Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Medicine and Health Sciences
Kavli Institute for Systems Neuroscience

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Volatile Relationships

Signal interactions of CO_2 and plant odorants in the primary olfactory processing center of the moth *Helicoverpa armigera*

Master's thesis in Neuroscience
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Abstract

Carbon dioxide is known to be an important molecule in many insects' sensory worlds, and the moth *H. armigera* is no exception. This study investigates the signal interactions between CO_2 and plant odorants in one distinct population of projection neurons in the primary processing center of the moth brain. This involved the use of calcium imaging to record neuronal activity in the glomeruli of the antennal lobes to compare calcium signal response strength to two different plant odor mixtures with and without additional stimulation with 1% CO_2. For improved results, neural labelling methods were validated with confocal imaging, and patterns of adaptation to both plant odor mixtures at a normal atmospheric level of CO_2 were observed before the full experiment was conducted. The results of this study show suppressed plant-odor signals during exposure to an elevated level of CO_2, suggesting that CO_2 may play a role in modulating such information processing. This also suggests that the primary and accessory odor pathways in the moth brain have intricate interactions that may be contributing to their precise navigation skills.

Abbreviations

AC – Anterior cell body cluster

ALT – Antennal-lobe tract

AL – Antennal-lobe

Ca – Calyx

CNS – Central nervous system

dALT – Dorsal antennal-lobe tract

dmALT – Dorsomedial antennal-lobe tract

GNG – Gnathal ganglion

lALT – Lateral antennal-lobe tract

LC – Lateral cell body cluster

LH – Lateral horn

LN – Local neuron

LPO – Labial-palp pit organ

LPOG – Labial palp pit organ glomerulus

mALT – Medial antennal-lobe tract

MB – Mushroom body

MC – Medial cell body cluster

MGC – Macroglomerular complex

mlALT – Mediolateral antennal-lobe tract

OB – Olfactory bulb

OSN – Olfactory sensory neuron

PN – Projection neuron

PVOC – Plant volatile organic compounds

tALT – Transverse antennal-lobe tract

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1. Introduction

Chemosensation – the ability of an organism to sense chemicals in its environment – is the first of the sensory systems to have developed in evolutionary history. Since then, the basic principles of smell and taste have been remarkably well-conserved. Many organisms express the same or similar genes in their chemosensing neurons (Poncelet & Shimeld, 2020). Given the absolute importance of the ability to detect chemicals and behave accordingly, to the point that it is universally retained across all organisms even when sight and sound devolves (Stoddart, 1980), it is not so surprising that the olfactory and gustatory systems of invertebrates and vertebrates have more similarities than differences.

The role of olfaction in an invertebrate is, like in a vertebrate, an essential tool for survival. While a human does not have the same sensory organs as an invertebrate, there are olfactory structures in invertebrates that are homologous to structures found in humans. Research on a highly conserved system with many parallels to the systems found in other organisms, despite even the convergent evolution that took place, has the tremendous benefit of being applicable and translatable across a diverse range of species. Studying the neuroethology of any organism has inherent value, of course, but it is also important to realize that what is learned about olfaction in a relatively simple invertebrate can be applied to a wide range of vertebrates too.

There is also an increasing demand for research on invertebrates such as insects due to the ongoing climate crisis. Climate change is having a significant impact on animal biodiversity across the globe (Dirzo et al., 2014), and a major concern is the rate of loss in insect biomass, which has been reported as high as 75% in certain areas of Germany in the span of less than thirty years (Hallmann et al., 2017). Other recent studies have shown more ambiguous results regarding both rate of loss and causation, particularly in that anthropogenic drivers other than climate change such as habitat loss seem to be more detrimental currently

(van Klink et al., 2020). Nevertheless, the decline in insect populations is a salient topic that calls for continued attention. Agriculture is reliant on insects, which makes a deeper understanding of insect sensation and behavior fundamentally important for food production. Insects play diverse roles in the lives of plants – usually as beneficial pollinators, but sometimes as detrimental pests – and studying these organisms is intrinsically valuable to the agricultural industry.

There are additional gains to be had, too. The study of the heliothine moth *Helicoverpa armigera*, a global pest insect comprising a subfamily of polyphagous and globally invasive noctuid moths, helps not only to further the development of less harmful pest control methods, but also to give a deeper insight into the evolution of olfaction and the impacts of rising levels of atmospheric CO_2.

Despite its relevance and importance, CO_2 signaling is not well-studied in the moth brain. Therefore, this research could be valuable to further investigations on signal interactions between CO_2 and plant odors within insect brains, and it could give new insights into the signal interactions that occur in the more complex brains of mammalians.

1.1. Odor Detection in the Moth Olfactory System

Many of a moth's behaviors are guided by the chemical cues in their dynamic, odor-rich environments. There are several functions an odorant could fulfill – for example, they could trigger repulsion or attraction, or they could induce dispersion or aggregation. The sheer diversity of odorants a moth encounters necessitates three specialized olfactory subsystems: one dedicated to plant odors, one dedicated to pheromones, and one dedicated to CO_2. Most biologically relevant odorants are transmitted by plants or other conspecific or heterospecific insect species, and these two subsystems are most pertinent when discussing the primary

olfactory system. Description of the third subsystem, the CO_2 sensitive "accessory olfactory system", can be found in a later section.

Plant-insect communication is primarily achieved through the release of plant volatile organic compounds (PVOCs). Plant volatiles typically consist of a plant's primary and/or secondary metabolites (de Bruyne & Baker, 2008). Because of the tremendous diversity found among plant life, a high sensitivity and specificity to these volatiles is required of the moths that are reliant on plants as food sources and hosts. Biologically relevant PVOCs have been identified to allow researchers to stimulate moths with applicable plant odor blends, and heliothine moths are notably receptive to a broad band of plant odorants, including remarkable sensitivity to terpenoids (Røstelien et al., 2005; Bruce et al. 2005). This may help to explain the success of some of these moths as prolific agricultural pests.

A powerful example of moth-to-moth communication, meanwhile, is the male sensitivity to female pheromone blends. Heliothine moths are noted to be particularly sensitive and selective to sex pheromones (de Bruyne & Baker, 2008; Berg & Mustaparta, 1995). A male moth can detect the pheromones of a female moth across significant distances, and not only that, but discriminate between pheromones from a conspecific or heterospecific female and respond accordingly (reviewed by Zhang et al., 2015). The female-produced pheromone will cause attraction of conspecific males, but at the same time repel a heterospecific male. Notably, male moths have specialized trichoid sensilla that detect compounds released by female moths (Kaissling, 2019). Some research supports the existence of autodetection, or the ability of a female moth to detect the conspecific pheromones emitted by other females, based on behavioral changes during or after exposure (reviewed by Holdcraft et al., 2016). However, it is still largely assumed that the pheromone subsystem is more characteristic of the male moth.

1.1.1. Anatomical Organization

Periphery

In all Lepidopterans, odor detection starts with their antennae, which serve as their primary structures of peripheral olfaction. Antennae across different families and subfamilies of moths are morphologically diverse, but a common denominator is the arrangement of porous, innervated hairs that can be found on them. These hairs are called olfactory sensilla, and while they are chiefly associated with the antennae, they are also located on other areas of a moth's body. Sensilla in Lepidopterans similarly have been observed to take on different shapes, sizes, and quantities, and multiple morphologies are commonly found on a single antenna with a meaningful distribution (Flower & Helson et al., 1974; Hallberg et al., 1994). All sensilla share the primary purpose of housing olfactory sensory neurons (OSNs). Once an odor molecule, which is typically fat-soluble, passes through one of the pores on the antenna, odorant binding proteins (OBPs) carry it through the water-based sensillum lymph (Altner & Prillinger, 1980; Steinbrech, 1996).

As previously mentioned, heliothine moths have evolved to maximize their detection of different plant volatiles and discriminate between conspecific and heterospecific pheromones. A great advantage with studying these moths is that many biologically relevant odors – not only pheromones, but also plant odors – have been identified (reviewed by Mustaparta, 2002; Zhao et al., 2014). Thorough studies using single-cell recording linked to gas chromatography have shown that OSNs tuned to plant odorants are narrowly tuned and respond to one key compound in a plume (Røstelien et al., 2005; Stranden et al., 2003). Likewise, the specialized OSNs of the male moth are narrowly tuned to detect precise sex pheromone compounds (de Bruyne & Baker, 2008). Typically, an OSN in a heliothine moth will have a primary odorant that a strong preference is shown for and some related odorants

that evoke weaker responses (Røstelien et al., 2005). This tuning is determined by the olfactory receptor protein expressed by a given OSN (Vosshall et al., 2000). When odorants are detected, the OSNs send the information through their axons, forming antennal nerves that ipsilaterally transmit information to the antennal lobes (ALs).

Antennal Lobes

The ALs are prominent spherical structures that serve as the primary olfactory center in the insect brain. They are homologous to the olfactory bulbs (OBs) in mammalian brains (Hansson, 2000), with activity in the AL demonstrating a clear link to olfactory behavior (Kuebler et al., 2012). As with the OBs, the ALs have a chemotopic organization characterizing this sensory system (Vosshall et al., 2000, Korsching, 2002). This means that OSNs expressing the same type of odor receptor, which are distributed throughout the antenna, send their sensory axons to one (or two) spherical structures in the ALs called glomeruli (Vosshall et al., 2000). Thus, each AL glomerulus seems to represent one distinct molecular stimulus. Like in other noctuid moths, the ALs in *H. armigera* are organized into ca. seventy-nine glomeruli (Anton & Homberg, 1999, Zhao et al., 2016), each representing different primary odorants. Generally, glomeruli that receive signals from OSNs tuned to PVOCs are called ordinary glomeruli, while signals from sources other than plants terminate in glomeruli specialized for this kind of sensory input. One example is the macroglomerular complex (MGC) in the male moth. Despite its naming convention, not all the glomeruli in the AL receive input from the antennae. Lepidopterans have a labial palp pit organ glomerulus (LPOG) dedicated to CO_2 sensory processing (Kent et al., 1986), which will be discussed more in-depth in the next section.

Notably, there is a prominent sexual dimorphism in the ALs of Lepidopterans – a male moth possesses an MGC that processes signals evoked by female sex pheromones (Berg et al., 2014). In *H. armigera*, the MGC is composed of three different glomeruli (Skiri et al., 2005), each of which is connected to male-specific OSNs tuned to female-produced compounds. Meanwhile, a female moth has a complex with one enlarged glomerulus called the female-specific complex (Fc; Zhao et al., 2016; Ian et al., 2022). This structural difference reflects the stereotypical role of the female moth as the emitter of sex pheromones and the male moth as the receiver.

The AL contains three main neuron types. These can be categorized as 1) local interneurons (LNs), which are involved with internal signal processing inside the AL, 2) the projection neurons (PNs), which communicate to brain areas and neurons higher in the hierarchy, and 3) the centrifugal neurons (CNs), which send information to rather than from the AL in a top-down manner (Homberg, 1988; Kymre et al., 2021). The PNs are essential for sending signals to the outside of the AL, and they have been found to take on a diverse range of morphologies. Additionally, there are three cell body clusters in the AL: the lateral, medial, and anterior cell body clusters (LC, MC, AC) (Homberg, 1988; Kymre et al., 2021; Ian et al., 2016). LN somata are exclusively located in the LC and PN somata can be found in all the clusters, while CN somata are usually located in the protocerebrum (Homberg et al, 1988; Kymre et al., 2020).

