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Antennal sensory neurons in the central nervous system of the semi-aquatic backswimmer *Notonecta glauca* (Hemiptera)

Master's Thesis

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Submitted by

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List of abbreviations

AG	abdominal ganglion
AL	antennal lobe
AMMC	antennal mechanosensory motor center
AN	antennal nerve
CB	central body
GNG	gnathal ganglion
LA	lamina
LAL	lateral accessory lobe
LOX	lobula complex
LSCM	laser scanning confocal microscope
MB	mushroom body
ME	medulla
N. glauca	*Notonecta glauca*
NGS	normal goat serum
OL	optic lobe
OSN	olfactory sensory neuron
PAS	papilla sensillum
PBS	phosphate buffered saline
PBST	phosphate buffered saline with triton X-100
PFA	paraformaldehyde
PFAT	paraformaldehyde with Triton X-100
PG	posterior ganglion
PIP	peg in pit
PL	protocerebral lobe
PN	projection neuron
PR	protocerebrum
proTG	pro thoracic ganglion
SCo	sensilla coeloconica
Sch	sensilla chaetica
SEM	scanning electron microscope
SN	sensory neuron
ST	sensilla trichodea
TRS	trichoid sensillum

Abstract

Secondarily water adapted insects can sense the environment from a wide range of sources that are crucial for their survival and reproduction. The knowledge of sensory neurobiology of aquatic insects remains rudimentary. Most of the studies were focused on the external morphology of the sensory organs or on the ethological and entomological perspectives. This study focuses on the brain organization of semi-aquatic backswimmer *Notonecta glauca* (Hemiptera: Notonectidae), the neuronal pathway of antennal sensory neurons (SNs), and their projection pattern in the central nervous system (CNS). The neuropil labelling with the Lucifer Yellow and immunohistochemistry with a synapsin antibody in combination with confocal laser scanning microscopy and digital 3D reconstruction revealed a glomerular antennal lobe (AL). The anterograde backfill experiments using a fluorescent dye showed that antennal sensory neurons (SNs) target glomeruli in the antennal lobe (AL) which indicates the presence of an olfactory pathway. SNs also project the medial protocerebral lobe (PL), the gnathal ganglion (GNG), the prothoracic ganglion (proTG), and the posterior ganglion (PG). Such a projection pattern indicates that the antennae of this semi-aquatic bug play a major role to detect environmental cues and to exert motor function. The external morphology of sensilla on the antennae and proboscis were characterized by scanning electron microscopy (SEM). The presence of both mechano- and chemosensory sensilla indicating that the antenna of *Notonecta glauca* is a multimodal sensory organ. These findings are discussed in the context of the rich behavioral repertoire of Notonectidae. Moreover, the research on semi-aquatic insects in their transition from land to water might help in understanding the evolutionary trajectories for multimodal encoding mechanisms in this aquatic insect.

Zusammenfassung

Die Insekten, die sekundär an das Wasser angepasst sind, können ihre Umgebung aus einer Vielzahl von Quellen wahrnehmen, was sowohl für das Überleben als auch die Reproduktion unabdingbar ist. Das Wissen über die sensorische Neurobiologie aquatischer Insekten ist weitestgehend rudimentär. Die meisten Studien haben sich entweder auf die externe Morphologie sensorischer Organe oder auf ethologische und entomologische Perspektiven fokussiert. Diese Studie konzentriert sich auf die Organisation des Gehirns des semi-aquatischen Rückenschwimmer, *Notonecta glauca* (Hemiptera: Notocetidae), die Nervenbahnen der sensorischen Neuronen der Antenne (SN) und ihrer Projektionsmuster in das zentrale Nervensystem (CNS). Die Färbung der Neuropile mit Hilfe des Farbstoffes Lucifer Yellow und die Immunohistochemie mit einem Synapsin-Antikörper in Kombination mit konfokaler Lasermikroskopie und digitaler 3-D-Rekonstruktion zeigte eine glomeruläre Organisation des Antennallobus (AL). Anterograde Färbungsexperimente mit floureszenten Farbstoffen zeigten sensorische Neuronen der Antenne, die direkt in die Glomeruli des Antennallobus verlaufen und damit Hinweise auf den Präsenz olfaktorischer Nervenbahnen liefern. Die sensorischen Neuronen wiesen ebenfalls Projektionen in den medial-protocerebralen Lobus (PL), dem gnathalen Ganglion (GNG), dem pro-thorakalen Ganglion (proTG) und dem posterioren Ganglion (PG) auf. Ein solches Projektionsmuster deutet darauf hin, dass die Antenne des semi-aquatischen Rückenschwimmers eine große Rolle in der Detektion umgebender Reize und der Ausübung motorischer Funktionen spielt. Die externe Morphologie der Sensillen auf den Antennen und dem Proboscis wurden durch Rasterelektronenmikroskopie (SEM) bestimmt. Der Präsenz von mechano- und chemosensorischen Sensillen deuten darauf hin, dass die Antenne von *Notonecta glauca* ein multimodales sensorisches Organ ist. Diese Ergebnisse werden im Kontext des vielseitigen Verhaltens der Notonectidae diskutiert. Die Forschung von semi-aquatischen Insekten kann zu einem weiteren Schritt für das Verständnis evolutionärer Mechanismen im Verlauf des Übergangs von Land zu Wasser in Wasserinsekten beitragen.

1. Introduction

1.1. Phylogenetic position of *Notonecta glauca*

Notonecta glauca is a member of Notonectidae, which is the second most diverse water bugs family. It comprises of 400 species within 11 genera in the monophyletic infraorder Nepomorpha of the suborder Heteroptera under the insect order Hemiptera. Hemiptera are hemimetabolous insects, typically developing via a series of 5 nymphal instars (Polhemus & Polhemus, 2008). The backswimmers (Notonectidae) are predatory aquatic insects. They are one of the top predators in aquatic ecosystems (Nowińska & Brożek, 2019). Their abundance in many freshwater systems and their unusual morphological specializations, for example, specialized mouthparts, elongated rear-leg, and large complex eye (Polhemus, 2009; Khila et al., 2014) for adaptation to new habitats, made this group an interesting research specimen for the entomologists, and ethologists (Polhemus & Polhemus, 2008).

Predatory lifestyle has made the infraorder Nepomorpha as one of the most specialized groups among the suborder Heteroptera. The Nepomorpha, with about 2400 species worldwide (Polhemus & Polhemus, 2008) were classified as secondary aquatic bugs (Popov, 1971).

1.2. *Notonecta glauca's* transition from land to aquatic habitats

The whole aquatic Nepomorpha group started undergoing diversification about 263 million years ago in the late Permian (Ye et al., 2020). It is also demonstrated that they are the divergence of proto-heteropteran terrestrial groups, Protoochterids (Vélez, 2006) during the mid-Triassic period (Popov, 1971). Nearly all aquatic insects possess the tracheal system (Chapman, 1998). This fact supports the idea that these insects are secondarily adapted to aquatic habitat (Pritchard et al., 1993). Notonecta uses its extremely extended hind legs for swimming under the water like an oar, which is phylogenetically related to the terrestrial milkweed bug Oncopeltus (Khila et al., 2014) (Figure: 1). A conserved *Ultrabithorax* gene expression and function in the rear-leg have also been found between terrestrial and aquatic Heteropterans (Khila et al., 2014) (Figure: 1). In 2009, Strausfeld et al. reported that several Hemiptera has adopted an aquatic lifestyle independently (Strausfeld et al., 2009).

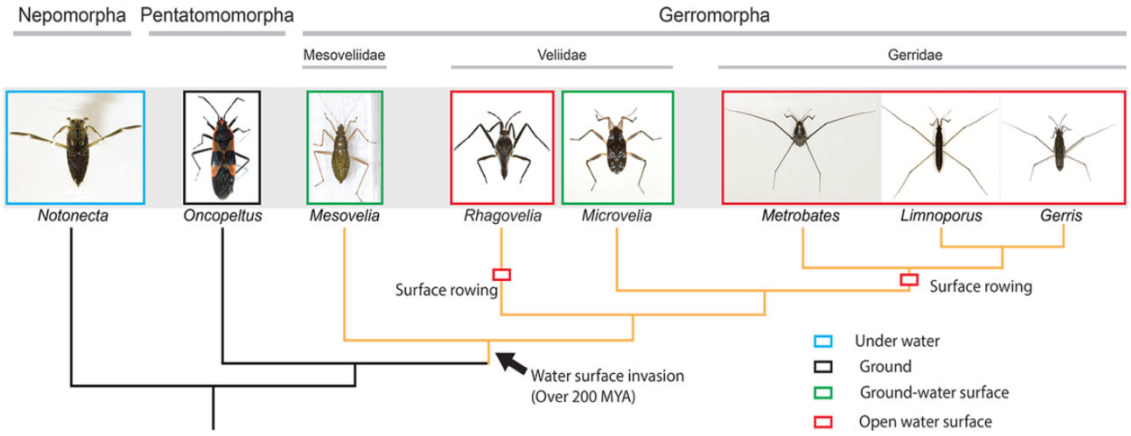

Figure 1: Semi-aquatic insects and outgroups: Phylogenetic relationships and diversity in relative leg length. The backswimmer Notonecta which had made the transition to the life under water, is highlighted in blue. The ground walking Oncopeltus, which is only found in land and used here as a terrestrial outgroup, is depicted in black lines. Mesoveliid Mesovelia and the veliid Microvelia, both water-surface walking semi-aquatic insects, are shown in green. Three water-surface rowing gerrids Metrobates, Gerris, and Limnoporus are shown in red. Small red boxes represent the evolution of Surface rowing's evolution as a novel mode of locomotion is represented by small red box. Adapted from (Khila et al., 2014).

1.3. Olfactory neurobiology of arthropods

Insects are extensively dependent on their antennal sensory system for behavior and for their response according to environmental stimuli. Among different sensory modalities' olfaction is significant, e.g., for feeding, enemy avoidance, and finding mating partner. In insects, peripheral olfactory sensory neurons (OSNs) that are present on the antennae are the first detection center for volatile chemical cues. The peripheral signals are then conveyed to the central nervous system (CNS) to induce behavioral responses (Figure: 2).

The OSNs project their axon throughout the antenna to the deutocerebrum and thus send stimuli information at the antennal lobe (AL) level for further processing. The AL, the first integration center of volatile odor information (e.g., Strausfeld et al., 1998; Hansson & Anton, 2000) generally houses several spherical glomeruli that are formed by sensory neurons (SNs) axon terminals, local interneuron arbor, and projection neuron (PN) (reviewed in Galizia & Rössler, 2010). The AL then transfers the chemical information to the higher brain center including the mushroom body (MB) and the lateral protocerebrum (PR) for further processing (reviewed in Galizia & Rössler, 2010) (Figure: 2).

Comprehensive studies on the olfactory pathway along with general brain organization of flies, cockroach, honeybee, moth, locust, and dragonflies have been done in the last two decades (Strausfeld et al., 1998; Schachtner et al., 2005; Galizia & Rössler, 2010; Hansson & Stensmyr, 2011; Martin et al., 2011; Strausfeld, 2012; Rebora et al., 2013; Rybak & Menzel, 2018).

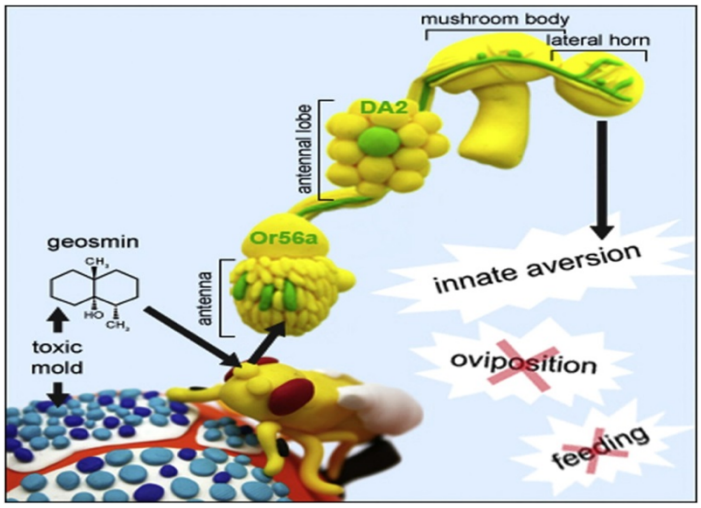

Figure 2: General circuitry of chemical sensing in the central nervous system of an insect. Volatile chemicals first bind to odorant receptors placed in the sensilla on the antenna. Stimuli then conveyed to the antennal lobe and subsequently to higher centers of the brain neuropil such as mushroom body and lateral protocerebrum for further processing. For example, the volatile toxic chemical geosmin triggers the neuron's circuit to exert avoidance behavior in *Drosophila melanogaster* (Stensmyr et al., 2012).

