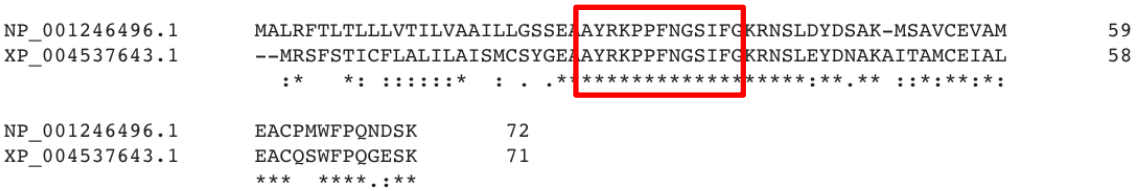
34- Proctolin



35- RYa/RYa2



36- SIFa



37- SK-1/SK-2

```
NP_524845.2         MGPRSCTHFATLFMPL-WALAFCFLVVLPIPAQTTSLQNAKDDRRLQELESKIGGEIDQ-        58
XP_004529342.1      --MAQAVNWSNLTRKVFILLAVCALGNSVLANDLDSIK-----SELHDVRSNGGGLSSSG        53
                        ...::::.*   :   **.* *    :  :  *::      .*::::.*: **  ..

NP_524845.2         -PI---ANLVGPSFSLFGDRRNQKTMSFGRRVPLISRPIIPIELDLLMDNDDERTKAKIF        114
XP_004529342.1      LPLGSTNDINGR-SLYANHRRMDRMYGFGPKMLQISRSKIPIELDLLVENEDG-DRSKIF        111
                     *:    :: *      ..** ::  .** ::  ***  ********::*:*   ::***

NP_524845.2         DDYGHMRFGKRGGDDQFDDYGHMRFGR--       141
XP_004529342.1      DDYGHMRFGKRGGEDQFDDYGHMRFGRSA       140
                    **********:**********
```

38- Trissin

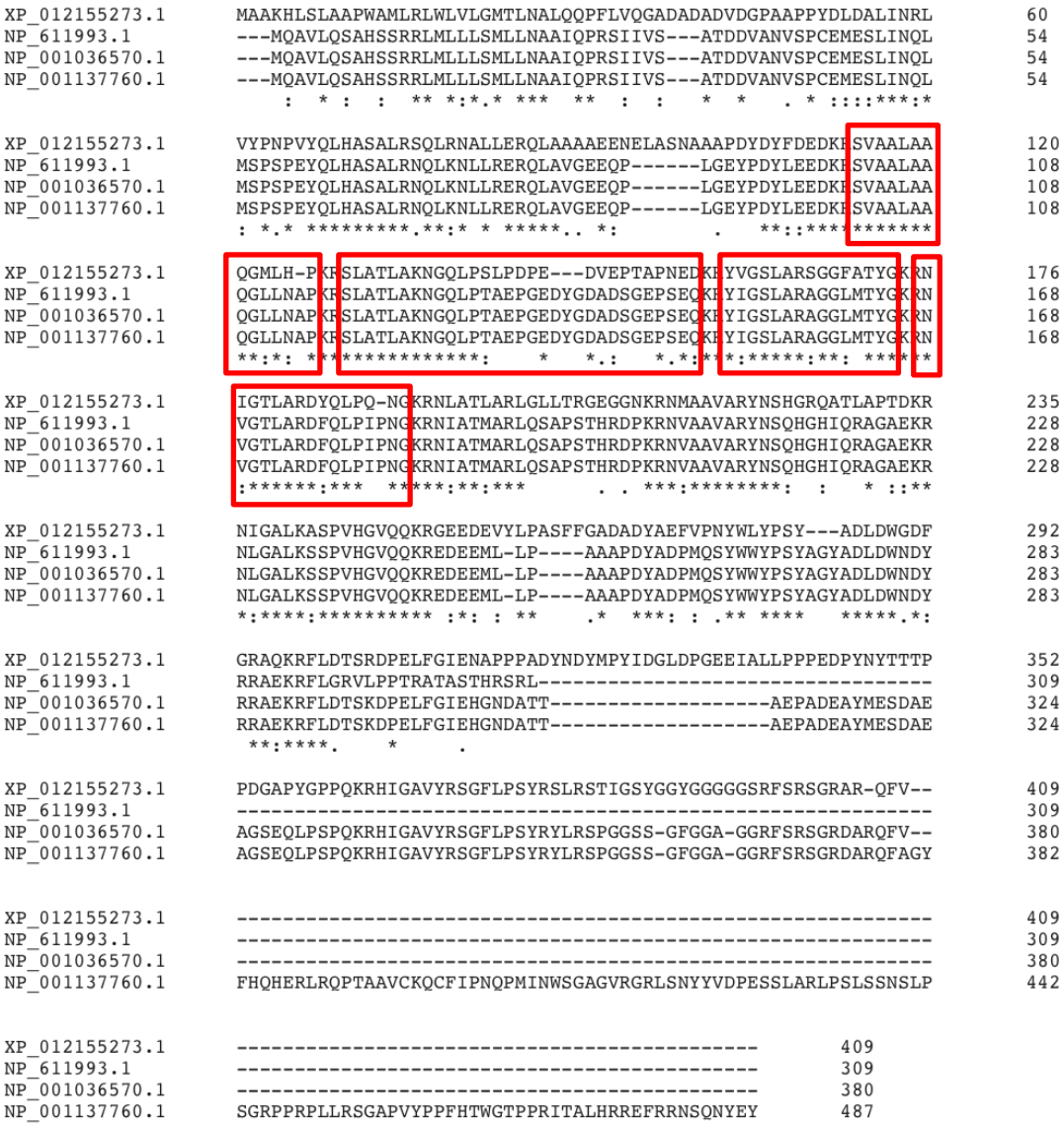
```
CLUSTAL O(1.2.4) multiple sequence alignment


NP_650471.2         MTKTTMHWLAHFQIILLCI----------------------------WLMC----PPSSQ        28
XP_012157482.1      MREEMRYKLLHCYLSLLCSIMLFLHNPITPCALHSKYFNTPTDDETLFGMEPTDIFERKR        60
                    * :   : * *  : ***                               : *        .:

NP_650471.2         AIKCDTCGKECASACGTKHFRTCCFNYLRKRSDPDALRQSSNRRLIDFILLQGRALFTQE        88
XP_012157482.1      SMTCDSCGNECTNACGTKNFRTCCFNYLRKRNDPGAMNSISNKRLIDFILLQGRAAMYTL        120
                    :::.**:**:**:.*****:***********.**.*:.. **:************ :

NP_650471.2         LRERRHNGTLMDL-------GLNTY------YP--------------------       108
XP_012157482.1      DEHLNTGDPVVVADMRSDENGKGEYGGDGKAYSATDSGHRIMNGRHNLQHMMV       173
                     .. . .. ::         * . *      *
```

39- NPLP1/APK/MTYa/IPNa



40- NPLP1-4

```
NP_652352.1         MFKLLVVVFAALFAAALAVPAPVARANPAPI----PIASPEPAPQYYYGASPYAYSGGYY      56
XP_012159653.1      MFKLFVVLFAALFAFAAAGPSPVARPAPLPVASPHPAPAPAPAPQFYYGGYASPYSYAY-      59
                    ****:**:****** * * *:****  * *:    *  :* ****:***.    ** .*

NP_652352.1         DSPYSYYG   64
XP_012159653.1      -SGYPYYG   66
                     * * ***
```