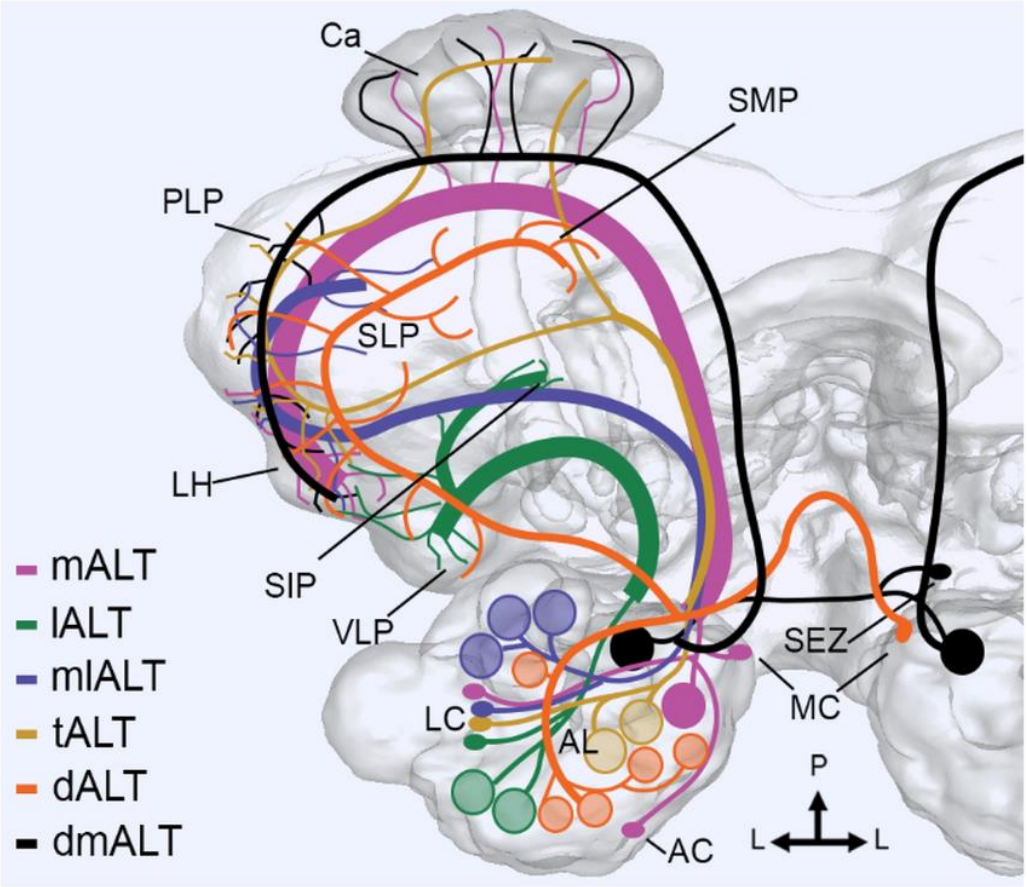
Antennal Lobe Tracts

Lepidopterans have several antennal-lobe tracts (ALTs; Fig. 1) that are responsible for sending information via their projection neurons from the antennal lobe to the higher centers of the brain. The tracts observed in *H. armigera* are the medial, lateral, mediolateral,

transverse, dorsal, and dorsomedial ALT (mALT; lALT; mlALT; tALT; dALT; dmALT; Homberg et al, 1988; Ian, Berg, Lillevoll, & Berg, 2016). It is here that the principle of divergence is seen as the ALTs communicate from the second to the third order of the sensory hierarchy. Investigations of the ALTs in heliothine moths have shown that the PNs directly targeting the calyces (Ca) of the mushroom bodies (MBs) – prominent structures responsible for learning and memory processes – originate primarily from the mALT, and only minor direct innervation connects the lALT and the tALT to this distinct region (Ian et al., 2016a).

Figure 1

An overview of the ALTs found in H. armigera



Note. Labeled are the medial, lateral, mediolateral, transverse, dorsal, and dorsomedial ALTs (mALT; lALT; mlALT; tALT; dALT; dmALT). Also labeled are the following brain regions: calyx (Ca), superior medial protocerebrum (SMP), superior lateral protocerebrum (SLP), superior intermediate protocerebrum (SIP), posteriorlateral protocerebrum (PLP), ventrolateral protocerebrum (VLP), subesophageal zone (SEZ), lateral horn (LH), antennal lobe (AL), anterior cell cluster (AC), medial cell cluster (MC), and lateral cell cluster (LC). Figure sourced from Kymre et al. (2021a).

The PNs of the ALTs are organized into two major roots within the AL: dorsal, which belongs exclusively to the mALT and has its somata housed in the MC, and ventral, which has its somata housed in the LC (Ian et al., 2016a). The mALT contains approximately 400 axons, yet it is more homogenous compared to other tracts, which tend to show a high PN diversity and have many other output areas in the protocerebrum (Homberg et al., 1988). The mALT seems to be the most pertinent of the tracts when focusing on the Cas and ALs, but all ALTs have some relevancy in the intricate moth brain olfactory hierarchy.

1.2 Detection of CO_2 in the Moth Accessory Olfactory System

Many insects use CO_2 as an important multifunctional cue. For lepidopteran insects, CO_2 is so essential that the labial palps – the part of their body associated capable of sensing that molecule – is considered fundamental to the taxonomy of their order (Kirstensen, 1984). The high sensitivity to CO_2 is achieved by the labial pit organ (LPO) housed within the labial palps of moths (Kent et al., 1986). The LPO has sensilla of its own, but – unlike the sensilla of the antennae – they are dedicated to the detection of CO_2. As is the case with olfactory sensation linked to the antennae, this detection communicates information about the insect's environment that will then modulate its most essential behaviors: specifically, locating food (Guerenstein & Hildebrand, 2008) and finding host plants for oviposition (Stange et al.,

1995). Emissions of CO_2 from plants are an indication of nutritious value. These signs can be utilized by moths of all ages, for example when young *H. armigera* moth larvae feed upon the most metabolically active parts of plants (Rasch & Rembold, 1994) and when the adult moth *Manduca sexta* finds freshly bloomed flowers emitting an elevated level of CO_2 for optimal nectar consumption (Thom et al., 2004).

The wide morphological variation across Lepidoptera suggests that the structure of the labial pit organ may correlate with the importance of CO_2 detection to the individual. For example, female and male *M. sexta* (Lepidoptera: Sphingidae) moths have large LPOs with no apparent sexual dimorphism in size (Kent et al., 1986), which was suggested by Guerenstein et al. (2003) to indicate a general importance of CO_2 detection to both sexes of moths belonging to that species. Additionally, Chen & Hua (2014) have proposed that the elongated, sexually dimorphic labial palps observed in the moth *Cactoblastis cactorum* (Lepidoptera: Pyralida) could assist with oviposition. Regardless of these variations, Lepidoptera is known to be universally sensitive to slight changes in CO_2 in their environment (Stange, 1992; KC, 2020) and responses to CO_2 are known to be consistent across the order (Bogner et al., 1986).

The LPO has receptor cells that, while traveling to the left and right ALs, create the labial palp nerve with their axons (Kent et al., 1986). Several distinct morphological types of projections have been observed in *H. armigera*: bilateral, unilateral, and contralateral, with the implication that contralateral is the weakest of the arrangements (KC et al., 2020). A divergence occurs at the level of the labial neuromere of the subesophageal ganglion (Kent et al., 1986) before the LPO connects with the AL glomerulus called the labial pit organ glomeruli (LPOG; Kent et al., 1986; Guerenstein et al., 2004). Importantly, this projection of the LPO to the LPOG is exclusive (KC et al., 2020). The LPOG was studied by Bogner er al. (1986), where it was noted that molecules with a high similarity to CO_2 such as CS_2 fail to

produce strong responses, and that CO_2 as a stimulus does not show many signs of adaptation as compared to ordinary odorants. This leads to the conclusion that the LPO receptor cells and/or LPOG are both highly selective and resilient to adaptation.

1.3 Imaging Techniques

The main research questions in this study are designed to be explored with the use of calcium imaging, with confocal imaging being used for additional questions and data points.

1.3.1 Calcium Imaging

Calcium imaging is a popular technique in neuroscience and similar fields that allows for indirect measurements of neuronal activity. It relies on the pervasiveness of calcium ions (Ca^{2+}) in molecular cell biology as an intracellular second messenger. Calcium signals are essential to the function of a neuron. The level of intracellular calcium ion concentrations is approximately 100nM while at rest, and neural activity can cause the level of Ca^{2+} to increase tenfold (Berridge et al., 2000). It is a broadly known fact that intracellular Ca^{2+} concentrations have a strong correlation to neural activity, and the relationship between calcium signaling and neural signaling means that, if one can monitor the calcium ion influx and efflux, the neural signaling taking place can be assumed.

This necessitated a means by which to visualize and measure calcium ion concentrations. In this study, fura-2 dextran – a ratiometric calcium indicator dye developed by Grynkiewics et al. (1985) – will be used. Ratiometric dyes such as fura-2 can be excited by two separate wavelengths of light. Depending on whether the dye has bound to a calcium ion or not, fura-2 was found to be maximally excited at 340nm and 380 nm (Grynkiewics et al. 1985). The dye is called ratiometric, or dual, because the relative emissions are calculated

to find the calcium concentrations. This is known to control for issues such as bleaching. In addition, fura-2 dextran is membrane-impermeable, allowing for a localized staining when undertaking neural circuit tracing procedures.

Using calcium imaging with fura-2 dextran is a valid methodology for this study as this combination can be used to give spatially and temporally high data consisting of quality calcium time traces. This method will also produce a larger amount of data when compared to single-neuron studies, providing an overview of the whole calcium dynamic in the AL at the time of recording. Calcium imaging also pairs well with the olfactory coding mechanisms being studied, as a population-level investigation is appropriate when investigating signaling in a well-organized brain structure.

1.3.2 Confocal Imaging

Confocal imaging is a form of fluorescent microscopy that uses a pinhole to sharpen image resolution by blocking blurred, unfocused light. It allows for high-quality sectioned imaging. In studies on heliothine moths, it has been used in conjunction with single-cell intracellular stainings and mass stainings. This is an excellent way to gain high-resolution images on a microscale that are not limited to the surface level.

One of the great strengths of calcium imaging is its ability to be used in tandem with other methods. Many previous studies on olfaction in *H. armigera* employ a combination of electrophysiology, calcium imaging, and confocal imaging techniques to address a research question. This study will, like the previous ones conducted in this lab, take advantage of two imaging methods to better understand the staining protocol and relevant neuroanatomical details.

1.4 Aims

Chemical cues represent a significant part of most insects' sensory worlds. The current study will use *Helicoverpa armigera* (Lepidoptera: Noctuiade; Heliothinae) as the model organism to understand olfactory signal interactions in the central nervous system (CNS) due to *H. armigera*'s role as a pest and previous usage as a research subject for olfactory processing. The aim of the study is to investigate a potential interaction between CO_2 and plant odor signals in the primary processing center of olfaction in the moth brain. Calcium imaging and confocal imaging will be used with adult *H. armigera* with several more specific goals in mind.

Specific aims

1. To learn the staining protocols for successful PN labelling.
2. To use calcium imaging measurements of the primary olfactory center to investigate the putative strength of adaptation to repeated plant odors stimulations.
3. To use calcium imaging to investigate the potential signal interactions during signal processing of plant odor blends concurrently with CO_2.
4. To use confocal imaging to validate fura-2 labelling methods and visualize retrograde staining of relevant ALTs.
5. To run and interpret data analyses of time traces.

2. Methodology

2.1 Insects

For all parts of this experiment, male and female adult virgin *H. armigera* (Lepidoptera: Noctuiade; Heliothinae) were used 3 to 5 days after emergence from their pupal stages. Pupae were delivered from Andermatt Group AG (Grossdietwil, Switzerland). The pupae were

sorted by sex and segregated in two climate-controlled chambers which were kept at 24°C and ~80% humidity. Adults were moved into cylindrical containers with *ad libitum* access to 10% sucrose. To avoid space-related stressors, larger containers (approx. 20 x 12 cm) held a maximum of eight moths and smaller containers (approx. 20 x 10 cm) held a maximum of five. According to Norwegian animal welfare regulations, there are no restrictions regarding the experimental use of Lepidoptera. Nonetheless, care was taken to follow good animal husbandry and limit unnecessary harm to the moths.

2.2 Staining

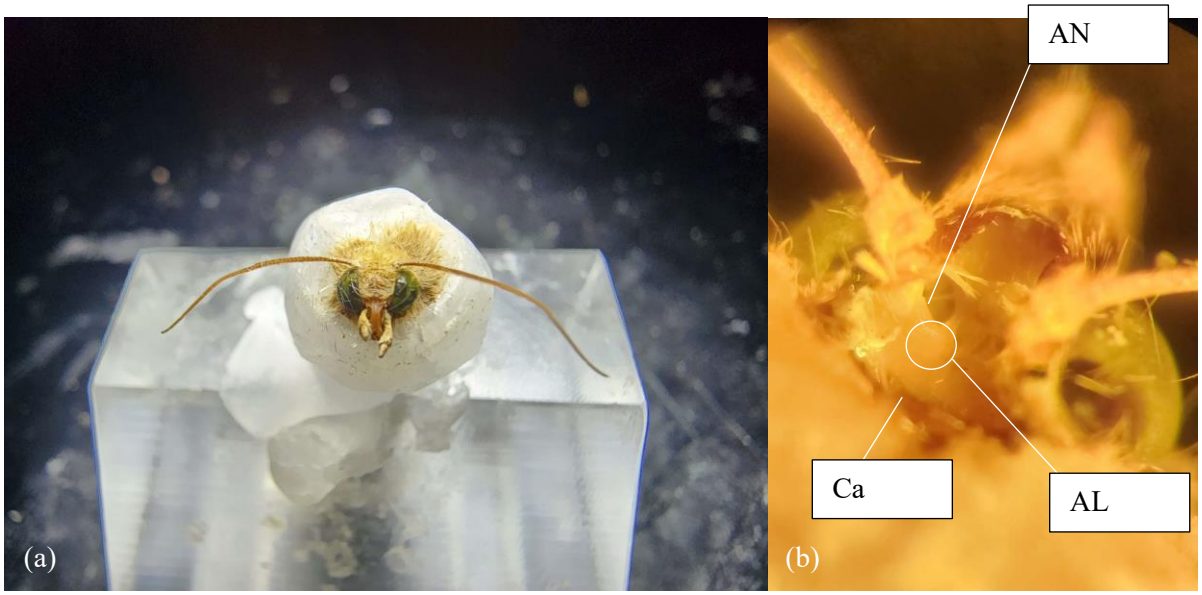
Moths were restrained in plastic tubes and fixed in place with wax (Kerr, Utility Wax Rods Round) beneath a stereo microscope (Figure 2a; Leica M60 Series, 1.0x objective). The head and proboscises were immobilized. Stability of the head ensures ease of access to the calyces, accuracy during dye application, and clearer images during calcium imaging. The labial palps were left uncovered to ensure the labial pit organs would be able to detect CO_2. Additionally, it was essential to the experiment that antennae remained undamaged and able to detect odorants. After careful removal of the head scales with wet tissue paper and forceps, a small opening was cut into the backs of the head capsules with a scalpel to expose the dorso-posterior part of the brain – particularly, the calyces. Trachea and fine membrane were gently removed with fine forceps. Two small crystals of calcium indicator dye (Fura dextran, Potassium Salt, 10,000 MW, Anionic; Invitrogen) were applied to each calyx using sharp electrodes, which were made with a Model-97 micropipette puller (Sutter Instrument Co., USA).