1.4. Sensory appendages: antennae and proboscis

Most insects possess a pair of crucial head appendage widely known as antennae which is used for sensing external environment. The antennae carry many small hairs or peg-like sensory cuticular appendages, called sensilla (singular sensillum). The sensilla host a number of sensory neurons (SNs), equipped with receptors to sense and process information about the environment (Steinbrecht, 2007) which were previously reported as complex multimodal organs (Chapman, 1998). These sensilla bulge from the cuticle of the exoskeleton of e.g., antenna, leg, labium, ovipositor, reproductive organ, or proboscis (an elongated tubular mouthpart). Thus, insects can sense touch, taste, temperature, humidity, vibration, movement of wind and CO_2 using those specialized structures. The antennae of some insects are the essential organs for their survival and reproduction (Keil, 1999).

Sensilla have a diverse structure according to their function (Figure: 5B) e.g., differentiation of host, location of food source, avoidance of predation, finding out mating partner, evidence of toxic chemical and oviposition. Different types of sensilla have been classified according to their functions (Figure: 5B). For example, olfactory, tactile, gustatory, tactile gustatory, temperature, and humidity sensilla (Chapman, 1998). The olfactory sensilla are characterized by small pores on the cuticle. These pores allow odorants to diffuse to the lymph along their shaft, also called wall-pored sensilla (Keil & Steinbrecht, 1984).

1.5. General brain organization of insects

The insect's brain is classically structured into a dorsally located protocerebrum (PR), medially situated deutocerebrum (DR), and ventrally situated tritocerebrum. The PR consists of the optic lobe (OL), central body (CB), the mushroom body (MB), protocerebral bridge (PB), and lateral accessory lobe (LAL). The DR is composed of the antennal lobe (AL), a dorsal lobe also known as the antennal mechanosensory and motor center (AMMC) (Figure: 3) (e.g., Kollmann et al., 2011). Hemipteran insects possess a central nervous system (CNS) that is tightly attached to the sub-esophageal, or gnathal ganglion (GNG). Primary antennal afferents ventrally and ipsilaterally branch in either in a glomerular or aglomerular AL, (Hanström, 1928, Pflugfelder 1937) and in other neuropils of the brain. Most of the aquatic Hemiptera have a prominent visual system with three optic neuropils named lamina (LA), medulla (ME) and lobula complex (LOX) in the PR (Pflugfelder 1937; Barrozo et al., 2009; Xie et al., 2019). In some cases, the MBs are well developed but in the case of so-called anosmic (lacking sense of smell) insects such as *Dytiscus marginalis* (diving beetle; Coleoptera) or *Notonecta undulata* (backswimmer; Hemiptera) a reduced or complete loss of calyces has been reported (Strausfeld et al., 1998). A well-defined CB with two sub lobes and a PB has been described in Hemiptera species (Pflugfelder 1937; Barrozo et al., 2009; Xie et al., 2019). The complete brain organization of terrestrial hemipteran species were described previously (Rossi Stacconi et.al., 2014, Barrozo et al., 2009; Xie et al., 2019,) which showed that the central brain is divided into the PR including OL, CB, MB, the deutocerebrum with AL, dorsal lobe, AMMC, and the tritocerebrum. The GNG or sub-esophageal ganglion is positioned at the posterior part of the head capsule followed by the prothoracic ganglion (pro-TG). A paired connective joins the posterior

ganglion (PG) in the ventral side away from the proTG has been reported in a terrestrial Hemipteran mirid bug *Apolygus lucorum* (Figure: 3) (Xie et al., 2016).

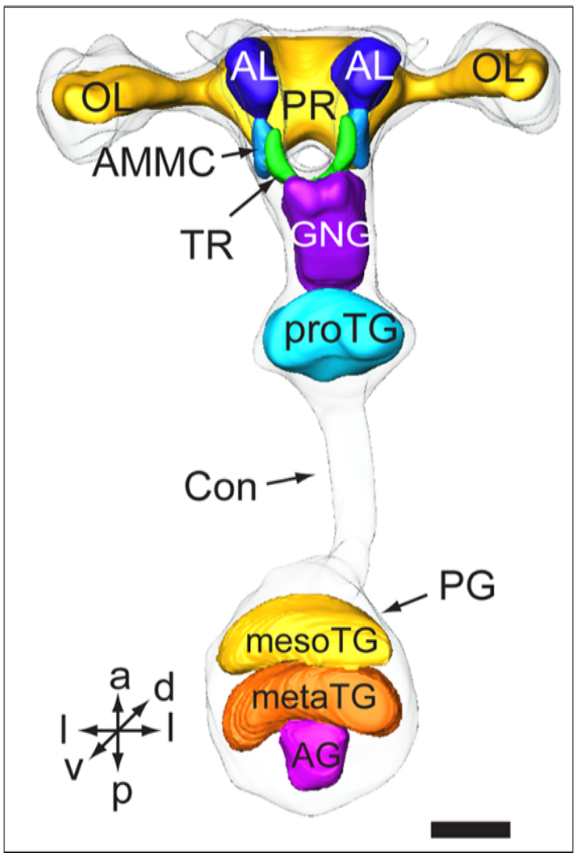

Figure 3: General brain organization of a terrestrial hemipteran insects. Ventral view of three-dimensional reconstruction model of CNS of terrestrial mirid bug *Apolygus lucorum* showing the location and relative size of all major neuropils throughout the CNS and ventral nerve cord. AG: abdominal ganglion, AL: antennal lobe, AMMC: antennal mechanosensory and motor center, Con: connective, GNG: gnathal ganglion, mesoTG: mesothoracic ganglion, metaTG: metathoracic ganglion, OL: optic lobe, PR: protocerebrum, PG: posterior ganglion, proTG: prothoracic ganglion, TR: tritocerebrum. Directions: a: anterior, d: dorsal, l: lateral, p: posterior, v: ventral. Scale bar: 100 µm. Adapted from (Xie et al., 2016).

1.6. Target areas of antennal sensory neuron in the CNS

Basically, antennal nerves form a complex meshwork in the dorsal and ventral part of the brain and divided into different axonal tracts that carry sensory neurons (SNs) of different sensing modalities (Galizia & Rössler, 2010). The relevant sensory cues e.g., odor, temperature, taste, and vibration are first detected at the periphery by SNs housed in different sensilla of the antennae. The peripheral signal is then conveyed to the central nervous system (CNS) to induce different behavioral responses (Figure: 2). Axons of the antennal sensory neurons project into the different brain regions. The projection pattern

varies depending on the different functional sensory organs. For example, the ALs generally receive the sensory innervation from olfactory sensory neurons (OSNs). Among others, in honeybees, 3-5 antennocerebral tracts connect the primary olfactory center AL with higher brain center, mushroom body, and lateral horn of protocerebrum (Hansson & Anton, 2000; Galizia & Rössler, 2010; Rebora et al., 2013). The antennal mechanosensory motor center (AMMC) receives afferents from the mechanosensory sensilla while sub-esophageal ganglion receives afferents from the gustatory sensilla. (Xie et al., 2016).

In some insects' axons from CO_2 and thermo-sensory sensilla project to specific regions of the AL as well as in the protocerebral lobe (PL) (Bräunig & Krumpholz, 2013). In American cockroaches, some glomeruli have been found to receive axons from the thermoreceptors and other sets of glomeruli are innervated by afferents from hygroreceptors (Nishikawa et al., 1995). In the case of the american grapevine leafhopper *Scaphoideus titanus* and *Hyalesthes obsoletus,* it has been demonstrated that the sexual communication is processed by sensory cells present in the pedicel that target AMMC in the brain (Mazzoni et al., 2010). AMMC mostly receives the terminal arborization from mechanosensory neurons that are mostly located on the scape and pedicel of the antennae (Homberg et al., 1989). Moreover, the AMMC has also been found to receive afferents from an auditory organ, named as Johnston's organ in fruit fly and honeybee, to process auditory information (Kamikouchi et al., 2006). A map of afferents from gustatory sensilla in the *Drosophila* CNS was published in 2014 which revealed that the taste neurons project in the thoracic-abdominal ganglia and sub-esophageal ganglion (Kwon et al., 2014).

1.7. Mushroom body

Arthropods mushroom bodies (MBs) are the prominent higher-order neuropils that entail the cross-sensory integration and memory formation which are mainly associated with olfactory pathways of arthropods (Strausfeld et al., 2009). It is composed of processes of densely packed neurons that make a prominent shape, as reflected from the name 'Mushroom body' (Rybak & Menzel, 1993; Menzel, 2014). The size, shape and complexity of MBs differ between taxa, as well as among statuses of species of communal insects (Strausfeld et al., 1998). The dendrites of Kenyon cells form a cap- or cup-like regions called calyces at the top of the posterior part of the peduncles which receive afferents from

the ALs. The mushroom body plays two vital roles, first- the memory building, and second-rely on higher brain centers information from sensory afferents (Strausfeld et al., 1998).

1.8. Antennal lobe and Glomeruli

The antennal lobes (ALs), the primary olfactory center of the brain is similar to the olfactory bulbs of vertebrates. Receptor neurons provide input from the antennae to the ALs of insects. ALs are generally subdivided into some roughly spherical profile termed as glomeruli as it is in vertebrate's olfactory bulbs (Anton & Homberg, 1999). The number of these glomeruli vary extensively from species to species (Boeckh et al., 1990), although within the same clade the number remains almost stable (Schachtner et al., 2005). The highest number of glomeruli has been found in Caelifera (Orthoptera) ranging from 1000 to 3000 (Schachtner et al., 2005) and the least number of glomeruli has been counted is approximately 13 in male *Scaphoideus titanus* a homopteran species (Rossi Stacconi et al., 2014). The usual number of AL glomeruli that occur in the same or different layer is 40-450 (Hansson & Anton, 2000; Nishikawa et al., 2008; Galizia & Rössler, 2010; Kelber et al., 2010). Additionally, reduced or aglomerular AL have been reported in anosmic (lacking sense of smell) insects (Strausfeld et al., 1998; Kristoffersen et al., 2008).

1.9. Main questions and hypothesis

N. glauca is a semi-aquatic insect with dispersal behavior during a threat of fish predation (Baines et al., 2015). Those phenomena gave rise to the idea that they might possess a strong and well-developed vision and mechanical sense to sense and explore the surroundings. A recent study indicates that, olfaction also plays a role in the escaping behavior of Notonectidae (Ferzoco et al., 2019). Interestingly several studies by Strausfeld et al. claimed that secondarily water adapted semi-aquatic bugs *Notonecta undulata* are anosmic (lacking sense of smell) since mushroom body (MB) of these insects lack a calyx which is mainly associated with olfactory pathways (Strausfeld et al., 1998, 2009). While Hanström in 1928 and Pflugfelder in 1937, reported the presence of olfactory glomeruli in semi-aquatic backswimmer, *Notonecta glauca;* Strausfeld et al. (1998) reported aglomerular antennal lobes in *Notonecta undulata*; another species of the Notonectidae. It was discussed by Strausfeld et al. (1998) that *Notonecta undulata* lost their AL glomeruli

and odor sensitivity on their antennae. In contrast, in 2019 a behavioral study was done on two co-occurring semi-aquatic backswimmer relatives to find out whether these insects use chemical cues to minimize their predation risk. That study showed that *N. undulata* reduces their activity after sensing the chemical cues to escape from their predator Belostomatidae (Ferzoco et al., 2019). This controversy raises several questions. Are the *Notonecta glauca* able to smell? Do they possess olfactory glomeruli? If their MB do not possess calyx, where in the CNS do, they process olfactory information?

The primary hypothesis of this study is "secondarily water adapted Hemiptera do not have olfactory sense because they lack olfactory brain structures such as olfactory glomeruli and mushroom body calyx which are hallmarks of the olfactory pathways". This statement was formulated by Strausfeld et al., (1998, 2009) and is supported by behavioral studies on Notonectidae (Freund & Olmstead, 2000).

