The Expression and Function of Gustatory Receptors in the Honeybee (*Apis mellifera*)

By

Nicola Kay Simcock

A thesis submitted to the Faculty of Medical Sciences, Newcastle University

In fulfilment of the requirements for the degree of Doctor of Philosophy

September, 2014.

Newcastle University

Abstract

The ability to accurately assess the chemical environment is vital to the honeybee and as bees mature, their demands upon their chemical senses change. While honeybee olfaction has been intensively studied, the physiological mechanisms of bee gustation have only recently come to light. Robertson and Wanner (2006) were the first to identify honeybee gustatory receptors (Grs) and in comparison to other insect species, honeybees possess surprisingly few Gr genes (*Apis*: 10 Gr genes). The current project aimed to assess the expression of honeybee Gr genes and relate this to selection of the two most concentrated components of floral nectar; sugars and amino acids. The behavioural experiments demonstrated that bees are able to differentiate between the two major floral monosaccharides, with both newly emerged and forager bees exhibiting a slight fructose preference over glucose. Additionally, while no individual amino acid solution was preferred over sucrose alone, newly emerged bees were most willing to consume an eight amino acid mixture, probably due to its protein-resemblance, a major dietary component for young bees. Interestingly, the analysis of anatomical receptor gene expression discovered all 10 Gr genes in every gustatory appendage assayed (mouthparts, tarsi and antennae). All receptor genes were additionally expressed internally (gut and brain) indicating that, as in other insect species, honeybee Grs may play a role in nutrient sensing and feeding regulation. Some differential Gr gene expression was discovered between newly emerged and forager bees, indicating altered gustatory sensitivity with task differentiation. Finally, the expression of Gr genes in the forager brain were dependent on the nutritional status of the individual as well as nutritional experience. The current study demonstrated that AmGr3 may be acting as a nutrient sensor, with altered gene expression following starvation or changes in diet.

Acknowledgements

Firstly I would like to say a huge thank you to my primary supervisor Prof. Jeri Wright, the single most enthusiastic scientist I have ever met and the only person to ever hive-five when looking at graphs. Without her continued guidance, encouragement and support, this thesis would have provoked considerably fewer swear words and ultimately would not have been possible. I would also like to thank my second supervisor Dr. Luisa Wakeling for all her insight, reassurance and unparalleled molecular knowledge over the last 4 years.

I must acknowledge all of the bee lab members, past and present for creating such a unique and entertaining ‘work’ environment. Those challenging lab days don’t feel so bad when you’re surrounded by people that appear just as oblivious, but keep showing up anyway. A special thanks to Dan Stabler, Kerry Simcock and Eileen Power for their help with the HPLC.

I must thank Newcastle University Faculty of Medical sciences for the financial means to carry out this project. Additionally I am grateful to Prof. Hugh Robertson for providing the honeybee Gr gene sequences and helping to answer my many queries. Many thanks to Prof. Diane Ford, Dr. Claire Rind, Dr. Candy Rowe and Dr. Peter Simmons for their much appreciated help and insight.

Finally I would like to thank my family. Primarily my sister Kerry, who has begrudgingly played the role of lab-slave and whose help has been vital to completing this work. Also my grandma, who has dutifully provisioned me with every bee article from the paper over the last 3 years. Also my friends, who have kept me fed, entertained, distracted, motivated and adequately supplied with gin, when the need was great, thanks to all of you.

Table of contents

Thesis title i

Abstract Ii

Acknowledgements Iii

List of figures X

List of Tables Xii

Abbreviations xiii

Chapter 1.0. General introduction 1

1.1. The chemosensory system 1

1.2. Gustatory receptors and their expression 1

1.3. Insect taste ability 5

1.3.1. Sweet taste *6*

1.3.2. A pinch of salt *8*

1.3.3. Bitter taste *10*

1.3.4. Water and hygroreceptors *12*

1.3.5.Carbon dioxide and pheromone receptors *12*

1.3.6. Gustation: not just gustatory receptors *13*

1.4. Concentration dependency 14

1.5. What about protein? 15

1.5.1 Amino acids: detection and response *15*

1.5.2. Amino acid receptor and sensors *17*

1.6. Chemoreceptor plasticity and it's evolutionary significance 18

1.7. Conclusions and future work 21

1.8. General aims of thesis 21

1.8.1. Psychophysics of taste and dietary selection *22*

1.8.2. Gustatory receptor location and life cycle expression ***23***

1.8.3. The importance of bee nutrition on the expression of the Grs ***23***

2.0. Chapter 2.0. Methods **25**

2.1. Introduction **25**

2.2. Honeybee capture and restraint **25**

2.3. Mouthparts assay **25**

2.4. Feeding preference assay: methods **27**

2.5. Feeding preference assay: solutions **28**

2.5.1. Sugar diet assays ***28***

2.5.2. Sugar choice assays ***28***

2.5.3. Regulation of sugar intake assays ***28***

2.5.4. Amino acid choice assays ***29***

2.6. Haemolymph collection **30**

2.7. Haemolymph collection following satiety feeding **31**

2.8. Preparation of haemolymph for HPLC analysis **31**

2.9. Satiety feeding and artificial increase of haemolymph sugars **32**

2.10. High performance liquid chromatography for carbohydrate analysis **32**

2.11. Molecular biology **33**

2.11.1. Honeybee selection ***33***

2.11.2. Sample collection ***34***

2.11.3. Sample preparation ***35***

2.11.4. Primer design and reverse transcription polymerase chain reaction (RT-PCR) ***36***

2.11.5. Sequencing ***42***

2.11.6. Revere transcription quantitative-PCR (RT-qPCR) **42**

2.12. Scanning electron microscopy **43**

2.12.1. Tissue collection **43**

2.12.2. Fixation and dehydration **43**

2.12.3. Coating and microscopy **44**

2.13. Phylogenetic tree construction **44**

2.14. Statistical methods **45**

Chapter 3.0. Sugar regulation in the honeybee **47**

3.1. Introduction **48**

3.2. Materials and methods **53**

3.2.1. Feeding assay and solutions **53**

3.2.2. Sugar feeding and haemolymph collection **53**

3.2.3. Haemolymph sugar manipulation **53**

3.2.4. Statistical methods **54**

3.3. Results **55**

3.3.1. Specific sugar diets affects survival **55**

3.3.2. Honeybee haemolymph sugars vary with nutritional input **60**

3.3.3. Honeybee haemolymph sugars are rapidly regulated **61**

3.3.4. Honeybees demonstrate a general fructose preference **61**

3.3.5. An artificial increase in honeybee haemolymph sugar can affect satiety feeding **67**

3.4. Discussion **70**

3.5. Conclusion **77**

Chapter 4.0. Amino acid preference **78**

4.1. Introduction **79**

4.2. Materials and methods **85**

4.2.1. Honeybee collection and mouthparts assay ***85***

4.2.2. Amino acid feeding preference assay ***85***

4.2.3. Statistical methods ***85***

4.3. Results **86**

4.3.1. Age affects consumption of sucrose-amino acid solutions ***86***

4.3.2. The majority of amino acid-sucrose solutions are consumed at the same rate as sucrose alone ***88***

4.3.3. Combining amino acids increases phagostimulatory effect on newly emerged but not forager bees ***90***

4.4. Discussion **92**

4.5. Conclusion **99**

Chapter 5.0. Comparative anatomical expression of gustatory receptors between newly emerged and forager honeybees (*Apis mellifera)* **100**

5.1. Introduction **101**

5.2. Materials and methods **110**

5.2.1. Phylogenetic tree construction ***110***

5.2.2. Honeybee capture and dissection ***110***

5.2.3. Sample preparation, RNA extraction, DNase treatment and reverse transcription ***110***

5.2.4. Relative expression using polymerase chain reaction (PCR) ***110***

5.2.5. Scanning electron microscopy ***111***

5.2.6. Statistical analyses ***111***

5.3. Results **112**

5.3.1. Phylogenetic tree analysis: AmGr1-3 are orthologous to Drosophila sugars receptors ***112***

5.3.2. Internal expression of most honeybee gustatory receptors is highest in the forager brain ***114***

5.3.3. Gustatory receptor expression is widespread but diverse across both newly emerged and forager honeybees ***115***

5.4. Discussion **122**

5.5. Conclusion **129**

Chapter 6.0. The influence of diet on gustatory receptor expression in the forager honeybee brain **130**

6.1. Introduction **131**

6.2. Materials and methods **136**

6.2.1. Honeybee capture and harnessing for hunger condition ***136***

6.2.2. Honeybee free-feeding on alternate diets condition ***136***

6.2.3. Sample dissection, extraction and PCR ***136***

6.2.4. Statistical analyses ***137***

6.3. Results **138**

6.3.1. Hunger significantly increases mRNA expression of AmGr3 in the honeybee brain ***138***

6.3.2. A monosaccharide diet significantly decreases the mRNA expression of AmGr2 in the honeybee brain ***138***

6.3.3. The addition of amino acids to a sucrose diet alters the mRNA expression of a select few gustatory receptors in the honeybee brain ***138***

6.4. Discussion **144**

6.5. Conclusion **153**

Chapter 7.0. General discussion **154**

7.1. Sugar regulation in the honeybee **154**

7.2. Amino acid preferences **155**

7.3. Comparative anatomical expression of gustatory receptors between newly emerged and forager honeybees **156**

7.4. The influence of diet on gustatory receptor expression in the forager honeybee brain **156**

7.5. Limitations **158**

7.6. Future research 160

Appendix 1.0 **162**

References **174**

List of Figures

Figure 1.1: (A) Taste sensilla locations of *Drosophila*. (B) SEM of honeybee antennal taste sensilla **4**

Figure 2.1: Dorsal view of the honeybee head **26**

Figure 2.2: Plastic Boxes for choice assays **28**

Figure 3.1: Consumption volumes for bees on 0.7 M sugar 96 h diets. **56**

Figure 3.2: Consumption volumes for bees on 1.4 M sugar 96 h diets **57**

Figure 3.3: Percentage survival over 96 h on one of seven diets **58**

Figure 3.4: Haemolymph sugar concentration after *ad libitum* feeding **63**

Figure 3.5: Haemolymph sugar concentrations after satiety feeding **64**

Figure 3.6: Sugar preference index between glucose and fructose **65**

Figure 3.7: Sugar preference index following 48 hour sugar provision **66**

Figure 3.8: Sugar consumption following sugar injections. **69**

Figure 4.1: Volume consumed of eight amino acid solutions **87**

Figure 4.2: Preference index between sugars and amino acids. **89**

Figure 4.3: Preference index between sugar and an amino acid mixture. **91**

Figure 5.1: Phylogenetic tree of *Apis* and *Drosophila* Gr protein sequences **113**

Figure 5.2: Internal expression of *Apis* Gr mRNA in brain and guts **116**

Figure 5.3: Expression of Gr mRNA across the *Apis* anatomy. **117**

Figure 5.4: SEM of forager honeybee mouthparts. **118**

Figure 5.5: SEM of forager honeybee proboscis **119**

Figure 5.6: SEM of forager honeybee antennae. **120**

Figure 5.7: SEM of forager honeybee fore-tarsus **121**

Figure 5.8: SEM of five tissues comprising extended honeybee proboscis. **125**
Figure 6.1 mRNA expression of *Apis* Grs following hunger treatment **140**
Figure 6.2: mRNA expression of putative *Apis* sugar Grs after specific diets. **141**
Figure 6.3: mRNA expression levels of *Apis* unknown Grs after specific diets **142**
Figure 6.4: Schematic showing potential expression and functioning of *Apis* Grs **149**

List of Tables

Table 2.1: Honeybee sugar diet provision and choice assay **29**

Table 2.2: Experimental solutions in amino acid choice assay **30**

Table 2.3: Diet duration prior to dissection for RT-qPCR **34**

Table 2.4: *Apis* Gr primer table. **37**

Table 3.1: Repeated measures ANOVA for volume consumption. **59**

Table 3.2: Cox regression for survival of forager bees on specific diets **59**

Table 3.3: Cox regression for survival of newly emerged bees on specific diets **60**

Table 4.1: Classification and description of fly amino acid classes **84**

Table 4.2: Honeybee preference for amino acid or sucrose solutions **96**

Table 5.1: Analysed *Drosophila* Grs with known or speculated functions. **104**

Table 5.2: GZLM for Gr expression in brains and guts of honeybees. **114**

Table 6.1: : GZLM of *Apis* Gr gene expression in the forager brain **143**

Abbreviations

µl	Microlitre
µM	Micromole
7-TM	Seven transmembrane domain
AA	Amino Acid
AgGr	*Anopheles gambiae* Gustatory receptor
AL	Antennal lobe
AmGr	*Apis mellifera* Gustatory receptor
AmHsTRPA	*Apis mellifera* Transient Receptor Potential A channel
Ca2+	Calcium
cDNA	Complementary deoxyribonucleic acid
CNS	Central Nervous System
CO_2	Carbon dioxide
Ct	Threshold cycle
d	Day
D: L	Dark: Light
DEG/ENaC	Degenerin/epithelial Na+ channels
Df	Degrees of freedom
DH 31	Diuretic hormone 31
DmGr	*Drosophila melanogaster* Gustatory receptor
DNA	Deoxyribonucleic acid
DSO	Drosophila Stress Odorant
gDNA	Genomic deoxyribonucleic acid
GPCR	G-protein Coupled Receptor
Gr	Gustatory receptor
GRN	Gustatory Receptor Neuron
GZLM	Generalised Linear Model
h	Hour
HPLC	High performance Liquid Chromatography
IAA	Indispensable Amino Acids
IR	Ionotropic Receptors
JH	Juvenile Hormone

KCl	Potassium chloride
LSD	Least Significant Difference
LSO	Labral Sense Organ
LTK	Locustatachykinin
M	Molar
ml	Millilitre
mM	Milimole
mRNA	Messenger RNA
Na^+ channel	Sodium channel
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
ng	Nanograms
NPF	Neuropeptide F
OBP	Odorant-Binding Protein
OD	Optical Density
Or	Olfactory receptor
PCR	Polymerase Chain Reaction
PER	Proboscis Extension Reflex
PI	Preference Index
PLC	Phospholipase C
PPK	Pickpocket
Ppm	Part per million
RH	Relative Humidity
RNA	Ribonucleic acid
RNAi	RNA interference
Rpm	Rotations per minute
RT	Room Temperature
RT-PCR	Reverse transcription-Polymerase Chain Reaction
RT-qPCR	Reverse Transcription – quantitative polymerase chain reaction
s	Seconds
SC	Secondary compound
SOG	Suboesophageal Ganglion

SPI	Sugar Preference Index
SS III	Superscript III
TNT	Tetanus toxin
V	Volts
Wk	week

Chapter 1.0. General introduction

1.1. The Chemosensory system

Animals assess and react to environmental conditions using an assembly of sensory systems that are well adjusted to the detection and processing of external stimuli. The chemical senses, for example, are heavily relied upon by the majority of animals and a diversity of behavioural outputs can be elicited following the detection of volatile or soluble chemical stimuli. For instance, the perception of long distance olfactory cues can be used in chemotaxis toward a potential food source, conspecific pheromone identification or detection of volatile warning signals. Gustation, however, is often involved in behaviours closely following those mediated by olfaction, such as, ingestion of edible foods, oviposition in suitable locations or copulation with a mate. As the chemical senses play a vital role in the majority of animal behaviours it is understandable that work deciphering their mechanisms has increased rapidly in recent years. (For reviews see: Stocker, 1994; Smith, 2001; Matsunami and Amrein, 2003; Scott, 2005; Lemon and Katz, 2007; De Brito Sanchez, 2011; Liman et. al., 2014). While mammalian studies are useful to help us understand the mechanisms of our own sensory systems, in depth analysis can prove troublesome due to the scale and complexity of neuronal architecture. Therefore, it is advantageous to study organisms that respond to a comparable diversity of stimuli and represent this sensory signalling in a simpler system. Insect models are ideal candidates due to their easily accessible and amenable chemosensory systems that can be studied with an ever increasing number of molecular, electrophysiological and behavioural techniques.

1.2. Gustatory receptors and their expression

In the depths of sensory research, gustation has usually fallen behind olfaction when it comes to acquiring knowledge in the chemical senses. Vertebrate gustatory receptors (Grs) are recognised as G-protein coupled receptors (GPCRs, Hoon et al., 1999) and due to their similarity as 7-transmembrane domain proteins (7-TM), insect Grs were also initially thought to be GPCRs (Clyne et al., 2000; Scott et al., 2001). Recently however, in-depth molecular studies have revealed that both insect olfactory receptors (Ors) and Grs

comprise an inverted membrane topology compared to mammalian GPCRs (with intracellular N-terminus and extra cellular C-terminus, Benton et al., 2006, Zhang et al., 2011). A series of experiments including G-protein signalling inhibitors, gamma subunit suppression, null mutants and RNA-interference (RNAi), has demonstrated that both G-protein dependent and independent signal transduction pathways function in gustatory coding, suggesting a potential role for ligand-regulated cation channels (Ishimoto et al., 2005; Ueno et al., 2006; Kain et al., 2010; Sato et al., 2011). Such studies highlight the complexity of the insect gustatory system.

Due to their similarities in molecular structure the identification of Grs, has mostly been a useful addition to molecular work on Ors. In their review on the subject, Amrein and Thorne (2005) point out that, due to exceptionally low expression of Gr mRNA in insect taste neurons, molecular techniques such as *in situ* hybridisation are often fruitless and consequently Grs have been relatively understudied. However, a variety of studies have been devoted to the depiction of the insect gustatory system and with progression in molecular techniques, the identification of chemoreceptors is on the rise.

In early gustatory work on insects researchers merely speculated the location of Grs (For review see Frings and Frings, 1949) and after the general gustatory anatomy was decided, work progressed toward deciphering receptor ligands and the behavioural responses they mediated. Due to modern techniques such as scanning electron microscopy (SEM), quantitative PCR (RT-qPCR) and electrophysiology, we now have an in depth knowledge of the structure and location of many species' Grs, in addition to chemical coding.

Molecular biology in particular has influenced the most recent gustatory work and has accounted for recent focus on the limited number of insects whose genome has been sequenced. For example, Clyne and colleagues (2000) first identified a large gene family in *Drosophila* that encoded GPCRs as candidate Grs. Since this preliminary work, a total of 68 *Drosophila* Grs encoded by 60 genes have been described (Dunipace et. al., 2001; Scott et. al., 2001; Robertson et. al., 2003; Thorne et. al., 2004). As genome sequencing continues, genes encoding seven transmembrane domain proteins have helped identify Grs in an increasing number of additional species (*Anopheles gambiae*: Hill et. al., 2002; *Apis mellifera*:

Robertson and Wanner, 2006; *Anopheles aegypti*: Kent et. al., 2008; *Tribolium castaneum*: Tribolium Genome sequencing consortium, 2008; *Bombyx mori*: Wanner and Robertson 2008; *Daphnia pulex*, Penalva-Arana et. al., 2009; *Acyrthosiphon pisum:* Smadja et. al., 2009; *Camponotus floridanus* and *Harpegnathos saltator*: Bonasio et. al., 2010, Zhou et. al., 2012; *Linepithema humile:* Smith et. al., 2011a).

Gustatory receptor genes are expressed in gustatory receptor neurons (GRNs) and often housed in hair–like structures called sensilla, which extend axons directly to the central nervous system (CNS, Mullin et. al., 1994), usually the suboesophageal ganglion (SOG). Upon dissection, Dethier (1955) discovered that the sensilla on the labellum and tarsi of the blowfly *Phormia regina* contained a number of bipolar neurons in addition to supporting cells that were later associated with the secretion of the surrounding lymph (Morita, 1992). These neurons come into contact with the environment via a terminal pore and each sensillum can be activated by chemical and tactile stimuli indicating the presence of a mechanoreceptor, which was later confirmed (Dethier, 1955; Falk, 1976).

Early work proposed the presence of a sugar and a non-sugar (most likely salt) receptor in each sensillum (Dethier, 1953, 1955), which was confirmed using electrophysiology, along with a separate water neuron (Hodgson, 1957; Mellon and Evans, 1961; Evans and Mellon, 1962). A fourth cell in the sensilla was speculated to additionally respond to salt and remained uncharacterized for many years (Dethier and Hanson, 1968). It is now understood that between two and six sensory neurons exist in each sensillum in most insect species and that the fourth cell is an additional salt cell that responds to high levels of salts and other aversive substances (Whitehead and Larsen, 1976b; Siddiqi and Rodrigues, 1980; Fujishiro et. al., 1984; Wieczorek and Wolff, 1989; Meunier et. al., 2003; Zhang et. al., 2013). The four cells are now commonly classified by the responses elicited from their general ligand groups; sugar (S cell), water (W cell), low salt concentrations (L1 cell) and high salt concentrations (L2 cell). But not all of these neurons function independently and it is thought that each GRN can express more than one Gr on its surface (Tanimura and Shimada, 1981; Meunier et. al., 2003; Hiroi et. al., 2004; Weiss et. al., 2011).

Dependent on species, the sensilla that house the GRNs can take a number of different forms, such as hairs, pegs or bristles. Their location, generally restricted to typical gustatory appendages (mouthparts and tarsi), can also be species specific e.g. wing margins and female genitalia of *Drosophila* (Stocker, 1994, see Figure 1.1A). Recently, Grs have also been revealed on the antennal tip of some insects suggesting a possible role in olfaction as well as gustation. Scott and colleagues (2001) discovered three Grs on the antennae of *Drosophila*. This finding was unexpected, not just because the majority of antennal sensilla house olfactory receptors (Ors) but because the GRN axonal projections were traced to the antennal lobe (AL), the primary olfaction processing centre and not the usual SOG (Stocker, 1994; Scott et. al., 2001). Gustatory receptors located on the antennal tip of the honeybee (Figure 1.1B) have also been discovered and they are thought to aid the insect in foraging (for review see: De Brito Sanchez, 2011).

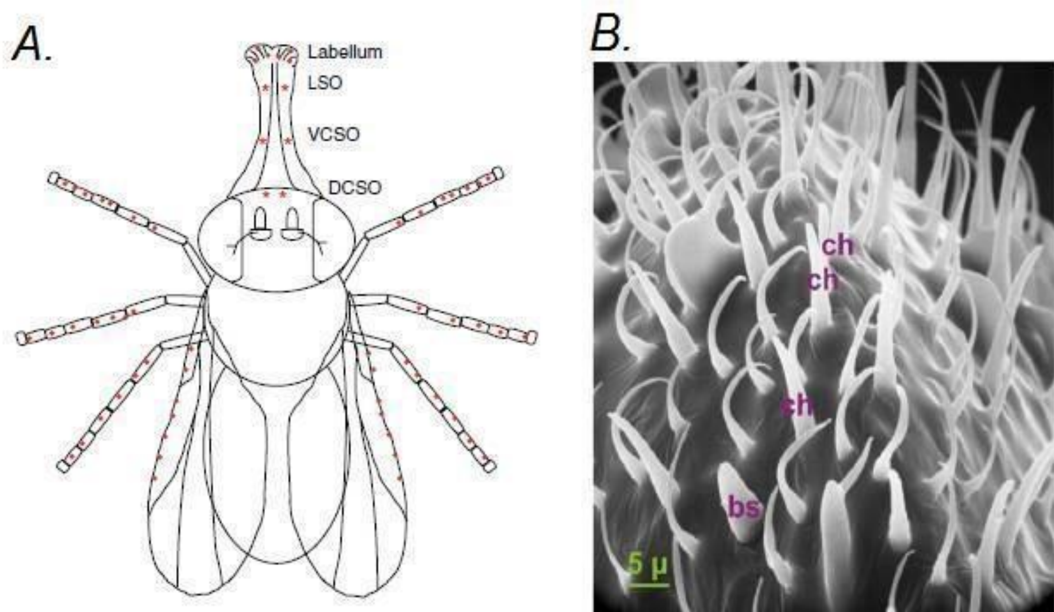

Figure 1.1. **A.** Taste sensilla locations, represented by red dots, on the anatomy of *Drosophila.* Sensilla can be found in similar locations on the majority of insect species and each house gustatory receptor neurons (GRNs) which detect chemical ligands via the receptors on their surface (taken from Amrein and Thorne, 2005). **B.** Scanning electron micrograph of the taste sensilla, hair-like chaetic (ch) and cone-like basiconic (bs) on the antennal tip of the honeybee (*Apis mellifera*, taken from De Brito Sanchez, 2011).

For food identification and assessment, it is understandable that Grs are present in high proportions on the appendages most likely to come into contact

with food. However, only recently the location of Grs have been speculated and indeed confirmed in some less obvious anatomical areas.

Many insects are known to orient towards specific foodstuffs containing nutrients, particularly when deficient and Grs are important in the detection of these nutrients (For review see Waldbauer and Friedman, 1991 and Browne, 1993). For example: *Drosophila* larvae have the primary goal of locating and consuming enough food in order to grow and develop into an adult and therefore, unlike in their adult form, larvae express Grs throughout their whole body (Stocker, 1994; Scott et. al., 2001). However, if nutrients are not immediately detectable on consumption, animals will require a sensory mechanism to alert them following ingestion. With this in mind, internal Grs may also act as nutritional 'sensors' allowing animals to respond appropriately to maintain nutritional homeostasis. Unsurprisingly internal expression of Grs has been discovered in some vertebrate and invertebrate species, primarily in the brain (Thorne and Amrein, 2008, Ren et. al., 2009; Singh et. al., 2011; Miyamoto et. al., 2012), thought to be involved in the central regulation of feeding, detecting nutrient levels in the blood and haemolymph. Additionally Grs have been discovered in the gut, detecting ingested nutrients and allowing for more efficient gut motility and secretion of digestive enzymes and hormones (Wu et. al.,2003; Dyer et. al., 2005; Wu et. al., 2005a; Mace et. al., 2007; Margolskee et. al., 2007; Park and Kwon, 2011; Behrens and Meyerhof, 2011; Sato et. al., 2011). Interestingly, bitter Grs have also been discovered in the nasal cavities and airway epithelia of rodents and humans (Finger et. al., 2003; Shah et. al., 2009; Deshpande et. al., 2010) and are involved in the detection of inhaled respiratory irritants. Detection of bitter stimuli from noxious compounds, potentially food items, by Grs, helps to elicit changes in respiration rate, nasal ciliary movement and bronchospasm in order to remove potentially harmful stimuli (Finger et. al., 2003; Shah et. al., 2009; Deshpande et. al., 2010).

1.3. Insect taste ability

We know gustatory receptors exist, but how do they function in order to create the sensory perception of taste? Does each receptor bind to only one specific ligand or does stimulation require more general chemical stimuli?

Gustatory receptor specificity has come under scrutiny over recent years and much progress has been made. Dependent on the species, an insect's taste ability can vary, along with the number of separate Grs they possess and the genes encoding them. As mentioned earlier, *Drosophila* possess 68 Grs (Dunipace et. al., 2001; Scott et. al., 2001; Robertson et. al., 2003; Thorne et. al., 2004) and when comparing this repertoire to other species *Drosophila* appear to have a relatively elaborate gustatory system. For example, honeybees only possess a meagre 10 receptors (Robertson and Wanner, 2006) which has been speculated as a trait of Hymenoptera (De Brito Sanchez, 2011) as some ant species also possess few receptors (*H. saltator*: 17 Gr genes, Bonasio et. al., 2010, Zhou et. al., 2012), although this is not the case for all ant species (*L. humile*: 116 Gr genes, Smith et. al., 2011a; *Pogonomyrmex barbatus*: 73 Gr genes, Smith et. al., 2011b). Having fewer Grs could mean that bees have poor taste acuity; however, increasing behavioural and electrophysiological studies demonstrate a broad tastant detection system in the honeybee (For review see De Brito Sanchez et. al., 2007; De Brito Sanchez, 2011).

1.3.1. Sweet taste

For the detection and coding of soluble chemical stimuli in general there are four distinct groupings. Not only have these groups been preferentially selected for study, but they also appear to be primarily represented by GRNs in insect sensilla (W cell, S cell, L1 and L2 cells). Sugars (carbohydrates) are an important dietary component for all animals, including insects. In primary work on the blowfly it was speculated that an S receptor stimulated by sugars was present, which elicited an acceptance response (Dethier, 1953; Hodgson, 1957). This speculation has since been confirmed (Mellon and Evans, 1961; Fujishiro et. al., 1984), along with the discovery of an L receptor which was stimulated by non-sugars and mediated a rejection response.

With the use of electrophysiology the majority of studies focused on a small subset of sugars, primarily sucrose, glucose and fructose. For example, honeybees were found to respond to sugars "linearly to the log of solute concentration", with sucrose always proving most stimulating (Whitehead and Larson, 1976b). Similarly in blowflies, more impulses per second were recorded from the sugar cell when presented with sucrose than four other sugars (D-

arabinose, glucose, fructose and sorbose) across a range of concentrations (Omand and Dethier, 1969). This same study also found that the combination of glucose and fructose in solution had a synergistic effect on firing rate of the sugar cell, which provided an early insight into differential Gr sugar-binding (Omand and Dethier, 1969). The idea of ligand-specific Grs was confirmed following short-term receptor ablation studies which demonstrated complete depression of sugar-specific responses in both fleshflies and fruit flies (Shimada et. al., 1974; Tanimura and Shimada, 1981). Inadvertently these authors were also the first to identify a correlation between genetic manipulations and 'sweet' ligands long before Grs in any insect were identified.

Tanimura and colleagues (1982) recognised differential taste sensitivity to trehalose between two genetically different *Drosophila* groups due to the *Tre* gene on the X-chromosome, whilst sensitivity to other sugars remained unaffected. Following the publication of the sequenced *Drosophila* genome and identification of putative Grs (Clyne et. al., 2000) it was not long before a convincing trehalose-specific receptor was identified. Dahanuka and team (2001) used *Drosophila* mutants that had undergone deletions in the *Gr5a-tre1* genomic region to demonstrate a severely reduced electrophysiological and behavioural response toward trehalose following gene alteration. Ueno and colleagues (2001) indicated a direct disruption to the expression of Gr5a mRNA when deletions were performed at the Tre locus. In addition, an *in vitro* study by Chyb and team (2003) established that changes to intracellular calcium (Ca^{2+}) levels occurred in cells expressing the Gr5a receptor immediately after the introduction of trehalose. This study also pointed out that Gr5a receptors are located in the majority, if not all, of *Drosophila* labellar sugar GRNs which was good evidence for the overlap of separate sugar binding Grs (Chyb et al., 2003).

The potential for Gr overlap was worth pursuing as receptors with similar gene sequences to Gr5a had subsequently been identified (Gr61a and the gene cluster Gr64a-f), with a potential role in sugar detection (Chyb et. al., 2003). The co-expression was confirmed and a selection of sugar Grs were characterised. *Drosophila melanogaster* gustatory receptor 64a (DmGr64a) was discovered to be primarily co-expressed with Gr5a and all positive responses to sugar solutions were depressed in *Gr5a;Gr64a* double mutants (Dahanukar et. al., 2007). Unlike

vertebrate sugar receptors that function as obligate heterodimers (Nelson et al., 2001) both Gr5a and Gr64a maintained individual functions responding to separate sugar sub-groups, suggesting potential dimerization with other members of the sugar Gr family (Dahanukar et al., 2007). Jiao and colleagues (2007) confirmed that the receptors most similar to Gr5a (Gr64a-f and Gr61a), were co-expressed in GRNs with Gr5a, in addition to identifying Gr64a as the receptor necessary for the detection of sucrose, glucose and maltose. Interestingly, further work by Slone and colleagues (2007) paralleled these findings by showing that deletion of the six Gr64(a-f) genes diminished behavioural responses to a number of sugars with the exception of fructose. They also established that proboscis extension reflex (PER) responses to trehalose were no longer possible despite the possession of a functioning Gr5a receptor (Slone et. al., 2007). In later work Gr64f was recognized as the receptor that must be co-expressed both with Gr5a to detect trehalose and with Gr64a for glucose, maltose and sucrose detection (Jiao et. al., 2008). This finding indicates Gr64f as a 'general' sugar receptor that must act in conjunction with other, more specific, receptors and additionally, that some Gr genes act as multimeric complexes. This independent and dimer functioning is thought to provide residual functioning in case any receptor becomes non-functional (Dahanukar et al., 2007).

Mapping of Gr5a in *Drosophila* has demonstrated a much higher proportion of neuronal expression compared to any other Gr (Thorne et. al., 2004), highlighting the importance of carbohydrate perception (Thorne et. al., 2004; Wang et. al., 2004). Whilst expression of Grs often overlap, each receptor was only thought to be expressed in a single neuron within a sensilla (Scott et. al., 2001; Thorne et. al., 2004). However, unlike the other receptors, *Gr5a* is known to exist on a few GRNs within the same sensilla (Thorne et. al., 2004). Similar 'sweet' GRNs within a sensilla may point to *Drosophila*'s heightened ability to differentiate between chemically similar sugars, particularly as Gr expression can differ within and between sensilla (Chyb et. al., 2003; Thorne et. al., 2004; Wang et. al., 2004).

1.3.2. A pinch of salt

The idea that receptors need to work in conjunction to detect a particular chemical may be useful in deciphering the mechanisms of salt perception. Early

chemosensory work determined that within most sensilla (those containing four GRNs), at least one neuron is responsible for salt detection (Dethier, 1953, 1955; Hodgson, 1957), now generally referred to as L1 cells; which are primarily considered as low salt detectors (for review see Amrein and Thorne, 2005). Salt activation also occurs in both the S and L2 cells in addition to the L1 cells (Hodgson, 1957; Meunier et. al., 2003; Hiroi et. al., 2004). In 2003, Meunier and his group demonstrated that the relatively unknown GRN L2, housed in *Drosophila* prothoracic leg sensilla, was responsible for the detection of bitter stimuli along with high salt concentrations (400 mM NaCl). However, ablation studies demonstrated that whilst responses to aversive stimuli such as caffeine were diminished when neurons expressing GR66a were ablated, responses to high salt concentrations remained unaffected (Wang et. al., 2004). This same highly concentrated salt solution was further found to activate the L2 neurons in the labellar i-type sensilla (houses only two separate GRNs) in addition to stimulating what was thought to be a sugar cell at lower concentrations (NaCl up to 50 mM, Hiroi et. al., 2004). It was concluded that this 'attractant' neuron within the i-type sensilla had the combined function of both an S+L1 cell (Hiroi et. al., 2004).

To date, evidence for the direct involvement of gustatory receptors in salt detection is lacking, however alternative mechanisms have recently been investigated. The involvement of amiloride-sensitive degenerin/epithelial Na+ channel (DEG/ENaC) pickpocket genes (*PPK11* and *PPK19*) in the perception of low salt concentrations, for both larval and adult *Drosophila,* have been discovered (Liu et. al., 2003). More recently the mechanisms mediating differential responses to low and high salt concentrations—by L1 and L2 chemosensory neurons respectively—in *Drosophila* labellar sensilla have also been investigated. In behavioural assays flies are known to preferentially ingest solutions containing 50 mM NaCl, while actively rejecting all solutions ≥200 mM NaCl (Zhang et. al., 2013). Zhang and colleagues (2013) assessed the recently identified (Benton et. al., 2009) ionotropic glutamtate receptor family (or ionotropic receptors, IR) and uncovered a specific IR and Na+ channel (IR76b) responsible for the detection of low salt concentrations in L1 sensilla. The loss of IR76b disrupted the behavioural attraction toward low salt concentrations, without affecting aversive behaviours toward high salt concentrations. Double labelling experiments uncovered that there was no

overlap in expression of IR76b channels in either Gr5a- or Gr66a-expressing neurons, indicating that salt detection neurons are separate from both the 'sugar' and 'bitter' neurons. While this channel operates within gustatory receptor neurons, it does so independent of gustatory receptors.

1.3.3. Bitter taste

Mapping of GRN projections highlighted a number of Grs which are distinct from the sugar neurons. Wang and colleagues (2004) were the first to point out a lack of overlap between Gr5a-and Gr66a-expressing neurons and suggested separate roles in alternate taste categories. In turn, the analysis of eight Gr genes identified two groups based on GRN projections and expression patterns (Thorne et. al., 2004). The first consisted solely of Gr5a, but the second was larger, consisting of seven receptors (Gr66a, Gr22e, Gr32a, Gr22b, Gr22f, Gr28be and Gr59b), often co-expressed within the same GRN (Thorne et. al., 2004). Central projections maintained the peripheral segregation, with axons terminating in a separate area of the SOG to all GR5a-expressing GRNs. A role in bitter taste detection was indicated following ablation studies using a UAS-tetanus toxin light chain (TNT) reporter. The ablation of Gr66a- or Gr22b-expressing GRNs resulted in a diminished ability to avoid caffeine accurately, without affecting sugar preference (Thorne et. al., 2004). Avoidance of quinine, berberine, denatonium or benzoate also remained unaffected, which suggests the function of these two Grs is not for 'general bitter tasting' (Thorne et. al., 2004). However, as with the majority of Gr ablation studies, elimination of the specified Gr is achieved through inactivation of the entire neuron (as seen in Scott et. al., 2001; Thorne et. al., 2004; Wang et. al., 2004). As a consequence, the functioning of any additional receptors expressed alongside the target receptor will also be abolished. Loss of caffeine avoidance behaviour following neuronal ablation attributes caffeine detection to Gr66a and Gr22b. However, without investigating the function of all remaining Grs—a daunting task for the 68 in *Drosophila*—it will remain difficult to depict the exact mechanisms of any single receptor.

More targeted Gr ablation methods are possible. Moon and his team (2006) obtained convincing evidence for Gr66a in caffeine perception via Gr66a locus deletion from the *Drosophila* genome and subsequent lack of caffeine aversive behaviours (Moon et. al., 2006). A later study identified that such caffeine-averse

behaviours are also reliant on the co-expression of Gr93a along with Gr66a (Lee et. al., 2009). Another more specific approach involves the use of RNA interference (RNAi) which targets and deactivates the receptor directly and has been shown to work successfully in previous Gr inactivation studies (Bray and Amrein, 2003; Ozaki et. al., 2011).

In addition to *Drosophila*, a variety of insects are able to detect soluble bitter stimuli, compounds often occurring as plant secondary compounds (SC) providing defence against herbivores (For review see Chapman, 2003). A large family of putative bitter receptors has recently been identified in the Silk moth (*Bombyx mori*), which are thought to be evolutionary significant as both the adult moth and its larvae often encounter bitter plant compounds (Wanner and Robertson, 2008). However, this is not the case for all insects, the honeybee is not thought to be able to detect bitter substances on the antenna (De Brito Sanchez et. al., 2005) despite being able to reject bitter solutions in behavioural assays (Ayestaran et. al., 2010; Wright et. al., 2010). Bitter stimuli are however thought to inhibit the responses to sugar and salt solutions in theses insects (De Brito Sanchez et. al., 2005; De Brito Sanchez, 2011).

Simple activation of Grs housed in 'bitter-sensitive' neurons is not the only mechanism employed in the gustatory system for bitter detection. Work on *Drosophila* has revealed that responses of the sugar and water cells are also inhibited directly by bitter substances and occasionally, this inhibition occurs even without L2 activation (Meunier et. al. 2003). As in olfactory neurons, lateral inhibition, "the sustained response of one olfactory receptor neuron (ORN) is inhibited by the transient activation of a neighbouring ORN" (Su et. al., 2012) may be responsible, which has previously been indicated in mammals (Vandenbeuch et. al., 2004). However, recent work in *Drosophila* has revealed the presence of an odorant binding protein (OBP), secreted into the sensilla endolymph, as integral to this sugar-neuron suppression (Jeong et. al., 2013). While the loss of OBP49a did not alter action potentials to sugar or bitter tastants separately, the suppression of the sugar neuron from a combination of sugar and bitter tastants was impaired (Jeong et. al., 2013). As many toxic solutions are recognised as bitter tasting (Glendinning, 2007) this is a valuable 'failsafe', ensuring that even when masked in a sugary solution, bitter substances are unlikely to go unnoticed. Additionally,

the suppression of sugar GRNs via the activation of bitter GRNs is also thought to mediate the aversive response toward low pH carboxylic acids (Charlu et. al., 2013).

Activation and inhibition, working separately and in concert, once again brings into question the function of individual Grs. From the array of recent evidence, acceptance or rejection behaviours no longer appear to be the direct result of simple ligand binding to a single receptor.

1.3.4. Water and hygroreceptors

As early as 1955, Dethier identified measurable reactions to water in the blowfly and with little previous work on the subject, the presence of a water cell was speculated (W cell). Using electrophysiology, the water cell was confirmed and subsequently found to be inhibited by non-electrolytes and inorganic electrolytes such as NaCl (Mellon and Evans, 1961; Fujishiro et. al., 1984; Inoshita and Tanimura, 2006). Whilst water detection is necessary for all insects, not every insect sensillum contains a water cell, for instance *Drosophila* i-type sensilla contain only two separate GRNs, as previously mentioned (Hiroi et. al., 2004). Neither of these cells respond to water or KCl; which is commonly attributed to the activation of the water cell (Haupt, 2004; Hiroi et. al., 2004; De Brito Sanchez et. al., 2005). Many insects have an alternate method of identifying a water source using specific hygroreceptors (honeybee: Lacher, 1964; Yokohari et. al., 1982; moth (caterpillar): Dethier and Schoonhaven, 1968; cockroach: Loftus, 1976, fruit fly: Thorne and Amrein, 2008). However, hygroreceptors are often used in the assessment of environmental humidity and therefore the role of the water GRN is solely attributed to water identification for consummatory purposes.

1.3.5. Carbon dioxide and pheromone receptors

It is beneficial and necessary for an insect's sensory system to detect and mediate the avoidance of potentially harmful stimuli. In *Drosophila* this involves the volatile 'stress odorant' (DSO) emitted by conspecifics (Suh et. al., 2004). Following vigorous shaking or electrocution fruit flies release DSO which elicits avoidance behaviours in other nearby flies. Carbon dioxide (CO_2) has been identified as a main component of DSO and calcium imaging demonstrates that both DSO and CO_2 activate Gr21a-expressing neurons (Suh et. al., 2004). This work indicates a role in olfaction, as unlike the majority of other Grs, Gr21a's

axonal projections terminate in the antennal lobe (AL) like olfactory receptors. Subsequent studies also identified Gr63a as a co-receptor in chemosensory neurons responsible for CO_2 sensitivity (Jones et. al., 2007, Kwon et. al., 2007), activation of which generally mediates acceptance behaviours (Fischler et. al., 2007).

Carbon dioxide detection is also present in other insects, as CO_2 receptor orthologs have recently been identified in a number of other species (Robertson and Kent, 2009). However, as Robertson and Kent (2009) point out, these highly conserved genes are not present in hymenopteran species (honeybee, parasitoid wasp, some ant species: Zhou et. al., 2012), despite both honeybees and some ant species being able to detect CO_2 (Seeley, 1974; Kleineidam and Tautz, 1996). Therefore it is likely that hymenoptera may have developed an alternate, or species-specific detection mechanism.

In addition to avoiding potential threats, some insects use gustatory receptors to aid potential mate identification. Male flies possess more chemosensory sensilla on foreleg tarsi than females, which indicate a role in pheromone detection and mating behaviours (Stocker, 1994, Bray and Amrein, 2003). Bray and Amrein (2003) identified Gr68a expression within these sensilla and observed that neuronal ablation and targeted RNAi receptor disruption resulted in a diminished ability to perform successful male courtship (Bray and Amrein, 2003). Additionally, the loss of Gr32a and Gr33a is known to enhance homosexual behaviour in flies (Miyamoto and Amrein, 2008; Moon et. al., 2009) and RNAi knock down of Gr39a leads to a reduction in male to female courtship (Watanabe et. al., 2011).

1.3.6. Gustation: not just gustatory receptors

In addition to gustatory receptors mediating taste sensitivity, an increasing number of studies have investigated the role of ionotropic receptors (IRs), as the expression of IRs was recently discovered in general taste organs (Benton et. al., 2009; Croset et. al., 2010; Zhang et. al., 2013; Koh et. al., 2014). Koh and team (2014) investigated a specific group of IRs termed the IR20a clade. This clade is thought to be primarily involved in olfaction, however some members were also expressed in a series of ‘orphan taste neurons’ that lack any gustatory receptors. These IRs are thought to be highly conserved and span a range of species (Croset

et. al., 2010). Subsequently the role of IRs in gustatory sensitivity adds a further level of complexity to determining the mechanisms and function of the gustatory system.

1.4. Concentration dependency

The encoding of concentration has been relatively neglected when it comes to the study of insect gustation. As previously mentioned, the main concentration dependent change in coding we know of, occurs in salt detection. The same salt can activate the S and L1 cell at low concentrations or the L2 cell at high concentrations (Meunier et. al., 2003; Hiroi et. al., 2004) and as discussed, is mediated in part by IR76b (Zhang et. al., 2013).

As seen, Gr5a-expressing neurons are routinely accepted as detecting trehalose and a variety of other sugars, just as Gr66a-expressing neurons are thought to detect bitter substances and highly concentrated salts. However, following ablation of these neurons, responses to highly concentrated sugars and salts remained unaffected (Wang et. al., 2004), suggesting that the receptors responsible for their detection do not reside solely on these neurons. Miyakawa (1982) attempted to annotate specific *Drosophila* larvae Grs by mixing a low concentration of one solution with a high concentration of another. Whilst the receptor definition was not exact, it did reveal an interesting finding about how the concentrations of different sugars are detected. It was found that 0.01 M sucrose and fructose could be detected in glucose up to 0.9 M whereas 0.01 M NaCl was still identifiable in 1.3 M glucose (Miyakawa, 1982). A heightened ability to detect lower concentrations of one solution over another may be represented physiologically e.g. a greater proportion of receptor expression. Indeed this appears to be the case in olfaction as observed by Hallem and colleagues (2004), an increase in olfactory stimulus intensity sees an increase in receptor number activation.

Neuronal responses change as solute concentration increases (Hiroi et. al., 2002; Dahanukar et. al., 2007). It may be possible that, as with salts, when a particular stimulus becomes highly concentrated it becomes aversive. Hiroi and colleagues (2002) demonstrated potential for this theory in sensilla recordings with increasing sugar concentrations. In general, an increase in sugar concentration is

reflected in spike number per second, however for trehalose, the increase from 500 mM to 1000 mM actually sees a drop in spike number (Hiroi et. al., 2002). Although relatively minor, this drop may indicate a concentration preference threshold for trehalose, beyond which aversive behaviours are triggered.

1.5. What about protein?

Carbohydrates, a major energy source for all animals, have been studied extensively in the form of sugars. Potentially toxic compounds, commonly perceived as 'bitter', have also been investigated. However protein, a nutrient all insects rely on for growth and survival has been relatively neglected in existing chemosensory research. Whist current knowledge indicates little likelihood of a separate protein GRN; it is yet to be determined whether individual protein receptors occur within the insect gustatory system. Early work by Dethier (1955) established that the blowfly will readily consume protein, particularly in early life stages. Similar responses have been demonstrated in a variety of insects and it is thought that attraction to some proteins occurs via sugar cell activation (for review see Chapman, 2003). Some insects are even known to self-select their own protein to carbohydrate ratios (Simpson et. al., 2004; Lee, 2007; Lee et. al., 2008; Altaye et. al., 2010). Therefore, it is reasonable to assume that these insects would possess the sensory ability to detect the nutrients they need in order to carry out accurate dietary selection.

Judging gustatory responses to proteins can be troublesome due to their range of chemical compositions. In order to accurately assess gustatory sensitivity toward protein it proves more reliable to record responses elicited by their individual components, amino acids (AAs).

1.5.1. Amino acids: detection and response

There exists a set of around ten essential AAs that are similar for all insect species (e.g. for the honeybee: De Groot, 1953). They are necessary to survival, cannot be built up or broken down from other compounds and must be sourced directly from food. These AAs represent relatively high priority nutrients in dietary selection and as a result, the majority of AA gustation studies have focussed on these ten. Amino Acids exist in two forms, the L-amino acids, which are the major components of proteins and the most likely form insects are to encounter and their

D-enantiomers, which are less common. As you would expect, the L-AAs are most stimulating to insects. This was demonstrated in a study of seven D-AAs which were shown to elicit severely diminished neuronal responses in the fleshfly (*Boettcherisca peregrine*) compared to their L-isomers (Shimada, 1978). As current research stands, what we know about the taste of AAs has mostly been obtained using behavioural assays and electrophysiology with very little molecular input.

As early as 1965 we knew that some AAs acted as feeding stimulants in houseflies and were thought to be the main components of the common artificial protein solution, casein hydrolysate (Robbins et. al., 1965). The first in depth assessment of gustatory responses to individual AAs was carried out by Shiraishi and Kuwabara (1970) in an attempt to establish which GRNs were activated by AAs and whether a separate AA-specific GRN existed in the blowfly or fleshfly. Recordings from GRNs within labellar sensilla were taken following presentation of 19 separate AAs, including the ten essentials. As a result, four distinct classes of AAs were defined (Shiraishi and Kuwabara, 1970). Class 1 comprised AAs that failed to stimulate any GRN (responses no different to distilled water, six in total), class 2 AAs inhibited all three GRNs at high concentrations (five in total), class 3 AAs were 'salt cell stimulators' as well as inhibiting the water cell (proline and hydroxyproline) and finally, class 4 AAs stimulated the sugar cell of flies (valine, leucine, isoleucine, methionine, phenylalanine and tryptophan, Shiraishi and Kuwabara, 1970). Interestingly, all sugar stimulating AAs were essential. A follow up to this study was carried out in 1973 using only blowflies which tried to correlate behavioural data to the electrophysiological responses (Goldrich, 1973). The study was a success for all but three of the selected AAs, the differences attributed to differing methodologies. Once again, this correlative study noted a lack of separate AA GRN, however no individual Grs were investigated (Goldrich, 1973).

Since this preliminary work, natural preferences for AAs in a variety of insects have been demonstrated. Some ant species are known to preferentially select artificial nectars containing AAs over sucrose-only controls (Lanza and Krauss, 1984; Lanza, 1988). This same preference also occurs in a number of other species and is often enhanced when insects have been pre-fed sugar only diets (fleshflies: Potter and Bertin, 1988; Rathman et. al., 1990, honeybees:

Inouye and Waller, 1984; Kim and Smith, 2000; Carter et. al., 2006; Bertazzini et. al., 2010, cabbage white butterfly: Alm et. al., 1990). An interesting study recently established that the gustatory system is likely responsible for the major sensory perception of AAs in honeybees. Proboscis extension reflex (PER) assays demonstrated that only a select few AAs could be detected via olfaction and only at concentrations higher than would be naturally encountered (Linander et. al., 2012). Further in depth physiological and molecular work must be carried out in order to establish the exact detection mechanisms, but uncovering this information is only likely once an entire set of insect Grs have been defined.

1.5.2. Amino acid receptor and sensors

The existence of a unique AA Gr was put into question following the discovery that mammalian AA detection may occur separately from gustation. In 2000, a GPCR was identified in rats that was 'structurally distinct' from Grs and contained a glutamate-binding domain which lent itself to the idea of a 'glutamate receptor' responsible for the perception of umami (the perceived flavour of protein, Ikeda, 1909; Chaudhari et. al., 1996, 2000). More recently however, Nelson and team (2002) have proposed that the combination of T1R1 and T1R3 mammalian gustatory receptors function in mice and humans as a heterodimer and broadly tuned AA sensor that can detect the 20 'standard' AAs but not their D-enantiomers.

In addition to a gustatory receptor for AAs, a number of species have also been found to express an AA sensing pathway comparable to that originally discovered in yeast. This pathway operates to prevent animals becoming deficient in indispensable amino acids (IAA), by mediating increased foraging for foods containing IAAs and developing conditioned aversions to foods that do not (For reviews see: Hao et. al., 2005; Gietzen and Rogers, 2006; Gietzen et. al., 2007). The mammals using this pathway are thought to possess an IAA 'sensor', often housed in the anterior piriform cortex (APC), which functions independently of the chemosensory system. This pathway is thought to be highly conserved and was identified in a number of species through behavioural and physiological assays demonstrating an ability to avoid IAA deficient diets, often correlated with activation in the IAA sensor (chicks: Firman and Kuenzel, 1988, rats: Markison et. al., 1999; Cota et. al., 2006, mice: Karnani et. al., 2011). Whilst a central AA

sensor may be possible in insects it is unlikely to operate independently as many studies have demonstrated behavioural preferences or aversions to AAs over very short time frames e.g. using consumption and PER data (Miyakawa, 1982; Inouye and Waller, 1984; Kim and Smith, 2000; Carter et. al., 2006). Exhibiting AA detection over such a short time suggests a gustatory role allowing almost immediate detection of AAs.

1.6. Chemoreceptor plasticity and its evolutionary significance

The ability to alter gustatory sensitivity to certain foods is necessary throughout an insect's life cycle and often involves physical or neuronal plasticity, in addition to hormonal influence. Differential sensitivity can be encompassed in short term changes, e.g. sensory specific satiety following ingestion of a particular nutrient (Dethier, 1961; for review see Rolls 1986). Longer term changes also occur, often accompanied by physical alterations, such as an increase in sensilla number as a reflection of food availability and chemical diversity (Chapman and Thomas, 1978; Chapman and Fraser, 1989; Rogers and Simpson, 1997; Bernays and Chapman, 1998; Opstad et. al., 2004).

Commonly, the changing physiological needs associated with progression from juvenile to adult are reflected in altered sensitivity towards certain foodstuffs. A good example concerns the difference in attraction toward sugar between adult and larval *Drosophila* (Miyakawa et. al., 1980). Once adult flies have been fed to satiety with a sugar solution they are no longer attracted toward that sugar. However, this appetite suppression does not occur in larvae as they continually exhibit chemotaxis toward sugar solutions despite being fed to repletion (Miyakawa et. al. 1980). This constant sugar preference reflects the larvae's need to consume enough nutrients to fuel the approaching adult transformation which is metabolically costly.

Whilst there is a distinct lack of studies demonstrating how gustatory sensitivity alters between larvae and adults, GRN mapping has allowed us to visualise the plasticity associated with changing gustatory systems. Scott and team (2001) observed that Gr21b expression occurs extensively over the *Drosophila* larval body, but can only be attributed to a single neuron in the Labral Sense Organ (LSO) of the adult fly. Alternatively, Gr32d1 only appears in a single

neuron in the larval terminal organ despite being expressed in a multitude of neurons across the adult proboscis.

