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Sensory projection of the antennal pathway in the brain of the benthic water bug *Aphelocheirus aestivalis*

Bachelor Thesis

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III. List of Abbreviations

A.aest.	*Aphelocheirus aestivalis*
AG	Abdominal ganglion
AL	Antennal lobe
AMMC	Antennal mechanosensory and motor center
AN	Antennal nerve
CB	Central body
COS	Sensilla conical
CUS	Sensilla cupola-like
GNG	Gnathal ganglion
LA	Lamina
LAL	Lateral accessory lobe

LOX	Lobula complex
CLSM	Confocal laser scanning microscopy
HMDS	Hexamethyldisilazane
MB(s)	Mushroom body(ies)
ME	Medulla
NGS	Normal goat serum
OL(s)	Optic lobe(s)
OSNs	Olfactory sensory neurons
PAS	Sensilla papilla
PBS	Phosphate-buffered saline
PBSTx	Phosphate-buffered saline with Triton X-100
PES	Sensilla peg
PFA	Paraformaldehyde
PG	Posterior ganglion
PL	Protocerebral lobe
PN	Projection neuron
proTG	Prothoracic ganglion
RT	Room temperature
SA	Sensilla ampullacea
SB	Sensilla basiconica
SCa	Sensilla campaniformia
SCh	Sensilla chaetica
SEM	Scanning electron microscopy
SN(s)	Sensory neuron(s)
SPl	Sensilla plate-like
STS	Sensilla stars-like
TRS	Sensilla trichoidea

IV. Abstract

The antennae of insects play a major role in detecting environmental cues. This study focuses on the investigation of a primary sensory center in the benthic water bug *Aphelocheirus aestivalis*. Given the reduced solubility of volatile organic compounds (VOCs), the altered optic density of the medium and the constant river stream within the habitat of the predatory bug its sensory perception must adjust to those conditions. Its brain is strongly fused and supraesophageal ganglion, suboesophageal ganglion and the prothoracic ganglion form a whole complex. Combining confocal laser scanning microscopy with fluorescent nerve fillings of the antenna and immunohistology a glomerular organization of the antennal lobe was identified. The antennal sensory pathway revealed target areas and arborizations in the protocerebral lobe, the gnathal ganglion and the prothoracic ganglion.

V. Zusammenfassung

Die Antennen von Insekten spielen eine wichtige Rolle in der Detektion von Umweltreizen. Diese Studie fokussiert sich auf die Untersuchung eines primären sensorischen Zentrum in der benthischen Wasserwanze *Aphelocheirus aestivalis*. Die geringe Löslichkeit von volatile organischen Substanzen (VOCs), die sich unterscheidenende optische Dichte des Mediums und der konstante Strom des Flusses im Lebensraum der räuberischen Wanze sorgen dafür, dass sich ihre sensorische Wahrnehmung daran anpassen muss. Das Gehirn zeigt starke Verschmelzungen, wobei das supraesophageales Ganglion, das subesophageale Ganglion und das prothorakale Ganglion einen Komplex bilden. Durch die Kombination von konfokaler Lasermikroskopie mit floureszenten Nervenfüllungen der Antenne und Immunhistologie konnte eine glomeruläre Organization des Antennallobus identifiziert werden. Der antennale sensorische Weg hat auch Zielregionen und Verzweigungen im protozerebralen Lobus, dem gnathalen Ganglion und them prothorakalen Ganglion gezeigt.

1 Introduction

1.1 *Aphelocheirus aestivalis*

Aphelocheirus aestivalis (*A. aest.*) is an aquatic bug distributed over most parts of Europe (Miguélez et al., 2020; Roca-Cusachs et al., 2020; Živić et al., 2007) and partly in south western asia (e.g., Turkey and Georgia) as well as in northern Africa (Papáček, 2012). Aphelocheirids inhabit lakes and upper or mid sections of steams with undisturbed environmental conditions. They have been investigated as an ecological indicator for the quality of river habitats (Pardo et al., 2014). Their spatial distribution depends mainly on dispersion through watercourses (Papácek et al., 2009), because most specimen are brachypterous.

1.1.1 Description of the species

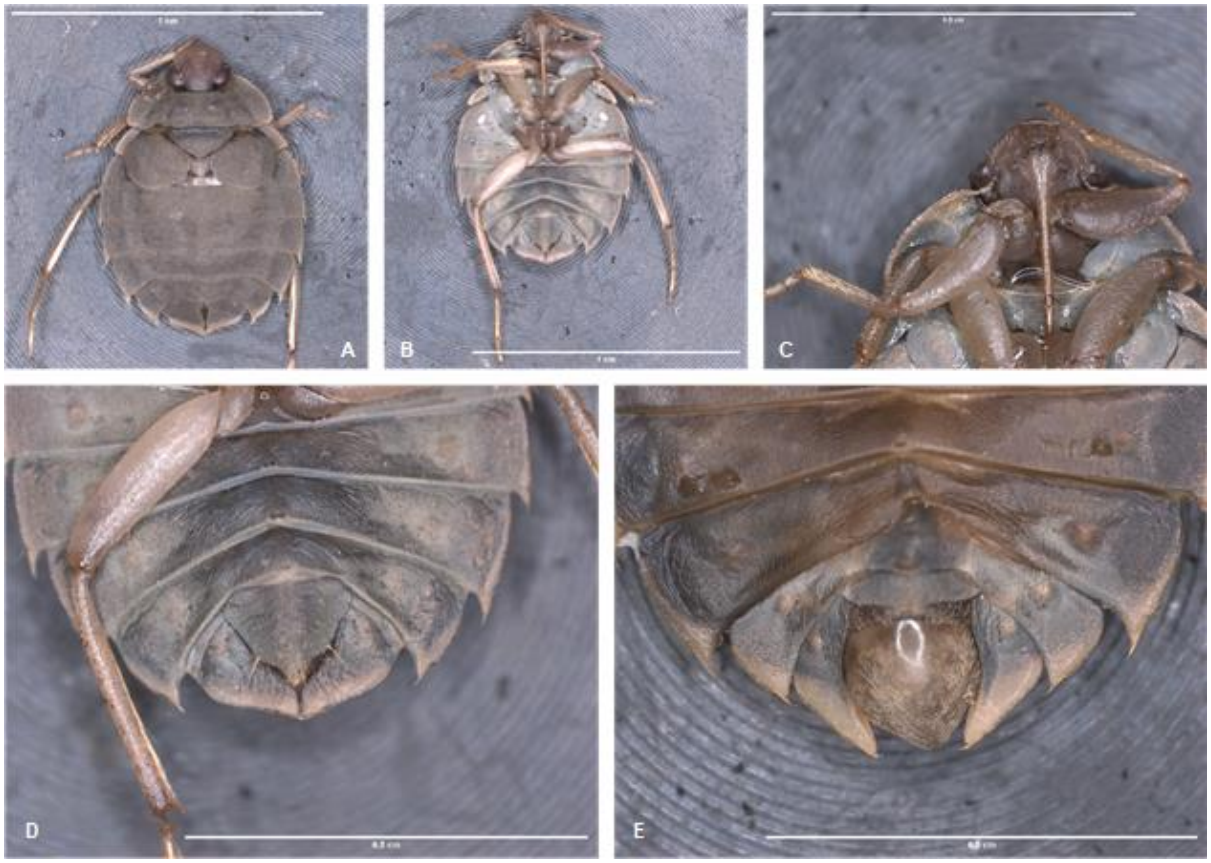

Figure 1: Habitus of A. aest.; Images taken with a Axio Zoom.V16; A-D: Female specimen; A: Dorsal view; B: Ventral view; C: Dorsal view on head region; D: Abdominal segments and female subgenital plate; E: Abdominal and male genital segment

A. aest. is a representative of the family of the Aphelocheiridae within the infraorder of Nepomorpha (water bugs) belonging to the order of Hemiptera, the "true bugs". The nepomorpha are generally known as "water bugs" and combine over 2000 different species. The family of Aphelocheiridae is a sister group to the Potamocorida. The age of the evolutionary divergence of the Aphelocheirids are estimated at 41.3-33.9 million years, one of the youngest families in the nepomorphan order (Ye et al., 2020).

The water bug has a flattened ovate shape reaching a body length of 8.5 to 11 [mm]. Its labium is relatively long compared to other nepomorphan species reaching the metasternum. The slender antennae consist of scape, pedicel and two flagellomeres. *A. aest.* possess "rosettes" surrounding the ventral abdominal spiracles, which is unique within the order of nepomorpha. All Aphelocheirids have a plastron, which allows oxygen extraction directly from the water via diffusion of dissolved oxygen into a layer of air held by hairs on its body. This respiratory system enables their submerged life in streams and lakes. The species is generally brachypterous, but alary polymorphisms are common (Schuh & Slater, 1995).

1.1.2 Evolutionary transition from terrestrial to aquatic habitats

Although it is yet to proof that the Aphelocheiridae or other nepomorphan species are secondarily adapted there are certain indications of its transition. Sharma argues that it is probable that the wing origin is most likely of terrestrial nature or within the transition to it. He states that the ancestors of winged insects didn't have aquatic nymphs, referring to the notion of aquatic nymphs being a transitional step to wing evolution (Sharma, 2019). Another indication is the tracheal respiration, which depends on gas exchange. Aquatic insects possess very different respirational mechanism like having breathing tubes, also called siphon, a plastron or using air bubbles in order to survive in aquatic habitats (e.g., *Notonecta glauca*, *A. aest.*,, *Corixida sp.*). The breathing tube and air bubble mechanism both depend on directly contacting atmospheric oxygen. The plastron consists of an air layer within small hairs on the body surface but depends on a high oxygen level in the water (Jones et al., 2018; Seymour et al., 2015), which results in a restriction of choices of habitats rather than being a pure advantage. All those adaptions seem to be better suited on a terrestrial lifestyle with more than enough atmospheric oxygen. Therefore, I hypothesize that the probability of these breathing mechanisms to originate from terrestrial ancestors is way higher.

