CRISPR-CAS9 KNOCKDOWN OF OCTOPAMINE BETA RECEPTOR SUBTYPE 2 TO
UNDERSTAND ITS ROLE IN HONEY BEE APPETITE REGULATION


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ABSTRACT

CRISPR-CAS9 KNOCKDOWN OF OCTOPAMINE BETA RECEPTOR SUBTYPE 2 TO UNDERSTAND ITS ROLE IN HONEY BEE APPETITE REGULATION

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Honey bee (*Apis mellifera*) foragers rely heavily on rapidly changing energetic demands during their foraging activities, which is reflected by the amount of trehalose that is in the hemolymph, and are particularly susceptible to stressors while foraging away from the hive. To meet their dynamic needs of energy while foraging, we suspect that these bees have a direct link between hemolymph trehalose levels and appetite regulation, mediated by octopamine levels in the brain. In order to establish a cause-and-effect relationship between octopamine and appetite regulation, we used CRISPR-Cas9 vectored by baculovirus, to knockdown the octopamine beta subtype 2 receptor in the brain, and then measured appetite levels after starvation, using the Proboscis Extension Response (PER) assay. We found that at three days post injection, there were significantly lower appetite levels and octopamine beta subtype 2 receptor gene expression, while there was higher GFP (Green Fluorescence Protein) signal in the brain. Taken together, our findings suggest that we successfully delivered the CRISPR-Cas9 system and knockdowned the octopamine beta subtype 2 receptor in the brain that is likely to be found in the Kenyon cells. In addition, we established that the octopamine beta subtype 2 receptor is involved in appetite regulation of the honey bee and this is likely to be independent of the glucose-signaling pathway found in vertebrates. With this proof of concept established, other targets for gene editing in adult honey bees are now possible using the CRISPR-Cas9 system.

# ÖZET

## OKTOPAMİNE BETA RESEPTÖR ALTTÜR 2'NİN BAL ARISINDA İŞTAHIN DÜZENLENMESİNDEKİ ROLÜ ANLAMAK İÇİN CRISPR-CAS9 SİSTEMİYLE NAKAVT EDİLMESİ

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Anahtar Kelimeler: Apis mellifera, OA, AmOctβ2, CRISPR/Cas9, bakulovirüs, PER, qRT-PCR, konfokal görüntüleme

Bal arısı (*Apis mellifera*) toplayıcıları, sırasında büyük ölçüde hızla değişen enerji ihtiyaçlarına dayanarak yiyecek arama faaliyetlerini yerine getirirler. Özellikle kovandan uzakta yiyecek ararken stres faktörlerine karşı hassas olmalarına sebep olan enerji durumu hemolenflerinde bulunan trehaloz seviyelerine bağlı olarak belirlenir. Toplayıcıların tozlaşma aktivitesi süresince değişen enerji seviyelerini, hemolenflerindeki trehaloz seviyeleri ve beyindeki oktopamin seviyeleri arasındaki direkt bağlantıyla iştah düzenlenmesi yoluyla dengelediklerini düşünüyoruz. Oktopamin ve iştahın düzenlenmesi arasındaki sebep-sonuç ilişkisini kurabilmek için toplayıcıların beyninde bakulovirüs vektörüyle Oktopamin Beta Reseptörü Alt Tip 2 (*AmOctβ2*) reseptörünün CRISPR (Düzenli Aralıklı Palindromik Tekrar Kümeleri) sistemi kullanarak nakavt edilmesini amaçladık. Sonrasında arıların iştah seviyelerini Hortum Uzatma Tepkisi (PER) ile test ettik. Enjeksiyondan 3 gün sonra CRISPR enjekte edilen arıların iştah seviyelerinde ve Oktopamin Beta Reseptörü Alt Tip 2'nin gen ifadesinde kontrol gruplarına kıyasla istatistiksel olarak anlamlı bir düşüş ile birlikte CRISPR enjekte edilen arıların beyinlerindeki GFP (Yeşil Floresan Protein) sinyalinde artış gözlemledik. Çalışmanın sonuçları, CRISPR-Cas9 sisteminin başarılı bir şekilde beyindeki hücrelere ulaştığını, Kenyon hücrelerde de bulunma ihtimalinin yüksek olduğunu ve *AmOctβ2*'nin nakavt edildiğini destekler niteliktedir. Ayrıca, Oktopamin Beta Reseptörü Alt Tip 2 (*AmOctβ2*)'nin dahil olduğğu iştah düzenleme mekanizmasının omurgalılardan farklı olarak glukoz sinyalleşme yolağından bağımsız olduğunu tespit ettik. Bu metodun uygulanabilirliğinin kanıtlanmasıyla, CRISPR-Cas9 sistemi yetişkin bal arılarındaki diğer genlerin düzenlenmesi hedeflenebilir.

# ACKNOWLEDGEMENTS

*To my family…*

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| | |
|---|---|
| AcNPV | *Autographa californica* nucleopolyhedrovirus |
| AL | Antennal Lobe |
| AmOAβR1-4 3 and 4 | Apis Mellifera Octopamine Beta Receptor Subtypes 1, 2, |
| cAMP | Cyclic adenosine monophosphate |
| Cas 9 | CRISPR-Associated Protein 9 |
| cDNA | Complementary DNA |
| CRISPR Repeats | Clustered Regularly Interspaced Palindromic |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DNA | Deoxyribonucleic acid |
| DPI | Days Post Injection |
| DSB | Double Stranded Breaks |
| GABA | Gamma-aminobutyric acid |
| GFP | Green Fluorescent Protein |
| GPCR | G Protein-Coupled Receptor |
| GP64 | Baculovirus envelope protein |
| gRNA | Guide RNA |
| GRS | Gustatory Response Score |
| HDR | Homology-Directed Repair |
| ILP | Insulin-Like Peptide |
| MB | Mushroom Body |
| NHEJ | Non-Homologous End Joining |
| OA | Octopamine |

| | |
|---|---|
| OctαR | α-adrenergic like octopamine receptor |
| OctβR | β-adrenergic like octopamine receptor |
| PER | Proboscis Extension Response |
| qPCR | Quantitative Polymerase Chain Reaction |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| RNP | Ribonucleoprotein |

# 1. INTRODUCTION

## 1.1 Honey Bee Health

The western honey bee (*Apis mellifera*) is a social insect which provides pollination services for approximately 80% of the flowering plants and crops. Honey bees are also producers of honey, propolis, royal jelly and beeswax. One-third of the global food supply relies on the pollination activities of honey bees (Ye, et al. 2020). Therefore, we are highly dependent on honey bees for food security and our well-being. Turkey has a geographical advantage in terms of biodiversity as it bridges Asia and Europe continents, its ecosystem includes plants and valuable crops that are pollinated by honey bees. A total of 8 million beehives are recorded in Turkey and Turkey ranks second for honey production, which totals to approximately 100,000 tons. However, there is even more room for improvement in regard to honey production per hive, even for countries that are top honey producers such as Canada, Mainland China, Brazil, and the US (FAO, 2020). The lack of production is somewhat attributed to poor bee health. In addition, there is a global decline of honey bee health that is attributed to pesticide exposure, poor nutrition, habitat loss, climate change, and lack of genetic diversity (Wilfert et al., 2016; Dennis & Kemp, 2016). Many of these stressors tend to converge along metabolic pathways of the honey bee that can cause dysregulation of them, which is associated with a lack of energy and nutrition, along with the inability of the honey bee colony to survive (Mayack et al. 2022).

The forager class of honey bees provide energy and nutrients to the whole colony as well as the accumulation of bee products which humans benefit from. Foragers serve the hive by bringing in adequate nutrition in the form of pollen which is rich in proteins and fat, and nectar rich in simple carbohydrates. Forager bees are relatively higher in susceptibility to stressors such as temperature and parasites because of their foraging activity outside of the hive (Bordier et al., 2018). Moreover, honey bee foraging is one of the most energy demanding activities in the animal kingdom (Mayack et al., 2019) and foraging distances for honey bees may be as far as 7-9 kilometers away from hives (Couvillon et al., 2015). The flight muscles of honey bees may reach up to 42 °C to sustain flight while the ambient temperature is 12 °C (Stabentheiner & Kovac, 2016). Therefore, there is a need to maintain endothermy during their foraging trips and this thermoregulation results in high energy costs.

During flight, metabolic rates of flying insects can be 170 times higher compared to their resting state (Bartholomew & Casey, 1978). Consequently, maintaining energetic homeostasis for foragers in particular is challenging as their energetic needs are extremely dynamic. Honey bee flight is mainly fueled by carbohydrates and trehalose, because forager bees have a low amount of fat stores for better foraging efficiency. Therefore, to meet rapidly changing energetic demands there should be mechanisms for rapid responses in appetite regulation for foraging honey bees (Akulku et al 2021).

## 1.2 Appetite Regulation in Honey Bees

Appetite regulation is one of the main mechanisms involved in balancing energy metabolism (Hainerová & Lebl, 2010). Honey bees have different characteristics of energy storage compared to vertebrates, as they have a limited fat storage. This is possibly due to their much smaller size (Candy et al., 1997). Glucose, fructose, and trehalose sugars are found in the hemolymph of honey bees. Among these sugars, it is known that trehalose is the one which fluctuates depending on the energetic state of the honey bee (Mayack & Naug, 2013).
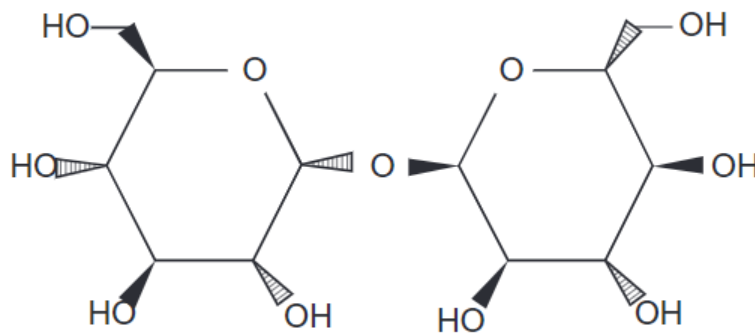


*Figure 14. Skeletal formula of trehalose Adapted from (Luyckx & Baudouin, 2011)*

Trehalose is made up of two glucose molecules (Luyckx & Baudouin, 2011). Even if honey bees feed on glucose, sucrose or fructose, energy is stored in the form of trehalose in their hemolymph. Dynamic changes in trehalose levels indicate changes in the hunger state of a honey bee. When honey bees are satiated, they have higher amounts of glucose and fructose in their hemolymph which leads to synthesis of trehalose (Blatt & Roces, 2001). In the case of hunger from starvation, trehalose is broken down into two glucose molecules to supply the cells their source of energy (Blatt & Roces, 2001). Therefore, lower trehalose levels in the

hemolymph of foragers are involved in increased appetite in honey bees. Appetite regulation has shown to be directed by increase in octopamine signaling in the honey bee brain in response to lowered trehalose levels in the hemolymph (Mayack et al. 2019) and this signaling pathway is thought to be independent of the glucose-insulin signaling pathway as ILP1 (Insulin like protein 1) and ILP2 (Insulin like protein 2) gene expressions showed no change in response to lowering trehalose levels in the hemolymph (Ghanem et al., In Review).

