MAPPING THE DISTRIBUTION OF THE OCTOPAMINE BETA RECEPTOR SUBTYPE-2 IN THE HONEYBEE (*Apis mellifera*) BRAIN

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By

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ABSTRACT

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Molecular Biology, Genetics and Bioengineering, MSc Thesis, June 2021

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Keywords: Immunohistochemistry, biogenic amines, honeybee brain, high performance liquid chromatography, honeybee appetite mechanism, trehalose-octopamine relationship

In invertebrates, hemolymph substitutes the role of blood in vertebrates. The fluctuating sugar levels in the hemolymph provides a crosstalk between the neurotransmitters in the brain and the energetic need of honeybees. Therefore, trehalose, the major sugar content of the honeybee hemolymph may play an important role in appetite regulation of the honeybee. In this thesis, we investigated the relationships between 4 major neurotransmitters (octopamine, tyramine, dopamine, and serotonin) and different sugar levels in the hemolymph with high performance liquid chromatography techniques. According to our findings, the most significant changes in biogenic amines and the appetite occur in the oldest honeybee age class, which is foragers. A lowering of trehalose increases octopamine levels, lowers tyramine, and corresponds with an increase in appetite levels. We did not observe any significant changes of Insulin-Like Protein 1 and 2 gene expression after manipulating trehalose and glucose levels suggesting that the appetite regulation, we observed is independent of glucose-insulin signaling pathway

A secondary aim was to design and optimize an antibody for the octopamine beta receptor subtype 2, so that we could map this receptor throughout the honeybee brain. The distribution of the octopamine beta receptor subtype 2 will provide insights to its possible function based on its spatial location in the bee brain. The final map will be used to indicate the localized injection sites required for CRISPR-Cas9 knockdown of the receptor and confirm whether octopamine plays a causal role in appetite regulation.

ÖZET

BAL ARISI (*Apis mellifera*) BEYNİNDEKİ OKTOPAMİN BETA RESEPTÖRÜ ALTTÜR-2 DAĞILIMININ HARİTALANDIRILMASI

İREM AKÜLKÜ

Moleküler Biyoloji, Genetik ve Biyomühendislik, Yüksek Lisans Tezi, Haziran 2021

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Anahtar kelimeler: Immünohistokimya, biyojenik aminler, bal arısı beyni, yüksek performanslı sıvı kromatografisi, bal arısı iştah mekanizması, trehaloz-oktopamin ilişkisi

Omurgasızlarda hemolenf sıvısı, omurgalılardaki kanın görevini üstlenmektedir. Hemolenfteki değişken şeker oranları, bal arısının enerji ihtiyacı ile beyindeki nörotransmiterler arasında iletişimi sağlamaktadır.Dolayısıyla hemolenfteki ana sakkarit olan trehaloz, bal arısı iştah mekanizmasında önemli bir rol oynuyor olabilir. Bu tezde arı beyninde bulunan 4 nörotransmiter (oktopamin, tiramin, dopamin, serotonin) ile hemolenfteki farklı şeker miktarları arasındaki ilişki yüksek performanslı sıvı kromatografisi (HPLC) ile incelendi. Bulgularımıza göre, biyojenik aminler ile iştah arasındaki en kayda değer değişim en yaşlı bal arısı sınıfı olan tarlacı arılarda gözlemlendi. Trehaloz seviyesindeki azalma, oktopamin seviyesinde artışa ve tiramin seviyesinde azalmaya sebep oldu; bu durum da iştah seviyesinin artması ile sonuçlandı. Hemolenfteki glikoz ve trehaloz seviyeleri manipüle edildikten sonra analiz edilen insülin-benzeri protein 1 ve 2 seviyelerinde kayda değer herhangi bir değişim gözlemlenmedi. Tüm bu sonuçlar göstermektedir ki, gözlemlerimize göre iştah mekanizması glikoz-insülin sinyal yolağından bağımsız olarak düzenlenmektedir.

Tezin ikinci kısmında oktopamin beta reseptörü alttür 2'nin arı beyninde dağılımını haritalandırmak için immünohistokimya deneyi optimize edildi. Henüz bu deney tamamlanmamış olmasına rağmen, pek çok aşamanın optimizasyonu tamamlandı. Elde edilen son harita, gelecek aşamalarda bu reseptörün *in vivo* knockdown'ı için CRISPR-Cas9 enjeksiyon bölgelerini gösterecektir. Ayrıca bu reseptörün dağılımı, reseptörün arı davranışına etkisi konusuna ışık tutacaktır.

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I am also grateful to my beloved family. They are the main reason that I was able to go further. They always supported me in every decision I have ever made, and in the things I was wrong. Without their unique love, support, and efforts, I would not be the way I am now.

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In memory of my beloved professor Özgür Gül…

Sevgili hocam Özgür Gül'ün anısına…

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µ	Micro
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
AADC	Amino acid carboxylase
AKH	Adipokinetic hormone
AL	Antennal lobe
ANOVA	Analysis of variance
APC	Endocrine cells
br	Basal ring
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CNS	Central nervous system
CRISPR	Clustered regularly interspaced short palindromic repeats
CS	Conditional stimulus
DAPI	4′,6-diamidino-2-phenylindole
DILP	Drosophila insulin-like peptide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
g	Gram

GPCR	G protein-coupled receptor
H	Hour
HPLC	High pressure liquid chromatography
IGF	Insulin/Insulin-like growth factor
IIS	Insulin/Insulin-like growth factor signaling
ILP	Insulin-like peptide
IPC	Neurosecretory cells
JH	Juvenile hormone
KLH	Keyhole limped hemocyanin
LC	Lateral calyces
L-DOPA	3,4-dihydroxyphenylalanine
LO	Lobula
L-Phe	Phenylalanine
L-Tyr	Tyrosine
m	Milli
m	Meter
M	Molar
MC	Medial calyces
ME	Medulla
min	Minute
NCBI	National Center for Biotechnology Information
NGS	Normal goat serum
Oa	Octopamine
OaβR2	Octopamine beta receptor subtype 2

OctαR	α-adrenergic like octopamine receptor
OctβR	β-adrenergic like octopamine receptor
p	Pico
PAH	Phenylalanine hydroxylase
PAM	Protocerebral arterior medial
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Triton X-100
PCR	Polymerase Chain Reaction
PER	Proboscis Extension Reflex
PFA	Paraformaldehyde
PPL	Protocerebral posterior lateral
RNA	Ribonucleic acid
SOG	Suboesophageal ganglion
TBH	Tyramine beta hydroxylase
TDC	Tyrosine decarboxylase
TH	Tyrosine hydroxylase
TOR	Rapamycin
TPH	Tryptophan hydroxylase
Trp	Tryptophan
U	Unit
US	Unconditional stimulus
UV	Ultraviolet
vl	Vertical lobe
vmb	Forefront view
χ^2	Chi-square

1. INTRODUCTION

1.1.The Anatomy of Honey Bee Brain Mushroom Bodies

The nervous system of the honey bee consist of three main parts which are the cerebral ganglion (the brain), a suboesophageal ganglion and a ventral nerve chord. An average worker bee brain has approximately 850000 neurons and almost half of them can be found in optical lobe (Witthoft 1967). Protocerebrum, deutocerebrum and tritocerebrum are the main three parts of the bee brain that are connected to each other by neuropilar interactions. The protocerebrum is the largest part of the brain and it includes ocellar tract neurophiles, central complex, lateral protocerebrum, pars intercerebralis, and most importantly, mushroom bodies (G. Bicker, Schäfer, and Rehder 1987).

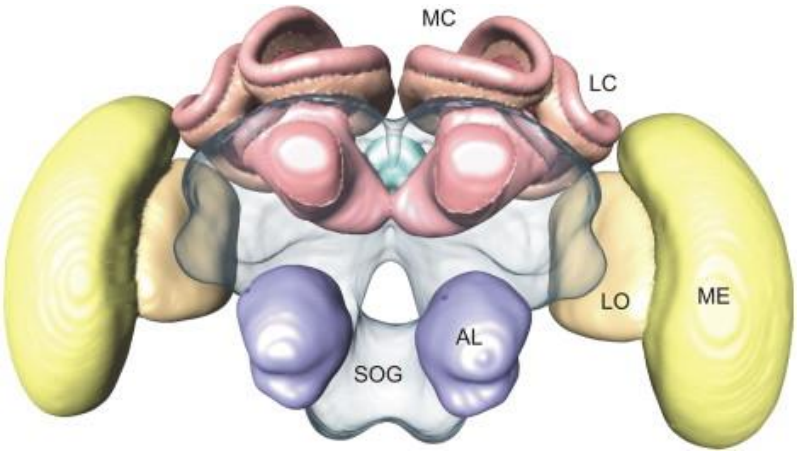

Figure 1: 3D structure of honeybee brain. The yellow color represents the medulla and lobula (ME and LO, respectively); blue color indicates the primary olfactory neurophil, which is antennal lobe,AL. Pink represents the paired mushroom bodies, MC and LC are

medial and lateral calyces. Transparent blue structures are protocerebral lobe and suboesophageal ganglion (SOG) (Menzel, Leboulle, and Eisenhardt 2006) **.**

Mushroom bodies or corpora pedunculata are thickly packed, paired structures, in the dorsal protocerebrum. They are highly developed in social insects, such as honeybees. The most important part of the mushroom body is called calyces, the cup-shaped structures. Each body contains lateral and medial calyces, α- and β- lobes, and all these 4 structures are connected to each other by the pedicula. Calyces are made of approximately 170000 intrinsic neurons that their dendrites are branched out to the cup-shaped structure of the calyces. Almost all the external stimuli come to calyces, which are the sensory input regions; and leave from α- and β- lobes, which are described as output regions (Mobbs 1982; Mobbs 1984; Schürmann 1974).

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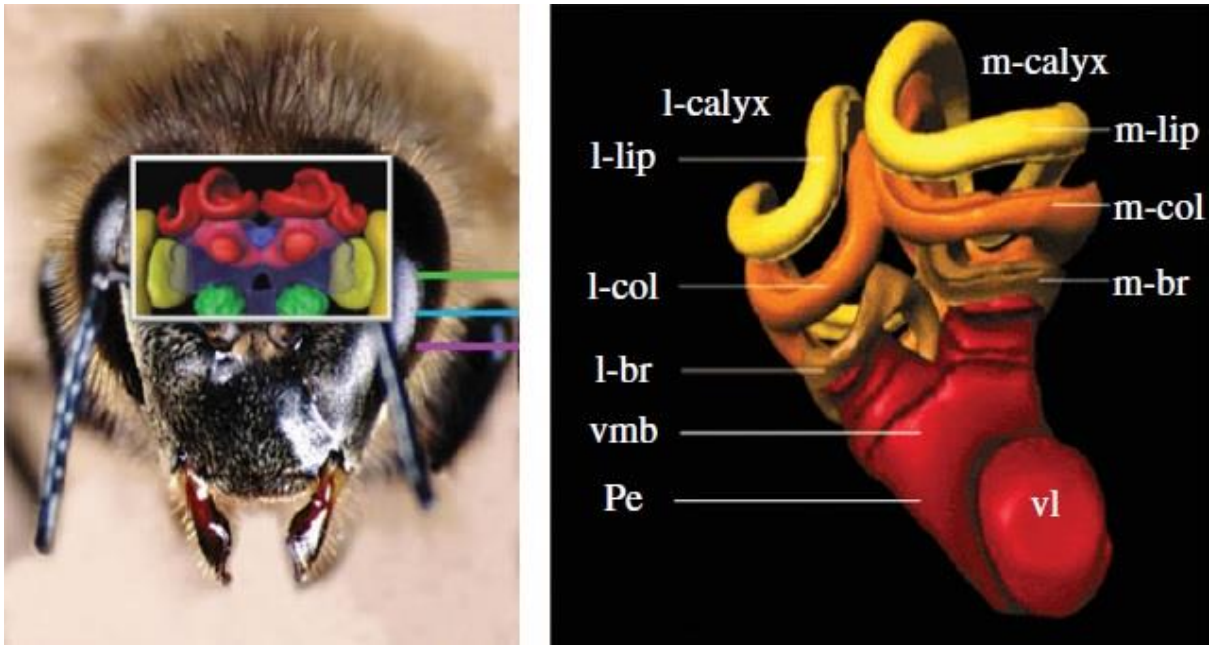


Figure 2: Forefront view (vmb) of the mushroom body. Pe, pedicula; lateral (l) and medial (m) calyces, collar (col), lip (lip) and the basal ring (br); vertical lobe (vl)(Avarguès-Weber and Giurfa 2013).

