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The olfactory pathway in the brain of the large milkweed bug *"Oncopeltus fasciatus"*

Bachelorarbeit

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List of Abbreviation

AL	Antennal lobe
ACT	Antenno-cerebral tract
AMMC	Antennal mechanosensory and motor center
CA	Calyx
CC	Central complex
CNS	Central nervous system
FI	Focal injection
GABA	Gamma-aminobutyric acid
glo	Glomeruli
GNG	Gnathal ganglion
KC	Kenyon cell
lam	Lamina
LN	Local interneuron
LSCM	Laser scanning confocal microscope
LY	Lucifer-Yellow
lob	Lobula
MB	Mushroom body
med	Medulla
MR	Micro Ruby
MSN	Mechano sensory neuron
NGS	Normal goat serum
O. fac.	*Oncopeltus fasciatus*
OSN	Olfactory sensory neuron
PBS	Phosphate-buffered-saline
PBSTX	Phosphate-buffered-saline with Triton X-100
PC	Protocerebrum
PFA	Paraformaldehyde
PL	Protocerebral lobe
PN	Projection neuron
PG	Posterior ganglion
SEM	Scanning electron microscope
SOG	Suboesophageal ganglion
TG	Thoracal ganglion

Abstract

This thesis aims to provide an overview of the 3 stages in the olfactory pathway, antenna, antennal lobe, and mushroom body, for the land-living bug *Oncopeltus fasciat*us (*O. fac.*), and also tries to optimize the backfill method for the use with *O. fac.*.

This study represents the first comprehensive examination of the olfactory pathway in the insect species *Oncopeltus fasciatus*. The olfactory pathway in *O. fac*. initiates with olfactory sensilla located on the distal segment of the antennae. Olfactory sensory neurons (OSNs) within these sensilla project into the outer layer of the glomeruli within the antennal lobe (AL). 114 glomeruli were identified per AL. Projection neurons (PNs), found both in the outer layers or in the hole glomeruli, project through an identified antenno-cerebral tract (ACT) in the calyx. The entire mushroom body (MB) has been reconstructed. The MB consist of one calyx, one pedunculus and 7 lobes and 987 Kenyon cells per hemisphere. This description confirms the presence and alignment of all olfactory pathway stages in *Oncopeltus fasciatus,* consistent with the established model.

Further reconstruction of the neuropiles in *O. fac.* imply that the olfactory pathway assumes an above-average significance in comparison to other land-living bugs. Consequently, it is reasonable to infer that in this insect olfaction holds a crucial role.

Furthermore, a modified version of the backfill method was developed, which allows to determine the projection from the antennae of *O. fac.*. This method is based on the lowering the temperatures on the cutting site before cutting the antenna. Initial optimisation attempts have largely worked successfully and further demonstrate the potential of this method.

1 Introduction

1.1 Insect olfaction

Olfaction is the primary sense of processing smells. For most insects, olfaction is a key modality for controlling their basic behaviour. Insects are exposed to a large variety of chemical odors. By encoding these chemical components, insects learn to improve specific behavioural patterns. E.g., the detection of food sources, avoidance of predators and the search for mating partners. This fact opens a broad research interest, for the insect olfaction, in two main directions. Firstly, researchers may to compare their studies, models, and data of the insect olfactory system to higher vertebrates. Secondly the research is mostly motivated by using the information to supervise and manage harmful insect species in regions with an ecologically unstable environment. (Reference [1])

1.2 *Oncopeltus fasciatus*

The large milkweed bug is a land-living insect of the family Lygaeidae. It mostly inhabits north and central America and lives in fields and meadows containing milkweed or dogbane [2]. Its adult form has a length between 1,0-1,5 cm. Typically identified is its orange back and wing colour, which is patterned by a black X-shaped mark. Like all insects *O. fac.* body is divided in three segments the head, thorax, and abdomen.

Prominent at the head is a proboscis which the bug uses to drink liquids and drink liquids. *O. fac.* feeds mostly from seed. Anterior on the underside of the head two antennae are placed. The visual organs the ocellus and compound eyes are pairwise located on the top and the side of the head. The thorax is mostly covered the Pronotum and the Scutellum. Shortly behind this shield are two wings. The Gender of the animal can be identified by features at the bottom abdomen side. There are eleven segments. The third segment is different from sex to sex. If there is a difference in the shape to the other segments then the animal is female, else it's a male. [2].

O. fac. is not a pest-insect, but it is suitable as a model for Heteropteran, due to its easy way to rear, to handle, for its short development time and a good experimentality.

1.3 Structure of the insect central nervous system

The central nervous system of insects is principally a ventral nerve cord. The nervous system is divided in two brain hemispheres. It has therefore mirrored nerve cords (ganglia) on both brain hemispheres located along the body, which are connected by connectives. As the name implies, the entire nervous system is located on the ventral side of the insect, as shown in the figure 1.

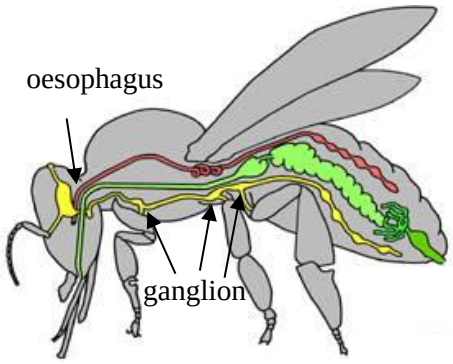

Fig 1: Schematic of the internal organs in *Apis mellifera*. Yellow: nervous system, green: digestive tract, red: central circulatory system [3]

A distinctive characteristic of insect brains is that the oesophagus, as part of the stomach extends between the first two ganglia. For this reason, the first ganglion is called "supraoesophageal ganglion" and the second ganglion is called "suboesophageal ganglion" (SOG). The supraoesophageal ganglion is significantly larger than all other ganglia and receives the most inputs within the nervous

system. Due to its significance in the central nervous system (CNS) the "supraoesophageal ganglion" is referred to as the brain.

It is assumed that the brain is the product of an evolutionary fusion of three ganglia, the protocerebrum, the deutocerebrum and the tritocerebrum [4].

The protocerebrum (PC) is the largest part of the brain. This part of the brain contains the protocerebral lobe and the paired optic lobes (OL), laterally. The OL is the primary visual centre and receives input from the ommatidia of the facet eyes. The OL is subdivided into the lamina, medulla, and lobula. Furthermore, the PC incorporates the paired mushroom bodies, which are responsible for multimodal sensory input, and a non-paired Central Complex (CC). The CC are medial and occupy both brain hemispheres in the brain. The deutocerebrum contains the antennal lobes (AL) and the antenno mechanosensory motor centre (AMMC). The deutocerebrum processes all antennal sensory information and is responsible for the muscular control of the antennae [1].

The Tritocerebrum is not explored more deeply yet. It is known, that the tritocerebrum is the smallest of the 3 sections. In most insects it is located posteriorly in the brain and lies directly above the oesophagus. The tritocerebrum is connected to the attached deutocerebrum and the lower SOG. It has been shown that the tritocerebrum receives input from the deutocerebrum, protocerebrum and primary sensory input from the mouthparts. Further posterior in the nervous system are various small ganglia, which are partly responsible for the mechanical control of the legs called thoracic ganglion (TG) and posterior ganglion (PG)[4].

1.4 The olfactory pathway in insects

The general structure of the olfactory pathway in insects is well investigated and also well conserved through the species. The olfactory system is divided in three main parts. The first is the antenna, the second is the antennal lobe (AL) and the third is the mushroom body (MB) as part of the protocerebrum (PC) [1].

1.4.1 Antenna

The first processing stage of the olfactory pathway. The antenna is covered with numerous sensilla, which look like hairs. There are different types of sensilla which can be categorized by their function in olfactory, mechano and hygro sensilla. I will focus on olfactory sensilla.

The basic morphology of the olfactory sensilla is similar across species. Usually, the sensilla are hollow cuticula-tubes, filled with lymph fluid. The walls are interspersed with fine openings (wall pores). Dendrites of olfactory sensory neurons (OSN`s) are located in the liquid. (See Fig. 2)

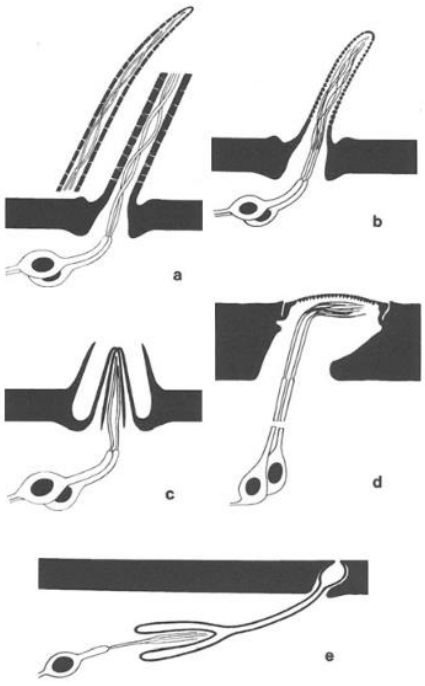

Fig. 2: Schematic of different olfactory sensilla types of *Helicoverpa armigera.* Indication of the path of an olfactory sensory neuron. Sensilla types: **a** s.tricodeum, **b** s.basiconicum, **c** s.coeloconicum, **d** s.placodeum, **e** s.ampullaceum.

Source: Hansson et al. (1999) [1]

Odor specific receptor on the membrane of the OSN dendrites transduce the chemical stimulus into an action potential (AP). The information will be forwarded trough the axons down the antenna in the brain. The antennal Nerve (AN) consist of OSNs, which later enter the antennal lobe (AL) and mechanosensory sensilla (MSN), which enter the AMMC.

1.4.2 Antennal lobe

The antennal lobe (AL) is the second stage of the olfactory system, but the first brain region, to process the olfactory information from the antennae. Usually, the AL in land-living Hemiptera has a spherical shape, filled with a globelike substructure called glomeruli. These structures are innervated by a high number of axons and dendrites of olfactory sensory neurons (OSNs), projection neurons (PNs) and local interneurons (LNs). The interconnection between these forms a network that encodes the odor information.

Local interneurons mostly originate outside the antennal lobe in the so called large lateral cellcluster (LCL) and extend only into the AL [1]. These neurons arborise in many or all glomeruli. Synapses of the LNs interconnect with all 3 neurotypes (LNs, PNs and OSNs) in the AL. The synapses of this neuron type usually use gamma-aminobutyric acid (GABA) as their neurotransmitter. GABA is believed to have an inhibitory neurotransmitter effect.[5]

Projection neurons are the only neurons in the AL which project to the protocerebrum. They extend with axons in the protocerebrum and terminate with in the calyxes (CA) or the lateral horn (LH). PNs can be uni-, oloigo- or multiglumerular. As soon as the PNs leave the AL, the PNs organize into several fibre tracts called antenno-cerebral tracts (ACT). These tracts can be categorized in groups according to their location in the brain. The inner antenno-cerebral tract (IACT) is biggest and most important of the ACTs and connects first with the calyx and second with the lateral horn. (See Figure 3)

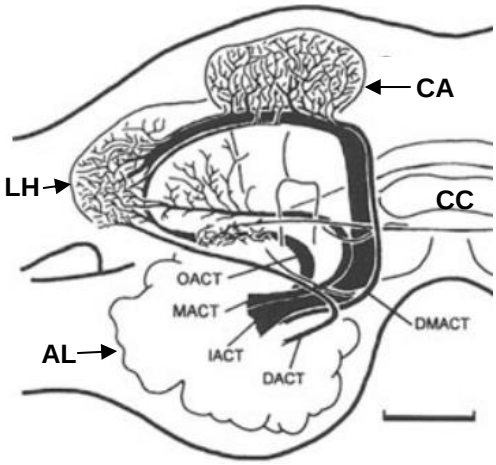

Fig. 3: Fiber tracts connecting antennal lobe (AL) with Protocerebrum in *Manduca sexta* (sphinx moth). Different ACT tracts: inner antenno-cerebral tract (IACT), middle antenno-cerebral tract (MACT), outer antenno-cerebral tract (OACT), dorsal antenna-cerebral tract (DACT), dorso-medial antenna-cerebral tract (DMACT). AL, antennal lobe; CA calyx; CC, central complex; LH, lateral horn. Scale bar: 200µm. Source: Hansson et al. (1999) [1]

1.4.3 Mushroom body

The mushroom body (MB) is the third stage of the olfactory pathway. The function of the MB is still discussed controversial, but studies showed a correlation between the MB size and the complexity of insect social behaviour. The MB is also believed to play a large role in memory formation. [1]

This neuropil has a very high structural variability across species and is therefore not clearly morphologically characterizable. The MB is part of the Protocerebrum, and is subdivided into the calyx, the peduculus and the lobes. PN's input the olfactory information from the AL in the calyx, trough the ACT. In the calyx PNs and the so called

“Kenyon cells” (KC) are interconnected. Through the densely, with Kenyon Cells, packed fibre tract named peduncles, the MB stretches through the brain to the lobes. The KCs again interconnect in the lobes. The lobes are divided into α- β- and γ-lobes, and usually form a finger-like structure [1]. The number of lobes and “Kenyon cells” is different between species and is therefore a big statistic research interest.

1.5 Research Interest

In this scientific work, a full characterization of all the stages described in the introduction (Chapter 1.4), of the olfactory pathway, for the land-living bug *Oncopeltus fasciatus*, will be pursued using histologically established methods.

Furthermore, it was found in preparatory experiments, that for land bugs of this size, the traditional "backfill" method does not yield the desired data. For this reason, a better understanding should be gained in order to optimize the method.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Tab. 1: List of the used chemicals

Chemical	Supplier
Ethanol	Carl Roth, Germany
Glycerol	Carl Roth, Germany
Micro Ruby	
Methyl-Salicylat	Sigma Aldrich, Germany
Normal Goat Serum	Cell Signaling, USA
Paraformaldehyd	Carl Roth, Germany
Phosphate-buffered-saline	Sigma Aldrich, Germany
Triton X-100	Sigma Aldrich, Germany

2.1.2 Solutions

Tab. 2: List of the used solutions

Solution	Preparation method	Storing
Dye solution (backfill)	2.5% Micro Ruby in 0.1M PBS	21ºC
Dye solution (Focal injection)	5% Micro Ruby in 0.1M PBS	21ºC
PFA	4% Paraformaldehyd 96% Aqua dest.	-20ºC
PBS	0.1 M phosphate-buffered-saline	4 ºC
PBSTX	0.2% Triton X-100 in 0.1M PBS	4 ºC

2.1.3 Antibodies

2.1.3.1 Primary antibodies

Tab. 3: List of the used primary antibodies

Primary antibody	Supplier	Storing
Mouse anti Synapsin	DSHB	-20 ºC
Polyclonal Rabbit anti GABA	Sigma	-20 ºC

2.1.3.2 Secondary antibodies

Tab. 4: List of the used secondary antibodies

Secondary antibody	Excitation maximum	Supplier
Goat anti mouse	546 nm	Invitrogen, USA
Goat anti rabbit	488 nm	Invitrogen, USA

2.1.4 Equipment

Tab. 5: List of the used equipment

Equipment	Supplier
Axio Zoom V16	Carl Zeiss, Germany
CCU-010	Safematic GmbH, Switzerland
coverslip	Carl Roth, Germany
Imager M2	Carl Zeiss, Germany
Gemini Leo 1530	Carl Zeiss, Germany
LSM 880	Carl Zeiss, Germany
medical syringes	Braun, Germany
microscope slide	Carl Roth, Germany
orbital shaker KS260	IKA, Germany
pipettes	GILSON, USA
razor blades	Personna, USA
reaction vessels (different sizes)	Carl Roth, Germany
scale	Sartorius, Germany
SEM stub ½”	EMZ Jena, Germany
scalpel	GRIFF INDUSTRIES, Great Britain

Equipment	Supplier
scissors	FST, German
stereomicroscope	Olympus, Japan
tweezers	Dumont, Switzerland

2.1.5 Software

Tab. 6: List of the used software

Software	Supplier
Adobe Photoshop CS	Adobe Systems
Amira 5.3	Fei, Visualization Science Group
Autodesk Fusion 360 (Education License)	Autodesk Inc.
Image J 1.53t	Public Domain
Microsoft Office	Microsoft Corporation
Microsoft Paint	Microsoft Corporation
ZEISS ZEN 2 Imaging Software	Carl Zeiss, Germany

2.2 Methods

As shown in Figure 4, all methods except SEM can be combined in their sequence. Since all staining methods use florescent dyes, the emission spectrum must not overlap. The possible dye-channels are 488nm 564nm and 633nm, depending on the microscope used.