**1.2 Honey Bee Brain**

An adult honey bee brain comprises of three regions: the protocerebrum, deutocerebrum and tritocerebrum. The protocerebrum includes two optic lobes that are involved in the transfer of visual image information to the higher-order processing centers of the brain, which are known as the mushroom bodies and the central body. The deutocerebrum consists of antennal lobes, which have odorant receptors, and the tritocerebrum contains two lateral lobes, which provide connections to the labrum and digestive tract (Scheiner et al., 2006).

Foraging relies on improved learning and memory in order to associate the nectar and pollen rewards with the plants that they visit. This activity is also highly dependent on appetite regulation. Octopaminergic cells which extend throughout the brain, including the mushroom bodies, antennal lobes and subesophageal ganglion, are regions that are likely involved in appetite regulation. Cell bodies of an octopaminergic neuron type, the ventral unpaired medial, are in the subesophageal ganglion, which controls the mouth parts (Roeder, 2020), and these neurons receive input from sucrose sensitive receptors (Rein et al., 2013). There is also evidence of a subset of octopaminergic neurons called OA-VPM4 in *Drosophila* that promotes sucrose acceptance behavior documented from the Proboscis Extension Response (PER) assay (Youn et al., 2018).

Antennal lobes (AL) within the glomeruli region, serve as a connection region for a set of neurons that is found in the honey bee brain. The AL provides the connection of interneurons in the brain and sensory neurons. Octopamine activity was detected in the glomeruli of AL, which makes this region likely to be involved in appetite regulation in honey bees. Moreover, network models of the appetitive odor learning propose that the octopamine receptor AmOA1 is located in the inhibitory neurons, and they vary in density. In this model, octopamine

mediates the potentiation of each glomerulus to change the response to each odor and appetitive behavior, across different honey bees (Rein et al., 2013).

Mushroom body (MB) and calyx regions of the honey bee brain are also important in appetitive learning which are the higher processing units. Kenyon cells found in MB are involved in olfactory learning and memory (Roeder, 2020). Neurons in the MB of *Drosophila*, the octopamine receptor isoform OAMB, is active in the octopamine signaling of appetitive olfactory learning (Kim et al., 2013). Octopamine injection to the calyces substituted the sugar reinforcement in appetitive behavior, as measured by the proboscis extension reflex in honey bees (Hammer & Menzel, 1998). This indicates that the calyces are involved in octopamine signaling as well as appetitive behavior regulation.

Neuropils in the honey bee brain are composed of a million neurons. For better understanding of social behaviors directed by the connections of neurons in different brain regions, genetic engineering methodologies are needed (Kohno & Kubo, 2019; Leboulle et al., 2022). Deformed wing virus results in learning deficits and changes in behavior (Iqbal and Mueller, Proc Biol Sci 2007). Behavioral changes and malfunctioning of the brain regions due to diseases can be studied with regulation of gene expression and gene editing approaches. There have been previous attempts for development of vectors such as lentivirus plasmid to introduce modifications in these neurons (Leboulle et al., 2022). However, no viral vectors are found to be efficient in transfecting the honey bee neurons. In this study, we used the baculovirus as a viral vector for transfection of the neurons in the honey bee brain.

### 1.3.1 Honey Bees as A Model Organism For Neurobiology

Honey bees have a true organized brain, yet it is much simpler than the human brain. In addition, there have been numerous studies on honey bee physiology, neurobiology, and behavior to establish a basic foundation for future neurological studies (Chen et al., 2021; Hu et al., 2019; Kaya-Zeeb et al., 2022; Roth et al., 2019). Therefore, the honey bee is an amenable model organism for the development of genetic engineering tools that is required for understanding neurobiology and neurological disorders such as a dysregulation in appetite.

## 1.3 Biogenic Amines in The Honey Bee Brain

In insects, physiological responses to stress involve the biogenic amine dopamine as well as tyramine and octopamine catecholamines which are analogs of epinephrine and norepinephrine in vertebrates (Even et al., 2012; Pflüger et al., 2004). Biogenic amines are types of phenolamine and they act as a neurotransmitter and neurohormone. They bind to their receptors that belong to the G protein-coupled receptors superfamily (GPCRs). Following the binding of biogenic amines, biogenic amine receptors alter the concentration of intracellular Ca2+-($[Ca2+]i$) or 3′,5′-cyclic adenosine monophosphate ($[cAMP]i$) within the cell (Blenau et al., 2020). These transient changes led by secondary messengers are involved in the signaling pathways in the neurons which regulate behaviors (Balfanz et al., 2014).
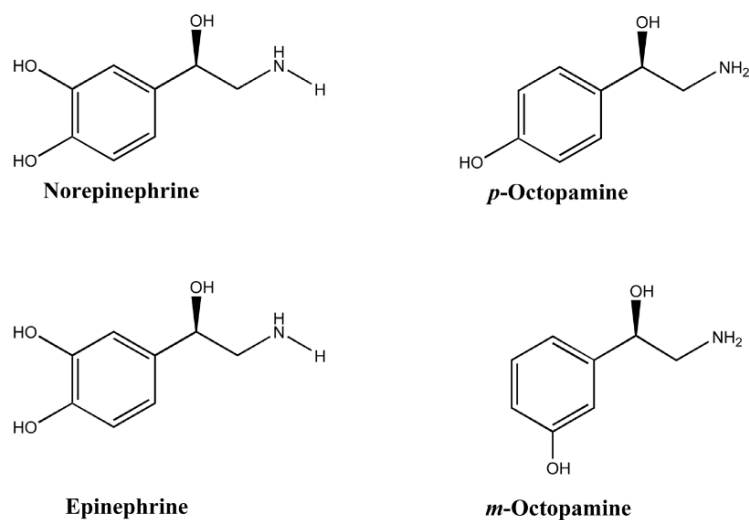


*Figure 15. Skeletal formula of biogenic amines octopamine Adapted from (Farooqui, 2007)*

Among biogenic amines, octopamine is a highly conserved regulatory hormone (Roeder et al., 2003) which elevates the $Ca^{2+}$-($[Ca^{2+}]_i$) or 3′,5′-cyclic adenosine monophosphate ($[cAMP]_i$) that is involved in modulation of neurological and physiological phenomena, such as neuromuscular transmission (Malamud et al., 1988), lipid and carbohydrate metabolism (Orchard et al. 1993), and responsiveness of sensory receptors (Scheiner et al., 2006). Octopamine acts as a neurochemical in flight muscles. In addition, octopaminergic neurons are expected to be clustered within the flight muscles (Kaya-Zeeb et al., 2022). Although the phenological effects of biogenic amines are known, metabolic pathways they are actively involved in and their functional link to appetite and energetic state are not well-studied.

A decrease in hemolymph trehalose levels causes an increase in octopamine neurotransmitter levels in the honey bee brains, which is also linked to an increase in appetite (Ghanem et al. In Review; Akülkü et al., 2021; Scheiner et al., 2002). However, there has yet to be a cause-and-effect relationship established between octopamine in the bee brain and whichever octopamine receptors are involved in the regulation of increased appetite levels of honey bee foragers. There is evidence that this appetite regulation pathway may have evolved independently of the glucose-signaling pathway found in vertebrates (Ghanem et al., In Review). Octopamine receptors are classified as α-types (alpha-adrenergic-like octopamine receptors) and β-types (beta-adrenergic-like octopamine receptors) and they are found to be similar in function to adrenergic receptors in vertebrates which sense analogs of octopamine and tyramine (catecholamines; adrenaline and noradrenaline) (Evans & Maqueira, 2005). Among these, 2 α-types: *AmOctαR1* and *AmOctα2R* are functionally characterized (Blenau et al., 2020; Sinakevitch et al., 2011). Both are involved in Ca2+ oscillations which is a common function with an orthologous receptor of *Drosophila melanogaster* (Balfanz et al., 2014; Blenau et al., 2020). Four of the *β*-type octopamine receptors were also characterized and similar to α-types, they show adenylyl cyclase activity in an octopamine-dependent fashion. Activity of *AmOctαR1* is validated on GABAergic neurons located in several regions of the honey bee brain, which are the mushroom bodies, the antennal lobes, and the central complex. These receptors exhibit a signaling role in inhibitory pathways in the olfactory learning and memory neuropils of the honey bee brain (Balfanz et al., 2014).

The *β*-types of the OA receptor (*AmOA1βR1-4*) have also been identified from the honeybee genome and *Drosophila* orthologs (Hauser et al., 2006). Heterologously expressed octopamine receptors *AmOAβR1-4* might have unique modulatory functions as they are activated in response to ranging concentrations of different agonists. In addition, expression of splice variants *AmOAβR3/4* is found to be changing in an age-dependent manner, and *AmOAαR1* is correlated with complex behaviors of social tasks independent of age (Reim and Scheiner, 2014). Relative abundance of these beta receptors was obtained using the RNA-Seq approach and according to the mRNA transcription profiles of 20 forager bees, *AmOA1-α*, *AmOAβ2R*s and *AmOAβ4R* are present in the honeybee brain, while the expression data of *AmOAβ3R* was low (Trapnell et al. 2010). This specific subtype has been studied in *Drosophila* and it is involved in appetite regulation in larvae (Zhang et al., 2013). Expression of *AmOAβ3R* might be high in larval state in the honey bees as well, but in the adult stage there is very little

expression of this subtype so the role in response to starvation might be switched to other subtypes after developmental stages are completed.

*AmOAβ2* was found to be expressed more than the other three beta receptor subtypes in the adult bee brain. *AmOARα1* and *AmOARβ2* are involved in thermoregulation through their activity in flight muscles. They are predominantly expressed in the flight muscles and are higher, especially in the older bees. These receptors are involved both in reception and transfer of the signals mediated by octopamine (Kaya-Zeeb et al., 2022). *Octβ2R* in *Drosophila* functions in the growth of neuromuscular junction in development as well as in response to starvation. Signaling is accomplished by the autoregulatory function of this receptor, in which octopaminergic neurons initiate a cAMP-dependent cascade (Koon et al., 2011; Koon & Budnik, 2012). However, the regulatory roles of these receptors in physiological and behavioral pathways in honeybees remained to be addressed. Therefore, the CRISPR-Cas9 system was devised to reveal the function of these beta receptors, in particular the octopamine beta receptor subtype 2, as the CRISPR-Cas9 system has higher specificity and stability, which can target splice variants of a single receptor subtype, in comparison to RNAi (Huang et al., 2018).