1.2.Appetite Regulation in Insects

Appetite is referred as the nominative concept of craving to eat or the sensitiveness of hunger. Appetite is also known to be defined as the potential of feeding or the willingness to feed (Egecioglu et al. 2011). Typically, it is correlated hunger or level of satiation which is best defined as the actual energetic state or need of the organism (Vucetic and Reyes 2010). In most of the vertebrates the appetite regulation is maintained by the signals between the liver and the brain. In case of hunger, the first signal starts in the stomach by fluctuation levels of nutrients such as glucose, lipids or amino acids (Rønnestad et al. 2017). For example, leptin is one of the most important hormones in human energy homeostasis and the leptin receptors can be found in hypothalamus and in the human hindbrain. The fluctuating nutrient levels trigger the leptin, insulin and glucagon pathways in mammals and the relation between the nutrients and the hormones regulate the appetite levels in mammals (Morley and Levine 1985; Fisher, Yagaloff, and Burn 1999; Valassi, Scacchi, and Cavagnini 2008; Hamann and Matthaei 1996).

On the other hand, invertebrates have hemolymph instead of blood as it contains components of the circulatory as well as the lymph system (Kanost 2009). Hemolymph is known as a homeostasis regulator of fluid in the insect body because it is constantly bathing the organs and flowing around the insect with its open circulatory system. Changes in the internal state of the individual can therefore be monitored by the substances and the amount of them in the hemolymph. Therefore hemolymph, just like blood in vertebrates, provides a communication link between the internal energetic state and the central nervous system (CNS) (Klowden 2008). Consequently, it may play an important role the crosstalk between the sugar levels in the hemolymph, indicating the individuals energetic state and neuronal control of appetite regulation (Mayack et al. 2019).

Unlike in vertebrates, trehalose is the primary sugar found in invertebrates in comparison to glucose (Fairbairn 1958). Trehalose is known to have other physiological functions besides energy storage, which is typically the first one to come to mind (Elbein et al. 2003). The levels are incredibly dynamic and the fluctuating levels themselves could therefore act as a signal of the energetic state of the individual (Paul et al. 2008).We hypothesize that, this is communicated via octopamine, a neurohormone which is known

to be correlated with trehalose and appetite levels in forager honeybees. If this is the case, then it is plausible that there is no need for the glucose-insulin signaling pathway to regulate appetite in forager honeybees. Relying on trehalose instead would be more direct and efficient means of regulating appetite as it would serve as a buffer against starvation because the lowering of trehalose levels during physical activity precedes the lowering of glucose levels in hemolymph (Blatt and Roces 2001).

1.3.Trehalose and Octopamine

Trehalose is a non-reducing disaccharide molecule that forms from two D-glucose molecules. The majority of trehalose is found in bacteria, fungi, and insects, and vertebrates are not able to synthesize trehalose. Insect hemolymph contains approximately 70-80% trehalose and serves several functions. The fluctuating trehalose levels regulate insect appetite and it is catabolized in high-energy-requiring tasks such as flight (Elbein 2010). The high concentration of trehalose is linked to honeybee appetite, as it governs honeybee foraging recurrence as well as foraging decision making (Y. Wang et al. 2012; Mayack and Naug 2011; 2013). It also provides freeze and dehydration resistance (Paiva and Panek 1996). Many organisms that exist in low temperature habitats are dependent on the presence of natural antifreeze molecules such as glycerol or trehalose. It is discovered that larval stage of sawflies can survive -40^{o}C temperatures and high concentrations of trehalose are present in the organism, while glycerol is absent (Richards et al. 2002).

Octopamine is an important highly conserved neurohormone and neurotransmitter in the insect nervous system. It has pleiotropic effects, so octopamine regulates many different physiological events such as various behaviors like learning, memory, maintenance of rhythmic behaviors, activation or inhibition of endocrine glands, and lastly, the transfer of lipids and carbohydrates (Farooqui 2007). In the honeybee, octopamine is also associated with social behaviors such as the recognition of other hive members and hygienic behavior (Schulz, Barron, and Robinson 2002; Schulz and Robinson 1999;

Spivak et al. 2003; Robinson et al. 1999). More specifically, octopamine has neuromodulatory roles in olfactory learning and memory, division of labor, sting responses to environmental stimuli and vision in *Apis mellifera* (Schwaerzel, Monastrioti, and Scholz 2003; Schulz, Barron, and Robinson 2002; Burrell and Smith 1995; Erber, Pribbenow, and Bauer 1993).

Octopamine shows structural similarity with norepinephrine in vertebrates, but it differs from norepinephrine by the absence of a hydroxyl group of the phenol ring. Based on this analogy, it is demonstrated that insect octopaminergic system corresponds to the "fight or flight" response that the vertebrate noradrenergic system elicits (T Roeder 1999; Gray 2004). During this response, octopamine functions as activator of flight muscles, and the mobilization of food stores. Therefore, this biogenic amine plays some role in the maintenance of energetic homeostasis when the insect is under stress and is known to be involved as a general stress response in insects. Starvation is a form of stress, but the explicit connection between octopamine and appetite regulation has yet to be elucidated.

Even though the appetite levels due to starvation increase in all ages for honeybee, the most significant change is observed in foragers. The reduced levels of trehalose are associated with an elevation of octopamine levels in the bee brain. Among other hemolymph sugars, glucose and fructose, the connection between trehalose and octopamine is the most pronounced (Mayack et al. 2019). We therefore predict that the octopamine beta receptors will play a role in appetite regulation and this regulation will have age dependent effects, so we may only see this connection within the foraging age class (the oldest age class) of the honeybee.

1.4.Glucose-Insulin Pathway

Metabolism and the feeding-related behavior are both governed by the insulin/insulin-like growth factor signaling (IIS) pathways. Food consumption and the high nutrient store content promote the insulin synthesis or insulin-like peptides (ILPs) and reduces the functioning of adipokinetic hormone (AKH), the insect equivalent of mammalian

glucagon. IIS also enhances the rapamycin (TOR) pathway and juvenile hormone (JH) (Ament et al. 2008).

In addition, ILPs plays an important role in metabolic regulations such as growth, lifespan, and machinery of insulin/insulin-like growth factors (IGF) signaling. Most of the IGFs are produced by the neurosecretory cells (IPC) in the fly brain. The mechanistic regulation of the pathway differs in adults and larvae. In adult *Drosophila melanogaster* it has been shown that the IPC cell-autonomous sensing of circulating glucose relates to *Drosophila* ILP (DILP) release. Glucose-related DILP production is mediated by several neurotransmitters and neuropeptides that are released from intestine and adipocytes. On the other hand, glucose-sensitive endocrine cells, that can produce AKH hormone, in intestine and fat body regulates the larval-IPCs (Nässel and Broeck 2016).

In adult *Drosophila*, the DILP release directly activates the IPCs in the brain. IPCs developed an cell-autonomous-glucose-sensing-capacity so that they activate DILP release in presence of sugar meal (Park et al. 2014). On the contrary, larval *Drosophila* IPCs are not able to sense the nutrient levels in hemolymph autonomously. The nutrient-sensing occurs in neighboring cells of the ring-gland that can secrete AKH, endocrine cells (APCs), adipocyte cells in the fat body or gut endocrine cells. The APCs are capable of responding to glucose and trehalose and they promote AKH release (Kim and Rulifson 2004; Ren et al. 2015).

Octopamine and serotonin hormones are also involved in neuroendocrine cell cycle (Luo et al. 2014; 2012). It is demonstrated that IPCs can express serotonin and octopamine receptors and they can interact with the neurons of pars intercerebralis region in the *Drosphila* brain. Knockdown of serotonin receptor causes a reduction in starvation resistance and nutrient intake; additionally, it elevates the glucose, trehalose, and glycogen levels in the hemolymph of fed *Drosophila* flies. Knockdown of the octopamine receptor in IPCs resulted in an increase of starvation resistance and nutrient uptake, but does not affect the circulating sugar levels in fed flies (Nässel and Broeck 2016). This suggests that in solitary insects normally there is a connection between sugar levels in the hemolymph, appetite regulation, and mobilization of food stores via ILP. However, in a foraging honeybee we hypothesize that the regulation of appetite can be accomplished via octopamine independent of this ILP signaling pathway.

1.5.Octopamine Alpha vs Octopamine Beta Receptors

In this study, it is hypothesized that fluctuating trehalose levels are linked to appetite via octopamine. To demonstrate a cause and effect relationship between octopamine and appetite regulation, the Octβ subtype 1-4 receptors are going to be knocked-down by the CRISPR-Cas9 system to demonstrate the connection. Therefore, our immunohistochemistry staining to map the distribution of these receptors will identify the injection sites for the CRISPR-cas9 system. Octopamine receptors of insects are a subclass of G protein coupled receptors (GPCRs). They are classified based on the increase or decrease of cyclic adenosine monophosphate (cAMP) levels by secondary messenger pathways and the generation of calcium signals in intracellular space. G-protein coupled receptors (GPCR) are the largest membrane receptors in eukaryotic cells. The cell surface provides communication between environmental stimuli such as light or nutrients and the intracellular components like secondary messengers, cellular structures, etc. GPCRs contain 7 transmembrane-spanning segments that have the N -terminus in the extracellular space and 3 extracellular loops that identify and recognize the ligand molecule. The C-terminal intracellular region and 3 intracellular loops interact with the G protein (J. V Zhang et al. 2013). The 7 intermembrane segments form a circular structure which is the binding site of most of the neurotransmitter ligands (Waxham 2004).

The most up-to-date classes of insect octopamine receptors are α-adrenergic-like octopamine receptors (OctαRs), the β-adrenergic-like octopamine receptors (OctβRs) and the octopamine/tyramine (or tyraminergic) receptors (Peter D. Evans and Maqueira 2005). It is demonstrated that the honeybee (*Apis mellifera)* genome has homolog genes to the *Drosophila* octopamine receptor genes. Fruit fly and honeybee octopamine receptors cluster in 2 groups. The first group governs intracellular calcium levels and the other one regulates cyclic AMP production. In other words, high octopamine levels increase cyclic AMP in the honeybee brain (Sinakevitch, Mustard, and Smith 2011; Balfanz et al. 2005). The alpha octopamine receptors have been shown to play a role in odor appetitive learning and memory. These have been in the antennal lobe region of the brain supporting their role in classical conditioning using odor (Farooqui et al. 2003; Farooqui, Vaessin, and Smith 2004). Whether they are utilized in appetite regulation

independent of odor conditioning, and what the role the beta octopamine receptors play in maintaining energetic homeostasis, has yet to be investigated in the honeybee. Determining their distribution within the brain will aid in providing insights on what functions they may serve in relation to honeybee behavior.

1.6.Dopamine and Its Role in Invertebrate Behaviors

Dopamine, tyramine and octopamine are L-tyrosine (L-Tyr) derived biogenic amines and they have crucial roles in different insect behaviors. Especially dopamine, which has important roles in scouting, motor abilities, arousal, and memory formation. It also contributes to exoskeleton formation via the melanization and sclerotization pathways (Verlinden 2018).

Phenylalanine (L-Phe) is converted to L-Tyr by phenylalanine hydroxylase (PAH). This hydroxylation reaction results in L-Tyr that to be converted into 3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH). L-DOPA is the precursor of dopamine. In addition to dopamine formation, L-Tyr takes the precursor role of tyramine and octopamine under the control of aromatic amino acid carboxylase (AADC) and tyramine beta hydroxylase (TBH), respectively (P. D. Evans 1985; Peter D. Evans and Robb 1993; Thomas Roeder 2005; Verlinden et al. 2010). For the visual of dopamine, tyramine and octopamine production; please see **Figure 3.**

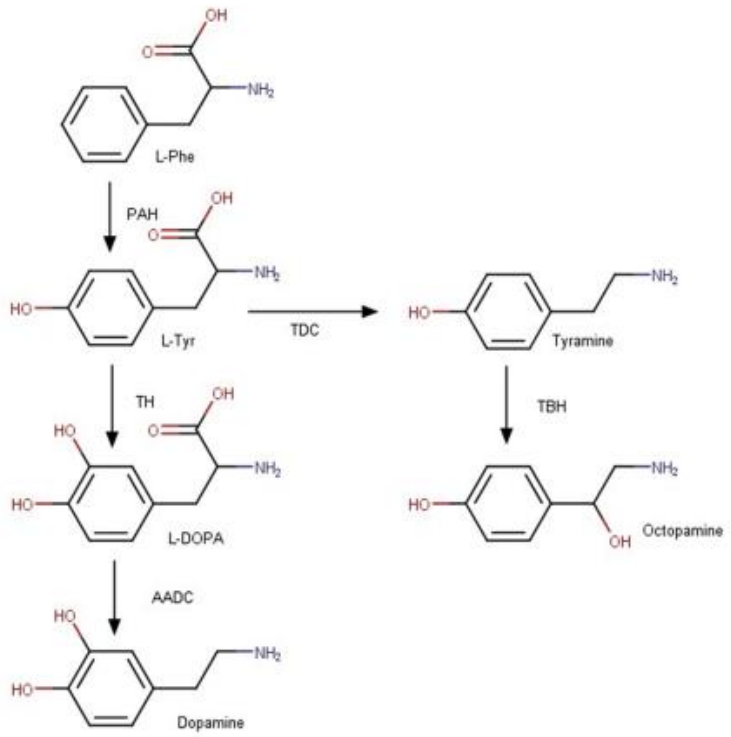


Figure 3: Tyrosine derived biogenic amine formations flow. L-Phe, phenylalanine; PAH, phenylalanine hydroxylase; L-Tyr, tyrosine; TH, tyrosine hydroxylase; L-DOPA, 3,4-dihydroxyphenylalanine; AADC, aromatic amino acid decarboxylase; TDC, tyrosine decarboxylase; TBH, tyramine beta hydroxylase (Verlinden 2018)**.**

Dopaminergic neurons in insects synthesize and release dopamine. Then the dopamine is converted into other compounds via different subsequent signaling pathways in postsynaptic cells (K. H. Wang, Penmatsa, and Gouaux 2015). Special parts of mushroom bodies called protocerebral anterior medial (PAM) and protocerebral posterior lateral (PPL) dopaminergic neuron-clusters have important roles in learning and motivation in *Drosophila melanogaster* (Mao and Davis 2009). Additionally, specific neurons in before-mentioned clusters also intervenes aversive and appetitive reinforcement such as different sugar and toxin-containing treatments (Yamagata et al. 2016).