As suggested, progression through the insect's life cycle is likely to be accompanied by a change in priorities often reflected in consummatory behaviour. For example, Dethier (1961) demonstrated a distinct difference in protein consumption between male and female blowflies, not only as they grew but also as their physiological needs changed. Female flies displayed an increased need for protein in the days immediately prior to oviposition in order to supply adequate provisions to their young. Another example involves the changing sensitivities observed in insects that are organised in a task-differentiated system, such as honeybees. Honeybee hives mostly consist of females, however, for a fairly short period each year a small population of males also exist, their main role concerning reproduction. The female bees can be split into two separate castes, the queens, who are primarily responsible for reproduction and the sterile workers, which carry out most other tasks. While all members of the same caste, the adult female workers undergo a level of task differentiation referred to as age-polyethism. As the bees' age they tend to progress through a range of occupations, younger bees, or nurses, remain within the hive carrying out tasks such as brood rearing and general hive maintenance (Winston, 1987). When the bees reach a certain age they become foragers, leaving the hive to collect nutritious provisions for the colony (Winston, 1987). With food-collection being the priority for foragers it would benefit them to possess an expanded gustatory system better adapted to the selection of nutrients. This theory has been validated by observations that older bees are able to modulate their gustatory responses to fluctuating sucrose concentrations more quickly than younger bees, who demonstrated less gustatory plasticity (Ramirez et. al., 2010). A difference in chemosensory sensitivity between foragers and nurses has also been observed in associative learning tasks, as forager bees demonstrate an increased acquisition rate following satiation compared to nurse bees (Ben-Shahar and Robinson, 2001). Such changes are likely to be the result of a combination of factors, primarily hormonal, but with potential for differences in gustatory receptor expression as well.

For many phytophagous insects annual alterations in gustatory sensitivity are important due to seasonal changes in food availability. For instance, as

nutritional plant choices become seasonally limited, many insects must undergo a trade-off between nutritional content and potential toxin consumption. Toxic nectar, for example, is relatively common (Adler, 2000), but when a wide variety of food sources are available many insects exhibit conditioned aversions to toxic foods, distinguishable by their bitter taste. However, as food choice becomes restricted or food deprivation increases, some insects overcome their aversion to toxins in food (London-Shafir et. al., 2003; Singaravelan et. al., 2005; Wu et. al., 2005b). A need to consume nutrients becomes more important than avoidance of toxins and this is reflected in decreased sensitivity to certain bitter compounds.

Alteration in gustatory sensitivity also occurs as a result of nutritional exposure or a function of existing nutritional state. This can be a direct, externally induced desensitisation effect, for instance; exposure of the *Manduca sexta* bitter cell to caffeine can desensitise this cell to further caffeine exposure and reduce subsequent neuronal responses (Glendinning et. al., 1999). A similar response reduction can also be observed in the locust, *Locusta migratoria,* whose gustatory sensitivity is altered as a result of haemolymph changes in nutrient content. Following direct injection of AAs into the maxillary palp "nutrient specific changes" are observed in the Grs on the mouthparts and subsequent responses to AA solutions are severely reduced (Simpson and Simpson, 1992). In contrast, sensitivity to nutrients can be rapidly regulated by internal nutritional state as demonstrated in the blowfly. Enhanced gustatory sensitivity was observed when flies were nutritionally deprived; however, this sensitivity was rapidly diminished following feeding (Omand, 1971).

As we can see the insect gustatory system is far from static, the sensitivities by which it operates can be rapidly changed as a function of both internal and external cues. Despite our knowledge in changing gustatory sensitivity we still know relatively little about alterations that occur at the Gr level. Whilst we have seen that Gr expression can alter over the life cycle of an insect (Scott et. al., 2001) we still have no evidence to link this expression change to an alteration in neuronal sensitivity or behavioural output. In addition, whilst we know gustatory sensitivity alters as a result of pre-feeding history (Dethier, 1961; Omand, 1971; Miyakawa et. al., 1980; Potter and Bertin, 1988; Rathman et. al., 1990), we have no knowledge as to how expression of both receptor neurons and Grs themselves

are altered in response to diet switching or diet restrictions. If an insect is confined to one particular diet for a length of time will it up-regulate receptor expression that preferentially responds to that diet or will expression be down-regulated due to the constant exposure? More gustatory work on long-term dietary studies will be needed in order to depict the exact functions of peripheral sensitivity alterations.

1.7. Conclusions and future work.

The gustatory systems at our disposal have, so far, been limited by molecular advances, with *Drosophila* leading the way in gustatory research. While we can appreciate that molecular work into Gr expression has proven challenging and time consuming, we still do not have a sufficient view of how the system is functioning until all current Grs are studied in depth.

As a priority individual receptors should be the focus as opposed to GRNs. Gustatory receptor neuron ablation studies have proved problematic due to the uncertainty of which Grs are also being affected. In addition to this, the functioning of individual Grs need to be assessed in terms of preferential ligands as well as concentration encoding.

1.8. General aims of thesis

Gustatory properties of food are important sensory stimuli for a variety of animals and are often used as a final means of differentiating between nutritious or potentially harmful food sources. Gustatory systems can often be incredibly complex in order to accommodate for the range of food available, making them difficult to study, especially in mammals. Honeybees are often used as a model animal for ourselves (Menzel, 1983) and gustation is one of the many modalities they possess that we are able to study due to its relatively simple neuronal representation. However gustation, as a chemical sense, has been relatively understudied in these useful insects and further work needs to be carried out in order to fully decipher how honeybees encode soluble chemical stimuli. With a drastically reduced set of gustatory receptors in comparison to *Drosophila* (Robertson and Wanner, 2006) honeybees provide an even simpler alternative to

the existing animal model without, as far as we know, being overly limited in their sensory capabilities.

The majority of the existing work on honeybee gustation has surrounded sensitivities to sugars, as the major dietary component and bitter substances as a representation of toxins. Despite being the second most concentrated component of floral nectar and a major source of protein (Baker and Baker, 1973), amino acids have mostly been ignored as to their role in gustation. This project plans to fill the gap in knowledge on honeybee gustation and combine molecular techniques with behaviour to determine how the Grs are functioning in relation to sugars and amino acids.

1.8.1. Psychophysics of taste and dietary selection

In order to understand how the gustatory system is functioning, it is first necessary to understand what compounds the system is able to perceive and which compounds are actively chosen. The initial aim of this thesis is to understand honeybee gustatory sensitivity toward amino acids, however this will not be possible without also considering sugars, for two reasons. Firstly, amino acids are never encountered without sugar by nectar forgers in a natural context and therefore investigating responses toward pure amino acids will give little insight into honeybee food perception in the wild. Secondly, provisioning bees with amino acid diets, lacking carbohydrates will quickly result in mortality, making separate amino acid assessment challenging. Therefore my gustatory sensitivity assessment will investigate both sugars and amino acids.

The sugar assessment will involve the three most common nectar sugars: sucrose, glucose and fructose. While floral nectar is known to vary widely in composition of these three sugars (Wykes, 1952), little is known about whether this composition affects honeybee choice. Does the honeybee perceive all sugars equally? Will one floral saccharide be preferentially detected and chosen over another? And is this choice mediated by honeybee sugar regulation or previous sugar consumption? This thesis aims to investigate these questions, initially using a dietary confinement assay. Honeybees are exposed to individual sugars to assess their regulation in the haemolymph and effect on survival. Secondly, subjects will undergo a choice assay in which they are able to select between two saccharides to determine a preference and whether this preference is altered

dependent on previous sugar feeding. The role of haemolymph sugar levels will also be assessed by artificially raising sugar levels and recording the subsequent acceptance of dietary sugars.

Honeybee preference for individual amino acids will also be investigated using the same choice assay. Amino acids have been selected from the taste classes originally defined by Shiraishi and Kuwabara (1970) and this thesis aims to determine whether behavioural responses by bees parallel the neuronal detection of AAs as seen in flies. Two amino acids from each of the four taste classes have been chosen, including a selection of both essential and nonessentials, to determine whether the necessity of consumption in the honeybee diet mediates selection. Amino acids will be added to a sucrose solution and bees will be given a choice between that and sucrose alone over a four day period, allowing assessment of AA selection over time. Amino acids will additionally be combined in order to assess whether honeybees prefer a solution more representative of floral nectar.

1.8.2. Gustatory receptor location and life cycle expression.

In their analysis of the 10 honeybee Grs, Robertson and Wanner (2006) demonstrated Gr expression in a number of anatomical locations in the forager honeybee. However, these locations were relatively general e.g. head, body (Robertson and Wanner, 2006). Therefore this thesis aims to assess more specific external gustatory appendages (antenna, galea, labial palps, glossa, fore-tarsi, mid-tarsi and hind-tarsi), in addition to internal expression (brain and gut), with the hope that Gr location may help in determining the role of each Gr. Additionally, honeybees are subject to age-polyethism in which the task differentiation is usually defined by age (Winston, 1987). Part of this age and task differentiation relates to the detection and assessment of potential food items. Therefore this thesis aims to assess expression of the 10 honeybee Grs in both the forager and newly emerged bees to determine whether Gr expression is plastic and potentially modulated by either the age or the occupation of the adult.

1.8.3. The importance of bee nutrition on the expression of the Grs.

Finally, as previously mentioned, it is beneficial for some animals to alter sensitivities toward certain chemical stimuli. This alteration may be a function of external nutrient availability or internal nutritional stores. Very little work to date

has investigated these changing sensitivities at the gustatory receptor level in any animal. Observing changes in receptor expression following specific nutrient exposure may also help in the identification of specific Gr ligands. Therefore the final aim of this thesis is to determine whether the expression of honeybee gustatory receptors is altered following provision of specific diets. These diets were determined from the behavioural results obtained for both sugars and amino acids.

2.0. Chapter 2. Methods

2.1. Introduction

A full description of all methods and materials used for the entire project are described in this chapter. Each subsequent chapter contains a brief overview of the chapter-specific methods, however detailed information for all experiments can be found below.

2.2. Honeybee capture and restraint

Forager (≈3 wk old) adult worker honeybees (*Apis mellifera Buckfast*) were collected returning to three hives situated outdoors at Newcastle University, Newcastle upon Tyne (UK) between April and September each year from 2011-2013. A wire mesh excluder was placed in front of the entrance of the colony to ensure that only returning foragers were collected. Pollen collectors were avoided. Newly emerged adult bees were collected from two brood frames taken from the outdoor hives and stored in a mesh brood box (275 mm X 440 mm X 140 mm) in an incubator at 34°C. The incubator was kept humid via the addition of a small tray of water. Each day bees that emerged from the frame in the previous 24-36 h were removed to ensure the age of the experimental bees did not exceed 48 h.

Bees were captured in individual phials and cold-anesthetised until movement was at a minimum (~2 min). Following anesthetisation, bees were restrained as described in Wright and Smith (2004); briefly, each bee was placed in a modified 1 ml pipette tip with one strip of duct tape placed between the head and thorax and one around the abdomen for restraint, ensuring full use of antennae and mouthparts (Figure 2.1.).

Subjects were left to acclimatise at room temperature (RT) for 20 min then fed to satiety (until mouthparts were lowered and solution refused) with 1 M sucrose to ensure a comparable nutritional state between individuals. Following feeding, bees remained at RT without any further access to food or water for approximately 20-24 h in a humidified box.

2.3. Mouthparts assay

A gustatory assay for assessing the sensitivity of the sensillae of the honeybee's mouthparts (Wright et. al., 2010) was used to test the bees' sensitivity to amino acid and sugar solutions. An initial gustatory assay for the proboscis

extension reflex (PER) (Page et. al., 1998) was used to assess the bees' motivation to respond to antennal stimulation with experimental solutions. Following the starvation period subjects were tested for motivation using a 0.4 µl droplet of 1 M sucrose solution, presented to the antennae. All subjects that failed to elicit a response to the motivation solution were excluded from subsequent trials (approximately 10%). Approximately 10 min after the 1 M sucrose motivation test all bees were supplied with an experimental solution, first to the antenna and then the mouthparts for consumption. The volume of the solution consumed was measured using a 0.2 ml Gilmont micrometre syringe (Gilmont Instruments). Each bee was only provided with one experimental solution and the volume consumed was recorded once the mouthparts were lowered and the solution was refused. Eight amino acids were tested: DL-arginine, glycine, trans4-hydroxy-L-proline (from herein referred to as hydroxyproline), DL-lysine monhydrochloride, DL-methionine, DL-phenylalanine, DL-proline and threonine (all >97% purity, Sigma-Aldrich, except threonine: >99% Alfa Aesar) and were fed to bees at 10 µM. This concentration was chosen as representative of floral nectar (Gottsberger et. al., 1984). Amino acids pH range 5.53 – 6.16. Amino acids were also combined with 100 mM sucrose and fed to bees. Sucrose alone (100 mM, pH: 5.93) was used as a positive control and deionised water (pH: 5.45) alone as a negative control.

Figure 2.1. Dorsal view of the head of a restrained forager honeybee with splayed mouthparts. Photo courtesy of Dr S. Kessler (personal communication).

2.4. Feeding preference assay: methods

Forager honeybees (*Apis mellifera Buckfast*) were collected returning to three hives situated at Newcastle University, Newcastle Upon Tyne (UK) between April and September (2011-2012). Newly emerged bees were collected from two brood frames taken from the outdoor hives and stored in a mesh brood box (275 mm X 440 mm X 140 mm) in an incubator at 34°C (see 2.2. Honeybee capture and restraint).

Bees were captured in individual phials and briefly cold-anesthetised for approximately 1 min. Twenty to thirty subjects were immediately placed in plastic boxes (110 mm x 200 mm x 60 mm, Really Useful Box, Figure 2.2.) and allowed to recover at room temperature (RT) for approximately 1 h without access to food. Experimental solutions were added and boxes were placed in a temperature controlled room 34 ±1°C, 60 ±5% relative humidity (RH) and kept under a D:L 22h:2h light regime to replicate in-hive conditions.

Holes were drilled into the lid of each box (3 mm) to allow for gas exchange and five 12 mm holes (two on one side and three on opposite side) were drilled to allow 2 ml microcentrifuge tubes to be inserted horizontally. Experimental solutions were added to tubes and bees gained access via four 3 mm holes drilled vertically into the top of each. One water tube remained available to the bees at all times. All experimental tubes except the water tube were re-positioned randomly after daily replacement to remove any location bias.

To record consumption, tubes were weighed every 24 h and replaced by a new tube containing a fresh solution. Bees were kept in boxes for 4 days (96 h) and dead insects were counted daily (without removal). The mean daily individual consumption was calculated as an average across the viable insects that remained from the previous 24 h.

A minimum of five replicates were run for each condition (approximately 100-150 bees total). An identical evaporation box (minus honeybees) was run simultaneously with experimental boxes in order to account for tube weight loss due to evaporation. Evaporation rates were calculated daily and subtracted from the experimental volumes prior to individual consumption calculations. Density

measurements were taken for each solution and volumes were calculated by multiplying the weight of volume loss in each tube by these.

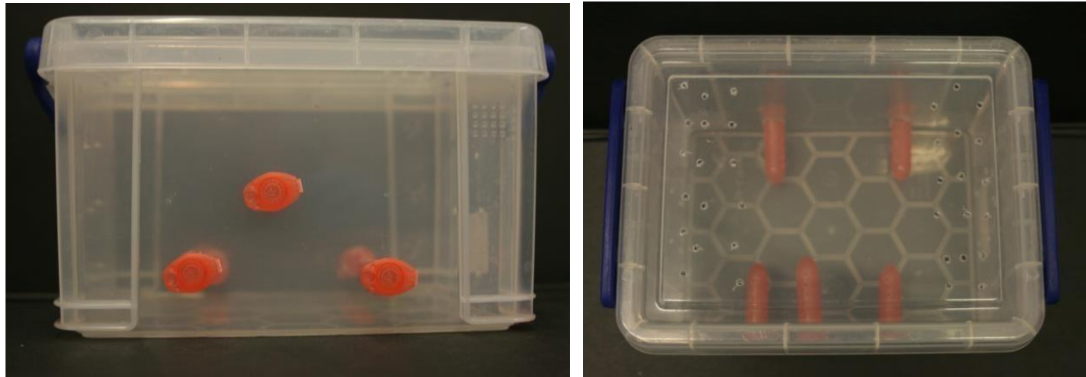

Figure 2.2. Plastic box in which 20-30 bees were placed for specific diet provision and behavioural choice assays

2.5. Feeding preference assay: solutions

2.5.1. Sugar diet assays

In order to assess survival on sugars, both forager and newly emerged bees were restricted to a specific sugar diet for 96 h, in which four tubes were filled daily with a specific sugar solution, the fifth always contained water only. The solutions were as follows: 0.7 M sucrose, 0.7 M glucose, 0.7 M fructose, 1.4 M glucose, 1.4 M fructose or 0.7 M glucose and 0.7 M fructose combined.

2.5.2. Sugar choice assays

A Behavioural choice assay was carried out in which a 0.7 M glucose solution and 0.7 M fructose solution were added to two separate microcentrifuge tubes in each box, the fifth tube contained water only. Boxes were run for 96 h and solutions were replaced daily.

2.5.3. Regulation of sugar intake assays

Feeding was carried out in boxes as stated above (see 2.5.2. Sugar choice assays) with only one type of sugar solution (either 0.7 M glucose or 0.7 M fructose) being provided for 48 h. Each day, the sugar tubes were weighed and replaced. After 48 h, the test solutions (0.7 M glucose and 0.7 M fructose, Table 2.1.) were placed in the box.

Table 2.1. Honeybee diet provision and choice assay. Dietary sugars were offered to bees in boxes over a consecutive 96 h period. Sugars were split evenly across four microcentrifuge tubes with a fifth always containing water only.

Available diet for first 48 h	Available diets for second 48 h
0.7 M glucose	0.7 M glucose 0.7 M fructose
0.7 M fructose	0.7 M glucose 0.7 M fructose

2.5.4. Amino acid choice assays

Behavioural choice tests for foragers and newly emerged bees consisted of two solutions (one amino acid in sucrose and the other sucrose alone) added to two microcentrifuge tubes in each box (see 2.4. Feeding preference assay: methods), the fifth tube contained water only (Table 2.2.). Eight amino acids were tested: DL-arginine, glycine, hydroxyproline, DL-lysine monhydrochloride, DL-methionine, DL-phenylalanine, DL-proline and threonine (all >97% purity, Sigma-Aldrich, except threonine: >99% Alfa Aesar). Tubes were weighed and refilled daily with positions being randomised to remove potential location bias. Amino acids were added from a 1 mM stock solution in 0.7 M sucrose to a final concentration of 10 µM in 0.7 M sucrose for each amino acid. Additionally a combination of all eight experimental amino acids to a final concentration of 80 µM in 0.7 M sucrose was tested against 0.7 M sucrose alone in order to observe any additive effects of amino acids.

Table 2.2. Experimental solutions added to two tubes in the behavioural choice assay boxes, tubes were re-filled daily with these solutions and weighed daily to assess consumption.

Foragers and newly emerged	
Experimental solution 1	**Experimental solution 2**
0.7M sucrose	10μM glycine in 0.7M sucrose
0.7M sucrose	10μM lysine in 0.7M sucrose
0.7M sucrose	10μM threonine in 0.7M sucrose
0.7M sucrose	10μM arginine in 0.7M sucrose
0.7M sucrose	10μM hydroxyproline in 0.7M sucrose
0.7M sucrose	10μM proline in 0.7M sucrose
0.7M sucrose	10μM phenylalanine in 0.7M sucrose
0.7M sucrose	10μM methionine in 0.7M sucrose
0.7M sucrose	80μM AA Mix: 10μM glycine, 10μM lysine, 10μM threonine, 10μM arginine, 10μM hydroxyproline, 10μM proline, 10μM phenylalanine, 10μM methionine in 0.7 M sucrose

2.6. Haemolymph collection

Following 96 h feeding on the 0.7 M sucrose, 0.7 M glucose or 0.7 M fructose solutions, both foragers and newly emerged bees, were collected and harnessed (see 2.2. Honeybee capture and restraint). Haemolymph was collected from these subjects by creating a small incision immediately above the median ocellus using a 1.1 mm X 40 mm needle (BD Microlance). Collection took place using a 10 μl capillary tube (Hirschmann) inserted into the head capsule to withdraw as much haemolymph as possible. Due to bees having fed *ad libitum* prior to harnessing, collection volumes were often small, therefore all samples were pooled from a minimum of five bees to a minimum volume of 2 μl and added directly to a microcentrifuge tube containing 2 μl 0.1 M perchloric acid (Sigma-Aldrich). All volumes over 2 μl were matched with perchloric acid (1:1 ratio) and subsequently stored at -20°C until further processing. Six pooled samples were collected in total for each treatment group and analysed for sugar composition using HPLC (see 2.10. HPLC for carbohydrate analysis).

2.7. Haemolymph collection following satiety feeding

Following capture, harnessing and 24 h starvation (see 2.2. Honeybee capture and restraint) honeybees were fed to satiety with 1 M sucrose. The time taken for each bee to feed was recorded in order to gauge the change in sugar levels from the initiation and termination of feeding. When each bee reached satiety, it was placed in a rack with other bees. At a specific time point after feeding, haemolymph was collected from each bee by making a small incision using a 1.1 mm X 40 mm needle (BD Microlance) above the median ocellus. Haemolymph was collected by inserting a 10 µl capillary tube (Hirschmann) into the head capsule. Each capillary tube was placed in the head capsule for a total of 2 min after the specified time point. The haemolymph was sampled at one of the following time points: 30 s, 1 min, 3 min, 5 min, 10 min, 20 min, and 60 min, after feeding to satiety. Haemolymph was also collected from a subset of bees prior to feeding (time point zero). A minimum of 1 µl of haemolymph was collected and was immediately added to 1 µl 0.1 M perchloric acid, any volume greater than 1 µl was subsequently matched with an equal volume of 0.1 M perchloric acid (1:1 ratio). Any sample less than1 µl was discarded, as was any haemolymph available after the 2 min collection time in order to standardise all samples. Haemolymph samples were taken from approximately 10 bees per treatment group and analysed using HPLC (see 2.10. HPLC for carbohydrate analysis).

2.8. Preparation of haemolymph for HPLC analysis

Haemolymph samples were centrifuged for 10 min at 14,000 rpm (Eppendorf centrifuge 5424), 1 µl of haemolymph was removed and diluted 1:200 with nanopure water (Fisher Scientific). Diluted samples were then filtered through a Puradisc sample preparation syringe filter, nylon 0.45 µm pore, 4 mm diameter (Whatman). High performance liquid chromatography was used to measure concentrations of specific sugars (glucose, fructose, sucrose and trehalose) (See 2.10. HPLC for carbohydrates).

2.9. Satiety feeding and artificial increase of haemolymph sugars

Bees were harnessed, fed to satiety with 1 M sucrose and left overnight (see 2.2. Honeybee capture and restraint). Approximately 18-24 h afterwards, each bee was injected with 1 µl of sugar solution to artificially raise haemolymph sugar concentrations. A small incision was made directly above the median ocellus using a 1.1mm X 40mm needle (BD Microlance) and bees were injected with a specified sugar solution or a water control using a 1 µl Hamilton syringe (Hamilton). Any bees that bled post-surgery were excluded from the experiment. A 'no-injection' control was performed in order to define the minimum length of time, post-surgery, taken for bees to resume normal feeding. Twenty bees were fed 0.7 M sucrose either 10, 20 or 30 min following a water injection and consumption volumes were compared to a 'no-injection' control using an analysis of variance (ANOVA). Twenty min post-injection was the minimum time in which satiety feeding did not differ significantly from the no-injection treatment (ANOVA $F_{1, 81} = 5.40$, $P = 0.007$, LSD *Post hoc:* $P = 0.272$) therefore all subsequent consumption recordings were made 20 min following experimental injection. The experiment followed a full factorial design in which groups of approximately 20 bees were injected with one of three sugars: sucrose, glucose or fructose, at one of four concentrations: 100 mM, 150 mM, 300 mM or 400 mM and then fed to satiety with one of the same three sugars, all 0.7 M (36 treatment groups in total). Each set of injections were always matched with a group of control water injections to check injection precision. All injections were performed blind, the experimenter was unaware of the solution being injected into the head capsule.

2.10. High performance liquid chromatography for carbohydrate analysis

High performance liquid chromatography was used to measure concentrations of specific sugars (glucose, fructose, sucrose and trehalose). HPLC analysis was conducted by injecting 20 µl of sample via a Rheodyne valve onto a Carbopac PA-100 column (Dionex, Sunnyvale, California, USA). Sample components were eluted from the column isocratically using 100 mM NaOH flowing at 1 ml/min. The chromatographic profile was recorded using pulsed amperometric detection (ED40 electrochemical detector, Dionex). Elution profiles were analysed using PeakNET software package (Dionex, Breda, The

Netherlands). Daily reference curves were obtained for glucose, fructose, sucrose and trehalose by injecting calibration standards with concentrations of 10 ppm. for each sugar.

2.11. Molecular biology

2.11.1. Honeybee selection

Honeybee samples were split into two categories: control (unmanipulated) samples and experimental samples. For control samples, forager (≈3 wks old) adult worker honeybees (*Apis mellifera Buckfast*) were collected returning to one hive situated outdoors at Newcastle University, Newcastle upon Tyne (UK) between July and September, 2013. Newly emerged bees (≈24 h old) were collected from two brood frames taken from the outdoor hive and stored in a mesh brood box (275 mm X 440 mm X 140 mm) in an incubator at 34°C. Honeybees were captured individually in small plastic phials and immediately returned to the laboratory for anesthetisation.

For experimental samples, forager honeybees only were captured as above and approximately 20 subjects were transferred into plastic boxes (see 2.4. Feeding preference assay: methods). Honeybees were restricted to one of six diets provided via four modified microcentrifuge tubes, a fifth always containing water alone. Diets were refilled daily and honeybees were maintained on specific diets for a 96 h duration (see Table 2.3.). All diet reagents were >99% purity (Sigma-Aldrich). In experimental samples the 'sucrose' treatment (0.7 M sucrose) was used as a control with mRNA expression levels of all gustatory receptors (Grs) being normalised to 1 for this diet. Gustatory receptor mRNA levels in all remaining experimental samples are expressed as a proportion relative to the control treatment. Only brains were dissected from foragers in the experimental treatment.

An additional sample group of forager honeybees were collected from the hive and harnessed (see 2.2. Honeybee capture and restraint). Restrained bees were then fed 10 µl of 0.7 M sucrose solution by hand using a 0.2 ml Gilmont micrometre syringe (Gilmont instruments). Honeybees were subsequently starved for 24 h and the sample tissues (brains) collected represented a 'hunger' control

(this was the minimum volume and concentration of sugar I could use in order to maintain a viable sample size over 24 h).

Table 2.3. Specified diets and duration of diet for forager honeybees prior to dissection and experimental sample collection for RT-qPCR (Reverse Transcription – quantitative Polymerase Chain Reaction).

Diet	24h	96 h
0.7 M sucrose		X
0.7 M glucose		X
0.7 M fructose		X
10 μM hydroxyproline in 0.7 M sucrose		X
10 μM glycine in 0.7 M sucrose		X
10 μM methionine in 0.7 M sucrose		X
10 μl of 0.7 M sucrose (hunger)	X	

2.11.2. Sample collection

Following capture, subjects were transferred onto ice until cold-anesthetised. Dissections took place under a light microscope; all traces of RNase were removed from the dissection area and tools using RNaseZAP solution (Sigma-Aldrich). For 'hard' tissues 75 body parts were collected for a sample, however due to the time of dissection not all body parts came from the same subjects. Body parts collected: both antennae (2 samples), both galea (2 samples), both labial palps (2 samples), individual glossa (1 sample), 6 tarsi separated into pairs: fore-tarsi (2 samples), mid-tarsi (2 samples) and hind-tarsi (2 samples). Tarsi consisted of five tarsomeres, including basitarsus, distal pretarsus and tarsal claws. External body parts were only collected from the 'control' forager and newly emerged honeybees (see 2.11.1. Honeybee selection). Dissected body parts were immediately transferred into 500 μl of TRIzol reagent (Invitrogen) and transferred to -80°C until homogenisation. Samples were homogenised by hand using an Eppendorf micropestle (Sigma-Aldrich) and a further 500 μl of TRIzol was added to the samples (1 ml total). A maximum of two samples were collected for each of the external body parts.

Due to ease of RNA extraction of 'soft' tissues only five whole brains and guts (from the crop to the rectum) were collected for each sample, these were immediately transferred into 1 ml of TRIzol reagent, without undergoing any homogenisation and placed in -80°C until further processing. A total of four samples were collected for each of the brains and guts from ‘control’ (unmanipulated) honeybees. Only four brain samples were collected from the forager honeybees in the ‘experimental’ groups (see 2.11.1. Honeybee selection).

2.11.3. Sample preparation

Total RNA extraction followed the TRIzol reagent protocol (Invitrogen) with a few modifications. Pure chloroform (200 µl) was added (Sigma-Aldrich) and the samples were shaken vigorously by hand for 15 s then incubated (RT for 3 min). Samples were centrifuged for 15 min at 12,000g, 4°C (phase separation) and the subsequent aqueous phase was removed in full and added back into 750 µl of TRIzol. The chloroform step was repeated, however extra care was taken to avoid any phenol carry over by removing only the top 80% of subsequent aqueous phase. Isopropanol (500 µl, Sigma-Aldrich) was added to samples along with 2.5 µl of a co-precipitant (Glycoblue, Ambion) before being placed in -80°C overnight. Samples underwent a double ethanol (75%, Fisher Scientific) wash and the remaining extraction steps followed TRIzol protocol with samples being re-suspended in 20 µl of RNase and DNase-free water (AccuGENE, Lonza). One microliter of the extracted RNA was transferred onto a Nanodrop spectrophotometer ND-1000 in order to determine the total RNA yield for DNase treatment. A total of 2000 ng of sample RNA (4500 ng for brain and gut samples) were treated with RNase-free DNase (Promega) following the manufacturer’s instruction and 1 µl of the subsequent sample was assessed on the spectrophotometer, blanking the instrument first with a “DNase treated” water sample. RNA concentration varied from 0.3-4.2µg and optical density (OD) 260/280 was >1.8, 260/230 was >1.8 and 1000 ng of RNA from all samples were added to the reverse transcription reaction. cDNA synthesis was carried out on a Techne TC-5000 PCR machine following the manufacturer’s protocol for Superscript III reverse transcriptase (Invitrogen). cDNA samples were then transferred to -20°C until further processing.

2.11.4. Primer design and reverse transcription polymerase chain reaction (RT-PCR).

Oligonucleotides were manually designed to all 10 honeybee gustatory receptor sequences provided by Hugh Robertson (supplementary to Robertson and Wanner, 2006) with an optimal size of 20bp (17-27 range), 50% GC content (30-65% range) see Table 2.4. Where possible primers were designed to span an exon boundary in order to minimise gDNA contamination. Two reference genes were selected to ensure successful reverse transcription. Reference genes were further required for quantification to control for variations in cDNA across samples, both genes have been previously published as appropriate reference genes for RT-qPCR on honeybees; *A. mellifera ribosomal protein S8* (RS8) as used in Robertson and Wanner (2006) and *A. mellifera ribosomal protein 49* (RP49) as used in Ament et. al. (2011). All PCR results were analysed using both reference genes and after ensuring that no changes in reference gene expression was occurring across tissues, RP49 was used in all final analyses. End-point PCR was carried out as a check for successful DNase treatment and primer validation, reactions contained; 1 µl of forward and reverse primers (5 µM), 10 µl RNase-free water, 12.5 µl MyTaq HS Mix DNA polymerase (Bioline) and 0.5 µl cDNA. The program comprised:

	95°C	1 min initial denaturation
35 cycles	95°C	15 s denaturation
	X*^{o}C	15 s annealing
	72°C	10 s elongation
	4°C	Final hold

*The annealing temperature X can be found in Table 2.4.

Samples were held at 4°C until further processing. PCR products were then run for 40 min at 65 V on a 2% agarose gel (Promega) against hyperladder IV (Bioline) to check product size.

Table 2.4. Gustatory receptor primer table, including forward and reverse sequences and corresponding annealing temperatures for all 10 honeybee gustatory receptors and two reference genes (ribosomal protein 49 and ribosomal protein S8).

Gustatory receptor/ Ribosomal protein	RNA accession number	Length of amplicon	GC content	Primer length	Tm according to Eurofins	Primers designed 5'-3'	Location in whole genome shotgun sequence	Annealing Temerature for RT-PCR (°C)
AmGr1	NW_003378096.1	340	40% 45%	20 20	53.2 55.3	Forward primer: ATCGATAATCCACGGTTACT Reverse primer: CAGTTGTCTCGTTAAGGTTG	Amel_4.5 Group5.14. Range FOR: 1: 1184039 to 1184059 Range REV: 1: 1184514 to 1184534	55
AmGr2	NW_003378145.1	109	47% 47%	19 19	54.5 54.5	Forward primer: CGCTCAAATATTCGGCATG Reverse primer: GGCGATGAAACCTGAATAC	Amel_4.5 Group5.18. Range FOR 1: 490325 to 490344. Range REV: 1: 490413 to 490432	55

AmGr3	NW_003377939.1	171	33% 48%	21 21	52 57.9	Forward primer: GCGTACTTGTATTACTACTTA Reverse primer: GGAAAGGAGAGCCAACAATAC	Amel_4.5 Group8.6. Range FOR: 1: 695189 to 695210. Range REV: 1: 695339 to 695359	55
AmGr4	NW_003377876.1	168	47% 50%	17 18	50.4 53.7	Forward primer: CATCGTTTGCAACAACC Reverse primer: GCCTGCGAAAATTGTAGG	Amel_4.5 Group13.3. Range FOR: 1: 1552 to 1569. Range REV: 1: 1701 to 1719	55
AmGr5	NW_003378176.1	217	50% 50%	20 18	57.3 53.7	Forward primer: GTACGATCGATCGAGAAACG Reverse primer: CTGCATTGCGTGCAATTG	Amel_4.5 Group1.34. Range FOR: 1 76932 to 76952. Range REV: 1: 77282 to 77300.	55

AmGr6	NW_003377880.1	209	44% 50%	18 18	51.4 53.7	Forward primer: CAGATGAATGTTTCCGTG Reverse primer: CGAATACAAGAGCGAGTC	Amel_4.5 Group3.3. Range FOR: 1: 379638 to 379656. Range REV: 1: 379828 to 379846.	55
AmGr7	NW_003378095.1	190	44% 47%	18 19	51.4 54.5	Forward Primer: GGCAACATTATTTGCGAG Reverse primer: CTTGGATCATGACTACGAG	Amel_4.5 Group9.12. Range FOR 1: 1011871 to 1011988. Range REV: 1: 1012185 to 1012204.	55
AmGr8	NW_003378027.1	276	40% 58%	20 19	53.2 58.8	Forward primer: CAATACAGAAGTAGGCAAGA Reverse primer: GCACGTCATGTCCGTCACA	Amel_4.5 Group13.9. Range FOR: 1: 329776 to 329796. Range REV: 1: 3301 71 to 330190	50

AmGr9	NW_003378027.1	288	35% 50%	23 20	55.3 57.3	Forward primer: GCATTTAGAGGAGAAACATTTAG Reverse primer: GCGTCATAAAGGGTCCACTT	Amel_4.5 Group13.9. Range FOR: 1: 331301 to 331324. Range REV: 1: 3315V68 to 331588	53
AmGr10	NW_003378093.1	306	45% 59%	20 17	55.3 55.2	Forward primer: CTGACAAGATAATAGAGGCG Reverse primer: ATTCGCCTGATGAGCCG	Amel_4.5 Group4.13. Range FOR: 1: 1963155 to 19631574. Range REV: 1: 19638 56 to 1963879.	55
RP49	AF441189.1	100	41% 30%	22 27	56.5 57.4	Forward primer: GGGACAATATTTGATGCCCAAT Reverse primer: CTTGACATTATGTACCAAAACTTTTCT	Amel_4.5 Group11.16. Range FOR: 1: 945031 to 945053. Range REV: 1: 945103 to 945130.	60

RPS8	NM_001011604.3	182	555 65%	20 20	59.4 63.5	Forward primer: GGTGCGAAACTGACTGAAGC Reverse primer: TCCTCACGACCGCACTGTCC	Amel_4.5 Group9.10.1: Range FOR 1751793 to 1751813. Range REV: 1751519 to 1751539	60

2.11.5. Sequencing

PCR products were diluted to 200 ng/µl in 20 µl with RNase and DNase free water (AccuGENE, Lonza) and sent for forward sequencing to Genevision (INEX Business Centre, Herschel Building, Newcastle Upon Tyne) with the corresponding forward primer (20 µl, 3.2 µM). Once products were confirmed to match our receptor gene sequences and the corresponding insect genome (see Appendix 1), samples were then analysed quantitatively. Sequencing ensured that the expected product was being amplified and that samples were not contaminated with genomic DNA.

2.11.6. Reverse transcription quantitative-PCR (RT-qPCR)

Quantitative real time-PCR was performed on a Roche LightCycler 480, each reaction contained 7 µl RNase-free water, 1 µl of each forward and reverse primer (5 µM), 10 µl LightCycler SYBR Green I Master (Roche) and 1 µl cDNA and was run in a 96 well plate (Starlab). Each sample was run in duplicate for 50 cycles with the following cycling parameters:

	95ºC	5 min initial denaturation
50 Cycles	95ºC	15 s denaturation
	55ºC	30 s annealing
	72ºC	1 min elongation

1 cycle melting	95ºC	5 s
	65ºC	1 min
1 cycle cooling	40ºC	10 s

All samples were normalized to the reference gene RP49 (Ament et. al. 2011). The standard curve, generated from 2 µl of cDNA from all samples (for control samples) and 4 µl of cDNA from all brain samples (for experimental samples), consisted of six serial dilutions (1:1, 1:2, 1:4, 1:16, 1:32) and was used

to calculate efficiency[1] values of target and reference gene primer sets. The efficiencies of the primers ranged between: 1.796 - 2.001. A melt curve analysis was additionally carried out on each plate to ensure single product quantification. To be able to compare separate Gr primer pair reactions for the control samples, a final plate containing a reaction for each set of primer pairs with a mixture of all forager brain samples in triplicate was used. Expression of gustatory receptor 1 (AmGr1) was randomly selected as the 'control' sample and the difference in expression levels between AmGr1 and each Gr gene was used as a ratio to multiply expression levels of the specific Gr gene across tissue samples.

Relative mRNA levels were expressed as a proportion relative to the forager brain expression levels which were always normalised to 1.

2.12. Scanning electron microscopy

2.12.1. Tissue collection

Following capture and experimentation (See 2.3. Mouthparts assay) forager honeybees were placed on ice and cold-anesthetised before removal of the whole head and fore-tarsi (five tarsomeres, including basitarsus, distal pretarsus and tarsal claws). Eight whole heads and four tarsi were collected in total. An attempt to extend and splay the mouthparts of each head was made for imaging. All samples were added to 50 ml falcon tubes (Fisher Scientific) containing 2% glutaraldehyde in Sorenson's phosphate buffer (TAAB Laboratory Equipment, Aldermaston).

2.12.2. Fixation and dehydration

Samples were maintained for 24 h in 2% glutaraldehyde in Sorenson's phosphate buffer. Following the 24 h fixation, samples were 'rinsed' three times in Sorenson's buffer by adding samples into a fresh tube of buffer for a minimum of 1 h. Samples underwent dehydration from an ascending concentration series of ethanol solutions. Initially samples were added to a 25% ethanol solution (Fisher Scientific) for 30 min, 50% ethanol for 30 min, 75% ethanol for 30 min,

---

[1] * "Theoretically, the number of templates should double after each cycle. In practice, the DNA increases by a factor of (1+*N*) where *N* is the cycle efficiency. Thus an efficiency of *N*=1 would imply a doubling of the DNA concentration." (Booth et. al., 2010).

100% ethanol for 1 h, again 100% ethanol for 1 h. Final dehydration took place in a Baltec critical point dryer with carbon dioxide (EM Research Services, Newcastle University).

2.12.3. Coating and microscopy

Whole dehydrated samples were mounted onto an aluminium stub using Achesons silver dag (Agar Scientific, Stansted) and dried overnight. The following day samples were gold coated, standard 15 nm, using a Polaron SEM coating unit (EM Research Services, Newcastle University).

Once prepared samples were examined using a Stereoscan 240 scanning electron microscope, housed within EM Research Services, Newcastle University. Digital images were collected using Orion6.60.6 software.

2.13. Phylogenetic tree construction

Each *Apis mellifera* gustatory receptor (AmGr) genomic nucleotide sequence (provided by Hugh Robertson, Robertson and Wanner, 2006) was analysed in a BLASTn search on the National Centre for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi?

PAGE_TYPE=BlastSearch&BLAST_SPE C=OGP__7460__9555) against the *Apis mellifera* genome (Amel_4.5 reference assembly top level). The protein sequence of the subsequent 'top hit' (which was always a 100 % match) was then added into a BLASTp search using the UniprotKB/Swiss-prot database in order to find well annotated sequences, with an expect threshold of 0.05. All returned sequences, from *Drosophila melanogaster* and the original *Apis* sequences, underwent a multiple alignment analysis in ClustalW and ClustalX version 2.0 using a BLOSUM62 matrix with default settings (Larkin et. al., 2007). Aligned sequences were subsequently used to build a phylogenetic tree in MEGA version 6 (Tamura et. al., 2013). Both neighbour joining and maximum likelihood (ML) analyses were constructed using a 500 replication bootstrap method. Once both outputs were confirmed to match, the final phylogenetic tree was constructed using a maximum likelihood analysis.

2.14. Statistical methods

All data analyses were performed using SPSS version 21.0.. Continuous data were analysed using a Univariate Analysis of Variance (ANOVA) with Least Significant Difference (LSD) *post hoc* comparisons. As a parametric test, an ANOVA assumes normality and therefore all data were analysed using a frequency histogram and found to be normally distributed prior to statistical analysis. Repeated measures ANOVA was used to analyse 96 h box data. During box diet experiments, water consumption did not differ between treatment for foragers (repeated measure ANOVA: $F_{8, 36} = 2.56$, $P = 0.052$) or newly emerged bees (repeated measures ANOVA: $F_{8, 36} = 1.45$, $P = 0.178$) and therefore water consumption was excluded from all analyses. A sugar preference index (SPI) was calculated for the choice tests by (solution 1 volume – solution 2 volume) / (solution 1 volume + solution 2 volume), a positive preference score indicates a solution 1 preference and a negative preference score indicates a solution 2 preference. A Generalised linear model (GZLM) was carried out on sugar haemolymph concentrations following satiety feeding. Survival analysis was carried out using a Cox regression with all diets compared to the 0.7 M sucrose diet.

Following RT-qPCR on control samples, relative mRNA expression was calculated using the 2ΔΔCt method (Livak and Schmittgen, 2001) against the reference gene RP49 where $\Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{target}$. Expression level of mRNA in the forager brains was used as the 'control' sample and expressed as 1 by:

AVERAGE (Σ(2ΔΔCt/AVERAGE2ΔΔCt))

This was done separately for every Gr, all other expression levels were normalised to this value.

Due to the small sample sizes, no statistical analyses were carried out on expression levels of any Gr in any tissue except brains and guts. Expression levels of gustatory receptor mRNA in brain and gut samples were analysed in SPSS version 21.0. A generalised linear model (GZLM) was carried out separately for each gustatory receptor with age (forager vs newly emerged) and body part (brain vs gut) used as independent variables. A pairwise comparison

was carried out with Sidak adjustment for multiple comparisons. Significant *P* value < 0.05.

Following RT-qPCR on experimental samples, relative mRNA expression was calculated using the 2ΔΔCt method (Livak and Schmittgen, 2001) against the reference gene RP49, as above. This was done separately for every Gr and the 0.7M sucrose diet was classed as the ‘control’ diet and normalised to 1, all other expression levels were normalised to this value. Expression levels of gustatory receptor mRNA in bee brains were analysed in SPSS version 21.0. A generalised linear model (GZLM) was carried out separately for each gustatory receptor with dietary treatment at 96 h as an independent variable. A *post hoc* pairwise comparison using Least Significant Difference (LSD) adjustment was additionally carried out between each diet.

Chapter 3.0. Sugar regulation in the honeybee

The honeybee diet consists primarily of carbohydrates. Whilst a whole range of sugars are usually present in floral nectar, most flower species consistently produce three main saccharides: sucrose, glucose and fructose. The exact concentrations of each sugar can vary widely between floral species and all three are phagostimulatory to the honeybee. However, little work has revealed whether bees are able to differentiate, either pre-or post-ingestively, between these main sugars. Additionally, it is unknown whether any one of these sugars provides a greater nutritional benefit to honeybee survival and whether bees regulate sugar consumption and metabolism. The present study revealed that honeybees are able to pre-ingestively differentiate between all three sugars, with sucrose appearing to be the most phagostimulatory and promoting the highest levels of survival in forager bees. Furthermore, sucrose levels in bee haemolymph most significantly influenced future dietary choice and may additionally regulate activation and inhibition of feeding via internal nutrient sensors. Between the monosaccharides, fructose appears most phagostimulatory, both at the periphery and over time. Both newly emerged and forager bees will consume fructose at a rate equal to, or greater than, glucose in a choice assay, even after pre-exposure to fructose alone. This work highlights that not all dietary sugars are treated equally by the honeybee and that the concentration of floral sugars provided in nectar may actively influence honeybee floral choice.

3.1. Introduction

All aspects of survival, from metabolic functioning and growth, to tissue repair and motility, are energetically costly. In order to meet energetic demands an animal must assess its current nutritional and metabolic status and adjust dietary input accordingly. This is accomplished by controlling the intake of essential macronutrients such as protein and carbohydrate (Simpson and Raubenheimer, 1997).

Carbohydrates are the primary energy source for most animal species. A range of carbohydrates are often incorporated into the diet, but the two most common saccharides that contribute to energy production and storage are glucose and fructose. In mammals, glucose can be converted to fructose via the polyol biochemical pathway (Lanaspa et. al., 2013), and ingested fructose can also to be converted to glucose, at least in humans (Feinman and Fine, 2013; Sun and Empie, 2012) and *Drosophila* larvae (Mishra et. al., 2013). For insects, trehalose, a disaccharide composed of two glucose units, is also important and often referred to as the major sugar in insect haemolymph (Wyatt and Kalf, 1957). Many studies have identified trehalose synthesis in the fat body from glucose alone (Candy and Kilby, 1961; Blatt and Roces, 2001; Arrese and Soulages, 2010), however Candy and colleagues (1997) additionally highlighted that other monosaccharides, such as fructose and mannose, may also contribute to the synthesis of trehalose.

The homeostatic regulation of blood glucose in mammals is currently thought to be mainly accomplished via the peptidergic signals, insulin and glucagon. Following a meal, glucose is actively transported across membranes in the intestine via membrane associated carrier proteins (Bell et. al., 1990) leading to an increase in blood sugar. This rise is rapidly detected and triggers a series of events which include a brain-centred glucoregulatory system (BCGS) and insulin secretion from pancreatic islets, to lower blood sugar levels. The BCGS promotes glucose disposal and uptake by muscle and adipose tissues, alongside glycogen synthesis. Additionally insulin acts on the liver, suppressing hepatic glucose synthesis (Schwartz et. al., 2013) and inhibiting glucagon production. Glucagon is synthesised and released in response to decreasing blood glucose

levels and works in an opposing manner to insulin. In contrast to fructose, glucose can be utilized directly by tissues, such as the muscles and brain, through its oxidisation to adenosine triphosphate (ATP).

Unlike glucose, the absorption of fructose from the intestinal lumen of mammals uses both passive and active mechanisms, making it a slightly slower process (Riby et. al., 1993; Sun and Empie, 2012). Once absorbed, fructose metabolism is carried out solely in the liver by the enzyme ketohexokinase (KHK), more commonly referred to as fructokinase. Much of the recent work on the dietary impacts of fructose has investigated its role in medical conditions such as obesity, metabolic syndrome and other related diseases (Johnson et. al., 2007; Tappy and Le, 2010). Fructose is more likely to be synthesized into fatty acids following its conversion to fructose–1–phosphate, as it bypasses an additional regulatory step in the glycolysis process undergone by glucose and therefore can be converted to fat more efficiently (Lyssiotis and Cantley, 2013).

We know less about how carbohydrate regulation is accomplished in insects, but some of the principles of regulation are the same. Insulin-like peptides (ILPs), sharing amino acid sequence similarity with mammalian insulin, were first identified in the silk moth; *Bombyx mori* (Nagasawa et. al., 1984) and have since been reported in several insect species including *Drosophila* (Ikeya et. al., 2002; for review see Gronke and Partridge, 2009), honeybees (Wheeler et. al., 2006) and mosquitos (Riehle and Brown, 2002; Krieger et. al., 2004; Riehle et. al., 2006). Insulin-like peptides are responsible for a range of physiological functions including glucose regulation (Masumura and colleagues, 2000). In *Drosophila*, seven ILP genes exist (*dilp1-7*) and their expression depends on anatomical location and stage of life cycle (Brogiolo et. al., 2001). Starvation experiments in larvae of *Drosophila* and *B. mori,* lead to the down-regulation of some of these ILPs (Masumura et. al., 2000; Ikeya et. al., 2002; Min et. al., 2008), suggesting that they are used to signal nutrient levels. When ILP-producing neurosecretory cells in the brain of *Drosophila* adults and larvae were ablated, fasting haemolymph glucose levels were elevated compared to flies with intact ILP cells (Rulifson et. al., 2002; Broughton et. al., 2005). These levels are comparable to those of diabetic mammals, lending support to insects as a good model for investigating sugar regulation. Some discrepancies do exist however, as the

function of ILPs in some species such as honeybees and mosquitos appear to work in the opposite way to mammalian insulin (Ament et. al., 2008; Brown et. al., 2008, respectively) highlighting sugar regulation as a diverse and complex mechanism.

A feedback pathway similar to mammalian glucagon also exists in insects. In mammals, when the blood glucose titre gets too low, glucagon is released and works to help increase glucose levels. In insects, low haemolymph sugars leads to the release of adipokinetic hormones (AKHs) from the corpora cardiac (CC) cells (Oudejans et. al., 1993; Van der Horst et. al., 1999). The role of AKHs in sugar homeostasis has been widely investigated, for example; Kim and Rulifson (2004) discovered that, following a period of starvation, *Drosophila* larvae, in which the AKH-producing cells of the CC had been ablated, displayed up to 75% lower haemolymph glucose levels than control larvae. Passier and colleagues (1997) also identified that the presence of trehalose along with glucose alone was enough to inhibit the release of specific AKHs in the locust, whereas the disaccharide sucrose had no such effect, suggesting that such peptidergic sugar signalling is specific to particular saccharides (e.g. glucose and trehalose).

Due to generally lower levels of fat storage in insects (Arrese and Soulages, 2010) the intake of sugars could be considered more important than for mammals. In mammals the production of triglycerides, fatty acids and glycogen is efficient and partly responsible for the growing obesity epidemic, however, fat-storage in insects is not the same. This is particularly true in the honeybee as fat stores are known to differ between the age and occupation of individuals (Toth et. al., 2005). In-hive bees possess much greater lipid and protein stores compared to foragers and depletion in these stores in partly responsible for the transition from nurse to forager (Toth et. al., 2005). While insects possess some glycogen stores and a fat body—functionally equivalent to mammalian liver and fat tissue—insect haemolymph sugars tend to act as a direct energy source and are often variable and relatively concentrated compared to mammals (Arrese and Soulages, 2010). Trehalose is thought to be the primary insect sugar and is considered a major energy store, rather than fat. Despite the synthesis and degradation of trehalose being under hormonal control, some studies have noted a lack of homeostatic control over trehalose levels themselves

(for review see: Thompson, 2003). While it is clear that glucose levels in the blood and tissues of mammals is strongly regulated, we do not know whether the homeostatic levels of such sugars in insects are equally maintained.

The primary job for the honeybee forager is to collect nutritious floral rewards and return them to the hive in order to meet colony demands. Carbohydrates are the main constituent of floral nectar and generally consist of three primary sugars: sucrose, glucose and fructose. The concentrations and volumes of each of these sugars can vary greatly dependent on floral species, season and time of day (Wykes 1952). In a review of nectar composition from 889 angiosperm species Percival (1961) categorised three major groups of nectars, namely: sucrose dominant, glucose and fructose dominant, or balanced nectars in which equal quantities of all three were detected. In the monosaccharide-dominant nectars, neither glucose nor fructose were identified as notably greater, with most nectars containing comparable quantities (Percival, 1961).

Studies of honeybee physiology have frequently reported high levels of sugar in honeybee haemolymph, despite some variability as a result of age, season, activity levels and previously consumed solutions (Bounaise, 1981; Arslan et. al., 1986; Crailsheim, 1998a; Fell, 1990; Leta et. al., 1996). It is these high levels of sugar that are thought to fuel the majority of honeybee flight (Beenakkers, 1969; Sacktor, 1970; Toth and Robinson, 2005; Rothe and Nachigall, 1989). Despite many floral nectars being rich in sucrose, there is very little, if any, sucrose found in the honeybee haemolymph (Fell, 1990; Abou-seif et. al., 1993; Woodring et. al., 1993; Blatt and Roces, 2001). As a disaccharide of glucose and fructose, sucrose appears to be broken down into these two components almost immediately after ingestion (Crailsheim, 1988b). Previous behavioural and electrophysiological studies have highlighted an attractive property of each of these saccharides, but many report a greater preference toward sucrose (Whitehead and Larson, 1976b), perhaps matching favourable nectar compositions. We know that both glucose and fructose are important in honeybee metabolism (Crailsheim, 1988b; Blatt and Roces, 2001), however we do not know whether bees demonstrate an active preference or regulatory mechanism between them.

In the current study, I first determined whether the sugars sucrose, fructose, and glucose equally supported survival of bees over a 96 h period. Bees clearly regulate their intake of carbohydrates when fed diets composed of sucrose and essential amino acids (Paoli et. al. 2014a) and so the current study investigated whether this was possible with sugars alone. Sucrose is metabolised into fructose and glucose by enzymatic hydrolysis. Whether or not animals can independently regulate their intake of glucose and fructose has rarely been tested. To investigate this issue, bees were offered a choice between glucose and fructose to test whether they were able to regulate their intake and if their choice was altered following a specific sugar diet. This study aimed to assess the importance of haemolymph sugar levels on future dietary decisions separately from any effects associated with the actual consumption of food; such as external chemosensor activation, food ingestion or food absorption. By artificially elevating the blood sugar levels in the honeybee via injection and assessing the subsequent effects on feeding, I tested whether elevation of fructose, glucose, or sucrose influenced feeding.

3.2. Materials and methods

3.2.1. Feeding assay and Solutions

Note: For detailed methods see 2.4. Feeding preference assay: methods and 2.5. Feeding preference assay: solutions.

Forager adult worker honeybees (*Apis mellifera Buckfast*) and newly-emerged adult bees were placed in plastic boxes for 96 h and provided with experimental solutions: 0.7 M sucrose, 0.7 M glucose, 0.7 M fructose, 1.4 M glucose, 1.4 M fructose or 0.7 M glucose and 0.7 M fructose combined. Total consumption was recorded daily. Additionally, two behavioural choice assays were carried out; the first, a choice between 0.7 M glucose and 0.7 fructose for 96h and the second allowed feeding on only one sugar (either 0.7 M glucose or 0.7 M fructose) for 48 h, followed by the choice between both for 48 h. The mean daily individual consumption was calculated as an average across the viable insects from the previous 24 h.

