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Sex differences in olfaction – description of antennal-lobe neurons in male and female moths based on systematic neuronal labeling

Master's thesis in Psychology

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Abstract

The dimorphism of antennal lobes in the male and female moths has been known for a long time. So far, the majority of studies on the moth olfactory system has been more focused on the male. The knowledge of the female output neurons connecting the primary olfactory center to higher centers are largely undescribed. By mass staining a population of projection neurons in both sexes, we visualized the olfactory parallel processing pathways in the *Helicoverpa armigera.* To illustrate the anatomical details of individual neurons, the intracellular staining technique was performed. This pilot neuron collection in female was used to proceed a comparison with the corresponding neurons previously reported in male. The results revealed that the male and female moth share the similar anatomical structures in the main antennal-lobe tracts (ALTs) in the mass staining experiments. It is noteworthy that the all individual neurons we stained in this study, including four projection neurons and one centrifugal neuron, showed isomorphic patterns. Moreover, our data suggested, unlike the second-order pheromone neurons in male having different protocerebral projections from the plant-odor neurons, the output neurons innervating the female-specific antennal lobe glomeruli shared the output regions with those plant-odor neurons.

Sammendrag

Dimorfismen til antennelobene i hann- og hun nattsvermeren har vært kjent i lang tid. Så langt har flertallet av studier på møllens luktesystem vært mer fokusert på hannen. Kunnskapen om hun nattsvermerens output-nevroner som forbinder det primære lukte senteret til høyere sentre, er stort sett ikke beskrevet. Ved massefarging av en populasjon av projeksjonsnevroner i begge kjønn, visualiserte vi de parallelle lukt prosessbanene i *Helicoverpa armigera*. For å illustrere de anatomiske detaljene til individuelle nevroner ble den intracellulære fargeteknikken utført. Denne pilot-nevronsamlingen hos hun nattsvermeren ble brukt til å utføre en sammenligning med de tilsvarende nevronene som tidligere har vært rapportert hos hanner. Resultatene avslørte at han og hun nattsvermere har de samme anatomiske strukturene i hoved antennelobe traktene (ALT) i massefargingeksperimentene. Det er bemerkelsesverdig at alle individuelle nevroner vi farget i denne studien, inkludert fire projeksjonsneuroner og ett sentrifugalt nevron, viste isomorfe mønstre. Videre foreslo våre data, i motsetning til andreordens feromon-nevroner hos hannen, som har forskjellige protocerebrale projeksjoner fra plantelukt-nevronene, delte outpu-neuronene som innerverer de hun-pesifikke antennelobe glomeruli utgangsområdene med de planteluktneuronene.

Preface and acknowledgement

The writing of this thesis and the experimental work have been conducted in the Chemosensory laboratory at the Department of Psychology (Norwegian University of Technology and Science). Working with this thesis has been a delightful and challenging experience and has given me the opportunity to studying the wondrous nervus system. I am truly astonished by the microscopes making it possible for us to see into the advanced neural system of the tiny insect brain. I would like to deeply thank my lovely supervisor Xi Chu and co-supervisor Ph. D. student Jonas Kymre Hansen, who has provided me with help, support and time with both experiments, writing and so much more. I would like to thank the other lab members Pramod KC, Elena Ian and leader of the Chemosensory lab Professor Bente G. Berg, for all the educational and joyful conversations and the good work environment they make in this lab. I also want thank Tom Knudsen for the insects and with all the help with all the computer when needed. Last, I would like to thank my loving mother for always stepping up to watch my two kids and dog during my master period. Writing this thesis during a pandemic has not been easy, but with the help from friends, family and lab members I made it in the end.

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Abbreviations

OSN	Olfactory sensory neuron
OR	Olfactory receptor
Orco	Co-reseptor
PN	Projection neuron
MGC	Macroglomerular complex
Fx	Female complex
LPOG	Labial-palp pit organ
OG	Ordinary glomeruli
ALT	Antennal-lobe tract
mALT	Medial antennal-lobe tract
mlALT	Medio-lateral antennal lobe tract
lALT	Lateral antennal-lobe tract
dmALT	Dorso-medial antennal-lobe tract
dALT	Dorsal antennal-lobe tract
tALT	Transverse antennal-lobe tract
LH	Lateral horn
AVLP	Anterior ventro-lateral protocerebrum
SIP	Superior intermediate protocerebrum
PLP	Posterior lateral protocerebrum
LN	Local interneuron
CN	Centrifugal neuron

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1. Introduction

1.1 Insects serve as model organism for olfactory studies

The olfactory system in both vertebrates and invertebrates is formed by a group of neuronal networks, processing chemosensory cues. This neural network transduce the environmental volatile chemicals into electric signals and computes this information to guide the animal in an everchanging landscape, whether the aim is finding a mate, avoiding predators or localizing food (Buck, 2000; Shepherd, 1974). Despite the evolution distancing the vertebrates and invertebrates, they share many organizational features with respect to this system. This includes not only the arrangement of sensory receptors in the periphery (reviewed by Benton, 2009), but also the organization of parallel and hierarchical processing along the central pathways. For instance, the peripheral olfactory level, in both humans and insects, contains sensory neurons targeting the primary olfactory center. These neurons have a uniform structure, i.e. small bipolar cells with a dendrite containing the receptor proteins and an unmyelinated axon conveying nerve impulses directly to the brain (reviewed by Ache & Young, 2005; Hildebrand & Shepherd, 1997). The primary olfactory center, the antennal lobe in insects and the olfactory bulb in humans, bear a striking similarity in synaptic organization, as sensory terminals make contact with second order neurons in characteristic spherical structures termed glomeruli (Shepherd, 1974). An assembly of parallel tracts connecting the antennal lobe (AL) with the second olfactory centers in moths (Homberg, Montague, & Hildebrand, 1988) matches the mammalian olfactory tract targeting the cortical regions in the temporal lobe (reviewed by Lledo, Gheusi, & Vincent, 2005). Insects have a strong behavioral response to smells (Berg, Zhao, & Wang, 2014; Sato & Touhara, 2008) Similarly, humans respond strongly to certain stimuli, such as rotten food, which induce behavior preventing us from eating it and getting sick. In addition, the strong ability to establish odor memory, known in mammals as well as insects, is linked to this distinct level of the olfactory pathway (Gottfried et al., 2004; Heisenberg, 2003).

As pointed out in the preface of "From neuron to Brain" written by Kuffler and Nicholls (1976) "Fortunately, in the brains of all animals that have been studied, there is apparent uniformity of principles for neurological signaling. Therefore, with luck, examples from a lobster or a leech will have relevance for our own nervous system". Insects have served as valuable model organisms in many studies (Hansson, 1999). Two of the outstanding advantages of using insects in chemosensory studies are listed below. First, the

insect has a relatively simple but functionally complex nervous system which is easily accessible. Second, unlike the mammalian olfactory epithelium embedded deeply in the nasal cavity, the insect`s organ for olfactory detection, the antennae, are a pair of protruding appendages on the head, that can be simply accessed for testing relevant odor stimulations.

Many moth species, including *Helicoverpa armigera*, the specie used in this thesis, have been extensively studied due to its astonishing sense of smell and the characteristic behavioral responses to smell (reviewed by Berg et al., 2014; Sakurai, Namiki, & Kanzaki, 2014). The quality and quantity of odor substances in the environment are encoded through the pattern of activation across neurons within the olfactory system. This stimulus is integrated in the olfactory centers of the brain, eventually leading to a change in the behavior. The behavior of the moth can be guided by either odor produced by plants or by other insects, the latter including both conspecific and interspecific cues. The odors produced by plants are used as cues for determining the location of food or selecting the optimal location for females to lay eggs, also called oviposition. Odors produced by insects are mainly involved in intraspecific communication, as pheromones. A male moth can detect a female moth from a great distance (Priesner, Witzgall, & Voerman, 1986). Even low amount of pheromone molecules is sufficient to evoke a behavioral flight response towards the plume. When entering the plume, the male initiates a characteristic zigzagging flight pattern, orienting the male in the direction of the conspecific female and continue this pattern until reaching the source. The male proceeds to engage in a short-range orientation of the female, resulting in mating (Hansson, 1999). Such behavioral response is also observed in females when they are seeking an ovipositing position, however, the female zig-zagging flight response towards the host-plant is mediated by odor (Schneiderman et al., 1986; Willis & Arbas, 1991). When reaching the odor source, they will choose based on individual preference to either lay eggs or continue searching for a “better” location.

1.2 The peripheral olfactory system in insects

The antenna is a whip-like appendage that has evolved to be an organ with the ability to detect not only chemical information about the environment but also gravitational, tactile, as well as hygro- and thermo-sensory information (reviewed by Dirks & Dürr, 2011). The antenna can be divided into three morphological units: two small basal segments, the scapus and the pediellus, along with a long distal flagellum. The olfactory receptors are mostly seated on the flagellum of the antenna, within one of several different types of hair-like

structures called sensilla (Imms, 1939). One olfactory sensillum contains one to several olfactory sensory neurons (OSN), depending on the type of sensillum. The sensilla on the male antennae, as compared to those on female, are greater in length, as well as more numerous. The trichodea on male antennae are specialized to detect long-distance sex pheromones. Females in most moth species are equipped with a modest number of short and medium length sensilla (Koh, Park, & Boo, 1995). For instance, the female sensillum trichodea have shown to respond to hostplant odor (Anderson, Hansson, & Löfqvist, 1995; Hansson & Löfqvist, 1989). Mainly two pheromone components are utilized in the male *H. armigera*,, the primary component is *cis*-11-hexadecenal (Z11-16:Al) and *cis*-9-hexadecenal (Z9-16:Al) is the secondary component (Kehat & Dunkelblum, 1990).