41- TRP

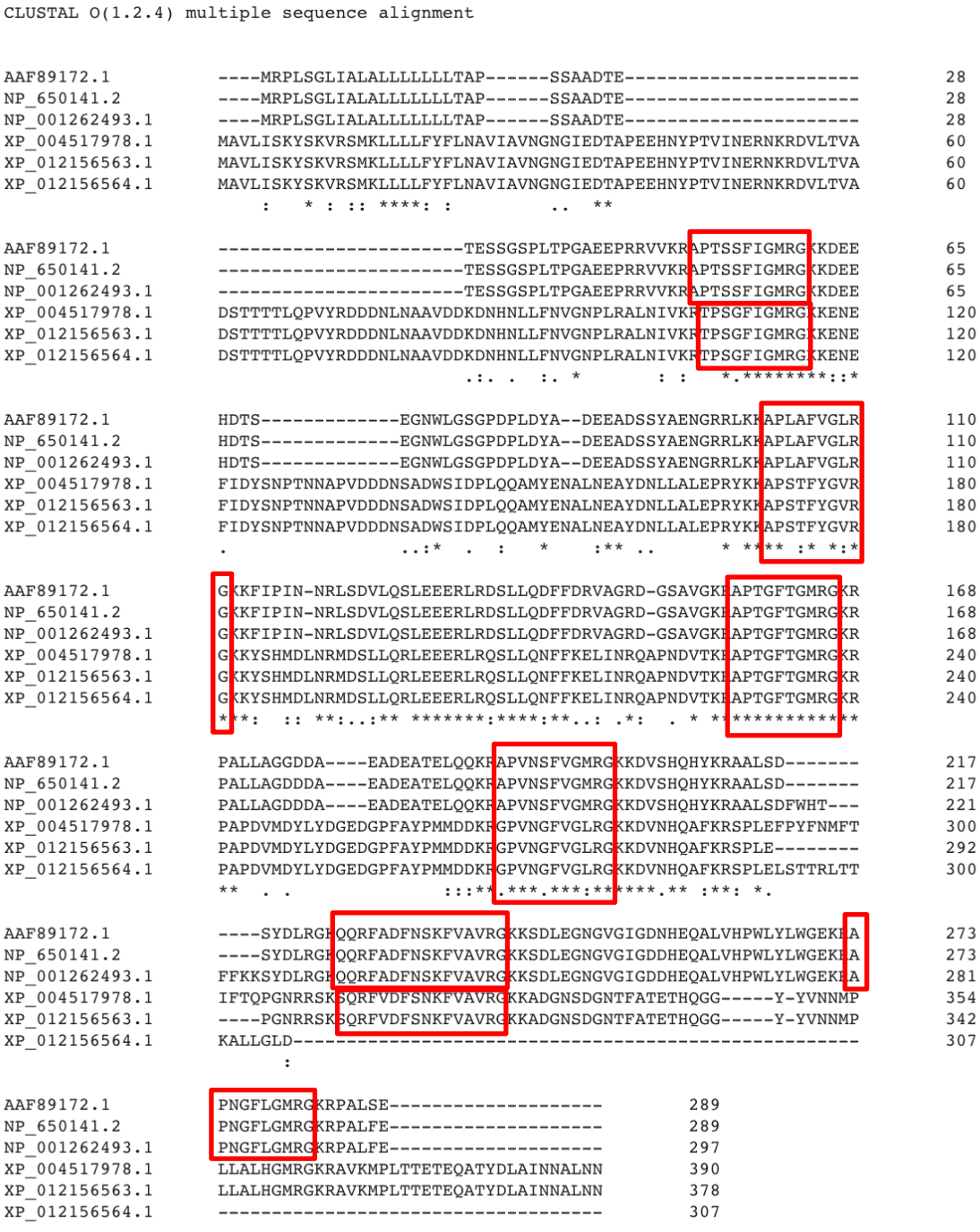
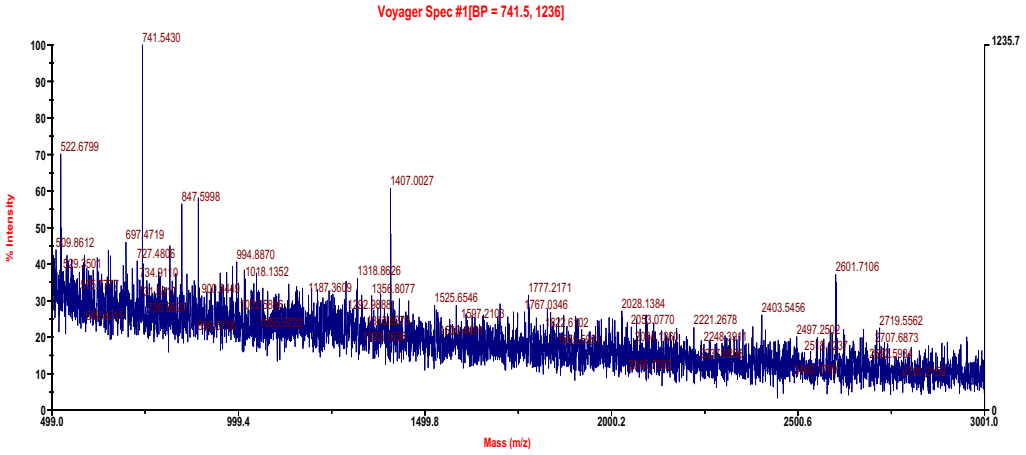


Figure 6.26 List of all *C.capitata* transcripts used to predict mature peptides that were subsequently used to analyse their similarity and level of identity analysis as presented in Table 6.2.

F21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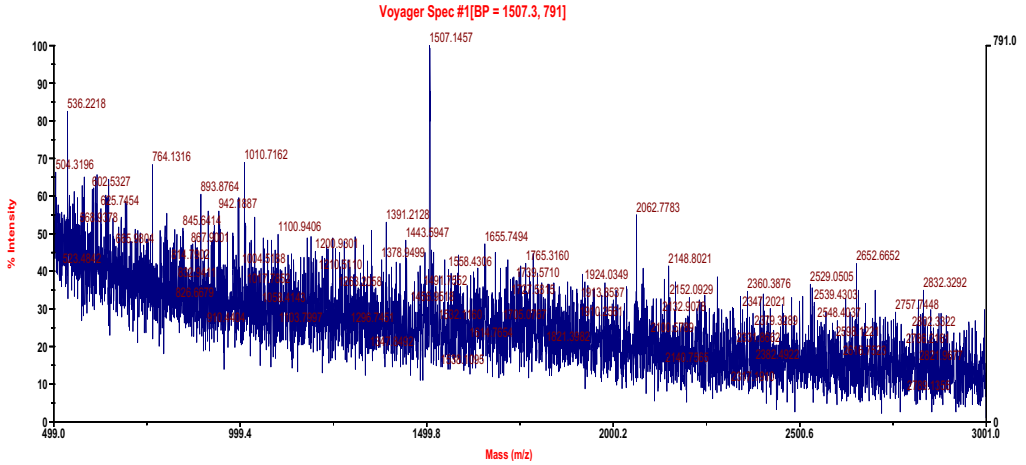
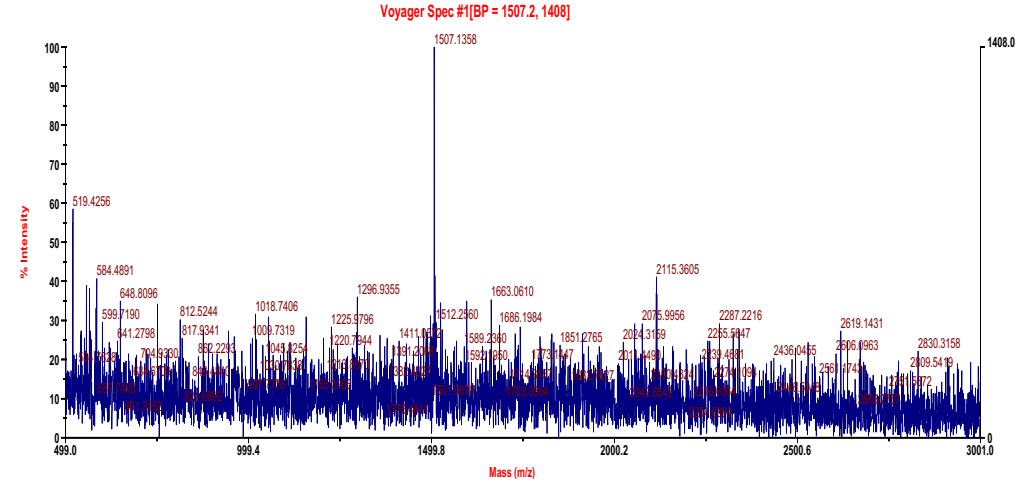
% Intensity
Mass (m/z)
741.5430
522.6799
847.5998
1407.0027
994.8870
1018.1352
1318.8626
1777.2171
2601.7106
2403.5456
2719.5562
499.0
999.4
1499.8
2000.2
2500.6
3001.0
1235.7