3.2.2. Sugar feeding and haemolymph collection

Note: For detailed methods see 2.6. Haemolymph collection and 2.7. Haemolymph collection following satiety feeding.

Following 96 h of 0.7 M sugar (glucose, fructose or sucrose) feeding, haemolymph was collected from both forager and newly emerged bees using a 10 µl capillary tube inserted into the head capsule. All samples were pooled and added directly to 1:1, 0.1 M perchloric acid. Samples were analysed using HPLC.

Additionally, haemolymph was collected from forager bees only, as above, at specified time points after satiety feeding with 1 M sucrose: 30 s, 1 min, 3 min, 5 min, 10 min, 20 min, and 60 min. Haemolymph was also collected from a subset of bees prior to feeding.

3.2.3. Haemolymph sugar manipulation

Note: For detailed methods see 2.9. Satiety feeding and artificial increase of haemolymph sugars.

Forager bees were injected with 1 µl of sugar (sucrose, glucose or fructose) at one of four concentrations (100 mM, 150 mM, 300 mM or 400 mM) directly above the median ocellus. Bees were then fed to satiety with one of the same three sugars, all 0.7 M (36 treatment groups in total). All consumption recordings were made 20 min following injection.

3.2.4. Statistical methods

All data analyses were performed using SPSS v. 21.0. Continuous data were analysed using an Analysis of Variance (ANOVA) with Least Significant Difference (LSD) *post hoc* comparisons. Repeated-measures ANOVA was used to analyse 96 h box data. During box restriction experiments, water consumption did not differ between treatment for foragers (repeated measure ANOVA: $F_{8, 36} = 2.56$, $P = 0.052$) or newly emerged bees (repeated measures ANOVA: $F_{8, 36} = 1.45$, $P = 0.178$) and therefore water consumption was excluded from all analyses. A sugar preference index was calculated for the choice tests, a positive preference score indicates a 0.7 M fructose preference and a negative preference score indicates a 0.7 M glucose preference. A generalised linear model was carried out on sugar haemolymph concentrations following satiety feeding. Survival analysis was carried out using a Cox regression with all diets compared to the 0.7 M sucrose diet.

3.3. Results

3.3.1. Specific sugar diets affects survival

Both forager and newly emerged honeybees consumed the same average volume of all solutions every day (Figure 3.1., respectively; repeated measures ANOVA, day: $F_{1, 36} = 0.27$, $P = 0.606$; $F_{1, 36} = 2.64$, $P = 0.113$). However, the volume consumed of each solution over 96 h was dependent on the diet available (Table 3.1.) for both foragers (Figure 3.1A, repeated measure ANOVA, treatment: $F_{3, 16} = 3.93$, $P = 0.028$) and newly emerged bees (Figure 1B, repeated measure ANOVA, treatment: $F_{3, 16} = 4.20$, $P = 0.023$).

When comparing the effects of concentration on glucose and fructose consumption (Figure 3.1., 3.2.), I observed differences between forager and newly emerged bees. Concentration (0.7 M or 1.4 M) did not affect the volume consumed of either fructose or glucose by newly emerged bees (Figure 3.1B, Figure 3.2B, repeated measure ANOVA, treatment: $F_{3, 16} = 1.38$, $P = 0.285$). An increase in the concentration of fructose (1.4 M) for forager bees also failed to affect volume consumption (Figure 3.1A, Figure 3.2A, repeated measure ANOVA, treatment: $F_{3, 16} = 3.00$, $P = 0.061$ LSD *Post hoc* $P = 0.586$). However, 1.4 M glucose was consumed at significantly lower volumes by forager bees than 0.7 M glucose, across the 96 h (Figure 3.1A, Figure 3.2A, LSD *Post hoc* $P = 0.01$).

In terms of survival, foragers had greater sensitivity to dietary sugars than newly emerged bees (Figure 3.3.). Forager bee survival peaked on the 0.7 M sucrose diet, in which 91.2% of subjects survived the 96 h experimental duration (Figure 3.3A). Only the 1.4 M glucose treatment matched this survival rate (Table 3.2.); foragers did not survive well on either of the 0.7 M monosaccharides alone or when given a choice between the two (0.7 M fructose vs. 0.7 M glucose). In contrast to foragers, all dietary treatments maintained the survival rate of newly emerged bees in comparison to 0.7 M sucrose alone (Figure 3.3B, Table 3.3.).

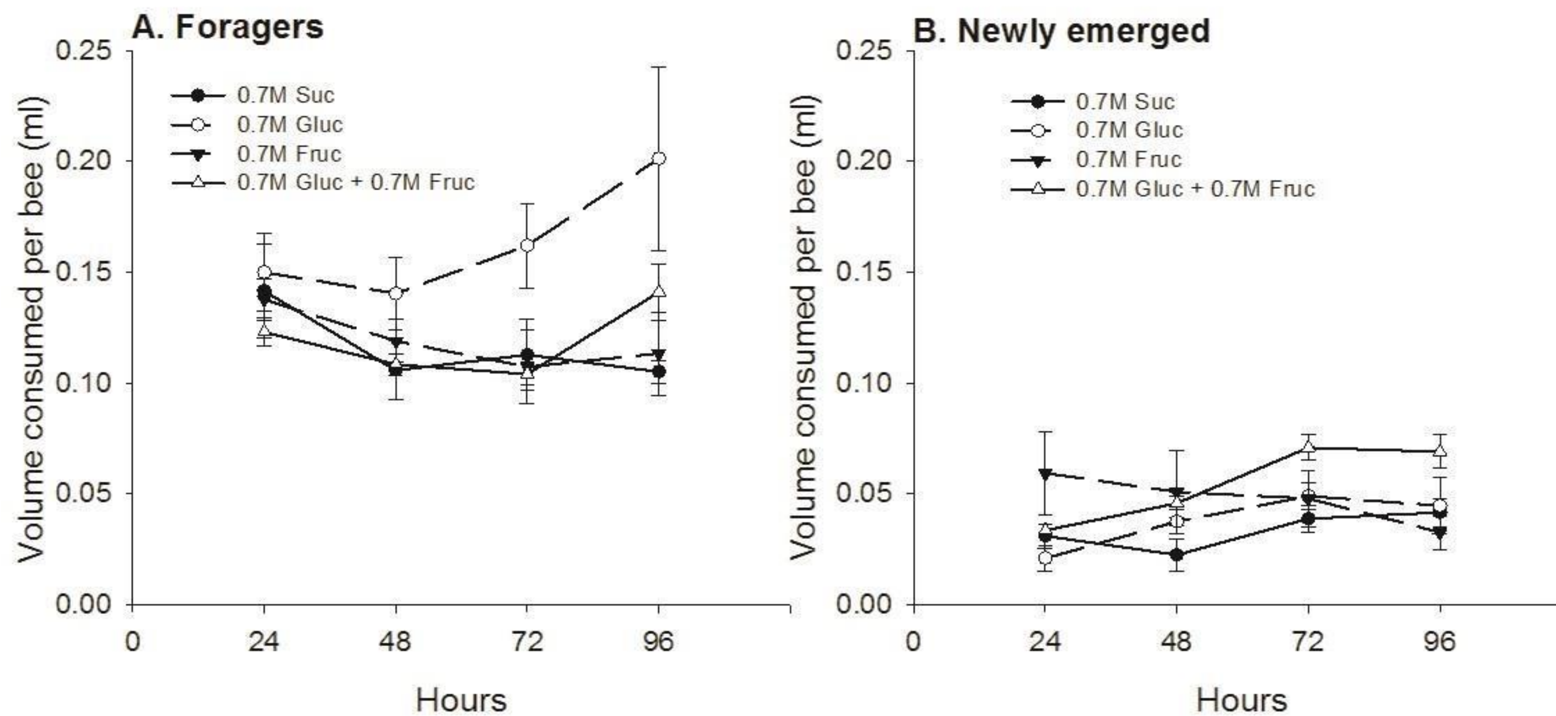

Figure 3.1. Newly emerged honeybees consume less sugar over 96 h than forager bees (repeated measures ANOVA, age: $F_{1, 78} = 16.00$, $P < 0.0001$). Consumption volumes ±SEM for honeybees restricted to one diet for 96 consecutive hours. Diets comprised either 0.7 M sucrose, 0.7 M glucose, 0.7 M fructose or an equal mixture of 0.7 M glucose and 0.7 M fructose and were provided in boxes via four microcentrifuge tubes, the fifth always containing water only. **A**. Forager bees (≈3 wks old), N=5 boxes. **B**. Newly emerged bees (≈24 h old), N=5 boxes.

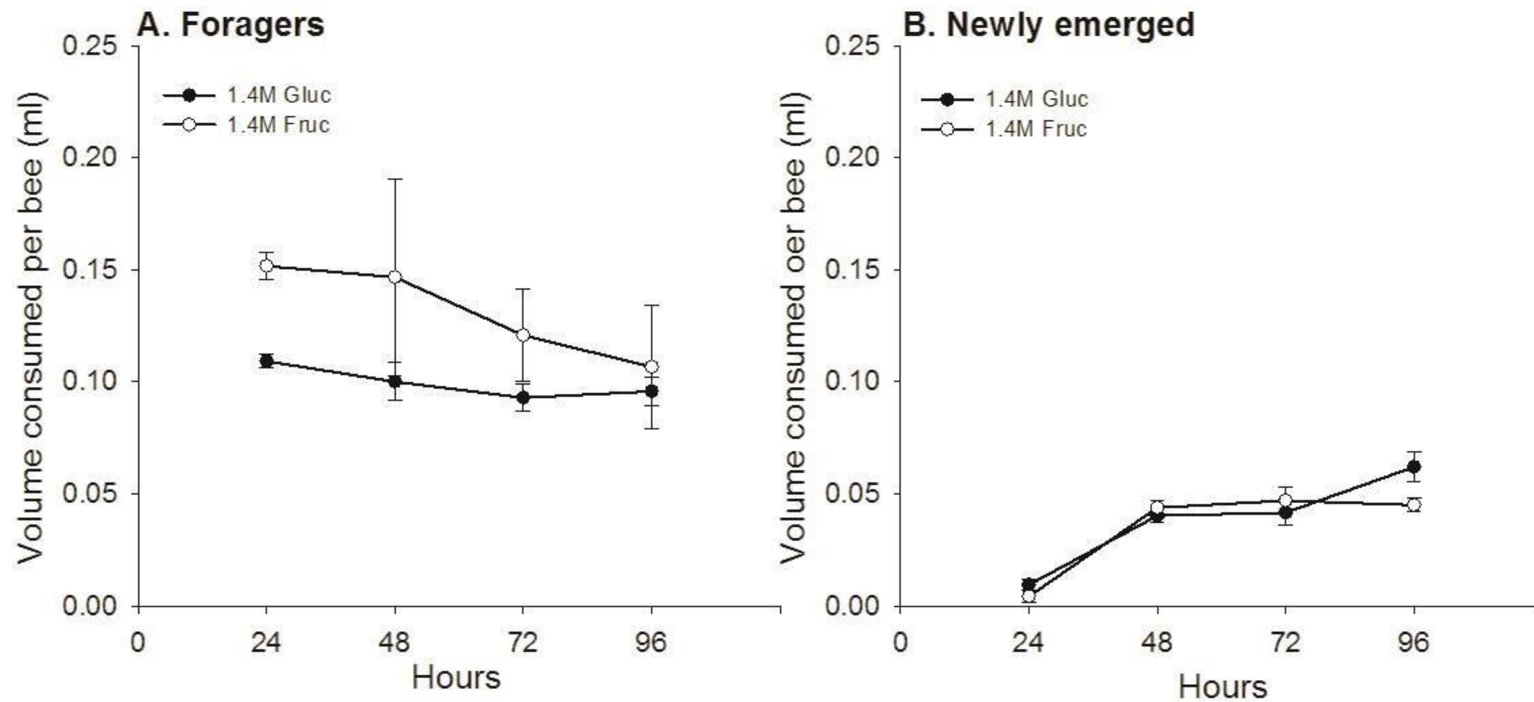

Figure 3.2. At a 1.4 M concentration forager bees generally consume glucose whereas newly emerged bees demonstrate no notable difference in consumption of glucose or fructose over time. Consumption volumes ±SEM for honeybees restricted to one diet for 96 consecutive hours. Diets comprised either 1.4 M glucose or 1.4 M fructose and were provided in boxes via four microcentrifuge tubes, the fifth always containing water only. **A.** Forager bees (≈3 wks old), no significant difference was found in sugar consumption over 96 h between treatments (repeated measures ANOVA, treatment: $F_{1,8} = 1.87$, $P = 0.209$), N=5 boxes. **B**. Newly emerged bees (≈24 h old), no significant difference was found in sugar consumption over 96 h between treatments (repeated measures ANOVA, treatment: $F_{1,8} = 0.42$, $P = 0.537$), N=5 boxes.

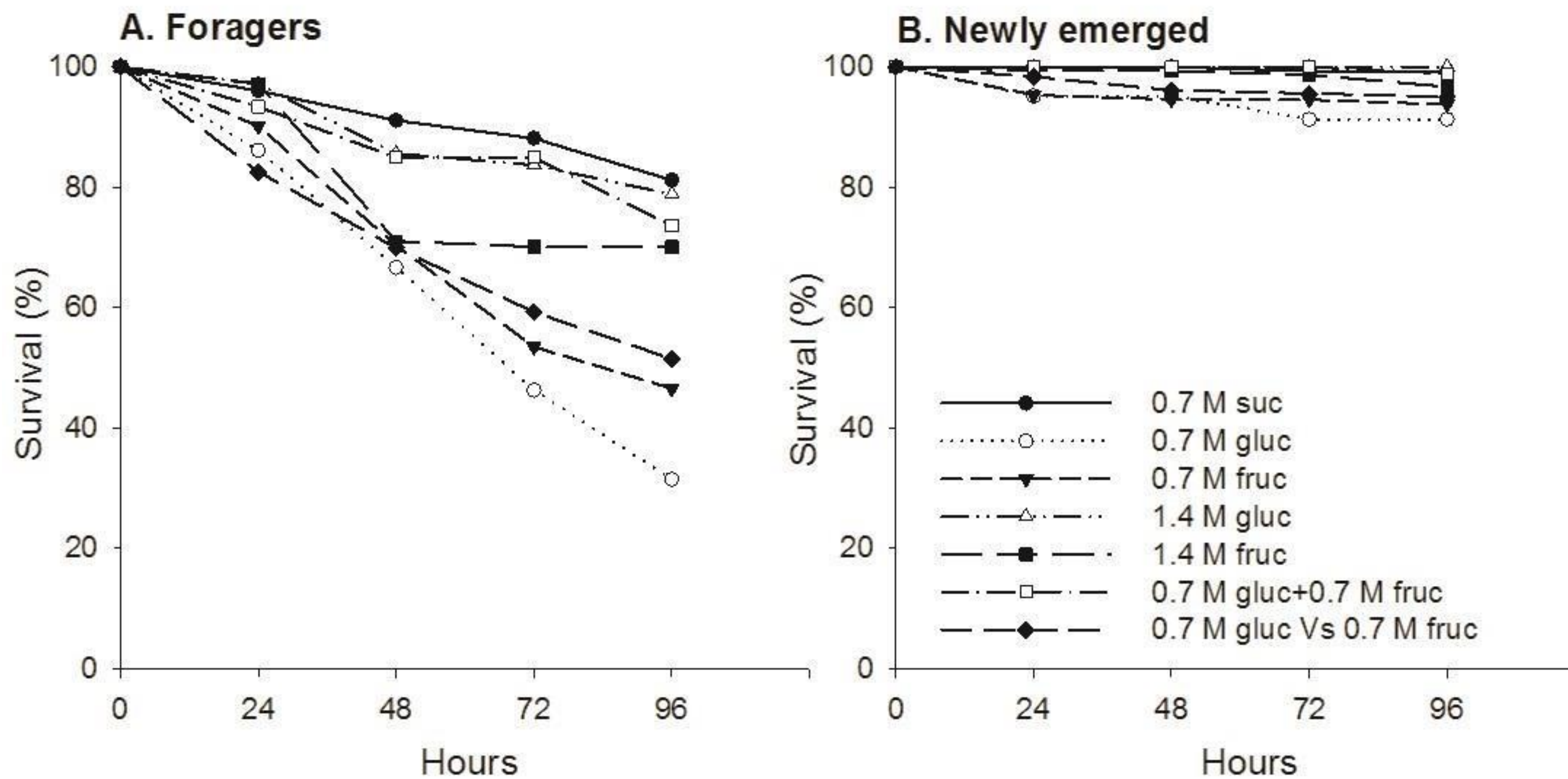

Figure 3.3. Survival rate between forager and newly emerged bees differed significantly between dietary treatments (Coxreg, age, $\chi_1^2 = 197.2$, $P < 0.001$). Percentage survival over 96 h when provided one of seven diets. **A**. Forager honeybees (≈3 wk old) N=5 boxes per diet. B. Newly emerged honeybees (≈24 h old), N=5 boxes per diet.

Table 3.1. Repeated measures ANOVA analysis comparing the volume consumed of different sugar solutions (glucose: gluc, fructose: fruc or sucrose: suc) by forager and newly emerged honeybees on specific diets. Significant, Least Significant Difference (LSD) *post hoc* values shown in bold.

Diet one	**Diet two**	**Forager honeybees: Repeated measures ANOVA LSD *Post hoc***	**Newly emerg honeybees: Repeated measures ANOVA LSD *Post hoc***
0.7 M suc	0.7 M gluc	***P* = 0.010**	*P* = 0.503
0.7 M suc	0.7 M fruc	*P* = 0.854	***P* = 0.048**
0.7 M suc	0.7 M gluc Vs 0.7 M fruc	*P* = 0.862	***P* = 0.005**
0.7 M gluc	0.7 M fruc	***P* = 0.015**	*P* = 0.164
0.7 M gluc	0.7 M gluc Vs 0.7 M fruc	***P* = 0.014**	***P* = 0.022**
0.7 M fruc	0.7 M gluc Vs 0.7 M fruc	*P* = 0.991	*P* = 0.295

Table 3.2. Cox regression analysis of survival by forager honeybees on specific sugar diets for 96 h (glucose: gluc, fructose: fruc or sucrose: suc). Significant values shown in bold.

Diet one	**Diet two**	**Cox regression** χ^{2}_{1}	**HR (Hazard ratio)**	**95% (confidence interval)**	**CI Significance**
0.7 M suc	0.7 M gluc	17.5	0.324	0.191-0.549	***P* < 0.001**
0.7 M suc	0.7 M fruc	4.32	1.46	1.022-2.095	***P* = 0.038**
0.7 M suc	0.7 M gluc + 0.7 M fruc	5.78	0.580	0.372-0.904	***P* = 0.016**
0.7 M suc	0.7 M gluc Vs 0.7 M fruc	11.1	0.455	0.286-0.722	***P* = 0.001**
0.7 M suc	1.4 M gluc	0.214	1.10	0.745-1.609	*P* = 0.644
0.7 M suc	1.4 M fruc	15.6	0.363	0.220-0.600	***P* < 0.001**

Table 3.3. Cox regression analysis of survival for newly emerged honeybees on specific sugar diets for 96 h (glucose: gluc, fructose: fruc or sucrose: suc).

Diet one	Diet two	Cox regression χ^2_1	HR (Hazard ratio)	95% (confidence interval)	CI Significance
0.7 M suc	0.7 M gluc	3.52	0.138	0.081-1.091	$P = 0.060$
0.7 M suc	0.7 M fruc	1.49	1.78	0.705-4.475	$P = 0.223$
0.7 M suc	0.7 M gluc + 0.7 M fruc	0.586	0.652	0.219-1.947	$P = 0.444$
0.7 M suc	0.7 M gluc Vs 0.7 M fruc	3.75	0.220	0.048-1.019	$P = 0.053$
0.7 M suc	1.4 M gluc	0.231	1.26	0.487-3.273	$P = 0.631$
0.7 M suc	1.4 M fruc	0.001	0.000	0.000	$P = 0.973$

3.3.2. Honeybee haemolymph sugars vary with nutritional input

The haemolymph sugar composition of both forager and newly emerged bees reflected that of the available diet (Figure 3.4.). Newly emerged bees had higher haemolymph concentrations of all sugars, across all treatments (1-way ANOVA, age: $F_{1, 120} = 11.30$, $P = 0.001$). Interestingly when restricted to 0.7 M sucrose alone, both forager and newly emerged bees demonstrated a very low sucrose concentration in the haemolymph, instead possessing an uneven split between the two monosaccharide components (Figure 3.4A). In general, fructose concentrations were higher in the haemolymph of all bees restricted to sucrose, although not significant for foragers (1-way ANOVA, sugar: $F_{3, 20} = 4.39$, $P = 0.016$, glucose*fructose LSD *post hoc*: $P = 0.385$) the effect was very clear in newly emerged subjects (1-way ANOVA, sugar: $F_{3, 20} = 17.85$, $P < 0.001$, glucose*fructose LSD *post hoc*: $P = 0.001$).

Following 96 h with access to 0.7 M fructose alone, fructose was the most concentrated haemolymph sugar in both foragers (approx. 70 mM) and newly emerged bees (approx. 125 mM), with negligible glucose and

sucrose concentrations (Figure 3.4B). Similarly, following 0.7 M glucose confinement, the most concentrated sugar in the haemolymph of both forager and newly emerged honeybees reflected that of the provided diet i.e. glucose (Figure 3.4C). The concentration of all haemolymph sugars was also comparable between foragers and newly emerged bees after glucose provision (glucose box, 2-way ANOVA, age: $F_{1, 40} = 1.64$, $P = 0.208$, age*sugar: $F_{3, 40} = 1.20$, $P = 0.321$).

3.3.3. Honeybee haemolymph sugars are rapidly regulated

When bees are fed to satiety with 1 M sucrose, the concentration of sucrose in forager honeybee haemolymph increased very rapidly (Figure 3.5.). Within 3 min of feeding, sucrose levels had dropped and concentrations of fructose and glucose in the haemolymph began to rise at the same rate to their maximal concentration around 200 mM. The concentration of haemolymph sugars was dependent on the time post-feeding (GZLM, sugar*time interaction: $\chi_2^1 = 55.6$, $P < 0.001$).

3.3.4. Honeybees demonstrate a general fructose preference

When given a choice, forager bees preferred 0.7 M fructose over 0.7 M glucose (Figure 3.6., repeated measure ANOVA, sugar: $F_{1, 8} = 6.12$, $P = 0.038$). Foragers also appeared to follow a 24 h cyclic preference pattern, consuming more fructose over glucose on the first day then switching to equal consumption of both sugars the next, before starting the cycle over again. In contrast, newly emerged bees demonstrated a slight preference for 0.7 M fructose that increased over time (Figure 3.6., repeated measure ANOVA, sugar: $F_{1, 8} = 3.89$, $P = 0.084$).

Fructose preference was apparent in both forager and newly emerged bees during a choice test following 48 h of specific sugar provision (Figure 3.7.). Pre-feeding foragers with 0.7 M glucose caused them to slightly prefer 0.7 M fructose, although not significantly (Figure 3.7A, repeated measure ANOVA, sugars: $F_{1, 8} = 4.38$, $P = 0.070$). Newly emerged bees on the other hand, showed a distinct switch in consumption toward fructose over glucose after pre-feeding with glucose (repeated measures ANOVA, sugar: $F_{1, 8} = 17.60$, $P = 0.003$). Unexpectedly, when bees were pre-fed fructose for 48 h, they did not switch to glucose. Foragers chose

both sugars equally (Figure 3.7B, repeated measures ANOVA, sugar: $F_{1, 8} = 1.58$, $P = 0.244$), whereas newly emerged bees continued to prefer fructose (repeated measures ANOVA sugar: $F_{1, 8} = 18.08$, $P = 0.003$).

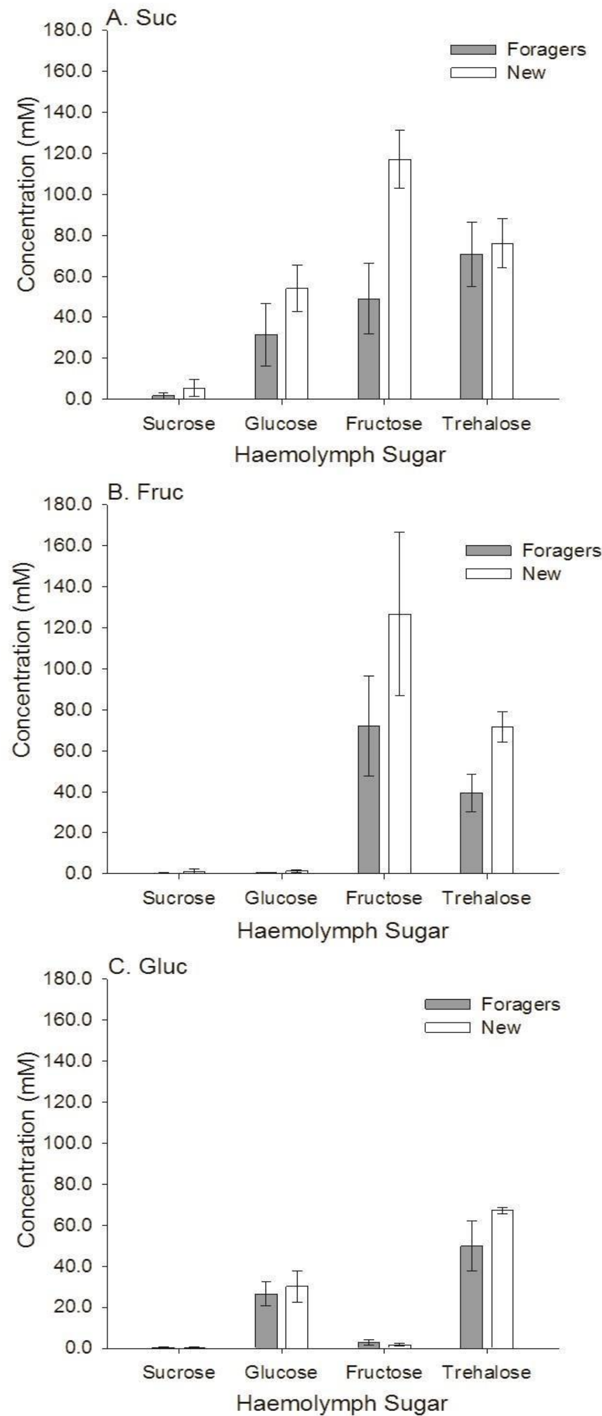

Figure 3.4. In both forager and newly emerged honeybees only haemolymph trehalose concentration appears to be regulated and maintained over time independent of nutritional input (respectively, 2-way ANOVA, forager, treatment: $F_{2,\ 15} = 1.56$, $P = 0.236$, newly emerged, treatment: $F_{2,\ 15} = 0.31$, $P = 0.741$). Haemolymph concentrations (mM) ± SEM of four major sugars (sucrose, glucose, fructose and trehalose) following 96 h specific sugar provision. **A.** Forager (F) and newly emerged (NE) bees were kept in boxes and restricted to 0.7 M sucrose alone, with water access, N=6 pooled samples. **B**. Forager and newly emerged bees were restricted to 0.7M fructose alone, with water access, N=6 pooled samples. **C.** Forager and newly emerged bees were restricted to 0.7 M glucose alone, with water access, N=6 pooled samples.

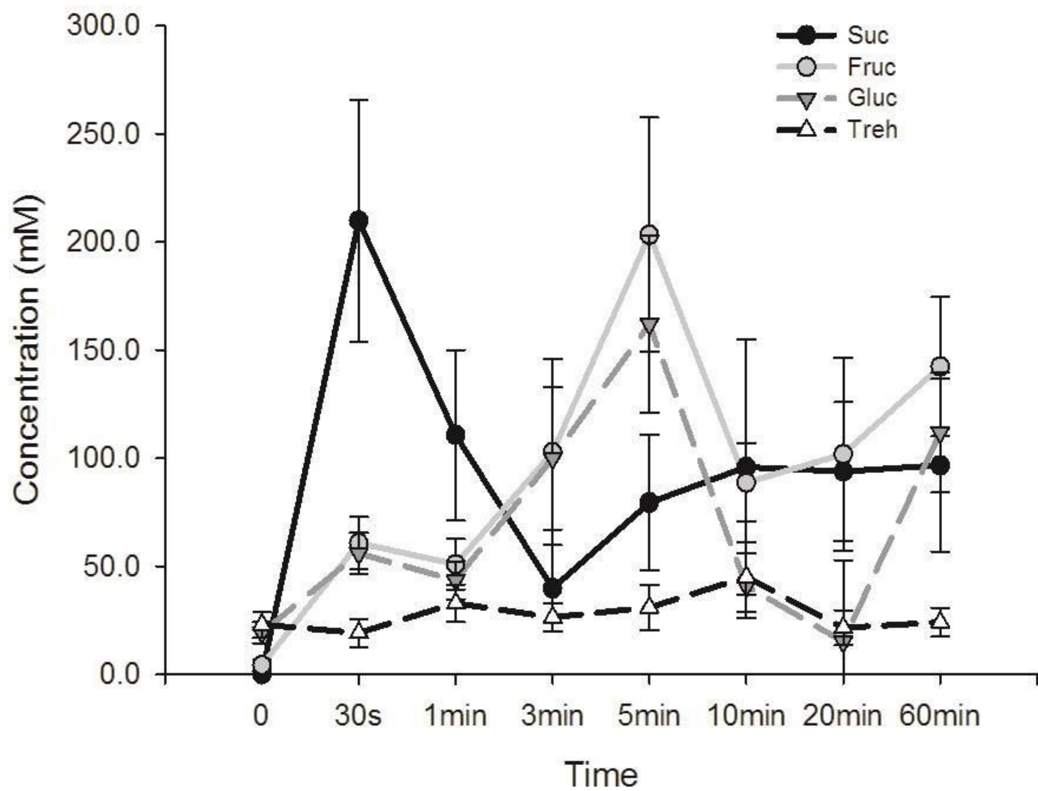

Figure 3.5. Concentration of all forager honeybee haemolymph sugars were variable except trehalose, which remained stable up to 1 h after feeding to satiety with 1M sucrose (trehalose GZLM, LSD *post hoc*, time: zero*all other time points: $P > 0.05$). Concentrations (mM) ± SEM of four major sugars (sucrose, glucose, fructose and trehalose) at specified time points after feeding to satiety (30s, 1 min, 3 min, 5 min, 10 min, 20 min, 60 min), time point zero indicates no prior feeding. All haemolymph removals were taken at the indicated time point from the completion of satiety feeding. The average time taken to feed to satiety was 64 s. Concentration was dependent on both the sugar (GZLM, sugar; $\chi_3 = 31.2$, $P < 0.001$) and the time after feeding (GZLM, time; $\chi_7 = 38.7$, $P < 0.001$). Each time point N=8-10 individuals.

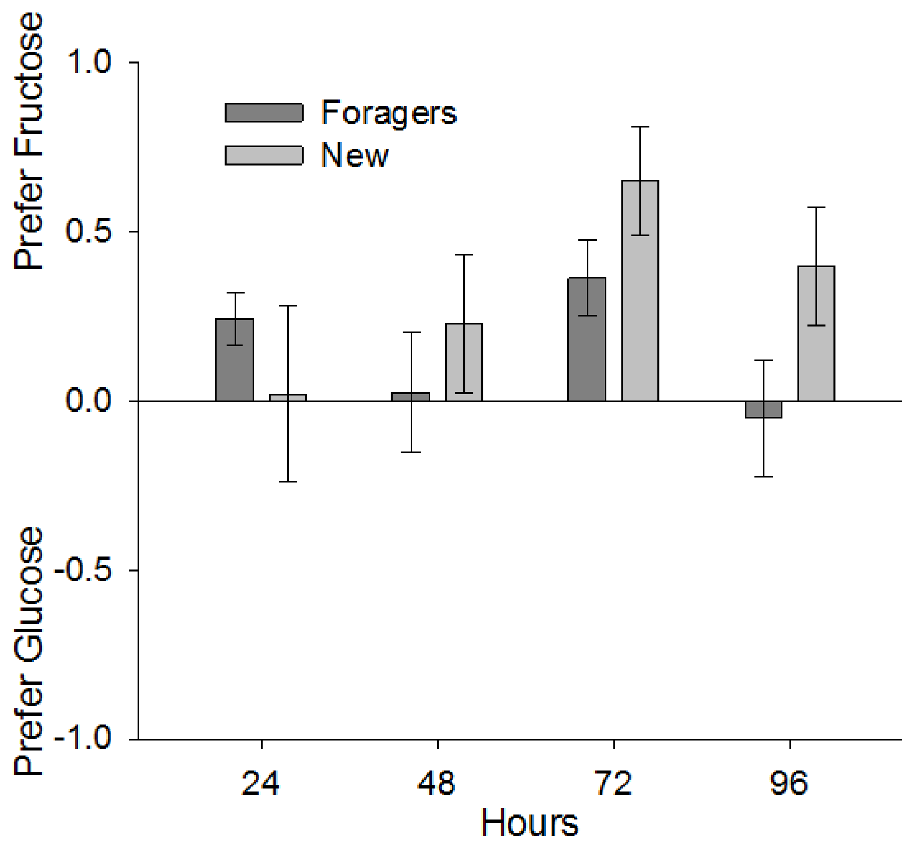

Figure 3.6. In general both forager and newly emerged (New) bees tend to prefer fructose over glucose when given a choice. Sugar preference index (SPI) ± SEM demonstrating feeding choice made by honeybees kept in boxes for 96 consecutive hours between 0.7 M glucose and 0.7 M fructose (Foragers: ≈3 wk old N=5 boxes, Newly emerged: ≈24 h old, N=5 boxes). Positive SPI indicates fructose preference, negative SPI indicates glucose preference.

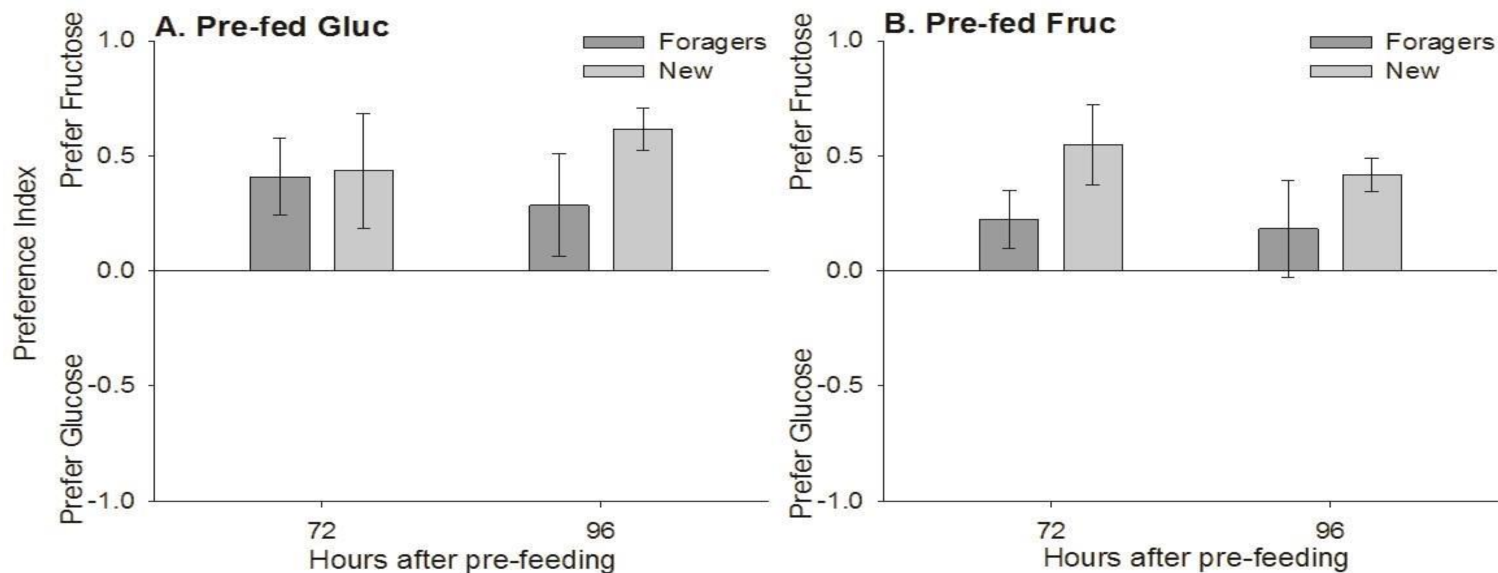

Figure 3.7. Regardless of prior diet provision, both newly emerged and forager bees tend to prefer fructose over glucose in a choice assay. Sugar preference index (SPI) ± SEM reflecting the proportion of sugar consumed in a two-sugar choice assay by honeybees following 48 hour of specific sugar provision. Positive SPI indicates fructose preference, negative SPI indicates glucose preference. **A.** Forager and newly emerged (New) bees were restricted to 0.7 M glucose only diet for 48 consecutive hours, followed by a further 48 h choice between 0.7 M glucose and 0.7 M fructose. Foragers N = 5 boxes, newly emerged N = 5 boxes **B.** Forager and newly emerged bees were restricted to a 0.7 M fructose only diet for 48 consecutive hours, followed by a further 48 h choice between 0.7 M glucose and 0.7 M fructose . Foragers N = 5 boxes, newly emerged N = 5 boxes.

3.3.5. An artificial increase in honeybee haemolymph sugar can affect satiety feeding

Forager bees in the no-injection control group consumed different sugar volumes dependent on the solution offered (Figure 3.8D, 1-way ANOVA, sugar: $F_{2, 81} = 8.48$, $P < 0.001$). In general, bees consumed more of the 0.7 M sucrose, although this did not differ significantly from 0.7 M fructose (LSD *post hoc*: $P = 0.090$). Forager bees consumed the least 0.7 M glucose (LSD *post hoc* glucose*sucrose: $P < 0.001$, glucose*fructose: $P = 0.003$).

An injection of sucrose, glucose, or fructose into the haemolymph of forager honeybees affected the volume of sucrose consumed (Figure 3.8A). Consumption of 0.7 M sucrose was dependent on both the injected sugar and the concentration of that sugar (2-way ANOVA, injection*concentration: $F_{6, 363} = 2.16$, $P = 0.047$). None of the injected sugars, at the lowest concentration (100 mM), altered the volume of sucrose consumed in comparison to the water control. In contrast, injections at the two highest concentrations (300 mM and 400 mM), of all sugars, significantly decreased the volume of sucrose consumed by the bees (Figure 3.8A).

A different pattern emerged as a result of sugar injection when bees consumed 0.7 M fructose. Once again, both the sugar injected and its concentration were found to significantly affect the volume consumed (Figure 3.8B, 2-way ANOVA, injection*concentration: $F_{5, 367} = 4.79$, $P < 0.001$). Interestingly, sucrose injection significantly decreased fructose consumption, whereas fructose injection had no effect, at any concentration. Only glucose injections mimicked the effect seen during experimental sucrose feeding (Figure 3.8A), as the two highest concentrations significantly reduced the volume of fructose consumed (Figure 3.8B).

For 0.7M glucose satiety feeding (Figure 3.8C) both, injected sugar and its concentration, significantly affected volume consumption independently (respectively, 2-way ANOVA: $F_{2, 313} = 30.41$, $P < 0.001$, $F_{3, 313} = 3.70$, $P = 0.012$). Similarly to fructose feeding, sucrose injection at all concentrations significantly reduced consumption of glucose. However, neither fructose nor glucose

injections affected glucose consumption at any of the four experimental concentrations (Figure 3.8C).

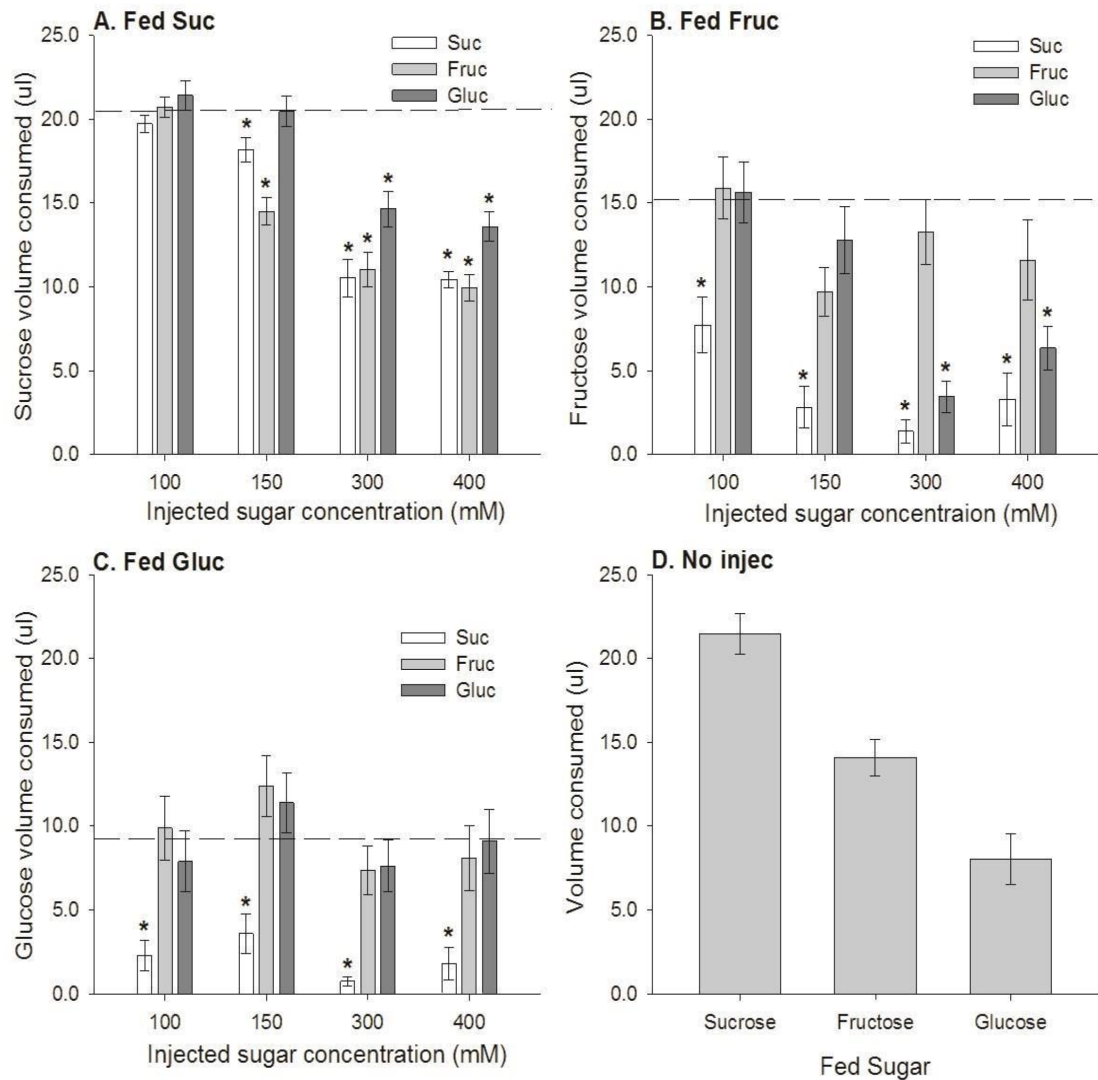

Figure 3.8. By artificially increasing the sucrose concentration of forager honeybee haemolymph, subsequent consumption of some sugars are drastically reduced. Volume consumed ± SEM of a specific sugar by forager honeybees 20 min following either; 1 µl injection of a sugar or water solution (performed blind), or no injection. **A.** Volume of 0.7 M sucrose consumed following injection with one of three sugars (sucrose, glucose or fructose) at one of four concentrations (100 mM, 150 mM, 300 mM or 400 mM). Hatched line indicates sucrose consumption following a water-only control injection, minimum N=19. * indicates significant difference in sucrose consumption between the sugar injection and water control (ANOVA LSD *post hoc* $P < 0.05$). **B**. Volume of 0.7 M fructose consumed following injection with one of three sugars at one of four concentrations. Hatched line indicates fructose consumption following a water-only control injection, minimum N=18. * indicates significant difference in fructose consumption between the sugar injection and water control (ANOVA LSD *post hoc* $P < 0.05$) **C**. Volume of 0.7 M glucose consumed following injection with one of three sugars at one of four concentrations. Hatched line indicates glucose consumption following a water-only control injection, minimum N=18. * indicates significant difference in glucose consumption between the sugar injection and water control (ANOVA LSD *post hoc* $P < 0.05$). **D**. Volume of a 0.7 M sugar (sucrose, fructose or glucose) consumed by un-injected bees, minimum N=19.

3.4. Discussion

These experiments demonstrate that honeybees 1) can distinguish glucose and fructose using taste; 2) regulate their intake of each sugar separately. Despite having the same caloric content and often being found in equal amounts in floral nectars (Percival, 1961) and honey (Kamal and Klein, 2011), when honeybees were given a choice between glucose and fructose, they had a slight fructose preference that was most pronounced in younger bees. This preference persisted after bees were restricted to glucose or fructose. Additionally, when offered each sugar in a simple feeding assay, forager bees were willing to drink approximately 50% greater volume of fructose over glucose, which was unexpected, and indicates a pre-ingestive differentiation by the bees towards these two sugars.

Like other insects, honeybees assess potential food items using gustatory receptors housed in chemo-sensitive sensilla on their gustatory appendages (Robertson and Wanner, 2006). All gustatory sensilla categorised to date are known to house between two and six (generally four) gustatory receptor neurons (GRNs) that are named according to the general taste property to which they are receptive (Whitehead and Larsen, 1976b; Siddiqi and Rodrigues, 1980; Fujishiro et. al., 1984; Wieczorek and Wolff, 1989; Meunier et. al., 2003; Zhang et. al., 2013). Very little information currently exists on the function or potential ligands of gustatory receptors in the honeybee, however it is thought that bee sensilla house the same GRNs as other insects and that their 'sweet' GRNs are able to detect dietary sugars (Whitehead and Larson, 1976a, b). A number of glucose/fructose combination experiments have led researchers to speculate distinct gustatory domains for glucose and fructose detection in some insect species (Omand and Dethier, 1969; Schmidt and Friend, 1991; Kessler et. al., 2013). Additionally, recent work into sugar ligands in *Drosophila* have demonstrated that separate gustatory receptors exist, able to detect fructose and glucose independently (Miyamoto et. al., 2012; Miyamoto et. al., 2013 respectively). If honeybees possess similar receptors in their own repertoire this may afford the bee a unique taste percept of each sugar rather than a general 'sweet' taste across all sugars. In humans, fructose is perceived as sweeter than glucose (Hanover and White,

1993) and if the bee is able to taste sugars separately this may also be true in the bee. Indeed, a higher response rate toward high fructose concentrations compared to glucose has been noted in honeybee galea sensilla (Whitehead and Larsen, 1976b), perhaps indicating fructose as the more attractive sugar, contributing to the general preference observed.

Honeybees clearly demonstrate a fructose preference when offered a choice between fructose and glucose and unexpectedly, following 48 h fructose provision, this preference persists, particularly in young bees. A similar fructose preference has also been demonstrated in bumblebees (*Bombus terrestris*, Mommaerts et. al., 2013). Similarly to honeybees in the current study, free-flying bumblebees showed an initial preference to sucrose rather than glucose or fructose, to which the authors attributed an 'innate' preference, alongside the fact that bumblebees are known to forage on sucrose-rich floral nectar (Mommaerts et. al., 2013). Mommaerts and colleagues (2013) demonstrated a certain level of plasticity in the *Bombus* gustatory system as bees previously exposed to fructose for 72 h significantly increased their response rate toward a fructose solution. Therefore bumblebees, like honeybees, also alter gustatory preferences dependent on experience. Such plasticity could be mediated via a number of influential factors, such as hormones, biogenic amines or changes in receptor expression levels. Further work is required to investigate the exact mechanisms eliciting this change and Chapter 6 focusses on gustatory receptor changes in the honeybee.

In addition to fructose and glucose, relatively high sucrose concentrations are common in honeybee pollinated plants, with many species exhibiting sucrose-dominant nectar (Percival, 1961). Like many animals, honeybees are adept at selecting foods that offer a nutritional advantage and avoiding foods with potential deleterious consequences following ingestion (Altaye et. al., 2010, Wright et. al., 2010). Whitehead and Larson (1976b) performed physiological recordings from the galea sensilla of adult worker honeybees to these same three sugars across a range of concentrations. Their work demonstrated that sucrose always elicited the greatest frequency of spikes from the 'sugar' cell (Whitehead and Larson, 1976b). The combination of glucose and fructose increased the spiking frequency above that expected by simple addition of spike number from each sugar alone.

Despite this synergism from monosaccharide combination, the response observed was still lower than sucrose alone. The current study demonstrates that, at least in terms of survival, sucrose offers the greatest nutritional advantage compared with the other tested sugars, likely due to sucrose being a disaccharide of both glucose and fructose and therefore offering a higher energetic value at the same concentration.

Alternatively, a fructose preference could be a result of a separate post-ingestive detection and regulatory mechanism for glucose and fructose, acting via internal nutrient sensors. Recent work by Miyamoto and colleagues (2012) using a calcium imaging technique identified gustatory receptor 43a (DmGr43a) as a narrowly tuned fructose receptor in the taste organs of *Drosophila melanogaster.* Further work uncovered the expression of this receptor in the brain of the fly which responded to increasing fructose levels in the haemolymph, the same levels observed following carbohydrate ingestion (Miyamoto et. al., 2012). This Gr activation was also closely coupled with feeding and by insertion and activation of a temperature-dependent ion channel (TRPA1) the team could artificially activate the receptor $Gr43a^{GAL4}$ brain neurons and subsequently shutdown feeding (Miyamoto et. al., 2012). It was concluded that DmGr43a acts as a nutrient sensor in the *Drosophila* brain which responds to fluctuating fructose levels in the haemolymph, activating or inhibiting feeding. A DmGr43a ortholog has been identified in the honeybee (AmGr3, Robertson and Wanner, 2006), highlighting the potential for a similar mechanism, which was tested in the present study by artificially elevating sugar levels in the head capsule of the forager bee via injection. While the injection of fructose into the head significantly reduced sucrose feeding, it had no effect on either fructose or glucose feeding, indicating that fructose alone is unlikely to mediate feeding responses. Concentrated glucose injection suppressed sucrose and fructose feeding, but it did not affect glucose consumption. Elevating sucrose levels however, had a significant influence on all sugar feeding indicating that haemolymph sucrose concentration may regulate feeding in the honeybee.

In *Drosophila*, fructose haemolymph levels fluctuate greatly after a large meal, as opposed to glucose and trehalose which are regulated to a relatively stable concentration (Miyamoto et. al., 2012). This dramatic fructose fluctuation

is directly sensed by DmGr43a and is thought to shut down feeding (Miyamoto et. al., 2012). As shown, fructose fluctuations are common in honeybee haemolymph, as a result of satiety feeding or different diets, however they are also usually matched with glucose fluctuations. However, feeding bees to satiety with sucrose led to a rapid influx of sucrose in the haemolymph, a potential satiety signal that may have been responsible for the termination of feeding. Additionally, sucrose feeding over 96 h leads to minimal sucrose levels in the blood and with *ad libitum* access to the sucrose it is unlikely that feeding would be inhibited. This signal could involve a Gr in the bee brain acting as a nutrient sensor, as in *Drosophila* (Miyamoto et. al., 2012), or alternatively, as bee haemolymph is rapidly mixed throughout the body (Crailsheim, 1985), such a sensor may be located elsewhere. The expression of potential gustatory receptors across the honeybee anatomy is further investigated in Chapter 5.

While glucose and fructose are thought to be absorbed from the gut passively and metabolised at similar rates (Crailsheim, 1988b), the main difference between them concerns their involvement in trehalose synthesis. Trehalose is a disaccharide of two glucose molecules and while less common in vertebrates, is often referred to as the major haemolymph sugar in insects (Wyatt and Kalf, 1957; Treherne, 1958; Sacktor, 1968; Thompson, 2003). Trehalose is an important energy store for honeybees, as a non-reducing sugar it proves less reactive than glucose and rapid metabolism and synthesis has previously been noted in a number of studies (Gmeinbauer and Crailsheim, 1993; Woodring et. al., 1993; Crailsheim, 1998a). In a study investigating the absorption and utilization of sugars by the honeybee, Gmeinbauer and Crailsheim (1993) observed that some of the [^{14}C]glucose fed to bees following an exhaustive flight, was consequently detected as trehalose in the haemolymph within two minutes of ingestion. Whilst glucose to fructose conversion occurs in insects (Maurizio, 1965; Candy et. al., 1997), none of the ingested [^{14}C] was detected as fructose indicating that fructose synthesis, if apparent, is less rapid (Gmeinbauer and Crailsheim, 1993). Degradation of trehalose leads to a flux in haemolymph glucose, but not fructose and therefore acts as an additional ‘glucose-storage sugar’ (Becker et. al., 1996; Blatt and Roces, 2002).

In the current study, trehalose concentration in the haemolymph appears to be undergoing strict regulation. The 96 h feeding studies demonstrate that honeybee haemolymph sugars are affected by nutritional input as observed previously by Maurizio (1965) and Blatt and Roces (2002). When bees are fed a diet of glucose or fructose, each respective sugar became more concentrated in the haemolymph than the corresponding monosaccharide after 96 h in both newly emerged and forager honeybees. The only haemolymph sugar to remain relatively constant across treatments was trehalose. Furthermore, forager bees fed to satiety with 0.7 M sucrose maintained a trehalose concentration between 25-50 mM. This concentration is slightly lower than those fed sucrose *ad libitum* and reflected that bees were starved 24 h prior to satiety feeding, as honeybees are known to rapidly deplete haemolymph stores when denied access to food (Woodring et. al., 1993). This concentration range, when compared to an almost 200-fold increase of other sugars following satiety feeding, is relatively unchanged. A study by Bounaise and Morgan (1985) injected trehalose directly into bee haemolymph and whilst a marked increase in trehalase activity was noted, trehalose values were rapidly returned to a normal range. Similarly, in the current study trehalose levels remained stable despite a large flux in haemolymph glucose following satiety feeding, indicating that trehalose synthesis was being regulated. Trehalose is known to play a role in feeding regulation in other insects (Friedman et. al., 1991) and therefore strict trehalose regulation may be vital to maintaining honeybee homeostasis.