Each sensillum has several pores on the surface allowing the odor molecules to diffuse into the hemolymph. Here the odor molecule is transported by distinct odorant binding proteins to the receptors seated on each cilia of the OSN (Kanaujia & Kaissling, 1985). Each OSN expresses one olfactory receptor (OR) gene, following the "one OSN, one receptor" rule. All olfactory neurons in insects also express a universal co-receptor (Orco) with the common OR, working together as a functional unit (reviewed by Fleischer et al., 2018). The functional OR-Orco complex form a ligand-gated ion channels, where the OR binds the odor molecule and the Orco is important for generating action potentials (reviewed by Kaupp, 2010). The odor signal is forwarded along a single basal axon, projecting directly into the AL via the antennal nerve (Hildebrand & Shepherd, 1997). In correspondence with the pheromone production in female moth, different OR expression between male and female was reported by Liu et al. (2012), in which they illustrated that *H. armigera* has three male specific ORs and two female specific ORs.

1.3 The primary olfactory center in insects

The sensory neurons project their axonal terminals into the AL, where they form dense synaptic contacts with the second-order output neurons within spheroidal neuropilar subcompartments called glomeruli. OSNs expressing the same receptor type targets one glomerulus (Hildebrand & Shepherd, 1997). Information processing of a given odor is mapped by the activation of a selective group of glomeruli, making a chemotopic map of the "odor space" (Vosshall, Wong, & Axel, 2000). The number of glomeruli in the AL varies among species, though in general most insect species have about 50 -160 glomeruli, the number is always found to be consistent within species (Hansson & Anton, 2000; Rospars,

1983). In *H. armigera*, the number of glomeruli is recently found to be 81 in females and 79 in males (Zhao et al., 2016).

The glomeruli are classified into three groups regarding their functions. The first group of the specific glomeruli involved in reproductive activity are sexual dimorphic: the macroglomerular complex (MGC) in males, and the female complex (Fx) in females. The MGC in *H. armigera* comprises three glomeruli, the largest is the cumulus, and to smaller units, the dorsomedial anterior and the dorsomedial posterior, all of which contribute to the detection of the female produced components (Berg et al., 1998). The Fx in the same species consists of five glomeruli: one central large female glomerulus and four smaller units (F1-F4) (Zhao et al., 2016). The study in *Manduca saxta* showed that those units in Fx responded to odors of host plants, that might be associated with female specific ovipositing behavior (King, Christensen, & Hildebrand, 2000; Willis & Arbas, 1991). The second group consists of only one glomerulus, the labial-palp pit organ glomerulus (LPOG), which is located most ventral in the AL and mainly receives CO_2 signals from the labial pit organ of the labial palp, a section located at the mouthpart (Chu et al., 2020; KC et al., 2020; Zhao et al., 2013). The third group, i.e. the ordinary glomeruli (OG), is formed by a large number of glomeruli encoding various plant odor information. The OG group mentioned here also include the posterior complex, which is a cluster of 10 glomeruli located just posterior to the MGC, away from the rest of OG (Zhao et al., 2016). In many moth species including *H. armigera*, the anatomical studies have demonstrated that the OG are sexually isomorphic (Galizia, Sachse, & Mustaparta, 2000; Zhao et al., 2016).

1.4 Parallel olfactory pathways

In most insect species, the AL output neurons, known as projection neurons (PNs) connect the AL to higher processing centers via three main parallel pathways (Fig. 1). These main tracts termed as the medial, mediolateral, and lateral antennal-lobe tracts (mALT, mlALT, and lALT, respectively) are found in many insect species (Ito et al., 2014). In Lepidoptera, there are also minor ALTs, including the dorsal (dALT), dorsomedial (dmALT), and transverse (tALT) tracts (Homberg et al., 1988; Ian et al., 2016; Ian et al., 2016; Kanzaki et al., 1989; Namiki & Kanzaki, 2011; Rø, Müller, & Mustaparta, 2007). The main output regions of the projection neurons confined to these ALTs are the calyces of mushroom body, lateral horn (LH), anterior ventrolateral protocerebrum (AVLP) and column in the superior intermediate protocerebrum (SIP).

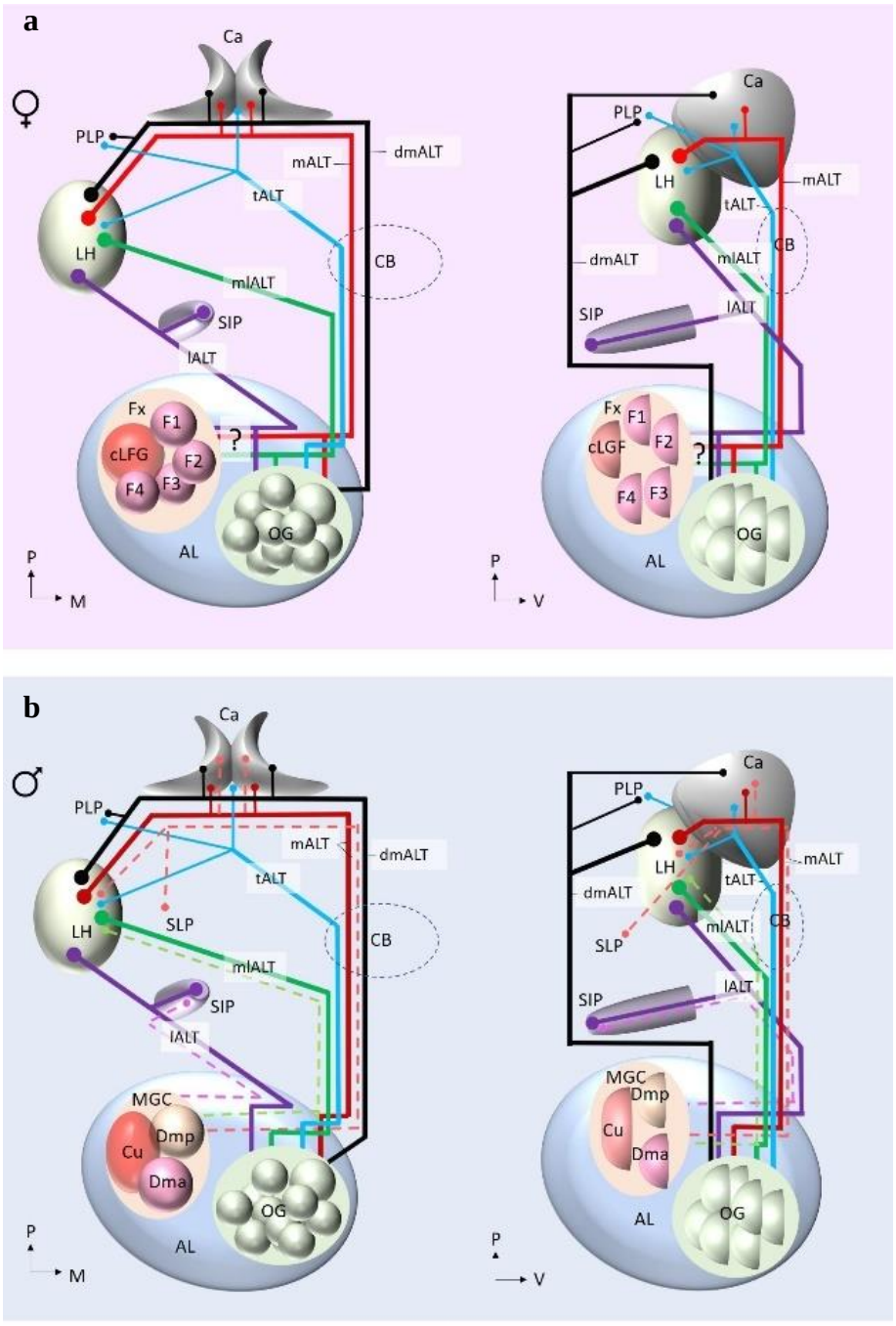

Figure 1. Schematic overview of the antennal-lobe tracts (ALTs). **a.** Olfactory processing in female moth brain. L*eft:* dorsal view; Sagittal view *right* panel. Antennal-lobe (AL) with the female complex (Fx) and the ordinary glomeruli (OG). The projections from the Fx is unknown. The medial ALT (mALT) target the calyces (Ca) and further projects to the lateral horn (LH). The transverse ALT (tALT) targeting the Ca, posterior lateral protocerebrum (PLP), and LH. The medial-lateral ALT (mlALT) projects to the LH. The lateral ALT (lALT) projects to the LH and to the column of the superior inter-mediate protocerebrum (SIP). The dorsomedial ALT (dmALT) target the Ca, PLP and LH Sagittal view of the ALTs in female. **b.** Olfactory processing in male moth brain. L*eft:* dorsal view; *Right:* Sagittal view. The male AL comprises the OG and the male specific, macro glomerular complex (MGC). Projections from the OG are the same as in female (described above). Projections from the MGC are marked with dashed lines. The MGC neurons projecting via the mALT targets the Ca, LH and the superior lateral protocerebrum (SLP). MGC neurons projects to the LH via the mlALT, all lALT neurons from the MGC target the SIP. Cu, cumulus; Dmp, dorsomedial posterior unit; Dma, dorsomedial anterior unit; CB, central body; P, posterior; M, medial; V, ventral.