1.10. Goals of the project

This study focuses on the anatomical organization of the central nervous system (CNS) of semi-aquatic backswimmer *Notonecta glauca* and their antennal sensory neurons (SNs) projection in the CNS by a combination of antennal backfilling methods and immunohistochemistry. Besides, the morphological characterization of sensory structures of the antennae and proboscis using SEM, this study will provide neuroanatomical evidence for an olfactory pathway as well as provide the data to compare between hemipteran species of different life habitats (water and land). The findings of this study will provide an anatomical base for future functional analysis of aquatic bug's olfactory neurobiology.

2. Materials and Methods

2.1. Materials

2.1.1. Insect

Adult male and female *Notonecta glauca* Linnaeus, 1758 (Hemiptera: Notonectidae) were originally collected using a landing net during June-July 2020 from a small natural lake situated beside the Max Planck Institute for Chemical Ecology Jena (50°54'39.4"N 11°34'10.1"E) Jena, Thüringen, Germany. Three male and four female specimens were successfully dissected for the antennal backfill experiments. Two male and two female insects were effectively labelled with fluorescent dye Lucifer Yellow for neuropil visualization. Immunostaining with monoclonal antibody SYNORF1 was performed on two female insects. Sex identification of insects was done based on genitalia structures. No permission was needed for the use of *Notonecta glauca* in these experiments.

2.1.2. Chemicals

Table 1: List of Chemicals

Name of Chemicals	**Suppliers**
Ethanol	Roth, Karlsruhe, Germany
Glycerol	Roth, Karlsruhe, Germany
Triton X-100	Sigma Aldrich, Steinheim, Germany
Paraformaldehyde	Roth, Karlsruhe, Germany
Tetra-methyl-rhodamine dextran with biotin	Molecular Probes, Invitrogen, Eugene
Methyl salicylate	Sigma Aldrich, Steinheim, Germany
Lucifer Yellow	Sigma Aldrich, Steinheim, Germany
Normal Goat serum	Cell Signaling Technology, Massachusetts, USA

2.1.3. Solutions

Table 2: List of used solutions

0.1 M PBS
0.1M PBS + 0.1% Triton X-100
Ringer’s solution
Fixative solution (4% PFA)
PFAT (4% PFA + 0.2% Triton)

2.1.4. Antibodies

Table 3: List of Antibodies

Antibody	Name	Suppliers
Primary	Monoclonal mouse synapsin SYNORF1	Developmental Studies Hybridoma Bank,
Secondary	Goat anti-mouse Alexa Fluor 546	Thermo Fisher Scientific, Darmstadt, Germany

2.1.5. Equipment

Table 4: List of equipment

List of the used equipment	Suppliers
Laser Scanning Confocal Microscope (LSCM)	Zeiss, Jena, Germany
Field emission scanning electron microscopy (FESEM)	Zeiss, Jena, Germany
Axio Zoom	Zeiss, Jena, Germany
Stereo Microscope	Olympus, Tokyo, Japan
Gold sputtering machine	Suppliers name not found
Vortexer	Scientific Industries, Bohemia, NY
Cover glasses	Paul Marienfeld, Lauda-Königshofen, Germany
Pipettes	Gilson, Middleton, WI
Paintbrush	Ted Pella Inc, Redding, CA
Fine forceps	Fine Science Tools, Heidelberg, Germany
Magnetic stirrer with heating	Imlab Bvba, Boutersem, Belgium
Microscope slides	Paul Marienfeld, Lauda-Königshofen, Germany
Minutien pins	Fine Science Tools, Heidelberg, Germany
Micro scissor, Razor blade, Sylgard, Ultrasound cleaner, Glass vials	Suppliers name not found

2.1.6. Software

Table 5: List of used Software

Software	Dealer
Adobe Photoshop CS	Adobe Systems
Amira 5.6.0	Thermo Fisher Scientific
Mendeley	London, UK
Fiji-Image J	Public Domain
Microsoft Office	Microsoft Corporation
Microsoft Paint	Microsoft Corporation
ZEISS ZEN 2 Imaging Software	Zeiss, Jena, Germany

2.2. Methods

2.2.1. Insect rearing

The laboratory colony of *Notonecta glauca* was kept in a glass aquarium (30cm×20cm×120cm) with water, flora, and fauna from the collecting site. The insects were fed on daphnia, and mosquito larvae under the conditions of 28±1°C, 60% relative humidity, and a 12 h:12 h illumination regime. A water bubbler was used to facilitate aeration.

2.2.2. Neuropil labelling with Lucifer Yellow

For general visualization of the brain anatomy and olfactory glomeruli, the staining experiments with Lucifer Yellow solution (LY, 4% aqueous solution; Sigma- Aldrich, Steinheim, Germany) were conducted. The head capsules were opened in 0.1 M phosphate-buffered saline (PBS) (pH 7.2) and the exposed brains were dissected and fixed in 4% paraformaldehyde (PFA) for 2 hours at 4 °C. After washing 3 × 15 minutes in PBS, the preparations were dehydrated in an ascending ethanol series (50%, 70%, 80%, 90%, 100%, 10 minutes each) and rehydrated in a descending ethanol series (90%, 80%, 70%, and 50%) to facilitate dye penetration into the dissected brains. Specimens were subsequently rinsed in PBS and incubated overnight at 4 °C in LY solution (1 µL LY/500 µL PBS). LY-treated specimens were postfixed in PFA solution (4% in PBS) for the second time, for 3 h at room temperature. All specimens were subjected to a final treatment of 3 × 20 minutes washes

in PBS, dehydrated in an ascending ethanol series (50%, 70%, 80%, 90%,100%, 10 minutes each), and cleared in methyl salicylate (Sigma-Aldrich, Steinheim, Germany). The transparent brains then transferred to the microscope slides (HistoBond, Paul Marielfeld, Germany) and then, covered with high precision coverslip (Paul Marienfeld, Lauda Königshofen, Germany).

2.2.3. Anterograde fills of antennal nerves

To examine the projection pathway of sensory neurons (SNs) from the antennal sensilla, backfill staining experiments were performed using neural tracer Micro-Ruby (Tetra-methyl-rhodamine dextran with biotin (Molecular Probes, Invitrogen, Eugene, OR). The animals were immobilized in dental wax dish using permanent adhesive with the ventral side facing up. The forelegs of insects were cut off to avoid disturbance during "pool" creation. The "pool" around the antennae was created using Vaseline with the aid of a micro syringe. Both antennae were cut obliquely at the base of the scapus to expose most of the antennal nerves. The open antennal scape was then exposed to distilled water for 30 minutes to avoid dehydration. 1 µL of fluorescent dye Miro-Ruby (Tetra-methyl-rhodamine dextran with biotin, Molecular Probes, Invitrogen, Eugene, OR) were then placed into the pool by using a pipette (Gilson, Middleton, WI) at the cut end after removing the remaining distilled water. The pool was covered with Vaseline and the animals were placed in the dark box with a moist filter paper, at 4°C overnight, for allowing the transportation of the dye through the antennal nerve into the brain. After the animals were briefly cooled on ice, the brains and the ventral nerve cords were dissected out in Ringer's saline. The dissected brains were then transferred into 4% paraformaldehyde in 0.1 M Phosphate-buffered saline (PBS, pH 7.4) to be fixed overnight at 4°C. Following a rinse in 0.1 M PBS at room temperature (3×15 minutes), the brains specimens were dehydrated with an ascending ethanol series 50%, 70%, 80%, 90%, and two times 100%, 10 min, each), cleared in methyl salicylate (Sigma-Aldrich), and mounted in a perforated aluminium slide with two glass coverslips (Paul Marienfeld, Lauda Königshofen, Germany).

2.2.4. Immunohistochemistry

To visualize the brain neuropils, anti-synapsin antibody staining was performed. The dissected brains and the ventral nerve cords were transferred into 4% paraformaldehyde in 0.1 M Phosphate-buffered saline (PBS, pH 7.4) to be fixed overnight at 4°C. Following rinse (6X 15 minutes) in 0.1 M PBS at room temperature, preincubation was performed with 5% Normal Goat Serum (NGS) (Cell Signaling Technology, Massachusetts, USA) in 0.1M PBS containing 1% Triton X-100 (PBS-Tx; 0.1 M, pH 7.4) overnight at 4°C. After blocking with NGS the brains were shortly rinsed in PBS-Tx at room temperature. The primary antibody monoclonal mouse synapsin SYNORF1 (Developmental Studies Hybridoma Bank, University of Iowa) at a concentration of 1:50 (with 2% NGS in PBS-Tx) was applied at 4°C for 2 days. Following six rinses in PBS (20 minutes, each), incubation in the secondary antibody, Cy2-conjugated goat anti-mouse (Invitrogen, Eugene, OR; dilution 1:300 with 1% NGS in PBST), was performed for 2 days at 4°C. Finally, the brains were washed for 6×20 minutes in PBS. Then the specimens were dehydrated with an ascending ethanol series 50%, 70%, 80%, 90%, and two times 100%, (10 minutes, each). After that, the brain specimens were cleaned in methyl salicylate (Sigma-Aldrich) and mounted in a perforated aluminium slide with two glass coverslips (Paul Marienfeld, Lauda Königshofen, Germany).

2.2.5. Sample preparation for SEM

The antennae and the proboscis of adult insects were dissected in 0.1 M PBS (pH 7.2) solution. Specimens were subjected to ascending ethanol series 20%, 30%, 50%, 70%, 90%, 100% (10 minutes each) for dehydration after being cleaned in the ultrasonic cleaner. The basal part of the antennae and proboscis were glued onto an electron microscopic stub. The whole antennae and proboscis were covered with gold-coated film using a gold sputtering machine.

2.2.6. Imaging

2.2.6.1. Scanning Electron microscopy

High-resolution images of different sensilla from the antennae and proboscis were obtained using Zeiss LEO-1530 field emission scanning electron microscopy under vacuum and acceleration voltage of 10 KeV at Electron Microscopic Zentrum (EMZ) Jena. Secondary electron was detected for image acquisition with magnification ranging from 25X to 40,000X.

2.2.6.2. Laser Scanning Confocal Microscope

The mounted brain preparations were visualized with a laser scanning confocal microscope, ZEISS LSCM 880 with Airyscan (Carl Zeiss, Jena, Germany) equipped with 10X, 20X, 40X water and oil immersion objectives. The pinhole aperture of the LSCM was set at one Airy unit for all the experiments. Argon 488 and He/Ne 543 lasers were used to visualize the stained samples. Excitation wavelengths of 488 nm for Lucifer Yellow (Alexa 488), 543 for rhodamine dextran, 556 for the CY2 fluorophore were used. Image stacks were performed using an optical section thickness between 0.5 to 5 µm depending on the numerical aperture and resolution of the objective.

2.2.7. Image processing/ Data analysis

AMIRA 5.6.0 (Thermo Fisher Scientific), Image J (Public Domain), ZEISS ZEN 2 Imaging Software (Zeiss, Jena, Germany), and Adobe Photoshop CS (Adobe Systems) were used for three-dimensional reconstruction and adjustment of the confocal image stacks for contrast and brightness, changes in channel hue, maximum intensity projections, and rotations. For three-dimensional reconstruction, each neuropil was labelled by using the Amira software (AMIRA 5.6.0, Thermo Fisher Scientific). The neuropil structures were labelled using the segmentation editor, including the “brush” and “interpolate” tools, and resulting label fields were rendered three-dimensionally using the “surfaceGen” tool. Maximum intensity projection views of the nerve fiber were done using the inverse option of the “Projection view” tool. The Amira tool "MaterialStatistics" was used to measure the volume of each reconstructed brain neuropil. The volume data of one male and one female

N. glaucas brain neuropils were subjected to numerical analysis. The brain-neuropil ratios were calculated by using Microsoft Excel.

The number and width of different sensilla were measured using Photoshop CS5 image processing software, and the length of each antennal segment was measured using Fiji-Image-J software. Pictures were adjusted for brightness and contrast. The directions of the neuropils were assigned according to the body axis of the animal.

2.2.8. Terminology

All the ganglia and neuropil structure of the CNS were named according to a previous study on hemipteran insects (Xie et al., 2016). For the terminology of antennae and sensilla, this study followed the nomenclature used for sensilla of aquatic hemipteran insects (Brozek, 2013; Ahmad et al., 2016; Nowińska & Brożek, 2019, 2020).