F23


Voyager Spec #1[BP = 1507.3, 791]
% Intensity
Mass (m/z)
1507.1457
536.2218
764.1316
1010.7162
893.8764
1391.2128
1443.5947
1655.7494
2062.7783
2652.6652
2832.3292
499.0
999.4
1499.8
2000.2
2500.6
3001.0
791.0

F24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% Intensity
Mass (m/z)
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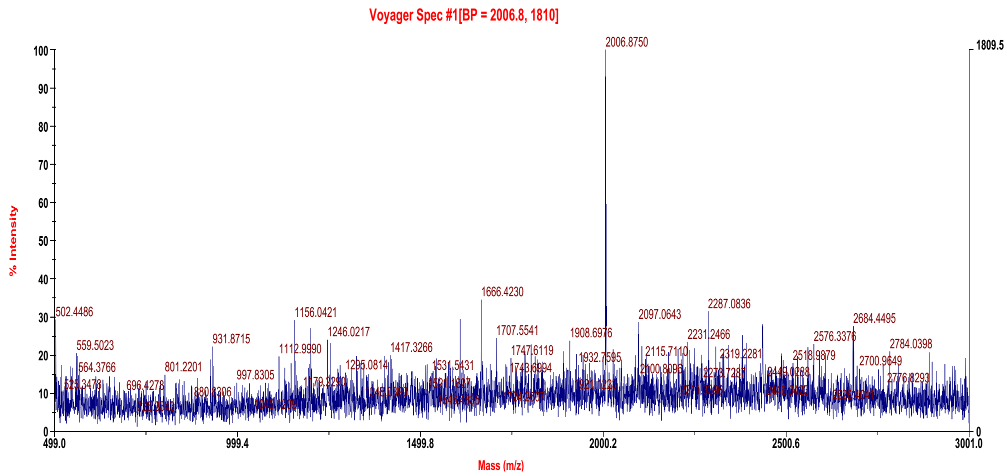
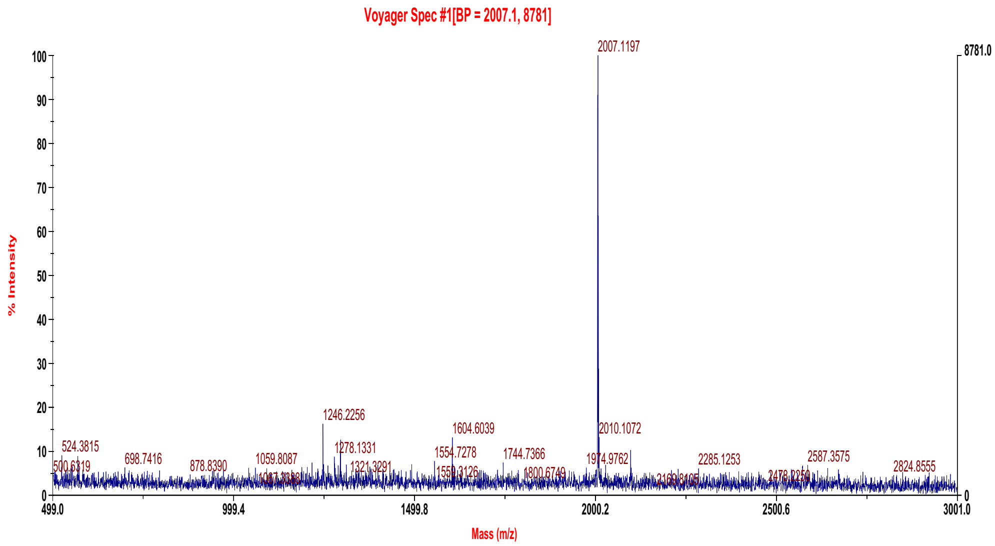
1663.0610
2115.3605
2619.1431
2830.3158
499.0
999.4
1499.8
2000.2
2500.6
3001.0
1408.0

F25


Voyager Spec #1[BP = 2006.8, 1810]
2006.8750
1809.5
% Intensity
Mass (m/z)
1666.4230
502.4486
1156.0421
2097.0643
2287.0836
2684.4495
559.5023
931.8715
1246.0217
1707.5541
1908.6976
2231.2466
2576.3376
2784.0398
1112.9990
1417.3266
1747.6119
2115.7110
2319.2281
2518.9879
564.3766
801.2201
997.8305
1531.5431
1932.7595
2700.9649
696.4278
2776.4293
499.0
999.4
1499.8
2000.2
2500.6
3001.0

F26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2007.1197
8781.0
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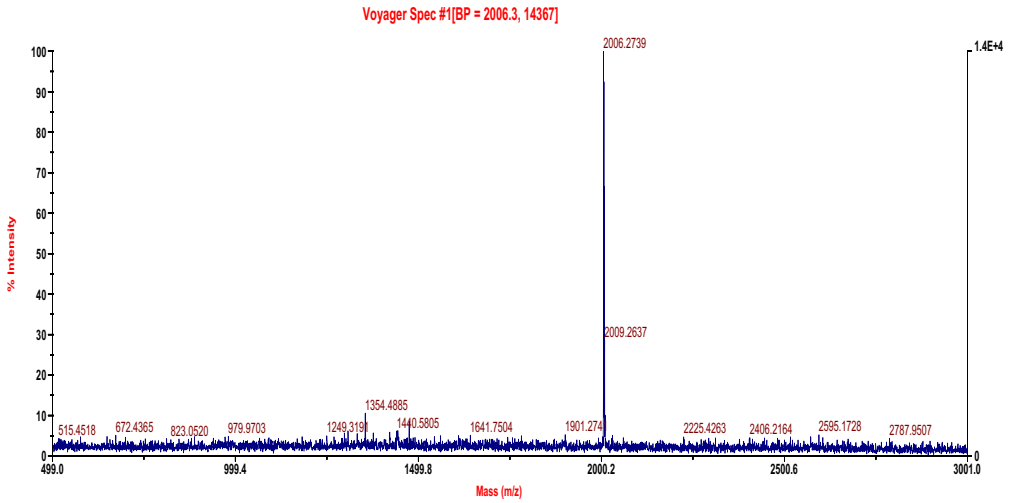
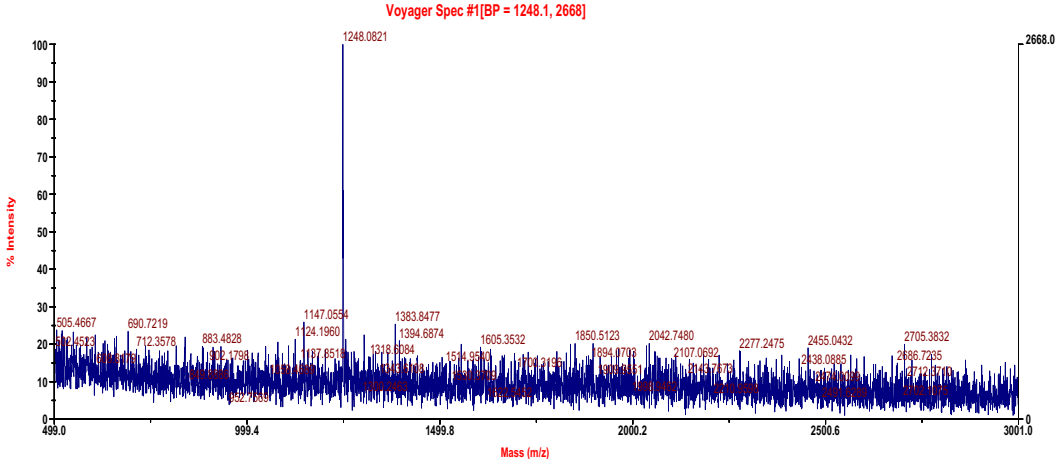
698.7416
1059.8087
1554.7278
1744.7366
1974.9762
2285.1253
2587.3575
500.6319
878.8390
1321.3291
1550.3126
1800.6749
2824.8555
499.0
999.4
1499.8
2000.2
2500.6
3001.0