The most prominent tract between the AL and the protocerebrum is the mALT (Homberg et al., 1988). It exits the AL towards the posterior part of the protocerebrum and turns to the anterior edge of the calyces of the mushroom bodies, which are a pair of cup-shape neuropils located in the superior protocerebrum on the top of the pedunculus of the mushroom body (Fig.1). A portion of mALT-axonal branches enter this region and innervate the cup areas with a considerable number of terminals that make synaptic contact with Kenyon cells. The mALT then continues its path laterally and turns anteriorly to the LH. This tract consists of mostly uniglomerular PNs, meaning they arborize within one glomerulus. Medial-tract PNs originating from the MGC in males target inner cup of the calyces and a wide area in the lateral protocerebrum. The output region of MGC PNs processing of the primary pheromone vs. OG PNs processing general odorants are spatially separated (Homberg et al., 1988; Kymre et al., 2020; Zhao et al., 2014).

Another prominent tract, the lALT, is formed by both uni-and multi-glomerular PNs. Compared to the above-mentioned mALT, the lALT has a thicker axonal bundle and exits from the AL more ventrally (Ian et al., 2016). The output regions of the lALT are varied in different insect species. In honeybee or ant, the PNs in the lALT project to the calyces via the LH (Galizia & Rössler, 2010; Homberg et al., 1988; Ian et al., 2016). In moths, a part of lALT PNs target the calyces via LH, while a remaining part of the fibers in lALT project to the column, a region embedded SIP, situated between the vertical lob of the mushroom body and the anterior optic tubercle (Ian, et al., 2016; Rø et al., 2007).

The mlALT is the third prominent tract, but with a thinner bundle when compared with the mALT or the lALT. It consists of mostly multiglomerular PNs. This tract exits the AL together with the mALT but turns lateral at the anterior boundary of the central body, from where the axons proceeds to the LH and the superior lateral protocerebrum (Homberg et al., 1988; Ian et al., 2016; Kymre et al., 2021). In addition to the three main tracts, there are three minor tracts also carrying signals from AL to the protocerebrum, namely the tALT, the dmALT and dALT. The tALT turns lateral from the mALT at the posterior edge of the central body, where it splits into subbranches targeting calyces, posterior lateral protocerebrum (PLP) and the LH. This minor tract is formed by relatively few PNs, among them a small portion is bilaterally projected into the protocerebrum. A recent study illustrated that over half of the PNs in this tract originate from the LPOG, the glomerulus processing the CO_2 signals (Chu et al., 2020). The dmALT containing even less axons, it exits the AL dorsomedial and follows a path parallel to the mALT, albeit more dorsally. Most PNs confined to the dmALT target the calyces and the LH (Ian et al, 2016; Kanzaki et al., 1989;

Kymre et al., 2020). Last tract is the dALT, it exits the AL dorsal to the lALT, proceeding lateral targeting several neuropils of the lateral protocerebrum. The somas of PNs confined to this tract are located close to the midline in the contralateral hemisphere (Kymre et al., 2021).The main axon fibers of the dALT exits the AL projects lateral, mediodorsally to the lALT and targets the LH and several other neuropils of the protocerebrum.

In addition to the PNs, two other categories of neurons contribute in the odor information processing within the AL, namely local interneurons and centrifugal neurons (CNs). The local interneurons, which are mainly inhibitory (Berg, Schachtner, & Homberg, 2009), innervate multiple glomeruli in the AL (Seki & Kanzaki, 2008). They sharpen the contrast between activated and quiet glomeruli, and are important for the synchronization of firing in the projection neurons (Christensenet al., 1993; Galizia & Menzel, 2000; MacLeod & Laurent, 1996). Moreover, several CNs also innervate the glomeruli and participate in the olfactory coding by adjusting the activity in the glomeruli based on information gathered from the protocerebrum (reviewed by Anton & Homberg, 1999). The CNs appear to integrate signals from various sensory networks and convey the feedback information to the AL, leading to shape the corresponding olfactory information (Zhao & Berg, 2009; Zhao et al., 2013). The composition of these neural types, the OSN, PNs, local interneurons and CNs, profiles the activity pattern in and between glomeruli, making a chemotopic map of "odor space" (reviewed by Hildebrand, 1995; Hildebrand & Shepherd, 1997).

1.5 The aim of this study

In moth, the morphology and function of olfactory system have been extensively studied in male, due to the specific pheromone system (Sakurai et al., 2014). Little attention was paid onto the secondary olfactory neurons in the opposite sex. As the plant odor evoked behavior is largely similar in males and females, a general impression of morphological difference between two sexes was that the output patterns of PNs innervating the OG are the same. However, a systematic comparison is required, particularly the projection patterns from the Fx remain largely undescribed. In this thesis we aim to investigate and compare the general morphology of the parallel olfactory pathways in the male and female *H. armigera* and its projection neurons of the antennal-lobe tracts. A series of systematically designed mass staining in the AL will be conducted in combination confocal microscopy. We aim to apply dye in different areas of the AL; (i) the whole AL including Fx/MGC units and OG, (ii) the medial part of the AL targeting the OG output neurons exclusively, and (iii) the

ventral part of the AL, including the OG and LPOG Further, we will explore individually stained neurons confined to the ALTs in female, this to compare a more detailed morphology with male ALT neurons found in previously reported studies. Our goal is to gain more knowledge of the female secondary olfactory system and to get a better understanding of the neural differences and communalities in male and female *H. armigera*.

2. Method

2.1 Insects

Male and female moths of *H. amigera* (Lepidoptera; Noctuidae, Heliothinae) was used. The pupae were obtained from Keyun Bio-pesticides (Henan, China). Male and female pupae were placed in separate chambers in climate cabinets at 24°C and 60 % air humidity with light on at 18:00 and light off at 8:00. After the moth emergence, they were carefully transferred into the housing arena - a plexiglas cylinder bedded with soft tissues and constant access to 10% sucrose solution. Each cylinder contained maximum 8 same-sex insects.

Lepidoptera uses for experimental purposes have no restriction according to the Norwegian law of animal welfare (see www.lovdata.no/dokument/NL/lov/2009-06-19-97). Still, the insects were always protected from unnecessary stress or harm, and they were carefully inspected. The housing arena was cleaned on a daily basis.

2.2 Mass staining

In order to compare the projection pattern of antennal lobe output neurons between males and females,mass staining experiments were systematically conducted by applying fluorescent dye to the male and female one of the two antennal lobes. The insect was first placed in a small plastic tube (~3 cm long), where we pre-placed dental wax (Kerr Corporation, Romulus, MI, USA) on one end. The tube restricted the movement from the body and kept the entire head exposed. Under a stereo microscope, the head was further immobilized by applying dental wax in the gap between the tube and head capsule. After the scales were removed, the cuticle and trachea were carefully removed to expose the brain (Fig. 2). To prevent the brain from drying out, Ringer solution (NaCl: 150mM, KCl: 3mM, N-tris (hydroxymethyl)-methyl-2-amino-ethanesulfonic acid: 10mM, $CaCl_2$: 3mM sucrose: 25mM, pH: 6.9) was applied when necessary. The mass staining was manually applied. A fine borosilicate glass needle pulled by a horizontal puller (P97; Sutter Instruments, Novarto, CA, USA) tip-coated with a thin layer of vaseline was used to apply the fluorescent dye biotinylated dextran-conjugated tetramethylrhodamine (3000 mw, micro-ruby, Molecular Probes) into the target brain area. The brain was oriented dorsally and the needle tip covered with fluorescent dye crystals was inserted in one of the antennal lobes manually. To prevent dye overloading and expanding to the neighbor regions, the Ringer solution was immediately

applied to rinse away excess dye. The preparation was then covered with a small piece of tissue, soaked with a generous amount of Ringer solution to keep the brain hydrated. The preparation was placed inside a dark box for at least 1 hour at room temperature or kept overnight at 4 °C, in order to allow neuronal transportation of the dye.

2.3 Intracellular staining

To investigate single neuron morphology in females compared to those found in males, iontophoretic labeling of the antennal lobe PNs was conducted. The insect was prepared as previous described in mass staining section. A chloritized silver wire was inserted in the eye, working as a reference electrode. Glass electrodes with a resistance between 80 and 300 MΩ, was tip-filled with a droplet of the 4% micro-ruby solution, and further back-filled with 0.2M K+-acetate. The electrode was lowered into the AL by using the micromanipulator. Once stable contact with a neuron was obtained, 1-2 nA depolarizing current pulses of 200 ms were applied at 1 Hz for 10-15 minutes, to inject the dye into the neurons. The insects were then kept in 4°C overnight.