Earlier work assessing haemolymph sugar levels have highlighted a large range in concentrations (Wyatt, 1961; Bounaise, 1981; Arslan et. al., 1986; Fell, 1990; Blatt and Roces, 2002) leading some authors to assume a lack of sugar homeostasis in the honeybee. Whilst trehalose levels are clearly regulated, the current results also observed high variability in other haemolymph sugars dependent on experiment and nutritional input. Following satiety feeding there was a huge peak in sucrose concentration, which differs from a number of previous studies that have noted a lack, or very minimal detection, of sucrose in honeybee haemolymph (Fell, 1990; Abou-seif et. al., 1993; Woodring et. al., 1993; Blatt and Roces, 2001). This peak of sucrose, however, was rapidly reduced (within 3 min) which indicates an efficient breakdown to glucose and

fructose by sucrases (Huber and Thompson, 1973). In the pea aphid, efficient activity of sucrases have been noted in the regulation of osmotic pressure of the aphid body fluids (Karley et. al., 2005). As the honeybee diet consists of highly concentrated carbohydrate solutions, a rapid sucrose break-down may also prove vital in the bee. Furthermore, variability among sugar levels may also be explained by differences in both the race of bees and the time of year in which the haemolymph was collected (Bounaise, 1980).

When fed to satiety, unmanipulated foragers honeybees consume higher volumes of 0.7M sucrose than glucose or fructose. As a disaccharide of these two sugars, sucrose, at the same molarity, is double the carbohydrate concentration of either sugar alone and therefore physiologically more rewarding. Doubling the concentration of either monosaccharide to 1.4 M significantly increases the likelihood of survival. Interestingly, while the combination of 0.7 M glucose and 0.7 M fructose improved the survival compared to either sugar alone, it did not increase survival to the level of sucrose, which corresponds with the neuronal responses obtained by Whitehead and Larson (1976b). This suggests that the sucrose solution itself has some form of additional physiological benefit for the honeybee.

Taking the experimental methods into account there may be concern that forager survival in the current study was affected by the bee's ability to obtain enough sugar in order to maintain metabolic functioning. Honeybees are known to only excrete in flight (Winston, 1987) and due to the experimental boxes used, bees were unable to sustain flight for any length of time. Over 96 h, very little excrement was noted in the boxes which could indicate bees were swollen with waste, subsequently affecting their ability to consume enough sugar solution to survive. However, as sugar consumption did not decline over time this indicates that waste excretion had no influence on sugar intake.

Unlike foragers, survival rates did not differ significantly between treatments for newly emerged bees, which is attributable to differences in physiology. New bees generally exhibit lower metabolic rates (Harrison, 1986) and therefore haemolymph sugar utilisation may not have been as rapid in the young bees accounting for the higher haemolymph sugar levels detected over 96

h in all three dietary sugar groups. Additionally, new bees are known to possess greater glycogen and lipid stores (Toth et. al., 2005; Toth and Robinson, 2005), making them less reliant on immediate nutritional input for survival and explaining the reduced volume consumption of all experimental solutions compared to forager bees.

3.5. Conclusion

Honeybees rely on carbohydrates for primary metabolic functions and to fuel flight (Beenakkers, 1969; Neukirch, 1982; Rothe and Nachtigall, 1989; Gmeinbauer and Crailsheim, 1993). A highly efficient regulatory system is beneficial for the bee in order to optimally utilise carbohydrates and be prepared to meet changing metabolic demands. Out of the three experimental saccharides in the present study, sucrose appears most rewarding to forager honeybees in terms of survival and consumption, primarily because of its existence as a disaccharide of glucose and fructose. Therefore I would expect sucrose dominant nectars to be most phagostimulatory for honeybees in the wild. Sucrose also has the greatest influence on future food choice through circulating haemolymph levels and could be a regulator of sugar consumption. Further work will be needed to determine the exact role of haemolymph sucrose levels in satiation and feeding along with the importance of stable trehalose haemolymph levels. Both circulating haemolymph sugar levels and specific sugar for consumption affects volume consumed, as some sugars e.g. sucrose, appear more important for consumption than others. Investigating the pre-ingestive detection mechanisms for these individual sugars will shed light on the importance of pre-and post-ingestive mechanisms in sugar regulation.

Chapter 4.0. Amino acid preferences

Carbohydrates, obtained from nectar and protein from pollen, are the two main components of the honeybee diet. Floral nectar however, contains a range of nutritional compounds, the second most concentrated of which are amino acids. With no direct benefit to the honeybee diet, amino acid provision in nectar is thought to aid pollinator attraction. The current work demonstrates that amino acids are mildly phagostimulatory to honeybees; however not all amino acids are accepted equally indicating that bees may be able to pre-ingestively differentiate between them. Furthermore, as honeybees mature, their dietary requirements shift primarily from amino acid and protein, to carbohydrate consumption and the current work aimed to assess whether this shift was represented in dietary choice. Both newly emerged and forager honeybees demonstrate either a neutral or slightly adverse response toward eight single amino acid-sucrose solutions compared to sucrose alone. However, when all eight amino acids are combined, the newly emerged bees—those most in need of protein—prefer to consume this solution over 96 h, whereas forager bees continue to accept both solutions at the same rate.

4.1. Introduction

As a primary macronutrient, protein is required in every animal diet and mediates vital bodily processes such as immunity (Chandra, 1997; Alaux et. al., 2010), fecundity (Dethier, 1961; Sang and King, 1961; Mevi-Schütze and Erhardt, 2005) and somatic growth (Borer et. al., 1979; Ito and Inokuchi 1981). Amino acids (AAs) are the 'building blocks of proteins' and therefore essential for life. Unsurprisingly, animals seek these nutrients and actively reject diets that lack them (Gietzen, 1993; Koehnle and Gietzen, 2005; Gietzen and Aja, 2012; Bjordal et. al., 2014). One major goal of nutritional research concerns understanding the selection and regulation of dietary protein. Following a series of exclusion experiments two main categories of dietary AAs; essential and non-essential AAs (excluding non-protein AAs), were first identified in rats (Rose, 1938). As the name suggests, essential AAs (often referred to as indispensable amino acids, IAAs) are a necessity for every animal diet, they cannot be synthesised from any other substance and therefore must be obtained directly in food. Following similar procedures to those on rats (Rose, 1938), ten essential AAs have been identified for most animals: arginine, phenylalanine, valine, threonine, methionine, leucine, isoleucine, lysine, tryptophan and histidine, which only differ marginally dependent on species (Almquist, 1947; Albanese, 1950). Due to the ease of dietary manipulation, herbivorous insects have often been central to studies of protein regulation and Moore (1946) was the first to define the same 10 mammalian essential AAs for insects in one study on the carpet beetle (*Attagenus*). A later in-depth protein requirement study concluded the same essential AAs for the honeybee (*Apis mellifera*) (De Groot, 1953), a major plant pollinator.

Many nutritional regulation studies have implemented behavioural choice assays in which insects choose between solutions containing or lacking protein/AAs. Often these studies indicate a sex-specific skew in AA preference, with females of many species more commonly selecting AA solutions, which has been attributed to their role in oogenesis (Sang and King, 1961; Erhardt and Rusterholz 1998; Alm et. al., 1990, Mevi-Schütze and Erhardt, 2005). Not all insects however, require AAs for egg production. Foraging honeybees for example, collect protein-rich pollen from angiosperms, however, foragers are

sterile females that exist in a well-structured eusocial community, where only the queen can procreate. The protein requirements of a honeybee hive have been studied in-depth and protein is a vital resource to provision the young (De Groot, 1953).

Newly emerged bees are responsible for general hive maintenance (Winston, 1987) before becoming ‘nurse bees’, eating protein-rich bee bread (Crailsheim, 1990) and feeding royal jelly and pollen to larvae that require high quantities of protein (Hrassnigg and Crailsheim, 2005). Herbert and colleagues (1977) demonstrated the importance of protein consumption for newly emerged bees. By adding a specific protein quantity (23%) to a sucrose diet they subsequently observed an increase in the number of brood raised. In their first eight days of life bees increase their total body weight, protein and nitrogen content, midgut proteolytic activity and incorporation rates of AAs into protein (Crailsheim, 1986; Moritz and Crailsheim, 1987; Winston, 1987). As the bees approach foraging age however (~2-3 wks old), their protein content and requirements decrease (Crailsheim, 1986), primarily requiring protein for tissue maintenance and repair, the thorax and flight muscles in particular. Even though the foragers’ protein demand is relatively low compared to the nurse bees, these insects must still locate and collect protein resources to return to the hive.

Honeybees meet their protein needs from the consumption of pollen and are thought to be attracted towards particular pollen sources dependent on both the AA complement and their previous experience with that pollen (Cook et. al., 2003). Amino acids however, are also present in floral nectar. First discovered by Ziegler (1956) in a limited number of plants, a more detailed analysis of 266 angiosperm species was undertaken, confirming that AAs are ubiquitous in floral nectar and are the second most concentrated component behind sugars (Baker and Baker, 1973). While a certain degree of constancy exists (Baker, 1977; Baker and Baker, 1973, 1977, 1986; Gardener and Gilman, 2001), not all floral AA complements are the same and a number of authors have speculated that plant visitors are driving selection for specific AAs rather than AA content in general (Baker, 1977; Baker and Baker, 1973, 1977; Lanza and Krauss, 1984; Carter et. al., 2006). This hypothesis has led authors to investigate both pre-and post-ingestive assessment of AAs by insects.

With the theory that nectar AAs enhance taste, a number of studies have tested this hypothesis in bees. In a free-flying experiment Alm and colleagues (1990) trained bees to collect artificial nectar from flowers that offered either a sugar-only nectar or a plant mimic nectar (*Lantana camara*) that contained a mixture of AAs. The team discovered that bees would consume significantly greater volumes of the AA nectar (Alm et. al., 1990). Whilst such AA mixtures do provide a more natural replica of floral nectar, other studies have focussed on assessing whether all AAs are judged similarly by bees or whether attraction is influenced by dietary demand, for example essentials versus non-essentials.

In associative learning assays honeybees have not only been found to respond positively towards an odour representing single AA solutions (Kim and Smith, 2000; Simcock et. al., 2014), but these positive responses are also dependent on both the specific AA and the bees' feeding history (Simcock et. al., 2014). This result indicates that attraction towards AAs is not equal and can be modulated in honeybees (Simcock et. al., 2014). In a study assessing 24 individual AAs, Inouye and Waller (1984) discovered that some dilute AA solutions were preferred over sucrose alone (12 AAs). However, this did not apply to all AA solutions (sucrose was preferred to glycine, GABA, hydroxyproline and tyrosine) and only one (phenylalanine) was consistently preferred to the sucrose control at all concentrations, with the most concentrated solution consumed at the highest volume (~1000 mg/ 100 ml).

Positive results in such brief exposure assays indicate that AAs may influence the 'taste' of nectars. One of the major studies into AA taste properties in insects was carried out by Shiraishi and Kuwabara (1970) on two fly species (the fleshfly, *Boettcherisca peregrina,* and the blowfly, *Phormia regina)* using electrophysiology. By presenting a suite of 19 AAs to the labellar chemosensory sensilla of the flies, the team were able to categorise four distinct AA classes subject to their influence upon the four gustatory receptor neurons (GRNs) housed within the sensilla (see Table 4.1.). A subsequent study by Goldrich (1973), on blowflies only, confirmed these findings for all but four AAs (alanine, aspartic and glutamic acids, and valine), the differences being attributed to methodological approaches; specifically the sensilla from which the recordings were taken.

In addition to pre-ingestive detection, a number of post-ingestive detection mechanisms have also been investigated to determine how insects successfully acquire their essential AAs. When deprived of a particular nutrient, such as protein, many animals become attracted/responsive toward foods that contain that nutrient or avoid/reject foods that lack it, demonstrated in vertebrates (Firman and Kuenzel, 1988; Murphey and King, 1989; Murphy and Pearcy, 1993; Gibson et. al., 1995; Fromentin and Nicolaidis, 1996; Koehnle et. al., 2003; Hao et. al., 2005; Gietzen and Rogers, 2006) and invertebrates alike (Simpson and Abisgold, 1985; Rathman et. al., 1990; Simpson et. al., 1991; Simmonds et. al., 1992; Toshima and Tanimura, 2012).

Whilst obtaining AAs, through either pre-or post-ingestive mechanisms, clearly has its benefits, this is not always the case. High protein and AA diets have recently been correlated with increased mortality of some insect species, including the honeybee (Grandison et. al., 2009, Dussutour and Simpson, 2012; Paoli et. al., 2014a). Paoli and team (2014a) allowed bees to ascertain their own intake target by selecting between solutions varying in their protein to carbohydrate ratio (P:C ratio), with the protein provided as a mixture of all 10 essential AAs (De Groot, 1953). The essential AA intake target was age dependent, as forager bees consistently prioritised carbohydrate intake (P:C: 1:250), whereas newly emerged bees preferred a diet consisting of ~1:50 P:C ratio that became more carbohydrate biased as the bees aged (Paoli et. al., 2014a). Exposure to a high level of essential AAs however, was shown to significantly reduce the lifespan of foragers (Paoli et. al., 2014a; Paoli et. al., 2014b).

Whilst their effects can vary, AA ingestion is important and many animals, including the honeybee (Kim and Smith, 2000; Carter et. al., 2006; Bertazzini et. al., 2010) have demonstrated a specific attraction towards them, particularly following protein deprivation. In the current study I used honeybees to investigate a number of aspects to AA selection. Under the hypothesis that bees can detect AAs I assessed honeybee taste preferences to investigate whether AA behavioural responses match those originally noted by Shiraishi and Kuwabara (1970) in flies. Additionally, the age division in honeybees is also matched by their dietary requirements, with young bees possessing a considerably greater need

for protein than foragers (Winston, 1987; Paoli et. al., 2014a). With this in mind I compared feeding responses between newly emerged and forager bees to assess whether a greater need for AAs would be portrayed via a greater attraction towards them. Additionally, attraction toward individual AAs has previously been demonstrated by bees (Inouye and Waller, 1984; Kim and Smith, 2000, Bertazzini, et. al., 2010), although individual AAs are never encountered in nature. Therefore I aimed to determine whether bees would be more attracted towards an AA mixture, as expected from nectar compositions, or whether the presence of only a select few AAs are needed to increase nectar attractiveness.

Table 4.1. Classification and description of L-type amino acids belonging to four distinct taste classes (modified from Shiraishi and Kuwabara, 1970)

Class 1 Amino acids: did not stimulate any chemoreceptor cell	**Class 2 Amino Acids: inhibit non-specifically the discharges from three kinds of chemosensory cells**
Glycine Alanine Serine Theronine Cystine Tyrosine	Aspartic Acid Glutamic Acid Histidine Arginine Lysine
Class 3 Amino acids: Stimulated the salt receptor cell	**Class 4 Amino acids: Stimulated the sugar receptor cell.**
Proline Hydroxyproline	Valine Leucine Isoleucine Methionine Phenylalanine Tryptophan

4.2. Materials and methods

4.2.1. Honeybee collection and mouthparts assay

Note: For detailed methods see 2.2. Honeybee capture and restraint and 2.3. Mouthparts assay.

Forager and newly emerged honeybees (*Apis mellifera Buckfast*) were collected at Newcastle University in individual phials and cold-anesthetised. Bees were restrained as described in Wright and Smith (2004) then fed to satiety with 1 M sucrose and left for 18-24 h in a humidified box. Proboscis extension reflex (PER) (Page et. al., 1998) was used to assess motivation with a 0.4 µl droplet of 1 M sucrose solution. All bees responding positively to the motivation test were supplied with one of nine experimental amino acid solutions and the volume consumed was recorded. Separate groups were additionally fed sucrose only as a positive control and deionised water as a negative control.

4.2.2. Amino acid feeding preference assay

Note: For detailed methods see 2.4. Feeding preference assay: methods and 2.5. Feeding preference assay: solutions.

Forager and newly emerged bees were placed in boxes and provided with two experimental solutions in microcentrifuge tubes (one of nine experimental amino acid solutions or sucrose alone, five boxes for each age group). Tubes were weighed every 24 h for 96 h and dead insects were counted daily. The mean daily individual consumption was calculated as an average across the viable insects from the previous 24 h, minus evaporation. An identical evaporation box (minus honeybees) was run simultaneously.

4.2.3. Statistical methods

All data analyses were performed using SPSS v. 21.0. Continuous data were analysed using a Univariate Analysis of Variance (ANOVA) with Least Significant Difference (LSD) *post hoc* comparisons. Repeated measures ANOVA was used to analyse 96 h box data. A preference index (PI) was calculated for the choice tests, a positive preference score indicates a sucrose preference and a negative preference score indicates an AA+sucrose preference.

4.3. Results

4.3.1. Age affects consumption of sucrose-amino acid solutions

On average, foragers ate more solution than newly-emerged bees (Figure 4.1., Univariate ANOVA, age: $F_{1, 624} = 11.50$, $P = 0.001$). Newly emerged bees did not significantly increase consumption following the addition of sucrose to amino acid solutions (Figure 4.1A, Univariate ANOVA, sucrose addition: $F_{1, 312} = 3.46$, $P = 0.064$). Forager honeybees on the other hand, consumed more of amino acid-sucrose solutions than pure amino acids (Figure 4.1B, Univariate ANOVA, sucrose addition: $F_{1, 312} = 7.04$, $P = 0.008$). The class of the amino acid failed to influence the volume consumed for both newly emerged (Univariate ANOVA, class: $F_{3, 312} = 2.18$, $P = 0.090$) and forager honeybees (Univariate ANOVA, class: $F_{3, 312} = 0.85$, $P = 0.466$).

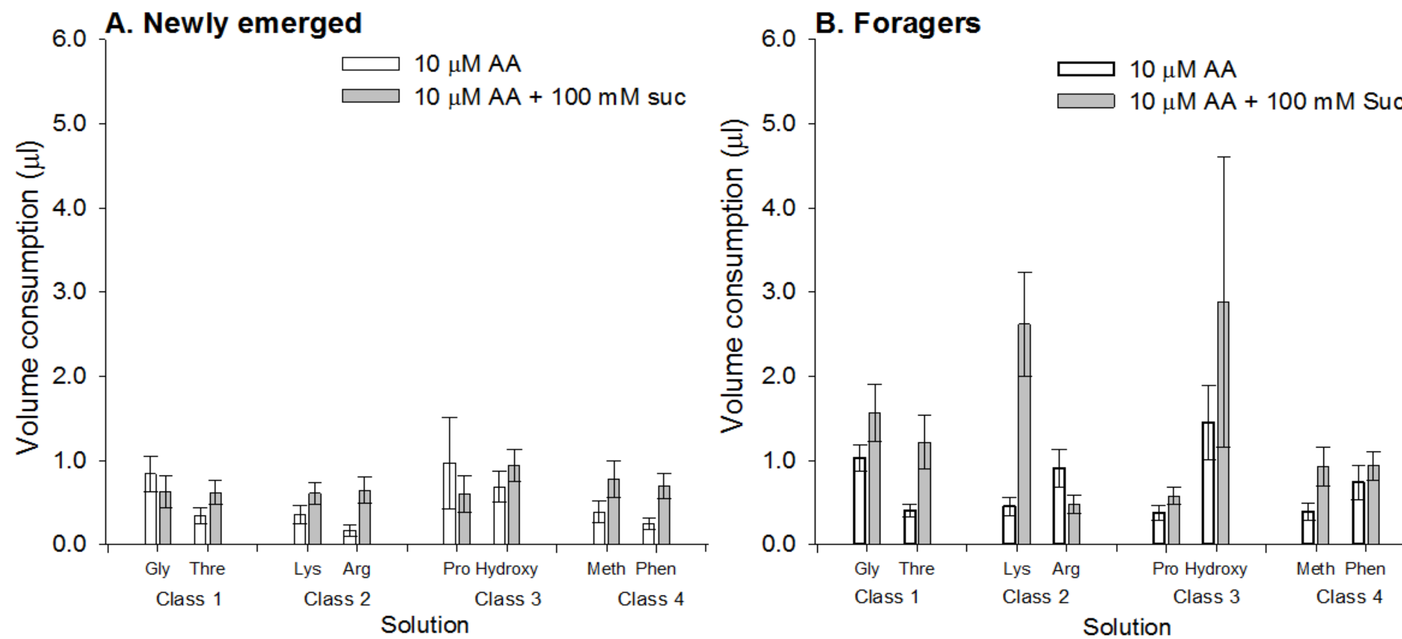

Figure 4.1. Forager honeybees consumed greater volumes of pure amino acids and those dissolved in sucrose, than newly emerged bees. Volume consumed (µl) ± SEM of eight pure amino acid solutions (10 µM: glycine, threonine, lysine, arginine, proline, hydroxyproline, methionine and phenylalanine) across the four fly taste classes (Shirashi and Kuwabara, 1970), or the same amino acids added to 100 mM sucrose. **A**. Volume consumed by newly emerged bees, All N =20. **B.** Volume consumed by forager honeybees, All N = 20.

4.3.2. The majority of amino acid-sucrose solutions are consumed at the same rate as sucrose alone

Newly emerged bees consume more sucrose over 96 h (Figure 4.2A) when given the choice between sucrose alone and either of the two class 1 amino acids in sucrose (Figure 4.2A, glycine, repeated measures ANOVA, Solution: $F_{1, 8} = 10.00$, $P = 0.013$; Figure 4.2B, threonine, repeated measures ANOVA, Solution: $F_{1, 8} = 6.50$, $P = 0.034$). However, newly emerged bees consumed the same volumes of sucrose alone and all remaining amino acid-sucrose solutions across the remaining three amino acid classes (Figure 4.2C, class 2: Lysine and arginine; Figure 4.2E, class 3: proline and hydroxyproline and Figure 4.2G, class 4: methionine and phenylalanine).

Forager bees consumed the same volume of class 1, class 2 and class 4 amino acid-sucrose solutions as sucrose alone (respectively: Figure 4.2B, class 1: glycine and threonine; Figure 4.2D, class 2: lysine and arginine; Figure 4.2H, class 4: methionine and phenylalanine). The class 3 amino acids (proline and hydroxyproline) were an exception to this. Whilst the forager bees consumed the same volumes of proline-sucrose as sucrose alone (Figure 4.2F, repeated measures ANOVA, solution: $F_{1, 8} = 0.04$, $P = 0.847$), they demonstrated a strong preference for sucrose alone over hydroxyproline-sucrose (repeated measures ANOVA, solution: $F_{1, 8} = 9.57$, $P = 0.015$).

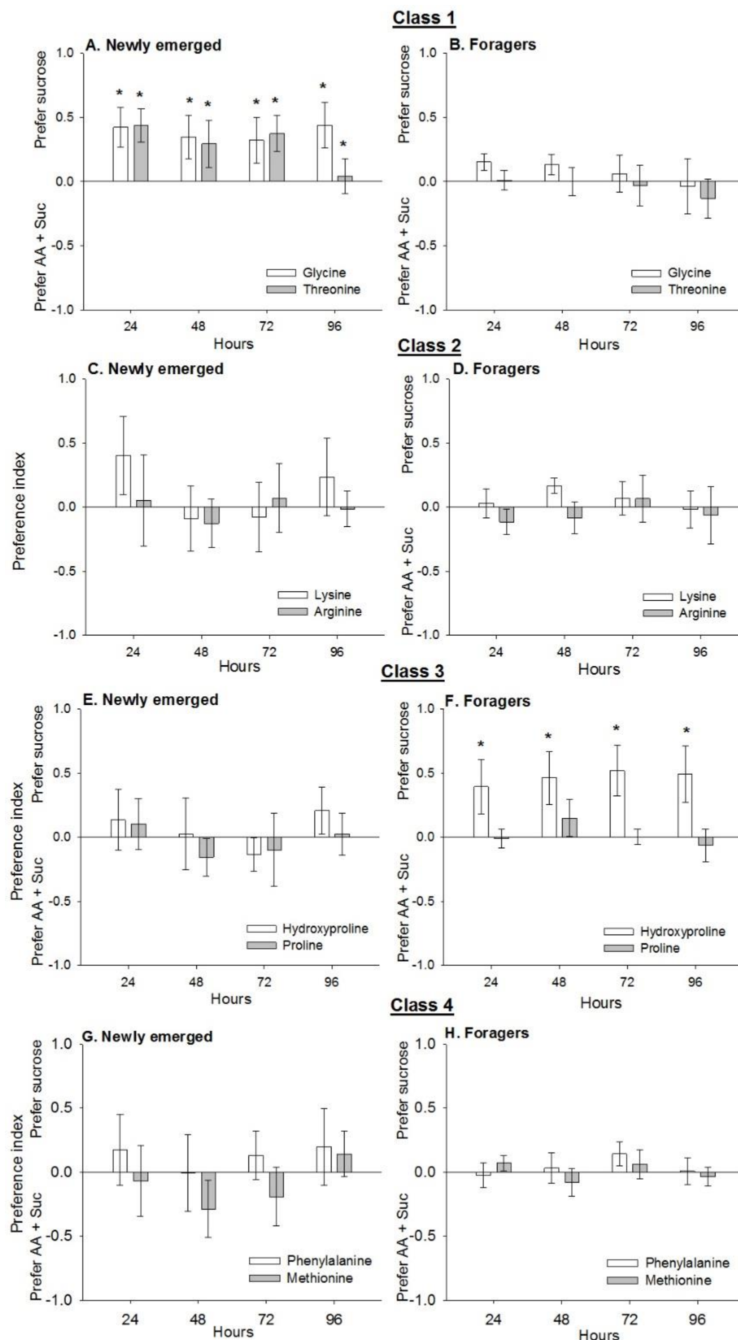

Figure 4.2. Over 96 h, most amino acid-sucrose solutions are consumed at the same volume as sucrose alone. Preference index ± SEM for forager (all N = 5 boxes) and newly emerged bees (all N = 5 boxes) given a choice between either 0.7 M sucrose alone or a 10 μM amino acid in 0.7 M sucrose. **A**. newly emerged bees: consume less of both class 1 amino acids (glycine and threonine) in sucrose than sucrose alone whereas there is no difference in consumption for **B.** forager bees **C.** newly emerged and **D.** forager bees: consume both experimental class 2 amino acids (lysine and arginine) at the same volume as sucrose alone. **E.** Newly emerged bees: consume class 3 amino acids (proline or hydroxyproline) in sucrose solution at the same volume as sucrose alone, whereas **F.** forager bees: consume more sucrose alone than hydroxyproline in sucrose. **G.** newly emerged and **H.** forager bees: consume both experimental class 4 amino acids (phenylalanine and methionine) in a sucrose solution at the same volume as sucrose alone. * indicates a significant preference for one solution over 96 h (Repeated measures ANOVA).

4.3.3. Combining amino acids increases phagostimulatory effect on newly emerged but not forager bees.

When all eight experimental amino acids are combined and offered in a sucrose solution against sucrose alone, a difference in consumption was observed between newly emerged and forager bees (Figure 4.3., repeated measures ANOVA, age: $F_{1, 8} = 5.80$, $P = 0.043$). Forager bees consumed both solutions (8 AA mix in sucrose and sucrose alone) at a similar volume (repeated measures ANOVA, solution: $F_{1, 8} = 3.92$, $P = 0.083$), whereas newly emerged bees consumed more of the amino acid mixture over sucrose alone, across the 96 h experimental duration (repeated measures ANOVA, solution: $F_{1, 8} = 7.43$, $P = 0.026$).

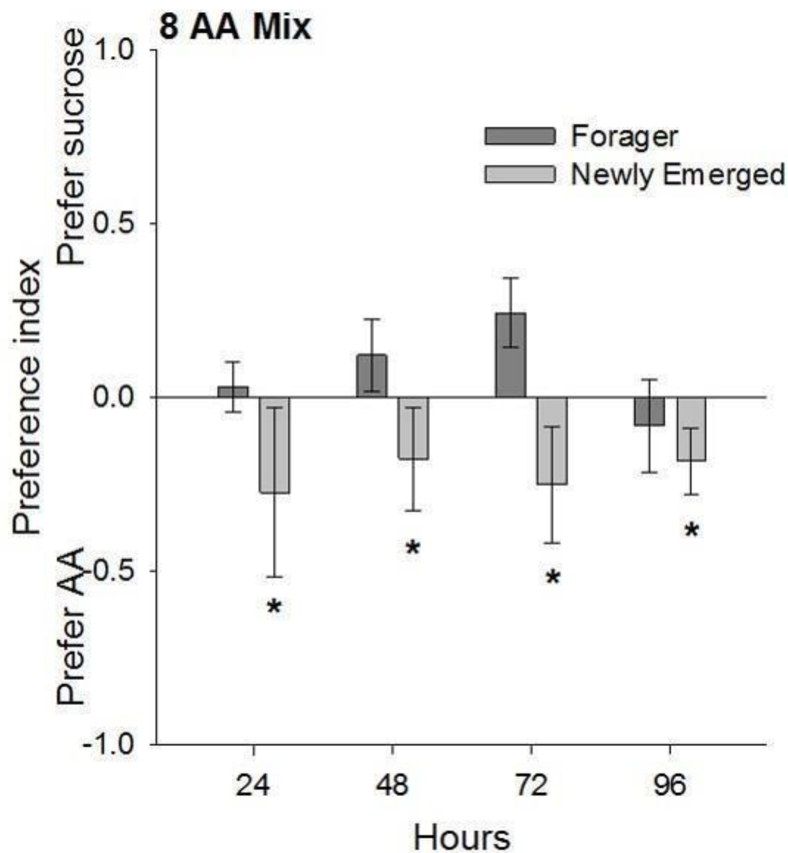

Figure 4.3. Only newly emerged bees consume a greater volume of the eight amino acid experimental mixture in sucrose over sucrose alone when given a choice. Preference index for forager and newly emerged bees given a choice between either 0.7 M sucrose alone or a 80 µM mixture of eight amino acids (glycine, threonine, lysine, arginine, proline, hydroxyproline, methionine and phenylalanine) in 0.7 M sucrose, over 96 h. Foragers N = 5 boxes, newly emerged N = 5 boxes. * indicates a significant preference for one solution over 96 h (Repeated measures ANOVA).

4.4. Discussion

In the current study I observed low volume consumption of single AAs dissolved in water or 100 mM sucrose by honeybees, independent of age. Although foragers were generally willing to consume slightly more of the AA solutions than newly emerged bees, the volumes were still relatively low compared to the maximal 60 µl capacity of the honeybee crop (Núñez, 1982).

I found no evidence to support the hypothesis that bees possess the AA taste classes defined in flies (Shiraishi and Kuwabara, 1970; Goldrich, 1973). Newly emerged bees consume the same volumes of all eight pure AAs and these volumes barely alter even with the addition of sucrose. Whilst foragers are more likely to consume AAs if they have been added to a sucrose solution (which more accurately represents the composition of floral nectar, Baker and Baker, 1973) the responses do not seem to follow the pattern expected from the fly AA taste classes. For example, phenylalanine and methionine, AAs belonging to taste class 4, are known to activate the sugar cell in the blowfly and fleshfly (Shiraishi and Kuwabara, 1970, Goldrich, 1973). The behavioural responses recorded by Goldrich (1973) indicated a high percentage of blowflies responding to class 4 AAs (including phenylalanine and methionine) via a positive PER, which would usually initiate feeding. If these taste classes existed for honeybees I would expect the class 4 AAs to be more phagostimulatory than the remaining classes and therefore be consumed in greater volumes. Consumption however, is no greater than any other AA offered in the behavioural choice assay. Responses toward these taste classes may be species specific as some studies have noted similarities to the fly taste classes, for instance; a distinct consummatory response is elicited in cockroaches in the presence of class 4 AAs (Sugarman and Jakinovich, 1986), but this is not true for all species (10 species of caterpillars, Dethier and Kuch, 1971).

Foragers did demonstrate a preference for specific AAs in 100 mM sucrose (glycine, lysine and hydroxyproline), unlike newly emerged bees. As worker honeybees reach foraging age they must leave the hive and source food such as nectar, to return to their nest mates (Winston, 1987). In order to do this, the worker bees undergo some physiological changes including increased proboscis and antennal sensitivity that allows the efficient detection of dilute nectar solutions

(Page et. al., 1998; Pankiw and Page, 2000). This increase in sensitivity may explain the increased consumption of AA sugar solutions when compared to newly emerged bees. Following electrophysiology with AAs on the tarsal GRNs of the tsetse fly, Van der Goes Van Naters and Den Otter (1998) concluded that general AA detection in these insects was possible, but discrimination between different AAs was not. Here I see that foragers consumed over double the volume of some AAs compared to others, suggesting that discrimination between specific AAs is possible for foraging honeybees.

Amino acid-deprived animals are known to increase sensitivity toward the presence of specific AAs in food (Simpson and Abisgold, 1985; Rathman et. al., 1990; Simpson et. al., 1991; Simmonds et. al., 1992; Toshima and Tanimura, 2012), however this sensitivity may not always be permanent. For example, when lysine-or threonine-deficient rats were given 40 min access to solutions containing glycine, lysine or threonine they demonstrated no difference in their response towards any solution, recorded as rate of licking. However, during a long duration test (5-6 d), rats ate more of solutions containing the deficient AA (Markison, 1999), demonstrating an ability to discriminate between AA solutions and to respond positively toward the AA they require. This ability has also been investigated in some insect species, for example, when *Drosophila* are deprived of AAs for six days they consume significantly more of an AA solution than non-deprived flies (Toshima and Tanimura, 2012). In these studies, animals were deprived of protein or AAs for six days or more, whereas the bees in the current study were only deprived for 24 h, although I had no knowledge of the bee's diet prior to catching. Such a brief duration without AAs may not have been sufficient to elicit a clear AA-orientated gustatory response. Whilst this test does not rule out AA sensing, it does show that gustatory sensitivity toward AAs in honeybees is not high, particularly without AA deprivation.

In general, bees never preferred a single amino acid containing solution to sucrose alone when assayed over 96 h. The amount eaten depended on the age of the bees and which amino acid was in solution. There was no distinction between responses toward essential and nonessential amino acids by newly emerged or forager honeybees. Out of the eight AAs tested in the present study, five are essential to the honeybee diet and therefore I expected higher

consumption rates than sucrose alone. This prediction however was unsubstantiated, with no obvious attraction toward essential AAs, a result also noted in a previous study on forager honeybees (Inouye and Waller, 1984). In a recent free-flying study however, a preference for essential AAs was noted, although the authors observed mostly ‘neutral or mildly deterrent responses’ from honeybees towards their 20 experimental AA solutions (Hendriksma et. al., 2014), similarly to the present study (see Table 4.2.). Hendriksma and colleagues (2014) concluded that, due to the low AA concentrations and the low number of positive responses obtained from honeybees, AAs in nectar are unlikely to play a role in honeybee nutrition, although they may influence plant-pollinator interactions.

Pollen is primarily identified as the major honeybee protein source, with AA concentrations far higher than those found in nectar (Baker and Baker, 1986; Winston, 1987; Avni et. al., 2014). A number of studies have demonstrated the efficiency with which the hive itself and individual bees can adjust to changes in pollen stores, highlighting the importance of this nutrient (Fewell and Winston, 1992; Camazine, 1993; Pernal and Currie, 2001; Calderone and Johnson, 2002). As pollen provides protein and a supply of essential AAs for honeybees in a natural setting, this removes any nutritional dependency for AAs in nectar. A study by Cook and colleagues (2003) indicated that honeybees may select pollen sources dependent on the essential AA content, an ability that must be learnt. In their study on oil seed rape *Brassica napus* (OSR) and field bean *Vicia faba* (FB) pollens, honeybees demonstrated a lack of innate preference between the two, perhaps indicating an inability to pre-ingestively assess AA content (Cook et. al., 2003). Only after foraging experience on each pollen type did honeybees demonstrate a clear preference for OSR, which contains the highest levels of the 3 most limiting essential AAs in the honeybee diet: valine, leucine and isoleucine (De Groot, 1953).

Honeybee preferences for AAs in nectar however, are unclear and choices appear dependent upon the specific methodology implemented. For example, in two free-flying experiments on forager bees (Inouye and Waller, 1984; Hendriksma et. al., 2014) and the current study on newly emerged bees, glycine appears to be phagoinhibitory and aversive to honeybees. However in Kim and

Smith's (2003) physical restriction study, a persistent glycine preference was observed and correlated with increasing concentration. Furthermore, the addition of glycine to a reward solution during the associative conditioning assay did in fact improve honeybee learning (Kim and Smith, 2003). However, the effect of AAs on learning may not necessarily be dependent on pre-ingestive or gustatory input. Chalisova and colleagues (2011) demonstrated a significant improvement in honeybee short and long-term memory when certain AAs are injected 30 mins prior to a conditioning assay. An additional study investigating the impact of AAs on honeybee learning found that the inclusion of three separate AAs in a reward solution (proline, methionine and phenylalanine) presented during a learning paradigm, significantly reduced positive responses (Simcock et. al., 2014), whereas isoleucine did not. Amino acid pre-feeding however, actually decreased responses towards a sucrose only solution, whereas pre-feeding with proline or isoleucine proved to reinforce learning toward themselves (Simcock et. al., 2014). As in the current study the authors observed specific responses dependent on the AA and concluded that a select few AAs are able to influence the mechanisms signalling hunger or nutritional sufficiency (Simcock et. al., 2014). In the current study, while most AA-sucrose solutions are consumed at the same volume of sucrose alone, a small number are avoided and this differs for newly emerged and forager honeybees.

Newly emerged bees actively avoid consuming a solution containing either glycine or threonine in comparison to sucrose alone. Both of these AAs belong to taste class 1 which reportedly fail to stimulate any receptor cell in the two fly species and presumably are 'undetected' (Shiraishi and Kuwabara, 1970). A lack of detection in newly emerged bees in unlikely however, as the glycine is added to a 0.7 M sucrose solution, I would expect both solutions to be consumed at equal rates. Therefore I can conclude that both glycine and threonine are phagoinhibitory to newly emerged honeybees but not foragers. The present assay is not sufficient to detect whether these AA solutions are pre- or post-ingestively aversive as bees are known to exhibit aversive behavioural responses toward toxic solution within a 60 min period (Wright et. al., 2010) and therefore the earliest measurement in the current study, 24 h, could potentially be an interaction of the two. Although, as both solutions were consumed by newly emerged bees,

albeit in small amounts, in the drink assay, this suggests a more prominent postingestive influence.

Foragers on the other hand, only avoid hydroxyproline in sucrose solution. The exact reasoning for this aversion is unclear, however this is the only non-proteinogenic AA used in the current study. Additionally, in the drink assay, hydroxyproline was in fact consumed at the highest volume in combination with 100 mM sucrose, which is considerably more dilute than in the choice assay. Current investigations into protein intake at the expense of carbohydrate have noted a strong honeybee carbohydrate bias (Altaye et. al., 2010; Paoli et. al., 2014a) particularly for older bees (Paoli et. al., 2014a). Here, forager honeybees are continuingly selecting sucrose alone over the consumption of hydroxyproline.

Table 4.2. Newly emerged and forager honeybees preference for 10 μM amino acid solutions in 0.7 M sucrose in a choice assay with 0.7 M sucrose alone over 96 h. 0: consumed the same volume of AA solution as sucrose, -: consumed less AA solution than sucrose alone.

Amino acid in 0.7 M sucrose	Class as defined by Shiraishi and Kuwabara (1970)	Preference compared to 0.7 M sucrose alone	
		Newly Emerged	Foragers
Glycine	1	-	0
Threonine	1	-	0
Arginine	2	0	0
Lysine	2	0	0
Proline	3	0	0
Hydroxyproline	3	0	-
Methionine	4	0	0
Phenylalanine	4	0	0

Newly emerged bees consumed a greater quantity of the eight-AA mixture than sucrose alone, whereas foragers demonstrated no distinction between the two. Newly emerged bees require more protein than foragers (Haydak, 1970; Paoli et. al., 2014a), particularly for their continued post-emergence tissue development and growth (Winston, 1987). As proteins exist as a combination of multiple AAs our AA mixture more closely resembles protein than any one AA alone. As discussed, newly emerged bees demonstrated no attraction, compared with sucrose alone, toward any single AA in sucrose. In *Drosophila* the addition of methionine alone to a sugar diet benefits fecundity without a negative influence on life span as seen with multiple AAs (Grandison et. al., 2009). In the present study I observed no preference and no obvious benefit from the addition of any single AA, but the combination of AAs proves more phagostimulatory for newly emerged honeybees.

Forager honeybees on the other hand, demonstrated no difference in consumption of an AA mixture and sucrose alone. In a similar study by Alm and colleagues' (1990) forager honeybees were trained to collect artificial nectar solutions from feeders in the field and, contrary to the present study, consistently consumed more AA solution than sugar alone at the feeders. The reason for these contrasting results could be attributed to two distinct differences in experimental design. Firstly, the free-flying experiment presumably assayed bees that collected solutions from the experimental feeders which they then returned to the hive, likely distributing them amongst hive-mates (Wainselboim and Farina, 2000). In the present study the experimental bees were prevented from returning to the hive and so therefore bees may alter intake dependent on the hive needs versus their individual survival requirements. Secondly, in Alm and colleagues study (1990) the AA mixture used was a replica of an angiosperm nectar know to be visited by honeybees: *Lantana camara* (Goulson and Derwent, 2004). Therefore, the foragers may have previously come into contact with the actual plant nectar and possess a pre-existing bias towards that nectar, increasing consumption of the replica. As previously mentioned, such a bias does occur after honeybees come into contact with previous pollen sources (Cook et. al., 2003) and is thought to be mediated through pollen olfactory information (Arenas and Farina, 2014). Additionally, AA pre-feeding also influences associative learning of specific AAs

(Simcock et. al., 2014), but whether such a mechanism is possible for nectar is unknown. The AA mixture in the present study is not known to replicate any existing floral nectar and its novelty may have actually reduced its attraction to forager honeybees, although not below the acceptance level of sucrose alone and such neophobia is unlikely to persist for 96 h.

4.5. Conclusion

The array of amino acids present in floral nectar are often not essential to the diet of floral visitors and therefore are thought to have evolved to aid pollinator attraction and fidelity (Baker, 1977; Baker and Baker, 1973, 1977; Lanza and Krauss, 1984; Carter et. al., 2006). This is particularly apparent for one of the most successful pollinator species; the honeybee, as all necessary AA nutrients are obtained from pollen (Winston, 1987; Crailsheim, 1990). In previous work a high attraction toward AA solutions has been noted in the honeybee (Alm et. al., 1990; Kim and Smith, 2000; Bertazzini et. al., 2010), however the current study observed no single AA in sucrose was actively preferred over sucrose alone, however single AAs in nature are very rare. Additionally, only newly emerged bees demonstrated a clear preference for a combination of AAs in sucrose over sucrose alone whereas foragers continued to accept the AAs at the same rate. However, the responses toward AA solutions did differ over time between newly emerged bees and foragers that perhaps indicates a change in dietary needs and a separate detection system for individual AAs. Both newly emerged and forager honeybees will consume small volumes of both pure AAs and those added to sugar, however, the AAs tested here did not appear to match the 'taste classes' originally specified in flies (Shirashi and Kuwabara, 1970). While further work is needed to differentiate exactly how these AAs are being detected by the honeybee, whether pre-or post-ingestively, it is likely that bees respond toward these compounds differently from flies due to a significant difference in dietary requirements (Zhang et. al., 2011).

Chapter 5.0. Comparative anatomical expression of gustatory receptors between newly emerged and forager honeybees (*Apis mellifera*).

The honeybee diet primarily consists of nectar and pollen collected from a host of floral species and dietary requirements often shift as the bee matures. Together these resources contain a variety of nutritional compounds such as sugars, amino acids, fatty acids, phenolics, minerals etc. With such a diverse diet one might expect a similarly diverse gustatory system, however, in comparison to other insects, the honeybee possesses a severely reduced set of gustatory receptor (Gr) genes. Ligands for these receptors are yet to be identified, however some orthologs have been identified in other species that may aid the determination of Gr function. In the present work the expression of gustatory receptor genes was assessed across the honeybee anatomy. All 10 Gr genes were discovered in all gustatory appendages, in addition to internal expression, which may indicate a role in nutrient sensing and central feeding regulation. Furthermore, Gr gene expression demonstrated differential expression between tissues of newly emerged and forager bees demonstrating plasticity within the gustatory system that may adapt dependent on the differing roles of bees within the hive.

5.1. Introduction

In comparison to insect olfaction, gustation has been relatively understudied. Recent advances in molecular techniques have allowed significant progress to be made in deciphering gustatory mechanisms. As an increasing number of genomes are determined more members are added to conserved gustatory gene families, aiding functional assessment and ligand identification.

In insects, pre-ingestive assessment of tastants is primarily carried out via gustatory receptor neurons (GRNs) housed in hair-like sensilla. Sensilla can take a number of forms such as hairs, pegs or bristles and depending on the species and anatomical location, can house between two and six GRNs (Dethier and Hanson, 1968; Whitehead and Larsen, 1976a; Siddiqi and Rodrigues, 1980; Nayak and Singh, 1983; Stocker, 1994; Meunier et. al., 2003). These GRNs are generally described according to the tastants they detect. The four main types are the S cell (sugars), the W cell (water), the L1 cell (low salt concentrations) and the L2 cell (high salt concentrations and aversive stimuli). Unsurprisingly, GRNs are expressed in all typical gustatory appendages of insects such as the mouthparts and tarsi, in addition to some less obvious species-specific areas, such as the wing margins and ovipositor of *Drosophila* (Stocker, 1994). The surface of each GRN usually expresses one or more 7-transmembrane gustatory receptor(s) (Grs, Chyb et. al., 2003; Dahanukar et. al., 2007; Jiao et. al., 2007), some of which are activated simultaneously and function as heterodimers (Jiao et. al., 2008; Lee et. al., 2009).

More recently, some studies have observed internal expression of insect Grs. For example, Park and Kwon (2011) discovered 12 out of the total 68 Grs expressed in the enteroendocrine cells of the *Drosophila* midgut. The expression of these 12 receptors were co-localised with three regulatory peptides (neuropeptide F, NPF; locustatachykinin, LTK and diuretic hormone 31, DH31), suggesting a role in food uptake and nutrient regulation. Similarly, the fructose receptor BmGr9 in the silk moth, *Bombyx mori*, is also expressed in the gut, further indicating a role for insect Grs in feeding regulation (Sato et. al., 2011). Interestingly, the ortholog to the silkmoth receptor in *Drosophila*, DmGr43a, functions in the mouthparts as a narrowly tuned fructose receptor, but it is also expressed and functional in the adult fly brain (Miyamoto et. al., 2012). Activation

of the brain receptor was coupled with haemolymph fructose levels leading Miyamoto and colleagues (2012) to conclude that DmGr43a acts as a 'nutrient sensor' in the brain and is directly responsible for feeding regulation in the fly.

As genome sequencing becomes more efficient, determination of Grs for more insect species and work deciphering their function is on the rise. As the first fully sequenced insect genome, *Drosophila* has led the way in Gr identification, the functions of which are slowly being uncovered (Dunipace et. al., 2001; Scott et. al., 2001; Robertson et. al., 2003; Thorne et. al., 2004). *Drosophila* has provided a useful comparative for the identification of other insect Grs, including the honeybee. Therefore, comparison with receptors first identified in *Drosophila* is a good starting point for the functional assessment of any insect gustatory receptor (for a general overview of the current known functions of *Drosophila* Grs see Table 5.1.).

The majority of *Drosophila* Grs are thought to be involved in the detection of 'bitter' or aversive substances (Table 5.1.). In an extensive study investigating the role and function of the *Drosophila* gustatory system toward bitter compounds, Weiss and colleagues (2011) mapped the labellar sensilla and identified 4 distinct bitter-sensing taste classes of bitter GRNs. Some neurons are broadly tuned to a wide variety of bitter tastants and generally express more receptor types (e.g. bitter GRN in the S-a and S-b sensilla, each responding to at least 16 different tastants and expressing up to 28 different Grs), whereas others are more narrowly tuned (responding to as few as 5 tastants and expressing only 6 Grs, Weiss et. al., 2011). The high receptor numbers involved in bitter taste detection was speculated as a function of the number and diversity of natural bitter compounds likely experienced by the fly (Weiss et. al., 2011).

Even though the bitter receptor family appears to be highly expanded in *Drosophila*, the sugar and CO_2 receptors are thought to be more highly conserved among insect species (Isono and Morita, 2010). Additionally, the existence of bitter receptors is thought to relate directly to the specific feeding approach of the insect. For example, evolutionary studies of five *Drosophila* species have supported the idea that the switch from host generalist to host specialist is associated with a greater and more rapid Gr gene loss (McBride, 2007; McBride

and Arguello, 2007). This reduction in Gr genes was also hypothesised to be non-random and primarily associated with genes responsible for bitter substance detection. McBride and Arguello (2007) reason that specialists are likely to encounter a reduced set of potentially toxic pathogens, therefore an expanded bitter Gr family is not necessary. Similarly Robertson and Wanner (2006) originally attributed the low number of honeybee Grs to a lack of expansion of certain Gr lineages. Due to the mutualistic relationship between honeybees and angiosperms, the bees do not need to accurately detect toxic compounds as the hosts provide pollinator-attractant rewards rather than costly toxins as a deterrent (Robertson and Wanner, 2006).

The level of overlap in the binding properties of Grs used to detect specific ligands such as sugars is poorly understood. In *Drosophila*, DmGr61a and the DmGr64a-f complex function in *Drosophila* sugar perception (Dahanuka et. al., 2001; Ueno et. al., 2001; Chyb et. al., 2003; Wang et. al., 2004; Slone et. al., 2007; Jiao et. al., 2007; Dahanuka et. al, 2007). However, following a DmGr64 complex knock-down, the reintroduction of just 2 receptors (DmGr64a and DmGr64f) appears to fully restore sugar sensing (Jiao et. al., 2008), which brings into question the function of the other 5 receptors. The co-expression of many of these receptors indicates their function as co-receptors, which is additionally thought to provide a “residual function” in case any receptor becomes non-functional (Dahanukar et. al., 2007). Montell (2009), however, highlights the lack of knowledge for fatty acid and amino acid detection by the remaining Grs, an ability that other insects, including the honeybee, may also possess. A recent study however has demonstrated the involvement of sugar GRNs in the detection of fatty acids, although this occurs through a phosopholipsae C (PLC) - signalling mechanism rather than the Grs themselves (Masek and Keene, 2013).

Table 5.1. Analysed *Drosophila* gustatory receptors with known or speculated functions.

Gustatory receptor	Function/general taste category	Reference
Gr68a, Gr32a	Pheromone detection and/or bitter detection	Bray and Amrein, 2003; Wang et. al., 2011; Park and Kwon, 2011
Gr21a, Gr63a	CO_2 detection	Suh et. al., 2004; Faucher et. al., 2006; Jones et. al., 2007; Kwon et. al., 2007
Gr5a, ,Gr61a, Gr64a-f	Sugar detection	Dahanuka et. al., 2001; Ueno et. al., 2001; Chyb et. al., 2003; Wang et. al., 2004; Slone et. al., 2007; Jiao et. al., 2007; Dahanuka et. al, 2007
Gr64e	Glycerol detection and sugar detection	Wisotsky et. al., 2011
Gr8a, Gr22b, Gr22d, Gr22e, Gr22f, Gr28a, Gr28b.a, Gr28b.d, Gr28b.e, Gr32a, Gr33a, Gr36a, Gr36b, Gr36c, Gr39a.a, Gr39a.b, Gr39a.d, Gr39b, Gr47a, Gr57a, Gr58b, Gr59a, Gr59b, Gr59c, Gr59d, Gr66a, Gr89a, Gr92a, Gr93a, Gr93b, Gr98b, Gr98c, Gr98d	Bitter detection	Thorne et. al., 2004; Moon et. al., 2006; Park and Kwon, 2011; Weiss et. al., 2011.
Gr10a/b, Gr63a	Potential olfactory role	Scott et. al., 2001.
Gr28b.d	Thermosensing	Ni et. al., 2013

Early work on honeybee gustation identified gustatory sensilla chaetic (or taste-hairs) on all mouthparts (glossa, labial palps and galea), antennae and tarsi and sensilla basicona (or taste-pegs) in the same regions excluding the antenna and glossa (Whitehead and Larsen, 1976a). Interestingly, what was described as a "peculiarly shaped sensillum trichodeum" was observed on honeybee mandibles and noted to only contain one sensory neuron (Whitehead and Larsen, 1976a). After categorising the different sensilla, Whitehead and Larsen (1976b) used a tip recording technique to record from the galea sensilla and further categorise the bees' physiological responses to a number of tastants. They observed a higher firing rate toward sugar over salt solutions, across a range of concentrations, with sucrose always eliciting the greatest number of spikes (Whitehead and Larson, 1976b). Very similar results were also obtained from labial palp sensilla (Whitehead, 1978). While less excitatory than sucrose, a mixture of glucose and fructose elicited a greater number of spikes from galea sensilla than expected by the simple addition of spikes from either sugar alone (Whitehead and Larsen, 1976b). This synergism between glucose and fructose is not restricted to the honeybee and has been demonstrated in a number of other insect species (blowfly: Dethier et. al., 1956; Omand and Dethier, 1969; mosquitos; *Culiseta inornata*: Schmidt and Friend, 1991, *Anopheles aegypti*: Ignell et. al., 2010, *Anopheles gambiae:* Kessler et. al., 2013). Furthermore this may hint at the involvement of multiple receptors, perhaps indicating receptor co-expression functioning as heterodimers, as observed in *Drosophila* (Chyb et. al., 2003; Dahanukar et. al., 2007; Jiao et. al., 2007, Lee et. al., 2009).

Electrophysiological studies on honeybee sensilla have demonstrated a high affinity for sugars in the mouthparts (Whitehead and Larsen, 1976b; Whitehead, 1978), tarsi (De Brito Sanchez et. al., 2008, 2014) and antennae (Haupt, 2004). Behavioural work has established a greater sensitivity for tastants on the antennae as compared to the tarsi (Marshall, 1935; De Brito Sanchez, 2008) and the proboscis (Whitehead and Larsen, 1976b). An electrophysiological study identified antennae sensilla responses toward sucrose as low as 0.1% (Haupt, 2004) demonstrating high sensitivity and variability, as the response dynamics from sensilla on the same antennae were not identical. It is yet to be

determined whether this heightened antennal sensitivity is reflected in proportion of Grs.

Detection of bitter substances is a more controversial issue. Work investigating sensitivity toward bitter tastants on the antennal tip of the honeybee demonstrated a lack of both behavioural and physiological responses to salicin stimulation and only very minor responses to quinine (De Brito Sanchez et. al., 2005). The authors therefore concluded that bees lack any receptors for bitter taste detection at the antennal tip (De Brito Sanchez et. al., 2005). While loss in bitter taste receptors has been demonstrated in some insects (McBride, 2007; McBride and Arguello, 2007) and the low number of honeybee Grs has been speculated as a reduced need to avoid toxins, we know that some bee-pollinated floral species do contain bitter secondary compounds (Detzel and Wink, 1993; Kretschmar and Baumann, 1999; Liu et. al., 2007; London-Shafir et. al., 2003; Singaravelan et. al. 2005). The occurrence of such compounds in nectar is known to both attract and deter honeybees (Liu et. al., 2007; London-Shafir et. al., 2003; Singaravelan et. al., 2005). Honeybees are additionally known to alter their preference for ‘toxic’ resources dependent on the availability of alternative food sources (Singaravelan et. al., 2005; Tan et. al., 2007).

