University of Southampton

Faculty of Environmental and Life Sciences

Institute of Life Sciences

Investigation of cue dependent feeding behaviour in *Caenorhabditis elegans* reveals a critical mechanosensory dependent microcircuit in the control of pharyngeal pumping

by

Samah Zarroug

Thesis for the degree of Doctor of Philosophy

December 2018

University of Southampton

Abstract

Faculty of Environmental and Life Sciences

Institute of Life Sciences

Doctor of Philosophy

Investigation of cue dependent feeding behaviour in *Caenorhabditis elegans* reveals a critical mechanosensory dependent microcircuit in the control of pharyngeal pumping

Samah Zarroug

Both external and internal sensory cues are critical for modulating feeding behaviour. *C. elegans* provides a powerful model to study feeding behaviour, since one can reduce a multimodal cue to its component parts to show how different modalities impact the execution of simple behaviours. On food, worms pump at high levels but display a dramatic reduction in pumping rate in the absence of food. The main aims of the studies reported herein were twofold, 1) to examine behavioural plasticity in feeding behaviour in *C. elegans* and 2), again in in *C. elegans,* to determine which sensory modalities regulate feeding behaviour.

Plasticity in feeding behaviour was studied by examining innate and acquired behavioural responses of *C. elegans* to olfactory cues in on- and off-food contexts (Chapters 3-4). Alcohols affected chemotaxis in a dose-dependent manner but reduced pharyngeal pumping in the presence and absence of food. These results were followed up by investigating plasticity of chemotaxis and pharyngeal pumping to butan-1-ol and butanone. Only pre-exposing worms to pairing of butanone and food enhanced chemotaxis to butanone in subsequent tests, without altering pharyngeal pumping to butanone. These findings indicate that a specific component of the olfactory modality regulates chemotaxis and is subject to plasticity.

The modalities that regulate feeding behaviour of *C*. *elegans* were investigated by behavioural analysis which revealed elevated pharyngeal pumping to a mechanosensory cue presented in the form of microspheres (beads). These beads selectively stimulated

pumping if they were small enough to be ingested by the worms. Therefore, the physical contact and an enteric response are critical to drive pumping rate (Chapter 5). However, these mechanosensory cues that drive pharyngeal pumping failed to evidence plasticity in the pharynx as a consequence of a novel pairing between paradigm-based on olfactory and mechanosensory cues (Chapter 7).

Genetic studies revealed that serotonin and dopamine were critical determinants in the mechanical stimulation of pumping (Chapter 6). Despite having normal feeding behaviour on food, *dop-4 (tm1392)* mutants failed to respond to beads. Further mutant analysis revealed that the pharyngeal neurones, I2, were critical in the bead-induced pumping rate, since ablation of I2 neurones occluded the response to beads. A novel assay that scored the change in pump rate as worms entered food indicated that dopamine released outside the pharynx acted via DOP-4 receptors, expressed in I2 neurones, to accelerate the rate at which worms reached maximal pump rate on food. This evidenced a previously undiscovered dopaminergic volume transmission in the control of food-induced pharyngeal pumping and provides a distinct contribution to multiple mechanisms that allow the rapid increase in pumping as the worm transits from and off- to an on-food environment. These observations highlight a role for dopamine in the anticipation of nutrition provided by the ingestion of a potential food source in the worm.

Table of Contents

Abstract ..... **i**

Table of Contents ..... **iii**

List of Tables ..... **ix**

List of Figures ..... **x**

DECLARATION OF AUTHORSHIP ..... **xv**

Acknowledgement ..... **xvii**

Definitions and Abbreviations ..... **xix**

Chapter 1: General introduction **1**

1.1 Sensory cues are important drivers of behaviour 1

1.1.1 The *C. elegans* as a model to study molecular and cellular determinants in cue-dependent behaviour 2

1.1.2 Sensory-dependent behavioural plasticity 2

1.2 *Caenorhabditis elegans* 3

1.2.1 The life cycle of *C. elegans* 4

1.2.2 Environmental regulation of development 5

1.3 Advantages of *C. elegans* as a model organism 8

1.4 The nervous system of *C. elegans* 8

1.4.1 Organization of synaptic transmission and modulation 9

1.5 Feeding behaviours in *C. elegans* 17

1.5.1 The fundamentals of *C. elegans* locomotion behaviour 18

1.5.2 The pharynx of *C. elegans* 23

1.6 The regulation of *C. elegans* feeding behaviour is coupled to input from its environment 31

1.6.1 An introduction to sensory neurones 32

1.6.2 Sensory modalities 32

1.7 The role of biogenic amines in regulating sub-behaviours of feeding 41

1.7.1 The role of serotonin in regulating locomotion behaviour 41
1.7.2 The role of serotonin in regulating pharyngeal pumping 42
1.7.3 The role of glutamate in locomotion behaviour 43
1.7.4 The role of glutamate in regulation pharyngeal pumping 43
1.7.5 The role of dopamine in locomotion behaviour 44
1.7.6 The role of dopamine in pharyngeal pumping 44
1.7.7 The role of tyramine and octopamine in the modulation of locomotion 45
1.7.8 The role of tyramine and octopamine in the modulation of pharyngeal pumping 46
1.8 Behavioural plasticity in *C. elegans* in response to sensory cues 46
1.9 Aim of the study 48
Chapter 2: General Materials and Methods 51
2.1 Solutions and chemicals 51
2.2 Strains used 54
2.3 *C. elegans* maintenance and husbandry 56
2.4 Behavioural assays statistical analysis 64
2.5 Microinjection of *C. elegans* 65
2.6 Assessing calcium levels in I2 neurones in the presence of dopamine 66
2.7 Crossing worms to identify *gur-3 (ok2246); N2; Is[Pflp15::GCaMP3]* strain 67
2.8 Molecular biology 71
Chapter 3: The effect of volatile alcohols on innate feeding behaviour 87
3.1 Introduction 87
3.2 Results 88
3.2.1 Effect of alkyl chain length on alcohol chemotaxis 88
3.2.2 Pharyngeal pumping during alcohol chemotaxis in the absence of food 93
3.2.3 Determination of pharyngeal pumping in the presence of alcohol and food 97
3.2.4 The distal effect of alcohols on pharyngeal pumping 97
3.2.5 The proximal effect of alcohols on pharyngeal pumping 99
3.3 Discussion 101

3.3.1 The impact of alcohols on chemotaxis index depends on the number of the chain length 101

3.3.2 Alcohols affect chemotaxis in a dose-dependent manner 101

3.3.3 Alcohol modulation of pharyngeal pumping is independent of their effect on chemotaxis..... 102

3.3.4 Alcohols suppress pharyngeal pumping in the absence and presence of food..... 103

3.3.5 Conclusion..... 104

Chapter 4: Investigating pharyngeal pumping in olfactory cue-dependent acquired behaviour 107

4.1 Introduction..... 107

4.2 Results 108

4.2.1 Chemotaxis to butan-1-ol did not increase following pre-exposure to butan-1-ol and food..... 108

4.2.2 Chemotaxis to butan-1-ol remained unchanged when butan-1-ol is added to food on agar..... 108

4.2.3 Preconditioning worms to butan-1-ol and food did not affect pharyngeal pumping when butan-1-ol was added to agar..... 111

4.2.4 Preconditioning worms to butan-1-ol and food does not affect pharyngeal pumping and chemotaxis when butan-1-ol is added inside of the lid..... 113

4.2.5 The dose-dependent effect of butanone on chemotaxis and pharyngeal pumping in the absence of food 115

4.2.6 Determining pharyngeal pumping rate in response to butanone during a chemotaxis assay in the absence of food..... 115

4.2.7 Butanone-induced chemotaxis plasticity following conditioning worms in the presence of food and butanone for 90min..... 116

4.2.8 Butanone-induced chemotaxis plasticity following pre-conditioning regime had no effect on pharyngeal pumping 119

4.2.9 Butanone-induced chemotaxis plasticity following chronic exposure to butanone and food..... 121

4.3 Discussion..... 124

4.3.1 Chemotaxis to butanone, but not butan-1-ol, increases following pre-conditioning of worms to butanone in the presence of food 124

4.3.2 Butanone did not induce pharyngeal pumping dependent plasticity following conditioning of worms to butanone in the presence of food 125

Chapter 5: Defining the sensory modalities that stimulate feeding behaviour in *C. elegans*..... 127

5.1 Introduction 127

5.2 Results 128

5.2.1 The rate of pharyngeal pumping depends on the density of bacteria, *E. coli* OP50..... 128

5.2.2 Olfactory cues do not affect pharyngeal pumping on food 131

5.2.3 Mutations of the ciliated sensory neurones affect pharyngeal pumping in the absence of food 133

5.2.4 *C. elegans* exhibits normal feeding behaviour on UV-radiated bacteria 135

5.2.5 Mechanosensory cues lead to a transient elevation in pumping rate..... 137

5.2.6 Physical contact with ingested beads is required for sustained and elevated pharyngeal pumping 140

5.2.7 Large, non-ingestible beads do not elevate pumping rate 143

5.2.8 Ingestible beads do not affect body bends 145

5.2.9 Bacterially conditioned media do not elevate pumping behaviour 147

5.3 Discussion 149

5.3.1 The absence of olfactory cues reduces pharyngeal pumping 149

5.3.2 Mechanosensory cues increase pumping rate..... 149

5.3.3 Beads-induced pharyngeal pumping does not affect locomotion 151

5.3.4 Pumping behaviour on beads was slower compared to on food 151

5.3.5 Conclusion 152

Chapter 6: Characterisation of mechanosensory-mediated pharyngeal pumping . 153

6.1 Introduction 153

6.2 Results 154

6.2.1 Exogenous dopamine increases pharyngeal pumping in the absence of food 154

6.2.2 Dopamine and serotonin signalling modulate bead-mediated pumping rate…… 156

6.2.3 Dopamine receptors, DOP-3 and DOP-4, are required for bead-induced pumping rate in response to beads and for dopamine-mediated pharyngeal pumping 159

6.2.4 Defining the circuit that underpins the bead-induced pharyngeal pumping 162

6.2.5 The FMRF-amide-like neuropeptide mutants, *flp-15*, does not respond to the beads stimulated pumping 168

6.2.6 Expression of wild-type dop-4 partially rescued bead- and dopamine-mediated pumping rate in *dop-4* mutant 170

6.2.7 Expression of wild-type dop-4 in I2 partially rescued in part elevated pumping rate 172

6.2.8 Physical contact with food is required for elevated pharyngeal pumping ... 174

6.2.9 Serotonergic and dopaminergic signalling are required for elevated pumping rate upon entry to food 176

6.2.10 The FMRF-amide-like neuropeptide, FLP-15, is required upon entry to food…… 178

6.2.11 DOP-4 receptors are critical for reaching maximum pumping rate upon entry to food 180

6.2.12 Expression of wild-type dop-4 in I2 partially rescued the half-time required to reach the maximum pumping rate upon entry to food 182

6.3 Discussion 185

6.3.1 Mechanosensory cues in the pharynx increase the pumping rate in a dopamine- and 5-HT-dependent manner 186

6.3.2 Receptor expression defines key determinants 187

6.3.3 FLP-15 is critical in beads-mediated pumping and on entry to food 189

6.3.4 UNC-31 and EAT-4 regulates mechanosensory-dependent pharyngeal pumping 189

6.3.5 Bead-induced pumping acts through I2 neurones to elevate pharyngeal pumping 190

6.3.6 Dopamine disinhibits the pharynx to increase pharyngeal pumping upon entry to food 190

Chapter 7: Investigating plasticity in chemosensory responses upon prior pairing of mechanosensory cue and odour 195

7.1 Introduction 195

7.2 Results 196

7.2.1 Beads-induced pharyngeal pumping does not alter butanone chemotaxis index following incubation of worms in the presence of beads and butanone 196

7.3 Discussion 199

7.3.1 Dopaminergic determinants primitive to food reward 199

7.3.2 Dopaminergic signalling of feeding plasticity 199

Chapter 8: General Discussion 201

8.1 Beads, a paradigm for mechanosensory modality, reveal a complex role of mechanoreception in pharyngeal pumping 202

8.2 A positive feedback from the gut to the central nervous system is a conserved mechanism in feeding behaviours 203

8.3 Dopamine volume transmission is a significant determinant of feeding 204

8.4 Physiological relevance of the dopamine-mediated disinhibition 206

8.5 Serotonin acts in parallel to dopamine to stimulate pharyngeal pumping 206

8.6 A complex interplay of modalities and outputs allow a worm to successfully feed 209

8.7 Pharyngeal pumping-induced plasticity may require more than one modality 209

8.8 Conclusion 211

Appendices 213

References 219

List of Tables

Table 1: Mutant strains used in this study.....55
Table 2: A summary of the effect of alpha-carbon hydrolate alcohols on locomotion versus on-food and off-food. 105
Table 3: Colour coding key based on the largest absolute effect of alcohols on chemotaxis and pumping rate.....105

List of Figures

Chapter 1

Figure 1.1: The life cycle of C. elegans at 20°C.....5
Figure 1.2: The pharynx of the C. elegans……..9
Figure 1.3: A schematic representation of neurotransmitter pathways in C. elegans.....13
Figure 1.4: Pseudocoelom and coelomocytes in C. elegans hermaphrodite……..15
Figure 1.5: Neuronal control of C. elegans locomotion…… 19
Figure 1.6: Locomotory behaviour in response to food availability.....21
Figure 1.7: A schematic representation of pirouette and weathervane (curve) behaviour. 23
Figure 1.8: The pharynx of C. elegans. A) The anatomy of the pharynx. 25
Figure 1.9: The positions of pharyngeal nuclei…….. ..27
Figure 1.10: The locations of mechanosensory neurones that respond to low threshold body touch…….. 34
Figure 1.11: Sensory neurones that respond to gentle nose touch…….. 35
Figure 1.12: The chemosensory neurones in C. elegans.....38
Figure 1.13: The sensory neurones that detect and modulate ambient oxygen levels…...40
Figure 1.14: The carbon dioxide sensory neurones. 41
Figure 1.15: The theoretical scheme of behavioural plasticity.....48

Chapter 2

Figure 2.1: Verification of gur-3 (ok2246) homozygote in GFP-positive offspring by gel electrophoresis. 71
Figure 2.2: Gel electrophoresis of the amplified dop-4 cDNA……74
Figure 2.3: Restriction digest of a dop-4 cDNA in the TOPO vector by gel electrophoresis.....77
Figure 2.4: Restriction mapping of the dop-4 following NB19 ligation……..83
Figure 2.5: Verification of dop-4 DNA cosmid by gel electrophoresis……86

Chapter 3

Figure 3.1: Determining the dose-dependent effect of alpha-carbon alcohols on chemotaxis index. *92*

Figure 3.2: Determining the effect of dose of compound on pharyngeal pumping rate during chemotaxis assay in the absence of food. *96*

Figure 3.3: The effect of alcohols on pharyngeal pumping when alcohols were presented on the agar……*98*

Figure 3.4: The effect of alcohols on pharyngeal pumping rate when the alcohol was placed on the lid of the agar plate……..*100*

Chapter 4

Figure 4.1: Chemotaxis to butan-1-ol did not enhance chemotaxis following conditioning worms in the presence of food and butan-1-ol……....*110*

Figure 4.2: Preconditioning worms to butan-1-ol in the presence of food has no effect on pharyngeal pumping during a chemotaxis assay. *112*

Figure 4.3: Butan-1-ol conditioning assay when worms are in contact with the food and the odorant is placed on agar plugs on the lid of the plate...... *114*

Figure 4.4: The effect of dose of butanone on CI was assessed by a chemotaxis assay..... *116*

Figure 4.5: C. elegans enhanced their chemotaxis index to butanone following pre-conditioning worms to butanone and food...... *118*

Figure 4.6: Pre-exposure of worms to butanone and food did not modify pharyngeal pumping in a subsequent test..... *120*

Figure 4.7: Prolonged exposure to butanone and food induced chemotaxis plasticity without accompanying any changes to pharyngeal pumping in subsequent tests…123

Chapter 5

Figure 5.1: Pharyngeal pumping depends on the number of colonies of the bacterial culture……..130
Figure 5.2: Distal olfactory cues do not regulate pharyngeal pumping on-food.....132
Figure 5.3: Cilia-deficient mutant, osm-6, selectively regulates the off-food pump rate…134
Figure 5.4: The effect of non-viable bacteria on pharyngeal pumping rate……...136
Figure 5.5: A burst in pump rate was observed when worms were in contact with ingestible beads……139
Figure 5.6: The distribution of fluorescent beads in worms fed with ingestible (0.5µm) of beads…….....140
Figure 5.7: Pumping rate on beads is dose- and time-dependent……..142
Figure 5.8: Intestinal distention and defecation from worms subjected to protracted feeding with ingestible (0.5µm) of beads for 90 min.....143
Figure 5.9: Non-ingestible beads do not elevate pumping rate……144
Figure 5.10: Ingestible and non-ingestible beads do not affect worm's locomotion…….146
Figure 5.11: Bacterially conditioned medium had no effect on pumping rate in the absence of food.....148

Chapter 6

Figure 6.1: Dopamine increases pharyngeal pumping in the absence of food…… 155
Figure 6.2: Pharyngeal-induced pumping in cat-2 (e1112) and tph-1 (n4622) on food, dopamine, 5-HT in response to exogenous 5-HT, dopamaine and beads. 158
Figure 6.3: DOP-3 and DOP-4 are selectively required for bead-induced and dopamine-induced pharyngeal pumping……..162
Figure 6.4: eat-4 (ky5) and unc-31 (e169) mutants occlude bead and dopamine-dependent pharyngeal pumping……..165
Figure 6.5: I2 neurones are required for bead-mediated pharyngeal pumping.....167
Figure 6.6: flp-15 (gk1186) mutants are deficient in the bead- and dopamine-induced pumping rate…….. ..169
Figure 6.7: Transgenic rescue of the dop-4 mediated bead- and dopamine-induced pharyngeal pumping…… 171

Figure 6.8: Cell-specific expression of dop-4 in I2 neurones in dop-4 mutant background. 173
Figure 6.9: Food triggers an immediate increase in pharyngeal pumping rate as worms enter the food area…….. 175
Figure 6.10: Comparison of food entry-induced pumping in dopamine-deficient mutant (cat-2) and serotonin-deficient mutant (tph-1)……177
Figure 6.11: Comparison of food entry induced pumping in the FMRF-amide-like neuropeptide mutant, flp-15 (gk1186). 179
Figure 6.12: DOP-4, is required as the worm enters the food lawn…… .181
Figure 6.13: Cell-specific expression of wild-type dop-4 cDNA in I2 neurones partially rescued entry to food elevated pumping rate…….....183
Figure 6.14: An overview of the approach used to delineate the mechanosensory-dependent pharyngeal pumping……...185
Figure 6.15: Mechanosensory neurones in C. elegans……..…....187
Figure 6.16: The role of mechanosensation in C. elegans in driving feeding behaviour..192

Chapter 7

Figure 7.1: Pre-conditioning worms in the presence of beads and butanone do not alter subsequent chemotaxis to butanone……198

Chapter 8

Figure 8.1: The mechanosensory cues from food are both 5-HT and dopamine dependent……. .208
Figure 8.2: The main sensory modalities for chemotaxis towards butanone versus the sensory modalities required for stimulating pharyngeal pumping. 210

DECLARATION OF AUTHORSHIP

I, Samah Zarroug declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Investigation of cue-dependent feeding behaviour in *Caenorhabditis elegans* reveals a critical mechanosensory dependent microcircuit in the control of pharyngeal pumping.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signed: .....

Date: .....

Acknowledgement

First of all, I would like to thank my supervisors Professors Lindy Holden-Dye, Vincent O' Connor and Dr Steven Glautier for their expert supervision and incredible support and encouragement throughout my time at the University of Southampton. Their critical appraisal and scientific input during this project was absolutely invaluable. I would like to thank each one of them for their patience and compassion during and after my pregnancy, whilst writing this thesis.

I would like to thank Dr Fernando Calahorro for carrying out the microinjection procedure on my behalf and for his support and assistance whilst carrying out the calcium imaging and molecular biology experiments. I would like to thank Dr Nico Dallière for his help and advice in the lab and in particular for his assistance with the beads experiment. I would like to thank Dr Anna Crisford and Dr James Dillon for sharing their valuable knowledge and expertise and for their help in the lab.

I would like to thank Professor Robert Walker and his students for sharing their dopamine preliminary data with me. I would like to thanks Irina Vasilescu for carrying out the food entry assay on my behalf. I would like to thank Dr Katrin Deinhardt for giving me the permission to use her calcium imaging laboratory. I would like to thank Mark Willet for his assistance and support in the calcium imaging laboratory.

I would like to thank the Institute of Life Sciences, University of Southampton (ifLS) for funding my postgraduate project. Additionally, I would like to thank The Gerald Kerkut Trust for funding the last six months of my PhD. I would not have been able to carry on without their generous support.

I would like to thanks all the administrative staff at the Centre of Biological Sciences and School of Psychology for providing help and support throughout my PhD project.

I would like to thank all my colleagues in the wormlab (previous and current students) for their support and help throughout my time at the University.

A special thanks to Hassan for his patience and support during my PhD project and whilst writing this thesis. To my son, Barra, thank you for behaving well whilst producing this thesis. A huge thanks to my siblings Osman and Sarah for their support and for looking after Barra whilst producing this thesis.
Last but not least, I would like to thank my mother for all the sacrifices that she has made and still making for myself and my siblings. I am ever grateful for her ongoing encouragement and support which keep me going!

Definitions and Abbreviations

5-HT: 5-Hydroxytryptamine

ACh: acetylcholine

ADL: Chemosensory neurones in *C. elegans*

ADE: Dopaminergic sensory neurones in *C. elegans*

ATP: adenosine tri-phosphate

ALM and AVM: mechanosensory neurones

AQR: oxygen sensory neurones in *C. elegans*

ARS: area restricted search

ASE: Chemosensory neurones in *C. elegans*

ASH: polymodal nociceptive sensory neurones in *C. elegans*

AWA/ AWC: odour-sensory neurones in *C. elegans*

AWB: odour-sensory neurones in *C. elegans*

BSA: bovine serum albumin

CEP: Dopaminergic sensory neurones in *C. elegans*

CI: Chemotaxis index

C. elegans: *Caenorhabditis elegans*

CGC: *Caenorhabditis elegans* genetics centre

CNS: central nervous system

DCV: dense core vesicle

DNA: deoxyribonucleic acid

E. coli: *Escherichia* coli

FLP: FMRF-amide-like neuropeptide

GABA: γ-aminobutyric acid

GFP: green fluorescent protein

LAS: local search area

LB: Luria Broth

NGM: nematode growth medium

NMJ: neuromuscular junction

PCR: polymerase chain reaction

PLM and PVM: mechanosensory neurones

PNS: pharyngeal nervous system

PPM: pump per min

PQR: Oxygen sensory neurones

PDE: Dopaminergic sensory neurones in *C. elegans*

RNA: ribonucleic acid

RIP: ring and pharyngeal interneurons

SNARE: soluble NSF attachment protein receptors

UV: ultra violet

URX: oxygen sensory neurones

Chapter 1: General introduction

1.1 Sensory cues are important drivers of behaviour

To allow adaptation, animals must be able to detect and process changes in their external and internal environments. External environmental conditions include presence or absence of food, detection of extreme temperatures, presence or absence of light or predators, whereas internal conditions include nutritional status (fed or starved). The nervous system gathers this information from the sensory systems, which detects relevant entities and events to execute appropriate responses (Manita et al., 2015). The combination of external sensory inputs by the nervous system, with inputs from the internal physiological state, allow the animal to control its behaviour by executing the appropriate responses. In mammals, the integration of sensory information is a "top-down" control of perception, in which a feedback from higher-order regions regulate the activity of primary sensory cortex (Manita et al., 2015). Neurological disorders such as sensory-processing disorder, schizophrenia, attentional deficit hyperactivity disorder (ADHD), bulimia nervosa and autism spectrum disorders exhibit disruption and changes in sensory and neuro-modulatory circuits as a result of abnormal processing and integration of sensory information (Carmona et al., 2015; Clince et al., 2016; Green et al., 2017; Takarae et al., 2016). In human, the analysis of underlying neural mechanisms of sensory processing and integration is limited to functional imaging and physiological studies (Sánchez-Alcañiz and Benton, 2017). Conversely, the use of reductionist approach permits the use of model organisms to allow manipulation of the central nervous system at cellular and molecular level (Sánchez-Alcañiz and Benton, 2017). For instance, a recent study used a range of techniques to delineate the local microcircuit involved in the "top-down" regulation of visual cortex processing in mice (Zhang et al., 2014). Nevertheless, this type of detailed analysis has not been used to define the neuronal mechanisms underpinning perceptual decision-making in freely-moving animals (Ghosh et al., 2017).

1.1.1 The *C. elegans* as a model to study molecular and cellular determinants in cue-dependent behaviour

An advantage of using the nematode, *Caenorhabditis elegans (C. elegans),* is due to the ability of manipulating several components of the nervous system at cellular and molecular level, which bridges the gap on studies of sensory integration when combined with both physiological and behavioural measurements in freely-moving animals. The nervous system of *C. elegans* has 302 neurones with a defined connectivity (White et al., 1986) and a cell-specific genetic manipulation which can be carried out in many of these neurones, e.g. (Bhatla and Horvitz, 2015). Although *C. elegans* has a simple nervous system, the worm can respond to a wide range of sensory modalities such as taste (Bargmann and Horvitz, 1991), smell (Bargmann et al., 1993), temperature (Hedgecock and Russell, 1975), oxygen and carbon dioxide (Bretscher et al., 2008; Gray et al., 2004), touch (De Jager et al., 1998) and light (Edwards et al., 2008). Additionally, the worm can detect and respond to internal cues such as nutritional state (Lemieux et al., 2015). As in higher animals, *C. elegans* can detect and respond to one cue (single sensory) or multiple cues (multisensory), in combination with the internal state (contextual response, fed or starved), which influences *C. elegans* decision-making (Bono and Villu Maricq, 2005; Sengupta, 2007).

1.1.2 Sensory-dependent behavioural plasticity

As described above, animals do not only respond to stimulus in their environment, but they must respond to changes in their environment to alter their response to that stimulus. Behavioural plasticity is a change in the behaviour of an organism in response to either an external or internal stimulus presented under specific environmental conditions (Gluck et al., 2007). The capacity to learn plays a crucial role in survival and therefore many studies have been carried out to show how animals have to change their behaviour (Li and Liberles, 2015). For example, in order to survive, animals have to adapt to find new food sources if current food sources are diminished and learn to find a new territory if they were attacked by predators. These tasks require anticipation of new and important events in the environment and the learned behavioural responses are vital for survival (Li and Liberles, 2015). Suppression of behavioural responses also occur due to a learned mechanism from previous experience, for example, animals avoid areas where predators or harm are present (Fendt and Fanselow, 1999). Learning to refrain from responding is also as critical

as learning to make new responses. For example, consumption of harmful food, which results in illness, can lead to avoidance of this food and cues associated with it by the animal (Boakes et al., 1997; Revusky and Parker, 1976). Therefore, environmental cues provide information for the organism which assists in avoiding harm and learning new responses to aid survival. For adaptive behaviour to occur, sensory information must be relayed into the existing output circuits that drive behaviour (Naumann et al., 2016). This means that the animal encodes the valence of stimulus by forming a connection between the executed behaviour and the stimulus to make accurate predictions of future events and change its behaviour accordingly. Plasticity can occur at synaptic, cellular or molecular level, before it can occur at behavioural level (Schaefer et al., 2017).

More studies are needed to elucidate the cellular and molecular mechanisms underlining behavioural plasticity. Due to difficulties in investigating this in the mammalian system, *C. elegans* provides a useful model to investigate sensory-dependent detection of environmental cues, pathways of integration and routes by which plasticity may be imposed.

1.2 *Caenorhabditis elegans*

C. elegans is a free living, transparent, nematode worm (length is 1mm) (Brenner, 2003). In the wild, the life cycle of *C. elegans* is marked by either feast or famine (Barrière and Félix, 2005, 2007). *C. elegans* was isolated as a dauer, non-feeding state larval, (Barrière and Félix, 2005, 2007) from compost and rich soils in humid regions (Andersen et al., 2012; Kiontke et al., 2011). Fertile and feeding stages of *C. elegans* have been found in rotten fruits, flowers and thick herbaceous plant stems (Félix and Duveau, 2012). These rotten materials are a good source of bacteria, which is *C. elegans'* main food. Chemicals released bacteria, which are detected by *C. elegans* chemosensory systems (see section 1.6.2), allow them to differentiate between food sources and pathogens (Bargmann and Horvitz, 1991; Bargmann et al., 1993; Zechman and Labows, 1985). Nonetheless, the detection of harmful bacteria is not an innate response (Zhang et al., 2005). *C. elegans* can avoid pathogenic bacteria as a learned behaviour due to association with illness or repellent odour (Zhang et al., 2005). Therefore *C. elegans* is able to modify its behaviour according to changes in its environment. This could be used to model important aspects of conditioned taste aversion described in mammals (Boakes et al., 1997; Revusky and Parker, 1976).

In the laboratory, *C. elegans,* the Bristol strain (N2), are maintained in Petri-dishes containing Nematode Growth Medium (NGM), which are seeded with *E. coli* OP50 as a source of food. Furthermore, *C. elegans* is a self-fertilised organism, capable of producing about 7 eggs/hr, in which all off-spring are genetically identical (Brenner, 2003). *C. elegans* produces a small percentage of males (~0.2%) due to a miss-segregation of the sexual chromosomes, as *C. elegans* has X chromosome only (Brenner, 2003). Males have a distinct morphology to hermaphrodites, as they possess a specialised tails required for mating.

1.2.1 The life cycle of *C. elegans*

C. elegans is a hermaphrodite, which reproduces by self-fertilisation or fertilisation by males. In the presence of optimum conditions, the worm develops from a hatched egg to adulthood in 4 larval stages (L1- L4, *Figure 1.1*). This developmental process is defined by moults. Once they reached adult stage, wild type worms can live for additional 10-15 days (Wood, 1988).

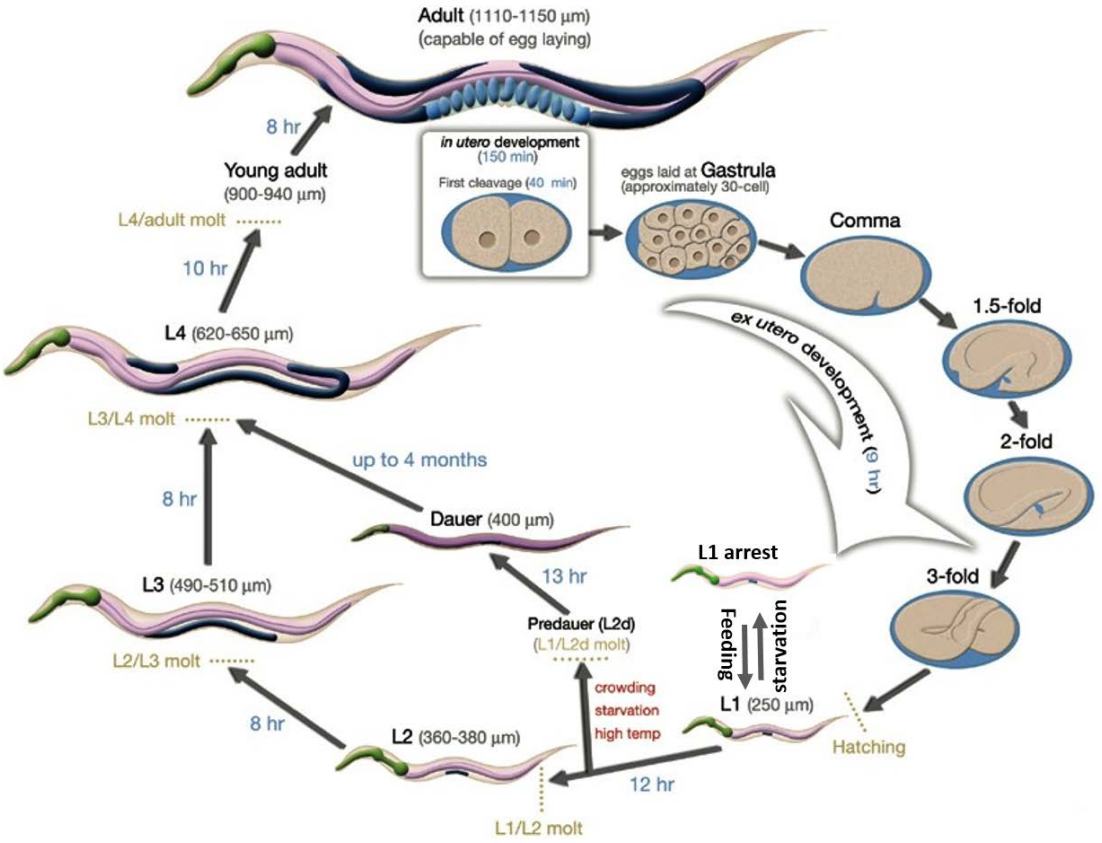


Figure 1.1: The life cycle of C. elegans at 20°C. *The development of C. elegans from eggs to adult stages undergoes four larval stages (L1-L4). The worm reaches adult fertile stage in 3 days at 20°C. In harsh conditions, the worm enters a dauer stage from L2 stage, which is an inactive stage to promote survival under unfavourable conditions. The image was obtained from wormatlas.org.*

1.2.2 Environmental regulation of development

The development of *C. elegans* is influenced by temperature and the availability of food. For instance, arrested development is observed in the absence of food (Golden and Riddle, 1984; Klass, 1977).

1.2.2.1 L1 arrest

The nervous system of the worm develops during embryogenesis (Sulston et al., 1983), however, embryonic worms are unable to sense external environmental cues due to the presence of the impermeable eggshell (Sengupta, 2007). Once hatched, worms are able to sense and respond to external stimuli.

The first larvae stage (L1) worms that hatch in the absence of food enter a developmental stage known as L1 arrest (Johnson et al., 1984). In this stage, worms do not continue their development and can survive for weeks without undergoing morphological changes (Baugh, 2013; Johnson et al., 1984). Nonetheless, L1 arrested worms undergo metabolic adaptation and increased stress resistance (Baugh, 2013; Johnson et al., 1984). Once L1 arrested worms encounter food, they emerge from their developmental arrest and continue their development by directly entering the second larvae stage (L2) (*Figure 1.1*).

Unlike embryonic worms, which feeds on maternal stores, larvae feeding is controlled by the availability of nutrients. L1 arrest is known as starvation-induced quiescence (Baugh, 2013). Thus, the environmental cue that triggers L1 developmental arrest is the prolonged absence of food cues, which leads to starvation (Baugh, 2013).

1.2.2.2 Dauer arrest

In the presence of abundant food, newly hatched worms can develop to gravid adults by continuing to grow and pass through the four larvae stages (L1- L4, *Figure 1.1*). However, under stressful conditions and inadequacy of food, late stage L1 worms enter an alternative stage known as dauer and can survive in this state for up to four months (Hu, 2007). In this stage, worms do not reproduce or feed as this moult generates a cuticle that surrounds the worm's body and mouth, allowing worms to survive harsh conditions (Hu, 2007). When food becomes abundant, worms emerge from the dauer and continue their development by entering directly the fourth larval stage.

The external environmental conditions that drives the first larval worms into a dauer stage are crowding, high temperature (above 27°C) and limited food availability. *C. elegans* constitutively release a pheromone known as daumone, which measures the levels of food and population density (Albert et al., 1981).

Dauer formation is an independent physiological process to L1 arrest, since dauer formation is an alternative stage to third larva stage in response to limited food, high temperature and crowding (Hu, 2007). Furthermore, population density is sensed by daumone, which is a mixture of ascarosides and it is the main inducer of dauer formation (Golden and Riddle, 1982, 1984; Ludewig and Schroeder, 2013).

C. elegans hermaphrodite has 302 neurones, 60 of which are ciliated sensory cells, located in the worm's head and responds to different environmental conditions. The amphid sensory neurones ASI, ASG and ADF (see section 1.6.1 below) have been implicated in dauer formation, since laser ablation of these neurones in L1 stage transiently triggered dauer formation, even in the presence of food (Bargmann and Horvitz, 1991). The additional laser ablation of ASJ caused worms to permanently enter the dauer stage. Therefore, these neurones promote development and dauer recovery, since hormones that promote growth are secreted by these neurones. DAF-7, a TGF-β-related peptide produced by ASI neurones, inhibits dauer formation and promotes development by acting as a neuroendocrine signal (Ren et al., 1996; Schackwitz et al., 1996). Furthermore, the regulation of insulin plays a major role in dauer formation through DAF-2, an insulin-related ligand produced by ADF and ASG (Li et al., 2003; Pierce et al., 2001). Moreover, DAF-28 , expressed in ASI and ASJ, and serotonin, released by ADF, have been reported to prevent dauer formation (Sze et al., 2000). Overall, the amphid sensory neurones receive sensory input from external cues and produce hormones that regulate growth and developmental choice.

1.2.2.3 Temperature regulation of development

In the wild, *C. elegans* is found in decaying plant and compost heaps environments with great differences in local temperature gradients (Andersen et al., 2012; Kiontke et al., 2011). The differences in temperature allow *C. elegans* to survive and reproduce in a wide range of temperatures between 12-25°C in the wild (Anderson et al., 2011; Schulenburg and Félix, 2017; Tattersall et al., 2012).

In the laboratory, the temperature of cultivation affects reproduction, growth and life span of *C. elegans*. The maximum egg production was recorded at 20°C, whereas the lowest was reported at 25°C or below 10°C (Klass, 1977). The maximum life span was recorded at 10°C, while the shortest reported at 25°C or above (Klass, 1977). The life cycle (from

eggs to fertile adult stage) is 2 days at 25°C, 3 days at 20°C and a week at 15°C. Overall, high temperatures reduce the life span of worms, while low temperatures increase it.

AFD ciliated sensory neurones are the main neurones that detect temperature fluctuations and levels of CO_2 (Bretscher et al., 2008). Other chemosensory neurones, such as ASI, ASJ and AWC have been implicated in thermosensory, play a minor role in regulating temperature in *C. elegans* (Goodman and Sengupta, 2017).

1.3 Advantages of *C. elegans* as a model organism

In contrast to complex systems such as mammals, *C. elegans* has a simple nervous system with a defined connectome and mapped nervous system (White et al., 1986). Nonetheless, new studies have been working on re-wiring the worm's connectome using new technologies (Bumbarger et al., 2013; Varshney et al., 2011). Some of the functional synapses were recently discovered (Bhatla and Horvitz, 2015). As mentioned previously (see section 1.1 above), *C. elegans* can respond to wide array of sensory modalities (Bono and Villu Maricq, 2005; Hobert, 2003). Furthermore, they can perform a range of innate behaviours such as chemotaxis to food sources, foraging and feeding. In addition, they are able to modify their behaviours in response to external or internal stimulus (acquired behaviour - plasticity). For instance, worms avoided an attractant such as salt when it was presented in the absence of food for prolonged hours; worms migrated at a great efficiency following paired presentation of an olfactory cue (butanone) and food (Saeki et al., 2001; Torayama et al., 2007). Overall, *C. elegans* provide a powerful model for the delineation of sensory- dependent behaviour in both innate or learnt behaviours (see section 1.1.).

1.4 The nervous system of *C. elegans*

The nervous system of the hermaphrodite *C. elegans* has 302 neurones, whereas males have 385 neurones (Sulston and Horvitz, 1977; Sulston et al., 1980). The pharyngeal nervous system (PNS) is isolated from the somatic nervous system (CNS) by basal membrane and both systems are connected by a pair of RIP interneurons (*Figure 1.2*) (White et al., 1986). *C. elegans* has an invariant cell lineage, about 6000 chemical synapses, 1400 neuromuscular junctions and 890 electrical synapses (White et al., 1986).

The majority of neurones have their cell bodies localised in a region called the nerve ring in the head (White et al., 1986), whereas the rest of neuronal cell bodies are found in the ventral cord and the tail ganglia.

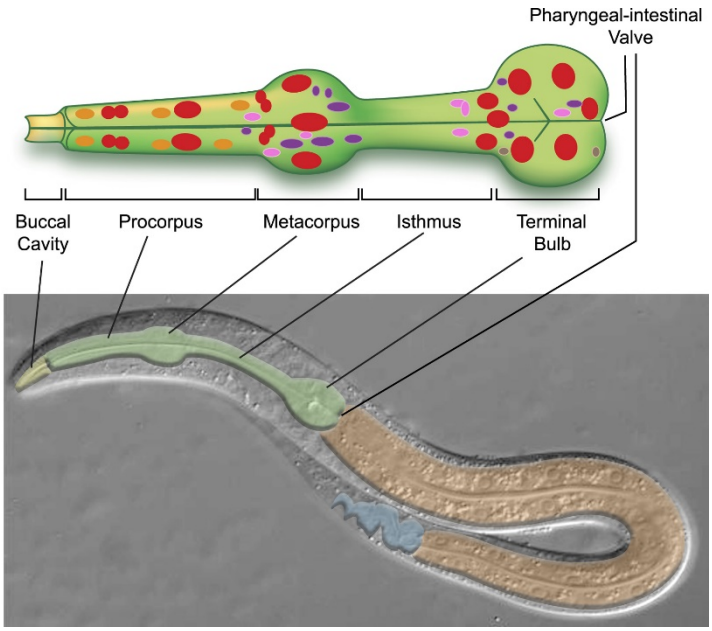


Figure 1.2: The pharynx of the C. elegans. *The pharynx of C. elegans consists of buccal cavity, anterior gut (pharynx, green colour), posterior gut (intestine, orange colour) and hindgut (blue colour). There is a pharyngeal-intestinal valve (not shown) that connects the pharynx and intestine. The pharyngeal nervous system is separated from the central nervous system by a basal membrane (not shown). The close-up panel shows the nuclei within the pharynx. The purple colour is neurones; pink colour is marginal cells and brown glands; red colour is muscles and orange colour is epithelial. Image was obtained from Mango, S.E. The C. elegans pharynx: a model for organogenesis (January 22, 2007), WormBook, ed. The C. elegans Research Community, WormBook.*

1.4.1 Organization of synaptic transmission and modulation

The nervous system of *C. elegans* shares similar key features to the neurochemistry of the nervous system of the higher organisms. There are sets of genes encoding key components of the mammalian nervous system are found in *C. elegans* (Hobert, 2013). *C. elegans* can transmit information through the classical neurotransmission (small molecule)

and neuropeptides. Moreover, the nervous system has neurotransmitters such as acetylcholine, dopamine, 5-Hydroxytryptamine (5-HT, or serotonin) and gamma-aminobutyric acid (GABA). *C. elegans* does not have adrenaline or noradrenaline, however, tyramine and octopamine are considered invertebrate orthologues of adrenergic signalling (Alkema et al., 2005; Roeder et al., 2003; Sanyal et al., 2004).

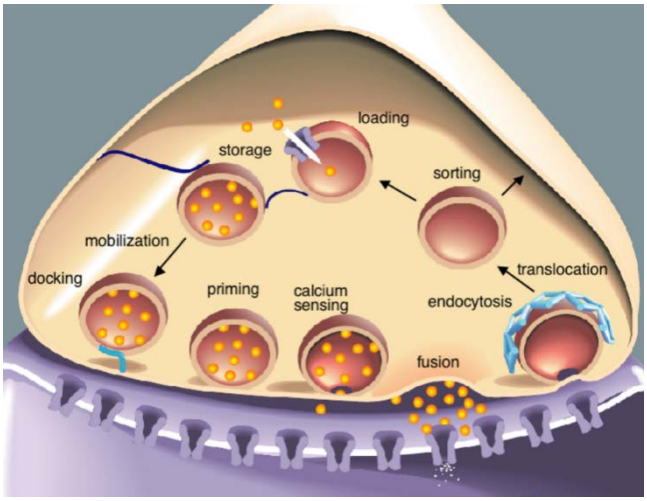
1.4.1.1 Small molecule neurotransmission

During nerve transmission, exocytosis of neurotransmitters occurs by transporting pre-stored synaptic vesicles to the synaptic cleft. These vesicles undergo a process of events before they dock with the pre-synaptic plasma membrane. These molecular events include translocation, sorting, loading of neurotransmitters into vesicles, storage and mobilisation of synaptic vesicles. The docked vesicles undergo priming, a process in which the vesicles become fusion competent (*Figure 1.3A*). During the priming event, SNARE (soluble NSF attachment protein receptors) proteins on the plasma membrane, syntaxin (*unc-64*) and SNAP-25 (*ric-4*), collectively mediate membrane fusion by bringing the vesicles closer to the plasma membrane by forming a tight complex membrane (Chen and Scheller, 2001). The SNARE proteins form α-helical bundle structure called SNARE complex (Sutton et al., 1998). The presynaptic UNC-13, is an orthologue of the mammalian MUNC-13-1, is a critical priming factor, since *unc-13* mutants showed defects in neurotransmission (Betz et al., 1997; Brenner, 2003; Hobert, 2013; Rose and Baillie, 1980). Syntaxin has a closed configuration, which prevents the formation of SNARE complex (Richmond et al., 2001). UNC-13 binds to the N-terminal of the syntaxin, which allows the open configuration of syntaxin, thus facilitating the formation of SNARE complex (Richmond et al., 2001).

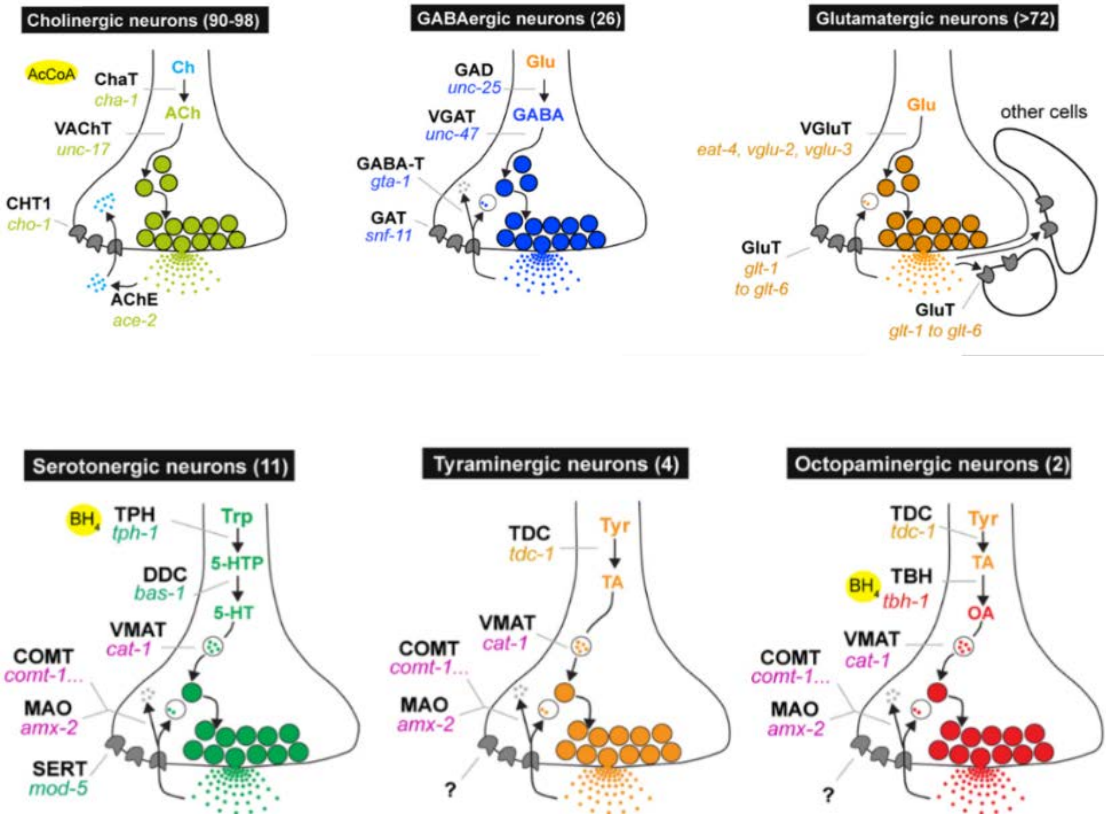
The primed vesicles are clamped at the neurotransmitter release site in a hemi-fused state that halt the vesicles fusion until an increase in the intracellular calcium triggers the release of the neurotransmitter (Richmond et al., 2001). Neuronal depolarisation allows the opening of the voltage-gated calcium channel, resulting in a rise in intracellular Ca^{2+}. This rise in the intercellular Ca^{2+} is sensed by synaptotagmin, a synaptic vesicle protein (Nonet et al., 1997). The calcium channel subunits that contribute to the calcium signalling in *C. elegans* at the synapse include EGL-19 (L-type calcium α-subunit), UNC-2 (N-type calcium subunit) (Richmond et al., 2001), UNC-36, which is an auxiliary of subunit of the UNC-2 (Schafer et al., 1996) and NCA-1 and NCA-2 (α-type calcium subunit) (Humphrey et al., 2007; Yeh et al., 2008).

The neurotransmitter uptake and/or degradation occurs following release to allow regulation of the signalling strength. The reuptake and release of neurotransmitter is modulated in *C. elegans* by the SLC transporter family, which are orthologues of the mammalian SLC family (He et al., 2009). Figure 1.3 below shows the biosynthesis, release, uptake and transporters for neurotransmitters in *C. elegans.* For example, *mod-5* (SLC6 family) 5-HT, *cho-1* (SLC5 family) which transports choline, the by-product of acetylcholine, *dat-1* (SLC6 family) for dopamine, and 6 transporter genes, the *glt* gene (SLC1 family) for the reuptake of glutamate (Hobert, 2013). Most transporters are found expressed where their neurotransmitter is made, with the exception of glutamate transporters, which are expressed in multiple sites away from glutamate-producing cells such as muscle tissues in *C.elegans* (Mano et al., 2007).

A


loading
storage
sorting
mobilization
translocation
docking
priming
calcium sensing
endocytosis
fusion

B


Cholinergic neurons (90-98)
AcCoA
ChaT
cha-1
Ch
ACh
VAChT
unc-17
CHT1
cho-1
AChE
ace-2
GABAergic neurons (26)
GAD
unc-25
Glu
VGAT
unc-47
GABA
GABA-T
gta-1
GAT
snf-11
Glutamatergic neurons (>72)
Glu
other cells
VGluT
eat-4, vglu-2, vglu-3
GluT
glt-1
to glt-6
GluT
glt-1 to glt-6
Serotonergic neurons (11)
BH4
TPH
tph-1
Trp
5-HTP
DDC
bas-1
5-HT
VMAT
cat-1
COMT
comt-1...
MAO
amx-2
SERT
mod-5
Tyraminergic neurons (4)
TDC
tdc-1
Tyr
TA
VMAT
cat-1
COMT
comt-1...
MAO
amx-2
?
Octopaminergic neurons (2)
TDC
tdc-1
Tyr
TA
BH4
TBH
tbh-1
OA
VMAT
cat-1
COMT
comt-1...
MAO
amx-2
?

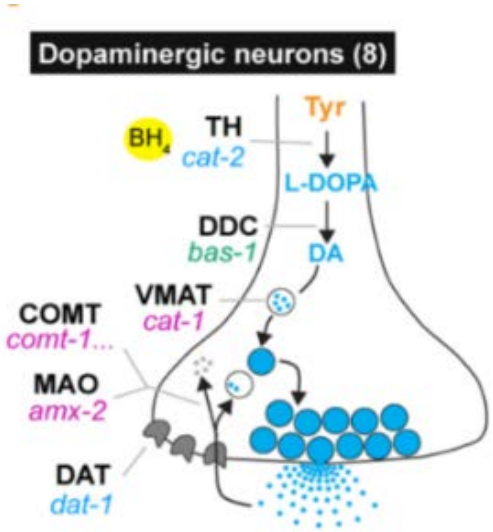


Figure 1.3: A schematic representation of neurotransmitter pathways in C. elegans.

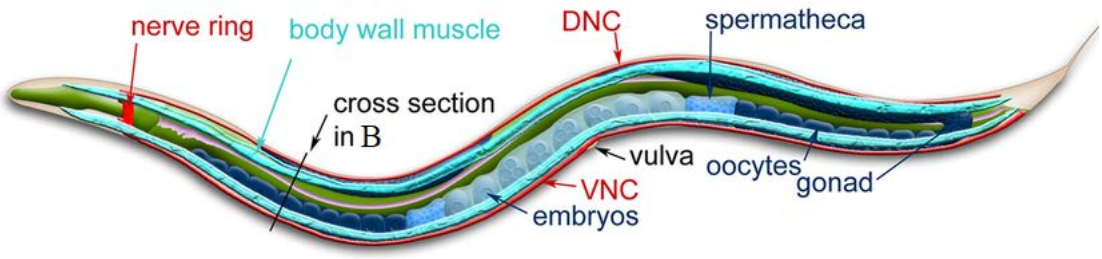
A) The cycle of synaptic vesicles involves loading and storing of neurotransmitters into the vesicles, before mobilising and translocating the vesicles to the nerve terminal plasma membrane, where they dock to the active zone. A further step involves priming of the docked vesicles, in which vesicles become fusion competent. An increase in intracellular Ca^{2+} binds to calcium sensors upon neuronal excitation, which triggers vesicle fusion and neurotransmitter release into the synaptic cleft. As a result, a neurotransmitter binds and activates receptors in the post-synaptic neurones. Finally, vesicle members and proteins are retrieved by clathrin-mediated endocytosis and recycled back to begin another cycle of synaptic vesicle transmission. The diagram was obtained from (Richmond, 2005). B). Each cartoon depicts the steps of neurotransmitter synthesis, vesicular uptake and reuptake or degradation. The number in brackets represents the number of neurones expressing each neurotransmitter. Diagram was obtained from (Hobert, 2013).

1.4.1.2 Neuropeptides transmission in *C. elegans*

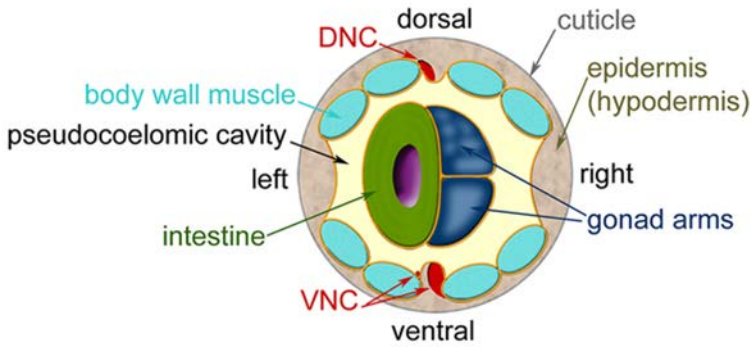
In addition to synaptic (classical) neurotransmission, neuropeptides are major transmitters found in *C. elegans.* However, unlike classical neurotransmitters, they modulate volume transmission thus, their release and signalling is not restrained to actives zones or to adjacent synapses (Salio et al., 2006). Furthermore, neuropeptides are found stored in the

terminal nerve and coexist with low molecular weight neurotransmitters in neurones (Salio et al., 2006). This variation in storage and coexistence permits both slow and fast transmission to occur (Salio et al., 2006). In *C. elegans,* pseudocoelom, a fluid-filled body cavity found inside the external body wall of *C. elegans* that bathes the internal organs (Wood, 1988), can mediate distal diffusion to allow indirect signalling. There are 3 pairs of coelomocytes (*Figure 1.4*), in which one is positioned at the dorsal site in the posterior side of the body, one is on the ventral side close to the head and one is in close proximity from the released peptides. The role of the coelomocytes are to detect released peptides, act as reporters of pseudocoelom contents and immune role (Fares and Greenwald, 2001; Sieburth et al., 2007).

A



B



C

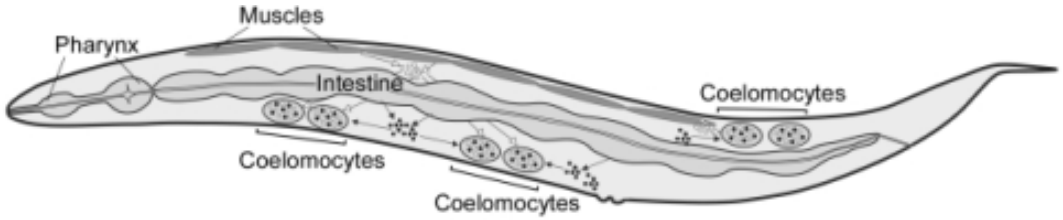


Figure 1.4: Pseudocoelom and coelomocytes in C. elegans hermaphrodite. *A) A representation of the major anatomical features of the C. elegans hermaphrodite. B) A cross section of (A) showing the pseudocoelomic cavity. The Image was obtained from wormatlas.org, based on (Altun et al. 2002-2015) and (Corsi et al., 2015). C) The location of coelomocytes in C. elegans. There are 3 pairs of coelomocytes in the pseuodocelomic cavity. One pair is located close to the head, while another pair is found anterior to the vulva, close to the mid-body, and the third pair is found in the posterior part of the body. Image was obtained from (Schwartz et al., 2010).*

1.4.1.2.1 Neuropeptide-encoding genes

In the *C. elegans* genome, there are 122 genes encoding more than 250 neuropeptides (Holden-Dye and Walker, 2013; Li and Kim, 2010). They are divided into three categories: 31 genes encode FMFR-amide-related peptides (FLP), 40 genes encode insulin-like neuropeptide (INS) and 51 genes encode neuropeptide-like protein (NLP), also known as non-insulin, non-FMRF-amide related neuropeptide which is a heterogeneous family (Li and Kim, 2010). FLPs are expressed in all neuronal cells and in 150 out of the 302 neurones in *C. elegans*, whereas INSs are mainly restricted to amphid neurones. On the other hand, NLPs are found to be expressed in the nervous system (pharyngeal and amphid neurones) and outside the nervous system, where they can act as anti-microbial agents (Couillault et al., 2004; Li and Kim, 2008).

1.4.1.2.2 Neuropeptides biosynthesis and processing

In *C. elegans,* the production mechanism of neuropeptides is similar to that found in mammals. The neuropeptide genes produce large inactive form of neuropeptides known as propeptides or proproteins (Li and Kim, 2010). These propeptides contain more than one peptide. Their processing takes place in dense core vesicles, where they are stored along with their processing enzymes and transported along the axon (Li and Kim, 2010).

Proprotein convertases are the enzymes responsible for the cleavage of inactive polypeptides such as propeptides. In mammals, the first proprotein convertase was the furin enzyme, which is encoded by the *fur* gene (Fuller et al., 1989). In *C. elegans,* the proprotein convertase is known as the kex2/subtilisin-like proprotein convertases (kpc). They consist of 4 genes encoding enzymes: kpc-1; egl-3/kpc2; aex-5/kpc-3 and bli-4/kpc-

4, which are all involved in the cleavage of the propeptides (Husson et al., 2006). The main domains of the KPCs are: 1) the subtilisin-like catalytic domain containing the endopeptidase function and 2) the P domain, which is a unique to the KPCs and a highly conserved sequence. This domain is essential for folding and maintaining of the catalytic site. In addition, the P domain plays a critical role in regulating calcium dependence and acidic pH-dependence of the enzymatic activity (Zhou et al., 1998).

Following the cleavage by the proprotein convertase, further cleavage occurs at the C-terminal basic residue of propeptides by carboxypeptidase (Li and Kim, 2010). Subsequently, most neuropeptides are modified by a process known as amidation.

1.4.1.2.3 Neuropeptide release

Neuropeptides are released by exocytosis from UNC-31 (the homologue of the mammalian Ca^{2+} activated proteins for secretin (CAPS)) and dense core vesicles (Ann et al., 1997; Grishanin et al., 2004). The release of neuropeptides from dense core vesicles depends on an increase in intracellular calcium throughout the nerve terminal (Salio et al., 2006). Unlike the mammalian system where CAPS have been found to regulate glutamate vesicular-mediated release (Jockusch et al., 2007), *unc-31* did not play a role in the regulation of vesicular-mediated neurotransmission in *C. elegans* (Jockusch et al., 2007). The process of neuropeptide release from dense core vesicles involves the same molecular core functions as of vesicular transmission (docking, priming and Ca^{2+} dependence release).

1.4.1.3 Postsynaptic receptor transduction

The released neurotransmitter binds to its receptor in the targeted neuron post-synaptically. Ligand-gated ion channels (LGIC) and G-protein coupled receptors (GPCRs) are the main types of postsynaptic receptors. Fast transmission involves the activity of LGIC receptors, which can be either inhibitory or excitatory receptors, hence leading to either hyperpolarisation or depolarisation, respectively. Upon the binding of the ligand to LGIC transmembrane receptors, ions (e.g. Na^+, K^+, Ca^{2+} and Cl^-) are allowed to pass through the membrane. Excitatory events are associated with the binding of cation channels such as ionotropic glutamate receptors (iGluRs) and acetylcholine nicotinic receptors (nAChRs) (Brockie, 2006; McKay et al., 2004). However, inhibitory events occur by activating ligand-gated chloride channels such as $GABA_A$ receptors or glutamate-gated chloride channels (Brockie, 2006; Schofield et al., 1987). On the other hand, a slow acting

neurotransmitter act by binding to GPCR, in which neurotransmitters can act at more than one site and affect a number of neurones. At synaptic sites, neurotransmitters are degraded or taken up rapidly. Nonetheless, neurotransmitters can avoid degradation by acting on non-junctional receptors if the released neurotransmitter has not formed junctional complexes with other neurones (Sarter et al., 2009). For instance, 5-HT is released in response to food from the pharyngeal neuron, NSM, to regulate locomotion by activating MOD-1, which is found in the nerve ring and hence has no direct contact with NSM (Flavell et al., 2013). Moreover, 5-HT released from ADF, which is located outside the pharyngeal system, regulates pharyngeal pumping rate by acting at a distance through SER-5 receptor on AVJ (Cunningham et al., 2012).

The neurotransmitter can function as both fast excitatory or inhibitory transmitter and a modulatory transmitter. For example, 5-HT can act as fast neurotransmitter via MOD-1, a serotonin-gated chloride channel, or as modulatory neurotransmitter via metabotropic receptors namely, SER-4, SER-5 and SER-7 (Komuniecki et al., 2004).

1.5 Feeding behaviours in *C. elegans*

Feeding behaviour in *C. elegans* is linked to locomotion and pharyngeal pumping behaviours.

Detection of food cues is critical for feeding behaviour and the process of decision-making. The worm assesses its environment for its suitability for development and survival (see section 1.2.1). This is achieved by recruiting the central nervous system of the worm, which is able to detect the presence and absence of food cues in the environment through their sensory systems. For instance, chemosensory neurones such as ASE detect water-soluble molecules (Bargmann and Horvitz, 1991), volatile chemicals (Bargmann et al., 1993), physical presence of food (Sawin et al., 2000), oxygen and carbon levels (Bretscher et al., 2008; Gray et al., 2004). The detection of food cues in the environment is integrated by the central nervous system to execute the appropriate behavioural response. This is achieved by regulating the two arms of feeding behaviour in *C. elegans*, i.e. locomotory and pharyngeal pumping behaviours. For example in the wild, the larvae of *C. elegans* display a nictation behaviour, in which the worm stands on its tail, so that flying insects can carry them to explore new food sources (Lee et al., 2012). The pharyngeal pumping responds directly to the presence of food by stimulating pumping rate or in the

absence of food by cessation of the pumping rate. The sub-behaviours of feeding (the locomotion and pharynx) are discussed in this section.

1.5.1 The fundamentals of *C. elegans* locomotion behaviour

The locomotion behaviour is critical for the worm's survival as it allows worms to forage for food and avoid noxious environments (Gray et al., 2005; Hilliard et al., 2002; Milward et al., 2011). The presence and absence of food regulates the worm's locomotory behaviour (Flavell et al., 2013; Sawin et al., 2000). The worm crawls on solid surfaces, whereas they thrash in liquid medium. On solid surfaces, the locomotory behaviour is characterised by a forward and backward sinusoidal propulsion, where a sequence of waves travel along the worm's body (Bono and Villu Maricq, 2005). The worm lay on either sides (left or right), and thus allowing the waves to form by contraction and relaxation of the dorsal and ventral longitudinal body wall muscles (Bono and Villu Maricq, 2005). Unlike the slow crawling behaviour observed on solid surfaces, thrashing in liquid comprises much faster wavelength undulations (Cohen and Sanders, 2014). The dorsal and ventral longitudinal muscles consist of 95 cells which are controlled by excitatory signals at the cholinergic neuromuscular junction (NMJ) and inhibitory signals at the GABAergic NMJ (De Jager et al., 1998).

The forward movement is controlled by excitatory and inhibitory dorsal neurones (DB, DD) and ventral neurones (VB, VD). DA and DD neurones are excitatory, whereas VA and VD neurones are inhibitory and control backward movement (Figure 1.5). These neurones are interconnected, to allow the execution of a series of coordinated movements in a particular direction. For instance, when particular regions of the ventral body wall muscle contract via cholinergic motor neurones (e.g. VA), the opposite dorsal body wall muscle relaxes via GABAergic motor neurones (De Jager et al., 1998). Furthermore, laser ablation studies found AVB and PVC interneurons abolish forward movement and posterior touch avoidance, whereas AVA and AVD interneurons abolished anterior touch avoidance and reversal movement (De Jager et al., 1998; White et al., 1986). These interneurons were named command interneurons because they control forward and backward movements (*Figure 1.5*).

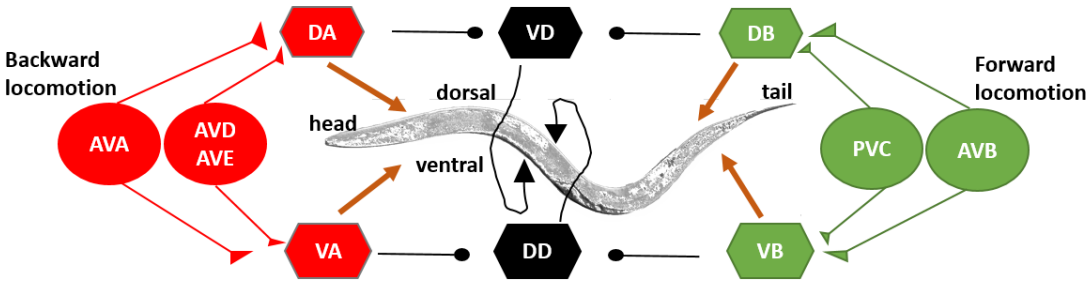


Figure 1.5: Neuronal control of C. elegans locomotion. *The dorsal and ventral body wall muscles receive excitatory signals (orange arrows) from cholinergic motor neurones (VA, VB, DB and DA) and inhibitory signals (black arrows) from GABAergic motor neurones (DD and VD). The motor neurones VA and DA (red hexagon) initiate backward locomotion, while VB and DB (green hexagon) initiate forward locomotion. The command interneurons (AVA, AVD, AVE, PVC and AVB) coordinate forward and backward locomotion by providing information to the motor neurones. Adapted from Chalfie et al. 1988 and De-Bono and Mariq, 2005.*

1.5.1.1 Neurotransmitters regulation of core C. elegans locomotion

Acetylcholine and GABA are two neurotransmitters which are critical for *C. elegans* locomotion. Acetylcholine is a major excitatory neurotransmitter at the NMJ in *C. elegans.* Additionally, it is important for development, since a null mutant for synaptic vesicle acetylcholine transporter (*unc-17)* is lethal (Abizaid et al., 2006). Acetylcholine is a vital neurotransmitter in initiating and maintaining coordinated, sinusoidal waves for both forward and backward movement contractions. Furthermore, it is involved in the oscillator circuit by controlling the rate of wave initiation. It plays a role in movement by coordinating body bends, since hypomorph *unc-17* mutants showed uncoordinated phenotype (Brenner, 2003; Rand and Russell, 1984).

GABA is a major inhibitory neurotransmitter at the neuro-muscular junctions, thus counteracts the actions of acetylcholine. GABAergic signalling detects environmental cues by chemosensory neurones by regulating head swings (Jorgensen, 2005). This was investigated by the ablation of four GABAergic RME neurones, which showed a significant increase in head swinging and thus suggested that GABAergic signalling limits the number of head swinging during exploratory behaviour.

1.5.1.2 Modulation of locomotion in the presence of food

When on a bacterial lawn, *C. elegans* locomotion is described by two major states roaming and dwelling (Ben Arous et al., 2009). The roaming state is characterised by increased speed and low frequency of turns that allows the worm to explore the food lawn. In a dwelling state, the worm displays a reduced speed and high frequency of turns (*Figure 1.6*). The dwelling state allows the worm to remain within a small area of food lawn. Dwelling and roaming states are considered as decision-making strategy, where the worm explores the food lawn and the quality of food (Ben Arous et al., 2009). They reported that worms spent more time dwelling when the food was in high concentrations and high quality (Ben Arous et al., 2009). Dwelling was not observed in the absence of food; however, worms spent more time roaming in the presence of low food quality (Ben Arous et al., 2009).

Moreover, further results have shown that dwelling is induced by internal nutritional cue (Gruninger et al., 2008). This was investigated by growing the worms in aztreonam-treated bacteria (*E. coli* OP50), which is an antibiotic that interferes with bacterial cell division and results in long chains of undivided bacteria (50µm) (Gruninger et al., 2008). Aztreonam-treated bacteria had a low nutritional value for the worms, since wild-type worms feeding on aztreonam-treated bacteria spent 75% of their time roaming compared to wild-type worms feeding on *E. coli* OP50. Even though the bacterial concentration was high and worms could receive both mechanosensory and chemosensory cues from food similar to worms growing in regular *E. coli* OP50 (Gruninger et al., 2008). Furthermore, defective cilium mutants, *che-2* - which have altered sensory perception and do not roam on food - have displayed normal locomotory behaviour when stimulated. When these mutants were grown in aztreonam-treated bacteria, they showed increased roaming time compared to the same mutants grown in regular OP50 (Ben Arous et al., 2009). Furthermore, *che-36* mutants - which are defective in AWC olfactory neurones - spent more time roaming relative to wild-type worms in the presence of regular food lawn (Ben Arous et al., 2009).