## 1.4 The CRISPR-cas9 system

The CRISPR-Cas9 system consists of a guide RNA (gRNA), which is around 20 nucleotides in size. The gRNA assists in directing the Cas9 enzyme, which acts as genetic scissors. Cas 9 is a nuclease, and it provides double stranded breaks (DSB) at the target site. Then, DNA repair mechanisms are activated and either non-homologous end joining (NHEJ) occurs or homology-directed repair (HDR) provides the repair of the cleaved region. NHEJ facilitates the repair, but it is error prone, so it can result in frameshift or point mutations from the ends being joined imperfectly (Gurumurthy et al., 2016).  However, in HDR, a homologous template is provided for insertion and thus the process is highly precise for knocking in a gene as opposed to knocking down or out a gene (Mengstie & Wondimu, 2021).

Loss-of-function mutations achieved by CRISPR-Cas9 followed by NHEJ activity enables observation of changes in the interested phenotype. CRISPR-Cas9 systems serve in understanding the roles of individual genes not only *in vitro*, but also *in vivo* studies (Tschaharganeh et al., 2016). Depending on the target tissue, different delivery techniques such

as viral vectors and nanoparticles are available for the transportation of the CRISPR-Cas9 system for *in vivo* applications (Javaid & Choi, 2021; Liang et al., 2015). These vectoring methods improve the effectiveness of the system *in vivo* because relying on a passive mechanism such as diffusion is not sufficient in live organisms for the targeting of specific tissues of interest within the organisms (Mengstie & Wondimu, 2021).

### 1.5.1 CRISPR-Cas9 as a tool for gene knockdown in honey bees

Gene delivery and genome editing methods have been studied on honey bee embryos mainly for the purpose of producing transgenic lines. CRISPR-Cas9 systems have the advantage of specificity, and they are more effective compared to other methods such as using transposons and siRNAs (Gurumurthy et al., 2016). The first studies on creating transgenic honey bees included conducting transfection of external DNA that was introduced to honey bees through injections and electroporations. The first attempt was a transfection of a linearized plasmid and sperm mixture into fertilized eggs, followed by artificial insemination of queen. The DNA was propagated for three generations; however, it was not integrated into the genome of the honey bee (Robinson et al., 2000).

Then there was delivery of EGFP (enhanced green fluorescent protein) into the honey bee adult brain, which was accomplished through electroporation of a EGFP- containing plasmid in two separate studies. Expression of GFP (green fluorescent protein) was confirmed in the honey bee brain with immunohistochemistry, but there was around 50% mortality of the adult bees that underwent the electroporation treatment, and the distribution of the transfection was limited to the edges of the bee brain (Schulte et al., 2013; Kunieda & Kubo, 2004). The drawback of this study is that it caused around 50% mortality from the electroporation treatment. There have been several studies using RNAi for gene knockdowns in the honey bee brain, but its effects are transient, lasting for around a maximum of 48 hours (Guo et al., 2018).

The first application of CRISPR-Cas9 on honey bees was reported by Kohno et al. (2016), where 57 embryos were injected directly with sgRNA and Cas9, at 50 ng/µl and 1 µg/µl concentrations, respectively. This is the most basic way of introducing the CRISPR-Cas9 system which is typically effective for cells grown in the lab (Lino et al., 2018). The knockdown of MRJP1 (*major royal jelly protein 1*) was targeted as its mutations are not

supposed to interfere with the normal development of honey bees (Drapeau et al., 2006). A total of 6 queens were developed from the injected embryos. From these queens, there were a total of 161 male offspring and one of the queens were scanned for gene editing in which 20 were successfully knocked down for the MRJP1 target (Kohno et al., 2016). Afterwards, the mKast (middle-type Kenyon cell-preferential arrestin-related protein) gene was targeted where they were successful in achieving the production of somatic mosaic queens and two more generations of drones and workers with genetic manipulations. However, these bees had to be maintained indoors due to legal restrictions limiting the power of conducting genetic manipulations via gene editing of queen honey bees (Suenami et al., 2018).

Due to these restrictions, direct F0 generation was targeted for gene editing and the sex determination pathway was examined using the CRISPR/Cas9 system. A mixture of the Cas9 mRNA or protein (400 to 2,000 ng/µl) and sgRNA, in a molar ratio of 1:2 to 1:0.75, was injected into embryos. Doublesex (dsx) mutants exhibited smaller reproductive organs and feminizer (fem) mutants lost their ability to respond to worker nutrition driven size control. Consequently, the size polyphenism of the reproductive organ in females and genes involved in sex determination was successfully revealed. The injection site of embryos was changed later on and this resulted in 100% genome editing efficiency (Roth et al., 2019).

A similar study where sgRNA and Cas9 were delivered through egg injections, targeting MRJP1, resulted in biallelic knockout of honey bee embryos. A total of 200 ng/ml MRJP1 sgRNA and 200 ng/ml Cas9 protein were injected into honey bee embryos, then the gene editing was validated by Sanger sequencing where the clones revealed 73.3% and 76.9% efficiency for MRJP1 and PAX6, respectively, for biallelic knockout mutants (Hu et al., 2019).

In a recent attempt guide RNA was delivered through injections to honey bee eggs in Ribonucleoprotein (RNP) form which is a complex of ribonucleic acid (RNA) and RNA-binding proteins. A total of 2 - 3 µl of 2.5 µM RNP solution were used, but high mortality was recorded in the injected eggs (Chen et al., 2021). Although successful gene delivery and CRISPR applications were performed on honey bee embryos, larvae, pupae, workers, queens and drones, mainly through injections, maintenance of a transgenic line could not have been achieved. Engineering of CRISPR multigene constructs (Mansouri et al., 2016), constructs with "dead" cas9, dCas9, for transcriptomics (Chen et al., 2016) and inducible genome editing systems which could be on and off under different chemical conditions (Dow et al., 2015) could be used to figure out the roles of a number of genes involved in signaling pathways. In addition,

a viral delivery method could improve the gene editing for adults that may have development issues if the gene editing was performed at the embryonic stage. Moreover, specific tissues and locations of adult bees can be penetrated more easily using a viral vector.

**1.5 Baculovirus as a vector system**

Baculovirus could potentially be an optimal delivery method of the CRISPR-Cas9 system because it presents a high infectivity rate for insect cells leading to higher transduction efficiencies (Kost, Condreay, & Jarvis, 2005). The lentivirus is widely used for vectoring the CRISPR-Cas9 complex for mammalian cells; however, it has low transduction efficiency which is approximately 20% for honey bee cells (M. M. Chan et al., 2010). In addition, the lentiviral delivery was performed *in vivo* as well and injected into the brains of adult bees, but its transfectivity, especially on neurons, was limited and the GFP signal was only coming from the surrounding glial cells of the kenyon cells. Therefore, lentivirus seems to be more suitable as a transfection agent for somatic cells, which is not applicable for targets in the bee brain (Leboulle et al., 2022).

Baculovirus, which is also called *Autographa californica* nucleopolyhedrovirus (AcNPV), is known to infect the Alfalfa Looper moth, *A. californica*. The AcNPV genome is double stranded, and it is not an integrating virus (Schaly et al., 2021; Kim et al., 2021).

Baculovirus is engineered for gene delivery applications of different hosts. Its efficiency as a vector was demonstrated in mosquitoes (Naik et al., 2018), the beet armyworm (Han Y, 2018) as well mammalian cells (Schaly et al., 2021). This vector has also been injected into queen honey bee pupae to start a transgenic germline. The $1\times10^5$ Infectious Unit (IFU) of baculovirus was successful in delivering EGFP and the GP64 envelope protein-containing plasmid, however, the $1\times10^6$ IFU concentration led to high mortality of the honey bees (Ikeda et al., 2011). A high AcNPV titer was also reported to be potentially lethal for lepidopteran insects as well (O'Reilly et al., 1998).

EGFP delivery enclosed within an AcNPV plasmid was also performed on honey bee larvae, pupae, and adults (Ansari et al., 2016). A total of 0.2 µl and 5 µl of ($10^6$ PFU/ul) viral solution was applied to the larvae and pupae. A fluorescence signal was obtained in live pupae 4 days

after injection, but 5 µl of injection solution interfered with the metamorphosis of pupae. On the other hand, when the viral titer was decreased to 0.2 µl, 2 out of 8 pupae were able to survive and undergo metamorphosis. Researchers concluded susceptibility to mortality when using a high titer (5 µl) of vector, which emphasizes the need for optimizing the viral titer for a particular target. In addition, viral transfection of adult honey bees was not achieved in this study (Ando et al., 2007). Therefore, it is important to use the minimal amount of baculovirus required for successful delivery of the CRISPR-Cas9 system because the infectivity of this virus is very high for insects.

# 1. AIM OF THE STUDY

The main aim of the study is to determine whether octopamine beta receptor subtype 2 (*AmOctβ2*) is involved in appetite regulation. This was tested by knocking down *AmOctβ2* with a CRISPR-Cas9 system and then measuring the appetite levels of the honey bees, using the PER assay, after delivering the baculovirus vector with an ocellar tract brain injection. The second aim of our study was to test whether baculovirus is an effective gene delivery tool for the honey bee brain. Transfection regions of the baculovirus vector were validated by imaging the honey bee brain tissue and using a GFP reporter gene integrated in the vector. The effectiveness of the vector and CRISPR-Cas9 system was also validated by conducting gene expression assay using qPCR.