In olfactory associative learning paradigms, honeybees will initially consume a ‘reward’ solution containing toxins, rejection of which in latter trials is attributed to post-ingestive malaise (Ayestaran et. al., 2010; Wright et. al., 2010). However, Wright and colleagues (2010) demonstrated that the pre-ingestive taste of some compounds is also influential, as the majority of experimental bees (~80%) refused to accept a sucrose solution containing relatively concentrated quinine at any stage of a learning assay. A similar result was achieved using free-flying bees in a colour association assay, in which the rejection of a stimulus paired with quinine was significantly enhanced, through pre-ingestive as opposed to post-ingestive mechanisms (Avarguès-Weber, 2010). Additionally, the presence of caffeine in honeybee-pollinated plants is thought to have evolved to secure pollinator fidelity and is now known to actually enhance honeybee memory for floral odours (Wright et. al., 2013). As all these previous studies show a clear ability for honeybees to detect ‘bitter’ compounds, there is potential that this occurs via a ‘bitter’ gustatory receptor.

In addition to sugar and bitter responses, both of which could be represented by specific Grs, honeybees also respond behaviourally and physiologically to salts (NaCl, KCl, LiCl: Whitehead and Larsen, 1976b; De Brito Sanchez et. al., 2005). The likelihood of a separate 'salt Gr' however, is unlikely, as work exploring salt detection in *Drosophila* has attributed salt sensitivity to Na+ channels, pickpocket genes and ionotropic receptors (Liu et. al., 2003; Zhang et. al., 2013).

To date, no ligands have been identified for the honeybee Grs. The function of only three receptors can be speculated from detailed assessment of other insect orthologs. Phylogenetic similarities exist between AmGr1 and AmGr2 and all eight sugar receptors in both *D. melanogaster* (DmGr64a-f, DmGr61a and DmGr5a, Chyb et. al., 2003) and the mosquito, *Anopheles gambiae* (AgGr14-21, Hill et. al., 2002; Kent et. al., 2008), indicating a likely role in sugar detection (Robertson and Wanner, 2006). Orthologs have also been identified for AmGr3 which is more unique. AmGr3 belongs to a highly conserved lineage that only includes one receptor from each member species (Robertson and Wanner, 2006). The corresponding orthologs in this lineage from the silk worm (*Bombyx mori,* BmGr9, Sato et. al., 2011), fruit fly (*Drosophila melanogaster*, DmGr43a, Miyamoto et. al., 2012) and cotton Bollworm (*Helicoverpa armigero, HaGr9,* Xu et. al., 2012) have all been identified as fructose receptors, indicating a role for AmGr3 in fructose detection (Sato et. al., 2011; Miyamoto et. al., 2012). As previously mentioned, DmGr43a also functions as a nutrient sensor within the fly brain, a role that is also possible for AmGr3 in the honeybee.

As for the remaining 7 honeybee Grs, there is little information as to what they may detect. In their original discovery, Robertson and Wanner (2006) noted some weak support for AmGr4 and AmGr5 as orthologs to the DmGr28a/b complex, now thought to be involved in bitter tastant detection, however, the remaining Grs had no orthology to any fly Gr. There is potential for the remaining Grs to carry out honeybee-specific functions as noted in other insects. For example, the females of some butterfly species are known to respond to specific tastants that signify potential oviposition sites (Ozaki et. al., 2011; Ryuda et. al., 2013). The ligand for one specific Gr (PxutGr1) of the swallowtail butterfly (*Papilio xuthus)* was discovered to be the oviposition-stimulant: synephrine. As worker

honeybees are sterile it is unlikely that they will possess a specific receptor involved in egg laying, however there are a variety of other chemical compounds that influence honeybee behaviour, particularly cuticular hydrocarbons (CHCs, Chaline, et. al., 2005; Dani et. al., 2005) or the 'queen pheromone'. However, as the olfactory receptor AmOr10 is already known to detect the main component of queen substance; 9-oxo-2-decenoic acid (Wanner et. al., 2007), this may indicate pheromone detection relies on the olfactory system, however both olfactory and gustatory receptors may be involved, similarly to *Drosophila* (Ferveur, 2005).

While determining the ligands for each honeybee Gr may prove challenging, examining receptor expression throughout the bee life cycle may supply additional information. Honeybees are usually separated by both age and task (Winston, 1987). Such behavioural segregation may potentially influence, or indeed be influenced by, Gr expression. In her review on honeybee taste perception, De Brito Sanchez (2011) speculates the potential for differing Gr numbers between different castes. Differential Gr expression has also been previously identified in a number of insect species. For example, *Drosophila* larvae possess fewer Gr genes than adults (Mishra et. al., 2013) and whilst capable of sugar detection, larvae do not express any of the eight adult primary sugar receptor genes (Gr5a, Gr61a, Gr64a-f). Mishra and colleagues (2013) discovered that larval sugar sensitivity relies solely on the fructose-sensing Gr43a to detect all dietary sugars. The authors propose this differential gene expression as environmentally appropriate due to the "complex environment of adult *Drosophila*" versus the limitations of egg location and hatching site imposed on the larvae (Mishra et. al., 2013). Differential expression of two Gr genes (CfGr9 and CfGr54) has additionally been noted between the minor and major worker caste in the ant, *Camponotus floridanus,* thought to aid each ant's particular lifestyle (Zhou et. al., 2012). Differences in gustatory sensitivity as a function of age has additionally been identified in the mosquito (*A. gambiae*) as the spike asymptote reached from GRNs in five day old subjects was around twice as high as those at zero days old (Kessler et. al., 2013).

Unsurprisingly, differences in various gene expressions in the brain of nurse and forager bees have been discovered; some solely attributable to behavioural changes and some to age (Whitfield et. al., 2003). Gustatory

sensitivity also alters following behavioural changes and while assumed to be a result of various hormonal interactions, we currently do not know the influence on Gr expression (Page et. al., 1998; Amdam et. al., 2006).

The current work aims to assess the difference in Gr expression between two ages of bees, the newly emerged and foragers. Gustatory receptor expression was analysed in nine anatomical locations, both external (antenna, mouthparts, tarsi) and internal (brain and guts). If honeybee Grs function as dimers then similar receptor expression is expected in any one location. As forager bees leave the hive to locate and assess potential food items, I expect to see an expanded Gr repertoire, with higher Gr numbers compared to younger bees.

5.2. Materials and methods

5.2.1. Phylogenetic tree construction.

Note: For detailed methods see 2.13. Phylogenetic tree construction.

Each *Apis mellifera* gustatory receptor (AmGr) genomic nucleotide sequence was analysed in BLASTn then added into a BLASTp search using the UniprotKB/Swiss-prot database. All returned sequences, from *Drosophila melanogaster* and the *Apis* sequences, underwent a multiple alignment analysis and were used to build a phylogenetic tree in MEGA version 6.0 (Tamura et. al., 2013).

5.2.2. Honeybee capture and dissection.

Note: For detailed methods see 2.11. Molecular biology.

Forager and newly emerged honeybees (*Apis mellifera Buckfast*) were captured individually, cold-anesthetised and immediately dissected under a light microscope.

For 'hard' tissues, 75 body parts were collected per sample, a maximum of 2 samples were collected for each body part (antennae, galea, labial palps, glossa, fore-tarsi, mid-tarsi and hind-tarsi) and were transferred into 500 µl of TRIzol reagent. Due to good RNA yield from 'soft' tissues, five whole brains and guts were collected for each sample (4 samples for each), these were immediately transferred into 1 ml of TRIzol reagent.

5.2.3. Sample preparation, RNA extraction, DNase treatment and reverse transcription.

Note: For detailed methods see 2.11.3. Sample preparation.

Total RNA extraction followed the TRIzol reagent protocol (Invitrogen) with a few modifications. Samples were treated with RNase-free DNase (Promega) following manufacturers instruction. A total of 1000 ng of RNA were added to the reverse transcription reaction following the manufacturer's protocol for Superscript III reverse transcriptase.

5.2.4. Relative expression using polymerase chain reaction (PCR).

Note: primers were designed manually, for detailed methods see: 2.11.4 Primer design and Reverse transcription polymerase chain reaction (RT-PCR).

End-point PCR was carried out as a check for successful DNase treatment and primer validation. PCR products were diluted and sent for sequencing with the corresponding forward primer, for details see: 2.11.5. Sequencing.

Quantitative real time-PCR was performed on a Roche LightCycler 480, each reaction contained 7 µl RNase-free water, 1 µl of each forward and reverse primer (5 µM), 10 µl LightCycler SYBR Green I Master and 1 µl cDNA, each sample was run in duplicate.

All samples were normalized to the reference gene RP49 (Ament et. al. 2011). Relative mRNA levels were expressed as a proportion relative to the forager brain expression levels which were always normalised to 1.

5.2.5. Scanning electron microscopy

Note: for detailed methods see 2.12. Scanning electron microscopy.

Forager honeybees were cold-anesthetised before removal of the whole head and fore-tarsi. Samples were fixed using 2% glutaraldehyde in Sorenson's phosphate buffer, 'rinsed' three times in Sorenson's buffer and dehydrated in ethanol before the final dehydration took place in a Baltec critical point dryer with carbon dioxide. Samples were mounted and gold coated and finally examined using a Stereoscan 240 scanning electron microscope.

5.2.6. Statistical analyses

Following RT-qPCR, relative mRNA expression was calculated using the 2ΔΔCt method (Livak and Schmittgen, 2001). Expression level of mRNA in the forager brains was used as the 'control' sample and expressed as 1 by:

AVERAGE (Σ(2ΔΔCt/AVERAGE2ΔΔCt))

This was done separately for every Gr.

Due to the small sample sizes, no statistical analyses were carried out on expression levels of any Gr in any tissue except brains and guts. Expression levels of mRNA in brain and gut samples were analysed in SPSS version 21.0. A generalised linear model (GZLM) was carried out separately for each Gr with age and body part used as independent variables. A pairwise *post hoc* comparison was carried out with Sidak adjustment for multiple comparisons. Significant P-value < 0.05.

5.3. Results.

5.3.1. Phylogenetic tree analysis: AmGr1-3 are orthologous to Drosophila sugar receptors

Using the manually annotated UniProtKB/Swiss-Prot protein database, the 10 *Apis* mellifera gustatory receptor protein sequences returned a total of 20 similar *Drosophila melanogaster* sequences (Figure 5.1.). The UniProtKB/SwissProt database was selected in order to identify well-annotated sequence data that could be used to infer potential functions of the *Apis* receptors.

The gustatory receptors AmGr1 and AmGr2, are closely clustered within the branch containing the eight sequences for the *Drosophila* sugar receptors (DmGr5a, DmGr64a-f and DmGr61a, Chyb et. al., 2003; Dahanukar et. al., 2007; Jiao et. al., 2007, Lee et. al., 2009), suggesting a sugar-detection role for AmGr1 and AmGr2. Additionally AmGr3 appears most closely related to the fructose receptor DmGr43a (Miyamoto et. al., 2012). The remaining *Apis* receptors are not orthologous to any *Drosophila* receptor. The common ancestor of AmGr4 and AmGr5, DmGr66a, is known to be present in all 'bitter'-sensitive gustatory receptor neurons (GRNs) in *Drosophila* (Wang et. al. 2004) implying a potential role in bitter-sensing, particularly caffeine (Moon et. al., 2006). AmGr6 lies near to AmGr10, however both remain on distinct branches with the closest *Drosophila* homologs as Gr68a, thought to be involved in pheromone detection and male courtship (Bray and Amrein, 2003) and Gr32a, also thought to be involved in pheromone detection, male-male aggression and bitter-sensing (Miyamoto and Amrein, 2008; Wang et. al., 2011). AmGr7 also exists on a solitary branch, however it appears to share a common ancestor with all *Drosophila* and *Apis* putative sugar receptors, perhaps indicating a role in sugar detection. AmGr8 and AmGr9 are unusual in that they are segregated and do not branch particularly close to any *Drosophila* receptor and therefore may provide sensitivity to a honeybee-specific compound.

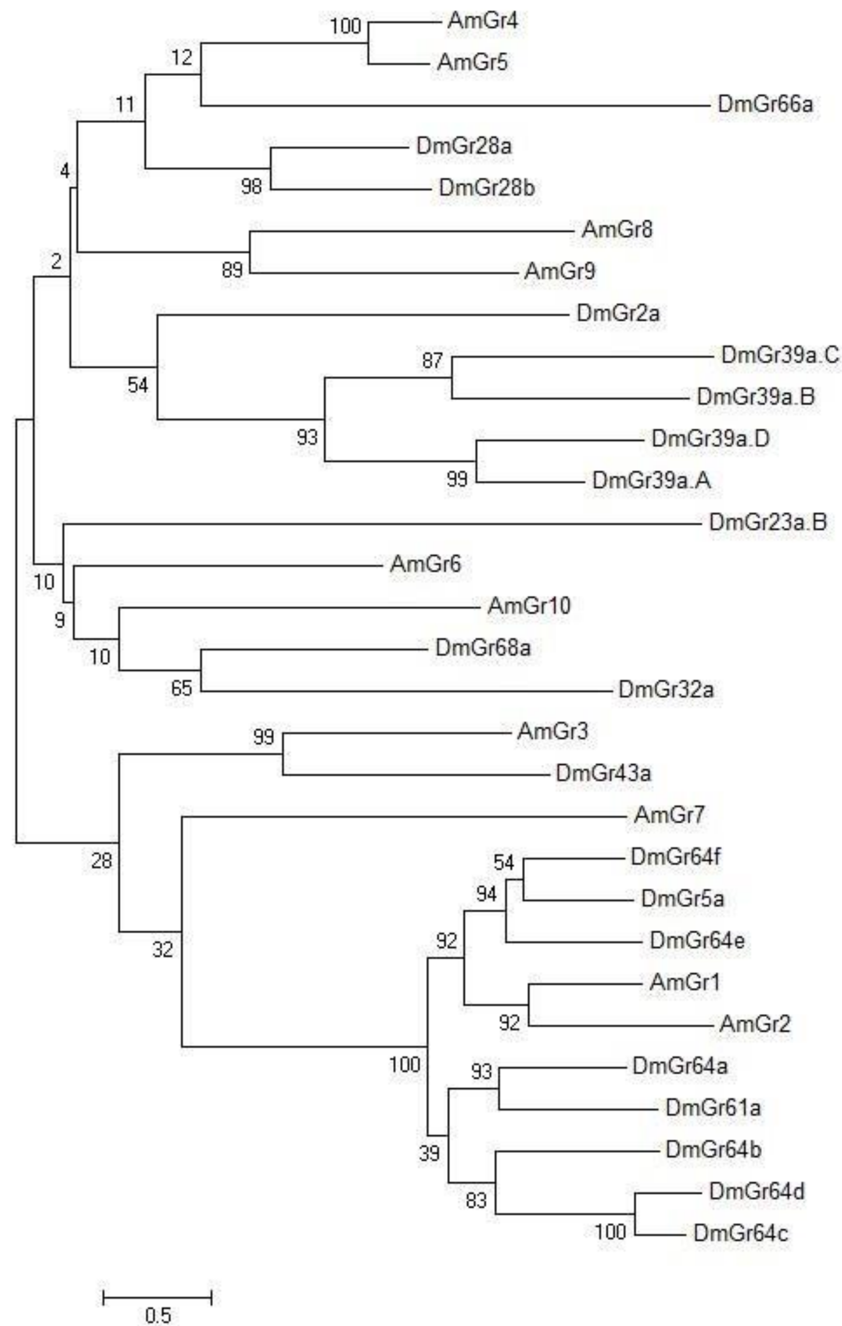

Figure 5.1. Phylogenetic tree of *Apis mellifera* (Am) and homologous *Drosophila melanogaster* (Dm) gustatory receptor protein sequences analysed using a maximum likelihood (ML) analysis, constructed using a 500 replication bootstrap method in MEGA 6.0 (Tamura et. al., 2013). The bootstrap value, shown next to branches, demonstrates percentage of trees in which the associated taxa clustered together. Tree is drawn to scale and branch length represent the number of amino acid substitutions per site.

5.3.2. Internal expression of most honeybee gustatory receptors is highest in the forager brain

The internal assessment of gustatory receptor gene expression revealed that all 10 Grs are expressed in both the brain and gut of newly emerged and forager honeybees (note: AmGr9 mRNA expression was detected at levels too low for confident quantification across samples).

The mRNA expression levels for all honeybee gustatory receptors, except AmGr2 and AmGr8, were dependent on both the age of the bee and the anatomical location of expression (Table 5.2.). Both age and body part independently influenced the mRNA expression of AmGr2, whereas expression levels of AmGr8 was solely dependent on the age of the individual (Table 5.2.).

Table 5.2. GZLM for gustatory receptor expression in brains and guts of newly emerged and forager honeybees with age and body part as independent variables for a full factorial analysis. All *P*-values < 0.05 are shown in bold.

	Age			**Internal body part**			**Age*Internal body part**		
	X_2	**df**	**Pvalue**	**X_2**	**df**	**Pvalue**	**X_2**	**df**	**Pvalue**
AmGr1	8.299	**1**	**0.040**	1.984	**1**	0.159	10.022	**1**	**0.020**
AmGr2	5.282	**1**	**0.022**	4.262	**1**	**0.039**	3.553	**1**	0.059
AmGr3	24.953	**1**	**<0.001**	22.303	**1**	**<0.001**	41.938	**1**	**<0.001**
AmGr4	30.629	**1**	**<0.001**	18.069	**1**	**<0.001**	20.424	**1**	**<0.001**
AmGr5	98.510	**1**	**<0.001**	115.253	**1**	**<0.001**	93.354	**1**	**<0.001**
AmGr6	10.810	**1**	**0.001**	25.546	**1**	**<0.001**	14.241	**1**	**<0.001**
AmGr7	33.283	**1**	**<0.001**	23.994	**1**	**0.001**	23.486	**1**	**0.001**
AmGr8	5.620	**1**	**0.018**	2.050	**1**	0.152	1.751	**1**	0.186
AmGr10	42.681	**1**	**<0.001**	30.599	**1**	**<0.001**	35.686	**1**	**<0.001**

Expression levels of mRNA for all but one receptor gene (AmGr8) were greater in the brains of forager honeybees compared to forager guts, or indeed any internal expression in newly emerged bees (Figure 5.2.). Whilst AmGr8 mRNA expression was highest in forager gut tissue, this expression was highly variable (Figure 5.2H). Brain mRNA expression was significantly different between forager and newly emerged bees for the majority of Gr genes, however expression levels in the guts of the two groups was similar (Figure 5.2.).

5.3.3. Gustatory receptor expression is widespread but diverse across both newly emerged and forager honeybees.

Note: AmGr9 mRNA expression levels were detected in all tissue types in both groups however levels were too low to include reliable expression values.

Expression of mRNA for all 10 honeybee gustatory receptors was observed in all tested tissue types in both forager and newly emerged bees. Gustatory receptor mRNA expression levels were generally higher across tissues in forager bees (Figure 5.3A) compared to newly emerged bees (Figure 5.3B). The mRNA for some gustatory receptors, notably AmGr4 and AmGr5, demonstrated relatively stable expression levels across all tissue types, while others, such as AmGr1 and AmGr3, were more variable between tissues. In both honeybee groupss, the putative fructose receptor (AmGr3) was expressed at the highest level across tissues (Figure 5.3.) followed by the remaining candidate sugar receptors (AmGr1 and AmGr2).

The primary difference between newly emerged and forager bees was the relatively high expression, of a number of Grs, in the forager brains, whereas most Gr expression appeared relatively low in the newly emerged brains.

In terms of specific tissue types; the labial palps (Figure 5.4A) and the galea (Figure 5.4B) of the mouthparts possessed the greatest combined expression of all Grs in both forager and newly emerged bees, whereas the guts and hind-tarsi possessed the lowest. In the proboscis, all gustatory receptors were expressed at the lowest levels in the glossa (Figure 5.5.), compared to the other mouthparts, for forager bees and all except AmGr10 in the newly emerged bees (Figure 5.3B). Gustatory receptor expression levels in the antennae (Figure 5.6.) were almost double, for every Gr, in newly emerged bees compared to foragers. Tarsal expression across both groups demonstrated a general decrease in total Gr expression from the fore-tarsi (Figure 5.7.) to the hind-tarsi (anteroposterior).

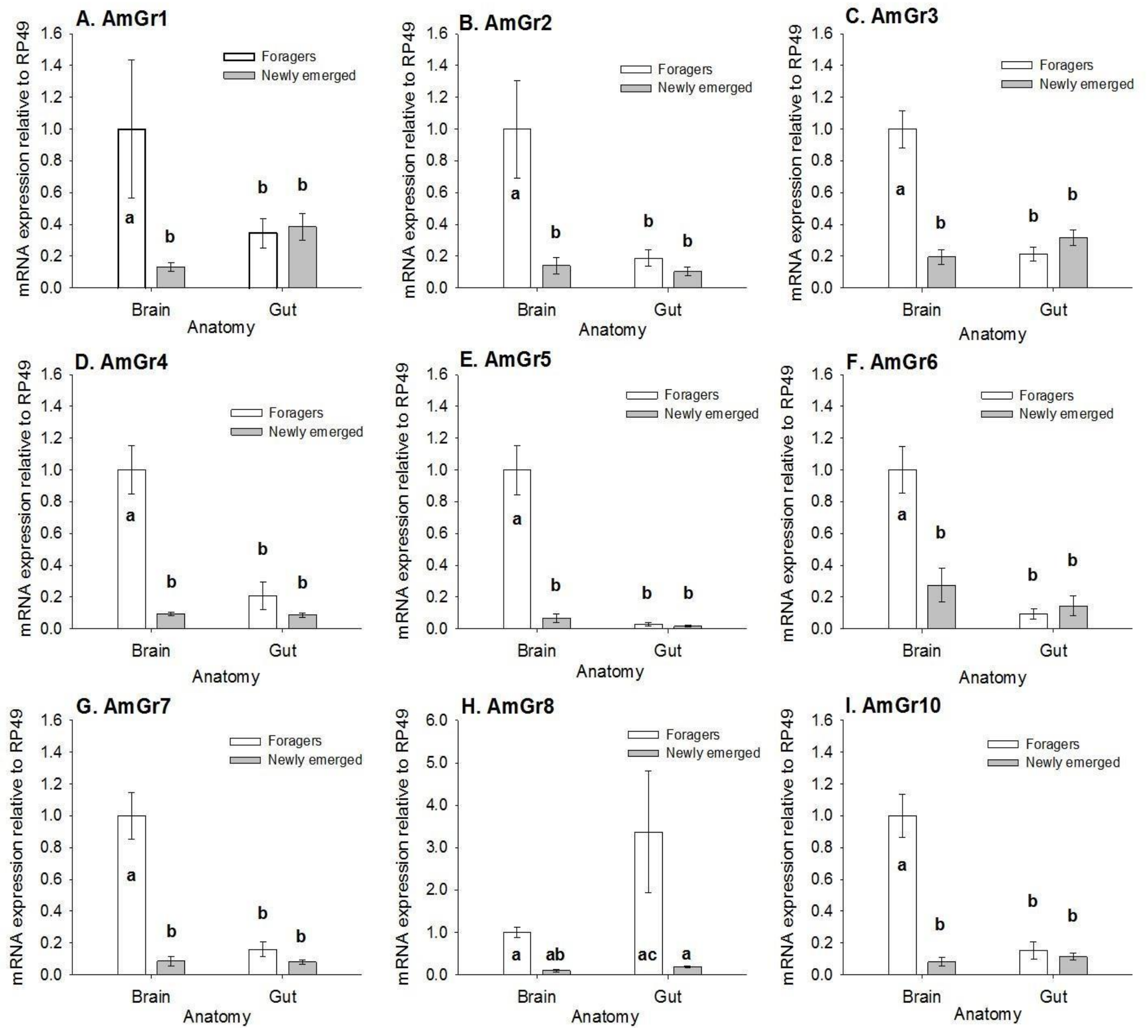

Figure 5.2. Internal expression levels of most gustatory receptor mRNA is greatest in the forager honeybee brain. Internal expression of mRNA for *Apis mellifera* gustatory receptors (AmGr): in newly emerged and forager bees, brain and gut tissues **A**. AmGr1 (N=4), **B**. AmGr2 (N=4), **C**. AmGr3 (N=4), **D**. AmGr4 (N=3-4), **E**. AmGr5 (N=4), **F**. AmGr6 (N=4), **G**. AmGr7 (N=4), **H**. AmGr8 (N=4), **I**. AmGr10 (N=4). a, b, c represent GZLM pairwise comparison, Sidak $P < 0.05$.

A. Forager	Gr1	Gr2	Gr3	Gr4	Gr5	Gr6	Gr7	Gr8	Gr10
Brain	1.00	17.39	33.24	0.30	0.06	7.14	2.02	0.51	18.25
Guts	0.34	3.29	7.11	0.06	0.00	0.68	0.32	1.73	2.82
Antennae	15.43	10.63	29.61	0.52	0.09	5.48	4.96	0.91	6.64
Galea	18.54	7.13	18.69	0.34	0.06	18.67	15.43	9.15	11.23
Labial Palps	16.04	7.88	38.86	2.05	0.29	41.80	17.43	20.08	29.60
Glossa	3.55	6.68	23.16	0.10	0.07	4.27	1.14	4.78	14.53
Fore-legs	21.29	19.85	43.20	9.41	0.10	8.05	21.87	10.87	18.90
Mid-legs	15.15	11.28	60.90	4.91	0.06	8.38	8.62	2.79	10.51
Hind-legs	2.21	4.97	21.49	2.54	0.03	1.40	2.17	1.41	4.01

B. Newly Emerged	Gr1	Gr2	Gr3	Gr4	Gr5	Gr6	Gr7	Gr8	Gr10
Brain	0.13	2.45	6.48	0.03	0.00	1.97	0.17	0.05	1.48
Guts	0.39	1.81	10.57	0.03	0.00	1.03	0.16	0.10	2.07
Antennae	46.93	21.73	45.60	1.17	0.07	5.60	5.22	1.80	3.28
Galea	10.69	3.96	20.77	NA	NA	16.19	12.97	21.99	3.54
Labial Palps	26.79	6.23	23.56	1.06	0.10	26.30	15.02	8.82	7.89
Glossa	2.24	3.01	9.26	NA	0.11	3.97	0.53	1.45	15.03
Fore-legs	17.03	9.87	57.15	NA	NA	8.31	18.68	3.55	6.94
Mid-legs	6.02	3.01	21.60	0.43	0.04	2.31	3.83	0.79	2.57
Hind-legs	1.51	1.74	17.97	0.16	0.01	0.85	1.15	0.42	3.92

Figure 5.3. Expression of gustatory receptor mRNA levels varied with anatomical location and specific Gr in both newly emerged and forager honeybees. All expression values are relative to the expression of the reference gene RP49 and are normalised to AmGr1 in the forager brain. **A.** Expression levels across the un-manipulated forager anatomy (≈2-3 wk old). **B.** Expression levels across the un-manipulated newly emerged honeybee (≈24 h old) anatomy (NA represents unavailable data.). Note: AmGr9 mRNA expression levels were detected in all tissue types in both groups however levels were too low to include reliable expression values.

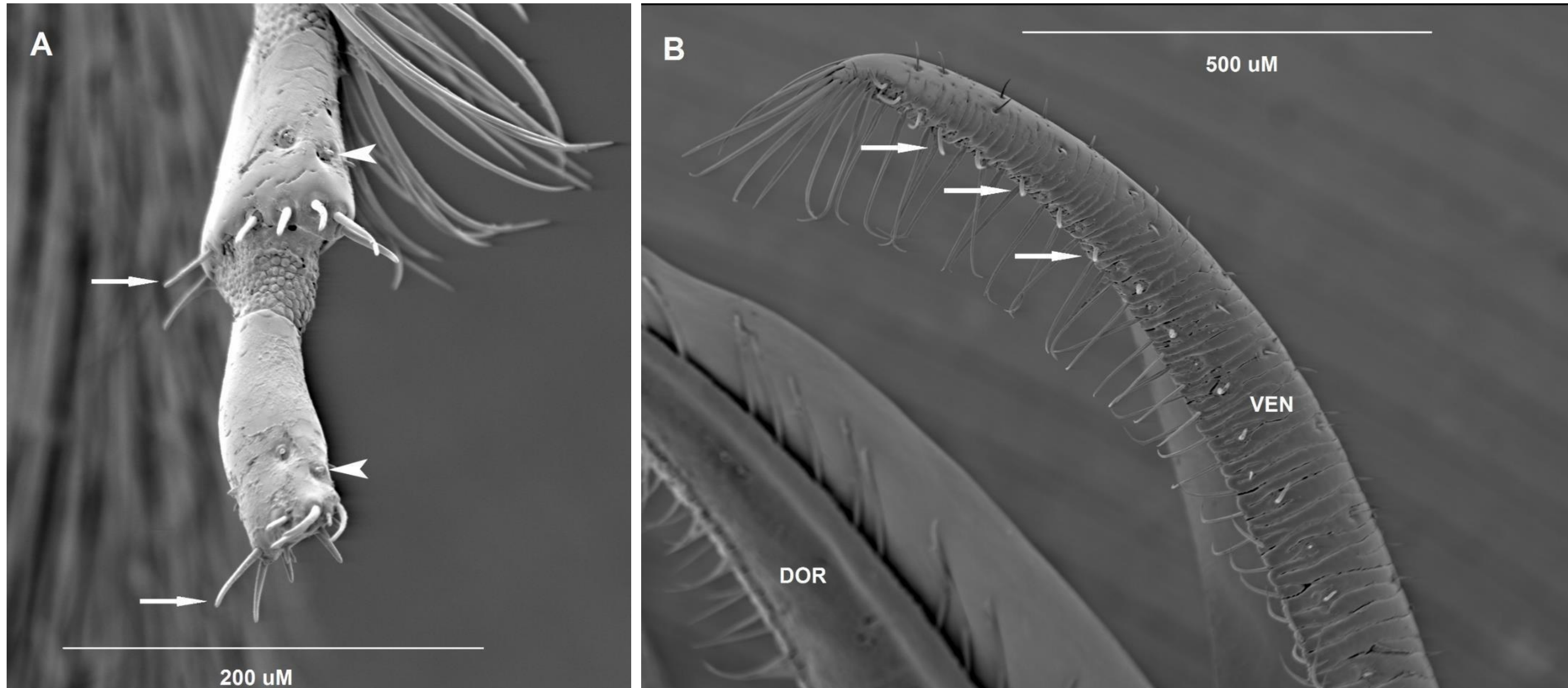

Figure 5.4. Mouthparts of an adult forager honeybee as shown by scanning electron microscopy. **A.** The tip of a labial palp. Arrows indicate sensilla chaetica and arrow heads indicate sensilla basiconica, both of which house gustatory receptor neurons (GRNs) and gustatory receptors (Grs). **B.** The ventral (VEN) and dorsal (DOR) side of galea of the two maxillae. Arrows indicate sensilla chaetica that house gustatory receptor neurons.

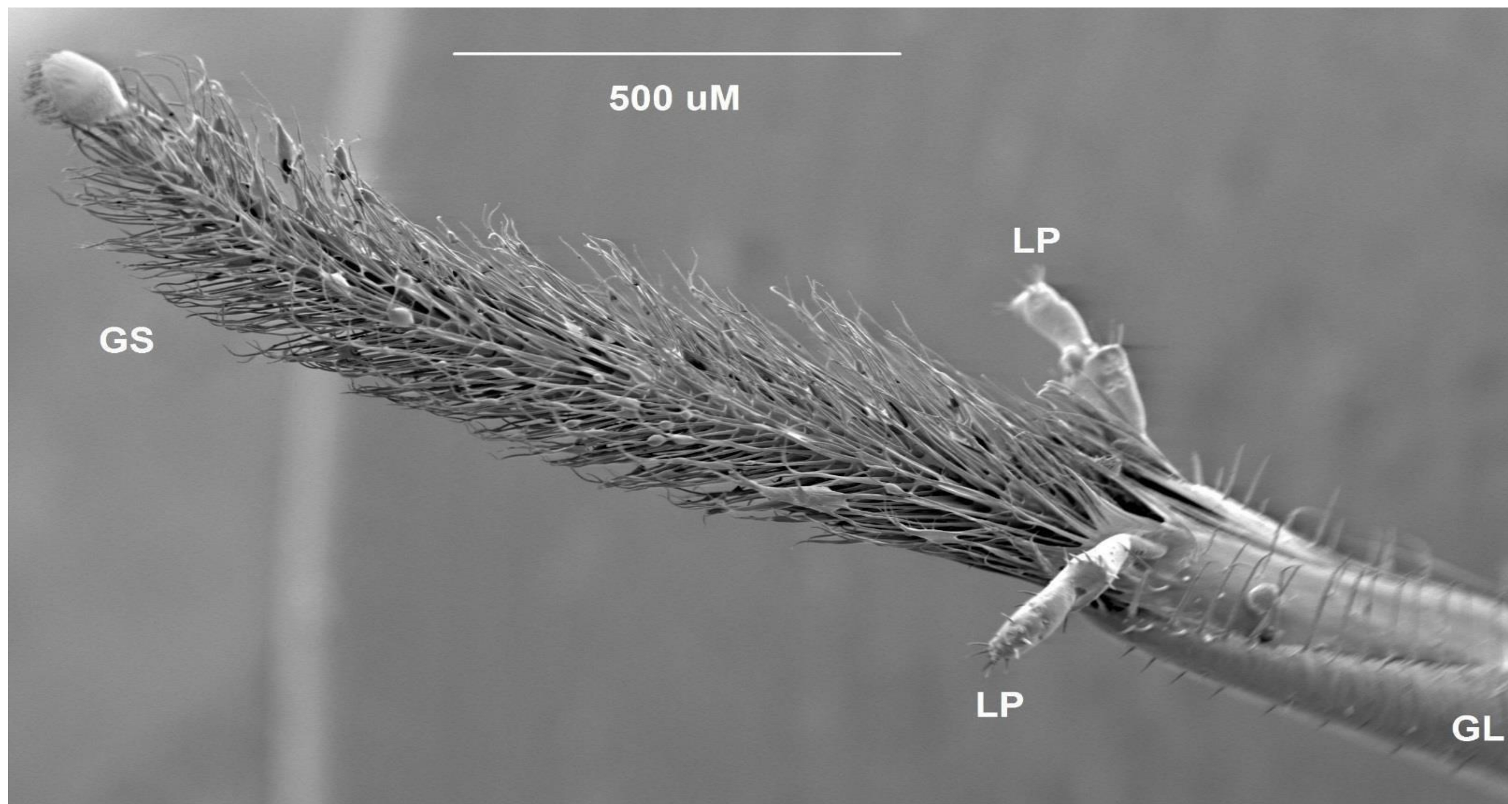

Figure 5.5. Proboscis of an adult forager honeybee revealed by scanning electron microscopy. Comprising: the extended glossa (GS), two labial palps (LP) and the left-hand galea (GL). All sections contain sensilla that house gustatory receptor neurons.

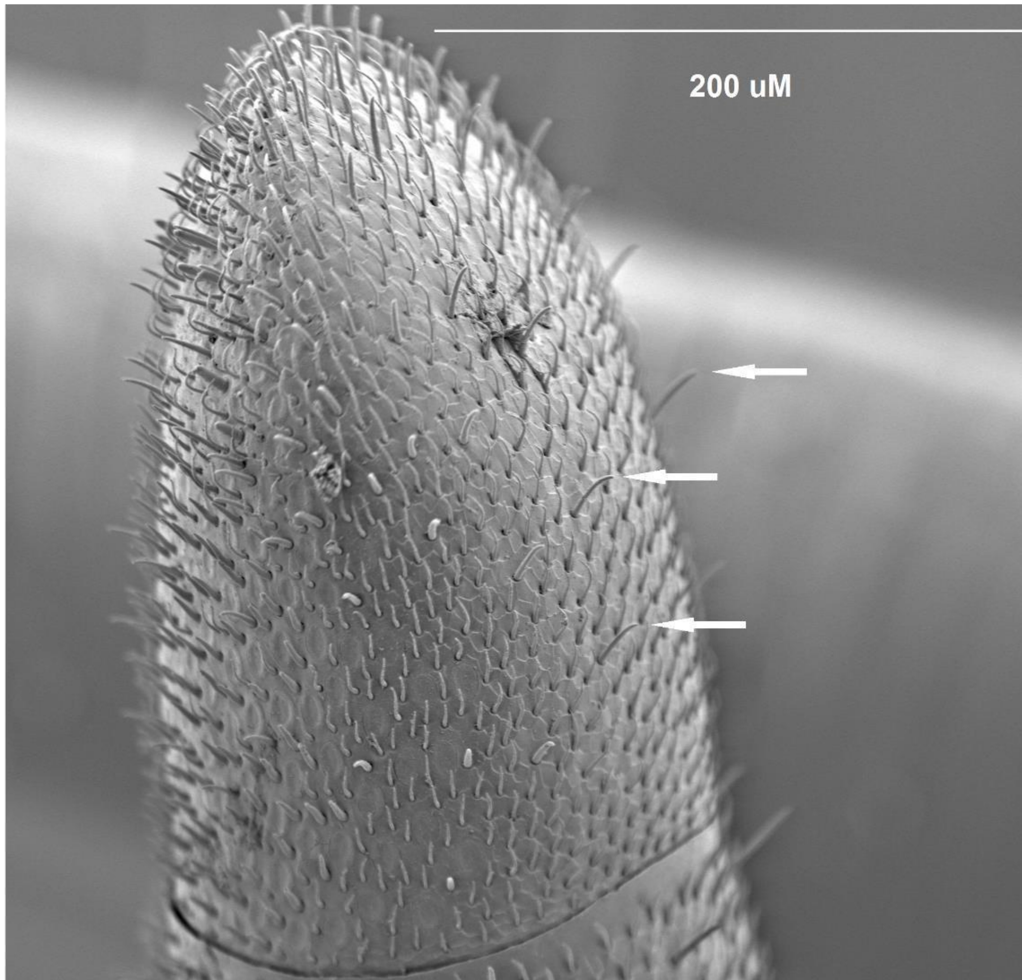

Figure 5.6. Scanning electron micrograph of the 9th and 10th segments of the forager honeybee antennae (apical tip). Gustatory receptor neurons are thought to be housed in the sensilla chaetica (indicated by arrows) surrounded by sensilla trichodea which house the olfactory receptor neurons.

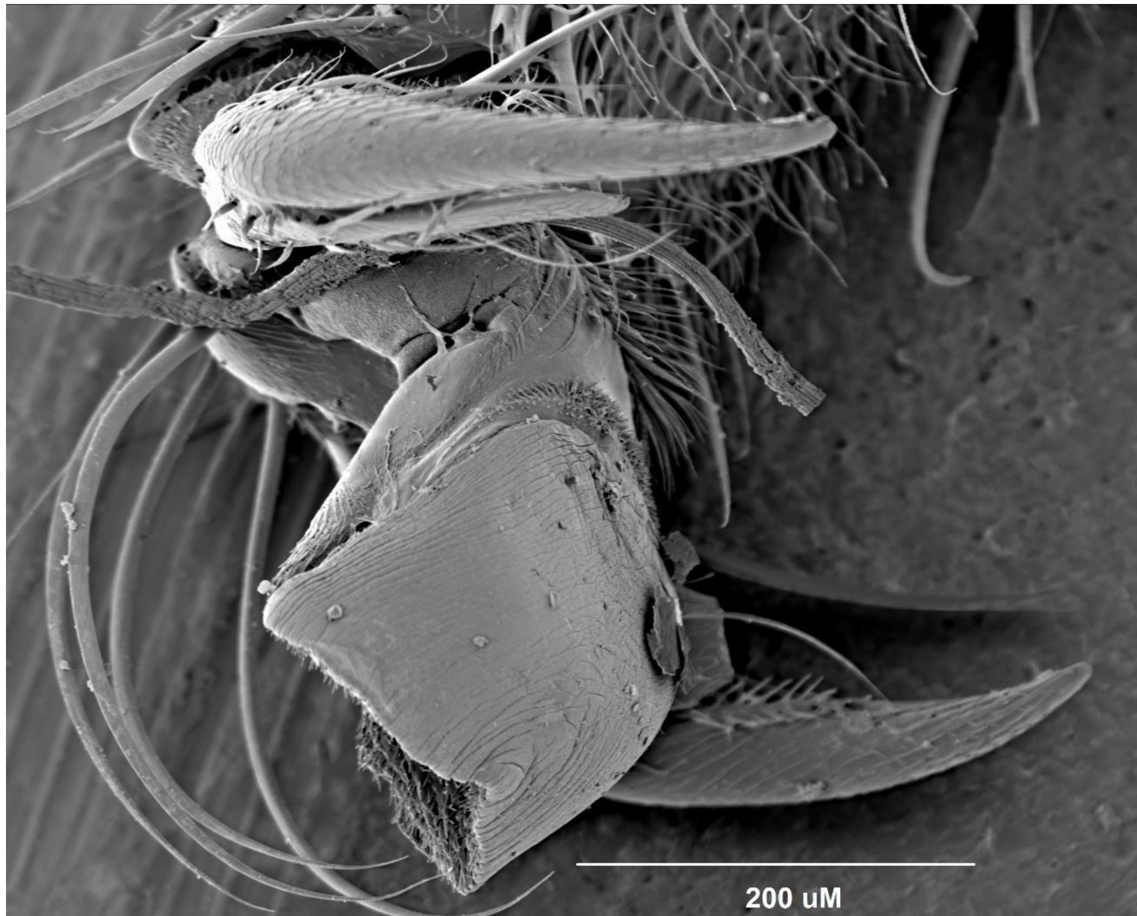

Figure 5.7. The apical tarsal claw of the fore-tarsus of an adult forager honeybee shown by scanning electron microscopy. The majority of the honeybee Grs show the highest mRNA expression levels in the fore-tarsi compared to the mid- and hind-tarsi. Expression also tends to be higher in forager fore-tarsi than newly emerged fore-tarsi.

5.4. Discussion

The current phylogenetic analysis between *Apis* and *Drosophila* gustatory receptor proteins matches very closely to those originally identified by Robertson and Wanner (2006). The 2006 paper noted close clustering of AmGr1 and AmGr2 to the eight candidate sugar receptors in both *Drosophila* (DmGr5a, DmGr61a, DmGr64a-f) and *Anopheles* (AgGr14-21), based on DmGr5a as a trehalose receptor (Chyb et. al., 2003; Robertson and Wanner, 2006). Additionally they suggested that AmGr3 is very similar to DmGr43a, which they noted may indicate it plays a 'conserved role' in these insects (Robertson and Wanner, 2006). We now know that the DmGr43a-family primarily function as fructose-sensors (Sato et. al., 2011; Miyamoto et. al., 2012; Xu et. al., 2012); AmGr3, therefore, may also play a similar role in bees.

The only other similarity that Robertson and Wanner (2006) noted was some 'weak bootstrap support' between AmGr4 and AmGr5 and the *Drosophila* DmGr28a/b complex. This weak support is also apparent in the present analysis along with DmGr66a, now known to function as a bitter-taste detector and more specifically a caffeine receptor (Thorne et. al., 2004; Wang et. al., 2004; Moon et. al., 2006). Expression of the Dm28a/b complex has similarly been identified in labellar bitter neurons (Weiss et. al., 2011). More recently however, Gr28b.d has also been indicated in thermosensing (Ni et. al., 2013). In the honeybee, thermosensing is attributed to a Transient Receptor Potential A channel (AmHsTRPA) which additionally functions in sensing insect anti-feedants (Kohno et. al., 2010), however an extant *Apis* Gr may also possess a similar role.

Similarly to the original findings, the remaining five *Apis* receptors do not cluster confidently with any *Drosophila* receptor and are only supported by very weak bootstrap values. AmGr6 and AmGr10 diverge closest to DmGr68a and DmGr32a, both of which are involved in pheromone detection (Bray and Amrein, 2003; Agrawal and Riffell, 2011); however, Gr32a is also involved in bitter-taste (Park and Kwon, 2011). These receptors may also function in bee pheromone detection and only match weakly with *Drosophila* receptors due to species specific differences. AmGr7 sits between the general sugar receptors and the fructose receptors and could also function as a sugar-detecting receptor. AmGr8 and AmGr9 separate out near the top of the tree with no close matches.

Identification of the actual ligands for these receptors will require knock-in or knock-out studies. However, we do know the honeybee diet is highly varied; nectar and pollen contain a variety of sugars, amino acids, fatty acids, vitamins, phenolics, alkaloids and other plant secondary compounds, (Percival, 1961; Baker and Baker, 1973) any of which could be detected by one or more of these unknown receptors.

Surprisingly, expression of all 10 honeybee Grs were observed in all tissues analysed, including internal expression in the brain and gut. (AmGr9 was detected in all tissues; however the expression levels were too low for valid quantification). Internal Gr expression has been identified in a number of species (Park and Kwon, 2011; Sato et. al., 2011; Miyamoto et. al., 2012) and all current work indicates that these receptors fulfil a 'nutrient-sensor' role. The greatest level of 'internal' expression for the majority of honeybee Grs (excluding AmGr8) was noted in the forager brain. The transition from in-hive bees to foragers is associated with a series of physiological and hormonal changes (Fluri et. al., 1982; Winston, 1987; Huang et. al., 1994; Wagener-Hulme et. al., 1999). My data suggest that these changes also influence the forager bee's gustatory system and its internal ability to detect nutrients.

When young bees emerge from their cells as adults, development continues for the next 8-10 days and within the first few hours of emergence, bees begin to consume pollen to aid development and growth (Winston, 1987). Young bees in the current experiment were not supplemented with any pollen in the ~24 h between emergence and dissection. While the lack of protein may have influenced Gr expression in newly emerged bee brains, this appears unlikely as some peripheral tissues demonstrate Gr expression comparable to that of foragers. However, the nutritional status of the individuals may be key. A recent study observed that high-fat diet-induced obese rats express significantly lower levels of the mammalian sweet taste receptor, T1R3, in taste buds, compared to control rats (Chen et. al., 2010). As previously observed (see Chapter 3), newly emerged bees consume significantly less of sugar solutions compared to foragers while still maintaining comparable, if not elevated, levels of haemolymph saccharides. The young bees' existing nutritional stores and fat body (Toth and Robinson, 2005; Toth et. al., 2005) afford them a relatively high nutritional status

which could potentially maintain internal Gr expression at low levels, similarly to obese rats (Chen et. al., 2010). Decreasing the nutritional status of these individuals, or indeed reducing the nutritional stores, as seen in forager bees, could therefore result in increased expression levels.

Unexpectedly, expression of all Grs were observed in the antennae of both newly emerged and forager bees. Despite previous studies observing *Drosophila* Grs in the antenna, only a small number were detected and generally these were thought to play a role in olfaction as the GRNs were mapped to the antennal lobe (Scott et. al., 2001; Suh et. al., 2004). One antennal Gr, DmGr21a, was additionally revealed to detect CO_2 (Suh et. al., 2004; Jones et. al., 2007; Kwon et. al., 2007). In the honeybee, all Grs are expressed in the antennae and interestingly, some Grs within the antennae (AmGr1, AmGr2, AmGr3, AmGr4 and AmGr8) appear to be differentially expressed between groups, with newly emerged bees demonstrating an almost 2 fold increase in expression compared to foragers. The three candidate sugar receptors (AmGr1, AmGr2 and AmGr3) in particular, are notably enhanced in the antennae of newly emerged bees, perhaps indicating a greater sensitivity for sugars in the young bees. From an ecological perspective this idea is difficult to interpret. Newly emerged bees generally spend the first few days of life consuming honey and pollen within the hive before developing into nurse bees and caring for the brood (Winston, 1987). The foragers on the other hand must leave the hive and collect carbohydrate-enriched nectar, therefore high sensitivity toward sugar solutions would be beneficial. However, as previously mentioned, nectar contains a range of variable nutritional compounds, many of which are essential to the honeybee diet (e.g. 10 essential amino acids, De Groot, 1953). Therefore reduced sugar sensitivity may benefit the forager, allowing enhanced sensitivity toward more dilute and less common substances.

Antennal stimulation with sugars is a major component of the popular Proboscis Extension Reflex (PER), a common behavioural tool used to study honeybees in the laboratory (Bitterman et. al., 1983; Menzel, 1983). Honeybees efficiently elicit a positive behavioural response upon antennal sugar stimulation (Bitterman et. al., 1983; Haupt et. al., 2004) and therefore carbohydrate sensitivity mediated via AmGr1, AmGr2 and AmGr3, is appropriate. Bitter antennal

stimulation, on the other hand, has not been investigated as thoroughly, with zero to little, behavioural effects observed, as a result of bitter tastants (De Brito Sanchez et. al., 2005). To date, the most likely bitter-sensitive Grs are considered to be AmGr4 and AmGr5 and the low expression levels of both these receptors in newly emerged and forager honeybees could explain the low antennal sensitivity to bitter stimulation.

Gustatory receptor expression in the honeybee mouthparts demonstrates a similar general trend between newly emerged and forager bees. With only a few exceptions, Gr expression was greatest in the labial palps, and lowest in the glossa, with galea expression levels only marginally behind those in the palps. Gustatory receptor differentiation between groups appears to be Gr-dependent, although expression levels do appear to be slightly higher generally in forager bees. While the antennae of bees carry out initial nutritional assessment, detection of potential food items will elicit PER (Haupt et. al., 2004), so the food can be further assessed and consumed. Sampling of food with individual mouthparts is unlikely however, as all five mouthparts tend to rest together and form a 'straw-like' apparatus, with which solutions are imbibed (see Figure 5.8.). However, the lateral curvature at the tip of each labial palp indicates a potential food-assessment role and may explain the higher levels of Gr expression.

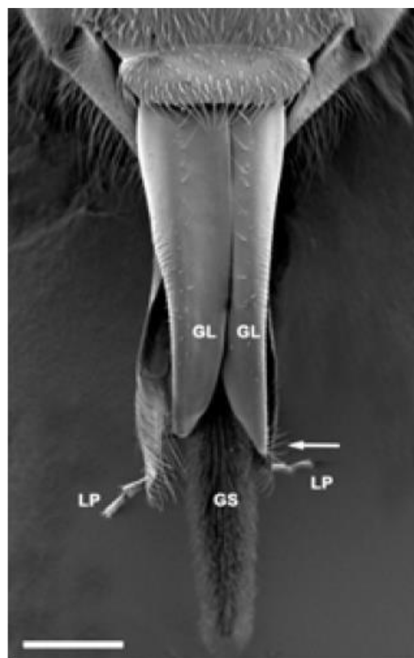

Figure 5.8. Taken from Wright et. al., 2010. Electron micrograph of the five tissues comprising the extended proboscis of the adult forager honeybee. Galea: GL, Labial Palps: LP and Glossa: GS. Scale bar: 500 μm

Honeybees are most likely to detect nutritional compounds with their fore-tarsi and least likely with their hind-tarsi which explains the general decreasing anteroposterior Gr expression levels. The fore-tarsi are known to elicit PER upon sugar stimulation (Takeda, 1961; De Brito Sanchez et. al., 2008) and so, high expression of the three candidate sugar receptors is appropriate (AmGr1, AmGr2 and AmGr3). However, in their study of tarsal sensitivity toward sucrose, De Brito Sanchez and colleagues (2008) noted that the tarsal sucrose sensitivity was lower than the antennal sucrose sensitivity in forager bees. This discovery is particularly interesting as all Grs, including the three candidate sugar receptors, are expressed at lower levels in the antenna compared to the fore-tarsi of foragers. There are a number of possible explanations for this finding. Firstly, gustatory sensitivity may not be proportionally represented by Gr expression level. Furthermore, sweet tastant sensitivity may be reliant on other receptors in addition to Grs, such as ionotropic receptors (Benton et. al., 2009).

A recent study investigating the behavioural and physiological responses to tastants in forager fore-tarsi discovered a lack of sucrose and bitter sensitivity in the tarsomeres—only salt solutions elicited a response—whereas strong responses to sucrose in the tarsal claw were recorded (De Brito Sanchez et. al., 2014). Unfortunately, as the current study incorporated the tarsal claw and the five apical tarsomeres into the fore-tarsi analysis, this response differentiation cannot be compared with Gr expression. Similarly to the antennae, no direct neuronal response was recorded from tarsal sensilla as a result of bitter stimulation, leading the group to conclude a lack of bitter taste receptors in honeybee tarsi (De Brito Sanchez et. al, 2014). However, as bitter tastant stimulation elicited some behavioural responses, the team proposed that bitter sensitivity is mediated via the inhibition of sugar receptors rather than bitter receptor stimulation (De Brito Sanchez et. al, 2014). Sugar neuron inhibition by bitter tastants occurs in a number of insect species (Meunier et. al., 2003; Kessler et. al., 2013) and similarly, sugar solutions have also been reported to inhibit bitter neuronal responses in some insects (Cocco and Gelndinning, 2012). Charlu and colleagues (2013) observed that carboxylic acids, while exciting the bitter neuron, additionally inhibited the sugar neuron, an ability, the team suggest; that allows *Drosophila* to avoid acidic food items even when the bitter neuron is

compromised. Receptor inhibition in the honeybee is therefore feasible, without the necessity of possessing a distinct receptor for any specific ligand.

Gustation is an important sense primarily used for the recognition and assessment of potential food items. The honeybee possesses an apparent reduced gustatory receptor repertoire, however, both vertebrate and invertebrate studies demonstrate that a sensitive gustatory system is not necessary for the detection of nutritious food. For example, de Araujo and colleagues (2008) used *trpm5*$^{-/-}$ knockout (KO) mutants, which lack sweet taste transduction machinery, to demonstrate that learned preferences toward sucrose solutions can develop in mice dependent solely on the caloric content of the food. This preference was paired with dopaminergic activity in the ventral striatal region, known to play a role in the brain's reward circuit (review: Gutierrez and Simon, 2011). Similar ventral striatal activity was also noted on the consumption of sweet foods (de Araujo et. al., 2008). In an olfactory learning paradigm, *D. melanogaster* were able to accurately select between non-sweet nutritious and non-sweet, non-nutritious sugars (Fujita and Tanimura, 2011), an ability further enhanced following food deprivation (Burke and Waddell, 2011; Dus et. al., 2011). However, the flies were able to learn positive associations more efficiently when the food was both sweet and nutritious (Fujita and Tanimura, 2011). These studies demonstrate that gustatory input, especially from sweet foods, is an important sensory stimulus that aids more efficient learning of nutritious food items.