1.5.1.3 Modulation of locomotion in the absence of food

In the absence of food, the worm exhibits an exploratory behaviour known as Local Area Search (LAS) or Area-Restricted Search (ARS, *Figure 1.6*) (Gray et al., 2005; Hills, 2004). This behaviour is observed following immediate removal from food also known as early

phase (Gray et al., 2005). The LAS behaviour is characterised by increased turns and reversals displayed within the first 15 min of removal from food. Worms exhibit this behaviour to explore a previous area where they encountered food, in which AWC neurones seemed to be required for this food-seeking behaviour (Chalasani et al., 2007). Worms switch from ARS to dispersal behaviour, which is observed following prolonged food deprivation (Gray et al., 2005; Hills, 2004). Gray et al. 2005 found that worms switch from ARS or LAS behaviour to dispersal after 12 minutes (*Figure 1.6*). It involves forward runs with significant reduction in reversals, allowing worms to explore a large area for food rather than being confined to a small area as seen in LAS behaviour.

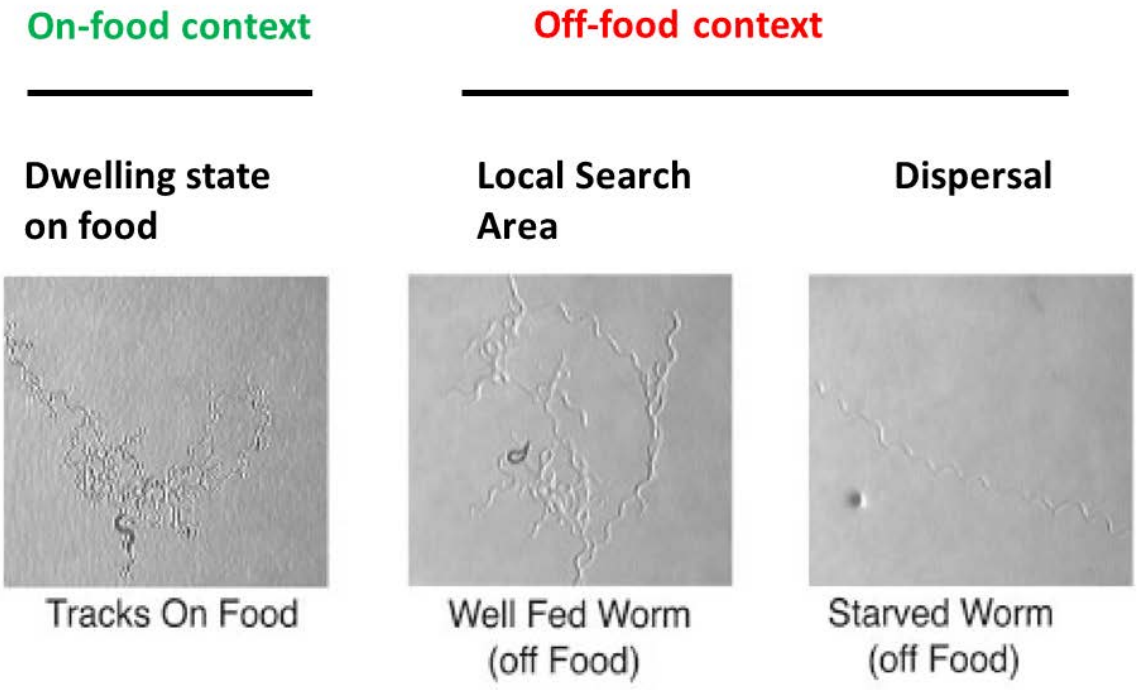


Figure 1.6: Locomotory behaviour in response to food availability. *The tracks of an individual worm on food and on agar (without food) after 1 (well fed worm – the image in the middle) and 12 minutes (starved worm – the right-hand side image) from removal from food. In the presence of food, worms which were in a dwelling state were characterised by increased turns and reduced speed. After 1 min of removal from food, worms displayed LAS, where there were increased turns and reversals. LAS behaviour permits worms to search for food in a restricted area. After prolonged food deprivation, worms displayed long forward movement known as dispersal. Dispersal is favoured in food-deprived worms to allow worms to forage for food in distant areas. The diagram was obtained from (Gray et al., 2005).*

1.5.1.4 Modulation of locomotion as the worm undergoes chemotaxis to food-related cues

In contrast to on-food or off-food locomotory behaviours, worms show an exquisite ability to detect distal food sources by olfaction through the execution of efficient chemotaxis towards food cues (Bargmann and Horvitz, 1991; Bargmann et al., 1993; Ward, 1973). These exploratory behaviours execute directed locomotion, depending on the modality of worm navigation, namely chemotaxis, thermotaxis or oerotaxis to favourable conditions. These behaviours are characterised by forward movement interrupted by transient backward movement or head turning, which allows change of direction. This behaviour is known as random-biased walk, which was first described in bacterial chemotaxis. Gray and colleagues (2005) reported that reversals are defined by a number of head swings before the worm reorients and changes direction. The largest reorientation in direction is generated in a sharp omega turn, as the worm's turn event resembles the Greek letter Ω. A pirouette is an event marked by sharp reversals; however, frequency of pirouette depends on the presence of an attractant cue or food. An increase in pirouette frequency is observed in the absence of food or attractant, but decreases in favourable environmental conditions (Pierce-Shimomura et al., 1999).

In addition to the use of pirouettes, a weathervane or klinotaxis strategy is observed as the worm navigates towards a spatial gradient of an attractant (*Figure 1.7*) (Iino and Yoshida, 2009; Yoshida et al., 2012). Weathervane strategy is characterised by a gradual curved forward locomotion towards a higher concentration of an attractant such as salt or isoamyl alcohol. In contrast to weathervane, pirouette is observed when the worm senses a decrease in the concentration of an attractant (*Figure 1.7*), which results in the worm choosing a random forward direction (Yoshida et al., 2012).

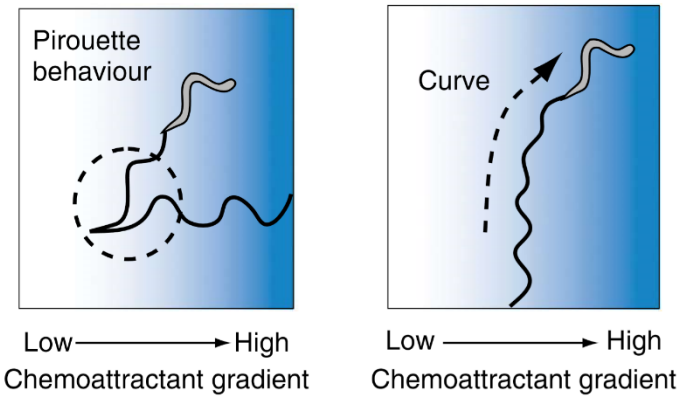


Figure 1.7: A schematic representation of pirouette and weathervane (curve) behaviour. *The blue shading represents the concentration gradient of a chemoattractant. Dotted circle represents a pirouette behaviour which is characterised by reversal and omega-turn. Pirouette behaviour is favoured when the worm perceives a low concentration of chemoattractant gradient. However, when the worm senses high concentration of chemoattractant, worms switch from pirouette to weathervane behaviour, where the worm curves towards the higher concentration of chemoattractant. The diagram was obtained from (Yoshida et al., 2012).*

1.5.2 The pharynx of *C. elegans*

The nematode worm is a bacterivore organism, in which bacteria is ingested and passed to the intestine through the pharynx. The pharynx is a tube-like, neuromuscular pump found in the head of the worm (*Figure 1.8*). The structure of the pharynx has been reconstructed from serial sections of electronmicrographs (Keane and Avery, 2003). The organ is 100 µm long and 20 µm wide, where its anterior structure is connected to buccal cavity and the posterior end is connected to the intestine. The pharynx is separated from the rest of the body by a basal lamina membrane, which surrounds the entire pharynx. Furthermore, there is another connection between the pharyngeal nervous system and central nervous system through the extra-pharyngeal neurones, RIP (see section 1.4).

The pharynx consists of 34 muscle cells, 9 epithelial cells, 20 neurons, 9 marginal cells and 5 gland cells (Keane and Avery, 2003). There are eight different classes of pharyngeal muscle cells (PM 1-8), which makes the pharynx's 3 main functional

compartments: the corpus, the isthmus and the terminal bulb (Figure 1.8) (Keane and Avery, 2003).

Pumping begins as synchronous contraction and relaxation of the three pharyngeal muscles (corpus, anterior isthmus and terminal blub). Upon contraction of the pharyngeal muscles, the lumen of the pharynx opens allowing the drawing of suspended bacteria in liquid into the pharynx from the external environment (Seymour et al., 1983). Bacteria particles are concentrated in the lumen and the liquid is expelled through the mouth, before transferring bacteria into the intestine (*Figure 1.8*). A second feeding motion, known as isthmus peristalsis, is a posterior moving wave of muscle contractions of the posterior isthmus. This motion is thought to allow the transport of bacterial particles to the terminal bulb and enhances nutritional transfer by facilitating the action of digestive enzymes that break down the bacteria (Avery, 2003).

The corpus and terminal blub can still contract, even if the pharyngeal neurones were laser ablated (Avery and Horvitzt, 1989). This is because *C. elegans* muscles are electrically coupled by gap junctions, which is similar to vertebrates' cardiac muscle cells. The *C. elegans* genome encodes a family of gap junction-forming proteins found in invertebrates, known as innexins (Phelan et al., 1998). *eat-5*, a mutant lacking one of the innexins proteins, exhibits non synchronised contractions of the corpus and terminal blub (Starich et al., 1996).

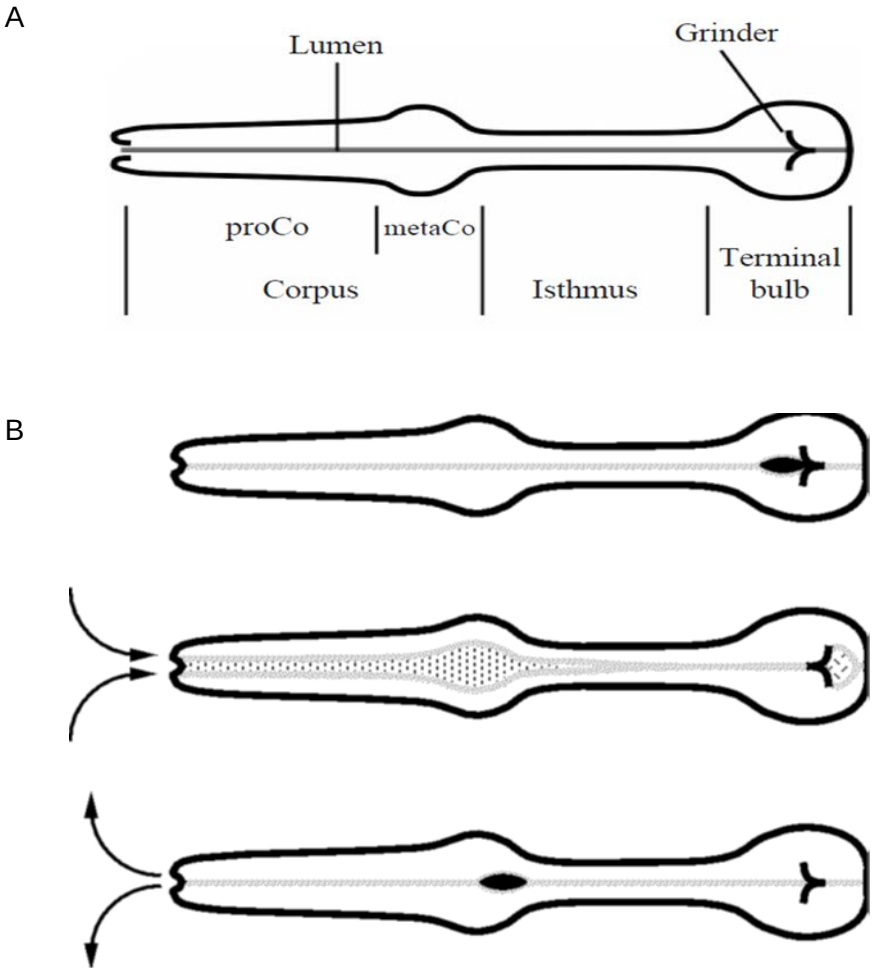


Figure 1.8: The pharynx of C. elegans. A) The anatomy of the pharynx. *A Side view of the pharynx where the main functional regions of the pharynx are shown. B) The cycle of pharyngeal pumping. A single pharyngeal pumping begins with the contraction of corpus, anterior isthmus and terminal blub. This causes the opening of lumen where suspended bacteria in liquid are drawn to the mouth. Simultaneously, the terminal bulb muscles contracts, where the grinder plates inverts, to crush the bacteria and allow passage to the intestine. Relaxation is followed by returning the grinder into its resting position and expels liquid from the corpus and anterior isthmus. The diagrams was obtained from (Avery and Horvitzt, 1989; Avery and You, 2012; Raizen and Avery, 1994)*

1.5.2.1 The pharyngeal nervous system

The pharynx has its own nervous system separated from the somatic nervous system by a basal lamina membrane (see section 1.4 - *Figure 1.2*). The basal lamina has two holes (one on either side), which connect the pharyngeal and extra pharyngeal nervous system via RIP neurones (Avery and Horvitzt, 1989; Franks et al., 2006). RIP neurones are connected to pharyngeal neurones I1 via gap-junctions (Keane and Avery, 2003).

There are 20 pharyngeal neurons, which are divided into 14 different types (Figure 1.9) (Albertson and Thomson, 1976). The pharyngeal neurones can be either single or bilateral (Albertson and Thomson, 1976). The neurons consist of five motor neurons (MC & M1-M5) and six classes of interneurons (I1-I6). There are pharyngeal neurosecretory, motor and sensory neurones (NSM), and motor interneuron (MI) (Avery and Horvitzt, 1989; Keane and Avery, 2003).

There are 3 main classical fast neurotransmitters found in the pharynx, which are 5-HT, glutamate, acetylcholine. Nevertheless, the pharynx expresses receptors for neurotransmitters that are not made locally such as dopamine, DOP-3 receptors, which are expressed in NSM neurones and DOP-4 receptors, which are expressed in I1, I2 and NSM (Sugiura et al., 2005), suggesting that these neurotransmitters can act on the pharynx by volume transmission.

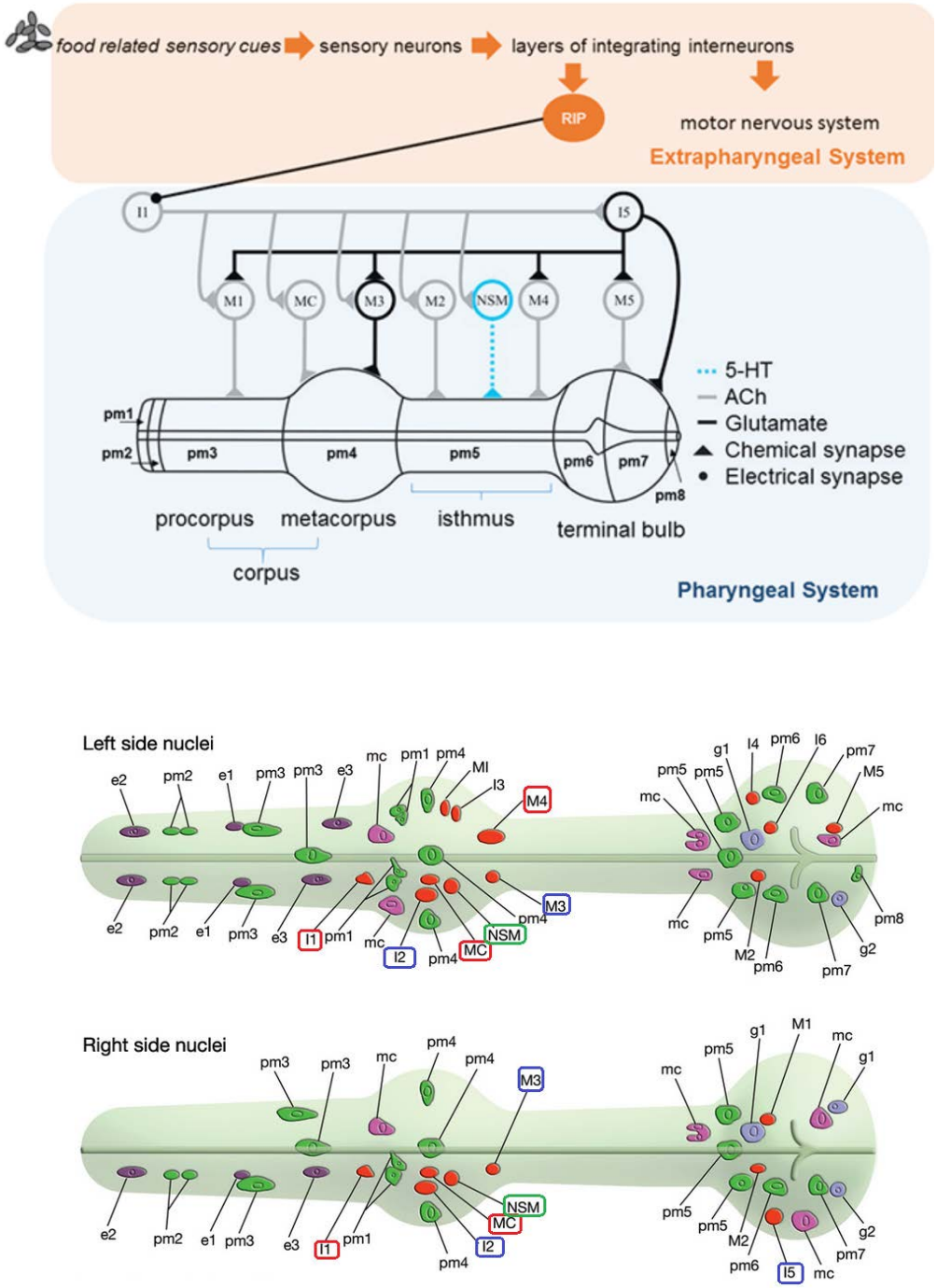


Figure 1.9: The pharyngeal nervous system. *A) The pharynx of C. elegans is composed of a corpus, isthmus and terminal bulb. Its nervous system is isolated from the rest of the body by a basal membrane, and only physically linked to the somatic nervous system by the RIP-I1 gap junction. Additionally, neurotransmission can occur via neurohormone and do not require neuronal connectivity (Flavell et al., 2013). The image was obtained from (Dallière et al., 2017). B) Pharyngeal muscle nuclei indicated in green (pm1-6); neuron nuclei are shown in red (MC, NSM, M1, I1, I2, I3, I4, I5 and*

I6); gland nuclei are shown in lavender (g1 and g2); marginal cell nuclei are shown in fuchsia (mc) and epithelia nuclei are in purple (e1, e2, e3). Some cells are bilateral found left side and right side such as MC neurones or single such as I4. Neurones highlighted in rectangular boxes are neurones known to control pharyngeal pumping in the presence of food. Red boxes: MC, I1 and M4 are cholinergic neurones; green boxes are serotonergic neurones; and M3, I2 and I5 are glutamatergic neurones. The images were obtained from wormatlas.org.

1.5.2.2 Modulation of pharyngeal pumping

1.5.2.2.1 Regulation of pharyngeal pumping in the presence and absence of food

The transparency of *C. elegans* allows a visual count of pumping rate in a given period of time using a dissecting microscope. In the presence of food, worms dwell and constitutively pump at a rate of 250 ppm(pumps/min) (Walker et al., 2002). When the worm is transferred to a non-food plate, they exhibit an immediate cessation in pumping rate in response to mechanosensation-mediated picking (Dallière et al., 2016). After recovery from picking, pumping rate gradually increases but remains low (15-50 ppm) (Dallière et al., 2016). However, there are no measures of pumping as the worm transits from an off-food to an on-food arena.

However, the timing of pumping is directly controlled by the neurones. The motorneurons (M3, M4 and MC) play a critical role in regulating pharyngeal pumping rate. During the core pump, MC has synapses with pm3 and pm4 and mediates fast pumping rate. MC laser-ablated mutants have a starved phenotype due to markedly reduced food intake and pumping rate, which leads to a retarded development (Avery and Horvitzt, 1989; McKay et al., 2004; Raizen and Avery, 1994). The glutamatergic motor neurones, M3 initiates relaxation of the pharynx by controlling pump duration by acting on the pm3 (Raizen and Avery, 1994). Laser-ablation studies showed that the duration of pharyngeal contraction increases, leading to reduced pharyngeal pumping (Raizen et al., 1995). Pump duration is mediated via glutamate actions on the glutamate-gated chloride channel, AVR-15, which is expressed on pm4 and pm5 (Dent et al., 1997). The cholinergic motorneuron, M4, is critical for isthmus peristalsis as M4 synapses through the posterior part of pm5. The growth of M4 laser-ablated worms was reduced, as the worms could not ingest bacteria

due to relaxation of the posterior part of the isthmus and lumen closure (Avery and Horvitzt, 1989).

1.5.2.2.2 Myogenic regulation of pharyngeal pumping

Laser ablation studies identified a clear dependence for physiological regulation from within the pharyngeal nervous system. However, following the total ablation of the pharyngeal nervous system, the pharynx continued to pump slowly (Avery and Horvitzt, 1989). This suggests that the pharynx has a myogenic activity, meaning that the timing of pumping is controlled by the muscle itself and the neurons modulate the excitability of the muscle.

1.5.2.2.3 Extra-pharyngeal regulation of pharyngeal pumping

The pharyngeal neurones I1 are connected to extra-pharyngeal neurones RIP via a pair of bilateral gap junctions, which is thought to be the only connection between the pharyngeal nervous system and the central nervous system (Keane and Avery, 2003). This connection may provide a route to allow detection of food cues by sensory system extrapharyngeally to modulate pumping rate (Dallière et al., 2016). The laser ablation of RIP worms did not affect pharyngeal pumping in the presence of food (Dallière et al., 2016). Furthermore, electron micrograph (EM) reconstruction revealed that I1 interneurons had chemical synapses to MC (Keane and Avery, 2003). I1 is the only pharyngeal neuron that is connected to the extra-pharyngeal nervous system, specifically from the nerve ring. When I1 is laser-ablated, MC can still fire in the presence of food and serotonin (Avery and You, 2012). Therefore, these data suggest that extra-pharyngeal chemosensory system does not have an input in regulating pharyngeal pumping via synaptic connectivity, or they could exert their action through neurohormone signalling (Dallière et al., 2016).

1.5.2.2.4 The neuronal regulation of core pharyngeal pumping behaviour

1.5.2.2.4.1 5-HT regulation of pharyngeal pumping

In wild-type worms, the addition of exogenous 5-HT in the absence of food stimulates pharyngeal pumping to levels comparable to that observed on food (Sze et al., 2000). Furthermore, the addition of 5-HT to food elevates pumping rate by 15% (Avery and Horvitz, 1990; Niacaris, 2003). In the absence of food, the serotonin receptor, SER-1, is responsible for stimulating pharyngeal pumping in response to exogenous 5-HT (Srinivasan et al., 2008).

Mutant analysis studies have shown that *tph-1* mutants display a reduced pumping rate in the presence of food, however, *tph-1* pumps at similar rate to wild-type worms in the absence of food (Sze et al., 2000). 5-HT plays a major role in regulating pharyngeal pumping in the presence of food by controlling the excitation-relaxation cycle of pharyngeal pumping. In the presence of food, 5-HT release results in pharyngeal muscle excitability by acting on MC neurones to stimulate the release of acetylcholine (Avery and Horvitzt, 1989). Simultaneously, 5-HT acts on M3 neurones to increase the release of glutamate, which speeds up the relaxation phase by controlling the pump duration (Avery, 2003). Thus, on food, 5-HT stimulates pharyngeal pumping by increasing the activity of pharyngeal neurones MC and M3, which in turn stimulates the contraction-relaxation cycle of the pharynx, hence the pump duration is shortened (Niacaris, 2003). Furthermore, 5-HT regulates isthmus peristalsis through M4 neurones (Song and Avery 2012). The serotonin receptor, SER-7, which is expressed in MC and M4 neurones, is responsible for the increased pumping rate and isthmus peristalsis (Song and Avery, 2012).

1.5.2.2.4.2 Acetylcholine regulation of pharyngeal pumping

Acetylcholine is crucial for development since it is an excitatory neurotransmitter at the neuromuscular junctions. Out of 6 cholinergic neurones present in the pharynx, MC is the most investigated neuron and it plays an important role in maintaining high pump rate in the presence of food. As mentioned above, 5-HT release in response to food leads to increased pumping rate by stimulating the activity of MC (Niacaris, 2003). This occurs when MC neurones release acetylcholine on nicotinic receptors in the neuromuscular junction at the pharyngeal muscle (Niacaris, 2003; Raizen et al., 1995). M3 neurones release acetylcholine, which activates the nicotinic cholinergic receptor EAT-2 on pm4 (McKay et al., 2004). These observations were further corroborated by mutant analysis investigations which showed a reduced pharyngeal pumping in the presence of food in MC-ablated and *eat-2* mutant worms (McKay et al., 2004; Raizen et al., 1995).

1.5.2.2.4.3 Glutamate regulation of pharyngeal pumping

Glutamate is expressed in the pharyngeal nervous system and plays an important role in sustaining a high pump rate in the presence of food (Serrano-Saiz et al., 2013). The glutamate vesicular transporter, *eat-4* (Lee et al., 1999), displays a low pharyngeal pumping in the presence of food. As mentioned in section 1.5.2.2.4.1, the release of 5-HT in response to food stimulates the release of glutamate from the pharyngeal neurones, M3 (Niacaris, 2003). The action of glutamate in food dependent pumping depends on AVR-15, a glutamate-gated chloride channel, which is expressed on pm4 and pm5 pharyngeal muscles (Dent et al., 1997). As a result, *eat-4* mutants display a reduced pharyngeal pumping in the presence of food (Greer et al., 2008; Lee et al., 2008; Li et al., 2012).

On the other hand, *eat-4* mutants show an increased pharyngeal pumping in the absence of food, suggesting that glutamate signalling is required for a distinct regulation to that observed on food and inhibits pharyngeal pumping in the absence of food (Dallière et al., 2016). Dallière and colleagues (2016) have found that glutamate released from I2 neurones are involved in reducing pharyngeal pumping in the absence of food, since I2-ablated worms displayed an aberrant increase in pump rate in an off-food context (Dallière et al., 2015, unpublished data). Additionally, M3 pharyngeal neuron, which is implicated in maintaining high pump rate in the presence of food, showed to have a selective role in regulating pharyngeal pumping (Dallière et al., 2015, unpublished data). This was further supported by *che-2*, a mutant with non-functional M3 neurones, showed a distinct reduction in pumping rate on-food, whilst maintaining a normal pharyngeal pumping in the absence of food (Dallière et al., 2015, unpublished data).

1.6 The regulation of *C. elegans* feeding behaviour is coupled to input from its environment

As *C. elegans* navigate in its environment, external sensory cues are detected and integrated by its nervous system to evoke appropriate responses. This communication can be from sensory neurones directly to motor neurones or through complex circuits, which can involve other sensory neurones and interneurons before relaying information to the motor neurones (Bono and Villu Maricq, 2005).

Herein, the sensory modalities and the neurones and neurotransmitters implicated in each modality will be reviewed.

1.6.1 An introduction to sensory neurones

The *C. elegans* nervous system detects and processes information of its external and internal environment through sensory neurons. There are 60 well characterised ciliated sensory neurones found in the head (amphids) and the tail of *C. elegans* (Perkins et al., 1986; Ward, 1973). These can be either a single or multisensory neurones that can detect theromosensation (see section 1.2.2.3), gustation, olfaction or mechanosensation. The amphid chemosensory organs are innervated by invaginations of cuticle localised in the head region, where 12 sensory neurones are found in each of them. These amphid sensory neurones have a non-motile cilium at their dendritic terminal end, which allows direct contact with the external environment (Perkins et al., 1986). Nonetheless, there are sensory neurones that function in the absence of cilia such as mechanosensory neurones, CEP, ADE and PDE (Sawin et al., 2000).

Sensory signals are processed through different cell signalling pathways, which involves G-proteins and ion channels that are either cyclic GMP or belong to transient receptor potential (TRP) channel superfamily. The TRP channel superfamily are a large group of channels that functions in vertebrate pain sensation, insect phototransduction and in non-neuronal pressure sensation and osmo-sensation. The chemoreceptors in *C. elegans* have seven transmembrane domains and are distantly related to the family I protein-coupled receptors (GPCRs). The GPCRs are involved in recognising odorants (Troemel et al. 1996; Sengupta 1995) while TRP channel plays a role in mechanosensory, thermosensation and proprioception in *C. elegans*.

1.6.2 Sensory modalities

1.6.2.1 Mechanosensation

In the absence of food, worms increase their locomotory rate to allow foraging for food over a large area, however, upon entry to food, worms reduce their locomotory rate, increase pharyngeal pumping and egg laying (Sawin et al., 2000). It has been previously reported that the physical detection of food particles activates the dopaminergic neurones CEP, ADE and PDE (*Figure 1.10A*), which mediates the slowing response observed when

the worm enters a food lawn (Sawin et al., 2000). The CEP-laser ablated worms did not reduce their locomotory behaviour upon entry to food, whereas the ablation of all dopaminergic neurones CEP, ADE and PDE had the same effect as seen in CEP-laser ablated worms alone (Sawin et al., 2000).

In touch avoidance behaviour, the mechanosensory neurones detect low threshold touch to the body stimuli to anterior (AVM, ALM) and posterior (PLM, PVM) regions of the *C. elegans* body (*Figure 1.10A*). The sensory information is passed to forward and backward command interneurons (AVA, AVD, and AVE). Subsequently, command interneurons synapse to forward and backward motor neurones to induce an escape response from a harsh stimuli (De Jager et al., 1998). However, a recent study has shown that the command interneurons AVA plays a major role in triggering backward locomotion more than AVB, AVD and AVE (Piggott et al., 2011). Nonetheless, the ablation of AVA, AVD and AVE reduced backward locomotion without completely abolishing them. Therefore, these interneurons may contribute to, but are not essential for spontaneous or touch-induced reversal.

C. elegans can respond to non-localised touch stimulus, known as plate-tap response (Chiba and Rankin, 1990). In this behaviour, the anterior touch circuit ALM and AVM neurones detect the stimulus which converge the sensory signals to AVB interneurons, thus, facilitating a backward movement in response to a tap on the culture plate (Chiba and Rankin, 1990). In addition to modification of locomotor behaviour, a gentle touch controls and inhibits pharyngeal pumping, egg laying and defecation (Syntichaki and Tavernarakis, 2004). These responses are thought to be produced due to synaptic connections between the touch neurones and CEP, motor neurones HSN and RIP interneurons (Syntichaki and Tavernarakis, 2004).

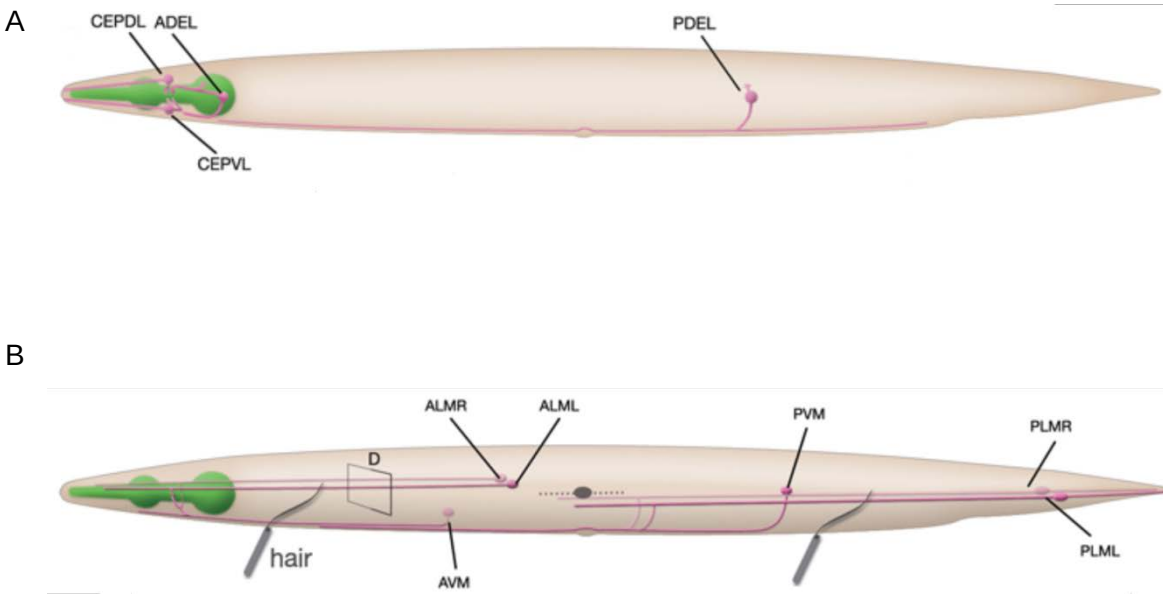


Figure 1.10: The locations of mechanosensory neurones that respond to low threshold body touch. *A) mechanosensory neurones which detect mechanical stimulus in C. elegans. They are dopaminergic-producing neurones (CEP, ADE and PDE). B) A gentle touch with a hair activates the touch receptors (ALM, AVM, PVM and PLM) neurones, which mediate avoidance response to mechanosensory stimulus. Image was obtained from wormatlas.org.*

Other types of somatosensation include high threshold touch to the head or nose region. ASH, FLP and OLQ (*Figure 1.10A*) sensory neurones respond to head-on nose stimulus (Kaplan and Horvitz, 1993). This is due to the presence of ciliated endings of these neurones in the nose, which facilitates backward movement (Kaplan and Horvitz, 1993). ASH and FLP neurones detect harsh mechanosensory cues to the head, whereas OLQ neurones are thought to enhance this mechanoreception (Chatzigeorgiou and Schafer, 2011). ASH and FLP mediate backward movement by signalling to AVB, AVD and AVA interneurons (Chatzigeorgiou and Schafer, 2011). In the case of low threshold nose touch OLQ and IL1 synapse to NR motor neurones and head muscles, which mediate inhibition of lateral foraging movements of the heads and head-withdrawal reflex (Hart et al., 1995). CEP and OLQ neurones can indirectly detect gentle nose touch in the FLP head nociceptors via the RIH interneurons (Chatzigeorgiou and Schafer, 2011). Therefore, FLP neurones can respond to low threshold touch and facilitate an avoidance behaviour (Chatzigeorgiou and Schafer, 2011).

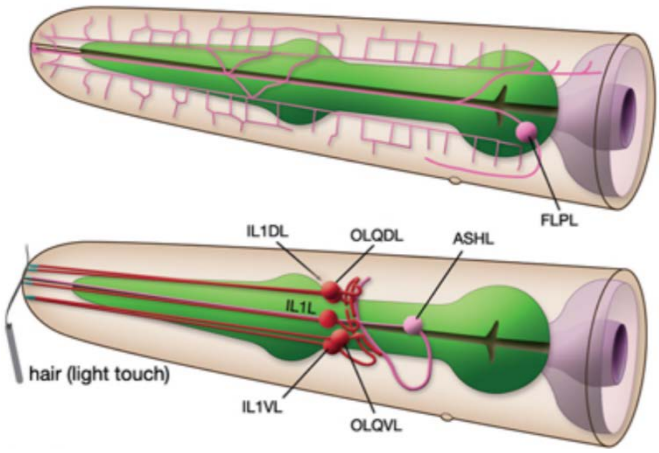


Figure 1.11: Sensory neurones that respond to gentle nose touch. *FLP (top drawing) and ASH, IL1 and OLQ neurones (bottom drawing). Image was obtained from wormatlas.org.*

1.6.2.2 Nociception/ repulsion

C. elegans is able to sense harmful and toxic stimuli in their environment and avoid them to allow survival. These aversive cues include attractive odours at high concentrations, high osmotic strength, light and harsh mechanical touch (see section 1.6.2.1), low pH levels and extreme high or low temperatures (Tobin and Bargmann, 2004). Furthermore, *C. elegans* avoids quinine, sodium dodecyl sulphate (SDS) and heavy metals (e.g. copper) (Sambongi et al., 1999; Tobin and Bargmann, 2004). The polymodal ASH nociceptor neurones detect harmful stimuli, as the cilia of ASH neurones are exposed to the outside environment, which allows the quick detection of stimulus to mediate an avoidance response by reversing and turning away upon encountering a harmful stimuli (Perkins et al., 1986; Troemel et al., 1997). ASH neurones require the activation of TRPV channels, OSM-9 and OCR-2 (Tobin and Bargmann, 2004). The glutamatergic ASH neurones are polymodal sensory neurones, which generate specific cue-response signalling by activating different NMDA and non-NMDA glutamate receptors (Mellem et al., 2002). For instance, ASH neurones detect mechanical stimuli, such as nose touch, which is mediated by the non-NMDA receptors, GLR-1 and GLR-2 in AVA neurones, and thus, facilitating avoidance response (Mellem et al., 2002). However, osmotic avoidance

behaviour is detected by ASH neurones which activates GLR-1, GLR-2 and NMR-1 glutamate receptors in postsynaptic neurones to mediate avoidance behaviour (Mellem et al., 2002).

Besides ASH neurones, AWB neurones detect and facilitate repulsion to high concentrations of volatile chemicals (Troemel et al., 1997). ODR-10 is a G-protein-coupled receptor that detects diacetyl. However, when ODR-10 is expressed in AWB neurones, diacetyl becomes an aversive cue and when it is expressed in AWC neurones (see 1.6.2.3.2 below), diacetyl is an attractant (Troemel et al., 1997; Wes and Bargmann, 2001). These findings indicate that dedicated sensory neurones are linked to a behavioural response, in which AWA and AWC result in attraction (see 1.6.2.3.2), whereas AWB results in repulsion (Bargmann, 2006). Similar findings have been reported in studies in the sweet and bitter taste systems in mammals, which indicates that intrinsic chemosensory preferences in animals are hard wired to mediate behavioural response (Mueller et al., 2005).

1.6.2.3 Chemosensation in *C. elegans*

Bacteria, the main food source of *C. elegans*, release water-soluble and volatile alcohol by-products, which are utilised by *C. elegans* chemosensory neurones to chemotax towards bacteria (Bargmann and Horvitz, 1991; Bargmann et al., 1993; Grewal and Wright, 1992; Zechman and Labows, 1985). In this section the gustatory and olfactory modalities are reviewed separately below.

1.6.2.3.1 Gustation (taste cues)

C. elegans chemotaxis to water-soluble molecules such as cations, anions, cyclic nucleotides, biotin, and amino acids was first described by (Ward, 1973). More chemicals have been screened since Ward's findings (Bargmann and Horvitz, 1991; Dusenbery, 1974). However, the number of water-soluble chemicals detected by *C. elegans* is low compared to that of volatile chemicals (Bargmann, 2006).

The sensory neurones that mediate chemotaxis to water soluble molecules are ASE, ASG, ASK, ASI, ASJ and ADF (*Figure 1.12*) (Bargmann, 2006; Sengupta, 2007). However, ASE plays a major role in water-soluble molecules, whilst other sensory neurones have a minor role, as per previous observations which showed that the ablation

of ASE pair neurones reduced, but not completely abolished, chemotaxis efficiency to Na^+, Cl^-, cAMP, biotin, and lysine (Bargmann and Horvitz, 1991). However, the ablation of all amphid and phasmid neurones except ASE neurones did not affect the chemotaxis of *C. elegans* to these chemicals (Bargmann and Horvitz, 1991; Kaufman et al., 2005). This because sensory neurones can be asymmetrical at the receptor expression level even though they are symmetrical at the structural level as revealed by gene expression studies (Yu et al., 1997). This was further demonstrated in another study which showed that ASE neurones on the left and right have different functions from each other. For instance, the ASEL neurones detect sodium ions whereas ASER detects chloride and potassium ions (Pierce-Shimomura et al., 2001).

1.6.2.3.2 Olfaction

Bargmann and co-workers (1993) screened the ability of *C. elegans* to detect volatile compounds all related to the alcohols, ketones, aldehydes and fatty acids that emanate from metabolizing bacteria (Bargmann et al., 1993). Out of 120 volatile chemicals tested, 50 of them were attractive including alcohols, ketones, aldehydes, amines, esters organic acids, aromatic and heterocyclic compounds. It indicates that in the wild, volatile chemicals are used for long distance chemotaxis, whereas water-soluble molecules are used for short distance chemotaxis, since the diffusion through air is much faster than through the agar (Bargmann, 2006). The two main bilateral amphid neurones involved in the detection of attractive volatile chemicals are AWA and AWC neurones (*Figure 1.12*) (Bargmann et al., 1993). AWA senses diacetyl, 2,4,5-trimethylthiazole and pyrazine, whereas AWC senses butanone, benzaldehyde, isoamyl alcohols, 2,3-pentanedione, and 2,4,5-trimethylthiazole (Bargmann et al., 1993).

Similar to ASE (left and right) neurones, AWCL and AWCR neurones have distinct functions from each other, in which the AWCL (AWC^{ON}) detects butanone and AWCR (AWC^{OFF}) detects 2,3-pentanedione. Both AWCL and AWCR sense benzaldehyde and isoamyl alcohols (Wes and Bargmann, 2001). Hence, without AWC asymmetry, worms cannot differentiate between butanone and benzaldehyde (Wes and Bargmann, 2001). It was reported that the differentiation between the two AWC neurones is random from animal to animal, however, there is coordination in which each animal has one neuron of each type (Troemel et al., 1997).

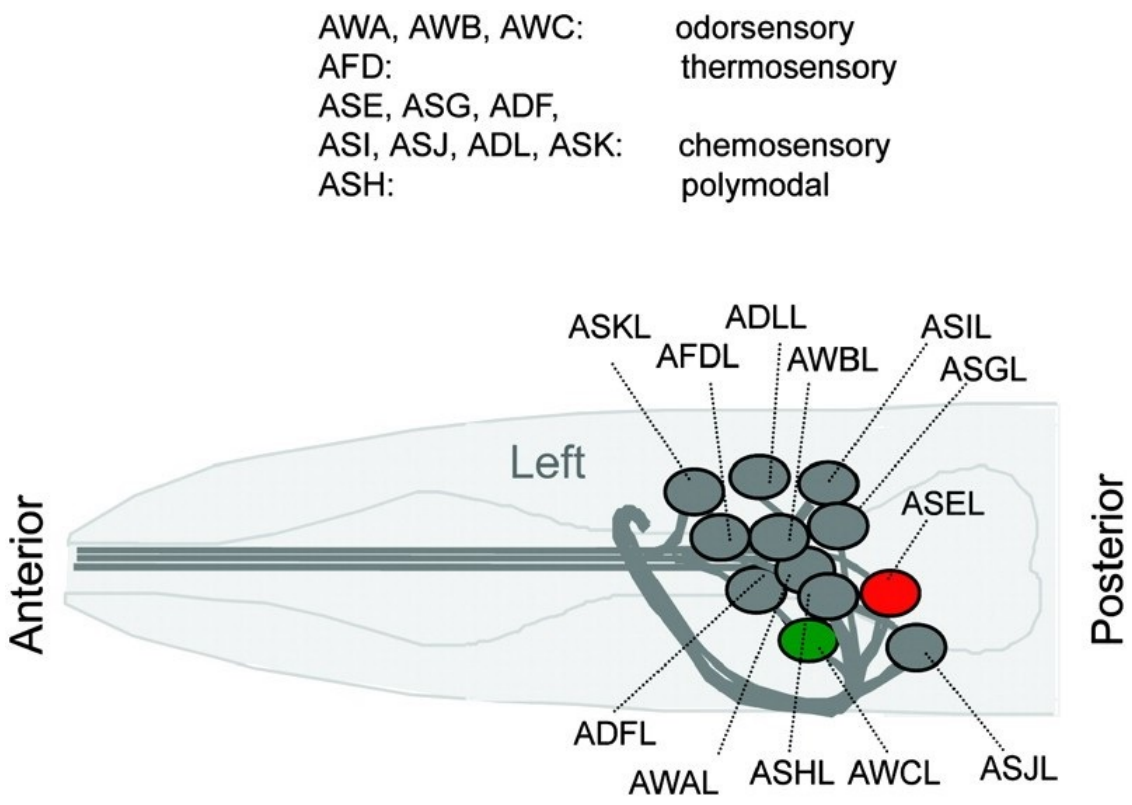


Figure 1.12: The chemosensory neurones in C. elegans. *AWA and AWC detect attractive odours, whereas AWB detects aversive odours. ASE, ASG, ASK, ASI, ASJ and ADF detect attractive water-soluble compounds. ASH neurones are polymodal neurones and detect aversive compounds. AFD detects fluctuations in temperatures (see section 1.2.2.3). The image was obtained from (Ortiz et al., 2006).*

1.6.2.3.3 Chemosensory neurones to behaviour

In the absence of food, water-soluble or volatile chemicals are sensed by dedicated sensory neurones, in which sensory information is passed to a layer of interneurons. The AIY interneurons receive sensory inputs from AWA, AWC and ASE sensory neurones, whilst AIA interneurons receive sensory inputs from ASK, ASG and ASE, AWC, ASI, ADL and ASH sensory neurones (White et al., 1986). Following immediate removal from food, the sensory neurones AWC, ASK and AFD relay sensory information to AIB and RIB interneurons. For example, AWC neurones have basal activity in the absence of odour, which is stimulated in the absence of odour and inhibited following the addition of odour (Chalasani et al., 2007). These interneurons synapse to command AVA interneurons to increase the frequency of reversals (Gray et al., 2005). However, in prolonged food deprivation, ASI sensory neurones may inhibit reversals and omega turns through the inhibition of the AIY interneurons, which in turn inhibits RIM interneurons, AVA command motor neurones and head motor neurones (Gray et al., 2005). Thus, facilitates dispersal to increase the area of food search.

1.6.2.4 Oxygen sensation (aerotaxis)

C. elegans exchange gases by diffusion through the cuticle and via the tissue bathing pseudocoelomic fluid (Van Voorhies and Ward, 2000). In the wild, *C. elegans* lives in an environment with rapid shifts in oxygen concentration between 0% and 21%. Ambient oxygen may be an indicative of the presence of food to *C. elegans, since C. elegans* are found in compost, where oxygen is consumed by microbes (Cheung et al., 2005; Gray et al., 2004; Rogers et al., 2006). Under laboratory conditions with oxygen gradient from anoxic conditions to saturated oxygen levels, *C. elegans* favoured oxygen concentrations between 7-14%, whilst avoiding extreme oxygen levels (Cheung et al., 2005; Gray et al., 2004; Rogers et al., 2006).

Oxygen is sensed by a network of neurones, most notably, major oxygen-sensing neurones AQR, PQR and URX, as well as minor oxygen-sensing neurones, including, SDQ, ALN, PLN and BDN interneurons, which express guanylate cyclase (*gcy-32*, *-34*, *-35*, *-36*, and *-37*) (Cheung et al., 2004; Gray et al., 2004; White et al., 1986; Yu et al., 1997). AQR and PQR have ciliated endings (*Figure 1.13*); however, the AQR dendritic endings are free within the pseudocoleomic cavity, whereas PQR dendritic endings are located close to the pseudocoelom. Furthermore, URX are enclosed by plasmid socket

cell and located close to the pseudocoelom. Collectively, the locations of these major neurones suggest that they regulate pseudocoelom fluid and its oxygen level since they are positioned inside or within a close proximity to the pseudocoelom (Rogers et al., 2006). Similarly, soluble guanylate cyclases expressed in SDQ, BDU, ALN and PLN interneurons bind molecular oxygen and have a similar function to AQR, PQR and URX neurones (Cheung et al., 2004; Yu et al., 1997). Aerotaxic responses of hypoxia and hyperoxia are regulated by the presence and absence of food (Cheung et al., 2005; Gray et al., 2004; Rogers et al., 2006). Oxygen-sensory neurones converge sensory information to command AVA interneurons, which mediate avoidance by inducing a backward movement (Rogers et al., 2006). Avoidance of hyperoxic conditions is mediated by the neuropeptide receptor NPR-1, the transforming growth factor-β (TGF-β)-related protein, DAF-7 and the release of serotonin from ADF neurones (Chang et al., 2006; Cheung et al., 2005).

Figure 1.13: The sensory neurones that detect and modulate ambient oxygen levels*. Major oxygen-sensing neurones are URX, AQR and PQR and minor-oxygen sensing neurones BDUL, SDQL, ALNL and PLNL. The image was obtained from wormatlas.org*.

1.6.2.5 Carbon dioxide sensation

The natural habitat of *C. elegans* has a wide range of CO_2 levels. High levels of CO_2 above 9% can cause slow development, deterioration of muscles and reduced fertility (Bretscher et al., 2008). *C. elegans* mediates an avoidance response to CO_2 when CO_2 is

above 0.5% (Bretscher et al., 2008). AFD, BAG and ASE neurones sense ambient CO_2 levels (*Figure 1.12*, Figure 1.13 & *Figure 1.14*) (Bretscher et al., 2011). The response is mediated by the activation of soluble guanylate cyclases in BAG and TAX-2/TAX-4 cGMP-gated heteromeric channels (Bretscher et al., 2011). AFD neurones respond to CO_2 by changes to modifying Ca^{2+} levels, for example low levels in CO_2 lead to an increase in Ca^{2+} (Bretscher et al., 2008).

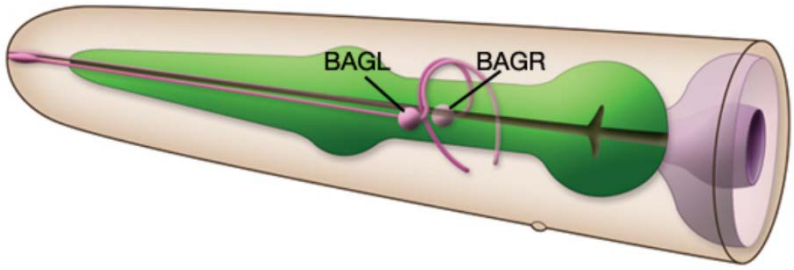


Figure 1.14*: The carbon dioxide sensory neurones. Bilateral neurones are located in the head region of C. elegans.*

1.7 The role of biogenic amines in regulating sub-behaviours of feeding

Neurotransmitters play a major role in regulating feeding behaviours in *C. elegans* as discussed in (sections 1.5.1.1 & 1.5.2.2.4). In this section the role of biogenic amines in regulating sensory-cue dependent feeding behaviour has been discussed by focusing on their role in modulating both locomotion and pharyngeal pumping.

1.7.1 The role of serotonin in regulating locomotion behaviour

The neurotransmitter 5-HT is synthesised in NSM and I5 pharyngeal neurones and in six non-pharyngeal neurones, ADF, HSN, males-specific neurones (CP 1-6, R1, R2 & R3), ventral cord motor neurones that innervate the muscles of vulva and ventral body muscles

(VC4 &5), RIH (nerve ring interneuron) and AIM (interneuron in ventral ganglion) (Chase and Koelle 2007). Exogenous application of 5-HT to wild-type *C. elegans* inhibits defecation and locomotory rate, whilst stimulates pharyngeal pumping and egg laying (Horvitz et al. 1982; Segalat et al. 1995; Weinshenker et al. 1995; Waggoner et al. 1998; Sawin et al. 2000; Rogers et al. 2001; Niacaris and Avery 2003). The serotonin receptors, SER-4 and 5-HT-gated chloride channel, MOD-1, regulate 5-HT-dependent reduced locomotion (Ranganathan et al. 2000).

The NSM neurones have morphologically defined sensory endings in the lumen of pharynx and may detect the presence of food (Chase and Koelle 2007). A study found that 5-HT released from the NSM neurones stimulate dwelling behaviour in the presence of food (Flavell et al. 2013). Furthermore, MOD-1 appears to be the main receptor required for dwelling behaviour on food (Flavell et al. 2013). In a separate study, food-deprived worms displayed an enhanced slowing response when re-entering food, in which starved worms completely inhibited their locomotion rate (Sawin et al. 2000). MOD-1 is required for the enhanced slowing response (Sawin et al. 2000). This behaviour depends on the 5-HT pathway, since starved *bas-1* mutants, which encodes serotonin and dopamine synthetic aromatic amino acid decarboxylase, did not show enhanced slowing response when entered a food lawn. This behaviour is thought be independent from the basal slowing response, which utilises the dopamine signalling as *cat-2* mutants did not exhibit normal enhanced slowing response behaviour.

Furthermore, *tph-1* mutants, which encodes tryptophan hydroxylase enzyme for the synthesis of 5-HT, have a defective exploratory behaviour in the absence of food (Gray et al. 2005). These mutants displayed increased short reversals compared to wild-type worms during dispersal and local area search. Therefore, overall, 5-HT plays an important role in regulating the worm's locomotion behaviour in the presence and absence of food.

1.7.2 The role of serotonin in regulating pharyngeal pumping

Serotonin plays a major role in regulating core pharyngeal pumping, which has been discussed in section 1.5.2.2.4.1. Moreover, a study has found that the presence of an attractive odorant increases pharyngeal pumping in the presence of food (Li et al., 2012). It has been reported that MOD-1, a 5-HT gated chloride channel, is required for the diacetyl- mediated increased pharyngeal pumping on food (Li et al., 2012).

1.7.3 The role of glutamate in locomotion behaviour

Glutamate plays an important role in regulating foraging behaviour in *C. elegans*. In the absence of food, glutamate is required for LAS behaviour as *eat-4* mutants, which encodes a vesicular glutamate transporter, showed a deficit in high-angled turn and omega-turn (Hills 2004; Chalasani et al. 2007). GLR-1 and GLR-2, ionotropic glutamate receptors, and GLC-3, glutamate- gated chloride channel, are critical for omega turns and high-angled turns following removal of food (Hills 2004; Chalasani et al. 2007). Furthermore, the chemosensory neurones, AWC, play a role in LAS behaviour, which was shown by partial rescue of LAS deficit in wild-type *eat*-4 (Chalasani et al. 2007). This reinforces the role of LAS in the absence of food because AWC olfactory neurones are activated in response to removal of food odours (Chalasani et al. 2007). The glutamatergic chemosensory neurones, ASK, are responsible for reducing forward locomotion and stimulating reversals in the absence of food (Wakabayashi et al. 2004; Gray et al. 2005). Moreover, ASK and AWC modulate LAS behaviour through activation of AIB interneurons (Gray et al. 2005).

1.7.4 The role of glutamate in regulation pharyngeal pumping

Mechanosensory stimulus have been shown to reduce pharyngeal pumping (Avery, 2003; Keane and Avery, 2003). Harsh touch of the worm's tail completely inhibits pumping rate for a short time before pumping rate gradually recovers. Pumping rate is reduced by 90% following mechanical stimulus (Dallière et al., 2016; Keane and Avery, 2003). Mutant analysis has shown that both the innexin gene, UNC-7 and glutamate signalling via both glutamate gated-chloride channels AVR-14 and AVR-15 are involved in this behaviour (Keane and Avery, 2003). The detection of mechanosensory inputs is thought to be detected by extra-pharyngeal neurones which must pass to the pharyngeal nervous system either directly via connections between I1 and RIP neurones or indirectly via humoral pathway (Keane and Avery, 2003). According to published data, AVR-14 expression pattern indicates it is found in extra-pharyngeal neurones such as mechanosensory neurones ALM, PLM and PVD (Dent et al., 1997, 2000). However unpublished observation from our own lab showed AVR-15 was expressed in pharyngeal muscles pm4 and pm5 (Dallière et al., 2015, unpublished data). thus inhibition of pumping

rate in response to mechanical stimulus may be exerted through AVR-15 found in pharyngeal muscles.

1.7.5 The role of dopamine in locomotion behaviour

Dopamine is a context-dependent modulator of food in *C. elegans* and higher animals. In *C. elegans,* dopamine is synthesised in eight bilateral extra-pharyngeal neurones, CEP, ADE and PDE in hermaphrodite and in additional six bilateral neurones, R5A, R7A and R9A in male *C. elegans* (Sulston et al., 1975). The dopamine-releasing neurones are mechanosensory neurones, as the ablation of these neurones results in the worm's inability to respond to mechanical stimulus (Hills, 2004; Sanyal et al., 2004; Sawin et al., 2000). In the presence of food, dopamine is required for reducing locomotion rate (Sawin et al., 2000), whereas in the absence of food, dopamine mediates an increase in frequency turns (Hills, 2004).

The role of dopamine has been reported in decision-making behaviours and memory in *C. elegans*. For instance, in a choice test, when worms were presented with attractive compound, such as diacetyl, and an aversive compound, such as Cu^{2+}, a large number of worms moved towards the attractive compounds (Wang et al., 2014). This behaviour is controlled by the dopamine receptors, DOP-1 and DOP-4. Furthermore, dopamine signalling is required for enhanced avoidance response following pre-exposure to a repellent compound, such as 2-nonane-1-ol, in subsequent chemotaxis assay. Another study has shown that avoidance of soluble repellents was enhanced in the presence of food (Elefteriou et al., 2015). This response is facilitated by dopamine, since the food sensing is directly sensed by dopamine neurones. The enhanced repellent is partially mediated by DOP-4 expressed in ASH (Elefteriou et al., 2015).

1.7.6 The role of dopamine in pharyngeal pumping

There is no evidence for dopamine regulation of feeding in *C. elegans* and limited studies showed that the addition of exogenous dopamine to food does not alter pharyngeal pumping (Rani and Srivastava, 2018). In contrast, there indication that it might contribute off-food pumping, since *cat-2* mutants showed an increased pharyngeal pumping in the absence of food (Dallière et al., 2016).

1.7.7 The role of tyramine and octopamine in the modulation of locomotion

Tyramine is synthesised by the enzyme tyrosine decarboxylase from tyrosine (TDC-1) (Alkema et al. 2005). Tyramine is found in 3 cells, RIC, RIM and four uterine cells known as UV1 cells (Alkema et al. 2005). Tyramine can be converted to octopamine via the enzyme tyramine ß-hydroxylase (TBH-1) (Alkema et al. 2005). Since TBH-1 is expressed in RIC cells, it is thought that only RIM and UV1 cells release tyramine (Alkema et al. 2005).

Tyramine is required for the sensory-dependent inhibition of head oscillations that mediates the escape response. *tdc-1* mutants showed no reduction in head oscillations when animals reverse in response to light touch to the anterior part of the body (Alkema et al. 2005). Anterior touch to the worm's body is detected by the ALM/AVM mechanosensory neurones which promote tyramine release from the RIM neurones through the activation of the AVD and AVA backward locomotion command neurones (Alkema et al. 2005).

Furthermore, tyramine is required for the inhibition of egg laying and spontaneous body reversals (Alkema et al. 2005). This indicates that tyramine is released in the absence of food and in search for new food sources when food sources are diminished (Alkema et al. 2005; Rex et al. 2004). These behaviours were only observed in *tdc-1* mutants and were not found in *tbh-1* mutants.

Octopamine is synthesised by TBH-1 from tyramine in the RIC interneurons and gonadal sheath cell body (Alkema et al. 2005). The role of octopamine in locomotion is not well-understood. However, a recent study investigated the effect of octopamine in liquid using microfluidic devices (Churgin et al. 2017). They tested octopamine-deficient mutant, *tbh-1*, which displayed increased quiescence, dwelling and decreased roaming in fasting conditions. The application of exogenous octopamine attenuated defects in quiescence and roaming, but not dwelling. Churgin et al. 2017 found that SER-3 and SER-6 mediate exogenous octopamine effects, since *ser-3* and *ser-6* mutants did not respond to exogenous octopamine. Therefore, these data suggest that octopamine plays a role in promoting locomotion in the presence of food as well as in fasting conditions.

1.7.8 The role of tyramine and octopamine in the modulation of pharyngeal pumping

In the presence of repellent odours or unpleasant smells, octopamine and tyramine were released to reduce pharyngeal pumping in the presence of food (Li et al. 2012). However, there was no effect reported in the absence of food (Greer et al. 2008; Li et al. 2012). Furthermore, exogenous addition of 2mM tyramine inhibited pharyngeal pumping by 60% (Greer et al. 2008; Li et al. 2012). SER-2 receptor, which is expressed in the pharynx is responsible for this reduction (Li et al. 2012). This receptor is expressed in pharyngeal muscles, pm1 and pm2, and pharyngeal neuron, NSM. Moreover, TYRA-2 a distinct tyramine receptor is found in MC and NSM neurones (Rex et al. 2005). A recent study has found that TYRA-2 inhibits off-food pharyngeal pumping in response to a repellent (Fu et al. 2018).

Similarly, exogenous application of octopamine inhibited pharyngeal pumping in the absence of food (Horvitz et al. 1982). The octopamine receptors, SER-2, is expressed in the pharyngeal muscle (Carre-Pierrat et al. 2006). Observation of EPG recording showed that octopamine inhibited pharyngeal pumping by increasing the action potential of the pharynx, thus leading to the suppression of M3 neurones (Rogers et al. 2001; Niacaris and Avery 2003).

1.8 Behavioural plasticity in *C. elegans* in response to sensory cues

C. elegans express many forms of behavioural plasticity often involving either short-term or long-term changes in the behaviours such as locomotion. They reveal plasticity at the level of sensory detection, integration and output level of the executing behaviour.

C. elegans has the ability to learn and remember cues from the environment. These learning behaviours can be divided into non-associative and associative learning. Non-associative learning involves presenting the stimulus cue only with no association with an unconditioned stimulus. For instance, a study has found that prolonged exposure of *C. elegans* to an attractant odour, benzaldeyhdye, in the absence of food led to a reduced chemotaxis efficiency by 25% after 30 min and a further reduction in chemotaxis was observed after 1hr by 50% (Colbert and Bargmann, 1995). However, prolonged exposure

to benzaledyde did not affect chemotaxis to the attractant odorant, diacetyl, which indicates that plasticity occurs at a molecular level (Colbert and Bargmann, 1997). Additionally, Bernhard and van der Kooy (2000) have reported that the worm can habituate and adapt to odorants presented and that both behaviours depend on the volume of the odorant and the time of exposure. For instance, worms conditioned in the presence of 5 µl of 100 % diacetyl for 30, 60 and 2 hr reduced their chemotaxis efficiency to a point source of diacetyl by 20%, 30% and 50%, respectively, compared to naïve worms (Bernhard and Van Der Kooy, 2000).

On the other hand, associative learning involves presenting a stimulus cue in the presence of either a positive (appetitive, e.g. eliciting approach and/or feeding behaviours) or a negative cue (aversive, e.g. eliciting avoidance and/or withdrawal and/or other avoidance behaviours) (Sasakura et al., 2013). For instance, when diacetyl, which is ordinarily an attractant olfactory cue. was presented in the presence of an aversive cue, acetic acid solution, worms avoided diacetyl in subsequent chemotaxis test (Morrison et al., 1999). In another study, butanone is an attractant olfactory cue (Bargmann et al., 1993), when butanone is presented in the presence of food, worms increased their chemotaxis to butanone in the following tests (Torayama et al., 2007). Torayama and colleagues have shown that this plasticity is butanone specific since benzaldehyde, an attractant olfactory cue, did not increase chemotaxis when worms were conditioned in the presence of benzaldehyde and food. They attributed this differences to sensory neurones, since butanone is sensed by AWC^{ON} while benzaldehyde is sensed by AWC^{OFF} neurones (Torayama et al., 2007). Furthermore, pre-exposure to butanone and 5mM of serotonin did not enhance chemotaxis efficiency, suggesting that 5-HT is not required for butanone-induced plasticity, although 5-HT mimics the presence of food. This indicates that there is a multisensory integration mechanism involved (Ardiel and Rankin, 2010).

Food is a powerful appetitive cue for *C. elegans* and guides its behaviour. Thus, investigating behavioural plasticity in *C. elegans* seems physiologically relevant (*Figure 1.15*) since food is associated with environmental cues to drive foraging and feeding behaviours. Besides the well-mapped connectome of *C. elegans,* a single or multiple sensory cue can be determined and linked to output behaviours in innate and acquired behaviours (Sasakura et al., 2013).

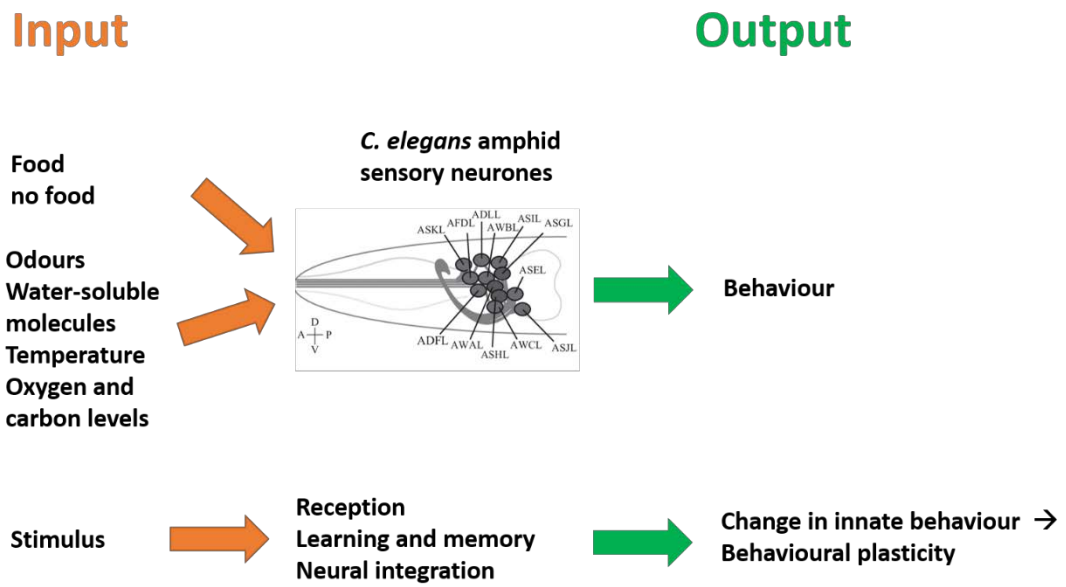


Figure 1.15: The theoretical scheme of behavioural plasticity. *The diagram was adapted from (Sasakura et al., 2013).*

1.9 Aim of the study

As detailed above, the pharyngeal pump is a core behaviour that underpins the worm's feeding. Its response to external environment and food are well described and easily assayed. Sensory modulation of the pharynx is clear and exemplified by testing different types of food or food-related cues. However, the modalities and drives of this are poorly understood. Along with foraging for food, the integrity of pumping is an important determinant of the worm's nutritional state. Thus one might predict that plasticity of pharyngeal pumping would be important, however this has been subjected to limited investigation. This thesis is based on the hypothesis that the biological organisation of the pharynx supports its use as a route to tractable investigation of the rules that govern plasticity. The value of this is reflected in the idea that enhanced or reduced pharyngeal functions represent tractable models of appetitive and suppressive plasticity. For this reason, the thesis aims to:

1. Define environmentally salient mimics of food associated modalities to define determinates of feeding behaviour.

2. Investigate if these food-related cues can execute plasticity in pharyngeal function
3. Utilise the paradigms defined in aims 1 and 2 to define the molecular and cellular determinates of sensory driven modulation of pharyngeal function and plasticity.

Chapter 2: General Materials and Methods

2.1 Solutions and chemicals

2.1.1 4l Nematode Growth Media (NGM)

Stock	**Amount to add**
Agar	80g
NaCl	12g
Bactopeptone	10g
dH_2O	q.s. 3888l

A large magnetic stirrer bar was placed inside a 5l glass bottle. Following autoclave, the bottle was left to cool down while stirring and the following reagents were added:

Stock	**Volume to add**
Cholesterol (4 mg/ml) in cholesterol	4ml
1M $CaCl_2$	4ml
1M $MgSO_4$	4ml
1M Potassium phosphate	100ml

2.1.2 S-basal

Stock	Amount to add
NaCl	5.85g
K_2HPO_4	1g
NH_2PO_4	6g
dH_2O	q.s.1l

Mix well, aliquot into 10 bottles at 100 ml and autoclave. Using sterile technique, add 1 ml cholesterol (5 mg/ml).

2.1.3 M9

Stock	Amount to add
KH_2PO_4	3g
Na_2HPO_4	6g
NaCl	5g
dH_2O	q.s. 999l

Then add 1ml of 1M $MgSO_4$. Mix well, aliquot into 10 bottles at 100ml and autoclave.

2.1.4 Phosphate Buffer

Stock	Amount to add
K_2HPO_4	23g
NH_2PO_4	118.12g
dH_2O	q.s.1l

Mix well, aliquot into 10 bottles at 100ml and autoclave.

2.1.5 Luria Broth agar

Stock	Amount to add
Bacto-agar	15g
Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	10g
dH_2O	q.s. 475ml

Mix well and autoclave.

2.1.6 Freezing buffer

Stock	Amount to add
NaCl	2.925g
KH_2PO_4	3.4g
Glycerol	150g
1M NaOH	2.8ml
dH_2O	q.s. 500ml

Mix well, aliquot into 5 bottles at 100 ml and autoclave. Using sterile technique, add 30µl 1M $MgSO_4$ to each bottle before use.

2.1.7 Dent's solution

Stock	Amount to add
glucose	0.18g
Hepes	0.115g
NaCl	0.818g
KCl	0.044g
$CaCl_2$	0.044g
$MgCl_2$	100µl
dH_2O	q.s. 100ml

A small magnetic stirrer bar was added to the beaker. While stirring, adjust pH to 7.4 using sodium hydroxide.

2.1.8 Worm lysis buffer

Stock	**Volume to add (ml)**
1M Tris HCl	1
1M KCl	5
NP40 (45% v/v)	1
Tween 20 (45% v/v)	1
Gelatin 10X (2mg/ml)	10
dH_2O	q.s. 100 ml

Mix well, aliquot 1ml into Eppendorf tubes and freeze. Before use, a fresh lysis buffer and proteinase K mix was prepared; 10µl of 10 mg/ml Proteinase K was added to 90µl of lysis buffer to give a final volume of 100µl.

2.2 Strains used

C. elegans, N2 wild-type Bristol strain and mutants thereof, were used in this study. The mutants used are listed in table 1 below.

Table 1: Mutant strains used in this study.