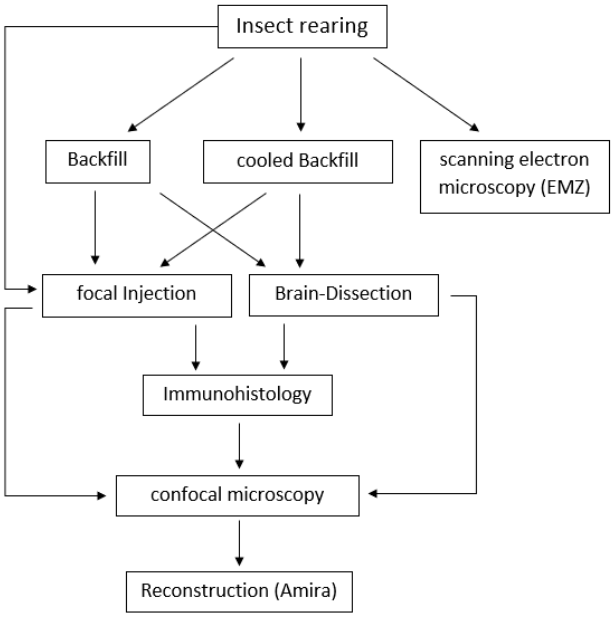

Fig 4: Flowchart with all possible combinations of the used methods

2.2.1 Insect rearing

Only *Oncopeltus fasciatus* Adult (male and female) were used in the experiments, which were kept in an established laboratory colony in an 15cm-by-15cm-by-15cm cage at room temperature. The bugs were fed sunflower seeds. For water supply, they were fed with a 2-ml microcentrifuge tube filled with water and sealed with absorbed cotton. The colony is a subsidiary of another existing colony of the Max-Planck-Institute Research Group "Predators and Toxic Prey" under Kati Barthold.

2.2.2 Scanning electron microscope

For a closer observation of different antenna parts, the scanning electron microscopy (SEM) was used. This Method was done in cooperation with the Electron Microscopy Centre of the University Hospital in Jena (EMZ). First the antenna was cut off. The antenna tip was dehydrated in an increasing ethanol series (30%, 50%, 70% 90%, 99%, 100%, 100%, 10 min each). The preparation was then handed over to the EMZ. Subsequently, a critical point drying was carried out, by the Institute. The Two antennas were attached in standing position on a ½" SEM Stub with conductive glue. Then a gold sputtering was performed whit a CCU-010 after this the preparations were scanned. All images were obtained with a scanning electron microscope (Gemini Leo 1530).

2.2.3 Backfill

For the identification of the antennal lobes, the backfill technique is an established method. The basic idea is that the dye should selectively reach the neuropil-region, in which the dye, like the information, passes through the nerve into the brain. Therefore, the nerve was selectively damaged and then the damaged tissue was infused with dye.

In preparation for the method, the insect was immobilized. The legs were cut off and the insect was fixed with plasticine on a flat plate in dorsal position. Scissors were then used to make a targeted cut on the insect's antennae. A small volume bound by a circle of Vaseline was placed around the cut. The purpose of this Vaseline pool was to allow liquids to get applied to the cut section. Water was first completely filled into the pool for 10 minutes. Then the water was replaced by about 1µl of the dye solution (2.5% Micro Ruby in 0.1M phosphate-buffered-saline (PBS)) The pool was capped with Vaseline. The entire experiment was placed in a darkened box. A paper tissue dipped in water was placed in this box to humidify the air and thus preventing the preparation from drying out. In the end, the box was kept in the refrigerator at 4°C for 2h-1 day.

The experiment was then further processed in the brain dissection or focal injection (See Figure 4).

2.2.4 Cooled backfill

This method is an optimized version of the backfill technique, which was developed due to problems with the original method.

As in the original method, the insect was immobilized as a preparation, by cutting off the legs. The insect was then fixed in the dorsal position with plasticine on a device specially developed for the method, as seen in the Figure 5. Attention should be given that the antennae of the bug are overhanging. The insect was then cut open horizontally at the abdomen with a scalpel.

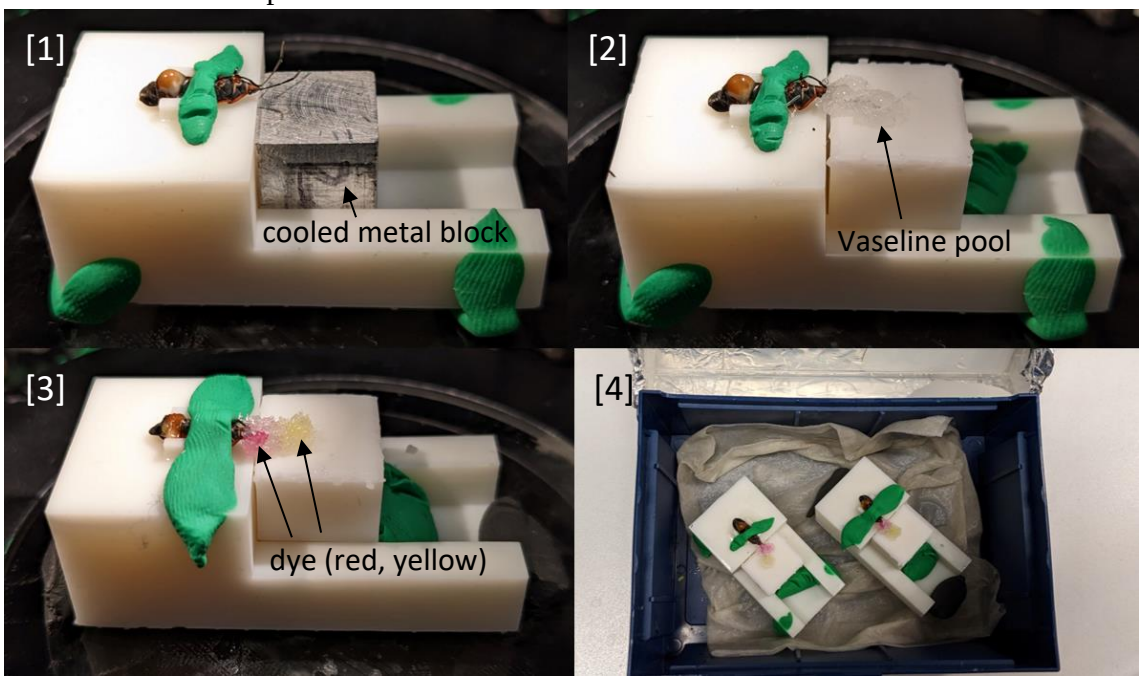

Fig 5: Sequence of pictures, of the most important working steps of the cooled backfill method, performed on a bug-mount. (1) Immobilization, abdomen cut, cooled block placement. (2) Vaseline-Pool, water soaking. (3) Infusion with a dye solution, (4) Storage.

As shown in Fig. 5, a previously cooled metal block was placed under the antennae. The shape of the block and the cutting technique was varied for optimization tests. For the experiment, two different shapes of an aluminium block were used. The first version was a 1,3cm-by-1,3cm-by-1,6cm cube. The second version was an L-shaped block with dimensions x 1,2cm, y 1,2cm and z 1,6cm with a thickness of 4,5cm. These blocks were cooled to a temperature of -196 °C by liquid nitrogen. The cut was then performed. Two cutting techniques were performed. The vertical cut, which cuts through the a to damage the nerve in the antenna and the horizontal cut, which cuts the sensilla with a scraping move.

After the cut, the aluminium block is replaced by a platform. On this, as in the original method, a Vaseline trough was sprayed around the cut. Water was then added to the trough for ten minutes at room temperature, which was then replaced with a Micro-Ruby solution. The solution is a 2.5% Micro-Ruby water mixture. The trough was sealed with Vaseline and stored in a darkened box with a wet cloth in the refrigerator at 4°C for 2h-1 day. Brain preparation then took place.

The experiment was then further processed in the brain dissection or focal injection (See Figure 4).

2.2.5 Brain dissection

The first step, if not already done in previous methods, was to immobilize the insect by cutting off its legs. Then, the insect was embedded in wax in ventral position. The insect is then completely submerged with 0.1M PBS. For the next steps the embedded insect was placed under a stereomicroscope. Using a scalpel and tweezers, the pronotum is removed, and all muscles and glandular tissues are removed until the ganglia are visible. Then the insect head was cut open and the head shell was removed. The now visible tissue was carefully removed until the brain was visible. The tracheas on the surface of the brain were removed neatly. The antennal nerves and ganglionic nerves were then cut. Very carefully, with tweezers, the brain was then peeled out of the head. For fixation the dissected brain was transferred into 4% Paraformaldehyde (PFA) in 0.1M phosphate-buffered-saline (PBS) and was kept in a fridge at 4ºC.

The brain can be used in immunobiological methods or scanned with confocal microscopy (See Fig. 4).

2.2.6 Focal injection

For the identification of the antenna-cerebral tract (ACT), the method of focal injection was used. The basic idea of this method is similar to a backfill. In both methods a specific damaged part of neurons is infused with dye to trace the projections of it in the brain. In contrast to backfill, in this method a primary damage does not target a nerve, but rather a region within the brain e.g., a neuropil or a soma-cluster and exposed to dye.

Similar to brain dissection, the first step, if not already done in previous methods, was to immobilize the insect by separating the legs and embedding the insect in a ventral position in wax. The insect was then completely covered with 0.1M PBS. For the next steps the embedded insect was placed under a stereomicroscope. The head of the bug was opened and the tissue overlying the brain was removed. Afterwards the PBS was removed, with a scalpel and tweezers. The electrodes were filled with 1µl dye solution (5% Micro-Ruby in 0.1 M PBS). The tip of the electrode was pierced into the targeted brain region. In my experiments the calyx was pierced. The insect was then again carefully covered with 0.1M PBS. For 10 minutes to 2 hours the head was placed under a light impermeable cover. The cover was removed, and the antennal nerves and ganglionic nerves were cut. Very carefully, with tweezers, the brain was then peeled out of the head. For fixation the dissected brain was transferred into 4% Paraformaldehyde (PFA) in 0.1M PBS and was kept in a fridge at 4ºC.

The brain can be further used in immunobiological methods or scanned with confocal microscopy (See Fig. 4).

2.2.7 Immunohistology

To visualize the central neuropils in the brain, anti-synapsin-antibody staining was performed. To visualize the local interneurons in the antennal lobe, an anti-GABA-antibody staining was performed. The protocol is based on the immunohistological staining protocol used by Gui Ying Xie et al. [6].

After the fixation of the brain in the brain dissection (See methods chapter 2.2.5) or the focal injection (See methods chapter 2.2.6), the brain was again transferred into 0.1 M phosphate-buffered-saline (PBS) and washed for 10 minutes. The washing was repeated

two times. Then the brain was dehydrated in Ethanol with rising concentration 30% 50% 70% (per step 10 minutes) and then rehydrated in 50% and 30% Ethanol (per step 10 Minutes). Then the brain was washed in 0.3M PBSTX (Trition X-100 in 0.1M PBS (See Tab. 2) for 10 minutes. The next step was a preincubation with 5% normal goat serum (NGS) (Cell Signaling, USA) in 0.3M PBSTX for 2h or overnight at 4°C. The used primary antibody solution is a mouse anti-synapsin-antibody, at a concentration 1:20 with 1% NGS in 0.3M PBSTX or a goat anti-GABA-antibody at a concentration 1:1000 with 1% NGS in 0.3M PBSTX. 1µl primary antibody solution is applied to the brain and incubated for 2-4 days at room temperature and placed on an orbital shaker (KS260). After this the brain was washed 3x in PBSTX (20 minutes each). The used secondary antibody is dependent on the animal of the primary antibody. A goat anti-mouse-antibody, at a concentration 1:200 in 0.3M PBSTX is used for the synapsin-staining. A goat anti-rabbit-antibody, at a concentration 1:200 in 0.3M PBSTX is used for the GABA-staining. The secondary antibody carries a fluorescent marker, for this reason all the subsequent procedures are performed under an aluminium foil cover. 1µl secondary antibody solution was applied to the brain and again incubated for 4-6 days at room temperature and placed on an orbital shaker. The last step was washing the brain 4x with 0.1M PBS on an orbital shaker for 20 Minutes each.

The dyed brain can be scanned with confocal microscopy (See Fig. 4).

2.2.8 Laser scanning confocal microscopy

Laser scanning confocal microscopy (LSCM) is used to visualize the experiments mentioned above. The basic function of LSCM is to stimulate a certain point in the tissue to emit light and to exclude all light that does not come from this focal point. This allows individual planes to be sharply focused deep in the tissue [7].

A very simplified description of the basic principles of this type of microscope is the following. The set-up shown in Fig.__ is used to enable the features of this type of microscope. A prominent feature is the use of a built-in interference-specific mirror, also known as a dichroic mirror, which allows both the excitation beam and the emission beam to be guided in a single beam path. Another striking feature is the installation of a pinhole lens. This enables the visualization of individual layers in tissue samples. The effect of optical bending in optical set-ups such as microscopes creates the so-called Airy disk. This Airy disk can be manipulated by using the pinholes.

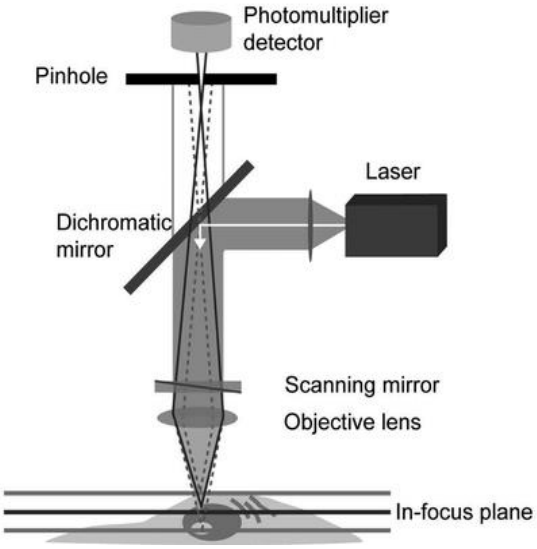

Fig 6: Components of a confocal microscope. Source: Amicia D. Elliot (2021) [7]

By narrowing the pinhole, the amount of light hitting the detector field is reduced. This enables control of the amount of light that reaches the detector to focus on the emitting point in the tissue. The light portion of the Airy disk that is necessary for focusing is called the Airy unit. By moving the scanning Mirow, each point of the scanning area is scanned. This produces an image of a single

layer. By repeating this process for different layers, a stack of images can be produced, which can be combined to form a block [7].