1.2 Sensory appendages of *Aphelocheirus aestivalis*

The detection of environmental stimuli is essential for survival. While humans have sensory organs like eyes, ears, nose, and skin to gain information arthropods have sensory appendages with structures called sensilla. Those cuticular extensions otherwise called sensory hair or sensory peg are responsible for signal reception. They are widely distributed over the whole body, but especially focused on antenna, mouthparts, which will be focused on in this study. The shapes and varying receptors in the sensilla determine the perceived stimuli modality (Altner & Linde Prillinger, 1980; Dietrich, 1964; Hallberg & Hansson, 1999; Mciver, 1975; Slifer, 1970).

1.2.1 The antenna

Antennae are paired structures on the head capsules, which are equipped with a huge amount of sensilla. The antenna is segmented into scape, pedicel and flagellum, which is additionally segmented into a differing amount of flagellomeres. Sensilla distributed all over the antenna are receptive for various signal modalities. Studies on the ultrastructure of sensilla categorize them functionally into mechano-, chemo-, thermo-, hygroreceptive (Altner & Linde Prillinger, 1980; Hallberg & Hansson, 1999; Liu et al., 2021; Nowińska & Brożek, 2019, 2020; Shields, 2004; Zacharuk, 1980). The antenna of *A. aest.* has previously been investigated by (Nowińska & Brożek, 2020) and permission has been granted to use and show the Figure 2.

The antenna of *A. aest.* possesses a sparse amount of sensilla in comparison to other insect species (e.g., *Drosophila melanogaster, Apis mellifera, Locusta migratoria, Notonecta glauca*). The authors have identified sensilla plate-like (SPl), sensilla basiconica 2 and 3 (SB2, SB3), sensilla campaniformia (SCa), and sensilla ampullacea (SA). Sensilla basiconica 2 are distributed over both flagellomeres, while the singular SB3 is on the tip of the second flagellomere (Fig 3). Studies have shown that sensilla basiconica are involved in the olfactory system of different arthropod species (Dutt Parashar et al., 1994; Lopes et al., 2002; Tichy & Barth, 1992). They typically have a round shape and possess either a flexible or inflexible socket. Additionally numerous wall pores are found on its cuticle. Sensilla campaniformia (SCa) are characteristically nonporous and considered to serve mechanosensory functions (Chapman, 1998; Gupta, 1992; Tuthill & Wilson, 2016). Deeply sunken small pegs are called sensilla ampullacea (SA) or sensilla styloconica (Keil, 1999). A study in leaf-cutting ant *Atta sexdens* has shown CO_2-responses of SA (Kleineidam et al., 2000). Others considered it to possess a hygro- and thermosensitive function in *Camponotus rufipes*, *Libellula depressa*, and Solpugids (Bauchhenss, 1983; Nagel & Kleineidam, 2015; Piersanti et al., 2011). The sensilla plate-like (SPl) possess a

plate-like shape. Compared to sensilla placodea in other insects SPl have uneven edges. Wall pores on sensillar surfaces are generally considered to serve chemosensory functions (Nowińska & Brożek, 2020).

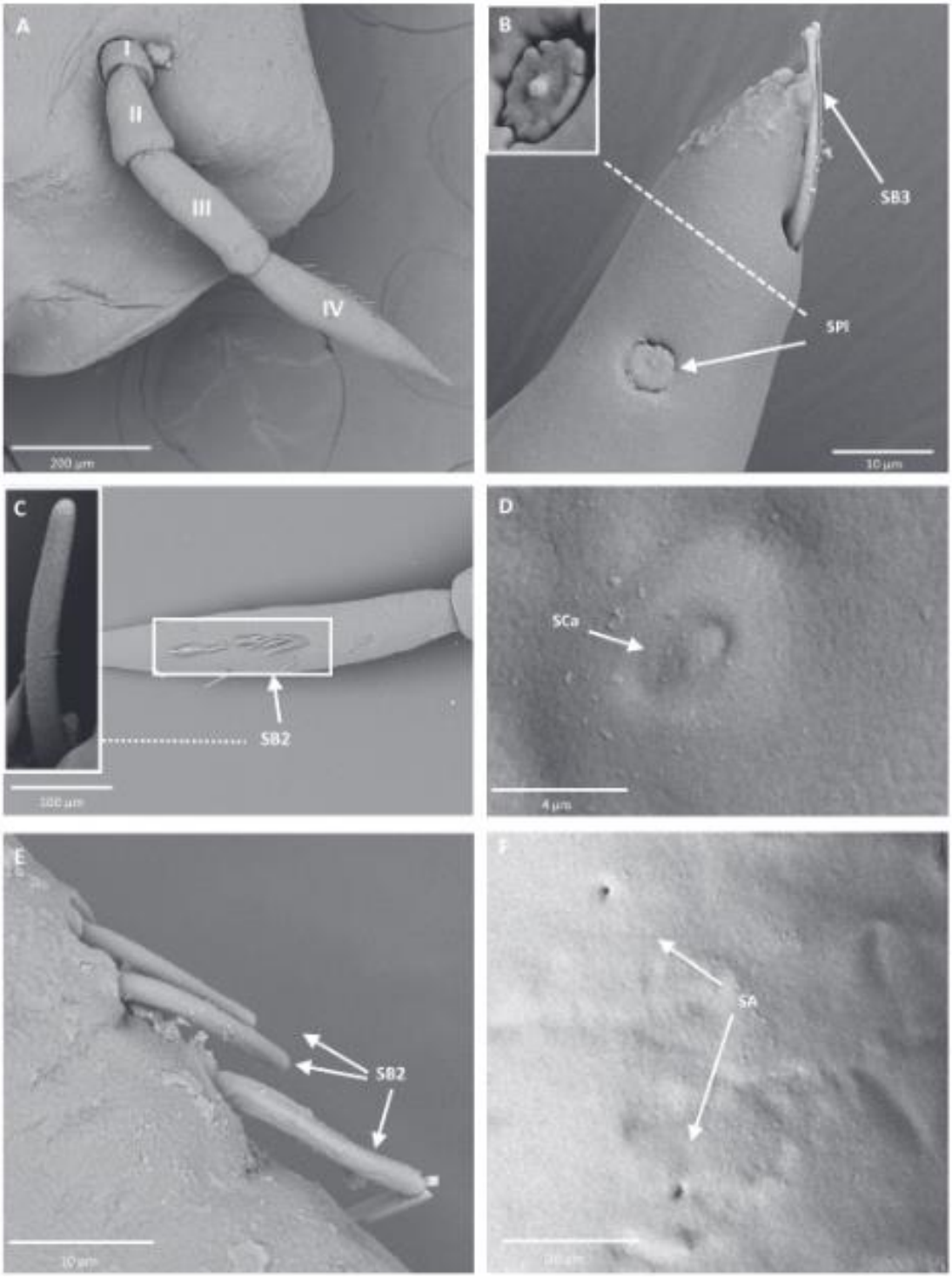

Figure 2: Sensillar distribution on the antenna of A. aest.; A: Segmentation of the antenna; B-F: Sensilla types and their distribution; SB2/3: sensilla basiconica 2/3; SPl: sensilla plate-like; SCa: sensilla campaniformia; SA: sensilla ampullaceal; Figure from Nowinska & Brozek 2020

1.2.2 The mouthparts

The feeding process of insects vary on the specialization of their mouth parts. They are generally equipped with paired mandibles and maxillae, and a labium. *A. aest.* as a representative of the hemipteran order possesses a rostrum, which is a modification of the labium forming a sheath around the stylet-like mandibles and laciniae, a defining feature of the whole order (Rolf G. Beutel et al., 2014). The apparatus is capable of piercing plant tissues and sucking liquids in phytophagous species, whereas predators, such as *A. aest.*, can pierce the cuticles of their prey. Detection and recognition of such prey can be mediated by different sensory systems. Besides the optical and antennal system, the mouthparts play a key role in this task and therefore, carry numerous types of sensilla, which in turn are considered to be part of the mechanosensory or chemosensory system. While olfaction is mostly associated with the antennal sensory system, taste reception generally is associated with the sensilla on the mouthparts of insects and have been studied extensively (e.g., (Gabriela De Brito Sanchez, 2011; Guo et al., 2018; Hallem et al., 2006)). Additionally, the mouthparts also contain mechanosensory sensilla, which might be linked to prey detection or feeding behaviors. The types of sensilla on the mouthparts of *A. aest.* have already been investigated and are shown as a figure from (Brozek, 2013). Permission to show or usage of the figure has been granted.