Strain name	Gene	Allele	Supplier	References
CB1112	*cat-2*	*e1112*	CGC	J.Sulston's lab, University of Oxford, UK
MT6308	*eat-4*	*ky5*	CGC	Leon Avery's lab, Virginia Commonwealth University, USA
DA509	*unc-31*	*e169*	CGC	Leon Avery's lab, Virginia Commonwealth University, USA
PR811	*osm-6*	*p811*	Horvitz's lab (MIT)	Joseph Gary Culotti's lab, Mt. Sinai Hospital Research Institute, Canada
VC2504	*flp-15*	gk1186	CGC	Donald Gordon Moerman's lab, University of British Columbia, Canada
MT21421	*N2; Is[Pflp-15::Csp-1b]*		Horvitz's lab (MIT)	Horvitz's lab, Massachusetts Institute of Technology, USA
	N2; Is[Pflp-15::GCaMP3]		Horvitz's lab (MIT	Horvitz's lab, Massachusetts Institute of Technology, USA
	gur-3; Is[Pflp-15::GCaMP3]		Generated by crossing	
LX645	*dop-1*	*vs100*	CGC	Michael Robert Koelle's lab, Yale University, USA
LX702	*dop-2*	*vs105*	CGC	Michael Robert Koelle's lab, Yale University, USA
LX703	*dop-3*	*vs106*	CGC	Michael Robert Koelle's lab, Yale University, USA

FG58	*dop-4*	*tm1392*	CGC	Denise M Ferkey's lab, State University of New York, USA
MT14984	*tph-1*	*n4622*	CGC	H. Roberts Horvitz's lab, Massachusetts Institute of Technology, USA
RB1756	*gur-3*	*ok2246*	CGC	*C. elegans* Gene Knockout Project at the Oklahoma Medical Research Foundation, part of the International *C. elegans* Gene Knockout Consortium,
	dop-4(tm1392); Ex[Pdop-4::dop-4]		Generated by microinjection	
	dop-4(tm1359);Ex[Pflp15::dop-4]		Generated by microinjection	

2.3 *C. elegans* maintenance and husbandry

2.3.1 Maintenance of NGM plates

C. elegans were cultured on Nematode Growth Medium (NGM, see section 2.1.1) at 20°C and kept in an incubator. The plates were sealed with parafilm to prevent cross-contamination. Worms were maintained by either picking L4 or adults into fresh plates every 3 days or by removing an agar plug from a population plate and transferring it onto a fresh plate.

2.3.2 Maintenance of food source

C. elegans is grown on *E. coli* strain OP50, which is used as a food source (Brenner, 2003). Single colonies of *E. coli* OP50 was isolated from a streaked plate of Luria Bertani (LB, see section 2.1.5) agar. A single colony was aseptically removed from the streaked plate and inoculated into LB broth. The LB broth OP50 culture was grown overnight at 37

°C in incubator shaker, before seeding NGM plates. The OD of OP50 = 0.8. Otherwise, if it was not required for immediate use, the LB broth culture was stored at 4 °C and used for a maximum of 2 weeks.

2.3.3 Long-term storage of worms

A mixed age population of nearly starved worms was washed with 2ml volume of M9 solution (see section 2.1.3) before transfer to a 15ml Falcon tube. A freezing buffer of 2ml (see section 2.1.6) was added (1:1 M9/freezing buffer proportion) and 1ml of the mix was transferred into 4 cryovial tubes and placed at –80ºC. Following 4 days of storage, one of the cryovials was thawed to check worm survival.

2.3.4 Alcohols

Methanol, ethanol, propan-1-ol, butan-1-ol, 2-butanone, pentan-1-ol, hexan-1-ol, heptan-1-ol, octan-1-ol & nonan-1-ol were purchased from Sigma Aldrich, UK. These chemicals have a percentage purity of ≥ 98%. Alcohols were diluted in 100% ethanol with exception of ethanol, which was diluted in double distilled water. The following dilutions were used: $1\mu l \times 10^{0}$, $1\mu l \times 10^{-1}$, $1\mu l \times 10^{-2}$, $1\mu l \times 10^{-3}$, $1\mu l \times 10^{-4}$, $1\mu l \times 10^{-5}$ and $1\mu l \times 10^{-6}$.

2.3.5 Behavioural assays

2.3.6 Chemotaxis assay

The chemotaxis assay was performed on L4+1 age synchronised worms, as described by (Bargmann et al., 1993). Fifteen adult worms (L4+1) were picked into NGM plates (6 cm) seeded with 100µl of OP50 and were left to lay eggs for 4 h before being removed. Worms were incubated at 20ºC and were checked to ensure that worms had reached L4 stage 24 hr before assaying them. This stage was identified via the presence of white patch in the area of the vulva.

On the test day, L4+1 worms were washed with M9 and transferred to an Eppendorf tube before they were centrifuged for 10 seconds at 10 K rpm in a bench top centrifuge (Eppendorf 5417C). The supernatant was removed and a fresh M9 was added to the

Eppendorf tube. The washing step was repeated three times. After the last wash, the supernatant was removed and worms were added to the chemotaxis plates at the defined start point. Before the adding the worms to the plates, on the back of the 9 cm chemotaxis assay plate, and with a marker pen, the regions of the worms' starting point, odorant (tested alcohol), and control were clearly marked. The spots are 1.9 cm away from worms' start point and 1 cm away from the edge of the plate. Subsequently, worms were pipetted to the plate and excess M9 was removed using a filter paper. Subsequently, 1µl of sodium azide (1M) was added to the odorant and control spots. Immediately, 1µl of ethanol was added to the control spot and 1µl of the tested odorant (tested alcohol) was added to the odorant's spot. Sodium azide was added to prevent the worms from leaving the spots due to adaptation to odour (Bargmann et al., 1993). The chemotaxis index for each volatile was calculated using the following formulae:

$$\boldsymbol{Chemotaxis\ index} = \frac{\boldsymbol{no.of\ worms\ on\ (O) - no.of\ worms\ on\ (C)}}{\textbf{total no. of worms on the plate}}$$

2.3.7 Measurement of pharyngeal pumping rate in the presence and absence of food

Pharyngeal pumping rate was recorded in pumps per minute by direct visual observation of the movement of the grinder in the terminal blub under the dissection microscope at a magnification of X40. Counts were made against an alarm minute using and click-counter. A pharyngeal pump was counted as a single movement of the anterior and posterior of the grinder.

2.3.8 Conditioning assays

2.3.8.1 Short-term conditioning assays

Worms were conditioned in the presence of either butan-1-ol or butanone to investigate the effect of preconditioning regime on subsequent chemotaxis or pump rate. Please refer to chapters 4 and 6 for more details on the conditioning regime for each alcohol.

In general, synchronised L4+1 worms were obtained as described (see section 2.3.6). L4+1 worms were conditioned for either 60 or 90 min. The alcohols were added either on the agar or inside the lid opposite to the worms. Pharyngeal pumping was recorded during the first 30 min of the conditioning phase. After the conditioning the chemotaxis index was calculated and conditioned worms were washed with M9 and transferred to testing plates. Testing plates were prepared as described in section 2.3.6. After 60 min, the chemotaxis index of both conditioned and control worms to either 1µl × 10^{-1} butan-1-ol or 1µl × 10^{-1} butanone was calculated.

Following the conditioning treatment, worms were picked onto an unseeded 6cm NGM plate and left for 1 min as a cleaning step to get rid of bacteria. The cleaned worms were transferred onto a clean unseeded 9cm NGM plate and tested against either 1µl × 10^{-1} butan-1-ol or butanone. Ten minutes after transfer to the testing plate, pharyngeal pumping was recorded.

2.3.8.1 The effect of long-term exposure to butanone from eggs to L4 +1 on subsequent chemotaxis

Approximately 200 age synchronised eggs were obtained as described (see section 2.3.6). The exposure to butanone was continued from egg until worms reached L4+1 stage. The lids and agar plugs were changed every 24 h for 3 days. Control group were eggs developed in the presence of food without pre-exposure to butanone. There is only one conditioning group, butanone + food. For this group, 6µl of undiluted butanone was distributed on 6 agar plugs placed on the inside of the lid directly above the OP50 lawn, so that worms are in contact with food but not butanone. The development of the worms was observed throughout the pre-conditioning phase and their development was not affected by the presence of butanone (data not shown). At this point both groups were rinsed from their plates and washed 3 times with M9 before being placed on a chemotaxis assay plate with 1µl ×10^{-1} butanone and chemotaxis indices were obtained after one hour.

For assessment of pharyngeal pumping, worms were sampled from each groups of conditioned groups. Following the conditioning treatment worms were picked from the control and butanone + food groups onto an unseeded 6 cm NGM plate and left for 1 min as a cleaning step to get rid of bacteria. Subsequently, the worms were picked onto another 9 cm unseeded NGM plate for testing against 1µl $\times 10^{-1}$ butanone. Five minutes after transfer to the testing plate, 1 µl $\times 10^{-1}$ butanone was added to the inside of the plate lid. Pharyngeal pumping was recorded for 1 min for each worm at 10, 30 & 60 min after addition of butanone.

2.3.9 Determining pharyngeal pumping in the presence of ingestible and non-ingestible microspheres

2.3.9.1 Ingestible microspheres

A stock of 3.64 x 10^{11} particles/ ml in DI sterile water stock solution of Fluoresbrite® YG Carboxylate Microspheres, ingestible beads, 0.50µm (Polysciences, Inc.), were used to investigate the effect of mechanical cue on pharyngeal pumping in an off-food context.

2.3.9.2 Non-ingestible microspheres

To assess the effect of external mechanical cue on pump rate of food-deprived worms, a stock of 2.10 x 10^{8} particles/ml Fluoresbrite® YG Carboxylate Microspheres, non-ingestible beads, 6.0µm (Polysciences, Inc.) were used in the assay.

2.3.9.3 10 min ingestible beads assay

A dilution of the beads stock solution was performed to obtain a concentration of 1×10^{9} particles/ml. Medium plates (6 cm) were used for the beads assay by pouring 10 ml of NGM and leaving it to set for 3 days without seeding them with OP50. Instead, 68.7µl of 10^{9}/ml beads in S-basal solution (6.87×10^{8} beads/cm^{2}) was added to the plate. Beads were spread evenly over whole surface of the plate by manually swirling the plate. This was verified by microscopic inspection of the plate to ensure the worms were always in contact with them. Synchronised L4 + 1 worms were transferred onto a cleaning plates

prior to transfer them onto the bead-seeded plates. Pumping rate was assessed every minute for 10 min in total.

2.3.9.4 30-120 min ingestible beads assay

A 12-well plate was seeded with 68.7µl of 10^7 beads/ml (6.87×10^6 beads/cm^2) diluted in S-basal on each well, while control plate was supplemented with S-basal only. The plate was left to dry in the fume hood for 1 hr. Synchronised L4+1 worms were picked onto cleaning plate (unseeded) for 1 min before they were transferred onto the assay plate. Pharyngeal pumping was recorded every 30 min for a total of 2 hours.

2.3.9.5 Dose-dependent effect of beads on pump rate (30-120 min assay)

Different densities of beads were used to study the effect of bead concentration on the pumping rate. A stock of 1×10^9, 1×10^8 and 1×10^7 beads/ml (6.87×10^8, 6.87×10^7 and 6.87×10^6 beads/cm^2, respectively) diluted in S-basal were prepared and 68.7µl of each dilution was added to 12-well plate. Synchronised L4+1 worms were picked onto a cleaning plate (unseeded) for 1 min before transfer onto the assay plate. Pharyngeal pumping was recorded every 30 min.

2.3.9.6 Pumping rate on non-ingestible beads assay

A stock of 1 x 10^8 particles/ml of 6.0µm non-ingestible beads were diluted into S-basal to obtain the following dilutions: 1×10^7, 1×10^6 and 1×10^5 beads/ml, 68.7µl was removed from each dilution and added to a 12-well plate to give the following densities on each well: 6.87×10^6, 6.87×10^5 and 6.87×10^4 beads/cm^2, respectively. The assay plates were left to dry in the fume hood for 1 hr before they were used. Synchronised L4+1 worms were picked onto a cleaning plate for 1 min prior to transfer them onto assay plate. Pharyngeal pumping was recorded every 30 min for a total of 2 hours following removal from food.

2.3.9.7 Assessment of body bends in the presence of beads

The plates were prepared as described in section 2.3.9.5. The assessment of body bends was defined by observing the area just behind the pharynx bending in the opposite direction and returning to its original direction (S-shape). Body bends were assessed after 30, 60, 90 & 120 min after being placed on beads.

2.3.10 Determining pharyngeal pumping in the presence of bacterial supernatant

A single bacterial colony was inoculated into 10ml LB broth and incubated at 37°C in an incubator shaker overnight. Bacteria were diluted to an OD was 0.8 and 1ml of the culture was filtered using 0.2 µm filter. 68.7µl of filtered bacterial supernatant, beads diluted in bacterial supernatant and beads diluted in S-basal were added to 12-well plate. The plate was left to dry for 1 hr before adding the worms to the wells. Synchronised L4+1 worms were picked onto a cleaning plate for 1 min prior to transfer onto assay plate. Pharyngeal pumping was recorded every 30 min for a total of 2 hours following removal from food.

2.3.11 The effect of titrating OP50 culture on pumping rate

The dose-dependent effect of *E.coli* OP50 on pharyngeal pumping was assessed. A single colony was inoculated into a fresh 10 ml LB Broth. The culture was incubated at 37º C in an incubator shaker overnight. The OD of the culture was 1.0. A 1 in 10 dilutions were performed to be obtain the following dilutions (1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9}, 1×10^{-10}) and 100µl from each diluent was added to separate wells of 12-well plate. The plates were incubated and left to dry overnight. L4+1 worms were picked onto unseeded plate to remove bacteria before transfer onto assay plates. Pharyngeal pumping rate was assayed 10 min after worms were placed on the varying dilution of bacteria.

The number of colonies in the serial dilution was determined by streaking out 100µl of each diluent onto an LB agar that was then incubated at 37°C overnight.

2.3.12 Assessing pharyngeal pumping in the presence of UV-killed bacteria

A single *E. coli* OP50 bacterial colony was inoculated into 10ml LB broth and incubated at 37°C in an incubator shaker overnight. OD of OP50 was 0.8 when measured the following day. The assay plates (6 cm) were seeded with 50µl OP50 and left at room temperature for 48 hr to allow bacterial multiplication. Subsequently, a radiated bacterial suspension obtained by placing one of the plates in UV-light incubator set at 3000 joules for 30 min. Control plates were untouched at room temperature. To confirm that bacteria was killed by UV-light, a sample was taken from treated and control plates and streaked onto separate LB agars. The streaked plates were kept in an incubator at 37°C overnight. The number of colonies on each plate was counted the following day. As expected, colonies were observed on control plates only (see Chapter 5, section 5.2.4, *Figure 5.4*).

Synchronised L4+1 worms were picked onto a cleaning plate for 1 min before transfer to assay plates. Pharyngeal pumping was recorded 10 min after transferring onto testing plates.

2.3.13 Food entry assay

To investigate the effect of entry to food on pump rate, assay plates (6 cm) were prepared with bacteria-free in the centre of the plate (3.5 cm in diameter). A band of 600µl *E. coli* OP50 was placed around the circumference. L4+1 worms were picked onto a cleaning plate for 1 min and then placed onto non-food area of the assay plates. Pumping frequency was measured until worms entered the food and for 5 min on food using an online counter (http://wormweb.org/countdown). The online counter allows to changes in pumping rate in seconds rather than using a conventional counter, Worms enter food at the head end, and food entry is defined visually as the point when the top of the nose of one worm touched the boundary of the bacteria lawn.

2.3.14 Determining the effect of exogenous dopamine application on pumping rate

Pharyngeal pumping was assessed in the presence of dopamine and 5-HT in intact and cut-head worms with exposed pharynxes.

2.3.14.1 The effect of dopamine and serotonin on pharyngeal pumping in intact worms on NGM plate

Large plates 9 cm containing 20ml of NGM were prepared and left to set for 3 days. A stock of 20mM dopamine and 40mM 5-HT were dissolved in M9 buffer. Subsequently, 2ml of dopamine or 2.5ml of 5-HT were added to assay plates to give final concentration of 2mM and 5mM of dopamine and 5HT, respectively. M9 buffer was added to control plates. The plates were covered with foil, as dopamine and 5-HT are light sensitive. The plates were incubated at room temperature overnight. L4+1 worms were picked onto a cleaning plate for 1 min before transfer to assay plates. Pharyngeal pumping was recorded 10, 30 & 60 min following removal from food.

2.3.14.2 The effect of dopamine and serotonin on pharyngeal pumping in cut head preparations

Dopamine (30µM) and 5-HT (1µM) were prepared in Dent's solution to investigate the effect of dopamine and 5-HT in cut heads. L4+1 worms were picked into Dent's solution and the heads were cut with a blade just behind the grinder (see introduction *Figure 1.2*). The cut heads were transferred to a fresh Dent's solution and their baseline pumping rate was recorded, before transferring the worms to dopamine (30µM) or 5-HT (1µM) plates. Pumping rate was recorded every 10 minutes for 1 hr in the presence of dopamine.

2.4 Behavioural assays statistical analysis

Statistical analyses were made using GraphPad Prism software. Statistical significance was set at $p<0.05$. One-Sample Student's *t*-test was used to investigate the effect of alcohols on chemotaxis index against 0. Independent Sample *t*-test was used to assess the significance difference of alcohols on off-food pump rate compared to control. One-way ANOVA was used to investigate the effect of alcohols on pump rate in the presence of food.

For butan-1-ol and butanone conditioning assays, a one-way ANOVA with Bonferroni post-test was used to test for significant difference in chemotaxis indices or pump rates between all treated groups. For long-term butanone conditioning assay, independent samples *t*-test was used to test for significance difference in chemotaxis indices or pump rates between all treated groups.

To investigate the effect of beads on pumping rate, two-way ANOVA with Bonferroni post-hoc test was used to test for significant difference between strains, wild-type compared to mutant, over time following removal from food. Similarly, the same statistical analysis was used to investigate the effect of bacterial supernatant on pumping rate.

A one-way ANOVA with Bonferroni post-test was to assess the influence of bacterial (*E. coli* OP50) dose-dependent on pumping rate. Student's *t*-test was used to test the effect of UV-killed bacteria on pumping rate.

A two-way ANOVA with Bonferroni post-test was used to compare the entry on food phenotypes of assessed mutants to control, N2-wild- type worms over time. The same statistical test was used to compare effect of dopamine and 5-HT on mutants to wild-type worms.

2.5 Microinjection of *C. elegans*

To generate transgenic worms, plasmid/cosmid DNA was microinjected into the worm's (L4+1) gonad. The injected DNA was a wild type copy of the defective gene used to rescue a mutant.

Pmyo-3::gfp (L3785) was used as a co-injection marker and lines selected based on GFP in the body wall muscle. The injection mix contained 50ng/µl of the plasmid DNA (or 15ng/µl of the *dop-4* cosmid (C52B11.4)) and 30ng/µl of the marker plasmid in a final volume of 20µl. Subsequently, the mix was centrifuged for 30 min at 14k rpm/min before injection. A 15µl of the supernatant was transferred into clean tube.

For injection, agarose pads were prepared and 1 droplet of 2% agarose solution was added to a microscopic slide. Immediately, another slide was placed on top to flatten the agarose. L4 worms were picked the day before injection to obtain fertile adults (L4+1). Before injection, the needles were prepared by pulling the aluminosilicate glass capillaries (1mm diameter) on 2000 Sutter Instrument electrode puller (program 99). The procedure

of the microjections was conducted according to Michael Koelle's microinjection protocol (http://medicine.yale.edu/lab/koelle/protocols/index.aspx). The needle was filled with the mix by capillary action. To prevent drying, the worms were immersed in Halocarbon oil. The injection mix, containing the DNA, was injected into the worm's gonads using the microinjection system (An Eppendorf TransferMan NK2 manipulator was attached to the eppendorf FemtoJet pressure system). The needle was visualised under NikonEclipse TE200 microscope (Japan) X40 magnification. Worms were injected individually and then transferred onto a clean, seeded plate following the injection procedure. The injected worms were kept at 20°C until progeny was observed. Subsequently, fluorescent worms were transferred onto a clean, seeded plates to maintain the worms with the extra-chromosomal arrays.

2.6 Assessing calcium levels in I2 neurones in the presence of dopamine

The genetically encoded calcium indicator GCaMP3 (Tian et al., 2009) was used to report neuronal activity of the pharyngeal neurones, I2, in the presence of dopamine. The transgenic strain N2; Is[Pflp15::GCaMP3] expresses GCaMP3 under the control of *flp-15* promoter was used to measure calcium level in the pharyngeal neuron I2 (Bhatla and Horvitz, 2015). L4+1 intact worms were glued on one side on a 3 cm unseeded NGM plate using Histoacryl Blue. A 300µl of M9 was added to record baseline response before adding dopamine (dissolved in M9 buffer) to give a final concentration of 2mM.

2.6.1 Optimisation of experimental conditions for measuring calcium levels in I2 neurones

Immobilization of worms by Histoacryl Blue completely inhibited pharyngeal pumping of the worm (data not shown). Furthermore, Preliminary findings have shown that I2 neurones are activated by UV light, which concealed the effect of dopamine on I2, if any. Therefore, the wild-type GCaMP strain , N2; Is[Pflp15::GCaMP3], was crossed into *gur-3 (ok2246)* (see section 2.7) to generate *gur-3; Is[Pflp-15::GCaMP3]* transgenic strain to abolish activation of I2 neurones by light. In addition, the preparation of cut heads were used instead of intact worms to avoid the use of glue on worms.

Synchronised L4+1 intact worms were placed in 3.5 cm without NGM containing Dents solution and their heads were cut to expose the pharynx. Subsequently, the worms were placed into a fresh and clean (200µl) Dent's solution. The baseline pumping rate was measured for 10min before adding dopamine (dissolved in Dent's solution) to give a final concentration of 30µM. Calcium levels were measured for 30 min following the addition of dopamine. Successively,

2.6.2 Imaging for I2 responses to dopamine

To record calcium levels in I2 neurones in response to dopamine, an inverted microscope with a 40X air objective was used. Imaging was done by stimulating the I2 neurones using 10% light intensity at 475 nm with exposure time of 100-200 ms and recording at what wave length at 5 frames per second. Videos were recorded using an EMCCD camera and analysed using ImageJ software.

2.7 Crossing worms to identify *gur-3 (ok2246); N2; Is[Pflp15::GCaMP3]* strain

gur-3 (ok2246); N2; Is[Pflp15::GCaMP3] mutant strain was generated by crossing N2; Is[Pflp15::GCaMP3] and *gur-3 (ok2246).*

2.7.1 Male generation

3 plates where 5 N2; Is[Pflp15::GCaMP3] L4 were picked and incubated at 28ºC for either 5, 5.5 or 6 hr. The plates were then stored for 3 days at 20ºC.

2.7.2 Cross

Ten males N2; Is[Pflp15::GCaMP3] positively selected for GFP expression were picked onto a plate with 4 *gur-3*(*ok2246)* adult worms (L4+1) and placed on a drop of 5µl OP50 and incubated at 20ºC for 3 days.

Ten GFP-positive males worms of the F1 generation were picked and crossed with either 4 L4 +1 *gur-3* (*ok2246)* hermaphrodite and placed on a drop of 5µl OP50 and incubated at 20ºC for 3 days. Hermaphrodite worms (30 single F2 YFP-positive) were selected and left to self-fertilise for 2-3 days (F3). Single-worm PCR was used to genotype the F2 parents.

2.7.3 Genotyping to identify homozygote GFP-positive *gur-3 (ok2246)* by single worm PCR

2.7.3.1 Lysis step

10µl of proteinase K containing lysis buffer (1µl of proteinase K for 100µl of lysis buffer) was place into 0.2µl PCR tube. Each worm was picked into PCR tube incubated at -80°C with lysis buffer for 1 hr before being placed in a thermostatically-controlled heating block (PCR machine model) under the following conditions:

65°C	1 hr
95°C	15 min
4°C	∞

Following lysis procedure of the residual reagent, Oligo (MWG Eurofins) and PCR reagents was added.

2.7.3.2 PCR

The PCR reaction was done using Expand Long template PCR system (Roche, Sigma-Aldrich, cat no: 11681834001). Recipes were designed following the manufacturer's instructions.

Primers

Forward primer	gur-3_geno_Fwd	GGCGGTGAGAGTAAGCTTTG
Reverse primer	gur-3_geno_Rev	AAATGGACGTCACCAAGGAG

Forward primer	dop-4_geno_Fwd	GTGAGGCGGCTGTTT
Reverse primer	dop-4_geno_Rev	TCAGTGCAAGGCACG

2.7.3.3 PCR reaction

The following components were brought together and mixed.

	Stock unit	Final unit	Volume (µl)/tube
F. primer	5 µM	0.3 µM	3
R. primer	5 µM	0.3 µM	3
Buffer 1	10 X	1 X	5
dNTPs	2mM	0.2 mM	5
Expand Long enzyme	150 U	2.25 U	0.75
DNA template			10
ddH_2O			24.25
Total volume			50

Following the addition of the above ingredients, the contents were lysed in the same heating block using the following cycling parameters

Reaction step	Temperature (ºC)	Time (min)
Initial denaturation	94	2 min
Denaturation Annealing Extension	94 56 72	2 0.5 4 35 cycles
Final extension	72	10
Soak	4	∞

2.7.3.4 Gel electrophoresis

10µl of the PCR was resolved on a 0.7% (w/v) agarose gel prepared in TBE buffer (45mM Tris borate, 1mM EDTA). 5µl of 10,000x Gel Red (Biotium; Cambridge, UK) was added to the molten agarose. Once it had cooled to ~ 50°C, the mixture was added to a gel mount.

10µl of PCR sample + 2µl of 5X loading buffer (5% bromophenol blue, 30% glycerol in dH2O) were loaded per well. 5µl of 1 kb DNA ladder (Promega, UK) was used. DNA amplicons and marker and were visualised using an Alpha Imager UV transilluminator.

Offspring of strains homozygote for *gur-3 (ok2246)* with 980kb deletion were selected (*Figure 2.1*). The strains *gur-3 (ok2246)* homozygote GFP-positive offspring were maintained and frozen down.

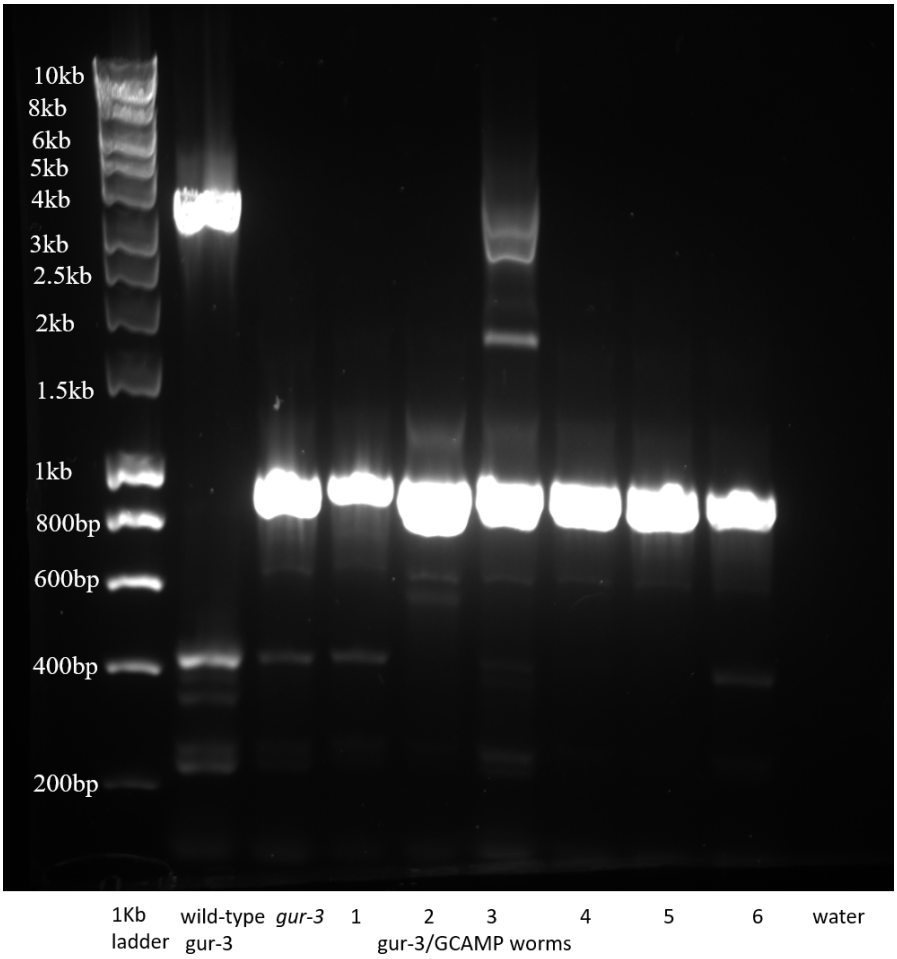


Figure 2.1: Verification of gur-3 (ok2246) homozygote in GFP-positive offspring by gel electrophoresis.

2.8 Molecular biology

2.8.1 Generation of *Pflp-15::dop-4* construct

To provide evidence for the critical role of DOP-4 receptors expressed in I2 in the mechanical stimulation of pharyngeal pumping in a rescue experiment, a construct under the control *flp-15* promoter was prepared using TOPO cloning of Taq polymerase-amplified PCR product into gateway entry vector.

2.8.1.1 Construction of pENTRY_dop-4 gateway entry vector

2.8.1.1.1 PCR amplification of *dop-4* cDNA

Primers were designed (Eurofins Genomics, Germany) to flank the *dop-4* cDNA and include the Start and Stop codon (shown in red in the sequences below).

Forward primer	dop-4_Fwd	5' AATGTTGGCTTACGGGTC 3'
Reverse primer	dop-4_Rev	5' CTATTCAATTGAAGTATTCGGCG 3'

The PCR was performed with Phusion DNA polymerase and CG buffer (NEB, UK) as indicated below:

	Stock unit	Final unit	Volume (µl)/tube
F. primer	5µM	0.5µM	5.00
R. primer	5µM	0.5µM	5.00
5X CG Buffer	10 X	1 X	10.00
dNTPs	2mM	0.2mM	5.00
Phusion enzyme	2 U/µl	1 U	0.50
DNA template	300 ng/µl	300	1.00
dH_2O			23.50
Total volume			50.00

The PCR cycles were as follow:

Reaction step	Temperature (ºC)	Time (min)
Initial denaturation	98	30
Denaturation	94	10
Annealing	56	0.5
Extension	72	1.5
Final extension	72	10
Soak	4	∞

The amplicons from this reaction was resolved on 0.7% agarose gel (*Figure 2.2*). 8µl of the total amplification volume + 2µl of 5X loading buffer was loaded into the gel. 5µl of 1 kb Bioline Hyper Ladder 1 was used.

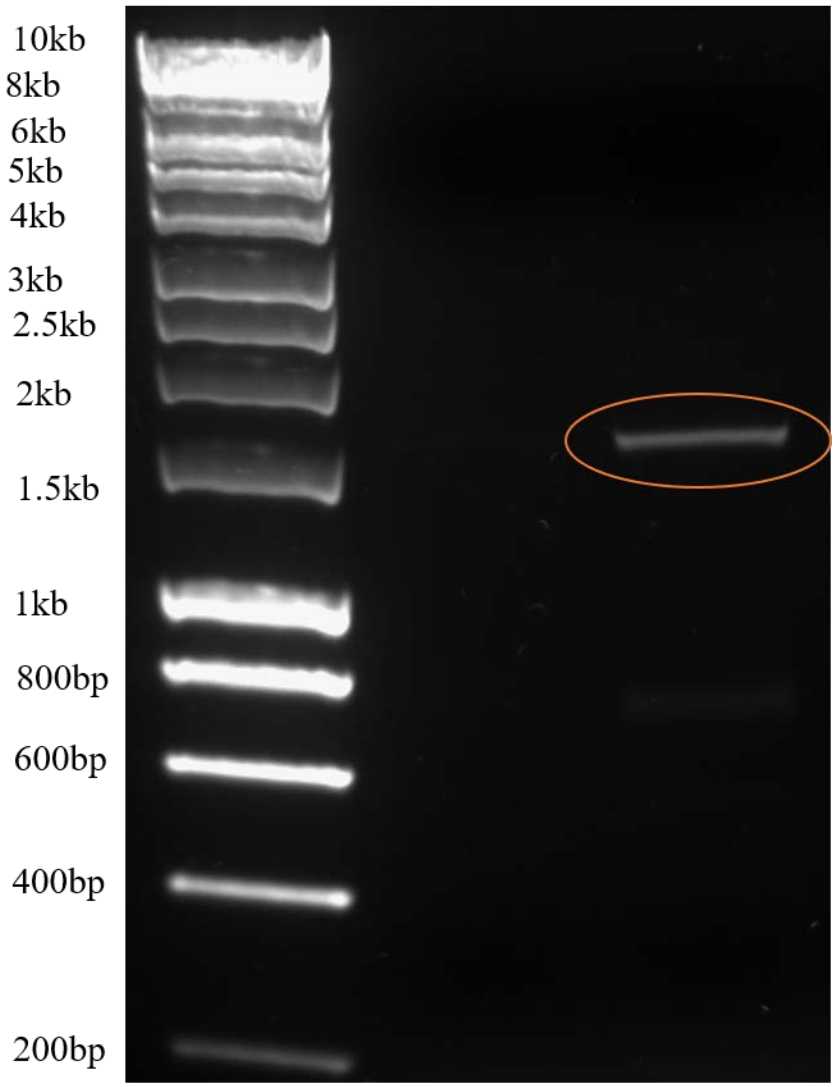


Figure 2.2: Gel electrophoresis of the amplified dop-4 cDNA. *A 0.7% agarose gel was loaded with 8µl of PCR product + 2µl of 5X loading buffer. The expected band (circled in orange) was excised.*

2.8.1.1.2 Gel purification

The remainder of the reaction was resolved on a 0.7% agarose gel and a band at 1.5 kb was excised under UV light with a sterile razor blade and immediately transferred to a clean Eppendorf tube. The DNA was eluted from the excised gel into 20µl of ddH20 using Gel Purification Kit according to the manufacturer's instruction (Qiagen).

2.8.1.1.3 Taq reaction

A Taq polymerase sub-reaction was performed on the gel-purified DNA to add a nucleotide 'A' overhang to the *dop-4* cDNA purified PCR product.

Taq pol. Buffer 10X	3µl
dATPs 100mM	1.5µl
Taq polymerase	0.5µl

The above reaction was incubated at 72˚C for 15 min in a thermocycler.

2.8.1.1.4 TOPO Ligation of dop-4 cDNA into a topovecor

The amplified products with the A overhangs were subjected to ligation reaction into pCR®8/GW/TOPO plasmid using the TA cloning kit performed according manufacturer's protocol (Invitrogen Catalogue Numbers = K2500-20).

ddH_2O	0 µl
Salt Solution	1 µl
PCR Product	4 µl
pCR®8/GW/TOPO® plsmid (Invitrogen)	1 µl
Final volume	6 µl

The reaction was incubated for 15 min at room temperature and immediately placed on ice.

2.8.1.1.5 Bacterial transformation

TOP-10, chemically-competent bacteria, were thawed on ice. 4µl of either the TOPO cloning reaction or the salt solution from the pCR®8/GW/TOPO® kit (negative control) were added to bacteria and incubated for 30 min on ice. Heat shock was performed by immersing the reaction mixture in water (42ºC) for 30 seconds before immediately placing it back on ice for 2min. 250µl of room temperature SOC medium was added and the reaction mixture and incubated with shaking for 1 hour at 37ºC. 50µl and 250µl volumes were spread over LB agar plates containing a final concentration of 100µg/ml Spectinomycin. Plates were incubated at 37ºC overnight to allow transformants to grow. There were no colonies found in the salt solution plates (control). On the other hand, colonies were observed in the plates containing bacteria treated with ligation product. Colonies were picked into 7.5ml of LB containing 100µg/µl Spectinomycin overnight in incubator shaker at 37ºC.

2.8.1.1.6 Restriction mapping of putative dop-4 cDNA

2.8.1.1.6.1 Mini-preps of dop-4 cDNA

Qiagen column kit was used to purify high quality plasmid dop-4 cDNA. Positive colonies from TOPO ligation (see section 2.8.1.1.4 above) were picked and cultured in 10 ml of LB containing Spectinomycin (100µg/µl) at 37ºC overnight.

2.8.1.1.6.2 Restriction digest

Restriction digest was performed to confirm the presence of the *dop-4* cDNA insert. The restriction enzymes, *Msc1* and *BmgB1*, described below produced a band at 1.5kb, corresponding to the predictive dop-4 cDNA.

Master Mix

Plasmids from individual colonies were incubated with restriction enzyme as shown below:

Stock	Volume (µl)
NEB *MScI* 5000 U/µl	1.0
NEB *BmgBI* 5000 U/µl	1.0
Buffer 3.1 (10X)	5
Plasmid	5.9
ddH_2O	37.1
Final volume	50

The digests were incubated 2.5 hours at 37ºC. A 2.5µl of 5X loading buffer was added to the digest (10µl) before being resolved on a 0.7% agarose gel. 5µl of Bioline Hyper Ladder 1 was used to determine the size of the fragments.

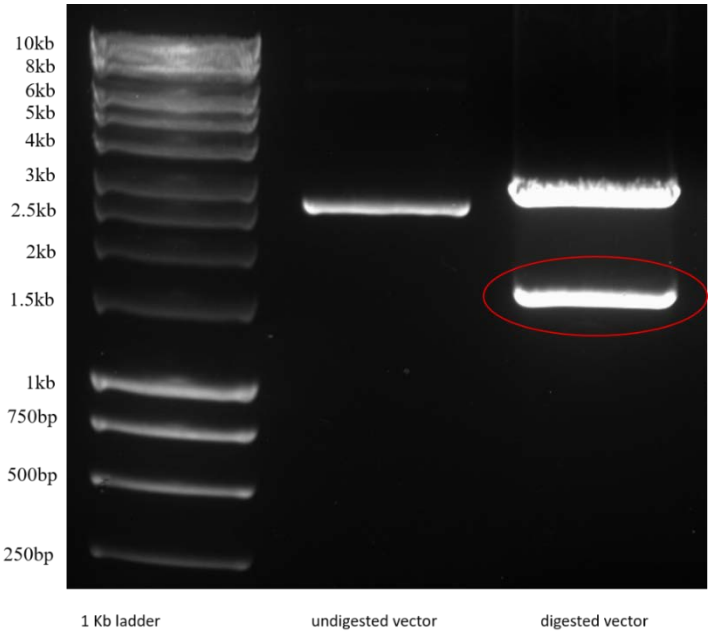


Figure 2.3: Restriction digest of a dop-4 cDNA in the TOPO vector by gel electrophoresis. *10µl of DNA mixed with 2.5µl of 5X loading buffer was loaded on a 0.7% agarose gel. A 1.5 kb band of dop-4 cDNA insert (circled in red) was observed in*

the gel. The restriction enzymes, MSc1 and BmgB1, were used to cut the plasmid at the recognition site to release the dop-4 cDNA insert (1.5 kb). The EcorI digest plasmid should give two bands as expected; dop-4 cDNA at 1.5kb and the plasmid at 2.8kb.

2.8.1.1.6.3 Sequencing

Qiagen column kit was used to purify high quality plasmid DNA from a colony in which the MSc1 and BmgB1digestion described above described an insert of 1.5kb corresponding to the predictive dop-4 cDNA. The authenticity of the dop-4 cDNA clone was verified by sequencing (Eurofins Genomics, Germany) on both forward and reverse strands (*Figure 2.3*).

2.8.1.2 Generation of dop-4 cDNA in pNB19 construct

Following the amplification of *dop-4* cDNA PCR product using the pENTRY plasmid, the *dop-4* cDNA was prepared to be inserted into the NB19 plasmid.

2.8.1.2.1 Restriction digest of dop-4 cDNA & NB19 plasmids

An individual digest for TOPO, containing *dop-4* cDNA, and NB19 plasmids were performed as shown in the table below. The digestion reaction was incubated for 4 hours at 37ºC before resolving it on a 0.7 % agarose gel. The 10µl digest volume was supplemented with 2.5µl of 5X loading buffer before the total volume was added to the gel. 5µl of 1 kb Bioline Hyper Ladder 1 was used to identify the size of the fragments.

dop-4 TOPO plasmid digest	
ddH_2O	11.9µl
NEB *MScI* 5000 U/µl	1µl
NEB *BmgBI* 5000 U/µl	1 µl
BSA 10X 1µg/µl	1µl
dop-4 Topo-plasmid (731.7 ng/µl)	4.10µl
NEB Buffer 3.1 (10 X)	2µl
Total volume	20 µl

NB19 plasmid digest	
ddH_2O	11.9µl
NEB MScI 5000 U/µl	1µl
NEB BmgBI 5000 U/µl	1µl
BSA 10X 1µg/µl	1µl
NB19 plasmid (802.2 ng/µl)	4.10µl
NEB Buffer 3.1 (10 X)	2µl
Total volume	20µl

2.8.1.2.2 Gel Purification

A 2.5µl loading buffer (5X) was added to the two separate digests before resolve on a 0.7% agarose gel. The expected band sizes for *dop-4* cDNA is 1.5kb and 5.4kb for NB19 plasmid were confirmed against the DNA ladder before excise the bands under UV light with a clean razor blade and placed into separate Eppendorf tubes. The DNA was eluted from the excised gels into 20µl of ddH_20 using Gel Purification Kit according to the manufacturer's instruction (Qiagen).

2.8.1.2.3 De-phosphorylation

Shrimp Alkaline Phosphatase (SAP) (USB, product number: 78390) was used to remove phosphate group from 5'-ends of the NB19 DNA. The procedure was used according to the manufacturer's protocol. The reaction was prepared in 20µl as follow:

	Final Stock	Final volume (µl)
NB19 DNA (empty plasmid)	1 µg	3.43
10X SAP reaction buffer	1X	2
SAP	1 unit/µl	1
Water, nuclease-free	N/A	19
Final volume		20

Subsequently, the reaction mixture was incubated at 37°C for 60 min. The reaction was terminated by heating it at 65°C for 15 min in a thermocycler to inactivate SAP.

2.8.1.2.4 Ligation reaction

The purified *dop-4* cDNA and the de-phosphorylated NB19 plasmid were ligated using T4 ligase (Promega, UK). The ligation reaction ratio was 3:1 (insert to plasmid):

ddH_2O	3.12µl
De-phosphorylated NB19 plasmid (59.7 ng/ µl)	1.68µl
dop-4 cDNA (110.9 ng/ µl)	1.2µl
T4 Ligase	0.5µl
T4 Buffer 10 X	1µl
PEG (6000) 20%	2.5µl

The ligation reaction was incubated for 6 hours at room temperature (20°C). Two separate control plates were prepared; one with all the above components except PEG and the

second control plate contained all the above components except of adding water (ddH_2O) instead of T4 enzyme and buffer.

2.8.1.2.5 Bacterial transformation

DH5α chemically competent bacterial cells were thawed on ice. 5µl of each ligation reaction (1 treated and 2 controls) was added to bacteria and incubated for 30 min on ice. Heat shock was performed by transferring the reaction mixture to water (42ºC) for 30 seconds before immediately placing it back on ice for 2 minutes. 250µl of room temperature SOC medium were added and the bacteria were incubated at 37ºC and shaken for 1 hour. Subsequently, 50µl and 250µl of the mix were spread over LB agar plates containing Ampicillin (50µg/ml).

Subsequently, the plates were left to dry for 10 min and incubated at 37ºC overnight to allow successful transformants to grow. There were no colonies observed on control plates (bacteria transformed with ligation reactions that contained no T4 ligase enzyme). Colonies were observed on plates containing bacteria transformed with the reactions made in the presence of T4 enzyme.

2.8.1.2.6 Restriction mapping of dop-4 insert orientation

The ligation reaction of *dop-4::NB19* is a blunt-ended ligation. Therefore, a restriction mapping was needed to differentiate between the required orientation of 5'– 3' compared to 3'-5' and empty vector. The following reaction steps were performed:

2.8.1.2.6.1 Crude mini-prep of DNA of dop-4::NB19 construct

14 colonies from *NB19::dop-4* (T4) ligation were picked and cultured in 10ml of LB containing Ampicillin (50µg/ml) and incubated at 37ºC overnight. Crude mini-prep was carried out as described 2.8.1.1.6.1). Potential plasmid containing colonies were identified by restriction digest as indicated below.

2.8.1.2.6.2 Restriction digest

Following the purification of *NB19::dop-4* plasmid, the plasmid was digested using *KpnI* (Promega). The restriction enzyme, *Kpn1*, cuts at 2 sites if *dop-4* cDNA was taken by the NB19 plasmid (regardless of *dop-4* orientation) and cuts once if it an empty plasmid. The expected band sizes are: empty plasmid: 5.4kb, corrected orientation (5' – 3'): 6.5kb and 445bp and wrong orientation (3'– 5'): 5.4kb and 1.5kb (Figure 2.4).

Master Mix

Individual colonies were incubated with restriction enzyme as shown below:

Stock	Volume (µl)
Kpn1 ((12U/µl)	0.5
Buffer J 10X	1
BSA 10X (1µg/µl)	1
Crude-prep product	1
ddH_2O	5.5
Final volume	10

The digests were incubated 2 hours at 37ºC. A 2.5 µl of 5X loading buffer was added to the digest (10µl) before being resolved on a 0.7% agarose gel. 5µl of Bioline Hyper Ladder 1 used to determine the size of the fragments.

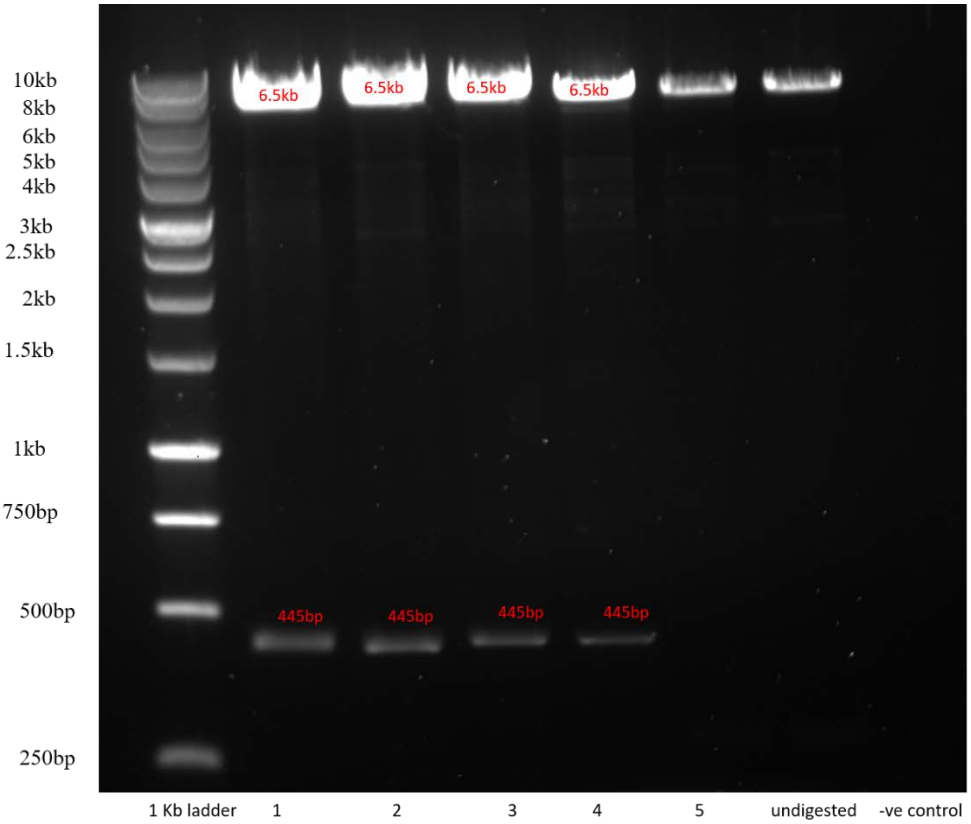


Figure 2.4: Restriction mapping of the dop-4 following NB19 ligation. *The ligated products were digested by KpnI restriction enzyme and loaded on a 0.7% agarose gel. A 10µl of DNA mixed with 2.5µl of 5X loading buffer was loaded onto a 0.7% agarose gel. 5µl of 1kb ladder (Promega, UK) was added. The expected bands in the right direction 5' -3' were 445bp and 6.5kb. Undigested band was NB19 vector and negative control used was water.*

2.8.1.2.6.3 Mini-preps and sequencing

Qiagen column kit was used to perform mini-preps on a colony exhibiting the digestion of dop-4 band (1.5 kb). The authenticity of the *NB19::dop-4* plasmid was verified by sequencing (Eurofins Genomics, Germany) and confirmed *dop-4* cDNA on the forward strand.

2.8.2 DOP-4 cosmid rescue

2.8.2.1 Supply of cosmid

Cosmid (C52B11.4, 40 kb in size) was received in LB culture from The Wellcome Trust Sanger Institute.

2.8.2.2 Bacterial transformation and mini-preps

A sample was taken from the cosmid stock and streaked onto LB containing ampicillin (50µg/ml) of. The plates were incubated at 37ºC overnight. Qiagen column kit was used to perform mini-preps on selected colonies.

2.8.2.3 PCR

The PCR reaction was done using GoTaq® G2 Hot Start Green Master Mix (Promega, cat no: M7422). Recipes were designed following the manufacturer's instructions.

2.8.2.4 Primers

Forward primer	*dop-4*_geno_Fwd	GTGAGGCGGCTGTTT
Reverse primer	*dop-4*_geno_Rev	TCAGTGCAAGGCACG

2.8.2.5 PCR reaction

The PCR reaction was performed with Phusion DNA polymerase and CG buffer (NEB, UK) as followed:

	Stock unit	Final unit	Volume (µl)/tube
F. primer	5µM	0.5µM	5.00
R. primer	5µM	0.5 µM	5.00
5X CG Buffer	10 X	1 X	10.00
dNTPs	2mM	0.2mM	5.00
Phusion enzyme	2U/µl	1 U	0.50
Cosmid	146ng/µl	146ng	1.00
Nuclease-free H_2O			23.50
Total volume			50.00

The contents were mixed and subjected to the following cycling parameters in a regulated heating block (machine).

Reaction step	Temperature (ºC)	Time
Initial denaturation	98	30 sec
Denaturation	98	10 sec
Annealing	56	30 sec
Extension	72	1.5 min
Final extension	72	10 min
Soak	4	∞

The reaction was resolved on 0.7% gel to confirm the authenticity of the dop-4 cosmid. Genomic DNA from wild-type and *dop-4 (tm1392)* null mutant were assessed, as positive and negative controls, respectively, alongside DNA from *dop-4* cosmid. The expected

bands are as follow: wild-type dop-4 = 1.5kb, *dop-4* = 1kb and cosmid 1.5kb, respectively (*Figure 2.5*).

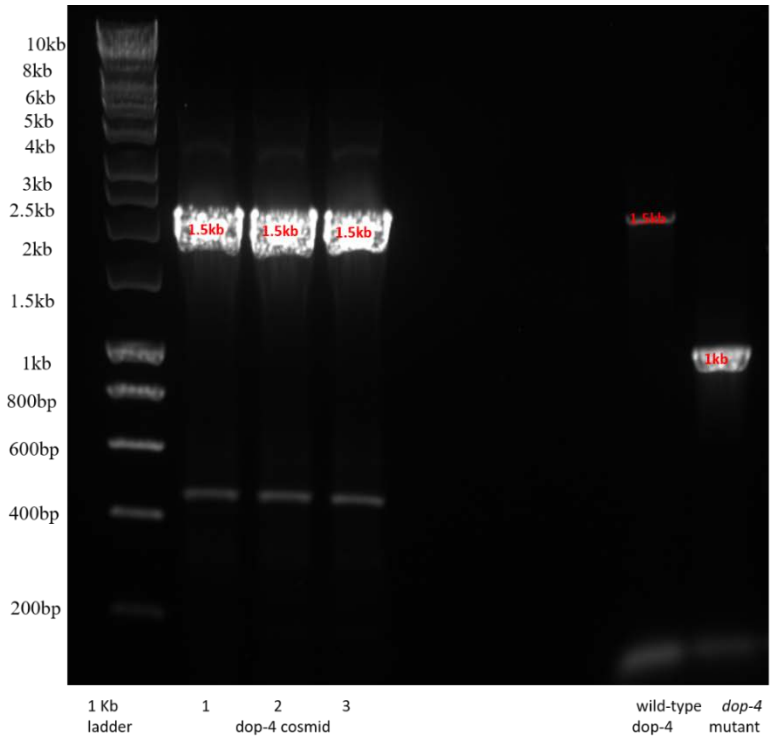


Figure 2.5: Verification of dop-4 DNA cosmid by gel electrophoresis. *Genomic DNA from wild-type and dop-4 (tm1392) worms were amplified. The bands expected are indicated in red. 1.5 kb for dop-4 cosmid and wild-type dop-4 and 1kb for dop-4 mutant.*

Chapter 3: The effect of volatile alcohols on innate feeding behaviour

3.1 Introduction

Bacteria and other microbes living in the soil release chemicals such as small alcohols, ketones and esters as metabolic by-products (Dainty et al. 1985; Zechman and Labows 1985). These chemicals can act as cues to organisms (e.g. *C. elegans*) seeking and assessing the suitability of bacteria as a food source (Bargmann 2006).

A chemotaxis assay can be used to measure the responses of *C. elegans* to chemicals. The chemotaxis assay, is based on the worms' innate behaviour to locate food and avoid toxins in their environment. The chemotaxis assay can be used for populations or single worms to determine behaviour. The chemotaxis population assay is measured by establishing a gradient of compound from a point source and observing the accumulation of worms at the compound source. Chemotaxis assays have been widely used to measure chemosensory behaviours, such as water-soluble molecules or volatile alcohols in *C. elegans*. They were first developed by Ward (Ward 1973) to measure detection of various metals by *C. elegans* and later modified by (Bargmann et al. 1993). Furthermore, the assay has been used to investigate plasticity (Ardiel and Rankin 2010) in *C. elegans*. Volatile cues are well suited to be investigated in a chemotaxis assays. Unlike water-soluble chemicals which diffuse through the agar in order to establish a gradient (Ward 1973), volatile alcohols diffuse through the air and quickly form gradients detected by *C. elegans*, thus, worms immediately orient their movement towards the source of the volatile alcohol. This is qualitatively demonstrated by observing the accumulation of worms under the odorant source when the odour is added inside of the lid of a Petri dish with the worms present on the agar below (Bargmann et al. 1993). They also showed that chemotaxis efficiency is independent of whether the odorant was placed on the lid or the agar. For that reason, the test is quick and efficient and is completed within 1 hr of placing the odorant on the agar or inside of the lid (Bargmann et al. 1993).

The dependent variable in chemotaxis assays is usually a measure of locomotion e.g. measuring the speed at which the worm approaches a point source, or by calculating the distribution of a population of worms at the odorant or food spot compared to their starting point. Therefore, chemotaxis assays reflect on the foraging aspects of feeding behaviour. In contrast, there has been little investigation of how these environmentally salient cues (volatile alcohols) modulate the pharyngeal pumping that executes the ingestion arm of feeding behaviour.

Since *C. elegans* is found in fermenting environments where volatile alcohols are released and given the established observations that alcohols drive a chemotaxic response, it is also of interest to understand how these fermentation products produce responses that modulate the pharyngeal system that supports feeding.

Is this chapter I describe the responses of *C. elegans* to volatile alcohols, Methanol to Nonan-1-ol, by analysing their effect on chemotaxis and pumping rate. The aim of screening these alcohols is to select a suitable odorant cue for subsequent conditioning experiments to cue-dependent plasticity in pharyngeal pumping as an important sub-behaviour in the overall feeding behaviours. To achieve this, two distinct contexts were investigated, pharyngeal pumping in presence and absence of food. First the ability of a chemical series of alcohols to elicit chemotaxis was determined and subsequently the effect on pharyngeal pumping in the context of this assay was investigated.

3.2 Results

3.2.1 Effect of alkyl chain length on alcohol chemotaxis

The selective effect of alcohols to produce an attractant or aversion chemotaxic response has been shown to depend on the dose and chemical structure of the cue (Bargmann et al., 1993). To provide a systematic characterisation of this a chemical series of alcohols with an increasing chain length were tested in a chemotaxis assay. Alcohols are organic compounds in which the hydroxyl (-OH) functional group is bonded to a saturated carbon atom. The assay involved placing 1µl×10^{-6} M, 1µl×10^{-5} M, 1µl×10^{-4} M, 1µl×10^{-3} M, 1µl×10^{-2} M, 1µl×10^{-1} M, and 1µl×10^{0} M on the agar for each alcohol, as described in Methods section 2.3.6. The compounds were methanol, ethanol, propan-1-ol, butan-1-ol, pentan-1-ol, hexan-1-ol, heptan-1-ol, octan-1-ol, and nonan-1-ol. For each alcohol tested the chemotaxis assay result represents the mean of 2 experiments in which 150 worms were studied for each compound across the range of doses specified. The data allow the

identification of the most potent alcohols either negatively or positively affecting chemotactic behaviour by the calculation of a chemotaxis index for each dose. The essay measures chemotaxis relative to ethanol control. An index of 0 indicates a neutral effect, a positive value up to a maximum of +1 implies an attractive cue when all the worms are on the agar position at which the alcohol was placed (*Figure 3.1A*). In contrast, a negative value up to a maximum -1 indicates an aversive cue when all the worms are on the control position i.e. the ethanol spot.

Methanol had a low positive CI at an undiluted dose only ($p < 0.0001$, *Figure 3.1*B) with a small significant difference from zero ($p = 0.037$) indicating it is an attractant at this high dose. Lower doses of methanol had no effect on CI. Ethanol is a neutral compound as its chemotaxis indices were not significantly different from zero ($p = 0.563$, *Figure 3.1C*). Additionally, all the doses tested were not significantly different from zero ($P > 0.05$, *Figure 3.1C*). Therefore, as described previously there is no effect of ethanol verifying its suitability as a neutral cue.

At low doses ($1\mu l \times 10^{-6}$ – $1\mu l \times 10^{-2}$ M) propan-1-ol had no impact on CI ($P > 0.05$, *Figure 3.1D*). However, at high doses, $1\mu l \times 10^{-1}$ M and $1\mu l \times 10^{0}$ M, the CI was positive indicating propanol is an attractant ($p = 0.027$ & 0.013, respectively, *Figure 3.1D*). High doses ($1\mu l \times 10^{-2}$ – $1\mu l \times 10^{0}$ M) of butan-1-ol had a large positive CI, thus indicating this is a robust attractant cue, with maximal attraction observed at 10^{-2} with an average CI of 0.78 ($p < 0.05$, *Figure 3.1E*).

Pentan-1-ol had a small positive CI over all the doses range tested with a peak attractiveness at 10^{-2} ($p = 0.004$, *Figure 3.1F*). The 6-carbon chain alcohol, hexan-1-ol, showed a clear shift from a negative CI of -0.17 at undiluted dose, to a positive CI of 0.43 at $1\mu l \times 10^{-1}$ M as doses increased (*Figure 3.1G*). There was a significant peak CI observed at $1\mu l \times 10^{-3}$ M and $1\mu l \times 10^{-2}$ M, with average chemotaxis indices of 0.77 and 0.83, respectively ($P = 0.041$, *Figure 3.1G*).

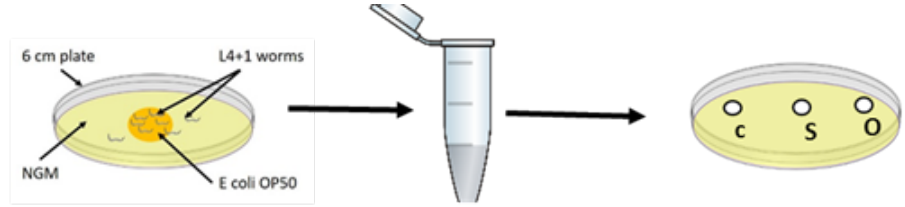
Heptan-1-ol did not give a positive CI at any of the doses tested, since none of the doses tested were significantly different from zero. Thus, the analysis indicates it is a neutral cue ($p = 0.667$, *Figure 3.1H*).

Octan-1-ol had a dose-dependent effect on CI ($p = 0.001$, *Figure 3.1I*). At $1\mu l \times 10^{-1}$ M and $1\mu l \times 10^{0}$ M ($P = 0.033$, *Figure 3.1I*) the CI was negative indicating it is an aversive cue, whilst at lower doses there was no significant effect indicating it is neutral ($P > 0.05$, *Figure 3.1I*).

Finally, the longest carbon chain tested, nonan-1-ol had no effect on CI. Nonan-1-ol is an aversive cue, as indicated by a negative chemotaxis at higher doses (*Figure 3.1I*), where it has a positive to no effect at lower doses. However, there is no significant differences detected.

Overall, these data on the effect of different chain length alcohols on CI showed a shift from attractants for the lower chain length to aversion for the longer chain length compounds.

A

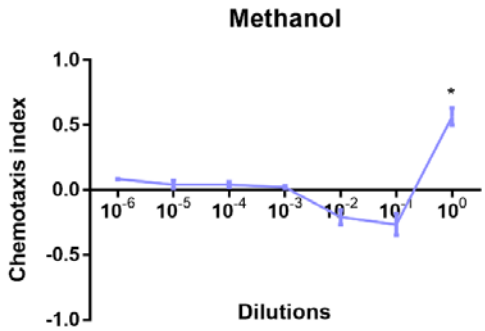


Synchronised well-fed adult worms

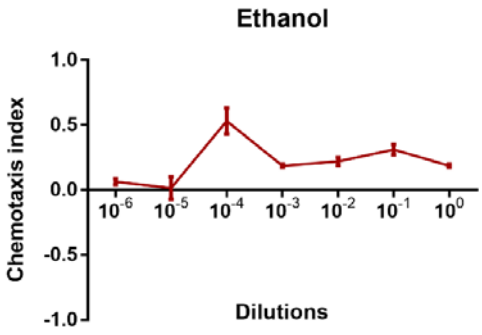
Washed with M9 (X3)

Testing phase = 9 cm plate
S = worms' starting point
O = alcohol spot
C = control (ethanol) spot

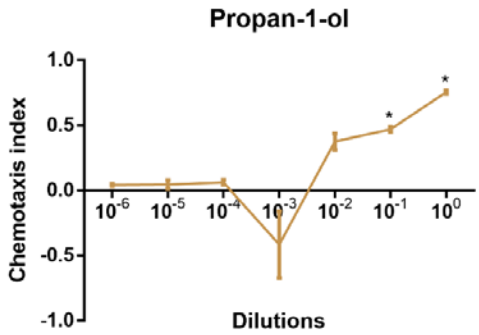
B



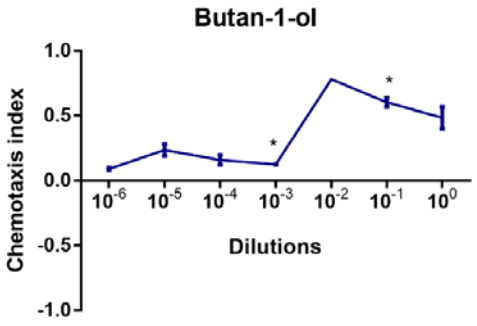
C



D



E



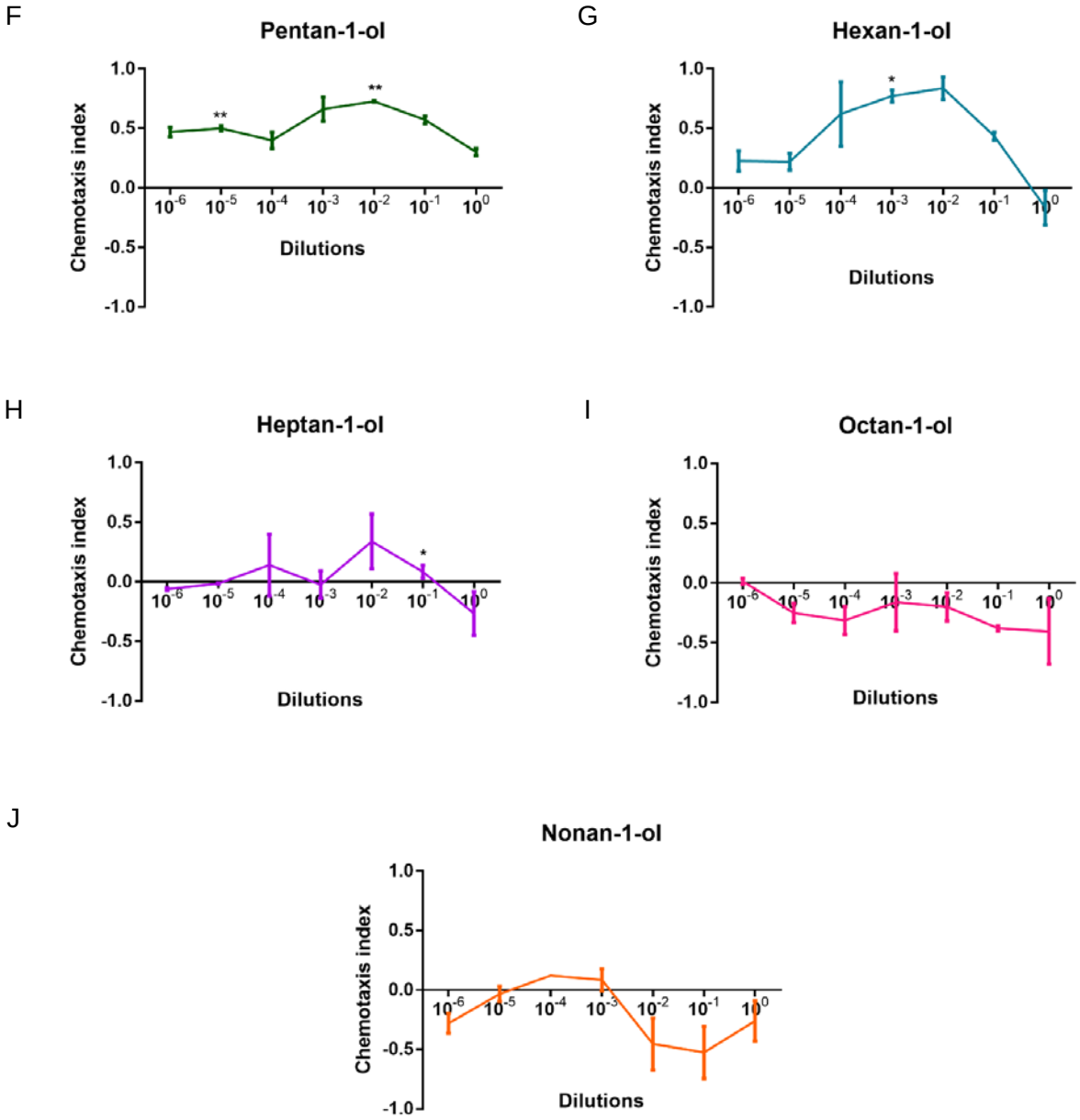


Figure 3.1: Determining the dose-dependent effect of alpha-carbon alcohols on chemotaxis index. *A) A schematic diagram of the layout of the chemotaxis experiment. Approximately 150 L4+1 worms were washed and placed on start point within equidistant from the odorant's and control (ethanol) on 9 cm test plates. One microliter of sodium azide was placed on both control's and odorant's sides. At the end of the test (60 min) the chemotaxis index was calculated. B-J) A chemotaxis index above 0 = attractive odorant, below 0 = repulsive odorant and at 0 is neutral. The experiment was repeated twice. One-sample t-test for relative significance effect of dose from zero. Significance difference from*

*zero is indicated for each dose. *** p < 0.001, ** p < 0.01 & * p < 0.05. Error bars represents mean± S.E.M*

3.2.2 Pharyngeal pumping during alcohol chemotaxis in the absence of food

The analysis above highlights that straight chain alcohols mimic aspects of a *C. elegans* food cue and may be detected to prompt foraging strategies important in *C. elegans* feeding behaviour. To investigate if this chemosensory detection of alcohols impact on the pharyngeal system, pharyngeal pumping was scored by visual observation as the worm approached the odorant (alcohol). It has been previously shown that placing an L4+1 worm on a non-food arena resulted in inhibition in pumping rate initially due to mechanical stimulus from picking (Dallière et al., 2016). Following recovery from picking, a two phase response to absence of food was recorded (Dallière et al., 2016). An early phase between 0-120 min, in which an inhibition of pumping rate was observed, whereas a late phase between 120-500 min, in which pumping rate remained inhibited in general, but a great variation in pumping rate was observed (Dallière et al., 2016). In view of this study, the pharyngeal pumping was assessed in the presence of odour and absence of food, while the worms undergoing a chemotaxis towards a point source of alcohol. Pharyngeal pumping was measured within the first 30 min of assay. This was compared to pump rates of worms at the start point, which are treated as control and they were 1.9cm away from alcohol point source (*Figure 3.2A*). Additionally, pumping rate was recorded within 0.5 cm away from the alcohol spot, thus the worms were not in contact with the alcohols, as the aim was to investigate the effect of olfaction on pumping rate.

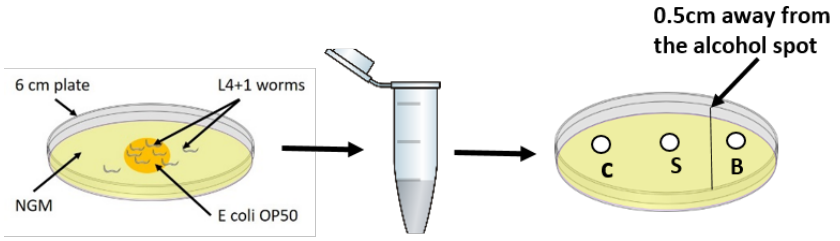
At undiluted dose of methanol (1µl $\times 10^{0}$), pumping rate within 0.5cm away from methanol, was inhibited (31± 3 ppm, p = 0.037, *Figure 3.2B*) compared to worms at the starting point (41± 2 ppm), with 1.9cm away from the odorant. At lower doses of 1µl $\times 10^{-1}$ - 1µl $\times 10^{-6}$ M, there was no differences in pumping rate between worms near the methanol point source and the worms at the starting point. Ethanol had no impact on pumping rate at all doses tested, when pumping rate was measured within 0.5cm away from ethanol (p > 0.05, *Figure 3.2C*). This observation further supports the use of ethanol as a neutral compound in chemotaxis assay. Conversely, propan-1-ol at doses of 1µl$\times 10^{0}$ M (17±3 ppm, p = 0.037), 1µl$\times 10^{-1}$ (27±5 ppm, p = 0.037) and 1µl$\times 10^{-1}$ (31±4 ppm, p < 0.0001) reduced pharyngeal pumping compared to worms at the starting point (42±4, *Figure 3.2D*).