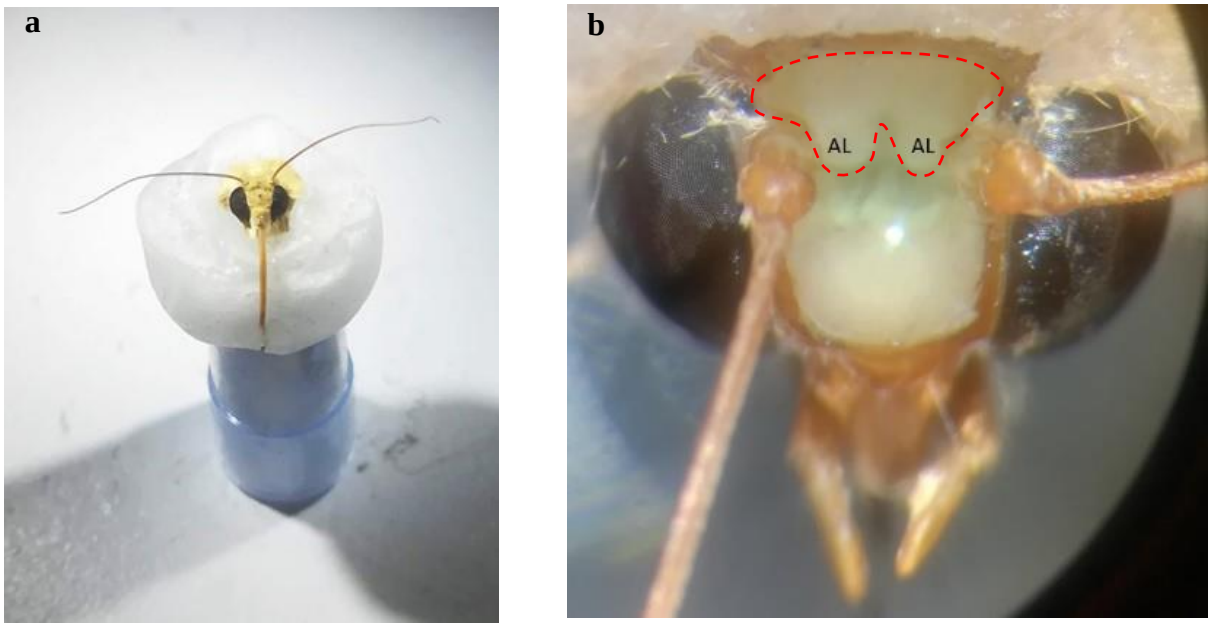

Figure 2. **a.** Immobilized insect restricted in a plastic tube with dental wax. **b.** Exposure of the moth brain (red dash line) and the antennal lobe (AL) in each hemisphere after removing the cuticle and the trachea.

2.4 Dissection and dehydration

The brains were dissected from the head capsule under a microscope. The antennas, proboscis and the labial palps was cut off with a micro-scissor before the eye capsule was removed with a small razor knife and wiped dry with a tissue. The cuticle between the eyes and head was then cut to make the dissection easier. After the cuticle and trachea was removed with fine forceps, the dissected brain was fixated in 4 % paraformaldehyde solution for 60 minutes in a small plastic container. The dehydration was then performed with a series of ethanol solutions for 10 minutes each (50%, 70%, 90%, 96% and 2 x 100%). The brain with stained neuron(s) was placed in an aluminum plate embedded with methyl salicylate covered by decker glass plate and kept in the fridge prior to imaging.

2.5 Confocal microscopy

Whole brains containing stained neurons were imaged dorsally or frontally by using a confocal laser scanning microscope (LSM 800 Zeiss, Jena, Germany), equipped with a C-Apochromat 10x/0.45 water objective on mass staining preparations and a Plan-Neofluar 20x/0.5 objective on intracellular staining preparations. Micro-ruby staining was excited with a HeNe laser at 553 nm and the fluorescent emission passed through a 560 nm long-pass filter. In addition to the fluorescent dyes, the autofluorescence of endogenous fluorophores in the neural tissue was imaged to visualize relevant structures in the brain containing the stained neurons. Since many autofluorescent molecules in the tissue are excited at 493 nm, images were obtained using the 493 nm argon laser in combination with a 505-550 nm band pass filter. Serial optical sections with resolution of 1024 x 1024 pixels were obtained at 2-9 µm intervals through the entire depth of brain. The confocal images shown in this thesis were edited in ZEN 2.3 (blue edition, Carl Zeiss Microscopy GmbH, Jana, Germany).

3. Results

In total, mass staining was performed in 32 individual insects, i.e. 19 females and 13 males. For the preparations to be further analyzed, we selected the preparations with AL labelled successfully, i.e. to clearly visualize three main ALTs (mALT, mlALT, lALT) and to examine the respective neuropils. It resulted in a total of 12 preparations (eight females and four males) with adequate mass labelling of the majority of those AL output neurons were analyzed, in which seven are presented in the results. In addition, 27 female moths were used in iontophoretic labeling to visualize the detailed morphological feature of individual neurons. Five of them were selected for comparing the neuronal projection with the same neuron subtype previously stained neurons in male moths.

3.1 Parallel olfactory processing in male and female moth

Application of the dye in the antennal lobe visualized the ALTs formed by AL projection neurons in both males and females and made it possible to obtain an overview of each tract and its connected neuropils. The mass staining data provided rich detailed information on the parallel processing pathways (Ian et al., 2016).

We first mass stained the entire AL, including the sexual dimorphic and isomorphic region. Four tracts were visualized: mALT, mlALT, lALT and tALT. The similarities of projection pattern of the AL neurons between female (Fig. 3a) and male (Fig. 3b) are listed below. First, the most prominent tract leaving the AL, mALT, contained AL projection neurons first projecting to the calyces of the mushroom body, then continuing the projection to the LH. The other prominent tract, lALT, was stained as a thick bundle leaving the AL more ventrally to the mALT projects to the column and LH. From the mass staining we obtained, the number of PNs projecting to the column appeared to be larger than that projecting to the LH. A number of lALT PNs showed enlarged terminals at the medial part of the LH. In addition to these two output regions, a few lALT PNs also targeted the AVLP. The neurons confined to the mlALT bending their axons from the anterior edge of central body send their axonal projections to the LH. The mlALT splits in two sub-branches on its lateral course, one targeting the posterior LH and the second targeting more ventral. The tALT was the least stained bundle, visualized in both preparations, turning lateral posterior to the central body. Generally, the PNs confined to the tALT are comparable between female and male, due to the manually staining, a clearer projection was shown in the female preparation. Here, the

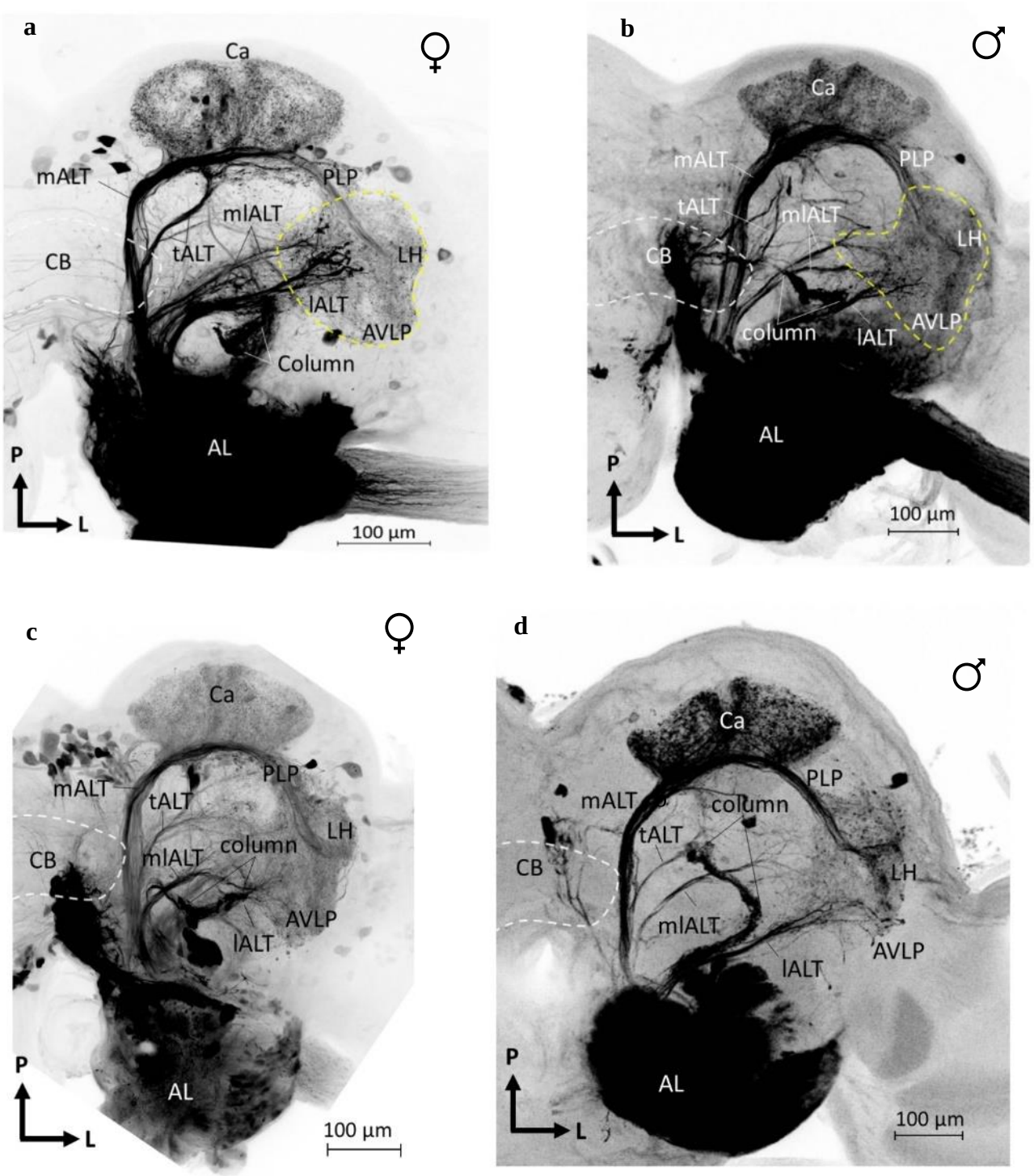

Figure 3. Confocal image of mass staining of the antennal-lobe (AL) visualizing the morphology of the antennal-lobe tracts (ALTs) in the ipsilateral hemisphere in male and female in dorsal view. **a, b.** Labeling of the whole AL in male and female, showing the medial ALT (mALT), lateral ALT (lALT), medial-lateral ALT (mlALT) and transverse ALT (tALT) .The neurons of the tracts innervating the calyes (Ca) of the mushroom-bodies, the lateral horn (LH). tALT the posterior lateral protocerebrum (PLP). The lALT neurons targeting the column in the superior inferior protocerebrum (SIP) and the anterior ventrolateral protocerebrum (AVLP). The neurons coming from the MGC in the male **(b)** visualize the projections going to lateral protocerebrum (dashed line), which is different in the female **(a)**. **c.** Dye application to the medial part of the AL in female, showing the tract and the respective regions of the protocerebrum as seen in **a. d.** A dorsofrontal view male brain hemisphere after applying dye to the OG, showing the mALT, mlALT, lALT and the tALT, additional to a clear view of the column. CB, central body; P, posterior; L, lateral.

tALT splits into two branches anteriorly to the calyces, where one branch targets the calyces and the other sending its terminals in the PLP. Although our male preparation did not illustrate the tALT as strong as that in the female preparation, it is very unlikely that the tALT is fundamental different from female to male. The main difference of AL neurons' projection between female and male is in the medial part of the lateral protocerebral region, where the projection of AL neurons in the female moth showed a more restrict pattern than the male moth (see yellow dash lines in Fig. 3). This is coincided with the previously reported data that many male MGC-PNs confined to the mALT projected into superolateral protocerebrum instead of the LH (Kymre et al., 2021).