Once the staining procedures were performed, the prepared moths were stored in moist opaque boxes with a ringer solution (in mM: 150 NaCl, 3 CaCl2, 2 KCl, 25 sucrose,

and 10 N-tris (hydroxymethyl)-methyl-2-amino-ethanesulfonic acid, pH 6.9) covering their brains. They were left to sit for either approximately 4 hours at room temperature or approximately ten hours overnight in a refrigerator set to ~5 °C. After an appropriate time window, the insects were removed from their boxes and a larger opening was made in the cuticles of their heads to expose the ALs, done under a yellow light filter to minimize light exposure to the indicator dye (Figure 2b).

Figure 2.

Moths in different stages of preparation



Note. (a) A partially wax-fixed moth. (b) A moth with most of the brain exposed, including the calyces (Ca), antennal nerve (AN), and antennal lobes (AL).

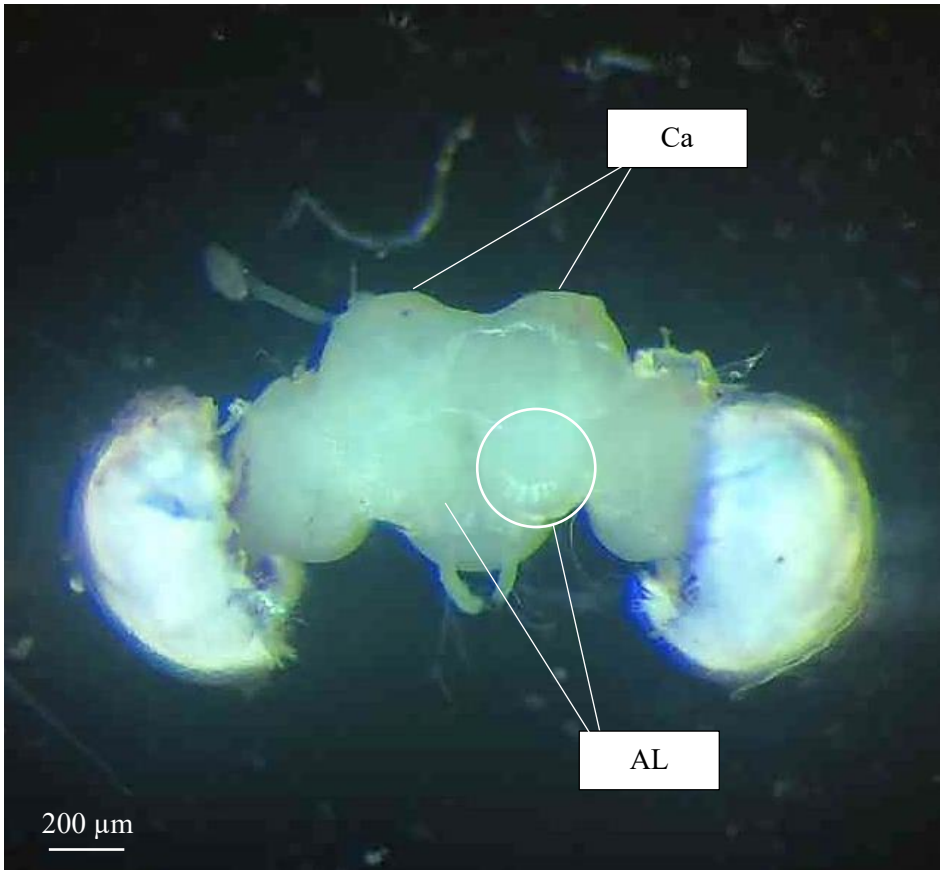
2.2.1 Validation of Staining

To better visualize the anatomical structure of the brain and the previously described staining technique, several brains were stained in an identical manner using a sharp electrode tip with

small micro-Ruby (Dextran, Tetramethylrhodamine and biotin, 3000 MW, Lysine Fixable; Invitrogen) instead of fura-2. Brains were dehydrated using an increasing ethanol series (50%, 70%, 90%, 100%, and 100%) with ten minutes for each series. Afterwards, the brains were mounted on slides for imaging (Fig. 3).

Figure 3.

A dissected moth brain



Note. Dissected moth brain. The calyces (Cas) and antennal lobes (AL) are indicated.

The prepared brains were imaged using a confocal laser scanning microscope (LSM 800, Zeiss, Jena, Germany). The following objectives were equipped: C-Apochromat

10x/0.45 water objective, C-Apochromat 10x/0.3 air objective, and Plan-Neofluar 20x/0.5 air objective. These were used while scanning each brain with a HeNe laser at 561 nm. Emitted light was filtered through a 560 to 600 nm band pass filter. For all confocal scans, serial optical sections with a resolution of 1024 × 1024 pixels were obtained at 1.5–9 μm intervals through the entire depth of brain. The confocal images shown in this study were edited in ZEN 2.3 (blue edition, Carl Zeiss Microscopy GmbH, Jena, Germany). Small adjustments were made to improve the quality of the images.

2.3 Calcium Imaging

Each moth was positioned anterodorsally beneath the microscope objective with Ringer solution covering the brain and the objective. An Olympus BX51WI epiflourescent microscope equipped with a 20X Olympus XLUMPLFLN Objective (1.00 NA, 2.0 mm WD) and a Hamamatsu CMOS camera (ORCA-Flash 4.0 V2 C11440-22CU) was used for this process. An LED system was used for the light source, which was equipped with a 340/380 nm LED (Omicron-Laserage Laserprodukte GmbH, Rodgau-Dudenhofen, Germany). The setup had a 410 nm short-pass filter and a 410 nm dichroic mirror as well as a 440 nm long-pass filter for the emitted light. Wavelengths 340 nm and 380 nm were used together for the excitation light at regular intervals, while wavelengths of approximately 505 to 520 nm were used at maximum for the emission light. Separation of the emission and excitation light was achieved with additional dichroic mirrors and emission filters (490-530). One AL at a time was brought into focus. First, the AL was monitored for any visible spontaneous activity, and then the stimulations began. Recordings were done via Live Acquisition V2.6.0.35 (TILL Photonics) with a TILL Photonics imaging control unit. The binning size for all images was 4x4. Irrespective of the stimuli being applied, all recordings lasted 10 second with 100 ms per frame, resulting in a total of 100 frames per recording. The stimulation window onset was at

the 3 second mark and lasted for 2 seconds, or 200 ms. All stimulations were performed 60 seconds apart.

2.3.1 Plant Odor Repetition

Plant odor mixtures ADE and CDE, displayed in Table 1, were chosen based on the results of an unpublished pilot study conducted by the same laboratory that the present research has taken place in (Table 1) wherein mixtures ADE and CDE reliably evoked strong calcium signals. Additionally, CO_2 was theorized to have suppressive responses that would emphasize the difference between similar odor coding, and so ADE and CDE's overlapping components were desirable to test that supposition. The outcome is to be addressed in the discussion section. The two unique components, A (methyl benzoate) and B (geraniol), are both floral odorants, with the former being a notable emission from snapdragon plants (Dudareva et al., 2000), and the latter being a key component in rose oil (Xiao et al., 2017). As a final note, the biological relevance of these odors was established by Røstelien et al. (2005), and all stimuli presented are guaranteed to be reliable attractants for *H. armigera*.

The first stimulation protocol performed were the plant odor repetition trials (Table 2). Moths under the microscope were exposed to a two-second puff of air within the ten-second experiment window via a stimulation tube attached to the stimulus controller (SYNTECH Stimulus Air Controller Type CS-55). At the end of the tube was a stimulus vial placed approx. 3 centimeters away from the target antenna. These were created by pipetting 20 µl of plant odor code ADE or plant odor code CDE onto small pieces of filter paper inside glass vials, and they were sealed with parafilm (Bemis) when not in use. All odorants and solvents were acquired from Sigma-Aldrich (Missouri, US). Each had a final cumulative concentration of 0.1%.

Table 1.

Exact components of plant odor mixtures.

Code	Solvent	Stimulus
ADE	Mineral oil	Methyl benzoate (aromatic compound) trans-Pinocarveol (Bicyclic MT) Caryophyllene oxide (Sesquiterpenes)
CDE	Mineral oil	Geraniol (Monoterpenes) trans-Pinocarveol (Bicyclic MT) Caryophyllene oxide (Sesquiterpenes)

Table 2

Experimental design for plant odor repetitions.

Plant Odor	Repetitions				
ADE	ADE	ADE	ADE	ADE	ADE
	ADE	ADE	ADE	ADE	ADE
CDE	CDE	CDE	CDE	CDE	CDE
	CDE	CDE	CDE	CDE	CDE

2.3.2 CO_2-Plant Odor Pairing

The second stimulation protocol was performed similarly, but with a different combination of stimulants (Table 3). For the first two repetitions, moths were exposed to a pairing of CO_2 (1% CO_2, Linde) and either ADE or CDE. To ensure CO_2 was present in the tube for these stimulations, the stimulus controller was run twice once the CO_2 tube was introduced to the setup. The stimulus tube was pointed away from the insect to avoid unnecessary exposure or adaptation. The procedure was otherwise identical to the repetitions. To make sure CO_2 was not lingering in the tubes for the next three stimulations – which consisted of three post-CO_2 exposure plant odor stimulations with no CO_2 pairing – the stimulus controller was again run twice after disconnecting the CO_2 line. The plant odor was kept consistent throughout each set of five (only using CDE or ADE for the full experiment).

Table 3

Experimental design for plant odor repetitions during and after exposure to CO_2.

Plant Odor	**CO_2 %**	**Repetitions**				
ADE	1% CO_2	CO_2+ADE	CO_2+ADE	ADE	ADE	ADE
CDE	1% CO_2	CO_2+CDE	CO_2+CDE	CDE	CDE	CDE

2.4 Analysis

The recordings obtained from the calcium imaging procedures were exported from the TILL Photonics software and imported into PyVIEW, a Python-based open-source software developed by Kumaraswamy et al. (2023) for the specific purpose of simple, user-friendly analysis of calcium imaging data. There, the raw data was examined, during which ROIs

marking potential individual glomeruli were created by hand. The average relative change in fluorescence of each ROI was saved in CSV files. A baseline shift to zero was performed using Microsoft Excel by subtracting the mean of the frames recording spontaneous activity from the frames falling within the stimulus window to normalize the stimulation window data. SPSS was used to perform statistical analyses on the stimulation window data stored in the CSV files – specifically, repeated measure ANOVAs were run with repetitions as the levels and stimulation as the measure. The variable of response strength was assumed to follow normal distribution. The protocol was arranged and then compared as a three-factor repeated measures ANOVA (Table 4).

Table 4

Three-Factor Repeated Measures ANOVA

Between Factor: ***Plant Odor***	Between Factor: ***CO_2 Protocol***	Within Factor**: *Repetition***				
ADE	Protocol 1 (1% CO_2)	CO_2+ADE	CO_2+ADE	ADE	ADE	ADE
	Protocol 2 (Atmospheric Level [0.04% CO_2])	ADE	ADE	ADE	ADE	ADE
CDE	Protocol 1 (1% CO_2)	CO_2+CDE	CO_2+CDE	CDE	CDE	CDE
	Protocol 2 (Atmospheric Level [0.04% CO_2])	CDE	CDE	CDE	CDE	CDE

Note. Here, the between-factors were plant odor (ADE or CDE) and CO_2 Protocol (application of 1% CO2 or no application of CO_2 resulting in normal atmospheric level).