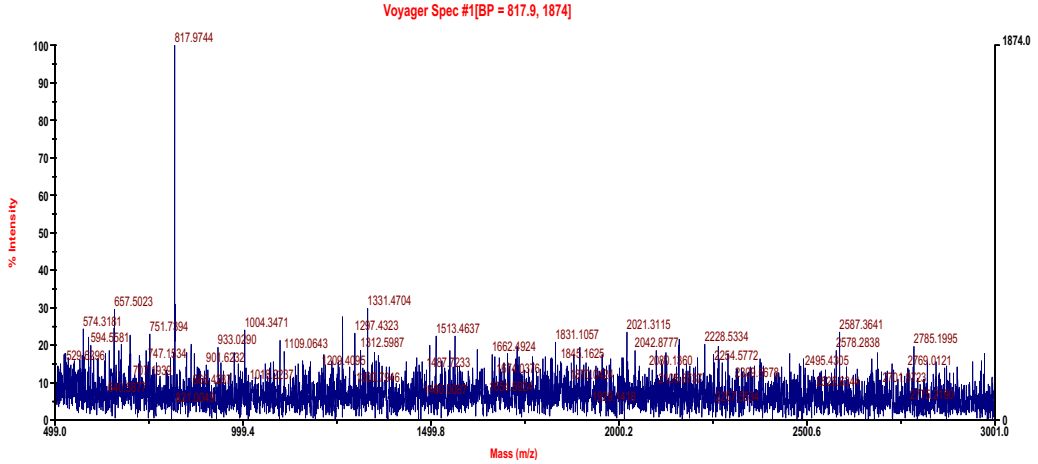
F27


Voyager Spec #1[BP = 2006.3, 14367]
% Intensity
Mass (m/z)
2006.2739
2009.2637
1354.4885
1.4E+4

F28


Voyager Spec #1[BP = 1248.1, 2668]
% Intensity
Mass (m/z)
1248.0821
2668.0

F29


Voyager Spec #1[BP = 817.9, 1874]
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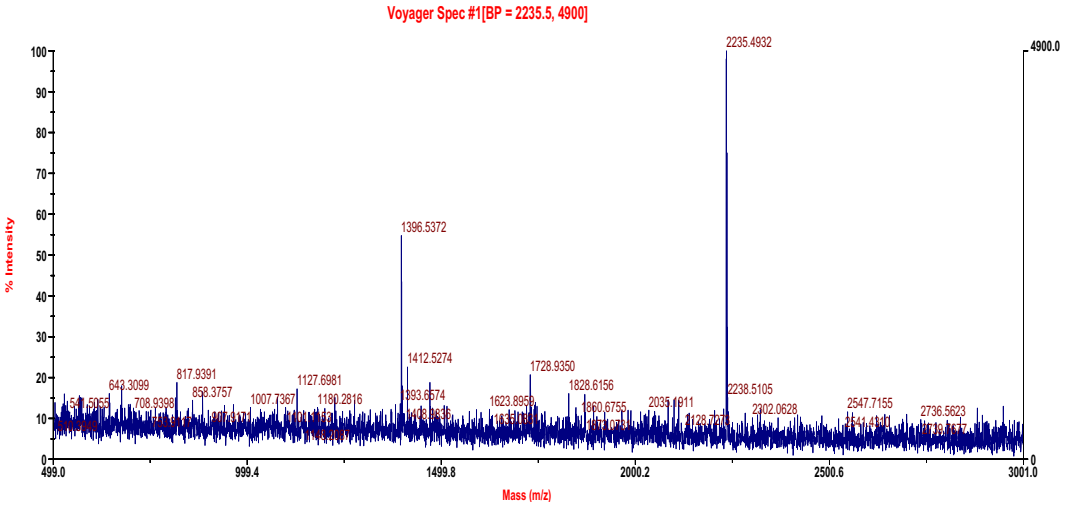
Mass (m/z)
817.9744
1874.0

F30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% Intensity
Mass (m/z)
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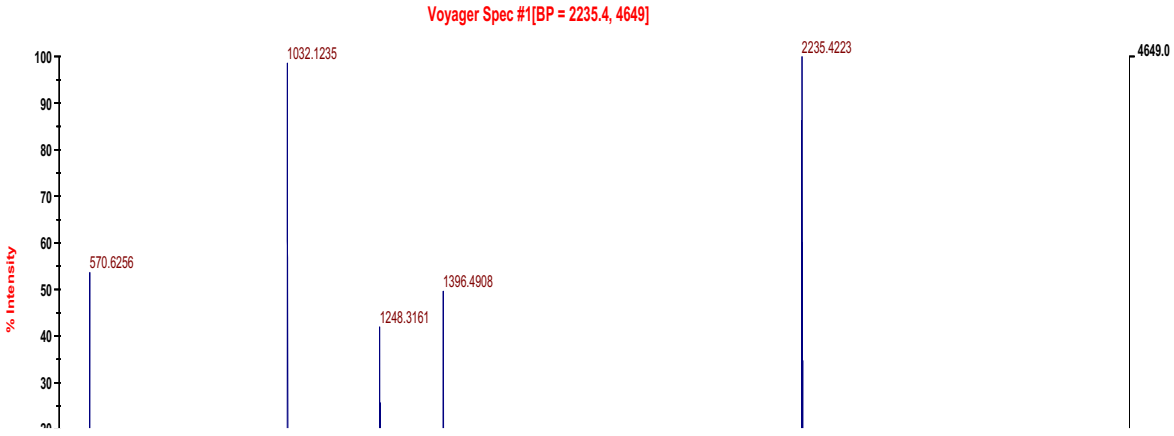
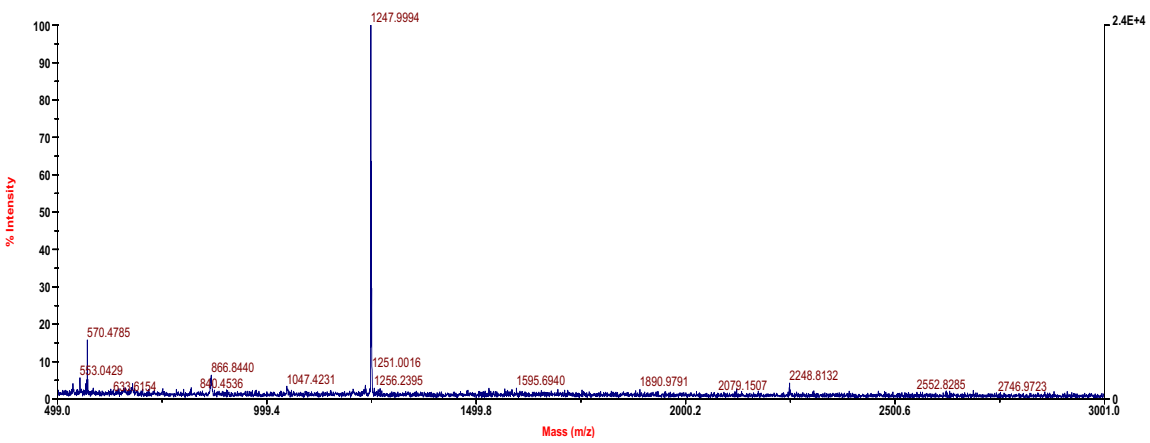
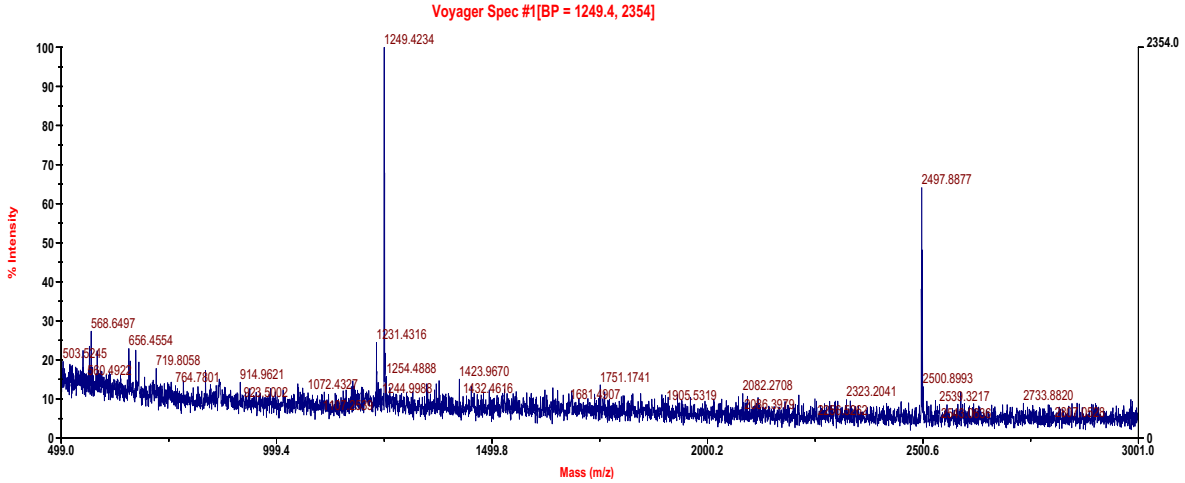
1828.6156
817.9391
1127.6981
643.3099
858.3757
708.9398
1007.7367
1180.2816
1623.8959
2238.5105
2302.0628
2547.7155
2736.5623
499.0
999.4
1499.8
2000.2
2500.6
3001.0