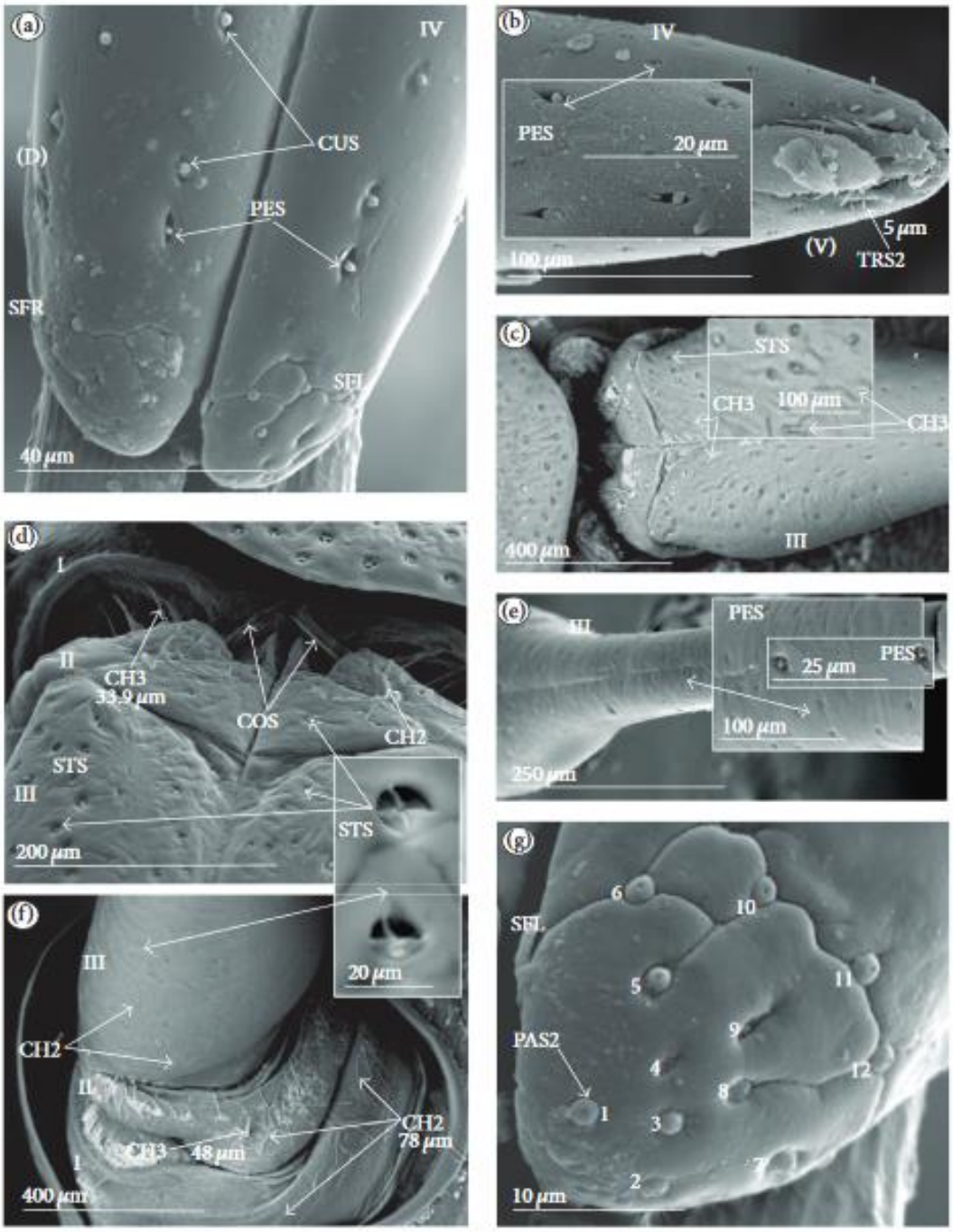

Figure 3: Sensillar distribution on the proboscis of A. aest.; CUS: *sensilla cupola-like, PES: peg, STS: sensilla stars-like CH2, CH3: sensilla chaetica 2, 3, COS: sensilla conical, TRS: trichoid sensilla, PAS2: sensilla papilla 2*

Sensilla are distributed widely over the mouthparts. The author has categorized the sensilla functionally in tactile sensilla cupola-like (CUS), peg (PES), stars-like (STS), chaetica 2 and 3 (CH2, CH3), and conical (COS); trichoid sensilla (TRS) as gustatory and tactile; papilla 2 (PAS2) as solely gustatory.

1.3 Insect brain and olfaction

1.3.1 General organization of an insect brain

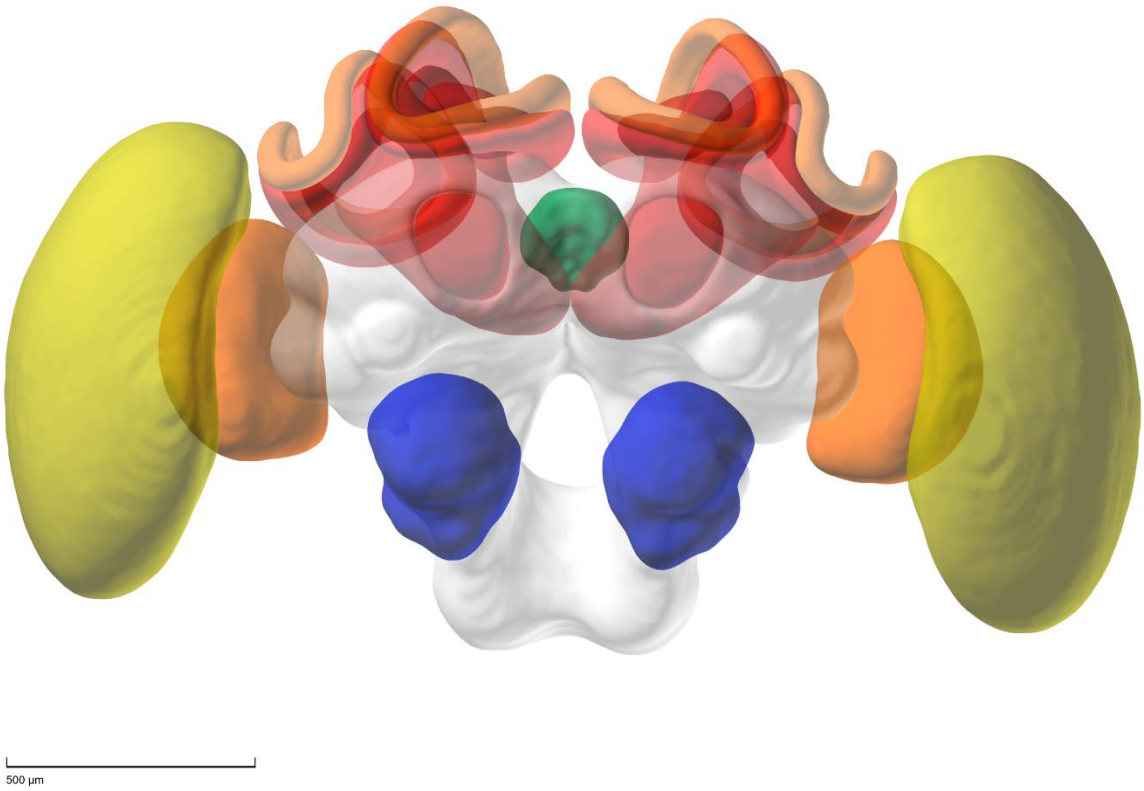

Figure 4: Brain of honeybee Apis mellifera; Image taken from insectbraindb.org; yellow: Medulla, orange: LOX, blue: Antennal lobe, red: mushroom body and calyx, green: central body

The first part of an insect brain called supraesophageal ganglion (SEG) is divided into 3 parts. The protocerebrum (PC) is dorsally situated relative to the esophagus and includes the optic lobes (OL), the central body (CB), the mushroom bodies (MBs), the protocerebral bridge (PB) and the lateral accessory lobe (LAL) and the lateral horn (LH). The deutocerebrum (DC) consisting of the antennal lobe (AL) and the antennal mechanosensory and motor center (AMMC) is positioned between the PC and the tritocerebrum (TC). The suboesophageal ganglion (SOG) lies ventrally to the esophagus and is connected by circumoesophageal connectives to the SEG. The nervous system continues as a postcephalic ganglionic chain. Each segment of thorax and abdomen have a pair of ganglia which are often fused dependent on taxa (Rolf G. Beutel et al., 2014).

1.3.2 General organization of a hemipteran brain

Dependent on the taxa hemipteran brains show effects of fusions. The circumoesophageal connectives might be massively reduced, which means that SOG or gnathal ganglion (GNG) and SEG are tied together. As a result of miniaturization or as a developmental characteristic brains of smaller insects (e.g., Strepsiptera) tend to shift towards the direction of the posterior thoracic and anterior abdominal region (Beutel et al., 2005). In some hemipteran taxa (e.g., Pentatomidae, Naucoridae) not only a shift to the thoracic region occurs, but also large fusions of the ventral ganglionic chain, which then appear as a compact mass. The paired ganglia of the forelegs are tightly attached to the SEG and GNG, as a potential result of the shift towards the thorax.

1.3.3 Neurobiological basis of the olfactory system in arthropods

Perception is indispensable for all living organisms. Thus, insects heavily rely on sensory organs to detect environmental cues. One of those, the antennal sensory system, allows a constant input of environmental stimuli to alter foraging, predation, mating behavior as well as escaping responses accordingly. Olfaction plays a central role within this system, which not only allows detection of volatile organic compounds (VOCs), but also the recognition of said cues. VOCs are detected by contact sensation of olfactory receptors (ORs) which are expressed in olfactory sensory neurons (OSNs). Those receptors are situated within structures called sensilla on the antenna as the or one of the first detection centers of the olfactory system. The OSNs convey these signals to the central nervous system (CNS) for further processing and ultimately triggering a response. The primary olfactory center of the CNS is the sphere-shaped antennal lobe, a part of the deutocerebrum (Hansson & Anton, 2000). Incoming signals are processed and transformed and then received by projections neurons (PNs). The olfactory information is carried by the PNs to higher brain centers, like the mushroom body (MB) or lateral horn (LH)(Tanaka et al., 2012).

1.3.3.1 The mushroom bodies

The mushroom bodies are prominent structures in the insect brain which are associated with learning and memory building. Extensive studies in model organisms like the fruit fly *Drosophila melanogaster* (e.g., (Busto et al., 2010; Davis, 2011)) and honeybee *Apis mellifera* (e.g., (Giurfa M & Sandoz JC, 2012; Menzel, 1999)) stated that the MBs play a fundamental role at encoding, storing and retrieval of memories. MBs are a pair of densely packed bilaterally symmetric neuropils, but their shape and size vary among taxa (Rybak & Menzel, 1993; Strausfeld

et al., 1998; Yang et al., 1995). A cluster of cells also called Kenyon cells, form a cup-like region on top of the pedunculus, which is part of the MB. Dendrites of the Kenyon cells arborize in those cup-like region and later form the input region for afferent signals from various neuropils of the protocerebral lobe (PL) including Lobula complex (LOX), medulla (ME) or ALs (Ehmer & Gronenberg, 2002; Hansson & Anton, 2000).