3. Results

This study explored the external morphology of antennal sensory structures (sensilla) and the internal brain morphology of *Notonecta glauca* to find out characteristic features of sensory receptors and their brain projections (Figure: 4 and 9). The antennae and the proboscis were investigated under scanning electron microscopy to characterize the sensilla types and their distribution. Several types of mechano-sensory sensilla, a putative chemosensilla type, and some contact chemosensory sensilla were identified according to their morphological features (Figure: 7 and 8). Background staining with Lucifer Yellow and immunohistochemistry along with confocal laser scanning microscopy and digital reconstruction on the dissected brains revealed major neuropils e.g., the antennal lobe (AL), antennal mechanosensory motor center (AMMC), lateral accessory lobe (LAL), central body (CB), the mushroom body (MB), optic lobe (OL), protocerebral lobe (PL), gnathal ganglion (GNG), prothoracic ganglion (proTG), posterior ganglion (PG) and their organization (Figure: 9,10, and 11). The tracing of projection pathways of antennal sensory neurons (SNs) using the antennal backfilling method with a fluorescent dye exposed that, antennal sensory neurons target the AL, the AMMC, the medial PL, the GNG, proTG and the PG (Figure: 13 and 15). The three-dimensional digital reconstruction of AL revealed the presence of distinct glomeruli in the AL (Figure: 9 and 12).

3.1. External morphology of *Notonecta glauca*

Notonecta glauca is a semi-aquatic bug and has a light brown body with a black triangle or scutellum, on the back (Figure: 4A). A pair of hind legs longer than the other two pairs is used as oars during swimming (Figure: 4A). This bug reaches a length of 13-15 mm. Adult females have larger body sizes than adult male (Svensson et al., 2000). These bugs are known as backswimmer or water boatman since they swim upside down and breathe on the water surface. They rest passively on the water surface. Two antennae are concealed in a grove behind the eyes (Figure: 4B). The length of the antenna is approximately 1 mm and it carries numerous sensilla on its surface (Figure: 4C and D). They have a long proboscis in their mouthpart (Figure: 4B). Two very large complex eyes cover almost half of the volume of the head (Figure: 4B). Notonectidae spends most of their time underwater by anchoring themselves to submerged vegetation as well as can fly to find new habitats or

if their water habitat dries. They are predatory on other aquatic insects like mosquito larvae or anything they can dominate (Zhang et al., 2012). They use their strong proboscis to suck out the body fluid of their prey as well as a defense weapon.

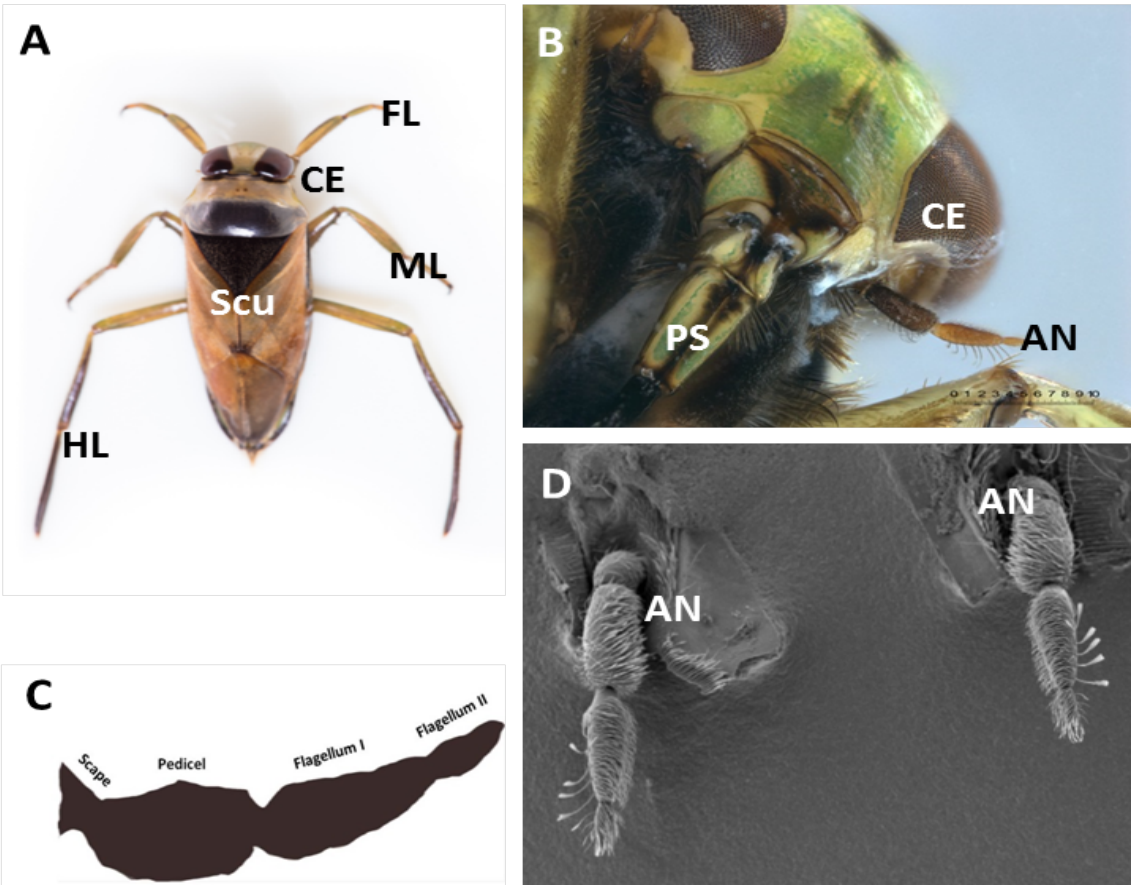

Figure 4: External morphology of *Notonecta glauca*. A. Dorsal appearance of the body of adult *Notonecta glauca* showing complex eyes and all three pairs of legs. **B.** A close view of the head of adult *N. glauca* and visible sensilla on the antenna that arises from just beneath the complex eye. Mouthparts consist of labial sensilla and a strong proboscis. **C**. Schematic diagram of antenna showing different antennal segments. **D.** Complete overview of both left and right antenna of female *N. glauca* under SEM. ML: mid leg, HL: hind leg, FL: fore leg, CE: complex eye, PS: proboscis, AN: antenna, Scu: scutellum.

3.2. Antennal structures

Antennae structure and sensilla distribution of *Notonecta glauca* has not been described so far, but the type and distribution of labial sensilla were described recently (Brozek, 2013). Besides, the details of antennal sensory structure of other aquatic Hemiptera are available (Nowinska & Brozek, 2019, 2020). In this study, antennas of *N. glauca* were examined under scanning electron microscope. Due to secondarily adaptation to water habitat, they bear very short antennae concealed in groves behind the eyes with many different type sensilla structures (Figure: 5A). The antennae consist of four segments (Figure: 4C and 6) with a very short scapus followed by the longest

antennomeres pedicel and two flagellums. The last flagellum is the shortest segment of the antennae (Figure: 4C and 5A). All the four segments bear diverse and significant amounts of sensilla which receive different signals from the environment (Figure: 7).

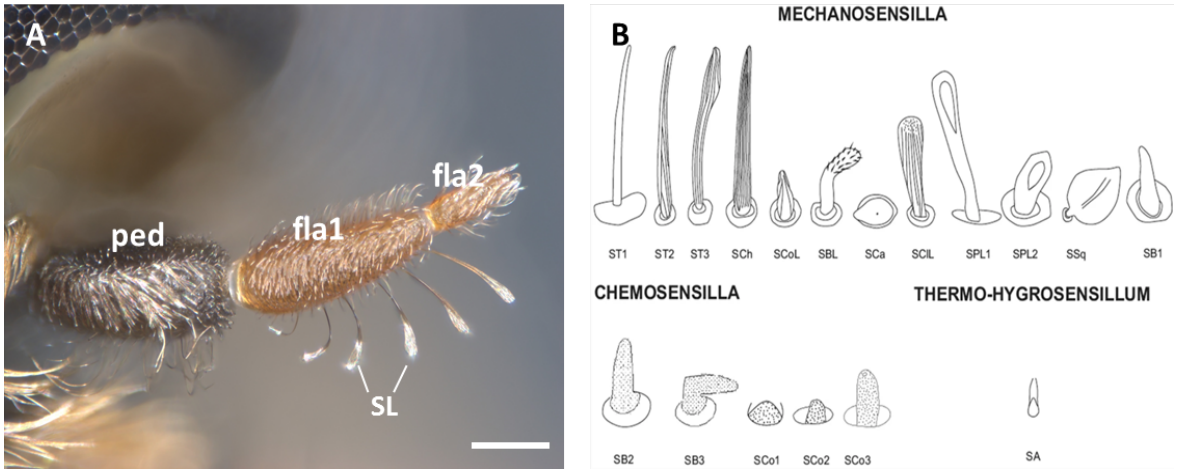

Figure 5: Antenna of *Notonecta glauca* and sensilla types in Nepomorpha. A. High magnification image of adult *N. glauca* antenna bearing different sensilla. **B.** Illustration of different groups of sensilla of aquatic bugs. ST1: sensilla trichodea subtype 1, ST2: sensilla trichodea subtype 2, ST3: sensilla trichodea subtype 3, SCh: sensilla chaetica, SCoL: sensilla cone-like, SBL: sensilla brush-like, SCa: sensilla campaniformia, SClL: sensilla club-like, SPL1: sensilla paddle-like subtype 1, SPL2: sensilla paddle-like subtype 2, SSq: sensilla squamiformia, SB1: sensilla basiconica subtype 1, SB2: sensilla basiconica subtype 2, SB3: sensilla basiconica subtype 3, SCo1: sensilla coeloconica subtype 1, SCo2: sensilla coeloconica subtype 2, SCo3: sensilla coeloconica subtype 3, SA: sensilla ampullacea, SL: sensilla leaflike, ped: pedicel fla: flagellum,. Scale bar: 200 µm. Figure B adapted from (Nowińska & Brożek, 2019)

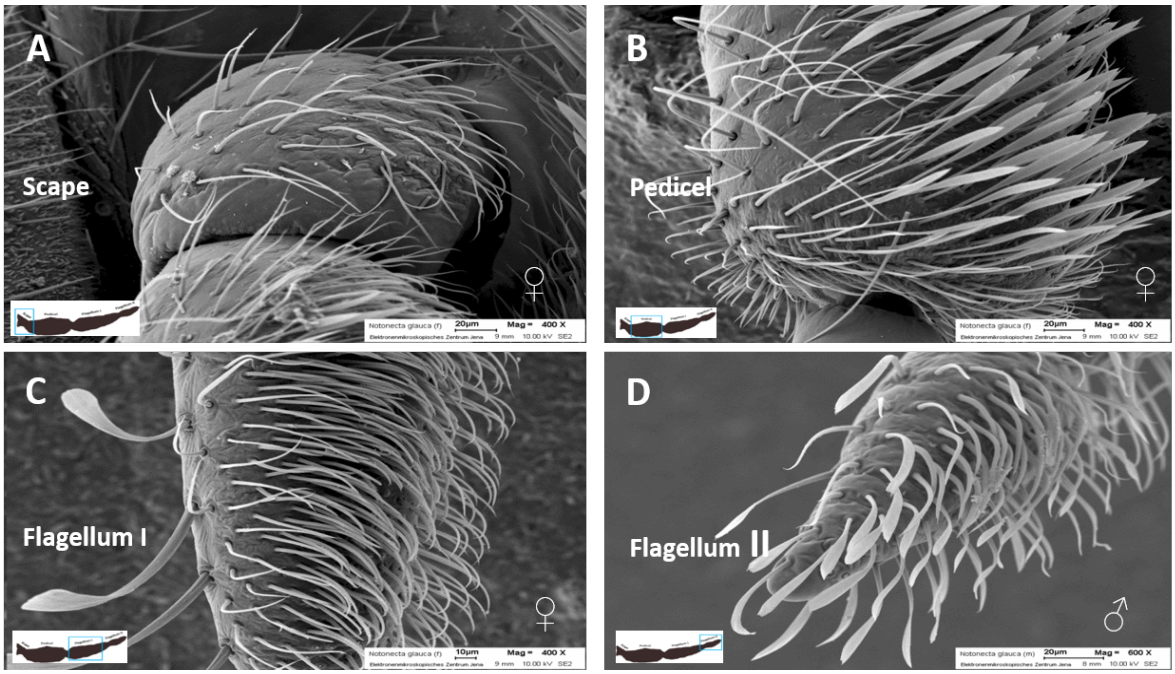

Figure 6: Scanning electron micrographs depicting four antennal segments of *Notonecta glauca.* A. Ventral view of first segment scape. **B.** A close dorsal view of the second segment pedicel. **C.** Lateral view of first flagellum. **D.** Lateral view of the last flagellum. Insets: Diagram of antennae marked in blue with respect to each antennal segment.