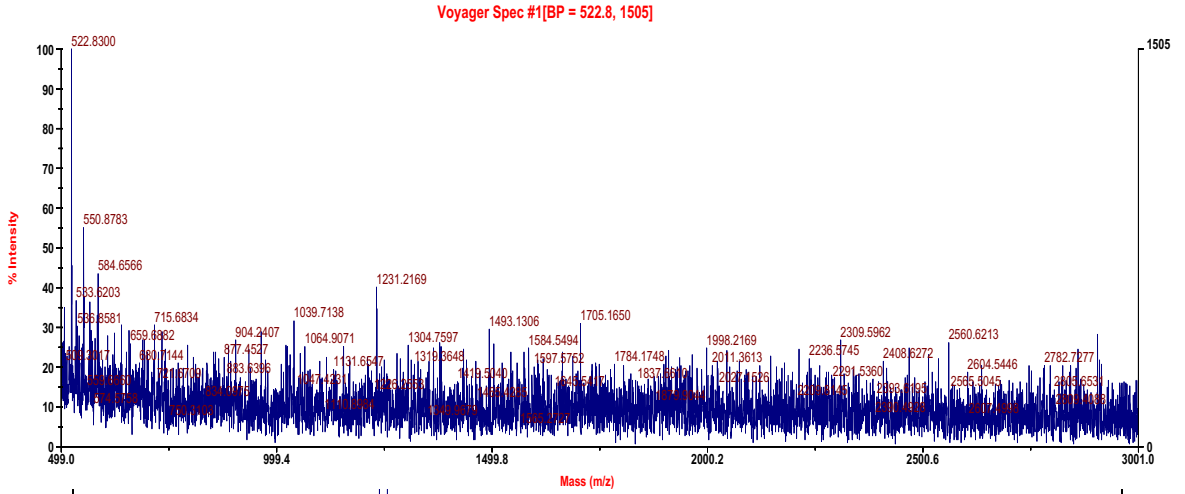
F31


Voyager Spec #1[BP = 2235.4, 4649]
% Intensity
1032.1235
2235.4223
4649.0
570.6256
1396.4908
1248.3161

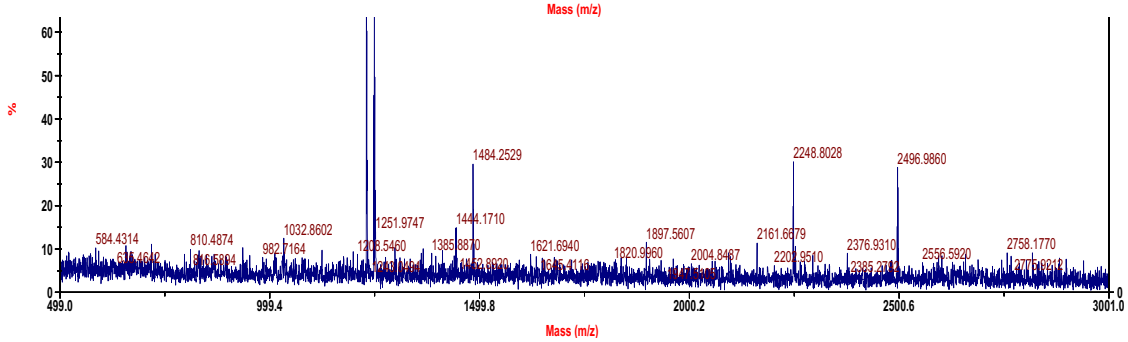
F32


Voyager Spec #1[BP = 1248.0, 23988]
% Intensity
Mass (m/z)
1247.9994
2.4E+4
570.4785
553.0429
866.8440
1047.4231
1251.0016
1256.2395
1595.6940
1890.9791
2079.1507
2248.8132
2552.8285
2746.9723
499.0
999.4
1499.8
2000.2
2500.6
3001.0

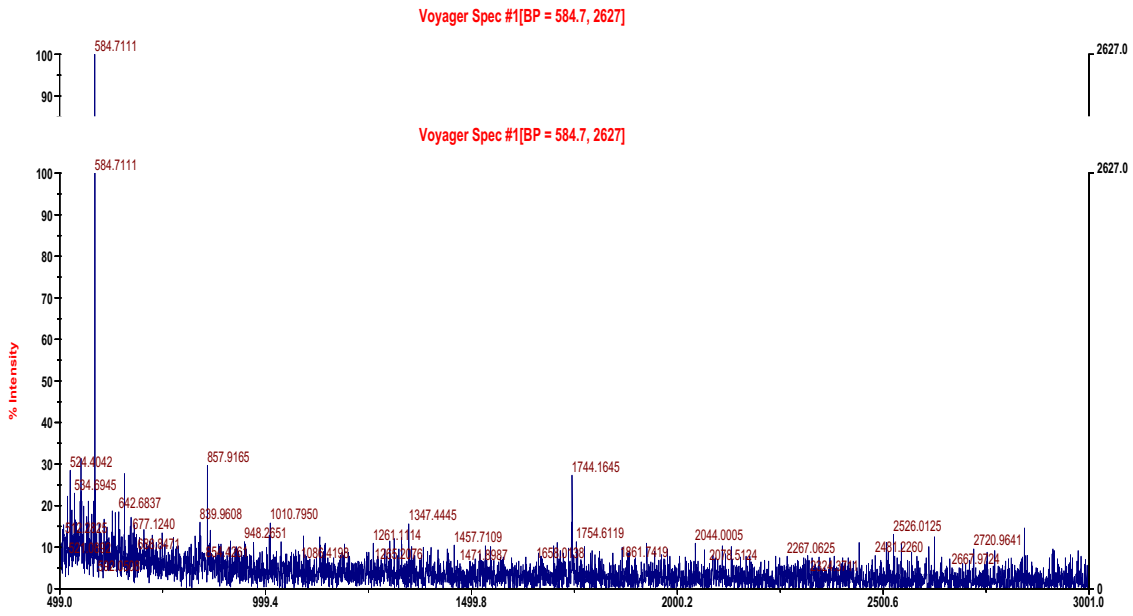

Voyager Spec #1[BP = 1249.4, 2354]
% Intensity
Mass (m/z)
1249.4234
2497.8877
568.6497
656.4554
719.8058
1231.4316
1254.4888
1423.9670
1751.1741
2082.2708
2323.2041
2500.8993
2733.8820


Voyager Spec #1[BP = 522.8, 1505]
% Intensity
Mass (m/z)
522.8300
550.8783
584.6566
1231.2169
1039.7138
1064.9071
1304.7597
1493.1306
1705.1650
1584.5494
1998.2169
2309.5962
2560.6213

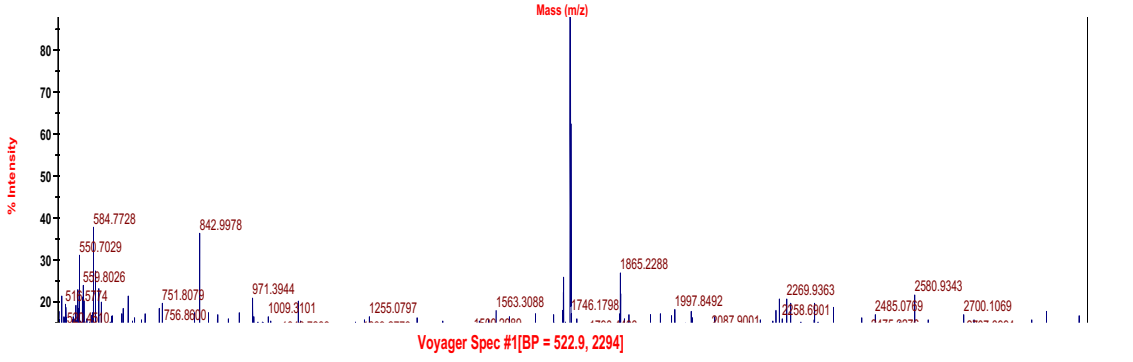
F33


%
Mass (m/z)
1484.2529
2248.8028
2496.9860
584.4314
1032.8602
1251.9747
1444.1710
1897.5607
2161.6679
2376.9310
2758.1770