1.3.3.2 The central complex

The central complex (CX) is a group of unpaired neuropils situated medially within the protocerebrum. It consists of central body lower, central body upper, a pair of noduli and the protocerebral bridge. The central complex itself is only indirectly connected to sensory brain areas with few exemptions given in the hawkmoth *Manduca sexta* (Heuer et al., 2012) and the cricket *Gryllus campestris* (Honegger & Schiirmann, 1975). The CX is widely connected to different regions in the protocerebrum (e.g., the lateral accessory lobes, parts of the optical system including anterior and posterior optic tubercles as well as mechanosensory and chemosensory input from the antenna and other body parts (Heinze et al., 2013; Homberg et al., 2011; Honkanen et al., 2019; Mota et al., 2011)). The current functional concept of the complex is that it plays a crucial role in motor control/ activity, spatial orientation, or navigation (Pfeiffer & Homberg, 2014).

1.3.3.3 The lateral accessory lobe

The lateral accessory lobe is a paired brain structure ventrolateral from the CX. They are both highly interconnected which why it was proposed to be part of the CX (Richter et al., 2010). The LAL is suggested to be a major output region after experimenting with electrolytic lesioning on cockroach and its effects on obstacle negotiation behavior (Harley & Ritzmann, 2010). The descending neurons (DNs) of the LAL has been examined in several studies within different insects (e.g., *Gryllus bimaculatus, Manduca sexta* (Kanzaki et al., 1991; Zorovic & Hedwig, 2013)). In *Drosophila melanogaster* activation of a LAL DN induced backwards walking (Bidaye et al., 2014). Therefore, as the functional role of the LALs are still not completely understood, there is a definite indication of its involvement in motor activity/ control (Namiki & Kanzaki, 2016).

1.3.3.4 The antennal lobes

The antennal lobes (ALs) are paired spherical neuropils. They are part of the deutocerebrum and are also primary olfactory centers of the brain, homologous to the olfactory bulb of vertebrates. The antennal lobes are innervated by sensory neurons of sensilla on the antenna. The ALs are generally subdivided into spherical segregated modules called glomeruli (Anton & Homberg, 1999). The amount of those subunits differs between taxa and have been reported for a range of insects like *Drosophila melanogaster* with around 50 (Grabe et al., 2015) or around 160 in *Apis mellifera* (Giovanni Galizia et al., 1999). Odor information received by the AL is transferred to higher brain regions like the Lateral Horn (LH) or MBs via multiple AL tracts called ALTs by projection neurons (PNs) (Shimizu & Stopfer, 2017).

1.4 Hypothesis

A. aest. is an aquatic insect living its whole life under water due to its plastron. The environmental perception in aquatic and terrestrial habitats differ tremendously. Water has differing physical properties including the difference in optical density altering the optical sense as well as the impeded movement for VOCs, because they are either not soluble in water or hard to distribute within the medium. Changing the conditions for the perception of cues for survival has an immense impact on each organism, which opens up certain questions. If those conditions are changed, how does an organism react to it in an evolutionary perspective? How does the change of usage in the perception of one sensory modality affect the others? There are countless examples of vertebrates and invertebrates with different adaptations to specific environments or lifestyles. Bats don't rely on heavily their optical sense, because either their perception using ultrasonic sound suits the night activity better or having its lifestyle shifted to the night certainly made the usage of optic signals disadvantageous. One could state that their ability to percept with ultrasonic sound can balance out their weak optical system. The same applies to proturans which lack eyes and antennae. How do they percept their environment? How do they forage? It is significant that each organism must be able to percept its environment. Therefore, the inability or the impediment of one sense should be balanced with another or it would turn into a disadvantage. *A. aest.* relies on its plastron for respiration, which in turn needs a high oxygen level in the water (Jones et al., 2018; Seymour et al., 2015). Those levels are achieved in rivers with strong streams, which is a reason for their frequent appearances in those habitats. Even though *A.aest* is benthic, the stream of a river should certainly should excited mechanoreceptors on the sensory appendages of the water bug frequently. The constant influx of air bubbles at locations with a higher river gradient and the dirt changes the ability to use its optical sense.

The hypothetical impediment of some of the senses raise question on the neuronal capacities and how exactly *Aphelocheirus aestivalis* can gain information about its environment. Hypothesis of this study is *A. aest.* should have definite differences from the general insect brain. The bad solubility of VOCs and the constant directional stream in its habitat should be an obstacle for its olfactory sense.

1.5 Goals of this study

The goal of this study is to investigate the brain organization of *A. aest.* especially in terms of the multimodal sensory projection of the different sensilla types in the brain. I want to find out the positions of antennal sensory projection in the brain. Another goal of this study is to focus on the olfaction especially. Therefore, the AL will be investigated thoroughly.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Table 1: List of chemicals

Chemical	Supplier
Ethanol	Roth, Germany
Glycerol	Roth, Germany
Paraformaldehyde	Roth, Germany
Methyl salicylate	Sigma Aldrich, Germany
Triton X-100	Sigma Aldrich, Germany
Lucifer yellow	Sigma Aldrich, Germany
Iodine	Sigma Aldrich, Germany
Normal Goat serum	Cell Signaling Technology, USA
Tetra-methyl-rhodamine dextran with biotin	Molecular Probes; Invitrogen, USA
Hexamethyldisilazane	Sigma Aldrich, Germany

2.1.2 Solutions

Table 2: List of solutions:

Solution	Description
PFA	4% Paraformaldehyde
PBS	0.1 M phosphate-buffered saline
PBSTx	0.2% Triton X-100 in 0.1 M PBS

2.1.3 Antibodies

Table 3: List of antibodies

Type	Antibody	Supplier
Primary	Synorf1 (monoclonal Mouse Synapsin)	Developmental Studies Hybrid-oma, USA
Primary	Polyclonal Rabbit anti-GABA	Sigma Aldrich, Germany
Secondary	Goat anti-Mouse Alexa Fluor 546	Thermo Fisher Scientific, USA
Secondary	Goat anti-Rabbit Alexa Fluor 488	Thermo Fisher Scientific, USA

2.1.4 Equipment

Table 4: List of equipment

Equipment	Supplier
Skyscan 2211	Bruker, USA

Axio Zoom.V16	Carl Zeiss, Germany
LSM 880	Carl Zeiss, Germany
Imager.M2	Carl Zeiss, Germany
Stereo Microscope	Olympus, Japan
Scanning electron microscope	Germany
Sputter Coater	
High-Precision cover slip	Carl Zeiss, Germany
Slides	
Minutien pins	
Sylgard dish	selfmade
Glass vials	
Pipettes	Gilson
Micro scalpel	Fine Science Tools, Germany
Micro scissor	Fine Science Tools, Germany
Forceps	Fine Science Tools, Germany
Razor blades	

2.1.5 Software

Table 5: List of software

Software	Version
Amira	5.6.0 / 6.0.1
Mendeley	1803
ImageJ (Fiji)	1.53k
Microsoft Office 365	
Zeiss Zen 2	
Helicon Focus 6	
Inkscape	

2.2 Methods

2.2.1 Insect collection and rearing

Insects were collected at 50°55'36.6"N 11°35'40.7"E in the Saale with a water net. Bugs were reared in an 40x25x25 [cm] aquarium at 25°C under 12:12-hour light:dark cycles and held on a *Gammarus spec.* diet. A water/air pump was equipped to induce streaming and infusion of oxygen for water aeration. Specimen were dissected/ used within 7 days.

2.2.2 Histology

Head capsules were opened in PBS and dissected brains were fixed in PFA for 2h at RT or overnight at 4°C. After washing 6x10 min in PBS brains were left overnight in a 4% LY solution at 4°C.

2.2.3 Immunohistochemistry

Head capsules were opened in PBS and dissected brains were fixed in PFA for 2h at RT or overnight at 4°C. To improve antibody penetration and hence better staining loose nerve ends were cut off close to the brain. After washing 6x10 min in PBS blocking was facilitated in a PBSTx-NGS (10%) solution for 1h at RT. Following the washing 6x10 min in PBSTx (0.2%) the brains were incubated for 72 hours at 4°C on a shaker in the primary antibody solution consisting of anti-mouse Synorf1 at a 1:30 and anti-rabbit GABA at a 1:1000 dilution in PBSTx-NGS (2%). The preparations were washed 6x10 min in PBSTx and then incubated in the secondary antibody solution consisting of Alexa Fluor goat anti-rabbit 488nm (excitation wavelength) and Alexa Fluor goat anti-mouse 546nm (excitation wavelength) both in a 1:200 dilution in a PBSTx solution for 24h at 4°C on a shaker.

2.2.4 Anterograde fills of antennal or proboscal nerves

Sylgard plates were poured with a centric cylindrical airy space. Needles were placed in midst of the airy space as a layer. Specimen were cooled down at 4°C and dorsally immobilized with needles. Either antennae or proboscis were cut at the scape with a micro scissor and surrounded by a layer of vaseline to create a hydrophobic surface. A 4% microruby solution was added and sealed with vaseline. The airy space was filled with freshwater and the whole apparatus was incubated at 4°C for 1 or 2 days. Afterwards brains of the specimen were exposed in PBS and fixed for either 2h or overnight in PFA at 4°C.

2.2.5 Micro-CT preparation and scan

Specimen was directly dehydrated in an ascending ethanol series (50%, 70%, 90%, 96%, 99.8%, 100%, 100%) and incubated in 0.15% Iodine in a 100% Ethanol solution afterwards. The specimen was cut at the second thoracic segment to fit the tube. Specimen was scanned with a pixel density of 1 µm/pixel.