As the mass staining of entire AL showed some projection difference between female and male, next, we asked what about the projection pattern of the neurons innervating the sexual isomorphic AL glomeruli. To answer this, we selectively mass stained the sexual isomorphic area in AL, i.e. the OG and the LPOG in both female and male moths (Zhao et al., 2016). As the exact location and function of the female complex were yet unclear, the dye crystals were applied into the medial and ventral part of AL in female. Whereas, the structure and function of male specific MGC was previously well studied, thus, we applied the dye in the male OG area and carefully avoided the MGC region. As shown in Fig. 3, all three main tracts were clearly stained in both female (Fig. 3c) and male (Fig. 3d). These OG neurons did not show morphological difference between two sex. These mass staining experiments revealed that the sex dimorphism of projection pattern is restricted in the PNs innervating the female/male-specific area.

Staining in the ventral part of the AL visualized a prominent lALT, portions of the lALT PNs project to the column in the SIP area (Fig. 4a-4b). The dmALT following the dorsal part of the midline projecting posterior are stained in both male and female. These neurons project close to the midline, dorsal to the central body targeting the calyces. Two bilateral tALT PNs were clearly labeled in the male, one of the male bilateral neurons appears to have innervation the LPOG of both ALs. The axon crosses the midline through the inferior AL commissure and splits in two sub-branches, one targeting the LPOG of the contralateral AL, the other projects via the tALT targeting the LH, having the same projecting patterns as in $Pt\_e_{(LPOG)}$ subtype (see Fig. C2 in Chu et al., 2020). The second PN crosses the midline through the lateral AL commissure where it projects directly to the LH via the tALT(see Fig. 4a), having the projecting pattern of a $Pt\_f_{(LPOG)}$ (see Fig. 5d in Chu et al., 2020). The female bilateral neurons are not as visible as in the male staining but are most likely the same type of neurons as seen in males, since the LPOG is stained in both preparations. Here one crosses

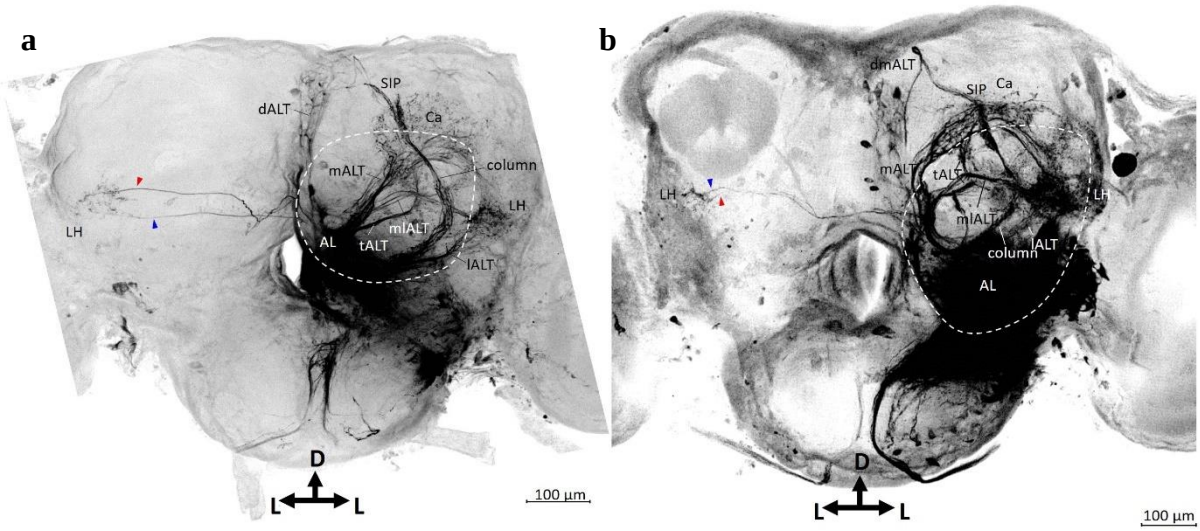

Figure 4. Confocal image of a frontal oriantated whole brain in a male insect visualizing, the antennal-lobe tracts (ALTs). Tracts included are the medial, mediolateral and the lateral the transverse ALT and the dorsomedial ALT (mALT, mlALT, lALT, tALT and dmALT), with the related protocerebral regions; lateral horn (LH) and the calyces (Ca) and the column in the superior inferior protocerebrum (SIP). In the contralateral protocerebrum two bilateral neurons were visualized (arrow heads). **b** Confocal image of whole brain in female insect. Two bilateral PNs are visual in the contralateral hemisphere (arrow heads). AL, antennal lobe; D, dorsal; L, lateral.

the inferior AL commissure and the other crosses the lateral AL commissure (see Fig. 4b), both targeting the contralateral LH (see Fig. 5d in Chu et al., 2020).

In one of the mass staining of mainly the medial part of the female AL we found some interesting bilateral neurons together with a highly prominent lALT (see Fig 5). The lALT target mainly the column and a portion of the lALT PNs targeted the AVLP (see Fig. 5). Additionally, two clearly labeled PNs were outstanding in this mass staining. One neuron project bilaterally and one within the ipsilateral protocerebrum. The bilateral PN send one axon over the midline though the lateral AL commissure. Further, this neuron project into the contralateral superior clamp of the inferior neuropils and projects ventrolateral to the PLP, having small sub-branches along most of the axon in the superior protocerebrum. A sub-branch of this neuron innervate the base of the calyces, dividing into two branches sending terminals in both cups of the calyces (see red arrowhead Fig. 5). The other neuron is also stained in this preparation (blue arrowhead Fig 5), the cell bodies in the protocerebrum is belonging to this neuron suggesting that it is a CN. The dendritic arborization neuron originated from the calyces, PLP, SIP, AVLP and the LH and projected to the AL via the lALT, having the same characteristic as a Cl_a subtype (Kymre et al., 2021).