Butan-1-ol also showed a marked reduction in pumping rate at high doses of $1\mu l \times 10^{0}$ M (10±1 ppm, $p < 0.0001$), $1\mu l \times 10^{-1}$ M (20±2 ppm, $p < 0.0001$) and $1\mu l \times 10^{-2}$ M (11±4, $p < 0.0001$) compared to worms at the starting point (29±3 ppm, *Figure 3.2D*). At lower doses ($1\mu l \times 10^{-3}$ - $1\mu l \times 10^{-6}$) butan-1-ol had no pumping rate compared to worms at the starting point. Furthermore, pentan-1-ol suppressed pumping rate at all doses with average pumping rate of 10±2 ppm ($p < 0.001$, *Figure 3.2F*) compared to worms at the starting point pumping rate at around 32±4 ppm. In case of hexan-1-ol, low doses of hexan-1-ol ($1\mu l \times 10^{-6}$ – $1\mu l \times 10^{-2}$) showed no effect on pump rate ($p > 0.05$, *Figure 3.2G*). While at a higher dose of $1\mu l \times 10^{-1}$, a reduced pumping was observed (7±2 ppm, $p = 0.002$) compared to worms at the starting point (27±4 ppm). At the undiluted dose of $1\mu l \times 10^{0}$, a reduction in pumping rate was observed but there was not significantly different to worms at the starting point.

Heptan-1-ol significantly reduced pharyngeal pumping at all doses tested (8±3 ppm, $p < 0.0001$, *Figure 3.2H*) compared to worms at the starting point (32±5 ppm). Similarly, at high doses ($1\mu l \times 10^{-2}$ – $1\mu l \times 10^{-0}$) of octan-1-ol, pumping rate was inhibited (10±3 ppm, $p < 0.0001$, *Figure 3.2I*) compared to worms at the starting point. Nonan-1-ol reduced pharyngeal pumping at doses $1\mu l \times 10^{-5}$ – $1\mu l \times 10^{-0}$ (7±2 ppm, $p < 0.0001$) compared to worms at the starting point. (30±4, *Figure 3.2J*). With the exception of the lowest dose (10^{-6}) which had no effect on pharyngeal pumping ($p = 0.83$).

In conclusion, short carbon chain alcohols have a modest effect on pharyngeal pumping at high doses only, when worms are undergoing chemotaxis to a point source of alcohol in the absence of food. Ethanol has no impact on pharyngeal pump rate. However, with exception to pentan-1-ol, which reduced pumping at all doses, medium-chain alcohols reduced pharyngeal pumping at high doses only. Similarly, long chain alcohols, heptan-1-ol and nonan-1-ol inhibited pharyngeal pumping while modest effect observed in the presence of octan-1-ol at high doses only. Taken together, a negative impact of straight chain alcohols on pharyngeal pumping is observed at increasing concentrations.

A

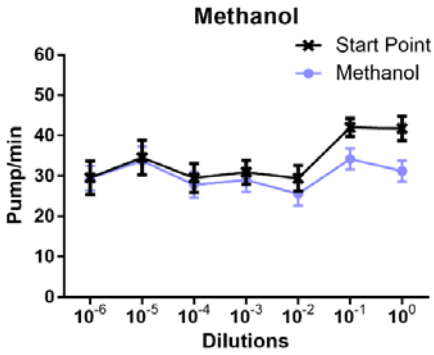


Synchronised well-fed L4+1 worms

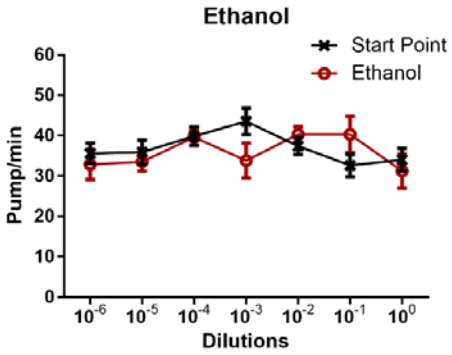
Washed with M9 (X3)

Testing phase
Plate = 9cm
S = worms' start point
O = odorant (alcohol)
C = control (ethanol= 1µL)

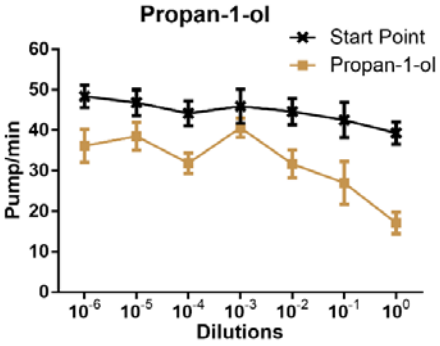
B



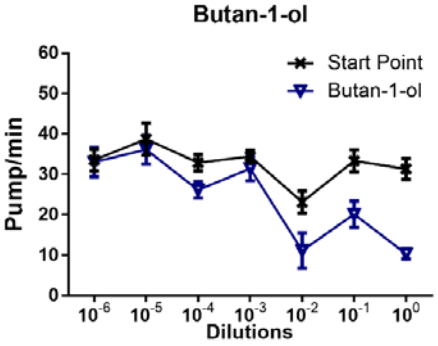
C



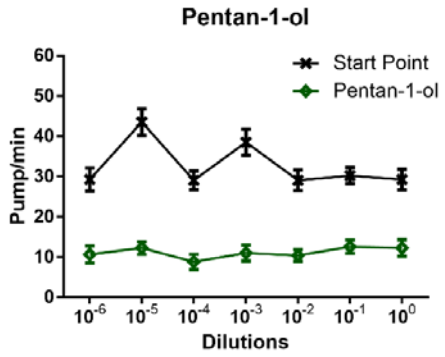
D



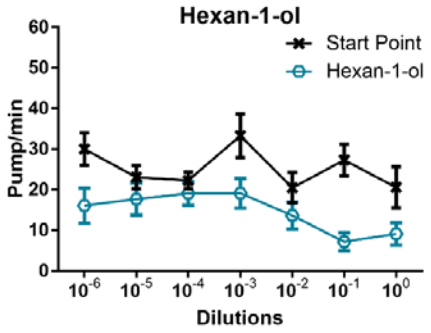
E



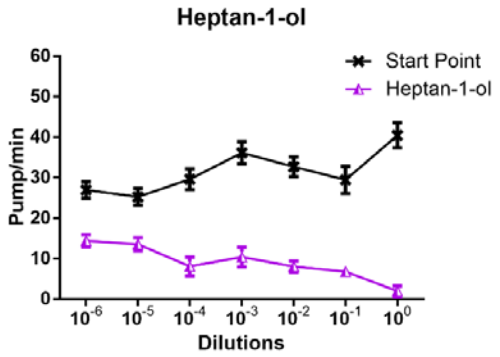
F



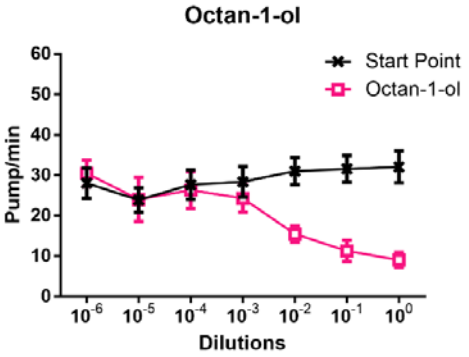
G



H



I



J

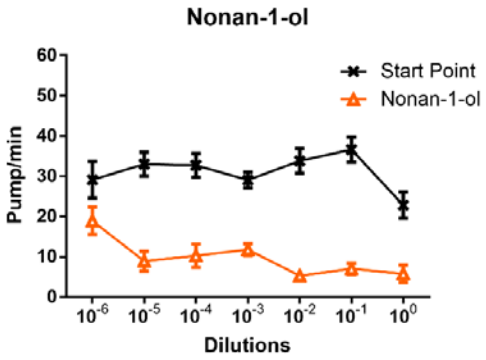


Figure 3.2: Determining the effect of dose of compound on pharyngeal pumping rate during chemotaxis assay in the absence of food. *A) A diagram represents the layout of the experiment. 150 L4+1 worms were placed on start point within equidistant from both the odorant's and control's spots (1.9 cm) testing plates (9 cm). Pharyngeal pumping rate was recorded within the first 30min of the chemotaxis assay (n=8-19 worms). The experiment was repeated twice. B-J) The effect of each dose of alcohol on pharyngeal pumping compared to worms at the starting point. A small effect on pump rate was observed in the presence of methanol and propan-1-ol, with no effect for ethanol. Medium and long chain*

alcohols (butan-1-ol – nonan-1-ol) reduced pharyngeal pumping compared to control. The experiment was repeated twice. Two-way ANOVA was used to analyse the overall differences between compound and control on pumping rate. The test was followed with post-hoc Bonferroni to investigate the effect of each dose of compound on pumping rate compared to start point (control). Error bars represent Mean±S.E.M.

3.2.3 Determination of pharyngeal pumping in the presence of alcohol and food

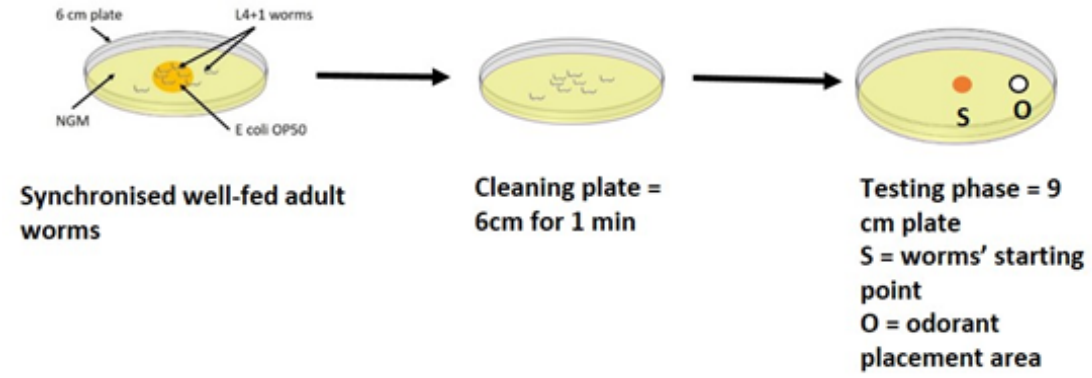
Pharyngeal pumping displays a clear context-dependent function in which pumping on food is upregulated via a number of neuromodulatory pathways (see Chapter 1: general introduction). To extend the investigation of the modulatory role of the alcohols listed above, their ability to modify pharyngeal pump rate on food was investigated.

In view of the data described above in which high concentrations of the medium and long chain alcohols modified pump rate, the focus for this investigation was high doses of alcohols on pumping rate in the presence of food. In these experiments worms were placed on an OP50 food lawn and the interaction with alcohol was tested by spotting undiluted alcohol at a distance similar to that used to drive a chemotaxis response (*Figure 3.3*), or worms were placed on a food lawn and alcohols were spotted inside of the lid with 0.7cm away above the worms (*Figure 3.4A*).

3.2.4 The distal effect of alcohols on pharyngeal pumping

To investigate the effect of alcohols placed at a distance on pumping rate in the presence of food, worms were first picked onto chemotaxis assay and placed on food at the centre of the plate (*Figure 3.3A*). Worms were left to recover from picking-mediated inhibited pumping rate to normal pumping rate with an average 250 ppm, which is line with previous studies. Subsequently, the odorant was added to the agar plate within 1.9cm away from the food lawn. pumping rate was recorded after 10 or 60 min, following the addition of the odorant. All pump rates were recorded on food. However, there was no effect on pumping rate after 10 or 60 min, when the odorant was placed on agar away from the worms (*Figure 3.3A*).

A



B

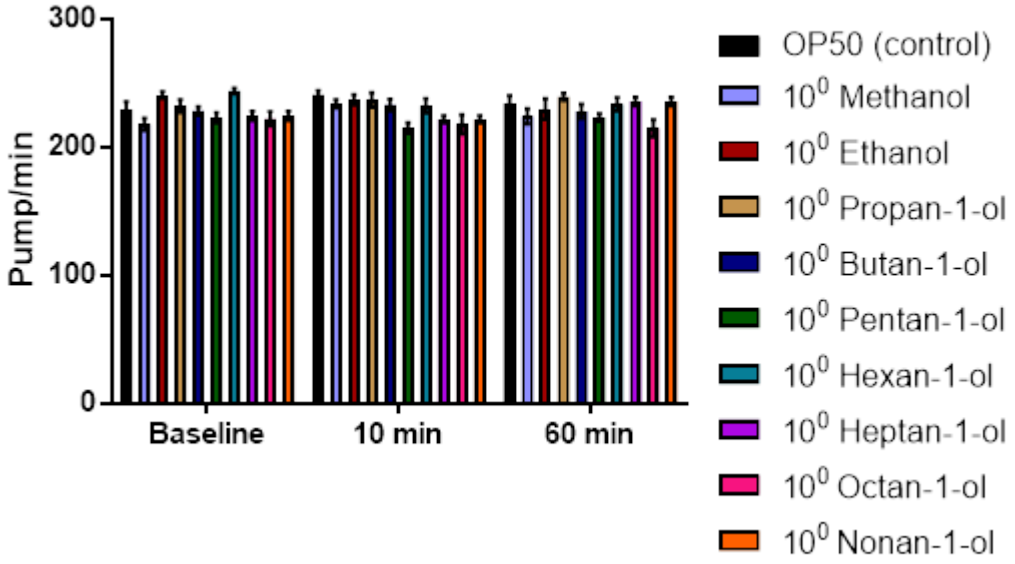


Figure 3.3: The effect of alcohols on pharyngeal pumping when alcohols were presented on the agar. *A) 15 L4+1 worms were picked and placed onto cleaning plate for 1 min before placed on a food lawn of a chemotaxis assay plate (1.9cm). The baseline pumping rate was recorded. Subsequently, alcohol was added to the agar plate within 1.9cm away from the food lawn (start point). Pumping rate was recorded 10 and 60 min after the addition of alcohols. B) One-way ANOVA followed by Bonferroni test revealed no significant ($p > 0.05$, $n = 10$ worms/ group) difference between treated groups and baseline control at 10 or 60 min on pharyngeal pumping. The experiment was repeated twice. One-way ANOVA followed by Bonferroni post-hoc test was used. Error bars represent mean±S.E.M.*

3.2.5 The proximal effect of alcohols on pharyngeal pumping

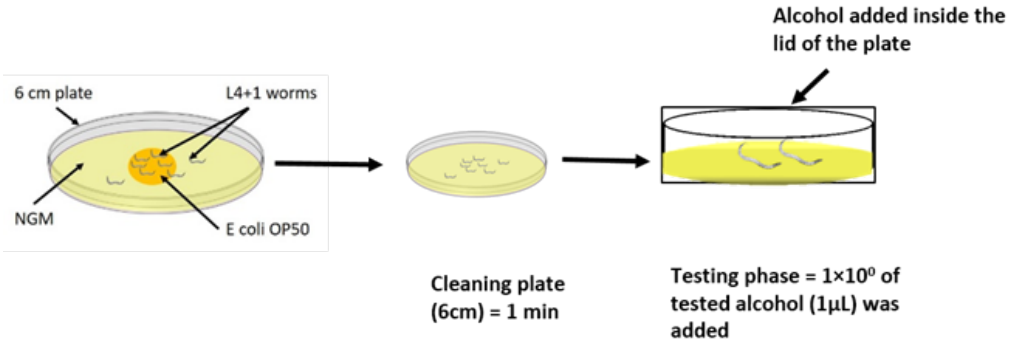
As described above (section 3.2.4), the addition of alcohol to the agar had no effect on pharyngeal pumping when it was placed at a distance from the worm. For these further experiments to examine the effect of a more proximal alcohol cue on pharyngeal pumping was tested. This was achieved by placing a spot of alcohol on the inside lid of the Petri dish. The alcohol was placed above the food lawn that had about 0.7cm clearance from the worms (*Figure 3.4A*). The lid was left on during the experiment to prevent the evaporation of alcohol. For the experiment, the baseline of pharyngeal pumping was recorded with an average of 250 ppm. Once the alcohol was added to the lid of the plate, pharyngeal pumping was measured at 10 and 60 min after the addition of the alcohols to parallel the time course used in the agar spot assay.

The addition of short carbon chain (methanol - propan-1-ol) did not affect pump rate compared to control (*Figure 3.4B*). Moreover, worms did not leave the food lawn during the assay. On the other hand, pharyngeal pumping rate was inhibited at 10 min in the presence of butan-1-ol (6±4 ppm, $p < 0.0001$) and pentan-1-ol (0±0 ppm, $p < 0.0001$) compared to baseline of 239 and 223 ppm, respectively. However, at 60 min pharyngeal pumping was restored to higher levels in the presence of butan-1-ol comparable to baseline pump rate (235±7, $p > 0.05$, *Figure 3.4*). While in the presence of pentan-1-ol, pharyngeal pumping increased but remained significantly low (53± 12, $p < 0.0001$). Furthermore, worms remained on food during the test in the presence of butan-1-ol and pentan-1-ol.

Hexan-1-ol, heptan-1-ol, octan-1-ol and nonan-1-ol completely inhibited pharyngeal pumping at 10 min (0±0 ppm, $p < 0.0001$) compared to baseline of 229, 244 and 247 ppm, respectively. Pumping rate was not restored pumping rate at the end of the test (60 min). Furthermore, worms moved away from the food lawn, at 60 min, 2-3 out 10 worms were on-food. The worms seemed alive because they moved when they were prodded.

In conclusion, these data suggest that short chain alcohols have a neutral effect on pumping rate regardless of the distance of alcohols from worms in the presence of food. Medium chain alcohols have a modest effect on pumping when added within a short distance (0.7cm), whereas long chain alcohols had toxic effect on worms as they inhibited their feeding behaviour when added within a short distance (0.7cm).

A



B

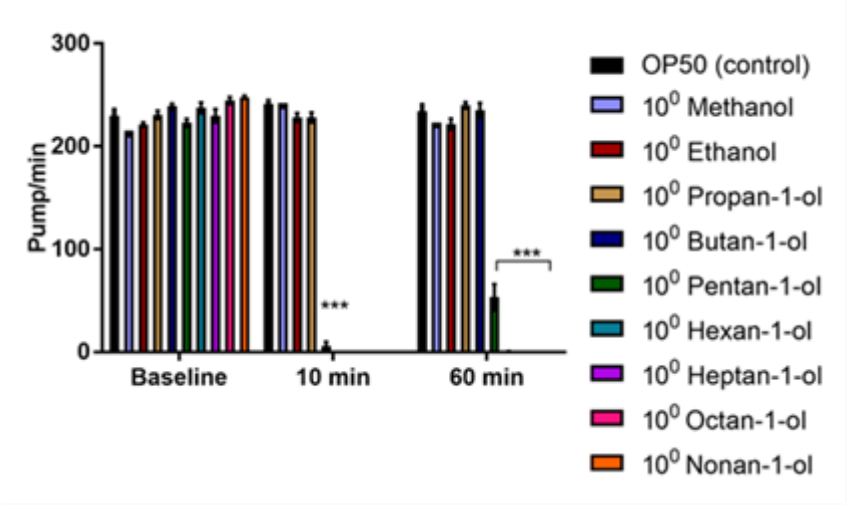


Figure 3.4: The effect of alcohols on pharyngeal pumping rate when the alcohol was placed on the lid of the agar plate. A) *15 L4+1 worms were picked and placed onto a clean seeded plate. The baseline pumping rate was recorded. Alcohols (odorant) as indicated in the inset were added to the lid of the agar plate within 0.7 cm away from food lawn. B) Pumping rate was recorded at 10 and 60 min after the addition of alcohols. There was no significant effect of methanol, ethanol and propan-1-ol on pharyngeal pumping rate (p>0.05, n= 10 worms/ group). The addition of butan-1-ol, hexan-1-ol, pentan-1-ol, heptan-1-ol, octan-1-ol and nonan-1-ol had significantly affected pharyngeal pumping rate (p<0.0001). The experiment was repeated twice. One-way ANOVA followed by Bonferroni post-hoc test was used. Error bars represent mean±S.E.M. ***p < 0.0001.*

3.3 Discussion

3.3.1 The impact of alcohols on chemotaxis index depends on the number of the chain length

In general, the chemotaxis assay findings indicate that *C. elegans* responses to short chain alcohols suggest they are weak attractants at high doses and neutral at low doses (*Figure 3.1*). While, the medium chain alcohol, butan-1-ol, pentan-1-ol and hexan-1-ol are attractive cues. In the case of long chain alcohols, octan-1-ol and nonan-1-ol are repellent cues whereas heptan-1-ol is an exception since it is a neutral compound. Hence, the data suggest that the length of carbon chain is an important determinant of the nature of the cue (neutral, attractant or repellent).

In chemotaxis assays, Bargmann & al. (1993) investigated the responses of wild-type *C. elegans* to 120 volatile compounds and observed a similar pattern to the data reported in this chapter. Their data suggest that as the carbon-chain increases in size, alcohols become either weaker attractants or repellents (Bargmann et al., 1993) with medium chain length compounds categorised as attractive cue to *C. elegans.*

Worms detect chemicals in the environment by sensory neurons in the ciliated amphid sensory organ located in the head region (see Chapter 1: section 1.6.1). The amphid gustatory neurons ASE detect water-soluble compounds (Bargmann and Horvitz, 1991). For volatile alcohols, AWA and AWC mediate attraction (Bargmann et al., 1993). AWB and ASH mediate avoidance to repellent compounds. Bargmann and colleagues (1993) showed that propan-1-ol and medium chain length alcohols may be sensed by both AWC^{ON} and AWC^{OFF} neurones. they have shown that when worms were pre-exposed to isoamyl alcohol had a reduced chemotaxis index to isoamyl alcohol as well as propan-1-ol, butan-1-ol and pentan-1-ol in subsequent chemotaxis assay. Therefore, the results might suggest that propan-1-ol, and medium chain alcohols are sensed by both AWC^{ON} and AWC^{OFF} neurones.

3.3.2 Alcohols affect chemotaxis in a dose-dependent manner

Although the carbon chain length is important in detecting the compound presented, the dose of the compound is also an important determinant of chemotaxis index. Propan-1-ol

is an attractant at high doses whereas medium chain alcohols have high chemotaxis indices at lower doses. For example, butan-1-ol has a chemotaxis of 0.78 at 10^{-2} and hexan-1-ol had a chemotaxis index of 0.83 at the same dose. Hexan-1-ol is a neutral cue at high dose. Pentan-1-ol has a peak attraction at the same dose. However, medium chain alcohols have a reduced chemotaxis index at high doses compared to low doses.

3.3.3 Alcohol modulation of pharyngeal pumping is independent of their effect on chemotaxis

High doses of alcohols inhibited pump rate in the absence of food and this was related to both increasing dose and chain length. Short chain alcohols have less impact on pump rate off-food, whereas medium carbon chain alcohols, butan-1-ol and hexan-1-ol have moderately reduced pump rates at high doses. However, pentan-1-ol is an exception to this generalisation as an inhibition was observed at all doses. Long chain alcohols inhibited pumping rate in similar pattern to pentan-1-ol. Interestingly, the inhibition of off-food pumping is independent of the ability of alcohols to positively modulate chemotaxis. For example, methanol and propan-1-ol are neutral cues but have a negative effect on pharyngeal pumping. Medium chain alcohols are attractive cues with high chemotaxis indices but reduced pharyngeal pumping. The reason for this is unclear, but it could be that reduced chemotaxis efficacy and pump rate at high doses indicate that several neurones play a role and that high concentrations of some alcohols are aversive, hence the negative impact on locomotion and pharyngeal pumping.

To further understand the inhibition of pump rate of high doses of alcohol, their impact on pharyngeal pumping in the presence of food was investigated. The experiment was carried in two formats; alcohols were placed on agar (1.9cm away from worms placed on food) or inside on the lid of plate (0.7cm away from worms on food) in a chemotaxis assay plate. The on-agar experiment revealed that alcohols do not affect pump rate when placed distantly from worms. This could mean that the odours released by the food lawn mask the presence of alcohols when presented at a distance, since the data above (*Figure 3.1)* showed that worms can detect and drive chemotaxis towards alcohols placed at a similar distance. On the other hand, the experiment in which the alcohols were placed adjacent to the worm by spotting the dose onto the lid of the Petri dish showed that diffusion of alcohols within a close proximity affected pump rate depending on the number of carbon in the chain (*Figure 3.4*). High doses of medium chain alcohols reduced pumping rate in the presence of food similar to that observed in absence of food (*Figure 3.2E-G*), which

suggests they might be associated with cross-sensitisation resulting in reduced pump rate.

A striking observation is that long chain alcohols that were aversive in the chemotaxis assay were also aversive in these pharyngeal experiments, inhibiting pumping on food and eliciting a food-leaving response. This is consistent with the reduction in pharyngeal pumping that has been associated with noxious environments and harmful bacteria (Li et al., 2012). For example, the aversive compound, quinine, suppresses pump rate when added inside of the lid of plate in the presence of food (Li et al., 2012). This reduced pumping rate is thought to activate ASH neurones, which leads to the release of octopamine from RIM/RIC interneurons, thus resulting in suppression of pharyngeal pumping on food.

3.3.4 Alcohols suppress pharyngeal pumping in the absence and presence of food

Worms in an off-food arena exhibit a low pump rate associated with the off-food context. This basal rate (average 30-40 pump/min) undergoes a modulation with increasing time that a worm finds itself off food. The time window of the current off-food experiments are consistent with the low steady-state levels of pharyngeal pumping observed by (Dallière et al., 2016).

Surprisingly, even though alcohol cues are released by bacteria and may provide a signal to allow worms to forage for feeding sites, they do not stimulate pharyngeal pumping in a similar fashion to that seen on food. This is most notable for butan-1-ol, which has a high CI but which does not stimulate pumping. This means the chemosensory pathways that may anticipate an interaction with food do not drive the pharyngeal response that is associated with the filter feeding and mastication of the food.

In the presence of food, our findings reveal no positive modulation of alcohols on pump rate. However, it was previously reported that increased pharyngeal pumping was observed following the addition of diacetyl to the inside of the lid in the presence of food by upregulating 5-HT release to facilitate feeding behaviour (Li et al., 2012).

C. elegans detect volatile alcohols and water-soluble compounds by chemosensory neurons, which are localised in the ciliated amphid. The role of cilia-deficient mutants (*che-3, osm-3, osm-6, che-3, che-12* and *che-13)* in modulation of pharyngeal pumping

was investigated (Dallière, et al, 2015, unpublished data). These genes play roles in either the formation and/or maintenance of cilia. These mutants all showed high pharyngeal pumping in the presence of food similar to N2 wild-type worms (Dallière, et al, 2015, unpublished data). These findings suggest that olfaction is not required for stimulating pharyngeal pumping rate in the presence of food but they could play a major role in reducing pharyngeal pumping when food is removed. The data from the current experiment further support the above findings that the smell of attractive odours (olfaction) modulate feeding behaviour in the absence of food and may be required for foraging behaviour to guide worms to locate food in the environment.

3.3.5 Conclusion

Having established precise measures of chemotaxis index and pharyngeal pumping, butan-1-ol, was selected as a cue in a pairing paradigm experiment with food to investigate the subsequent effect on chemotaxis and feeding behaviour. Butan-1-ol was chosen because its chemotaxis indices do not have a ceiling effect that allow comparison of chemotaxis efficiency before and after the pairing experiment with food. Additionally, unlike other attractants, butan-1-ol has a small effect on pumping rate, which allow investigation of impact of pairing with or without food on on-food and off-food context.

Table 2: A summary of the effect of alpha-carbon hydrolate alcohols on locomotion versus on-food and off-food.

Carbon length	Olfactory Cue	Overall effect on locomotion assay (type of cue)	Overall effect on pump rate assays		
			Off-food pump rate	On-food pump rate	
				On agar	On lid
Short carbon chain	Methanol				
	Ethanol				
	Propan-1-ol				
Medium carbon chain	Butan-1-ol				
	Pentan-1-ol				
	Hexan-1-ol				
Long carbon chain	Heptan-1-ol				
	Octan-1-ol				
	Nonan-1-ol				

Table 3: Colour coding key based on the largest absolute effect of alcohols on chemotaxis and pumping rate.

Chemotaxis	Pump rate	Key
Strong attractant CI>=0.75	stimulation	
Weak attractant 0.25<=CI<0.75	Moderate stimulation	
Neutral -0.25<=CI<0.25	No effect	
Weak repellent -0.75<=CI<-0.25	Moderate inhibition	
Strong repellent CI<-0.75	Complete inhibition	

Chapter 4: Investigating pharyngeal pumping in olfactory cue-dependent acquired behaviour

4.1 Introduction

In the previous chapter, it was shown that *C. elegans* can detect straight chain alcohols and that the impact of these alcohols on CI depends on the dose and number of carbon atoms. Moreover, *C. elegans* are attracted to medium chain alcohols (butan-1-ol – pentan-1-ol), with butan-1-ol as a potent attractant which has a minimal effect on pharyngeal pumping in the presence and absence of food.

Previous studies have shown that *C. elegans* can form simple associations, when a cue is paired with or without food, which results in altered CI. Naïve worms have an innate attraction towards NaCl, an attractive water-soluble cue (Saeki et al., 2001). However, when worms were conditioned for 4h in the presence of NaCl in a non-food context, worms navigate away from NaCl (Saeki et al., 2001). On the other hand, when NaCl was presented in an on-food context, worms increased their chemotaxis efficiency to NaCl in a subsequent test (Wen et al., 1997). Naïve worms chemoattract to NaCl by amphid chemosensory neurones, ASE, (Bargmann and Horvitz, 1991) while ASH mediates aversion. Tomioka et al. 2006 showed that ASE neurones play a major role in salt avoidance. However, other chemosensory neurones such as ASH, ADF, ASI might detect other environmental cues such as prolonged absence of food, which can be required to drive plasticity of salt avoidance behaviour (Hukema et al., 2006). Moreover, worms conditioned in the presence of propan-1-ol, an attractive olfactory cue, and HCl, an aversive gustatory cue, propan-1-ol was avoided in the subsequent chemotaxis assay (Amano and Maruyama, 2011). Although naïve worms chemoattract towards propan-1-ol. In a different conditioning paradigm, pre-exposure of worms in the presence of an

aversive olfactory cue, nonan-1-ol, and attractive gustatory cue, KCl, resulted in worms driving a positive chemotaxis towards nonan-1-ol in the following test (Amano and Maruyama, 2011). Moreover, worms enhanced their CI in a subsequent chemotaxis assay, when they were pre-exposed to butanone in the presence of food (Kauffman et al. 2011). These findings indicate that worms show an experience-dependent plasticity of a cue preference based on the nature of unconditioned stimulus, an aversive or an appetitive cue.

These studies have utilised CI to study odour preference learning in *C. elegans*. However, the impact of pre-exposing worms in the presence of an attractive cue on an on-food context on subsequent feeding behaviour in the absence of food has not been investigated. Therefore, based on the observation obtained in Chapter 3, that *C. elegans* can detect butan-1-ol and drive a positive chemotaxis toward this olfactory cue, we were interested to explore the utility of butan-1-ol in a conditioning paradigm in an on-food context. The ability of an appetitive conditioning paradigm to modulate and reflect an associative learning at the level of the pharyngeal system was investigated. This was done to probe the worm for a model of plasticity in feeding behaviour. In parallel, the impact of such a pairing paradigm on CI was studied.

4.2 Results

4.2.1 Chemotaxis to butan-1-ol did not increase following pre-exposure to butan-1-ol and food

The impact of pre-conditioning worms in the presence of food and butan-1-ol on CI and pharyngeal pumping to butan-1-ol was investigated by developing and optimising different conditioning paradigms.

4.2.2 Chemotaxis to butan-1-ol remained unchanged when butan-1-ol is added to food on agar

The first conditioning paradigm utilised the natural tendency of worms to navigate towards food and attractive cues (e.g. butan-1-ol (see Chapter 1 section 1.5.1). Therefore, food and butan-1-ol was added at a distance from worms' starting point to investigate if worms undergo behavioural changes in the presence of such combination on chemotaxis and

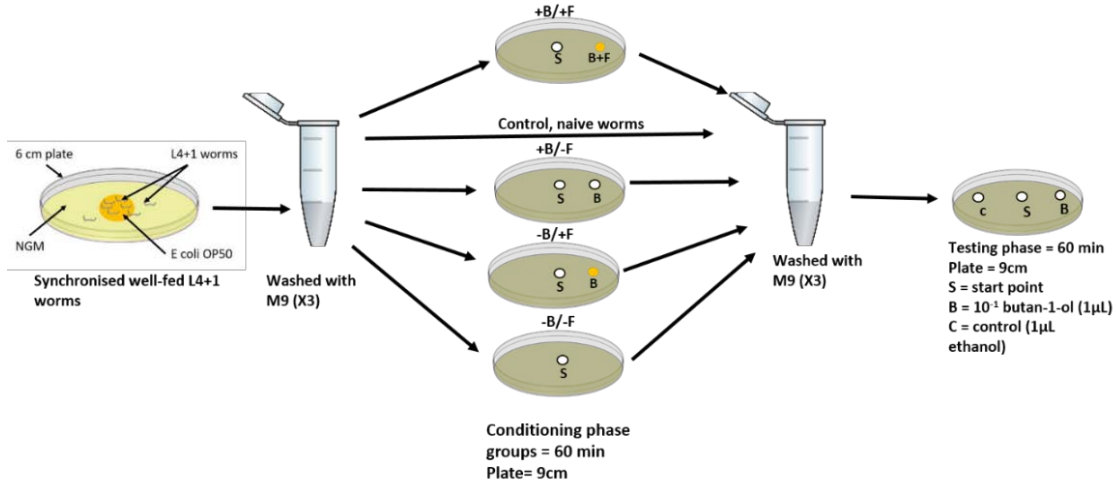
pharyngeal pumping. The timing of the conditioning phase set for 60 min was investigated. The conditioning timing was chosen based on previous data that observed plasticity in chemotaxis efficiency after prolonged exposure to a structurally similar compound, butanone (Torayama et al. 2007; Kauffman et al. 2011).

C. elegans were exposed to one of four different treatments; 1µl of 1×10^{-1} butan-1-ol and food, 1µl of $1\times10^{-}$ butan-1-ol only (in the absence of-food), food only and a blank plate (no food and no butan-1-ol). Worms were placed in the centre of the plate with 1.9cm distance from the conditioning spot (*Figure 4.1A*). Following the conditioning phase, worms were washed and transferred to the testing plate to measure their responses to 1µl of 1×10^{-1} butan-1-ol. After 60 min the CI was calculated for each group (chemotaxis assay- Chapter 2, section 2.3.6.).

Worms showed the highest CI (0.95 ± 0.03, *Figure 4.1*B) to a combination of food and butan-1-ol, measured during the conditioning phase compared to food alone. This reflects that worms respond to a combined cue, which supports that supplementing food with an appetitive odour (butan-1-ol) enhances CI compared to food alone group. In a similar manner, worms showed enhanced CI in the presence of food and butan-1-ol relative to butan-1-ol alone group, reinforcing the idea that the experimental design is not confounded by potential ceiling effects. Despite the modulatory role of butan-1-ol supplementation as a conditioning cue to the unconditioned food cue, there was no enhanced CI observed in the testing phase, when the different groups of worms were compared (*Figure 4.1B*). Worms conditioned in the absence of food and butan-1-ol for 60 min showed a positive CI index to butan-1-ol (CI = 0.55) in testing phase similar to that observed in naïve well-fed worms (CI = 0.52). Therefore this suggests that starvation has no impact on butan-1-ol response to naïve worms, as reported previously (Colbert and Bargmann, 1997).

Failure to observe a change in subsequent CI following this pre-conditioning regime, the conditioning time was extended from 60 to 90min to find the optimum conditioning time. However, a similar pattern was observed when conditioning time was extended to 90 min using the same conditioning regime and groups (data not shown).

A



B

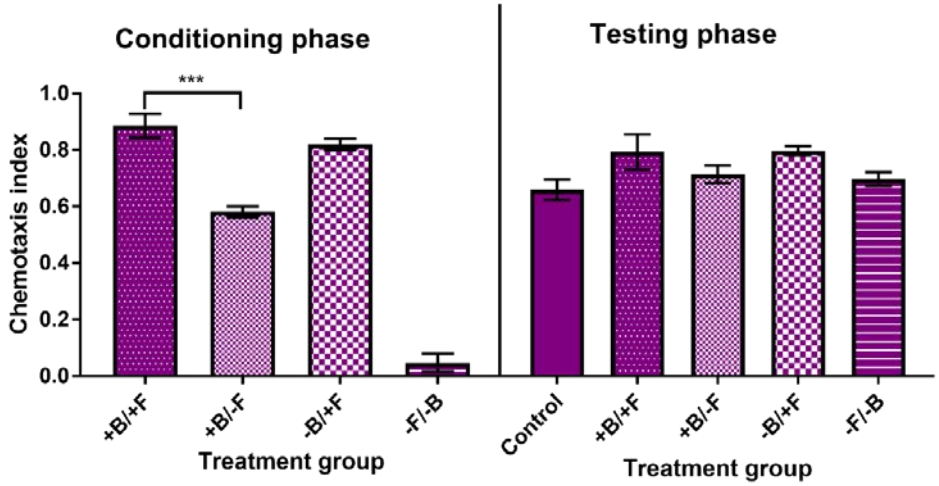


Figure 4.1: Chemotaxis to butan-1-ol did not enhance chemotaxis following conditioning worms in the presence of food and butan-1-ol. *A) A schematic representation of the butan-1-ol conditioning assay.150 L4+1 worms were washed with M9 and added to conditioning plates (9 cm) with or without OP50 patches (food) supplemented with or without butanol (1 µl of 1×10^{-1} M). Butan-1-ol was added / food was added to the plate (+F/+B), butan-1-ol was added/ no food was added, (+B/-F), no butan-1-ol was added/ food was added (-B/+F) and no butan-1-ol/no food added (-B/-F). Control group was tested with no previous exposure to butan-1-ol. After 60min, chemotaxis index to 10^{-1} butan-1-ol was calculated. (B) The effect of pre-conditioning worms to butan-1-ol in the presence of food for 60min on CI. Worms conditioned with butan-1-ol and food had a higher CI compared to butan-1-ol and no food (0.95 ± 0.03, p < 0.0001). In the testing, there was no significant difference between groups (p =*

0.286). The experiment was repeated 5 times. One-way ANOVA followed by Bonferroni post-hoc test was used. Error bars represent mean±S.E.M.

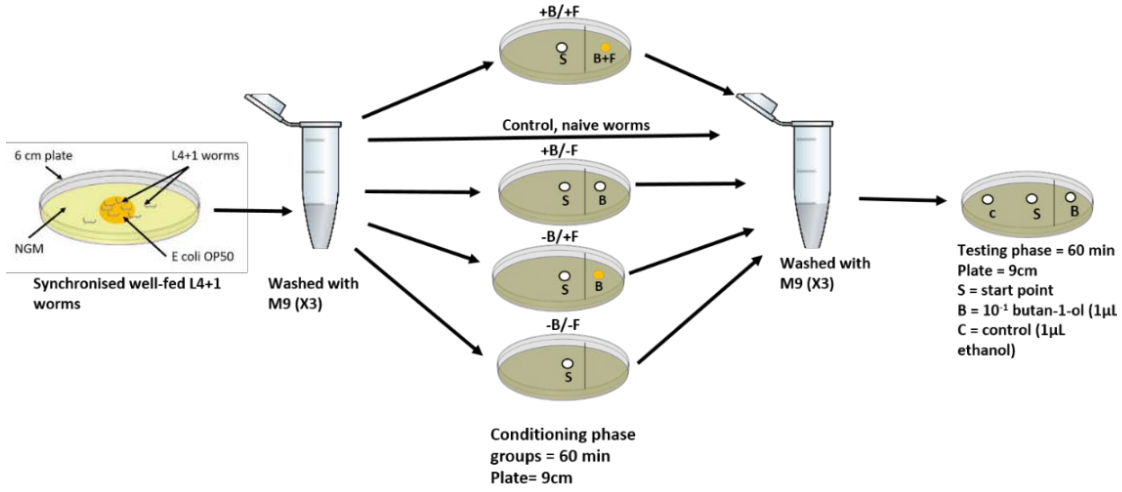
4.2.3 Preconditioning worms to butan-1-ol and food did not affect pharyngeal pumping when butan-1-ol was added to agar

The impact of conditioning worms in the presence of butan-1-ol and food on pharyngeal pumping was assessed during both conditioning and testing phases. Pharyngeal pumping was measured as worms approaching the conditioning spot in the conditioning phase and butan-1-ol spot in the testing spots (0.5cm distance from treated spot, *Figure 4.2A*). This was done to observe any underlying modification of butan-1-ol on pumping rate in the absence of food in both phases.

In the conditioning phase, butan-1-ol and food group showed no effect on pharyngeal pumping compared to off-food group, despite an enhanced CI to butan-1-ol and food (*Figure 4.2B*). Similarly, there was no effect on pharyngeal pumping observed between the treatment group in the conditioning and testing phase. Similar findings were observed when conditioning time was extended to 90min (data not shown).

These experiments highlight that *C.elegans* is able to detect the olfactory cue, butan-1-ol, but this was not able to drive behavioural plasticity in the testing phase at both pharyngeal pumping and chemotaxis level.

A



B

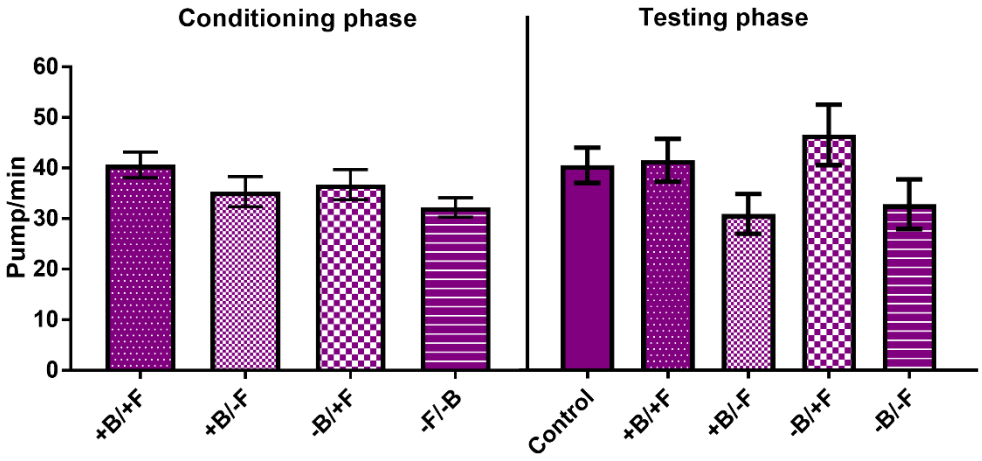


Figure 4.2: Preconditioning worms to butan-1-ol in the presence of food has no effect on pharyngeal pumping during a chemotaxis assay. *A) A schematic representation of the assay. 20 L4+1 worms were picked and placed onto a cleaning plate for 1 min before transfer to a seeded conditioning plate or a testing plate. Pharyngeal pumping is recorded 0.5cm away from the conditioning spot and butan-1-ol spot in the testing phase (represented in the diagram with a line). B) The effect of preconditioning worms to butan-1-ol and food for 60min on pharyngeal pumping. There was no significant difference between the groups in the conditioning phase (p = 0.176, n = 10-12 worms/group) and testing phase (p = 0.224, n = 10-12 worms/ group) compared to control group. The experiment was repeated 5 times. One-way ANOVA followed by Bonferroni post-hoc test was used. Error bars represent mean S.E.M.*

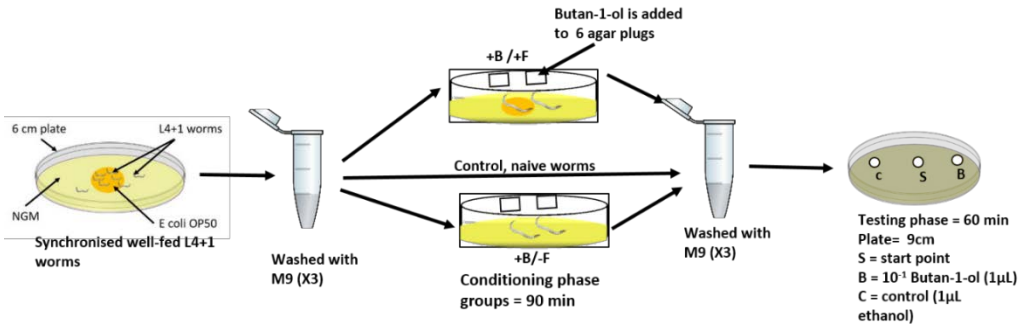
4.2.4 Preconditioning worms to butan-1-ol and food does not affect pharyngeal pumping and chemotaxis when butan-1-ol is added inside of the lid

The above format (*Figure 4.1* and *Figure 4.2*) describes an ability of the worms to detect the volatile alcohol butan-1-ol but this did not drive a subsequent enhanced CI. Previous experiments using distinct volatiles with related structure have been used to successfully drive plasticity at the level of CI readout. In these paradigms the cue was based on lid exposure above worms placed on food (*Figure 4.3*). We described this format in Chapter 3 by measuring pumping on food (section 3.2.5). We know that the state of worms on and off-food are distinct. To investigate if failure to condition is affected by the position of cue, we re-configured conditioning exposure. Worms were conditioned for 90 min by adding the odorant to agar plugs placed on the lid of the agar. This mimics the method adopted from (Torayama et al., 2007) which showed that the structurally related volatile, butanone, enhanced chemotaxis efficiency following preconditioning worms in the presence of butanone and food for 90 min.

We designed the experiment such that control, naïve non-conditioned worms were compared to worms placed on a food lawn with butan-1-ol added to agar plug inside of the lid. The third group were worms incubated in the absence of food and presence of butan-1-ol. As worms were on food, their CI during the conditioning phase was not investigated. Pharyngeal pumping rate was not recorded during the conditioning and testing phase, as the aim was to drive a positive chemotaxis-induced plasticity first.

Worms conditioned with 1, 2, 5, 6 or 7 µl of 1×10^{0} butan-1-ol for 90 min failed to show a change in chemotaxis to butan-1-ol in subsequent test when compared to control, non-conditioned worms (*Figure 4.3B*). In contrast worms conditioned with 10 µl butan-1-ol in the presence of food for 90 min (0.31 ± 0.03, *Figure 4.3B*), a reduced chemotaxis to butan-1-ol compared to control, non-conditioned group was observed. A similar effect was seen in worms conditioned in the absence of food (0.33 ± 0.03), worms also reduced their chemotaxis efficiency to butan-1-ol compared to control worms. A similar pattern was observed in the presence of 20 µl butan-1-ol in the presence of food (0.23 ± 0.02) and in the absence of food (0.18 ± 0.02, *Figure 4.3*).

A



B

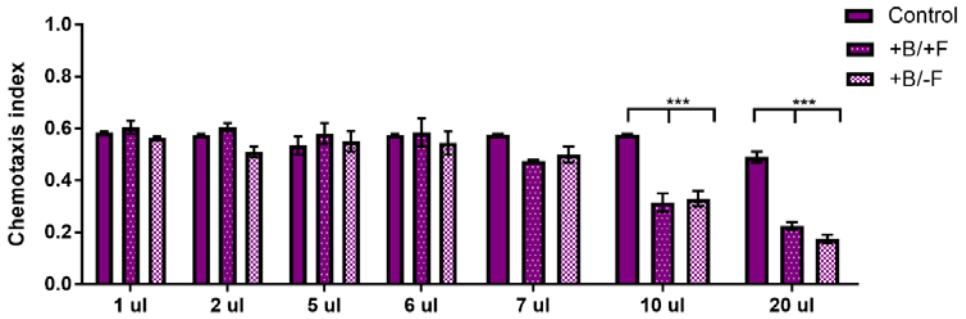


Figure 4.3: Butan-1-ol conditioning assay when worms are in contact with the food and the odorant is placed on agar plugs on the lid of the plate.

A) A schematic representation of conditioning assay. 150 L4+1 worms were washed and placed on either (+B/+F) 6cm plate with food in the presence of butan-1-ol added to two agar plugs inside of the lid, or 9cm plate without food in the presence of butan-1-ol (+B/-F). The conditioning time was 90 min. Control group were naïve, non-conditioned group. Following conditioning phase, worms were washed and placed on chemotaxis assay plate (9cm). After 60 min the CI to 10^{-1} butan-1-ol was calculated. (B) The effect of the amount of butan-1-ol in pre-conditioning assay on subsequent chemotaxis indices. The amount of 1-7µl of butan-1-ol used were not significantly different control (p = 0.99). The amount of 10µl reduced CI in groups +B/+F (0.31 ± 0.03, p = 0.0004) and –B/-F (0.33 ± 0.03, p = 0.0006) compared to control group. Similarly, to 20µl of butan-1-ol in groups +B/+F (0.23 ± 0.02, p = 0.0003) and –B/-F (0.18 ± 0.02, p < 0.0001). The experiment was repeated 4 times. Two-way ANOVA followed by Bonferroni test was used. Error bars represent mean ± S.E.M.

4.2.5 The dose-dependent effect of butanone on chemotaxis and pharyngeal pumping in the absence of food

The ability to enhance chemotaxis index following pre-conditioning worms to butanone has been established and is well described (Torayama et al. 2007; Kauffman et al. 2011; Stein and Murphy 2014). Therefore, butanone was included in conditioning experiments to investigate its ability to modulate pharyngeal pumping in post-conditioning test. Firstly, the effect of butanone on CI and pharyngeal pumping was assessed in naïve worms. the effect of dose of butanone on CI was assessed by a chemotaxis assay (*Figure 4.4A*).

The doses of 1×10^{-4}, 1×10^{-3}, 1×10^{-2} 1×10^{-1} and 1×10^{0} at 1µl of butanone were investigated. Butanone had a positive CI at undiluted dose only, indicating it is an attractant cue at this dose (*Figure 4.4B*). Lower doses had no effect on CI (*Figure 4.4B*), suggesting that worms did not detect butanone at lower doses.

4.2.6 Determining pharyngeal pumping rate in response to butanone during a chemotaxis assay in the absence of food

The analysis above supports the conclusion that butanone is an attractant compound to *C. elegans* and can mimic aspects of a *C. elegans* food cue and may be detected to prompt foraging strategies important in *C. elegans* behaviour. To investigate if this chemosensory detection of butanone impacts on the pharyngeal system, pharyngeal pumping was scored by visual observation as the worm approached the butanone, within 0.5cm away from the odorant's point source (*Figure 4.4C*).

The doses and volumes used were the same as indicated for chemotaxis assay in the above section 4.2.5. Pharyngeal pumping of worms approaching butanone's point source was compared to their starting point. Butanone did not affect pharyngeal pumping off-food (*Figure 4.4D*).

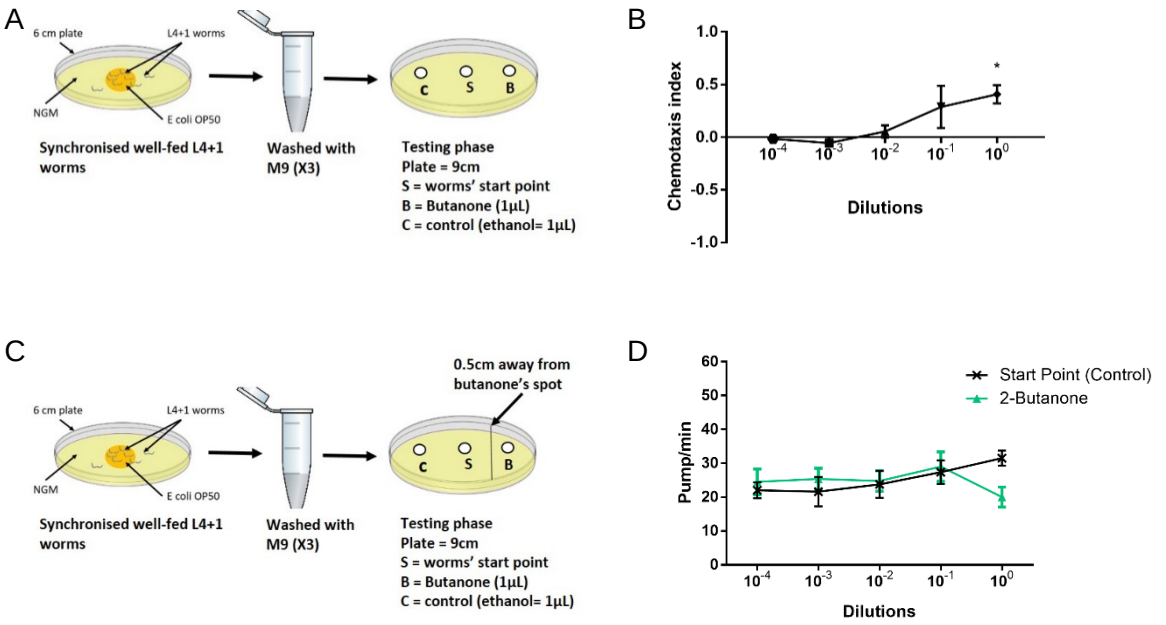


Figure 4.4: The effect of dose of butanone on CI was assessed by a chemotaxis assay. *A) A schematic representation of chemotaxis assay. 150 L4+1 well-fed worms were washed and transferred to 9cm chemotaxis assay plate and placed at start point (S). After 60 min CI was calculated. B) No significant difference detected at low doses of butanone ($p > 0.05$). At undiluted dose CI was significantly different from 0 ($p < 0.043$). One-sample t-test was used to compare chemotaxis indices against 0. C) A diagrammatic representation of pharyngeal pumping assay during chemotaxis. 20 L4+1 worms were picked onto a cleaning plate for 1 min before transfer to non-seeded testing plate. Pumping rate was measured within 0.5cm away from the butanone's point source and within the first 30 min of the assay. The assay was repeated 3 times. D) Pumping rate was assessed by comparing worms' approaching butanone's spot to worms at the start point. There was no significant difference between control and butanone at all doses ($p > 0.05$, n= 10-15 worms/group). The experiment was repeated 4 times. Two-way ANOVA followed by Bonferroni test was used. Error bars represent mean ± S.E.M.*

4.2.7 Butanone-induced chemotaxis plasticity following conditioning worms in the presence of food and butanone for 90min

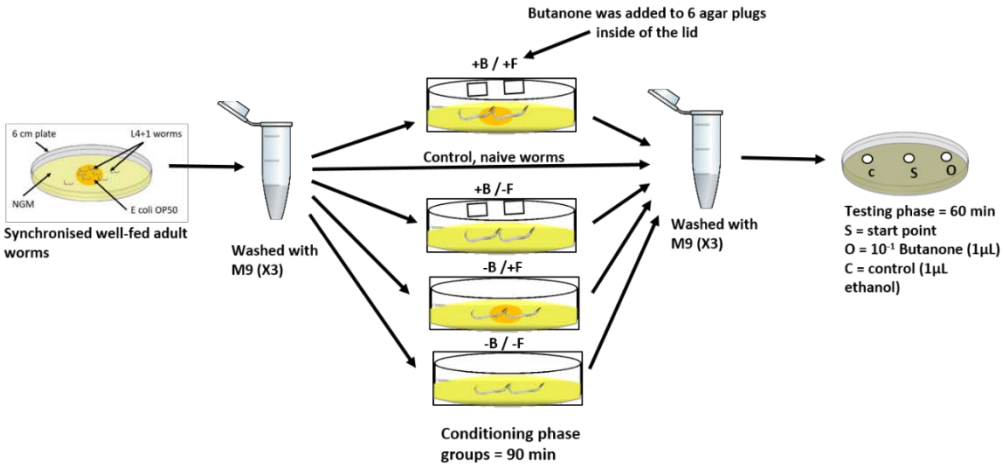
As described in the section 4.2.5 above, butanone has an attractant quality which can be used as a conditioning cue. Additionally, it has no distinct effect on the off-food pump rate as worms execute chemoattraction towards this cue.

Unlike other conditioning protocols (Kauffman et al. 2011; Stein and Murphy 2014), where conditioning is carried out in pre-starved worms, the protocol reported in (Torayama et al., 2007) was used since their conditioning assay was carried out in well-fed worms similar to butan-1-ol conditioning assay described in this chapter. To condition with butanone, worms were exposed using the agar plugs of 6µl of 1×10^{0} butanone. This exposed worms to the volatile while they reside on the food lawn for 90 min as represented diagrammatically in *Figure 4.5A*. These worms were then washed and transferred to testing plates to examine their chemotaxis to 1µl of 1×10^{-1} butanone. In the testing phase, worms showed enhanced chemotaxis to butanone compared to naïve, non-conditioned worms (0.52 ± 0.1, *Figure 4.5B*).

A second group of worms were conditioned in the presence of 30 µl 1×10^{0} butanone added to 6 agar plugs placed inside of lid in the absence of food (*Figure 4.5A*). Worms were not in contact with butanone. After 90min conditioning time, worms were washed and added to testing plates and their chemotaxis to 1µl of 1×10^{-1} was calculated. In the testing phase, butanone had a negative chemotaxis index (-0.24 ± 0.8, *Figure 4.5B*) compared to control, non-conditioned group. This suggests that butanone is an aversive cue when presented in the absence of food (Colbert and Bargmann, 1995).

Overall this suggests that butanone despite being a weak chemoattractant is able to act as a positive or negative modulator of post-conditioning behaviour. This will be dependent on the context in which the conditioning cue is applied, either on food or during prolonged starvation.

A



B

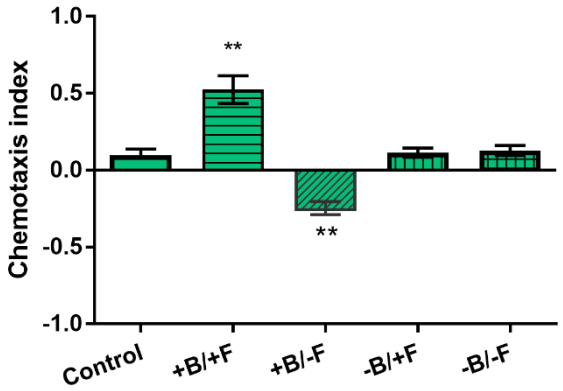


Figure 4.5: C. elegans enhanced their chemotaxis index to butanone following pre-conditioning worms to butanone and food.

A) A diagrametic represesntation of butanone conditioning asssay. B) 150 L4+1 worms were washed and transferred to 4 conditioning plates as follolw: Butanone +food (+B/+F) group – 6 µl of 1×10^{0} butanone added to 6 agar plugs inside of the lid of 6 cm while worms were placed on a food lawn opposite the butanone agar plugs. Butanone + no-food (+B/-F) group – 30 µl of 1×10^{0} butanone added to 6 agar plugs inside of the lid of 9cm while worms were placed on a non-food arena opposite the butanone agar plugs. Food in the absence of butanone (-B/+F) group – worms were conditioned in the presence of food and absence of butanone in 6 cm. No butanone and no food (-B/-F) group – worms were conditioned in the absence of both butanone and food in 9 cm plate. Conditioning time was 90 min. Subsequently, worms were washed and transferred to chemotaxis plates to measure their CI to 1 µl of 1×10^{-1} butanone in testing phase. B) Worms pre-conditioned in the presence of food and butanone

enhanced chemotaxis index to butanone in testing phase (p = 0.0001). Worms conditioned in the presence of butanone and absence of food reduced chemotaxis index to butanone in testing phase (p = 0.0007). –B/+F and –B/-F groups had no effect on CI compared to control (p = 0.99). The experiment was repeated 5 times. One-way ANOVA test followed by Bonferroni test was used. Error bars represent mean ± S.E.M.

4.2.8 Butanone-induced chemotaxis plasticity following pre-conditioning regime had no effect on pharyngeal pumping

The ability of butanone in inducing chemotaxis behavioural plasticity following pre-conditioning worms in the presence and absence of food provides a platform to investigate its impact on the pharyngeal system. The conditions used in the chemotaxis-induced plasticity were used in pharyngeal pumping assay. However, following the conditioning phase, worms were placed in a cleaning plate for 1 min to remove food residue before they were added to an assay plate with 1µl × 10^{-1} butanone added inside the lid of the plate (*Figure 4.6A*).

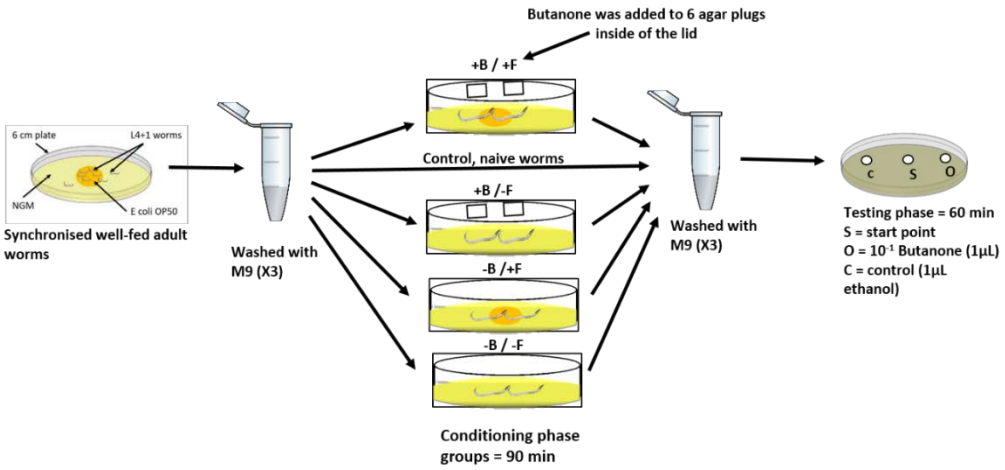
During the conditioning phase, worms conditioned in the presence of food and butanone showed a slight reduction (209 ± 3 ppm, *Figure 4.6B*) in pumping rate compared to food-only worms (242 ± 4 ppm). In the absence of food, Butanone had no effect on pumping rate in the absence of food (20 ± 3 ppm, *Figure 4.6B*) compared to worms pre-conditioned in the absence of food and butanone (27 ± 2 ppm). These worms showed a significant reduction in pumping rate in both butanone-only group and in no-butanone and no-food group compared to worms pre-conditioned in the presence of food. This low pump rate in the absence of food is consistent with previous observation (Dallière et al., 2016).

In the testing phase, butanone and food group or butanone and no-food group pumped similar to control worms (*Figure 4.6B*). A similar pattern was observed in food-only and no-butanone and no-food group. Therefore, this indicates that pre-conditioning worms in the presence of butanone and either in the presence or absence of food showed no positive or negative modulation of butanone on pharyngeal pumping in the testing phase.

The overall results indicate that worms modify their chemotaxis behaviours towards odours based on previous experiences. Although worms showed enhanced chemotaxis

efficiency to butanone following pre-exposure to butanone and food, but this was not reflected on the subsequent pharyngeal behaviour.

A



B

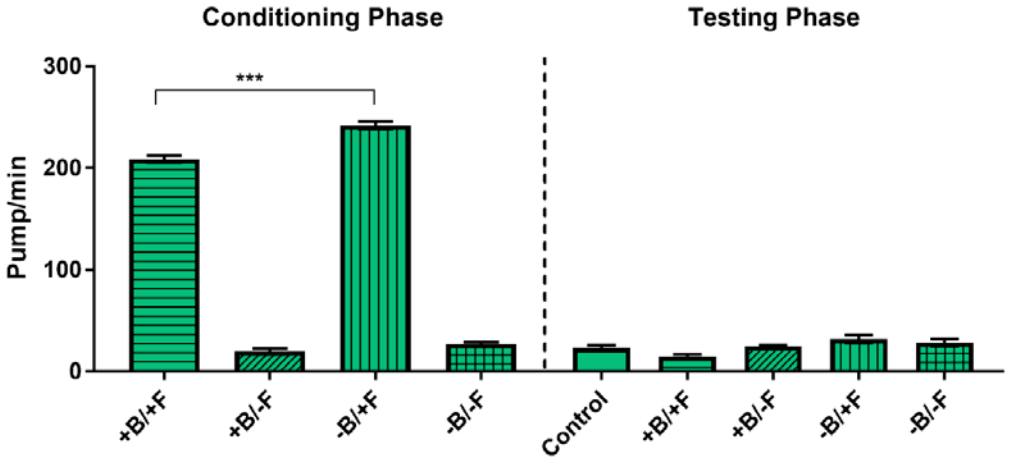


Figure 4.6: Pre-exposure of worms to butanone and food did not modify pharyngeal pumping in a subsequent test. *A) A schematic representation of butanone conditioning assay. 150 L4+1 worms were washed and conditioned for 90 min as follow: In the presence of 6 µl of 1×10^{0} butanone and food (+B/+F), in the presence of 30 µl of 1×10^{0} butanone and absence of food (-B/-F), in the absence of butanone and presence of food (-B/+F) and in the absence of butanone and food (-B/-F). Subsequently 10-15 worms were picked into cleaning plate for 1 min before transfer to non-seeded testing plate. 1 µl of 1×10^{-1} butanone was added to the inside of the lid after*

10min from removal worms from the cleaning plate. Pharyngeal pumping was measured 10 min after the addition of butanone.

B) In the conditioning phase, +B/+F showed a reduced pump rate compared to -B/+F group (209 ± 3 ppm, p < 0.0001, n =12 worms). In the testing phase, there was no significant difference between groups (n = 12-16 worms/ group). The experiment was repeated 3 times. One-way ANOVA test followed by Bonferroni test was used. Error bars represent mean ± S.E.M.

4.2.9 Butanone-induced chemotaxis plasticity following chronic exposure to butanone and food

The conditioning paradigm above utilized short-term exposure to butanone for 90 min. The test was successful in driving a post-conditioning effect represented by enhanced CI. However, this conditioning paradigm failed to modify subsequent modulation in the pharyngeal system. Therefore, a butanone conditioning across the entire developmental widow, from eggs to adulthood (L4+1 stage) was developed before investigating its subsequent effects on chemotaxis assay and pharyngeal pumping. A chronic exposure was used suggest that a taste preference emerges from long exposure (Donohoe et al., 2009; Song et al., 2013).

For the conditioning phase, eggs were laid onto OP50 and worms were exposed to butanone in agar plugs and 6 µl of 1×10^{0} butanone was added to the agar plugs (*Figure 4.7A*). The worms took 72 hours to develop to L4+1 stage and the plates were sealed to reduce evaporation. The presence of butanone through the entire developmental window did not affect worms' development, similar to that observed in worms developed in the presence of food and absence of butanone. Therefore, the observation suggests that both cohorts had reached a synchronized L4 population after 2 days since eggs were laid. These L4 were grown over night in the presence of the volatile before being tested in butanone-induced chemotaxis on the third day since eggs were laid (*Figure 4.7A*).

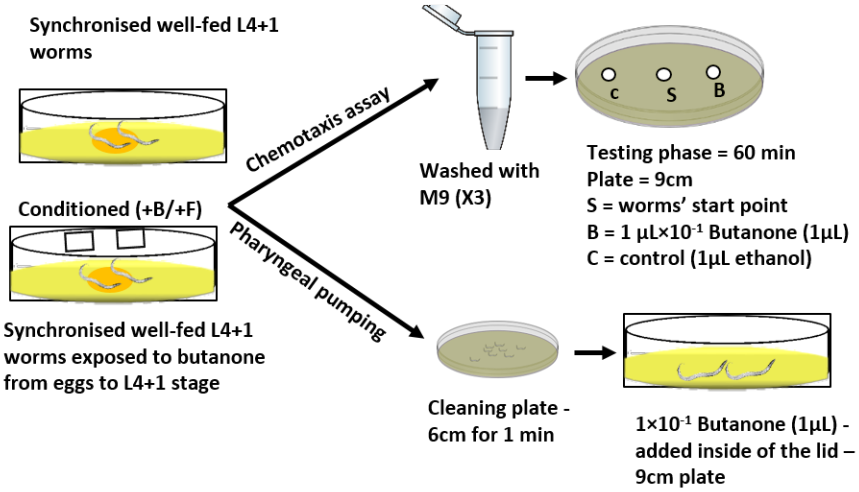
The long-term exposure of worms to butanone produced cohorts of worms that showed a pronounced chemotaxis efficiency (0.85 ± 0.1 ppm, *Figure 4.7B*) compared to control in the testing phase. This conditioning paradigm led to a highly significant increase in the CI in the testing phase. The mean value of butanone and food group (0.85 ± 0.1 ppm) is highly significant and shows a substantial elevation relative to the mean value (0.52 ± 0.1

ppm, *Figure 4.5B*) of the same group in the short -term conditioning paradigm. This observation indicates that adult worms that emerge from chronic exposure exhibits a strong plasticity-inducing effects compared to short-term exposure experiment (*Figure 4.5B*), as similar observation has been previously reported (Remy and Hobert, 2005).

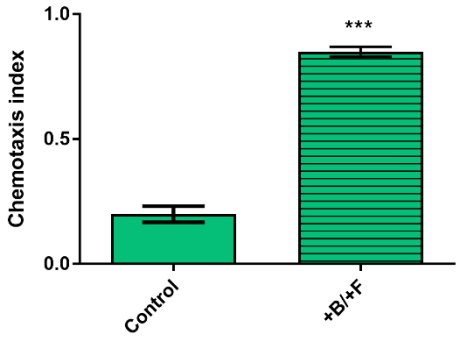
To address the effect of the chronic exposure on pharyngeal pumping, worms were picked from their conditioning plate to testing plat to assess their pharyngeal pumping after 10, 30 and 60 following the addition of butanone (1 µl 1×10^{-1} butanone, *Figure 4.7B*). Butanone failed to modulate pharyngeal pumping in response to butanone regardless of the duration of pre-exposure time in the testing phase (*Figure 4.7B*).