2.2.6 Sample preparation for CLSM

Every sample was washed in PBS 6x10min and then dehydrated in an ascending ethanol series for 10 minutes of 30%, 50%, 70%, 90%, 96%, 100%, 100% ethanol solutions. The brains were then cleared in methyl salicylate and mounted on 200µm slides with methyl salicylate and a high-precision cover slip.

2.2.7 CLSM Imaging

The whole mount brain was imaged in a Zeiss LSM 880 Confocal laser microscope with 10x, 10w, 20w, 40w objectives. Pinholes were set at 1 airy unit. The fluorescent dyes were excited with a Argon laser at 488 nm or He/Ne laser at 543 nm. The spacing between sections in a z-stack were adaptively changed between 0.5 µm and 2 µm depending on the numerical aperture and resolution of the objective.

2.2.8 Image processing

AMIRA 5.6.0/ 6.0.1 (Thermo Fisher Scientific), Image J (Public Domain), and ZEISS ZEN 2 Imaging Software (Zeiss, Jena, Germany) were used for three-dimensional reconstructions and adjustments of the confocal image stacks concerning contrast and brightness. Amira was used to produce maximum intensity projections and depth maps. Neuropil labeling was done in the segmentation editor of the Amira software with a brush tool and the interpolation feature. This labelfields were reconstructed by generating a surface and surface view. Maximum intensity projections were produced with the “Image Ortho Projection” Tool.

3 Results

In this study I investigated the spatial position of the neurosystem and the organization of the brain of *A. aest.* using a micro-CT scan, histological stainings with Lucifer Yellow (LY) and immunohistology with SYNORF1 antibodies. I also filled the antennal nerve with Microruby (MR) to trace the antennal sensory pathway and its projection into the brain.

3.1 Brain position and the sensory pathway to it

Using a micro-CT scan the whole head was imaged (Fig. 6, G) and the brain was reconstructed (Fig. 6, H) digitally with the Amira Software. The head of *A. aest.* has a flattened shape (Fig. 6, A-C). Parts of the posterior head capsule are inserted into the thoracal region (Fig. 6, C). The brain of *A. aest.* is heavily fused. The SEG and GNG are tightly attached. The GNG and PTG are also fused together. All three ganglia are forming a singular complex while at the same time connectives between those parts are reduced (Fig. 6, E-G). The pair of proboscal nerves run along close to the paired gland ducts and innervate the GNG (Fig. 6, C). The optic lobes are connected to the protocerebral lobe (PL) via optic nerve (ON) and positioned anterolateral to the PL close to the compound eyes (Fig. 6, H). The antenna is positioned underneath the compound eyes (Fig. 6, C) and its nerve runs close to the optic lobes and optic nerve until entering the SEG (Fig. 6, H). The oesophagus passes through the protocerebreal lobe in between the tritocerebral and deutocerebral regions (Fig. 6, E).

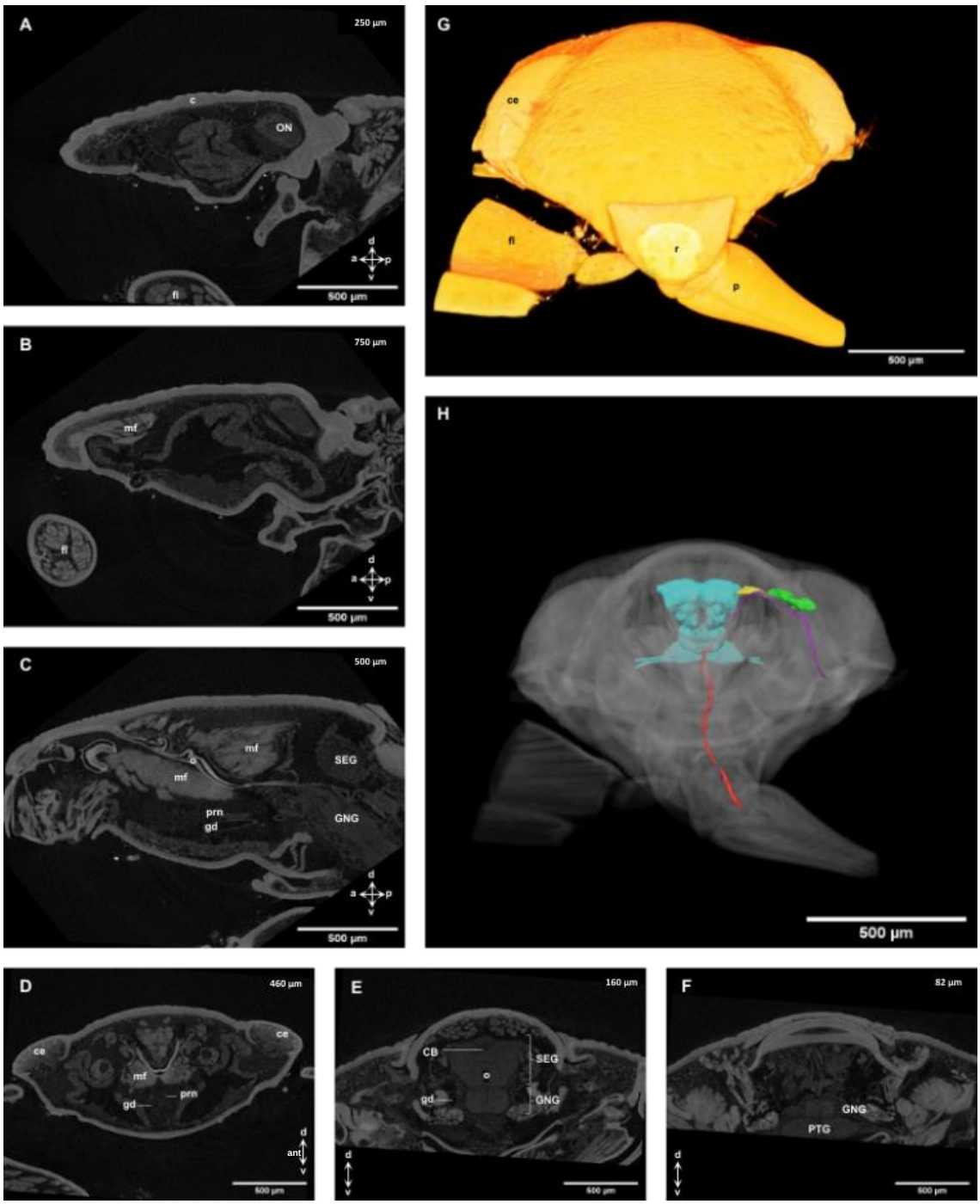

Figure 5: A-F: medial (A-C) and frontal sections (D-F) of a micro-CT scan of the head of A.aest.;; ant: antenna, c: cuticula, CB: central body, ce: compound eye, fl: foreleg, gd: gland duct, GNG: gnathal ganglion, mf: muscular fibers, o: oesophagus, ON: optic nerve, prn: proboscal nerve, PTG: prothoracic ganglion, SEG: supraesophageal ganglion,; a: anterior, p: posterior, d: dorsal, v: ventral; G: frontral view volren of the whole micro-CT scan; r: rostrum, p: proboscis, fl: foreleg, ce: compound eye; H: Frontal view on the reconstruction of the neurosystem of A.aest on the left hemisphere; turquoise: fused SEG and GNG, red: left proboscal nerve and innervation, violet: left antennal nerve and innervation, yellow: left optic nerve, green: left optic lobe; Section depths are indicated at the upper right corner