3.3. Sensilla types on the antennae and their distribution

In this study morphological analysis of the *N. glauca* antennae under SEM have been performed. Sensilla that arise from flexible sockets (Figure: 7) which were reported as mechanosensilla (Nowińska & Brożek, 2019) were of the greatest diversity. Sensilla trichodea long subtype 1 (ST1), sensilla paddle-like (SPL) and sensilla chaetica (SCh) were of most abundance (Figure 7A, C, and D). Sensilla with pores on the cuticle wall were absent. These types of sensilla were described chemosensory sensilla (Nowińska & Brożek, 2019). The presence of sensilla which are peg in pit grown over shallow open cavities of the cuticle bearing one pore at their tip previously named as sensilla coeloconica (SCo) were observed in this study (Figure: 7B). They have been found in groups on the third and fourth antennomere in both male and female antennal surface (Figure: 7 C and B). They arise from a flexible socket (Figure: 7B).

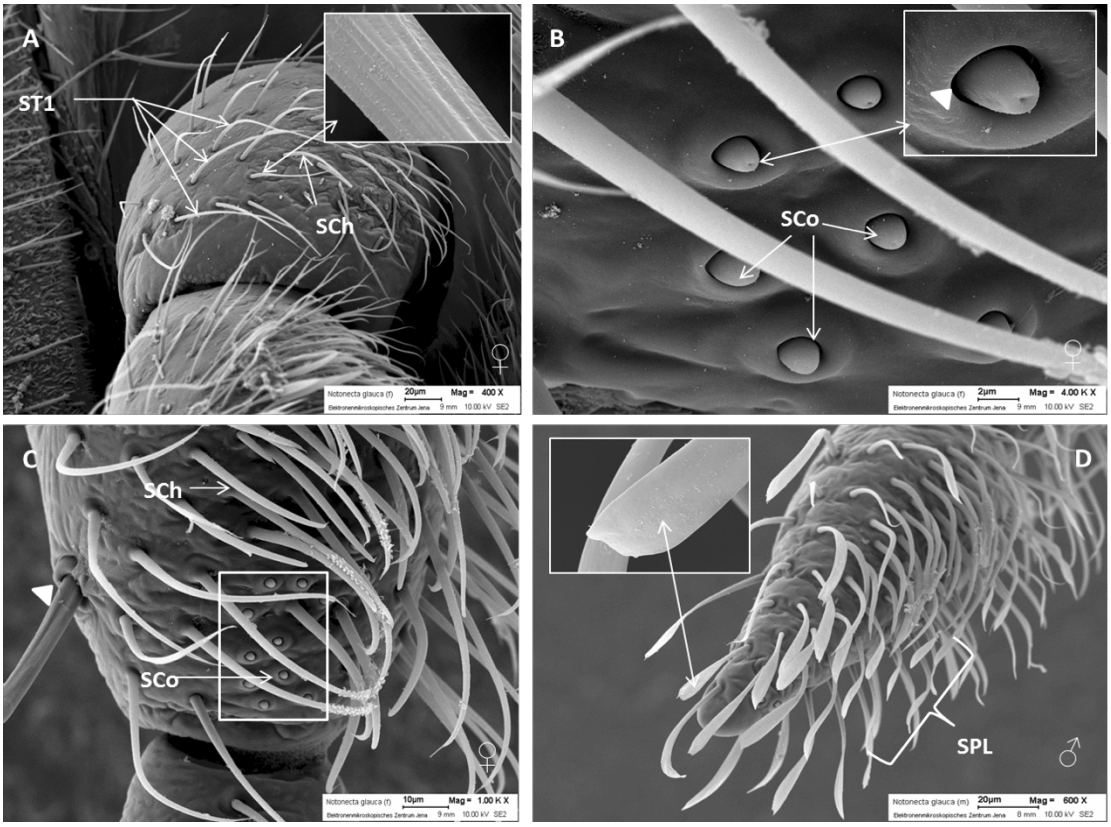

Figure 7: Sensilla types and distribution on the antennae of *Notonecta glauca*. A. Mechanoreceptive sensilla trichodea subtype 1(ST1) and sensilla chaetica (SCh). **B.** Chemo sensitive tip pore sensilla coeloconica (Sco). **C.** A group of mechanoreceptive sensilla chaetica (SCh) and putative chemosensory sensilla coeloconica (Sco). **D.** A group of mechanoreceptive paddle-like sensilla on the surface of the last segment. All insets showing the high magnification (30,000X-40,000X) image of the sensilla surface. The white triangle indicates the flexible socket of the sensilla.

3.4. Labial sensilla on the proboscis

The proboscis (an elongated tubular mouthpart) of *Notonecta glauca* was also examined under the SEM and it contains different types of sensilla (Figure: 8B, C, and D). The proboscis has four segments (Figure: 8A). The presence of several mechanosensitive sensilla chaetica (Sch) has been observed on the dorsal surface of the third and last segment (Figure: 8B, D). One pair of long trichoid sensilla (TRS) is placed dorsally close to the apex with a terminal pore which can be described as a contact chemoreceptive sensilla (Figure: 8B). Gustatory papilla sensillum (PAS) and chemo-sensitive peg in pit (PIP) sensilla have been found at the sensor field (at the tip) of the proboscis, (Figure: 8C) as previously described (Brozek, 2013).

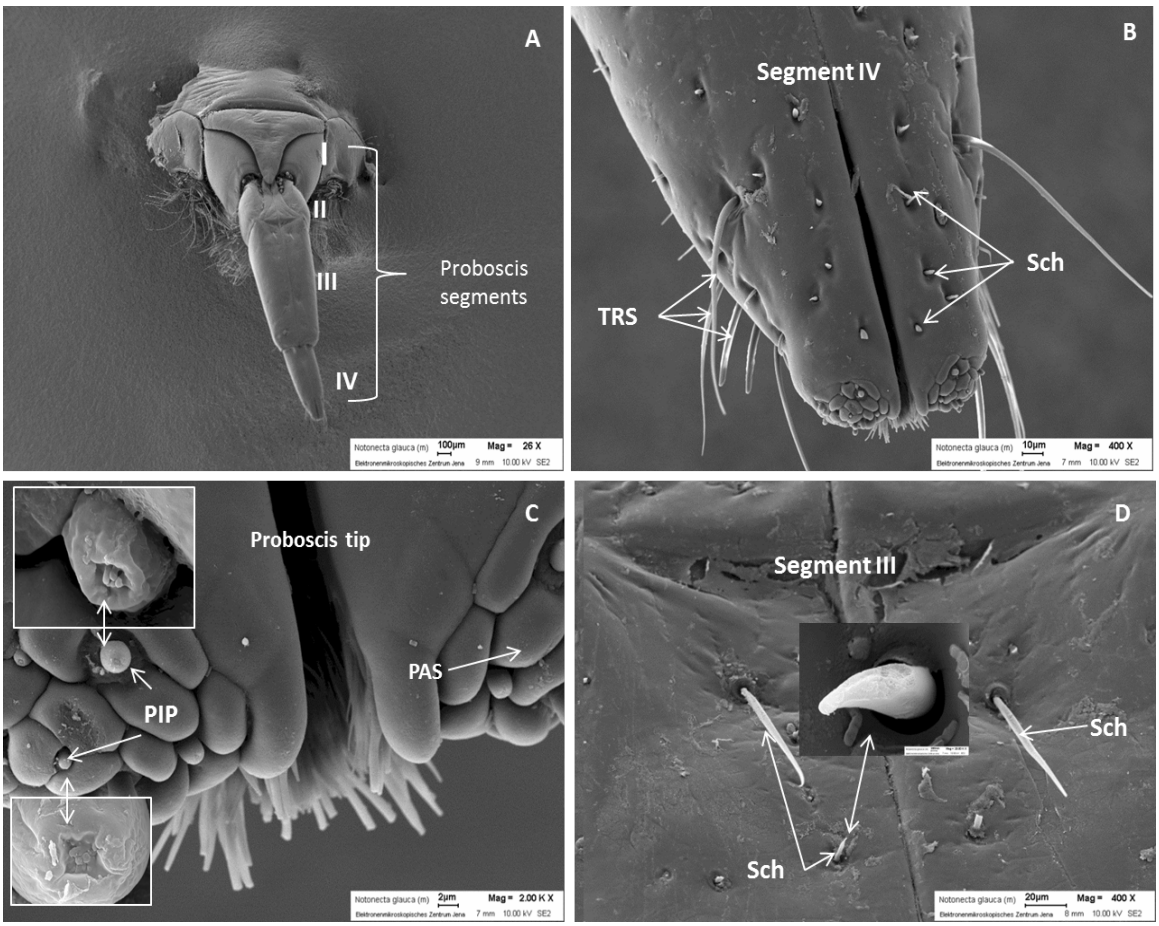

Figure 8: Sensilla types and distribution on the proboscis of *Notonecta glauca*. A. The complete view of the proboscis with all four segments visible. **B.** Contact-chemoreceptive trichoid sensilla (TRS) and mechanoreceptive chaetic sensilla (SCh) on the distal segment. **C.** Gustative papilla sensilla (PAS) and thermo- and hydro sensitive peg-in-pit sensilla (PIP) at the tip of proboscis. Insets: high magnification view of the peg-in-pit sensillum. **D.** Tactile Sensilla chaetica (SCh) on the third segment.

3.5. Summary of findings of antennal and proboscal sensilla

Different mechanoreceptive sensilla e.g., sensilla chaetica (Sch), leaf-like sensilla (SL), sensilla trichodea subtype 1 (ST1) and paddle-like (SPL) sensilla are present throughout the antenna of *Notonecta glauca* (Figure: 7). Putative chemosensilla, sensilla coeloconica (Sco) bearing a pore at the tip have been observed mainly in groups at both flagellums. Contact-chemoreceptive trichoid sensilla (TRS), mechanoreceptive chaetica sensilla (SCh), gustative papilla sensilla (PAS), and thermo- and hydro sensitive peg-in-pit sensilla (PIP) have been found in proboscis (Figure: 8).

3.6. Brain anatomy of *Notonecta glauca*

After opening the cuticle of the head capsule and removal of some pharyngeal muscle ventrally, with mouthparts facing up, the brain of *N. glauca* with the esophageal hole was exposed and was clearly visible (Figure: 9A). Three-dimensional reconstruction based on unambiguous border according to neuropil and synaptic density after background staining and synapsin staining revealed that the central brain is composed of a partly fused protocerebrum, deutocerebrum, and tritocerebrum.

The protocerebrum (PR) consists of a paired hemisphere of central brain structures and laterally attached are the large optic lobes (OLs) (Figure: 9). The dense neuropil such as the central complex (CX) embedded in the protocerebral lobe (PL) occupies the central part of the PR and is positioned above the esophagus. The CX is composed of well-defined units, upper and lower units form the central body (CB) (Figure: 9C and D). Two noduli and a protocerebral bridge (PB) are attached in the posterior of CB. A calyx deprived mushroom body (MB) with medial and vertical lobe has been identified (Figure: 10) which is similar to the previous description on the MB of another Notonectids species *Notonecta undulata* (Strausfeld et al., 1998). The OL, the visual information processing center in the brain, occupies the largest volume of the brain, consist of three main sub-compartments, named as lamina (LA) at the most periphery neuropil next to retina, an internally divided medulla (ME), and the lobula complex (LOX) (Figure: 9B, C, and D).

Ventrally to the PL lies the deutocerebrum that consists of the antennal lobe (AL), the antennal mechanosensory and motor center (AMMC) and the lateral accessory lobe (LAL) that have been identified in the proximity of each other at lateral side of the esophagus (Figure: 9C). The tritocerebrum is located ventrally of the AMMC on the opposite side of the esophagus. The gnathal ganglion (GNG) is highly fused with the tritocerebrum (Figure: 9A, B, and D) that contain some neuromeres from sensory afferents from the maxilla, the mandible, and the labium.