For the LSCM, a prepared and fixed brain is required (See Fig. 4). This is dehydrated in an alcohol series in 30 50 70 90 99.8 100 100 % (every 10 minutes) ethanol. The subsequent steps are carried out under a fume hood. The brain is then transferred to methyl salicylate. The brain is then transferred to methyl salicylate, which has the effect of making the brain completely transparent. The brain is placed with tweezers in a microscope slide filled with methyl salicylate and covered with a cover slip. The edges are sealed with glass glue to prevent drying out.

The slide is placed in the confocal microscope (LSM 880) and then scanned.

The results can then be processed using the image processing method (See Fig. 4).

2.2.9 Image processing

For adjustment of the confocal image stacks, for reconstruction of fibre tracts and neuropiles and maximum intensity projections, Amira 5.3 (Fei, Visualization Science Group) and ZEISS ZEN 2 Imaging Software (Zeiss, Jena, Germany) was used. One method that requires a detailed explanation is the visualization in Amira via a Voltex. This method assigns a volume to each pixel in a slice. This is defined by the pixel size and the thickness of a slice. This volume is given a colour, which is determined from the bit value of the pixel. All volumes are rendered together to form a three-dimensional body.

3 Results

3.1 Structure of the central brain

An analysis of the general organization of the brain into neuropils was performed by immunohistochemical staining of synapsin (See methods chapter 2.2.7). The expectation of those Experiments is that all regions in the brain rich in synapses, i.e., neuropils, will be stained and all regions that do not contain any synapses, i.e., fibre tracts or somata, will not be stained. By reconstructing the neuropils, their position in the brain can be elucidated. The results of this experiment have provided extensive information about the structure of the brain of *O. fac*. as shown in figures 7-9.

The nervous system of *O. fac*. is clearly a rope ladder nervous system (See Fig. 7). A large anteriorly supraoesophageal ganglion is situated, which is followed by a more ventral suboesophageal ganglion. This ganglion is directly connected to the thoracal ganglion and further posterior the posterior ganglion by two connectives. Both the TG and the PG receive two large nerve tracts from the legs. The TG receives input from the anterior pair of legs and the PG receives input from the rest of the legs. The whole nerve system has the following dimensions: X=1025µm, Y=1556µm, Z=452 µm.

The entire brain appears very compressed. The optic lobes are exposed anterior laterally from the Brain. The AL are ventral anterior exposed in the brain. The AL hide the posterior mushroom lobes from the anterior view.

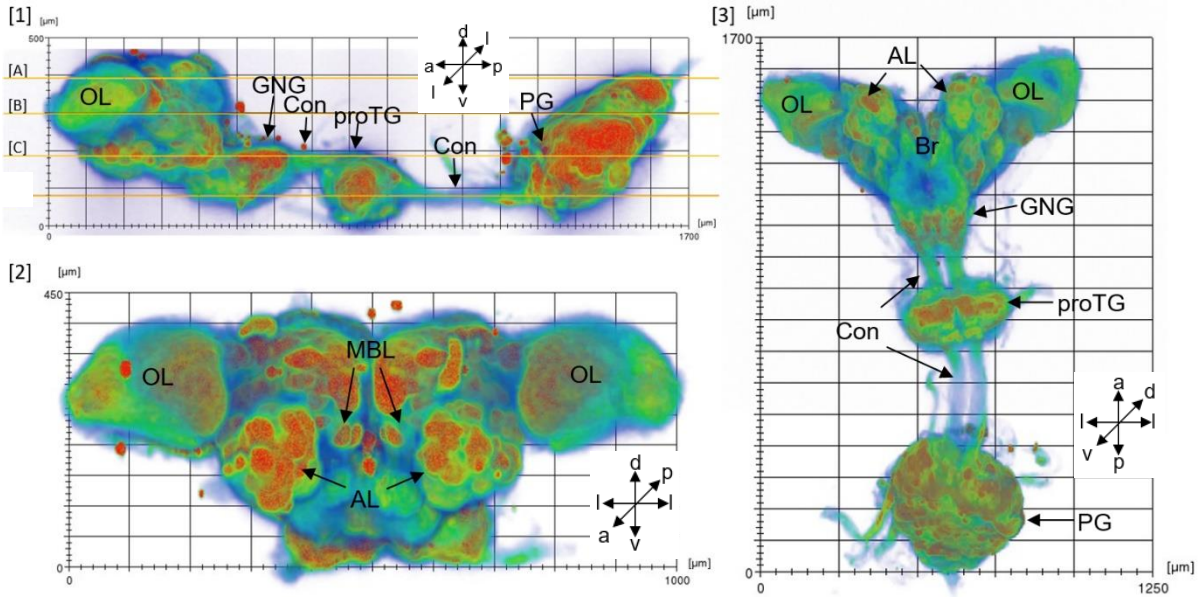

Fig 7: Three-dimensional confocal scan of an anti-synapsin antibody staining of *Oncopeltus fasciatus*. Pictures generated by a Voltex in Amira. (1) lateral view. (2) anterior view. (3) ventral view. (1) orange lines show the slices for figure 8. AL, antennal lobe; Br, central Brain; Con, Connectives; GNG, gnathal ganglion; MBL, lobes (mushroom body); OL, optic lobe; PG, posterior ganglion; TG, thoracal ganglion. Directions: a, anterior; d, dorsal; l, lateral; p, posterior; v, ventral.

For a better insight into the organization of neuropile in the brain, it is required to analyse the brain in slices with different depths (See Fig. 8)

The neuropil boundaries in the brain are mostly specifically recognizable. The deutocerebrum and protocerebrum can be clearly separated, but the neuropil limits of the tritocerebrum to the protocerebrum cannot. Further observations are the following. A subdivision of the OL into lamina, medulla and lobula based on their morphological characteristics is possible. Also, very well identifiable is the AL and the glomeruli in it. The protocerebrum is traversed by many unstained structures. These are fibre tracts. One of these fibre tracts connects the calyx and the mushroom lobes, therefore this has to be the pedunculus.

A second staining with anti-GABA antibodies was also performed on the brain in Fig. 8 (in green). This stained the soma-clusters which are visible as green dot clusters at the edge of the brain. Local interneurons in the AL, in which GABA is present as an inhibitory neurotransmitter, were not stained.

The reconstruction of the CNS has enabled the identification of the organization of the neuropils. (See Fig. 9).

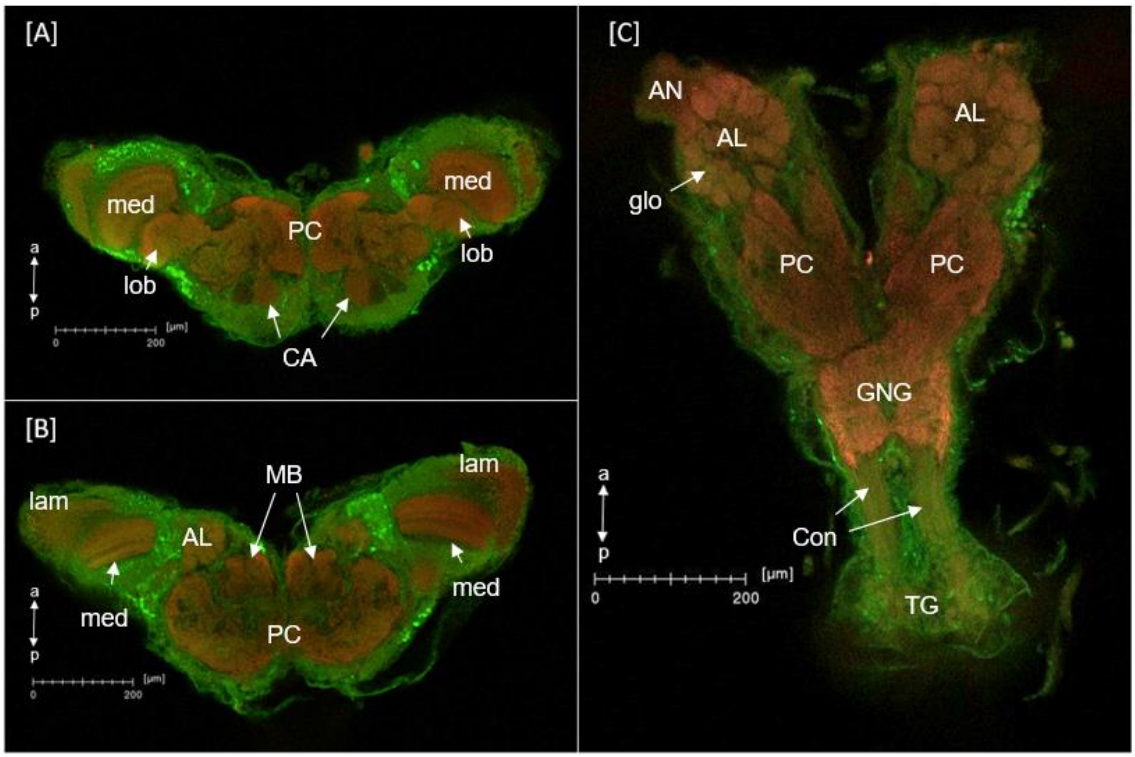

Fig 8: Slices of the central nervous system of *Oncopeltus fasciatus* from Figure 7.1. Slices in different depth. (A) 390 µm. (B) 300 µm. (C) 180 µm. Red channel (546 nm): anti-synapsin antibody staining. Green channel (488nm): anti-GABA antibody staining. AL, antennal lobe; AN antennal nerve; CA, calyx; Con, connectives; glo, glomeruli; GNG, gnathal ganglion; lam, Lamina; lob, lobula; MB, mushroom body; med, medulla; PC, protocerebrum; TG, thoracal ganglion. Directions: a, anterior; d, p, posterior.

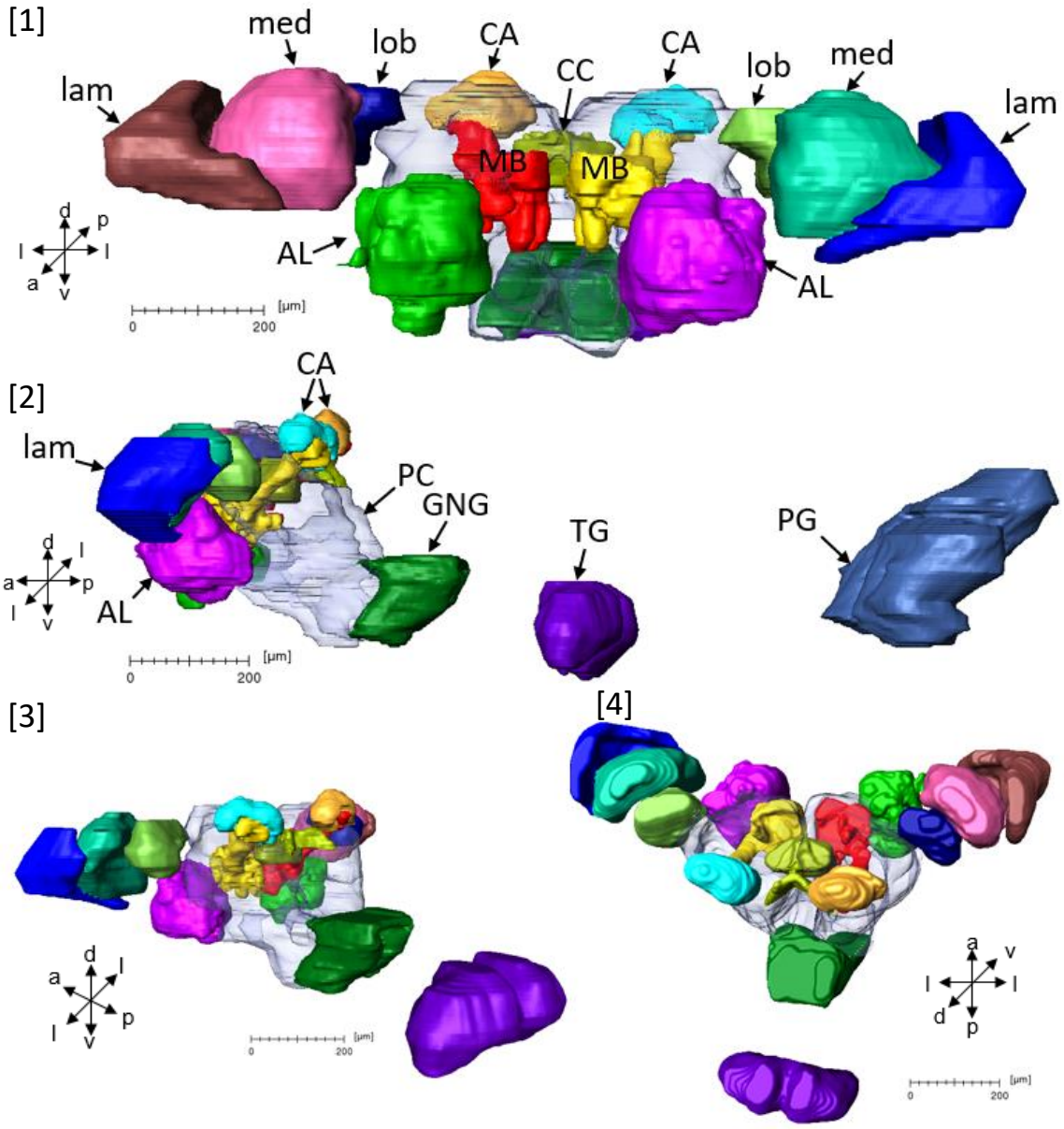

Fig 9: Three-dimensional reconstructions of the central nervous System of *Oncopeltus fasciatus*. (1) anterior view. (2) lateral view. (3) lateral-posterior view (4) dorsal view. AL, antennal lobe; CA, calyx; CC, central complex; GNG, gnathal ganglion; lam, Lamina; lob, lobula; MB, mushroom body; med, medulla; PC, protocerebrum; PG, posterior ganglion; TG, thoracal ganglion. Directions: a, anterior; d, dorsal; l, lateral; p, posterior; v, ventral.