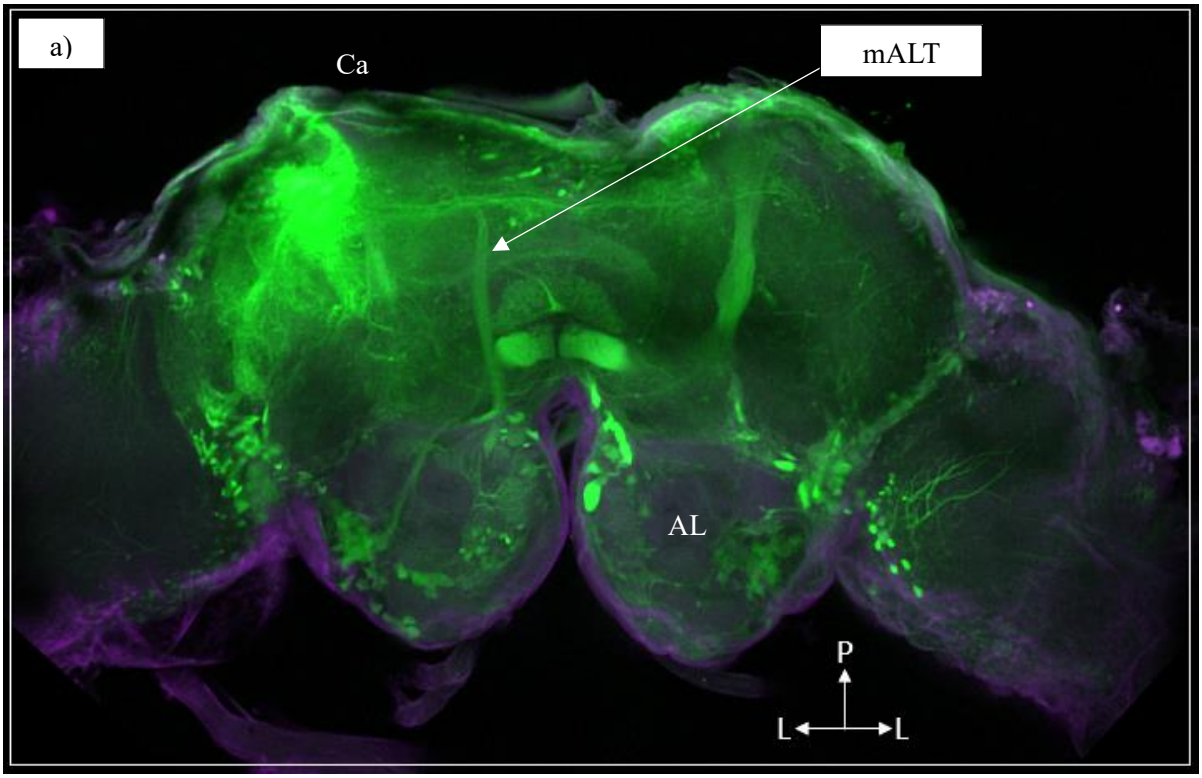
3. Results

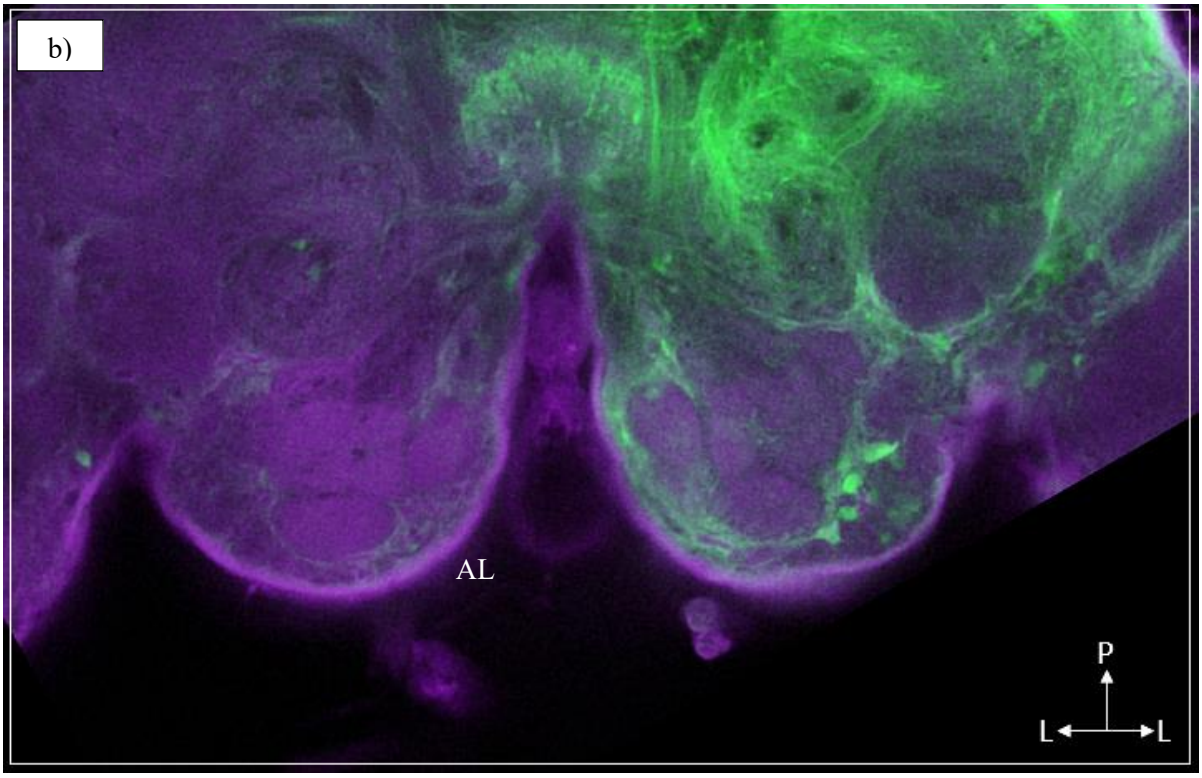
3.1 Labelling of medial antennal lobe tract

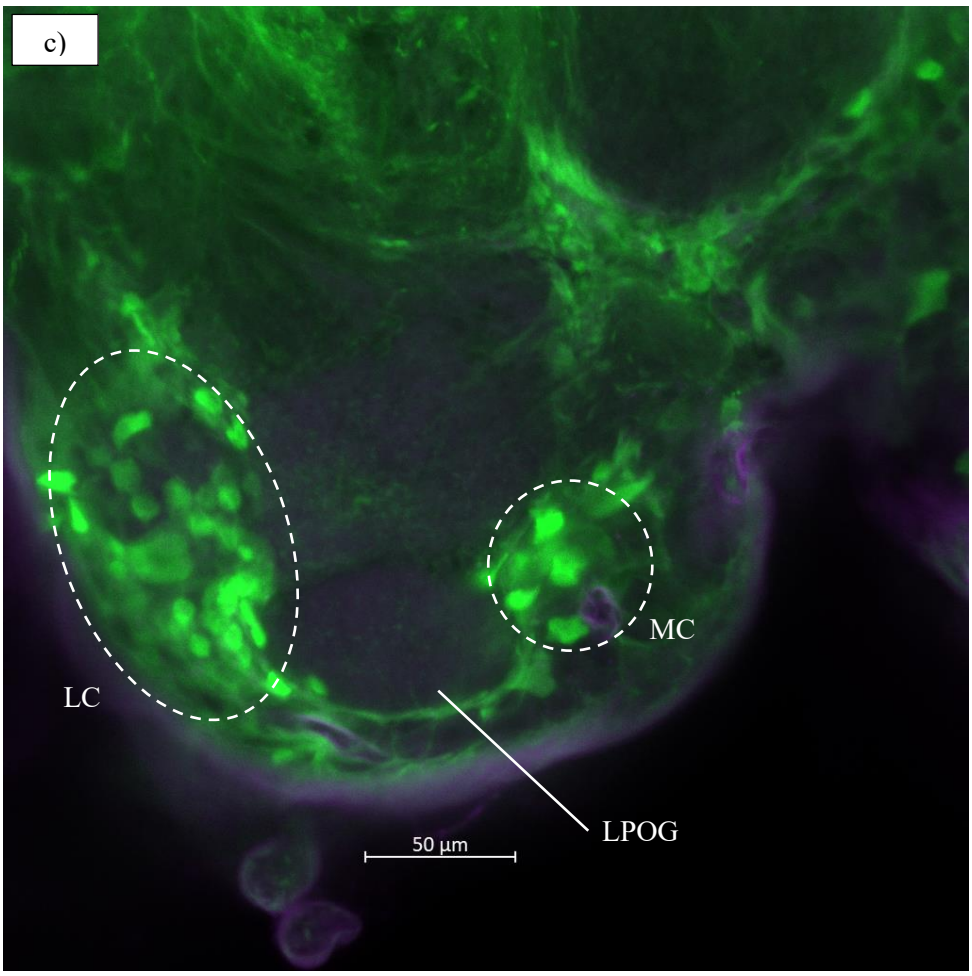
To perform retrograde labelling of the AL, calcium indicator dye fura-2 was applied to the calyces of moth brains and then imaged (Fig. 4). While the relevance of the mALT has already been discussed previously, it should be emphasized that a major advantage of dye application in the calyces is that most neurons stained by this technique are the axons of the uniglomerular AL PNs that form the mALT. The strong connectivity between the calyces and the ALs is what allows for such successful labelling. Calcium imaging is highly dependent on neural circuit tracing success, thus the need to validate the labelling methodology and ensure that the correct neurons are being stained.

Figure 4.

Labelling of mALT and validation of PN staining




b)
AL
P
L
L


c)
LC
MC
LPOG
50 µm

Note. (a) The medial antennal-lobe tract (mALT) is highly visible, indicated by the green pathways the dye takes from the calyces to the antennal lobes. This is indicative of a good staining. (b) A comparison of one successful staining (right hemisphere, saturated by green fluorescence) and one unsuccessful staining (left hemisphere, weak green saturation). The unsuccessful staining was a result of missing the calyx while applying dye. (c) Images of projection neuron somata in the antennal lobe. Labeling of the medial cell cluster (MC) and the lateral cell cluster (LC) further validates the sampling strategy as these clusters have been shown to contain mALT PNs (Homberg et al., 1988). Also, lack of staining in the labial palp pit organ glomerulus (LPOG) can be considered an additional validation of a successful mALT staining from the calyces.

Though there is strong connectivity allowing for this method to work, mastery of the technique remains important. If dye is not applied in an exact manner, the PN axons terminating at the calyces are unlikely to receive enough – if any – dye, and the AL will not be successfully labelled.

3.2 Repetitive Exposure to an Odor May Depreciate Calcium Signal

It has been firmly established that synaptic plasticity allows for adaptation during repeated exposure to a given stimulus. Since measuring odor-evoked responses during exposure to CO_2 involves repeated exposure to the same plant volatiles, which could cause adaptation, the first test conducted in this study was to determine the effect of repeated exposure to the same plant odorants without CO_2. Under these parameters, to further understand the putative adaptation pattern of repetitive plant odor stimulations, the calcium traces of AL PNs responsive to 10 repeated exposures of the same plant odor blend were obtained. Each of two plant odor blends with partially overlapping components were also applied so that the data consisted of repetitions of either 10 ADE or 10 CDE stimulations. The glomerular activities

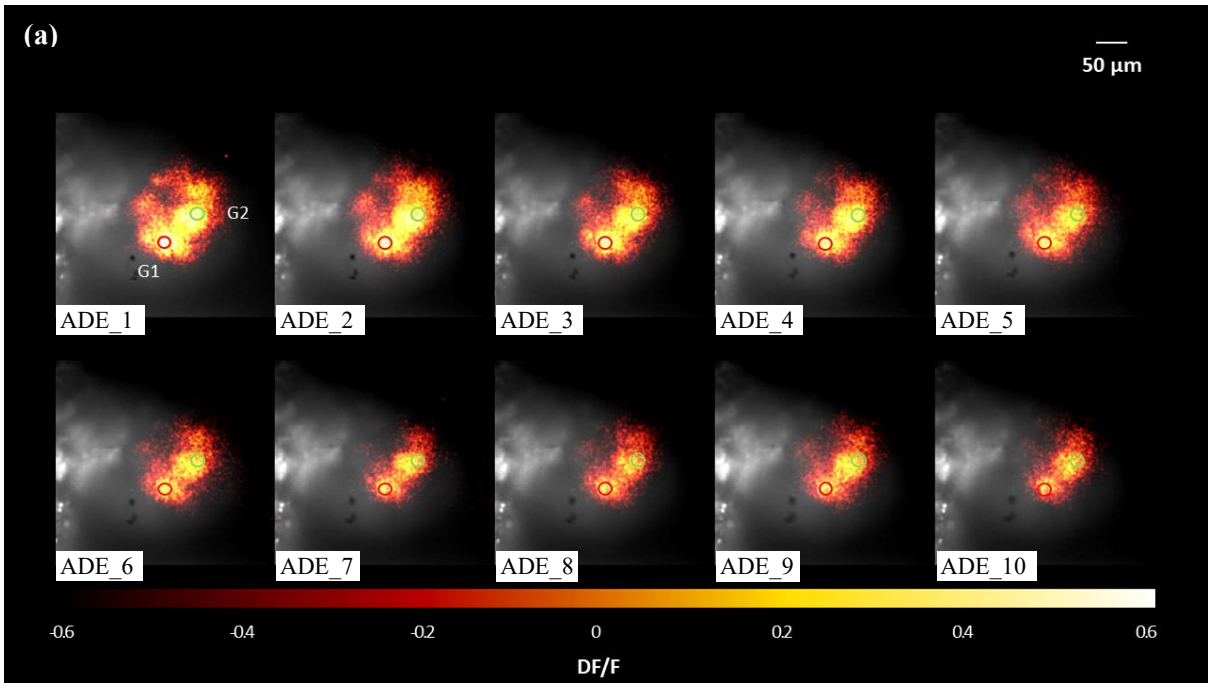
evoked by ADE and by CDE were analyzed first independently of each other and then together (n = 11 for both *CO_2 Protocol 1* groups combined).