The amount of energy storage is directly related to neuropeptide release, so that it either promotes or prevents nutrient uptake based on need (Marella, Mann, and Scott 2012). Dopaminergic neurons in mushroom bodies mediate the response thresholds against flavors (Masek and Keene 2016). Multiple research groups have demonstrated that octopamine released from a subgroup of PAM dopaminergic neurons have a significant role in short-term appetitive learning and octopamine-dependent memory in different

insect species (Mizunami and Matsumoto 2017; Owald and Waddell 2015; Perry, Baciadonna, and Chittka 2016; Schwaerzel, Monastrioti, and Scholz 2003).

1.7.Serotonin and Its Role in Insect Behaviors

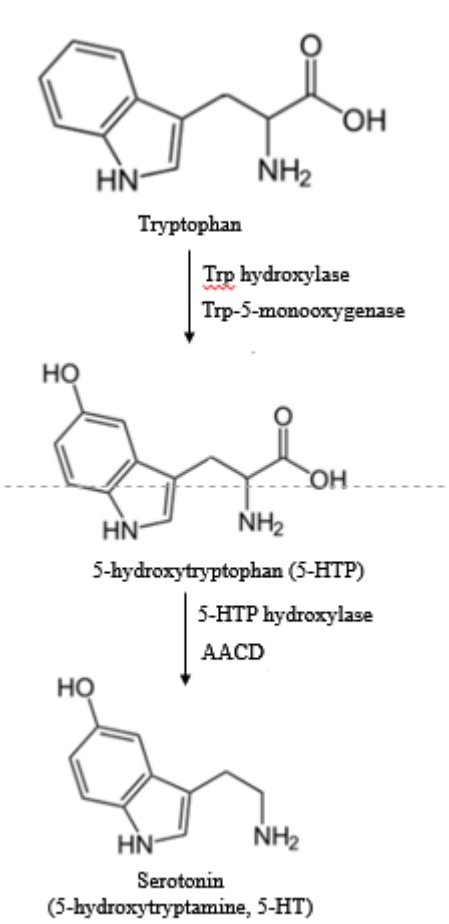


Figure 4: Biosynthesis of serotonin. Modified from (Vleugels, Verlinden, and Broeck 2015)

Serotonin, also known as 5-hydroxytryptamine (5-HT) is a tryptophan-derived biogenic amine neurotransmitter (Vleugels, Verlinden, and Broeck 2015). Serotonin is a monoamine, that has an aromatic core and a basic amine group bounded by an aliphatic chain. Biosynthesis of serotonin starts from tryptophan (Trp), that is converted into 5-hydroxytryptophan (5-HTP) by the enzyme called tryptophan hydroxylase (TPH) or so-called tryptophan-5-monooxygenase. Then 5-HTP substrate is catalyzed by 5-HTP carboxylase that resulted in 5-hydroxytryptamine (5-HT) also known as serotonin. 5-HTP carboxylase is the same enzyme as dopamine decarboxylase, which is AACD (Roberts and Fitzpatrick 2013; Kim-Ha, Smith, and Macdonald 1991).

All organisms that has CNS, serotonin is present as an intracellular signal. Also, in many insects, serotonin is an effective biogenic amine. In *D. melanogaster*, increased serotonin levels are directly proportioned with sleep duration (Yuan, Joiner, and Sehgal 2006). In addition to sleep cycle regulation, it also has important roles in appetite regulation in fruit fly. It has been demonstrated that declined serotonin levels resulted as an incline in appetite (Neckameyer 2010). Additionally, serotonin has significant effects on learning and memory, thus it regulates multiple insect behaviors in *Drosophila*. Inhibition in serotonergic pathways in fruit fly neurons lead to reduced appetitive

olfactory memory formation (Sitaraman et al. 2012). Another study has demonstrated that, decreased serotonin levels in fly brain results in reduction in memory formation under different temperature-dependent behavior tests (Sitaraman et al. 2008).

In *Apis mellifera*, several local serotonin injections resulted in different responses. For example, serotonin injection to the brain caused feeding inhibition, gut injection resulted in muscle contraction excitation. However, elevated serotonin level in honeybee hemolymph does not affect appetite (French et al. 2014). Another honeybee study suggested that, memory storage and retrieval has been decreased in pre-conditioning serotonin injected bees (Gerd Bicker and Menzel 1989; Menzel et al. 1999; Mercer and Menzel 1982).

2. AIM

We first aim to establish a connection between fluctuating hemolymph sugar levels, biogenic amines in the bee brain, and appetite levels of the honeybee. To accomplish this, we quantified the 4 major biogenic amines: octopamine, dopamine, serotonin and tyramine with high performance liquid chromatography (HPLC) from bees that were injected with sugars and enzyme inhibitors to manipulate their hemolymph sugar levels that then had their appetite levels measured by proboscis extension response (PER) assay. We assessed the physiological relationships across three age classes of the bee: newly emerged (<24h), nurse (1 week), and forager (4 weeks) bees. We also aim to map the distribution of the recently characterized octopamine beta receptor subtype 2 (OaβR2) in the honey bee forager brain by using immunohistochemistry methods and by doing so, gain a better understanding of its possible functions based on its spatial location in the brain. More specifically, information will also be used to locate injection sites for the knockdown of octopamine beta receptors so we can elucidate the cause and effect relationships between hemolymph sugar levels and the neurological regulation mechanisms of appetite control as the bee ages. For example, the honeybee may use fluctuating trehalose levels as an appetite regulatory signal via the octopamine neurohormone, independent of the glucose-insulin pathway. In this study, a custom-made primary peptide antibody with conjugated fluorescence protein was used. This will serve as a model for the development and staining of the three other octopamine beta receptor subtypes located in the honeybee brain. For counterstaining, the cell nuclei and the cytoskeleton will be stained with DAPI and phalloidin, respectively. Our main target in the honeybee brain is the mushroom body, which is a densely packed neuronal region in the insect brain and is responsible for processing olfaction-appetitive reinforced tasks.

3. MATERIALS AND METHODS

3.1.High Performance Liquid Chromatography and Sample Preparation

3.1.1. Sample Preparation of Freeze-dissected Honey Bee Brains

A 100 pg/µl of each synephrine (internal standard for serotonin and dopamine) and N-methylserotonin oxalate (internal standard for octopamine and tyramine) were prepared and added to a 0.2 M perchloric acid solution. Biogenic amine sample preparation was carried out according to methods found in literature (Mayack et al. 2019). A total of 50 µl 0.2 M perchloric acid solution was added to each frozen brain sample in 1.5 ml microcentrifuge tubes. The samples were thawed on ice for 30 s. Then the sample mixture was macerated using a sterile pestle. Samples were sonicated in ice water for 5 min. After sonication, the samples were placed on ice for 20 min and then centrifuged (Eppendorf, Germany) at 4˚C for 10 mins at 15,000 g. The supernatant of each sample was transferred to a spring loaded 100 µl glass insert placed inside a dark amber 1.5 ml glass microvial for HPLC analysis.

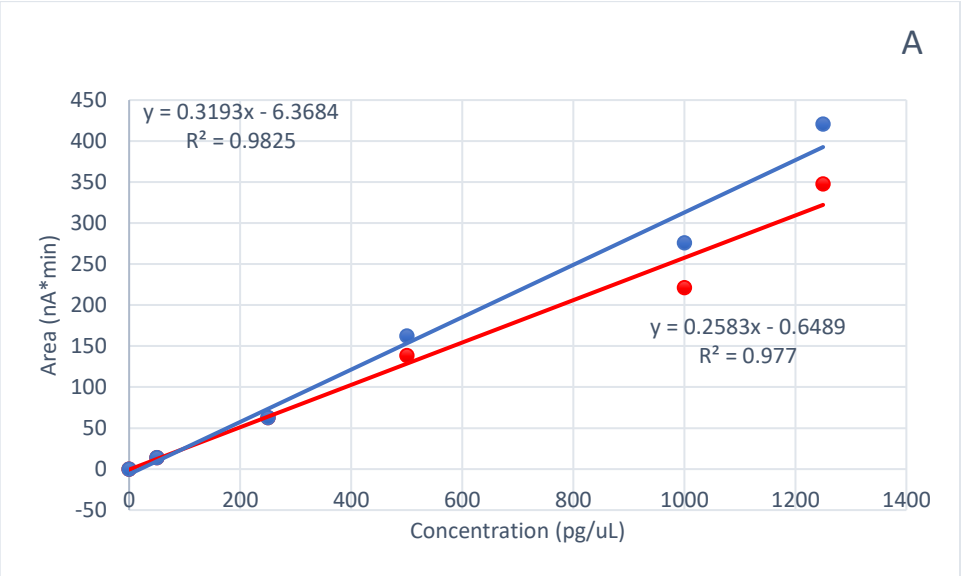
Table 1: Sample groups of appetite regulation HPLC experiment

	Newly Emerged	**Nurse**	**Forager**
Control	39	40	53
10% Sorbose	49	38	41
1.5 M Trehalose	50	40	45
Total	**138**	**118**	**139**

3.4.2. High Performance Liquid Chromatography

Table 2. External Standard Mixtures (ng/µl)

	Dopamine	**Serotonin**	**Octopamine**	**Tyramine**
A	800	0	50	200
B	400	25	0	100
C	200	125	250	0
D	100	250	500	800
E	50	500	1000	400
F	0	625	1250	50


A
y = 0.3193x - 6.3684
R² = 0.9825
y = 0.2583x - 0.6489
R² = 0.977
Area (nA*min)
Concentration (pg/uL)
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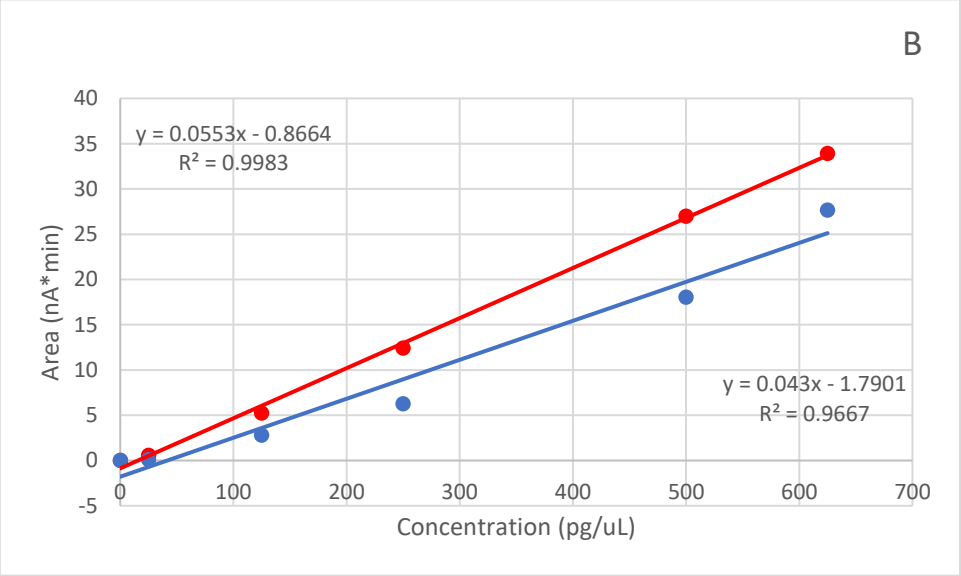
400
350
300
250
200
150
100
50
0
-50
0
200
400
600
800
1000
1200
1400