3.2 Olfactory sensilla

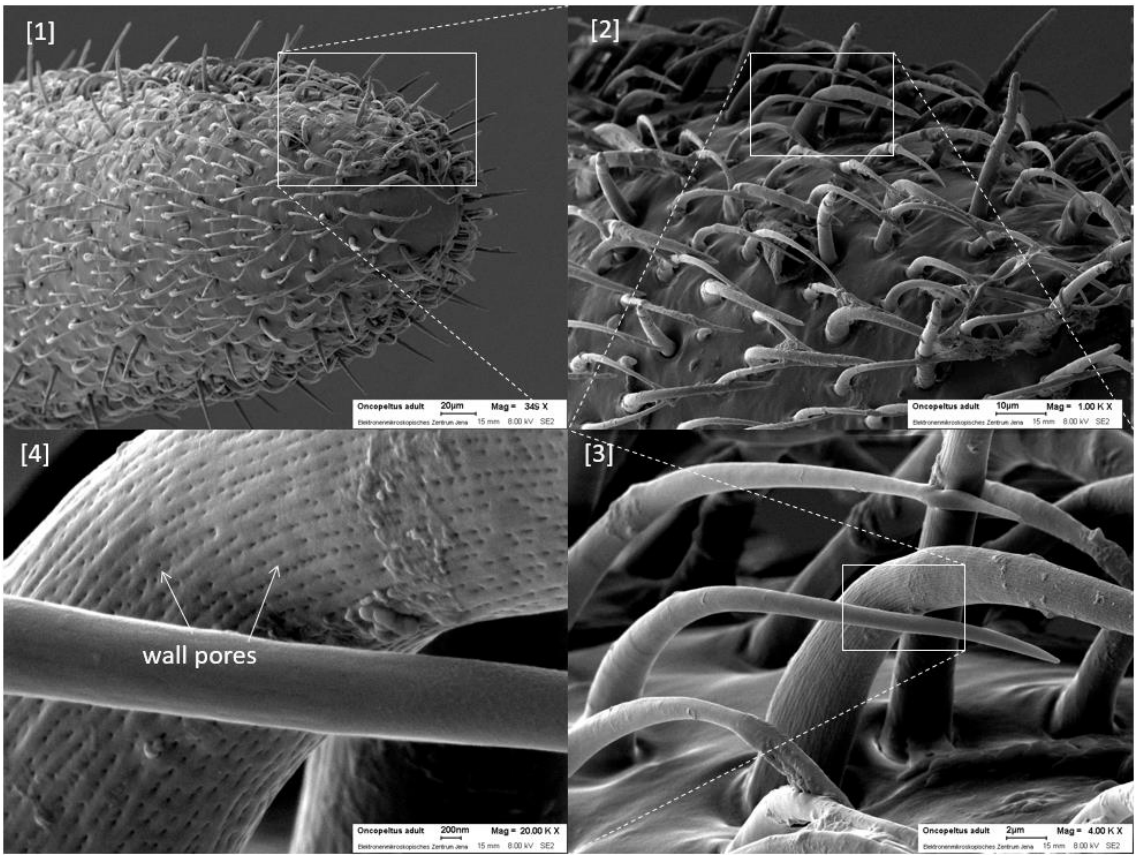

Fig 10: Scanning electron microscopy images of antennae tip and sensilla of *Oncopeltus fasciatus*. Image at different magnifications. (1) 394x, (2) 1.000x, (3) 4.000x, (4) 20.000x. (4) Small dots can be identified as wall pores.

As explained in the introduction chapter 1.4.1, the morphological structure of olfactory sensilla has been extensively studied. Identification is possible based on so-called wall pores, which are exclusively found on olfactory sensilla.

The antenna of *O. fac* is divided into 4 segments. The direction towards the tip is called anterograde and towards the head basal. All segments of the antenna are completely covered by sensilla. For this reason, all segments of both male and female animals were randomly tested for sensilla with wall pores. For each gender, 2 antennas were tested. The findings show that sensilla with wall pores were identified exclusively in the anterograde segment. This result is observed in both male and female animals and is therefore not sex-specific. One sensillum identified as olfactory can be seen in Fig 10. It is clearly visible that the entire sensilla is covered with wall pores. The identified wall pores have a diameter of 25 nm (N=3). In addition to this sensilla type, two other sensilla types were found based on morphological characteristics. A deeper identification of these was not conducted.

3.3 Backfill

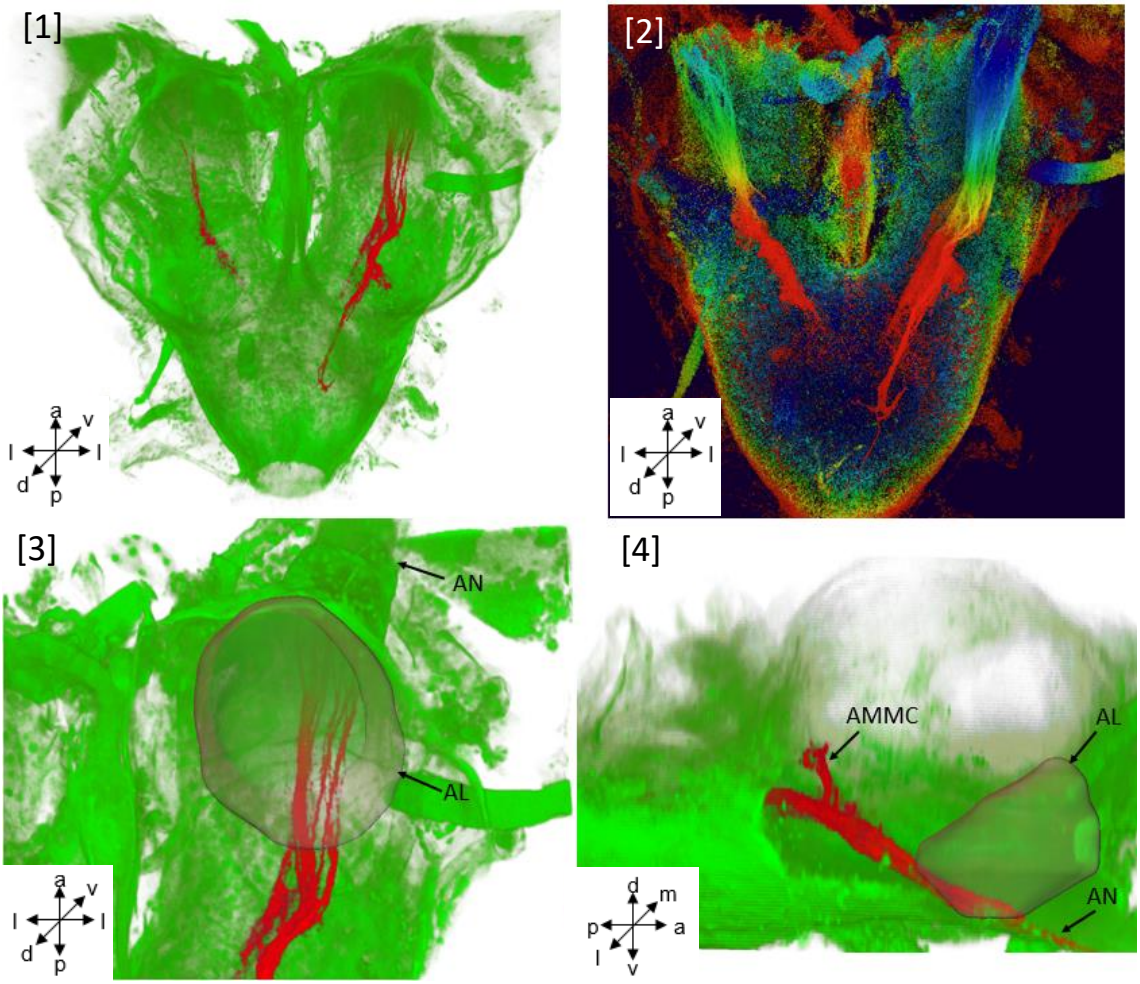

Fig 11: Confocal images of *Oncopeltus fasciatus*' brain after normal backfill. (1,2,4) images generated by a Voltex in Amira. (3) Intensity projection view (1-3) dorsal view. (4) lateral view. (2). AL, antennal lobe; AN, antennal nerve; AMMC, antennal mechanosensory and motor centre. Directions: a, anterior; d, dorsal; m, medial; l, lateral; p, posterior; v, ventral.

As explained in the introduction chapter 1.4.1-1.4.2, neurons of different modalities traverse the antennal nerve and branch into different neuropils in the brain. For example, OSN ramifies into the AL or MSN into the AMMC. It is likely that a backfill will affect the different types of neurons in the nerve and stain them.

As can be seen in figure 11.1, the backfill method does not give the anticipated results. After backfilling, the stained neurons do not disperse in the antennal lobule. The neurons extend from the AN entrance, which is located anterior ventrally, toward a posterior ventral position, as shown in figure 11.2. All neurons run ventrally past the AL. Some of the neuron's branch after a junction in the AMMC. Others extend into the gnathal ganglion. A few of them extend as far as the connectives, which connect the brain with the TG.

3.4 Cooled backfill

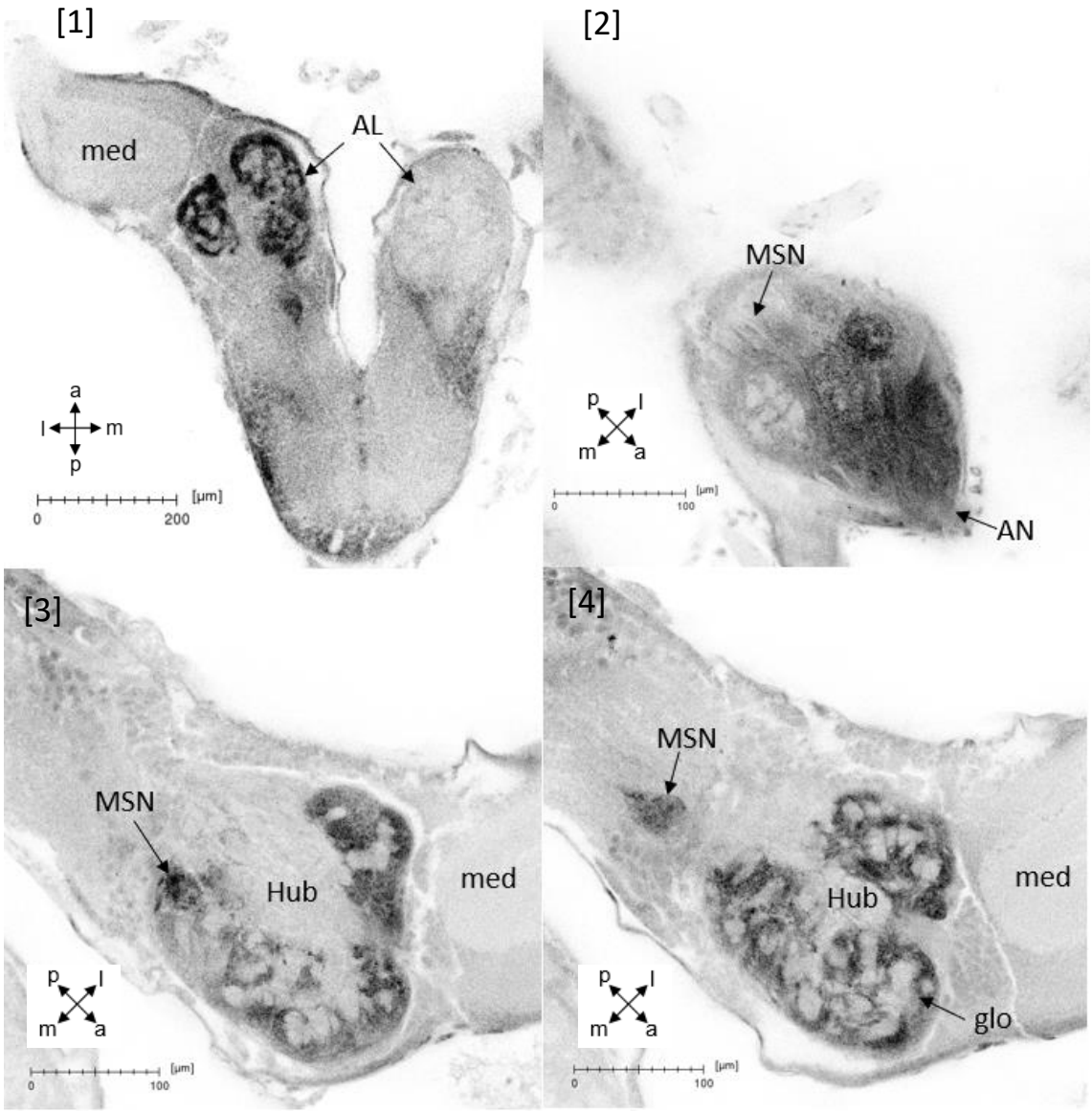

Fig 12: Confocal images of *Oncopeltus fasciatus*' brain after cooled backfill. Backfill parameter: Block 1 at -196ºC without a bug-mount, vertical cut on only left antenna. (See methods chapter 2.2.4). (1) overview picture, (2-4) pictures of the AL every 75µm, from ventral to dorsal. Used dye is Micro Ruby scanned at 546 nm. AL, antennal lobe; AN, antennal nerve; glo, glomeruli; med, Medulla; MSN, mechano-sensory neuron. Directions: a, anterior; m, medial; l, lateral; p, posterior.

Since a normal backfill did not give the intended results (See results chapter 3.3), a first experiment was performed with an optimized version of the backfill without a bug-mount (See methods chapter 2.2.4).

Through the cooled backfill, staining the antennal lobes (AL) was possible as depicted in Figure 12. Especially in Figure 12.1 it can be observed that the left AL is specifically stained compared to the right AL. A close inspection of the left AL at different depths (Figure 12.2-12.4) well visualizes the course of all neurons (MSN and OSNs) emerging out of the AN. A distinct staining of the outer layers of the glomeruli is visible. The inner structures of the glomeruli and the hub does not contain any dye.

Based on the positive data of the first experiment, the method was applied to a group of 6 *Oncopeltus fasciatus*. In at least 5 of them, dye was found in one of the antennal lobes.

The quality of the results is limited to a very diffuse staining in the AL. The major structures of the glomeruli are recognizable, but no differentiated fibres are visible. In addition, other neuropils are affected by nonspecific staining. It has been shown that especially the optic lobe is affected by such staining. Especially in the lamina a high concentration of dye is present. The medulla remains largely unstained as seen in Fig. 13. However, parallel striped staining on the outer layers of the medulla, which recurrently appear between different experiments, is noticeable.

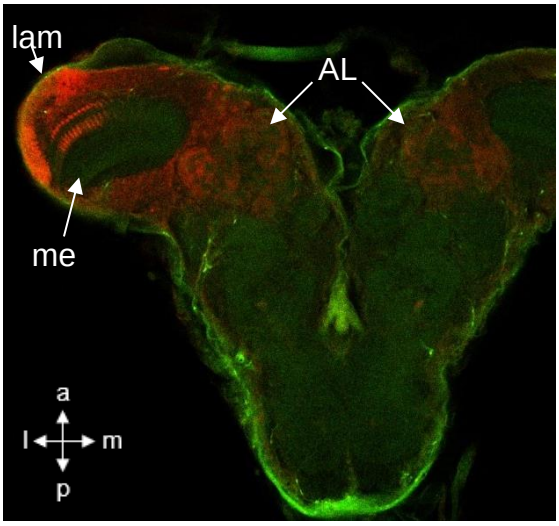

Fig 13: Confocal images of *Oncopeltus fasciatus'* brain after cooled backfill. Backfill parameter: Block 1 at-196ºC, vertical cut on both antennae, on a bug-mount. (See used method 2.2.4). Used dye is Micro Ruby (546 nm, red) and autofluorescence (488nm, green). AL, antennal lobe; lam, Lamina; med, Medulla. Directions: a, anterior; m, medial; l, lateral; p, posterior.