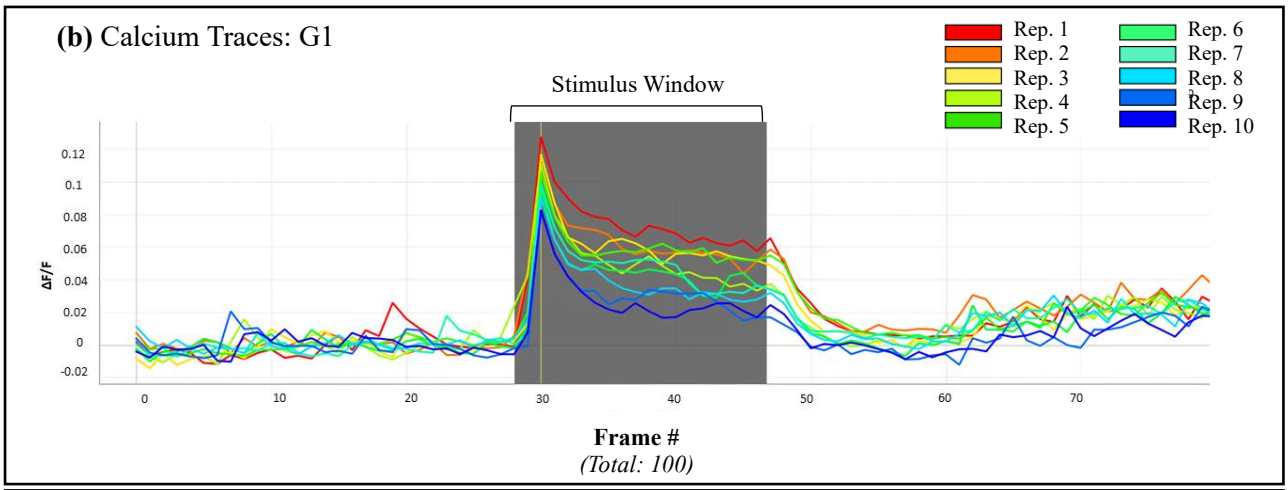
3.2.1 Example of adaptation to a single plant odor blend

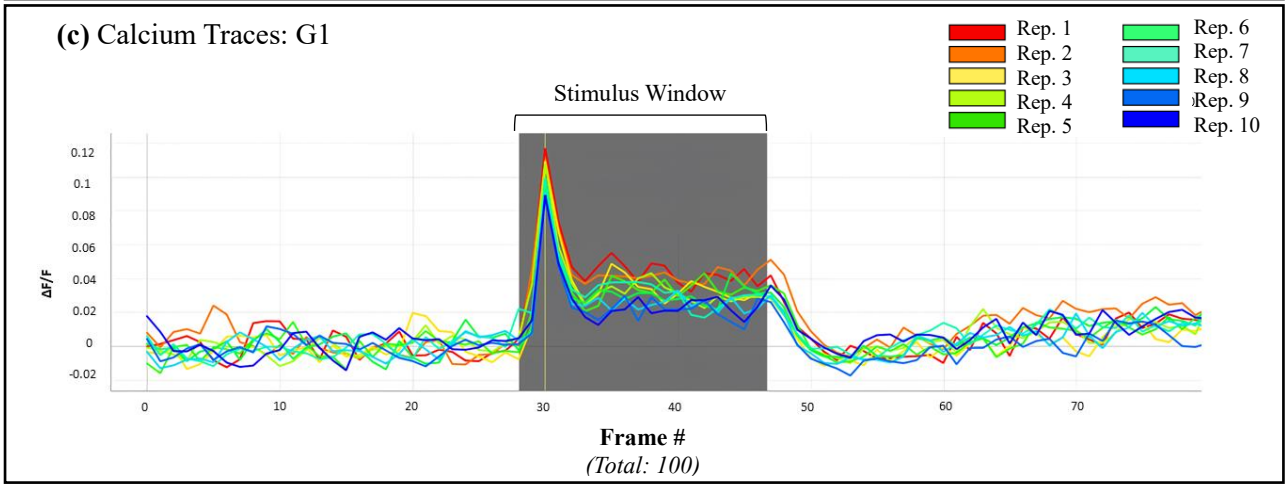
To evaluate plant odor blend ADE specifically (n = 5), 10 repetitions of ADE stimulations were analyzed for five different responsive glomeruli pooled across separate insects. The calcium traces were collected for a total period of 10 seconds, with a 3-second pre-stimulation, 2-second stimulation, and 5-second poststimulation period. There was an additional interstimulus interval of 1 minute. Within the AL presented, two glomeruli in the anterodorsal position exhibited clear activation. The imaged Ca^{2+} in glomerulus 1 (G1; marked red in Fig. 5A) showed a phasic tonic response, decreasing steadily after the initial stimulus. Meanwhile, the intracellular Ca^{2+} in glomerulus 2 (G2; marked green in Fig. 5A) decreases sharply after the spike. Variability is otherwise low. An example of the calcium traces during repetitive antennal stimulation with plant odor blend ADE is illustrated in Figure 5B and 5C. This data is presented only to provide a visual illustration of the calcium traces collected. Afterwards, the exact process used for the ADE stimulations was repeated for CDE (n = 6), either continuing with the same insect or switching to a new one depending on the condition of the insect's brain. The same AL was used whenever reasonably possible. In the given calcium traces, there appears to be a tendency of adaptation, but traces alone are not enough to decide. Further analysis is needed before drawing any conclusions.

Figure 5.

Example of repetitive exposure to ADE







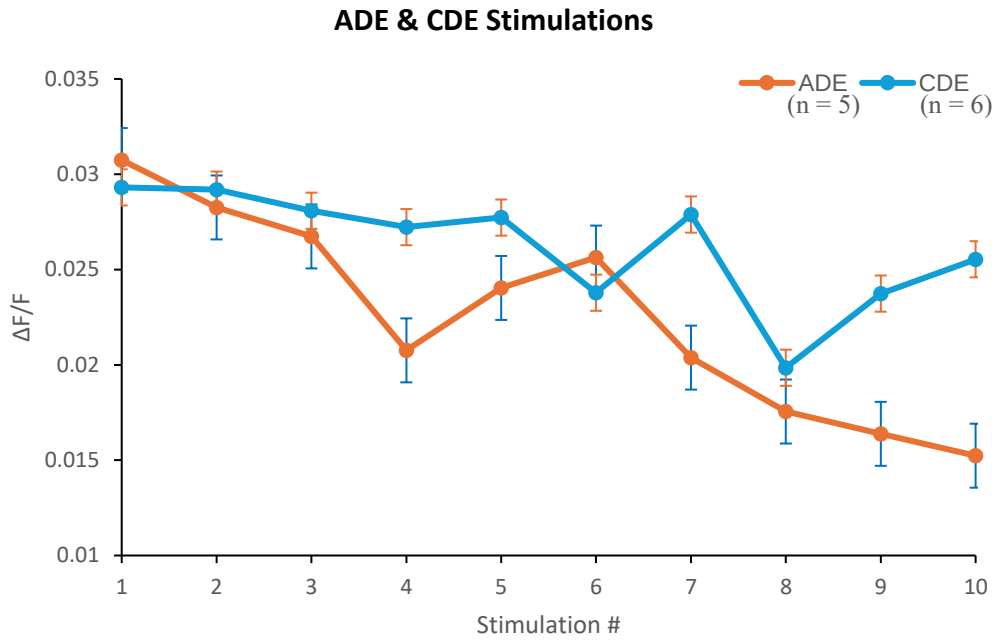
Note. (a) Antennal lobe heat maps showing the response pattern evoked by repeated exposure to ADE. (b-c) Individual calcium traces showing the ADE-evoked responses over the course of ten stimulations. Each graph corresponds to one ROI. The stimulus window duration is two seconds. Repetitions are labelled by line color, color-coded in chronological order.

3.2.2 Adaptation patterns during repetitive odor stimuli

The response amplitude of each glomerulus was computed by using the mean Ca^{2+} signal during the stimulation window subtracted by the corresponding data recorded during the pre-stimulation window. The response amplitudes were compared using a two-factor repeated measures ANOVA with one within-subject factor (*repetitions*) and one between-subject factor (*plant odor*). As demonstrated in Figure 5, analysis of the data gathered from the 10 repetitive stimulations showed a reduction in neuronal activity during repeated exposures ($F_{9,81} = 7.03$, $p < 0.001$, $\eta^2 = 0.11$). The post hoc analysis showed that, compared to the first trial, the adaptation to the stimuli occurred from the 4th trial (ps < 0.05). The adaptation pattern between plant odor mixtures (between-subject factor) showed no difference ($F_{1,9} = 0.35$, $p = 0.57$). Although the interaction of *plant odor* by *repetitions* was significant ($F_{9,81} = 2.06$, $p = 0.04$), the confidence interval showed no significance between these two factors across trials (ps > 0.05). Despite some individual repetition-by-repetition variation, repeated exposure to ADE and to CDE can be assumed to evoke similar adaptation patterns (Fig. 6).

Figure 6.

Comparison of responses during repeated ADE and CDE-stimulations



Note. This figure displays a reduction in ΔF/F across repetitive stimulations of plant odor mixtures ADE and CDE. This reduction is indicative of adaptation. The data is presented as the means of their respective groups (ADE or CDE), and the error bars represent the standard error.

3.3 Elevated levels of CO_2 effect plant odor signaling

To highlight the effect of CO_2 without interference from the adaptation observed in the first part of the experiment, CO_2 was applied only during the first two stimulations when there was no chance of adaptation occurring. Subsequent stimulations would consist only of plant odor blends at a normal atmospheric level of CO_2. With these modifications made, the second part of the experiment was run to test whether CO_2 showed any effect on the perception of plant odor on the projection neuron level, data from a total of 36 glomeruli from 5 individual insects were collected due to their responsiveness to the given stimuli. Since adaptation was

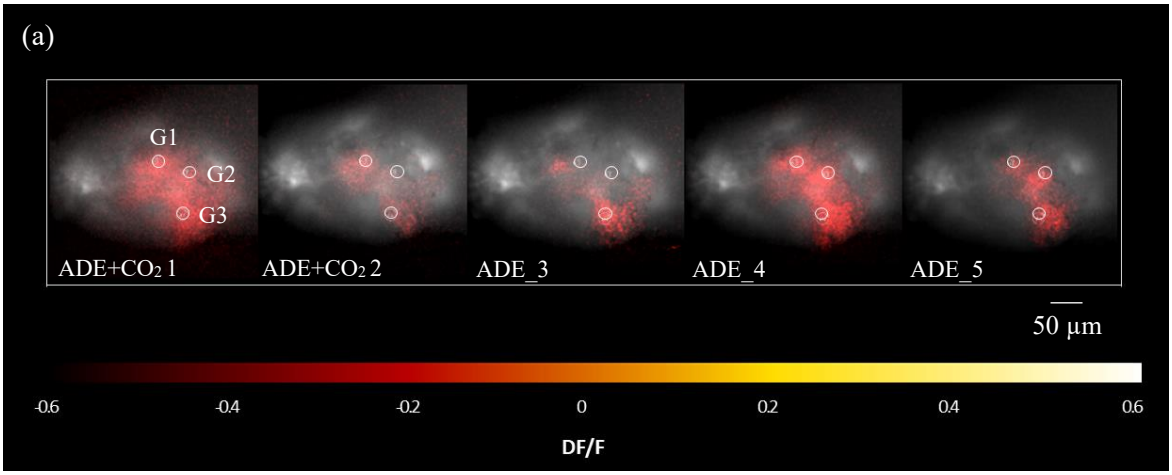
found to begin at the 4th repetition of odor stimuli, the data collection and analysis in this section was designed to comprise just 5 repetitions instead of 10.