Whilst comparisons with *Drosophila* orthologs prove a useful starting point in determining the function of honeybee Grs, these may not provide us with definitive ligand identification. In their receptor evolution paper, Kent and Robertson (2009) highlight the "convoluted" history that sugar receptors such as those of *Drosophila*, undergo. As a result of this complicated development they point out that, while remaining orthologous to Grs in the *Anopheles* genus of mosquitos, ligand specificity of these receptors cannot necessarily be transferred to other species (Kent and Robertson, 2009). Additionally, the eusociality of honeybees, incurs a notably different life cycle to most other insect species. Honeybees have developed a self-supporting and efficient colony structure, well adapted to its function. Such adaptations are also likely to have occurred in the bee's gustatory system. Honeybee Grs that possess orthologs may in fact have

developed an entirely new set of functions. A very recently discovered example of this can be seen in the hummingbird. While the humming bird survives solely on sugar-rich nectar, it actually lacks the mammalian sweet taste receptor T1R2 (Baldwin et. al., 2014). Recent analysis has revealed that these evolutionary prosperous animals have ‘repurposed’ the heterodimeric mammalian umami receptor, T1R1-T1R3, into a carbohydrate detector (Baldwin et. al., 2014), allowing the exploitation of an alternative nutritional niche. Similarly, the honeybee may have adopted the ‘less is more’ approach to gustatory coding, however further detailed analysis into gustatory sensitivity is needed before successful ligand identification for the 10 *Apis* Grs can be obtained.

5.5. Conclusion

Despite their similarities, in comparison to olfaction, the assessment and understanding of gustatory systems has been a much slower process. Upon their discovery in 2006, the main surprise concerning the honeybee gustatory receptors was their low number in comparison to other insect species (Robertson and Wanner, 2006). The 10 *Apis* Gr genes still remain the lowest number of any insect to date and since their discovery, very little additional work has revealed how these Grs function and the mechanisms underlying their expression and regulation. Here I show, unexpectedly, that these relatively few Gr genes are wide-spread across the bee anatomy, with expression observed in all major external gustatory appendages, in addition to internal expression. Additionally, these expression levels alter as the bees age, demonstrating differential expression across tissues. The most notable of these changes is the significantly greater expression of most Gr genes in the forager bee brain compared to the newly emerged, demonstrating potential for centrally regulated Gr function that may aid foraging. Furthermore, a phylogenetic analysis highlights that the function of only a few honeybee Grs can be speculated based on their sequence similarity to Grs in other insect species. While the honeybee Gr repertoire may be small, the lack of similarity to other species may highlight unique adaptation in bee gustatory coding allowing these pollinators to minimise Gr expression, without drastically limiting gustatory perception.

Chapter 6.0. The influence of diet on gustatory receptor expression in the forager honeybee brain.

As an increasing number of gustatory receptors (Grs) are discovered in different species, more work is being devoted to the identification of receptor ligands and how these receptors function to create an accurate perception of taste. Recently, a small number of studies in both vertebrates and invertebrates have revealed that taste perception is additionally subject to change dependent on the animal's nutritional state. Furthermore, this change is directly mediated through changing Gr expression levels. In the present study, a similar change in forager honeybee Gr genes was investigated in the brain of starved bees versus those with *ad libitum* access to sugar. Starved bees demonstrated a significantly elevated mRNA expression of one receptor (AmGr3), indicating a potential role in central feeding regulation. Additionally, exposure to different sugar and amino acid diets also affected the mRNA expression of various brain Grs. This change in Gr gene expression, as a result of nutritional experience rather than hunger state, is the first demonstrated in any animal. Nutrient-specific feeding and subsequent changes in Gr expression could potentially be used as a novel method for ligand identification, particularly in animals for which sophisticated molecular techniques are limited.

6.1. Introduction

To survive in a constantly changing environment, animals must locate and consume adequate nutrients to fuel their daily activities. The chemical senses are relied upon to efficiently locate and assess food sources for nutrients, and the gustatory system may be specifically adapted for the lifestyle of the species as a result of natural selection (e.g. Ozaki et. al., 2011). It is possible that there is plasticity within an animal's lifetime that could influence how the gustatory system detects nutrients, but this has not often been studied.

The insect gustatory system must adequately sense its specific environment, which can occur through adaptations in sensilla number, neuronal firing, or receptor expression. In insects, there is some evidence that what the gustatory system detects depends on an animal's nutritional environment. Locusts (*Locusta migratoria*) fed nutritionally adequate, yet chemically simple, synthetic diets during the final 2 developmental instars have significantly fewer chemosensilla on both their maxillary palps and antennae as adults (Rogers and Simpson, 1997). In comparison, locusts fed wheat (known to contain an array of nutritional and non-nutritional compounds) or a simple synthetic diet with added flavourings had more sensilla than those fed a simple diet (Rogers and Simpson, 1997). Similarly, grasshoppers (*Schistocerca americana*) raised on artificial diets—considered to be chemically impoverished—developed fewer antennal chemosensilla, of both olfactory (basiconic, coeloconic sensilla) and gustatory (trichoid sensilla) function, than grasshoppers provided with lettuce in their final larval stages (Bernays and Chapman, 1998). This disparity disappeared following the addition of salicin or other volatile compounds to the artificial diet (Bernays and Chapman, 1998). More recently, a similar study has linked this change in peripheral plasticity to behavioural output. Opstad and colleagues (2004) also noted that locusts raised on a mix of two complementary foods with different flavours exhibited up to a 20% greater number of maxillary palp chemosensilla compared to the insects fed a near-optimum, but singular, synthetic food. The team further investigated the role of differential sensilla number and discovered that following mild food deprivation, the presentation of 'marginally acceptable food' items was more likely rejected in locusts endowed with greater sensilla numbers (Opstad et. al., 2004). The team concluded that increased sensilla

numbers affords the insects' heightened gustatory responsiveness, resulting in a more accurate assessment of food in light of the nutritional status of the insect (Opstad et. al., 2004). While likely an increase in chemosensilla number would similarly demonstrate increased gustatory receptor expression, studies to confirm this idea are lacking.

Recent work has emerged that demonstrates Gr expression itself may also retain a level of plasticity without obvious physical alterations. Several recent studies in mammals and insects have shown that Grs are expressed internally and may function as nutrient sensors (Dyer et. al., 2005; Ren et. al., 2009; Chen et. al., 2010; Miyamoto et. al., 2013) and in some cases directly influence feeding (Miyamoto et. al., 2012). A study by Ren and colleagues (2009) demonstrated that the TIR1, TIR2 and TIR3 mammalian taste receptor genes are expressed in regions of the brain involved in nutrient sensing (such as: paraventricular and arcuate nuclei of the hypothalamus, hippocampus, the habenula, and cortex) and showed that starvation of mice specifically affected the expression of two receptors, *Tas1r1* and *Tas1r2* (Ren et. al., 2009). Further *in vitro* analysis revealed that exposure to a low-glucose medium elevated the expression of the 'sweet gene' *Tas1r2*, in hypothalamic cells, which could subsequently be reduced when an artificial sweetener was added to the medium (Ren et. al., 2009). This discovery led the team to conclude that Tas1r2 is a brain 'glucosensor', independent of intracellular glucose metabolism (Ren et. al., 2009). The following year a similar study was carried out using high-fat diet-induced obese rats, focussing on the periphery, observing Gr expression in taste buds (Chen et. al., 2010). The obese rats possessed significantly reduced expression levels of T1R3 mRNA in the taste buds, which was additionally linked to low preference ratios. Rats demonstrated less sensitivity toward, and willingness to accept, low sugar concentrations compared to either the controls or diet-restricted rats (Chen et. al., 2010).

A nutrient/satiety-mediated effect on Gr expression in insects has only recently been reported. Nishimura and colleagues (2012) investigated the motivation to feed between two strains of *Drosophila melanogaster* following a period of starvation. Interestingly, they discovered that similarly to mice and rats, mRNA expression of the sugar receptor, in this case DmGr64a, was elevated

following a 24 h starvation period in both fly strains (Nishimura et. al., 2012). However, the strain with the greatest elevation was also willing to consume lower sugar concentrations (Nishimura et. al., 2012).

Existing work investigating the effect of nutrition on Gr expression has primarily focussed on the role of sugars, which as a major carbohydrate source are vital for all animal nutrition. However, carbohydrates are not the only nutrient that animals must locate and therefore Gr expression, or indeed, internal nutrient sensors, may also demonstrate plasticity to aid alternate nutritional requirements. Protein is a macronutrient and could, like sugars, affect Gr expression. As previously discussed (see Chapter 4), several studies have explored gustatory sensitivity and dietary selection surrounding protein intake (Simpson and Abisgold, 1985; Rathman et. al., 1990; Simpson et. al., 1991; Simmonds et. al., 1992; Koehnle et. al., 2003; Gietzen and Rogers, 2006).

Carbohydrates are the major dietary component of the honeybee and while likely that bees possess at least three receptors responsible for their detection (AmGr1, AmGr2, AmGr3, see Chapter 5), bees also require protein and have previously demonstrated gustatory preferences toward some amino acids (Inouye and Waller, 1984; Alm et. al., 1990; Kim and Smith, 2000; Carter et. al., 2006; Bertazzini et. al., 2010; Hendriksma et. al., 2014). Expression of gustatory receptors with reference to nutritional intake is yet to be investigated in the honeybee. However, investigations of amine receptor expression are more common and have been correlated with chemosensory sensitivity. For example, Mcquillian and team (2012) observed a correlation between the "behavioural responsiveness" toward queen mandibular pheromone and the antennal expression levels of the dopamine receptor; *Amdop1.* The receptor *Amdop2* was more variable and seemingly unrelated to the age of the bees, whereas the expression of the dopamine receptor, *Amdop3*, and the tryamine receptor, *Amtyr3*, depended on the bee's age (McQuillian et. al., 2012). Potentially, changes in expression of these amine receptors could also correlate with changes in gustatory receptors, enhancing chemosensory sensitivity.

As one might expect, changes in gustatory sensitivity are apparent in the honeybee and occur as a function of genotype (Page et. al., 1998), task

differentiation (Pankiw and Page, 2000; Scheiner et. al., 2001), nutritional state (Wright et. al., 2010) and gustatory experience (Ramierez et. al., 2010; Chapter 3). In their study investigating chemosensory experience and sensitivity, Ramírez and colleagues (2010) noted that the gustatory responsiveness of pre-foraging aged bees was affected by the concentration of sucrose with which they were fed. Additionally, modulation of this responsiveness was much faster in forager bees, indicating a higher level of plasticity, compared to younger bees (Ramírez et. al., 2010). While still unknown, the honeybees' 'responsiveness' could reflect the proportion of peripheral Gr expression or plasticity in internal Gr nutrient sensors.

The following experiments aim to assess the influence of nutrition and nutritional state on the mRNA expression of Grs in the forager honeybee brain. Results from the previous chapter (Chapter 5) identified mRNA expression of all Grs in the forager brain. As mentioned, Grs located in the brain are thought to play a role in nutrient sensing and demonstrate plasticity with changing nutritional status (Ren et. al., 2009; Miyamoto et. al., 2012). Identifying Gr brain plasticity following dietary restriction in the honeybee will allow us to ascertain whether Gr expression can be used as a marker for nutritional status in the bee, as in the fly (Nishimura et. al., 2012). Similarly, the expression and activation of a *Drosophila* brain Gr, a honeybee ortholog, has been identified in the direct regulation of feeding (DmGr43a, Miyamoto et. al., 2012). If Gr expression in the brain is centrally regulating feeding in the same manner as the fly, I hypothesise that the expression of one or more nutrient sensing Grs will increase following food deprivation.

Additionally, assessing changes in Gr expression as a result of dietary input may help to identify a ligand, or particular function, of a honeybee gustatory receptor. To date the function of all 10 *Apis* Grs are unknown, however there is strong evidence to suggest that three of these receptors function in sugar detection (AmGr1, AmGr2, AmGr3, Robertson and Wanner, 2006, Chapter 5). By feeding bees different sugar diets this may show changes in the expression of these receptors which could indicate how they are functioning, for example, if AmGr1 and AmGr2 function as heterodimers, as seen in *Drosophila* (Jiao et. al., 2008; Lee et. al., 2009), there may be an equal change in expression level

between the two. I hypothesise that the putative sugar receptors will demonstrate differential expression levels as a result of differing sugar diets.

In addition to the three putative sugar receptors, there remain seven bee Gr genes that encode receptors with unknown functions. It is likely that their ligands are important nutritional components of the honeybee diet. Protein and AAs, are undoubtedly essential to all animal diets and as mentioned, honeybees are attracted toward some of these compounds (Alm et. al., 1990; Kim and Smith, 2000; Carter et. al., 2006; Hendriksma et. al., 2014). As in Chapter 4, when bees are given a choice between an amino acid-sugar or sugar alone there is varied responses. Some AAs appear as attractive as sugar (methionine), some are clearly 'aversive' in comparison to sugar (hydroxyproline) and the attraction toward others appears to change over time (glycine). Such attraction, aversion and temporal dynamics may similarly be represented at the level of the gustatory receptors. Therefore, feeding these compounds to bees and assessing Gr expression will help to identify potential 'amino acid' Grs. If honeybees are able to differentiate between AAs, rather than possessing a general 'amino acid sensor', then exposure to different AA diets may additionally alter change Gr expression.

6.2. Materials and methods

6.2.1. Honeybee capture and harnessing for hunger condition.

Note: For full methods see 2.2 Honeybee capture and restraint.

Forager honeybees (*Apis mellifera Buckfast*) were captured and cold-anesthetised then restrained as described in Wright and Smith (2004). Subjects were fed 10 µl of 0.7 M sucrose and dissected 24 h later. These bees represented a 'hunger' condition.

6.2.2. Honeybee free-feeding on alternate diets condition.

Note: For detailed methods see 2.4 Feeding preference assay: methods.

Following capture, twenty to thirty subjects were immediately placed in plastic boxes in which experimental solutions were provided via four modified microcentrifuge tubes. Bees underwent brain dissection after 96 h. Tubes were replaced every 24 h.

6.2.3. Sample dissection, extraction and PCR.

Note: For full methods see 2.11. Molecular biology.

Dissections took place under a light microscope. Whole brains were removed from each bee, a total of 4 samples were collected with 5 brains per sample. Brain tissue was immediately transferred into 1 ml of TRIzol and RNA extracted following the TRIzol reagent protocol with a few modifications. Samples were re-suspended in 20 µl of RNase and DNase-free water. A total of 4500 ng were treated with RNase-free DNase (Promega) following manufacturer's instructions. A total of 1000 ng of sample RNA was added to the reverse transcription reaction following the manufacturer's protocol for Superscript III Reverse Transcriptase.

End-point PCR was carried out as a check for successful DNase treatment and primer validation

Quantitative real time-PCR was performed on a Roche LightCycler 480, each reaction contained 7 µl RNase-free water, 1 µl of each forward and reverse primer (5 µM), 10 µl LightCycler SYBR Green I Master and 1 µl cDNA.

All samples were normalized to the reference gene RP49 (Ament et. al. 2011).

6.2.4. Statistical analyses

Following RT-qPCR, relative mRNA expression was calculated using the 2ΔΔCt method. Expression level of mRNA in the brains following 96 h 0.7 M sucrose restriction was used as the ‘control’ sample and expressed as 1 by:

AVERAGE (Σ(2ΔΔCt/AVERAGE2ΔΔCt))

This was done separately for every Gr, all other expression levels were normalised to this value. Expression levels of Gr mRNA in bee brains were analysed in SPSS v. 21.0. A generalised linear model (GZLM) was carried out separately for each Gr with dietary treatment at 96 h as an independent variable. A *post hoc* pairwise comparison using Least Significant Difference (LSD) adjustment was additionally carried out between each diet.

6.3. Results

Note: due to low expression levels, valid quantification could not be obtained for AmGr4, AmGr8 and AmGr9. For this reason, these receptors were not included in any of the subsequent analysis.

6.3.1. Hunger significantly increases mRNA expression of AmGr3 in the honeybee brain.

The expression of only one Gr was significantly altered as a result of receiving 10 µl of 0.7 M sucrose followed by 24 h starvation (AmGr3, Figure 6.1., GZLM, treatment: x_6^2 = 4.4, *P* = 0.617). AmGr3 mRNA expression level was significantly greater in hungry bees compared to the control (Figure 6.1, GZLM, *post hoc,* AmGr3*: P* = 0.001).

6.3.2. A monosaccharide diet significantly decreases the mRNA expression of AmGr2 in the honeybee brain

Honeybees possess three candidate sugar receptors (AmGr1, AmGr2, AmGr3, Figure 6.2.) and only the expression of one was significantly affected by feeding on different sugar diets (Figure 6.2C, GZLM, Diet: x_5^2 = 15.5, *P* = 0.008). AmGr2 mRNA expression was significantly decreased following feeding on either glucose (*post hoc* suc*gluc, *P* = 0.032) or fructose (*post hoc* suc*fruc, *P* = 0.023) for 96 h compared to sucrose. The mRNA expression of AmGr3 (Figure 6.2E), AmGr7 (Figure 6.3E) and AmGr10 (Figure 6.3G) also showed decreased expression levels following monosaccharide feeding however, these were not found to be significant (Table 6.2.).

6.3.3. The addition of amino acids to a sucrose diet alters the mRNA expression of a select few gustatory receptors in the honeybee brain

Out of the seven receptor genes analysed, the mRNA expression of three were significantly altered as a result of feeding all experimental amino acids in sucrose for 96 h compared to sucrose alone (AmGr2, AmGr3, AmGr10). The addition of AAs to a sucrose solution significantly reduced AmGr2 mRNA expression over 96 h (Figure 6.2D, Table 6.2.), most notably with the addition of glycine (post hoc suc*gly: *P* < 0.001). AmGr3 and AmGr10 mRNA expression in the forager brain was similarly dependent on diet (respectively, Figure 6.2E, F, GZLM, Diet: x_5^2 = 16.3, *P* = 0.006, Figure 6.3G, H, GZLM, Diet: x_5^2 = 21.3, *P* = 0.001), with all amino acid-sucrose feeding over 96h significantly reducing AmGr3 and AmGr10 mRNA expression (Table 6.2.).

AmGr6 mRNA expression was dependent on diet (Figure 6.3C, D, GZLM, Diet: χ_5^2 = 15.1, P = 0.010), however only feeding on glycine in sucrose for 96 h significantly reduced AmGr6 mRNA expression (Table 6.2., *post hoc* suc*gly$_{96}$: P = 0.002). Similarly, only feeding on glycine in sucrose significantly decreased AmGr7 expression in the forager honeybee brain (Figure 6.3E, F, GZLM, Diet: χ_5^2 = 5.9, P = 0.313, *post hoc*, suc*gly$_{96}$: P = 0.020, Table 6.2.).

The mRNA expression of AmGr1 was narrowly affected by diet over 96 h (Figure 6.2A, B, GZLM, Diet: χ_5^2 = 11.2, P = 0.048), however, expression levels were not significantly different in any diet condition compared to the sucrose only control (Table 6.2.). Furthermore, neither a monosaccharide diet nor an amino acid in sucrose diet altered AmGr5 expression levels over 96 h compared to sucrose alone in the forager brain (Figure 6.3A,B, GZLM, Diet: χ_4^2 = 3.9, P = 0.419 Table 6.2.).

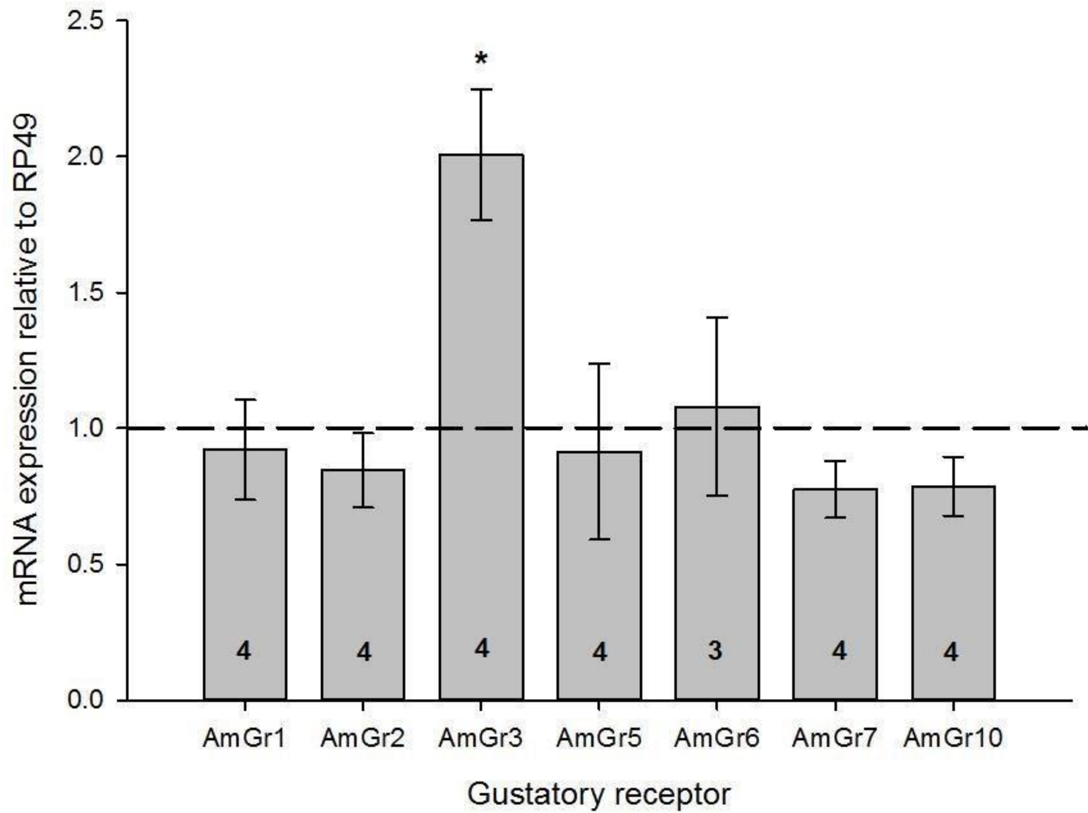

Figure 6.1. The mRNA expression of only one *Apis mellifera* gustatory receptor (AmGr3) was significantly different in the brain when bees were hungry. Bees received 10 µl of 0.7 M sucrose solution 24 h prior to brain dissection. Hatched line represented the control condition in which bees had *ad libitum* access to 0.7 M sucrose for 96 h, all expression values are relative to this control. *: comparison to control GZLM $P < 0.05$. N = displayed in each treatment bar (biological replicates).

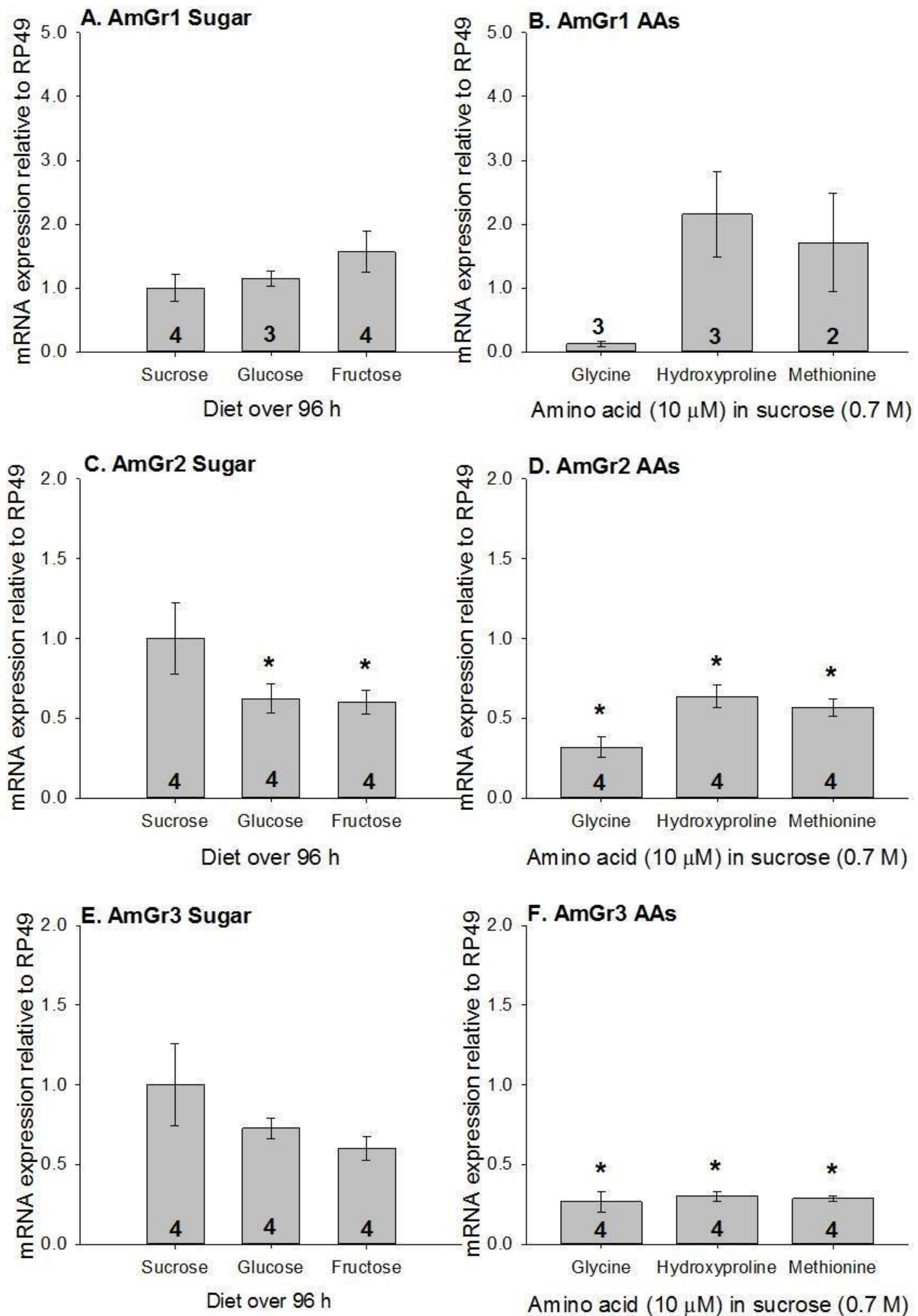

Figure 6.2. mRNA expression of putative sugar gustatory receptors (AmGr1, AmGr2 and AmGr3) in the forager honeybee brain changed dependent on a diet of sugars or amino acids (AAs). **A, C, E**. Bees had *ad libitum* access to one of three 0.7 M sugar diets for 96 h. **B, D, F.** Bees had *ab libitum* access to one of three amino acids (10 μM) added to a sucrose solution (0.7 M). N = displayed in each treatment bar (biological replicates). Note: all sample expressions are relative to the control sample (0.7 M sucrose for 96 h) for each Gr. *: GZLM $P < 0.05$.

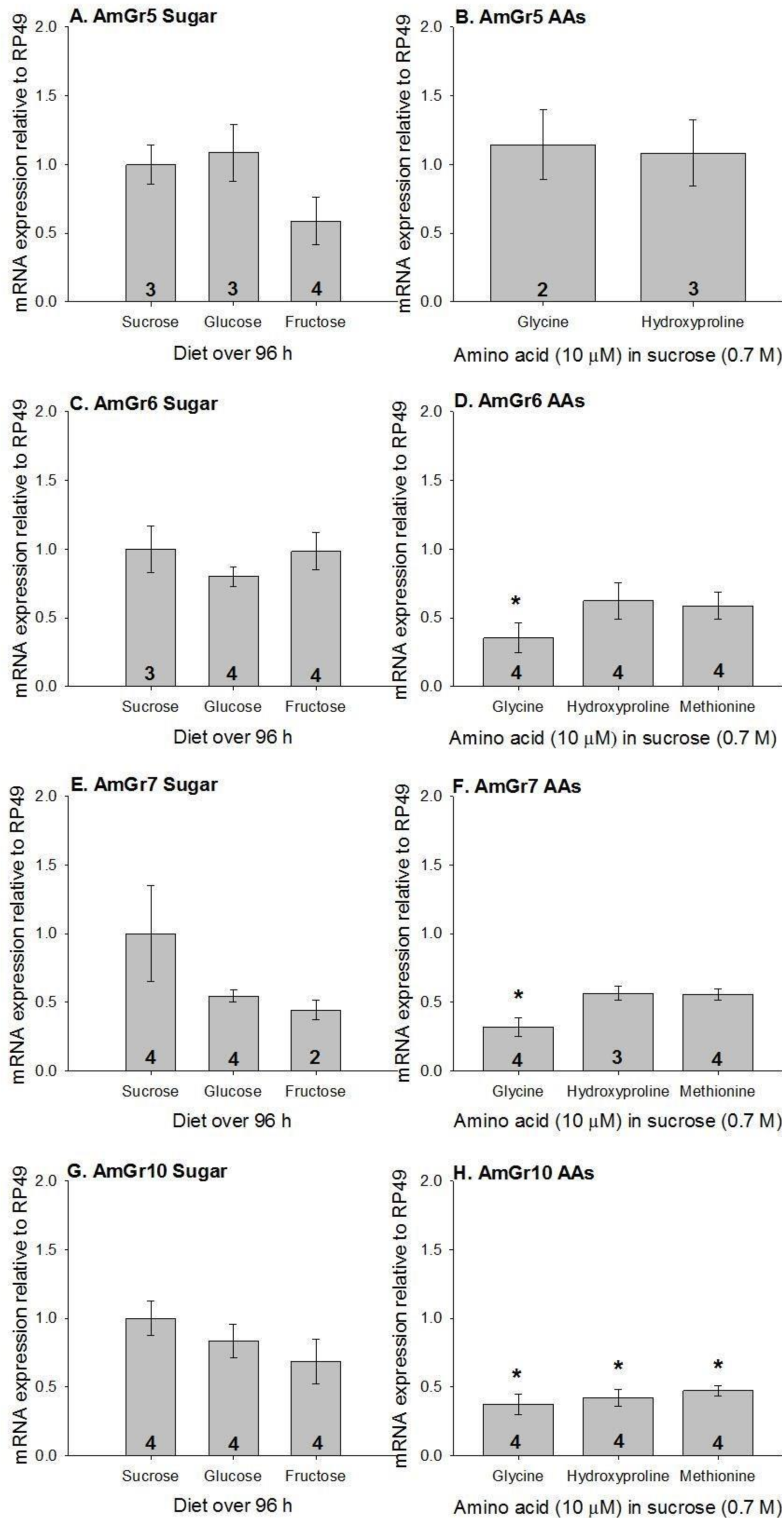

Figure 6.3. mRNA expression levels of gustatory receptors (Grs), with unknown function (AmGr5, AmGr6, AmGr7 and AmGr10), in the forager honeybee brain changed dependent on a diet of amino acids (AAs) in sucrose. **A, C, E and G**. Bees had *ad libitum* access to one of three 0.7 M sugar diets for 96 h. **B, D, F and H.** Bees were sampled after 96 h *ab libitum* feeding on one of three amino acids (10 µM) added to a sucrose solution (0.7 M).). Results for 96 h methionine treatment group are unavailable. N = displayed in each treatment bar (biological replicates). Note: all sample expressions are relative to the control sample (0.7 M sucrose for 96 h) for each Gr, all experiments were carried out and analysed at the same time *: GZLM $P < 0.05$.

Table 6.1. Expression of *Apis mellifera* gustatory receptor genes (AmGr) in the forager brain. GZLM *post hoc pairwise* comparisons between expression in bees fed 0.7 M sucrose and 5 experimental diets over 96 h. All significant comparisons ($P < 0.05$) are shown in bold. Note: results for AmGr5, methionine treatment group are unavailable (NA).

	***Post hoc* P-value (Diet* 0.7 M sucrose, 96 h)**						
96 h Diet	**AmGr1**	**AmGr2**	**AmGr3**	**AmGr5**	**AmGr6**	**AmGr7**	**AmGr10**
0.7 M glucose	0.813	**0.032**	0.107	0.804	0.351	0.121	0.340
0.7 M fructose	0.325	**0.023**	0.063	0.280	0.937	0.122	0.069
10 µM glycine in 0.7 M sucrose	0.157	**<0.001**	**0.001**	0.714	**0.002**	**0.020**	**<0.001**
10 µM hydroxyproline in 0.7 M sucrose	0.063	**0.039**	**0.003**	0.808	0.079	0.172	**0.001**
10 µM methionine in 0.7 M sucrose	0.331	**0.014**	**0.001**	NA	0.054	0.132	**0.002**

6.4. Discussion

Interestingly, not only were all 10 honeybee Grs discovered internally, but the current experiments also demonstrate that Gr expression itself is influenced by nutritional experience and hunger state. Recently, the mRNA expression of a *Drosophila* sugar receptor (DmGr64), known to be involved in the fruit fly's perception of sucrose and other sugars (Dahanukar et. al., 2007), was elevated in the mouthparts of hungry flies (Nishimura et. al., 2012). The current experiment investigated a similar change in expression in the honeybee brain in the hope of identifying potential Gr ligands and nutrient sensor(s).

A Gr recently identified in *Drosophila* as a narrowly tuned fructose receptor (DmGr43a), functions mainly as an 'internal nutrient sensor' (Miyamoto et. al., 2012). Using a Ca^{2+} imaging assay in an *ex vivo* brain preparation, Miyamoto and colleagues (2012) identified that DmGr43a responded to fructose but no other sugars. As the primary haemolymph sugars, both glucose and trehalose undergo strict regulation in flies (Lee and Park, 2004; Kohyama-koganeya et. al., 2008; Miyamoto et. al., 2012), whereas fructose, fluctuates dramatically depending on feeding in the fly. In the head capsule, haemolymph fructose exhibited a 3-10 fold increase in concentration, enough to activate DmGr43a-expressing brain neurons (Miyamoto et. al., 2012). Miyamoto and team (2012) proposed that activation of DmGr43a in a hungry fly acts as a positive reinforcer to promote feeding, whereas the same activation in a satiated fly is negative and mediates the inhibition of feeding. To test this hypothesis DmGr43a-expressing brain neurons were genetically silenced in satiated flies, resulting in up to a 30% increase in sugar consumption, indicating a regulatory role of DmGr43a in feeding (Miyamoto et. al., 2012).

AmGr3 is an ortholog to DmGr43a (Robertson and Wanner, 2006, Chapter 5). The mRNA expression of this Gr was significantly elevated following food deprivation in the forager honeybee brain. Orthology often implies conservation of function (Fitch, 1970) and the elevation of AmGr3 mRNA in hungry bees indicates a likely role in internal nutrient sensing. However, AmGr3 may not necessarily regulate feeding via fructose detection. Firstly, DmGr43a works well as a nutrient sensor as it responds toward highly fluctuating fructose levels in

Drosophila haemolymph while the other haemolymph sugars such as trehalose are tightly regulated. Fructose also fluctuates in honeybee haemolymph (see Chapter 3); however this fluctuation is often paralleled by glucose fluctuations. While responses toward fructose changes alone are possible, it is unlikely that feeding regulation would solely rely on these changes and be unaffected by the same changes in glucose. Additionally, in Chapter 3, fructose was directly injected into the honeybee head capsule, to artificially elevate haemolymph sugar concentrations and observe the subsequent effects on feeding. In these experiments, fructose injections only reduced sucrose feeding, whereas glucose or fructose feeding was unaffected, indicating that haemolymph fructose levels alone cannot control all aspects of feeding.

In my experiments, the mRNA expression of none of the other Grs varied in the brain following food deprivation, but this does not rule out their involvement in central feeding regulation. Miyamoto and colleagues (2012) study in *Drosophila* did not investigate changes in Gr expression, only the outcome of DmGr43a activation and loss (Miyamoto et. al., 2012) and so it remains unknown whether any change in Gr expression also influences feeding in the fly. Activation of Grs and the subsequent activation of the GRNs on which they are expressed may be sufficient to influence feeding in the bee and perhaps the fly. Changes in Gr expression may not be necessary to regulate feeding, and further work must be undertaken to determine the behavioural extent of Gr changes.

To date, a few studies, including my data, have demonstrated a correlation between hunger state, Gr gene expression and gustatory sensitivity (Ren et. al., 2009; Chen et. al., 2010; Nishimura et. al., 2012). No studies have determined the physiological mechanisms linking these elements. Figure 6.4. provides a diagram of the processes that could be occurring internally and externally in the bee as it transitions from a hungry state to a satiated state. When the concentration of nutrients drops in the haemolymph, activation of the Grs is less frequent. This could be a mechanism for signalling hunger that would eventually result in driving the animal to feed.

Haemolymph mediated signalling in the chemical senses is a relatively old theory. For example, electrophysiological recordings from the antennal

chemosensory neurons of female mosquitos (*Aedes aegypti*) noted a high frequency of responses toward the 'host-attractant substance', lactic acid, prior to feeding (Davis, 1984). These neurons subsequently elicited significantly fewer impulses following a blood meal (Davis, 1984). Transfusion of blood from fed females to non-fed females and the resulting decrease in sensitivity allowed Davis (1984) to attribute this change to a "Haemolymph-borne factor". More recently, the previously mentioned study by Ren and colleagues (2009) demonstrated that the hypothalamic expression of the 'sweet' *Tas1r2* gene was susceptible to change when exposed to sugars or sweeteners in the external cell medium. This highlights a change that would occur when sugars are present in mouse blood.

Following the drop in haemolymph nutrients (Figure 6.4.), the hunger state could result in an upregulation in Gr mRNA expression as seen for AmGr3 in the current experiment. While my experiments did not investigate a parallel upregulation in receptor protein expression, previous chemosensory studies have observed a comparable change in both Gr mRNA and protein expression (Ren et. al., 2009; Rund et. al., 2013). An increase in Gr expression is likely to elicit an increase in gustatory sensitivity. For example, both rodent studies demonstrating a change in Gr expression levels, centrally (Ren et. al., 2009) and at the periphery (Chen et. al., 2010), noted a complementary change in sensitivity. In high-fat diet-induced obese rats, a significantly reduced level of Gr mRNA expression in the taste buds was accompanied by a significantly reduced preference for low sucrose concentrations (0.01 M and 0.04 M) compared to control and chronically diet-restricted rats (Chen et. al., 2010). Additionally, a number of olfactory studies have identified changing sensitivity as a result of feeding-induced changes in Or expression. Studies carried out in *Anopheles gambiae* demonstrate that reduced sensitivity in the female olfactory system and the subsequent reduction in host-attraction, occurs within hours of a blood meal (Klowden and Lea, 1979; Davis, 1984; Brown et. al., 1994). Fox and colleagues (2001) observed a significant reduction in AgOr1 mRNA levels—a receptor now known to detect a certain component of human sweat (Hallem et. al., 2004)—in the antenna of female mosquitos 12 h after a blood meal, compared to pre-feeding levels.

A similar increase in Gr sensitivity as a result of elevated Gr expression (Figure 6.4.) is also likely to promote feeding. A recent example directly links an

increase in expression of the mosquito *(A. gambiae*) odorant binding protein, AgamOBP1, with increased olfactory sensitivity at regular daily intervals (Rund et. al., 2011; Rund et. al., 2013). These changes in OBP1 expression and sensitivity additionally correlate with changing blood-feeding behaviours. High expression levels pertain high sensitivity, which additionally correlates with high levels of blood-feeding (Rund et. al., 2013). Additionally, Nishimura and team (2012) used food-deprived *Drosophila* to demonstrate that the strain expressing the sugar receptor gene, DmGr64a, at the greatest levels additionally possessed the greatest sucrose sensitivity and preferably consumed a low-concentration sucrose solution.

As seen in Chapter 3, feeding would lead to an elevation in nutrient concentration in the haemolymph (Figure 6.4.). This change would be detected by Grs, such as AmGr3 and lead to feedback about state that would then result in satiety signalling. Figure 6.4. demonstrates a very simple signalling pathway, however there are likely to be multiple other factors simultaneously involved. As previously mentioned a host of biogenic amines influence chemosensory sensitivity in insects (McQuillian et. al., 2012; Inagaki et. al., 2012) and recently, rejection of essential amino acid deficient diets in *Drosophila* larvae was attributed to dopaminergic neurons (Bjordal et. al., 2014). Satiety signals are also likely mediated via some form of stretch receptor in the gut, as in mosquitos (Klowden and Lea, 1979). Furthermore, neuropeptides (Wu et. al., 2005b; Gonzalez and Orhard, 2008; Ament et. al., 2011) and insulin signalling (Wu et. al., 2006; Ament et. al., 2008) are also known to play a major role in feeding regulation. These separate elements may additionally impact Gr expression, however a number of studies are required to determine the extent of their input. Once satiated however, the expression of Grs (in this case AmGr3) is no longer upregulated and expression levels fall (Figure 6.4.). This satiety signal and decrease in Gr expression is likely to be behaviourally represented by an inhibition of feeding, resulting in a timely decrease of nutrients in the haemolymph.

Changes in Gr expression are certainly observable within 96 h, however it is probable that these changes occur over a much shorter time frame, such as the 12 h change observed for AgOr1, in the mosquito (Fox et. al., 2001), or the 45 min change in Gr expression in hypothalamic cells of the mouse brain (Ren et.

al., 2009). The current study highlights changes in Gr expression internally in the honeybee brain, however these same changes, or indeed more dramatic changes may synchronously occur at the periphery, as observed in the rat (taste buds on the tongue, Chen et. al., 2010) and *Drosophila* (proboscis, Nishimura et. al., 2012).

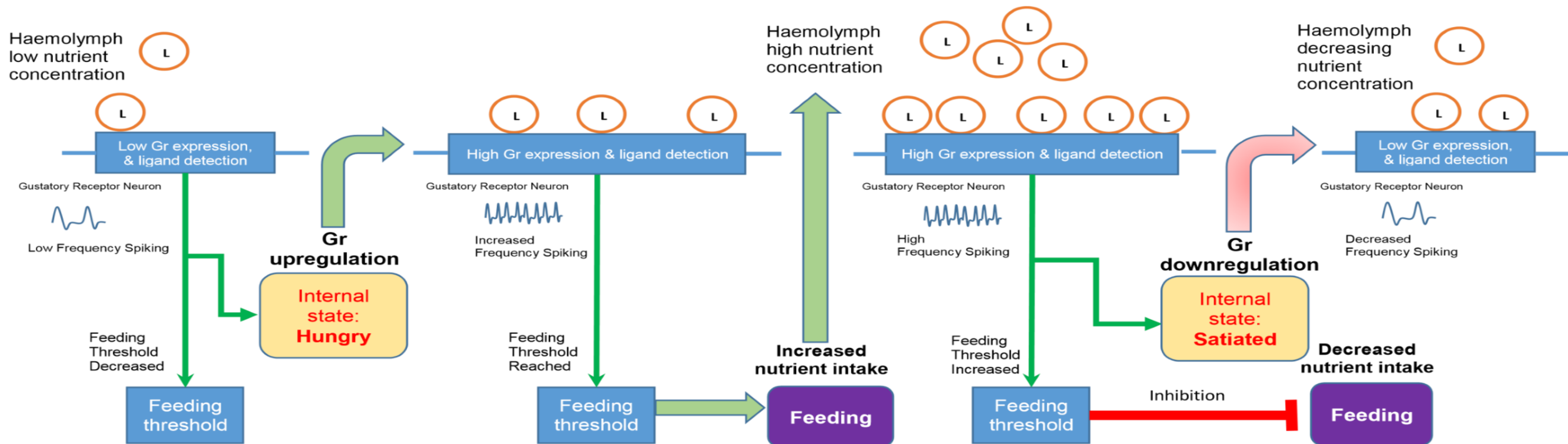

Figure 6.4. Schematic representing potential expression and functioning of gustatory receptors (Gr) in the forager honeybee. When the nutrient concentration of the haemolymph drops this decreases the likelihood of ligand (L) detection by the Grs. The spiking frequency of the gustatory receptor neuron (GRN) drops, the bee is 'hungry' and the feeding threshold is decreased. Gr expression is upregulated in order to increase ligand sensitivity. Upon ligand detection, GRN spiking increases, surpassing the feeding threshold which initiates feeding. As feeding commences, nutrient intake is increased and causes an increase in haemolymph nutrient content. The high Gr expression promotes maximal ligand detection and GRN spiking, the bee becomes satiated and Gr expression is downregulated. The feeding threshold rapidly increases, inhibiting feeding and subsequently lowering the nutrient concentration of the haemolymph. Once the haemolymph concentration reaches a minimum threshold the cycle will start again.

Robertson and Wanner (2006) originally identified three honeybee receptors (AmGr1, AmGr2 and AmGr3) as orthologs to what we now know are sugar receptors in *Drosophila*, a conclusion supported by the phylogenetic analysis in Chapter 5. As candidate sugar receptors, I expected that sugar-only diets would influence the expression of these receptors in the brain. However, only the mRNA expression of AmGr2 was significantly altered after different sugar diets. Following 96 h of fructose and glucose feeding, the expression of AmGr2 mRNA was reduced compared to its expression in bees fed with sucrose. The schematic in Figure 6.4. represents a potential change in Gr expression as a result of changing hunger state; however, we do not know if this change is a general nutritional effect or whether it is ligand-specific. To date, few studies have investigated the influence of specific ligands on Gr expression. In *Drosophila*, some sugar receptors detect multiple ligands (Dahanukar et. al., 2007; Jiao et. al., 2007) and a reduction in AmGr2 expression following both glucose and fructose feeding may indicate these sugars as candidate ligands for AmGr2. In Figure 6.4. the expression of candidate nutrient sensors decrease when nutrients become highly concentrated in the haemolymph. Similarly, AmGr2 expression decreases when both fructose and glucose are highly concentrated in the diet. Interestingly however, this expression level is elevated following sucrose feeding (a disaccharide of glucose and fructose), indicating that sucrose provision perhaps does not meet the detection threshold of either sugar alone in the haemolymph, causing AmGr2 elevation.

In *Drosophila*, there are eight Grs classified in the 'sugar receptor family' (DmGr5a, DmGr61a, DmGr64a-f). Additionally DmGr43a, whose structure is unrelated, can also sense fructose, as a member of a unique fructose-sensing family (Sato et. al., 2011; Miyamoto et. al., 2012). Sugar sensing in the fly is known to rely on the co-expression of sugar receptor genes (Jiao et. al., 2007) and in particular DmGr5a and DmGr64f, which are found in the majority, if not all, sugar GRNs (Dahanukar et. al., 2001; Dahanukar et. al., 2007). Co-expression of genes leads to the co-expression of proteins which function together as subunits in a homo-multimer (Kwon et. al., 2007; Jones et. al., 2007) or hetero-multimer complex (Dahanukar et. al., 2007; Slone et. al., 2007). Similarly, sugar sensing in the honeybee may also rely on the combined expression of Gr genes. Whilst, no

two of the three candidate sugar receptors demonstrated a similar change in mRNA expression after sugar feeding, all receptors were expressed together in the bee brain and therefore may still function as dimers.

As the second most concentrated component of floral nectar (Baker and Baker, 1973) and an essential component of the honeybee diet, amino acids were included in the current analysis in the hope of identifying a candidate AA receptor in the honeybee brain. In the AA dietary treatments, five out of the seven Grs assayed demonstrated a significant decrease in mRNA expression following 96 h of glycine feeding. Behavioural responses towards glycine feeding have been previously observed in honeybees, noted as both appetitive (Kim and Smith, 2000) and aversive (Hendriksma et. al., 2014). Kim and Smith (2000) observed honeybee attraction toward a sugar solution containing glycine and concluded that this represented the honeybees attempt to maintain a specific 'glycine-target' relative to other nutrients. In the current experiment I exposed the bees to glycine feeding over 96 h without any other nutrient aside from sucrose. Here I see a significant decrease in Gr expression (AmGr3, AmGr5, AmGr7 and AmGr10) after 96 h of glycine feeding, perhaps indicating the involvement of multiple Grs in central glycine sensitivity.

Toshima and Tanimura (2012), who investigated behavioural responses toward AA solutions and the effect of deprivation noted that, whilst physiological changes toward AAs can be found in locust nymphs after just 4h protein deprivation (Simpson et. al., 1991) several days are required to elicit AA preferences in *Drosophila*. They speculate the change in sensitivity and AA preference to be a result of peripheral changes in the sensory system and indicate a potential for an AA taste receptor (Toshima and Tanimura, 2012). Toshima and Tanimura (2012) also mention the possibility that glycine is primarily sensed internally. Flies actively select diets containing glycine in spite of the fact that it demonstrates no phagostimulation (flies failed to elicit PER upon tarsal stimulation) at the periphery (Toshima and Tanimura, 2012). This internally mediated glycine preference in the fly could be due to changing Gr expression, similarly to that observed in the bee brain, which could regulate dietary choice. In the bee, glycine may provide a general nutritional quality marker and therefore at

least five of the ten honeybee receptors respond to its presence in sugar solutions.

Additionally, feeding with methionine and hydroxyproline also reduced mRNA expression of AmGr2, AmGr3 and AmGr10. In *Drosophila,* multiple sugars can be detected by the same Gr (Slone et. al., 2007; Dahanukar et. al., 2007) and co-expression studies additionally reveal that multiple Grs are required to detect the same ligand (Dahanukar et. al., 2007; kwon et. al., 2007; Jones et. al., 2007; Slone et. al., 2007). A simultaneous reduction in Gr expression following amino acid provision indicates that general AA sensing may be dependent on the expression and activation of multiple Grs, primarily AmGr2, AmGr3 and AmGr10.

6.5. Conclusion

Although the current experiment has not confirmed any definitive ligands for the 10 honeybee Grs, or indeed whether the expression of these Grs mediate gustatory sensitivity, it has determined that Gr expression in the bee is subject to both hunger state and nutritional experience. The honeybee possesses at least three candidate sugar receptors alongside some of the remaining receptors in the brain that are susceptible to change as a result of AA feeding, indicating the bee is likely to detect at least some AAs. Within a colony, honeybee gustatory sensitivity can vary massively (Page et. al., 1998; Pankiw and Page, 2000; Scheiner et. al., 2001). Such sensitivity variation between age groups may not only depend on Gr expression, but changing Gr levels may additionally mediate nutrient-specific foraging. However, further work is required to elucidate the exact role of changing Gr expression and the time frame in which these changes occur.

Chapter 7.0. General discussion

The overarching aim of this thesis was to broaden what we know about gustatory perception in the honeybee. Since the discovery of the 10 *Apis* gustatory receptor genes (Robertson and Wanner, 2006), there has been little work carried out investigating how Gr expression is regulated in the bee, where the receptors are expressed and how they function.

7.1. Sugar regulation in the honeybee.

In this chapter (Chapter 3) I explored the selection and regulation of three common floral sugars by the honeybee: sucrose, glucose and fructose. I ascertained that separate pre-ingestive detection of these sugars is possible as the intake quantity in a simple drink assay significantly decreased from sucrose to glucose. Sucrose, the most phagostimulatory, indicated potential adaptation by the honeybee as this sugar also promoted the greatest survival amongst foragers. Therefore, I would expect sucrose dominant nectar to prove most attractive in a natural setting. Furthermore, the sucrose concentration of honeybee haemolymph proved most influential on subsequent sugar consumption. Additionally, large fluctuations in haemolymph sucrose may be detected via an internal nutrient sensor(s) and mediate activation and inhibition of feeding in the honeybee. As the three candidate sugar receptor genes (AmGr1, AmGr2 and AmGr3), along with all other *Apis* Gr genes, were detected internally in the gut and the brain (Chapter 5), one or more of these receptors is likely involved in haemolymph sucrose detection and subsequent feeding regulation. Additionally, honeybees prefer fructose over glucose consumption, both on initial presentation in a drink assay and over time in a 96 h choice assay. Surprisingly, this fructose preference even persists when bees are given a choice between the two sugars after being confined to a 48 h fructose-only diet. Whilst further work is required to reveal exactly why this preference in exhibited, the fact that bees appear able to pre-ingestively differentiate between these sugars may indicate that fructose is a more phagostimulatory pre-ingestive reward, whilst maintaining a comparable post-ingestive nutritional benefit. Indeed, both sugars maintained honeybee survival at a similar rate. Finally, despite trehalose commonly being

referred to as 'the primary insect sugar' (Wyatt and Kalf, 1975; Thompson, 2003) here we see that; whilst haemolymph trehalose concentrations are generally low across a number of dietary conditions, these levels are often maintained and indicate relatively strict trehalose regulation in the honeybee.

7.2. Amino acid preferences.

Amino acids are rife in floral nectar (Baker and Baker, 1973) and whilst there exists a list of 10 essential AAs that honeybees must obtain from their food to survive (De Groot, 1953), these floral sources do not appear to contribute toward bee nutrition (Hendriksma et. al., 2014). Honeybees source their dietary protein and AAs from pollen (Winston, 1987) and as a result, floral AA provisions are thought to have evolved as an additional pollinator attractor, particularly for honeybees (Baker, 1977; Baker and Baker, 1973, 1977; Lanza and Krauss, 1984; Carter et. al., 2006). To promote pollinator attraction and fidelity, AAs must first be detected in the nectar, perhaps pre-ingestively or following consumption, either via internal nutrient sensor(s) or feeding into the reward pathway like other floral components, such as caffeine (Wright et. al., 2013).

In Chapter 4 I investigated the dietary choices that both newly emerged and forager honeybees would make when given access to single AA solutions in sucrose or sucrose alone. Single AAs are virtually non-existent in nature and certainly lacking in nectar, and so, unsurprisingly no solitary AA proved more attractive than sucrose alone. When all eight of the experimental AAs were combined, the subsequent solution offered a more protein-like consistency and while forager bees continued to accept this solution at the same rate as sucrose alone, newly emerged bees demonstrated a clear AA mixture preference. This result complements the dietary provisions commonly found within the hive, with young bees requiring and consuming the most protein compared to foragers (Winston, 1987). Additionally, in a similar dietary selection study, young bees selected diets much more protein concentrated, whereas forager bees opted for a majorly carbohydrate-based diet (Paoli et. al., 2014a). In the current study the acceptance of the AA mixture in sucrose plus the sucrose alone may bring the foragers closer to their optimum carbohydrate-rich intake (Paoli et. al., 2014a).

When offered AA solutions (either pure or in sucrose) to consume, both newly emerged and forager bees did accept small quantities, however not all solutions were accepted equally between age—groups, indicating that bees may detect different AA compounds differently. With seven remaining undefined Gr genes still present in the honeybee (Chapter 5 and 6), there still exists the possibility for an AA sensitive Gr.

7.3. Comparative anatomical expression of gustatory receptors between newly emerged and forager honeybees.

Since the discovery of the 10 *Apis* gustatory receptor genes (Robertson and Wanner, 2006), no further work has investigated the expression, function or regulation of these relatively few Grs. The original study by Robertson and Wanner (2006) did quantify AmGr gene expression in a select few gustatory tissues, in addition to a phylogenetic analysis that identified existing orthologs and a potential role for these receptors. However, this work was only preliminary in terms of the potential scope of the honeybee gustatory system. In Chapter 5 I expanded upon these initial results and identified *Apis* Gr gene expression across all major gustatory appendages, including internal expression, which highlights the likely possibility for internal Grs as nutrient sensors, similarly to other insects (Sato et. al., 2011; Miyamoto et. al., 2012; Xu et. al., 2012). Additionally, the analysis between newly emerged and foragers identified that Gr genes appear to be differentiated between either age and/or task and thus demonstrate plasticity within the gustatory system that may aid the specific roles of different bees within the hive.