B
y = 0.0553x - 0.8664
R² = 0.9983
y = 0.043x - 1.7901
R² = 0.9667
Area (nA*min)
Concentration (pg/uL)
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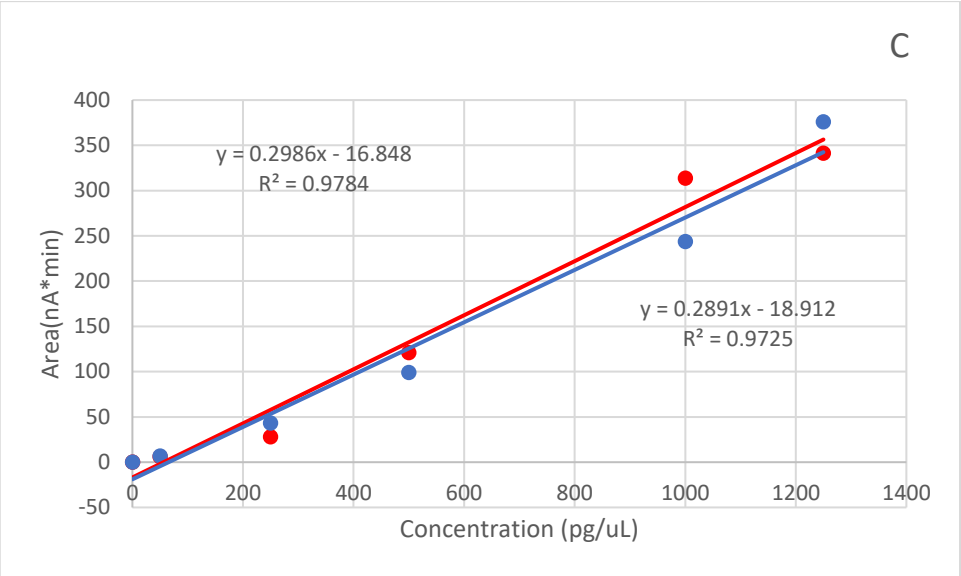
35
30
25
20
15
10
5
0
-5
0
100
200
300
400
500
600
700



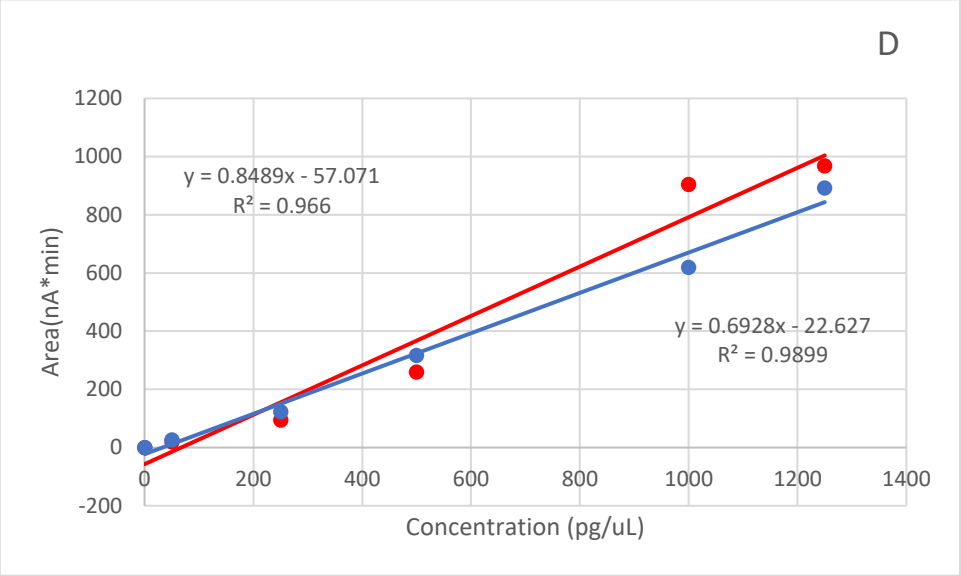


Figure 5: Standard curves of A)Dopamine B) Serotonin C) Octopamine D) Tyramine for HPLC analysis. Red and blue trendlines represent the pre- and post-runs to evaluate the degradation of biogenic amines. Area is converted into concentration based on the equations then the average results taken.

HPLC analysis for quantification of biogenic amines was conducted using a Thermo Scientific Dionex Ultimate 3000 HPLC system consisting of a SR-3000 solvent rack, a refrigerated ISO-3100BM pump, a WPS-3000TBSL analytical autosampler maintained at 5^{o} C, an 150 mm x 2.1 mm high-efficiency C18 reverse phase Acclaim 120 column maintained at 40^{o} C, and a 4-channel 6011RS Ultranalytical Cell electrochemical detector. Only two channels were used for detection: one channel was set to 650 mV for octopamine and tyramine, while other one was set to 350 mV for dopamine and serotonin. Mobile phase was kept at 4^{o} C until used. The flow rate of the mobile phase was set to 0.2

ml/min and injection volume was set to 2 µl. Peaks were integrated using the Chromeleon 7.2.10 software and quantities were calculated in pg on a per brain basis. The samples were run in batches of 24 that were randomized, while external standards were run before and after each run. A standard curve was constructed for the quantification of each biogenic amine. All biogenic amine external and internal standards were purchased from Sigma-Aldrich.

3.2.RNA Isolation for qPCR Analysis for Insulin-Like Protein (ILP) 1 and ILP-2

For the qPCR analysis, RNA isolation was performed using a One Step RNA Reagent (Bio Basic Inc) followed by purification from EZ-10 Spin Column Total RNA Mini-preps Super Kit (Bio Basic Inc) from following the manufacturers' instructions.

Briefly, bee bodies (approximately 10 mg) were placed in 1.5 ml microcentrifuge tubes. For each 10 mg, 1 ml of One Step RNA reagent was added. Then the bee body-reagent mixture was macerated with sterile pestle. Tubes were centrifuged at12,000 g for 10 min at 4^{o} C. Homogenized samples were incubated at room temperature for 5 min. Per 1 ml of solution, 200 µl of chloroform was added, and the tubes were shaken by hand for 15 s, these were then incubated at room temperature for 3 min. Following this step, the samples were centrifuged at 12,000 g at 4^{o} C for 15 min. The aqueous phase supernatant was taken and added to new 1.5 ml microcentrifuge tube for further isolation following the instructions in the EZ-10 Spin Column Total RNA Mini-preps Super Kit.

For each 100 mg of sample, 450 µl of RLT solution was added and the microcentrifuge tube was vortexed for 10 s. A total of 330 µl of 100% ethanol was added to the mixture and the sample was mixed gently. The final product was transferred into EZ-10 spin column in a 2 ml collection tube and centrifuged at 6,000 g for 1 min. Since our mixtures were high in volume, this step was performed twice. Flow-through was discarded, then 500 µl of RW solution was added on the column and centrifuged at 6,000 g for 1 min again. The flow-through was then discarded. A total of 500 µl of RPE solution was added onto the column and the tube was centrifuged at 6,000 g for 1 min. The flow-through was

discarded and the column was centrifuged again to get rid of residual buffer components. The column was then transferred into 1.5 ml sterile microcentrifuge tubes and 40 µl of RNAse-free water was added to the middle of the membrane. Samples were incubated at 50 ^{o} C for 2 min and then centrifuged at 8,000 g for 1 min. The eluted RNA's concentration and quality were measured with a ND1000 Nanodrop Spectrophotometer (Thermo Scientific, United States). Purified RNA samples were kept at -80 ^{o} C until needed.

3.3.cDNA Synthesis and Primer Validation

For the cDNA synthesis, a OneScript® cDNA Synthesis Kit with AccuRT Genomic DNA Removal Kit (ABM, Canada) was used according to the manufacturer instructions. Briefly, 2 µg of RNA template and 2 µl of 4X AccuRT reaction mix were mixed in a 0.2 mL microcentrifuge tube. The final volume was increased by 8 µl from adding nuclease free water. Samples were incubated at room temperature for 5 min. Then 2 µl of 5X AccuRT Reaction Stopper was added and the sample was mixed by gentle pipetting. When the samples were ready for synthesizing the first strand of cDNA, 1 µl 10 µM Random Primers and 1 µl of dNTP mix were added to the microcentrifuge tube and the final volume was increased by 14.5 µl from adding nuclease-free water. Tubes were then incubated at 65 ^{o} C for 5 min and then placed on ice for 1 min. After first strand was synthesized, 4 µl of 5X RT buffer, 0.5 µl RNAseOFF Ribonuclease Inhibitor and 1 µl of OneScript RTase was added to each sample and then incubated using a thermocycler set at 25 ^{o} C for 10 min,42^{o} C for 15 min and lastly 8 ^{o} C for 5 min. The final product was stored -20^{o} C until it was needed.

For the primer validation of ILP1 and ILP2 genes, conventional PCR was conducted using a ABM 2X PCR Taq Mastermix Kit (ABM,Canada). The protocol and thermocycler program settings are as follows:

Table 3: The components of PCR protocol

Component	Amount
cDNA Template	2 µl
Forward and Reverse Primers	1 µl of each
RNAse-free Water	8.5 µl
2X PCR Mastermix	12.5 µl
Final Volume	25 µl

Table 4: List of primers for gene expression analysis of *Apis mellifera* ILP-1 and ILP-2

Primer Name	Sequence
Apis mellifera ILP-2 Forward 1	GGTCGAACTTTGTCAAGTGCAT
Apis mellifera ILP-2 Reverse 1	TAGGAGCGCAACTCCTCTGT
Apis mellifera ILP-2 Forward 3	GGTCGAACTTTGTCAAGTGCATTA
Apis mellifera ILP-2 Reverse 3	TTAACGGGCACCGCAATAGG
Apis mellifera ILP-1 Forward 4	TGGTCGAACTTTGTCAAGTGCAT
Apis mellifera ILP-1 Reverse 4	GCAACTCCTCTGTCGTGCAA

Table 5: PCR cycles for primer validation

Step	Temperature (ºC)	Time (min)	Cycles
1	95	03:00	
2	95	00:30	x35
3	54	00:30	
4	72	01:30	
6	72	07:00	
7	4	∞	

For the gel electrophoresis 2 g of agarose dissolved 100 ml 0.5 X TBE buffer to make a 2% agarose gel. The mixture was heated in the microwave until the agarose was dissolved completely. The mixture was cooled and the gel was made using a caste. Before loading each well, 5 µl of each PCR product was mixed with 1 µl of a 6x GelRed Pre Stain with 6X DNA Loading Buffer (Biotium, United States). A low molecular weight DNA ladder (New England Biolabs, USA) was used as a reference. The gel electrophoresis was conducted at 100 V for 45 - 90 min. The gels were visualized by UV light using a Biorad Gel Doc EZ (Biorad, USA).

3.4.Reverse Transcription PCR (RT-PCR) for ILP-1 and ILP-2 Gene Expression

For the insulin-like protein 1 and 2 expression analysis, RT-PCR technique was utilized. For this experiment Blastaq Green 2x PCR Kit (ABM, Canada) was used and the manufacturer's instructions were followed. The ingredients were mixed in 96-well plate (Axygen, United States) as follows:

Table 6: The components of RT-PCR protocol

Component	**Amount**
cDNA template	1 µL
Forward and reverse primers	0.6 µL of each
Green BlasTaq dye	5 µL
Nuclease-free water	2.8 µL
Total	10 µL

RT-PCR was conducted via Roche Lightcycler 480 II (Roche, Switzerland) and the program was set to 50 cycles, the protocol is as follows:

Table 7: RT-PCR cycles for ILP-1 and ILP-2 gene expression analysis

Temperature (˚C)	Ramp Rate	Time (min)
95	2.2	00:30
60	2.2	00:30
72	4.4	01:00

3.5.Antibody Design

The immunohistochemistry staining antibody was provided by GenScript®, USA. Since our goal was the mapping the distribution of octopamine beta receptor subtype 2 in mushroom bodies of the honeybee brain, the rabbit polyclonal antibody was produced against the 23 amino acids in the C-terminus of this receptor (See Table 1). The main reason we chose the C-terminus end of the protein was to ensure that we avoid a section that spans the transmembrane region of the receptor, which would not be exposed for the antibody to attach (Sinakevitch, Mustard, and Smith 2011). The specificity of the target sequence was validated by a NCBI protein BLAST search. The manufacturer stated that to eliminate the poor immunogenicity of the peptide, the peptide is conjugated with a keyhole limped hemocyanin (KLH) carrier protein. For the quality control and the purification of the antibody, high performance liquid chromatography (HPLC) was used. Additionally, the indirect enzyme-linked immunosorbent assay (ELISA) was conducted as antibody validation method. The antibody was conjugated with an Alexa Fluor 488 fluorescent protein for confocal microscope visualization.