# 2. MATERIALS & METHODS

## 3.1 Baculovirus Plasmid Design

The CRISPR-Cas9 system was designed to knockdown *AmOctβ2* (Figure 3, adapted from Ghanem, 2021). The system was integrated into the baculovirus *Autographa californica nucleopolyhedrovirus* (AcNPV) genome. The AcNPV vector was prepared by the VectorBuilder company. They provided a specific Cas9 sequence based on *Drosophila melanogaster* as its genome is highly conserved compared to *Apis Mellifera*. Guide RNA for the CRISPR system was prepared with the Benchling program by selecting *Apis mellifera* as the target genome. The Octopamine beta-receptor 2 sequence needed for the gRNA design was obtained from literature (Balfanz et al., 2014). EGFP sequence was also inserted into the system as the reporter of gene editing.
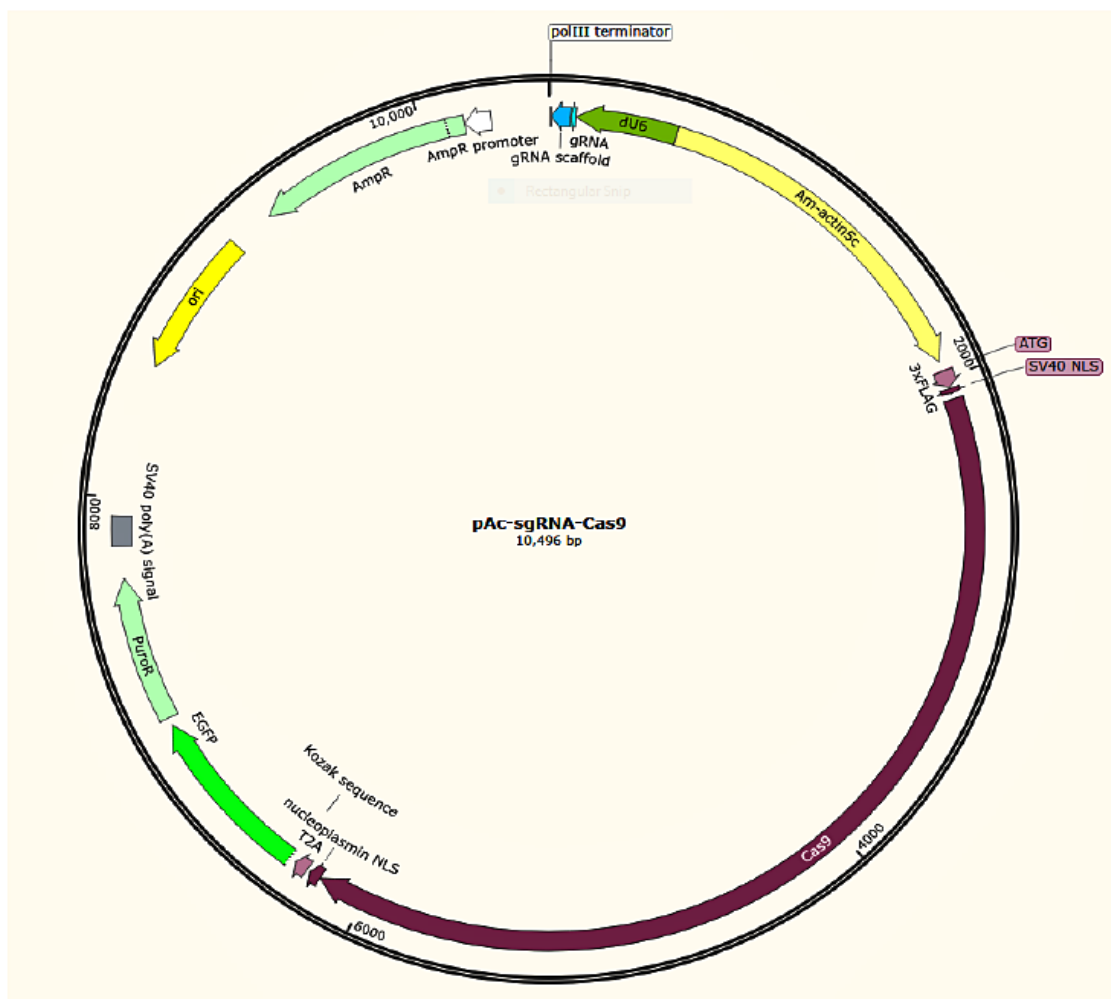


*Figure 16. pAC-sgRNA-Cas9 plasmid containing CRISPR system to target AmOctβ2. Figure adapted from Ghanem (2021).*

We determined the required amount for effective application of the baculovirus vector based on previous literature. Given that 5 µl of $10^6$ PFU/µl resulted in relatively high levels of mortality, while 0.2 µl of $10^6$ PFU/µl was found to have minimal impacts on honey bee mortality, we injected 2 µl of $10^7$ PFU/ml in preliminary experiments and then reduced this to 1 µl $10^7$ PFU/ml after observing high levels of mortality.

## 3.2 Honey Bee Collection, Harnessing, and Injections

From three different source colonies, we collected only pollen forager bees, to ensure that they were forager bees, for the experiment. We placed a wire mesh at the entrance of three different hives and individually captured 50 pollen foragers at a time in 20 ml glass liquid scintillation vials (Sigma-Aldrich, St. Louis, Missouri, United States). Then, we placed these vials on crushed ice for immobilization of the honey bees while they were being transported to the laboratory.

Next, we harnessed honey bees by placing them in cut plastic drinking straws which are approximately 5 cm in length. We placed their heads out of the straw (Figure 4). Then, we stabilized them with a 1 mm width duct tape strip by placing it in between their thorax and head. In this harnessed position, after 30 min of acclimation time, we fed them with 50% sucrose solution *ad libitum* to standardize their hunger levels (Mayack and Naug 2011).
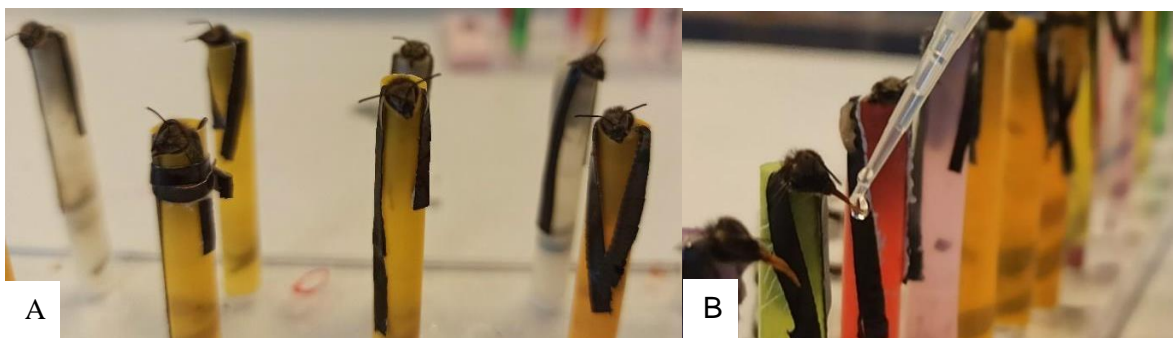


*Figure 4. Honey bees harnessed in cut straw pieces 5 mm in length. (A) Straws placed on a PER tray with up to 50 bees at a time, (B) Honey bees are being fed with 50% sucrose solution, 10 µl at a time, using a micropipette.*

Honey bees were divided into five groups as shown in Table 1 and injected with 1 µl of CRISPR, PBS (Phosphate-buffered saline), CRISPR+Sorbose, PBS+Sorbose and GFP (Green Fluorescent Protein). Up to 50 bees were injected at a time. CRISPR injected honey bees were classified as the test group while the others were considered control groups. We

performed medial ocellar tract injections (Figure 5), using a 10 µL Microliter Syringe (Hamilton, USA) placed in a right handed micromanipulator (WPI, Germany), to deliver 1 µl of the baculovirus vector, containing the CRISPR-Cas9 system ($10^6$ PFU/ul) (Søvik et al., 2016). Prior to this, the lens of the middle ocelli was removed using a micro scalpel with a 30-degree angle blade. After injection, the harnessed bees were once again fed *ad libitum*, to relieve the stress caused by injections. Then, they were placed inside an incubator at 25°C and 70% relative humidity when not being used for the experiment (Figure 6).

*Table 1. Honey bee treatment groups*
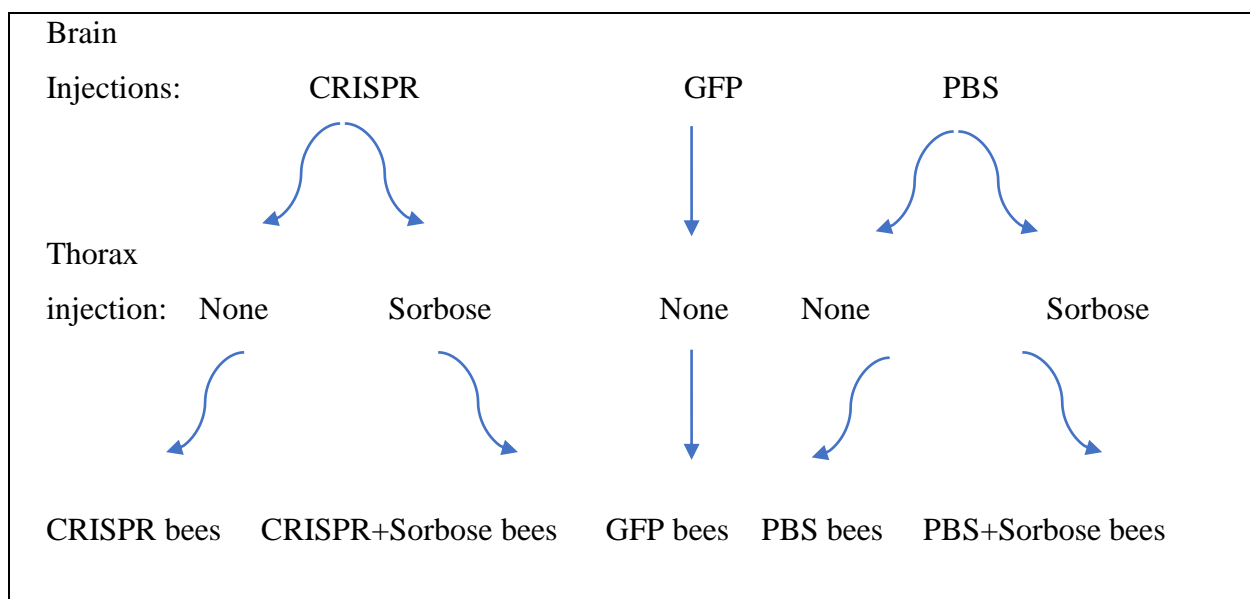
*Figure 5. Honey bee brain injections under the dissecting scope. (A) Harnessed honey bees were placed under the dissecting scope and ocellar tract injection was accomplished. (B) CRISPR, GFP and PBS solutions were injected with 10 µL Microliter Syringe (Hamilton USA) which is stabilized by micromanipulator (WPI, Germany)*

# Timeline of the Honey Bee Brain Injections

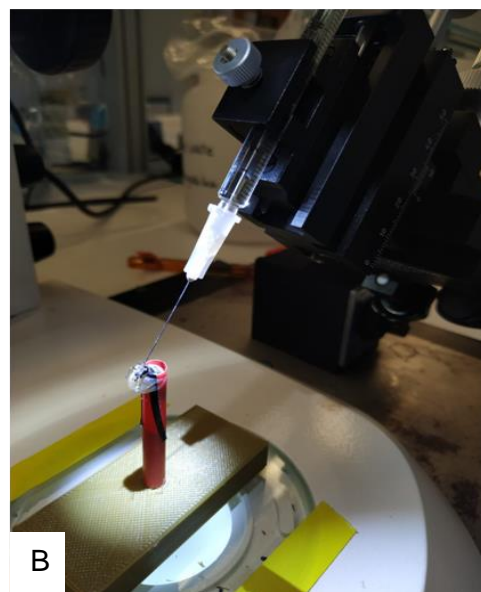| Injection Day | 1DPI | 2DPI | 3DPI |
|---|---|---|---|
| Honey Bee Brain Injections | 1 Day Post Injection | 2 Days Post Injection | 3 Days Post Injection |
| Ocellar tract injections of CRISPR, PBS, GFP solutions | 18 hours of starvation, PER Assay | 18 hours of starvation, PER Assay | 18 hours of starvation, PER Assay |
| Feeding after treatment | Feeding after treatment | Feeding after treatment | Flash-freezing honeybee heads in Liquid Nitrogen for RNA Isolation |
| | Extra feeding after 6 hours | Extra feeding after 6 hours | Honey Bee brain dissection for imaging |
| | | Only for Sorbose Treatment Groups: Sorbose thorax injections 10 min prior to PER Assay | |