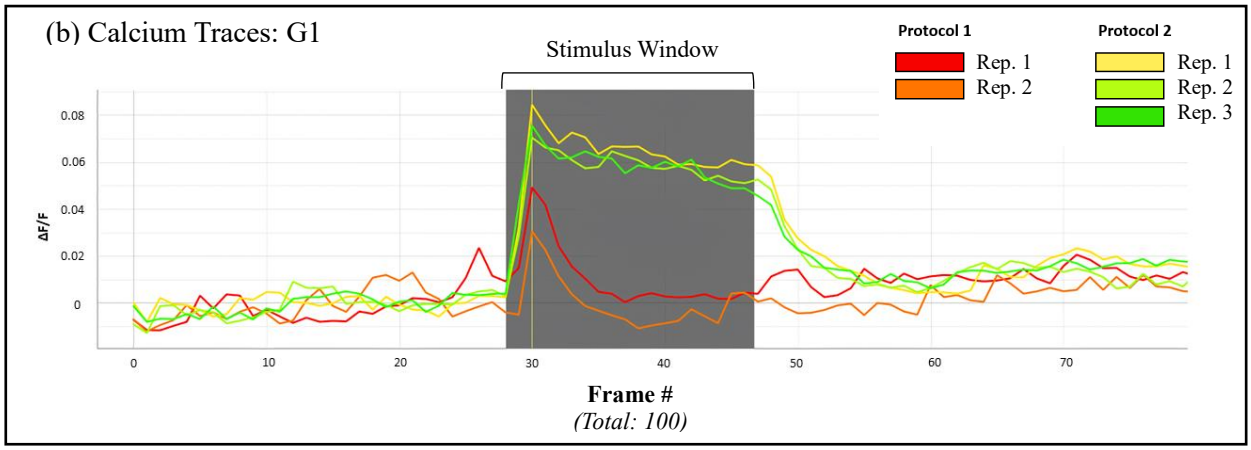
3.3.1 Example of projection neuron activity during CO_2 stimulation protocols

The data was collected when the insect was exposed to one of the two stimulation protocols: *CO_2 Protocol 1*, which included two air puffs with 1% CO_2 + a plant odor mixture followed by three plant odor puffs (n = 25), and *CO_2 Protocol 2*, which included five repetitions of a plant odor mixture alone at a normal atmospheric level of CO_2 (n = 11). For more details on these protocols, see Tables 3-4. One example of the data is shown in Figure 7. The figure illustrates heat maps of the antennal lobe of one insect with three glomeruli responding to the relevant stimuli (Fig. 7A) and the resulting calcium traces for one of the three glomeruli, G1 (Fig. 7B). As before, this is only meant to provide a visual illustration of what the processed data looked like. There is a tendency of suppression in the calcium traces but, again, further analysis is needed to confirm if an effect is present.

Figure 7.

Effects of CO_2 on plant odor signaling





Note. (a) Antennal lobe heat maps showing the response pattern in three glomeruli - G1, G2, and G3 - evoked by repeated exposure to ADE in two conditions. Repetitions 1 and 2 (two panels to the left) show the neurons' response to stimulation pairing ADE with CO_2 (Protocol 1) and repetitions 3-5 (three panels to the right) measure the same group of neurons' response to ADE alone (Protocol 2). (b) Individual calcium traces from one glomerulus, G1, showing the evoked responses to paired CO_2-ADE (red and orange trace) and ADE alone (dark green, light green, and yellow trace). The graph represents one ROI. The stimulus window duration is two seconds. Repetitions are labelled by line color, color coded chronologically.

3.3.2 Suppression observed during exposure to CO_2

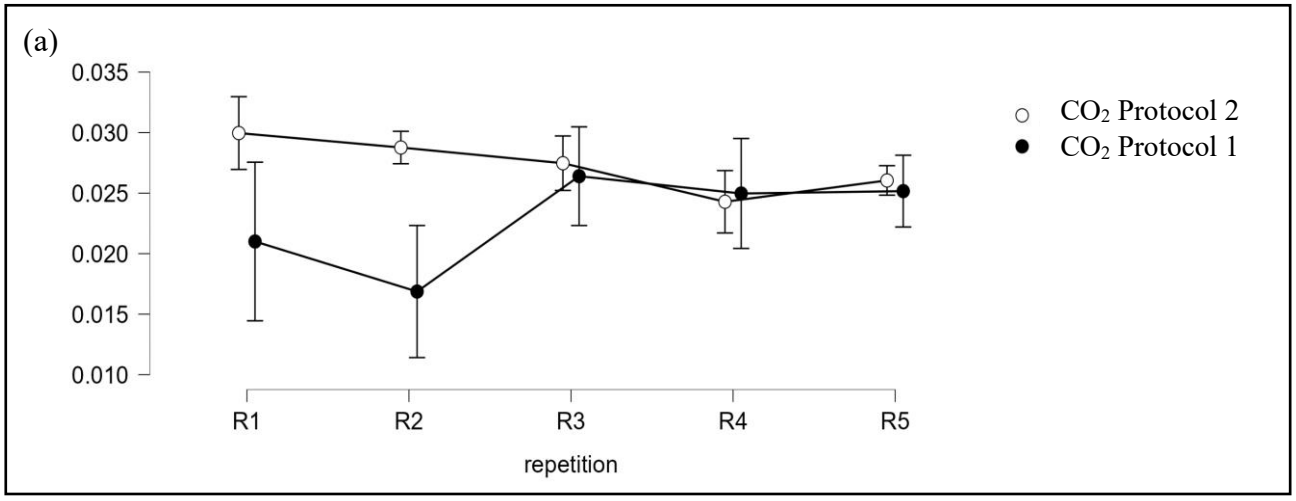
To analyze the data collected from the 36 glomeruli responding to the different antennal stimulation protocols, a three-factor repeated measures ANOVA test was run. The response amplitudes were compared to determine how three different factors affected the responses: one being repeated measures with one within-subject factor (*repetitions*) and two between-subject factors (*plant odor* and *CO_2 protocols* 1 and 2, respectively). Analysis of the data from the 5 trials, including each stimulation protocol, showed that the neuronal activity was not reduced during either of the two protocols ($F_{4,128} = 0.59$, $p = 0.67$). Neither '*plant odor*' nor 'CO_2 protocol' induced any change in response amplitude (plant odor: $F_{1,32} = 6.13$, $p = 0.44$; CO_2 protocol: $F_{1,32} = 0.76$, $p = 0.39$). Altogether, the data suggested that CO_2 did not

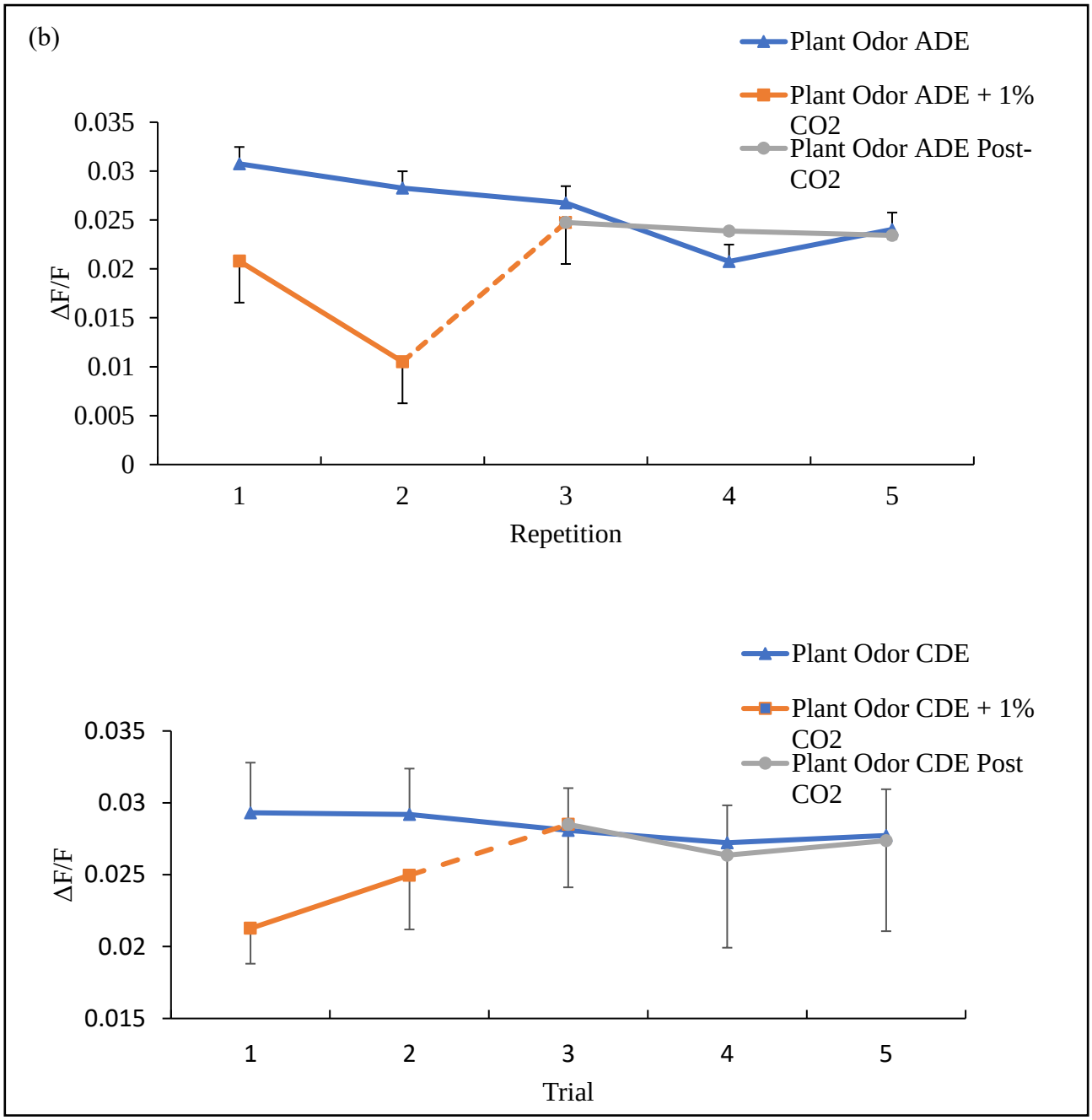
influence the responses to the plant odor stimulations. Interestingly, the interaction between *repetitions* x *CO_2 protocol* showed a tendency to be significant ($F_{4,128} = 2.29$, $p = 0.06$), and the effect only presented itself at the first two repetitions which were the CO_2 and plant odor mixture pairings (Fig. 8). It can be concluded that there is no long-lasting effect of CO_2, necessitating further analysis into the potential short-term effects.

While CO_2 Protocol 2 was consistent with its stimulations across each repetition, resulting in all five repetitions consisting of an identical treatment of one air puff with a given plant odorant, CO_2 Protocol 1 applied two different treatments under the label of one treatment. This was intentionally done to limit the effect of adaptation and to have an opportunity to run post hoc analyses on plant odor blend stimulations during and after CO_2 application to test for potential long-term effects from elevated CO_2 exposure. This did, however, mean that more analyses were required to understand how the CO_2 and plant odor pairings behaved.

Figure 8.

Response strengths of combined plant odor blends during CO_2 protocols





Note. (a) The combined mean neural activity for ADE and CDE during CO_2 protocol 1 (black) and 2 (white). (b) Individual mean responses for CDE and ADE showing no long-term effect of elevated CO_2 exposure.

To test for the true effect of CO_2 without interference from the three repetitions consisting only of the given plant odor blend, two additional analyses were employed. First,

an analysis of the data from the first two repetitions was conducted (Fig. 9). Coinciding with the previous result finding adaptation only after trial 4, the new analyses revealed no adaptation between these two repetitions ($F_{1,32} = 1.41$, $p = 0.24$), nor any response difference between the two plant odorants, ADE and CDE ($F_{1,32} = 0.91$, $p = 0.35$). It did, however, reveal a clear effect of elevated CO_2 concentrations on plant odors signaling (*CO_2 protocol*: $F_{1,32} = 7.00$, $p = 0.01$, $\eta^2 = 0.14$). This indicates that a higher level of CO_2 influenced the neural responses of the PNs of the AL.