7.4. The influence of diet on gustatory receptor expression in the forager honeybee brain.

Following on from the basic findings of Chapter 5, I pursued the mechanisms underlying Gr regulation and whether these receptor genes merely demonstrated plasticity as a function of honeybee age/task or whether the bees' specific gustatory environment additionally had an influence. For this work I only focussed on forager bees as gustatory plasticity is most notable in this age-group (Ramierez et. al., 2010). Since the discovery of insect Gr genes (Clyne et. al.,

2000) much of the existing work to date has primarily concerned *Drosophila* and has often implemented sophisticated molecular techniques in order to identify Gr ligands. Whilst the identification of possible honeybee Gr ligands was one optimistic aim of this thesis, the work was also aimed at understanding the timely expression and regulation of Grs and whether these genes underwent adaptations to promote efficient foraging in the honeybee.

Very few studies to date have demonstrated that Gr gene expression is susceptible to hunger state, with only one example in an insect (Ren et. al., 2009; Chen et. al., Nishimura et. al., 2012). The experiments in Chapter 6 have now expanded this short list to include the honeybee by revealing that the mRNA expression of AmGr3 is susceptible to significant increases when forager honeybees are starved.

The behavioural work carried out in Chapter 3 ascertained that the majority of honeybees were able to survive solely on one of the three common floral sugars, (sucrose, glucose and fructose) for 96 h, allowing investigation of Gr gene expression for the three candidate sugars receptors (AmGr1, AmGr2 and AmGr3). These dietary experiments identified that AmGr2 is most susceptible to gene expression changes following exposure to different sugar diets and additionally identified glucose and fructose as potential AmGr2 ligands. Furthermore, whilst no single AA compound elicited a clear preferential response from bees over sucrose alone (Chapter 4) I did see some differential behavioural outcomes e.g. acceptance at the same rate as sucrose over 96 h (methionine), avoidance compared to sucrose over 96 h (hydroxyproline) and a change from avoidance to equal preference over 96 h (glycine). Subsequently these three AAs were added to a sucrose solution and fed to forager honeybees in order to investigate any changes in Gr brain expression. Interestingly, (at least) five of the ten receptor genes exhibited significant changes in expression following the addition of at least one AA to sucrose, indicating that honeybee Grs may detect AA compounds and this detection may be mediated centrally. These experiments are the first, to my knowledge, to identify in any species, that gustatory sensitivity may change as a result of gustatory environment and previously consumed nutrients. While the results in Chapter 6 provide a good starting point to investigate this mechanism further, there is undoubtedly much more work to be

done. However, in the honeybee, an insect with which sophisticated molecular techniques are relatively limited, the present feeding and Gr expression experiments could prove a relatively easy step toward identification of Gr ligands.

7.5. Limitations

This thesis explores the role of gustatory receptors in encoding the perceptual quality of taste in the honeybee. However, this work has a number of limitations.

Firstly, while the behavioural assays explored how honeybees respond to different dietary nutrients, the pre-ingestive assessment of these nutrients was not isolated, particularly in the choice experiments as post-ingestive influences were likely involved. However, the behavioural assays commonly used to asses honeybee gustatory sensitivity, such as PER or drink assays, commonly involve feeding bees to normalise hunger state following capture and then starving bees to enhance motivation. Changes in feeding and starving e.g. volume, concentration and time can subsequently alter the motivational state of the animals and the resulting sensitivity. Furthermore, my *a priori* hypotheses; that both hunger state and nutritional experience may influence Gr expression and sensitivity, would confound such results as the behavioural representation of Gr expression. Therefore without a more controlled approach such as electrophysiology, I did not feel that these behavioural assays would benefit the current study.

Without an electrophysiological analysis of sensilla across the bee anatomy we are unable to ascertain the neuronal coding following Gr activation. Electrophysiology would help determine which ligands excite which GRNs and whether those GRNs demonstrate temporal firing dynamics. For example, early electrophysiological work in caterpillar larvae recorded both phasic and tonic firing components in six out of the eight discovered GRNs (Dethier and Crnjar, 1982). Only the ‘salt best’ cell was determined to fire during the phasic period (Dethier and Crnjar, 1982). These temporal firing dynamics could similarly enable the honeybee to code for different tastants, perhaps without requiring any change in Gr expression. Similarly, such dynamic firing patterns could be the key to tastant

concentration coding, as observed for bitter taste coding in fruit flies (Meunier et. al., 2003), an aspect unexplored in the current thesis.

Due to the difficulty of RNA extraction and resulting low sample numbers, the data gained on peripheral tissue expression between newly emerged and forager bees was not suitable for statistical analysis. Without expanding the sample number the current data can only be used to speculate Gr expression levels between age-gropus. However, analysis of the brain tissues did demonstrate significantly different expression levels between newly emerged and forager bees, emphasising that age/task-differentiation in Gr expression does occur. Additionally Gr mRNA expression was exceedingly low across tissues, a common problem with insect Grs (Amrein and Thorne, 2005). The low expression levels prevented the use of more detailed techniques, such as *in situ* hybridisation and therefore the ability to assess Gr expression on an individual level, hence the pooling of high numbers of peripheral tissues.

Chapter 6 gave a brief summary of some additional factors that are likely to be influencing Gr expression, sensitivity and functioning in the honeybee, such as biogenic amines, neuropeptides, insulin-like signalling pathways etc. However there is a whole host of additional mechanisms, cells and pathways that are likely to be involved, the investigation of which was beyond the scope of the current thesis. For instance, there may exist an alternative population of taste cells that do not express Grs, such as the Gal4 enhancer trap line discovered by Fischler and colleagues (2007) in the labellum taste pegs of *Drosophila*, E409, responsible for detecting the taste of CO_2. Additionally, in recent years an increasing number of studies have identified ionotropic receptor expression and function in gustatory neurons across insect species, including honeybees (Benton et. al, 2009; Croset et. al., 2010; Zhang et. al., 2013; Koh et. al., 2014). These IRs may act in conjunction with, or separately from gustatory receptors, with the potential to expand the bees' gustatory range massively. Furthermore, degenerin/epithelial sodium channel families such as pickpocket28 (*ppk28*)—responsible for water detection in *Drosophila*—may also be present in the bee (Cameron et. al., 2010). Nutrient sensing pathways are also possible, mediating the detection of specific groups of nutrients such as the anterior piriform cortex (APC), hypothalamic orexin/hypocretin neurons and mTOR signalling pathway, responsible for AA

detection in vertebrates, (Firman and Kuenzel, 1988; Hao et. al., 2005; Cota et. al., 2006; Gietzen et. al., 2007; Karnani et. al., 2011). While hormonal influences such as juvenile hormone (JH) and vitellogenin, are vital in the task transitions within the hive (Amdam and Omholt, 2003) and additionally known to influence gustatory perception in the honeybee (Amdam et. al., 2006; Wang et. al., 2012), these hormones may also influence task-specific changes in Gr expression (Chapter 5). While the current work only touches marginally on the variety of functions that the honeybee Grs may possess, it provides a starting place for the enhancement of knowledge in this understudied, yet complex area.

7.6. Future Research

In terms of understanding gustatory coding in the honeybee, the current work barely scratches the surface and therefore a number of directions could be taken to enhance this understanding with future work. In order to identify ligands for the 10 *Apis* Grs a much larger suite of compounds need to be tested on the gustatory appendages of the bee using behaviour, electrophysiology and expression studies. Ideally these compounds would be investigated in an intact animal and then be repeated in knock-out animals. As complex genetic silencing in the bee is not possible, siRNA designed to each Gr could be used and has been previously shown possible in the honeybee, delivered through feeding (Paoli et. al., 2014b). Injection of siRNA may additionally provide more targeted knockdown, a method that is commonly used in other insect species (Ozaki et. al., 2011). Following individual knock out of receptors a combination of siRNAs could be implemented to investigate potential Gr dimers.

As the current study assumes a change in protein expression as a result of changing mRNA expression, some form of protein analysis such as immunohistochemistry would be vital. Additionally, this technique would allow visualisation of Gr expression, allowing us to determine any Gr spatial segregation across gustatory appendages as seen in the three sensilla types of *Drosophila* (l; long, i; intermediate and s; short sensilla, Shanbhag et. al., 2001). Furthermore this technique will also allow us to assess whether GRNs in the gustatory appendages demonstrate heterogeneous Gr expression.

The only gustatory appendage not assayed in the current work was the honeybee mandible and interestingly the sensilla on the mandibles are thought to house only one GRN (Whitehead and Larsen, 1976a). Expression analysis and electrophysiology on the mandibles would be particularly interesting to reveal which Gr(s) are housed on this GRN and what ligands they respond to.

Finally, as sophisticated molecular techniques are limited in the bee and low Gr expression levels additionally rule out some techniques, *In vivo* expression systems such as cell lines e.g. Sf9 cells (Ozaki et. al., 2011) or human embryonic kidney cells (HEK293) transfected with inducible gustatory receptor constructs (Corcoran, et. al., 2014), will be important to investigate Gr function in depth. Additionally, an 'empty neuron' system used for Or studies (Dobrista et. al., 2003; Hallem et. al., 2004) is currently being developed for the gustatory system at Prof H. Amrein's lab in Texas, which could provide a useful 'gain-of-function' approach. A detailed investigation of Gr function could be carried out using this experimental technique in combination with calcium imaging to further establish the Gr ligands.

Appendix 1.0

Apis mellifera Gustatory receptor 1 (AmGr1)CLUSTAL O(1.2.1) multiple sequence alignment

```
Gr1_NCBI_seq              ATCGATAATCCACGGTTACTTCAAGGCGAAAGAATGCATCGAATTTCATCGAGAGCAGAG
Gr1_Original_seq          ATCGATAATCCACGGTTACTTCAAGGCGAAAGAATGCATCGAATTTCATCGAGAGCAGAG
Gr1_PCR_product_Seq       -------------------------TTGAACTAATGCATCGTA-TTCATCGAGAGCAGAG
                                                     ***. *********:* ****************

Gr1_NCBI_seq              TATTCTTGGCGTGTATTTCCAGATGCAATTTCCACAGATATTTTCTAGAACCTCGTACAG
Gr1_Original_seq          TATTCTTGGCGTGTATTTCCAGATGCAATTTCCACAGATATTTTCTAGAACCTCGTACAG
Gr1_PCR_product_Seq       TATTCTTGGCGTGTAT-TCCAGATGCAATTTCCACAGATATGTT---TTACCTCGTACAG
                          **************** ************************ **    :***********

Gr1_NCBI_seq              TTTATGGAAGGGAATATTGGTAGATATCATTAATATCCTCAGTACATTTTCGTGGAATTT
Gr1_Original_seq          TTTATGGAAGGGAATATTGGTAGATATCATTAATATCCTCAGTACATTTTCGTGGAATTT
Gr1_PCR_product_Seq       TTTATGGAAGGGAATATTGGTAGA--ACAGTAATATCCTCAGTACATTTTCGTGGAATTT
                          ************************  :** ******************************

Gr1_NCBI_seq              TGTGGATTTGTTTCTCATTCTTATCAGCATCGCTCTAACGGATCAATTTAGACAATTAAA
Gr1_Original_seq          TGTGGATTTGTTTCTCATTCTTATCAGCATCGCTCTAACGGATCAATTTAGACAATTAAA
Gr1_PCR_product_Seq       TGTGGATTTGTTTCTCATTCTTATCAGCATCGCTCTAACGGATCAATTTAGACAATTAAA
                          ************************************************************

Gr1_NCBI_seq              CAGCCGCTTGTATTCTATAAGAGGAAAGGCAATGCCGGAATGGTGGTGGGCCGAGGCAAG
Gr1_Original_seq          CAGCCGCTTGTATTCTATAAGAGGAAAGGCAATGCCGGAATGGTGGTGGGCCGAGGCAAG
Gr1_PCR_product_Seq       CAGCCGCTTGTATTCTATAAGAGGAAAGGCAATGCCGGAATGGTGGTGGGCCGAGGCAAG
                          ************************************************************

Gr1_NCBI_seq              AAGTGACTACAACCATTTGGCAACCTTAACGAGACAACTG
Gr1_Original_seq          AAGTGACTACAACCATTTGGCAACCTTAACGAGACAACTG
Gr1_PCR_product_Seq       AAGTGACTACAACCATTTGGCAACCTTAACGAGACAACTA
                          ***************************************.
```

Apis mellifera Gustatory receptor 2 (AmGr2)CLUSTAL O(1.2.1) multiple sequence alignment

```
Gr2_NCBI_seq            CGCTCAAATATTCGGCATGTTCCCAGTATCCGGAATTGGATCTTCATCGNTTATCAAACT
Gr2_Original_seq        CGCTCAAATATTCGGCATGTTCCCAGTATCCGGAATTGGATCTTCATCGTTATCAAAACT
Gr2_PCR_Product_seq     ------------------------AGC-CCTTGGGAATGGGACTTCATCGTTATCAAACT
                                                **   *  *     *    *      *    *****

Gr2_NCBI_seq            TCAATTCAAAATATTTTCACTTCTCACTATGTATTCAGGTTTCATCGCC-----------
Gr2_Original_seq        TCAATTCAAAATATTTTCACTTCTCACTATGTATTCAGGTTTCATCGCC-----------
Gr2_PCR_Product_seq     TCAATTCAAAATATTTTCACTTCTCACTATGTATTCAGGTTTAAACGCGCGACCAAAATT
                        ****************************************** * ***

Gr2_NCBI_seq            -------------------------------------
Gr2_Original_seq        -------------------------------------
Gr2_PCR_Product_seq     GGGTGCTTTTAAGAGACTTTCCATGAAAACCCTGGTA
```

Apis mellifera Gustatory receptor 3 (AmGr3)CLUSTAL O(1.2.1) multiple sequence alignment

```
Gr3_NCBI_seq            GCGTACTTGTATTACTACTTAGTGCGGA-AATTTGGGGTTTATGGAGAGATTTAAAAGAT
Gr3_Original_seq        GCGTACTTGTATTACTACTTAGTGCGGAA-ATTTGGGGTTTATGGAGAGATTTAAAAGAT
Gr3_PCR_Product_seq     ------------------------CGGCGAAATTGGGGGTTATGGAGAGATTT-AAAGAT
                                                ***.  *:****** ************** ******

Gr3_NCBI_seq            GGTTGGGAATATAGTACCAGATTAAAATCTCGAACAGCAGTAATCGCAACTTGTAGTGAT
Gr3_Original_seq        GGTTGGGAATATAGTACCAGATTAAAATCTCGAACAGCAGTAATCGCAACTTGTAGTGAT
Gr3_PCR_Product_seq     GGTTGGGAATATAGTACCAGATTAAAATCTCGAACAGCAGTAATCGCAACTTGTAGTGAT
                        ************************************************************

Gr3_NCBI_seq            GTGCTTGGGGTAATGAGTTTAACTGTGGTTTGTATTGTTGGCTCTCCTTTCC--
Gr3_Original_seq        GTGCTTGGGGTAATGAGTTTAACTGTGGTTTGTATTGTTGGCTCTCCTTTCC--
Gr3_PCR_Product_seq     GTGCTTGGGGTAATGAGTTTAACTGTGGTTTGTATTGTTGGCTCTCCTTTCAAA
                        ***************************************************.
```

Apis mellifera Gustatory receptor 4 (AmGr4) CLUSTAL O(1.2.1) multiple sequence alignment

```
Gr4_NCBI_seq             CATCGTTTGCAACAACCAATAAATCAAAAATATATTTTCAATCTTTTATTCTTCGTTTGT
Gr4_Original_seq         CATCGTTTGCAACAACCAATAAATCAAAAATATATTTTCAATCTTTTATTCTTCGTTTGT
Gr4_PCR_Product_seq      ----------------------------AACGCGGCTGGCATCTTTTCTTCTTCGTTTGT
                                                     **      *   ******* ************

Gr4_NCBI_seq             TTATTTAAATTCATTATATTTGTCGCTCTTTTATTTACTGAAATCATATATTTCAAACCT
Gr4_Original_seq         TTATTTAAATTCATTATATTTGTCGCTCTTTTATTTACTGAAATCATATATTTCAAACCT
Gr4_PCR_Product_seq      TTCATTAAATTCATTATATTTGTCGCTCTTTTATTTACTGAAATCATATATTTCAAACCT
                         **  ********************************************************

Gr4_NCBI_seq             GAACCAATTACATTATTAGGAAATCTCATACCTACAATTTTCGCAGGC-------
Gr4_Original_seq         GAACCAATTACATTATTAGGAAATCTCATACCTACAATTTTCGCAGGC-------
Gr4_PCR_Product_seq      GAACCAATTACATTATTAGGAAATCTTATACCTACAATTTTCGCAGGCAATGCTT
                         ************************** *********************
```

Apis mellifera Gustatory receptor 5 (AmGr5)CLUSTAL O(1.2.1) multiple sequence alignment

```
Gr5_NCBI_seq            GTACGATCGATCGAGAAACGAAAATAGAGGTAAAATGATATTTACAAATTCGATCGTCTT
Gr5_Original_seq        GTACGATCGATCGAGAAACGAAAATAGAGGTAAAATGATATTTACAAATTCGATCGTCTT
Gr5_PCR_Product_seq     -----------------------------GTAGTATGATATTTAC-AATTCGATCGTCTT
                                                     ***.:*********** **************

Gr5_NCBI_seq            AACGATTTCGTTGTTTTATATATTACGTACATCTTCTAGCTCGAACAATTTTCACTTCAA
Gr5_Original_seq        AACGATTTCGTTGTTTTATATATTACGTACATCTTCTAGCTCGAACAATTTTCACTTCAA
Gr5_PCR_Product_seq     -ACGATTTCGTTGTTTTATATATTACGTACATCTTCTAGCTCGAACAATTTTCACTTCAA
                         ***********************************************************

Gr5_NCBI_seq            TTGTTGCATCAAAAAGTGAAGTTTACAGCGAATGGATACTTCACATTAGATAATACTCTT
Gr5_Original_seq        TTGTTGCATCAAAAAGTGAAGTTTACAGCGAATGGATACTTCACATTAGATAATACTCTT
Gr5_PCR_Product_seq     TTGTTGCATCAAAAAGTGAAGTTTACAGCGAATGGATACTTCACATTAGATAATACTCTT
                        ************************************************************

Gr5_NCBI_seq            TTTCAATCGGTGAGCGAAAATTGGCCAATTATTATAAGTTTTGTTTGAATGCGGCGAATA
Gr5_Original_seq        TTTCAATCGGTGAGCGAAAATTGGCCAATTATTATAAGTTTTGTTTGAATGCGGCGAATA
Gr5_PCR_Product_seq     TTTCAATCGGTGAGCGAAAATTGGCCAATTATTATAAGTTTTGTTTGAATGCGGCGAATA
                        ************************************************************

Gr5_NCBI_seq            TACATTATTTGATATTTTTTATTATTGTTAGATGATTAATACAGTAACTACGTATATGGT
Gr5_Original_seq        TACATTATTTGATATTTTTTATTATTGTTAGATGATTAATACAGTAACTACGTATATGGT
Gr5_PCR_Product_seq     TACATTATTTGATATTTTTTATTATTGTTAGATGATTAATACAGTAACCACGTATATGGT
                        ************************************************ ***********

Gr5_NCBI_seq            GATTTTGTTTCAATTTCAAATGGAAATTTCAAATGAAAATGATAAATTCTGCAATTGCAC
Gr5_Original_seq        GATTTTGTTTCAATTTCAAATGGAAATTTCAAATGAAAATGATAAATTCTGCAATTGCAC
Gr5_PCR_Product_seq     GATTTTGTTTCAATTTCAAATGGAAACTTCAAATGAAAATGATAAATTCTGCAATTGCCC
                        ************************** *******************************.*

Gr5_NCBI_seq            GCAA-TGCAG--------------------------------------------------
Gr5_Original_seq        GCAA-TGCAG--------------------------------------------------
Gr5_PCR_Product_seq     GCCAATGCAGAAGAATTTATCATTTTCATTTGAAGTTTCCTTTGAAATTGAAACAAAATC
                        **.* *****
```

Apis mellifera Gustatory receptor 6 (AmGr6)CLUSTAL O(1.2.1) multiple sequence alignment

```
Gr6_NCBI_seq             AAAATGAATGTTTCCGTGGTACGATCTGTTAATAAAAAATGATGAAATCCATCCGAAAAG
Gr6_Original_seq         CAGATGAATGTTTCCGTGGTACGATCTGTTAATAAAAAATGATGAAATCCATCCGAAAAG
Gr6_PCR_Product_seq      --------------------------TGGCCA-TTAAAATG--ATGATCCATCCGA-AAG
                                                   **  .* ::******  .:.********** ***

Gr6_NCBI_seq             AATTGGCGCGTTACCCGGCGAAAAGGGTTCTCCCATTAACGAAGCCGCGGTTGAAACCGT
Gr6_Original_seq         AATTGGCGCGTTACCCGGCGAAAAGGGTTCTCCCATTAACGAAGCCGCGGTTGAAACCGT
Gr6_PCR_Product_seq      AATTGGCGCGTTACCCGGCGAAAAGGGTTCTCCCATTAACGTTGAAGTGGTTGAAACCGT

                         *****************************************::*..* ************

Gr6_NCBI_seq             TCGACGTTGAAACCACTTTCGCGGAAGTAACTCTGGCGAGAAAAGAGCACACGAGAAAAT
Gr6_Original_seq         TCGACGTTGAAACCACTTTCGCGGAAGTAACTCTGGCGAGAAAAGAGCACACGAGAAAAT
Gr6_PCR_Product_seq      TCGACGTTGAAACCACTTTCGCGGAAGTAACTCTGGCGAGAAAAGAGCACACGAGAAAAT

                         ************************************************************

Gr6_NCBI_seq             ACCACGGCCCCGACTCGCTCTTGTATTCG-------------------------------
Gr6_Original_seq         ACCACGGCCCCGACTCGCTCTTGTATTCG-------------------------------
Gr6_PCR_Product_seq      ACCACGGCCCCGACTCGCTCTTGTATTCGGAATAAAATATAAGACAAAAAATGAATGCGG

                         *****************************
```

Apis mellifera Gustatory receptor 7 (AmGr7)CLUSTAL O(1.2.1) multiple sequence alignment

```
Gr7_NCBI_seq             GGCAACATTATTTGCGAGTTTTATGAACCATTCGCAACGAAAGAATTTCAAGCAGAGATT
Gr7_Original_seq         GGCAACATTATTTGCGAGTTTTATGAACCATTCGCAACGAAAGAATTTCAAGCAGAGATT
Gr7_PCR_Product_seq      ----------------------GG----GATTCGCAC-G---ATGATTTCAGCAGAGATT
                                               .      *******. *   .:.:** .**********

Gr7_NCBI_seq             CGAGATTTCACTCTACAATTGATACAAAATCCAGTGGTATTTACGGCATACGGATTCTTC
Gr7_Original_seq         CGAGATTTCACTCTACAATTGATACAAAATCCAGTGGTATTTACGGCATACGGATTCTTC
Gr7_PCR_Product_seq      CGAGATTTCACTCTACAATTGATACAAAATCCAGTGGTATTTACGGCATACGGATTCTTC
                         ************************************************************

Gr7_NCBI_seq             AATCTGGATCACTCGTTTATCCAAGGGGTCATTGGAACGATCACCACGTATCTCGTAGTC
Gr7_Original_seq         AATCTGGATCACTCGTTTATCCAAGGGGTCATTGGAACGATCACCACGTATCTCGTAGTC
Gr7_PCR_Product_seq      AATCTGGATCACTCGTTTATCCAAGGGGTCATTGGAACGATCACCACGTATCTCGTAGTC
                         ************************************************************

Gr7_NCBI_seq             ATGATCCAAG--------------------------------------------------
Gr7_Original_seq         ATGATCCAAG--------------------------------------------------
Gr7_PCR_Product_seq      ATGATCCAAGAGAAAGTAGACATGATCCAAAGCAAAGGGGTCCTTGTAATTGTTGCCAGC
                         **********
```

Apis mellifera Gustatory receptor 8 (AmGr8)CLUSTAL O(1.2.1) multiple sequence alignment

```
Gr8_NCBI_seq            CAATACAGAAGTAGGCAAGATTACAAAGATAAACAGTAC-TCTTGGATTTGCAATTTTCT
Gr8_Original_seq        CAATACAGAAGTAGGCAAGATTACAAAGATAAACAGTAC-TCTTGGATTTGCAATTTTCT
Gr8_PCR_Product_seq     -------------GGCCAGGCGGCTG---------GAGCGCCTCAGATTTGCATTT-T-C
                                     ***.**.  .*:.         *:.*  ** .********:** *

Gr8_NCBI_seq            ATATCGTATATATTGTTATTTCTATTTCAATGATTGAAATTATTAGAGTTCAAATGAAAA
Gr8_Original_seq        ATATCGTATATATTGTTATTTCTATTTCAATGATTGAAATTATTAGAGTTCAAATGAAAA
Gr8_PCR_Product_seq     TATTCGTATATATTGTTATTTCTATTTCAATGATTGAAATTATTAGAGGCCA-ATGAAAA
                        :::*********************************************  ** *******

Gr8_NCBI_seq            AAATTGGCTCAAATATTCATAAAATTCTCGTGCACACTTTTGACGATCAAATTATAACGG
Gr8_Original_seq        AAATTGGCTCAAATATTCATAAAATTCTCGTGCACACTTTTGACGATCAAATTATAACGG
Gr8_PCR_Product_seq     AAATTGGCTCAAATATTCATAAAATTCTCGTGCACACTTTTGACGATCAAATTATAACGG
                        ************************************************************

Gr8_NCBI_seq            AGTTGGAGTTATTTTCTTTAGAAGTGCTGCAAAAAGACAATAAATTTATAATGTTTGGGC
Gr8_Original_seq        AGTTGGAGTTATTTTCTTTAGAAGTGCTGCAAAAAGACAATAAATTTATAATGTTTGGGC
Gr8_PCR_Product_seq     AGGTGGAATCATTTTCTTTGGAATAGCTGCATTTAAACGGACGGATTATAATGTTTGGGC
                        ** ****.* *********.*** :******:::*.**..:...:***************

Gr8_NCBI_seq            TTGAAATGGACTTGACTCTTGTGACGGACATGACGTGC----------------------
Gr8_Original_seq        TTGAAATGGACTTGACTCTTGTGACGGACATGACGTGC----------------------
Gr8_PCR_Product_seq     TTGATATGGACTTTACTCTTGTGACGAACATGACCTGCAAGACAATAGATTTATAATGTT
                        ****:******** ************.******* ***
```

Apis mellifera Gustatory receptor 9 (AmGr9)CLUSTAL O(1.2.1) multiple sequence alignment

```
Gr9_NCBI_seq             GCATTTAGAGGAGAAACATTTAGAAATCAGCGATGTCGTTCAATTGGTGAACGATACATT
Gr9_Original_seq         GCATTTAGAGGAGAAACATTTAGAAATCAGCGATGTCGTTCAATTGGTGAACGATACATT
Gr9_PCR_Product_seq      --------------------------CTTCCGATGTCGTTCATTG--GTGACGATACATT
                                                     : ************:*     .**********

Gr9_NCBI_seq             TATAATACATATTATAGTTTTGGTTATCACGACGTTTAGCACAATCACTTTCAATCTGTA
Gr9_Original_seq         TATAATACATATTATAGTTTTGGTTATCACGACGTTTAGCACAATCACTTTCAATCTGTA
Gr9_PCR_Product_seq      TATAATACATATTATAGTTCTGGTTATCACAACGTTTACCACAATCACTTTCAATCTGTA
                         ******************* **********.******* *********************

Gr9_NCBI_seq             TTTCTTCTTACTCAAAATGTATTCACCTAAAACTGAAAACATTAAGTTCTGGTTCATACC
Gr9_Original_seq         TTTCTTCTTACTCAAAATGTATTCACCTAAAACTGAAAACATTAAGTTCTGGTTCATACC
Gr9_PCR_Product_seq      TTTCTTCTTACTCAAAATGTATTCACCTAAAACTGAAAACATTAAGTTCTGGTTCATACC
                         ************************************************************

Gr9_NCBI_seq             GAATCTTGCCCCGGCTCTTTTTTTTTTTATTAAATTTGCCATGATAATTTGGATCTGCGA
Gr9_Original_seq         GAATCTTGCCCCGGCTCTTTTTTTTTTTATTAAATTTGCCATGATAATTTGGATCTGCGA
Gr9_PCR_Product_seq      GAATCTTGCCCCGGCTCTTTTTTTTTTTATTAAATTTGCCATGATAATTTGGATCTGCGA
                         ************************************************************

Gr9_NCBI_seq             GTCGACGACGAACGAGGCGAAGAAGATTAAGTGGACCCTTTATGACGC----
Gr9_Original_seq         GTCGACGACGAACGAGGCGAAGAAGATTAAGTGGACCCTTTATGACGC----
Gr9_PCR_Product_seq      GTCGACAACAAACGAGGCAAAAAAAATTAAGTGGACCCTTTATAACCAAAAA
                         ******.**.********.**.**.******************.** .
```

Apis mellifera Gustatory receptor 10 (AmGr10)CLUSTAL O(1.2.1) multiple sequence alignment

```
Gr10_NCBI_seq             CTGACAAGATAATAGAGGCGCATCGATCAATTAGGCATTCGACGTTACAACAGATATGGT
Gr10_Original_seq         CTGACAAGATAATAGAGGCGCATCGATCAATTAGGCATTCGACGTTACAACAGATATGGT
Gr10_PCR_Product_seq      -------------------------CTCAAGTTCGGCCTCGACGTT--CACTGATACGGT
                                                   .**** *: * . ********  .**:**** ***

Gr10_NCBI_seq             GGCTTCATTGTTCTCTAGCGAACGCGACCGAAATAATTAATTCCGTGTACGCGATCCAAT
Gr10_Original_seq         GGCTTCATTGTTCTCTAGCGAACGCGACCGAAATAATTAATTCCGTGTACGCGATCCAAT
Gr10_PCR_Product_seq      GGCTCCAAA-AAATCAAAAGAAGGCGACCGAAATAATTAAG----CGT---CCTTCCAAA
                          **** **:: ::.**:*..*** *****************      **   * :*****:

Gr10_NCBI_seq             TGTTGTTTTGGATCTCGTCTATGTCGTTCAACCTGATGTCGAGGATTTATTCGTTGAAAG
Gr10_Original_seq         TGTTGTTTTGGATCTCGTCTATGTCGTTCAACCTGATGTCGAGGATTTATTCGTTGAAAG
Gr10_PCR_Product_seq      TT--GTGTTGGTTGCAG----TG----AAAAGGAGATGTCGAGGATTTATTCGTTGAAAG
                          *   ** ****:*  .*    **    :.**  :**************************

Gr10_NCBI_seq             TGTTCAAATTATCGGATTACGGGAAGATCAGGGAATCAATGTTGGTGACTGATTGCGCGT
Gr10_Original_seq         TGTTCAAATTATCGGATTACGGGAAGATCAGGGAATCAATGTTGGTGACTGATTGCGCGT
Gr10_PCR_Product_seq      TGTTCAAATTATCGGATTACGGGAAGATCAGGGAATCAATGTTGGTGACTGATTGCGCGT
                          ************************************************************

Gr10_NCBI_seq             GGAACCTCGTATTGATCACCACCGTGTGCCACATGACGGCTCATCAGGCGAAT---
Gr10_Original_seq         GGAACCTCGTATTGATCACCACCGTGTGCCACATGACGGCTCATCAGGCGAAT---
Gr10_PCR_Product_seq      GGAACCTCGTATTGATCACCACCGTGTGCCACATGACGGCTCATCAGGCGAAATAA
                          ****************************************************:
```

Apis mellifera Ribosomal Protein S8 (AmRPS8)CLUSTAL O(1.2.1) multiple sequence alignment

```
RPS8_NCBI_seq              GGTGCGAAACTGACTGAAGCTGAAGAAGAAGTTTTAAATAAAAAACGTTCGAAAAAAGCA
RPS8_PCR_Product_seq       ------------------------------GGGGGCTCATAAAAACGTTCGAA-AAAGCA
                                                         *    .:.::************* ******

RPS8_NCBI_seq              GAAGCTAAATATAAAGCAAGGCAACGATTTGCTAAAGTTGAACCTGCTCTTGAGGAACAA
RPS8_PCR_Product_seq       GAAGCTAAATATAAAGCAAGGCAACGATTTGCTAAAGTTGAACCTGCTCTTGAGGAACAA
                           ************************************************************

RPS8_NCBI_seq              TTTGCTACAGGACGTGTTCTTGCTTGTATATCGAGTAGACCTGGACAGTGCGGTCGTGAG
RPS8_PCR_Product_seq       TTTGCTACAGGACGTGTTCTTGCTTGTATATCGAGTAGACCTGGACAGTGCGGTCGTAA-
                           *********************************************************.*

RPS8_NCBI_seq              GA
RPS8_PCR_Product_seq       --
```

Apis mellifera Ribosomal Protein 49 (AmRP49) CLUSTAL O(1.2.1) multiple sequence alignment

```
RP49_NCBI_Seq            GGGACAATATTTGATGCCCAATATTGGTTATGGAAGTAACAAAAAAACTCGTCATATGTT
RP49_PCR_Product_Seq     TCAGCCCACTTGTTGTATAATTGTTTGAGTTGGTGTCGTAGATCAAACTCGTCATATGTT
                             *    **        * * ** *   ***        *  ****************

RP49_NCBI_Seq            GCCAACTGGTTTTAGAAAAGTTTTGGTACATAATGTCAAG
RP49_PCR_Product_Seq     GCCAACTGGTTTTAGAAAAGTTTTGGTACATAATGTCAAA
                         ***************************************
```

References

Abou-Seif, M. A. M., Maier, V., Fuchs, J., Mezger, M., Pfeiffer, E. F., & Bounias, M. (1993). Fluctuations of Carbohydrates in Haemolymph of Honeybee (*Apis mellifica*) After Fasting, Feeding and Stress. *Hormone Metabolism Research*, 25(1): 4–8.

Adler, L. S. (2000). The ecological significance of toxic nectar. *Oikos*, 91(3): 409–420.

Agrawal, S., & Riffell, J. a. (2011). Behavioral neurobiology: the bitter life of male flies. *Current Biology*, 21(12): R470–2.

Alaux, C., Ducloz, F., Crauser, D., & Conte, Y. Le. (2010). Diet effects on honeybee immunocompetence. *Biology Letters*, 6(4): 562–565.

Albanese, A. A. (1950). Protein and Amino Acid Requirements of Mammals. Academic Press Inc., Publishers New York (pp. 1– 163).

Alm, J., Ohnmeiss, T. E., Lanza, J., & Vriesenga, L. (1990). Preference of cabbage white butterflies and honey bees for nectar that contains amino acids. *Oecologia*, 84(1): 53–57.

Almquist, H. J. (1947). Evaluation of Amino Acid Requirements by Observations on the Chick. *The Journal of Nutrition*, 34(5): 543–563.

Altaye, S. Z., Pirk, C. W. W., Crewe, R. M., & Nicolson, S. W. (2010). Convergence of carbohydrate-biased intake targets in caged worker honeybees fed different protein sources. *The Journal of Experimental Biology*, 213: 3311– 3318.

Amdam, G. V., & Omholt, S. W. (2003). The hive bee to forager transition in honeybee colonies: the double repressor hypothesis. *Journal of Theoretical Biology*, 223(4): 451–464.

Amdam, G. V., Norberg, K., Page, R. E., Erbera, J., & Scheiner, R. (2006). Downregulation of vitellogenin gene activity increases the gustatory responsiveness of honey bee workers (*Apis mellifera*). *Behavioural Brain Research*, 169(2): 201–205.

Ament, S. A, Corona, M., Pollock, H. S., & Robinson, G. E. (2008). Insulin signalling is involved in the regulation of worker division of labor in honey bee colonies. *Proceedings of the National Academy of Sciences of the United States of America*, 105(11): 4226–31.

Ament, S. A, Velarde, R. a, Kolodkin, M. H., Moyse, D., & Robinson, G. E. (2011). Neuropeptide Y-like signalling and nutritionally mediated gene expression and behaviour in the honey bee. *Insect Molecular Biology*, 20(3): 335–45.

Amrein, H., & Thorne, N. (2005). Gustatory perception and behavior in *Drosophila melanogaster*. *Current Biology*, 15(17): R673–84.

Arenas, A, & Farina, W. M. (2014). Bias to pollen odors is affected by early exposure and foraging experience. *Journal of Insect Physiology*, 66: 28–36.

Arrese, E. L., & Soulages, H. L. (2010). Insect Fat Body: Energy, Metabolism, and Regulation. *Annual Review of Entomology*, 22: 207–225.

Arslan, A., Standifer, L. N., & Don, H. (1986). Carbohydrates in Honey Bee Hemolymph. *Comparative Biochemistry and Physiology*, 84B(3): 363–367.

Avarguès-Weber, A., de Brito Sanchez, M. G., Giurfa, M., & Dyer, A. G. (2010). Aversive reinforcement improves visual discrimination learning in free-flying honeybees. *PloS One*, 5(10): e15370.

Avni, D., Hendriksma, H. P., Dag, A., Uni, Z., & Shafir, S. (2014). Nutritional aspects of honey bee-collected pollen and constraints on colony development in the eastern Mediterranean. *Journal of Insect Physiology*, In press.

Ayestaran, A., Giurfa, M., & de Brito Sanchez, M. G. (2010). Toxic but drank: gustatory aversive compounds induce post-ingestional malaise in harnessed honeybees. *PloS One*, 5(10): e15000.

Baker, H. G. (1977). Non-Sugar Chemical Constituents of Nectar. *Apidologie*, 8(4): 349–356.

Baker, H. G., & Baker, I. (1973). Amino Acids in Nectar and their Evolutionary Significance. *Nature*, 241: 543–545.

Baker, H. G., & Baker, I. (1977). Intraspecific Constancy of Floral Nectar Amino Acid Complements. *Botanical Gazette*, 138(2): 183–191.

Baker, H. G., & Baker, I. (1986). The Occurrence and Significance of Amino Acids in Floral Nectar. *Plant Systematics and Evolution*, 151: 175–186.

Baldwin, M. W., Toda, Y., Nakagita, T., O'Connell, M. J., Klasing, K. C., Misaka, T., Liberles, S. D. (2014). Evolution of sweet taste perception in hummingbirds by transformation of the ancestral umami receptor. *Science*, 345(6199): 929– 933.

Becker, A., Steele, J. E., & Wegener, G. (1996). The regulation of trehalose metabolism in insects. *Experientia*, 52: 433–439.

Beenakkers, A. M. (1969). Carbohydrate and fat as a fuel for insect flight. A comparative study. *Journal of Insect Physiology*, 15(3): 353–61.

Behrens, M., & Meyerhof, W. (2011). Gustatory and extragustatory functions of mammalian taste receptors. *Physiology & Behavior*, *105*(1): 4–13.

Bell, G. I., Kayano, T., Buse, J. B., Burant, C. F., Takeda, U., Lin, D., Fukumoto, H. Seino, S. (1990). The molecular biology of mammalian glucose transporters. *Diabetes Care*, 13(1): 198–208.

Ben-Shahar, Y. & Robinson, G. (2001). Satiation differentially affects performance in a learning assay by nurse and forager honey bees. *Journal of Comparative Physiology A: Sensory, Neural, and Behavioral Physiology*, 187(11): 891–899.

Benton, R., Vannice, K. S., Gomez-Diaz, C., & Vosshall, L. B. (2009). Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. *Cell*, 136(1): 149–62.

Bernays, E. A., & Chapman, R. F. (1998). Phenotypic plasticity in numbers of antennal chemoreceptors in a grasshopper: effects of food. *Journal of*

Comparative Physiology A: Sensory, Neural, and Behavioral Physiology, 183(1): 69–76.

Bertazzini, M., Medrzycki, P., Bortolotti, L., Maistrello, L., & Forlani, G. (2010). Amino acid content and nectar choice by forager honeybees (*Apis mellifera* L.). *Amino Acids*, 39(1): 315–8.

Bitterman, M. E., Menzel, R., Fietz, A., & Schafer, S. (1983). Classical conditioing of proboscis extension in honeybees (*Apis mellifera*). *Journal of Comparative Physiology*, 97(2): 107–119.

Bjordal, M., Arquier, N., Kniazeff, J., Pin, J. P., & Léopold, P. (2014). Sensing of amino acids in a dopaminergic circuitry promotes rejection of an incomplete diet in *Drosophila*. *Cell*, 156(3): 510–21.

Blatt, J., & Roces, F. (2001). Haemolymph sugar levels in foraging honeybees (*Apis mellifera carnica*): dependence on metabolic rate and in vivo measurement of maximal rates of trehalose synthesis. *The Journal of Experimental Biology*, 204(15): 2709–16.

Blatt, J., & Roces, F. (2002). The control of the proventriculus in the honeybee (*Apis mellifera carnica* L.) I. A dynamic process influenced by food quality and quantity? *Journal of Insect Physiology*, 48(6): 643–654.

Bonasio, R., Zhang, G., Ye, C., Mutti, N. S., Fang, X., Qin, N., Donahue, G., Yang, P., Li, Q., Li, C., Zhang, P., Huang, Z., Berger, S. L., Reinberg, D., Wang, J., Liebig, J. (2010). Genomic comparison of the ants *Camponotus floridanus* and *Harpegnathos saltator. Science*, 329(5995): 1068–71.

Booth, C. S., Pienaar, E., Termaat, J. R., Whitney, S. E., Louw, T. M., & Viljoen, H. J. (2010). Efficiency of the Polymerase Chain Reaction. Chemical Engineering *Science*, 65(17): 4996–5006.

Borer, K. T., Hallfrisch, J., Tsai, A. C., Hallfrisch, C., & Kuhns, L. R. (1979). The effect of exercise and dietary protein levels on somatic growth, body composition, and serum lipid levels in adult hamsters. *The Journal of Nutrition*, 109(2): 222–8.

Bounias, M. (1981). Inter-relations of trehalose glucose and fructose in workerbees from different races and hybrids. *Comparative Biochemistry and Physiology*, 69B: 471–477.

Bounias, M., & Morgan, M. R. J. (1985). Short-term adaptation of honeybee haemolymph disaccharidase following trehalose injection. *Comparative Biochemistry and Physiology*, 80(3): 451–454.

Bray, S., & Amrein, H. (2003). A putative *Drosophila* pheromone receptor expressed in male-specific taste neurons is required for efficient courtship. *Neuron*, 39(6): 1019–29.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., & Hafen, E. (2001). An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Current Biology*, 11(4): 213– 21.

Broughton, S. J., Piper, M. D. W., Ikeya, T., Bass, T. M., Jacobson, J., Driege, Y., Martinez, P., Hafen, E., Withers, D.J., Leevers, S.J., Partridge, L. (2005). Longer lifespan, altered metabolism, and stress resistance in *Drosophila* from ablation of cells making insulin-like ligands. *Proceedings of the National Academy of Sciences of the United States of America*, 102(8): 3105–10.

Brown, M. R., Clark, K. D., Gulia, M., Zhao, Z., Garczynski, S. F., Crim, J. W., Suderman, R.J., Strand, M. R. (2008). An insulin-like peptide regulates egg maturation and metabolism in the mosquito *Aedes aegypti*. *Proceedings of the National Academy of Sciences of the United States of America*, 105(15): 5716–21.

Brown, M. R., Klowden, M. J., Crim, J. W., Young, L., Shrouder, L. a., & Lea, A. O. (1994). Endogenous regulation of mosquito host-seeking behavior by a neuropeptide. *Journal of Insect Physiology*, 40(5): 399–406.

Browne, B. (1993). Physiologically induced changes in resource-oriented Behaviour. *Annual Review of Entomology*, 28: 1–25.

Burke, C. J., & Waddell, S. (2011). Remembering nutrient quality of sugar in *Drosophila*. *Current Biology*, 21(9): 746–50.

Calderone, N. W., & Johnson, B. R. (2002). The within-nest behaviour of honeybee pollen foragers in colonies with a high or low need for pollen. *Animal Behaviour*, 63(4): 749–758.

Camazine, S. (1993). The regulation of pollen foraging by honey bees: how foragers assess the colony's need for pollen. *Behavioural Ecology and Sociobiology*, 32(4): 265–272.

Cameron, P., Hiroi, M., John Ngai, J., & Scott, K. (2010). The molecular basis for water taste in *Drosophila*. *Nature*, 465(7294): 91–95.

Candy, D., Becker, A., & Wegener, G. (1997). Coordination and Integration of Metabolism in Insect Flight. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 117(4): 497–512.

Candy, D. J., & Kilby, B. A. (1961). The biosynthesis of trehalose in the locust fat body. *The Biochemical Journal*, 78: 531–6.

Carter, C., Shafir, S., Yehonatan, L., Palmer, R. G., & Thornburg, R. (2006). A novel role for proline in plant floral nectars. *Die Naturwissenschaften*, 93(2): 72– 9.

Châline, N., Sandoz, J. C., Martin, S. J., Ratnieks, F. L. W., & Jones, G. R. (2005). Learning and discrimination of individual cuticular hydrocarbons by honeybees (*Apis mellifera*). *Chemical Senses*, 30(4): 327–35.

Chalisova, N. I., Kamyshev, N. G., Lopatina, N. G., Kontsevaia, E. A, Urtieva, S. A, & Urtieva, T. A. (2011). Effect of encoding amino acids on associative learning of honeybee *Apis mellifera*. *Journal of Evolutionary Biochemistry and Physiology*, 47(6): 516–8.

Chandra, R. K. (1997). Nutrition and the immune system: an introduction. *The American Journal of Clinical Nutrition*, 66: 460–463.

Chapman, R. F. (2003). Contact chemoreception in feeding by phytophagous insects. *Annual Review of Entomology*, 48: 455–84.

Chapman, R. F., & Fraser, J. (1989). The chemosensory system of the monophagus grasshopper, *bootettix argentatus* bruner (orthoptera • acrididae). *Journal of Insect Morphology and Embryology*, 18(2): 111–118.

Chapman, R. F., & Thomas, J. G. (1978). The numbers and distribution of sensilla on the mouthparts of *Acridoidea*. *Acrida*, 7: 15–148.

Charlu, S., Wisotsky, Z., Medina, A., & Dahanukar, A. (2013). Acid sensing by sweet and bitter taste neurons in *Drosophila melanogaster*. *Nature Communications*, 4: 1-10.

Chaudhari, N., Yang, H., Roper, S., & Cartford, C. (1996). The Taste of Monosodium Glutamate: Membrane Receptors in Taste Buds. *The Journal of Neuroscience*, 16(12): 3817–3826

Chaudhari, N., Landin, A. M., & Roper, S. D. (2000). A metabotropic glutamate receptor variant functions as a taste receptor. *Nature Neuroscience*, 3(2): 113–9.

Chen, K., Yan, J., Suo, Y., Li, J., Wang, Q., & Lv, B. (2010). Nutritional status alters saccharin intake and sweet receptor mRNA expression in rat taste buds. *Brain Research*, 1325: 53–62.

Chyb, S., Dahanukar, A., Wickens, A., & Carlson, J. R. (2003). *Drosophila* Gr5a encodes a taste receptor tuned to trehalose. *Proceedings of the National Academy of Sciences of the United States of America*, 100: 14526–30.

Clyne, P. J., Warr, C.G. & Carlson, R. J. (2000). Candidate Taste Receptors in Drosophila. *Science*, 287(5459): 1830–1834.

Cocco, N., & Glendinning, J. I. (2012). Not all sugars are created equal: some mask aversive tastes better than others in an herbivorous insect. *The Journal of Experimental Biology*, 215(8): 1412–21.

Cook, S. M., Awmack, C. S., Murray, D., Williams, I., & Division, I. E. (2003). Are honey bees' foraging preferences affected by pollen amino acid composition? *Ecological Entomology*, 28: 622–627.

Corcoran, J. A, Jordan, M. D., Carraher, C., & Newcomb, R. D. (2014). A novel method to study insect olfactory receptor function using HEK293 cells. *Insect Biochemistry and Molecular Biology*, In Press: 1–11.

Cota, D., Proulx, K., Smith, K. A. B., Kozma, S. C., Thomas, G., Woods, S. C., & Seeley, R. J. (2006). Hypothalamic mTOR signaling regulates food intake. *Science*, 312(5775): 927–30.

Crailsheim, K. (1985). Distribution of haemolymph in the honeybee (*Apis mellifica*) in relation to season, age and temperature. *Journal of Insect Physiology*, 31(9): 707–713.

Crailsheim, K. (1986). Dependence of protein metabolism on age and season in the honeybee (*Apis mellifica carnica* POLLM). *Journal of Insect Morphology and Embryology*, 32(7): 629–634.

Crailsheim, K. (1988a). Intestinal transport of sugars in the honeybee (*Apis mellifera* L.). *Journal of Insect Physiology*, 34(9): 839–845.

Crailsheim, K. (1988b). Regulation of food passage in the Honeybee (*Apis Mellifera* L.). *Journal of Insect Physiology*, 34(2): 85–90.

Crailsheim, K. (1990). The protein balance of the honey bee worker. *Apidologie*, 21: 417–429.

Croset, V., Rytz, R., Cummins, S. F., Budd, A., Brawand, D., Kaessmann, H., Gibson, T. J., Benton, R. (2010). Ancient protostome origin of chemosensory ionotropic glutamate receptors and the evolution of insect taste and olfaction. *PLoS Genetics*, 6(8): e1001064.

Dahanukar, A, Foster, K., Van der Goes Van Naters, W. M., & Carlson, J. R. (2001). A Gr receptor is required for response to the sugar trehalose in taste neurons of *Drosophila*. *Nature Neuroscience*, 4(12): 1182–6.

Dahanukar, A., Lei, Y. T., Kwon, J. Y., & Carlson, J. R. (2007). Two Gr genes underlie sugar reception in *Drosophila*. *Neuron*, 56(3): 503–516.

Dani, F. R., Jones, G. R., Corsi, S., Beard, R., Pradella, D., & Turillazzi, S. (2005). Nestmate recognition cues in the honey bee: differential importance of cuticular alkanes and alkenes. *Chemical Senses*, 30(6): 477–89.

Davis, E. E. (1984). Regulation of sensitivity in the peripheral host-seeking behaviour by a haemolymph-borne factor in *Aedes aegypti*. *Journal of Insect Physiology*, 30(2): 179–183.

De Araujo, I. E., Oliveira-Maia, A. J., Sotnikova, T. D., Gainetdinov, R. R., Caron, M. G., Nicolelis, M. A. L., & Simon, S. A. (2008). Food reward in the absence of taste receptor signalling. *Neuron*, 57(6): 930–41.

De Brito Sanchez, G. M. (2011). Taste Perception in Honey Bees. *Chemical Senses*, 36(8): 675–692.

De Brito Sanchez, M. G., Giurfa, M., de Paula Mota, T. R., & Gauthier, M. (2005). Electrophysiological and behavioural characterization of gustatory responses to antennal "bitter" taste in honeybees. *The European Journal of Neuroscience*, 22(12): 3161–70.

De Brito Sanchez, G., Ortigão-Farias, J. R., Gauthier, M., Liu, F., & Giurfa, M. (2007). Taste perception in honeybees: just a taste of honey? *Arthropod Plant Interactions*, 1(2): 69–76.

De Brito Sanchez, M. G., Chen, C., Li, J., Liu, F., Gauthier, M., & Giurfa, M. (2008). Behavioral studies on tarsal gustation in honeybees: sucrose responsiveness and sucrose-mediated olfactory conditioning. *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology*, 194(10): 861–9.

De Brito Sanchez, M. G., Lorenzo, E., Su, S., Liu, F., Zhan, Y., & Giurfa, M. (2014). The tarsal taste of honey bees: behavioral and electrophysiological analyses. *Frontiers in Behavioral Neuroscience*, 8(25): 1–16.

De Groot, A. P. (1953). Protein and Amino Acid Requirements of the Honeybee (*Apis mellifera* L.). *Physiol. Comp. Oecol*, 3: 197–285.

Deshpande, D. a, Wang, W. C. H., McIlmoyle, E. L., Robinett, K. S., Schillinger, R. M., An, S. S., Sham, J. S. K., Liggett, S. B. (2010). Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction. *Nature Medicine*, *16*(11): 1299–1304.

Dethier, V. G. (1953). Summation and Inhibition Following Contralateral Stimulation of the Tarsal chemoreceptors of the Blowfly. *Biological Bulletin*, 105(2): 257– 268.

Dethier, V. G. (1955). The Physiology and Histology of the Contact Chemoreceptors of the Blowfly. *The Quarterly Review of Biology*, 30(4): 348– 371.

Dethier, V. G. (1961). Behavioral aspects of protein ingestion by the blowfly *Phormia regina meigen*. *Biological Bulletin*, 121(3): 456–470.

Dethier, V. G., & Crnjar, R. M. (1982). Candidate codes in the gustatory system of caterpillars. *The Journal of General Physiology*, 79(4): 549–69.

Dethier, V. G., & Hanson, F. E. (1968). Electrophysiological responses of the chemoreceptors of the blowfly to sodium salts of fatty acids. *Proceedings of the National Academy of Sciences*, 60(4): 1296–1303.

Dethier, V. G., & Kuch, J. H. (1971). Electrophysiological studies of gustation in lepidopterous larvae. I. Comparative Sensitivity to Sugars, Amino Acids, and Glycosides. *Zeitschrift Vergleichende Physiologie*, 72(4): 343–363.

Dethier, V. G., & Schoonhoven, L. M. (1968). Evaluation of evaporation by cold and humidity receptors in caterpillars. *Journal of Insect Physiology*, 14: 1049– 1054.

Detzel, A., & Wink, M. (1993). Attraction, detterence or intoxication of bees (*Apis mellifera*) by plant allelochemicals. *Chemoecology*, 4(1): 8–18.

Dobritsa, A. A., Van der Goes Van Naters, W., Warr, C. G., Steinbrecht, R. A., & Carlson, J. R. (2003). Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron*, 37(5): 827–41.

Dunipace, L., Meister, S., McNealy, C., & Amrein, H. (2001). Spatially restricted expression of candidate taste receptors in the *Drosophila* gustatory system. *Current Biology*, 11(11): 822–35.

Dus, M., Min, S., Keene, A. C., Young, G., & Suh, G. S. B. (2011). Taste independent detection of the caloric content of sugar in *Drosophila*. *Proceedings of the National Academy of Sciences*, 108(28): 11644– 11649.

Dussutour, A, & Simpson, S. J. (2012). Ant workers die young and colonies collapse when fed a high-protein diet. *Proceedings of The Royal Society B*, 279(1737): 2402–8.

Dyer, J., Salmon, K. S. H., Zibrik, L., & Shirazi-Beechey, S. P. (2005). Expression of sweet taste receptors of the T1R family in the intestinal tract and enteroendocrine cells. *Biochemical Society Transactions*, 33: 302–305.

Erhardt, A., & Rusterholz, H. (1998). Do Peacock butterflies (*Inachis io* L.) detect and prefer nectar amino acids and other nitrogenous compounds? *Oecologia*, 117(4): 536–542.