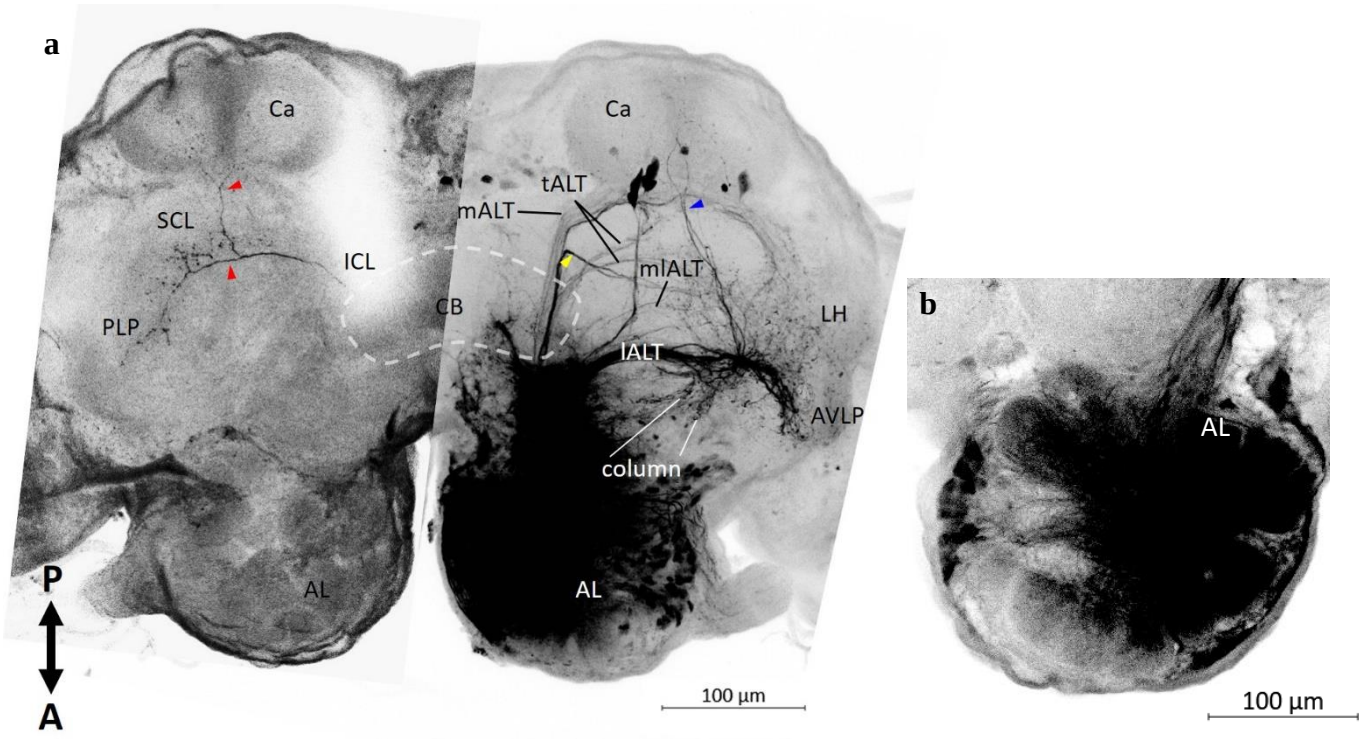

Figure 5. **a.** Dorsal projection view of female insect, showing the distinct pattern of the antennal-lobe tracts (ALTs) relative to dye application to the medial part of the antennal-lobe (AL). Confocal image of the whole brain showing projections in both hemispheres. The medial ALT (mALT) and mediolateral ALT (mlALT) and the transverse ALT (tALT). are weakly stained. Most prominent is the lateral ALT (lALT), targeting the LH and the anterior ventrolateral protocerebrum (AVLP). The sub-branching of the lALT targeting the column. One bilateral tALT PN crosses the brain`s midline medio-ventral to the central body (CB) (see red arrowhead). Another lALT neuron (arrowhead blue) targets the calyces (Ca). **b.** The dye (black area) was placed in the medio-ventral part of the AL. P, posterior; A, anterior.

3.2 Iontophoretic labeling of single female secondary olfactory projection neurons

3.2.1 Medial-tract neuron

One mALT PN was successfully stained in our experiment. As the dye was leaked around the staining site, the dendritic arborization of this neuron could not be determined. Based on the proception pattern this neuron belongs in the Pm_a subgroup (Homberg et al., 1988; Ian, Zhao, Lande & Berg, 2016; Kymre et al., 2021). This neuron innervates the calyces before terminating the LH. The axon sends four sub-branches into the two cups of the posterior part of the calyces, two branches into the medial, one in the lateral cup and one branch in between the cups. The neuron further innervates in the ventral part the LH.

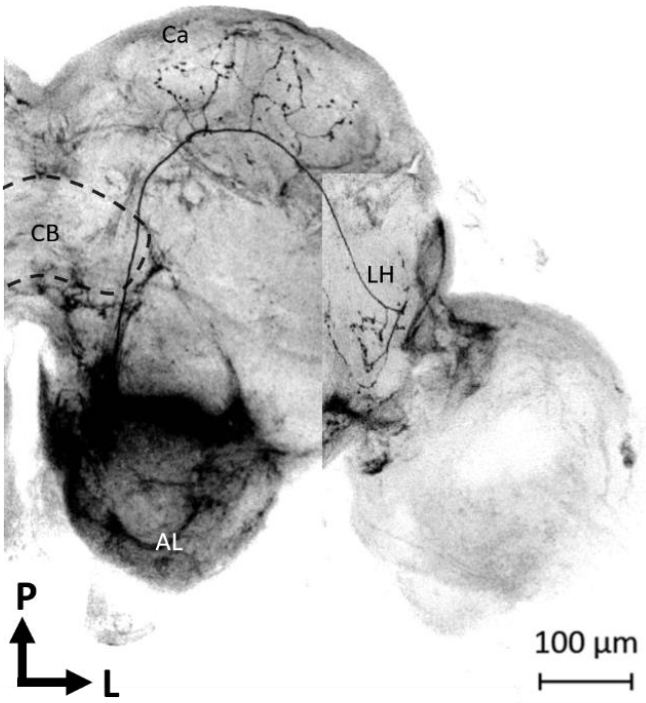

Figure 6. Confocal image of iontophoretic labeled medial antennal-lobe tract (mALT) projection neuron in female with innervation in the calyces (Ca) and the lateral horn (LH). AL; antennal lobe; CB, central body; P, posterior; M, medial.

3.2.2 Mediolateral-tract neurons

Two different types of unilateral mlALT PNs were stained. The first belonged to the most typical mlALT sub-type, termed Pml_a, which has previously been found in studies performed on male *Heliothis virescens* and *H. armigera* (Ian, Zhao, Lande & Berg, 2016; Kymre et al., 2021). This neuron enters the LH in the ventral part and has innervation in a large area of the LH with a high amount of belbs (see Fig. 6b).

The second mlALT PN was classified as a Pml_d neuron. It projected through the ventroposterior protocerebrum and LH and here inverted mostly the ventromedial part. Its sub-branch made a U-turn and passed on dorsomedial to the superolateral protocerebrum and the SIP, before finally ending up in the crepine and superomedial protocerebrum, close to the brain midline. The sub-branch has a broad contact region in the superior protocerebrum. A similar type of neurons is found in the male *H. armigera* (Kymre et al., 2021), the related moth *Manduca sexta* (Homberg et al., 1988), and has also been reported in the fly *Drosophila melanogaster* (Tanaka, Endo, & Ito, 2012).

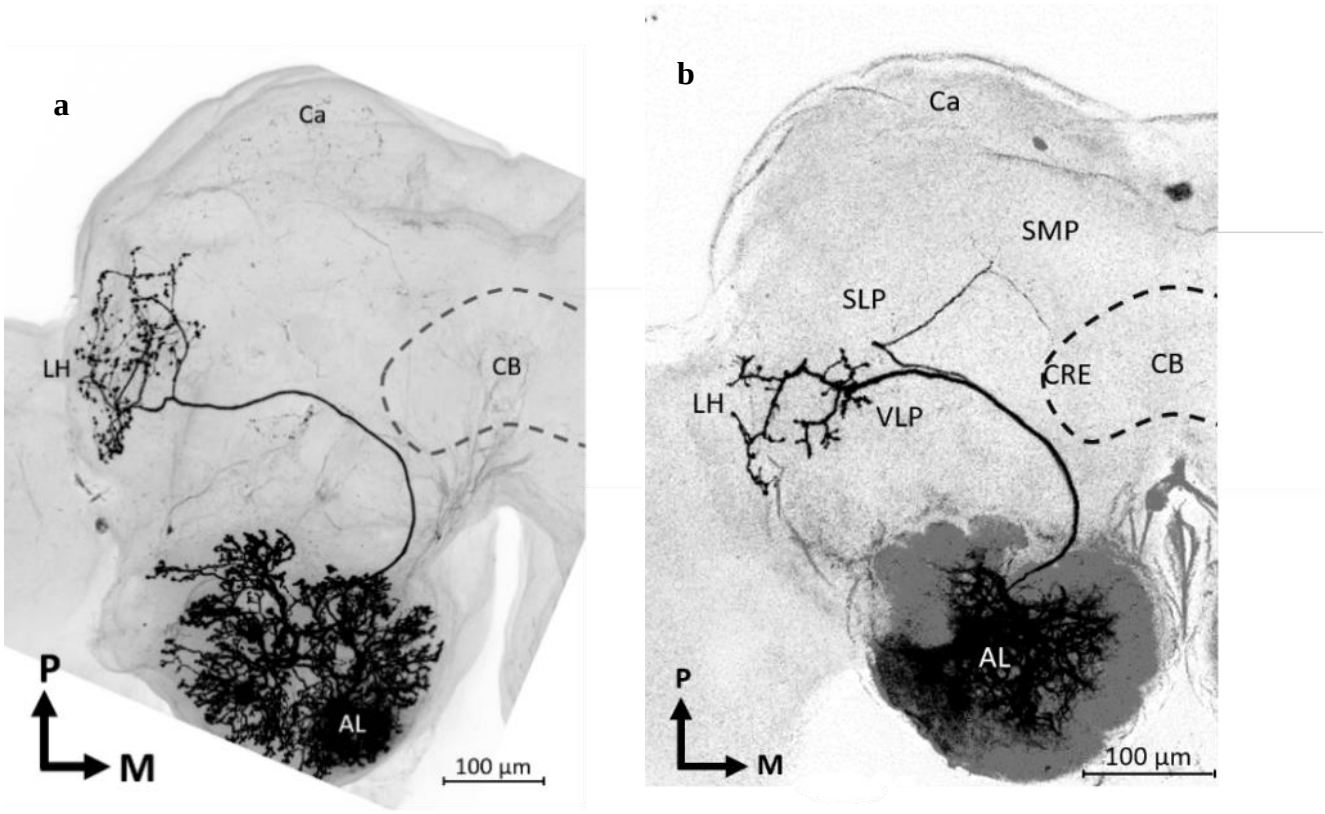

Figure 7. Confocal image of iontophoretic labeled medio-lateral antennal-lobe tract (mlALT) projection neurons **a.** Pml_a subtype in female, targeting the ventral part of the lateral horn (LH) with widely spread arborization terminals. **b.** Confocal image of an iontophoretic labeled medio-lateral antennal-lobe projection neuron Pml_d subtype, in female. The main axon targets the ventral part of the lateral horn (LH), the axon splits into a sub-branch projecting to the ventrolateral protocerebrum (VLP), superior lateral protocerebrum (SLP), superior medial protocerebrum (SMP) and the crepine (CRE). AL; antennal lobe; CB, central body; P, posterior; M, medial.