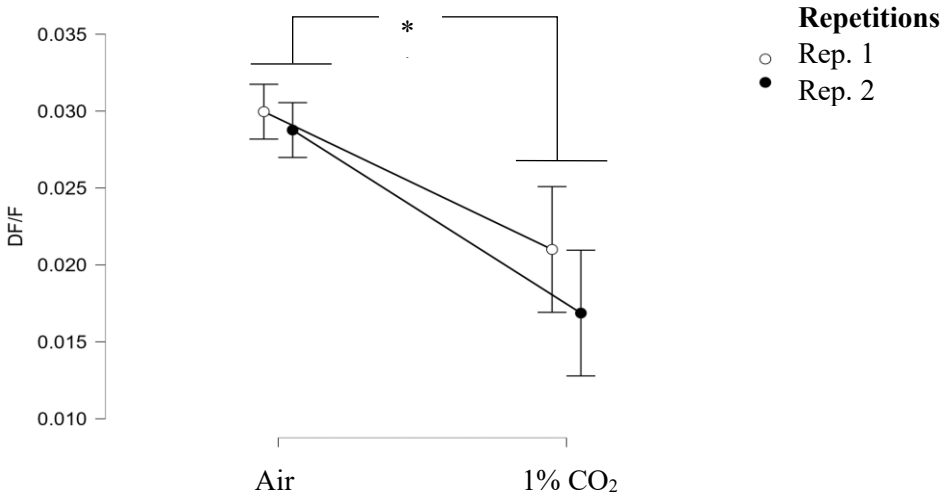
To confirm the CO_2 effect observed, a two-way repeated measures ANOVA was run by using only individual samples found in CO_2 Protocol 1 (n = 25), in which plant odors in both CO_2 conditions (1% vs. atmospheric level) were applied to the same insect. Here, the CO_2 effect is to be tested by a within-subject factor including 2 repetitions of a plant odor in 1% CO_2 followed by 3 repetitions of the same plant odor in air. The two plant odor mixtures, ADE and CDE, served as the between-subject factor. The results showed that there were significant differences in the responses across repetitions ($F_{4,92} = 2.46$, $p = 0.05$, $\eta^2 = 0.04$), i.e. potentially across CO2 conditions, whereas there were no significant differences across the plant odor mixtures ($F_{1,23} = 0.96$, $p = 0.34$).

Because the previous test found no significant difference between the plant odor mixtures, they were grouped together in the next analyses. A post-hoc contrast analysis demonstrated a highly significant contrast between the pair of first two repetitions with 1% CO_2 ($t(92) = 2.75$, $p < 0.01$) versus the pair of the third and fourth repetitions with air (Fig. 10B). On the other hand, no significant difference was observed within the first two stimulations ($t(92) = 0.99$, $p = 0.33$), neither within the third and fourth stimulation ($t(92) = 0.45$, $p = 0.66$). Overall, the data indicates a substantial difference in response strength from stimulating with 1% CO2 and plant odorants vs. ambient CO2 and plant odorants. Specifically, the response to the plant odor mixtures were substantially higher in the absence

of 1% CO2, which is particularly notable given the adaptation demonstrated in the repeated plant-odor protocol shown in the previous section.

Figure 9.

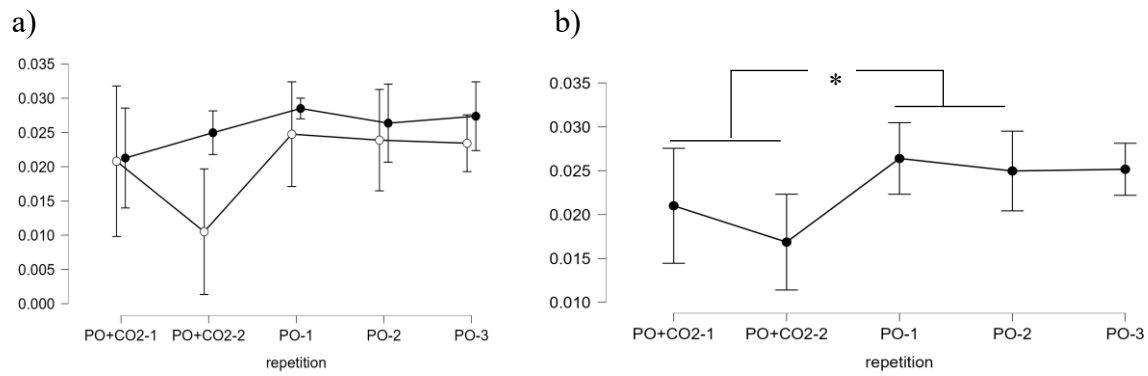
Potential CO_2 effect during the two first trials



Note. A new analysis with better temporal resolution, set up to highlight the short term – or, immediate – effect of CO_2. Suppression is evident when comparing the two pairs.

Figure 10.

Significant CO_2 effects found using contrast tests



Note. (a) ADE and CDE with CO_2 protocol 1 (white, ADE; black, CDE). (b) Contrast test. The means of plant odor blends with CO_2 protocol 1 are displayed, comparing the differences between data point pair 1 (repetitions 1-2) and data point pair 2 (3-4).

3.3.3 Individual effects of different plant odor mixtures during CO_2 protocols

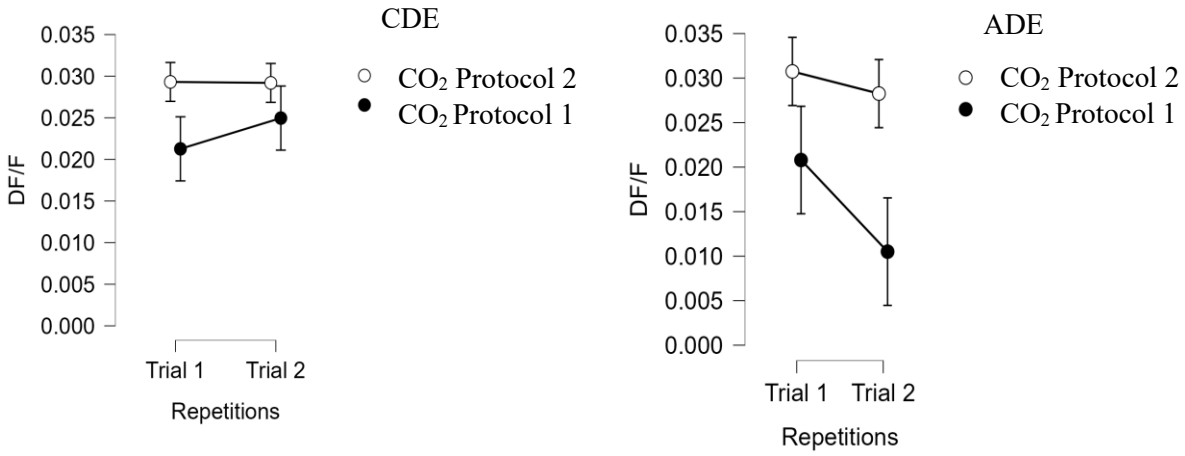
Thus far, the data has shown a general suppressive effect of CO_2 on plant odor processing in AL output neurons, and a general similarity between the plant odor mixtures' adaptation patterns and overall response to elevated CO_2 concentrations. However, based on the analysis cross comparing the data from the first two repetitions in both CO_2 protocols, there is an interaction effect between *repetitions* and *plant odor* ($F_{1,32} = 4.47$, $p = 0.04$, $\eta^2 = 0.02$). It appeared that each of the partially overlapped plant odor mixtures (with 67% identical components) influenced their adaptation pattern when there was an increased level of CO_2. This suggests that there may be a difference between ADE and CDE in the presence of 1% CO_2 after all.

Thus, an investigation of an exclusive selection of data comprising stimuli with 1% CO_2 alongside each of the two different plant odor mixtures (CO_2-ADE pairing: n=14; CO_2-CDE pairing: n = 11) was conducted. A two-factor repeated measure ANOVA was run to examine the putatively different results during 2 repetitions with 1% CO_2 as vehicle (within factor) when different plant odors (between factor) were applied. The outcomes showed no *repetition* effect ($F_{1,23} = 1.76$, $p = 0.20$), but an interesting plant odor effect ($F_{1,23} = 4.73$, $p = 0.04$, $\eta^2 = 0.11$) as well as an interaction of *repetition* x *plant odor* ($F_{1,23} = 7.92$, $p = 0.01$, $\eta^2 = 0.01$). This also suggests a difference in adaptation patterns between ADE and CDE during CO_2 protocol 1.

Another ANOVA analysis showed that the presence of CO_2 influenced the adaptation pattern differently in the two plant odor blends. The AL output neurons exhibited enhanced adaptation specifically to plant odor mixture ADE when CO_2 was introduced into the ambient environment, in contrast to when only air was present as the vehicle. As concerns the other plant odor blend, CDE, no such CO_2-induced change in adaptation was found (Figure 11). It can be concluded that adding CO_2 to both plant odor mixtures had only a short term effect on their adaptation pattern during repeated stimulations.

Figure 11.

Different patterns between protocols in CDE and ADE



Note. ADE is clearly showing more of a suppressive effect of CO_2 by itself. There is no significant change with CDE.

4. Discussion

This study investigated signal interactions occurring in the moth primary olfactory processing center during exposure to select plant odor blends under normal and elevated CO_2 conditions. Of particular interest was the possibility of CO_2 influencing glomerular activity during and

after exposure to a heightened concentration. While previous studies have shown behavioral changes under elevated levels of CO_2 (Rasch & Rembold, 1994; Stange et al., 1995; Thom et al., 2004; Guerenstein & Hildebrand, 2008), the precise effect and role of CO_2 in modulation of neural activity in the moth brain remains unknown. The data collected from the plant odor and CO_2 paired stimulations suggests that CO_2 may have a suppressive effect on plant odor evoked activity. Evidently, the increase of CO_2 during antennal stimulation with plant volatiles has a degree of effect on the calcium dynamics of the AL, which agrees with the body of literature showing a role of CO_2 in insect navigation and other behaviors.

4.1 Patterns of Adaptation

From the first experiments performed in this study, it was established that adaptation occurred during repeated exposure to a given plant odor blend. The adaptation was observed from the fourth repetition onward, and there was no significant difference between the adaptation patterns of the two partly similar plant odor blends utilized here. Meanwhile, no adaptation pattern could be established with CO_2 because the sensory neurons tuned to CO_2 have previously been shown not to adapt (Bogner et al., 1986). Olfactory adaptation is triggered by long-term exposure to ordinary odorants, especially at high concentrations, but not to sustained or repeated introduction to CO_2 (Bogner et al., 1986). This makes sense as a biological mechanism because CO_2 is omnipresent in the Earth's atmosphere, and if nocturnal moths using CO_2 to navigate to plants respiring CO_2 adapted to the constant presence of these molecules, then they would no longer be able to detect it. Responding to a change in concentration, however, allows a moth to be sensitive to minute changes. This is why only adaptation patterns for plant odor blends were of interest in the current investigation.

4.2 Paired PO-CO2 stimulation influenced the plant odor-evoked responses

Based on the results obtained, it was demonstrated that paired plant odor and CO_2 stimulation influenced the plant odor evoked responses in the AL output neurons, seemingly causing a suppressive effect. The issue of putative mechanisms underlying such inhibition is to be discussed.

4.2.1 Plausible Mechanisms of Suppression

Generally, three olfactory sub-arrangements constitute the olfactory systems of the moth brain: 1) the plant odor system, 2) the pheromone system, and 3) the CO_2 system. Interestingly, former studies by Ian et al. (2017) found that plant odorants and pheromones, when applied to the moth antenna together, will evoke long-term suppressed plant odor responses. This means that the suppressive effect of pheromones on plant odors lasted beyond the window of the paired stimulus. In contrast, the study carried out here found no long-term suppressive effect of CO_2. Despite this difference, the results from the present study can be said to be consistent with previous findings on CO_2 modulating neural responses to pheromones, reproductive behaviors, and feeding behaviors.

While the data in this study consists primarily of calcium traces from the AL, and that data has led to the conclusion that suppression was observed, that does not necessarily mean that the observed suppression must be happening within the AL exclusively. It is likely that there are mechanisms of suppression within the AL, but it is also possible that suppression mechanisms occur upstream of the recording site.

Suppression in the Antennal Lobe

The most likely site of suppression is within the AL. As mentioned in the introduction, a large number of LNs make local connections between AL glomeruli. Given that LNs play an inhibitory role in the AL network, these neurons are likely candidates for causing the suppression of plant odor signals. Notably, many of the LNs innervate the LPOG as well. In fact, a recent study in *H. armigera*, reported that among 67 morphologically identified LNs with normal multi-glomerular branching pattern, 45 displayed innervations in the LPOG (Kymre et al., 2021). The significant percentage of LPOG-innervating LNs indicate the importance of CO2 input for the local neural networks in the AL. The interneurons that connect the LPOG to other glomeruli would theoretically be able to inhibit the activity of plant-odor responding output neurons in ordinary glomeruli.