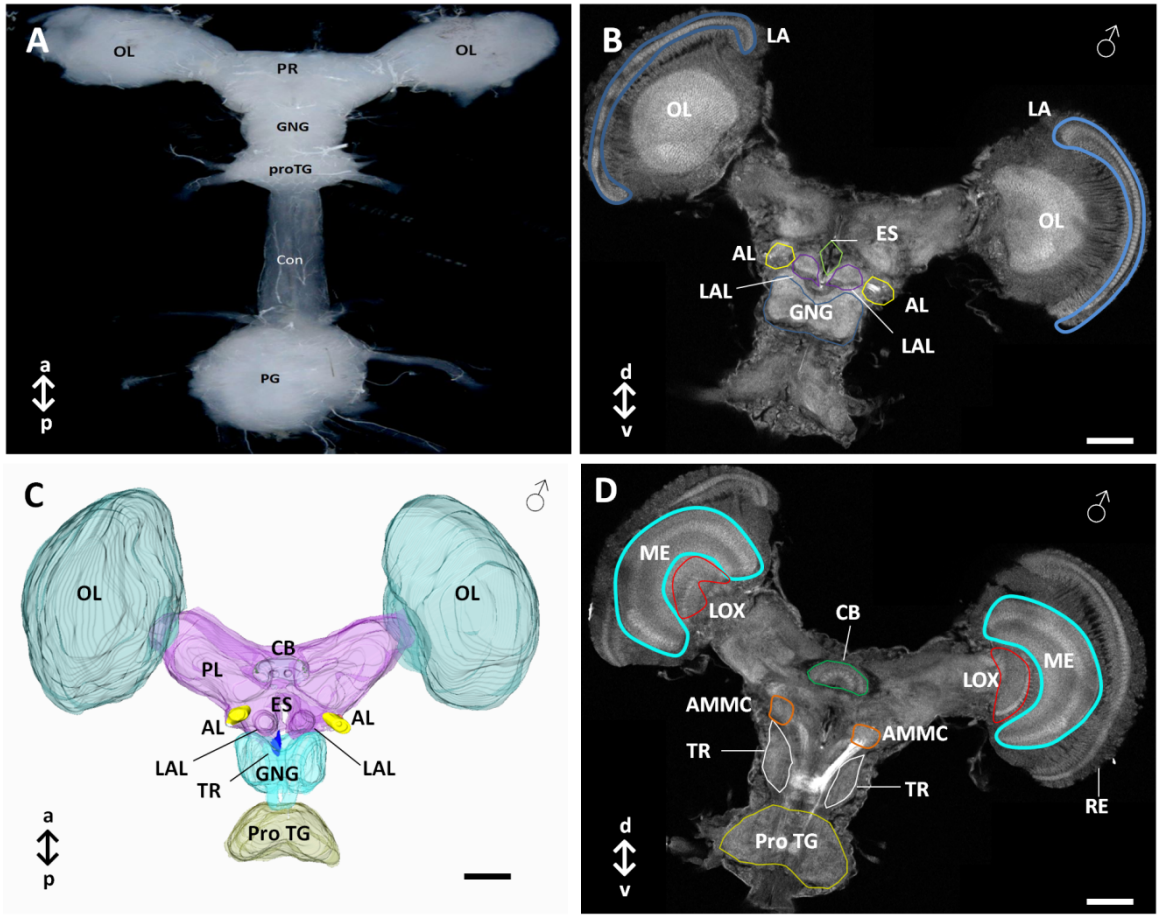

Figure 9: Brain organization of *Notonecta glauca*. A. Dissected central nervous system showing the protocerebrum region along with the optic lobes and all the ganglia. **B, D.** Confocal laser scanned image of the brain sections of different depths showing all major neuropils which were segmented based on different density and shape. **C.** Three-dimensional reconstruction model of CNS with major neuropil. AL: antennal lobe, Con: connective, GNG: gnathal ganglion, OL: optic lobe, PR: protocerebrum, PL: protocerebral lobe PG: posterior ganglion, proTG: prothoracic ganglion, TR: tritocerebrum RE: retina, CB: central body, LA: lamina, LOX: lobula complex, ME: medulla, ES: esophagus, AMMC: antennal mechanosensory and motor center. Directions: a: anterior, d: dorsal, p: posterior, v: ventral. Scale Bars: 200 µm.

Table 6: Absolute volume of prominent Neuropils. AL: antennal lobe, OL: optic lobe, MB: mushroom body, CB: central body, PL: protocerebral lobe.

Neuropils	Female (μm^3)	Male (μm^3)
Right OL	$7.08x\ 10^7$	$7.41x10^7$
Left OL	$7.08x\ 10^7$	*$7.41x\ 10^7$*
Right MB	$3.36x\ 10^5$	$2.09x\ 10^5$
Left MB	$3.18x\ 10^5$	$2.60x\ 10^5$
CB	$1.40x\ 10^6$	$9.33x\ 10^5$
Right AL	$2.89x\ 10^5$	$1.55x\ 10^5$
Left AL	$2.95x\ 10^5$	$2.20x\ 10^5$
PL	$4.25x\ 10^7$	$3.84x\ 10^7$
Central brain	$1.87x10^8$	$1.88x10^8$

Table 7: The relative size (i.e., size related to the volume of the central brain) of central brain neuropils. AL: antennal lobe, OL: optic lobe, and MB: mushroom body.

Neuropils	Female (%)	Male (%)
AL	0.31	0.20
OL	75.84	78.66
MB	0.35	0.25

3.7. Mushroom body

Fixation of female *N. glauca* brain with paraformaldehyde was done to induced background staining of major central neuropils. The fixed brain was then subjected to the confocal scanning. The central brain neuropils were unambiguously identifiable in all three dimensions. The MB is embedded in the PL with two sets of lobes that arise from the pedunculi at each side of the central body (CB) complex (Figure: 10A and B). The two lobes are assembled in medial and vertical direction. The axons of Kenyon cells form an unbranched pedunculus without any vertical lobe that extends towards the medial and vertical lobes (Figure: 10B). There is no clear evidence of calyces, but the small somata clusters are clearly seen (see arrows in Figure: 10B).

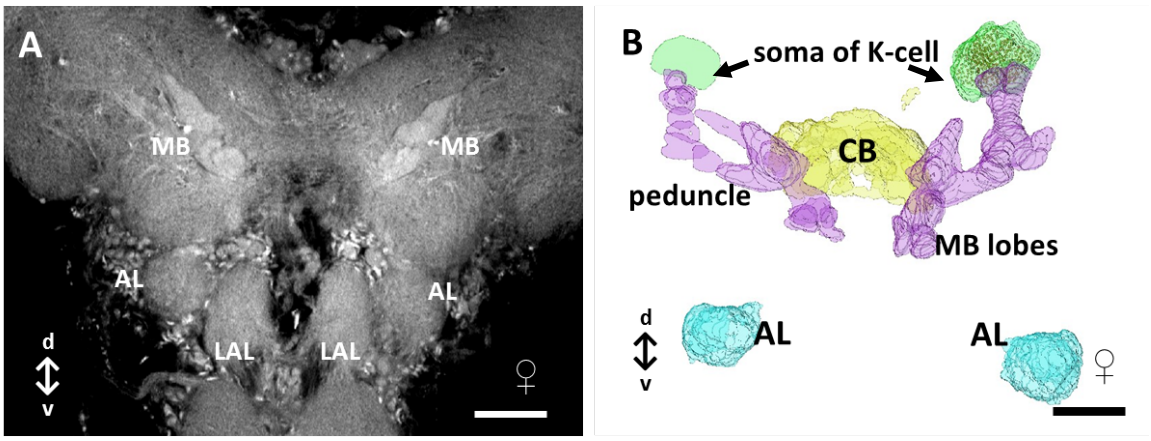

Figure 10: Mushroom body of *Notonecta glauca.* A. A ventral view of confocal laser scanned image of central brain region after paraformaldehyde fixation showing different neuropils including mushroom body (MB) lobes. **B**. Three-dimensional reconstruction of mushroom body lobes, peduncles and somata of Kenyon cells in anterior view. AL: antennal lobe, CB: central body, MB: mushroom body, LAL: lateral accessory lobe, K-cell: Kenyon cell. Directions: d: dorsal, v: ventral. Scale Bars: 100 µm.

3.8. Ventral nerve cord

In *Notonecta glauca,* the gnathal ganglion (GNG) is fused with the central brain at anterior side and with the prothoracic ganglion (proTG) at posterior side, form a complex of brain-GNG-proTG. A posterior ganglion (PG) is separated by a paired connective from the brain-GNG-proTG complex (see Figure: 3 and 9A). The proTG and the PG form the ventral nerve cord which is situated under the esophagus. The PG is a complex of three ganglionic compartments previously named the mesothoracic ganglion (meso TG), metathoracic ganglion (meta-TG), and abdominal ganglion (AG) (Figure: 11 and 3) (Xie et al., 2016).

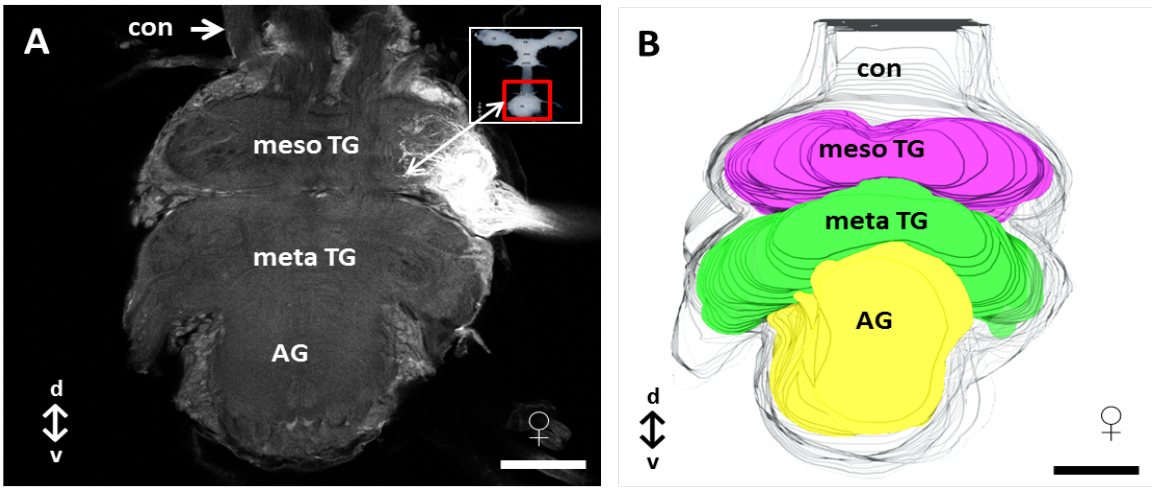

Figure 11: Posterior ganglion of *Notonecta glauca.* A. A ventral view of confocal laser scanned image of posterior ganglion showing different sub compartments. Inset shows the position of posterior ganglion with respect to the central brain. **B.** Ventral view of a three-dimensional reconstruction of posterior ganglion revealed the highly fused sub ganglions named as mesothoracic ganglion, metathoracic ganglion and abdominal ganglion. con: connective, mesoTG: mesothoracic ganglion, metaTG: metathoracic ganglion, AG: abdominal ganglion. Directions: d: dorsal, v: ventral. Scale Bars: 200 µm.

3.9. Spatial organization of Antennal lobe and glomeruli

The antennal backfill staining from the base of the scapus were performed to stain all the axons of afferent fibers housed on the whole antenna. This experiment revealed that many axonal terminal fibers clustered into the antennal lobe (AL) to form several spherical sub-compartments i.e., glomeruli, indicating that the AL is one of the major targets of antennal olfactory sensory neurons (Figure: 12A, and C). The glomeruli are relatively small spherical structures with a diameter around 8-12 µm (Figure: 12). Anti-synapsin antibody staining and backfill staining with Micro-Ruby (rhodamine dextran biotin) revealed that antennal nerves enter ipsilaterally to the AL and form at least two different sorting zones (Figure: 13). Thirty-four glomeruli at the left AL of a female and around thirty glomeruli at the right AL of a male *Notonecta glauca* were counted based on three-dimensional reconstruction according to the clear zones between glomeruli which are positioned in different layers of the AL (Figure: 12B and D). The exact number of glomeruli is yet to be disclosed because in some cases this study found the ambiguous borders of these small sub-compartments. In these cases, glomeruli seemed to be fused, or they formed a macro glomerulus, as indicated by asterisks in Figure 12 B and D. The background staining using the fluorescent dye Lucifer Yellow and the anti-synapsin staining also showed some

roughly spherical densities in AL of both sexes which were interpreted as glomeruli (arrow and yellow circles in Figure: 12E and F).