*Figure 6. Timeline of the Honey Bee Brain Injections*

## 3.3. Proboscis Extension Response (PER) Assay to Measure Appetite Levels

Each day, for 3 days, we measured their appetite levels using the Proboscis Extension Response (PER) assay, after 18 hours of starvation (Bitterman et al 1983). We performed the PER assay by touching a droplet of sucrose solution to the bee's antennae without feeding them. Scoring of the PER was binary, a PER score of 1, was recorded if the honey bee fully extended its proboscis in response to the droplet of sucrose solution and a score of 0, if honey bee showed no full proboscis extension. We recorded PER responses of individual honey bees to increasing concentrations of sucrose solutions: 0.1%, 0.3%, 1%, 3%, 10%, and 30% concentrations. A droplet of water was touched to the bee's antennae in between each sucrose solution to prevent desensitization. Honey bees which responded to all sucrose solutions, resulted in a Gustatory Response Score of 6 as the possible maximum value. If the honey bee did not respond to sucrose solutions, they had a score of 0. GRS for each honey bee was calculated by summing up the responses given to the gradually increasing sucrose solutions. The average GRS values of the honey bees which are in the same treatment group was

calculated. We fed the honey bees at the end of the assay *ad libitum* and kept them in an incubator until the next PER assay. We included an extra feeding time at 6 hours after each PER assay completed in 1 day after injection, 2 days after injection, and 3 days after injection. Those belonging to the CRISPR+Sorbose and PBS+sorbose treatment groups were injected with 1 μL of 1.5 M sorbose into their thorax 10 min prior to the PER assay on the first day. Sorbose is a competitive trehalose p-synthase enzyme inhibitor, so it can be used to lower trehalose levels in the hemolymph (Akulku et al. 2021), we therefore used this as a potential rescue treatment to confirm if the octopamine beta receptor subtype 2 is the only one involved in honey bee appetite regulation in response to the lowering of trehalose levels. After the third day, the honey bees were flash frozen in liquid nitrogen and their heads were stored at -80° C until further qPCR gene expression analysis.

### 3.4 qRT-PCR gene expression analysis of *AmOctβ2*

### 3.4.1 Homogenization, RNA isolation and DNAse Treatment

An EcoPURE Total RNA kit (EcoTECH Biotechnology, Turkey) was used for RNA isolation by following the manufacturer's instructions. Briefly, bee head samples were placed in a grinding specific, Safe-Lock 2 mL Eppendorf tube. Then, 300 μl of ECOPURE Lysis/Binding Buffer was mixed with 10% (3 μl) β-mercaptoethanol to inhibit the RNase activity in the samples. This mix was then added to each tube. A stainless-steel grinding bead was added to each centrifuge tube to be used in a tissue grinder (Qiagen, TissueLyser II).

The mixture was spun for 10 min in a microcentrifuge (Eppendorf, Germany) at 12,298 g (10,000 rpm) to pellet the debris. Then the supernatant was transferred to another 1.5 ml microcentrifuge tube, an equal amount of absolute ethanol (96-100%) was added to the supernatant, and the mix was vortexed for 10 s. An EcoPURE column was inserted into a collection tube, and the mixture was transferred to the column. The mixture was centrifuged at 12,298 G (10,000 rpm) for 30 s at room temperature (RT). Flow through was discarded, and EURX DNAse treatment protocol was followed using the manufacturer's instructions.

*Table 2. Reagents used in DNAse treatment*

| Reagents | 1 rxn (µl) |
|---|---|
| H2O | 8.6 |
| 10X Buffer II | |
| MgCl2 (50 mM) | 0.2 |
| DNAse I (Take out lastly) | 0.2 |
| Total | 10 |

A total of 10 µl DNAse treatment mixture was added on the column for each sample. This was followed with a 15-30 min incubation period at 37° C. A total of 300 µl of EcoPURE Wash buffer 1 was added to the column. The buffer was centrifuged at 12,298 G (10,000 rpm) for 30 s at RT. The flow through was discarded and 500 µl EcoPURE Wash buffer 2 was added to the column. The buffer was centrifuged at 10,000 rpm for 2 min at RT. The flow through was discarded and 200 µl of EcoPURE Wash buffer 2 was added to the column. To remove any residuals, the buffer was centrifuged at 10,000 rpm for 2 min at RT. The column was transferred to a sterile 1.5 ml microcentrifuge tube, and 35 µl of EcoPURE Elution buffer was added to the column. The solution was centrifuged at 10,000 rpm for 2 min at RT. Flow through was retained and the previous step was repeated. The column was discarded and eluted RNA was stored at -80°C.

### 3.4.2 Quality check

The purity and concentration of the extracted RNA was measured for each sample using a Nanodrop spectrophotometer (Hampton, USA) and gel electrophoresis.

### 3.4.3 cDNA Synthesis

The cDNA was synthesized using a OneScript Plus cDNA Synthesis Kit (abm, Canada), according to the manufacturer's instructions. RNA was standardized before synthesizing the

cDNA not to exceed 1 µg per sample. The reagents in Table 3 were used for each cDNA synthesis reaction.

*Table 3. cDNA Synthesis Reagents*

| Reagent | Volumes per rxn (ul) |
|---|---|
| Nuclease-free H2O | 13- (RNA volume) |
| 5X RT Buffer | 4 |
| dNTP | 1 |
| Primers | 1 |
| OneScript® Plus RTase | 1 |

## 3.4.4 qPCR reaction

Two different mixtures for the qPCR reaction were prepared with the reagents given in Table 4 for both the target gene (octopamine beta receptor sub-type 2) and the reference gene (Ribosomal protein 49 - RP49). The reference gene was known to be stable across tissues and within the brain of the honey bee, *Apis mellifera* (Lourenço et al., 2008).

*Table 4. Reagents used for qPCR reaction*

| Reagents | Volumes per rxn (ul) |
|---|---|
| Nuclease free $H_2O$ | 2.4 |
| Primers (F) | 0.3 |
| Primers (R) | 0.3 |
| BlasTaq 2X | 5 |
| cDNA | 2 |
| Total | 10 |

A total of 10 µl reactions were prepared with 2.4 µl of nuclease free water, 0.6 µl of forward and reverse primers (SenteBioLab, Ankara, Turkey), and 5 µl of Blastaq Green 2x master mix (Abm, Richmond BC, Canada). We used 2 µl of template cDNA.

The plate was placed in the thermocycler (Roche LightCycler 480 II). Parameters on the software were set to 45 quantification cycles (95$^\circ$C, 30 sec ramp rate 2.2, 55$^\circ$C ramp rate 2.2, 30 s, 60$^\circ$C ramp rate 4.4, 1 min). We checked the specificity of the primer sets with a melt curve analysis (5 s, 95 $^\circ$C, 1 min, 60 $^\circ$C) and all samples were run in technical triplicates. Gene expression of our target gene, *AmOctβ2*, was measured relative to the reference gene RP49 using the -ΔΔCT analysis method. Then the values were imported to JMP Pro v. 16 software to complete statistical analysis.

## 3.5 Brain Imaging

### 3.5.1 Fresh Brain Dissection and Microscopy Imaging Preparation

We placed the harnessed honey bee upside down, placing its head into a crushed ice box. Then a 100 µl of brain Ringer's solution (130 mM NaCl, 5 mM KCl, 4 mM MgCl2, 5 mM CaCl2, 15 mM Hepes, 25 mM glucose, 160 mM sucrose, pH 7.2) was placed on a beeswax glass petri dish dissecting plate. Then, we decapitated the honey bee head and immediately placed it on the brain Ringer's solution. Dissection of the brain was performed under a dissecting scope at 30X (Zeiss Stemi 305,). The bee head capsule was stabilized at three spots to the wax with 3 insect pins. Firstly, proboscis and mandibles were cut off. Then the chitin layer on the area near the neck was removed (Figure 7). The brain was removed from the head capsule by using a probe, micro scalpel, and micro scissors, while ensuring that we did not lose any parts of the brain tissue. We ensured not to grab or pluck off the brain tissue as a thread, because the brain is a fibrous structure, and it loses its original shape when pulled off.
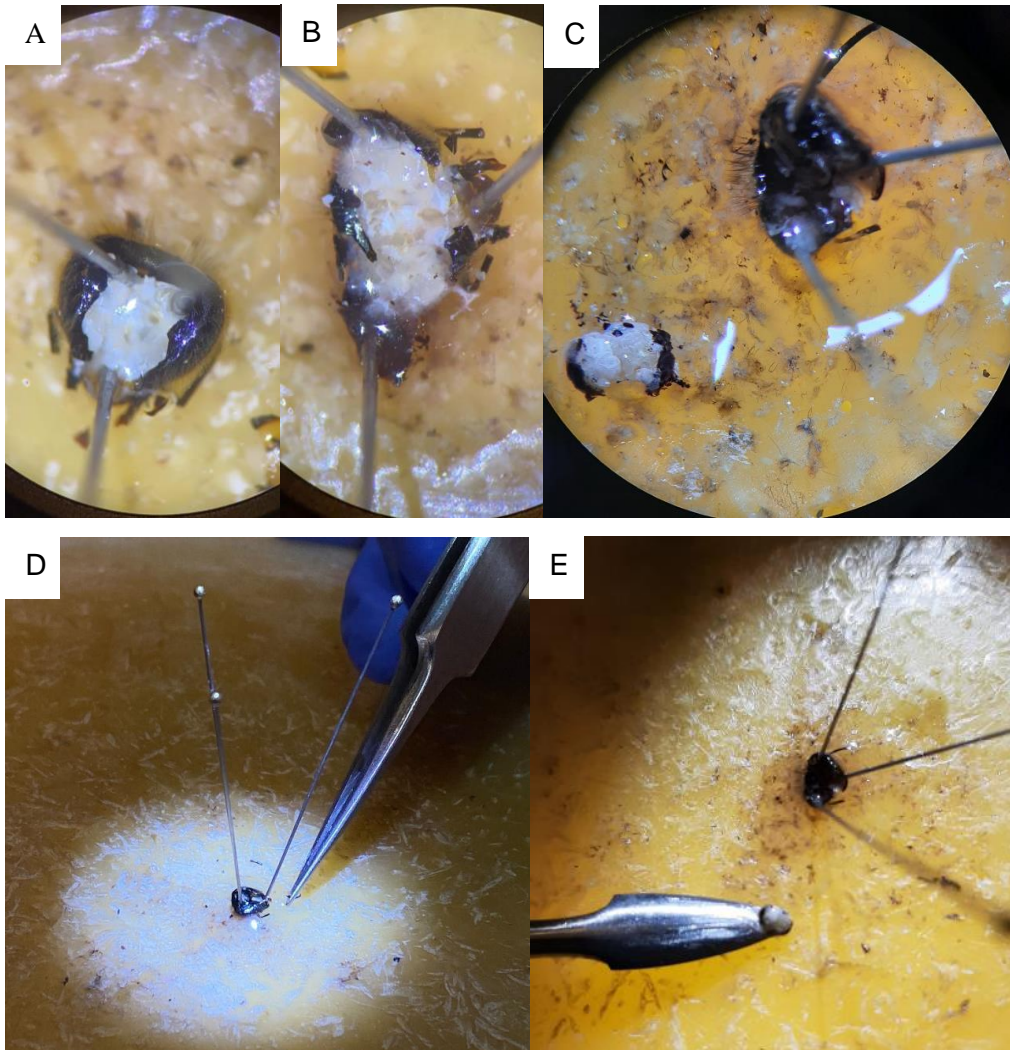
*Figure 17. Honey bee brain dissections on beeswax petri dish dissecting plate. Three pictures taken at different stages of brain dissections. (A) A honey bee head upon the removal of chitin layer at the neck site (30X), (B) a honey bee brain after detached from the inner sides of the chitin head capsule (30X), (C) a complete intact brain at the end of the dissection (30X), (D) A head capsule stabilized on the was dissecting plate with three needles and, (E) Whole brain dissected at the tip of the probe*