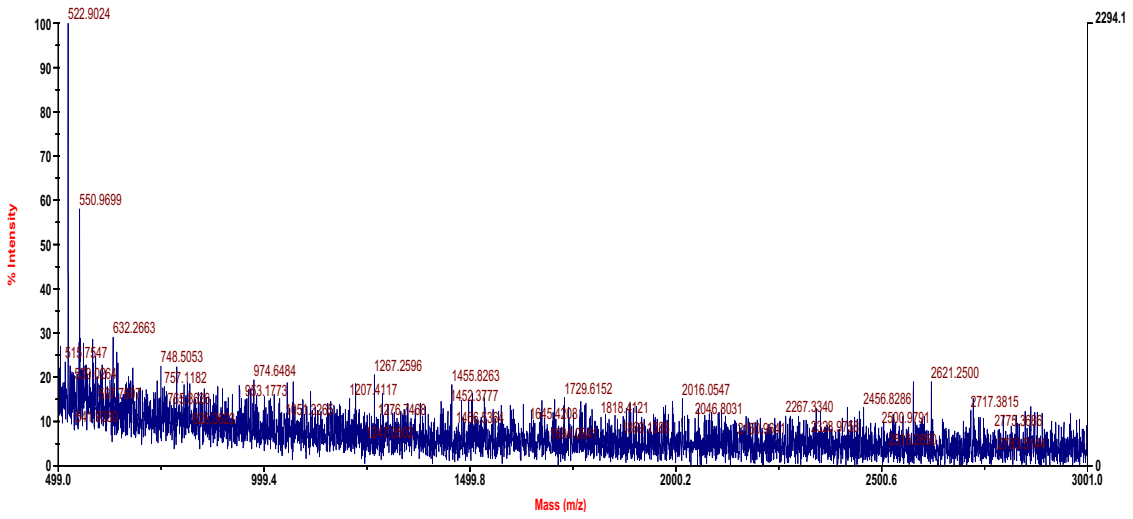
F34


Voyager Spec #1[BP = 584.7, 2627]
584.7111
2627.0
% Intensity
Mass (m/z)
524.4042
584.3945
642.6837
677.1240
839.9608
857.9165
948.2651
1010.7950
1261.1114
1347.4445
1457.7109
1744.1645
1754.6119
2044.0005
2267.0625
2526.0125
2720.9641

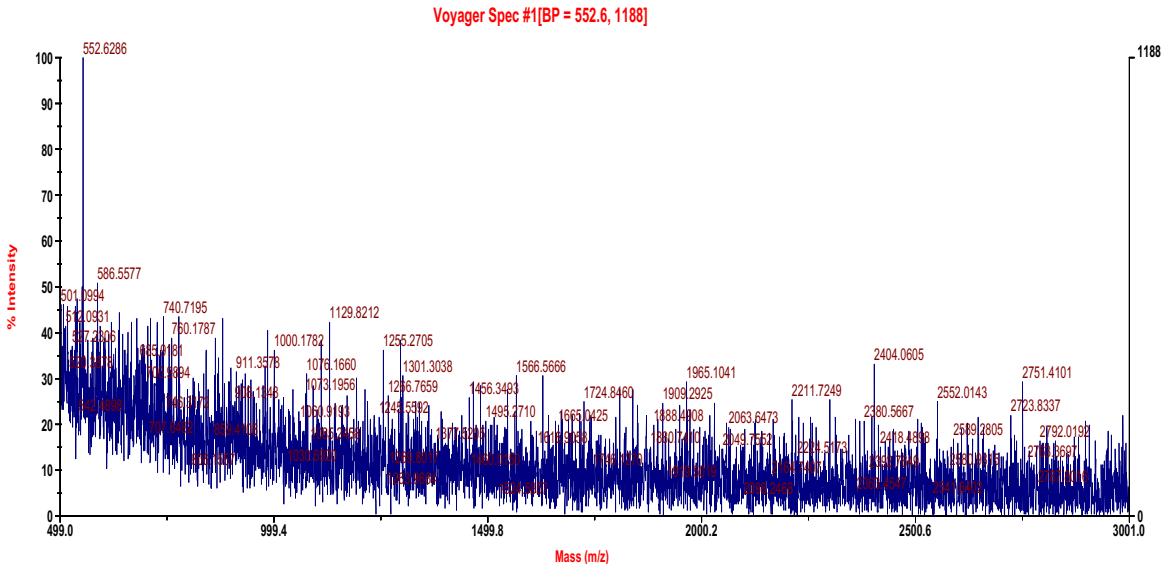
F35


% Intensity
584.7728
842.9978
550.7029
559.8026
751.8079
971.3944
1009.3101
1255.0797
1563.3088
1746.1798
1865.2288
1997.8492
2269.9363
2580.9343
2485.0769
2700.1069

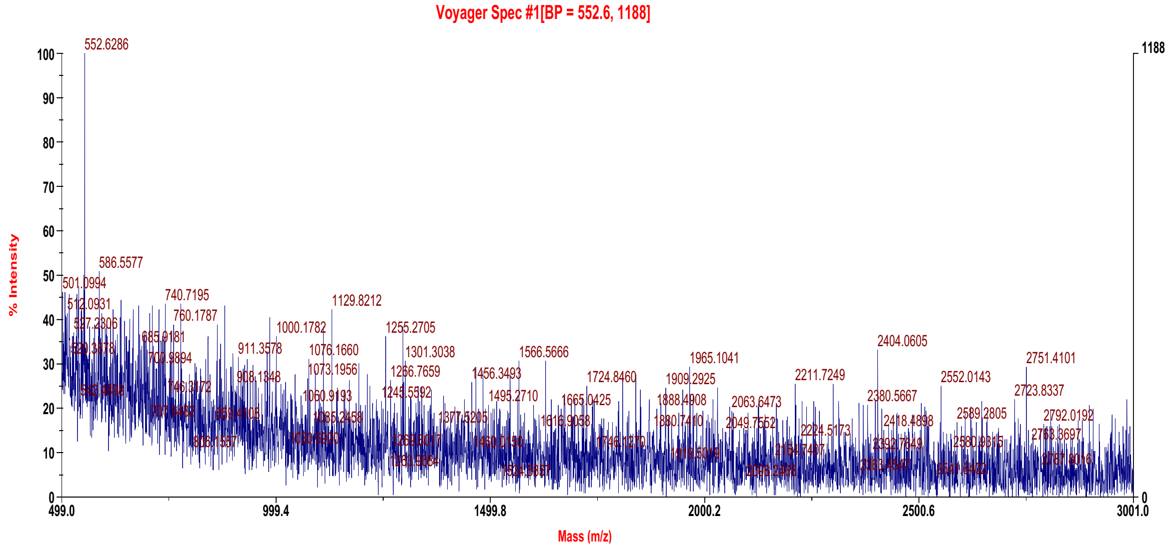
F36


Voyager Spec #1[BP = 522.9, 2294]
522.9024
2294.1
% Intensity
Mass (m/z)
550.9699
632.2663
748.5053
757.1182
974.6484
1267.2596
1455.8263
1729.6152
1818.4121
2016.0547
2267.3340
2456.8286
2621.2500
2717.3815