To obtain the theoretical understanding of the cut site of the cooled backfill in comparison to the normal backfill, these were examined in more detail under the scanning electron microscope (SEM). The experiment contained 8 antennae from 4 adult of *Oncopeltus fasciatus.* 4 were sectioned under normal backfill conditions and 4 under cooled backfill conditions (parameter: block 1, -196ºC). All cuts were made in the middle segment of the antenna.

This experiment shows that the cuts created by the original method are rough (See Fig. 14). The majority of these were also closed by debris. In addition, some of the antennas had remains of a viscous fluid, which also closed the openings. In contrast, the cuts made with a scalpel under cooled backfill conditions showed very clean and smooth cuts. Particularly noticeable were the unblocked openings. Furthermore, many of the cuts showed detached sensilla. Due to the location of the cut in the middle segment of the antenna, these sensilla must be non-olfactory.

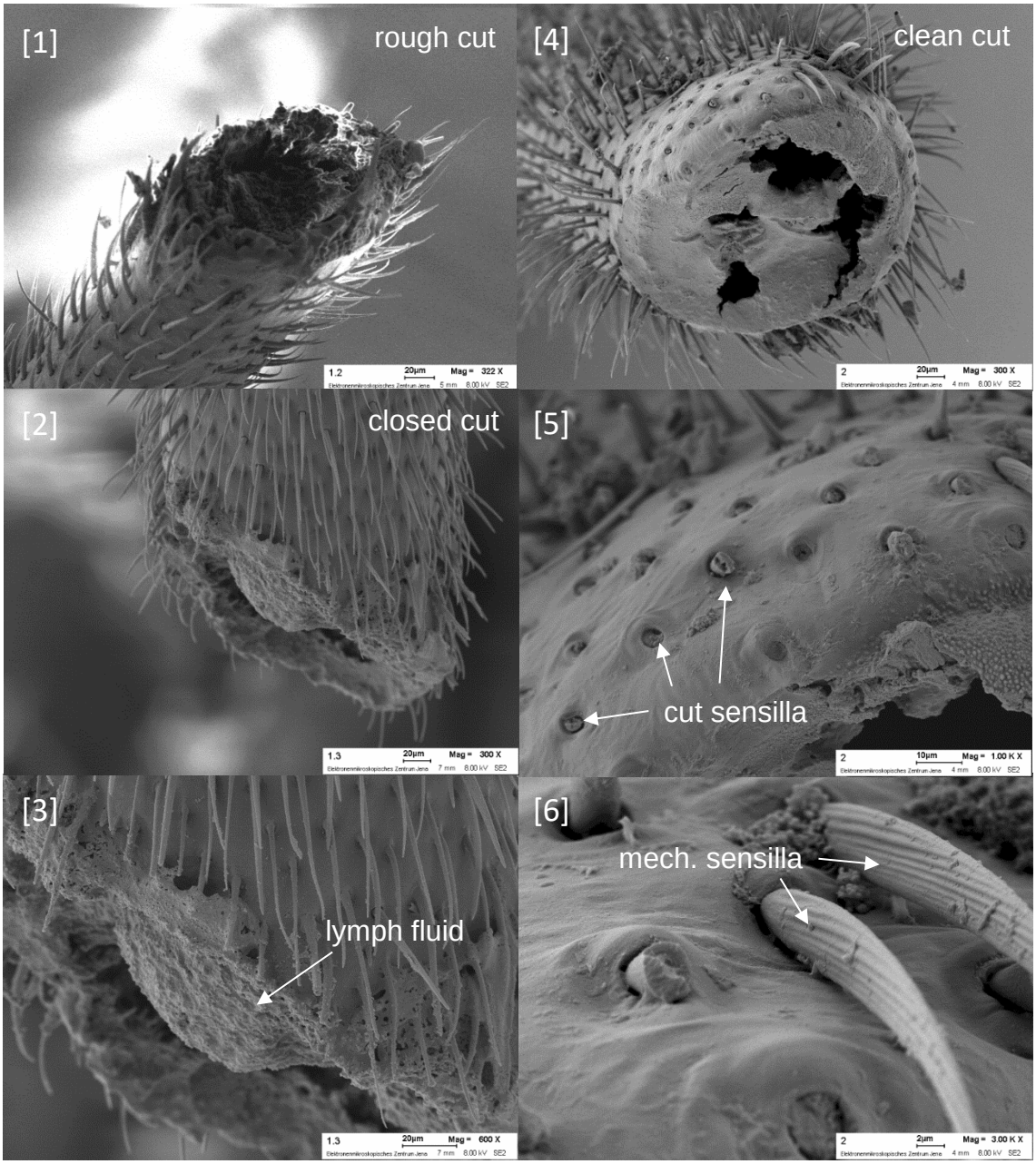

Fig 14: Scanning electron microscopy images of antennae cut and sensilla of *Oncopeltus fasciatus*. Image at different magnifications. (1) 322x, (2) 300x, (3) 600x, (4) 300x (5) 1000x (6) 2000x. (1-3) normal backfill cut at room temperature with scissor. (4-6) vertical cut with scalpel on Aluminium-Block 1 cooled to -196°C (See methods chapter 2.2.4).

3.5 Optimization of the cooled backfill method

A cooled backfill with a horizontal cut with block 2 at -196ºC produced the staining shown in Figure 15. The sequence of images shows the AL in different slides from ventral to dorsal. The staining shows a generally very weakly stained AL. Eight glomeruli are specifically stained. All but one are ventrally located near the AN. The last of the glomeruli is located laterally in a slightly dorsal position. Olfactory sensory neurons project from the AN and extend outside the AL to the glomerulus. In addition, staining of four single mechano sensory neurons (MSN) can be traced in the posterior direction.

As in the previous experiments, SEM should provide a better understanding of the processes at the interface (See Fig 16). The results show that a cut of only the sensilla is possible without damaging the antenna (See Fig. 16.1-16.2.). The damaged sensilla can be identified as olfactory and non-olfactory (See Fig. 16.3-16.5.)

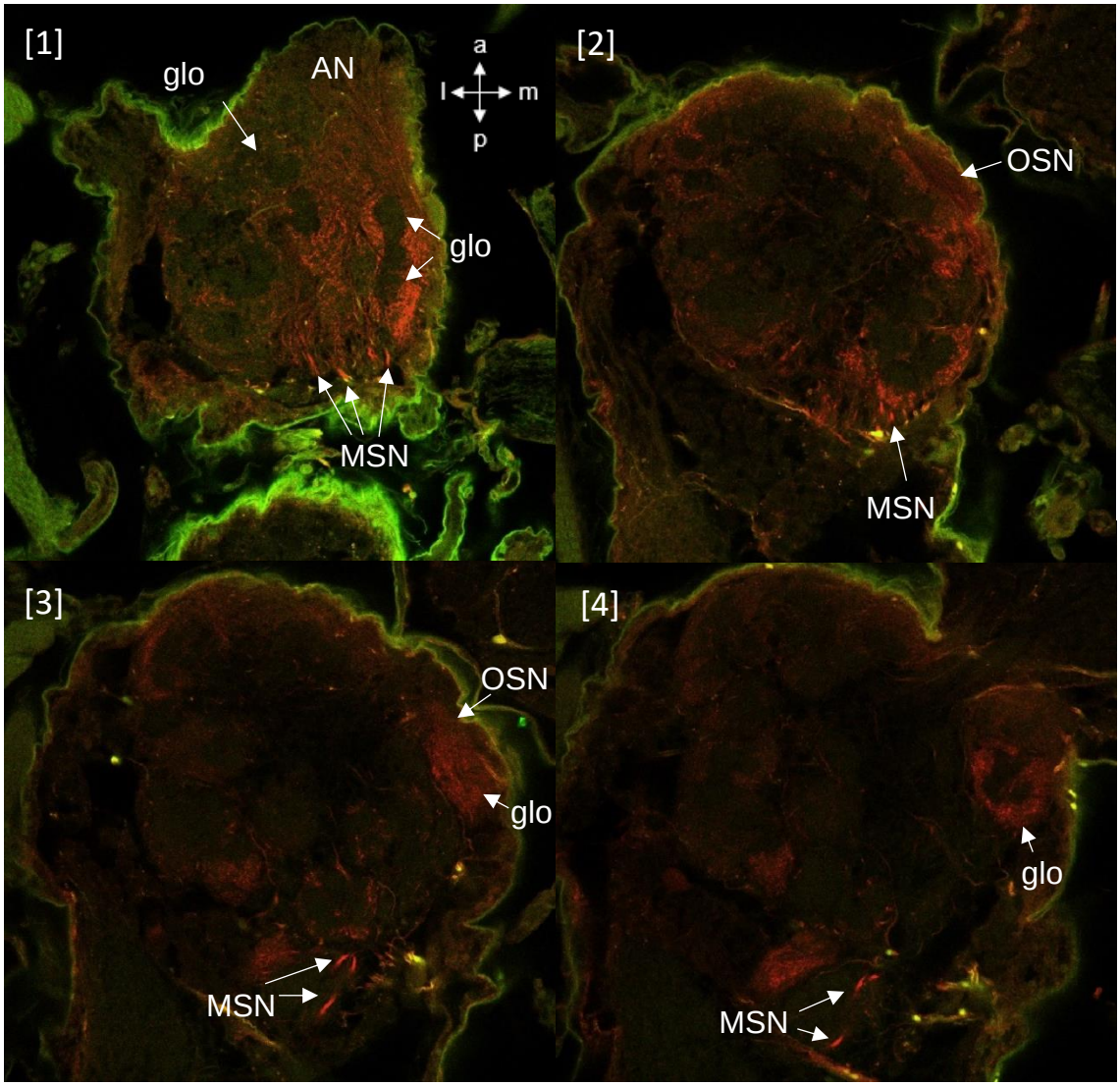

Fig 15: Confocal images in different depth of a backfill in *Oncopeltus fasciatus*. Backfill parameter: Block 2 at -196ºC, horizontal cut on both antennae (See methods chapter 2.2.4). Pictures every 150µm from ventral to dorsal. AL, antennal lobe; AN antennal nerve, glo, glomeruli; MSN, mechano-sensory neuron, OSN olfactory sensory neuron. Directions in all images equal: a, anterior; m, medial; l, lateral; p, posterior.

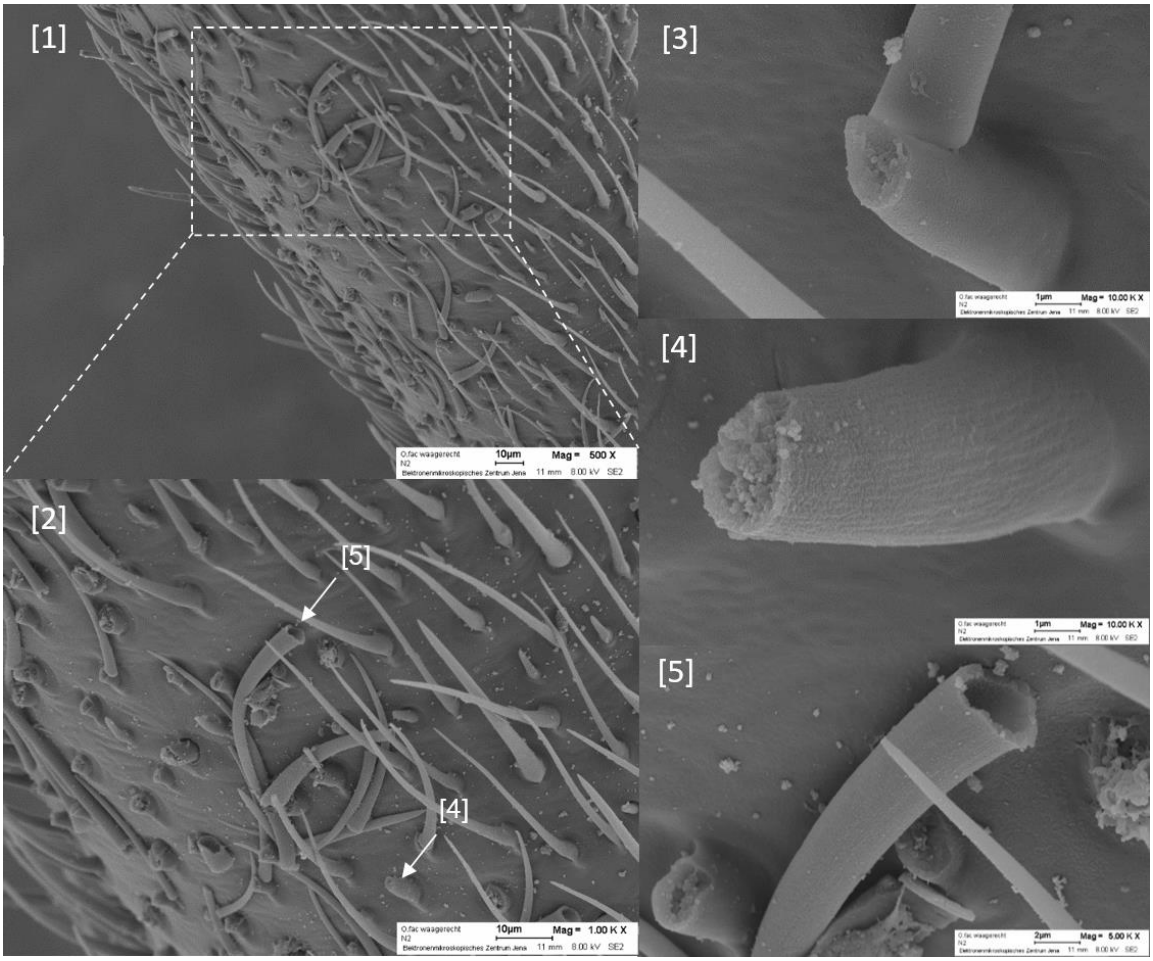

Fig 16: Scanning electron microscopy images of horizontal antennae cut of *Oncopeltus fasciatus*. Cut parameter: Block 2 at -196ºC, horizontal cut on both antennae (See methods chapter 2.2.4). (1) far overview of cut side(500x), (2) close overview of cut side (1.000x), (3) cut of non-olfactory sensilla (10.000x), (4) base of cut olfactory sensilla (10.000x), (5) top of cut olfactory sensilla (5.000x).