Additionally, we attempted to find a minimum a 15 amino acids long sequence which is common in all 4 subtypes of the octopamine beta receptor, however, our Clustal Omega alignment (Dineen et al. 2011) demonstrated that the common motifs among the subtypes are not long and specific enough to cover our needs. After that, we decided to map the

Oaß receptors individually. Subtype 2 was chosen based on its highest expression level, demonstrating that it would be the most abundant in the honeybee brain.

Table 8: The amino acid sequence of Octopamine beta receptor subtype 2. The 23 amino acid long target of the antibody is highlighted.

***Apis mellifera* Octopamine Beta Receptor Subtype 2 Amino Acid Sequence**
MTTIVTSSESSEVVSSVDVTTLLNGISTEDGQLGTNASYSSEEKLSVPVTIVKG CVLGSIIVTAVFGNLLVMVSVMRHRKLRIITNYFVVSLALADMLVAMFAMTF NASVQLTGKWLFGYFMCDVWNSLDVYFSTSSILHLMCISVDRYWAIVKPLK YPIIMTRRLAAYMLLACWILPAFISFVPIFMGWYTTAENSMRRQNHPEICEFK VNKIYVIFSSSVSFWIPCTIMTLTYVAVFKEPNRQEKQMHSRMGSVMLLSHRP SKDLNNLNGELNSAGSSKTLTLNEISTNHLHTPTKDKNIMKMKREHKAARTL GIIMGTFILCWLPFFLWYVITTLCGESCPCPDVVIALLFWIGYTNSALNPLIYAY FNRDFREAFKNTLQCAFCSLCRREPSDLEALDFRRPSLRYDLV-

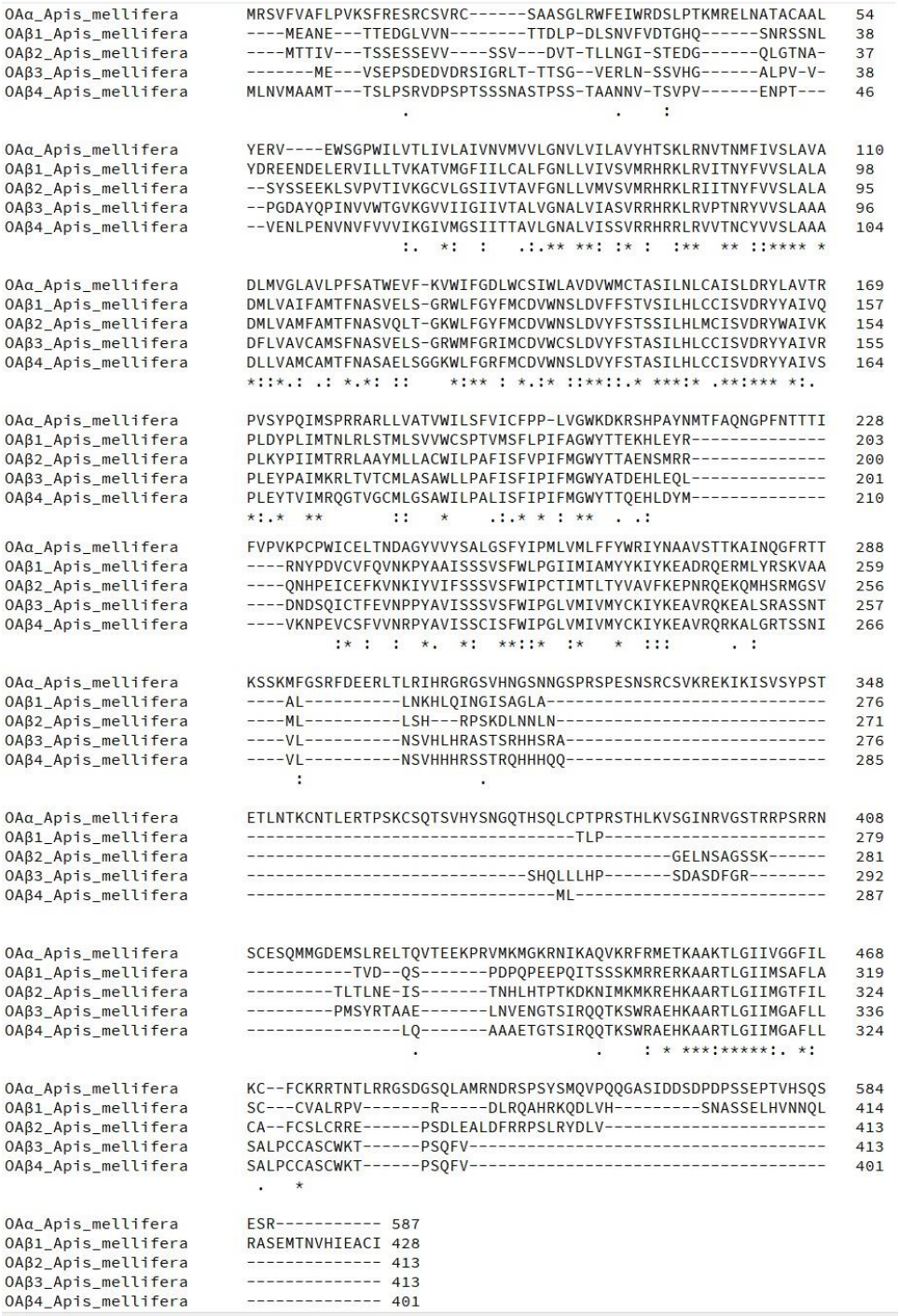


Figure 6. Multiple Sequence Alignment of Octopamine Alpha Receptor (OAα) and the four Octopamine Beta Receptors (OAβ)subtypes of *Apis mellifera*. Asterisks (*), colons (:), periods (.) indicates fully conserved amino acid sequences, conservation between groups of strongly similar amino acid properties, and conservation between groups of weakly similar amino acid properties, respectively. The specificity of the C-terminus region of the OAβ2 can be observed. The figure is adapted from Clustal Omega (Dineen et al. 2011).

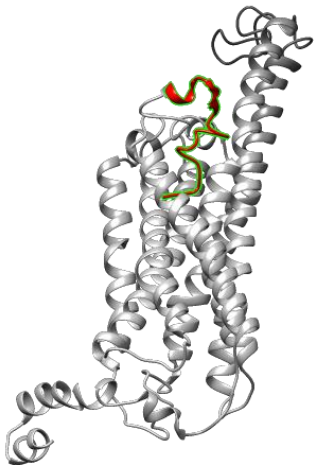


Figure 7. iTASSER-predicted (Roy, Kucukural, and Zhang 2010; Yang et al. 2014; Y. Zhang 2008) **3D structure of the octopamine beta receptor subtype 2.**The 23 amino acid target sequence at C terminus of the protein used for the antibody design is highlighted in red. Adapted from UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311 (Pettersen et al. 2004).

3.6.Solutions and Buffers

Cold physiological saline (PBS) was prepared by mixing 130 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 15 mM HEPES, 25 Mm glucose and 160 mM sucrose at a pH of 7.2.

For preparation of 4% paraformaldehyde (PFA) solution, 8 g of paraformaldehyde is weighed and mixed with double distilled water. The mixtures heated up to 60°C,1 M of NaOH was slowly added to the solution until it became clear in appearance. Then the solution was cooled to room temperature. Then, 20 ml of 10X PBS was added and the pH was adjusted to 7.4 by using 1 M HCl and NaOH. After the pH was adjusted, the mixture was brought up to a volume of 200 ml with double distilled water.

High performance liquid chromatography (HPLC) mobile phase was prepared by adding 75 mM sodium dihydrogen phosphate monohydrate, 1.7 mM 1-octanesulfonic acid sodium salt and 0.5 µL of 25µM Ethylenediaminetetraacetic acid (EDTA) tetrasodium tetrahydrate to 2L 10% acetonitrile-water (v:v) solution. The pH of the mobile phase was

titrated to 3.00 by using ortho-phosphoric acid. The final solution was then filtered through a 0.2 µm pore sized filter paper under vacuum pressure into 1L glass bottles. The mobile phase was then sonificated at room temperature for 20 mins.

A total of 4 external standards (dopamine, tyramine, octopamine, serotonin) and 1 internal standard (N-methylserotonin oxalate) were dissolved in 0.2 M perchloric acid. Each standard was prepared in 10 ng/µL stocks. For HPLC sample prep solution, N-methylserotonin oxalate stock diluted was diluted to a concentration of 100 pg/µL.

Table 9: The online tools and softwares that were used for the study

Program/Website/ Software Name	**Company / Website**	**Purpose**
Chromeleon 7	Thermo Fisher	Visualization and extraction of HPLC Data
iTASSER	https://zhanglab.dcmb.med.umich.edu/I-TASSER/	Protein 3D structure prediction
PHYRE 2	http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index	Protein 3D structure prediction
NCBI BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi	Local sequence alignment tool
Primer BLAST	https://www.ncbi.nlm.nih.gov/tools/primer-blast/	Primer design
Clustal Omega	https://www.ebi.ac.uk/Tools/msa/clustalo/	Multiple sequence alignment
UCSF Chimera	https://www.cgl.ucsf.edu/chimera/	Protein 3D structure

		visualizatio n
R	https://www.r-project.org/	Statistical Data analysis

3.7.Immunohistochemistry Staining of Octopamine Beta Receptor Subtype 2

Honeybee brains were then freshly dissected under a Zeiss (Oberkochen, Germany) dissecting microscope and for fixation incubated in 4% paraformaldehyde (PFA), at 4^{o} C, overnight. Then the brains were sectioned with a 10 µm thickness using a Thermo Fisher (Massachusetts, United States) NX50 Cryostar Cryotome that set at -20˚C. The different PBS-TritonX-100 (PBST) concentrations were used ranging from 0.1-1.0% for permeabilization, tissues were incubated in PBST for 60 mins at room temperature. This step was followed by incubation at 4% normal goat serum (NGS) in 0.2% PBST for 30 mins. Primary antibody dilutions were made from 1:50-1:100 with 0.2-0.5% PBST and tested. Invitrogen Alexa Fluor 488 anti-rabbit secondary antibody (Invitrogen, Thermo, United States) were used with either 1:250 and 1:500 dilutions. Tissues were incubated in primary antibody for ranging from overnight to 3 nights at 4˚ C, then with secondary antibody for 60 mins at room temperature. All tissues were washed 3 times with 1X PBS for 15 mins each. As counterstaining, 5 µg/ml 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) (Abcam,United Kingdom) along with 1U of Alexa Fluor 568 Phalloidin (Thermo Fisher, United States) to stain cell nuclei and f-actin respectively.