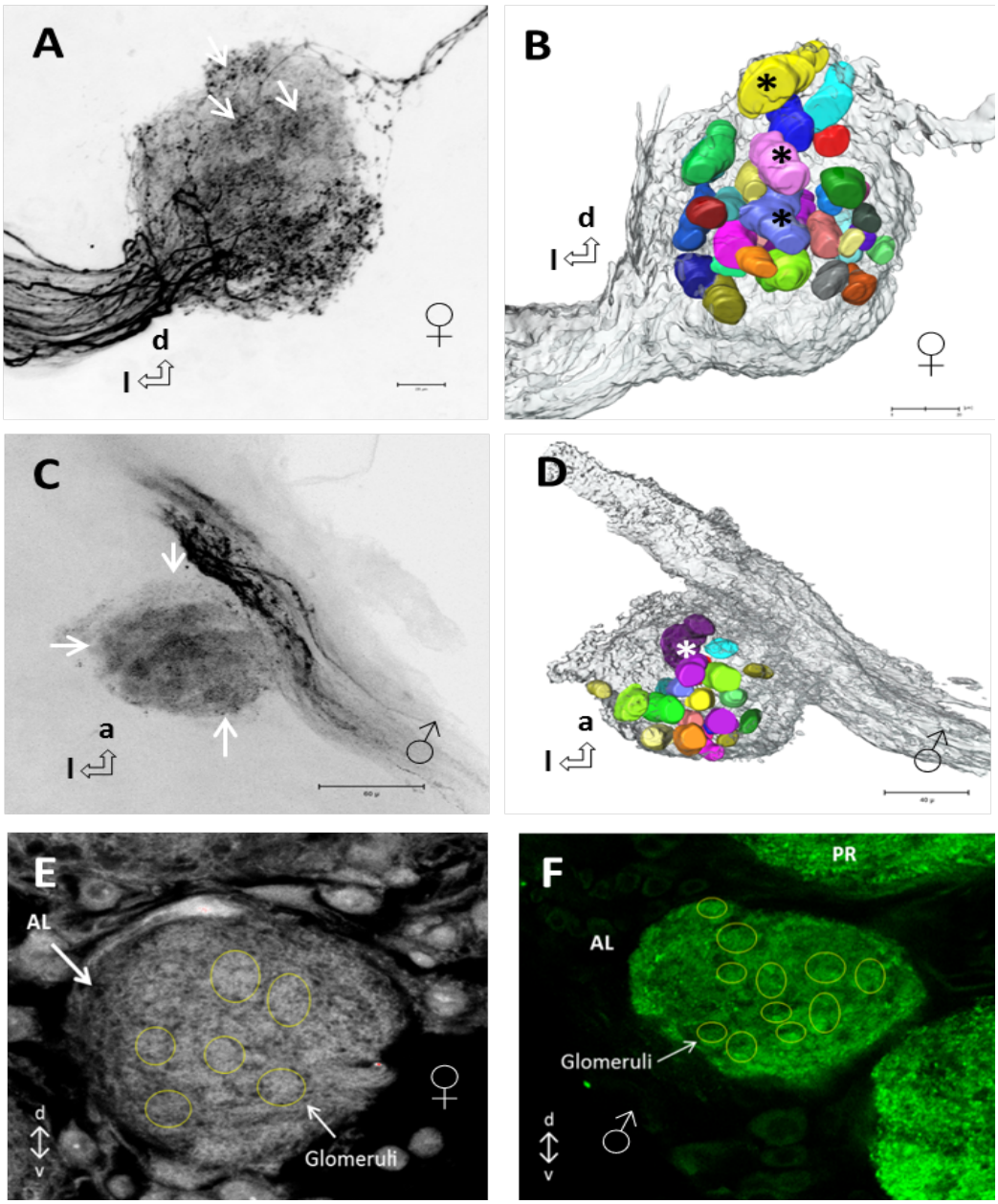

Figure 12: Confocal image and three-dimensional reconstruction of antennal lobe and glomeruli of *Notonecta glauca*. A and C. The maximum intensity view of the antennal nerve and AL after rhodamine dextran backfill showing the group of sensory axon terminals forming spherical knots in AL of female (A, B) and male (C, D) *N. glauca* respectively, (see arrows). **B and D.** The three-dimensional reconstruction of the AL (in transparency) and glomeruli of the same specimen were shown in A and C, respectively. The asterisks show putative macroglomeruli. **E and F.** Optical sections of AL after Lucifer Yellow staining (E) and anti-synapsin antibody staining (F) reveal dense spherical knots (yellow circles) which interpreted as glomeruli in both female and male *N. glauca* respectively. AL: antennal lobe, PR: protocerebrum. Directions: d: dorsal, v: ventral, l: lateral, a: anterior. Scale Bars: A and B: 20 µm, C: 60 µm, D: 40 µm.

3.10. Nerve fiber types in the antennal lobe

There are at least three nerve fiber types have been seen at the AL after backfilling the antennal nerve of male and female *N. glauca* with fluorescent dye rhodamine dextran (Figure: 13A-D). The Fiber type I is the thin axon terminals that end in glomeruli in the anterodorsal zone of the AL (arrows in Figure: 13A-D), while the Fiber type II forms a meshwork of thicker fibers in the posteroventral AL (area defined in broken line in Figure: 13A-E) and to the antennal mechanosensory and motor center (blue zone in Figure 13F). Thus, these two fiber types end in two spatially separated regions of the antennal lobe (Figure: 13F). Two distinct density zones within the antennal lobe were also identified after anti-synapsin staining (Figure: 13E). High-resolution reconstruction shows that some neurons from a third thick fiber types bypass the antennal lobe and project their axon to the medial protocerebral lobe and terminate at the region of anterior protocerebrum with bouton-like terminals (Figure: 14A and C).

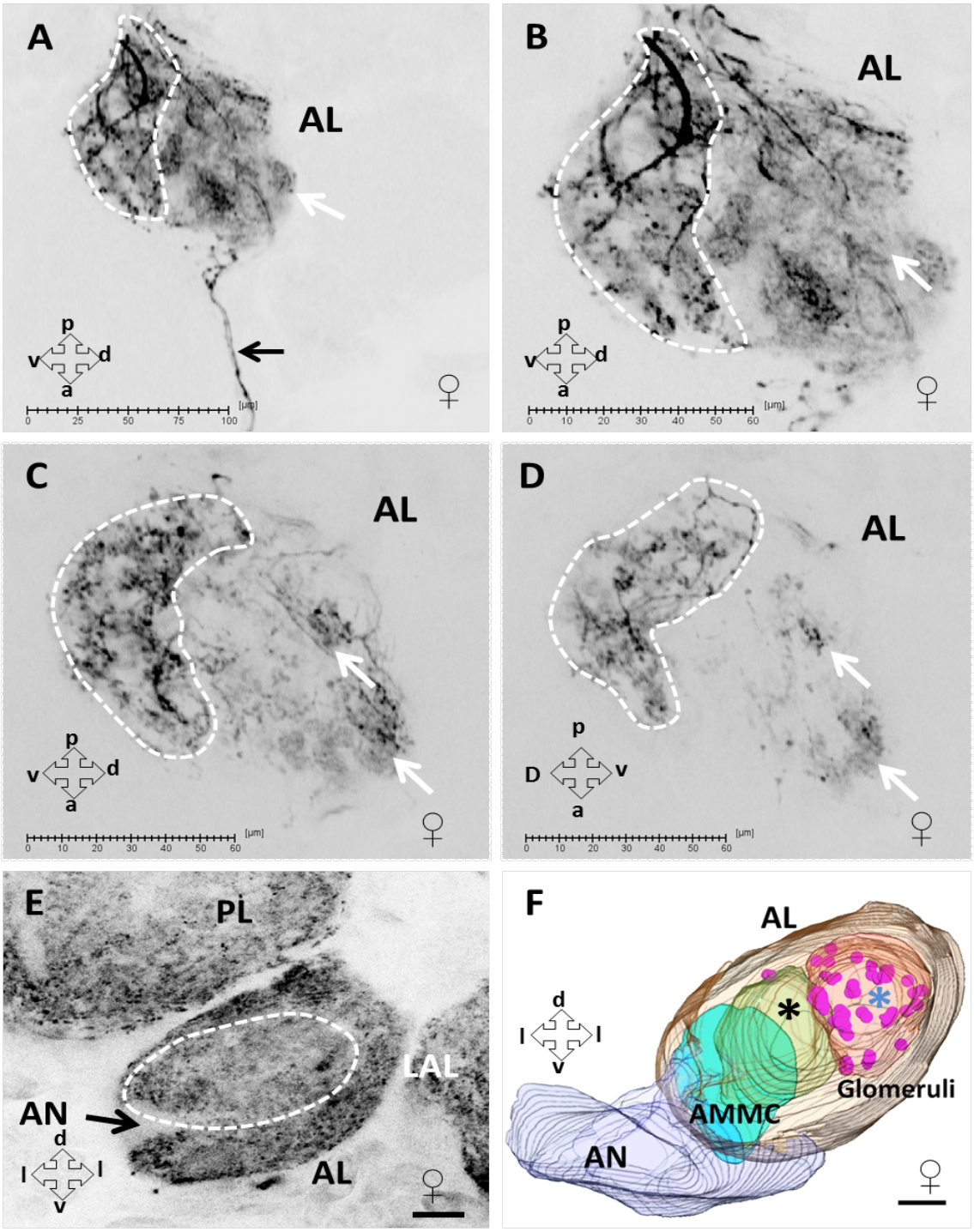

Figure 13: Nerve fiber types in the antennal lobe of female *Notonecta glauca*. A-D: The maximum intensity view of different optical sections in different depths of the antennal lobe. The backfill staining with rhodamine dextran shows two different nerve fiber types which are interpreted as the thermosensitive (marked with a broken line) and the olfactory fiber (arrow). The marked areas with broken lines show the thick fiber and the arrows show the thin fiber. **E:** Synapsin staining revealed the two zones of different density. **F:** The three-dimensional reconstruction of the antennal lobe after anti-synapsin staining shows two sorting zones within the antennal lobe. Blue and black asterisks show the thin and thick fiber zones, respectively. Pink dots represent the AL glomeruli. AN: antennal nerve, AL: antennal lobe, AMMC: antennal mechanosensory and motor center, LAL: lateral accessory lobe, PL: protocerebral lobe. Directions: l: lateral, a: anterior, p: posterior, d: dorsal, v: ventral. Scale bars in E and F: 100 µm.

3.11. Projection of antennal sensory neuron in CNS and ventral nerve cord

The successful backfill staining from the antennae shows a big bundle of axon from the antenna project to the AL where the staining is the strongest (Figure: 14A). Two or three neurons bypass the AL to project at the medial PL (Figure: 14A and C) where those neurons are finely arborized and form synaptic boutons along their terminal arborizations (Figure: 14 C). A fraction of the axon bundle supplies afferent to the AMMC (data not shown). Another bundle of the tracts projected downstream towards the gnathal ganglion (GNG), the prothoracic ganglion (TG) (Figure: 14B) and up to the posterior ganglion (PG) (data not shown). Two fiber tracts also pass the AL ipsilateral to the posterior region of the brain and project their axon towards the gnathal ganglion (blue arrows in Figure: 14C).