3.6 Glomerular organization of the antennal lobe

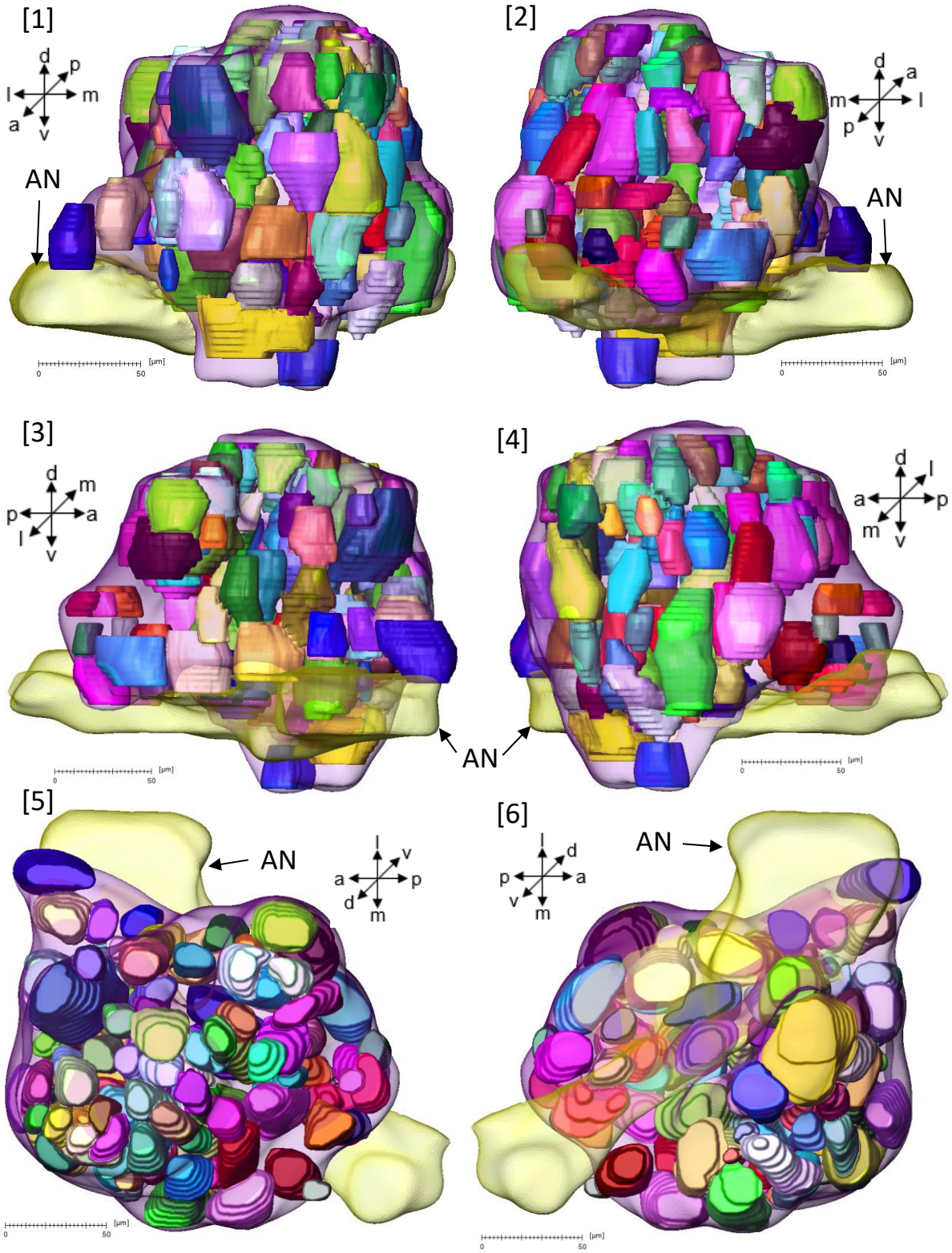

Fig 17: Three-dimensional reconstruction of the antennal lobe glomeruli. (1) anterior view, (2) posterior view (3) lateral view, (4) medial view, (5) dorsal view (6) ventral view. AN, antennal nerve. Directions: a, anterior; d, dorsal; m, medial; l, lateral; p, posterior; v, ventral.

Based on good immunohistochemical staining against synapsin (See Fig. 8), all glomeruli were reconstructed for an antennal lobe. (See Fig. 17). In the Al, 114 glomeruli were located. Noticeably, small glomeruli were present in an increased number dorsally. Glomeruli in the AL anteriorly showed substructure patterns. this could be an indication for an evolutionary clustering of smaller glomeruli. The reconstruction of the glomeruli also allows an analysis of the volume relation between glomeruli (See Fig. 18). The determined average volume of the glomeruli is 9.244,93 µm³.

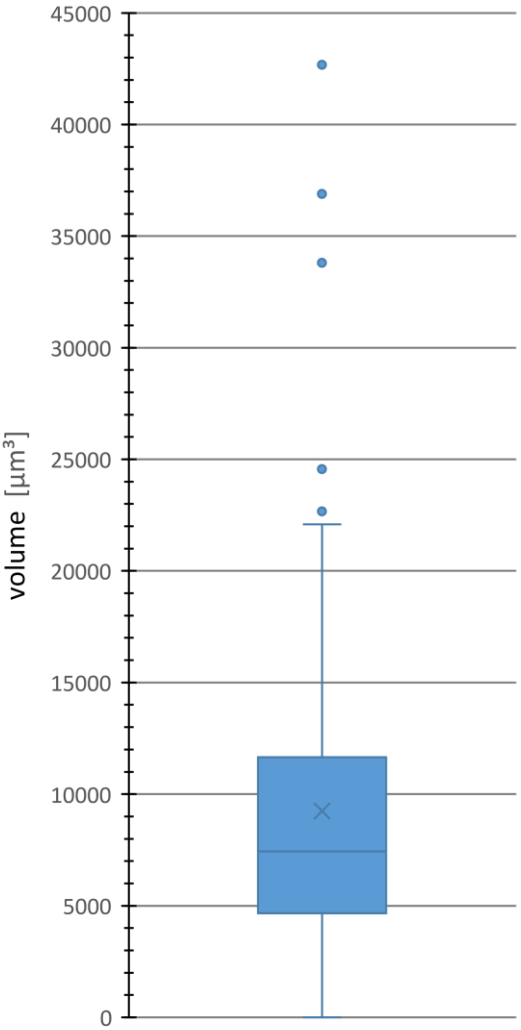

Fig 18: Box-Plot-Diagram of the volume of all glomeruli in an antennal lobe of *O. fac.* Number of glomeruli = 114. Median = 9.244,93 µm³.

3.7 Antenno-cerebral tract

To visualize the connection between the AL and the CA the called antenno-cerebral tract (ACT), the focal injection method was performed (See methods chapter 2.2.6). The florescent dye Micro Ruby was injected into the region of the calyx. This stained three individual neurons projecting from the AL (See Fig 19.4). The course of these neurons is clearly identifiable, and therefore reconstructed. As can be seen in Fig. 19.1-3, all 3 neurons originate in different glomeruli and extend through the same tract to the calyx, which passes close to the CC. The path of the neurons in the CA is not identifiable. The somata of the neuron from glomerulus 2 can be identified by its staining. It is located in the soma cluster posteriorly medial relative to the AL. Comparing the glomeruli (glo), it is noticeable that not all have the same staining. E.g., glo 1 is only stained in the outer layer of the neuron, while glo 2 is stained throughout the glomerulus (See Fig 19).

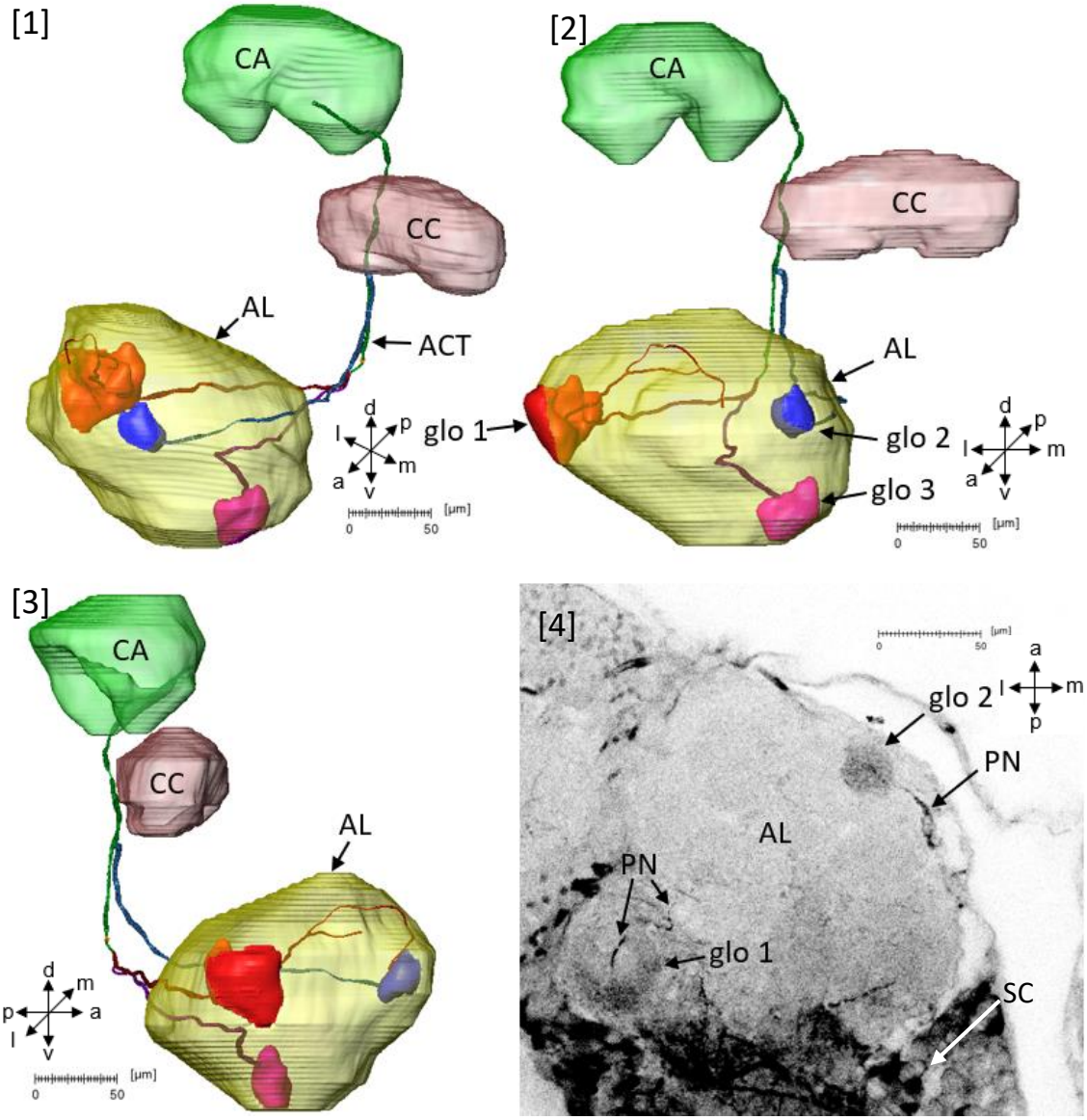

Fig 19: Antenno-cerebral tract (ACT) and confocal image of a focal injection on *Oncopeltus fasciatus.* Focal injection in the Calyx (CA) with 5% Micro Ruby. (1-3) reconstructions form different views: (1) anterior-medial view, (2) anterior view, (3) lateral view. (4) confocal image of the antennal lobe (AL). CC, central complex; glo, glomeruli; PN, projection neuron SC, soma cluster. Directions: a, anterior; d, dorsal; m, medial; l, lateral; p, posterior; v, ventral.

3.8 Mushroom body

Reconstruction of the mushroom body structure using the anti-synapsin antibody (See methods chapter 2.2.7) produced the following results, as shown in Figure 20. The structure is the right mushroom body, which has formed seven lobes. It is not possible to clearly classify these lobes into α-, β- and γ-lobes. In addition, the number of Kenyon cells was counted to be 987 (N=2).

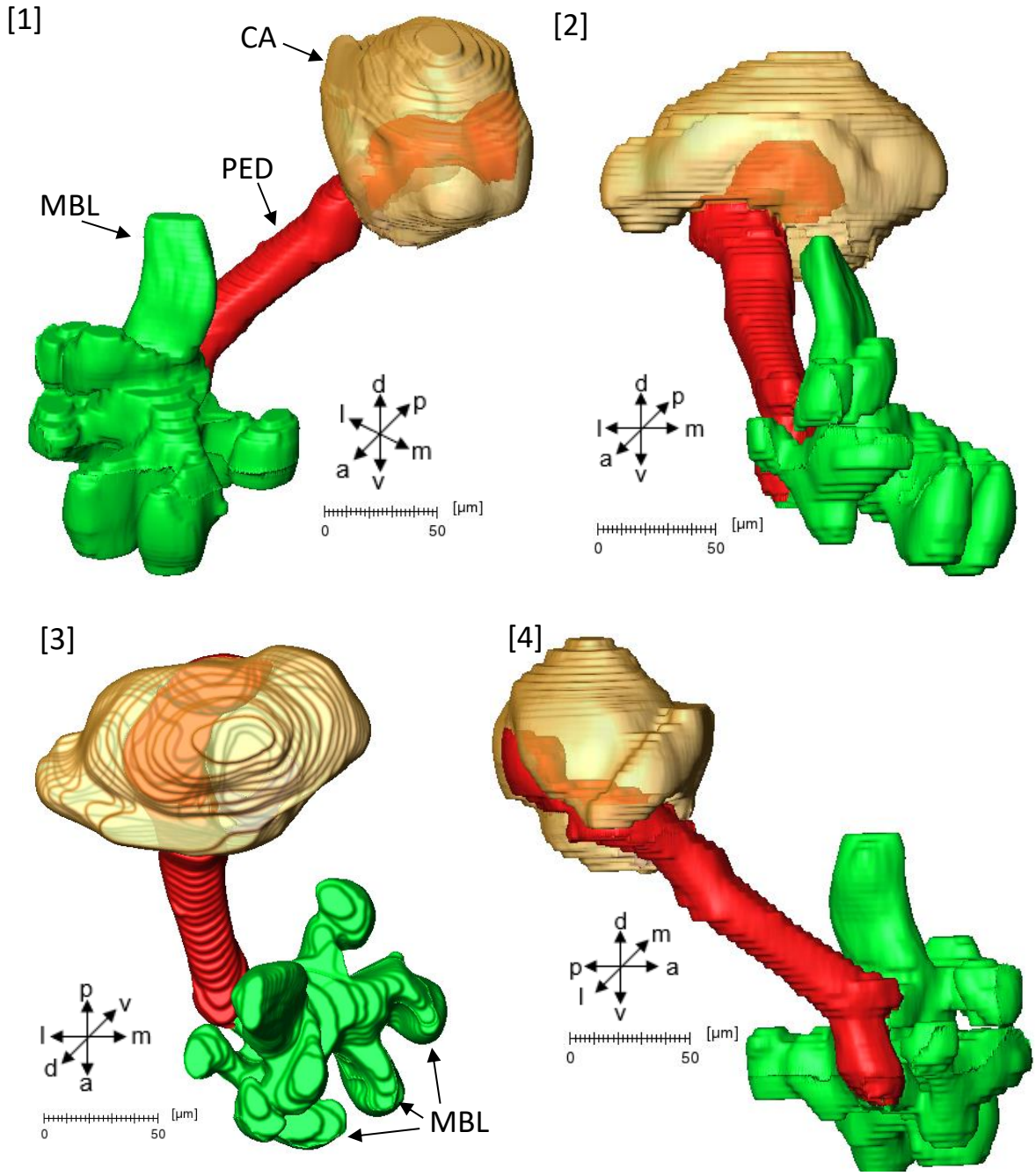

Fig 20: Three-dimensional reconstructions of *Oncopeltus fasciatus'* right mushroom body. (1) anterior-medial view, (2) anterior view, (3) dorsal view, (4) lateral view. CA, calyx; MBL, mushroom body lobes; PED, pedunculus. Directions: a, anterior; d, dorsal; m, medial; l, lateral; p, posterior; v, ventral.