Dissected fresh brains were placed in a 1.5 ml microcentrifuge tube for fixation. The tube contained 1 ml of 4% PFA solution and it was protected from UV light with aluminum foil. The brains were kept in 4% PFA overnight at 4° C. The brains then were kept in 15% sucrose-PBS and 30% sucrose-PBS solution, respectively, until it sank to the bottom of the tube. The brains were rinsed with PBS (1x) for 10 min and then embedded in low melting point agarose (4%). Later, the brains were sliced (50 µm thickness) with a Leica 1,000s vibratome at room temperature (Figure 8). These slices were kept in PBS (1x) at 4°C until further analysis.
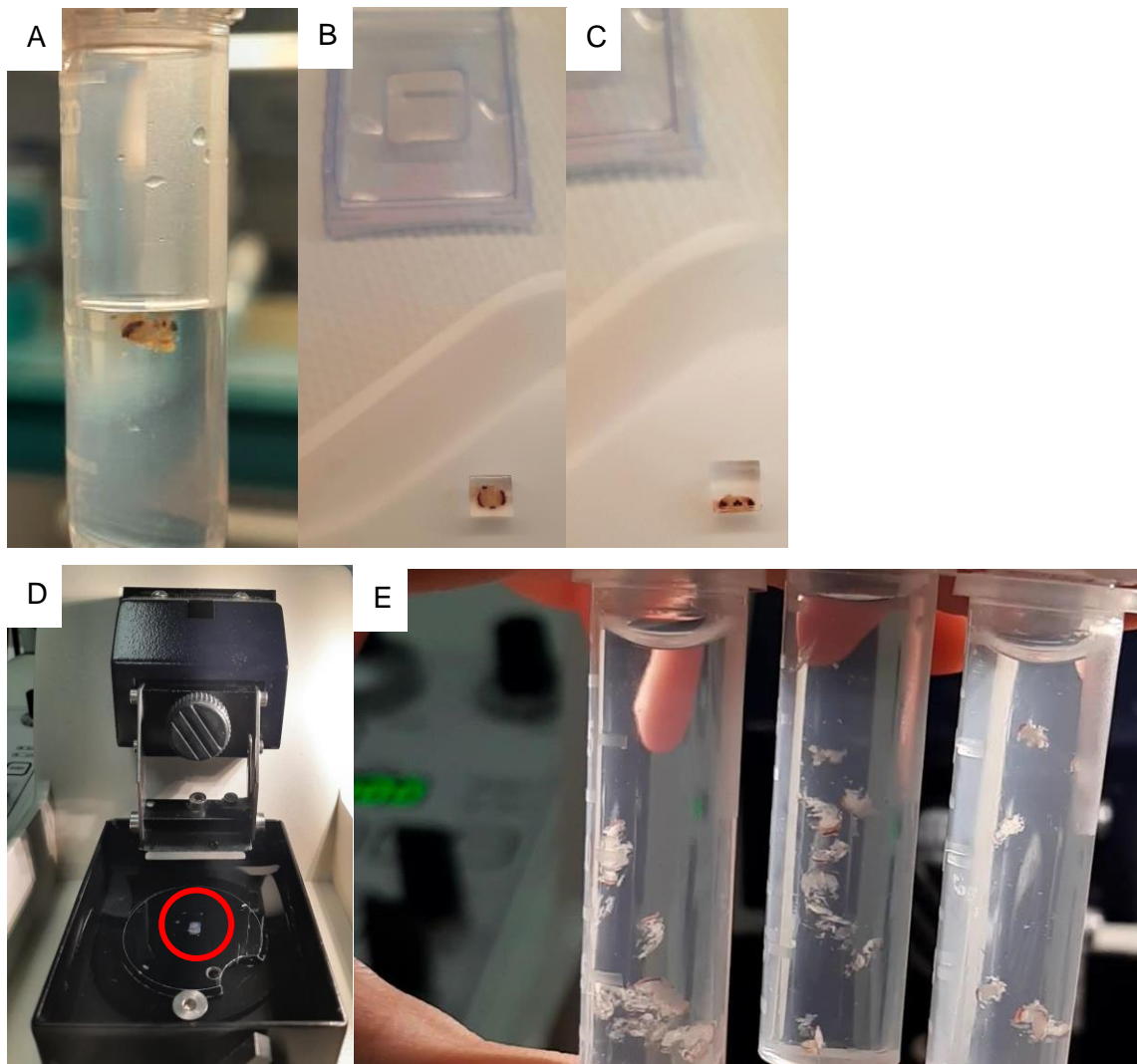
*Figure 18. Preparation of honey bee brain for brain imaging. (A) Honey bee brain kept in 15% sucrose solution, (B) Honey bee brain embedded in 4% Agarose (top view)), (C) Honey bee brain embedded and trimmed for slicing (back view)), (D) Embedded honey bee brain is glued on the vibratome specimen holder (brain shown within the circle) and (E) 50 µm honey bee brain slices were obtained.*

### 3.5.2 Staining and confocal imaging of the honey bee brain

A staining solution was prepared with 0.4 U (units), equivalent to approximately 2 µl of phalloidin, and 1 µl of DAPI per slice. Phalloidin enables visualization of the actin filaments and DAPI binds to DNA, making the nuclei of the neurons visible. Slices were kept in the staining solution for 20 min. Then, the brain slices were placed on a microscope slide and were mounted with 20 µl of methyl salicylate mounting media. The coverslips of the slides were then sealed with nail polish, and these were kept until dry.

Channels for the three different wavelengths, which were 405 nm for DAPI, 488 nm for GFP and 561 nm for Phalloidin, were set on a Carl Zeiss LSM 710 confocal microscope. Voltage gains of the samples, which is a setting used to determine the ratio of clear signals and background noises, were set at the same level for each imaging processing. Images of the whole brain were taken at 10x magnification.

## 3. RESULTS

### 4.1 Behavior Test: Proboscis Extension Response (PER) Assay

Across all days CRISPR honey bees (n=236) in comparison to PBS+Sorbose honey bees (n=135) had significantly lower appetite levels (Wilcoxon / Kruskal-Wallis Tests by treatment: $\chi2$=11.6088, df=4, P = 0.0205 Nonparametric Comparisons for Each Pair Using Wilcoxon Method: P < 0.0009).

GRS values obtained on day 1 post injection is significantly different than day 2 post injection and day 3 post injection (Wilcoxon / Kruskal-Wallis Tests by day: $\chi2$ = 88.8374, df = 2, P < 0.0001 Nonparametric Comparisons for Each Pair Using Wilcoxon Method: 3DPI-2DPI P < 0.0003, 2DPI-1DPI and 3DPI-1DPI P < 0.0001).

Thirdly, GRS values of each day were evaluated for different treatment groups within that day. On day 1 post injection there were no significant differences in GRS across CRISPR (GRS: 3.366 +/- 0.231) and GFP (GRS: 3.575 +/- 0.418) injected bees, but overall, there was a significant of treatment on appetite levels (Kruskal Wallis test for day 1 Wilcoxon / Kruskal-Wallis Tests by treatment: $\chi2$=12.5789, df=4, Prob>ChiSq 0.0135, Figure 5). There are significantly lower appetite levels in CRISPR treated bees (GRS: 3.366 +/- 0.231) in comparison to the PBS treated bees (GRS: 4.29 +/- 0.223) on day 1 post injection (Kruskal Wallis test for day 1 Wilcoxon / Kruskal-Wallis Tests by treatment: Nonparametric Comparisons for Each Pair Using Wilcoxon Method: p < 0.0008; Figure 9)

On day 2 post injection, the PBS+Sorbose treated bees, receiving an injection of sorbose into the thorax 10 min prior, has a significantly higher GRS (2.973 +/- 0.358) in comparison to the

GFP (GRS: 1.500 +/- 0.590 ), PBS (1.946 +/- 0.330), and CRISPR (2.141 +/- 0.280) treated bees (Kruskal Wallis test for day 2: χ2=8.0155, df=4, P = 0.0910, Figure 9)