In summary, butanone has shown to induce chemotaxis-dependent behavioural plasticity following short and long exposure (chronic). This modulation depends on the context whether butanone was paired with or without food. However, there was no evidence to suggest that butanone has any effects on pharyngeal pumping in the following testing phase.

A



B



C

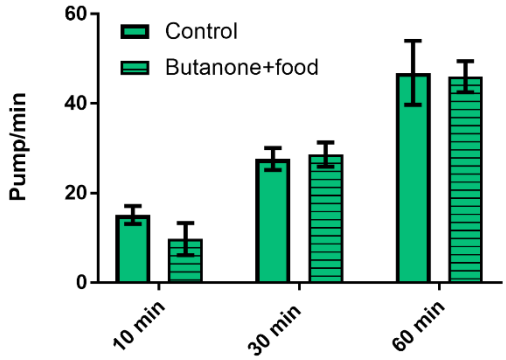


Figure 4.7: Prolonged exposure to butanone and food induced chemotaxis plasticity without accompanying any changes to pharyngeal pumping in subsequent tests.

A) Group +B/+F - 200 eggs were incubated in the presence of food and 6 µl 1×10^{0} butanone added to six agar plugs. The plugs and lids were refreshed every 24hours. Control group were egg incubated in the presence of food only. After 72 hr worms

reached L4+1 worms were washed and tested in chemotaxis assay. Their response to 1µl 1×10^{-1} butanone was calculated. Pharyngeal pumping assay – worms were conditioned as shown for chemotaxis assay except that 30 worms were picked into a cleaning non-seeded plate for 1 min before transfer to a non-seed assay plate. 1 µl of 1×10^{-1} butanone was added to the inside of the lid after 10min from removal of worms from the cleaning plate. Pharyngeal pumping was measured 10, 30 and 60 min after the addition of butanone. B) +B/+F had an increased CI (0.85 ± 0.1, p < 0.0001) compared to control (0.2 ± 0.03). Independent sample t-test was used. The experiment was repeated 4 times. C) Preconditioning worms to butanone and food does not affect pharyngeal pumping. In the testing phase, there was no significant difference between groups at any time tested (n = 15-22 worms/ group). The experiment was repeated 4 times. Two-way ANOVA followed by Bonferroni test was used. Error bars represent mean ± S.E.M.

4.3 Discussion

4.3.1 Chemotaxis to butanone, but not butan-1-ol, increases following pre-conditioning of worms to butanone in the presence of food

In this chapter the straight chain alcohol, butan-1-ol, was selected to investigate the impact of a pairing paradigm of butan-1-ol and food on chemotaxis index in subsequent test. In the environment, alcohols such as butan-1-ol alcohols are released by bacteria as a by-product and worms detect it by its sensory neurones to drive chemotaxis towards bacterial lawn (Bargmann, 2006; Hibbard et al., 1985; Zechman and Labows, 1985). Therefore, butan-1-ol is a relevant environmental cue that can be used in a pairing experiment. In spite of naïve *C. elegans* chemoattarction to butan-1-ol, chemotaxis to butan-1-ol was unchanged following conditioning worms in the presence of food and butan-1-ol. On the other hand, butanone enhanced CI using butan-1-ol conditioning paradigm (see section 4.2.7) as previously reported (Torayama et al. 2007; Kauffman et al. 2011; Stein and Murphy 2014).

Torayama et al. 2007 indicated that butanone-induced chemotaxis plasticity is unique to butanone, as worms conditioned in the presence of butanone and food did not enhance

their chemotaxis to benzaldehyde in the following test. Benzaldehyde is an attractant compound and when paired with food, worms did not increased their CI to benzaldehyde in the subsequent test (Torayama et al., 2007). Benzaldehyde and butan-1-ol are sensed by the AWC^{ON} and AWC^{OFF} neurones, whereas butanone is sensed by the AWC^{ON} neurones only (Bargmann et al., 1993; Wes and Bargmann, 2001). This indicates that butanone-induced chemotaxis plasticity depends on AWC^{ON} neurones.

Although butan-1-ol was unable to positively modulate CI, high volumes of butan-1-ol reduced CI following pre-conditioning worms in the presence and absence of food. There is evidence that worms show dose-dependent olfactory preference (Bargmann et al., 1993; Colbert and Bargmann, 1995; Luo et al., 2008; Taniguchi et al., 2014; Yoshida et al., 2012). High volumes of isoamyl alcohol results in reduced chemotaxis attraction and this behaviour is thought to be mediated by AWB neurones. As the volume of odorant increases, this can change, odours become less attractive or repellent. ASH neurones mediate aversion when higher volumes result in increased concentrations that could reached toxic levels (Colbert and Bargmann, 1995). Therefore, reduced chemotaxis to butan-1-ol following conditioning in the presence of a high volume of butanol suggest that this behaviour could be mediated by AWB which results in reduced attraction.

4.3.2 Butanone did not induce pharyngeal pumping dependent plasticity following conditioning of worms to butanone in the presence of food

The impact of butanone-induced plasticity on the pharyngeal system was investigated. Butanone is an attractive compound which had no effect on pharyngeal pumping in the absence of food in the chemotaxis assay although increased CI was observed following pairing of food and butanone. It was predicted that the presence of an appetitive cue might lead to increased pharyngeal pumping since chemotaxis-induced plasticity was observed in the butanone conditioning paradigm. Additionally, this stimulation of pharyngeal pumping was thought to be possible since worms maintain a low pumping rate in the absence of food giving a window to observe butanone-dependent activation of the pharyngeal system. Nonetheless, observations in this chapter suggest that butanone did not regulate pharyngeal pumping in the absence of food, even though an association between the odorant and food was formed at a chemotaxis level.

There is evidence in the literature suggesting olfactory modulation of the pharyngeal system in the presence of food. Li et al. 2012 reported increased pharyngeal pumping of worms in the presence of an attractive cue, diacetyl, whereas reduced pumping rate was observed in the presence of an aversive cue such as quinine or high volumes of an attractive cue, such as isoamyl alcohol. Furthermore, worms exposed to a bacterial lawn from the first larvae stage to adulthood showed increased pharyngeal pumping when tested on a familiar bacterial lawn compared to a new bacterial lawn (Song et al., 2013). In this current study butanone reduced pharyngeal pumping in the presence of food during the conditioning phase which could be due to exposure to a high volume of butanone as reported previously (Li et al., 2012). However, in the subsequent testing phase butanone did not affect pharyngeal pumping in the absence of food.

In this chapter, the data suggest a behavioural plasticity at the chemotaxis level with no plasticity in the pharyngeal system in the absence of food. This may indicate that olfaction is not required to drive feeding behaviour in the absence of food. This is supported by findings in Chapter 3 and Chapter 4 which showed that chemo-attractive straight chain alcohols, which are likely to be ecologically salient, did not have an impact on the pharyngeal system by stimulating pumping rate in the absence of food. Therefore, this suggests that olfaction is less important for the pharyngeal system but is critical for foraging behaviour and survival of worms as induced by chemotaxis-induced plasticity. Furthermore, the complexity of using food as unconditioned stimulus as it is a multimodal stimulus of olfactory, nutritional, gustatory and mechanosensory cues could prevent observing butanone-induced pharyngeal pumping plasticity.

Chapter 5: Defining the sensory modalities that stimulate feeding behaviour in *C. elegans*

5.1 Introduction

As it has been reported in Chapter 3 and 4, *C. elegans* can detect environmental cues and this drives chemotaxis towards them. However, in the case of butan-1-ol and butanone, these cues have no impact on pharyngeal pumping in naïve worms. In a pairing paradigm of butanone and food, a butanone-dependent olfactory plasticity in chemotaxis was observed without affecting the basal pharyngeal pumping rate. This inability to modify pharyngeal pumping following pre-exposure to butanone and food indicate that the sensory modalities that control feeding behaviour may be independent of those that modulate the locomotory response that underpins chemotaxis behaviour.

The worm's sensory system detects a range of sensory cues that signal the presence or absence of food and hence in turn worms execute the appropriate response (see Chapter 1). For instance, Olfaction allows the worms to respond to volatile cues and navigate in the environment to locate food at a distance (Bargmann et al., 1993). These cues are detected by chemosensory neurones, AWA and AWC. However, these neurones do not only sense the presence of odours but also respond to their absence. AWC neurones are activated following removal of odours (Chalasani et al., 2007). Moreover, worms sense water-soluble molecules, such as NaCl, through chemosensory neurones ASE (Bargmann and Horvitz, 1991; Ward, 1973). It was previously shown that *C. elegans* is attracted to low concentrations of NaCl and avoids high concentrations (Hukema et al., 2008). Nonetheless, worms avoid low concentrations of NaCl when paired with the absence of food (Saeki et al., 2001). Therefore, salt and other taste molecules are important cue in food detection. Worms can detect mechanical stimulus (see Chapter 1, section 1.5.1).

When worms come in contact with food, they reduce their locomotion rate compared to worms in the absence of food (Sawin et al., 2000). This basal slowing response is mediated by mechanosensory neurones. This behaviour is dependent on mechanosensory cues and worms can sense mechanosensory cues via dopaminergic CEP, ADE and PDE neurones. Other external environmental cues such as monitoring the levels of O_2 and CO_2 allow worms to escape extreme conditions when food diminishes and the environment becomes anaerobic (Bretscher et al., 2008). Besides the detection of external cues, *C. elegans* sense and modify their feeding behaviour if their internal nutritional state has been changed. For example, kynurenic acid has been identified as a nutritional cue, which is diminished in the absence of food (Lemieux et al., 2015). Starved worms increased their pharyngeal pumping rate on re-entry to food compared to well-fed worms. Moreover, their pharyngeal pumping remained high until kynurenic acid levels returned to normal.

Overall, the impact of the above sensory cues has been investigated with relevance to foraging behaviour. However, little is known about the effect of such sensory modalities on feeding behaviour in the presence and absence of food.

The aim of this chapter was to explore the impact of chemosensory and mechanosensory cues on feeding behaviour in the presence and absence of food. The starting point for this investigation was a systematic analysis of the density-dependence of the bacterial culture on feeding behaviour. The bacterial cue was then separated into chemical and mechansensory components using either bacterially conditioned media or inert beads to mimic these respective and distinct modalities and delineate their ability to stimulate feeding.

5.2 Results

5.2.1 The rate of pharyngeal pumping depends on the density of bacteria, *E. coli* OP50

C. elegans ingests and accumulates bacterial particles suspended in liquid into the pharynx and then crushes these at the grinder before passing the masticated bacteria into the intestine (see Chapter 1). In the presence of food worms display a pumping rate of 3.5-5 Hz, which translates to 250-275 ppm. A previous study reported that worms display a concentration-dependent effect of bacteria on pharyngeal pumping in liquid culture

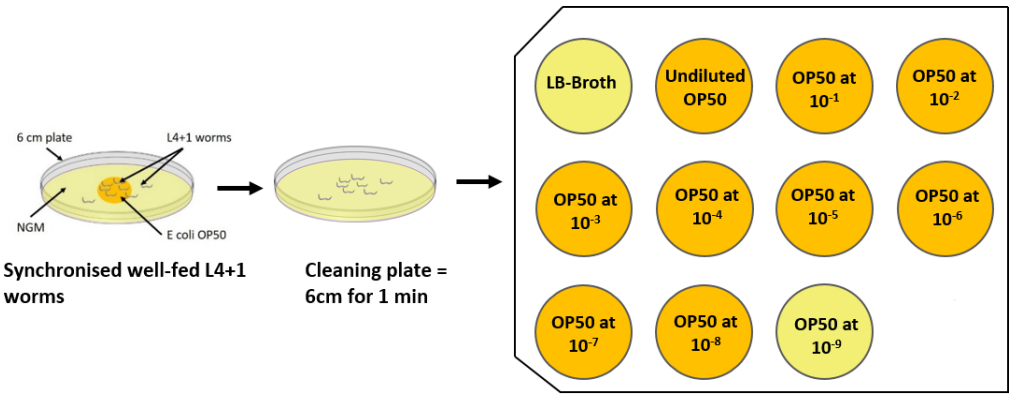
(Scholz et al., 2016). They showed that at low concentration of bacteria, worms pumped about 2 Hz, while at high concentration of bacteria pumping rate was 3.5 Hz. To understand the modalities that control feeding behaviour, the effect of OP50 (food) density on pumping rate was investigated over a greater range of bacterial densities and on NGM solid media.

A serial dilution (1 in 10) of bacteria was prepared and 100µl of each dilution was added and spread on the 12-well plate and incubated for 12-14 hours to allow bacterial growth (*Figure 5.1A*). Subsequently, to determine the number of bacterial colonies present in each well, a 100µl of each dilution was added onto LB agar and incubated at 37°C for 12-14 hours before counting the colony-forming unit (CFU) for each dilution. However, the number of bacteria may be higher because bacterial lawn can grow from the initial inoculation. Worms were picked into a cleaning plate before transfer to assay plates. This was carried out to find the number of colonies in the initial densities when plates were seeded.

Worms placed on LB broth showed pumping rate similar to that observed in the absence of food (~15 ppm, *Figure 5.1B*). Conversely, worms placed on undiluted concentration of bacterial lawn, with 86000000 CFU/ml, showed high pumping rate. At high doses of 1×10^{-1} – 1×10^{-5}, with colonies of 860000 – 860 CFU/ml (*Figure 5.1B*), worms displayed and sustained high (~250 ppm) pharyngeal pumping similar to the undiluted dose (*Figure 5.1B*). In the presence of 86 CFU at 1×10^{-6} dose, worms exhibited normal feeding rate. Interestingly, at dose of 1×10^{-5}, in which 10 colonies were present, worms showed a normal feeding behaviour similar to that observed at undiluted and high doses (*Figure 5.1B*). At the lowest dose of 1×10^{-8}, pump rate was lower than that observed at undiluted and highest does of OP50, however, pumping rate was still markedly higher than the control (LB Broth-off-food). At dose of 1×10^{-9}, worms pumped insignificantly different from worms on LB broth, which correlates with no colonies observed at this dose (*Figure 5.1B*).

In conclusion, worms are very sensitive to the presence of bacteria and they maintain high pumping rate even in the presence of low number of colonies (<1 colony per area). However, the presence of low number of colonies do not necessarily lead to low pumping rate since bacterial colonies can reproduce dense lawn from low number of colony densities, when seeded.

A



B

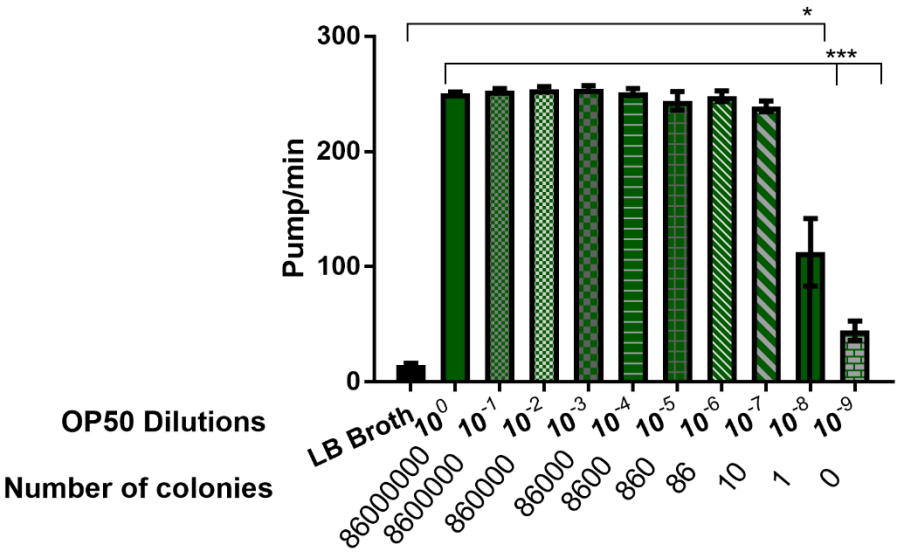


Figure 5.1: Pharyngeal pumping depends on the number of colonies of the bacterial culture. *A) The plates were seeded with a serial dilution of E. coli OP50 and left to incubated overnight. The number of bacteria associated with these are indicated. At the start of the experiment, 20 synchronised L4+1 worms were picked into a cleaning plate for 1 min before transfer into assay plates. Pharyngeal pumping rate was assayed after 10 min. B) Dilutions between 1×10^{-1} – 1×10^{-5} displayed and sustained high (250 ± 4 ppm) pharyngeal pumping similar to the undiluted dose. At a dilution of 1×10^{-8}, worms pumped significantly lower (112 ± 29 ppm, $p < 0.0001$) from worms on undiluted culture between 1×10^{0}. At a dilution of 1×10^{-8}, worms pumped similar to worm on LB broth (n= 10-15 worms/ group). The experiment was repeated 3 times. One-way ANOVA followed by Bonferroni test was used. Error bars represent mean± S.E.M.*

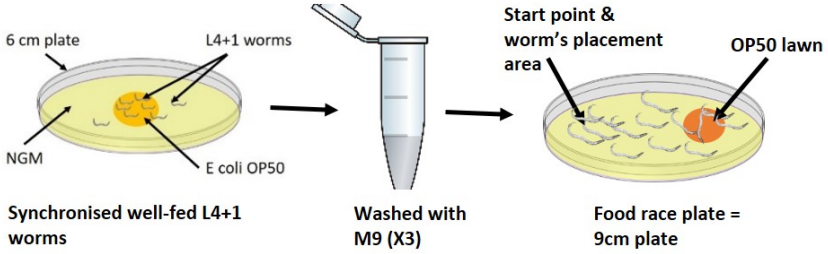
5.2.2 Olfactory cues do not affect pharyngeal pumping on food

In Chapter 3, the effect of environmentally salient cues, straight chain alcohols, on pumping rate in the absence of food revealed a reduced pumping rate at high doses. However, overall there was no effect of lower doses of these cues on pumping rate. Here, the effect of food odours released by the bacterial OP50 lawn on pumping rate was determined in the context of a food race assay. In this assay pharyngeal pumping was scored in freely moving worms as they navigated towards a point source of food.

Worms were placed in a non-food area and a source of *E. coli* OP50 (food) was placed on the opposite side of the plate (*Figure 5.2A*). Pharyngeal pumping was assessed every 10 min as worms navigated towards the food spot over the course of 2 hr. Additionally, the number of worms that reached the food lawn every 10 min was counted. Worms did not elevate their pharyngeal pumping as they approached the bacterial lawn, although after 2 hours 95% of worms reached the food lawn. Therefore, this indicates that worms detect olfactory cues released by the OP50 lawn and execute chemotaxis towards food did not stimulate pharyngeal pumping as worms were approaching the food source (*Figure 5.2B*).

This observation further supports findings reported in Chapter 3 that odours of food released by bacteria play a role in directing foraging behaviour, but do not cue the execution of control pharyngeal pumping the sub behaviours that brings about ingestion.

A



B

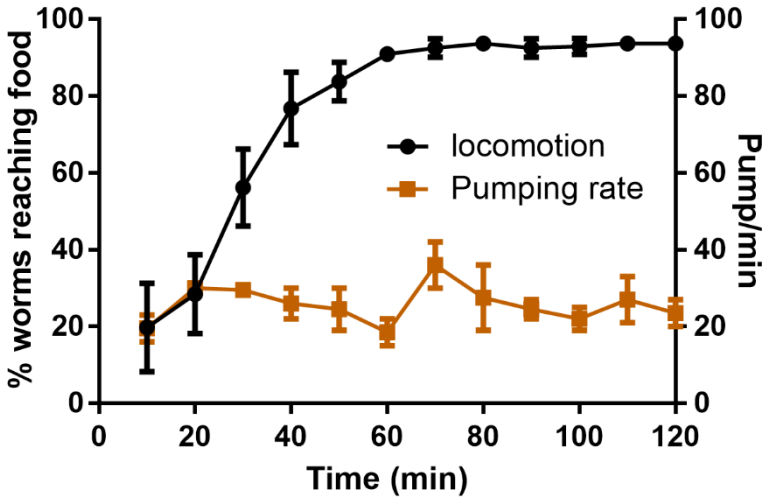


Figure 5.2: Distal olfactory cues do not regulate pharyngeal pumping on-food. *A) 100 L4+1 well-fed worms were washed with M9 buffer and transferred to food race plate (9 cm plate). OP50 (50 µl) was seeded 2 cm away from the edge of the plate and left at room temperature (12-14hr) to allow bacteria to grow. OP50 with* OD_{260} *= 0.8. B). Every 10 min the plate was observed for worms that were approaching, but not on, the food spot i.e. about 0.5 cm away from the food. The pumping rate was scored for 1 min. Additionally, the number of worms on the food lawn were counted and removed from the lawn every 10 min for 2 hr. After 2 hr, 95% of worms reached the food lawn. there was no increase in pharyngeal pumping rate as worm navigate towards the bacterial lawn (n = 26 worms). Both experiments were repeated twice. Error bars represent mean± S.E.M.*

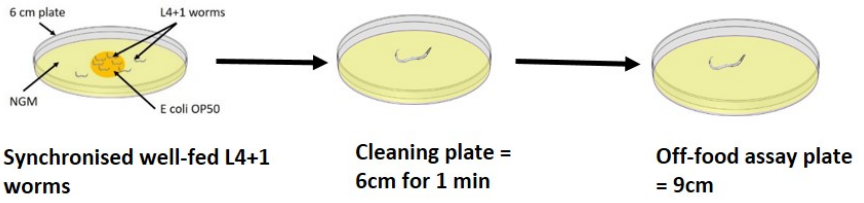
5.2.3 Mutations of the ciliated sensory neurones affect pharyngeal pumping in the absence of food

C. elegans exhibit chemotaxis towards a bacterial lawn by detecting a gradient of volatile and water-soluble cues (Bargmann et al., 1993; Ward, 1973). The chemosensory neurones, localised in the amphid and phasmid organs, are ciliated and their functions are linked to these cilia (Perkins et al., 1986). The role of sensory neurones in the regulation of feeding behaviour has been investigated using cilia-deficient mutants (Collet et al., 1998; Haycraft et al., 2003; Perkins et al., 1986; Wicks et al., 2000). The *osm-6* gene is a component of the intraflagellar transport (IFT) particle and is expressed in most sensory neurons (Collet et al., 1998) . The mutant *osm6 (p811)* is deficient in cilia and defective in chemosensory signalling. Therefore, pharyngeal pumping of this mutant was investigated in the context of the presence and absence of bacteria to address the role of chemosensory signalling in the regulation of pharyngeal pumping. In view of Chapter 3 data and *Figure 5.2*, these mutants would not increase pharyngeal pumping in the absence of food.

Pharyngeal pumping was assessed in the presence of food, before worms were picked onto a cleaning plate and then onto a non-food plate (*Figure 5.3A*). In the presence of food, *osm-6(p811),* showed a feeding behaviour similar to wild-type, N2, worms (*Figure 5.3B*) suggesting that sensory neurones of the central nervous system do not regulate pharyngeal pumping on food. However, in the absence of food, *osm-6* mutants showed an abnormally elevated pharyngeal pumping (196 ± 6 ppm) compared to the low pumping rate (20±5 ppm) exhibited by N2 worms (*Figure 5.3B*).

These findings suggest that ciliated sensory neurones inhibit pumping rate in response to the absence of food but surprisingly they make no contribution to the regulation of pumping on food.

A



B

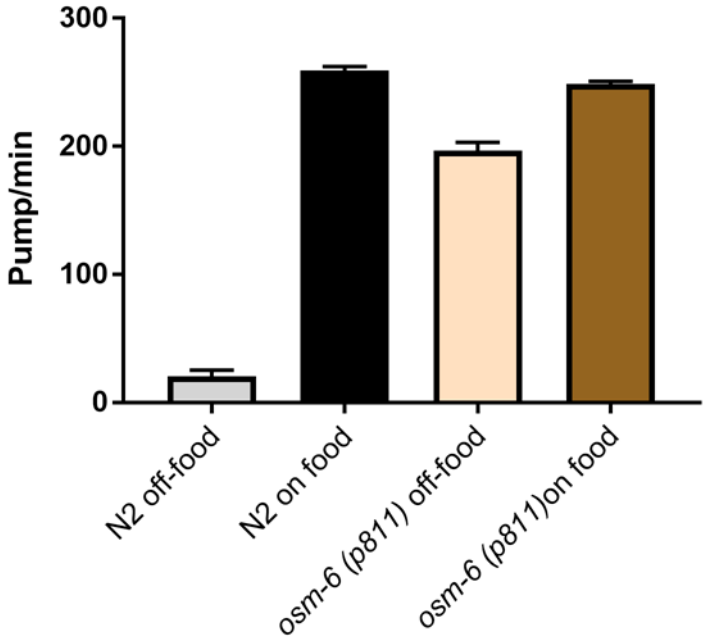


Figure 5.3: Cilia-deficient mutant, osm-6, selectively regulates the off-food pump rate. *A) Schematic representation of the off-food pumping rate assay. 20 L4+1 worms were picked into a cleaning plate for 1 min before transfer into 9cm assay plate. Pharyngeal pumping rate was assayed after 10 min from removal from food. B) osm-6 in the presence and absence of food. In the presence of food, osm-6 (p811) mutants pumped similar to wild-type N2, while in the absence of food osm-6, pumped significantly higher than N2 (201 ± 4 ppm, $p < 0.001$, n= 10 worms/ group). The experiment was repeated 3 times. One-way ANOVA followed by Bonferroni test was used. Error bars represent mean ± S.E.M.*

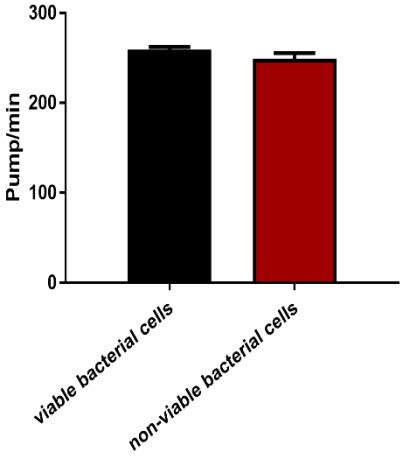
5.2.4 *C. elegans* exhibits normal feeding behaviour on UV-radiated bacteria

C. elegans feeds on bacteria and the by-products of bacteria metabolism is released and utilised by the worms to locate food in the environment (Bargmann, 2006; Bargmann et al., 1993; Hibbard et al., 1985; Zechman and Labows, 1985). As discussed in Chapter 3, the olfactory cues from food do not stimulate pharyngeal pumping in the absence of food (see Chapter 3 - section 3.2.2). However, here, we were interested in determining whether dead bacteria can stimulate pharyngeal pumping, since dead bacteria do not have an active metabolism to release cues. This will shed a light on how *C. elegans* perceive the presence of food in the absence of chemosensory cues.

A thick lawn of bacteria was obtained by seeding a plate with OP50 and incubating it at room temperature for 2 days to allow bacterial growth before bacteria were killed by UV light. Worms on non-viable bacteria showed a pump rate similar to that observed in the viable, non-irradiated bacterial lawn (*Figure 5.4A*). This indicates that worms feed at a normal rate on irradiated, dead bacteria. To confirm that this was not due to the survival of any bacteria on the irradiated lawn, both viable and non-viable lawns were, separately, streaked onto a LB agar and incubated at 37°C overnight. The non-viable bacteria lawn had no colonies formed on the agar relative to viable non-radiated control (*Figure 5.4B*).

Overall this data indicates that the worm may perceive food via a mechanical cue, since worms maintained a high pumping rate in the presence of non-viable bacteria.

A



B

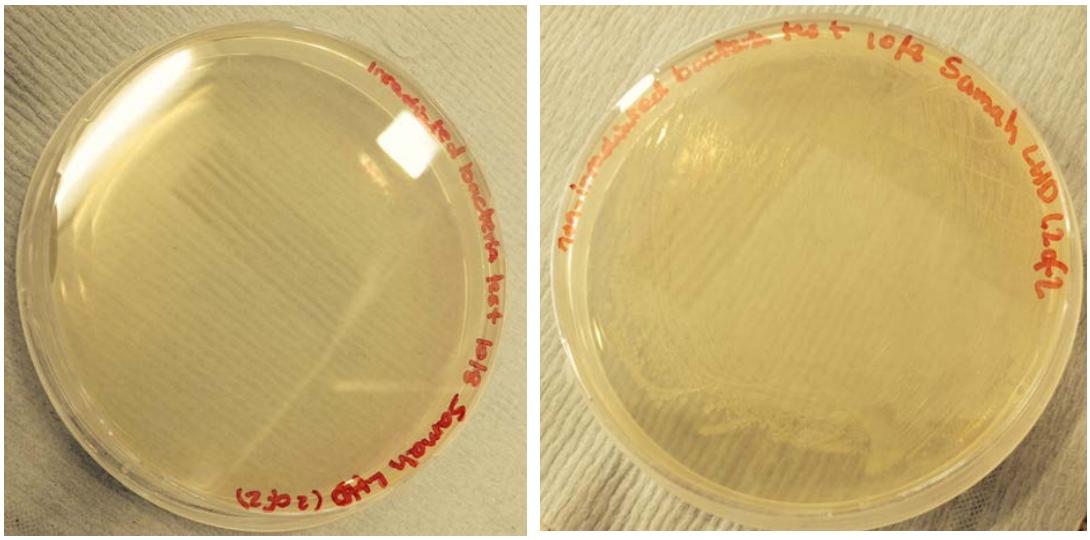


Figure 5.4: The effect of non-viable bacteria on pharyngeal pumping rate. *Assay plates (6 cm) were seeded with 6.4×10^{7} cells/ml in 50 µl and left at room temperature for 48 hr to allow bacterial growth. The plates were placed into the cross-linker and exposed to UV light set at 3000 joules for 30 min. 20 L4+1 worms were picked into a cleaning plate for 1 min and then transferred to assay plates. Pharyngeal pumping was recorded 10 min after transfer to test bacteria plate. A) Worms placed on control and radiated bacteria pumped insignificantly different from each other (p > 0.05, n =11-13 worms/ group). B) Authentication of non-viable bacteria. Radiated and irradiated bacteria (control) was streaked into LB agar and incubated at 37°C for 12-14. No viable colonies were observed in radiated bacteria (left) compared to control (right) where viable colonies were observed. The experiment was repeated twice.*

Student t-test was used to compare the control and treated groups. Error bars represent mean± S.E.M.

5.2.5 Mechanosensory cues lead to a transient elevation in pumping rate

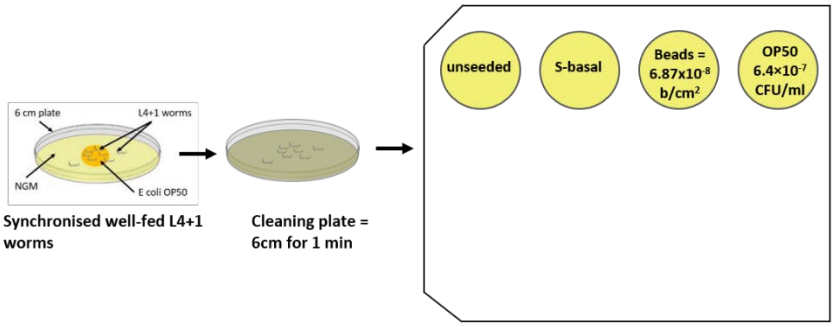
The data presented above show that cues detected via the worm's olfactory system have no influence on feeding rate. By elimination, this suggests an important role for the worm's somatosensory system i.e. mechanosensation and/or gustation. To test the role of mechanical cues, fluorescent polystyrene microspheres (beads) were used to mimic the physical presence of food (see Chapter 2, section 2.3.9 for details). The size of beads was the same size as the *E. coli* OP50 short axis, 0.5µm (Fang-Yen et al., 2009). Moreover, Fang-Yen et al. 2009 have previously shown the ingestion and accumulation of beads in the worm's intestine. Similar findings have been reported (Kiyama et al., 2012). To determine how worms behave in the presence of beads, pharyngeal pumping in the presence of beads was compared to off-food and on-food pumping rate. L4+1 worms were picked into a cleaning plate for 1 min before transfer onto a non-seeded plate, a plate with S-basal, an OP50 seeded plate or plate spread with beads (*Figure 5.5A*). Pharyngeal pumping was assessed every min for 10min.

In the absence of food, worms exhibited a low pump rate (~17 ppm), whereas when worms were placed on a plate seeded with OP50 they initially showed a low pumping rate for the first 5min after removal from cleaning plate (~0 – 150 ppm) before maintaining an expected on food pump rate averaging 250 ppm (*Figure 5.5B*). In the presence of beads, worms showed a transient increase in pumping rate at 2min (121 ppm, *Figure 5.5D*) followed by a low pumping rate at 3 min (59 ppm, *Figure 5.5D*) which was still significantly higher than worms in an off-food context (*Figure 5.5C*). Overall, worms in the presence of beads exhibited a burst in pumping rate but did not display a sustained elevated pumping rate over 10min of the assay. Nonetheless the overall average pumping rate was significantly higher (35 ± 4 ppm) compared to that observed in the absence of food (*Figure 5.5B&C*).

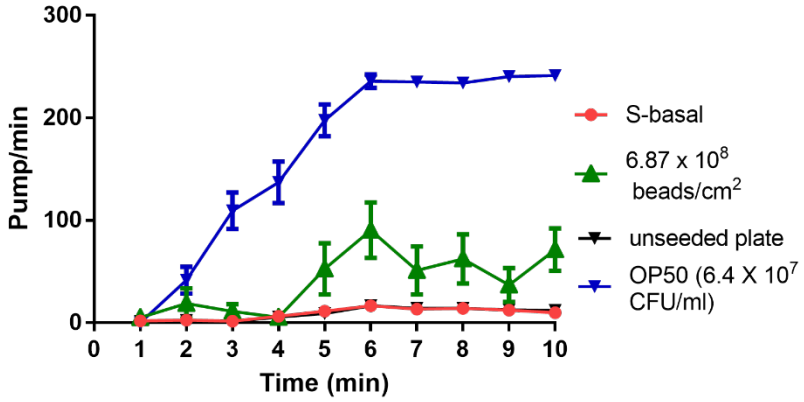
To understand this initial burst of pharyngeal pumping in the presence of beads, fluorescent images of the worms in contact with beads were taken after 10min. Fluorescence emanating from ingested beads was observed in the isthmus and terminal blub with a small modest intensity in the intestine (*Figure 5.6*).

In general, these findings suggest that the mechanical presence of beads in the pharynx stimulate pharyngeal pumping. However, this initial stimulation in pumping rate is transient.

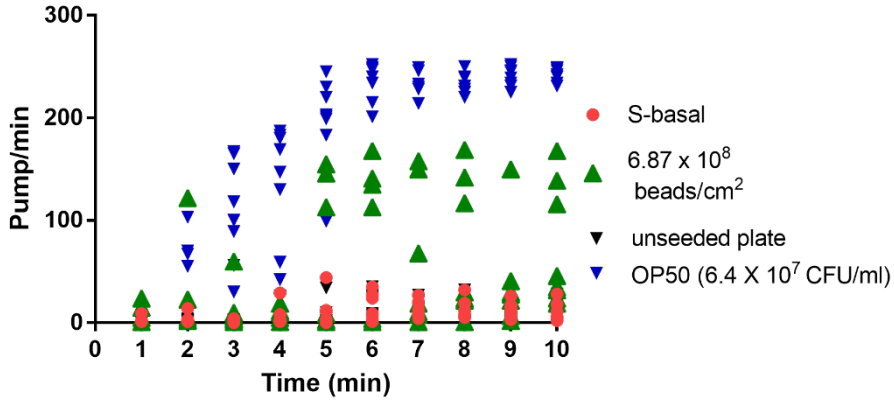
A



B



C



D

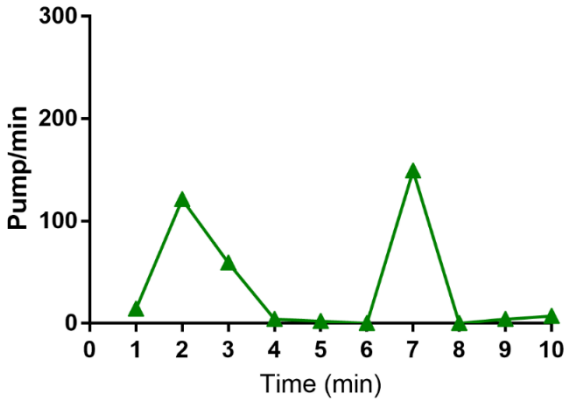
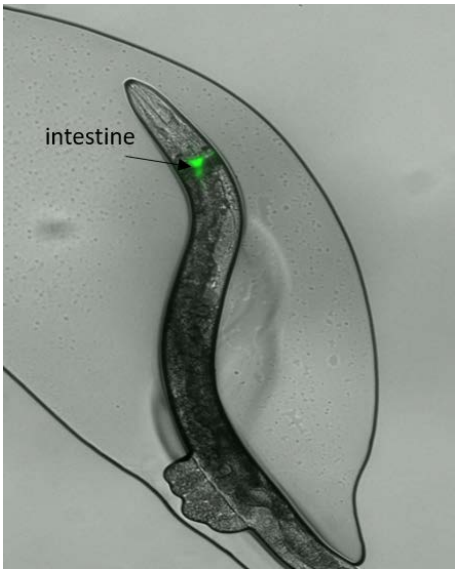


Figure 5.5: A burst in pump rate was observed when worms were in contact with ingestible beads. *A) A schematic representation of the assay. Beads were diluted in S-Basal. 15 L4+1 worms were picked and transferred to cleaning plate for 1 min before they were transferred to either a plate seeeded with OP50, unseeded plate, S-basal or a plate equally spread with 6.87×10^{8} beads/cm^{2} spread. B) In the presence of food, worms recovered on food pump rate 5 min (235 ± 4, n= 8) after being picked to the assay plate. In presence of s-basal worms exhibited pumping (10 ± 2 ppm, P = 0.99 that was not different from worms on an unseeded plate (9 ± 4 ppm). In the presence of beads, worms displayed high pumping rate compared to worms on s-basal and off-food plate (35 ± 4 ppm, p < 0.0001, n = 8). The experiment was repeated twice. C) A scatter diagram representing the transient increase of pumping rate in the presence of beads. D) A time line for single worm transitioning from low to high and high to low pumping rate during the assay.*

A



B

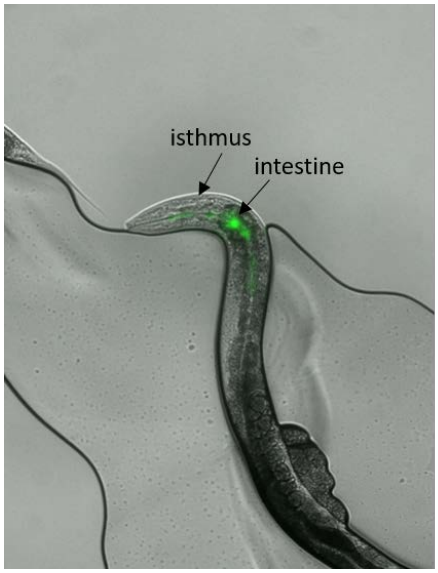


Figure 5.6: The distribution of fluorescent beads in worms fed with ingestible (0.5µm) of beads. *A & B) The representative images of worms exposed to Fluoresbrite® YG microspheres for 10 min. After 10 min of physical contact with beads, the worms were placed on an agarose pad and immobilised by adding 2 µl of 1M sodium azide, before placing a microscopic coverslip to image them. The beads were observed in the isthmus and terminal bulb of the pharynx. The anterior part of the intestine, just behind the terminal blub, also showed accumulation of beads. Number of worms = 2*

5.2.6 Physical contact with ingested beads is required for sustained and elevated pharyngeal pumping

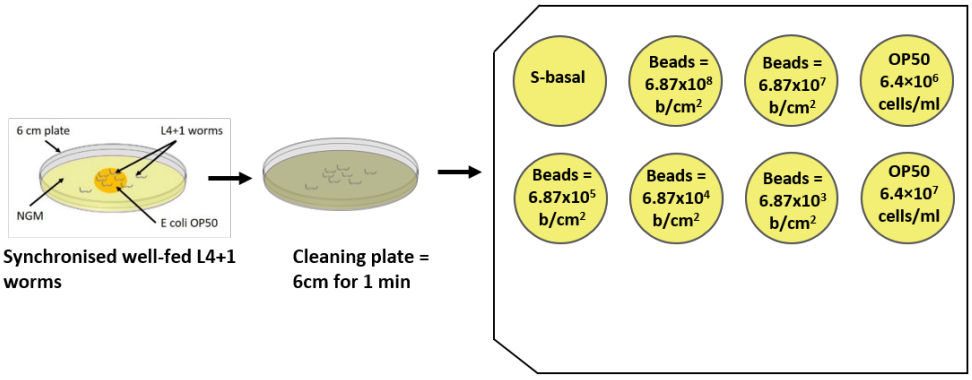
The above findings suggest that ingested beads of similar size to bacteria drive pharyngeal pumping in the absence of food i.e. they can mimic the presence of bacteria. However, this elevated pumping rate was short-lived. Therefore, the exposure of worms to beads was extended to 2 hr and the effect of time and dose of ingested beads on pumping rate were investigated. A stock of 3.64 x 10^{11} particles/ ml of ingested beads was used to prepare assay plates with increased densities 6.78 ×10^{3} - 6.78 ×10^{8} beads/cm^{2} to assess pharyngeal pumping (*Figure 5.7A*).

In the presence of ingestible beads, worms placed on high densities of 6.78 $\times 10^6$, 6.78 $\times 10^7$ and 6.78 $\times 10^8$ beads/cm^2 exhibited an elevated pumping rate compared to worms on off-food plate (*Figure 5.7B*). Moreover, worms showed a steady increase in pumping rate at these densities reaching maximum pumping rate at 90min (~200 ppm). Worms placed on the highest density of beads 6.78 $\times 10^8$ beads/cm^2 had a higher pumping rate than worms on 6.78 $\times 10^6$ and 6.78 $\times 10^7$ beads/cm^2. Overall, although worms on high densities of ingestible beads of 6.78 $\times 10^6$, 6.78 $\times 10^7$ and 6.78 $\times 10^8$ beads/cm^2 displayed an elevated pumping rate, their pumping rate was significantly lower than worms on food (~ 250 ppm, *Figure 5.7B*). Moreover, the beads induced a high and sustained pump rate but this required 90 min to reach steady state. This contrasts with the food reduced response in which 5min was needed to reach maximum pumping rate.

Furthermore, worms displayed defecation in the presence of ingestible beads (*Figure 5.8*) when worms were in contact with ingestible beads of 6.78 $\times 10^8$ beads/cm^2 for 90 min. The accumulation of beads was observed throughout the worm's gut, including the pharynx and intestine (*Figure 5.8*). Interestingly, high level of beads in the intestine was observed at 90min (Figure 5.8) compared to worms exposed to beads for 10min only (*Figure 5.6*).

In general, these observations suggest that worms increase their pharyngeal pumping in response to the presence of particles that are small enough to be ingested and this slow increase to a sustained pump rate is associated with accumulation and associated distension in the intestine.

A



B

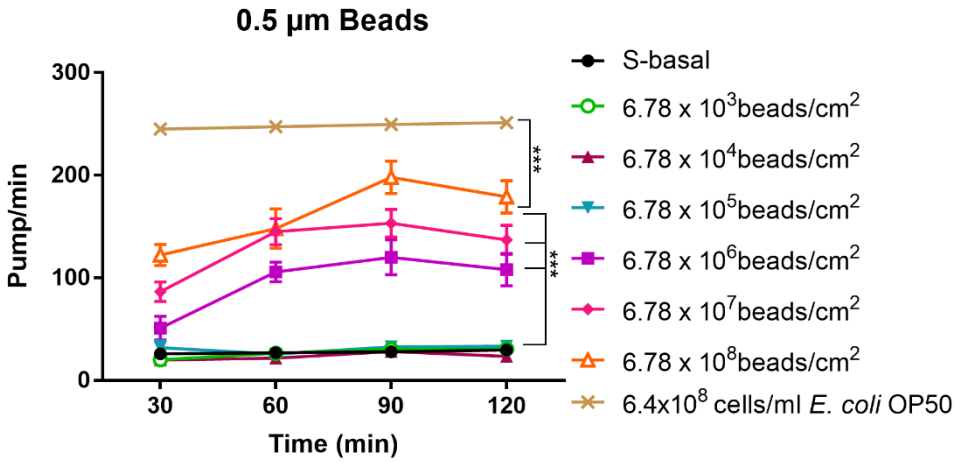


Figure 5.7: Pumping rate on beads is dose- and time-dependent. *A) A schematic diagram showing the experiment layout. 20 L4+1 worms were picked and placed onto cleaning plate for 1 min before placed onto a 12-well assay plate. B) Ingestible beads with 0.5 µm in diameter (equivalent to E. coli OP50 short-axis size = 0.5 µm). Worms placed on high densities of beads, 6.87x10^6 – 6.78x10^8 beads/cm^2 showed a sustained increase in pumping rate over 90 min. At these densities worms pumped significantly higher than worm off-food (120 ± 13, 153 ± 11 & 198± 11 ppm, p < 0.0001, n= 11-15 worms/ group) and pumped significantly lower than worm on food (248 ± 1 ppm). The experiment was repeated twice. Two-way ANOVA followed by Bonferroni test was used. Error bars represent mean ± S.E.M.*

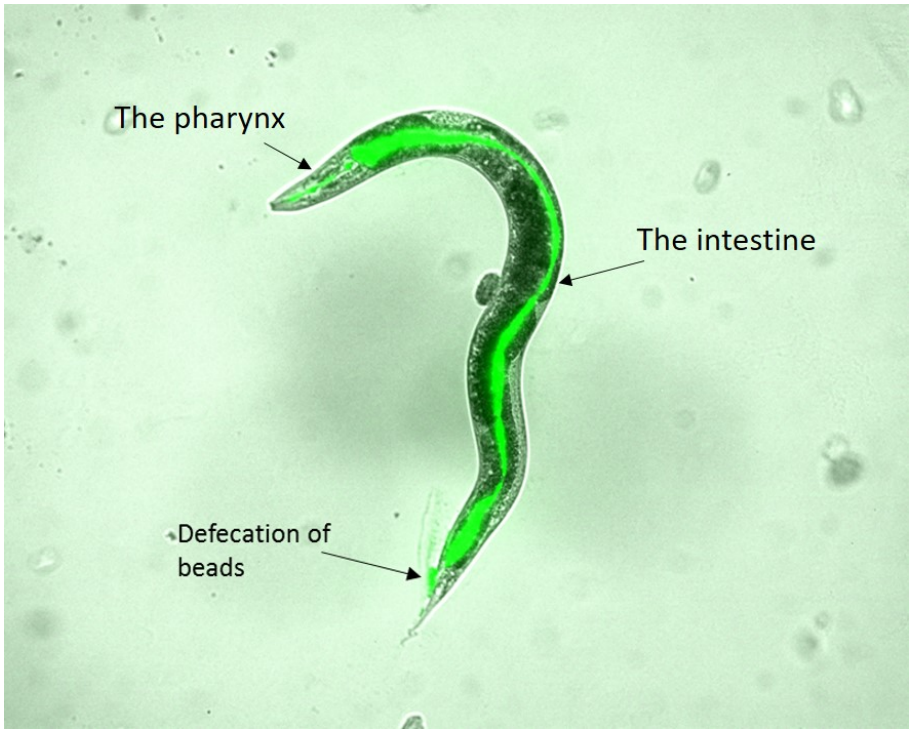


Figure 5.8: Intestinal distention and defecation from worms subjected to protracted feeding with ingestible (0.5µm) of beads for 90 min. *The image of worms exposed to Fluoresbrite® YG microspheres for 90 min. After 90 min incubation with beads, the worms were placed on an agarose pad and immobilised by adding 2 µl of 1M sodium azide, before placing a microscopic cover to image them. Fluorescence was observed accumulation of high density throughout the gut. Furthermore, the defecation of beads can be observed. Number of worms = 3.*

5.2.7 Large, non-ingestible beads do not elevate pumping rate

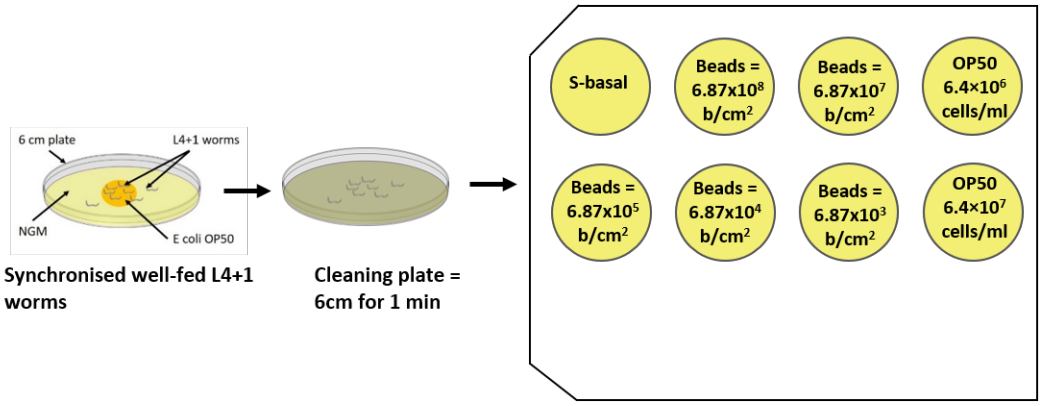
To understand the key elements of this mechanosensory cue the pharyngeal response to ingested beads (0.5µm) was compared to larger non-ingestible polystyrene microspheres (6.0µm). A stock of 2.10 x 10^8 particles/ml non-ingestible beads were used for the assay similar to the ingested beads experiment described above (*Figure 5.7A*).

In the presence of non-ingestible beads, worms did not stimulate their pharyngeal pumping and displayed pumping rate similar to off-food pumping rate (*Figure 5.7C*). The

increased contact of worms with non-ingested beads did not affect pharyngeal pumping. Moreover, the densities of 6.78 $\times 10^7$ and 6.78 $\times 10^6$ beads/cm^2 in non-ingestible did not elevate pumping (~15-40 ppm) compared to worms placed on the same densities of ingestible beads (~127 – 158 ppm, *Figure 5.9A&B*).

These data suggest further support the above findings (*Figure 5.7B*) that the physical presence of beads in the gut is required to drive increased pumping rate.

A



B

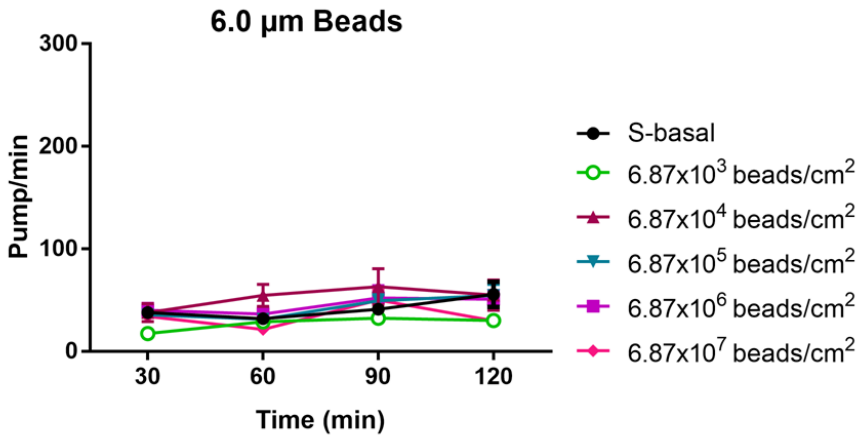


***Figure 5.9: Non-ingestible beads do not elevate pumping rate**. A) A schematic representation of the experiment. The experimental set-up used here is identical to that shown in Figure 5.7 with the exception of using large, non-ingestible beads of size 6µm. B) Worms on large beads did not show significant ($p > 0.05$, n= 9-12 worms/ group) differences from worms placed on unseeded plates. The experiment was repeated*

twice. Two-way ANOVA followed by Bonferroni test was used. Error bars represent mean ± S.E.M.

5.2.8 Ingestible beads do not affect body bends

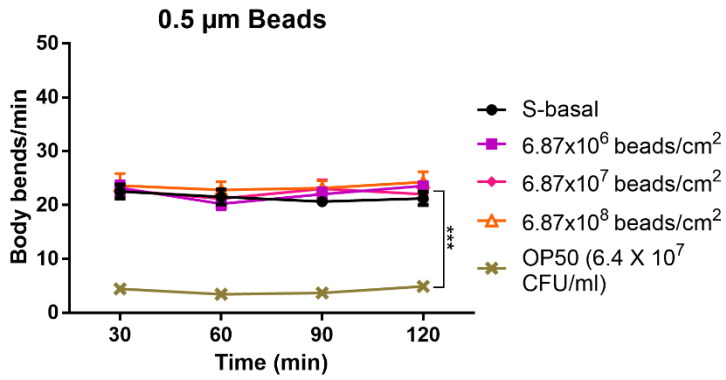
In addition to altering feeding behaviour according to the food context, worms also change their locomotory behaviour (see Chapter 1 sections 1.5.1 -1.5.1.4). In the presence of bacteria (food), worms switch between two patterns of locomotion, dwelling and roaming (Ben Arous et al., 2009). The dwelling state is manifest by a reduced speed and increased frequency turns to allow the worms to remain on food area. Dwelling is interleaved with periods of roaming in which worms exhibit increased speed with fewer turning events to permit exploration of the bacterial lawn (Ben Arous et al., 2009). It is thought the worms utilise these two behavioural states to assess their local environment (Ben Arous et al., 2009) because worms spend more time roaming in the absence of food or low-quality food and spend more time dwelling in the presence of food (Flavell et al., 2013). Moreover worms respond to the presence of food by reducing body bend frequency known as the slowing response (Sawin et al., 2000). This slowing response that follows contact with newly encountered food allows worms to spend more time on the food. Therefore, the effect of ingestible and non-ingestible beads on body bends frequency was investigated with a view to determining whether or not the sensory cue provided by ingestible beads underpins a coordinated behavioural response on food encompassing both elevated pumping and dwelling behaviour. Thus, the effect of ingestible beads and non-ingestible beads on locomotion was investigated. The body bends of worms on ingested and non-ingested beads were counted and compared to body bends of worms on food. Body bends is defined by observing the area just behind the pharynx bending in the opposite direction and returning to its original direction (S-shape). The densities that displayed the highest pumping in the presence of ingestible beads (Figure 5.7B) were investigated and compared to body bends of non-ingestible beads.

In the presence of ingestible beads, worms displayed high levels motility as assayed by body bend (21 ± 1 body bends/min, *Figure 5.10B*). Indeed, this is the level of motility observed when motility is measured in the absence of food (22 ± 2 body bends/min). Furthermore, the time of exposure to beads did not affect body bends even at 90min in the presence of 6.78×10^{8} beads/cm^{2}, which correlates with highest pumping rate (23 ± 2 body bends/min, *Figure 5.7B & Figure 5.10B*). Worms in the presence of food displayed a

basal slowing response which is characterised by reduced body bends as previously reported (Sawin et al., 2000). Worms incubated in the presence of small ingestible beads had the same rate of body bends as worms incubated in the absence of food (*Figure 5.10A*). similar findings were observed with the large beads (*Figure 5.10B*).

Overall, although ingested beads showed time and dose –dependent increased pharyngeal pumping. However, this was not reflected in the locomotion rate suggesting that this mechanical stimulation of pharyngeal pumping and locomotion rate is mediated through distinct neural circuits.

A



B

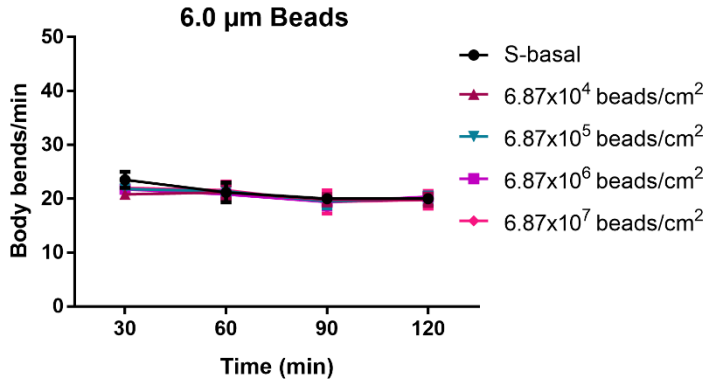


Figure 5.10: Ingestible and non-ingestible beads do not affect worm's locomotion. *The experimental set-up used here is identical to that shown in Figure 5.7. A) The effect of ingestible beads of 0.5µm motility as measured by body bends and compared to an OP50 patch of equivalent particular density. Worms on OP50 (23 ± 4 body bends, $p < 0.0001$, n =9-11 worms/ group) showed a marked reduction in motility relative to those*

incubated on S-basal on increasing density of ingestible beads. B). The number of body bends of worms incubated in the presence of 6 um beads was not different from number of body bends of worms in S-basal (off-food) (n= 10-15 worms/ group, p = 0.99). Each experiment was repeated twice. Two-way ANOVA followed by Bonferroni test was used. Error bars represent mean ± S.E.M.

5.2.9 Bacterially conditioned media do not elevate pumping behaviour

The observations that non-viable bacteria were sufficient to elevate pumping implicates mechanical and possibly gustatory cues in driving this behaviour. Additionally, the incubation of beads for prolonged period resulted in elevated pumping rate that is comparable to on food but not identical. These data suggest that an additional sensory modality, or modalities, sustain high pumping rate. Therefore, the effect of a potential gustatory cue on pumping rate was investigated over 2 hr period. This was conducted by filtering bacterial culture with OD_{260} of 1.0 using a 200 nm filter to trap bacterial cells. Worms were added to a 12-well plate prepared with either conditioned medium only, beads diluted in conditioned medium, fresh LB broth only or an unseeded plate. As a control, beads diluted in s-basal was used as a comparison.

In the presence of fresh LB broth, worms pumped at a similar rate (36 ± 2 ppm, *Figure 5.11*) to worms on a non-food plate which further support that fresh LB has no effect on pumping rate. Incubation of worms in conditioned medium only did not increase pumping rate in the absence of food (35 ± 3 ppm, *Figure 5.11*). However, worms incubated in beads diluted in conditioned medium pumped similarly to worms incubated in beads diluted in s-basal (at 30 min = 123 ± 2 ppm, at 60 min = 166 ± 3, at 90 min = 201 ± 4 ppm, at 90 min = 182 ± 5ppm, n= 10-14, *Figure 5.11*).

These findings indicate that a chemical gustatory cue or cues from the bacteria is not enough to drive pumping rate in the absence of food.

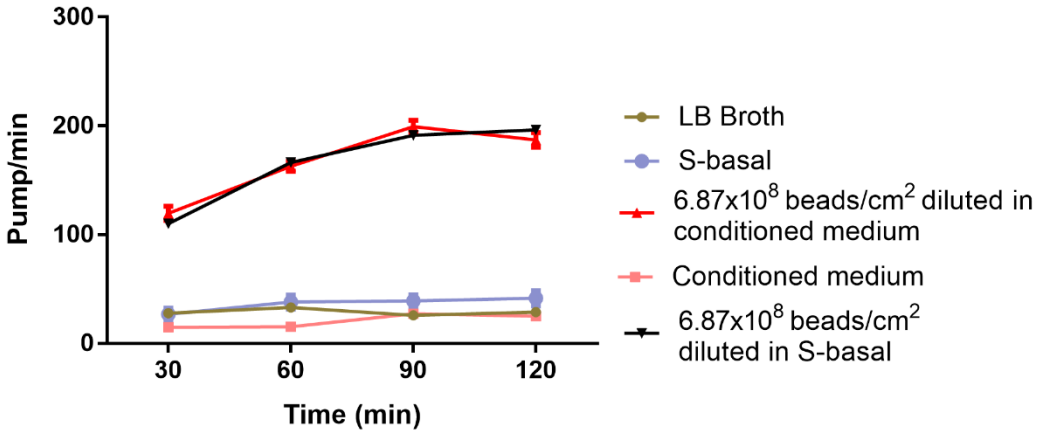


Figure 5.11: Bacterially conditioned medium had no effect on pumping rate in the absence of food. *A single colony was inoculated into a fresh 10 ml LB Broth. The culture was incubated at 37º C in an incubator shaker for 12-14 hr. To obtain bacterially conditioned medium, the LB culture was filtered using 200 nm filter to trap bacteria. The conditioned medium was added to assay plates. Beads were diluted in either conditioned medium or s-basal to obtain a density of 6.87×10^8 cells/cm^2. 20 L4+1 worms were picked and placed onto cleaning plate for 1 min before placed onto a 12-well assay plate. In the presence of conditioned medium, worms pumped at a rate insignificantly different from control ($p > 0.05$, $n = 13$ worms). In the presence beads diluted in conditioned, worms pumped at a rate insignificantly different from worms incubated in beads diluted s-basal (n= 10-14). The experiment was repeated 3 times. Two-way ANOVA followed by Bonferroni test was used. Data represent mean ± S. E.M.*

5.3 Discussion

5.3.1 The absence of olfactory cues reduces pharyngeal pumping

In this chapter the cue-dependence of pharyngeal pumping was investigated. The cilia-deficient mutant, *osm-6*, was used to investigate the role of chemosensory neurones in the modulation of pharyngeal pumping. *osm-6,* is expressed in ciliated sensory neurones and have been shown for their inability to avoid high concentrations of NaCl and fructose (Culotti and Russell, 1978). Here it was shown that the *osm-6* loss of function mutant exhibits normal pumping rate in the presence of food. This suggests that whilst functional ciliated neurones are required for worms to respond to and avoid noxious cues they are not involved in the elevation of pumping rate observed in the presence of food.

In the absence of food, *osm-6* mutants displayed an unexpected elevated pharyngeal pumping that approached the pharyngeal pumping rate in the N2 worms in the presence of food. This suggests that ciliated neurones play an important role in signalling the absence of food and are key upstream players in the circuit that inhibits pumping. These data provide important insight into the data shown in Chapters 3 and 4 i.e. the observations that environmentally-salient odours for innate and acquired chemotaxis behaviour were not sufficient to drive pumping rate or condition feeding behaviour.

5.3.2 Mechanosensory cues increase pumping rate

The data obtained in the chapter have shown that *C. elegans* is sensitive to the presence of bacteria, in which *C. elegans* maintained a high pumping rate even at low concentration (1×10^{-7}) with 10 CFU (*Figure 5.1*). Furthermore, that radiated, non-viable, bacteria had no effect on pharyngeal pumping, worms continued to pump at high rate, although cues released from bacteria were not present (*Figure 5.4*). Taken together, with the observations on *osm-6* mutants, this suggests that with respect to driving pharyngeal pumping the important sensory cues signalling the presence of food are mechanical and possibly, gustatory or nutritional-related.

To test the effect of mechanical cue on pumping rate, beads were used. To examine the effect of intrinsic detection of beads by the pharynx, ingestible beads were selected. It was previously reported that worms could ingest and elevate their pumping in response to beads with a diameter of less than 4.5µm (Fang-Yen et al., 2009). To allow direct

comparison to the worm's usual food source, *E.coli* OP50, beads with the same size of the bacterial short axis of 0.5µm was chosen (Fang-Yen et al., 2009).

The effect of short-term contact of worms with beads for 10 min was investigated. When worms were placed on beads, they showed a transient increase in pharyngeal pumping (*Figure 5.5C*). Individual worms showed transitions in pumping rate from very high pumping similar to on-food and low pumping rate similar to off-food (*Figure 5.5D*). Microscopic observation showed beads present in the isthmus and terminal blub of the pharynx and to a less extend in the anterior of the intestine. It was previously reported that the accumulation of beads was observed in the intestine after 15 min (Kiyama et al., 2012). This is further supported by increasing exposure time from 10 min to up to 2 hr. Worms displayed increased elevation in pumping rate that reached up 200 ppm after 90min of contact with beads (*Figure 5.7B*). Additionally, microscopic observation revealed high levels of beads accumulated inside the worm after 90min exposure correlating with the increased pharyngeal pumping. Moreover, worms displayed defecation of beads, although it was not seen in all animals possibly due to the reduced defecation drive that was reported to happen in the absence of food (Kiyama et al., 2012). Overall, this shows that to some extent at least the beads that mimic the size of *E. coli* may be ingested and transit through the gastrointestinal tract of the worm in a similar manner to bacteria.

To test if the presence of beads is perceived extrinsic to the worm, large, non-ingestible beads of 6.0µm were also investigated (Fang-Yen et al., 2009). In the presence of large beads, worms exhibited pumping rates similar to that observed in the absence of food (*Figure 5.9C*), suggesting that worms do not perceive the presence of beads outside the worm. It would seem that the accumulation of beads inside the pharynx could lead to the distension of the pharynx and, given the time-dependence of the behaviour that matches accumulation of beads in the post-pharyngeal gut, that this latter factor is critical to sustained increase in pharyngeal pumping observed in the presence of ingestible beads.

5.3.3 Beads-induced pharyngeal pumping does not affect locomotion

Despite the observation that ingestible beads could substitute for bacteria to some extent in terms of stimulating pharyngeal pumping there is no evidence that this mechanical cue alone is sufficient to trigger the locomotory behaviours observed when worms encounter food e.g. the basal slowing response. The effect of beads on locomotion was determined by counting the number of body bends. Neither ingestible nor non-ingestible beads had an effect on body bends relative to worms incubated off food. This indicates that beads-induced pharyngeal pumping is not associated with reduced locomotion. Sawin et al. 2000 reported that worms reduced their locomotion in response to Sephadex beads, which contradicts with findings in this chapter. Body bends were measured to correlate with the sustained pumping rate observed in the pharyngeal assay. However, they used a different paradigm to the one used here. The body bends were measured after 2min from transferring worms onto Sephadex beads plate. Moreover, the size of Sephadex beads used was 200 µm compared to 6 µm used in this experiment. Therefore, it could be that the locomotion rate was reduced due to the large size of the beads, which could have slowed down the movement of the worm on the agar. Therefore, the data suggest that the dopamine release in response to mechanical cue is distinct from the dopamine that drives the basal slowing response observed when worms encounter a new food source.

5.3.4 Pumping behaviour on beads was slower compared to on food

Although the mechanical cue from the ingestible beads increased the pumping rate in the absence of food, this rate was not as high as the on food pumping rate (*Figure 5.7B*). similarly, there was a density-dependent pharyngeal pumping in the presence of beads (*Figure 5.7B*). However, pumping rate was more gradual with the highest pumping rate was observed at 6.78×10^{-8} beads/cm^2 at 200 ppm, 6.78×10^{-7} at 153 ppm and 6.78×10^{-6} at 120 ppm. However, density-dependent effect of bacteria on pumping rate was very steep (*Figure 5.1*). Worms sustained high pumping rate even at very low density of bacteria (e.g. at 1×10^{-7} = 239 ppm). Therefore, this further indicate that there are other components in bacteria, which results in maintaining high pumping rate even at low concentrations. This suggests that the mechanical stimulation alone is not sufficient sustain a high rate of pharyngeal pumping. Therefore, gustatory and the nutritional cues might be important to

sustain elevated pumping rate. However, findings in this chapter suggest that bacterially conditioned medium did not have an impact on pumping rate in the absence of food. Therefore, other cues such as the nutritional cue from bacteria may be important to elevate and sustain high pumping rate.

5.3.5 Conclusion

Observations obtained from the cilia-deficient mutant, *osm-6,* indicate that olfaction does not play a role in stimulating pharyngeal pumping in the presence of food. On the contrary, the abnormally elevated pumping rate exhibited by the *osm-6* mutant suggest that these ciliated neurones respond to the absence of food odours and are involved in reducing pharyngeal pumping rate. These data indicate that the absence of olfactory cues plays a role in modulating feeding behaviour in the absence of food.

The observations with ingestible beads provide support for a model in which an important factor in driving elevated pumping rate is the mechanical distension of the pharynx following ingestion of bacteria. This is supported by the observation that large beads failed to increase pharyngeal pumping. Intriguingly the initial elevation in pumping rate in the presence of ingestible beads was not as rapid or as sustained as the response seen on bacteria. Clearly beads on their own are not a complete food mimic and this initial mechanosensory cue which results from the presence of beads in the pharynx and the anterior gut is only capable of transiently increasing pumping. However, overtime the accumulation of beads in the lower gastrointestinal tract led to a sustained elevation in pumping rate. This might be indicative of a second pathway that triggers pharyngeal pumping that emanates from the mechanical presence of bacteria in the posterior gut,

In conclusion, these data delineate an important role for mechanosensation in driving *C. elegans* feeding behaviour.

Chapter 6: Characterisation of mechanosensory-mediated pharyngeal pumping

6.1 Introduction

There are complex excitatory and inhibitory pathways that modulate pumping rates in the presence and absence of food (Dallière et al., 2016). Previous work has shown that e*at-4* mutants, which carries a loss of function mutation in a vesicular glutamate transporter, showed a reduced pumping rate in the presence of food (Greer et al., 2008; Lee et al., 2008) and increased foraging behaviour, which is defined by an increased body bends frequency (Lee et al., 2008). Dallière et al. 2016 showed that *eat-4* mutants showed a constitutive pumping in the absence of food. Moreover, *unc-31* mutants, which have a loss of function mutation in the orthologue of mammalian CAPS (calcium-activated protein for secretion), showed constitutive pumping and slow movement in the absence of food (Zetka and Rose, 1995), suggesting that both *eat-4* and *unc-31* mutants are required for reducing pumping rate in the absence of food. Additionally, a double mutant of *unc-31(e169);eat-4(ky5)* had an additive constitutive pumping in the absence of food (Dallière et al., 2016). This indicates that *unc-31* and *eat-4* mutants function in parallel pathways to inhibit pumping rate in the absence of food (Dallière et al., 2016).

These data suggest that the inhibitory tone, which actively suppresses feeding behaviour off-food, has signalling pathways that can activate the inhibitor tone. To better define the elements that stimulate pumping rate in response to beads, the molecular and cellular determinants of the on-beads effect were investigated.