3.2 Brain organization of *Aphelocheirus aestivalis*

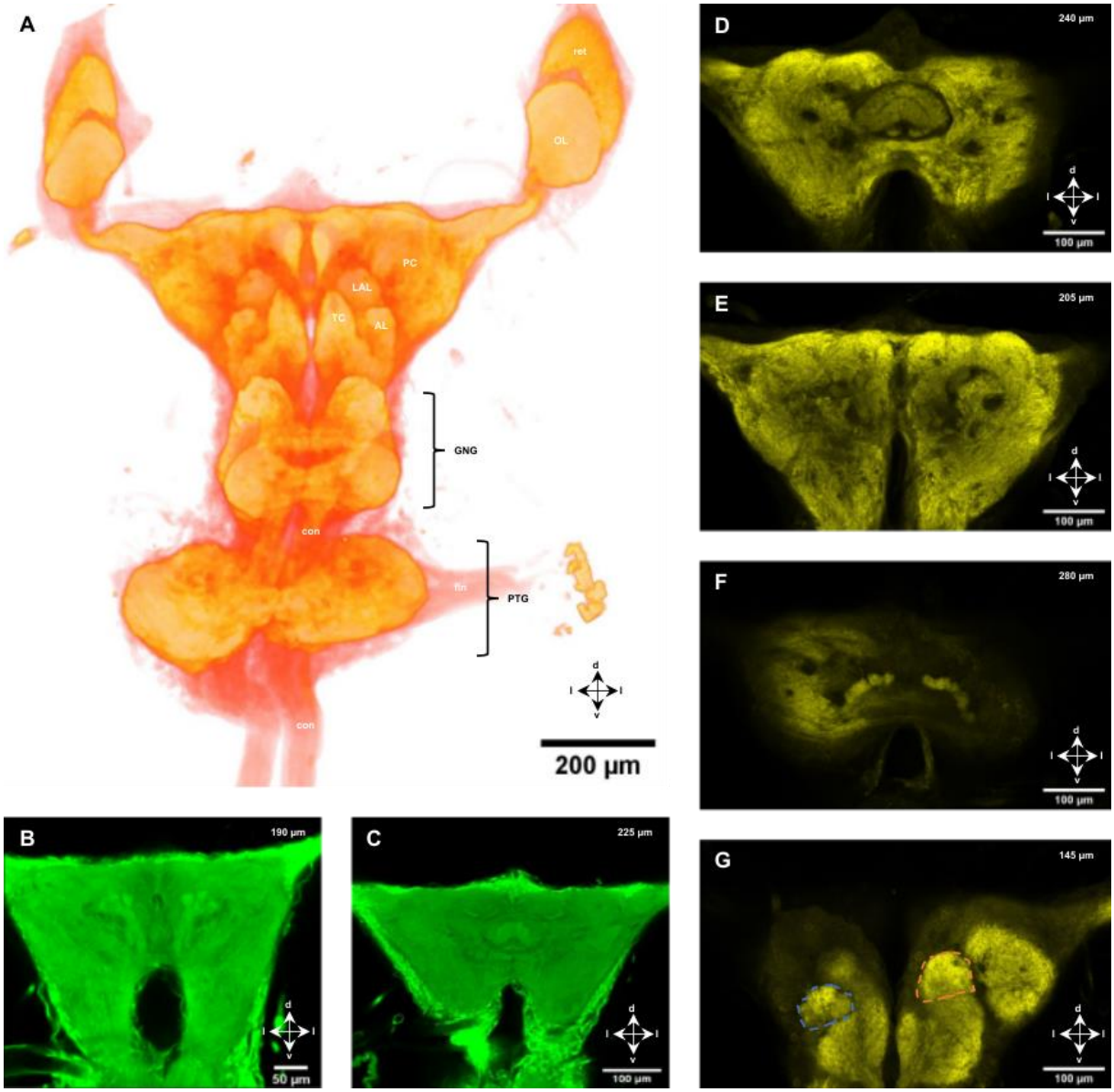

Figure 6: CLSM stack scans (B-G) of LY (B-C) and SYNORF1 (D-G) stainings of male A.aest. brains, all sections with an anterior view; A: Volren projection of a SYNORF1-Staining, scanning at excitation wavelength of 546 nm; B-C: View on mushroom bodies (B) and central body (C) in sections of a stack scan, scanning at excitation wavelength 488 nm; D-E: CLSM of SYNORF1-immunostaining at excitation wavelength 546; Sections of a stack showing CB (D), MB (E), PB (F), AL indication in blue and LAL indication in orange (G); Section depths shown at the upper right corner; AL: antennal lobe, con: connective, fln: foreleg nerve, GNG: gnathal ganglion, LAL: lateral accessory lobe, OL: optic lobe, PC: protocerebrum, PTG: prothoracic ganglion, TC: tritocerebrum; d: dorsal, l: lateral, v: ventral; section depths are indicated at the upper right corner

Histological stainings with LY and immunostainings with the SYNORF1 antibodies and 546 goat anti-mouse in combination with confocal light microscopy was used to visualize the general brain organization of *A. aest.* and its most prominent neuropils. The brain of *A. aest.* is divided into SEG or PL, GNG and PTG. All structures form a densely packed complex in which the connectives between PL and GNG and GNG and PTG seem to be immensely reduced (Fig. 7, A). The PL is additionally subdivided into PC, DC as well as TC. The TC is turned forward compared to other insects (e.g., *Apis mellifera, Locusta migratoria)* and is positioned anteromedial to the DC, pointing in the dorsal direction (Fig. 7, A). The GNG is tightly fused with the TC (Fig. 7, A). The mushroom bodies possess lobular structures but lack a calyx or cup-like region (Fig. 7, B, E; Fig. 8, A). The specific neuropils shown in Fig. 7 were digitally labeled. The surface was generated with the labelfields using Amira Software (Fig. 8). The CX is positioned in the center of the PL in between both brain hemispheres. The paired mushroom bodies are anterolaterally situated to the CB. The AMMC is very closely posterolaterally attached to the AL. The OLs, visual information processing centers of the brain, are divided into the distal lamina (LA), the medulla (ME) and the lobula complex (LO). The LALs are situation between MBs and ALs (Fig. 8).

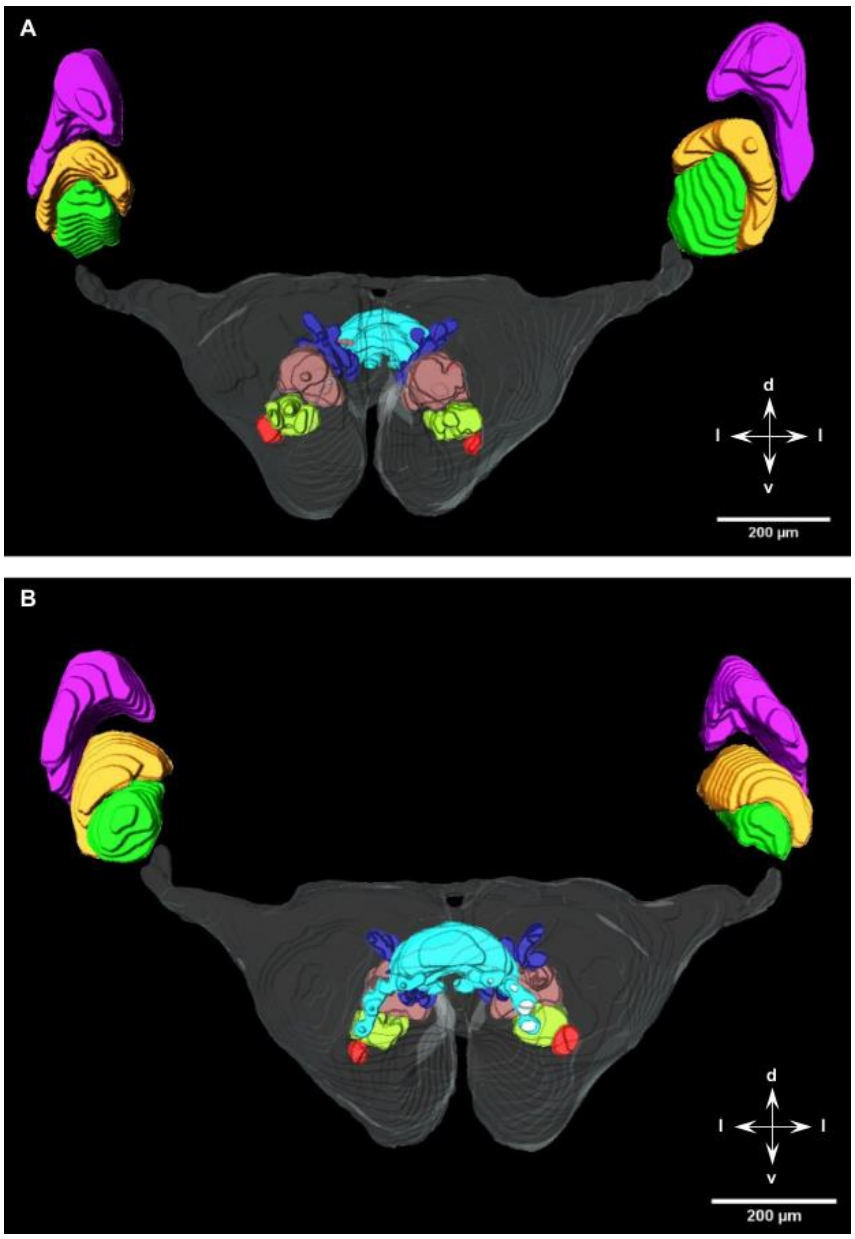

Figure 7: Labeling and Reconstruction of neuropils using synaptic immunostaining (SYNORF1); A: Anterior view on the PL; B: Posterior view on the PL; color indications: violet: Lamina, yellow: Medulla, neon green: LOX, turquoise: CX, dark blue: MB, light green: AL, brown: LAL, red: AMMC; d: dorsal, v: ventral, l: lateral

3.3 Spatial organization of the antennal lobe and GABA-activity

To investigate the specific spatial organization of the antennal lobe the AL was stained with anterograde nerve fillings at the antennal base, the scape. Successful backfills at the scape of the antenna stained the AL, which has different neuronal densities (Fig. 8, A, B). The densely packed region indicated in blue is positioned anterolateral to the loosely packed region (Fig. A, B, D) indicated in orange. The compact region below the AL indicated in violet shows the AMMC (Fig. 8, C). Double antibody staining with anti-GABA and SYNORF1 revealed separated GABAergic neuron clusters in the AL for the densely and the loosely packed region (Fig. 8, D-F). The soma clusters of these regions are indicated in Fig. 8 E and F.