Evans, D. R., & Mellon, D. (1962). Stimulation of a primary taste receptor by salts. *The Journal of General Physiology*, 45: 651–61.

Falk, R., Bleiser-Avivi, N., & Atidia, J. (1976). Labellar taste organs of *Drosophila melanogaster*. *Journal of Morphology*, 150(2): 327–341.

Faucher, C., Forstreuter, M., Hilker, M., & de Bruyne, M. (2006). Behavioral responses of *Drosophila* to biogenic levels of carbon dioxide depend on life-stage, sex and olfactory context. *The Journal of Experimental Biology*, 209(14): 2739–2748.

Feinman, R. D., & Fine, E. J. (2013). Fructose in perspective. *Nutrition & Metabolism*, 10(1): 45.

Fell, R. D. (1990). The qualitative and quantitative analysis of insect hemolymph sugars by high performance thin-layer chromatography. *Comparative Biochemistry and Physiology Part A: Physiology*, 95(4): 539–544.

Ferveur, J. F. (2005). Cuticular hydrocarbons: their evolution and roles in *Drosophila* pheromonal communication. *Behavior Genetics*, 35(3): 279–295.

Fewell, J. H., & Winston, M. L. (1992). Colony state and regulation of pollen foraging in the honey bee, *Apis mellifera* L. *Behavioural Ecology and Sociobiology*, 30(6): 387–393.

Finger, T. E., Böttger, B., Hansen, A., Anderson, K. T., Alimohammadi, H., & Silver, W. L. (2003). Solitary chemoreceptor cells in the nasal cavity serve as sentinels of respiration. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(15): 8981–8986.

Firman, J. D., & Kuenzel, W. J. (1988). Neuroanatomical regions of the chick brain involved in monitoring amino acid deficient diets. *Brain Research Bulletin*, 21(4): 637–642.

Fischler, W., Kong, P., Marella, S., & Scott, K. (2007). The detection of carbonation by the *Drosophila* gustatory system. *Nature*, 448(7157): 1054–7.

Fitch, W. M. (1970). Distinguishing homologous from analogous proteins. *Systematic Zoology*, 19(2): 99–113.

Fluri, P., Luscher, M., Wille, H., & Geri, L. (1982). Changes in weight of the pharyngeal gland and haemolymph titres of juvenile hormone, protein and vitellogenin in worker. *Journal of Insect Physiology*, 28(1): 61–68.

Fox, A. N., Pitts, R. J., Robertson, H. M., Carlson, J. R., & Zwiebel, L. J. (2001). Candidate odorant receptors from the malaria vector mosquito *Anopheles gambiae* and evidence of down-regulation in response to blood feeding. *Proceedings of the National Academy of Sciences of the United States of America*, 98(25): 14693–14697.

Friedman, S., Waldbauer, G. P., Eertmoed, J. E., Naeem, M., & Ghent, A. W. (1991). Blood trehalose levels have a role in the control of dietary selfselection by *Heliothis zea* larvae. *Journal of Insect Physiology*, 37(12): 919–928.

Frings, H., & Frings, M. (1949). The loci of contact chemoreceptors in insects. A review with new evidence. *American Midland Naturalist*, 41(3): 602–658.

Fromentin, G., & Nicolaidis, S. (1996). Rebalancing essential amino acids intake by self-selection in the rat. *British Journal of Nutrition*, 75(05): 669-682.

Fujishiro, N., Kijima, H., & Morita, H. (1984). Impulse Frequency and Action Potential Amplitude in Labellar Chemosensory Neurones. *Journal of Insect Physiology*, 30(4): 317–325.

Fujita, M., & Tanimura, T. (2011). *Drosophila* evaluates and learns the nutritional value of sugars. *Current Biology*, 21(9): 751–755.

Gardener, M. C., & Gillman, M. P. (2001). The effects of soil fertilizer on amino acids in the floral nectar of corncockle, *Agrostemma githago* (Caryophyllaceae). *Oikos*, 92(1): 101–106.

Gibson, E. L., Wainwright, C. J., & Booth, D. a. (1995). Disguised protein in lunch after low-protein breakfast conditions food-flavor preferences dependent on recent lack of protein intake. *Physiology & Behavior*, 58(2): 363–371.

Gietzen, D. W. (1993). Neural Mechanisms in the Responses Amino Acid Deficiency. *Journal of Nutrition*, 123(4): 610–625.

Gietzen, D. W., & Aja, S. M. (2012). The brain's response to an essential amino acid- deficient diet and the circuitous route to a better meal. *Molecular Neurobiology*, 46(2): 332–348.

Gietzen, D. W., Hao, S., & Anthony, T. G. (2007). Mechanisms of food intake repression in indispensable amino acid deficiency. *Annual Review of Nutrition*, 27: 63–78.

Gietzen, D. W., & Rogers, Q. R. (2006). Nutritional homeostasis and indispensable amino acid sensing: a new solution to an old puzzle. *Trends in Neurosciences*, 29(2): 91–99.

Glendinning, J. I., Ensslen, S., Eisenberg, M. E., & Weiskopf, P. (1999). Diet-induced plasticity in the taste system of an insect: localization to a single transduction pathway in an identified taste cell. *The Journal of Experimental Biology*, 202(15): 2091–102.

Glendinning, J. I. (2007). How do predators cope with chemically defended foods? *The Biological Bulletin*, *213*(3): 252–66.

Gmeinbauer, R., & Crailsheim, K. (1993). Glucose Utilization During Flight of Honeybee (*Apis mellifera*) Workers, Drones and Queens. *Journal of Insect Physiology*, 39(11): 959–967.

Goldrich, N. R. (1973). Behavioral responses of *Phormia regina* (Meigen) to labellar stimulation with amino acids. *The Journal of General Physiology*, 61(1): 74– 88.

Gonzalez, R., & Orchard, I. (2008). Characterization of neuropeptide F-like immunoreactivity in the blood-feeding hemipteran, *Rhodnius prolixus*. *Peptides*, 29(4): 545–558.

Gottsberger, G., Schrauwen, J. & Linskens, H. F. (1984). Amino Acids and Sugars in Nectar, and Their Putative Evolutionary Significance. *Plant Systematics and Evolution*, 145: 55-77.

Goulson, D., & Derwent, L. C. (2004). Synergistic interactions between an exotic honeybee and an exotic weed: pollination of *Lantana camara* in Australia. *Weed Research*, 44(3): 195–202.

Grandison, R. C., Piper, M. D. W., & Partridge, L. (2009). Amino-acid imbalance explains extension of lifespan by dietary restriction in *Drosophila*. *Nature*, 462(7276): 1061–1064.

Gronke, S., & Partridge, L. (2009). IGFs: Local Repair and Survival Factors Throughout Life Span. (D. Clemmons, I. C. A. F. Robinson, & Y. Christen, Eds.). Research and Perspectives in Endocrine Interactions (Vol. 105, pp. 105– 124). Berlin, Heidelberg: Springer Berlin Heidelberg.

Gutierrez, R., & Simon, S. a. (2011). Chemosensory processing in the taste - reward pathway. *Flavour and Fragrance Journal*, 26(4): 231–238.

Hallem, E. A., Ho, M. G., & Carlson, J. R. (2004). The molecular basis of odor coding in the *Drosophila* antenna. *Cell*, 117(7): 965–79.

Hanover, M. L., & White, J. S. (1993). Manufacturing, composition, and applications of fructose. *The American Journal of Clinical Nutrition*, *58*: 724S–732S.

Hao, S., Sharp, J. W., Ross-Inta, C. M., McDaniel, B. J., Anthony, T. G., Wek, R. C., Cavener, D. R., McGrath, B. C., Rudell, J. B., Koehnle, T. J., Gietzen, D. W. (2005). Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science*, 307(5716): 1776–8.

Harrison, J. M. (1986). Caste-Specific Changes in Honeybee Flight Capacity. *Physiological Zoology*, 59(2): 175–187.

Haupt, S. S. (2004). Antennal sucrose perception in the honey bee (*Apis mellifera* L.): behaviour and electrophysiology. *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology*, 190(9): 735–45.

Haydak, M. H. (1970). Honey bee nutrition. *Annual Reviews of Entomology*, 15: 143– 156.

Hendriksma, H. P., Oxman, K. L., & Shafir, S. (2014). Amino acid and carbohydrate trade-offs by honey bee nectar foragers and their implications for plant- pollinator interactions. *Journal of Insect Physiology*, In press.

Herbert, E. W., Shimanuki, H., & Caron, D. (1977). Optimum protein levels required by honey bees (hymenoptera, apidae) to initiate and maintain brood Rearing. *Apidologie*, 8(2): 141–146.

Hill, C. A., Fox, A. N., Pitts, R. J., Kent, L. B., Tan, P. L., Chrystal, M. a, Cravchik, A. Collins, F. H., Robertson, H. M., Zwiebel, L. J. (2002). G protein-coupled receptors in *Anopheles gambiae*. *Science*, 298(5591), 176–8.

Hiroi, M., Marion-Poll, F., & Tanimura, T. (2002). Differentiated response to sugars among labellar chemosensilla in *Drosophila*. *Zoological Science*, 19(9): 1009–1018.

Hiroi, M., Meunier, N., Marion-Poll, F., & Tanimura, T. (2004). Two antagonistic gustatory receptor neurons responding to sweet-salty and bitter taste in *Drosophila*. *Journal of Neurobiology*, 61(3): 333–42.

Hodgson, S, E. (1957). Electrophysiological studies of arthropod Chemoreception-II. Responses of labellar chemoreceptors of the Blowfly to stimulation by carbohydrates. *Journal of Insect Physiology*, 1: 240 - 247.

Hrassnigg, N. H., & Crailsheim, K. C. (2005). Differences in drone and worker physiology in honeybees (*Apis mellifera*). *Apidologie*, 36: 255–277.

Huang, Z. Y., Robinson, G. E., & Borst, W. D. (1994). Physiological correlates of division of labor among similarly aged honey bees. *Journal of Comparative Physiology A*, 174: 731–739.

Huber, R. E., & Thompson, D. J. (1973). Studies on a honey bee sucrase exhibiting unusual kinetics and transglucolytic activity. *Biochemistry*, 12(20): 4011–4020.

Ignell, R., Okawa, S., Englund, J. E., & Hill, S. R. (2010). Assessment of diet choice by the yellow fever mosquito *Aedes aegypti*. *Physiological Entomology*, 35(3): 274–286.

Ikeda, K. (1909). New seasonings. *Journal of Tokyo Chemical Society.*, 30: 820 – 836.

Ikeya, T., Galic, M., Belawat, P., Nairz, K., & Hafen, E. (2002). Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. *Current Biology*, 12(15): 1293– 1300.

Inagaki, H. K., de-Leon, S. B. T., Wong, A. M., Jagadish, S., Ishimoto, H., Barnea, G., Kitamoto, T., Axel, R., Anderson, D. J. (2012). Visualizing

neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. *Cell*, 148(3): 583–595.

Inoshita, T., & Tanimura, T. (2006). Cellular identification of water gustatory receptor neurons and their central projection pattern in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, 103(4): 1094– 1099.

Inouye, D. W., & Waller, G. D. (1984). Responses of Honey Bees (*Apis Mellifera*) to Amino Acid Solutions Mimicking Floral Nectars. *Ecology*, 65(2): 618–625.

Isono, K., & Morita, H. (2010). Molecular and cellular designs of insect taste receptor system. *Frontiers in Cellular Neuroscience*, 4(4): 1-16.

Ito, T., & Inokuchi, T. (1981). Nutritive effects of D-amino acids on the silkworm, *Bombyx mori*. *Journal of Insect Physiology*, 27(7): 447–453.

Jeong, Y. T., Shim, J., Oh, S. R., Yoon, H. I., Kim, C. H., Moon, S. J., & Montell, C. (2013). An odorant-binding protein required for suppression of sweet taste by bitter chemicals. *Neuron*, 79(4): 725–737.

Jiao, Y., Moon, S. J., & Montell, C. (2007). A Drosophila gustatory receptor required for the responses to sucrose, glucose, and maltose identified by mRNA tagging. *Proceedings of the National Academy of Sciences of the United States of America*, 104(35): 14110–1415.

Jiao, Y., Moon, S. J., Wang, X., Ren, Q., & Montell, C. (2008). Gr64f is Required in Combination with other Gustatory Receptors for Sugar Detection in *Drosophila. Current Biology*, 18(22): 1797–1801.

Johnson, R. J., Segal, M. S., Sautin, Y., Nakagawa, T., Feig, D. I., Kang, D.-H., Gersch, M. S., Benner, S., Sánchez-Lozada, L. G. (2007). Potential role of sugar (fructose) in the epidemic of hypertension, obesity and the metabolic syndrome, diabetes, kidney disease, and cardiovascular disease. *The American Journal of Clinical Nutrition*, 86(4): 899–906.

Jones, W. D., Cayirlioglu, P., Kadow, I. G., & Vosshall, L. B. (2007). Two chemosensory receptors together mediate carbon dioxide detection in *Drosophila*. *Nature*, 445(7123): 86–90.

Kamal, M. A., & Klein, P. (2011). Determination of sugars in honey by liquid chromatography. *Saudi Journal of Biological Sciences*, 18(1): 17–21.

Karley, A. J., Ashford, D. A., Minto, L. M., Pritchard, J., & Douglas, A. E. (2005). The significance of gut sucrase activity for osmoregulation in the pea aphid *Acyrthosiphon pisum*. *Journal of Insect Physiology*, 51(12): 1313–1319.

Karnani, M. M., Apergis-Schoute, J., Adamantidis, A., Jensen, L. T., de Lecea, L., Fugger, L., & Burdakov, D. (2011). Activation of central orexin/hypocretin neurons by dietary amino acids. *Neuron*, 72(4): 616–29.

Kent, L. B., & Robertson, H. M. (2009). Evolution of the sugar receptors in insects. *BMC Evolutionary Biology*, 9: 41-61.

Kent, L. B., Walden, K. K. O., & Robertson, H. M. (2008). The Gr family of candidate gustatory and olfactory receptors in the yellow-fever mosquito *Aedes aegypti*. *Chemical Senses*, 33(1): 79–93.

Kessler, S., Vlimant, M., & Guerin, P. M. (2013). The sugar meal of the African malaria mosquito *Anopheles gambiae* and how deterrent compounds interfere with it: a behavioural and neurophysiological study. *The Journal of Experimental Biology*, 216(7): 1292–306.

Kim, S. K., & Rulifson, E. J. (2004). Conserved mechanisms of glucose sensing and regulation by *Drosophila* corpora cardiaca cells. *Letters to Nature*, 431: 316– 320.

Kim, Y., & Smith, B. (2000). Effect of an amino acid on feeding preferences and learning behavior in the honey bee, *Apis mellifera*. *Journal of Insect Physiology*, 46(5): 793–801.

Kleineidam, C., & Tautz, J. (1996). Perception of Carbon Dioxide and Other "Air Condition" Parameters in the Leaf Cutting ant *Atta cephalotes*. *Naturwissenschaften*, 83: 566–568.

Klowden, M. J., & Lea, A. (1979). Abdominal distention terminates subsequent host- seeking behaviour of *Aedes*. *Journal of Insect Physiology*, 25: 583–585.

Koehnle, T. J., & Gietzen, D. W. (2005). Modulation of feeding behavior by amino acid-deficient diets: Present Findings and Future Directions. *Nutritional Neurocsience*, 10(1967): 147–158.

Koehnle, T. J., Russell, M. C., & Gietzen, D. W. (2003). Rats rapidly reject diets deficient in essential amino acids. *The Journal of Nutrition*, 133(7): 2331–2335.

Koh, T.-W., He, Z., Gorur-Shandilya, S., Menuz, K., Larter, N. K., Stewart, S., & Carlson, J. R. (2014). The *Drosophila* IR20a Clade of Ionotropic Receptors Are Candidate Taste and Pheromone Receptors. *Neuron*, 83: 1–16.

Kohno, K., Sokabe, T., Tominaga, M., & Kadowaki, T. (2010). Honey bee thermal/chemical sensor, AmHsTRPA, reveals neofunctionalization and loss of transient receptor potential channel genes. *The Journal of Neuroscience*, 30(37): 12219–12229.

Kohyama-Koganeya, A., Kim, Y. J., Miura, M., & Hirabayashi, Y. (2008). A *Drosophila* orphan G protein-coupled receptor BOSS functions as a glucose-responding receptor: loss of boss causes abnormal energy metabolism. *Proceedings of the National Academy of Sciences of the United States of America*, 105(40): 15328–15233.

Kretschmar, J. A., & Baumann, T. W. (1999). Caffeine in Citrus Fowers. *Phytochemistry*, 52: 19–23.

Krieger, J., Grosse-Wilde, E., Gohl, T., Dewer, Y. M. E., Raming, K., & Breer, H. (2004). Genes encoding candidate pheromone receptors in a moth (*Heliothis*

virescens). *Proceedings of the National Academy of Sciences of the United States of America*, 101(32): 11845–50.

Kwon, J. Y., Dahanukar, A., Weiss, L. A., & Carlson, J. R. (2007). The molecular basis of CO2 reception in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, 104(9): 3574–8.

Lacher, V. (1964). Elektrophysiologische untersuchungen an einzelnen rezeptoren fur geruch, kohlendioxyd, luftfeuchtigkeit und temperatur auf den antennen der arbeitsbiene und der drohne (*Apis mellifica* L.). *Zeitschrift Fur Vergleichende Physiologie*, 48: 587–623.

Lanaspa, M. a, Ishimoto, T., Li, N., Cicerchi, C., Orlicky, D. J., Ruzycki, P., Rivard, C., Inaba, S., Roncal-Jimenez, C. A., Bales, E. S., Diggle, C. P., Asipu, A., Petrash, J. M., Kosugi, T., Maruyama, S., Sanchez-Lozada, L. G., McManaman, J. L., Bonthron, D. T., Sautin, Y. Y., Johnson, R. J. (2013). Endogenous fructose production and metabolism in the liver contributes to the development of metabolic syndrome. *Nature Communications*, 4: 2434.

Lanza, J. (1988). Ant preferences for passiflora nectar mimics that contain amino acids. *Biotropica*, 20(4): 341–344.

Lanza, J., & Krauss, B. R. (1984). Detection of amino acids in artificial nectars by two tropical ants , *Leptothorax* and *Monomorium*. *Oecologia*, 63(3): 423–425.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, 23(21): 2947–2948.

Lee, G., & Park, J. H. (2004). Hemolymph sugar homeostasis and starvationinduced hyperactivity affected by genetic manipulations of the adipokinetic hormone- encoding gene in *Drosophila melanogaster*. *Genetics*, 167(1): 311– 323.

Lee, K. P. (2007). The interactive effects of protein quality and macronutrient imbalance on nutrient balancing in an insect herbivore. *The Journal of Experimental Biology*, 210(18): 3236–44.

Lee, K. P., Simpson, S. J., Clissold, F. J., Brooks, R., Ballard, J. W. O., Taylor, P. W., Sorna, N., Raubenheimer, D. (2008). Lifespan and reproduction in *Drosophila*: New insights from nutritional geometry. *Proceedings of the National Academy of Sciences of the United States of America*, 105(7): 2498– 503.

Lee, Y., Moon, S. J., & Montell, C. (2009). Multiple gustatory receptors required for the caffeine response in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, 106(11): 4495–500.

Lemon, C. H., & Katz, D. B. (2007). The neural processing of taste. *BMC Neuroscience*, 8(3): S5.

Leta, M. A., Gilbert, C., & Morse, R. A. (1996). Levels of haemolymph sugars and Body Glycogen of Honeybees (*Apis mellifera* L.) from Colonies Preparing to Swarm. *Journal of Insect Physiology*, 42(3): 239–245.

Liman, E. R., Zhang, Y. V, & Montell, C. (2014). Peripheral coding of taste. *Neuron*, 81(5): 984–1000.

Linander, N., Hempel de Ibarra, N., & Laska, M. (2012). Olfactory detectability of L- amino acids in the European honeybee (*Apis mellifera*). *Chemical Senses*, 37(7): 631–638.

Liu, F., Chen, J., Chai, J., Zhang, X., Bai, X., He, D., & Roubik, D. W. (2007). Adaptive functions of defensive plant phenolics and a non-linear bee response to nectar components. *Functional Ecology*, 21(1): 96–100.

Liu, L., Leonard, A. S., Motto, D. G., Feller, M. A., Price, M. P., Johnson, W. A., & Welsh, M. J. (2003). Contribution of *Drosophila* DEG/ENaC genes to salt taste. *Neuron*, 39(1): 133–146.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta CT)) Method. *Methods*, 25(4): 402–408.

Loftus, R. (1976). Temperature-dependent Dry Receptor on Antenna of Periplaneta. Tonic Response. *Journal of Comparative Physiology A*, 111: 153–170.

London-Shafir, I., Shafir, S., & Eisikowitch, D. (2003). Amygdalin in almond nectar and pollen – facts and possible roles. *Plant Systems Evolution*, 238: 87– 95.

Lyssiotis, C. A., & Cantley, L. C. (2013). F stands for fructose and fat. *Nature*, 502: 181–182.

Mace, O. J., Affleck, J., Patel, N., & Kellett, G. L. (2007). Sweet taste receptors in rat small intestine stimulate glucose absorption through apical GLUT2. *The Journal of Physiology*, 582: 379–392.

Margolskee, R. F., Dyer, J., Kokrashvili, Z., Salmon, K. S. H., Ilegems, E., Daly, K., Maillet, E. L., Ninomiya, Y., Mosinger, B., Shirazi-Beechey, S. P. (2007). T1R3 and gustducin in gut sense sugars to regulate expression of Na+ glucose cotransporter 1. *Proceedings of the National Academy of Sciences of the United States of America*, 104(38): 15075–15080.

Markison, S., Gietzen, D. W., & Spector, A. C. (1999). Essential amino acid deficiency enhances long-term intake but not short-term licking of the required nutrient. *The Journal of Nutrition*, 129(8): 1604–1612.

Marshall, J. (1935). On the sensitivity of the chemoreceptors on the antenna and fore-tarsus of the honey-bee, *Apis mellifica* L. *Journal of Experimental Biology*, 12: 17–26.

Masek, P., & Keene, A. C. (2013). *Drosophila* fatty acid taste signals through the PLC pathway in sugar-sensing neurons. *PLoS Genetics*, 9(9): e1003710.

Masumura, M., Satake, S., Saegusa, H., & Mizoguchi, A. (2000). Glucose stimulates the release of bombyxin, an insulin-related peptide of the silkworm *Bombyx mori*. *General and Comparative Endocrinology*, 118(3): 393–399.

Matsunami, H., & Amrein, H. (2003). Taste and pheromone perception in mammals and flies. *Genome Biology*, 4(7): 220.

Maurizio, A. (1965). Unteirsuchungen uber das zuckerbild der hamolymphe der honigbiene (*Apis mellifica* L.)- I. das zuckerbild des blutes erwachsener bienen. *Journal of Insect Physiology*, 11: 745–763.

McBride, C. S. (2007). Rapid evolution of smell and taste receptor genes during host specialization in *Drosophila sechellia*. *Proceedings of the National Academy of Sciences of the United States of America*, 104(12): 4996–5001.

McBride, C. S., Arguello, J. R., & O'Meara, B. C. (2007). Five *Drosophila* genomes reveal non-neutral evolution and the signature of host specialization in the chemoreceptor superfamily. *Genetics*, 177(3): 1395–416.

McQuillan, H. J., Barron, A. B., & Mercer, A. R. (2012). Age- and behaviourrelated changes in the expression of biogenic amine receptor genes in the antennae of honey bees (*Apis mellifera*). *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology*, 198(10): 753–61.

Mellon, J. D., & Evans, R, D. (1961). Electrophysiological evidence that water stimulates a fourth sensory cell in the blowfly taste receptor. *American Zoologist*, 1(3): 372–373.

Menzel, R. (1983). Neurobiology of learning and memory: the honeybee as a model system. *Die Naturwissenschaften*, 70(10): 504–511.

Meunier, N. Marion-Poll, F., Rospars, J. P., Tanimura, T. (2003). Peripheral Coding of Bitter Taste in *Drosophila*. *Journal of Neurobiology*, 56(2): 139–152.

Mevi-Schutz, J., & Erhardt, A. (2005). Amino acids in nectar enhance butterfly fecundity: a long-awaited link. *The American Naturalist*, 165(4): 411–419.

Min, K. J., Yamamoto, R., Buch, S., Pankratz, M., & Tatar, M. (2008). *Drosophila* lifespan control by dietary restriction independent of insulin-like signaling. *Aging Cell*, 7(2): 199–206.

Mishra, D., Miyamoto, T., Rezenom, Y. H., Broussard, A., Yavuz, A., Slone, J., Russell, D. H., Amrein, H. (2013). The molecular basis of sugar sensing in *Drosophila* larvae. *Current Biology*, 23(15): 1466–1471.

Miyakawa, Y. (1982). Behavioural evidence for the existence of sugar, salt and amino acid taste receptor cells and some of their properties in *Drosophila* larvae. *Journal of Insect Physiology*, 28(5): 405–410.

Miyakawa, Y., Fuiishiro, N., Kijima, H., & Morita, H. (1980). Differences in feeding response to sugars between adults and larvae in *Drosophila melanogaster*. *Journal of Insect Physiology*, 26: 685–688.

Miyamoto, T., & Amrein, H. (2008). Suppression of male courtship by a *Drosophila* pheromone receptor. *Nature Neuroscience*, 11(8): 874–876.

Miyamoto, T., Slone, J., Song, X., & Amrein, H. (2012). A Fructose Receptor Functions as a Nutrient Sensor in the *Drosophila* Brain. *Cell*, 151: 1113–1125.

Miyamoto, T., Wright, G., & Amrein, H. (2013). Nutrient sensors. *Current Biology*, 23(9): R369–373.

Mommaerts, V., Wäckers, F., & Smagghe, G. (2013). Assessment of gustatory responses to different sugars in harnessed and free-moving bumblebee workers (*Bombus terrestris*). *Chemical Senses*, 38(5): 399–407.

Montell, C. (2009). A Taste of the *Drosophila* Gustatory Receptors. *Current Opinion in Neurobiology*, 19(4): 345–353.

Moon, S. J., Köttgen, M., Jiao, Y., Xu, H., & Montell, C. (2006). A taste receptor required for the caffeine response *in vivo*. *Current Biology*, 16(18): 1812–1817.

Moon, S. J., Lee, Y., Jiao, Y., & Montell, C. (2009). A *Drosophila* gustatory receptor essential for aversive taste and inhibiting male-to-male courtship. *Current Biology*, 19(19): 1623–1627.

Moore, W. (1946). Nutrition of *Attagenus*. *Annals of the Entomological Society of America*, 39(3): 513–521.

Morita, H. (1992). T*ransduction process and impulse initiation in insect contact chemoreceptor. Zoological science*, 9: 1–16.

Moritz, B., & Crailsheim, K. (1987). Physiology of protein digestion in the midgut of the honeybee (*Apis mellifera* L.). *Journal of Insect Physiology*, 33(12): 923– 931.

Mullin, C. A., Chyb, S., Hollister, B., & Frazier, J. L. (1994). Review Neuroreceptor Mechanisms in Insect Gustation: a Pharmacological Approach. *Journal of Insect Physiology*, 40(11): 913–931.

Murphy, M. E., & King, J. R. (1989). Sparrows discriminate between diets differing in valine or lysine concentrations. *Physiology & Behavior*, 45(2): 423–30.

Murphy, M. E., & Pearcy, S. D. (1993). Dietary amino acid complementation as a foraging strategy for wild birds. *Physiology & Behavior*, 53(4): 689–98.

Nagasawa, H., Kataoka, H., Isogai, A., Tamura, S., & Akinori, S. (1984). Amino Terminal Amino Acid Sequence of the Silkworm Prothoracicotropic Hormone: Homology with Insulin. *Science*, 226(1983): 1344–1345.

National Centre for Biotechnology Information (NCBI). (2014). http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SP EC=OGP__7460__9555. Accessed: 01/2012 - 05/2014.

Nayak, S. V., & Singh, N. R. (1983). Sensilla on the tarsal segments and mouthparts of adult Drosophila melanogaster Meigen (Diptera - Drosophilidae). *Journal of Insect Morphology and Embryology*, 12(5): 273–291.

Nelson, G., Chandrashekar, J., Hoon, M. A., Feng, L., Zhao, G., Ryba, N. J. P., & Zuker, C. S. (2002). An amino-acid taste receptor. *Nature*, 416(6877): 199– 202.

Neukirch, A. (1982). Dependence of the Life Span of the Honeybee (*Apis meilifica*) upon Flight Performance and Energy Consumption. *Journal of Comparative Physiology*, 146: 35–40.

Ni, L., Bronk, P., Chang, E. C., Lowell, A. M., Flam, J. O., Panzano, V. C., Theobald, D. L., Griffith, L. C., Garrity, P. a. (2013). A gustatory receptor paralogue controls rapid warmth avoidance in *Drosophila. Nature*, 500(7464): 580–584.

Nishimura, A., Ishida, Y., Takahashi, A., Okamoto, H., Sakabe, M., Itoh, M., Takano- Shimizu, T., Ozaki, M. (2012). Starvation-induced elevation of taste responsiveness and expression of a sugar taste receptor gene in *Drosophila melanogaster*. *Journal of Neurogenetics*, 26(2): 206–215.

Nunez, J. (1982). Honeybee foraging strategies at a food source in relation to its distance from the hive and the rate of sugar flow. *Journal of Apiculture Research*, 21: 139–150.

Omand, E. (1971). A peripheral sensory basis for behavioral regulation. *Comparative Biochemistry and Physiology. A, Comparative Physiology*, 38(2): 265–87.

Omand, E., & Dethier, V. G. (1969). An electrophysiological analysis of the action of carbohydrates on the sugar receptor of the blowfly. *International Journal of Insect Morphology and Embryology*, 62(1): 136–143.

Opstad, R., Rogers, S. M., Behmer, S. T., & Simpson, S. J. (2004). Behavioural correlates of phenotypic plasticity in mouthpart chemoreceptor numbers in locusts. *Journal of Insect Physiology*, 50(8): 725–736.

Oudejans, R. C. H. M., Mes, I. T. H. M., Kooiman, F. P., & Van Der Horst, D. J. (1993). Adipokinetic Peptide Hormone Content and Biosynthesis During Locust Development. *Peptides*, 14: 877–881.

Ozaki, K., Ryuda, M., Yamada, A., Utoguchi, A., Ishimoto, H., Calas, D., MarionPoll, F., Tanimura, T., Yoshikawa, H. (2011). A gustatory receptor involved in host plant recognition for oviposition of a swallowtail butterfly. *Nature Communications*, 2: 542.

Page, R. E., Erber, J., & Fondrk, M. K. (1998). The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). *Journal of Comparative Physiology. A, Sensory, Neural, and Behavioral Physiology*, 182(4): 489–500.

Pankiw, T., & Page Jr, R. E. (2000). Response thresholds to sucrose predict foraging division of labor in honeybees. *Behavioral Ecology and Sociobiology*, 47(4): 265–267.

Paoli, P. P., Donley, D., Stabler, D., Saseendranath, A., Nicolson, S. W., Simpson, S. J., & Wright, G. A. (2014a). Nutritional balance of essential amino acids and carbohydrates of the adult worker honeybee depends on age. *Amino Acids*, 46(6): 1449–58.

Paoli, P. P., Wakeling, L. A, Wright, G. A, & Ford, D. (2014b). The dietary proportion of essential amino acids and Sir2 influence lifespan in the honeybee. *Age*, 36(3): 9649.

Park, J. H., & Kwon, J. Y. (2011). Heterogeneous expression of *Drosophila* gustatory receptors in enteroendocrine cells. *PloS One*, 6(12): e29022.

Passier, P. C., Vullings, H. G., Diederen, J. H., & Van der Horst, D. J. (1997). Trehalose inhibits the release of adipokinetic hormones from the corpus

cardiacum in the African migratory locust, *Locusta migratoria*, at the level of the adipokinetic cells. *The Journal of Endocrinology*, 153(2): 299–305.

Peñalva-Arana, D. C., Lynch, M., & Robertson, H. M. (2009). The chemoreceptor genes of the waterflea *Daphnia pulex*: many Grs but no Ors. *BMC Evolutionary Biology*, 9: 79.

Percival, M. S. (1961). Types of Nectar in Angiosperms. *New Phytologist*, 60(3): 235–281.

Pernal, S., & Currie, R. (2001). The influence of pollen quality on foraging behavior in honeybees (*Apis mellifera* L.). *Behavioral Ecology and Sociobiology*, 51(1): 53–68.

Potter, C. F., & Bertin, R. I. (1988). Amino Acids in Artificial Nectar: Feeding Preferences of the Flesh Fly *Sarcophaga bullata*. *American Midland Naturalist*, 120(1): 156–162.

Ramırez, G. P., Martınez, A. A. S., Fernandez, V. M., Bielsa, G. C., & Farina, W. M. (2010). The Influence of Gustatory and Olfactory Experiences on Responsiveness to Reward in the Honeybee. *PloS One*, 5(10): e13498.

Rathman, E. S., Lanza, J., & Wilson, J. (1990). Feeding Preferences of Flesh Flies (*Sarcophaga bullata*) for Sugar-only vs. Sugar-amino Acid Nectars. *American Midland Naturalist*, 1990(2): 379–389.

Ren, X., Zhou, L., Terwilliger, R., Newton, S. S., & Araujo, I. E. De. (2009). Sweet taste signaling functions as a hypothalamic glucose sensor. *Frontiers in Integrative Neuroscience*, 3(12): 1–15.

Riby, J. E., Fujisawa, T., & Kretchmer, N. (1993). Fructose absoption. *The American Journal of Clinical Nutrition*, 58: 748s–753s.

Riehle, M. A., & Brown, M. R. (2002). Insulin receptor expression during development and a reproductive cycle in the ovary of the mosquito *Aedes aegypti*. *Cell and Tissue Research*, 308(3): 409–420.

Riehle, M. A., Fan, Y., Cao, C., & Brown, M. R. (2006). Molecular characterization of insulin-like peptides in the yellow fever mosquito, *Aedes aegypti*:

expression, cellular localization, and phylogeny. *Peptides*, 27(11): 2547–60.

Robbins, W. E., Thompson, M. J., Yamamoto, R. T., & Shortino, T. (1965). Feeding Stimulants for the Female House Fly, *Musca domestica* Linneaus. *Science*, 147: 2–4.

Robertson, H. M., & Kent, L. B. (2009). Evolution of the Gene Lineage Encoding the Carbon Dioxide Receptor in Insects. *Journal of Insect Science*, 9(19): 1– 14.

Robertson, H. M., & Wanner, K. W. (2006). The chemoreceptor superfamily in the honey bee, *Apis mellifera*: Expansion of the odorant, but not gustatory, receptor family. *Genome Research*, 16: 1395–1403.

Robertson, H. M., Warr, C. G., & Carlson, J. R. (2003). Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster. Proceedings of the National Academy of Sciences*, 100: 14537–14542.

Rogers, S. M., & Simpson, S. J. (1997). Experience-dependent changes in the number of chemosensory sensilla on the mouthparts and antennae of *Locusta migratoria*. *Journal of Experimental Biology*, 200: 2313–2321.

Rolls, B. J. (1986). Sensory-specific Satiety. *Nutrition Reviews*, 44(3): 93–101.

Rose, W. C. (1938). The nutritive significance of the amino acids. *Physiological Reviews*, 13(1): 109–136.

Rothe, U., & Nachtigall, W. (1989). Flight of the honey bee. IV. Respiratory quotients and metabolic rates during sitting, walking and flying. *Journal of Comparative Physiology B*, 158: 739–749.

Rulifson, E. J., Kim, S. K., & Nusse, R. (2002). Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. *Science*, 296(5570): 1118–20.

Rund, S. S. C., Hou, T. Y., Ward, S. M., Collins, F. H., & Duffield, G. E. (2011). Genome-wide profiling of diet and circadian gene expression in the malaria vector *Anopheles gambiae*. *Proceedings of the National Academy of Sciences of the United States of America*, 108(32): E421–430.

Rund, S. S. C., Bonar, N. A., Champion, M. M., Ghazi, J. P., Houk, C. M., Leming, M. T., Syed, Z., Duffield, G. E. (2013). Daily rhythms in antennal protein and olfactory sensitivity in the malaria mosquito *Anopheles gambiae*. *Scientific Reports*, 3: 2494.

Ryuda, M., Calas-List, D., Yamada, A., Marion-Poll, F., Yoshikawa, H., Tanimura, T., & Ozaki, K. (2013). Gustatory sensing mechanism coding for multiple oviposition stimulants in the swallowtail butterfly, *Papilio xuthus*. *The Journal of Neuroscience*, 33(3): 914–24.

Shanbhag S. R., Park, S. K., Pikielny, C. W., Steinbrecht, R. A. (2001). Gustatory organs of Drosophila melanogaster: fine structure and expression of the putative odorant-binding protein PBPRP2. *Cell and Tissue Research*, 304(3): 423–437.

Sacktor, B. (1968). Trehalase and the transport of glucose in the mammalian kidney and intestine. *Proceedings of the National Academy of Sciences*, 60(3): 1007–1014.

Sacktor, B. (1970). Regulation of intermediary metabolism, with special reference to the control mechanisms in insect flight muscle. *Advances in Insect Phsiology*, 7: 267–347.

Sang, B. Y. J. H., & King, R. C. (1961). Nutritional requirements of axenically cultured *Drosophila melanogaster* adults. *Journal of Experimental Biology*, 38: 793–809.

Sato, K., Tanaka, K., & Touhara, K. (2011). Sugar-regulated cation channel formed by an insect gustatory receptor. *Proceedings of the National Academy of Sciences*, 108(28): 11680–11685.

Scheiner, R., Page, R. E., & Erber, J. (2001). The effects of genotype, foraging role, and sucrose responsiveness on the tactile learning performance of honey bees (*Apis mellifera* L.). *Neurobiology of Learning and Memory*, 76(2): 138– 50.

Schmidt, J. M., & Friend, W. G. (1991). Ingestion and diet destination in the mosquito *Culiseta inornata*: effects of carbohydrate configuration. *Journal of Insect Physiology*, 37(11): 817–828.

Schwartz, M. W., Seeley, R. J., Tschöp, M. H., Woods, S. C., Morton, G. J., Myers, M. G., & D'Alessio, D. (2013). Cooperation between brain and islet in glucose homeostasis and diabetes. *Nature*, 503(7474): 59–66.

Scott, K. (2005). Taste Recognition: Food for Thought. *Neuron*, 48: 455–464.

Scott, K., Brady, R., Cravchik, A., Morozov, P., Rzhetsky, A., Zuker, C., Axel, R. (2001). A Chemosensory Gene Family Encoding Candidate Gustatory and Olfactory Receptors in *Drosophila*. *Cell*, 104: 661–673.

Seeley, T. D. (1974). Atmospheric carbon dioxide regulation in honey-bee (*Apis mellifera*) colonies. *Journal of Insect Physiology*, 20(11): 2301–2305.

Shah, A. S., Ben-Shahar, Y., Moninger, T. O., Kline, J. n., & Welsh, M. j. (2009). Motile Cilia of Human Airway Epithelia Are Chemosensory. *Science*, *325*: 1131–1134.

Shimada, I. (1978). The Stimulating Effect of Fatty Acids and Amino Acid Derivatives on the Labellar Sugar Receptor of the Fleshfly. *The Journal of General Physiology*, 71: 19–36.

Shimada, I., ShiraishI, A., Kijima, H., & Morita, H. (1974). Separation of two receptor sites in a single labellar sugar receptor of the flesh-fly by treatment with p-chloromercuribenzoate. *Journal of Insect Physiology*, 20: 605–621.

Shiraishi, A., & Kuwabara, M. (1970). The effects of amino acids on the labellar hair chemosensory cells of the fly. *The Journal of General Physiology*, 56(6): 768– 782.

Siddiqi, O., & Rodrigues, V. (1980). Genetic analysis of a complex chemoreceptor. *Basic Life Sciences*, 16: 347–359.

Simcock, N. K., Gray, H. E., & Wright, G. A. (2014). Single amino acids in sucrose rewards modulate feeding and associative learning in the honeybee. *Journal of Insect Physiology*. In press.

Simmonds, S. J., Simpson, S. J. & Blaney, W. M. (1992). Dietary selection behaviour in *Spodoptera littoralis*: the effects of conditioning diet and

conditioning period on neural responsiveness and selection behaviour. *Journal of Experimental Biology*, 162: 73–90.

Simpson, S. J., & Abisgold, J. D. (1985). Compensation by locusts for changes in dietary nutrients: behavioural mechanisms. *Physiological Entomology*, 10(4): 443–452.

Simpson, S. J., James, S., Simmonds, M.S.J., & Blaney, W. M. (1991). Variation Chemosensitivity and the Control dietary selection in the Locust. *Appetite*, 17: 141–154.

Simpson, S. J., & Raubenheimer, D. (1997). Geometric analysis of macronutrient selection in the rat. *Appetite*, 28(3): 201–213.

Simpson, S. J., Sibly, R. M., Lee, K. P., Behmer, S. T., & Raubenheimer, D. (2004). Optimal foraging when regulating intake of multiple nutrients. *Animal Behaviour*, 68(6): 1299–1311.

Simpson, S. J., & Simpson, C. L. (1992). Mechanisms controlling modulation by haemolymph amino acids of gustatory responsiveness in the locust. *Journal of Experimental Biology*, 168: 269–287.

Singaravelan, N., Nee'man, G., Inbar, M., & Izhaki, I. (2005). Feeding responses of free-flying honeybees to secondary compounds mimicking floral nectars. *Journal of Chemical Ecology*, 31(12): 2791–2804.

Singh, N., Vrontakis, M., Parkinson, F., & Chelikani, P. (2011). Functional bitter taste receptors are expressed in brain cells. *Biochemical and Biophysical Research Communications*, 406(1): 146–151.

Slone, J., Daniels, J., Amrein, H., & Carolina, N. (2007). Sugar Receptors in *Drosophila. Current Biology*, 17: 1809–1816.

Smadja, C., Shi, P., Butlin, R. K., & Robertsonà, H. M. (2009). Large Gene Family Expansions and Adaptive Evolution for Odorant and Gustatory Receptors in the Pea Aphid, *Acyrthosiphon pisum. Molecular Biology and Evolution*, 26(9): 2073– 2086.

Smith, D. P. (2001). Drosophila Gustation: A Question of Taste. *Neuron*, 29: 551–554.

Smith, C. D., Zimin, A., Holt, C., Abouheif, E., Benton, R., Cash, E., et. al., (2011a). Draft genome of the globally widespread and invasive Argentine ant (*Linepithema humile*). *Proceedings of the National Academy of Sciences of the United States of America*, 108(14): 5673–5678.

Smith, C. R., Smith, C. D., Robertson, H. M., Helmkampf, M., Zimin, A., Yandell, M., et. al., (2011b). Draft genome of the red harvester ant *Pogonomyrmex barbatus*. *Proceedings of the National Academy of Sciences of the United States of America*, 108(14): 5667–5672.

Stocker, R. F. (1994). Cell & Tissue Review article: The organization of the chemosensory system in *Drosophila melanogaster*: a review. *Cell and Tissue Research*, 275: 3–26.

Su, C.-Y., Menuz, K., Reisert, J., & Carlson, J. R. (2012). Non-synaptic inhibition between grouped neurons in an olfactory circuit. *Nature*, 492(7427): 66– 71.

Sugarman, D., & Jakinovich, W. (1986). Behavioural gustatory responses of adult cockroaches, *Periplaneta americana* to D- and L-amino acids. *Journal of Insect Physiology*, 32(1): 35–41.

Suh, G. S. B., Wong, A. M., Hergarden, A. C., & Wang, J. W. (2004). A single population of olfactory sensory neurons mediates an innate avoidance behaviour in *Drosophila*. *Letters to Nature*, 431: 854–859.

Sun, S. Z., & Empie, M. W. (2012). Fructose metabolism in humans - what isotopic tracer studies tell us. *Nutrition & Metabolism*, 9(1): 89.

Takeda, K. (1961). Classical conditioned response in the honey bee. *Journal of Insect Physiology*, 6: 168–179.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, 30(12): 2725–2729.

Tan, K., Guo, Y. H., Nicolson, S. W., Radloff, S. E., Song, Q. S., & Hepburn, H. R (2007). Honeybee (*Apis cerana*) foraging responses to the toxic honey of *Tripterygium hypoglaucum* (Celastraceae): changing threshold of nectar acceptability. *Journal of Chemical Ecology*, 33(12): 2209–2217.

Tanimura, T., Isono, K., Takamura, T., & Shimada, I. (1982). Genetic Dimorphism in the Taste Sensitivity to Trehalose in *Drosophila melanogaster*. *Journal of Comparative Physiology A*, 147: 433–437.

Tanimura, T., & Shimada, I. (1981). Multiple Receptor Proteins for Sweet Taste in *Drosophila* Discriminated by Papain Treatment. *Journal of Comparative Physiology A*, 141: 265–269.

Tappy, L., & Le, K. (2010). Metabolic Effects of Fructose and the Worldwide Increase in Obesity. *Physiological Reviews*, 90: 23–46.

Thompson, S. N. (2003). Trehalose – The Insect "Blood" Sugar. *Advances in Insect Phsiology*, 31: 205–285.

Thorne, N., & Amrein, H. (2008). Atypical Expression of *Drosophila* Gustatory Receptor Genes in Sensory. *The Journal of Comparative Neurology*, 506: 548–568.

Thorne, N., Chromey, C., Bray, S., Amrein, H., Building, C., & Carolina, N. (2004). Taste Perception and Coding in Drosophila. *Current Biology*, 14: 1065–1079.

Toshima, N., & Tanimura, T. (2012). Taste preference for amino acids is dependent on internal nutritional state in *Drosophila melanogaster*. *Journal of Experimental Biology*, 215: 2827–2832.

Toth, A. L., Kantarovich, S., Meisel, A. F., & Robinson, G. E. (2005). Nutritional status influences socially regulated foraging ontogeny in honey bees. *The Journal of Experimental Biology,* 208(24): 4641–4649.

Toth, A. L., & Robinson, G. E. (2005). Worker nutrition and division of labour in honeybees. *Animal Behaviour*, 69(2): 427–435.

Treherne, J. E. (1958). The absorption of glucose from the alimentary canal of the locust *schistocerca gregaria* (Forsk.). *Journal of Experimental Biology*, 35: 297–306.

Tribolium genome sequencing consortium. (2008). The genome of the model beetle and pest *Tribolium castaneum. Nature*, 452:949-955.

Ueno, K., Ohta, M., Morita, H., Mikuni, Y., Nakajima, S., Yamamoto, K., & Isono, K. (2001). Trehalose sensitivity in *Drosophila* correlates with mutations in and expression of the gustatory receptor gene Gr5a. *Current Biology*, 11: 1451– 1455.

Van Der Horst, D. J., Van Marrewijk, W. J. A., Vullings, H. G., & Diederen, J. H. (1999). Metabolic Neurohormones: Release, Signal Transducion and Physiological Responses of Adipokinetic Hormones in Insects. *European Journal of Entomology*, 96: 299–308.

Van Der Goes Van Naters, W. M., & Den Otter, C. J. (1998). Amino acids as taste stimuli for tsetse flies. *Physiological Entomology*, 23(3): 278–284.

Vandenbeuch, A., Pillias, A.-M., & Faurion, A. (2004). Modulation of taste peripheral signal through interpapillar inhibition in hamsters. *Neuroscience Letters*, 358(2): 137–141.

Wagener-Hulme, C., Kuehn, J. C., Schulz, D. J., & Robinson, G. E. (1999). Biogenic amines and division of labor in honey bee colonies. *Journal of Comparative Physiology. A, Sensory, Neural, and Behavioral Physiology*, 184(5): 471–479.

Wainselboim, A., & Farina, W. (2000). Trophallaxis in the honeybee *Apis mellifera* (L.): the interaction between flow of solution and sucrose concentration of the exploited food sources. *Animal Behaviour*, 59(6): 1177–1185.

Waldbauer, G. P., & Friedman, S. (1991). Self-selection of optimal diets by insects. *Annual Review of Entomology*, 36: 43–63.

Wang, Z., Singhvi, A., Kong, P., & Scott, K. (2004). Taste representations in the Drosophila brain. *Cell*, 117(7): 981–991.

Wang, L., Han, X., Mehren, J., Hiroi, M., Billeter, J.-C., Miyamoto, T., Amrein, H., Levine, J. D., Anderson, D. J. (2011). Hierarchical chemosensory regulation of male-male social interactions in Drosophila. *Nature Neuroscience*, 14(6): 757– 762.

Wang, Y., Brent, C. S., Fennern, E., & Amdam, G. V. (2012). Gustatory perception and fat body energy metabolism are jointly affected by vitellogenin and juvenile hormone in honey bees. *PLoS Genetics*, 8(6): e1002779.

Wanner, K. W., Nichols, A. S., Walden, K. K. O., Brockmann, A., Luetje, C. W., & Robertson, H. M. (2007). A honey bee odorant receptor for the queen substance 9-oxo-2-decenoic acid. *Proceedings of the National Academy of Sciences of the United States of America*, 104(36): 14383–14388.

Wanner, K. W., & Robertson, H. M. (2008). The gustatory receptor family in the silkworm moth *Bombyx mori* is characterized by a large expansion of a single lineage of putative bitter receptors. *Insect Molecular Biology*, 17(6): 621–629.

Watanabe, K., Toba, G., Koganezawa, M., & Yamamoto, D. (2011). Gr39a, a highly diversified gustatory receptor in *Drosophila*, has a role in sexual behavior. *Behavior Genetics*, 41(5): 746–753.

Weiss, L. A., Dahanukar, A., Kwon, J. Y., Banerjee, D., & Carlson, J. R. (2011). The molecular and cellular basis of bitter taste in *Drosophila. Neuron*, 69(2): 258– 272.

Wheeler, D. E., Buck, N., & Evans, J. D. (2006). Expression of insulin pathway genes during the period of caste determination in the honey bee, *Apis mellifera. Insect Molecular Biology*, 15(5): 597–602.

Whitehead, A. T. (1978). Electrophysiological response of honey bee labial palp contact chemoreceptors to sugars and electrolytes. *Physiological Entomology*, 3(3): 241–248.

Whitehead, A. T., & Larsen, J. R. (1976a). Ultrastructure of the contact chemoreceptors of *Apis mellifera* L, (Hymenoptera • Apidae). *International Journal of insect Morphology and Embryology*, 5: 301–315.

Whitehead, T., & Larsen, J. R. (1976b). Electrophysiological responses of galeal contact chemoreceptors of *Apis mellifera* to selected sugars and electrolytes. *Journal of Insect Physiology*, 22: 1609–1616.

Whitfield, C. W., Cziko, A.-M., & Robinson, G. E. (2003). Gene expression profiles in the brain predict behavior in individual honey bees. *Science*, 302(5643): 296– 299.

Wieczorek, H., & Wolff, G. (1989). The labellar sugar receptor of *Drosophila*. *Journal of Comparative Physiology A: Sensory, Neural, and Behavioral Physiology*, 164: 825–834.

Winston, M. (1987). The Biology of the Honey Bee (1st ed., pp. 1–281). First Harvard University Press.

Wisotsky, Z., Medina, A., Freeman, E., & Dahanukar, A. (2011). Evolutionary differences in food preference rely on Gr64e, a receptor for glycerol. *Nature Neuroscience*, 14(12), 1534–1541.

Woodring, J., Boulden, M., Das, S., & Gade, G. (1993). Studies on Blood Sugar Homeostasis Honeybee (*Apis mellifera*, L.). *Journal of Insect Physiology*, 39(1): 89–97.

Wright, G. A., Mustard, J. A., Simcock, N. K., Ross-Taylor, A. A. R., McNicholas, L. D., Popescu, A., & Marion-Poll, F. (2010). Parallel reinforcement pathways for conditioned food aversions in the honeybee. *Current Biology*, 20(24): 2234– 2240.

Wright, G. A., Baker, D. D., Palmer, M. J., Stabler, D., Mustard, J. a, Power, E. F., Borland, A. M., Stevenson, P. C. (2013). Caffeine in floral nectar enhances a pollinator's memory of reward. *Science*, 339(6124): 1202–1204.

Wright, G. A., & Smith, B. (2004). Different Thresholds for Detection and Discrimination of Odors in the Honey bee (*Apis mellifera*). *Chemical Senses*, 29(2): 127–135.

Wu, S. V., Rozengurt, N., Yang, M., Young, S. H., Sinnett-Smith, J., & Rozengurt, E. (2002). Expression of bitter taste receptors of the T2R family in the gastrointestinal tract and enteroendocrine STC-1 cells. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(4): 2392–2397.

Wu, S. V., Chen, M. C., & Rozengurt, E. (2005a). Genomic organization, expression, and function of bitter taste receptors (T2R) in mouse and rat. *Physiological Genomics*, 22(2): 139–149.

Wu, Q., Zhang, Y., Xu, J., & Shen, P. (2005b). Regulation of hunger-driven behaviors by neural ribosomal S6 kinase in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, 102(37): 13289–13294.

Wyatt, A. G. R., & Kalf, G. F. (1957). The chemistry of insect hemolymph. *Journal of General Physiology*, 40(6): 833–847.

Wyatt, A. G. R., (1961). The biochemistry of insect hemolymph. *Annual Review of Entomology*, 6: 75–102.

Wykes, G. R. (1952). An investigation of the sugars present in the nectar of flowers of various species. *New Phytologist*, 51(2): 210–215.

Xu, W., Zhang, H. J., & Anderson, A. (2012). A sugar gustatory receptor identified from the foregut of cotton bollworm *Helicoverpa armigera*. *Journal of Chemical Ecology*, 38(12): 1513–1520.

Yokohari, F., Tominaga, Y., & Tateda, H. (1982). Antennal hygroreceptors of the honey bee, *Apis mellifera* L. *Cell and Tissue Research*, 226: 63–73.

Zhang, Y.-F., Huang, L.-Q., Ge, F., & Wang, C.-Z. (2011). Tarsal taste neurons of *Helicoverpa assulta* (Guenée) respond to sugars and amino acids,

suggesting a role in feeding and oviposition. *Journal of Insect Physiology*, 57(10): 1332–1340.

Zhang, Y. V, Ni, J., & Montell, C. (2013). The molecular basis for attractive salt taste coding in Drosophila. *Science*, 340(6138): 1334–1338.

Zhou, X., Slone, J. D., Rokas, A., Berger, S. L., Liebig, J., Ray, A., Reinberg, D., Zwiebel, L. J. (2012). Phylogenetic and transcriptomic analysis of chemosensory receptors in a pair of divergent ant species reveals sex specific signatures of odor coding. *PLoS Genetics*, 8(8): e1002930.

Ziegler, H. (1956). Untersuchungen uber die Leitung und Sekretion der Assimilate. *Planta*, 47: 447–500.