Suppression in the Periphery

As mentioned above, it is possible that suppression takes place upstream of the ALs as well. The effect observed might occur as soon as CO_2 molecules interact with the antennae of the moth. Since CO_2 is associated with a decrease in pH, it could be that a lower pH in the sensillum hemolymph influences the responding pattern in relevant first-order neurons identified by KC et al., 2020. Thus, a confirmational change at the very beginning of signal transduction might cause a cascading effect that would then be observed in the AL.

4.3 Possible Functions of Suppression

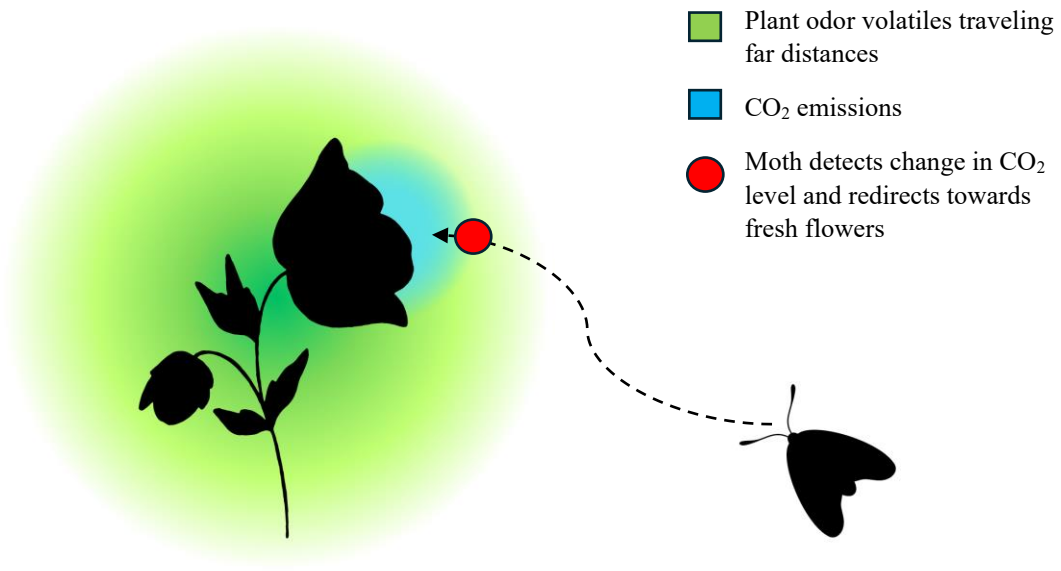
As previously mentioned in the methodology section, plant odor mixtures ADE and CDE were selected as they were known to evoke strong, reliable responses. Blends of plant odors are also more reflective of natural conditions, as it is rare to encounter one single, pure

odorant by itself. Two three-component mixtures with two overlapping components were also selected due to a hypothesis implying that a suppressive CO_2 effect, if present, would create a stronger contrast between two otherwise quite similar odor representations in the AL. However, a visual inspection of the heat maps and calcium traces across the ADE and CDE trials as well as the results of the majority of the statistical analyses show that these plant odor mixtures are not significantly different as regards the responses they induce. There are two limiting factors in trying to support the theory: 1) that the mixtures overlap too much (and that mixtures with only a small amount of overlap should have been chosen), and 2) that a 1% CO_2 concentration – quite high compared to the low percentage of CO_2 normally present in the air – is not strong enough to bring out the differences between the relevant response patterns. An alternative way to test the theory could be to use a variety of plant odor blends with different degrees of overlap or a 10% CO_2 concentration.

The suppression reflected in the findings of this study could represent a prioritization of high value signals. For example, a study in the mosquito showed that CO_2 is not only an attractant, but also a catalyst of optomotor responses that will then guide the mosquito to a host (Gillies 1980). This was later reviewed in the context of multimodal information interactions in insects which highlighted the importance of redundant information in sensory systems to maximize accuracy during navigation (Buehlmann et al., 2020). By the use of multimodal dues – in the case of a moth, signals from plant odor volatiles, conveyed via the primary olfactory pathway, and CO_2 via the accessory olfactory pathway – *H. armigera* could be creating some redundancy amongst their navigation cues and subsequently be receiving a cue to lock into the most important details relevant to their distance from a plant of interest. A simple example of how this might work is displayed in Figure 13.

Figure 13

CO_2-based navigation to quality flowers



Note. A figure showing the long-distance tracking of plant volatiles and the precise short-distance tracking of CO_2. This guidance system would allow a moth to locate the blooming flowers it needs while tuning out the plant odors it doesn't need. Image created with free Canva assets and Microsoft Office.

4.4 Methodological Considerations

The present study utilized neural circuit tracing and labelling as well as calcium and confocal imaging, which produced calcium signal data and neuroanatomical images of the moth brain and in particular the AL. The study design was set up to look for a CO_2 effect, but not for further exploration of this topic. Introducing CO_2 first and only twice was intended to avoid reduction of calcium signals in response to repetition of plant odor (which occurs after several repetitions), which could have been confused for reduction due to signal suppression.

4.4.1 Calcium imaging

Data from the present study consisted of fluorescence values recorded from projection neuron populations in the AL, which was representative of the calcium dynamics that took place in the AL during odor- and CO_2-evoked neuronal responses. Calcium imaging is a popular method of neuroscientific investigation thanks to its temporal accuracy, long duration, and data quantity, all of which greatly benefitted the data collection process. This imaging technique allowed for many repeated stimulations to be applied to the same insect over an extended period without signal loss if the insects remained in good physical condition. It also tracked neuronal responses in real-time, and resulted in large collections of glomerular activity across a population of PNs in the AL.

There are, however, some downsides to bear in mind while evaluating the study results. It is hard, if not impossible, to image specific glomeruli of interest. Additionally, only superficial glomeruli will be visible in recordings, as calcium imaging microscopy cannot be used for quality imaging of deeper structural layers. Only the general area of an AL that is visible to the camera will be recorded. Most of the work is done manually with educated guesses as to which angles are correct, and simply looking at the recordings does not give any information as to which glomeruli are active. Even in retrospect, data from this study cannot be used for accurate identification of which glomeruli and neurons were active.

It should also be considered that, while calcium flow correlates strongly with neural activity in a target area, the fluorescence was produced by a calcium-sensitive dye that is impermeable to cell membranes, and therefore labelled the medial-tract PNs specifically. The high specificity is a great advantage of this methodology, but it should also be said that the fluorescence only indicates which area has had the most flow when the increased levels of calcium ions occurred (the flow being what informs the greater dynamics). It does not show any activity occurring in local interneurons (which could be inhibiting projection neurons) or

the first-order neurons (where some of the inhibition could be occurring, for example if exposure to CO_2 causes a meaningful pH change).

4.4.2 Data Analysis

The study's main limitation is the low number of individual insects successfully stained and imaged within the narrow time window allotted to the present study's data collection stage. Consequently, the data displayed has low statistical power with a stronger likelihood of false positives. Oversimplified interpretations of findings are, however, always a concern in every study and has been factored into the study's interpretations.

Most of the statistical analyses for this study used a repeated measures ANOVA for testing. The data sets were complex and violated conditions of alternatives such as t-tests.

As a final note, the repeated measures design was overall appropriate for this study, but it did create some limitations. Since data collection in the CO_2 trials consisted of a protocol with five repetitions without elevated CO_2 and a protocol with three repetitions without elevated CO_2, the resulting data set was skewed towards CO_2 Protocol 2. This necessitated an array of post hoc tests to fully understand the effect of CO_2.

4.5 Further investigation

As mentioned, previous studies have shown that CO_2 is a key part of olfactory communication in many insects, and Lepidoptera is a powerful example. Results such as the ones found in this study may indicate the need to investigate the relationship between CO_2 and behaviors such as feeding, oviposition, and mating in greater depth to understand the nuances between different levels of CO_2 (high or low) and the behavioral effects observed.

Repetition studies looking further at CO_2 and plant odorant interactions would provide more insight into how these olfactory sub-systems function.

It has also been found that significantly increasing the level of CO_2 in the air not only leads to inhibition of pheromone-evoked signals in the brain of male *H. armigera*, but to a heightened release of pheromones in females (Choi et al., 2018). The current study used male and female moths, but the sample sizes are much too small for any meaningful comparisons to be made. A future study could address any potential sexually dimorphic suppressive effects of CO_2 on plant odor responses in male and female moths.

5. Conclusion

This study investigated the putative effect of elevated CO_2 levels on plant odor evoked responses in the moth brain. An experiment consisting of a test of adaptation and a test of two CO_2 treatments concluded that adaptation and suppression occurred in their respective experimental designs. Based on the results of this study, the following points were made:

I. Olfactory adaptation may begin at a fourth exposure to a given plant odor blend.
II. CO_2 may have a suppressive effect on plant odor responses.
III. The mechanism of suppression may be the interference of local interneurons in the antennal lobe.
IV. The suppressive effect of CO_2 may help fine-tune the navigation of a moth towards the freshest parts of plants.

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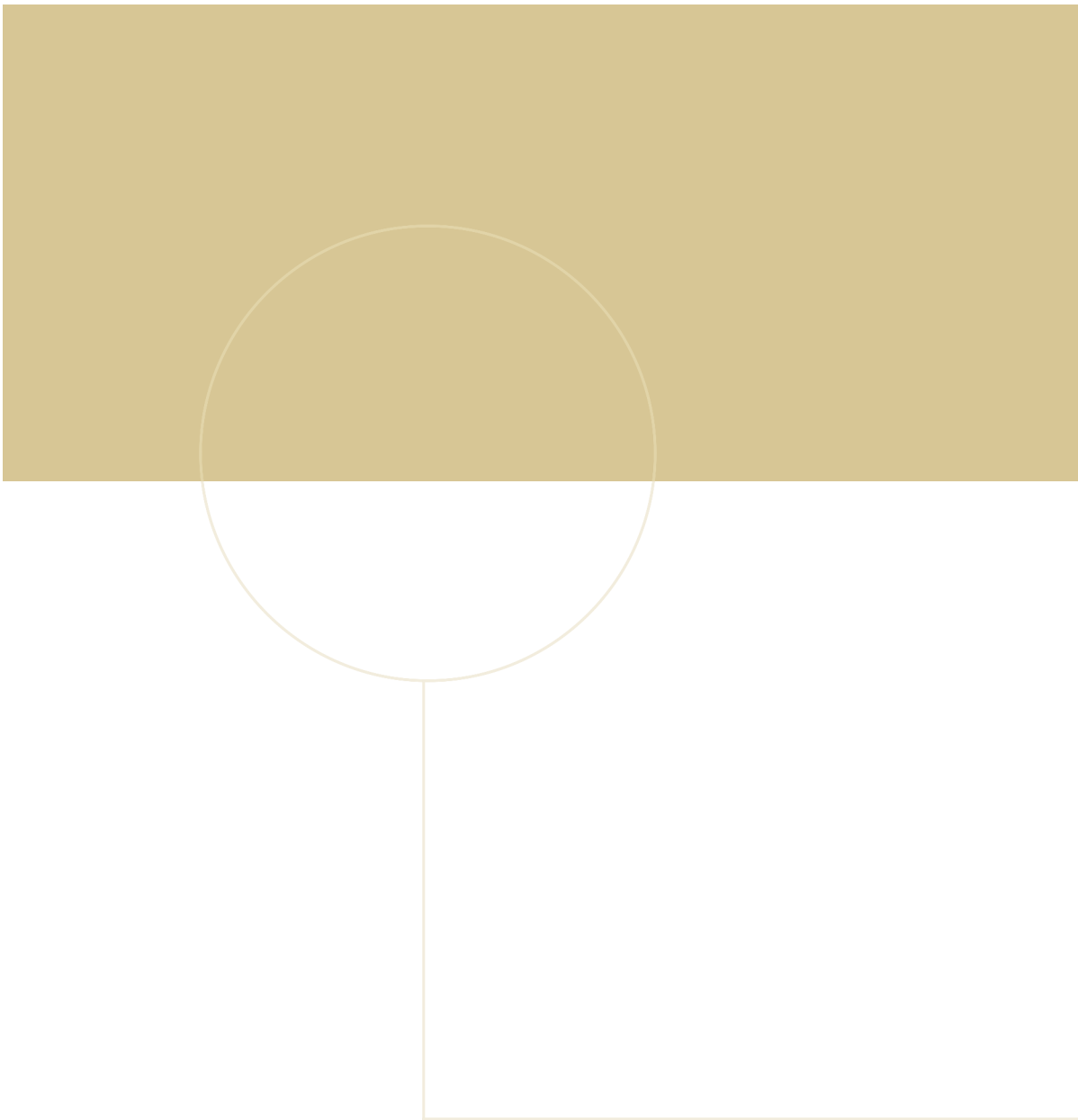

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