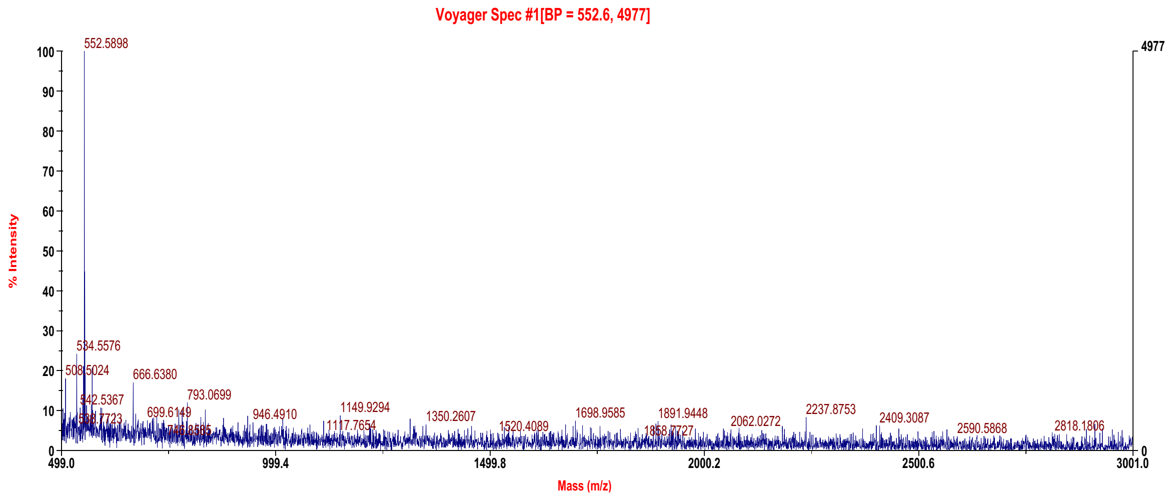
F39


Voyager Spec #1[BP = 552.6, 1188]
% Intensity
Mass (m/z)
552.6286
586.5577
740.7195
760.1787
1129.8212
1255.2705
1301.3038
1566.5666
1724.8460
1965.1041
2211.7249
2404.0605
2552.0143
2751.4101
2723.8337
499.0
999.4
1499.8
2000.2
2500.6
3001.0
100
90
80
70
60
50
40
30
20
10
0
1188

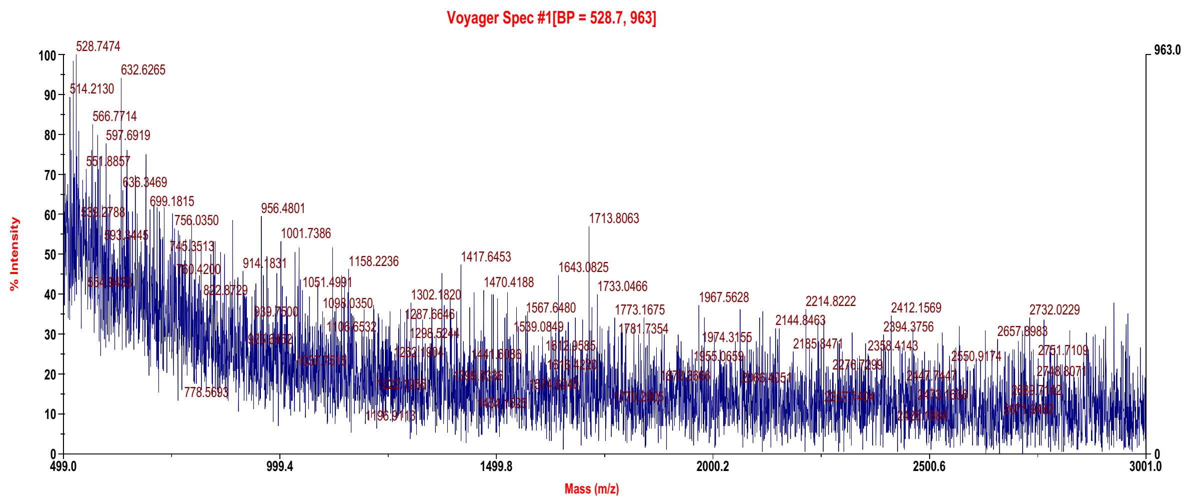
F40


Voyager Spec #1[BP = 552.6, 1188]
552.6286
586.5577
501.0994
740.7195
1129.8212
2404.0605
2751.4101
% Intensity
Mass (m/z)

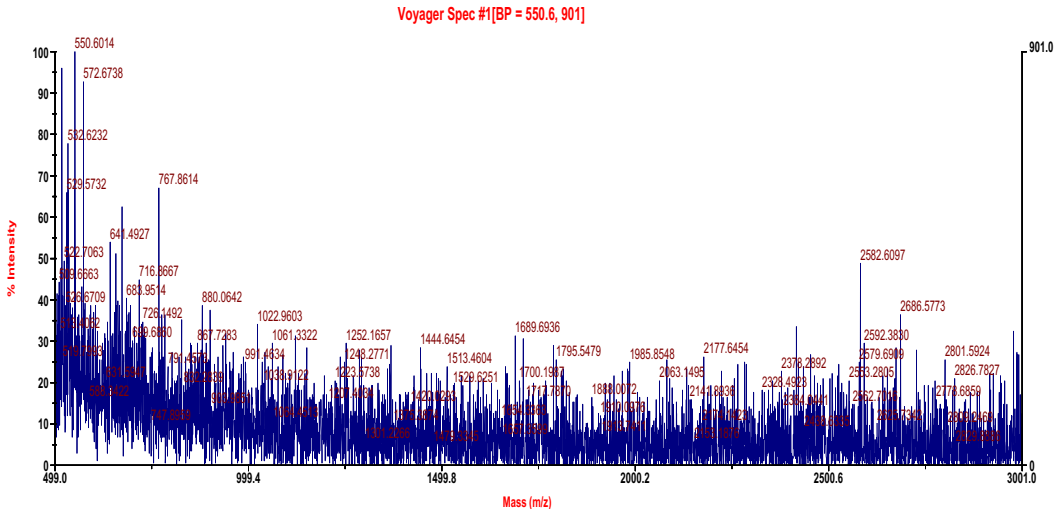
F41


Voyager Spec #1[BP = 552.6, 4977]
552.5898
534.5576
666.6380
793.0699
1149.9294
% Intensity
Mass (m/z)

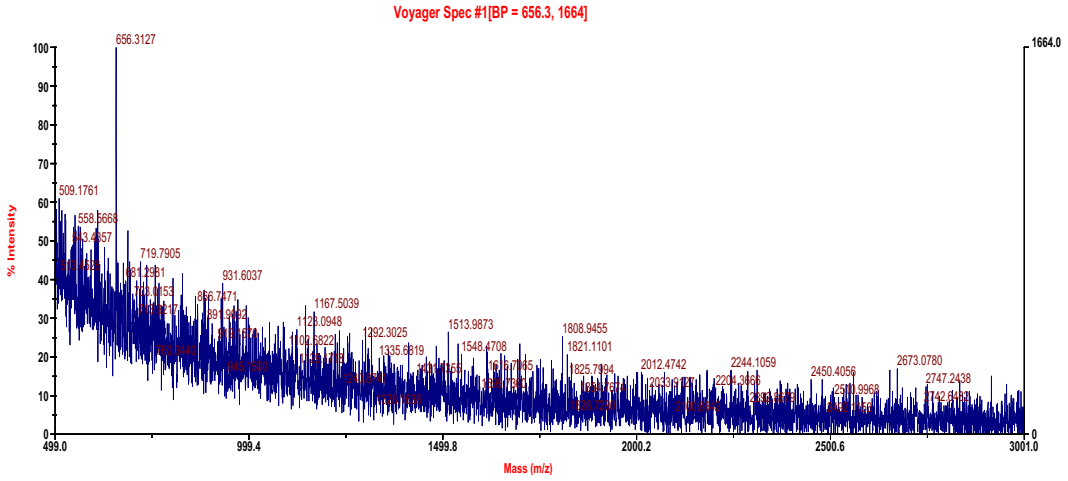
F43


Voyager Spec #1[BP = 528.7, 963]
528.7474
632.6265
514.2130
956.4801
1713.8063
% Intensity
Mass (m/z)