3.2.3 Lateral-tract neuron

Totally three lALT PNs were stained in this thesis. All three belonged to the Pl_a_uni sub-type (Fig. 8). The Pl_a_uni sub-type has previously been found in male *H. armigera* (Chu et al., 2020; Ian et al., 2016), along with several other lepidopteran species (Hansson, Anton, & Christensen, 1994; Homberg et al., 1988; Rø et al., 2007). The axons of these PNs exit the AL via the ventral root and follows the lALT path towards the lateral protocerebrum. However, the axons bend dorsomedial before reaching the LH, and then continues to the column of the SIP. Here, a great number of terminals were closely distributed around the main axon, making interaction with several protocerebral regions (arrows Fig. 6a-b).

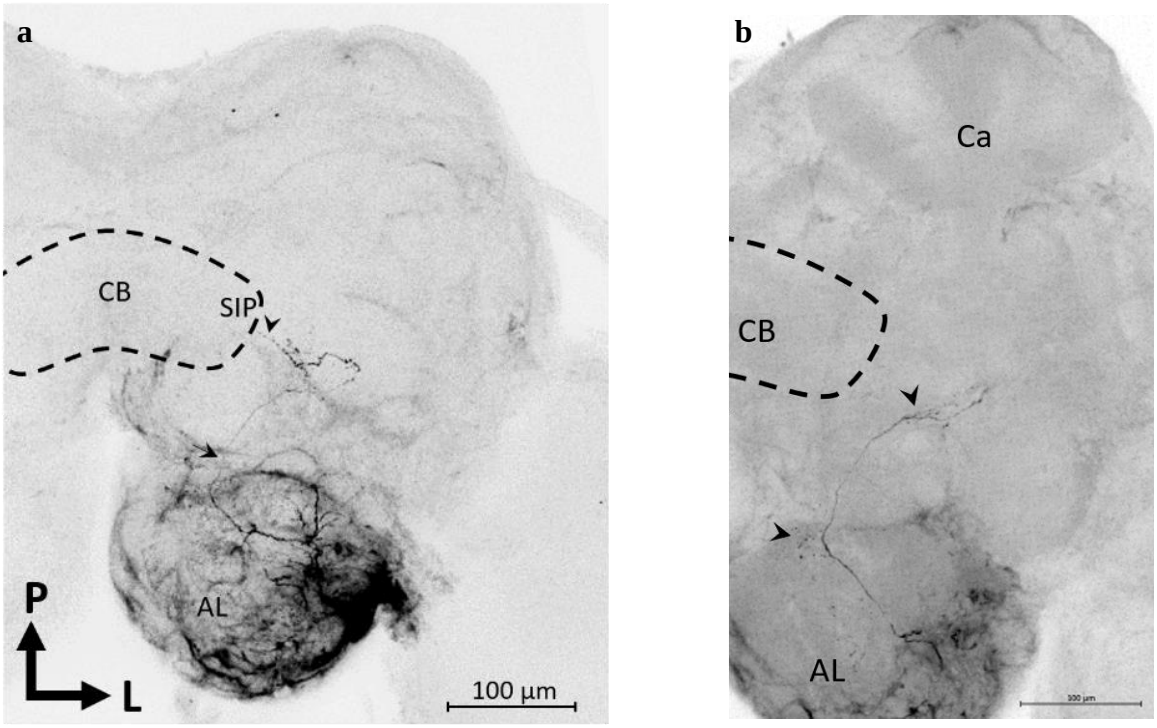

Figure 8. Confocal image of an iontophoretically labeled, unilateral lateral antennal-lobe projection neuron Pl_a_uni subtype. **a.** The Pl_a_uni neuron exits the antennal lobe (AL) via the ventral root, on the axon directly outside the AL a small portion neuron has bulbs (arrow) forming synapses. The neuron projects along the course of lateral antennal-lobe tract (lALT) and turn dorsally into the column of the superior intermediate protocerebrum (SIP; see arrowhead). **b.** A section of the axon of the same neurons, showing some of the sub-branches (arrows). CB, central body; Ca; calyces; P, posterior; L, lateral.

3.2.4 Centrifugal neuron

Additional to the PNs, one centrifugal neuron was stained when er applyed dye into the posterior part of the AL. This CN type has been identified in *H. virescens* and in the *H. armigera* (Kymre et al., 2021; Zhao, Pfuhl, Surlykke, Tro, & Berg, 2013). It follows the superior fiber system and is therefore termed Csfs. The CNs cell body is positioned dorsal to the central body, i.e. in the cell body rind along the midline (MCBR). The thick main axon projecting from the cell body, runs along the medial edge of the crepine of the inferior neuropils, turning dorsolateral towards the vertical lobe of the mushroom body (see Fig. 9). Here it extended an extensive number of dendritic branches into a large part of the superior part of the protocerebrum, including superiomedial protocerebrum, superior clamp, superiolateral protocerebrum, SIP and the creptine.

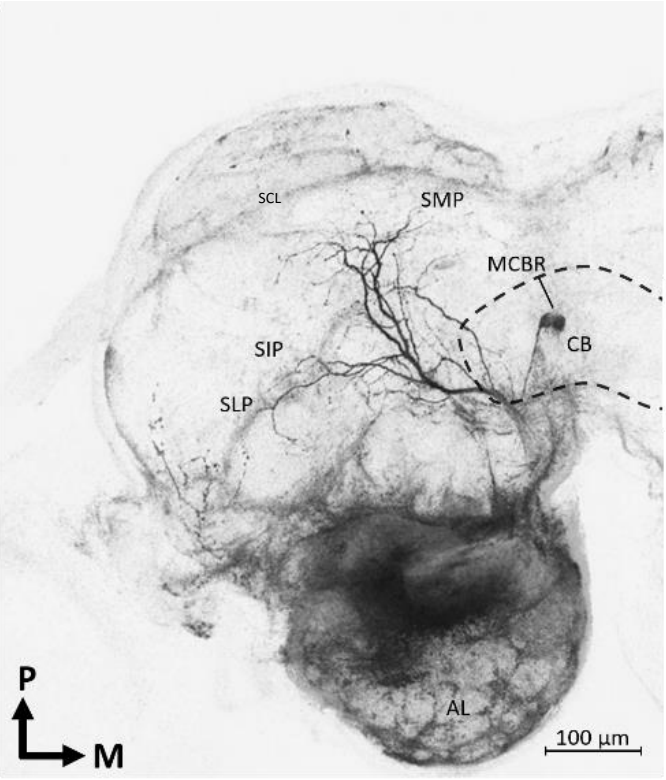

Figure 9. Confocal image of the centrifugal neuron called Csfs in female insect. The Csfs follows the superior fiber system. The dendrites run though the superior medial protocerebrum (SMP), superior intermediate protocerebrum (SIP), superior lateral protocerebrum (SLP), Superior clamp (SCL) and the crepine (CRE). The soma of the Csfs was in the cell body rind along the midline (MCBR). AL, antennal lobe; CB, central body; P, posterior; M, medial.

4. Discussion

In this thesis we presented a collection of anatomical presentations of male and female olfactory parallel pathways, in addition to a small collection of individual AL neurons from the female moth brain. These high-quality imaging data were used to compare the morphological properties of olfactory second order PNs between female and male. All five individual stained neurons in female are novel, which have only been reported in male, including one centrifugal neuron and 2 mediolateral tract projection neurons and lateral tract neuron.

4.1 The parallel antennal-lobe tracts in female and male moth brain

The mass staining experiments revealed that, generally, the projection patterns of the olfactory parallel tracts in the female moth brain are consistent with earlier findings in the male *H. armigera* as well as in other insect species (Ian et al., 2016; Rø et al., 2007). Five of six previously reported tracts have been included in our results. The mALT, mlALT, lALT, tALT, and dmALT. We failed to stain any neuron confined to the dorsal tract. This minor tract containing very few neurons that innervated most of the AL glomeruli (see Fig. 6b in Kymre et al., 2021), and thus make it rather less visible in the mass staining results.

To investigate the projections and the AL glomerular innervation of PNs, the dye applications were carefully designed. We compared mass staining of the AL in three different areas, (i) by inserting dye crystals in the entire AL including Fx/MGC units and OG, (ii) the dye crystals were applied restrict into the medial part of the AL resulting the staining of OG output neurons exclusively, and (iii) the dye was applied into the ventral part of the AL, including a part of OG and LPOG. An interesting feature is that when we compared the first two outcomes of mass staining preparations in female AL, little difference was revealed between the staining from the all AL glomeruli and that from medial AL glomeruli (Fig. 3a-b). This indicated that the female-specific projection of Fx neurons are consistent with those PNs innervating the medial located OG. This is in big contrast to the different projections between MGC PNs and OG PNs in male (Fig. 3c-d). One open question is the unknown ligands of female-specific neurons. As the clear separation of MGC and OG projections in male brain, the less segregation between Fx and OG output projections in female suggested the ligands tuned to OSNs innervating the Fx can be more plant related. In addition, the comparisons of ventral OG showed the projections confined to the dmALT and tALT as well as the lALT. The main commonality of projection between female and male brains in these

two preparations included that the visualization of the dmALT and the target region of the lALT is restrict within the column of SIP.

As shown in Homberg et al. (1988), the male *Manduca sexta* has a thicker mALT bundle than the female, a similar result in *H. armigera* was expected in our study. However, we did not observe such clear difference of the axon bundle thickness of mALT between the female and male. One of the reasons is the resolution of the confocal scanning is not sufficient to count the exact number of neuron axons confined to the mALT. Another reason is that the staining technique used here allowed the labeling of majority of the PNs but may not successfully label all AL output neurons. Based on the more detailed description of mALT PNs in Homberg et al. (1988), the number of mALT axons in male is ~ 400 including the male-specific MGC neurons, while the corresponding axon counting is ~360 in female. The extra 30-40 PNs were therefore assumed to be male-specific pheromone PNs. We argued that the number of male pheromone neurons could be underestimated, as the female specific PNs innervating the Fx might be attributed into the counting of the OG output neurons. Nevertheless, the underestimated amount of MGC PNs is very unlikely to alter the fact that the proportion between pheromone vs. plant odor mALT neurons is small. We expect that the female-specific projection neurons may also constitute a small proportion of OG neurons. Since the calyces one of the prominent output regions of the mALT PNs is highly related with memory and odor learning, the small proportion of female-specific neurons may indicate that the ligands of these neurons is less important in odor learning, but rather relevant to the innate behaviors.