3.9 Volume

Reconstruction of the neuropil from two anti-synapsin stains provided the volume data seen in Table 7. Evolutionarily, the volume of the brain changed between species. A larger brain allows for the development of complex social behaviour and the use of tools e.g., higher mammals, but at the cost of higher energy consumption. For this reason, the absolute volume value is compared between species (See Fig. 22). The volume of neuropils is proportional to their importance in the brain [8]. For this reason, the relative volumes of the neuropils in the brain are calculated (See Fig. 21) and compared with other species (See Fig. 23). The referring insects are the Moth *Heliothis virescens* due to its well published brain structures [9] and the Bug *Apolygus lucorum* due to its close relationship to *O. fac*. and well published volumes [10].

Tab. 7: Absolute and relative volumes of the neuropils of *Oncopeltus fasciatus*. Volume relative to sum of all neuropils (N=2).

neuropil	absolute volume in 10^6 µm^3	relative volume
antennal lobes	6,00	12,88%
mushroom bodies without calyces	1,48	3,17%
calyces	1,19	2,55%
central complex	0,61	1,31%
protocerebral lobe	18,31	39,29%
lamina	2,88	6,18%
medulla	4,73	10,14%
lobula	1,52	3,26%
gnathal ganglion	4,49	9,64%
thoracal ganglion	5,40	11,58%
complete Brain	46,62	100%

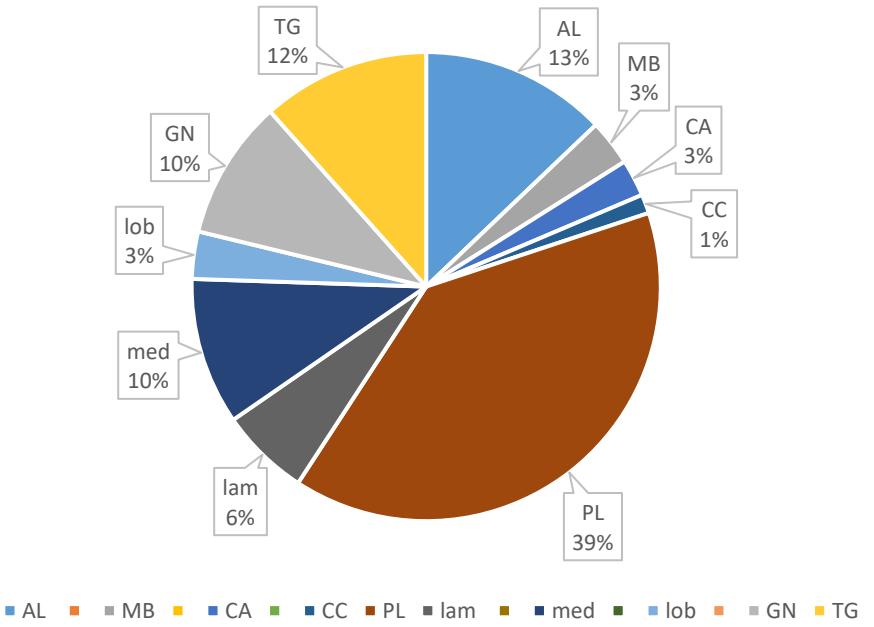

Fig 21: Relative volume of the neuropils in *Oncopeltus fasciatus*. Volume relative to sum of all neuropils (N=2). AL, antennal lobe; CA, calyx; CC, central complex; GN, gnathal ganglion; lam, lamina; lob, lobula; MB, mushroom body without calyx; med, medulla; PL, protocerebral lobe; TG, thoracal ganglion.

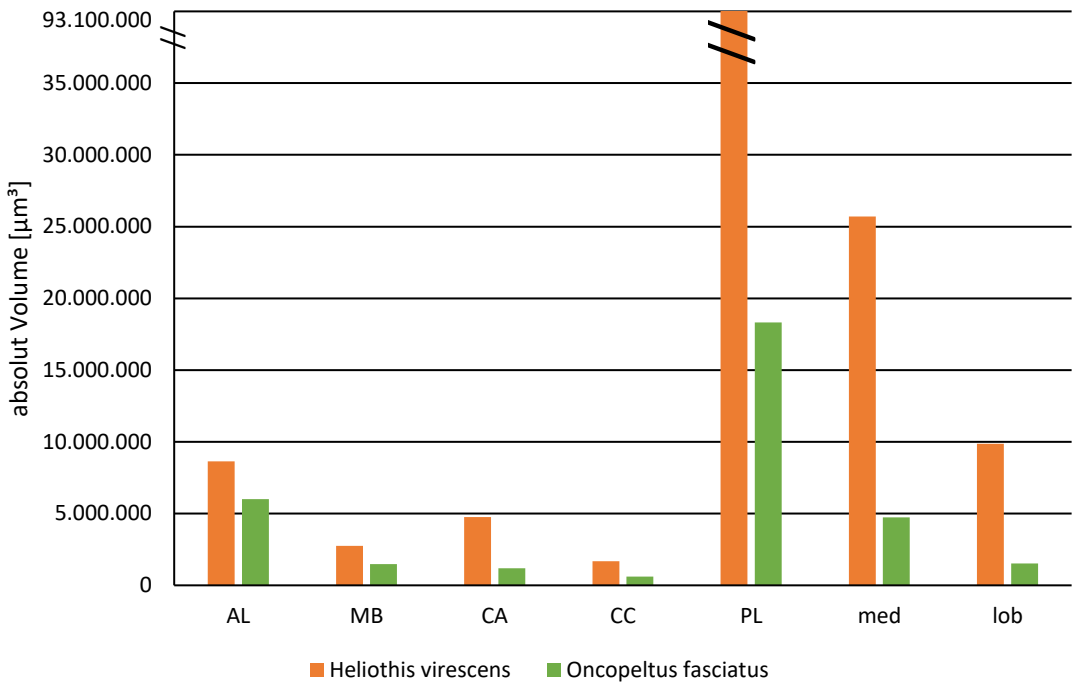

Fig 22: Comparison of the absolute volumes of prominent neuropiles Comparison between the species *Heliothis virescens* [9] and *Oncopeltus fasciatus***.** AL, antennal lobe; CA, calyx; CC, central complex; lob, lobula; MB, mushroom body without calyx; med, medulla; PL, protocerebral lobe.

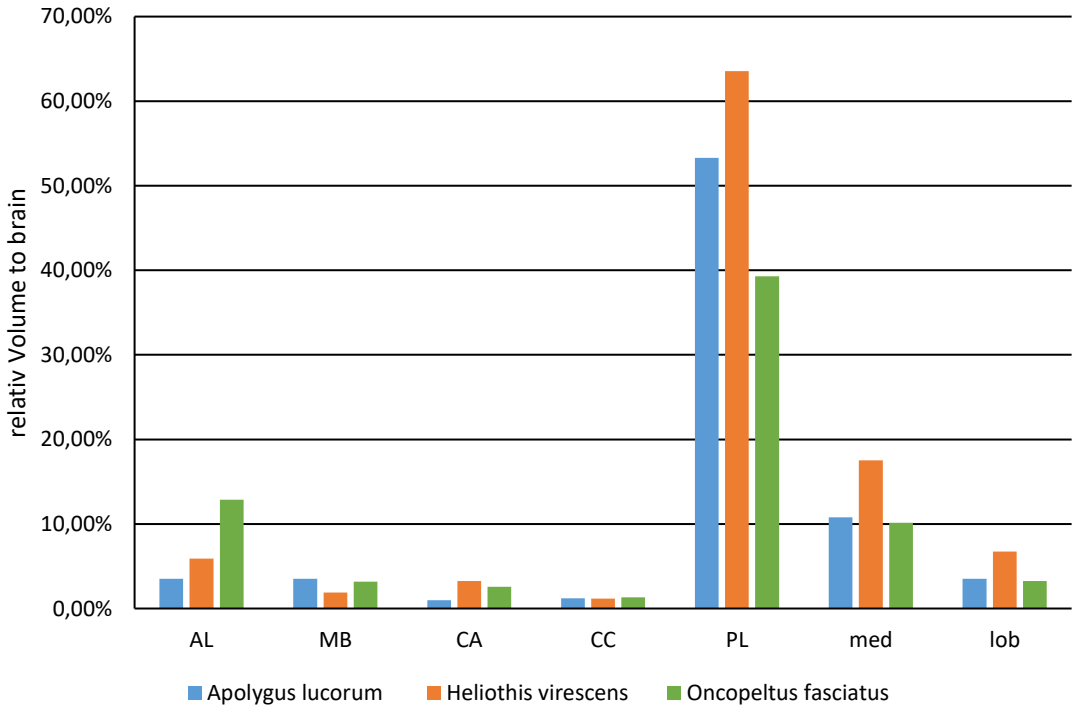

Fig 23: Comparison of the volume relative volumes to the brain of prominent neuropiles. Volume relative to all neuropils. Comparison between the species *Apolygus lucorum* [10], *Heliothis virescens* [9] and *Oncopeltus fasciatus.* AL, antennal lobe; CA, calyx; CC, central complex; lob, lobula; MB, mushroom body without calyx; med, medulla; PL, protocerebral lobe.

4 Discussion

The aim of the bachelor thesis is to characterize the in the introduction explained olfactory pathway for the land-living bug Oncopeltus using established methods. In addition, part of this work is to improve the methodological understanding of the backfill method to provide a possible solution to the problems associated with it.

For this reason, the discussion is divided into the methodological part, which deals with the validity of the methods used for the characterization of the olfactory pathway. And the result discussion, which explains the date based on the model of the olfactory pathway presented in the introduction and comparing the results with data from other insects.

4.1 Methodological discussion

4.1.1 Laser scanning confocal microscopy and fluorescence dyes

Confocal microscopy in combination with florescence markers is one of the most established methods in histology [7].

This method allows detailed resolution images of the insect brain, which form the basis of large parts of this thesis. This method has worked without problems. However, the method is limited in its use.

The microscope used is limited by a maximum ocular magnification of four times. For example, if the structures of a glomerulus are to be captured more precisely, this is not possible. In addition, the sample must be prepared almost transparently with Methyl-Salicylat in order to visualize the inner layers of the sample. Not every tissue can be made transparent. For example, the interior of the antenna cannot be visualized due to the light-impermeable cuticle. For this reason, the SEM method was used for this task.

4.1.2 Immunohistological methods

As explained in the method chapter 2.2.7, anti-GABA and anti-synapsin antibodies with their corresponding binding secondary antibodies were used. Both antibodies produced a specific staining. Parallel use of both antibody combinations did not cause crosstalk.

Difficulties were found, in the penetration of the antibodies into the brain. This can be assumed because four out of five synapsin stains showed a clear decrease in the dye concentration from the outside to the inside. This compromises the quality of the staining. A possible workaround is to increase the incubation time of the brain in the antibody solutions. It must be noted that the incubation time is limited by the stability of the brain. In case this does not have the expected effect, an alternative solution is to cut the brain embedded in agar with a vibratome into small slices after the brain dissection. This would, in theory, shorten the time required for the subsequent incubation by increasing the surface area and reducing the thickness of the brain.

In addition, the sensitivity of the GABA antibody is not sufficient to stain neurons in the neutrophils of the brain. An enhancement could be achieved by increasing the concentration.

4.1.3 Scanning electron microscopy

Scanning electron microscopy (SEM) was instrumental in providing a detailed view of the antennas. This would not have been possible in this form with any other method. Due to its few experimental steps, SEM has a constant, unproblematic good quality and served for this reason as a reference basis for the optimization of the backfill. A combination of

the backfill method and SEM performed on the same antenna is not possible, which prevents a qualitative comparison of the results. Instead, the comparison is quantitative.

4.1.4 Backfill

As already confirmed in preliminary experiments, the backfill method proves to be unsuitable for staining olfactory fibre tracts from the antennae (See results chapter 3.3). A big part of this work is to determine the cause of the problem with this method and to improve and optimize it accordingly. The relevant thoughts are the following.

Problems caused by the dye Micro Ruby can be excluded. Backfills with another florescent dye called "Lucifer Yellow" have been tried, but still without positive results.

Furthermore, it can be ruled out that *Oncopeltus fasciatus* is an exceptional phenomenon. Laboratory internal this problem has also occurred with the fire bug *Pyrrhocoris apterus*. *O. fac.* and *Pyrrhocoris apterus* show similarities in size and body structure. The problem may be due to the morphology of the bugs. In particular, the diameter of the antennae is significantly smaller than in other insects, for example, in comparison to Locust.

SEM images of the cut site indicate that the problem was caused by the sealing of the cut hole with debris and or fluid residues. Presumably this fluid was the highly viscous lymph fluid from inside the antenna. As already mentioned, the cause of this clogging could be the smaller dimensions of the antennae.

4.1.5 Cooled backfill

By identifying the problem that made the backfill method useless, the following theoretical thoughts were expressed and the cooled backfill was developed.

The exposing the antenna to cold temperatures causes the tissue to freeze and therefore to become fragile. A cut is then made with a sharp scalpel blade to break not cut the antenna. This procedure ensures that the cut is not crushed as usual but leaves a clean opening. The local low-temperature freezes liquids. This prevents residue in the fluid from sealing the openings after drying. The lymphatic fluid becomes liquid again only after the dye solution is added and is directly diluted by it. In order to clearly distinguish the modified method from the established backfill, it is referred to as cooled backfill.

To verify that a cooled backfill will work, the method was performed as described in the methods chapter 2.2.4. At this time without a bug-mount but instead on the edge of a flat surface. This experiment had caused a clear staining in the AL as seen in the results chapter 3.4. So, the theoretical processes worked in praxis. To further validate these data, the cut antennas were studied under SEM after a cooled backfill (Figure 14.4-14.6). These clearly showed that the resulting cut produced very clean and unsealed openings.

The problem-solving approach has shown to be a very promising alternative to backfill, but problems and improvements to this method were noted after the initial experimentation.

Firstly, problems occurred in the practicability of the experiment. These were mainly caused by the fact that the cooled block as well as the insect could slide uncontrolled during the experiment. To better fix the experiment, a simple bug-mount was designed in Fusion 360 (See Appendix 1) and printed using a 3D printer. The bug-mount made the experiment easier in the long run.

Furthermore, a mostly diffuse and or unspecific staining was observed in other neuropils as described in the results chapter 3.4. This staining significantly affects the quality of the backfill.

My theory suggests that ice crystals form in the antenna, which has contact with the extremely cooled metal block in the shape of a cube (block 1). These ice crystals puncture the membrane of the cells in the tissue. An effect similar to this occurs, for example, in the case of freezer burns. The dye can pass through these perforations from the nerves into the intermediate space between the cells of the antennal tissue. This theory is supported by the observations of the presence of dye in the head cavity after cooled backfills. Dye might enter the brain as a result of minimal damage during preparation. From experience, these occur especially during the peeling out of the OL. This theory is further supported by the position of the nonspecific staining which was mostly found in the OLs (See Fig. 13).

A smaller contact area between the antenna and the metal block would reduce the extent of the perforated tissue. For this reason, an improved metal block in metal block in L-shape (block 2) was used in the improvement experiments.

Another optimization exploits the morphology of olfactory sensilla. As described in the results chapter 3.4, sections of the cooled backfill have the feature of having cut sensilla. However, in this experiment, the sensilla were non-olfactory sensilla.

By cutting off only sensilla in a small area of the antenna by a horizontal cut, the antenna remains completely intact. By applying dye to this part of the antenna, the dye should penetrate through the hollow olfactory sensilla into the olfactory sensory neurons (OSNs). The smaller the number of severed sensilla, the more finely individual fibres could be traced.