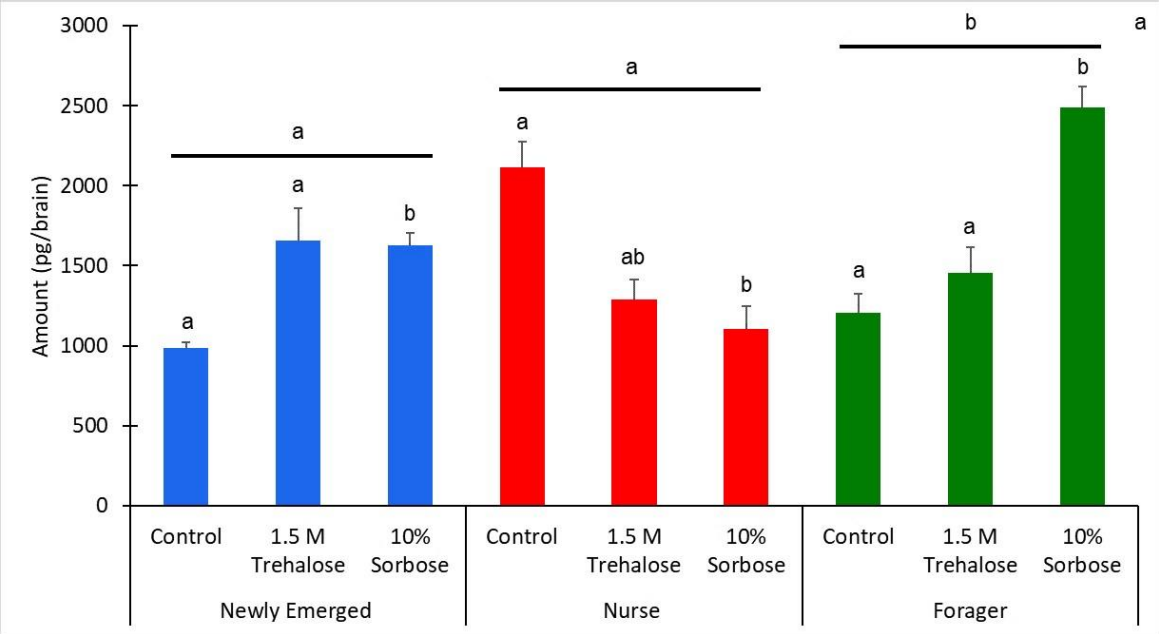
4. RESULTS

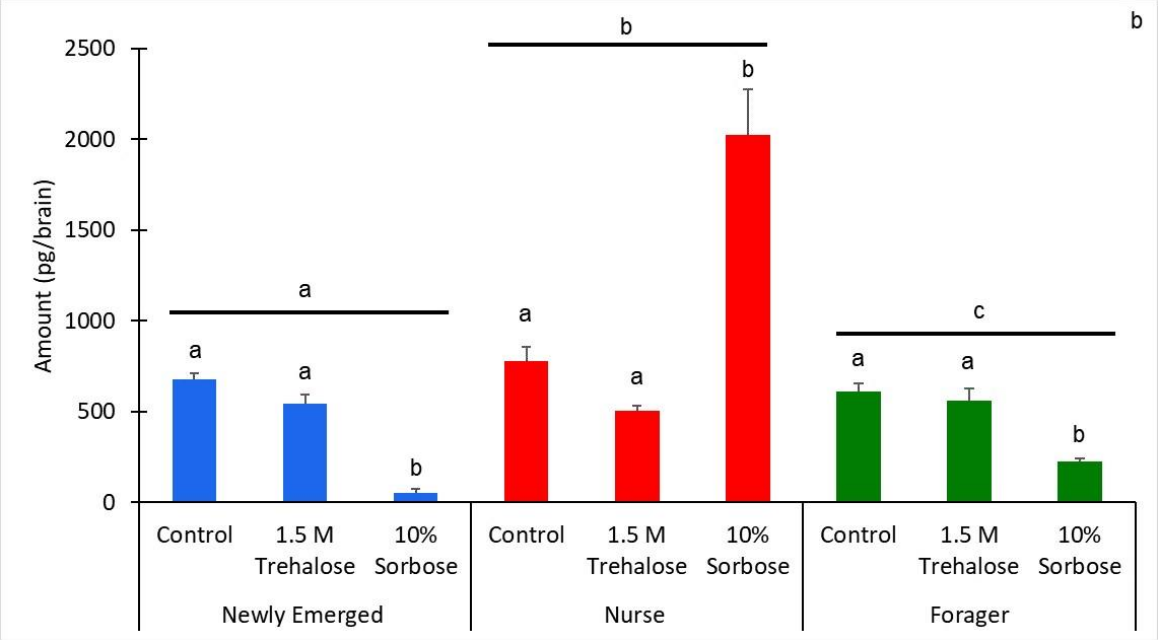
4.1.Appetite Regulation Experiment

The effect of the sugar injection treatment on the bees in relation to appetite is dependent upon age (Two-way ANOVA: $F_{10,745} = 14.08$, $P < 0.001$). Overall, there were significantly higher appetite levels found with forager bees in comparison to newly emerged and nurse bees. Surprisingly, in comparison to the control, newly emerged bees injected with fucose and sorbose had significantly lower appetite levels, while foragers had a significantly higher appetite level. There was no significant difference in appetite among the nurse bees from any sugar injection treatment ($F_{2,745} = 11.75$, $P < 0.001$; Appendix A, Figure S1).

The biogenic amine levels are age dependent (Kruskal Walls test: Octopamine $\chi^2_{2,398} = 15.18$, $P < 0.001$; Tyramine: $\chi^2_{2,398} = 24.47$, $P < 0.001$; Dopamine: $\chi^2_{2,398} = 95.23$, $P < 0.001$; Serotonin: $\chi^2_{2,398} = 129.27$, $P < 0.001$). The 10% sorbose sugar injections caused a significant increase in octopamine levels in the newly emerged and forager bees, but a significant decrease in nurse bees ($\chi^2_{2,398} = 27.78$, $P < 0.001$; $\chi^2_{2,398} = 33.70$, $P < 0.001$; $\chi^2_{2,398} = 21.73$, $P < 0.001$; Figure 2a). The opposite trend was found for tyramine where there was a decrease in newly emerged and forager bees injected with 10% sorbose, but an increase in nurse bees ($\chi^2_{2,398} = 65.84$, $P < 0.001$; Tyramine: $\chi^2_{2,398} = 28.98$, $P < 0.001$; Tyramine $\chi^2_{2,398} = 8.12$, $P = 0.02$, Figure 2b). There was a significant decrease in dopamine levels in newly emerged bees injected with 1.5 M trehalose ($\chi^2_{2,398} = 41.75$, $P < 0.001$), but an increase forager bees ($\chi^2_{2,398} = 19.18$, $P < 0.001$; Figure 2c). Interestingly, only in nurse bees was there an increase in dopamine from the 1.5 M trehalose injection, but an even higher increase in dopamine levels from the 10% sorbose injection ($\chi^2_{2,398} = 61.72$, $P < 0.001$; Fig 2c). In newly emerged bees there was a significant increase in serotonin levels for 1.5 M trehalose injected bees, while there was a decrease in the 10% sorbose injected bees ($\chi^2_{2,398} = 47.04$, $P < 0.001$). For nurse bees, there was a significant increase only with the 10% sorbose injected bees ($\chi^2_{2,398} = 20.64$, $P < 0.001$). In forager bees there

was a significant decrease in the 1.5 M trehalose injected bees ($\chi^2_{2,398} = 29.21$, $P < 0.001$; Fig. 2d).





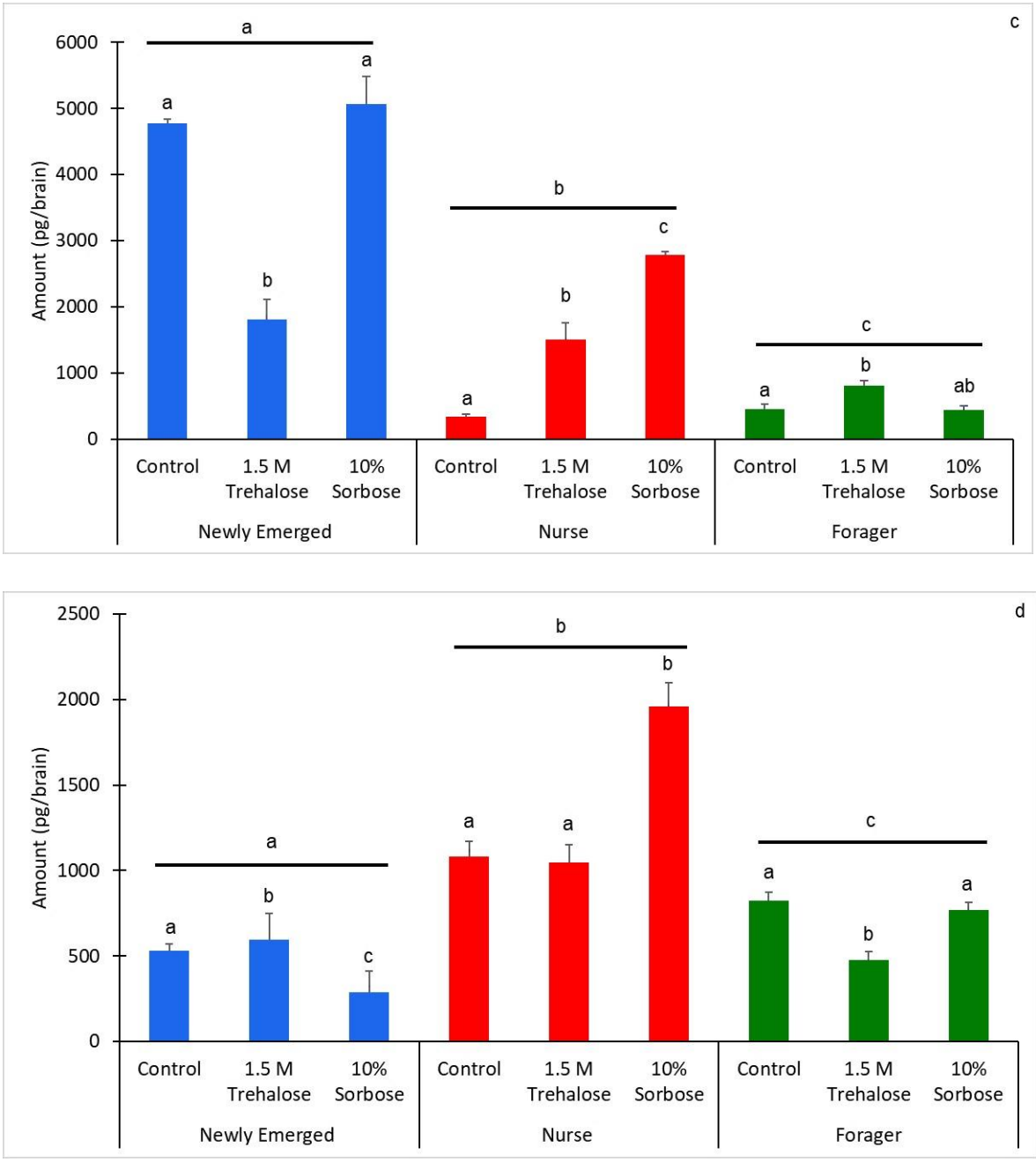


Figure 8: The biogenic amine levels on a per brain basis of (a) Octopamine, (b) Tyramine, (c) Dopamine, and (d) Serotonin, across the three honey bee age classes: newly emerged (blue), nurse (red), and forager bees (green). The bars represent means while the error bars represent standard errors. The letters above each bar represent significant differences across the treatments, while the different letters above the lines

represent significant differences across the age classes at the alpha = 0.05 level)

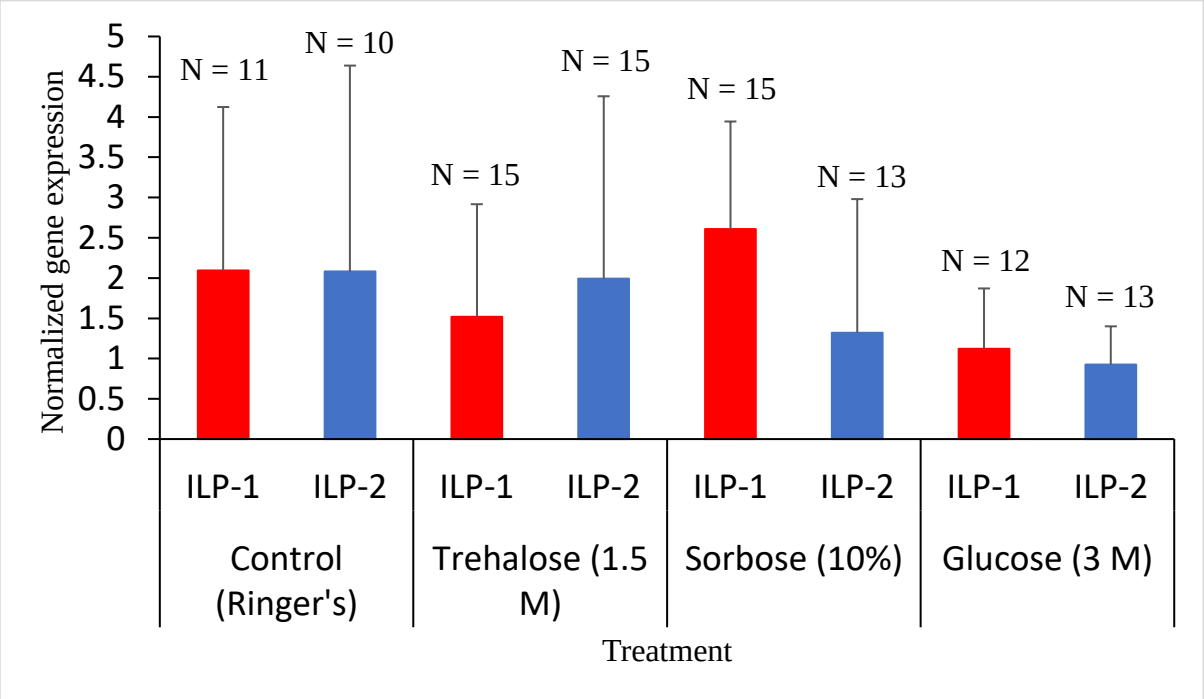


Figure 9. Gene expression analysis of Insulin Like Protein 1 (ILP-1) and ILP-2. Each bar represents the normalized gene expression means of either ILP-1 (red) or ILP-2 (blue), while the error bars represent the standard deviation. The sample sizes of each treatment are indicated above each error bar.