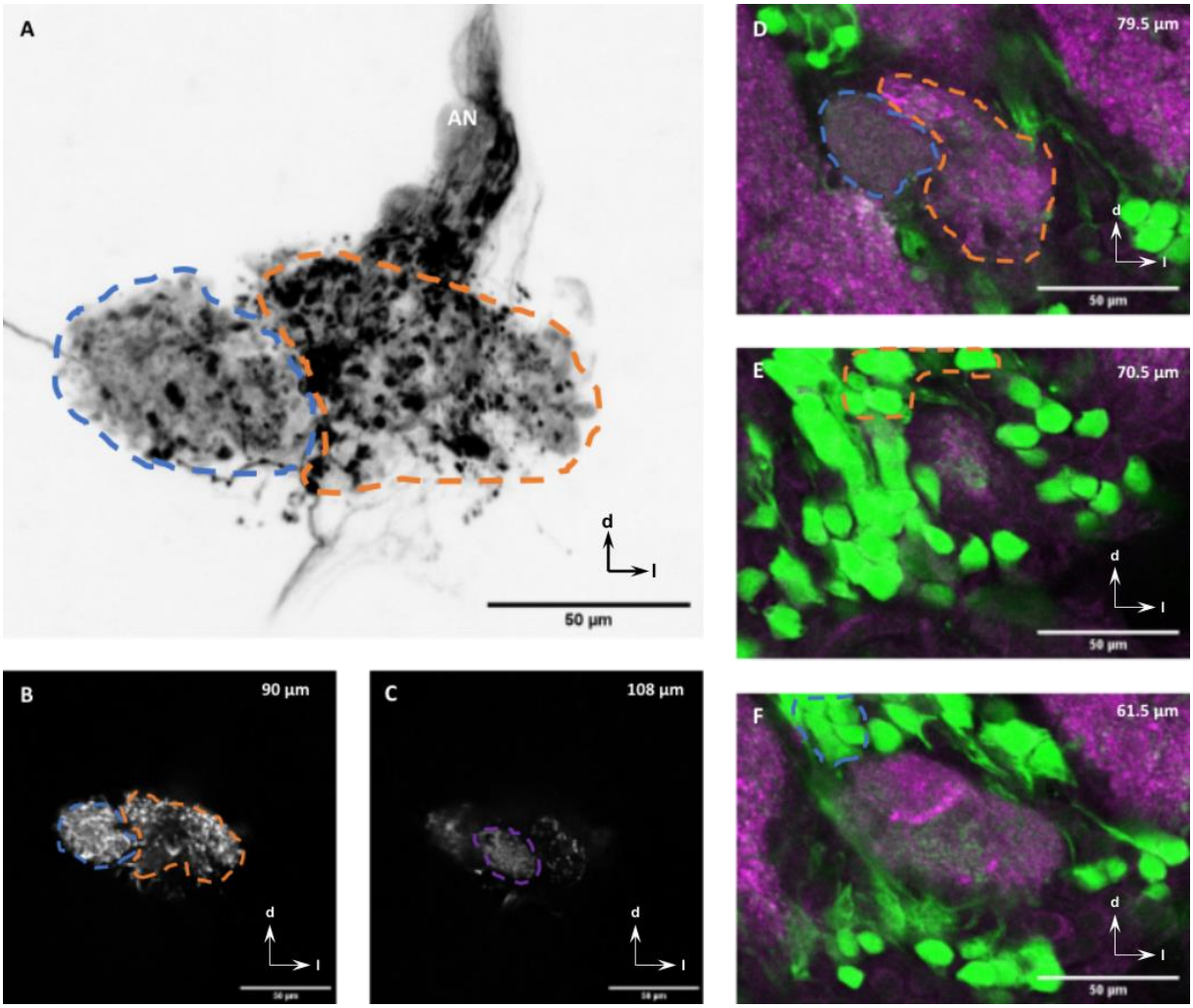

Figure 8: CLSM-Imaging of an antennal backfill at the scape of a male specimen (A-C) and an immunohistological staining with anti-synaptic staining (SYNORF1, indication in magenta) and anti-GABA staining (a-GABA, indicated in green) (D-F); blue and orange lines indicate a region and their GABAergic soma-cluster; violet lines indicate the AMMC; section depths are indicated on the upper right corner

The nerve filling also revealed a large cluster of axonal fibers entering the antennal lobe anter-odorsally (Fig. 8, A). The entering axons mainly terminate in the AL resulting in round or spherical shaped structures (Fig. 9, A-D). This indicates a major role of the AL in the antennal sensory system. The spherical shaped structures were labeled and reconstructed using Amira Software (Fig. 9, E, F).

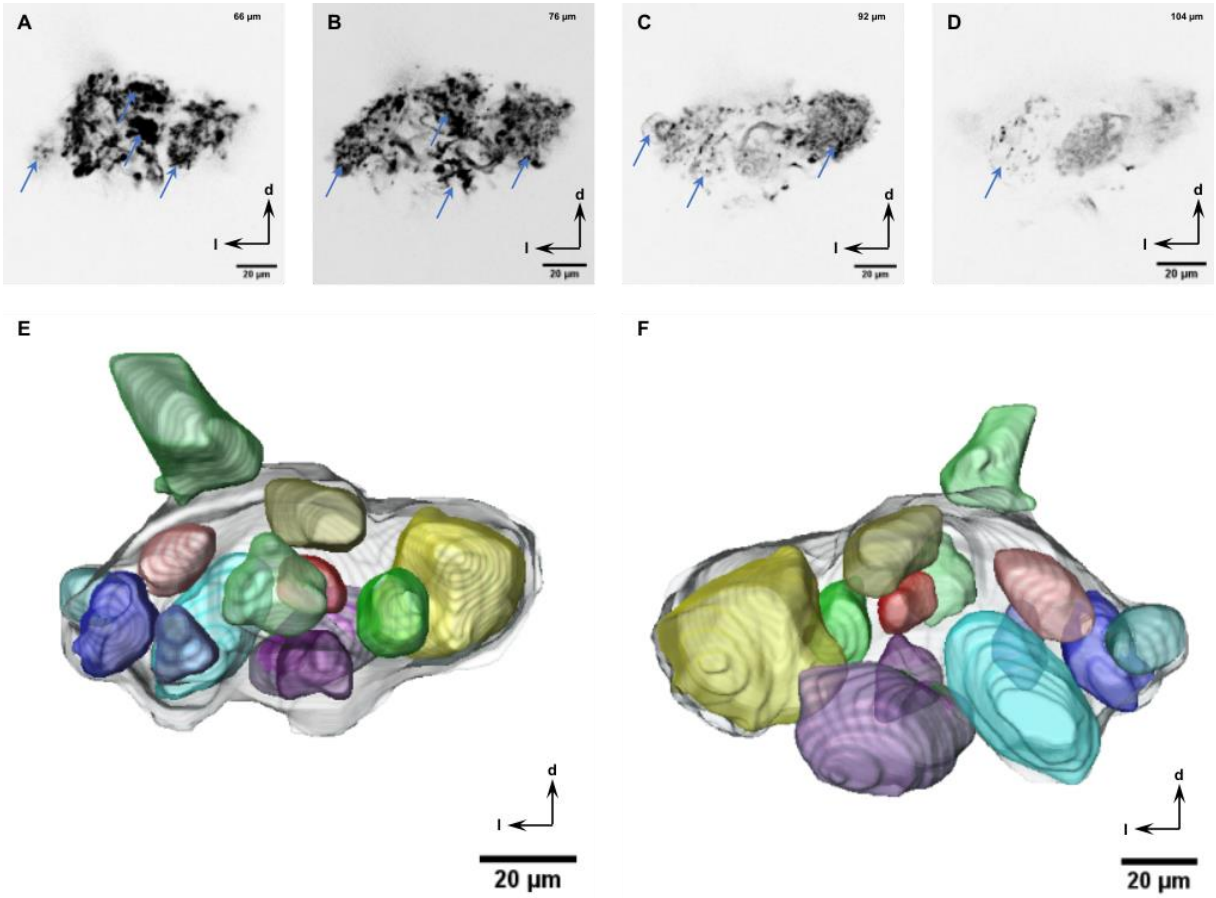

Figure 9: A-D: Anterior view on z-stack sections of an antennal backfill of a male specimen; E-F: Reconstruction of labeled axonal clusters; section depths are on the upper right corner; blue arrows point to dense axonal clusters in the antennal lobe

3.4 The antennal sensory pathway

I investigated the antennal sensory pathway by trying to fill all nerval fibers in the antenna, which are sensory neurons of the different sensilla. Successful nerve fillings revealed axonal bundles innervating the AL (Fig. 8, A; 10, A). Some tracts bypass the AL and project in the medial protocerebral lobe with a massive arborization (Fig. 10, C). Other tracts arborize in the tritocerebral region (Fig. 10, D), in the GNG (Fig. 10, E) and in the PTG (Fig. 10, F). The tritocerebral arborization is situated ventromedial in the posterior direction to the AL (Fig. 10, B, D). The protocerebral tract terminates laterodorsal to the mushroom bodies (Fig. 7, A; Fig 10, A, C).