The lALT appears to be isomorphic in *H. armigera*. From staining of the medial part of the AL (Fig. 5), the labeling in the protocerebrum is mainly within the area of column in both the female and male preparations. Considering the lALT is the most pronounced tract stained in this preparation, we proposed that the majority of the lALT PNs innervating the medial AL project to the SIP and AVLP. However, it is still worth mentioning that a few lALT neurons innervating the same area of the AL indeed project to the LH and AVLP. This is coincided with the previously reported single neuron staining (see Fig. 3d in Kymre et al., 2021). This suggest that the vast majority of these lALT neurons are less involved in odor memory. The lALT PNs targeting the column could serve a more direct role for fast control of key behaviors, requiring strength and sturdiness, as previous study found that all the MGC lALT neurons targeting the column have a robust response than the PNs confined to the mALT (Chu et al., 2020).

The dimorphism of PNs confined to the mlALT and the two minor tracts dmALT and the tALT is not illustrated in our results. A few dmALT PNs are labeled when applying dye to the dorsomedial part of the AL in both male and female (see Fig. 4). The results of our mass staining experiments show that the tALT is mostly stained when the ventral part of AL is labeled, particularly in the preparation where the LPOG was heavily stained. Two bilateral tALT LPOG neurons in both male and female are stained (see Fig. 4), corresponding to the previously reported neuron type Pt_e$_{(LPOG)}$ and Pt_f$_{(LPOG)}$. The LPOG output neurons are presented in both male and female (Chu et al., 2020), even the LPOG was found to be bigger in female (Zhao et al., 2016). Whether there is any difference of LPOG output neurons confined to the tALT between the female and male is still an open question. One of the reasons is that the morphological diversity of the neurons in this tract is huge (Chu et al., 2020).

4.2 Morphological feature and functional characterization of antennal-lobe neurons in female and male

The mass staining experiments provided a general similarity of AL output neurons between two sexes. In order to compare the anatomical details of the AL neurons, intracellular staining was performed to visualize the single neuron. A pilot comparison is conducted between our small collection of five individual neurons collected in female, together with previously stained individual neurons in male.

The morphologies of individual PNs presented here are in agreement with the general impression that projection patterns of OG output neurons are isomorphic. The mALT neuron with projection in the calyces and LH (Fig. 6) is a typical Pm_a subtype PN, which is the most common mALT PN subtype with dendritic arborization in the male OG (Ian et al., 2016; Kymre et al., 2021). Likewise, the PN in female confined to the lALT (Pl_a_uni subtype) targeting the column is also a common neuron type in male (Ian, Zhao, Lande & Berg, 2016). Interestingly, this subtype is also one of the frequently encountered one among the male-specific MGC output neurons in *H. armigera* (Chu et al., 2020). It appeared that the neurons in this subtype showed a unique morphology-function connection, i.e. the multiglomerular PNs mostly innervate the plant-odor subsystem Ian, Zhao, Lande & Berg, 2016), and the uniglomerular one innervate the pheromone subsystem (Chu et al., 2020). Therefore, we hypothesize that such connection between dendritic innervation and function

may also apply in the Pl_a_uni subtype in female. However, up to now, we have no sufficient data to support the hypothesis, the future data collection may shed more light on this issue.

In addition, the individually stained neurons confined to the mlALT also illustrated isomorphic to a large degree. The Pml_a subtype projecting to the LH was a very common mediolateral tract neuron type in male across several moth species with varied AL innervations (Homberg et al., 1988; Ian et al., 2016; Lee et al., 2019; Rø et al., 2007). The Pml_d neuron in female with wide-spread projection reported in our data has been found not only in the male conspecies (Kymre et al., 2021), but also in other male insect, such as *Manduca sexta* (Homberg et al., 1988) and *drosophila melanogaster* (Tanaka et al., 2012). This neuron type was visualized in several mass staining preparations in both male and female (Xi Chu et al., 2020). The Pml_d neuron labeled in male *H. armigera* had dendrites in all glomeruli including the MGC, meaning this neuron in male get information about pheromones and odor (see Fig. 4d in Kymre et al., 2021). The leaking staining in the female preparation made the determination of the dendritic arborization less clear, nevertheless we assume that the same subtype PN in female also innervates the all AL glomeruli, including the Fx.

The female centrifugal neuron labelled in the intracellular staining experiments, named “Csfs”, was previously reported in male *H. amigera* by Kymre et al. (see Fig 11, e1, e2 and 12 in 2021) and Zhoa et al. (2013; fig. 2). Mass staining done by others have shown two, to three Csfs cell bodies in the MCBR on both side of the midline (Kymre et al., 2021; Xin-Cheng Zhao et al., 2013). Zhoa et al. classified this neuron as a multisensory neuron, as it additionally responded to non-odor cues like sound pulses or air flow changes. They proposed that these kinds of neurons intergrade information from other modalities to shape the odor information coding, and thus acting as a feedback control.

4.3 The comparison between the male-specific MGC and female-specific Fx

In this section, we summarized our finding and several key publications on the issue of comparison of Fx and MGC from several perspective, including the anatomical and developmental features, the connective properties, as well as the functional characterizations. Despite the volume difference in the heteromorphic glomeruli, the isomorphism was found in several glomeruli within the posterior complex, the LPOG and the OGs. The fact that the glomeruli which are found in both sex varies in volume might indicate that the different odors elicit different behavior (Zhao et al., 2016).

The Fx are among the first glomeruli to develop immediately after the ingrowth of the first olfactory sensory axons, the same as in development of the MGC in male. (Rössler, Tolbert, & Hildebrand, 1998). The MGC and Fx share common features of development and possibly downstream connections mediating odor-modulated behavior (King et al., 2000; Rospars & Hildebrand, 2000). In addition, King and her colleagues (2000) showed in their experiments that the PNs originating from the lateral large female glomerulus in M. sexta responded to a very narrow molecular receptive range much like in the males MGC.

The function of the female-specific glomeruli is so far poorly understood (King, Christensen, & Hildebrand, 2000). The mass staining results in this study suggested that the Fx output neurons may share the same target regions with the neurons carrying plant odor information. This supports the wide-accepted theory about the function of Fx that the processing in these glomeruli is important for female-specific behavior, for instance upwind orientation and recognizing host plants and oviposition cues (King et al., 2000). This theory also well fitted with the male-tracking-female reproductive activities in moth. Although the male pheromone production may not be essential for mating searching, it is still an open question whether female moth can detect male pheromone(s).

4.4 Methodical consideration

In this thesis, a combination of mass staining and intracellular staining in both male and female moth was performed, in order to map the morphology of tracts and their respective neurons. A great benefit of the mass staining technique is that the dye is easy to apply and it offers the opportunity to map a wide-ranging neural projection of interest. Then again, the weakness of this method is the likelihood of not visualizing relevant neural structures, since the dye is to be injected in a highly specific area and may visualize unrelated neurons. The amount of dye applied is also difficult to estimate and may result in unclear outcomes and label unwanted structures. The tracts and structures had to be thoroughly traced and studied, then further compared with previous findings.

The intracellular iontophoretic labeling is a well-suited method to study details of the second order PNs. With success using this technique, the morphology of the projection is highly accurate and simple to tract. On the other hand, this is a very demanding and challenging method including the labeling and the scanning. It is also time-consuming, considering both the amount of practice needed, performing the experiments and the scanning of preparations. The success rate is relatively low, which is the reason why the amount of

intracellular staining preparations presented in this thesis is limited. Then again, the outcome results achieved are highly rewarding.

4.5 Future studies

The dimorphism within the olfactory parallel pathways are poorly studied. This kind of systematical study supplementing information about the female secondary olfactory system has never been done. Finding differences in female and male olfactory system can help understanding how the female choose her oviposition site and how neurological differences can be seen in different behavior or how the same neurons can have different function between the genders. This kind of experiments can gain more knowledge concerning the organization of the olfactory system. By observing the mass staining applied in the different regions of the AL, we can get an overview of the function of this spatial organization and to compare regions and relevant neurons processing either pheromone or odor information.

In future studies it would be beneficial to explore the detailed anatomy of the Fx in the *H. armigera*. It would also be interesting to analyze the neuron circuits morphology together with their function. By using calcium imaging, a method which allows us to measure the glomerular activity in the AL evoked by odor stimuli by using a calcium sensitive dye, it is possible to gain further knowledge of the female response pattern. Supplementary preforming intracellular recordings and staining of the Fx and its PNs.

5. Conclusion

- The 12 mass staining preparations analyzed in this thesis, showed similarities between the male and the female secondary olfactory neurons confined to different tracts.

- Protocerebral regions targeted by secondary projection neurons related to olfaction are consistent with previous findings.

- For the first time, individual neurons confined to medial, mediolateral and lateral parallel olfactory tracts are labeled respectively in female *H. armigera*.

- The lateral-tract projection neuron type targeting the ipsilateral column previously reported in male olfactory system is one of the most prominent types in female moth as well.

- The intracellular staining of olfactory neurons illustrated that the innervation pattern of projection neurons originating from ordinary glomeruli are highly isomorphic in the protocerebrum.

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