6.2 Results

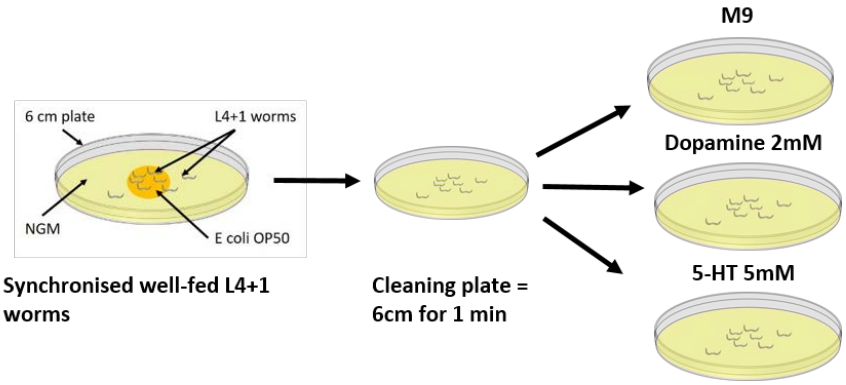
6.2.1 Exogenous dopamine increases pharyngeal pumping in the absence of food

In the previous chapter, we have shown that the stimulation of pharyngeal pumping in the absence of food was selectively achieved by 0.5µm beads that exhibited a time-dependent internal accumulation (Chapter 5 - *Figure 5.7B*).
This suggests that the elevation of pumping rate is mediated by detection through mechanical cues, like those that arise in the pharynx.
In view of previous observation, we considered a role for the biogenic amines, 5-HT, which had been shown to elevate food induced pumping and compared this to dopamine, which although important in mechanosensation, had not been implicated in regulation of pumping.

Pumping rate, which is low off-food, was first investigated in the presence of exogenous dopamine (2mM) or 5-HT (5mM) after 10, 30 and 60 minutes from removal from food (*Figure 6.1A*). The concentration of dopamine was chosen based on previous preliminary experiments that showed elevated pumping rate in the absence of food in response to exogenous dopamine (Kent et al. unpublished data). Furthermore, from literature, it has been reported that 5 &10 mM 5-HT was shown to elevate pumping in the absence of food to levels comparable to on-food pumping rate (Sze et al., 2000). Therefore, 5-HT was used as a positive control, and thus, the concentration was selected to be comparable to the dose of dopamine dose.
In the presence of dopamine, there was a steady increase in pumping rate over time (*Figure 6.1B*). Worms showed a significant increase in pumping rate compared to control worms on M9. The highest pumping rate was achieved after 60 minutes with pumping rate of 81 ppm (± 12) compared to a pumping rate of 57 ppm (±12) and 72 (±12) of worms exposed to dopamine for 10 and 30 minutes, respectively (*Figure 6.1B*). In contrast the exogenous application of 5mM 5-HT in the absence of food increased and sustained pharyngeal pumping over time to levels comparable to on-food pumping rate (10 min = 225 ppm ± 11, 30 min = 215 ppm ± 13 and 60 min = 226 ppm ± 12, *Figure 6.1B*). Overall, the results suggested that exogenous application of 2mM dopamine increased pharyngeal pumping in the absence of food. Pumping rate in the presence of dopamine was time-dependent and the highest pumping rate was achieved after 60 minutes, whereas in the presence of 5-HT an increase in pumping rate was achieved and sustained after 10

minutes. Additionally, from observations, worms on dopamine have increased locomotory behaviour compared to worms on 5-HT, which was significantly reduced.

A



B

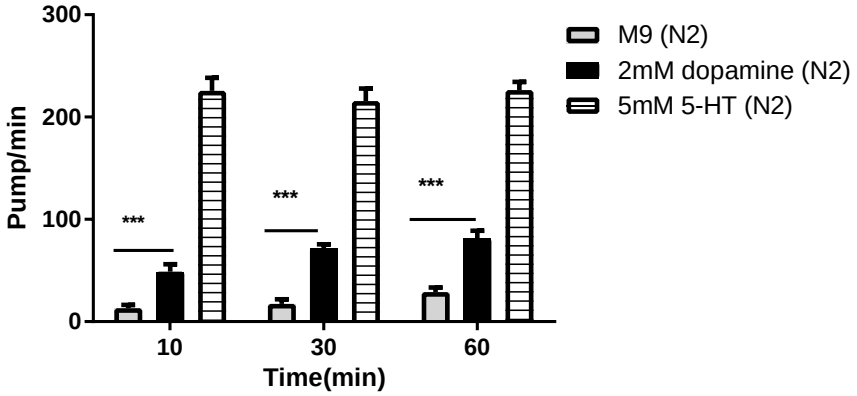


Figure 6.1: Dopamine increases pharyngeal pumping in the absence of food.
A) A schematic representation of the dopamine off-food assay. 20 well-fed L4+1 worms were picked onto a cleaning plate for 1 min before transferring onto M9, 2mM dopamine or 5mM 5-HT assay plates. Dopamine and 5-HT plates were prepared 12-14 hours befrore the assay and diluted in M9 buffer to give final concentration of 2mM dopamine or 5mM 5-HT. B) In the presence of dopamine, worms significantly increased pharyngeal pumping compared to worms in M9 after 10 min ($p = 0.012$, $n = 10 – 12$ worms/ group), 30 min ($p < 0.0001$, $n = 8$) and 60 min ($p < 0.0001$, $n = 10 – 11$ worms/ group). 5-HT significantly increased pharyngeal pumping compared to M9 after 10 min ($p < 0.0001$, $n = 10 – 12$ worms/ group), 30 min ($p < 0.0001$, $n = 9$ -10 worms/ group) and 60 min ($p < 0.0001$, $n = 13$ worms/ group). The experiment was repeated twice. Two-way ANOVA followed by Bonferroni post-hoc test was used. Data represented as Mean ± S.E.M.

6.2.2 Dopamine and serotonin signalling modulate bead-mediated pumping rate

To understand the mechanism controlling the bead-induced pumping rate, we conducted a mutant analysis. Mutants were tested on food, on beads, on dopamine or on 5-HT to determine how each mutant behaves in the different pump inducing contexts.

The findings from the previous chapter have revealed that ingestion of small beads (0.5 µm) stimulates pharyngeal pumping in the absence of food. Since exogenous dopamine can elevate pumping rate in an off-food context and dopamine is a key neurotransmitter in mechanosensory in *C. elegans*, dopamine signalling was a good candidate to test in bead-mediated pumping. Therefore, to investigate the effect of dopamine on bead-mediated pumping, we used dopamine-deficient mutant, *cat-2 (e1112),* which is important for dopamine synthesis since it encodes for tyrosine hydroxylase.

In the presence of food, *cat-2 (e1112)* mutants pumped at a similar rate relative to wild-type worms (*Figure 6.2A*). In the presence of S-basal or M9, all worms (wild type and mutants) showed reduced pumping as expected in the absence of food. However, there was a trend showing *cat-2 (e1112)* mutants on S-basal pumping lower than wild-type worms, but there was no statistical significant difference (*Figure 6.2B*). On beads, *cat-2 (e1112)* mutants pumped (67 ppm ± 8) higher than *cat-2 (e1112)* mutants on S-basal (29 ppm ± 3*, Figure 6.2B*). Nonetheless, *cat-2 (e1112)* mutants pumped significantly lower than wild-type worms (190 ppm ± 12) in the presence of beads (*Figure 6.2B*). In the presence of exogenous dopamine, *cat-2 (e1112)* worms pumped at a similar rate (37 ppm ± 10) to *cat-2* on M9 (46 ppm ± 4) and lower than wild-type worms (70 ppm ± 11, *Figure 6.2C*).

In view of the potent modulatory role of 5-HT, we investigated if there was a modification of 5-HT-induced pumping in *cat-2* mutants. However, these pumped at a similar rate in response to 5-HT (243 ppm ± 2, *Figure 6.2C*) relative to N2 worms (240 ppm ± 4, *Figure 6.2C*). Thus dopamine is not required for 5-HT stimulation of pumping, and may act either downstream or parallel to dopamine-induced pumping.

On the other hand, it was also important to test whether the stimulation of pumping by beads or dopamine was dependent on 5-HT. TPH-1 encodes tryptophan hydroxylase, which is a rate-limiting enzyme in the 5-HT biosynthesis. In the presence of food, *tph-1 (n4622)* showed the previously reported reduced pumping rate (148 ppm ± 5) compared to N2 worms (236 ppm ± 5, *Figure 6.2A*). In a similar way, on beads *tph-1 (n4622)* showed a pumping rate (22 ppm ± 3) similar to *tph-1 (n4622)* worms on S-basal (23 ppm ± 2) and a reduced pumping rate compared to wild-type worms (190 ppm ± 12) in

response to beads (*Figure 6.2B*). *tph-1 (n4622)* worms on dopamine showed an increased pumping rate (48 ppm ± 6) compared to *tph-1* worms on M9 (16 ppm ± 3), however, there was no significant differences between the groups. Furthermore, *tph-1(n4622)* worms pumped markedly lower than wild-type worms (70 ppm ± 11, *Figure 6.2C*). In the presence of 5-HT, *tph-1 (n4622)* worms showed a reduced pumping rate (163 ppm ± 5) compared to N2 worms (240 ppm ± 4, *Figure 6.2C*).

Overall, the data suggests that in the presence of food 5-HT, but not dopamine, is required to sustain high levels of pharyngeal pumping. The role for dopamine in stimulating pumping is only revealed by its ability to increase pharyngeal pumping in the absence of food. This appears to be linked to the effect of beads as the stimulation of pharyngeal pumping in the absence of food requires dopamine. Furthermore, the inability of *tph-1 (n4622)* mutants to increase pumping rate in response to beads indicates that the pharyngeal response to beads also involves 5-HT signalling. Combining the exogenous neurotransmitters in the face of the different neurotransmitter-deficient mutants suggest that the two transmitters act independently of each other to drive pharyngeal pumping.

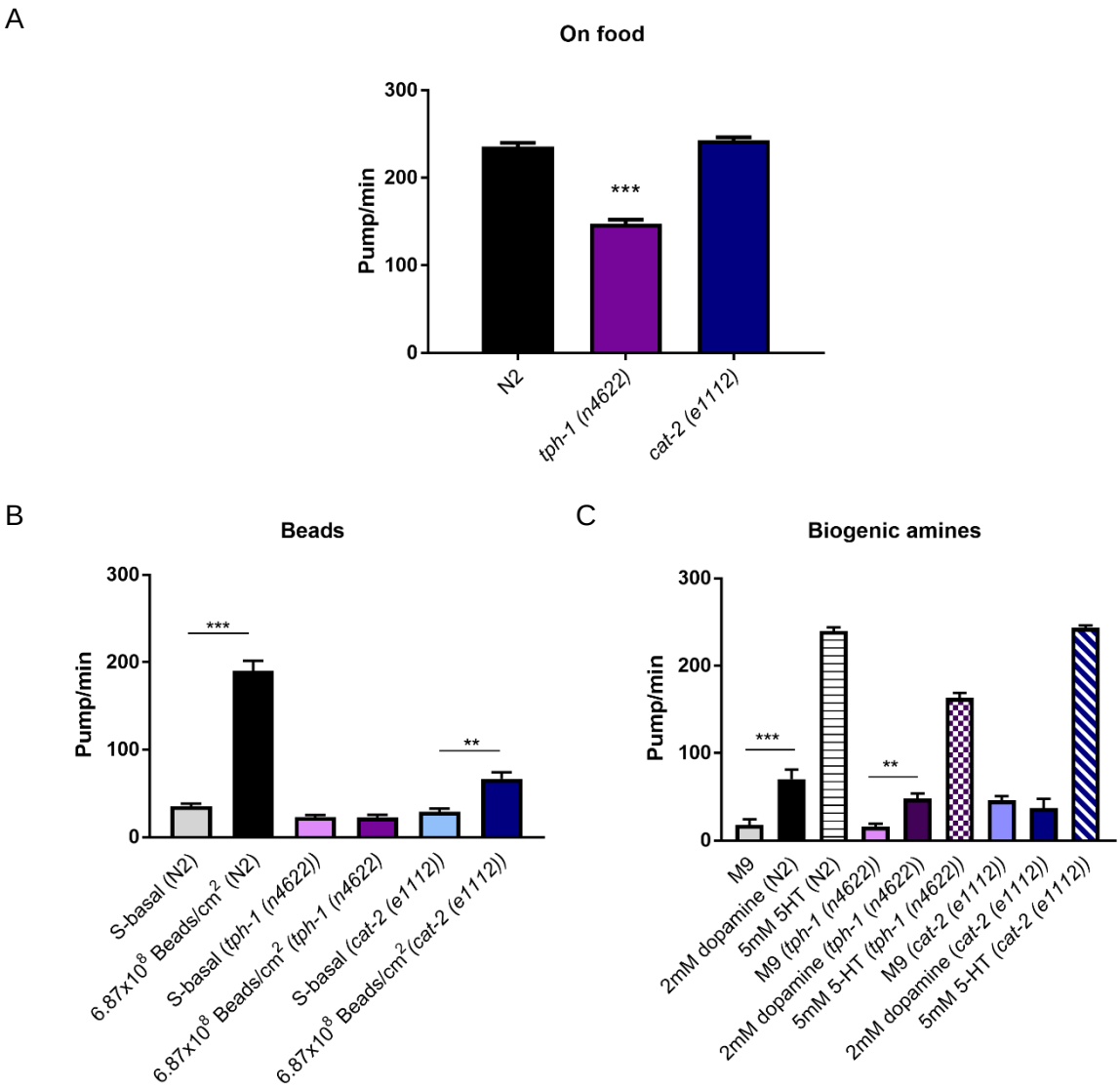


Figure 6.2: Pharyngeal-induced pumping in cat-2 (e1112) and tph-1 (n4622) on food, dopamine, 5-HT in response to exogenous 5-HT, dopamaine and beads.

15-20 L4+1 well-fed worms were picked into a cleaning plate for 1 min before transferring onto assay plates. For the schematic representations of the assays, see Figure 5.7A and Figure 6.1A. A) Comaprison of N2, cat-2 and tph-1 mutants pump rates on a food lawn. B) Comparison of wild-type and mutants pump rates after 90 min incubation on indicated density of beads. C) N2 and mutants pump rates in the presence of the indicated concentration of exogenous transmitter.

A) cat-2 mutants showed feeding behaviour similar to N2 ($p > 0.05$, n = 10 worms/ group). In contrast, tph-1 showed a reduction ($p < 0.0001$, n = 10 – 11 worms/ group) in pumping rate compared to N2. B) In the presence of beads, cat-2 pumped higher than cat-2 on S-basal ($p = 0.0024$, n = 13 worms), but lower than N2 worms ($p< 0.0001$) on beads. tph-1 mutants pumped at a similar rate to *tph-1* worms on S-basal ($p = 0.99$, n = 12 worms) and lower than N2 on beads ($p < 0.0001$). C) On dopamine, *cat-2 pumped at*

similar rate to cat-2 on M9 (p = 0.99, n = 6). tph-1 mutants pumped higher than tph-1 on M9 (p = 0.0017, n = 7 worms). On 5-HT, cat-2 mutants pumped at a high level (p < 0.0001, n =7 worms) compared to cat-2 worms on M9 and at similar rate to N2 on 5-HT (p = 0.99). Whereas tph-1 mutants showed increased pumping rate (p < 0.0001, n = 7 worms) compared to tph-1 on M9, but lower than that observed in wild-type worms (p = 0.0001). Each experiment was repeated twice. One-way ANOVA followed by Bonferroni post test was used. Data represented as Mean ± S.E.M.

6.2.3 Dopamine receptors, DOP-3 and DOP-4, are required for bead-induced pumping rate in response to beads and for dopamine-mediated pharyngeal pumping

The lack of pharyngeal stimulation of *cat-2* worms in response to beads or dopamine, suggests a role for dopaminergic signalling in the excitation of the pharyngeal system. To determine which dopaminergic receptor or receptors are required for the dopamine- and bead-mediated increased pumping rate, dopamine receptor mutants were investigated in the presence of food, beads, dopamine or 5-HT.

In the presence of food, the *cat-2* mutant retains an ability to elevate pump rate to a level seen in N2. In a similar way all the dopamine receptor mutants, *dop-1 (vs100), dop-2 (v105), dop-3 (vs106)* and *dop-4 (tm1392)* pumped at a rate similar to N2 worms when assessed on food (*Figure 6.3A*).

Next, the pump rate on S- basal and beads for each receptor was compared to N2 controls. All worms (wild type and mutants) on S-basal showed no differences in baseline off-food pump rate. In contrast there was a differential effect on *dop-4* with respect to bead-induced pumping *Figure 6.3D*. In the presence of beads, *dop-1 (vs100)* and *dop-2 (vs105)* worms pumped at a higher rate *(dop-1* = 161 ppm ± 7, *dop-2* = 179 ppm ± 10) compared to *dop-1 (vs 100)* and *dop-2 (vs 105)* worms on S-basal *(dop-1* = 35 ppm ± 5, *dop-2* = 27 ppm ± 5), which were similar to that observed in wild-type worms (182 ± 10, *Figure 6.3B*).

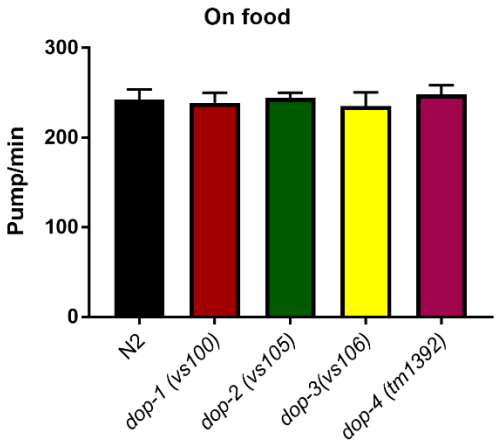
In the presence of exogenous dopamine, *dop-1 (vs 100)* and *dop-2 (vs105)* mutants showed an increased pumping rate (*dop-1* = 74 ppm ± 6, *dop-2* = 82 ppm ± 8) compared to *dop-1 (vs100)* and *dop-2 (vs105)* on M9 (*dop-1* = 20 ppm ± 4, *dop-2* = 17 ppm ± 6) and to wild-type worms (102 ppm ± 7, *Figure 6.3D*). In the presence of 5-HT, *dop-1 (vs100)* and *dop-2 (vs105)* mutants pumped higher compared to the same mutants on M9 (*dop-1* = 243 ppm ± 6, *dop-2* = 234 ppm ± 8) from N2 (102 ppm ± 7, Figure 6.3C). This indicates

that these receptors do not contribute to the bead- or dopamine-induced pharyngeal pumping.

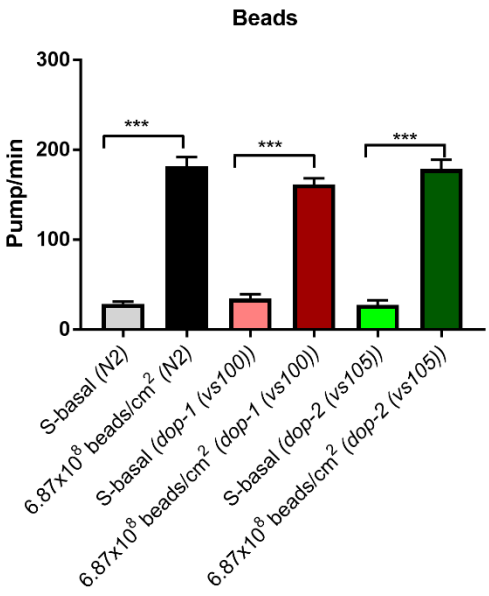
dop-3 (v106) mutants showed a reduced bead-induced pumping rate (93 ppm ± 13) compared to N2 (179 ppm ± 7). In a similar way when these mutants were tested for the ability to respond to exogenous dopamine they showed a reduced response (48 ppm ± 6) relative to N2 (101 ± 7, *Figure 6.3D & E*) controls. This suggests that *dop-3* may contribute to the dopamine-induced pumping. In the case of *dop-4 (tm1392)* mutants, there was a more complete loss of the bead-induced pumping (57 ppm ± 8) compared to N2 (179 ppm ± 7, *Figure 6.3D*), suggesting it is a major determinant of the dopamine-induced pumping as highlighted in *cat -2 (e1112)* mutants. Consistent with this, the induced pumping of the pharynx in response to exogenous dopamine was almost completely lost (18 ppm ± 3, *Figure 6.3D & E*) relative to N2 controls (102 ± 7). Previous experiments on pumping rate in response to exogenous transmitter and beads indicated that the 5-HT-dependence may be distinct, and this was reinforced by the observation that the elevated pumping in the presence of exogenous 5-HT was unaffected in any of the dopamine receptor mutants.

Overall, all dopamine receptors did not regulate on-food pumping rate. DOP-1 and DOP-2 receptors were not required for the bead-induced pumping rate in response to beads and dopamine. Nonetheless, the depression of pumping rate in the *dop-4 (tm1392)* mutants indicated a critical role for DOP-4 receptors in both bead- and dopamine- mediated pharyngeal pumping, whereas DOP-3 receptors had a modest effect on beads and dopamine.

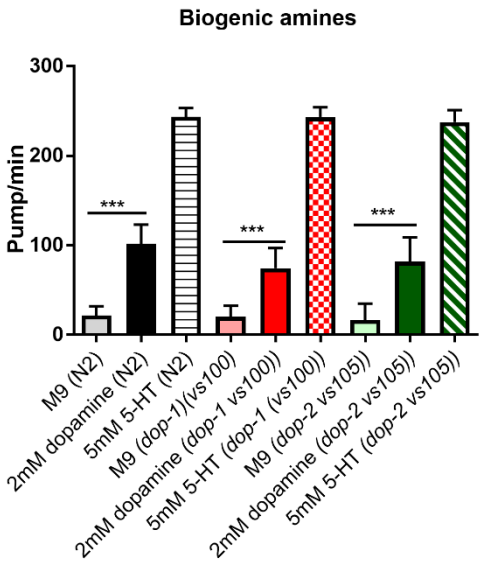
A


On food
Pump/min
300
200
100
0
N2
dop-1 (vs100)
dop-2 (vs105)
dop-3(vs106)
dop-4 (tm1392)

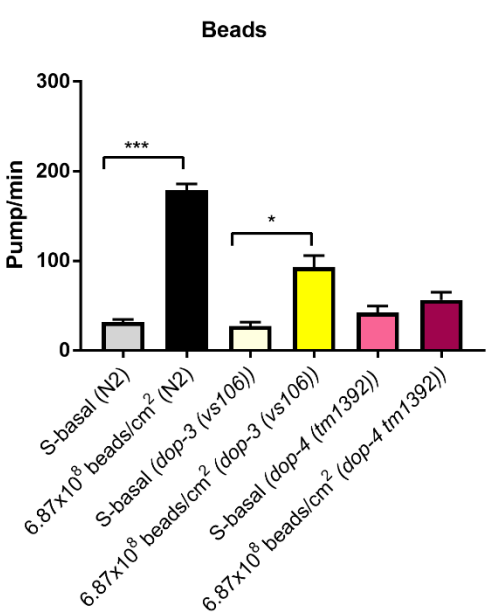
B


Beads
Pump/min
300
200
100
0
***
***
***
S-basal (N2)
6.87x10^8 beads/cm^2 (N2)
S-basal (dop-1 (vs100))
6.87x10^8 beads/cm^2 (dop-1 (vs100))
S-basal (dop-2 (vs105))
6.87x10^8 beads/cm^2 (dop-2 (vs105))

C


Biogenic amines
Pump/min
300
200
100
0
***
***
***
M9 (N2)
2mM dopamine (N2)
5mM 5-HT (N2)
M9 (dop-1)(vs100)
2mM dopamine (dop-1 vs100))
5mM 5-HT (dop-1 (vs100))
M9 (dop-2 vs105))
2mM dopamine (dop-2 vs105))
5mM 5-HT (dop-2 vs105))

D


Beads
Pump/min
300
200
100
0
***
*
S-basal (N2)
6.87x10^8 beads/cm^2 (N2)
S-basal (dop-3 (vs106))
6.87x10^8 beads/cm^2 (dop-3 (vs106))
S-basal (dop-4 (tm1392))
6.87x10^8 beads/cm^2 (dop-4 tm1392))

E

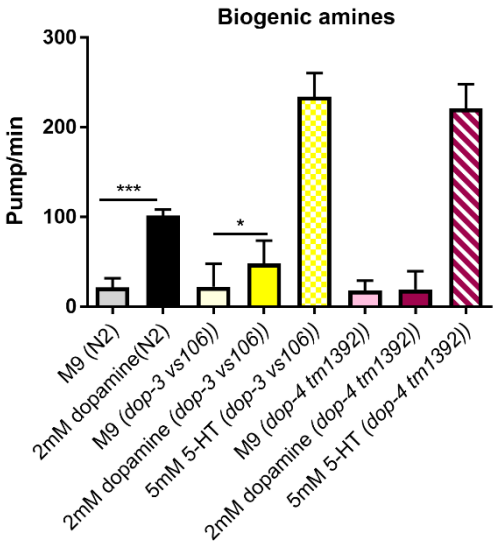

Biogenic amines
Pump/min
300
200
100
0
***
*
M9 (N2)
2mM dopamine(N2)
M9 (dop-3 vs106))
2mM dopamine (dop-3 vs106))
5mM 5-HT (dop-3 vs106))
M9 (dop-4 tm1392))
2mM dopamine (dop-4 tm1392))
5mM 5-HT (dop-4 tm1392))

Figure 6.3: DOP-3 and DOP-4 are selectively required for bead-induced and dopamine-induced pharyngeal pumping. *15-20 L4+1 well-fed worms were picked into a cleaning plate for 1 min before transferring onto assay plates. For the schematic representations of the assays see Figure 5.7A and Figure 6.1A. A) Comaprison of N2, dop1, dop-2, dop-3 and dop-4 mutants pumping rates on a food lawn. B) Comaprison of wild-type, dop-1 and dop-2 mutants pumping rates after 90 min incubation on indicated density of beads. C) N2, dop-1 and dop-2 mutants pumping rates in the presence of the indicated concentration of exogenous transmitter. D) N2, dop-3 and dop-4 mutants pump rates in the presence of the indicated concentration of exogenous transmitter. E) N2, dop-3 and dop-4 mutants pumping rates in the presence of the indicated concentration of exogenous transmitter.*

A) on food, all mutants pumped similar to N2 worms ($p = 0.99$, n = 10 - 14 worms). B) In the presence of beads, dop-1 and dop-2 mutants pumped higher relative to dop-1 and dop-2 on S-basal ($p < 0.0001$, n = 9-13 worms). C) On dopamine, dop-1 and dop-2 mutants showed similar pattern to N2 ($p > 0.05$, n = 12-14 worms). On 5-HT, similar pattern was observed in the presence of exogenous 5-HT ($p > 0.05$, n = 10 worms). D) On beads, dop-3 showed a modest effect ($p < 0.0001$, n =15 - 26 worms) compared to N2. E) On dopamine, dop-3 showed a modest reduction in pumping rate ($p = 0.036$, n = 16 worms) compared to dop-3 on M9. In dop-4 mutants pumped insignificantly different ($p = 0.999$, n = 11 worms) compared to dop-4 worms on M9. In the presence of 5-HT, both dop-3 and dop-4 pumped at a similar rate relative to N2 on 5-HT ($p = 0.99$, n = 10-11 worms). Experiment (A) was repeated twice and experiments (B, C, D & E) were repeated 3 times. One-way ANOVA followed by Bonferroni post test was used. Data represented as Mean ± S.E.M.

6.2.4 Defining the circuit that underpins the bead-induced pharyngeal pumping

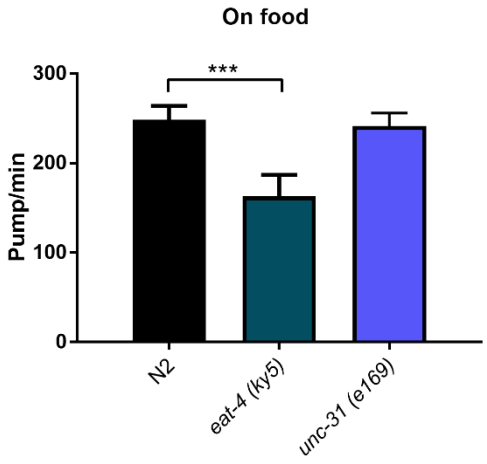
6.2.4.1 UNC-31 and EAT-4 are required for the bead-induced pharyngeal pumping

As indicated, there are two established routes to impose inhibition as highlighted by genetic regulation of the off food pump rate. One would hypothesise that the bead-induced pharyngeal pumping could be mediated by either inhibiting the glutamatergic pathway (EAT-4*)* or UNC-31 pathway. To test this, *unc-31 (e169)* and *eat-4 (ky5)* mutants were tested on food, dopamine or beads.

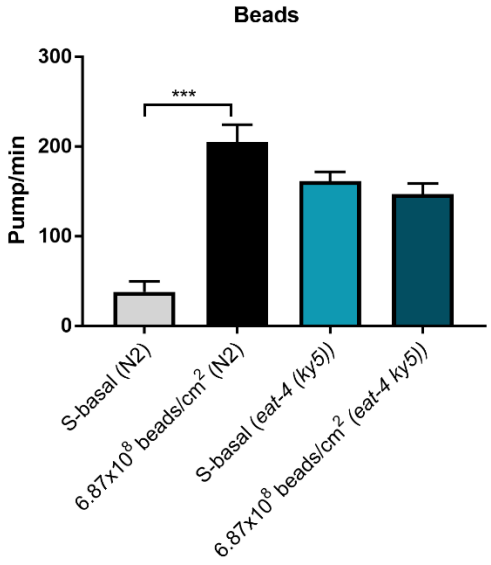
In the presence of food, *eat-4 (ky5)* mutants exhibited a reduced pumping rate (163 ppm ± 8) compared to N2 worms (*Figure 6.4A*). The role of glutamate on pumping rate in the presence of food has been discussed in details in Chapter 1.
In contrast, in the absence of food *eat-4 (ky5)* showed constitutive pumping off-food (*Figure 6.4B*), reinforcing the previous observation by (Dallière et al., 2016). Dalliere et al. (2016) concluded that the constitutive pumping off-food is mediated by a distinct pharyngeal circuit from the on-food circuit. *eat-4 (ky5)* mutants showed occluded response to beads, since *eat-4 (ky5)* worms on beads did not exhibit further increase (147 ppm ± 12) compared to *eat-4 (ky5)* worms on S-basal (162 ± 10, *Figure 6.4B*). Similar findings were observed in the presence of dopamine (*Figure 6.4C*). In contrast, in the presence of 5-HT, *eat-4 (ky5)* displayed a reduced pumping rate (*Figure 6.4C*). These observations suggest that *eat-4* mutants could have pumped at their maximum rate, since no further increase in pumping was observed in the presence of dopamine, 5-HT or beads. Nonetheless, the results further provide support to previous findings that 5-HT may activate the glutamatergic pharyngeal neurones M3 that control the pump duration by speeding up the relaxation phase (Zetka and Rose, 1995). Therefore, in consequence, *eat-4 (ky5)* mutants showed a reduced pumping rate in the presence of food may suggest that an intact M3 signalling is needed to reach maximum pumping rate.

Utilising *unc-31 (e169)* mutant allows investigation of distinct determinants of the inhibitory tone that acts in the off-food context. Indeed, in contrast to *eat-4 (ky5)* mutants, *unc-31 (e169)* mutants had a normal on-food pump rate consistent with this previous reported findings (Dallière et al., 2016) (*Figure 6.4A*). In the absence of food, *unc-31 (e169)* mutants showed the reported constitutively active off-food pumping rate, as previously reported by (Avery et al., 1993). Surprisingly, on beads, *unc-31 (e169)* pumped (157 ppm ± 13) at a similar rate to *unc-31 (e169)* worms on control plates (128 ppm ± 9), suggesting that *unc-31 (e169)* occluded the response to beads (*Figure 6.4D*). Similarly, on dopamine, no significant further increase in pumping rate (121 ppm ± 7) was observed above the baseline pumping rate on M9 (110 ppm ± 7, *Figure 6.4E*). In the presence of 5-HT, *unc-31 (e169)* mutants did not pump significantly different to wild-type worms (*Figure 6.4E*). This is consistent with the 5HT mimicking food and reinforces the observation that 5-HT acts independently from the dopamine signalling.
Overall, these findings indicate that although UNC-31 and EAT-4 pathways may inhibit pumping rate independently from each other, as previously reported by observation from the double mutant *eat-4::unc-31* (Dallière et al., 2016), the bead-induced pumping could utilises both UNC-31 and EAT-4 pathways to increase pumping rate.

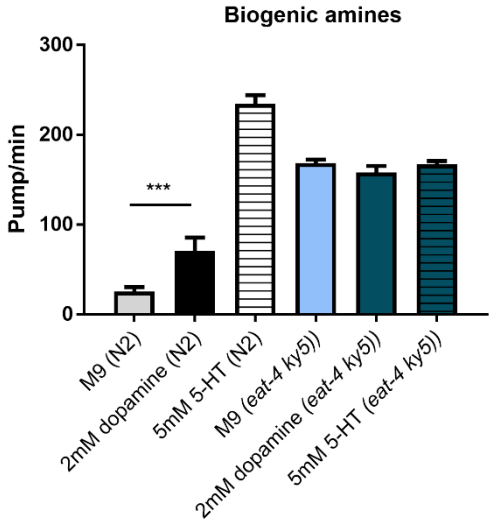
A


On food
Pump/min
300
200
100
0
***
N2
eat-4 (ky5)
unc-31 (e169)

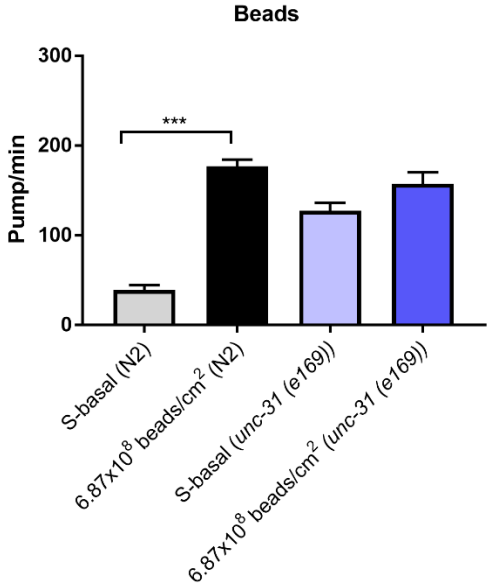
B


Beads
Pump/min
300
200
100
0
***
S-basal (N2)
6.87x10^8 beads/cm^2 (N2)
S-basal (eat-4 (ky5))
6.87x10^8 beads/cm^2 (eat-4 ky5))

C


Biogenic amines
Pump/min
300
200
100
0
***
M9 (N2)
2mM dopamine (N2)
5mM 5-HT (N2)
M9 (eat-4 ky5))
2mM dopamine (eat-4 ky5))
5mM 5-HT (eat-4 ky5))

D


Beads
Pump/min
300
200
100
0
***
S-basal (N2)
6.87x10^8 beads/cm^2 (N2)
S-basal (unc-31 (e169))
6.87x10^8 beads/cm^2 (unc-31 (e169))

E

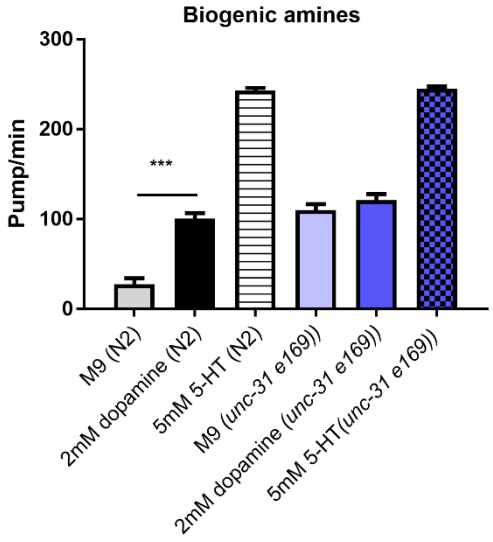

Biogenic amines
Pump/min
300
200
100
0
***
M9 (N2)
2mM dopamine (N2)
5mM 5-HT (N2)
M9 (unc-31 e169))
2mM dopamine (unc-31 e169))
5mM 5-HT(unc-31 e169))

Figure 6.4: eat-4 (ky5) and unc-31 (e169) mutants occlude bead and dopamine-dependent pharyngeal pumping.

A) On food, eat-4 mutant significantly ($p < 0.0001$, n = 10 worms) reduced pharyngeal pumping compared to N2. unc-31 mutant pumped insignificantly different to N2 ($p = 0.99$, n = 10 worms). B) On beads, eat-4 mutant pumped at a similar rate to eat-4 mutant in the presence of S-basal ($p = 0.99$, n = 11 worms). C) On dopamine, eat-4 mutants pumped insignificantly different to eat-4 on M9 ($p = 0.99$, n = 10 worms). In the presence of exogenous 5-HT, eat-4 mutant pumped significantly lower than N2 on 5-HT ($p = 001$, n = 10 worms). D) In the presence of beads, unc-31 pumped insignificantly different relative to unc-31 on S-basal ($p > 0.05$, n = 13-19 worms). E) In the presence of dopamine, unc-31 exhibited pumping rate similar to unc-31 mutants on M9 ($p > 0.05$, n = 15 worms). In contrast, unc-31 responded to 5-HT similar to N2 ($p > 0.05$, n = 10 worms). Experiment (A) was repeated twice and experiments (B, C, D & E) were repeated 3 times. One-way ANOVA followed by Bonferroni post test was used. Data represented as Mean ± S.E.M.

6.2.4.2 Ablation of pharyngeal neurones, I2, occluded the response to beads, dopamine and 5-HT

dop-4 (tm1392) mutants were severely impaired in response to exogenous dopamine and beads relative to a more modest effect on pumping rate on *dop-3 (vs106)* mutants. To identify the circuit that drives the dopamine and bead-mediated behaviour, a map of *dop-4* expression in *C. elegans* was analysed. DOP-4 receptors are expressed in extra-pharyngeal neurones such as vulva, rectal gland, intestine, PQR and in pharyngeal nervous system such as I2, and arguably, I1 neurones (Sugiura et al. 2005; Nikhil Bhatla, wormweb.org). Therefore, the role of I2 neurones in the bead-induced pharyngeal pumping was investigated.

To selectively investigate the cellular function of I2, the strain *N2; Is[Pflp-15::Csp-1b]* was investigated. This wild type worm with a selective ablation of I2 exhibited an on-food pump rate (242 ppm ± 4) similar to its N2 control (253 ppm ± 3, *Figure 6.5A*). In the presence of S-basal, the transgenic line *N2; Is[Pflp-15::Csp-1b]* showed an aberrant increase in pumping rate (126 ppm± 13) compared to N2 (43 ppm ± 7, Figure 6.5B). These worms exhibited the phenotype of constitutive pumping previously described in *unc-31 (e169)* and *eat-4 (ky5)* mutants (Dallière et al., 2016). In the face of this constitutive pumping, the presence of beads did not impose a further elevation in pumping (142 ppm ± 12) relative to N2 (208 ppm± 6, *Figure 6.5B*).

Similarly, exogenous application of dopamine, *N2; Is[Pflp-15::Csp-1b]* did not impose an increase in pumping on the constitutive pump rate (*Figure 6.5C*). This defines the *dop-4* expressing neuron I2 as a critical cellular determinant of the dopamine-dependent and bead-induced pumping. Importantly in the face of I2-ablated neurones (*N2; Is[Pflp-15::Csp-1b])*, 5-HT was able to induce high pumping rate (167 ppm± 6) compared to *N2; Is[Pflp-15::Csp-1b]* on M9 (107 ppm ± 4, *Figure 6.5C*). However, their pumping rate was lower than that observed in the intact N2 worms (215 ppm ± 11). This observation is surprising since I2 showed to have no effect on the on-food pumping rate (*Figure 6.5A*). Nonetheless, this observation reinforces the speculation above that dopamine and 5-HT act through independent cellular determinants and circuits.

Overall, the data suggest that I2 pharyngeal neurones are involved in the beads-induced pharyngeal pumping.

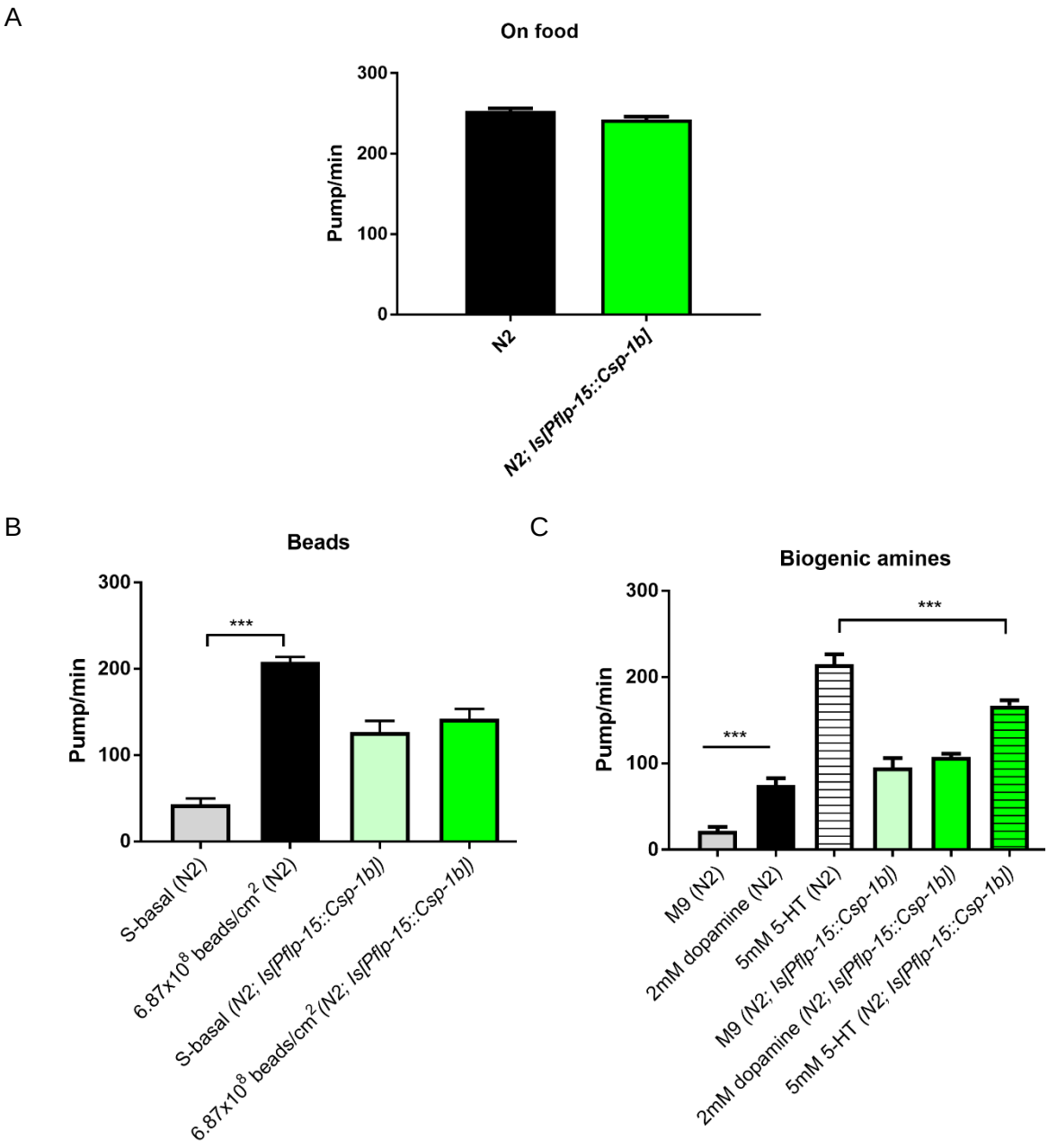


Figure 6.5: I2 neurones are required for bead-mediated pharyngeal pumping.
A) On food, the ablation of I2 neurons had no significant (p = 0.99, n =10 worms) effect on food compared to N2. B) On beads, N2; Is[Pflp-15::Csp-1b] mutants pumped insignificantly (p = 0.99 n= 10 worms) different relative to N2; Is[Pflp-15::Csp-1b] on S-basal. C) On dopamine, N2; Is[Pflp-15::Csp-1b] pumped at a similar rate to N2; Is[Pflp-15::Csp-1b] worms on M9 (p = 0.99, n = 10-12 worms). In the presence of exogenous dopamine, I2-ablated worms showed a significant reduction in pumping rate compared to N2 on 5-HT (p < 0.0001, n = 17 worms). Experiment (A) was repeated twice and experiments (B, C, D & E) were repeated 4 times. One-way ANOVA followed by Bonferroni post test was used. Data represented as Mean ± S.E.M.

6.2.5 The FMRF-amide-like neuropeptide mutants, *flp-15*, does not respond to the beads stimulated pumping

According to the findings above, the role of I2 neurones is critical for stimulating pharyngeal pumping in response to bead-induced pumping. Although I2 neurones are glutamatergic, they also express the NLP-3, NLP-8 and FLP-15 neuropeptides (Li et al., 1999; Nathoo et al., 2001). FLP-15 gene encodes FMRF-amide-like neuropeptide, which is expressed mainly in the pharyngeal neurones, I2*. flp-15 (gk1186)* had an inhibitory effect on pharyngeal pumping when applied exogenously to pharyngeal preparations (Papaioannou et al., 2005). Thus, *flp-15 (gk1186)* mutants were tested in the presence of beads, dopamine or 5-HT.

In the presence of food, *flp-15 (gk1186)* mutants exhibited pumping rate similar to wild-type worms (*Figure 6.6A*). *flp-15* mutants on beads pumped at a similar rate to *flp-15 (gk1186)* on S-basal and similar pattern was observed in the presence of dopamine (Figure 6.6B). Nonetheless in the presence of 5-HT, *flp-15 (gk1186)* mutants displayed pumping rates similar to that of wild-type worms (*Figure 6.6B*).

This suggests a critical role for FLP-15 neuropeptides in the bead-induced pumping rate.

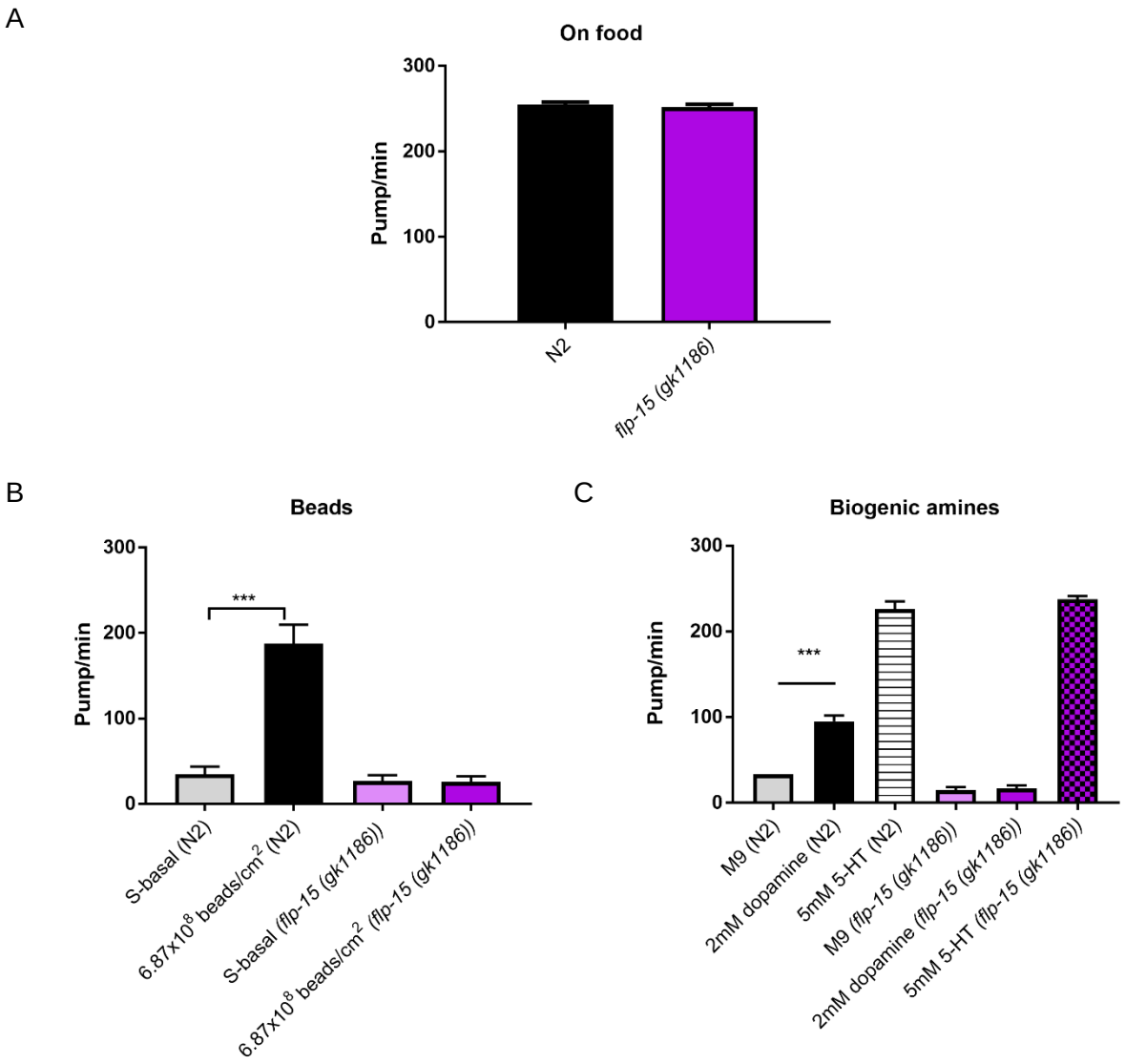


Figure 6.6: *flp-15 (gk1186)* mutants are deficient in the bead- and dopamine-induced pumping rate.

A) On food, *flp-15* mutants showed no significant ($p > 0.05$, n = 10 worms) difference in feeding behaviour compared to N2. B) On beads, *flp-15* mutants pumped insignificantly different ($p > 0.05$, n = 10 worms) to control on S-basal. C) On dopamine, *flp-15* worms were not significantly ($p > 0.05$, n = 14 worms) different to worms on M9. In the presence of exogenous 5-HT, *flp-15* pumped at a similar rate to N2 ($p > 0.05$, n= 12 worms). *Experiment (A) was repeated twice and experiments (B & C) were repeated 3 times.* One-way ANOVA followed by Bonferroni post test was used. Data represented as Mean ± S.E.M.

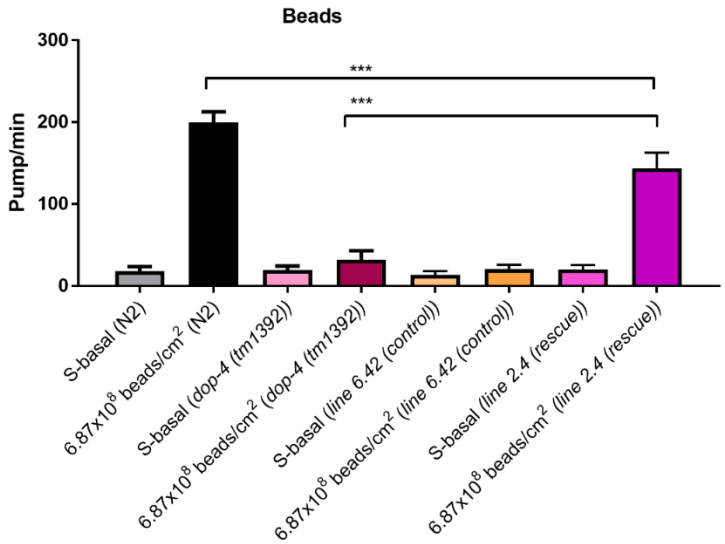
6.2.6 Expression of wild-type dop-4 partially rescued bead- and dopamine-mediated pumping rate in *dop-4* mutant

As shown above the response to dopamine or beads was lost in *dop-4* mutants (see *Figure 6.3B-E*). This suggests a critical role for DOP-4 receptors in the stimulation of the pharynx in response to beads. Hence, to investigate if the bead-induced pumping is mediated by DOP-4 receptors, a wild-type *dop-4* cDNA was transgenically expressed under the control of the putative native *dop-4* promoter in a *dop-4 (tm1392)* background. The transgenic line *dop-4(tm1359); Is[Pdop-4::dop-4]* was tested in the presence of beads or dopamine and compared to wild type and *dop-4* mutants (see Chapter 2 for description of the construct).

In the presence of beads, *dop-4 (tm1392)* mutants failed to elevate pumping rate compared to wild-type worms (*Figure 6.7A*), as expected. The line control (line 6.42) behaved similar to *dop-4 (tm1392)* mutants, in which line 6.42 worms did not respond to beads (*Figure 6.7A*). Conversely, rescue line 2.4 (*dop-4(tm1392); Is[Pdop-4::dop-4])* showed partial rescue demonstrated by increased pumping rate (144 ppm ± 19), which was significantly different from wild-type worms (199 ppm ± 13) and *dop-4* (32 ± 11) mutants. Similar pattern was observed in the presence of dopamine (*Figure 6.7B*), in which the rescue line showed partial rescue (74 ppm ± 8) compared to wild-type (102 ppm ± 8) and *dop-4* (19 ppm ± 3) worms.

Overall, these findings support the initial observations that the DOP-4 receptor is critical for the bead- and dopamine-induced pumping rate.

A



B

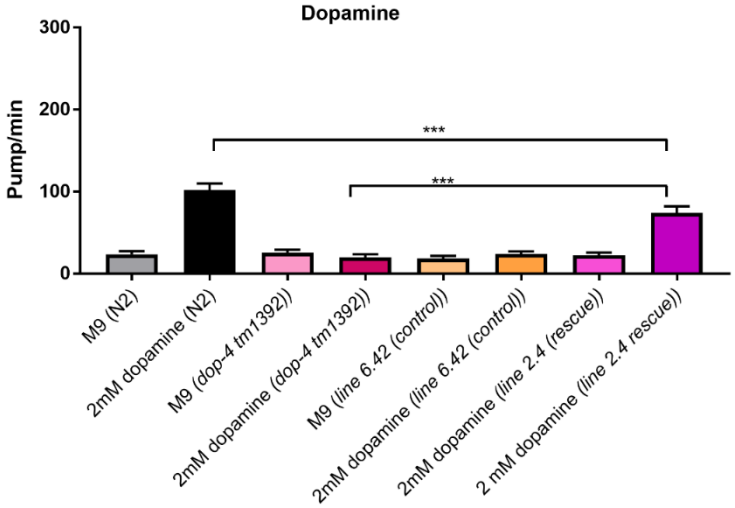


Figure 6.7: Transgenic rescue of the dop-4 mediated bead- and dopamine-induced pharyngeal pumping. *A) N2 dop-4 (tm1392) and indicated dop-4 rescue lines were investigated for their A) bead-induced pumping rate and B) dopamine-induced pumping. A) In the presence of beads, dop-4 (tm1392) showed reduction in pumping rate ($p < 0.0001$, n= 10 worms) worms compared to wild-type. Rescue line 2.4 (dop-4 (tm1395); Ex[Pdop-4::dop-4]) showed a higher pumping rate ($p < 0.0001$, n= 14 worms) relative to dop-4 (tm1395) but lower pumping rate than N2. B) In the presence of dopamine, dop-4 (tm1392) showed a reduced ($P < 0.0001$, n= 10 worms) pumping rate compared to wild-type. Line 2.4, dop-4 (tm1395); Ex[Pdop-4::dop-4] pumped significantly ($p < 0.0001$, n= 13 worms) higher compared to dop-4 (tm1395), but significantly lower than N2 ($p < 0.0001$). Each experiment was repeated 3 times. One-way ANOVA followed by Bonferroni post test was used. Data represented as Mean ± S.E.M.*

6.2.7 Expression of wild-type dop-4 in I2 partially rescued in part elevated pumping rate

The observation made above provides evidence that DOP-4 receptors play a pivotal role in the beads-induced pharyngeal pumping. However, DOP-4 receptors are expressed in I1, NSM and other non-pharyngeal neurones, vulva, rectal gland, intestine and PQR (Sugiura et al. 2005; Nikhil Bhatla, wormweb.org).

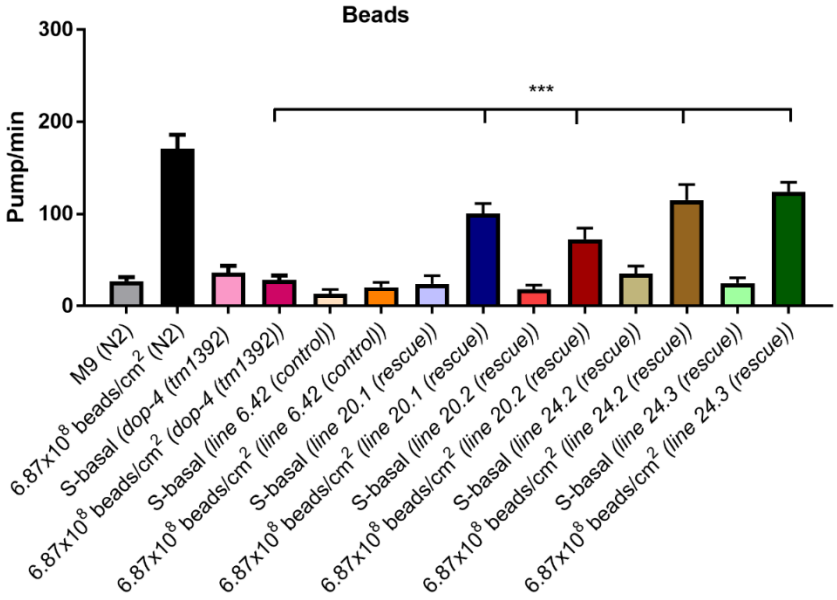
To better resolve this issue we investigated if wild-type dop-4 cDNA expressed under the flp-15 promoter in *dop-4(tm1392)* mutants impacted bead- and dopamine-induced pumping. The transgenic lines *dop-4(tm1392); Is[Pflp-15::dop-4]* (line 20.1, 20.2 and 24.3) were tested in the presence of beads or dopamine and compared to wild type and *dop-4 (tm1392)* mutants.

In the presence of beads, *dop-4* pumped significantly (36 ppm ± 7, *Figure 6.8A*) lower than N2. Rescue lines pumped significantly lower than N2 and significantly higher than *dop-4* mutants (line 20.1 = 100 ppm ± 11, line 20.2 = 72 ppm ± 12, line 24.2 = 115 ppm ± 6, and line 24.3 = 124 ppm ± 10, *Figure 6.8A*).

In the presence of dopamine, *dop-4 (tm1392)* pumped at a significantly low rate (21 ± 4, *Figure 6.8B*) compared to N2. Rescue lines pumped at significantly higher rate (line 20.1 = 30 ± 10, line 20.2 = 63 ± 6, line 24.2 = 64 ppm ± 10, and line 24.3 = 58 ppm ± 9) compared to *dop-4* mutants (28 ± 5) and significantly lower than N2 (170 ppm ± 14).

Overall, this data suggests a partial rescue of *dop-4* phenotype on beads and dopamine-induced pumping, since the rescue lines showed significant differences from wild-type worms and *dop-4* mutants. Nonetheless, this partial rescue indicates that activation of *dop-4* receptors in the I2 neurones is an important determinant in stimulating pharyngeal pumping in response to beads and dopamine.

A



B

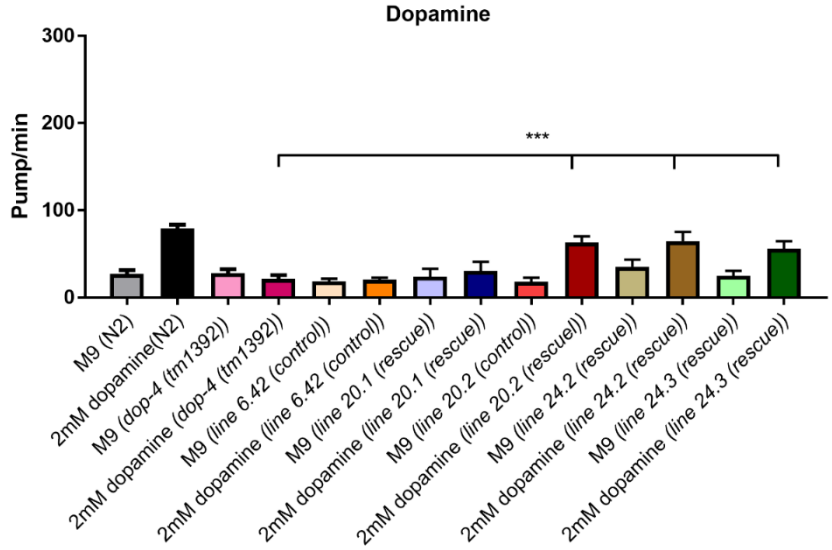


Figure 6.8: Cell-specific expression of dop-4 in I2 neurones in dop-4 mutant background. *A) In the presence of beads, dop-4 exhibited reduced pumping rate ($p < 0.0001$, n = 8 worms) than N2. Rescue lines pumped at lower rate relative to N2, but higher than dop-4 mutants ($p < 0.0001$, n = 9-10 worms). B) On dopamine, dop-4 pumped lower ($p < 0.0001$, n = 11 worms) than N2. Rescue lines pumped at higher levels ($p < 0.0001$, n = 9-10 worms) compared to dop-4 mutants, but at lower levels compared to N2. Each experiment was repeated 3 times. One-way ANOVA followed by Bonferroni post test was used. Data represented as Mean ± S.E.M.*

6.2.8 Physical contact with food is required for elevated pharyngeal pumping

According to the results described in Chapter 5, the bead-induced pumping is selectively achieved by the accumulation of ingestible beads in the gut. Additionally, in this Chapter, the incubation of *dop-4* mutants in the presence of beads did not lead to an increase in pharyngeal pumping compared to wild type (*Figure 6.3B*). This DOP-4 mediated response to beads was only resolvable by conducting experiments in the absence of food. In the presence of food, all the dopamine signalling mutants that were investigated showed a normal rate of pharyngeal pumping (*Figure 6.3*).

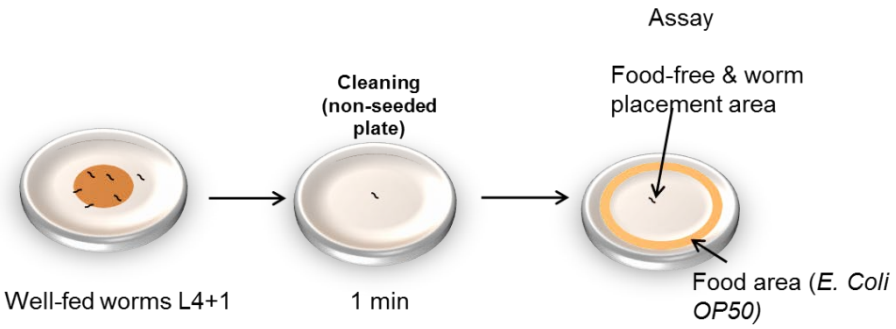
The experiments on pharyngeal pumping, so far, had looked at the steady state pumping on food and time-dependent modulation of pumping off food. However, measurement of pumping rate as the worm transitions between a no-food and a food environment. The observations from the chemotaxis assay showed that regardless of distance from food, feeding was sustained at a low rate off-food (see Chapter 3, section 3.2). Additionally, the physical interaction of the worm with ingested beads and activation of pumping suggests that bead-induced pumping might be mimicking the transition of worms from off-food to an on-food state.

Therefore, to probe for a physiological role of dopamine, a food entry assay was developed. An L4+1 was placed onto a no-food arena for 1 minute for cleaning, before transferring onto a food entry assay plate (*Figure 6.9A*). The worm was placed on a no-food area with a band of food around it. The worm's pumping frequency was counted off-food until the worm's nose was in contact with food, from which the pumping was counted, as accumulated pumps for up to 5 minutes

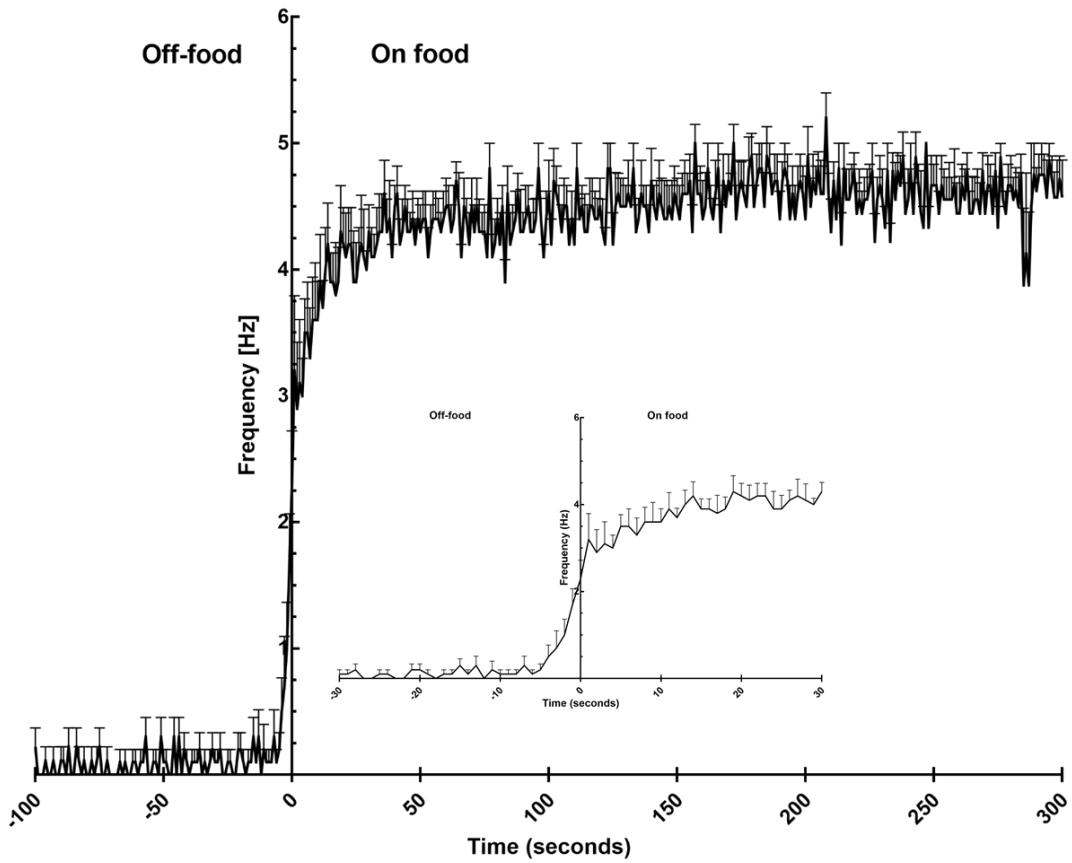
In the no-food arena, the worm displayed a low pump frequency (1 Hz, *Figure 6.11B*). However, upon entry to the food lawn, wild-type worms showed a rapid increase in pumping frequency, with a maximal frequency of 4-5 Hz reached in 20 seconds (*Figure 6.9B&C*). This steady state was the rate one normally would record when monitoring a worm on food.

These findings imply that direct contact with food triggers an elevated pumping rate.

A



B



C

	Off food	On food	Time to maximum pumping rate (sec)	t½ (sec.)	Number of worms tested
N2	0.20±0.05	4.58±0.03	20	1	10

Figure 6.9: Food triggers an immediate increase in pharyngeal pumping rate as worms enter the food area. *A) A cartoon representation of the food entry assay. A 6 cm NGM was prepared with a 3.5 cm food-free centre (3.5cm in diameter) and a band of 600 µl E. coli OP50 around the circumference. 1 Well-fed L4+1 worm was transferred onto non-seeded plate to remove bacteria for 1 minute before transferring onto assay*

plate. The worm was placed in the food-free centre and pumping rate was recorded: off-food, on entry to food and on food for 5 minutes using online counter. B) Frequency of pumping (Hz) in off food, upon entry and retention on the food lawn in wild-type worms. In the absence of food, worms showed low pumping frequency of about 1 Hz. There was a modest increase in pumping rate at 0 min before worms enter the food lawn, due to the presence of food residues (inset). A rapid increase in pumping was observed as the worm approached and contacted the food lawn. This had led to the worm staying on the food lawn and expressing a maximal steady state of 4 Hz. The experiment was repeated 3 times. C) The table shows the half-time of wild-type worms reaching maximum pumping rate. Data in Figure 6.9 are represented as mean±S.E.M..

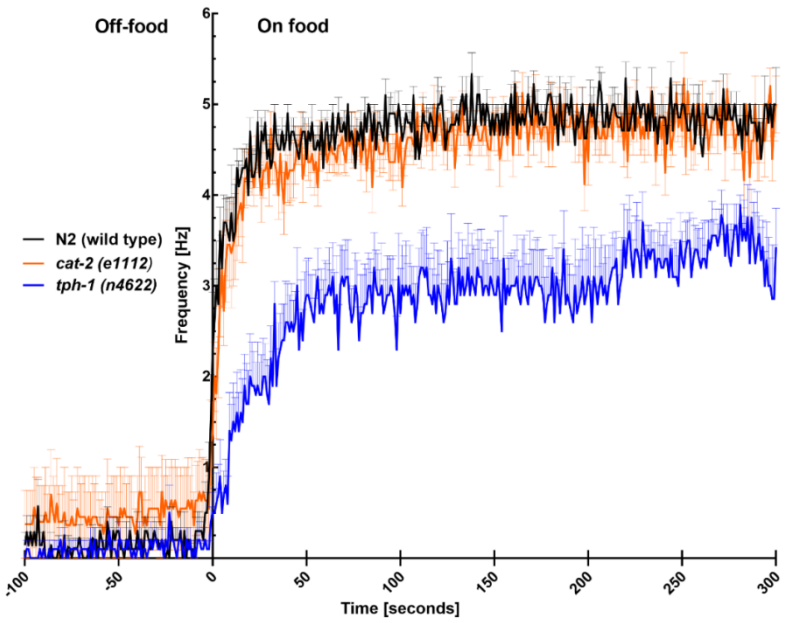
6.2.9 Serotonergic and dopaminergic signalling are required for elevated pumping rate upon entry to food

Serotonergic signalling plays a major role in feeding behaviour as discussed in Chapter 1. Therefore, it would be interesting to investigate mutants deficient in serotoninergic signalling, *tph-1,* on entry to food assay, since dopaminergic signalling was not required for on food pumping rate as shown in *Figure 6.2A*. Nonetheless, it was reported above that the dopamine signalling played a role in the earlier phases of feeding behaviour (see *Figure 6.2B&C*). Thus, *cat-2 (e1112)* and *tph-1 (n4622)* were investigated on the entry to food assay.