On day 3 post injection, there was significantly lower appetite levels from CRISPR injected bees (GRS: 0.927 +/- 0.226 ) in comparison to the GFP (GRS: 2.333 +/- 0.897), PBS (GRS: 2 +/- 0.429) and CRISPR+Sorbose (GRS: 1.928 +/- 0.666) treated bees (Kruskal Wallis test for day 3: χ2 = 10.0108, df = 4 P = 0.0402, Figure 9).
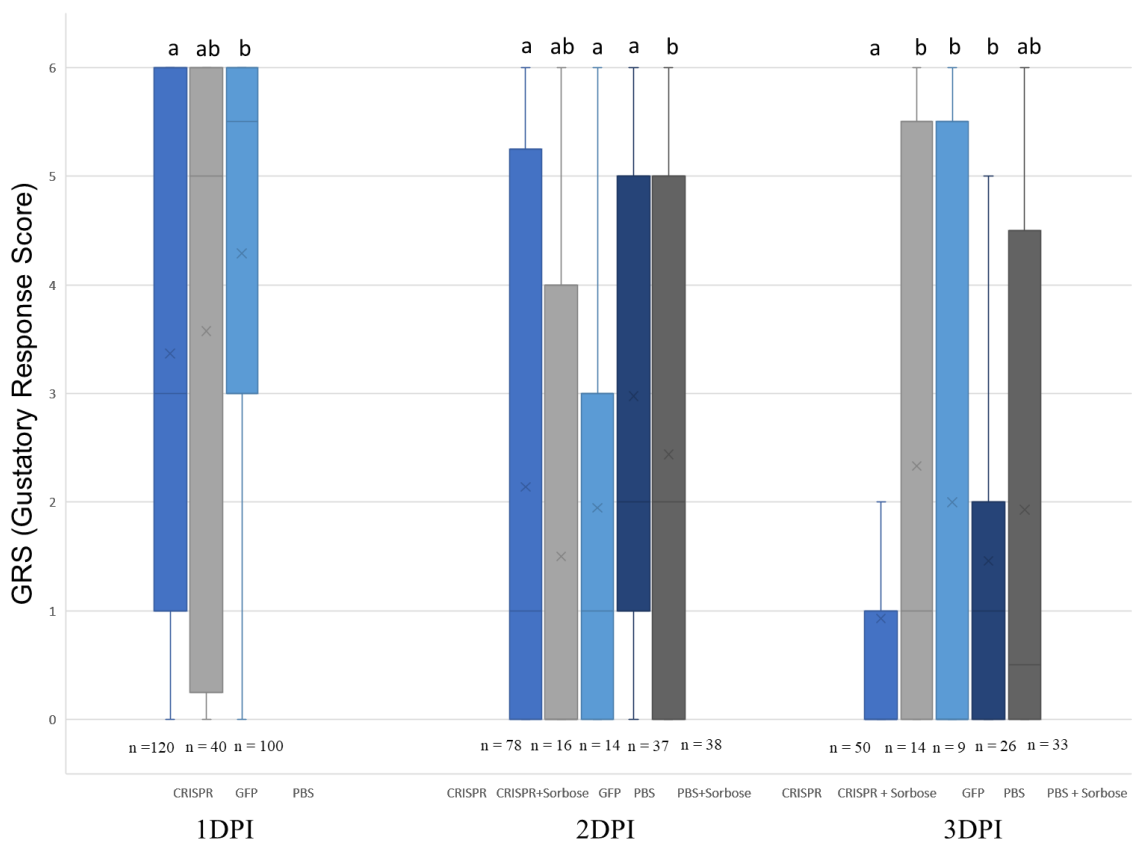


*Figure 19. GRS (Gustatory Response Score) values of different treatment groups by day. We calculated GRS of different treatment groups CRISPR, CRISPR+Sorbose, PBS, GFP and PBS+Sorbose at 1DPI, 2DPI and 3DPI. PBS+Sorbose and CRISPR+Sorbose treatment groups have 2DPI and 3DPI values as sorbose treatment was done at 2DPI. Each bar represents the GRS levels of treatments, error bars represent the standard error. The sample sizes are indicated above each bar, the letters below each bar represent significant differences across the treatments."X" marks on the bars represent the median while the top and bottom lines of each box represent the interquartile range within the group*

## 4.2 Gene expression analysis of AmOctβ2
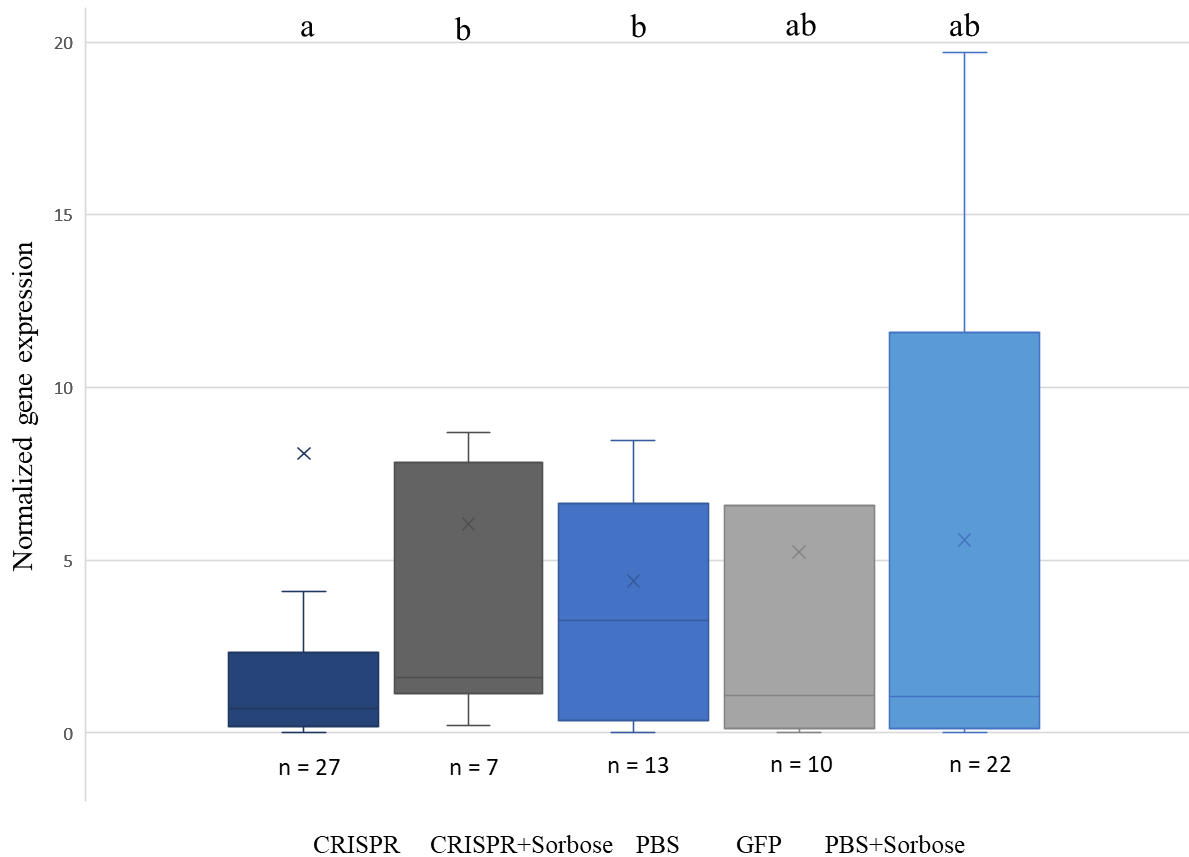


*Figure 20. Normalized gene expression of AmOctβ2 vs. Treatments at 3DPI. Mean and standard deviations of ΔΔCT values represent the normalized relative gene expression of AmOctβ2 vs. Treatments. We analyzed the gene expression levels of 5 different treatment groups: CRISPR, CRISPR+Sorbose, PBS, GFP and PBS+Sorbose. Each bar represents the fold differences in the gene expression of AmOctβ2 and RP49, error bars represent the standard error. The sample sizes are indicated above each bar, the letters below each bar represent significant differences across the treatments."X" marks on the bars represent the median while the top and bottom lines of each box represent the interquartile range within the group*

The normalized gene expression of CRISPR treated honey bees at the 3DPI (8.08 +/- 5.68) was significantly lower than the PBS ($2^{\wedge}$-**ΔΔ** cp: 4.39 +/- 1.43 ) and CRISPR+SORBOSE treated honey bees (normalized gene expression: 6.05 +/- 3.03) (Wilcoxon / Kruskal-Wallis Tests by treatment: $\chi^2 = 6.1114$, df = 4, Nonparametric Comparisons for Each Pair Using Wilcoxon Method: P < 0.0303 and 0.0453, respectively, Figure 10).

## 4.3 Brain Imaging

Whole brain 50 µm brain slices of noninjected and CRISPR injected honey bees were imaged under the confocal microscope. The whole brain area was captured in a rectangular area using the ZEN blue software 3.5 (Figure 11). The whole brain image of a noninjected honey bee brain had an arithmetic mean intensity of 4,416 for GFP signal while the whole brain image of CRISPR injected honey bee brain had a value of 6,774.



*Figure 21. Whole brain images of noninjected and CRISPR injected honey bees. (A) Noninjected honey bee brain image, slice thickness: 50 um. (B) CRISPR injected honey bee brain image, slice thickness: 50 um. Voltage gain:650 blue signals are for DAPI and green signals are for GFP.*

Particular regions were selected within the whole brain area for further analysis (Figure 12). The left antennal lobe (AL)of the noninjected honey bee brain had an arithmetic mean intensity of 5,69 for GFP signal while the AL of CRISPR injected honey bee brain had a value of 7,277.



*Figure 22. Left antennal lobe (AL) of noninjected and CRISPR injected honey bee brains. (A) AL of noninjected honey bee brain, slice thickness: 50 µm. (B) AL of CRISPR injected honey bee brain image, slice thickness: 50 µm. Voltage gain:650 blue signals are for DAP I and green signals are for GFP.*