F44

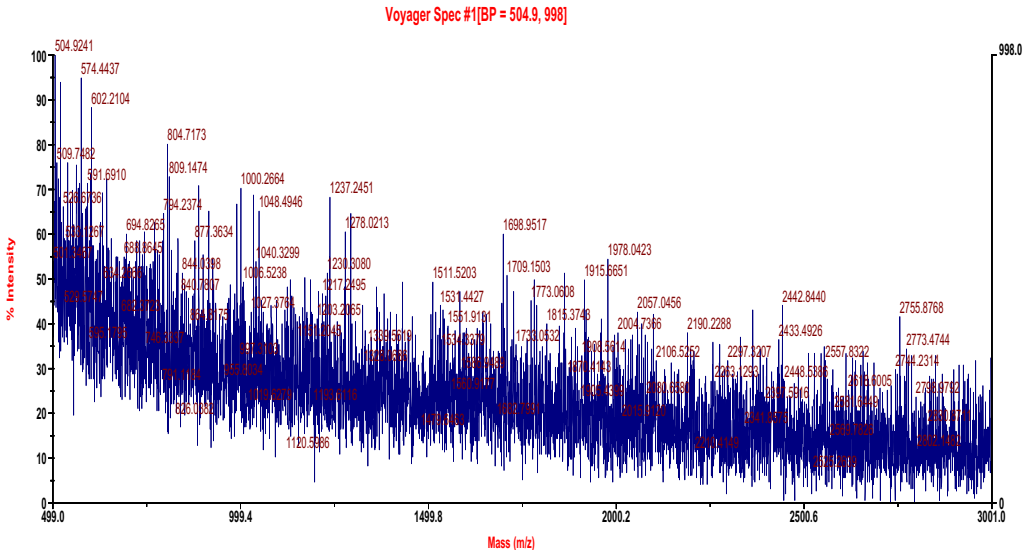


F53

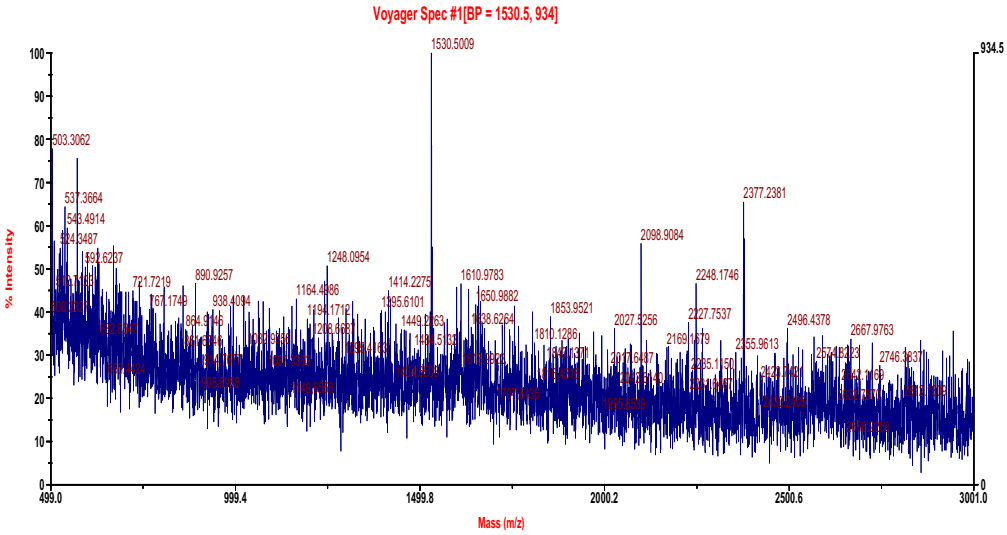


Supplementary figure 6.27. Brain samples dissected and separated with RT-HPLC, and analysed with MALDI-TOF analysis. (F- number of fraction). Methodology is described Chapter 6, *Ceratitis capitata* Neuropeptide.

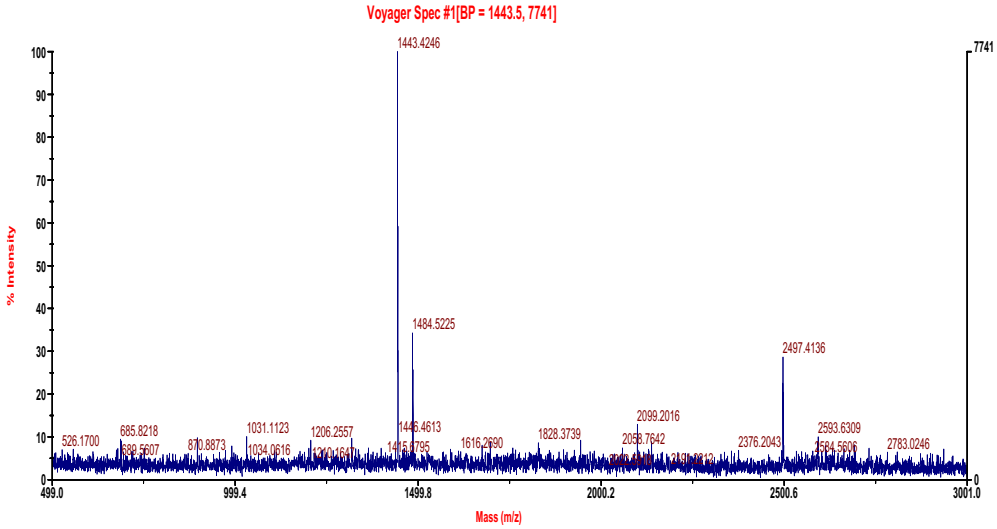
F31


Voyager Spec #1[BP = 504.9, 998]
% Intensity
Mass (m/z)
504.9241
574.4437
602.2104
804.7173
809.1474
1000.2664
1048.4946
1237.2451
1278.0213
1698.9517
1978.0423
2442.8440
2755.8768
2773.4744
998.0
499.0
999.4
1499.8
2000.2
2500.6
3001.0

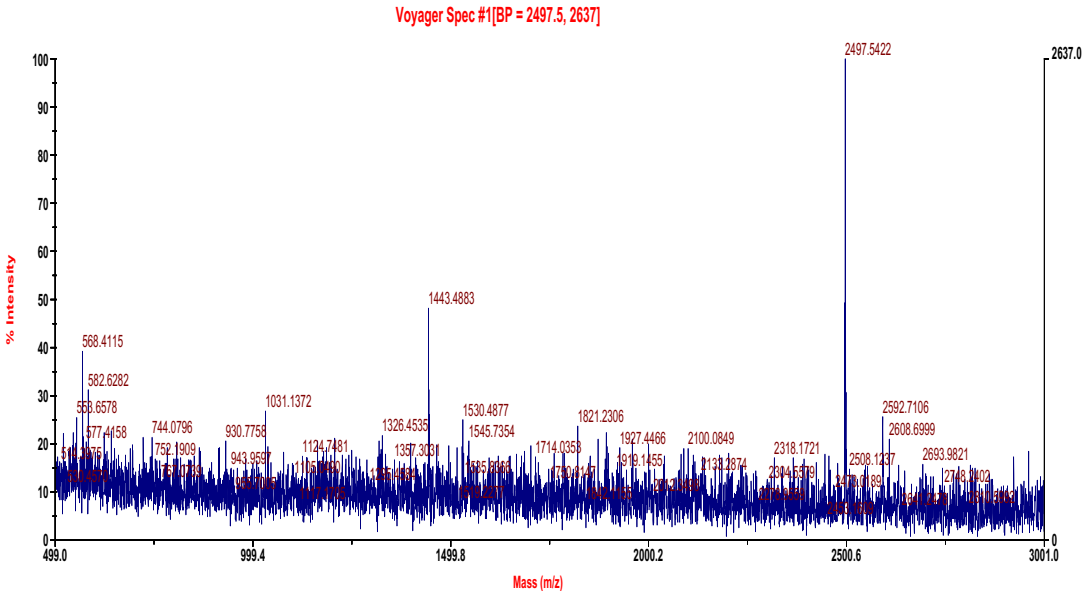
F32


Voyager Spec #1[BP = 1530.5, 934]
% Intensity
Mass (m/z)
1530.5009
934.5
503.3062
537.3664
543.4914
554.3487
721.7219
767.1749
890.9257
938.4094
1248.0954
1164.4986
1414.2275
1610.9783
1650.9882
1853.9521
2027.5256
2098.9084
2248.1746
2227.7537
2377.2381
2496.4378
2667.9763
2746.3637
2355.9613

F33


Voyager Spec #1[BP = 1443.5, 7741]
% Intensity
Mass (m/z)
1443.4246
7741
1484.5225
2497.4136
2099.2016
526.1700
685.8218
1031.1123
1206.2557
1446.4613
1828.3739
2376.2043
2593.6309
2783.0246

F34



Figures 6.28. Thoracico-abdominal ganglion samples dissected from *Ceratitis capitata*. Fractions collected from RT-HPLC were subsequently analysed with MALDI-TOF. (F- number of fraction).