The dopamine-deficient mutant, *cat-2 (e1112)* showed a delayed response ($t_{1/2}$ = 3 seconds) to the initial encounter to food compared to wild-type worms ($t_{1/2}$ = 1 second, *Figure 6.10A&B*). Additionally, *cat-2 (e1112)* reached the maximum pumping rate at 4 Hz in 16 seconds compared to wild-type worms which was 13 seconds (*Figure 6.10A*). In contrast serotonin-deficient mutant, *tph-1 (n4622),* showed a slow rise in pumping rate when worms encountered the food lawn ($t_{1/2}$ = 17 seconds) compared to wild-type worms ($t_{1/2}$ = 1 second). Furthermore, *tph-1* mutants did not reach the maximum pumping rate of 4 Hz, however, a maximum of 3 Hz was reached in 187 seconds compared to wild-type, which reached maximum at 4 Hz in 13 seconds (*Figure 6.10A&B*). Furthermore, *tph-1 (n4622)* mutants maintained a low pumping rate on food, which was consistent with reduced pumping rate observed in on food pumping rate reported in *Figure 6.2A*.

The data suggest that dopamine is selectively involved in triggering a rapid food contact-induced pumping, whereas 5-HT contributes to both the initiation and sustained pumping crucial for rapidly reaching the maximum steady state pumping rate.

A



B

	Off food	On food	Time to maximum pumping rate (sec)	t½ (sec.)	Number of worms tested
N2	0.25±0.06	4.80±0.12	13	1	10
cat-2 (e1112)	0.54±0.02	4.75±0.03	16	3	11
tph-1 (n4622)	0.13±0.01	3.4±0.04	187	17	9

Figure 6.10: Comparison of food entry-induced pumping in dopamine-deficient mutant (cat-2) and serotonin-deficient mutant (tph-1). *A) In the absence of food, tph-1 and cat-2 showed no significant difference ($p > 0.05$) in pumping rate compared to N2. Dopamine-deficient mutant (cat-2) showed a reduced pumping rate ($p = 0.002$) compared to N2 on entry to food, and it had reached maximum pumping slower compared to N2. In contrast, serotonin-deficient mutant (tph-1) showed a significant ($p < 0.0001$) reduction in pumping rate on entry to food and on food compared to N2. The experiment was repeated 4 times. B) The table compares the half-times of maximum pumping rate in cat-2 mutants and wild-type worms. It can be deduced from the table that cat-2 took longer to reach maximum pumping rate compared to N2. Data are represented as mean±S.E.M. One-way ANOVA followed by Bonferroni post test was used.*

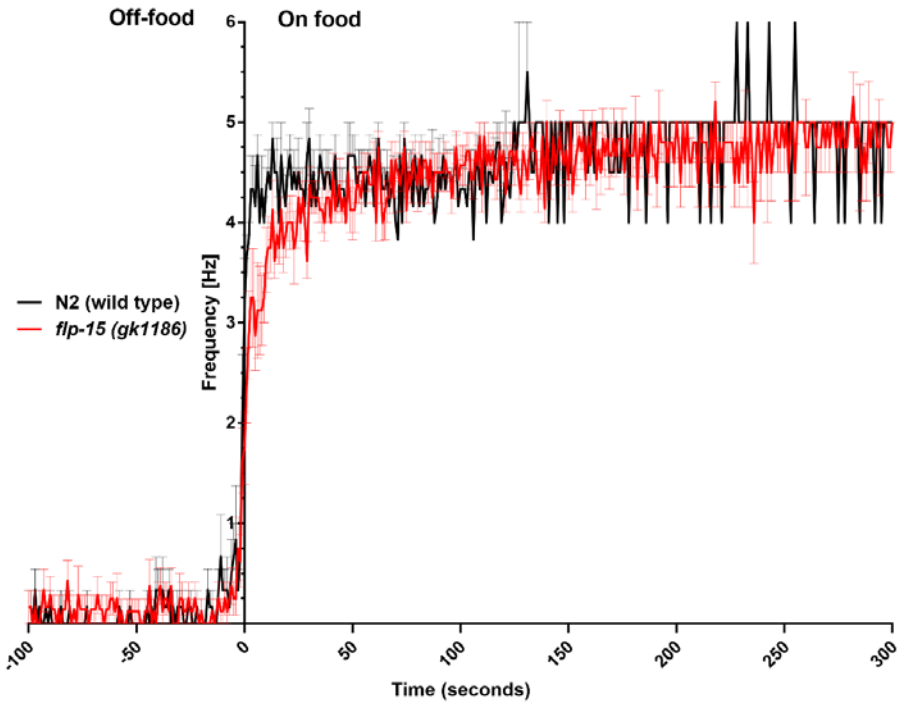
6.2.10 The FMRF-amide-like neuropeptide, FLP-15, is required upon entry to food

As previously shown, *flp-15 (gk1186)* mutants have a reduced pumping rate in the presence of beads (*Figure 6.6*). Hence we compared *flp-15 (gk1186)* mutant to N2 on food entry assay.

flp-15 (gk1186) mutants exhibited pump rates similar to wild-type worms when measured in the off-food context (*Figure 6.11A&B*). As worms entered the food, *flp-15* mutants had reached the maximum pumping rate of 4 Hz in 12 seconds compared to 3 seconds for wild-type worms (*Figure 6.11*A&B). However, *flp-15* had a t ½ = 1 second similar to wild-type worms.

This suggests that the FLP-15 neuropeptide may play a role as the worm enters the food lawn. This was further supported by the data reported in the presence of beads and dopamine, which suggests that FLP-15 is required for both the bead- and dopamine-induced pharyngeal pumping. This could mean that the activation of DOP-4 by dopamine in the I2 neurones may lead to the modulation of downstream neuropeptides in I2 neurones, including FLP-15.

A



B

	Off food	On food	Time to maximum pumping rate (sec)	t½ (sec.)	Number of worms tested
N2	0.20±0.08	4.40±0.10	3	1	7
flp-15 (gk1186)	0.51±0.05	4.30±0.09	12	1	8

Figure 6.11: Comparison of food entry induced pumping in the FMRF-amide-like neuropeptide mutant, flp-15 (gk1186) and wild-type (N2) worms. *A) In the absence of food, worms pumped in a similar manner ($p > 0.05$) to wild-type worms. On entry to food, flp-15 (gk1186) worms showed an increase in pumping rate similar to wild-type worms. The experiment was repeated 3 times. B) The table compares the half-times of maximum pumping rate in flp-15 mutants and wild-type worms. flp-15 mutants took longer to reach maximum pumping rate of 4 Hz in 12 seconds compared to wild-type in 3 seconds ($p = 0.0003$). Data represented as mean ± S.E.M. Student t-test was used.*

6.2.11 DOP-4 receptors are critical for reaching maximum pumping rate upon entry to food

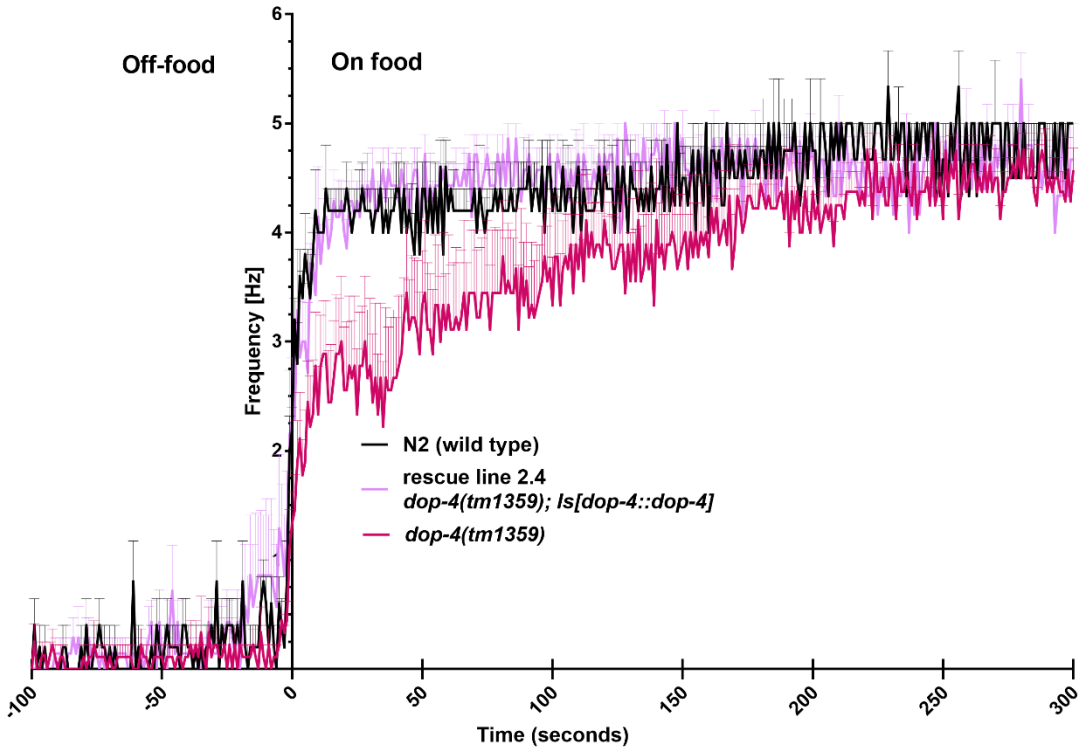
It was previously shown that *dop-4 (tm1392)* mutants showed a reduced pumping rate in the presence of beads and dopamine but not in the presence of food (*Figure 6.3A,D&E*). Since *dop-4 (tm1392)* mutants appeared to play a critical role in the bead- and dopamine-induced pharyngeal pumping (*Figure 6.7 & Figure 6.8*), I investigated the role of *dop-4 (tm1392)* on food entry assay.

dop-4 (tm1392) mutants reached a maximal pumping frequency of 4 Hz in 130 seconds compared to 10 seconds at 4 Hz observed in wild-type worms (*Figure 6.12A*). Furthermore, their initial response, as measured by the t ½, upon encountering food lawn was reduced (*Figure 6.12A*). *dop-4* mutants had a half-time of 6 seconds compared to 1 second for wild-type worms (*Figure 6.12A&B*). This was a more marked increase in t ½ and maximum pumping rate than that observed for *cat-2* (*Figure 6.10A&B*). However, it has been reported that the *cat-2* mutant is not null for dopamine, which explains the smaller effect compared to *dop-4 (tm1392)*.

These data suggest a critical role for DOP-4 receptors as worms enter the food lawn. Hence, to investigate this further, a wild-type DOP-4 was expressed under the control of *dop-4* promoter in a *dop-4 (tm1359)* background. The transgenic line *dop-4(tm1359); Is[Pdop-4::dop-4]* was tested in food entry assay and compared to wild type and *dop-4* mutants. The transgenic line *dop-4(tm1359); Is[Pdop-4::dop-4]* showed a significant increase in the initial rate of response when encountering the food lawn compared to *dop-4* mutants (*Figure 6.12A&B*). The transgenic line reached the maximum pumping rate in 1 second in a similar pattern to wild-type worms (*Figure 6.12A&B*).

Overall, this suggests that for the initial encounter of food lawn the activation of DOP-4 receptors is required to facilitate reaching the maximum pumping rate.

A



B

	Off food	On food	Time to reach maximum	t½ (sec.)	Number of worms tested
N2	0.22 ± 1.14	4.50 ± 0.22	10	1	9
dop-4 (tm1359)	0.17±0.03	4.47±0.02	130	6	9
dop-4(tm1359); Is[dop-4::dop-4] (line 2.4)	0.06±0.15	4.63±0.45	10	1	7

Figure 6.12: DOP-4, is required as the worm enters the food lawn. *A) In the absence of food, dop-4 mutants pumped at a similar rate to N2 ($p > 0.05$). On entry to food, dop-4 showed a significant ($p < 0.001$) reduction in pumping rate compared to N2. The transgenic line dop-4(tm1359); Is[dop-4::dop-4] (line 2.4) showed a significant increase in the time to reach maximum pumping rate compared to dop-4 mutants ($p < 0.0001$). The experiment was repeated 3 times. B) The table compared the half-times of maximum pumping rate in dop-4 mutants, the transgenic line and wild-type worms. The transgenic line dop-4(tm1359); Is[dop-4::dop-4] reached maximum pumping in a similar manner to N2 with t½ of 1 second compared to dop-4 mutants which was 6 seconds.*

Data in figure 6.12 are represented as mean±S.E.M. One-way ANOVA followed by Bonferroni post-hoc test was used

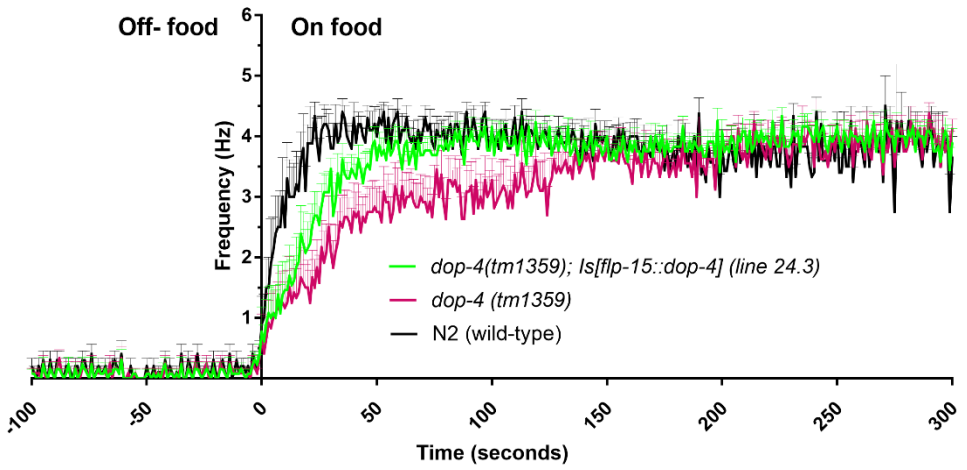
6.2.12 Expression of wild-type dop-4 in I2 partially rescued the half-time required to reach the maximum pumping rate upon entry to food

To investigate the efficiency to reach the maximum pumping rate upon entry to food via the inhibition of I2 neurones, by the activation of DOP-4 receptors in I2, the transgenic line *dop-4(tm1359); Is[Pflp-15::dop-4]* (line 24.3) was tested in a food entry assay.

The transgenic line *dop-4(tm1359); Is[Pflp-15::dop-4]* showed an increase in the rate of response in the initial encounter of food lawn compared *dop-4* mutants (*Figure 6.13A*). However, this rate was reduced compared to wild-type worms (*Figure 6.13A*). The transgenic line *dop-4 (tm1359); Is[Pflp-15::dop-4]* reached the maximum pumping rate in 15 seconds compared to 25 seconds for *dop-4* and 3 seconds for wild-type worms (*Figure 6.13A&B).* This suggests a partial rescue of wild-type *dop-4* gene in *dop-4* mutant background. Since this line was not integrated, it should be considered that mosaic expression might confound the analysis.

This data further supports that activation of DOP-4 receptors is important in reaching maximal pumping rate upon encountering food lawn. Additionally, it provides evidence for the observations that dopamine is released in response to ingestion of bacterial particles, which activates DOP-4 receptors, leading to the disinhibition of I2 to facilitate pumping.

A



B

	Off food	On food	Time to reach maximum	t½ (sec.)	Number of worms tested
N2	0.12 ± 0.01	3.77± 0.32	21	3	10
dop-4 (tm1359)	0.03±0.005	2.99±0.057	145	25	8
dop-4(tm1359); Is[flp-15::dop-4] (line 24.3)	0.03±0.007	3.58±0.04	46	15	20

C

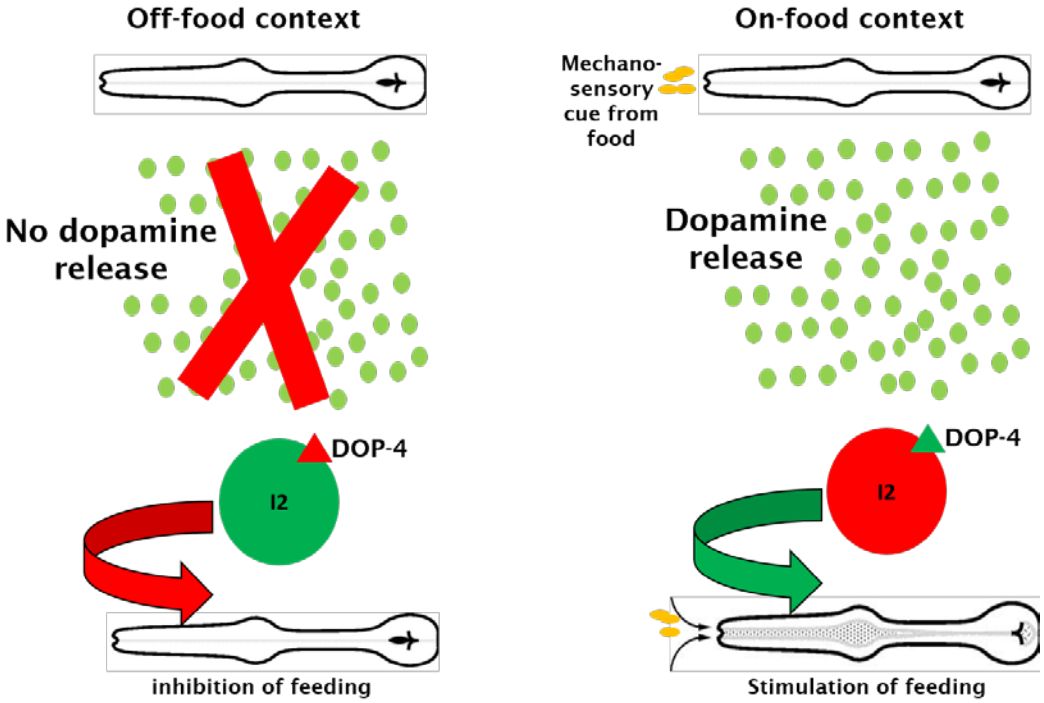


Figure 6.13: Cell-specific expression of wild-type dop-4 cDNA in I2 neurones partially rescued entry to food elevated pumping rate. *A) On entry to food, dop-4*

mutants were slower to reach maximum pumping rate on food compared to N2. Rescue lines reached maximum pumping rate significantly faster than dop-4 mutants ($p < 0.0001$), but significantly slower than wild-type worms ($p = 0.038$). The experiment was repeated 3 times. B) The table compares the half-times of maximum pumping rate in the transgenic line dop-4(tm1359); Is[Pflp-15::dop-4], dop-4 mutants and wild-type worms. The transgenic line reached maximum pumping in 15 seconds compared to dop-4 mutants with 25 seconds and N2 with 3 seconds. Data in figure 6.15 are represented as mean±S.E.M. One-way ANOVA followed by Bonferroni post-hoc test was used to compare dop-4 and dop-4(tm1359); Is[flp-15::dop-4] (line 24.3) to N2. C) Disinhibition of the glutamatergic neuron I2 by dopamine modulates feeding. In the absence of food, I2 neurones (green circle) are actively inhibiting pharyngeal pumping. In contrast, in the presence of food, the mechanosensory cues from food releases dopamine, which activates the DOP-4 receptors (green triangle) in the I2 neurones. This activation leads to inhibition of I2 neurones (red circle), thus, stimulating pharyngeal pumping.

6.3 Discussion

The observations in Chapter 5 showed that ingestion of small beads, but not large beads, elevated pumping rate in the absence of food. These data provide evidence that the physical presence of food in the pharynx is required to increase pharyngeal pumping. The results in this chapter reinforce this and reveal the neural circuit underpinning the mechanosensory induced pharyngeal pumping (*Figure 6.14*).

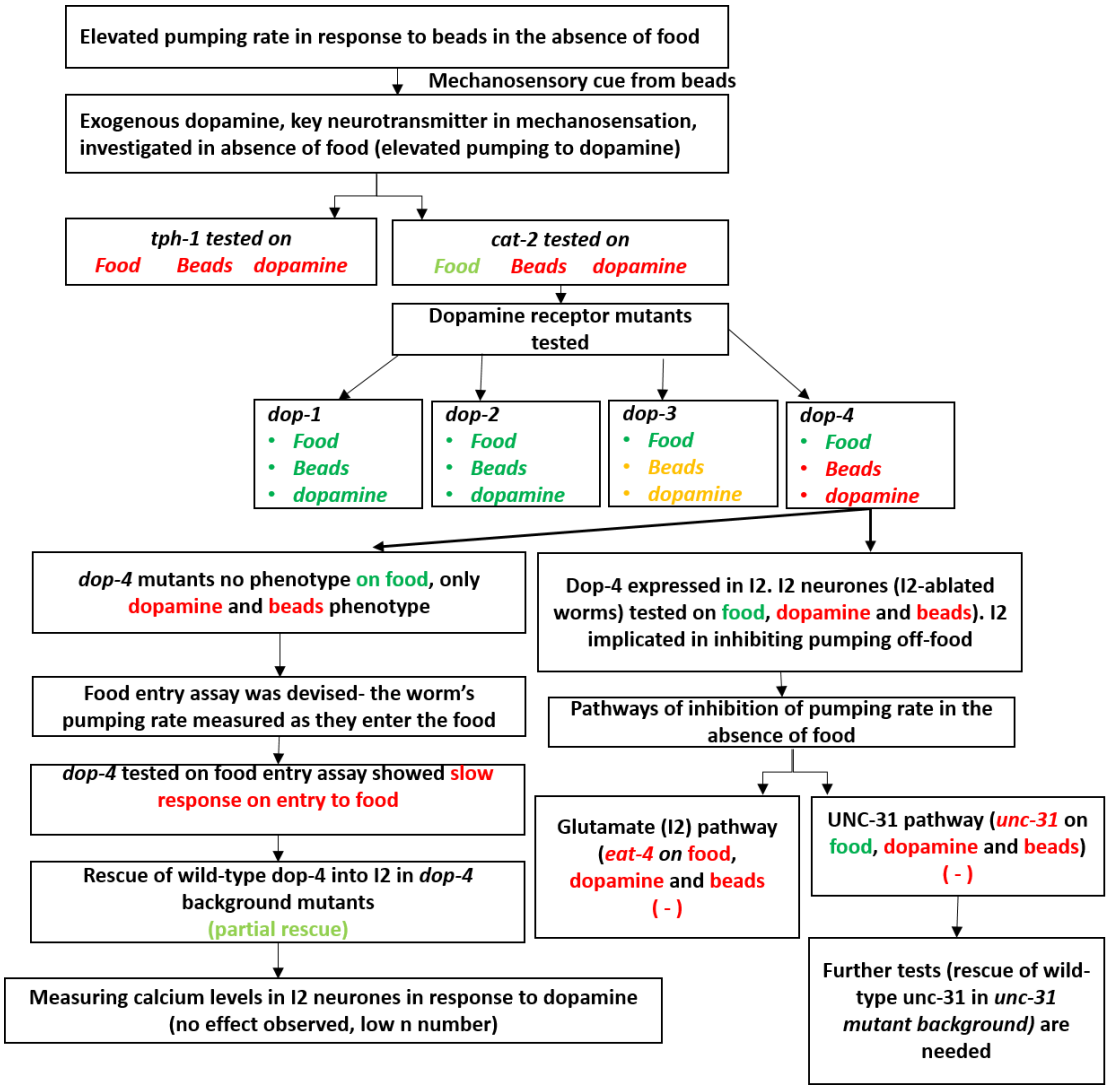


Figure 6.14: An overview of the approach used to delineate the mechanosensory-dependent pharyngeal pumping. *The elevated pumping rate in response to beads suggests an involvement of a mechanical cue. The response of worms to exogenous dopamine was investigated, since it is a key neurotransmitter in mechanosensation in C. elegans and had recently been shown to be an additional biogenic amine that increases pumping. An elevated pumping rate was observed in response to exogenous*

dopamine. The dopaminergic and serotonergic signalling both implicated in the bead-induced pumping. dop-4 mutants were used in bead-induced pumping and proved a critical role for DOP-4 receptors. However, additional investigations are required to determine the role of unc-31. Cell-specific rescue of dop-4 in I2 suggested that dopamine disinhibited I2 by activating dop-4 receptors within I2 neurones, resulting in inhibition of glutamate release, and thus, promoted feeding behaviour. Colour coding represents the effect of food, beads or exogenous dopamine or 5-HT on N2 or mutants. Green colour = no effect, yellow= moderate effect was observed, red colour = a significant effect was observed.

6.3.1 Mechanosensory cues in the pharynx increase the pumping rate in a dopamine- and 5-HT-dependent manner

Exogenous application of 2mM dopamine showed that dopamine increased pharyngeal pumping in the absence of food in both intact worms (*Figure 6.1B*) and cut heads preparation (appendix A). Dopamine is a critical context-dependent modulator in *C. elegans* (see Chapter 1). The role of dopamine in stimulating pharyngeal pumping in the absence of food is novel since there were no previous observations indicating a role for dopamine in feeding behaviour. It has been shown previously that dopamine plays a role in the modulation of food–related behaviour. For instance, dopamine is required for basal slowing response as worms enter the food (Sawin et al., 2000). Moreover, Local Area Search (LAS) that is initiated when the worms leave food is dependent on dopamine signalling. Exogenous application of dopamine increases high-angled turns frequency (Hills, 2004). Glutamate transmission is required for dopamine modulation of LAS behaviour since the exogenous application of dopamine rescued mutant deficient in dopamine (Hills, 2004). The application of exogenous dopamine, 5 or 30mM, had no effect on pumping rate on food (Rani and Srivastava, 2018). However, as aforementioned, dopamine induction of feeding acts in parallel to the circuits that likely react to distinct facets of food.

Unlike dopamine, the application of exogenous 5-HT in the presence of food increases pharyngeal pumping by 40 pumps/min (Avery and Horvitz, 1990) and in the absence of food, exogenous 5-HT showed a significant increase in pharyngeal pumping similar to that observed in the presence of food (Sze et al., 2000). However in comparison to 5-HT,

worms showed a modest increase in pumping rate in the presence of dopamine (*Figure 6.1B*) compared to 5-HT.

There are 8 bilateral dopaminergic neurones in *C. elegans,* which are sensory, activated by mechanical stimulation (*Figure 6.15*). The stimulation of pharyngeal pumping in response to dopamine provides evidence of the activation of dopaminergic neurones, which was also demonstrated by the ingestion of small beads. Accordingly, this mechanical stimulation of pharyngeal pumping in response to ingestion of beads mimics the mechanical cue from bacteria.

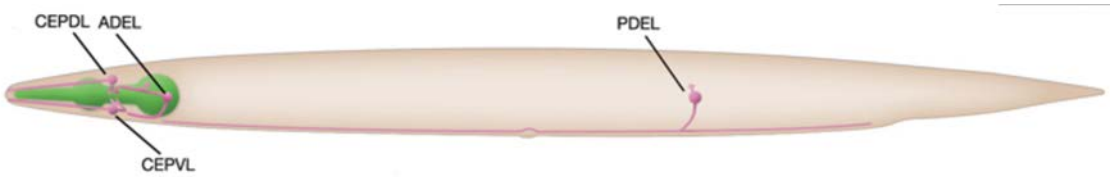


Figure 6.15: Mechanosensory neurones in C. elegans. *CEPs: there are four neurons of the cephalic sensilla which are located in dorsal & ventral, right & left, whereas the somas are located around the nerve ring. The sensory cell extends a ciliated dendrite to the tip of the nose and sensory information are processed in the nerve ring. ADE: anterior deirid neurons are bilateral and symmetric (located right & left), whereas the somas are located laterally over terminal bulb of pharynx. The sensory neuron extends a ciliated dendrite into the cuticle. PDEs: posterior deirid neurons are bilateral and symmetric (located right & left), whereas the somas are located sub-dorsally. The sensory neuron extends a ciliated dendrite into the cuticle and processes sensory information in the ventral nerve cord. Adapted from wormatlas.org.*

6.3.2 Receptor expression defines key determinants

The analysis of pumping rate in various mutants was conducted to delineate the effects of dopamine and serotonin in feeding behaviour. It revealed that *cat-2* mutants did not respond to beads (*Figure 6.2B*), therefore providing evidence for the role of dopamine in stimulating pharyngeal pumping. Thus, additional experiments were conducted to investigate the role of the four dopamine receptors in dopamine- and bead-mediated pharyngeal pumping. *dop-1* and *dop-2* mutants showed the ability to induce pumping in the presence of beads and dopamine (*Figure 6.3B&D*). *dop-1* is expressed in body wall

muscles such as acetylcholine motorneurones, interneurons, glutamatergic PVD, ALM mechanosensory neurones and unidentified head neurones (Chase et al., 2004; Kindt et al., 2007; Tsalik et al., 2003). On the other hand, *dop-2* is an auto-receptor, which is expressed in dopaminergic-releasing sensory neurones, CEP, ADE and PDE (Suo et al., 2009). The expression sites of these receptors and their non-involvement in the dopamine or bead-mediated pharyngeal pumping suggests they are upstream or downstream mediators. An important speculation is that the selective effect of the beads is via ingestion, suggesting that sensory modalities that trigger bead-mediated pumping are enteric. However, this needs to be cautioned against observations that extra-pharyngeal pathways, and thus receptors, could contribute to the modulation of pharyngeal function.

The *dop-4* mutant showed reduced response to beads and dopamine (*Figure 6.3C&E*). DOP-4 receptors are expressed in extra-pharyngeal neurones (Sugiura et al., 2005), but in contrast to others, also has clear pharyngeal expression of *dop-4*. Importantly existing studies indicate that the DOP-4 receptor is expressed in I2, NSM and arguably I1 (Sugiura et al., 2005). I2 neurones are a bilateral pair of pharyngeal neurones that release glutamate. I2s have been ascribed as pharyngeal neurons with a possible sensory function. They are involved in a light mediated inhibition of pumping rate on food (Bhatla and Horvitz, 2015). Furthermore, previous findings reported that I2 are important in inhibiting pumping in the absence of food by releasing glutamate. This conclusion is based on the observation that I2 null mutants showed an aberrant increase in pumping rate off-food (Dalliere et al. 2015, unpublished data). Additionally, in this Chapter, we have shown that I2 neurones play a major in bead-mediated pumping, since ablated I2 neurones, some worms occluded their response to beads and dopamine. Collectively, these data indicate that I2 harbours significant determinants for bead-induced pumping. However, DOP-4 receptors are also expressed in NSM, which indicate that both I2 and NSM are involved in the upregulation of pumping.
This is further supported by the 5-HT dependence of bead stimulation, since *tph-1* mutants failed to increase pumping rate in the presence of beads (*Figure 6.2*). However, the role of DOP-4 in NSM in the bead-mediated pharyngeal pumping is still unclear.
To further investigate the role of I2 and *dop-4* in beads-induced pumping, wild-type *dop-4* was rescued in *dop-4* mutant background. The expression of wild type *dop-4* gene in *dop-4* mutant background and cell-specific rescue of *dop-4* in I2 neurones rescued in part pumping rate in response to beads or dopamine (*Figure 6.7 & Figure 6.8*). However, this is not a complete rescue since these rescue lines are non-integrated. Therefore, the mosaic expression of the transgene may affect the results preventing a full rescue of the dop-4 wild-type phenotype. Additionally, it may suggest that other dopamine targets (e.g.

extra-pharyngeal or additional pharyngeal targets) might contribute. Overall it would appear that DOP-4 receptors play a central role in the bead-induced pumping and reinforces that there is a robust association between dopamine and I2 neurones in promoting pharyngeal pumping.

6.3.3 FLP-15 is critical in beads-mediated pumping and on entry to food

Furthermore, *flp-15* mutants failed to respond to beads and dopamine compared to wild-type worms (*Figure 6.6*) and showed a slower rise to reach maximum pumping rate compared to wild-type (*Figure 6.11*). This suggests a critical role for the *flp-15* neuropeptide in the mechanical excitation of the pharynx. However, these results are surprising since *flp-15* is associated with the inhibition of pharyngeal pumping (Papaioannou et al., 2005). One might suggest that the activation of DOP-4 could upregulate the release of other neurotransmitters I2 neurones such as FLP-15.

6.3.4 UNC-31 and EAT-4 regulates mechanosensory-dependent pharyngeal pumping

UNC-31 and EAT-4 play a critical role in regulating pharyngeal pumping in the absence of food. However, they act through distinct pathways to regulate off-food pumping rate, since *eat-4::unc-31* double mutants showed high pumping rate off-food (Dallière et al., 2016). Herein, based on I2 ablated worms' and *dop-4* mutants' behaviour in the presence of dopamine or beads, one would hypothesise that *eat-4* mutants would have no further increase in pumping rate on dopamine or beads (Dallière et al., 2016). While, one would expect that *unc-31* mutant would pump at a higher rate on beads and dopamine compared to *unc-31* mutant on M9 or S-basal. As expected, *eat-4* mutant occluded the response to dopamine and beads. However, surprisingly, *unc-31* mutants behaved in a similar way to *eat-4* mutants. The *unc-31* also occluded the bead-induced pumping rate (*Figure 6.4*). Similar results were observed in the presence of exogenous dopamine (*Figure 6.4*). Therefore, this data suggest that UNC-31 and EAT-4 play an important role in the mechanical stimulation of pharyngeal pumping.

6.3.5 Bead-induced pumping acts through I2 neurones to elevate pharyngeal pumping

The fact that glutamate release from I2 in an off food context provides a sustained inhibitory tone that has been mapped out (Dallière et al., 2015, unpublished data). The inhibition of pharyngeal pumping in the absence of food is mediated through AVR-14 (Dallière et al., 2015). In the current work I showed that DOP-4 allowed dopamine to stimulate pumping. Consequently, dopamine makes a strong case for imposing inhibitory modulation of glutamate, leading to the disinhibition of I2 and in turn stimulates pumping.

6.3.6 Dopamine disinhibits the pharynx to increase pharyngeal pumping upon entry to food

The observation that *dop-4* mutants did not show an increase in pumping rate in the presence of beads or dopamine, while showing normal feeding behaviour in the presence of food, is important as it suggests there is limited role for dopamine in the presence of food. Dopamine is known to offer preparatory modulation to transiting environment. Indeed, in worms dopamine is involved in basal slowing response to food (Sawin et al., 2000). Based on this, a food assay was devised to investigate the role of dopamine as the worm encounters the food lawn or as it transits from an off-food to an on food state. Wild-type worms showed a fast increase in pumping rate upon entry to food with maximum pumping rate reached in 13 seconds. Nonetheless, there are differences in the kinetics of the food entry response compared to wild-type worms incubated in beads. As reported before, wild-type worms showed an increase in pumping rate on beads within the first 10 min but a sustained and high pumping rate was observed after 30 min (see Chapter 5-*Figure 5.5* & *Figure 5.7*). This difference could be due to beads mimicking one modality of food, which is the physical presence of food, whereas food entry is a multi-modal response to olfaction, gustation, mechanosensation and nutritional value, which are all co-ordinated and utilised to produce the fast response upon entry to food.
cat-2 and *tph-1* mutants showed a slow pharyngeal response in the initial encounter of the food lawn (*Figure 6.10A&B*) compared to N2, indicating that the serotonergic and dopaminergic signalling are both involved upon entry to food. For reasons discussed, one would predict a different dependence to 5-HT and dopamine, and the nature of 5-HT response to food entry supports this. In contrast, dopamine selectively affects the fast phase but not the sustained increase in pumping. The data suggests dopamine is

released in response to ingestion of food or food-like particles. Also, *cat-2* mutants showed a blunted response to entry to food consistent with this. However, *dop-4* mutants were tested in the food showed a more marked reduction in pumping the food relative to *cat-2* (*Figure 6.12A*). However, like *cat-2*, *dop-4* mutant reached maximum pumping rate similar to wild-type when on food after approximately 3 minutes. These results agree with the observation that the *dop-4* mutant showed normal steady-state feeding behaviour when they are observed on food (*Figure 6.3A*). The expression of wild type *dop-4* in I2 neurones showed a partial rescue of the initial increase in the elevation of pumping rate as the worm encounter the food lawn (*Figure 6.12*). Based on these observations, the results obtained from beads, exogenous dopamine in intact worms and cut heads preparation provide evidence for the role for dopamine in the mechanical stimulation of the pharynx.

Volume transmission

As indicated, the data suggest that mechanical cues that utilise dopaminergic sensory neurones express a change in pharyngeal function. The most likely candidate dopaminergic neurons involved in this are those found in the worm's head, CEP and ADE (*Figure 6.16A&B*). However, these are anatomically isolated from the enteric nervous system. We provided evidence for ingestible beads triggering a dopamine effect, even though there was no evidence of dopamine-secreting neurones in the pharynx (Franks et al., 2006). However, dopamine neurons, particularly CEP mechanosensory neurones, have dendrites which extend to the tip of the nose within corresponding labial nerve (*Figure 6.16A*; *Figure 6.15*) and can directly respond to mechanical stimulation (Kang et al., 2010; Kindt et al., 2007). Nonetheless, all dopaminergic neurones have the ability to directly sense the presence of food (Elefteriou et al., 2015).

One explanation could be that bacteria and bacteria-sized particles provide a mechanosensory cue that activates these dendrites, allowing the release of dopamine. Alternatively, sensory cells within the enteric nervous system may be triggered to release transmitter that remotely act on dopamine neurons (*Figure 6.16C*). In the case of bead-induced pumping the time course would allow for accumulation of distension in the gut to allow distortion of associated dopamine mechanosensory neurones or transmitter-mediated transmission. Previous work in locomotory circuits have defined contraction based release from distal neurons leading to subsequent functionally modifying transmitters (Flavell et al., 2013).

In the case of dopamine mediated increases in pharyngeal function, the discrete localisation of dopamine outside of the pharynx requiring the downstream activation of

dop-4 receptors within the enteric neuron I2, indicates that this circuit is using volume transmission (*Figure 6.16C*).

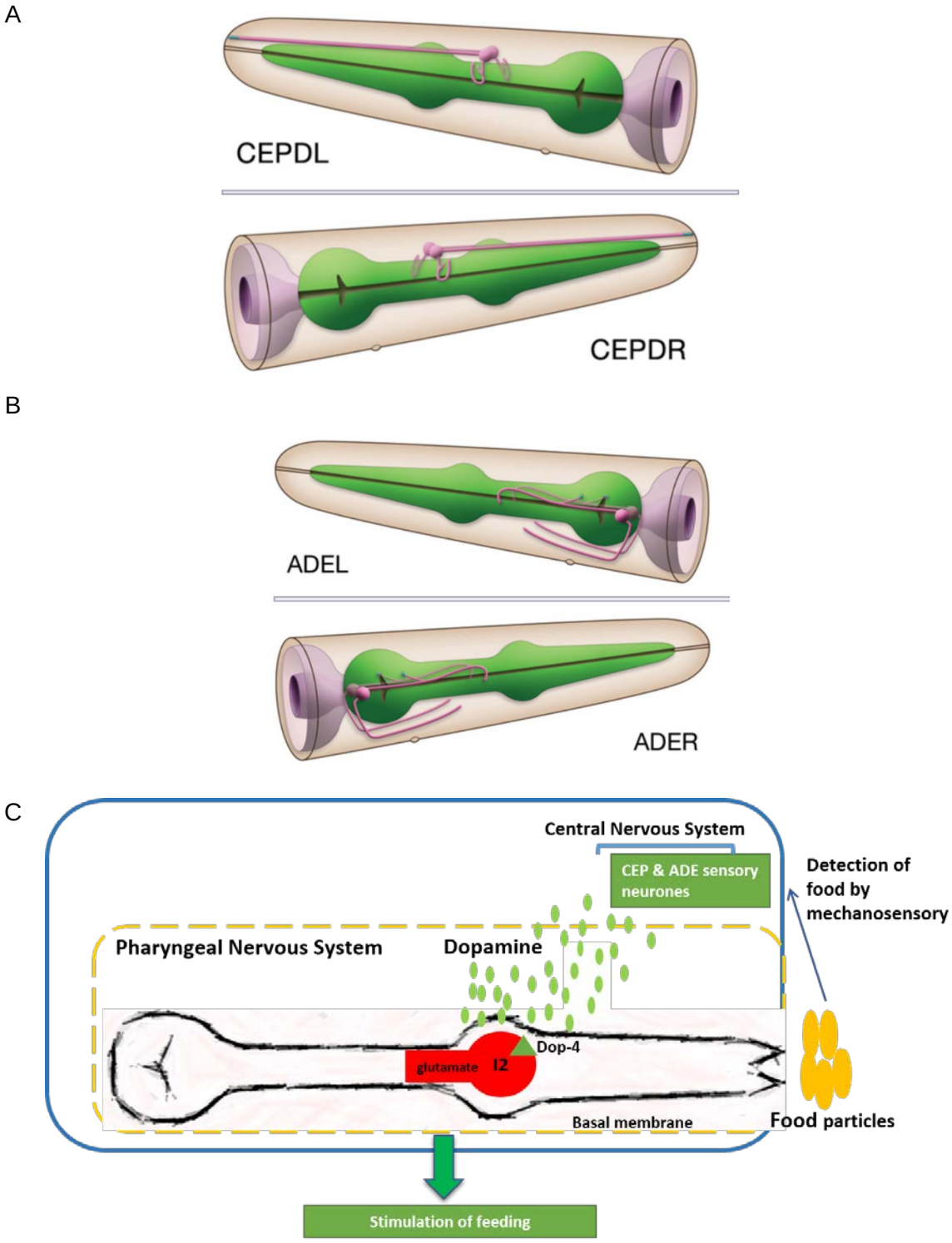


Figure 6.16: The role of mechanosensation in C. elegans in driving feeding behaviour.

A) A cartoon representation of the mechanosensory neurones, CEP. There are 4 bilateral cell bodies of CEP localised around the pharynx. The CEP dendrites are extended to the tip of the nose. Image obtained from WormAtlas.org. B) A cartoon representation of the mechanosensory neurones, ADE. C) A model of mechanosensation of food in response to mechanical cue. One suggestion is that food is detected by mechanosensory neurones, CEP or ADE, which leads to dopamine release. On the other hand, food may be detected within the pharynx by a neurotransmitter that can remotely activate dopamine. Dopamine can act as a neurohormone to activate DOP-4 receptors in I2. Activation of DOP-4 receptors disinhibits the I2 neurones, causing the inhibition of glutamate release, which increases pumping that promotes feeding. Red colour = inhibition, green colour = stimulation

Chapter 7: Investigating plasticity in chemosensory responses upon prior pairing of mechanosensory cue and odour

7.1 Introduction

The main findings in Chapter 4 revealed that olfactory conditioning with butanone induces a change in the efficacy of chemotaxis to a distal olfactory cue. Despite this plasticity in the worm's behaviour, this olfactory-mediated plasticity had no impact on pharyngeal function. Our observations above highlight that there is a distinct perception of food modalities when the worm contacts food rather than senses it as an olfactory cue at some distance. Interestingly beads and likely food induce a dopamine- dependent change in behaviour mediated by upstream release of dopamine, which mediates a downstream modulation of pharyngeal pumping. Previous studies indicate that bacteria presentation and removal engage a dopamine-dependent plasticity associated with motility behaviour that controls foraging strategy (see Chapter 1- General Introduction) (Hills, 2004; Sawin et al., 2000). This pointed to the notion that food induced changes in dopamine signalling might allow food cues to condition the pharynx. Therefore, one would question if a modification of the pairing approach used in Chapter 4 might reveal a food dependent change in plasticity. The principle of this was based on the notion that conditioning worms to an olfactory cue while incubating the worms on beads, a

physical mimic of food, might allow us to reveal plasticity or simple associative learning in the feeding behaviour.

7.2 Results

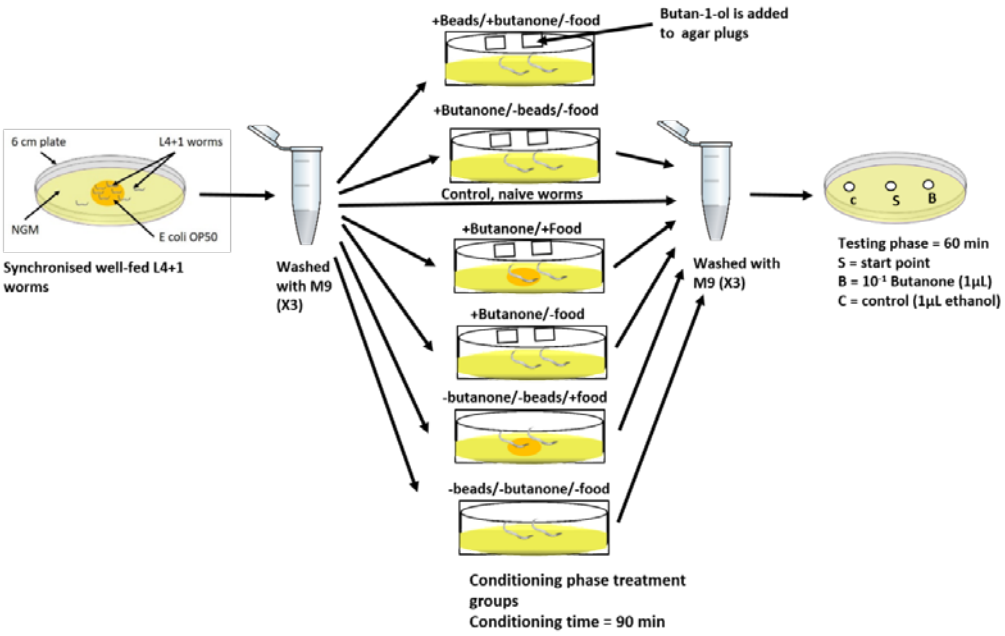
7.2.1 Beads-induced pharyngeal pumping does not alter butanone chemotaxis index following incubation of worms in the presence of beads and butanone

Synchronised L4+1 worms were pre-conditioned with $2.7x10^8$ beads/cm^2 beads in the presence of 6 µl× 10^0 butanone. These worms were compared to 6 µl× 10^0 butanone and food, 6 µl× 10^0 butanone only, food only or worms only (*Figure 7.1A*). The worms were pre-conditioned for 90 min, according to the previous conditioning paradigm discussed in Chapter 4, section 4.2.3, based on the ability of cue conditioning to induce plasticity in the circuits underpinning chemotaxis. Following the conditioning phase, all groups were washed and tested in a chemotaxis assay to 1µl× 10^{-1} butanone. Worms conditioned in the presence of beads and butanone did not show enhanced chemotaxis index to butanone relative to the control group or to worms conditioned in the presence of butanone only (*Figure 7.1B*). However, worms conditioned in the presence of butanone and food showed the highest chemotaxis index (0.46 ± 0.02) compared to control group consistent with previous observation reported in Chapter 4.

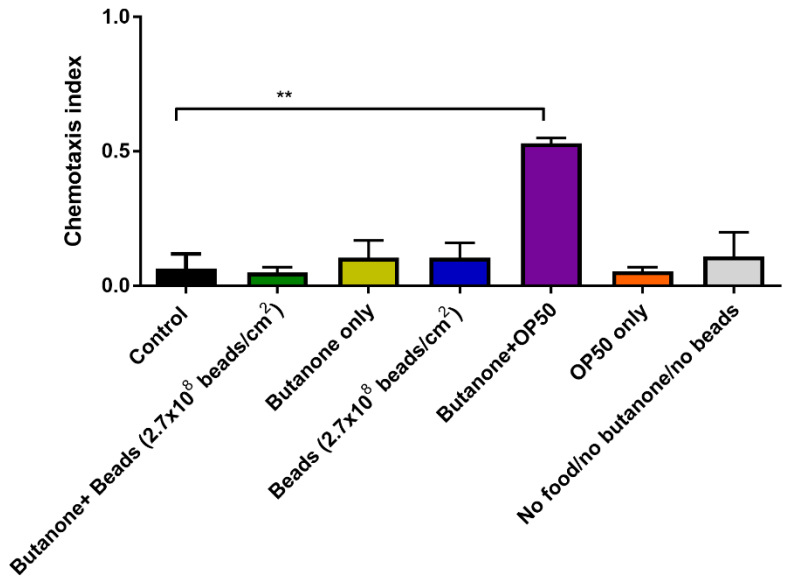
Pharyngeal pumping was recorded during the conditioning phase and testing phase. However, pumping rate was not affected following pre-conditioning worms in the presence of beads and butanone (*Figure 7.1C*).

Overall, the data suggest that although beads can increase pumping rate in the absence of food, the mechanical cue cannot alter subsequent chemotaxis or pharyngeal pumping rate. This suggests that the neurochemical changes associated with beads including dopamine release does not underlie plasticity.

A


+Beads/+butanone/-food
Butan-1-ol is added to agar plugs
+Butanone/-beads/-food
6 cm plate
L4+1 worms
NGM
E coli OP50
Control, naive worms
+Butanone/+Food
+Butanone/-food
-butanone/-beads/+food
-beads/-butanone/-food
Synchronised well-fed L4+1 worms
Washed with M9 (X3)
Washed with M9 (X3)
C
S
B
Testing phase = 60 min
S = start point
B = 10⁻¹ Butanone (1µL)
C = control (1µL ethanol)
Conditioning phase treatment groups
Conditioning time = 90 min

B


Chemotaxis index
1.0
0.5
0.0
**
Control
Butanone+ Beads (2.7x10⁸ beads/cm²)
Butanone only
Beads (2.7x10⁸ beads/cm²)
Butanone+OP50
OP50 only
No food/no butanone/no beads

C

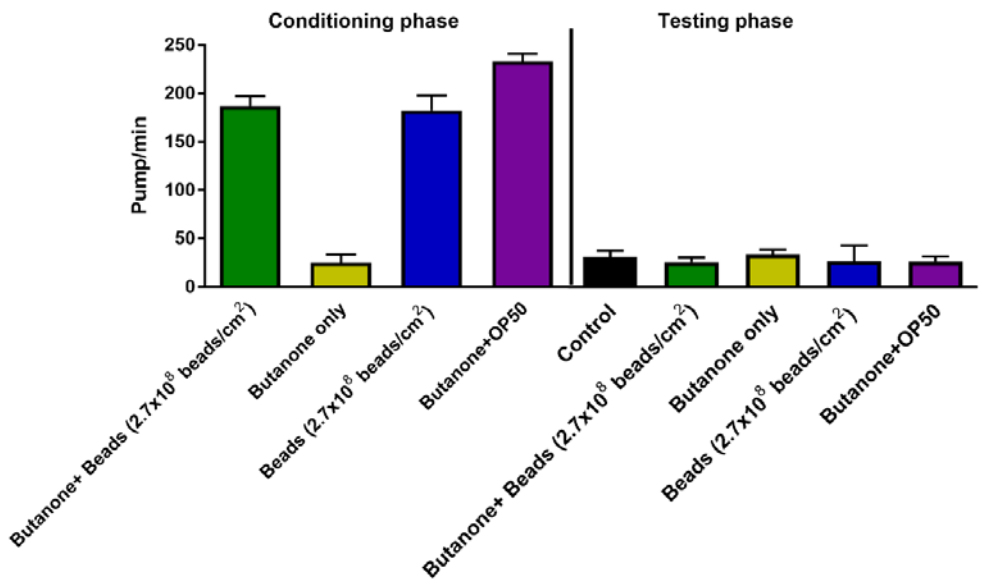


Figure 7.1: Pre-conditioning worms in the presence of beads and butanone do not alter subsequent chemotaxis to butanone. *A) A schematic representation of the assay. 150 synchronised L4+1 worms were washed and pre-conditioned as indicated in the presence of 2.7x10^8 beads/cm^2 beads+6 µl× 10^0 butanone, 6 µl× 10^0 butanone+food, 6 µl× 10^0 butanone alone, food alone or worms alone. Following the conditioning treatment, worms were washed and placed on chemotaxis plates and their response to 1µl× 10^{-1} butanone measured. B) Pre-conditioned worms in the presence of butanone and food had a significant high chemotaxis index (0.46 ± 0.02, p = 0.005) compared to control group (0.07± 0.05). Worms pre-conditioned in the presence of beads and butanone had a chemotaxis index similar to control group. The experiment was repeated twice. C) Pharyngeal pumping was recorded during the conditioning phase and testing phase. 20 L4+1 worms were picked and placed onto a cleaning plate for 1 min before placed onto a testing plate to 1 µl ×10^1 butanone. No significant difference between control and treatment groups was observed (p = 0.46, n= 8-10 worms/ group). One-way ANOVA test followed by Bonferroni post-hoc was used. Data represented as mean ± S.E.M.*

7.3 Discussion

7.3.1 Dopaminergic determinants primitive to food reward

As indicated, the bead experiments afford a way to delineate one modality of the multimodal food cue. We extended this to try delineating the mechanosensory-induced responses' physiological relevance. The modified food entry response suggests that the mechanosensory modality is utilised to trigger an increase in pump rate. This occurs in a much more rapid time course than seen on beads. This highlights two issues: a) the multimodal cue by food is an enriched modality and may utilise gustatory cues; b) the slow build up in pump rate in beads indicates that the mechanical drive is not as potent as the multimodal cue. Alternatively, the late pumping may reflect a post grinder response of the gut that involves a distinctively driven response. However, future work will be required to confirm it. Regardless of the dopamine-dependent cueing of food reflected in food entry suggest that the selective use of dopamine, in which an imposed inhibitory tone is rapidly halted by disinhibition, suggest that the worm and its embedded filter feeding is primed to respond to potential food sources. Interestingly, the speed of response would indicate that it is engaged before the worm has had the chance to assign if there is a nutritive return to the ingested particle. This would require the digestion of the food and interceptive assessment of benefit (reward) or caloric value. This has some parallels with the way dopamine is used in higher animals to respond to their environment (Wu et al., 2008). In terms of reward pathways, dopamine is central to our understanding. However, both predictive value and actual value are thought to be encoded by distinct dopaminergic signals.

7.3.2 Dopaminergic signalling of feeding plasticity

Although ingestion of beads can result in elevated pumping rate in the absence of food, a pre-conditioning of worms in the presence of beads and butanone (and absence of food) did not lead to the modification of chemotaxis index or pharyngeal pumping in response to butanone, compared to worms pre-conditioned in the presence of butanone and food (*Figure 7.1B&C*). Similar findings in

mammals showed that there is a difference between feeding for nourishment and food for reward (Szczypka et al., 2001). PET scans in humans showed that dopamine release from the dorsal striatum, but not the ventral, is associated with feeding (Small et al., 2003). Based on preliminary data of the beads conditioning experiment, dopamine release maybe associated with feeding, but not the reward aspect of food, since the conditions of the experiment were done in well-fed worms, in which feeding in this case was for nourishment. While feeding for reward might be associated with stress or following a long period of starvation. Nonetheless, further experiments are needed to confirm this.

Chapter 8: General Discussion

All animals, including *C. elegans*, navigate in complex environments, which have both favourable and toxic sources such as food or predators. These sources can be detected by one or more sensory modalities. Therefore, the animal must encode these sensory cue(s), integrate them into their nervous system to make decisions that aid survival and reproduction. Sensory-dependent behavioural paradigms have been developed to study sensory cue-dependent plasticity by taking advantage of the worm's ability to detect a range of modalities (Bono and Villu Maricq, 2005; Sengupta, 2007). Such paradigms can include: 1) singular cue to trigger a response; 2) complex cue leading to singular response. These studies have focused on chemotaxis index as a readout without investigating the impact of such cues on feeding behaviour. *C. elegans* feeds on bacteria by ingesting bacterial particles from the environment into the pharynx, where bacteria is ingested and ground before transfer to the intestine (Avery, 2003). In this thesis, I focused on pumping rate, which is a sub-behaviour associated with ingestion of food. The aim was to use the pharynx as a readout to identify how sensory modalities influence feeding behaviour and feeding-dependent plasticity. This study reported novel findings, in which I showed that pharyngeal pumping to butanone was unaffected when worms were pre-exposed to butanone and food in an experience-dependent behavioural plasticity (Chapter 4). I went on to identify that the mechanosensory detection of food, which is key in *C. elegans* pumping behaviour (Chapter 5). Finally, I delineated the cellular and molecular determinants in mechanosensory-dependent pumping behaviour, in which dopamine-dependent circuit utilised disinhibition of pumping to drive feeding response (Chapter 6).

Herein, I will be drawing a general overview of the main findings reported in this thesis and discuss the significance of sensory modalities in regulating feeding behaviour in *C. elegans*.

8.1 Beads, a paradigm for mechanosensory modality, reveal a complex role of mechanoreception in pharyngeal pumping

Observations obtained from Chapter 3 have shown that *C. elegans* detect environmentally-salient cue, such as volatile alcohols, at a distance and drive a potent innate chemosensory response (Chapter 3 - section 3.2.1). However, attractive alcohols did not stimulate pharyngeal pumping in the presence or absence of food (*Figure 3.2*, *Figure 3.3* & *Figure 3.4*). This was further evidenced by observation obtained from the cilia-deficient mutant (*osm-6)*, in which *osm-6 (p811)* mutants displayed an aberrant increase in pharyngeal pumping in the absence of food while maintaining a normal feeding behaviour on food (Chapter 5 – section 5.2.3). Overall, this indicates that olfaction does not play a role in stimulating pharyngeal pumping in the presence of food. On the contrary, the aberrantly elevated pumping rate exhibited by the *osm-6* mutants suggests that these ciliated neurones respond to the absence of food odours and are involved in reducing pharyngeal pumping rate on food. Moreover, these findings indicate that the absence of olfactory cues plays a role in modulating feeding behaviour in the absence of food. This was further evidenced by the observations which showed that, even in the presence of dead bacteria, worms maintained a high pumping rate (chapter 5 – section 5.2.4). Therefore, this indicates that mechanical cue from bacteria plays an important role in increasing pharyngeal pumping. Hence, gustatory or mechanosensory play a role in perceiving the presence of food.
To test this hypothesis, both modalities were investigated. One aspect of testing gustatory cue was investigated by using bacterially-conditioned medium in the absence of food to determine its effect on pumping rate (Chapter 5 – section 5.2.9). The results have shown no evidence of simple gustatory cue to stimulate pharyngeal pumping in the absence of food (Chapter 5 - section 5.2.9).

8.2 A positive feedback from the gut to the central nervous system is a conserved mechanism in feeding behaviours

The observations with ingestible beads provide support for the hypothesis that suggests mechanical distension of the pharynx following ingestion of bacteria is an important factor in driving elevated pumping rate (Chapter 5 – section 5.2.5 & 5.2.6). This is also supported by the observation that large beads failed to increase pharyngeal pumping in the absence of food (Chapter 5 –section 5.2.7). Intriguingly, the initial elevation in pumping rate in the presence of ingestible beads was not as rapid or as sustained as the response seen on bacteria. In the first 10 minutes of contact with beads, worms moved from low to high to low, or to high, pumping rate (Chapter 5 – *Figure 5.5*). Clearly, the beads on their own are not a complete mimic of food, and this initial mechanosensory cue which resulted in the presence of beads in the pharynx and the anterior gut is only capable of transiently increase pumping (Chapter – *Figure 5.5*). Mechanosensation in the mammalian system is critical for normal gastrointestinal tract function and distention is a key determinant for gastric motility (Alcaino et al., 2016; Brierley, 2010). In the mammalian system, the vagal gastro-oesophageal afferents, known as tension sensitive afferent, detect ingested food in the stomach by responding to stretch and distension of the stomach (Bielefeldt and Davis, 2007; Page et al., 2002, 2005). The vagal nerves in the stomach relay electrical signals, along with metabolic status such as insulin levels and secretion of the ghrelin hormone, to assess the state of emptiness or fullness of the stomach (Berthoud and Neuhuber, 2000). The stretch vagal afferent measures stretch of the stomach by the food that has been ingested and passed through pharyoesphageal canal to the stomach (Phillips and Powley, 2000). The ingested food starts to accumulate in the stomach, causing stretching, which in turns lead to the activation of the stretch vagal afferent neurons. This signal is transmitted to the brain (Phillips and Powley, 2000). The difference between stretch and tension is that stretch measures volume, whereas tension measures a change in the musculature without a change in volume. Lastly, the nutrient vagal afferent is found mainly in the intestine and responds to overall nutrient character (Dockray and Burdyga, 2011). Together, the

stomach provides physical signals to mediate satiety by providing information about the stretch and distension of the stomach, whilst the intestine provides nutritional-related signals, thus providing feedback about meal quality to the hindbrain (Amin and Mercer, 2016; Dockray and Burdyga, 2011).

However, overtime, the accumulation of beads in the lower gastrointestinal tract led to a sustained elevation in pumping rate (*Figure 5.7*). Due to the absence of food, defecation is greatly reduced (Epstein, 1990; Kiyama et al., 2012) resulting in the accumulation of beads in the intestine. This might be indicative of a second pathway that triggers pharyngeal pumping that emanates from the mechanical presence of bacteria in the posterior gut. In mammals, during the initiation of a meal, a hormone is secreted in the endocrine stomach cells known as ghrelin (Hosoda et al., 2006). This hormone signals positive feedback from the gut to the central nervous system to promote feeding. Ghrelin stimulates feeding by synthesising and releasing of agouti-related protein (AgRP) and neuropeptide Y in the arcuate nucleus neurones of the hypothalamus and hindbrain (Gil-Campos et al., 2006). In *C. elegans,* NPR-20 receptors were found expressed in the posterior gut, nerves of the head, tail and ventral nerve cord (Cardoso et al., 2012). Therefore, this receptor may have a role in regulating feeding behaviour (Cardoso et al., 2012). In future experiments, it would be interesting to test the role of NPR-20 mutants and investigate their response to beads.

8.3 Dopamine volume transmission is a significant determinant of feeding

A thorough investigation into the role of mechanosensation in driving feeding behaviour was conducted. This was achieved through the analysis of mutant *C. elegans* defective in specific neurotransmitter or neuropeptide signals utilising different contexts, such as the presence of food, the presence of beads or the presence of exogenous dopamine (Chapter 6). These results revealed the significance of dopamine, an important neurotransmitter in the mechanosensation, in regulating *C. elegans* feeding behaviour. *cat-2* mutants supported a role for

dopamine release, as *cat-2* mutants did not respond to exogenous dopamine or beads (see Chapter 6 –section 6.2.2). Further mutant analysis revealed that DOP-4 receptors expressed in the pharyngeal neurones, I2, play an important role in the mechanical stimulation of pumping rate (see Chapter 6 –sections 6.2.3, 6.2.4.2 & 6.2.7). Even though all the dopaminergic neurones are found outside the pharynx (Sulston et al., 1975), the presence of dopamine-releasing cells outside the pharynx proposes a role for CEP and ADE neurones in sensing the presence of food particles, which subsequently leads to the release of dopamine. CEP is the only dopaminergic neurones which are present at the tip of the worm's mouth (*Figure 6.15*) and play a critical role in mediating basal slowing response, when worms become in contact with food (Sawin et al., 2000). These suggest that CEP neurones may sense the presence of beads/ food particles, which allow the release of dopamine. ADE neurones are found posterior to the PDE and with no dendritic ends at the tip of the mouth, however, they are found wrapped around the pharynx, which may detect the distention of the pharynx as a result of the accumulation of food particles inside the pharynx. Alternatively, dopamine can act as a neurohormone by directly activating DOP-4 receptors in I2. Glutamate released from I2 mediates inhibition of pumping rate in the absence of food, as *eat-4* mutants and I2-ablated worms display a high pumping rate off-food, which is due to loss of glutamate signalling (Dallière et al., 2016). Thus, inhibiting the release of glutamate facilitates high pumping, and therefore, one would speculate that extra-pharyngeal dopamine activates DOP-4 receptors in I2 to inhibit of the release of glutamate (see Chapter 6 –section 6.2.4.1 & 6.2.4.2). Therefore, future experiments, investigating CEP- and ADE- ablated worms in the presence of beads might shed more light on the role of dopamine. Furthermore, partial rescue of DOP-4 in I2 suggests that other neurones where DOP-4 expressed (I1 and NSM neurones) should be investigated by specifically rescuing wild-type DOP-4 receptors in either I1 or NSM neurones.

Moreover, determining calcium activity of I2 neurones in wild type and *dop-4* mutants in the presence of beads in freely-moving animals would be interesting to provide further evidence to behavioural observations.

8.4 Physiological relevance of the dopamine-mediated disinhibition

Although *cat-2* and dopamine receptor mutants (*dop-3 & dop-4)* did not respond to beads, they showed a normal feeding behaviour on food (*Figure 6.2* & *Figure 6.3*). Therefore, it was speculated that dopamine may play a role in initiating feeding behaviour but not for sustaining pumping rate. This was further supported by observations obtained from food entry assay, which showed that *dop-4* mutants took longer to reach the maximum pumping rate when worms become in contact with food (see Chapter 6 –section 6.2.11). Expression of wild-type dop-4 in *dop-4 (tm1392)* background showed a full rescue of this behaviour in food entry assay (see Chapter 6 –section 6.2.11), whereas expression of wild-type dop-4 in I2 neurones in *dop-4 (tm1392)* background showed a partial rescue (see Chapter 6 – section 6.2.12). However, overall, these findings provide further support to previous observations (see Chapter 6 –section 6.2.6), which showed that DOP-4 receptors are critical for mechanical stimulation of pumping. Furthermore, this partial rescue seen in I2 neurones may suggest that other dopamine neurones expressed in NSM and I1 neurones are equally important. Overall, this data provide evidence for a role for dopamine signalling in appetitive signal in a context of newly encountered food.

8.5 Serotonin acts in parallel to dopamine to stimulate pharyngeal pumping

Mutant analysis has shown that *tph-1* mutants did not show an increase in pumping rate in the presence of beads (see Chapter 6 –section 6.2.2). Furthermore, food entry data have shown that serotonin signalling is important when worms become in contact with food (see Chapter 6 – section 6.2.9). These data suggest that 5-HT plays a role in the mechanical stimulation of the pharynx and on entry of food. However, in the presence of exogenous dopamine, *tph-1* mutants displayed a high pumping rate similar to wild-type worms (Chapter 6 –

section 6.2.2), indicating that dopamine signalling is not required for 5-HT-mediated pumping rate. The pharyngeal neurones, NSM, are the only serotonergic neurones expressed in the pharynx (Horvitz et al., 1982), which have proprioceptive endings in the pharynx acting as sensory neurones to detect the presence of bacteria inside the lumen (Axäng et al., 2008). A recent study has shown that serotonin is required for speeding up the slowing response observed upon entry of food, in which ADF and NSM neurones are both involved in this behaviour (Iwanir et al., 2016). Moreover, it was previously reported that 5-HT released from the pharyngeal neuron, NSM, in response to food promotes the dwelling behaviour (Flavell et al., 2013). Thus, these indicate that 5-HT may act in parallel to dopamine to stimulate pharyngeal pumping. Nonetheless, ADF are ciliated sensory neurones which release 5-HT in response to food and regulate pharyngeal pumping (Cunningham et al., 2012; Iwanir et al., 2016; Li et al., 2012). Therefore, overall, one would predict that the presence of bacterial particles inside the pharynx leads to dopamine and 5-HT release. The role of 5-HT would be to regulate the worm's locomotory behaviour and initiate feeding, when worms become in contact with food (*Figure 8.1*). Future experiments would determine the role of 5-HT in mechanical stimulation of beads. The approach would be by separately rescuing the expression of *tph-1* in NSM and ADF neurones.

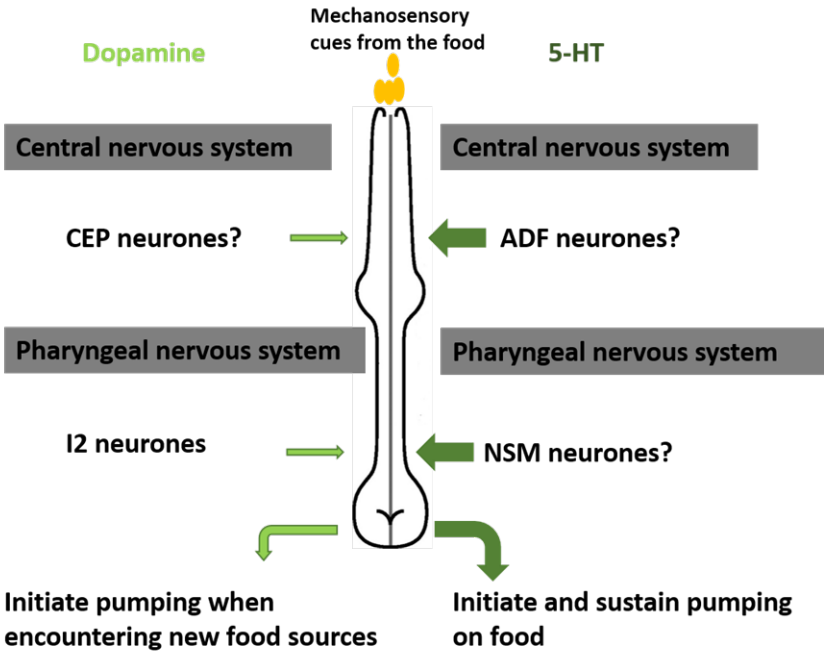


Figure 8.1: The mechanosensory cues from food are both 5-HT and dopamine dependent.