In a first experiment, this optimized method was tested. Staining according to the explained principle has been shown to produce a weaker and much more specific staining of individual glomeruli. Some single neurons are recognizable (See results chapter 3.5). The improved cooled backfill stained significantly fewer neurons in the brain. These results are similar to the observations of the SEM pictures (See Fig. 16). These show, that many olfactory sensilla were cut and therefore many OSN were cut on a small part of the antenna. I think that this initial attempt of optimization gives a good first impression of the potential of this method. I think that more practice and further prototyping will further improve this method. For example, horizontal slices at two locations with exposure to different florescent dyes could track the different projections of the regions in the AL.

The current state of the cooled backfill provides a method to determine the projections from the antenna into the brain of *O. fac.* However, it needs to be verified if this method is also an alternative to backfill in other insects. I think that the full potential of this method is not yet fully exhausted.

4.2 Result discussion

4.2.1 Location and structure of the central nervous system.

Based on successful immunohistological staining with anti-synapsin antibodies and segmentation, the largest parts of the nervous system of *Oncopeltus fasciatus* could be completely characterized morphologically. (See results).

The central nervous system (CNS) of *O. fac.* shares many similarities with the nervous system of the insect *Apolygus lucorum*, which was studied by Gui-Ying Xie et al. (2016,2019) [6,10]. For example, the location of the brain in the insects is nearly identical. In addition, the organization of neuropils in the brain of both species is very similar. The morphology of some neuropils, for example the mushroom bodies, is very distinctive.

Noticeably, the supraoesophageal and suboesophageal ganglion is strongly fused in both insects, to the degree that the tritocerebrum of *O. fac.* is not clearly identifiable.

It can be said that the brain of *O. fac.* is a rather normal brain, which extends on the ventral side of the body from the head to the region of the last pair of legs. The insect brain has also become very compressed in *O. fac.* evolutionary. One reason for this evolution is that, in order to fit into the small head, the brain must be smaller. Furthermore, the last two ganglia are fused to form the posterior ganglion. This can have various effects, for example, there is more space in the more posterior parts of the abdomen.

It must be noted that the position of the brain in the insect is only the result of observations during the dissection in the context of this work. These should be verified in further research, for example, using the method of computer tomography.

4.2.2 Volume discussion

Although the brain of *O. fac.* is a normal brain for a land-living bug, certain neuropils showed an increase or a decrease in size. In general, it can be said that enlargement or reduction correlates with importance in the brain.

The volume data were compared with an insect with very similar brain organization (*Apolygus lucorum* [10]) and a slightly less related insect species (*Heliothis virescens* [9]).

The comparison of the absolute volume between the species (See Fig. 22) shows that all neuropils in the brain of *Heliothis virescens* have a larger volume than the neuropils of *O. fac.*. Especially noticeable in this comparison is the PL and medulla, which differ by a factor of five between the two species. This comparison indicates that *Oncopeltus fasciatus*, with its reduced brain size, exhibits less complex behavioural patterns than the moth *Heliothis virescens*. I suspect that the evolutionary reduction in the size of the brain of *O. fac.* has led to a restriction of behavioural patterns to those that are essential for survival. Furthermore, it can be assumed that in Hemiptera an adaptation of the body to a flatter body occurred, and the brain was modified correspondingly. To answer the question why this evolutionary change has taken place in bugs requires additional study.

For the species *A. lucorum*, no absolute volume values are known, therefore a direct comparison is not possible. Due to the already explained high degree of relationship and close similarity between *O. fac.* and *A. lucorum*, it can be assumed that the volumes of both species are in a similar scale.

The relative volume describes the percentage of single neutrophils in relation to the total volume of the brain. This size can be used to estimate the importance of these in the brain. Differences between species reflect these evolutionary changes clearly (See Fig. 23).

It is notable that the neuropils belonging to the OL (med, lob) are a much smaller percentage of the brain in comparison of *Oncopeltus fasciatus* to *Heliothis virescens*. On the other hand, the two bug species have very similar proportions in these neuropils. This indicates that the land-living bugs receive less sensory input through the eyes than moths.

The CC shows a constant relative volume between species and therefore also a corresponding importance. No evolutionary change in this region is evident.

The neuropils of the olfactory pathway (AL, CA, MB) show substantial variations between all three species. *O. fac.* has a much larger AL compared to both species. The comparison of MB volumes shows that MB is more important in land-living bugs than in moths. Furthermore, *O. fac.* has a relative volume of the calyx that is higher than the average for land-living bugs. These results allow us to rank the importance of the olfactory pathway in *O. fac.* All three neuropils of the olfactory pathway are of significance in *O. fac.* This suggests that the processing and interpretation of olfactory stimuli is of great importance in *O. fac.* compared to the other two insects. This could suggest a stronger reliance on olfactory information, playing a crucial role in foraging, mate-finding or other behaviours.

The volume data analysed is, nevertheless, limited in comparison to the other data. Regions such as the AMMC or the lateral horn could not be clearly determined and for this reason were counted in the PL. It must be noted that this procedure affects the comparison of brain volumes. A sex-specific analysis of the brain volume data is also not possible due to the limited data gathered. With more synapsin antibody staining and subsequent reconstruction, this study could be expanded to include this type of analysis in the future.

4.2.3 Antenna

By using a SEM, wall pores could be detected on some of the sensilla. Therefore, these sensilla can be identified as olfactory (See results chapter 3.2). Research by Schmidt and Berg (1994) [11] supports these findings. As explained earlier, one sensilla type was identified morphologically by my results. The research team, on the other hand, was able to make a more precise identification, and classified 3 types of olfactory sensilla. These have minimal morphological differences but differ in the number of OSNs per sensilla. It was also found that the different types cannot be distinguished using SEM. This paper provides a much more detailed insight into the olfactory sensilla than the data I gathered. One finding not mentioned in the paper is that olfactory sensilla can only be found on the distal part of the antenna.

Nevertheless, the identification of the olfactory sensilla shown in the results proves that in *Oncopeltus fasciatus* olfactory perception starts on the antennae. This result corresponds to the model of the olfactory pathway explained in the introduction.

4.2.4 Antennal lobe

The AL as the primary olfactory centre in the brain in *O. fac.* was identified. In the AL, 114 glomeruli were counted. The number of glomeruli differs between species. For example, *Locusta migratoria* has 1000 glomeruli [12], whereas Drosophila melanogaster has only 62 glomeruli [13]. Animals with high numbers of glomeruli can discriminate many odor molecules because their number correlates with the number of odorant receptors. [14]

The results of the cooled backfill (See results chapter 3.4) proved that the AL receives projections from the antennas. It can be assumed that these projections are OSNs. These data fully support the existing model of the olfactory pathway.

By combining the knowledge obtained from the different experiments, it was also possible to make conclusions about the organization of the neuron types in the glomeruli in *O. fac.* The glomeruli show a division into an outer layer and an inner layer. Olfactory sensory neurons exclusively traverse the outer part of the glomeruli, recognizable by the cooled backfill results. PNs occur in two groups. Some PNs are organized only in the rim, whereas others are organized throughout the glomerulus (See results chapter 3.4). By the results of focal injection, a very speculative thesis could be made suggesting that a correlation of multiglomerularity and organization in the glomerulus, exists. Multiglomerular PNs were observed to be situated in the rim and monoglumerular PNs were present throughout the glomerulus (See results chapter 3.7). Because of the small database, this hypothesis needs to be investigated in the future by more focal injections. No conclusion can be made about the organization of the LNs because immunohistochemical staining's against GABA failed. More research is needed for a complete view of all neuron types in AL.

4.2.5 Antenno-cerebral tract

Results 1.1 show that neurons in the antenno-cerebral tract (ACT) could be stained. Due to its medial course in the brain, this tract could be identified as the dorso-medial antenno-cerebral tract (DMACT) or the inner antenno-cerebral tract (IACT) (See Fig. 3). However, a precise determination of the tract is not possible because neither the latheral horn nor other tracts were examined in more detail. An outlook could be to classify them further by more focal injections into the calyx. Further identification is not necessary for this work, because the evidence of an ACT proves the connection between AL and CA.

4.2.6 Mushroom bodies

The mushroom body of *O. fac.* was reconstructed with a high detail degree (See Fig. 20). As already explained, the morphology of the MB differs between insect species. This is also the case for *O. fac.* The calyx is attached in a U-shape to a peduculus. The calyx does not enclose the Kenyon cells as in other insects, instead the somacluster is nestled to the calyx at the edge of the brain. According to current research, the particular morphology has no significant effect on the function of the calyx or the MB in general.

Furthermore, the MB has seven lobes. In comparison *A. lucorum* has eight lobes [9], *Apis mellifera* two [15] and *Drosophila melanogaster* three lobes [16].

Especially *O. fac*. and *A. lucorum* as representatives of the Hemiptera show a higher number of lobes than other insects. In addition, 987 Kenyon Cells were counted per single calyx in *O. fac.* In comparison *Apis mellifera* has 170.000 KCs per brain hemisphere [15], *Drosophila melanogaster* has 2.500 KC [16] per brain hemisphere. *O. fac.* has significantly fewer Kenyon cells than other insects.

It is important to note that the exact relationship between the number of Kenyon cells or number of lobes to the behaviour is complex and is influenced by several factors, including the insect's specific species and habitat.

The research continues to study the correlations, so exact conclusions are not forthcoming. The data obtained provide a good basis for *O. fac.* on which further studies can be based.

The detection of the ACT verified the connection of the AL and the calyx. Consequently, it can be concluded that the calyx contains olfactory information, not primarily from the sensilla, but via the AL. The MB has been shown to be responsible for learning in other insects. For this reason, again, the assumption can be made that *O. fac.* is able to learn from smell. The existing model of the olfactory pathway related to the MB is supported by these data.

4.2.7 Olfactory pathway

The aim of this thesis is to describe the olfactory pathway. The olfactory pathway of *O. fac.* starts with the olfactory sensilla on the antennae. The OSNs in the sensilla project into the outer edge of the glomeruli of the AL. The AL forms the first station in the brain that processes olfactory information. The organization of LNs in the AL could not be demonstrated. There is evidence that PNs, which are present only in the rim as well as in the whole glomeruli, project through the ACT into the calyx. The calyx is connected to the lobes by the pedunculus. The MB is the second brain region that processes olfactory information in the brain. All the stages of the olfactory pathway explained in the introduction are present in *Oncopeltus fasciatus*. The sequence and interconnection of the stages do not differ from the existing model.

However, based on the volumetric observations, it can be assumed that the olfactory pathway plays an above-average role relative to other land-living bugs in the brain of *O. fac.* It can therefore be assumed that olfaction is an important sense for this insect to find its way in the environment.

This description is the first examination of the olfactory pathway for the insect *Oncopeltus fasciatus.* Therefore, this project has been successful and has achieved the desired objectives.

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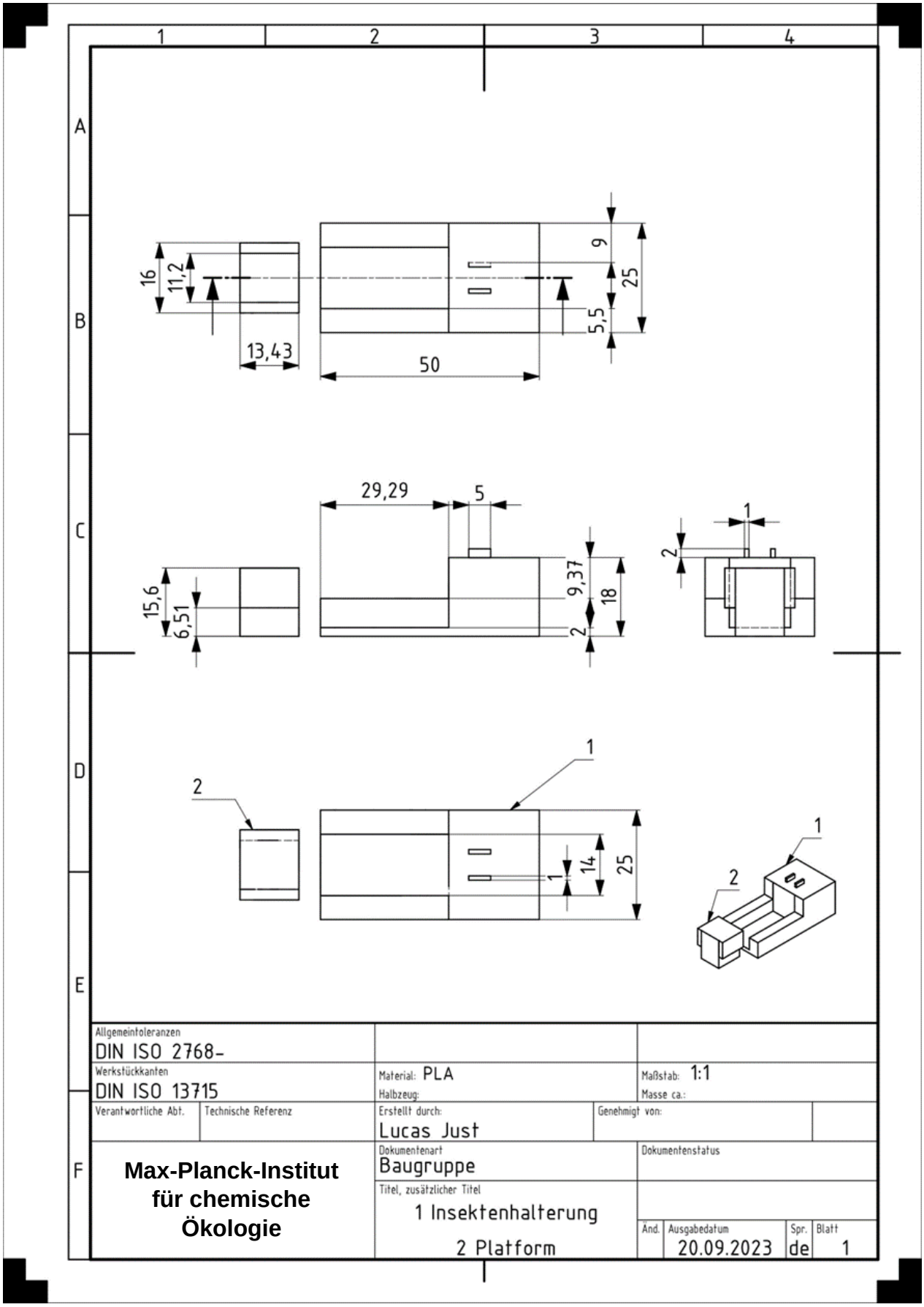
Appendix

Appendix 1: Document extract from the planning sketch of the bug-mount in German



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Selbstständigkeitserklärung

Ich, Lucas Just versichere, dass ich die vorliegende Arbeit selbständig und ohne unerlaubte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Alle Stellen, die inhaltlich oder wörtlich aus Veröffentlichungen stammen, sind kenntlich gemacht. Diese Arbeit lag in der gleichen oder ähnlichen Weise noch keiner Prüfungsbehörde vor und wurde bisher noch nicht veröffentlicht. Hiermit erkläre ich mich mit der Einsichtnahme in meine Abschlussarbeit im Archiv der Bibliothek der EAH Jena einverstanden.

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