The qPCR gene expression analysis of ILP-1 and 2 indicates there were no significant differences in response to a 1.5 M trehalose, 3M glucose or a 10% sorbose injection (Kruskal Wallis test by treatment: $\chi^2_{3,103} = 1.18$, P = 0.76). There was also no significant differences of gene expression between ILP-1 and ILP-2 across the treatments (Kruskal Wallis test by gene target $\chi^2_{1,103} = 0.75$, P = 0.39 ; Fig 8).

4.2.Immunohistochemistry Staining Experiment

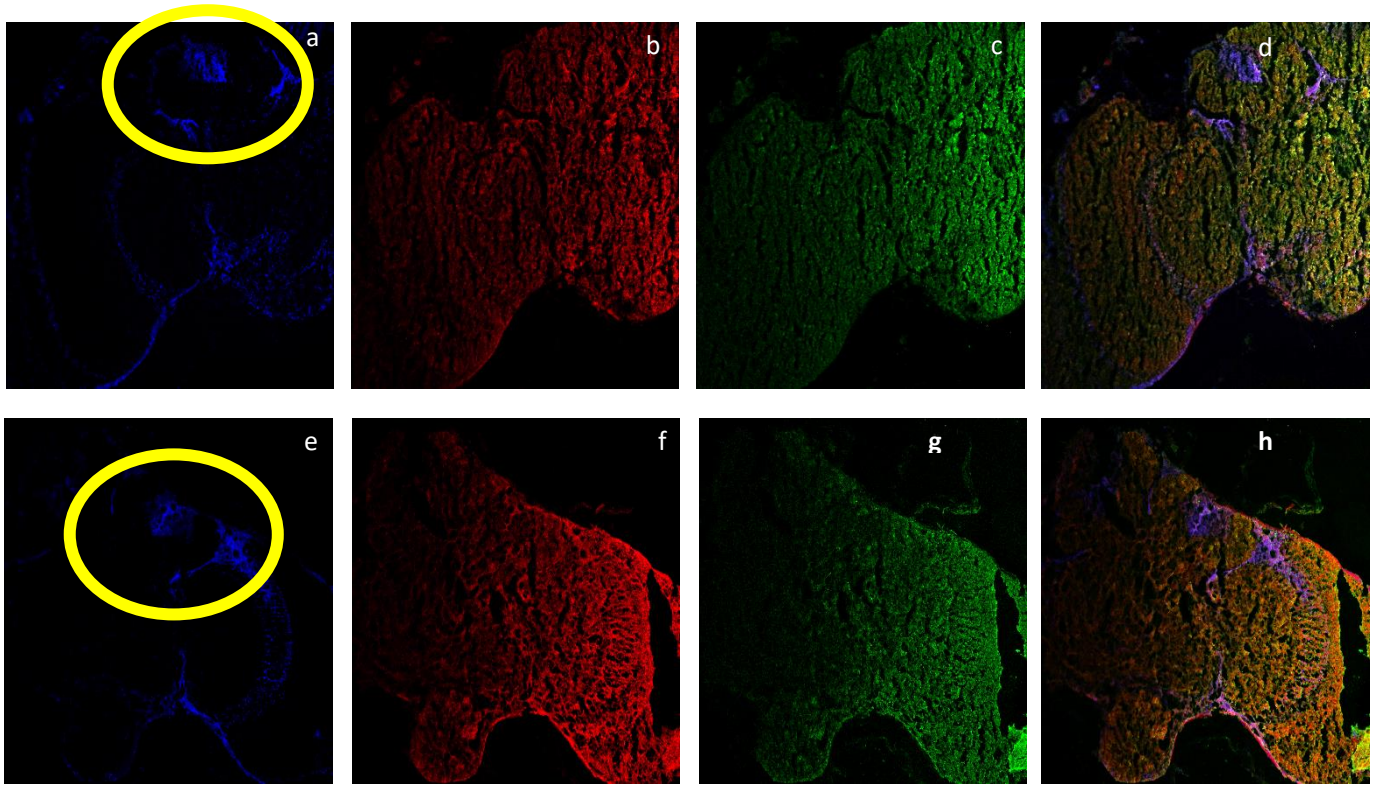


Figure 10: Troubleshooting confocal images of octopamine beta receptor subtype 2 with secondary antibody under 10X magnification. 10 µm of brain sections were stained with DAPI (blue), phalloidin (red) and goat anti-rabbit secondary antibody (green). Blue, red and green represents cell nuclei, f-actin and octopamine beta receptor subtype 2, respectively.

As a troubleshooting step to validate our custom-made antibody, we have conducted western blot analysis (Appendix A, Figure 2S) with the supplies and protocol that have been provided by KutluLab. We expected to see the our band-of-interest between 35-55 kDa bands because our receptor was expected to be 42.2 kDa (Balfanz et al. 2005). On analysis, we were able to see the band, however the color intensity was quite low. Also, we have seen 3 additional bands which might be either due to polymerization of proteins (130-250 kDa), or the cleavage of our target protein during SDS-PAGE protocols. Based on our result, we have suspected that our custom-made anybody binds to our target, but the affinity is low. For western blot experiment, mammalian anti-α-actin antibody was used as positive control, but bands have not showed up because the proteins were isolated from honeybee brain. We have interpreted that the mammalian actin and insect actin is structurally different.

.Figure 9 represents the final confocal images of our IHC staining troubleshooting. Both images were obtained from 10 µm thick honeybee brain tissues. The sectioning was done at -20° C using a cryotome. Sections were incubated in 1% PBST solution for 60 min to carry out the permeabilization process. The cross-section was then incubated in 1:50 diluted rabbit primary anti-OaβR2 antibody in 0.5% PBST overnight at 4 ˚C on Gyrorocker SSL3 shaker-rotator (Stuart, United Kingdom). The main difference between top and bottom images is the dilution ratio of Alexa Fluor 488 conjugated goat antirabbit secondary antibody which are 1:250 and 1:500 respectively. DAPI staining indicates the neuron nuclei which are found in honeybee brain. The most densely packed blue color indicates the mushroom bodies (indicated with yellow circle). Phalloidin staining visualizes the f-actin structure, which can be found the most in neuron axons and dendrites. Phalloidin coloring is almost as expected, however sectioning marks are also visible due to the cryotome blade. Since the blade disrupts the neuropils due to the freezing temperatures and mechanical cutting, the fine scale neuron structures such as the terminal ends of the dendrites are barely visible. Alexa Fluor 488 conjugated secondary antibody represents the green coloring, which are in close proximity to f-actin staining and their density differs in lobula and medulla region of the brain.This suggest that the designated antibody is specifically targeting the octopamine beta receptor subtype 2, which is found in the pre and post synaptic areas of the neuron, but was not able to be confirmed due to the loss of details in the fine structures of the neuron from the cryotome cross-sectioning.

5. DISCUSSION

We found that the effects of hemolymph sugar levels on appetite levels is age dependent. Surprisingly, fucose caused a significant lowering of appetite in newly emerged bees but a significant increase in appetite for forager bees. Fucose is a non-metabolizable sugar (Beverin, Sheppard, and Park 1971) so we expected not to see any effect of this sugar on appetite, but we suspect that due to its similarity in structure to glucose that it may have acted as a competitive inhibitor of enzymes acting on glucose. The fact that there is the opposite effect for newly emerged versus forager bees requires further investigation. Nurse bees on the other hand did not respond with an appetite change to any of the hemolymph sugar injections and we suspect this is due to their increased ability to buffer against energetic stress due to their larger fat stores in comparison to newly emerged and forager age classes (Ament et al. 2011). Foragers showed the largest increases in appetite overall from the sugar injection treatments with the 10% sorbose treatment. The 10% sorbose treatment has been shown before to lower the trehalose levels in honeybees by acting as a trehalase p-synthase enzyme inhibitor (Blatt and Roces 2002).We show that this lowering of trehalose levels in the hemolymph causes an increase in appetite in the forager age class. The fact that forager honeybees rely upon trehalose to fuel their foraging trips and this is the sole sugar that is fluctuating during foraging trips (Blatt and Roces 2001), supports the notion that a lowering of this may cause increase in appetite levels, specifically in the forager versus newly emerged and nurse class of honey bees. Under starvation, all bee age classes show increased appetite levels, but the foragers are the most significant among all three. It was stated that the newly emerged bees are not very susceptible to sucrose responsiveness and the responsiveness increase as the bee ages (Scheiner, Page, and Erber 2001; Behrends and Scheiner 2009).Our findings support this notion, newly emerged and nurse bees show relatively lower appetite levels across all the sugar injection treatments with the most dramatic changes in appetite being found in forager age class. The same was found with starving honeybees across age classes, the forager bees demonstrated the largest changes in appetite based on satiation state (Mayack et al. 2019). Therefore, we suspect that the forager honeybees are most sensitive to changes in their energetic state as indicated by their hemolymph sugar levels, while newly emerged and nurse bees are likely to use other physiological mechanisms to maintain

their energetic homeostasis. For example, newly emerged and nurse bees have been shown to readily mobilize their fat stores in response to energetic stress, while foragers are particularly susceptible to starving to death because they contain little to no fat stores, but in comparison to have a higher metabolic rate (Ament et al. 2011; Blanchard et al. 2000).

Trehalose injected forager bees were supposed to show lower appetite levels, but there was no significant decrease in their appetite levels. We suspect that trehalose levels are only used to safeguard against starvation and that other mechanisms must be at work to indicate satiation and lower the appetite levels of the honeybee. Recently, it has been found in *Drosophila melanogaster* that octopamine, which we have linked to lower trehalose levels, causes an increase in appetite despite the flies being satiated (Youn et al. 2018). Taking these findings together trehalose levels appears to be one way in which the bee can signal for increased caloric intake due to intense activity which has been shown to lower trehalose levels in the hemolymph.

It has been demonstrated that decreased levels of trehalose in honey bee hemolymph is inversely correlated with octopamine levels in the brain that results in higher appetite levels (Mayack et al. 2019). We show that there is a cause and effect relationship, as the forager bees with lowered trehalose levels due to a 10% sorbose injection, caused an increase in octopamine levels in the bee brain that corresponds with an increase in appetite levels as well. Farooqui (2007) has stated that the octopamine injection into *Apis mellifera* thorax resulted in higher response to sucrose (Farooqui 2007; Menzel, Leboulle, and Eisenhardt 2006; Scheiner, Page, and Erber 2001). Additionally, we found that there was also a lowering of tyramine levels in the brain. Tyramine acts as a general antagonist to octopamine and is also the precursor of the octopamine (Thomas Roeder 2005). Therefore, the lowering of tyramine may be just as functionally important as an increase in octopamine to increase the appetite levels in the forager honeybees. The lowering of tyramine may also result instead from the rapid increase in octopamine within a 10-minute time span and result from the rapid conversion of tyramine from increased tyramine beta-hydroxylase activity. Interestingly, the forager bees treated with 1.5 M trehalose show a significant increase in dopamine and lowering of serotonin levels in bee brain. This suggests that high hemolymph trehalose levels are likely to elicit behavioral changes in relation to their energetic state, but these are independent of appetite regulation. We find that in the newly emerged and nurse bees there are effects on the biogenic amine levels

due to fluctuating trehalose levels, but none of these appear to have a connection to appetite regulation. This supports the notion that trehalose levels are likely to play a number of behavioral and physiological roles besides the appetite regulation (Thompson 2003) and highlights the fact that, at least in honeybees, appetite regulation needs to be considered in an age-dependent manner.

In vertebrates the glucose-insulin signaling pathway is known to play a role in appetite regulation (Morley and Levine 1985; Valassi, Scacchi, and Cavagnini 2008). In honey bees because the glucose levels remain stable at the expense of trehalose we suspected that they may not rely on the glucose-insulin signaling pathway to regulate appetite levels. In the fruit fly *Drosophila melanogaster* and the mosquito *Aedes aegypti*, insulin/insulin-like signaling influences the production of yolk proteins like vitellogenin (Vg) (Gulia-Nuss et al. 2011; Shen et al. 2007; Jia, Chen, and Riddle 2004; Richard et al. 2005) as well as juvenile hormone (JH) synthesis (Tu, Yin, and Tatar 2005) that in turn has metabolic regulator effects. Increasing JH levels in honeybees can increase appetite levels and accelerate the transition from nurse to forager bee. Previously, ILP-1 but not ILP-2 levels, have been shown to decrease in response to double knockdown of Vg and JH even though it is suspected to be upstream of these two master regulators. Corresponding to this it was shown that the bees with lowered ILP-1 levels had increased glucose and trehalose levels in the hemolymph suggesting that there may be a connection between ILP-1 signaling and the regulation of sugar levels in the hemolymph (Y. Wang et al. 2012). However, we found that neither increased glucose, increased trehalose or lowered trehalose levels have any influence on ILP-1 and ILP-2 gene expression. We therefore suspect that the functioning of the insulin-glucose signaling pathway of forager honeybees may differ in relation to appetite regulation from the well-studied mammalian one. In summary, we can interpret that the appetite regulation of *Apis mellifera* via decreased trehalose levels and increased octopamine is independent of the glucose-insulin signaling pathway in forager bees. We suspect that this may result is a more rapid and precise regulation of the appetite levels in forager bees as octopamine is responding directly to the amount of trehalose in the bee hemolymph, while the glucose levels are remaining relatively stable are therefore not as a reliable indicator of the energetic state in real time (Mayack and Naug 2010; 2013; Blatt and Roces 2001). This has implications for forager bees because they are known to fill their crop with just enough nectar in order to make it to a specific flower patch. Overloading with nectar is known to increase weight

of the forager and would reduce their flight energetic efficiency (Wolf and Schmid-Hempel 1989). If a forager bee on the other hand runs out of energy to sustain flight it is likely to starve out away from the hive (Mayack and Naug 2013), so precise regulation of appetite for forager bees when foraging would be advantages for both survival at the individual level and foraging efficiency at the colony level.