On entry of food, the ingested food particles trigger both dopamine and 5-HT release. Ingested food particles may cause dopamine release through CEP or ADE neurones due to distention of the pharynx. Nonetheless, CEP may directly detect food particles via their dendritic endings that extend to the tip of the nose. Dopamine may act on the I2 neurones to inhibit glutamate release. Thus, initiating pumping rate as the worm enters a newly encountered food lawn. 5-HT released from ADF or NSM increases pharyngeal pumping when worms become in contact with food. However, unlike dopamine, 5-HT mediates both initiation and sustained pumping rate.

8.6 A complex interplay of modalities and outputs allow a worm to successfully feed

Together, these results suggest that chemosensory neurones sense the presence of bacteria by odours released from distal food source. A low pumping rate is maintained to sample the worm's environment (Avery and You 2012), whilst worms navigate towards the food lawn (chemotaxis). Once the worms come in contact with food, basal pumping rate accumulate bacteria in the pharynx, which leads to distension of the pharynx, allowing the detection of food within the pharynx by utilising the mechanosensory and gustatory modalities. This leads to removal of inhibition, which results in a high and sustained pumping rate. An apparent determinant is the dopamine-induced pumping, since the contribution of this circuit is not needed to sustain pumping on food. The value of this circuit is that it facilitates the initiation of pumping rate which is advantageous to the ecological niche of the worm, where the quantity and quality of the food is varied (Frézal and Félix, 2015). Hence, dopamine-induced pumping rate accelerates feeding, while the worms navigate between areas of low and high food quality. This notion was supported by the food entry assay data, which have shown that a sustained pumping rate was only achieved after 3 minutes from entry to food (Chapter 6 –section 6.2.8).

8.7 Pharyngeal pumping-induced plasticity was not feasible in an olfactory-based paradigm

The lack of an olfactory-induced pharyngeal plasticity may relate to the ecological niche in which the worms exist. *C. elegans* have a boom-and-bust lifestyle in which worms are found in either a feeding or non-feeding state (Frézal and Félix, 2015).

Plasticity within the pharyngeal system has been reported previously, in which familiar food resulted in an increased pharyngeal pumping following the training

trials (Song et al., 2013). Furthermore, starved worms showed increased pumping in a re-feeding trial compared to well-fed worms (Lemieux et al., 2015). In these studies, behavioural plasticity within the pharyngeal system was achieved in the presence of food. The current thesis failed to show conditioning of pharyngeal responses in fed worms or worms in which the pairing was achieved with a mechanic mimic of food intake. It could be that starvation or noxious associated conditions are the best routes to induce plasticity at the level of the pharynx. Therefore, the nematode worm, with a simple nervous system, is able to detect cues and integrate them based on the context, or the environment they live in, to drive appropriate behavioural response (*Figure 8.2*). Thus, *C. elegans* is a powerful model to investigate context-dependent behaviour and switch between different conditions.

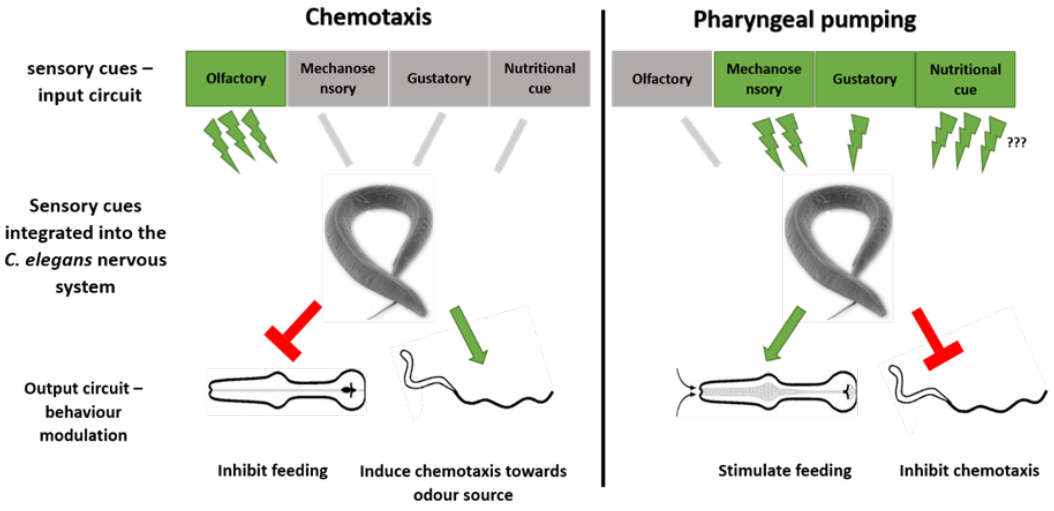


Figure 8.2: The main sensory modalities for chemotaxis towards butanone versus the sensory modalities required for stimulating pharyngeal pumping.

In the absence of food and presence of an attractant odour (butanone), olfactory cues are detected by chemosensory neurones, AWC/AWA. Sensory information is subsequently integrated into the C. elegans nervous system. The nervous system conveys information to output circuits to drive behaviour which is achieved by reducing turns and reversals to allow forward movement

(chemotaxis) towards the odour source. Pumping rate is inhibited due to the absence of food. In the presence of food, sensory cues from food (mechanosensory, gustatory and nutritional cues) are perceived and integrated into the nervous system to promote pharyngeal pumping. Once on food, locomotion rate is reduced to allow feeding and growth.

 *= the number of thunder bolts corresponds to the significance of sensory cue in behaviour modulation.*

8.8 Conclusion

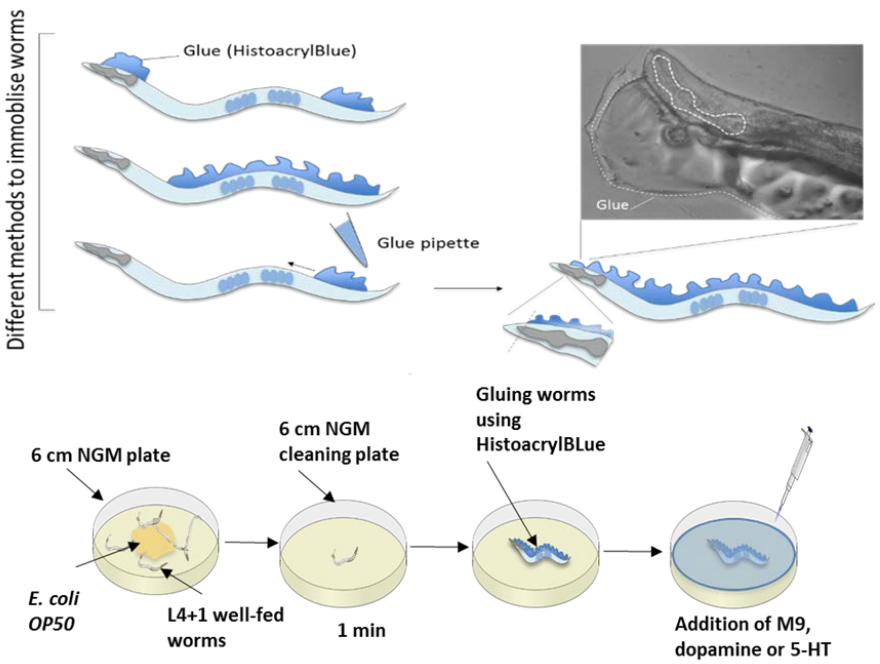
Overall, the study has revealed an unexpected complexity into the regulation of pharyngeal pumping which is clearly dependent on mechanosensory cues. Investigating pumping, in the context of transition, reinforce the key role of mechanosensory cues but also strongly reinforces the complexity and one can only resolve how additional cues, amongst the complex food signals, individually regulate pumping.

Feeding behaviour is known to undergo profound plasticity and mismatches and in this underlie critical disease states that clearly involve interaction between the gut and brain. Despite trying to recapitulate existing plasticity paradigms to evoke changes in feeding behaviour and design new paradigms based on data that emerged in the thesis, we were unable to show plasticity in pharyngeal pumping based on previous experience. As the limited insight that exists has come from paradigms based on starvation or aversive learning, perhaps feeding plasticity is restricted to times of stress, a possibility that might fit well with a boom bust filter feeder.

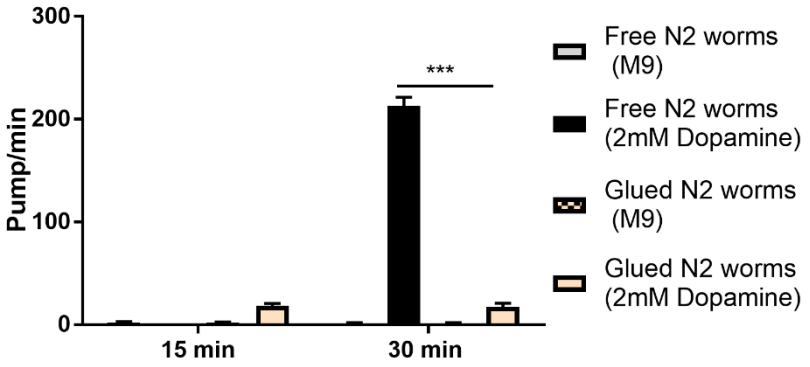
Appendices

Preliminary data for assessing calcium levels in the I2 in response to dopamine.

A



B



C

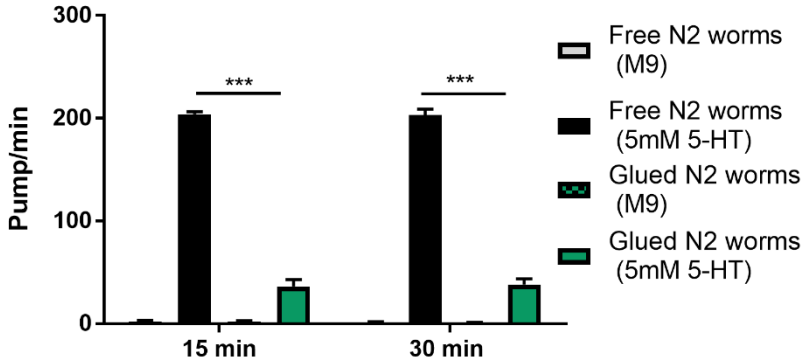
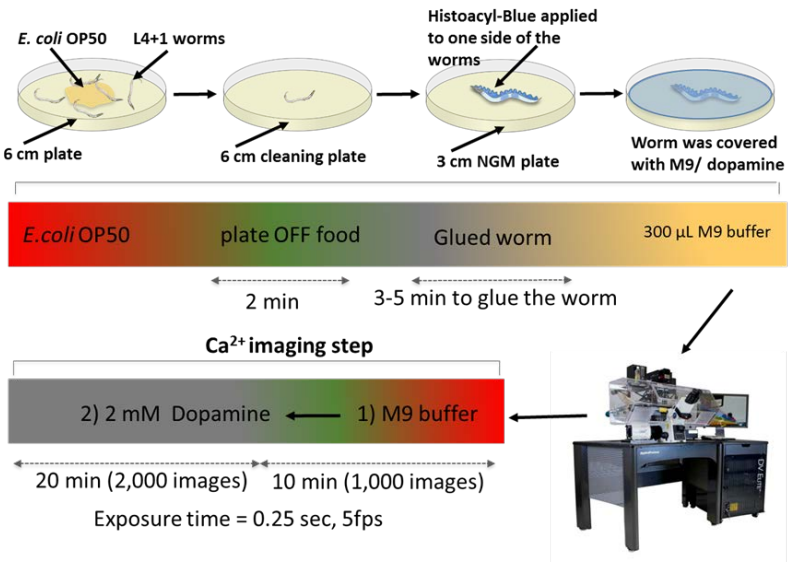
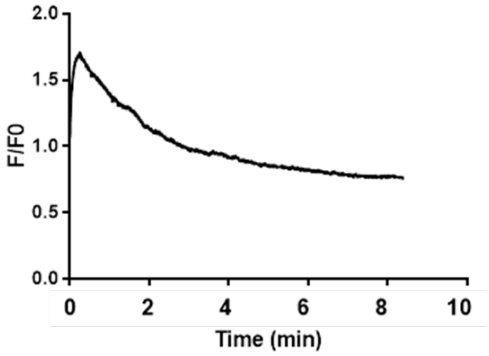


Figure 1: Immobilised worms have a reduced pumping rate in the presence of dopamine or 5-HT compared to control, free worms on pumping rate. *A) A schematic diagram showing different positions of application of histoacryl-Blue on the worm to immobilise it. 20 well-fed worms were picked onto a cleaning plate for 1 minute and then transferred onto 3 cm unseeded NGM plate for immobilisation. Worms were immobilised by gluing them to the NGM using histoacrylBLue, and then 300 µl M9 was added followed by the addition of dopamine or 5-HT at final concentration of 2mM or 5mM, respectively. B) In the presence of dopamine, due to increased thrashing activity, pumping rate was not observed in the first 15 minutes. Free worms in dopamine solution significantly (200 ± 8, $p < 0.0001$, n= 10 worms) increased pharyngeal pumping rate compared to control worms on M9 after 30minutes. In contrast, immobilisation of worms with histoacryl-Blue was characterised by a twitching of the pharynx, instead of pumping activity. However, their response to dopamine was significantly (18 ± 7, $p < 0.0001$, n = 15) reduced compared to free worms on dopamine. C) Free worms in 5-HT solution significantly (203 ± 5, $p < 0.001$, n= 9-10 worms/group) increased pharyngeal pumping compared to control worms in M9 after 15 minutes. Immobilised worms showed significant reduction in pumping rate (38 ± 6, $p < 0.0001$) compared to free worms. Similar pattern was observed after 30minutes in the presence of 5-HT.*

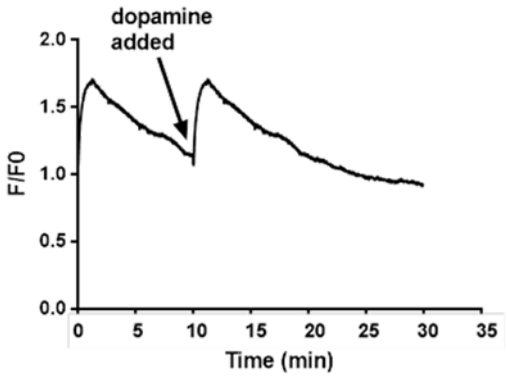
A


E. coli OP50
L4+1 worms
6 cm plate
6 cm cleaning plate
Histoacyl-Blue applied to one side of the worms
3 cm NGM plate
Worm was covered with M9/ dopamine
E.coli OP50
plate OFF food
Glued worm
300 µL M9 buffer
2 min
3-5 min to glue the worm
Ca2+ imaging step
2) 2 mM Dopamine
1) M9 buffer
20 min (2,000 images)
10 min (1,000 images)
Exposure time = 0.25 sec, 5fps

B


F/F0
Time (min)

C


dopamine added
F/F0
Time (min)

D

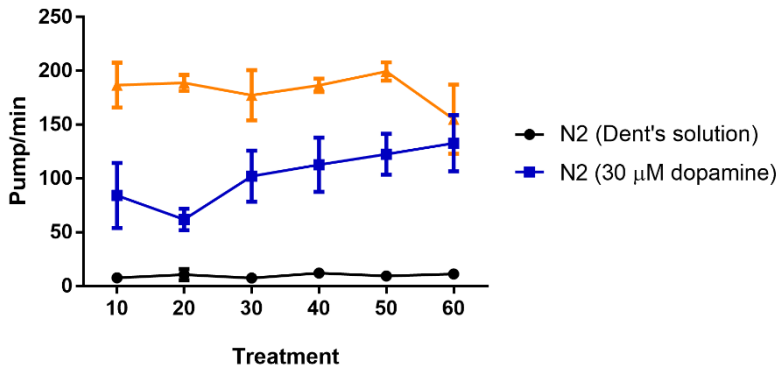

Pump/min
Treatment
N2 (Dent's solution)
N2 (30 µM dopamine)

Figure 3: Dopamine increases pharyngeal pumping in cut heads preparations. *A) 20 synchronised well-fed worms were placed in Dent's solution to isolate the head of the worm from the rest of its body and to expose the pharynx. The cut heads were transferred into non-seeded plate containing fresh Dent's solution. B) A baseline pumping rate was recorded before transfer into plates containing either dopamine (30 µM) or 5-HT (1 µM). C) A significant increase in pumping rate was observed in the presence of dopamine (89 ± 6, $p < 0.0001$, $n = 10$) compared to the control, cut heads in Dent's solution (8± 6, $n = 7$). In the presence of 5-HT, an elevated pumping rate was observed (166 ± 3, $p < 0.0001$, $n = 7$) compared to control.*

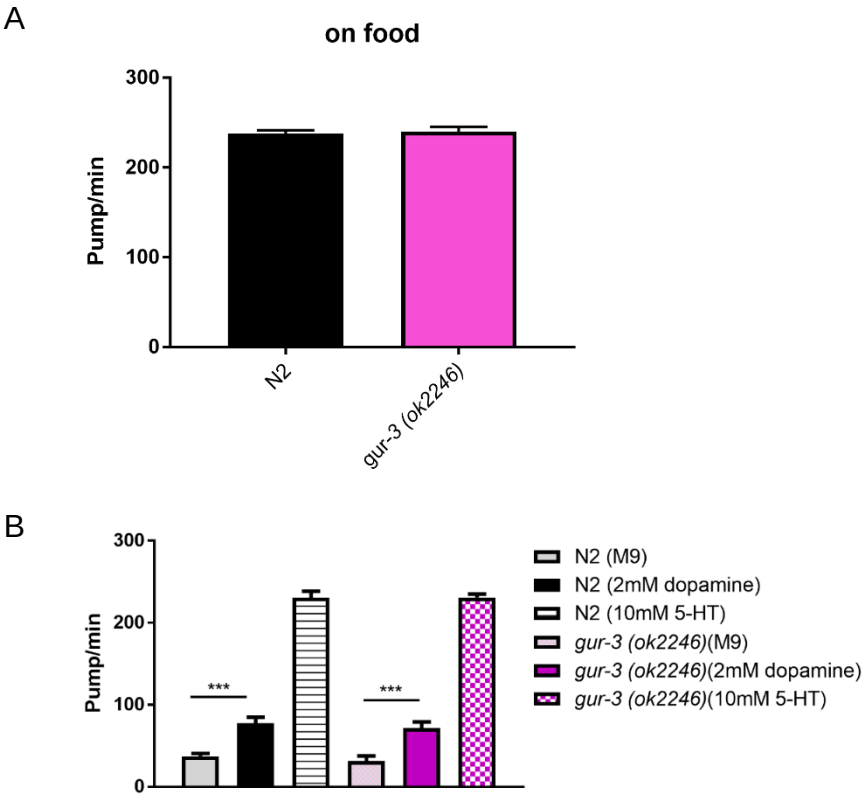


Figure 4: The gustatory receptor, gur-3 (ok2246), mutant displays increased pharyngeal pumping in the presence of exogenous 2mM dopamine and 5mM 5-HT*. For experimental design see Figure 6.1. A) gur-3 mutant pumped at a similar rate to N2 in the presence of food (p > 0.05, n =10 worms). B) N2 and gur-3 mutant pump rates in the presence of the indicated concentration of exogenous transmitter. gur-3 mutant showed high pumping relative to gur-3 on S-basal (p < 0.0001, n = 8-9 worms/ group). One-way ANOVA followed by Bonferroni post test was used. Data represented as Mean ± S.E.M.*

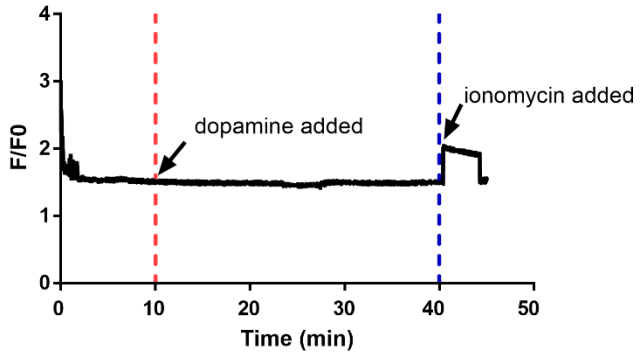


Figure 5: Exogenous dopamine did not increase calcium levels in I2.
Worms were picked into cleaning plate (6 cm) for 1 minute before transfer to assay plate (3 cm) containing Dent's solution. Dent's solution (100 µl) was added to cover the worm. Dopamine was added to give final concentration of 10µM. In the first 10 minutes, the baseline was recorded. An increased activity in calcium levels in the presence of Dent's solution was not observed. The addition of dopamine after 10 minutes did not increase calcium levels. Ionomycin (1µM) was added and small increase in calcium activity was observed (n = 1).

References

Abizaid, A., Liu, Z.W., Andrews, Z.B., Shanabrough, M., Borok, E., Elsworth, J.D., Roth, R.H., Sleeman, M.W., Picciotto, M.R., Tschöp, M.H., et al. (2006). Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite. J. Clin. Invest. *116*, 3229–3239.

Albert, P.S., Brown, S.J., and Riddle, D.L. (1981). Sensory control of dauer larva formation in Caenorhabditis elegans. J. Comp. Neurol. *198*, 435–451.

Albertson, D.G., and Thomson, J.N. (1976). The pharynx of Caenorhabditis elegans. Philos. Trans. R. Soc. Lond. B. Biol. Sci.

Alcaino, C., Farrugia, G., and Beyder, A. (2016). Mechanosensitive Piezo Channels in the Gastrointestinal Tract. Curr. Top. Membr. *79*, 219–244.

Alkema, M.J., Hunter-Ensor, M., Ringstad, N., and Horvitz, H.R. (2005). Tyramine functions independently of octopamine in the Caenorhabditis elegans nervous system. Neuron *46*, 247–260.

Amano, H., and Maruyama, I.N. (2011). Aversive olfactory learning and associative long-term memory in Caenorhabditis elegans. Learn. Mem. *18*, 654–665.

Amin, T., and Mercer, J.G. (2016). Hunger and Satiety Mechanisms and Their Potential Exploitation in the Regulation of Food Intake. Curr. Obes. Rep. *5*, 106–112.

Andersen, E.C., Gerke, J.P., Shapiro, J.A., Crissman, J.R., Ghosh, R., Bloom, J.S., Félix, M.A., and Kruglyak, L. (2012). Chromosome-scale selective sweeps shape Caenorhabditis elegans genomic diversity. Nat. Genet. *44*, 285–290.

Anderson, J.L., Albergotti, L., Ellebracht, B., Huey, R.B., and Phillips, P.C. (2011). Does thermoregulatory behavior maximize reproductive fitness of natural isolates of Caenorhabditis elegans? BMC Evol. Biol. *11*, 157.

Ann, K., Kowalchyk, J., Loyet, K., and Martin, T. (1997). Novel Ca2+-binding protein (CAPS) related to UNC-31 required for Ca2+-activated exocytosis. J Biol Chem. *272*, 19637–19640.

Ardiel, E.L., and Rankin, C.H. (2010). An elegant mind: learning and memory in *Caenorhabditis*

elegans. Learn. Mem.

Ben Arous, J., Laffont, S., and Chatenay, D. (2009). Molecular and sensory basis of a food related two-state behavior in C. elegans. PLoS One *4*, 7584.

Avery, L. (2003). Food transport in the C. elegans pharynx. J. Exp. Biol. *206*, 2441–2457.

Avery, L., and Horvitz, H.R. (1990). Effects of starvation and neuroactive drugs on feeding in Caenorhabditis elegans. J. Exp. Zool. *253*, 263–270.

Avery, L., and Horvitzt, H.R. (1989). Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of C. elegans. Neuron *3*, 473–485.

Avery, L., and You, Y.J. (2012). C. Elegans feeding. WormBook 1–23.

Avery, L., Bargmann, C.I., and Horvitz, H.R. (1993). The Caenorhabditis elegans unc-31 gene affects multiple nervous system- controlled functions. Genetics.

Axäng, C., Rauthan, M., Hall, D.H., and Pilon, M. (2008). Developmental genetics of the C. elegans pharyngeal neurons NSML and NSMR. BMC Dev. Biol. *8*, 38.

Bargmann, C. (2006). Chemosensation in C. elegans. WormBook 1–29.

Bargmann, C.I., and Horvitz, H.R. (1991). Chemosensory neurons with overlapping functions direct chemotaxis to multiple chemicals in C. elegans. Neuron *7*, 729–742.

Bargmann, C.I., Hartwieg, E., and Horvitz, H.R. (1993). Odorant-selective genes and neurons mediate olfaction in C. elegans. Cell *74*, 515–527.

Barrière, A., and Félix, M.A. (2005). High local genetic diversity and low outcrossing rate in Caenorhabditis elegans natural populations. Curr. Biol. *15*, 1176–1184.

Barrière, A., and Félix, M.A. (2007). Temporal dynamics and linkage disequilibrium in natural Caenorhabditis elegans populations. Genetics *176*, 999–1011.

Baugh, L.R. (2013). To grow or not to grow: Nutritional control of development during Caenorhabditis elegans L1 Arrest. Genetics *194*, 539–555.

Bernhard, N., and Van Der Kooy, D. (2000). A behavioral and genetic dissection of two forms of olfactory plasticity in Caenorhabditis elegans: Adaptation and habituation. Learn. Mem. *7*, 199–212.

Berthoud, H.R., and Neuhuber, W.L. (2000). Functional and chemical anatomy of the afferent vagal system. In Autonomic Neuroscience: Basic and Clinical, pp. 1–17.

Betz, A., Okamoto, M., Benseler, F., and Brose, N. (1997). Direct interaction of the rat unc-13 homologue munc13-1 with the N terminus of syntaxin. J. Biol. Chem. *272*, 2520–2526.

Bhatla, N., and Horvitz, H.R. (2015). Light and Hydrogen Peroxide Inhibit C.elegans Feeding through Gustatory Receptor Orthologs and Pharyngeal Neurons. Neuron *85*, 804–818.

Bielefeldt, K., and Davis, B.M. (2007). Differential effects of ASIC3 and TRPV1 deletion on gastroesophageal sensation in mice. AJP Gastrointest. Liver Physiol. *294*, G130–G138.

Boakes, R.A., Westbrook, R.F., Elliott, M., and Swinbourne, A.L. (1997). Context dependency of conditioned aversions to water and sweet tastes. J. Exp. Psychol. Anim. Behav. Process. *23*, 56–67.

Bono, M. de, and Villu Maricq, A. (2005). Neuronal substrates of complex behaviors in C. elegans. Annu. Rev. Neurosci. *28*, 451–501.

Brenner, S. (2003). Nature's gift to science (Nobel lecture). ChemBioChem *4*, 683–687.

Bretscher, A.J., Busch, K.E., and de Bono, M. (2008). A carbon dioxide avoidance behavior is integrated with responses to ambient oxygen and food in Caenorhabditis elegans. Proc. Natl. Acad. Sci. *105*, 8044–8049.

Bretscher, A.J., Kodama-Namba, E., Busch, K.E., Murphy, R.J., Soltesz, Z., Laurent, P., and de Bono, M. (2011). Temperature, oxygen, and salt-sensing neurons in C. elegans are carbon dioxide sensors that control avoidance behavior. Neuron *69*, 1099–1113.

Brierley, S.M. (2010). Molecular basis of mechanosensitivity. Auton. Neurosci. *153*, 58–68.

Brockie, P. (2006). Ionotropic glutamate receptors: genetics, behavior and electrophysiology. WormBook.

Bumbarger, D.J., Riebesell, M., Rödelsperger, C., and Sommer, R.J. (2013). System-wide rewiring underlies behavioral differences in predatory and bacterial-feeding nematodes. Cell *152*, 109–119.

Cardoso, J.C.R., Félix, R.C., Fonseca, V.G., and Power, D.M. (2012). Feeding and the rhodopsin family G-protein coupled receptors in nematodes and arthropods. Front. Endocrinol. (Lausanne). *3*, 157.

Carmona, S., Hoekzema, E., Castellanos, F.X., García-García, D., Lage-Castellanos, A., Van Dijk, K.R.A., Navas-Sánchez, F.J., Martínez, K., Desco, M., and Sepulcre, J. (2015). Sensation-to-cognition cortical streams in attention-deficit/hyperactivity disorder. Hum. Brain Mapp. *36*, 2544–2557.

Chalasani, S.H., Chronis, N., Tsunozaki, M., Gray, J.M., Ramot, D., Goodman, M.B., and Bargmann, C.I. (2007). Dissecting a circuit for olfactory behaviour in Caenorhabditis elegans. Nature *450*, 63–70.

Chang, A.J., Chronis, N., Karow, D.S., Marletta, M.A., and Bargmann, C.I. (2006). A distributed chemosensory circuit for oxygen preference in C. elegans. PLoS Biol. *4*, 1588–1602.

Chase, D.L., Pepper, J.S., and Koelle, M.R. (2004). Mechanism of extrasynaptic dopamine signaling in Caenorhabditis elegans. Nat. Neurosci. *7*, 1096–1103.

Chatzigeorgiou, M., and Schafer, W.R. (2011). Lateral Facilitation between Primary Mechanosensory Neurons Controls Nose Touch Perception in C. elegans. Neuron *70*, 299–309.

Chen, Y.A., and Scheller, R.H. (2001). Snare-mediated membrane fusion. Nat. Rev. Mol. Cell Biol. *2*, 98–106.

Cheung, B.H.H., Arellano-Carbajal, F., Rybicki, I., and De Bono, M. (2004). Soluble guanylate cyclases act in neurons exposed to the body fluid to promote C. elegans aggregation behavior. Curr. Biol. *14*, 1105–1111.

Cheung, B.H.H., Cohen, M., Rogers, C., Albayram, O., and De Bono, M. (2005). Experience-dependent modulation of C. elegans behavior by ambient oxygen. Curr. Biol. *15*, 905–917.

Chiba, C.M., and Rankin, C.H. (1990). A developmental analysis of spontaneous and reflexive reversals in the nematode Caenorhabditis elegans. J. Neurobiol. *21*, 543–554.

Clince, M., Connolly, L., and Nolan, C. (2016). Comparing and Exploring the Sensory Processing Patterns of Higher Education Students With Attention Deficit Hyperactivity Disorder and Autism Spectrum Disorder. Am. J. Occup. Ther. *70*, p1–p9.

Cohen, N., and Sanders, T. (2014). Nematode locomotion: Dissecting the neuronal-environmental loop. Curr. Opin. Neurobiol. *25*, 99–106.

Colbert, H.A., and Bargmann, C.I. (1995). Odorant-specific adaptation pathways generate

olfactory plasticity in C. elegans. Neuron *14*, 803–812.

Colbert, H.A., and Bargmann, C.I. (1997). Environmental signals modulate olfactory acuity, discrimination, and memory in Caenorhabditis elegans. Learn. Mem. *4*, 179–191.

Collet, J., Spike, C.A., Lundquist, E.A., Shaw, J.E., and Herman, R.K. (1998). Analysis of osm-6, a gene that affects sensory cilium structure and sensory neuron function in Caenorhabditis elegans. Genetics *148*, 187–200.

Corsi, A.K., Wightman, B., and Chalfie, M. (2015). A Transparent window into biology: A primer on Caenorhabditis elegans (June 18, 2015), WormBook, ed. The C. elegans Research Community. WormBook.

Couillault, C., Pujol, N., Reboul, J., Sabatier, L., Guichou, J.F., Kohara, Y., and Ewbank, J.J. (2004). TLR-independent control of innate immunity in Caenorhabditis elegans by the TIR domain adaptor protein TIR-1, an ortholog of human SARM. Nat. Immunol. *5*, 488–494.

Culotti, J.G., and Russell, R.L. (1978). Osmotic avoidance defective mutants of the nematode Caenorhabditis elegans. Genetics *90*, 243–256.

Cunningham, K.A., Hua, Z., Srinivasan, S., Liu, J., Lee, B.H., Edwards, R.H., and Ashrafi, K. (2012). AMP-activated kinase links serotonergic signaling to glutamate release for regulation of feeding behavior in C. Elegans. Cell Metab. *16*, 113–121.

Dallière, N., Holden-Dye, L., O'Connor, V., and Walker, R.J. (2015). Delineation of a gut brain axis that regulates context-dependent feeding behaviour of the nematode Caenorhabditis elegans. Centre for Biological Sciences, Faculty of Natural and Environmental Sciences, University of Southampton, Southampton , UK, SO17 1BJ.

Dallière, N., Bhatla, N., Luedtke, Z., Ma, D.K., Woolman, J., Walker, R.J., Holden-Dye, L., and O'Connor, V. (2016). Multiple excitatory and inhibitory neural signals converge to fine-tune Caenorhabditis elegans feeding to food availability. FASEB J. *30*, 836–848.

Dallière, N., Holden-Dye, L., Dillon, J., O'Connor, V., and Walker, R.J. (2017). Caenorhabditis elegans Feeding Behaviors.

Dent, J.A., Davis, M.W., and Avery, L. (1997). avr-15 encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in Caenorhabditis elegans. EMBO J. *16*, 5867–5879.

Dent, J.A., Smith, M.M., Vassilatis, D.K., and Avery, L. (2000). The genetics of ivermectin

resistance in Caenorhabditis elegans. Proc. Natl. Acad. Sci. *97*, 2674–2679.

Dockray, G.J., and Burdyga, G. (2011). Plasticity in vagal afferent neurones during feeding and fasting: Mechanisms and significance. Acta Physiol. *201*, 313–321.

Donohoe, D.R., Jarvis, R.A., Weeks, K., Aamodt, E.J., and Dwyer, D.S. (2009). Behavioral adaptation in C. elegans produced by antipsychotic drugs requires serotonin and is associated with calcium signaling and calcineurin inhibition. Neurosci. Res. *64*, 280–289.

Dusenbery, D.B. (1974). Analysis of chemotaxis in the nematode Caenorhabditis elegans by countercurrent separation. J. Exp. Zool. *188*, 41–47.

Edwards, S.L., Charlie, N.K., Milfort, M.C., Brown, B.S., Gravlin, C.N., Knecht, J.E., and Miller, K.G. (2008). A novel molecular solution for ultraviolet light detection in Caenorhabditis elegans. PLoS Biol. *6*, 1715–1729.

Elefteriou, A., Babatsikou, F., Rachiotis, G., Polikandrioti, M., and Tsavourelou, A. (2015). Occupation as a risk factor for carpal tunnel syndrome. Rev. Clin. Pharmacol. Pharmacokinet. Int. Ed. *29*, 49–56.

Epstein, H.F. (1990). Genetic analysis of myosin assembly in Caenorhabditis elegans. Mol. Neurobiol. *4*, 1–25.

Fang-Yen, C., Avery, L., and Samuel, A.D.T. (2009). Two size-selective mechanisms specifically trap bacteria-sized food particles in Caenorhabditis elegans. Proc. Natl. Acad. Sci. U. S. A. *106*, 20093–20096.

Fares, H., and Greenwald, I. (2001). Genetic analysis of endocytosis in caenorhabditis elegans: Coelomocyte uptake defective mutants. Genetics *159*, 133–145.

Félix, M.A., and Duveau, F. (2012). Population dynamics and habitat sharing of natural populations of Caenorhabditis elegans and C. briggsae. BMC Biol. *10*, 59.

Fendt, M., and Fanselow, M.S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. Neurosci. Biobehav. Rev. *23*, 743–760.

Flavell, S.W., Pokala, N., Macosko, E.Z., Albrecht, D.R., Larsch, J., and Bargmann, C.I. (2013). Serotonin and the neuropeptide PDF initiate and extend opposing behavioral states in C. Elegans. Cell *154*, 1023–1035.

Franks, C.J., Holden-Dye, L., Bull, K., Luedtke, S., and Walker, R.J. (2006). Anatomy, physiology

and pharmacology of Caenorhabditis elegans pharynx: A model to define gene function in a simple neural system. Invertebr. Neurosci. *6*, 105–122.

Frézal, L., and Félix, M.A. (2015). C. elegans outside the Petri dish. Elife *4*.

Fuller, R.S., Brake, A.J., and Thorner, J. (1989). Intracellular targeting and structural conservation of a prohormone-processing endoprotease. Science (80-.). *246*, 482–486.

Ghosh, D.D., Nitabach, M.N., Zhang, Y., and Harris, G. (2017). Multisensory integration in C. elegans. Curr. Opin. Neurobiol. *43*, 110–118.

Gil-Campos, M., Aguilera, C.M., Cañete, R., and Gil, A. (2006). Ghrelin: a hormone regulating food intake and energy homeostasis. Br. J. Nutr. *96*, 201.

Gluck, M.A., Mercado, E., and Myers, C.E. (2007). Learning and Memory: From Brain to Behavior.

Golden, J.W., and Riddle, D.L. (1982). A pheromone influences larval development in the nematode Caenorhabditis elegans. Science *218*, 578–580.

Golden, J.W., and Riddle, D.L. (1984). A pheromone-induced developmental switch in Caenorhabditis elegans: Temperature-sensitive mutants reveal a wild-type temperature-dependent process. Proc. Natl. Acad. Sci. *81*, 819–823.

Goodman, M.B., and Sengupta, P. (2017). The extraordinary AFD thermosensor of C. elegans. Pflugers Arch. Eur. J. Physiol. 1–11.

Gray, J.M., Karow, D.S., Lu, H., Chang, A.J., Chang, J.S., Ellis, R.E., Marletta, M.A., and Bargmann, C.I. (2004). Oxygen sensation and social feeding mediated by a C. elegans guanylate cyclase homologue. Nature *430*, 317–322.

Gray, J.M., Hill, J.J., and Bargmann, C.I. (2005). A circuit for navigation in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. *102*, 3184–3191.

Green, S.A., Hernandez, L., Bookheimer, S.Y., and Dapretto, M. (2017). Reduced modulation of thalamocortical connectivity during exposure to sensory stimuli in ASD. Autism Res. *10*, 801–809.

Greer, E.R., Pérez, C.L., Van Gilst, M.R., Lee, B.H., and Ashrafi, K. (2008). Neural and Molecular Dissection of a C. elegans Sensory Circuit that Regulates Fat and Feeding. Cell Metab. *8*, 118–131.

Grewal, P., and Wright, D. (1992). Migration of Caenorhabditis elegans larvae towards bacteria and the nature of the bacterial stimulus. Fundam. Appl. Nematol. *15*, 159–166.

Grishanin, R.N., Kowalchyk, J.A., Klenchin, V.A., Ann, K., Earles, C.A., Chapman, E.R., Gerona, R.R.L., and Martin, T.F.J. (2004). CAPS acts at a prefusion step in dense-core vesicle exocytosis as a PIP2binding protein. Neuron *43*, 551–562.

Gruninger, T.R., Gualberto, D.G., and Garcia, L.R. (2008). Sensory perception of food and insulin-like signals influence seizure susceptibility. PLoS Genet. *4*, e1000117.

Hart, A.C., Sims, S., and Kaplan, J.M. (1995). Synaptic code for sensory modalities revealed by C. Elegans GLR-1 glutamate receptor. Nature *378*, 82–85.

Haycraft, C.J., Schafer, J.C., Zhang, Q., Taulman, P.D., and Yoder, B.K. (2003). Identification of CHE-13, a novel intraflagellar transport protein required for cilia formation. Exp. Cell Res. *284*, 251–263.

He, L., Vasiliou, K., and Nebert, D.W. (2009). Analysis and update of the human solute carrier (SLC) gene superfamily. Hum. Genomics *3*, 195.

Hedgecock, E.M., and Russell, R.L. (1975). Normal and mutant thermotaxis in the nematode Caenorhabditis elegans. Proc. Natl. Acad. Sci. U. S. A. *72*, 4061–4065.

Hibbard, C.M., Dainty, R.H., Edwards, R.A., and Hibbard, C.M. (1985). Time course of volatile compound formation during refrigerated storage of naturally contaminated beef in air. J. Appl. Bacteriol. *59*, 303–309.

Hilliard, M.A., Bargmann, C.I., and Bazzicalupo, P. (2002). C. elegans responds to chemical repellents by integrating sensory inputs from the head and the tail. Curr. Biol. *12*, 730–734.

Hills, T. (2004). Dopamine and Glutamate Control Area-Restricted Search Behavior in Caenorhabditis elegans. J. Neurosci. *24*, 1217–1225.

Hobert, O. (2003). Behavioral plasticity in C. elegans: Paradigms, circuits, genes. J. Neurobiol. *54*, 203–223.

Hobert, O. (2013). The neuronal genome of Caenorhabditis elegans. WormBook 1–106.

Holden-Dye, L., and Walker, R.J. (2013). The Roles of Neuropeptides in Caenorhabditis elegans Including their Importance in the Regulation of Feeding and Metabolism. Protein Pept.

Lett. *20*, 636–646.

Horvitz, H.R., Chalfie, M., Trent, C., Sulston, J.E., and Evans, P.D. (1982). Serotonin and octopamine in the nematode Caenorhabditis elegans. Science (80-.). *216*, 1012–1014.

Hosoda, H., Kojima, M., and Kangawa, K. (2006). Review Biological , Physiological , and Pharmacological Aspects of Ghrelin. Transl. Res. *410*, 398–410.

Hu, P.J. (2007). Dauer. WormBook 1–19.

Hukema, R.K., Rademakers, S., Dekkers, M.P.J., Burghoorn, J., and Jansen, G. (2006). Antagonistic sensory cues generate gustatory plasticity in Caenorhabditis elegans. EMBO J. *25*, 312–322.

Hukema, R.K., Rademakers, S., and Jansen, G. (2008). Gustatory plasticity in C. elegans involves integration of negative cues and NaCl taste mediated by serotonin, dopamine, and glutamate. Learn. Mem. *15*, 829–836.

Humphrey, J.A., Hamming, K.S., Thacker, C.M.M., Scott, R.L., Sedensky, M.M.M., Snutch, T.P., Morgan, P.G.G., and Nash, H.A. (2007). A Putative Cation Channel and Its Novel Regulator: Cross-Species Conservation of Effects on General Anesthesia. Curr. Biol. *17*, 624–629.

Husson, S.J., Clynen, E., Baggerman, G., Janssen, T., and Schoofs, L. (2006). Defective processing of neuropeptide precursors in Caenorhabditis elegans lacking proprotein convertase 2 (KPC-2/EGL-3): Mutant analysis by mass spectrometry. J. Neurochem. *98*, 1999-2012.

Iino, Y., and Yoshida, K. (2009). Parallel Use of Two Behavioral Mechanisms for Chemotaxis in Caenorhabditis elegans. J. Neurosci. *29*, 5370–5380.

Iwanir, S., Brown, A.S., Nagy, S., Najjar, D., Kazakov, A., Lee, K.S., Zaslaver, A., Levine, E., and Biron, D. (2016). Serotonin promotes exploitation in complex environments by accelerating decision-making. BMC Biol. *14*.

De Jager, J.M., Potgieter, A.B., and Van Den Berg, W.J. (1998). Framework for forecasting the extent and severity of drought in maize in the free state province of South Africa. Agric. Syst. *57*, 351–365.

Jockusch, W.J., Speidel, D., Sigler, A., Sørensen, J.B., Varoqueaux, F., Rhee, J.S., and Brose, N. (2007). CAPS-1 and CAPS-2 Are Essential Synaptic Vesicle Priming Proteins. Cell *131*,

796–808.

Johnson, T.E., Mitchell, D.H., Kline, S., Kemal, R., and Foy, J. (1984). Arresting development arrests aging in the nematode Caenorhabditis elegans. Mech. Ageing Dev. *28*, 23–40.

Jorgensen, E.M. (2005). Gaba. WormBook.

Kang, L., Gao, J., Schafer, W.R., Xie, Z., and Xu, X.Z.S. (2010). C. elegans TRP Family Protein TRP-4 Is a Pore-Forming Subunit of a Native Mechanotransduction Channel. Neuron *67*, 381–391.

Kaplan, J.M., and Horvitz, H.R. (1993). A dual mechanosensory and chemosensory neuron in Caenorhabditis elegans. Proc. Natl. Acad. Sci. *90*, 2227–2231.

Kauffman, A., Parsons, L., Stein, G., Wills, A., Kaletsky, R., and Murphy, C. (2011). C. elegans positive butanone learning, short-term, and long-term associative memory assays. J. Vis. Exp. 1–10.

Kaufman, A., Keinan, A., Meilijson, I., Kupiec, M., and Ruppin, E. (2005). Quantitative analysis of genetic and neuronal multi-perturbation experiments. PLoS Comput. Biol. *1*, 0500–0506.

Keane, J., and Avery, L. (2003). Mechanosensory inputs influence Caenorhabditis elegans pharyngeal activity via ivermectin sensitivity genes. Genetics *164*, 153–162.

Kindt, K.S., Quast, K.B., Giles, A.C., De, S., Hendrey, D., Nicastro, I., Rankin, C.H.H.C.H., and Schafer, W.R. (2007). Dopamine Mediates Context-Dependent Modulation of Sensory Plasticity in C. elegans. Neuron *55*, 662–676.

Kiontke, K.C., Félix, M.A., Ailion, M., Rockman, M. V., Braendle, C., Pénigault, J.B., and Fitch, D.H.A. (2011). A phylogeny and molecular barcodes for Caenorhabditis, with numerous new species from rotting fruits. BMC Evol. Biol. *11*.

Kiyama, Y., Miyahara, K., and Ohshima, Y. (2012). Active uptake of artificial particles in the nematode Caenorhabditis elegans. J. Exp. Biol. *215*, 1178–1183.

Klass, M.R. (1977). Aging in the nematode Caenorhabditis elegans: Major biological and environmental factors influencing life span. Mech. Ageing Dev. *6*, 413–429.

Komuniecki, R.W., Hobson, R.J., Rex, E.B., Hapiak, V.M., and Komuniecki, P.R. (2004). Biogenic amine receptors in parasitic nematodes: What can be learned from Caenorhabditis elegans? Mol. Biochem. Parasitol. *137*, 1–11.

Lee, D., Jung, S., Ryu, J., Ahnn, J., and Ha, I. (2008). Human vesicular glutamate transporters functionally complement EAT-4 in *C. elegans*. Mol. Cells *25*, 50–54.

Lee, H., Choi, M.K., Lee, D., Kim, H.S., Hwang, H., Kim, H., Park, S., Paik, Y.K., and Lee, J. (2012). Nictation, a dispersal behavior of the nematode Caenorhabditis elegans, is regulated by IL2 neurons. Nat. Neurosci. *15*, 107–112.

Lee, R.Y., Sawin, E.R., Chalfie, M., Horvitz, H.R., and Avery, L. (1999). EAT-4, a homolog of a mammalian sodium-dependent inorganic phosphate cotransporter, is necessary for glutamatergic neurotransmission in caenorhabditis elegans. J. Neurosci. *19*, 159–167.

Lemieux, G.A., Cunningham, K.A., Lin, L., Mayer, F., Werb, Z., and Ashrafi, K. (2015). Kynurenic acid is a nutritional cue that enables behavioral plasticity. Cell *160*, 119–131.

Li, C., and Kim, K. (2008). Neuropeptides. WormBook 1–36.

Li, C., and Kim, K. (2010). Neuropeptide Gene Families in Caenorhabditis elegans. In In Neuropeptide Systems as Targets for Parasite and Pest Control. Advances in Experimental Medicine and Biology, Vol 692., (Springer, Boston, MA), pp. 98–137.

Li, Q., and Liberles, S.D. (2015). Aversion and attraction through olfaction. Curr. Biol.

Li, C., Kim, K., and Nelson, L.S. (1999). FMRFamide-related neuropeptide gene family in Caenorhabditis elegans. Brain Res. *848*, 26–34.

Li, W., Kennedy, S.G., and Ruvkun, G. (2003). *daf-28* encodes a *C. elegans* insulin superfamily member that is regulated by environmental cues and acts in the DAF-2 signaling pathway. Genes Dev. *17*, 844–858.

Li, Z., Li, Y., Yi, Y., Huang, W., Yang, S., Niu, W., Zhang, L., Xu, Z., Qu, A., Wu, Z., et al. (2012). Dissecting a central flip-flop circuit that integrates contradictory sensory cues in C. elegans feeding regulation. Nat. Commun. *3*, 776.

Ludewig, A., and Schroeder, F. (2013). Ascaroside signalling in C.elegans.

Luo, L., Gabel, C. V, Ha, H.-I., Zhang, Y., and Samuel, A.D.T. (2008). Olfactory behavior of swimming C. elegans analyzed by measuring motile responses to temporal variations of odorants. J. Neurophysiol. *99*, 2617–2625.

Manita, S., Suzuki, T., Homma, C., Matsumoto, T., Odagawa, M., Yamada, K., Ota, K., Matsubara, C., Inutsuka, A., Sato, M., et al. (2015). A Top-Down Cortical Circuit for

Accurate Sensory Perception. Neuron *86*, 1304–1316.

Mano, I., Straud, S., and Driscoll, M. (2007). Caenorhabditis elegans glutamate transporters influence synaptic function and behavior at sites distant from the synapse. J. Biol. Chem. *282*, 34412–34419.

McKay, J.P., Raizen, D.M., Gottschalk, A., Schafer, W.R., and Avery, L. (2004). eat-2 and eat-18 are Required for Nicotinic Neurotransmission in the Caenorhabditis elegans Pharynx. Genetics *166*, 161–169.

Mellem, J.E., Brockie, P.J., Zheng, Y., Madsen, D.M., and Maricq, A. V. (2002). Decoding of polymodal sensory stimuli by postsynaptic glutamate receptors in C. elegans. Neuron *36*, 933–944.

Milward, K., Busch, K.E., Murphy, R.J., de Bono, M., and Olofsson, B. (2011). Neuronal and molecular substrates for optimal foraging in Caenorhabditis elegans. Proc. Natl. Acad. Sci. *108*, 20672–20677.

Morrison, G.E., Wen, J.Y., Runciman, S., and van der Kooy, D. (1999). Olfactory associative learning in Caenorhabditis elegans is impaired in lrn-1 and lrn-2 mutants. Behav. Neurosci. *113*, 358–367.

Mueller, K.L., Hoon, M.A., Erlenbach, I., Chandrashekar, J., Zuker, C.S., and Ryba, N.J.P. (2005). The receptors and coding logic for bitter taste. Nature *434*, 225–229.

Nathoo, A.N., Moeller, R.A., Westlund, B.A., and Hart, A.C. (2001). Identification of neuropeptide-like protein gene families in Caenorhabditis elegans and other species. Proc. Natl. Acad. Sci. *98*, 14000–14005.

Naumann, E.A., Fitzgerald, J.E., Dunn, T.W., Rihel, J., Sompolinsky, H., and Engert, F. (2016). From Whole-Brain Data to Functional Circuit Models: The Zebrafish Optomotor Response. Cell *167*, 947-960.e20.

Niacaris, T. (2003). Serotonin regulates repolarization of the C. elegans pharyngeal muscle. J. Exp. Biol. *206*, 223–231.

Nicolas Dallière (2015). Multiple excitatory and inhibitory neural signals converge to fine tune Caenorhabditis elegans pharyngeal behaviour to food availability.

Nonet, M.L., Staunton, J.E., Kilgard, M.P., Fergestad, T., Hartwieg, E., Horvitz, H.R., Jorgensen, E.M., and Meyer, B.J. (1997). Caenorhabditis elegans rab-3 mutant synapses exhibit

impaired function and are partially depleted of vesicles. J. Neurosci. *17*, 8061–8073.

Ortiz, C.O., Etchberger, J.F., Posy, S.L., Frøkjær-Jensen, C., Lockery, S., Honig, B., and Hobert, O. (2006). Searching for neuronal left/right asymmetry: Genomewide analysis of nematode receptor-type guanylyl cyclases. Genetics.

Page, A.J., Martin, C.M., and Blackshaw, L.A. (2002). Vagal Mechanoreceptors and Chemoreceptors in Mouse Stomach and Esophagus. J. Neurophysiol. *87*, 2095–2103.

Page, A.J., Brierley, S.M., Martin, C.M., Price, M.P., Symonds, E., Butler, R., Wemmie, J.A., and Blackshaw, L.A. (2005). Different contributions of ASIC channels 1a, 2, and 3 in gastrointestinal mechanosensory function. Gut *54*, 1408–1415.

Papaioannou, S., Marsden, D., Franks, C.J., Walker, R.J., and Holden-Dye, L. (2005). Role of a FMRFamide-like family of neuropeptides in the pharyngeal nervous system of Caenorhabditis elegans. J. Neurobiol. *65*, 304–319.

Perkins, L.A., Hedgecock, E.M., Thomson, J.N., and Culotti, J.G. (1986). Mutant sensory cilia in the nematode Caenorhabditis elegans. Dev. Biol. *117*, 456–487.

Phelan, P., Bacon, J.P., Davies, J.A., Stebbings, L.A., and Todman, M.G. (1998). Innexins: A family of invertebrate gap-junction proteins. Trends Genet. *14*, 348–349.

Phillips, R.J., and Powley, T.L. (2000). Tension and stretch receptors in gastrointestinal smooth muscle: Re-evaluating vagal mechanoreceptor electrophysiology. Brain Res. Rev.

Pierce-Shimomura, J.T., Morse, T.M., and Lockery, S.R. (1999). The fundamental role of pirouettes in Caenorhabditis elegans chemotaxis. J. Neurosci. *19*, 9557–9569.

Pierce-Shimomura, J.T., Faumont, S., Gaston, M.R., Pearson, B.J., and Lockery, S.R. (2001). The homeobox gene lim-6 is required for distinct chemosensory representations in C. elegans. Nature *410*, 694–698.

Pierce, S.B., Costa, M., Wisotzkey, R., Devadhar, S., Homburger, S.A., Buchman, A.R., Ferguson, K.C., Heller, J., Platt, D.M., Pasquinelli, A.A., et al. (2001). Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse C. elegans insulin gene family. Genes Dev. *15*, 672–686.

Piggott, B.J., Liu, J., Feng, Z., Wescott, S.A., and Xu, X.Z.S. (2011). The neural circuits and synaptic mechanisms underlying motor initiation in C. elegans. Cell *147*, 922–933.

Raizen, D.M., and Avery, L. (1994). Electrical activity and behavior in the pharynx of caenorhabditis elegans. Neuron *12*, 483–495.

Raizen, D.M., Lee, R.Y.N., and Avery, L. (1995). Interacting genes required for pharyngeal excitation by motor neuron MC in Caenorhabditis elegans. Genetics *141*, 1365–1382.

Rand, J.B., and Russell, R.L. (1984). Choline acetyltransferase-deficient mutants of the nematode Caenorhabditis elegans. Genetics.

Rani, P., and Srivastava, R. (2018). Integration of metal organic framework with zeolite: A highly sustainable composite catalyst for the synthesis of γ-valerolactone and coumarins. Sustain. Energy Fuels *9*, e85874.

Remy, J.J., and Hobert, O. (2005). Neuroscience: An interneuronal chemoreceptor required for olfactory imprinting in C. elegans. Science (80-.). *309*, 787–790.

Ren, P., Lim, C.-S., Johnsen, R., Albert, P.S., Pilgrim, D., and L. Riddle, D. (1996). Control of C. elegans Larval Development by Neuronal Expression of a TGF-beta Homolog. Science (80-.). *274*, 1389–1391.

Revusky, S., and Parker, L.A. (1976). Aversions to unflavored water and cup drinking produced by delayed sickness. J. Exp. Psychol. Anim. Behav. Process. *2*, 342–353.

Richmond, J. (2005). Synaptic function. WormBook 1–14.

Richmond, J.E., Weimer, R.M., and Jorgensen, E.M. (2001). An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. Nature *412*, 338–341.

Roeder, T., Seifert, M., Kähler, C., and Gewecke, M. (2003). Tyramine and octopamine: Antagonistic modulators of behavior and metabolism. Arch. Insect Biochem. Physiol. *54*, 1–13.

Rogers, C., Persson, A., Cheung, B., and de Bono, M. (2006). Behavioral Motifs and Neural Pathways Coordinating O2 Responses and Aggregation in C. elegans. Curr. Biol. *16*, 649–659.

Rose, A.M., and Baillie, D.L. (1980). Genetic organization of the region around UNC-15 (I), a gene affecting paramyosin in Caenorhabditis elegans. Genetics *96*, 639–648.

Saeki, S., Yamamoto, M., and Iino, Y. (2001). Plasticity of chemotaxis revealed by paired presentation of a chemoattractant and starvation in the nematode Caenorhabditis

elegans. J. Exp. Biol. *204*, 1757–1764.

Salio, C., Lossi, L., Ferrini, F., and Merighi, A. (2006). Neuropeptides as synaptic transmitters. Cell Tissue Res. *326*, 583–598.

Sambongi, Y., Nagae, T., Liu, Y., Yoshimizu, T., Takeda, K., Wada, Y., and Futai, M. (1999). Sensing of cadmium and copper ions by externally exposed ADL, ASE, and ASH neurons elicits avoidance response in Caenorhabditis elegans. Neuroreport *10*, 753–757.

Sánchez-Alcañiz, J.A., and Benton, R. (2017). Multisensory neural integration of chemical and mechanical signals. BioEssays *39*.

Sanyal, S., Wintle, R.F., Kindt, K.S., Nuttley, W.M., Arvan, R., Fitzmaurice, P., Bigras, E., Merz, D.C., Hébert, T.E., van der Kooy, D., et al. (2004). Dopamine modulates the plasticity of mechanosensory responses in Caenorhabditis elegans. EMBO J. *23*, 473–482.

Sarter, M., Parikh, V., and Howe, W.M. (2009). Phasic acetylcholine release and the volume transmission hypothesis: Time to move on. Nat. Rev. Neurosci. *10*, 383–390.

Sasakura, H., Tsukada, Y., Takagi, S., and Mori, I. (2013). Japanese studies on neural circuits and behavior of Caenorhabditis elegans. Front. Neural Circuits *28*, 221-7.

Sawin, E.R., Ranganathan, R., and Horvitz, H.R. (2000). C. elegans Locomotory Rate Is Modulated by the Environment through a Dopaminergic Pathway and by Experience through a Serotonergic Pathway. Neuron *26*, 619–631.

Schackwitz, W.S., Inoue, T., and Thomas, J.H. (1996). Chemosensory neurons function in parallel to mediate a pheromone response in C. elegans. Neuron *17*, 719–728.

Schaefer, N., Rotermund, C., Blumrich, E.M., Lourenco, M. V., Joshi, P., Hegemann, R.U., Jamwal, S., Ali, N., García Romero, E.M., Sharma, S., et al. (2017). The malleable brain: plasticity of neural circuits and behavior – a review from students to students. J. Neurochem. *142*, 790–811.

Schafer, W.R., Sanchez, B.M., and Kenyon, C.J. (1996). Genes affecting sensitivity to serotonin in Caenorhabditis elegans. Genetics *143*, 1219–1230.

Schofield, P.R., Darlison, M.G., Fujita, N., Burt, D.R., Stephenson, F.A., Rodriguez, H., Rhee, L.M., Ramachandran, J., Reale, V., Glencorse, T.A., et al. (1987). Sequence and functional expression of the GABAA receptor shows a ligand-gated receptor super-family. Nature *328*, 221–227.

Scholz, M., Lynch, D.J., Lee, K.S., Levine, E., and Biron, D. (2016). A scalable method for automatically measuring pharyngeal pumping in C. elegans. J. Neurosci. Methods *274*, 172–178.

Schulenburg, H., and Félix, M.A. (2017). The natural biotic environment of Caenorhabditis elegans. Genetics *206*, 55–86.

Schwartz, M.S., Benci, J.L., Selote, D.S., Sharma, A.K., Chen, A.G.Y., Dang, H., Fares, H., and Vatamaniuk, O.K. (2010). Detoxification of multiple heavy metals by a half-molecule ABC transporter, HMT-1, and coelomocytes of Caenorhabditis elegans. PLoS One *5*, e9564.

Sengupta, P. (2007). Generation and modulation of chemosensory behaviors in C. elegans. Pflugers Arch. Eur. J. Physiol. *454*, 721–734.

Sengupta, P. (2013). The belly rules the nose: Feeding state-dependent modulation of peripheral chemosensory responses. Curr. Opin. Neurobiol. *23*, 68–75.

Serrano-Saiz, E., Poole, R.J., Felton, T., Zhang, F., De La Cruz, E.D., and Hobert, O. (2013). XModular control of glutamatergic neuronal identity in C. elegans by distinct homeodomain proteins. Cell *155*, 659–673.

Seymour, M.K., Wright, K.A., and Doncaster, C.C. (1983). The action of the anterior feeding apparatus of Caenorhabditis elegans (Nematoda: Rhabditida). J. Zool. *201*, 527–539.

Sieburth, D., Madison, J.M., and Kaplan, J.M. (2007). PKC-1 regulates secretion of neuropeptides. Nat. Neurosci. *10*, 49–57.

Small, D.M., Jones-Gotman, M., and Dagher, A. (2003). Feeding-induced dopamine release in dorsal striatum correlates with meal pleasantness ratings in healthy human volunteers. Neuroimage *19*, 1709–1715.

Song, B. -m., and Avery, L. (2012). Serotonin Activates Overall Feeding by Activating Two Separate Neural Pathways in Caenorhabditis elegans. J. Neurosci.

Song, B.M., Faumont, S., Lockery, S., and Avery, L. (2013). Recognition of familiar food activates feeding via an endocrine serotonin signal in Caenorhabditis elegans. Elife *2*, :e00329.

Srinivasan, S., Sadegh, L., Elle, I.C., Christensen, A.G.L., Faergeman, N.J., and Ashrafi, K. (2008). Serotonin Regulates C. elegans Fat and Feeding through Independent Molecular Mechanisms. Cell Metab. *7*, 533–544.

Starich, T.A., Lee, R.Y.N., Panzarella, C., Avery, L., and Shaw, J.E. (1996). eat-5 and unc-7 represent a multigene family in Caenorhabditis elegans involved in cell-cell coupling. J. Cell Biol. *134*, 537–548.

Stein, G.M., and Murphy, C.T. (2014). C. elegans Positive Olfactory Associative Memory is a Molecularly Conserved Behavioral Paradigm. Neurobiol. Learn. Mem. *115*, 86–94.

Sugiura, M., Fuke, S., Suo, S., Sasagawa, N., Van Tol, H.H.M., and Ishiura, S. (2005). Characterization of a novel D2-like dopamine receptor with a truncated splice variant and a D1-like dopamine receptor unique to invertebrates from Caenorhabditis elegans. J. Neurochem. *94*, 1146–1157.

Sulston, J.E., and Horvitz, H.R. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev. Biol. *56*, 110–156.

Sulston, J., Dew, M., and Brenner, S. (1975). Dopaminergic neurons in the nematode Caenorhabditis elegans. J. Comp. Neurol. *163*, 215–226.

Sulston, J.E., Albertson, D.G., and Thomson, J.N. (1980). The Caenorhabditis elegans male: Postembryonic development of nongonadal structures. Dev. Biol. *78*, 542–576.

Sulston, J.E., Schierenberg, E., White, J.G., and Thomson, J.N. (1983). The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev. Biol. *100*, 64–119.

Suo, S., Culotti, J.G., and Van Tol, H.H.M. (2009). Dopamine counteracts octopamine signalling in a neural circuit mediating food response in C. elegans. EMBO J. *28*, 2437–2448.

Sutton, R.B., Fasshauer, D., Jahn, R., and Brunger, A.T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. Nature *395*, 347–353.

Syntichaki, P., and Tavernarakis, N. (2004). Genetic models of mechanotransduction: the nematode Caenorhabditis elegans. Physiol. Rev. *84*, 1097–1153.

Szczypka, M.S., Kwok, K., Brot, M.D., Marck, B.T., Matsumoto, A.M., Donahue, B.A., and Palmiter, R.D. (2001). Dopamine production in the caudate putamen restores feeding in dopamine-deficient mice. Neuron *30*, 819–828.

Sze, J.Y., Victor, M., Loer, C., Shi, Y., and Ruvkun, G. (2000). Food and metabolic signalling defects in a Caenorhabditis elegans serotonin-synthesis mutant. Nature *403*, 560–564.

Takarae, Y., Sablich, S.R., White, S.P., and Sweeney, J.A. (2016). Neurophysiological

hyperresponsivity to sensory input in autism spectrum disorders. J. Neurodev. Disord. *8*.

Taniguchi, G., Uozumi, T., Kiriyama, K., Kamizaki, T., and Hirotsu, T. (2014). Screening of Odor-Receptor Pairs in Caenorhabditis elegans Reveals Different Receptors for High and Low Odor Concentrations. Sci. Signal. *7*, ra39.

Tattersall, G.J., Sinclair, B.J., Withers, P.C., Fields, P.A., Seebacher, F., Cooper, C.E., and Maloney, S.K. (2012). Coping with thermal challenges: Physiological adaptations to environmental temperatures. Compr. Physiol. *2*, 2151–2202.

Tian, L., Hires, S.A., Mao, T., Huber, D., Chiappe, M.E., Chalasani, S.H., Petreanu, L., Akerboom, J., McKinney, S.A., Schreiter, E.R., et al. (2009). Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat. Methods *6*, 875–881.

Tobin, D.M., and Bargmann, C.I. (2004). Invertebrate nociception: Behaviors, neurons and molecules. J. Neurobiol. *61*, 161–174.

Tomioka, M., Adachi, T., Suzuki, H., Kunitomo, H., Schafer, W.R., Iino, Y., Tomioka, M., Adachi, T., Suzuki, H., Kunitomo, H., Schafer, W. R., & Iino, Y., Tomioka, M., Adachi, T., Suzuki, H., et al. (2006). The Insulin/PI 3-Kinase Pathway Regulates Salt Chemotaxis Learning in Caenorhabditis elegans. Neuron *51*, 613–625.

Torayama, I., Ishihara, T., and Katsura, I. (2007). Caenorhabditis elegans integrates the signals of butanone and food to enhance chemotaxis to butanone. J. Neurosci. *27*, 741–750.

Troemel, E.R., Kimmel, B.E., and Bargmann, C.I. (1997). Reprogramming chemotaxis responses: Sensory neurons define olfactory preferences in C. elegans. Cell *91*, 161–169.

Tsalik, E.L., Niacaris, T., Wenick, A.S., Pau, K., Avery, L., and Hobert, O. (2003). LIM homeobox gene-dependent expression of biogenic amine receptors in restricted regions of the C. elegans nervous system. Dev. Biol. *263*, 81–102.

Varshney, L.R., Chen, B.L., Paniagua, E., Hall, D.H., and Chklovskii, D.B. (2011). Structural properties of the Caenorhabditis elegans neuronal network. PLoS Comput. Biol. *7*, e1001066.

Van Voorhies, W.A., and Ward, S. (2000). Broad oxygen tolerance in the nematode Caenorhabditis elegans. J. Exp. Biol. *203*, 2467–2478.

Walker, D.S., Gower, N.J.D., Ly, S., Bradley, G.L., and Baylis, H.A. (2002). Regulated disruption of inositol 1,4,5-trisphosphate signaling in Caenorhabditis elegans reveals new functions

in feeding and embryogenesis. Mol. Biol. Cell *13*, 1329–1337.

Wang, D., Yu, Y., Li, Y., Wang, Y., and Wang, D. (2014). Dopamine receptors antagonistically regulate behavioral choice between conflicting alternatives in c. elegans e115985. PLoS One.

Ward, S. (1973). Chemotaxis by the nematode Caenorhabditis elegans: identification of attractants and analysis of the response by use of mutants. Proc. Natl. Acad. Sci. *70*, 817–821.

Wen, J.Y.M., Kumar, N., Morrison, G., Rambaldini, G., Runciman, S., Rousseau, J., Kooy, D. Van Der, Der, D. Van, and van der Kooy, D. (1997). Mutations That Prevent Associative Learning in C . elegans. Behav. Neurosci. *111*, 354–368.

Wes, P.D., and Bargmann, C.I. (2001). C. elegans odour discrimination requires asymmetric diversity in olfactory neurons. Nature *410*, 698–701.

White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1986). The Structure of the Nervous System of the Nematode Caenorhabditis elegans. Philos. Trans. R. Soc. B Biol. Sci. *314*, 1–340.

Wicks, S.R., de Vries, C.J., van Luenen, H.G.A.M., and Plasterk, R.H.A. (2000). CHE-3, a Cytosolic Dynein Heavy Chain, Is Required for Sensory Cilia Structure and Function in Caenorhabditis elegans. Dev. Biol. *221*, 295–307.

Wood, W.B. (1988). Introduction to C. elegans Biology. The Nematode Caenorhabditis Elegans 1–16.

Wu, Y.L., Liang, J.Q., and Liu, Z.Z. (2008). Treatment for bioactive surface of titanium implants. J. Clin. Rehabil. Tissue Eng. Res. *12*, 2693–2696.

Yeh, E., Ng, S., Zhang, M., Bouhours, M., Wang, Y., Wang, M., Hung, W., Aoyagi, K., Melnik-Martinez, K., Li, M., et al. (2008). A putative cation channel, NCA-1, and a novel protein, UNC-80, transmit neuronal activity in C. elegans. PLoS Biol. *6*, 0552–0567.

Yoshida, K., Hirotsu, T., Tagawa, T., Oda, S., Iino, Y., Ishihara, T., Wakabayashi, T., Iino, Y., and Ishihara, T. (2012). Odour concentration-dependent olfactory preference change in C. elegans. Nat. Commun. *3*, 739.

Yu, S., Avery, L., Baude, E., and Garbers, D.L. (1997). Guanylyl cyclase expression in specific sensory neurons: a new family of chemosensory receptors. Proc. Natl. Acad. Sci. *94*,

3384–3387.

Zechman, J.M., and Labows, J.N. (1985). Volatiles of Pseudomonas aeruginosa and related species by automated headspace concentration--gas chromatography. Can. J. Microbiol. *31*, 232–237.

Zetka, M., and Rose, A. (1995). The genetics of meiosis in Caenorhabditis elegans. Trends Genet. *11*, 27–31.

Zhang, S., Xu, M., Kamigaki, T., Do, J.P.H., Chang, W.C., Jenvay, S., Miyamichi, K., Luo, L., and Dan, Y. (2014). Long-range and local circuits for top-down modulation of visual cortex processing. Science (80-.). *345*, 660–665.

Zhang, Y., Lu, H., and Bargmann, C.I. (2005). Pathogenic bacteria induce aversive olfactory learning in Caenorhabditis elegans. *438*, 179–184.

Zhou, A., Martin, S., Lipkind, G., LaMendola, J., and Steiner, D.F. (1998). Regulatory roles of the P domain of the subtilisin-like prohormone convertases. J. Biol. Chem. *273*, 11107–11114.