The right AL of the noninjected honey bee brain had an arithmetic mean intensity of 4,35 for GFP signal while the right AL of the CRISPR injected honey bee brain had the value of 7,712 (Figure 13).
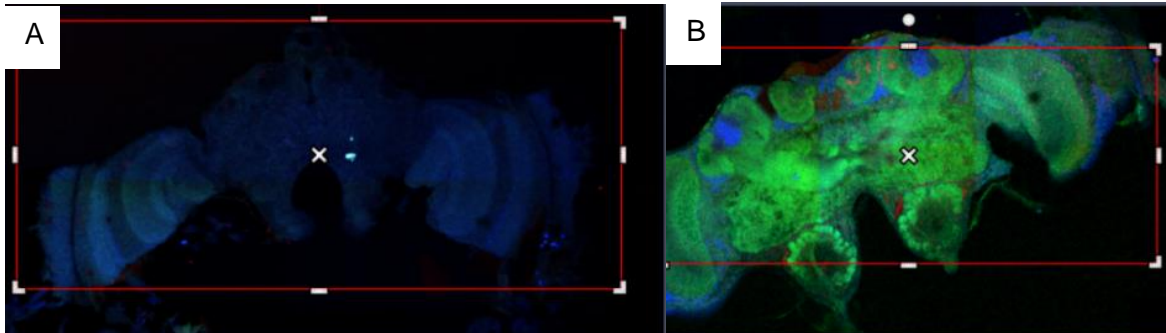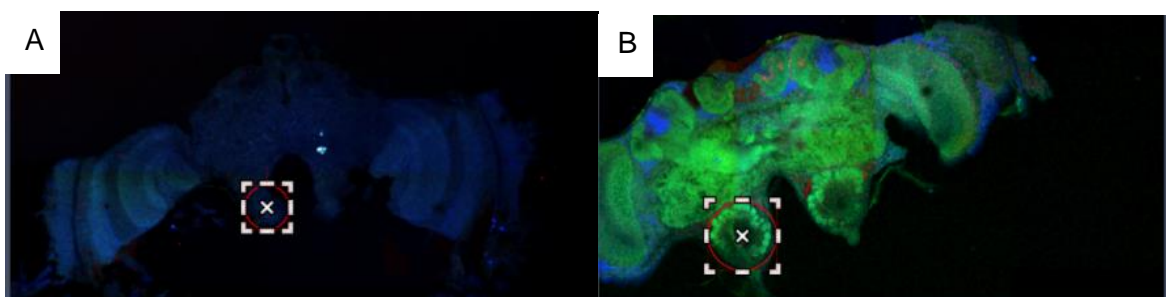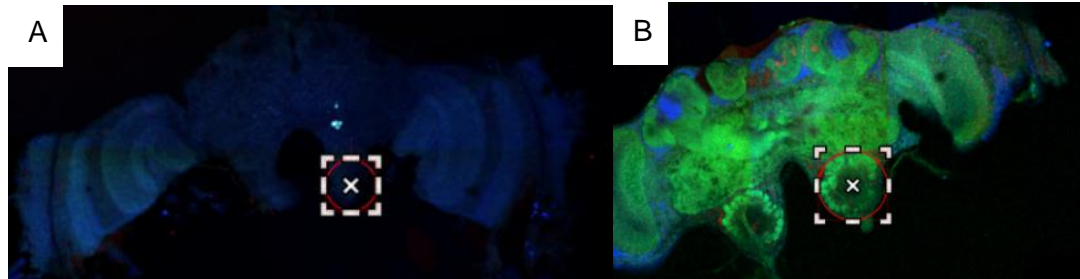


*Figure 23. Right Antennal lobe (AL) of noninjected and CRISPR injected honey bee brains. (A) AL of noninjected honey bee brain, slice thickness: 50 um.(B) AL of CRISPR injected honey bee brain image, slice thickness: 50 um. Voltage gain:650 blue signals are for DAPI AND green signals are for GFP.*

## 4. DISCUSSION

In this study, a novel gene delivery method is applied to honey bees through brain injections. Transfectivity of the viral vector was observed throughout all regions of the honey bee brain. Therefore, we suspect that the baculovirus was successful in infecting the neurons of the honey bee brain. The effectiveness of the CRISPR-Cas9 system was also supported by the behavioral data by a lowering of appetite in starved bees and was validated both with a significant lowering of octopamine beta receptor subtype 2 gene expression as well as higher GFP signal coming from the brain of treated bees emanating from the confocal microscopy images.

We first injected 2 µl of our viral vector, however, this viral titer resulted in high mortality (22.2%) of GFP-plasmid injected honey bees (for example 2 out of 9 honey bees were dead 1 day after injection). GFP has been used as a reporter to measure gene expression and cell tracking, however, it is claimed that it can potentially interfere with *in vivo* experimental data. GFP has been shown to cause cytotoxicity as well as immunogenicity at the *in vivo* and cellular level. Therefore, in future studies, the GFP reporter could be removed in order to increase the survival of the treated bees in the experiment. In addition, the widespread nature of the infection in the honey bee brain suggests that a lower dose could have been used that may achieve the same level of desired effect in terms of knocking down a neural target to determine its function.

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After our observation that either GFP or the baculovirus in high load led to high mortality, we decreased the injection volume from 2 µl to 1 µl and we added an extra feeding time, which was 6 hours after the PER assay each day after injection. However, we ended up with a low sample size of GFP treated honey bees because we had a limited amount of GFP-containing plasmid (200 µ). Moreover, the use of a microinjector could have been used for more precise injections, thereby conserving our limited material. We did not find a significantly lower AmOctβ2 gene expression of the CRISPR treated bees in comparison to the GFP injected bees on day three post injection. We believe that this is due to the small sample size for the GFP treated bees. In general, harnessed bees are stressed and do not survive for very long under these conditions (Finkelstein et al., 2019). In addition, these bees were starved over time adding an additional stressor which could have contributed to the high mortality that we observed. Using 2 µl of the baculovirus vector was shown to be safe in a previous study for larvae, and we injected 10 times less concentration of the virus in comparison to this to decrease its toxicity. Based on our imaging results, it appears that even a lower dose of baculovirus could have been used, although we have injected 5 times less amount of viral material of the previously used 5 µl, which has shown to result in high mortality (Ando et al., 2007). Lastly, 0.2 µl of baculovirus vector was shown to be safe in the same study for larvae, and we injected 10 times less of the virus to decrease mortality, however we still observed a relatively high level of mortality that was due to the virus itself. Fine tuning of the viral titer may result in an increased survival of the injected harnessed honey bee in future experiments.

Images of CRISPR injected honey bee brains belonging to the third day after injection displays the GFP expression and the spread of the vector to the many different regions of the brain which was remarkable as this vector appears to be a promising gene delivery tool for genetic honey bee brain manipulations. The GFP signal obtained from the CRISPR injected honey bee brain is higher than the signals obtained from a noninjected honey bee brain using the same confocal imaging settings. This supports the idea that there was infection of the baculovirus on the third day after injection. Our finding corresponds with previous studies that have observed the infection of the baculovirus to be around 48 - 72 hours after injection. In addition, our timeline is in accordance with the confocal images obtained after the AcMNPV injection into the *S. exigua* larval brain where the infection of baculovirus was observed starting two days after injection (Han, Y., 2018). Infection might have started two days after injection in our experiment, but we only observed the knockdown after three days post injection as we know it

would take time to see the downstream effects from the CRISPR-Cas9 system. The GFP expression in the honey bee brain after baculovirus injection could be tracked over time in the future to ascertain the infection timeline of the virus.

The GFP imaging from three days post injection revealed that indeed the baculovirus was widespread throughout the honey bee brain, within the calyces reaching the glomeruli at the antennal lobe region of the brain, which is far away from the ocellar tract injection site, located on the top of the honey bee brain. The antennal lobe region is where octopamine alpha receptor is involved in appetitive learning and memory (Farooqui, 2007; Rein et al., 2013) , so we suspected this might be a crucial region of the brain to be able to reach with the CRISPR-Cas9 system. For the processing of sensory perception, we know that this occurs in the mushroom bodies of the honey bee brain (Kim et al., 2013) and this region was also reached by the baculovirus vector. Together our findings demonstrate a successful knockdown of *AmOctβ2* gene expression in the honey bee brain three days after injection and by doing so, we revealed its functional role in appetite regulation.

Overall, there was a decreasing trend of appetite levels from the CRISPR injected bees across the three days after injections and there was dramatic effect of lowering appetite levels in comparison to the PBS and GFP injected controls on day three post injection, taken together this suggests that the maximum effect of the CRISPR-Cas9 treatment was on day three post injection. The fact that we observed significantly lower gene expression of *AmOctβ2* in the CRISPR-Cas9 injected bees versus the PBS injected controls on day three post injection further supports this notion. We suggest that the octopamine beta subtype 2 receptor is involved in appetite regulation of the honey bee which is likely to be independent of the glucose-signaling pathway found in vertebrates (Ghanem et al., In Review). Supporting this, the counter-regulatory role of octopamine in glucose deficit has been established where an increase in glucose concentration of hemolymph was not observed neither in hungry nor in sated bees, which emphasizes the presence of an alternative pathway acting on appetite independently from glucose (Buckemüller et al., 2017).

The CRISPR treated bees also had significantly lower appetite versus the PBS+Sorbose treatment on day two post injection, but not on day three post injection. We believe that this is due to the toxic cumulative effect of sorbose that was somewhat mediated by the injection of the CRISPR-Cas9. On each day, starting on day two post injection, we injected them with

sorbose which is known to act within about 10 minutes after injection. Therefore, on day two post injection this treatment elevated appetite levels significantly higher than the CRISPR treated bees and controls as expected (Akulku et al 2021), but on the third day due to the toxic effect the bees were too weak to extend their proboscis even though they may have been hungry. On day three post injection it appears that the CRISPR treatment has possibly mediated this toxic effect somewhat and these bees continued to have higher appetite levels in comparison to the CRISPR treated bees on day three post injection. Neither the PBS injection nor the GFP injection resulted in significantly different appetite levels over the three days, which supports the idea that CRISPR alone is the acting agent responsible for the lowered appetite observed. The higher appetite levels on day three post injection for the CRISPR + Sorbose injected bees versus the CRISPR injected bees suggests that there may be other pathways or octopamine receptor subtypes involved in appetite regulation, stemming from a lowering of trehalose levels, besides the pathway mediated by octopamine beta receptor subtype two. Causal relationships were established between starvation, hemolymph sugar levels, octopamine, and change in the appetite of the foragers (Mayack et al., 2019). Our results support the role of octopamine in the appetite regulation because we found that with the knockdown of *AmOctB2* the honey bee's appetite response was significantly decreased. Construction of the same baculovirus plasmid could assist in the knockdown of the other octopamine B receptor isoforms. Alternative pathways of the glucose-signaling pathway can be examined in the future with the help of technological breakthroughs in imaging as well as genome engineering, which include whole-brain functional imaging, electron microscopy, multigene delivery constructs and inducible gene editing systems (Lin et al., 2019; Winding et al., 2023). These methods enable tracking of the gene editing systems and mapping of the receptors on the honey bee brain.

Studies measuring different conditions of appetitive behavior, the proboscis extension, and different feeding or starvation conditions, aid in understanding the factors involved in the effects of starvation. Such research also aids in answering questions such as how the nervous system senses requirements of the body and this impacts external stimuli that communicate with the internal state of individuals. Organisms with a true brain share the common function of the brain as a sensor of nutrients and the changing levels of neurochemicals. Then the activity of those forms the central and peripheral neural circuits, inputs, and outputs of which can be changed temporarily and reversibly by a number of environmental factors (Lin et al., 2019).

Starvation in *Drosophila* has been shown to induce hyperactivity and foraging behavior. Response to starvation was required and was sufficient in the presence of neurons releasing octopamine. Energy regulation is claimed to be regulated by the central nervous system and octopamine as the neural substrate that links metabolic state of the organism with the specific behavioral change (Yang et al., 2015). There is evidence in *Drosophila* that the biogenic amines octopamine and tyramine play a key role in modulating metabolic processes and behaviors, which are fundamental to meet physiological needs. Octopamine-deficient flies showed increased body fat as well as reduced physical activity and lifespan, which emphasizes the central role of octopamine in balancing metabolic modes. These findings support the notion that the analogs of octopamine and tyramine, which are adrenaline and noradrenaline, are candidates of signaling systems associated with metabolic disorders such as obesity or diabetes in humans (Li et al., 2016).

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