As a troubleshooting step to validate our custom-made antibody, we have conducted western blot analysis. We expected to see our band-of-interest between 35-55 kDa because our receptor was expected to be 42.2 kDa (Balfanz et al. 2005). On analysis, we were able to see the band, however the color intensity was quite low. Also, we have seen 3 additional bands which might be either due to polymerization of proteins (130-250 kDa), or the cleavage of our target protein during SDS-PAGE protocols. Based on our result, we have suspected that our custom-made anybody binds to our target but the affinity is low. Our primary antibody for the octopamine beta receptor subtype 2 was conjugated with 488 nm fluorescent protein, however secondary antibody was needed to be able to detect the signal with the confocal microscopy. An Alexa Fluor 488 conjugated secondary antibody was kindly provided by Kutlu Lab at Sabancı University Nanotechnology Research and Application Center, and we were able to detect our amplified signal. However, based on our results, we have concluded that the cryotome is not a sufficient sectioning tool because it disrupts the neuropils of the honeybee brain, preventing the confirmation of whether there is specific tagging of the proteins located in the membrane of the neurons. We suspect that the lower temperatures to freeze the brain tissue, required for making cross sections using the cryotome, is not suitable for our primary antibody as the freezing is known to affect the structure of proteins and change its conformation. We have ordered additional Alexa Fluor 488 conjugated secondary antibody to optimize secondary antibody detection, but the delivery has been delayed due to pandemic regulations. We are currently using a vibrating microtome at room temperature, and increasing the cross-section thickness, so that free-floating immunohistochemistry techniques can be applied to improving the signal to noise ratio for the mapping of the subtype 2 octopamine beta receptor.

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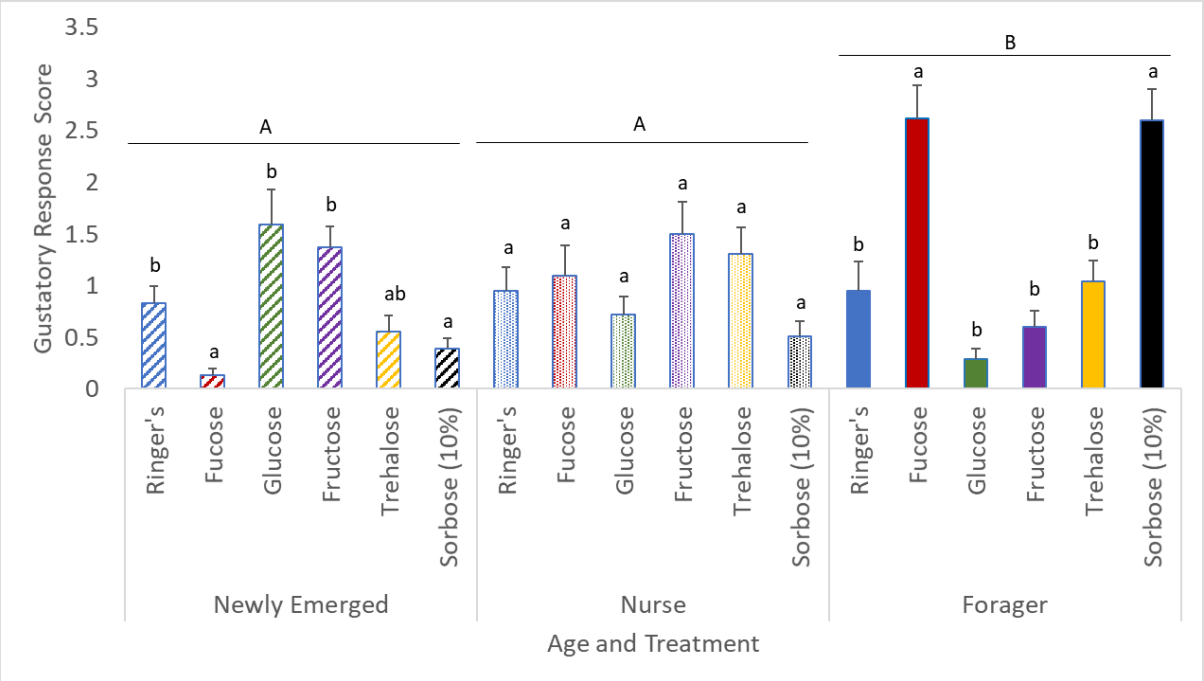
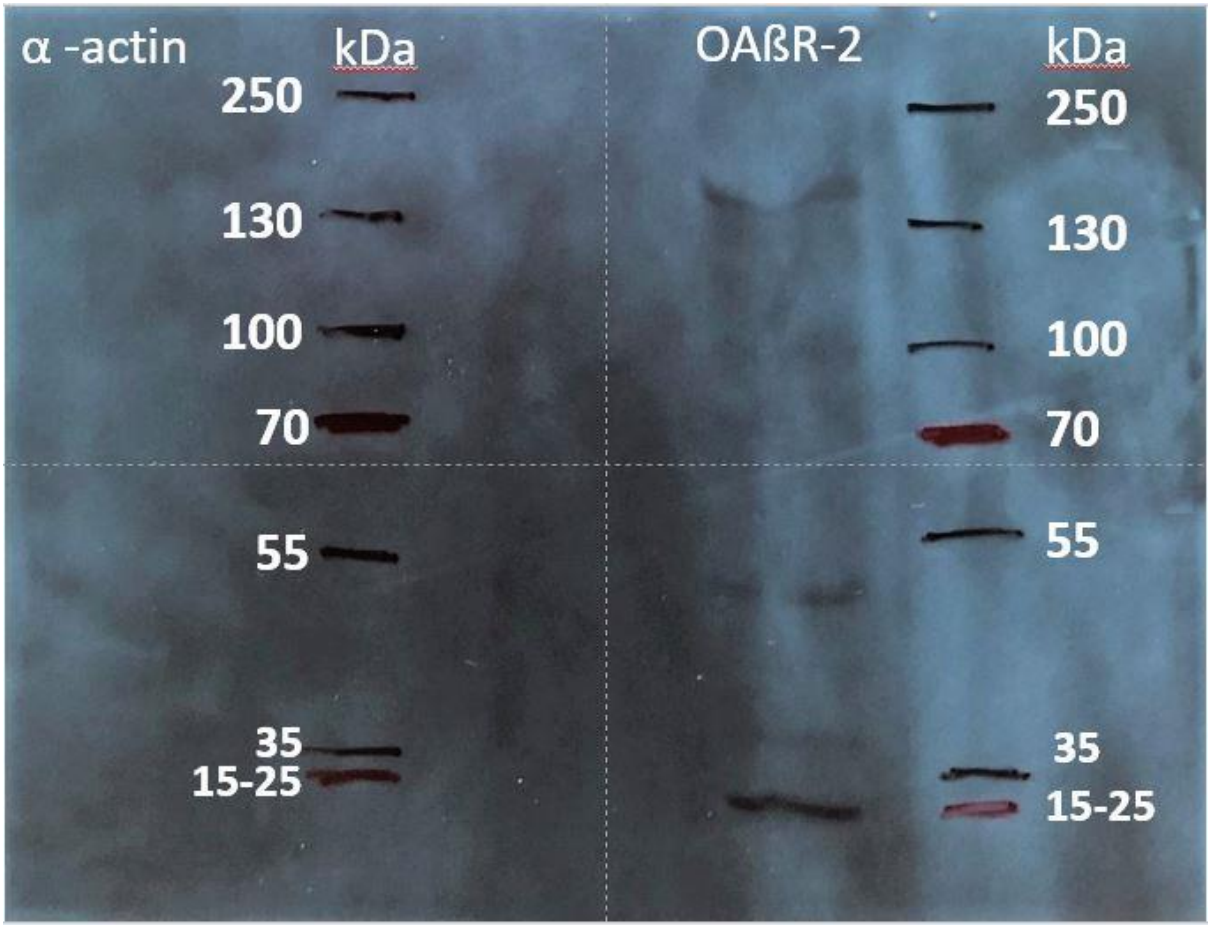
APPENDIX A



Supplementary Figure 1S. The gustatory response scores of sugar-injected newly emerged, nurse and forager bees. The bars represent the mean gustatory response score which is an index used to measure appetite based on the proboscis extension response assay, while the error bars represent standard error. The letters above each bar indicates significant differences within each age class while the letters above each line segment indicate the significant differences across the age classes of the bees at the alpha= 0.05 level.



Supplementary Figure 2S: Western blot results of anti-OaßR2 antibody. From right to left, positive control with anti-α-actin antibody, protein ladder, anti-OaßR antibody, protein ladder, respectively. Numbers indicate the molecule weights of the ladder.

APPENDIX B

Chemicals

1-Octanesulfonic Acid Sodium Salt	Sigma, Germany
Alexa Fluor 568 Phalloidin	Thermo Fisher, USA
DAPI	Abcam, UK
Dopamine	Sigma, Germany
EDTA Tetrasodium Tetrahydrate	Sigma, Germany
GelRed DNA Loading Dye, 6X	Biotium, USA
Glucose	Sigma, Turkey
HEPES	Sigma, Germany
Hydrochloric Acid	Sigma, Turkey
Magnessium Chloride	Sigma, Turkey
N-methylserotonin Oxalate	Sigma, Germany
Octopamine	Sigma, Germany
Paraformaldehyde	BioBasic, USA
Perchloric Acid	Sigma, Turkey
Potassium Chloride	Sigma, Turkey
Serotonin	Sigma, Germany
Sodium Dihydrogen Phosphate Monohydrate	Sigma, Germany
Sodium Hydroxide	Sigma, Turkey
Sodium Hydroxide	Sigma, Turkey
Sucrose	Sigma, Turkey
Synephrine	Sigma, Germany
Triton X-100	Sigma, Germany

Tyramine	Sigma, Germany

APPENDIX C

List of the Equipment

Autocleave	HiClave HV-110, Hirayama, Japan
	Priorclave, UK
Balance	Ohaus, Switzerland
Centrifuge	5425 Eppendorf, Germany
	5424R, Eppendorf, Germany
Column	Acclaim 120 C18, Thermo Fisher Scientific,USA
Cryotome	CryoStar NX50 Cryostat, Thermo Fisher Scientific, USA
Deep freeze	-80 ˚C, Forma ULT Freezer, Thermo Fisher Scientific, USA
HPLC System	HPLC Ultimate 3000, Thermo Fisher Scientific, USA
Ice Machine	AF20, Scotsman Inc., USA
Magnetic Stirrer	MSH-D, Witeg, Germany
Microinjector	Hamilton Syringe, Sigma Aldrich,USA
Microliter pipettes	Eppendorf, Germany
Microscope	LSM 710, Zeiss, Germany
Microwave oven	Arçelik, Turkey
pH Meter	Ohaus, Switzerland

Refrigerator	Bosch, Germany
Shaker-rotater	Gyrorocker SSL3, Stuart, UK
Solvent Filtration Pump	GM0.050b, Biobase, China
Spectrophotometer	NanoDrop 2000, Thermo Fisher Scientific, USA
Thermo Cycler	Proflex PCR System, ABM, USA
	Light Cycler 480 II, Roche, Switzerland
Ultrasonic Bath	Sonorex, Bandelin,Germany

APPENDIX D

Molecular Biology Kits

2X PCR Taq Mastermix Kit	ABM, Canada
EasyChrom Glutamate Assay Kit	BioAssay Systems, USA
EZ-10 Spin Column Total RNA Minipreps Super Kit	BioBasic Inc., Canada
OneScript cDNA with AccuRT Genomic DNA Removal Kit	ABM, Canada
One Step RNA Reagent Kit	BioBasic Inc., Canada