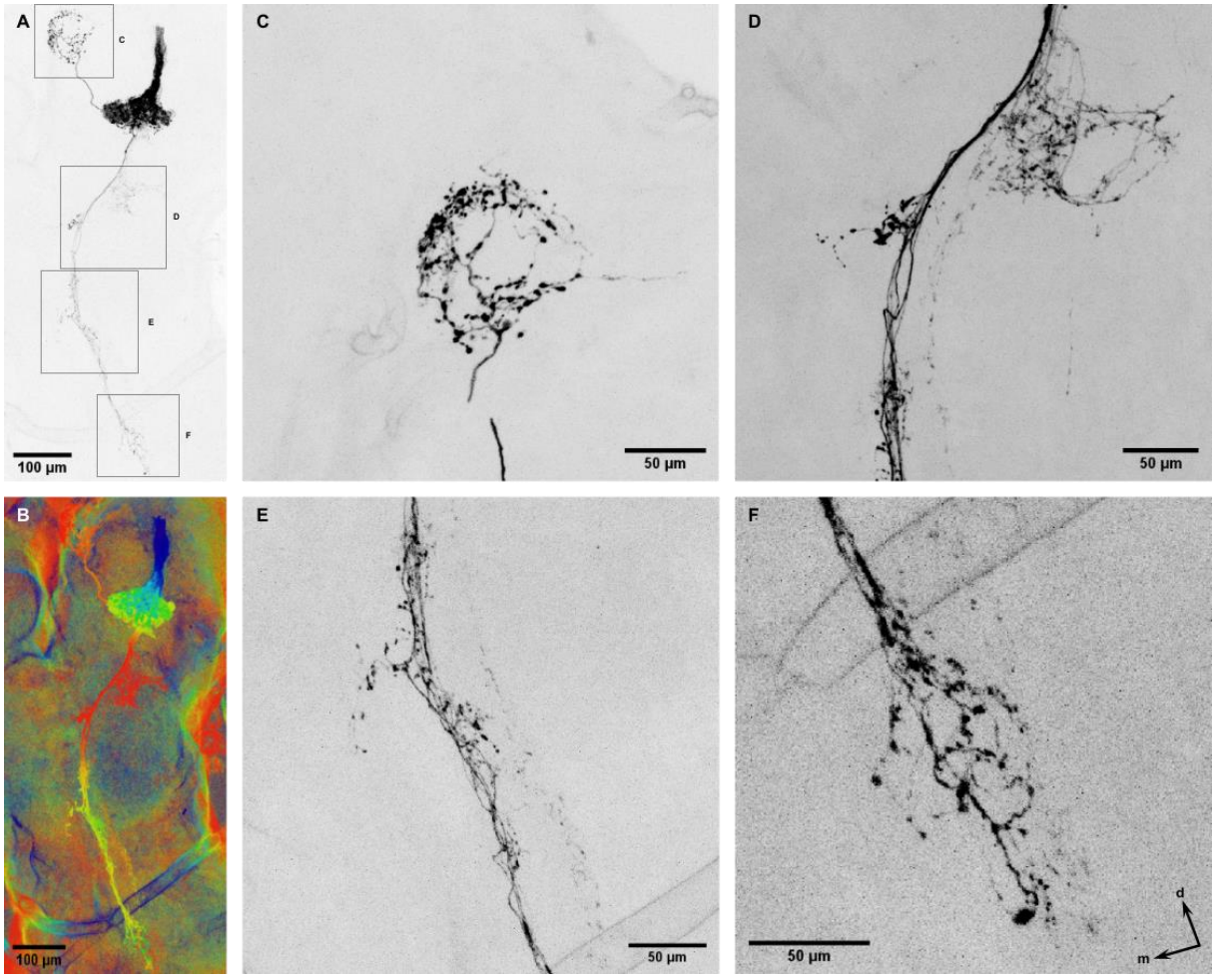

Figure 10: A-F: CLSM imaging of an antennal backfill at the scape of a male specimen; A: inverse maximum intensity projection of the whole antennal sensory pathway; B: Depth map of the maximum intensity projection with height -> depth being indicated in blue to red; C-F: Arborization of the antennal sensory pathway without AL; directions indicated in F apply to all images

4 Discussion

Using the micro-CT, histological- and immunostainings and anterograde backfills as well as reconstruction via Amira Software the brain anatomy of *A. aest.* and its sensory pathway to and in the brain was characterized. The organization of its brain and their neuropils were identified with a special focus on the antennal lobe.

4.1 Brain organization of *Aphelocheirus aestivalis*

A. aest. has the characteristics of a hemipteran species. The head is shifted into the thoracal region (Fig. 5, B, C) and many regions of the brain have been fused (Fig. 6, A; 5, H). The connecting nerve cords between PL and GNG are reduced and potentially embedded into the TC. The brain is also shifted and positioned exactly between head capsule and prothorax (Rolf G. Beutel et al., 2014). This shift potentially results of miniaturization or the flattened head shape.

The brain of *A. aest.* possesses all general brain structures including the OLs which are subdivided into LA, ME, and LOX, paired MBs except for missing calyces, a CX, ALs with attached AMMC and LALs (Fig. 7). Even though the brain regions are fused, the PL, GNG and PTG are easily distinguishable (Fig. 6, A). The same applies to the subregions of the SEG/PL: PC, DC and TC.

The size of sensory neuropils can be an indicator for the number of neurons participating in each sensory system. Therefore, volume analysis could do to follow up this study. The optic lobes of *A.aest*. seem to be rather small compared to the brain of *Apis mellifera* (Fig.4; 6, A; 7, A). Generally large OLs indicate large involvement of the optical system in the behavior of a given insect. The small OLs could be a sign that *A. aest.* is more dependent on different sensory systems.

The AMMC is a center for processing of mechanical cues (e.g., vibration, stream direction). Backfills of the antenna have shown that axon bundles of the antennal sensory pathway terminate in the AMMC of *A. aest.* (Fig. 8, C; 9, D). These arborization might be SNs of SCa located on the antenna signaling mechanosensory information.

The MBs of *A. aest.* don't possess calyces (Fig. 7, 8). Other aquatic insects are also reported to lack a calices but are still able to sense olfactory stimuli (Rebora et al., 2012; Strausfeld et al., 1998). Another study has also reported learning ability in hemiellipsoid bodies a crustacean species. The MBs are homologous to the HBs and therefore, the MBs of *A.aest.* could be structured differently compared to the general organization (Maza et al., 2016).

4.2 Sensillar types and the sensory pathway of the antenna

In this study I try to combine the knowledge about sensilla types on the antenna (Fig. 2) and their sensory function with the projection pattern into regions of the brain (Fig. 10). Investigations on AL revealed a glomerular organization in the AL (Fig. 8 E) and successful backfillings at the scape resulted in distinct arborizations in the brain (Fig. 10). Clusters of sensory neuron axons enter the AL anterodorsally and either terminate in glomeruli or arborized in the PL, GNG or PTG.

The antenna of *A. aest*. possesses various sensilla even though they are sparsely distributed (Nowińska & Brożek, 2020) compared to other insect species (e.g., *Drosophila melanogaster*, *Apis mellifera*). Nowinska et al. have identified sensilla, which can functionally be distributed to different signal modalities. The SB2 and SB3 have olfactory functions in other insects (Dutt Parashar et al., 1994; Tichy & Barth, 1992). The SCa are considered to serve mechanosensory function (Chapman, 1998; Gupta, 1992) and the SA is associated with either CO_2-responses (Kleineidam et al., 2000) or hygro- and thermosensitive function (Bauchhenss, 1983; Piersanti et al., 2011). SPl might be an indication of an autapomorphy within the nepomorphan order and the function remains unknow, but it was hypothesized that they are chemosensory, because they have wall pores on the surface (Nowińska & Brożek, 2020).

The sensory pathway of the antenna is dependent on the sensillar types on the antenna. Therefore, sensory neurons for different modalities like OSNs should target or arborize in different regions. The antennal backfill has shown various arborizations in the whole neurosystem (Fig. 10, A-F). The spherical structures in the AL, which are formed by the cluster of axons from the sensory neurons of the antenna can be interpreted as glomeruli (Fig. 8, E, F). The glomeruli in ALs are generally associated with olfaction (Anton & Homberg, 1999). Hence, the termination of clusters of axons from the antenna in the glomeruli of the AL might be SNs of multiporous SB2 and SB3 or SPl (Fig. 3, 8). The density of axonal fibers in the AL (Fig. 10, A) compared to afferents entering GNG or PTG (Fig. 11 E, F) hint towards the AL being the major target area of the antennal sensory pathway.

The SCa and their mechanosensory function might result in projections into the AMMC (Fig. 8; 9, D) as an indication of mechanosensory neuron (MSN) terminations (Kristoffersen et al., 2008; Rossi Stacconi et al., 2014).

The spatial zones indicated in Fig. 8, A are potentially similar to the two zones in the study of Piersanti 2011. The authors hypothesized that one of the regions positioned very similarly to the spatial organisation of the reconstructed zone in the specimen are processing thermal or

hygral stimuli (Piersanti et al., 2011). Combining this with the findings of SA on the antenna and studies showing its function the thermosensory or hygrosensory system it is possible to explain this projection with input SNs of SA.

The projection to the protocerebral region (Fig. 10, C) has been also reported in the *Cataglyphis sp.* as an afferent of the Johnston organ (Grob et al., 2021). This indicates mechanosensory activity in the protocerebral lobe with a tract of the antennal sensory pathway.

4.3 Olfaction in *A. aest.*

The mushroom bodies play an important role in learning and memory formation. Even though MBs are mainly associated with olfaction, the calyces also receive visual input both from the LOX and ME in *Apis mellifera* (Ehmer & Gronenberg, 2002). The absence of calyces, which are major input regions for afferents of other PL neuropils to the MBs (Fig. 7, E; Fig. 8, A) might indicate either a loss of olfactory processing in the brain or a change of the way olfactory signals are integrated in the brain. A study in *Libellula depressa* has shown that despite the lack of glomeruli in the ALs and MB calyces olfactory neurons are present, which strongly supports its olfactory function (Rebora et al., 2012). In another experiment with a crustacean species *Neohelice granulate* it was investigated whether MBs and hemiellipsoid bodies (HBs), which are homologous to the MBs, have a common origin. They compared HBs with calyxless MBs and revealed altered neuronal responses after training (Maza et al., 2016). In combination with the existence of SB2 and SB3 on the antenna of *A. aest.* which are proven to serve olfactory functions in other insects (Dutt Parashar et al., 1994; Lopes et al., 2002; Tichy & Barth, 1992) this might be a clear indication of potential olfactory perception. Although it needs to be proven with electrophysiological or behavioral experiments in the future.

5 Outlook

Given the investigated projections of the antennal sensory pathway into the brain, behavioral experiments could prove certain functions of sensilla. Another study could measure volumes of all the neuropils and compare the ratios of neuropil/ whole brain volume to other insects.

6 Conclusion

In this study the organization of the brain of *Aphelocheirus aestivalis* was investigated. The brain show signs of fusions, which are also characteristic for hemipteran species. Additionally, to the fusion, the whole head and therefore, the brain is shifted towards the prothorax. The ALs revealed a glomerular organisation with differing neuronal densities and functions. The antennal sensory pathway indicates tracts for different signal modalities, terminating in specific regions. The antennal sensory pathway combines perception and signaling of mechano-, thermo-, hygrosensory and olfactory stimuli.

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Declaration of Authorship

I, Ngoc Anh Luu, hereby declare that this thesis was prepared based on my own experimental work and confirm that I am the sole author of this thesis. All direct and indirect ideas and information that I used are cited in text and acknowledged as standard referencing practices in the bibliography. I also confirm that, according to the best of my knowledge, my thesis does not violate any proprietary right and this thesis has never been used for any achievement or examination purpose and has not been published in any language.

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