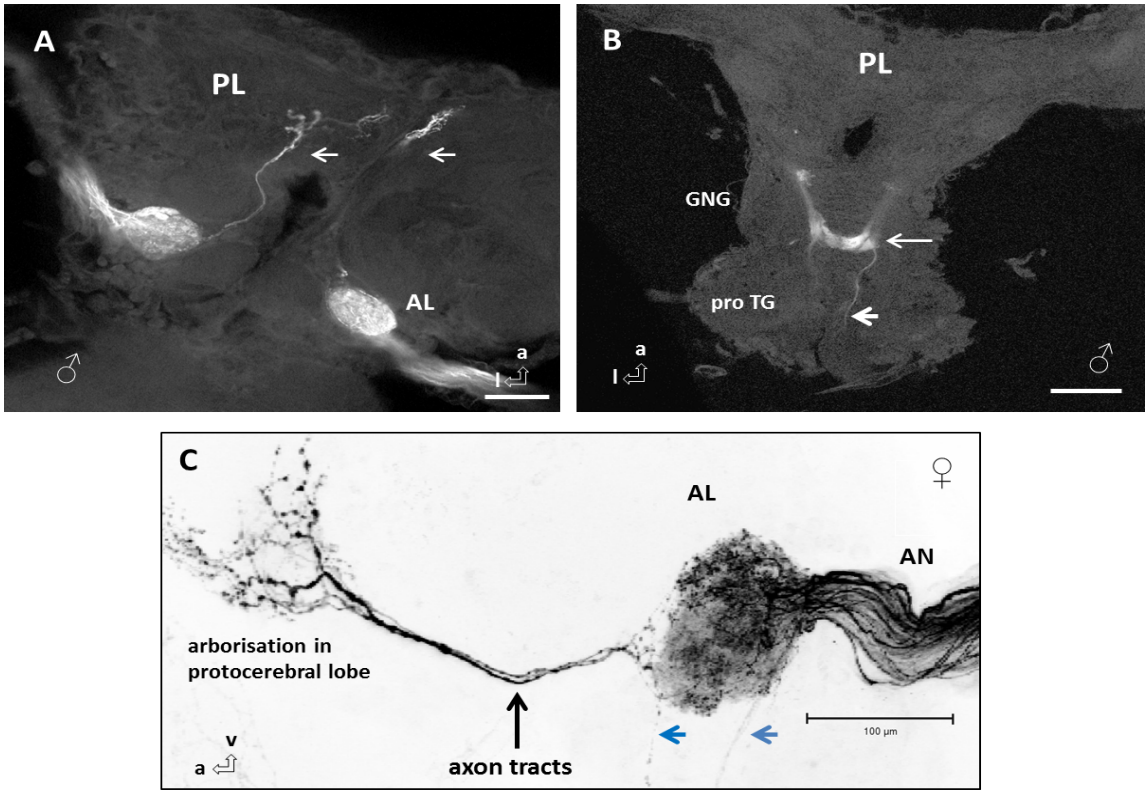

Figure 14: Target areas of the sensory afferents from the antennal nerve in the central nervous system of *Notonecta glauca*. A. An optical section of an upstream brain region after the rhodamine dextran backfill shows that the ipsilateral antennal nerves enter the antennal lobe (AL). Bundle of the axon projects to AL. From AL 2-3 neurons projects to the medial protocerebral lobe (arrows). **B.** Confocal section after the antennal backfill with the rhodamine dextran reveals the projection of the antennal sensory neurons in the gnathal ganglion (thin arrow) and then to the prothoracic ganglion (thick arrow). **C**. Maximum intensity projection view of the antennal sensory neurons in the AL where dense axon terminals are forming spherical knots and axon tracts are finely arborised in the medial protocerebral lobe. Blue arrows indicate two fibers projecting towards gnathal ganglion. AL: antennal lobe, AN: antennal nerve, PL: protocerebral lobe, GNG: gnathal ganglion, pro TG: prothoracic ganglion. Directions: a: anterior, l: lateral, v: ventral. Scale bars: A and B: 200 µm.

3.12. Summary of the target areas of the antennal nerve

A set of seven preparation of anterograde backfill with rhodamine dextran (Micro-Ruby) from the base of the antennae revealed the major target areas of the antennal nerve in the central nervous system and ventral nerve cord. The antennal afferents project the antennal lobe and the antennal mechanosensory and motor center. From the antennal lobe, a bundle of axon tracts ventures and finely arborized at the medial protocerebral lobe. A thick bundle of tracts projects in the gnathal ganglion and descends further down towards the prothoracic ganglion and up to the posterior ganglion. An unintentional backfill from the thoracic leg revealed their nerve projection in the prothoracic ganglion. No afferents have been observed into the mushroom body.

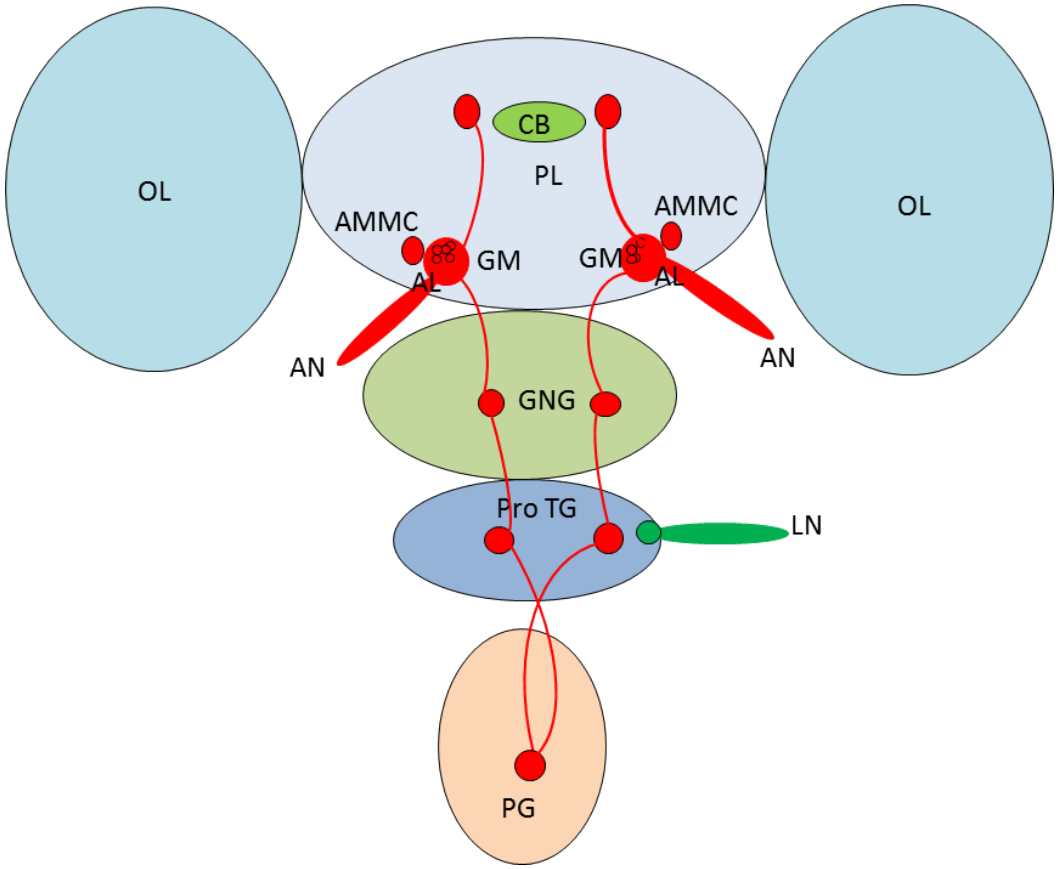

Figure 15: A schematic summary of the target areas of sensory afferents from the antennal nerve and the leg nerve of *Notonecta glauca.* The major targets are the antennal lobe, the gnathal ganglion and the prothoracic ganglion. Projections in the medial protocerebral lobe, the antennal mechanosensory and motor center, and up to the posterior ganglion have been seen in all brain specimens after the antennal backfill staining. A backfill from the thoracic leg shows that afferents from the leg nerves project to the proTG (in green) and do not ascend to the higher brain. The red and green circles show the target areas of the antennal nerve and the leg nerve in different brain regions, respectively. AL: antennal lobe, AMMC: antennal mechanosensory and motor center, GNG: gnathal ganglion, OL: optic lobe, PL: protocerebral lobe, PG: posterior ganglion, proTG: prothoracic ganglion, LN: leg nerve, AN; antennal nerve, GM: glomeruli, CB: central body

4. Discussion

The antennal sensory structures and brain organization were characterized by using SEM and neuropil labelling methods. In addition to this, the neuronal pathways in *Notonecta glauca* were described by using anterograde tracing of antennal sensory neurons. The probable role of the putative olfactory sensilla, brain neuropils organization, glomerular antennal lobe structure, and the antennal sensory fibers with their projection in different neuropils are discussed in the following sections:

4.1. Antennal and proboscal sensilla in *Notonecta glauca*

The secondarily water-adapted insects must encounter a wide variety of physical and chemical conditions which have a significant effect on their behavior and physiology e.g., oar-like use of legs, modified eyes (Figure: 4A and B), which help to see at both under and above water by using specialized photoreceptors (Immonen et al., 2014), and that differs from terrestrial insects (Crespo, 2011). Among the other physiological systems, the environment affects the sensory systems highly as insects rely on their sensing modality to exert their behavioral responses (Crespo, 2011). The aquatic insects are more challenged to use the chemical cue for hunting, avoiding predators, finding mating partners etc. in water due to the environmental conditions e.g., turbidity, reduced light transmission, and habitat complexity etc. (Bronmark & Hansson, 2000).

In this context, the antennae are the most exposed sensory organs to the external environment and specialized receptors housed in antennal sensilla perform olfactory, tactile, taste and thermo-hygro sensation in aquatic insects. The aquatic insects can perceive organic or inorganic chemicals, dissolved in water by tip-pored taste sensilla or if airborne, perceived by multi-porous wall-pored sensilla (Müller et al. 2020; Nowińska & Brożek, 2020). In aquatic animals, these distinct structural differences and their correlation to taste and olfaction in water is still unclear, but those descriptions are still used based on the insect's behavioral response and the organization and the location of the sensilla (Zacharuk, 1980; Mollo et al., 2014).

For example, a behavioral experiment on *Laccophilus maculosus,* a diving beetle, revealed that this beetle responded in air at a significantly lower fraction of a given chemical concentration compared to the concentration required to produce a similar response in water. A similar experiment on another diving beetle *Graphoderus occidentalis* that led to the conclusion that the multiporous sensilla may function as olfactory organs in low concentration of chemicals both in air and water (Jensen & Zacharuk, 1992).

During this study, a gross morphological analysis of *N. glauca* antennae was done for the first time with the help of scanning electron microscopy (SEM) (Figure: 6 and 7), although the detailed morphological study of the sensory organs of the mouthpart labium has been reported (Brozek, 2013). The sensilla structures vary depending on their functions. Also, different types of sensilla features have been described, such as socket type, surface, shape, existence and the location of pores which are the most common characteristic features (Figure: 5B) (Brozek, 2013; Nowińska & Brożek, 2019, 2020). In this study, SEM showed no evidence of wall-pored sensilla on the antennae. Pores were only found at the tip of small sensilla that are singly situated in a peg in a pit grown over the shallow open cavities on the cuticle of the antenna (Figure: 7B and C). This is typical feature for gustatory sensilla (Nowińska & Brożek, 2019, 2020). Probably, these so-called coeloconica sensilla are used for the chemosensation in water (Zacharuk, 1980). The explanation of the olfactory sense in aquatic insects is not clear because olfaction and taste in water is still a vogue (Crespo, 2011; Mollo et al., 2014; Nowińska & Brożek, 2020). However, the present data of the sensilla morphology, especially the presence of sensilla coeloconica (this study Figure: 7B) also demonstrates a previous claim of the existence of the olfactory sensilla in some species of aquatic bugs. (Nowińska & Brożek, 2019). Another study reported that the sensilla coeloconica possess olfactory receptors (Mazzoni et al., 2010). It has been proven that the chemoreceptors in the tip-pore sensilla of insects can be stimulated by the volatile odorants (discussed by Müller et al., 2020). Moreover, a similar projection target of the receptor neurons at glomerulus-like sub-compartments in the CNS of dipterans such as *Drosophila melanogaster* were innervated by the sensory neurons housed in both the olfactory and gustatory sensilla (discussed by Müller et al., 2020)

The neuropil staining of the brain of *N. glauca* revealed the presence of glomeruli in the AL (Figure: 12 and 13F), in contrast, the absences of the olfactory sensilla basiconica or trichodea with wall-pores on their antenna arise the question of whether the olfactory cues

perceived by gustatory sensilla in this insect, an assumption based on findings on spider's tip-pore sensilla that exert both olfactory and gustatory function (Foelix & Chu-wang 1973a; Foelix, 2011; Müller et al., 2020). Alternatively, the AL glomeruli may receive sensory input from the sensilla other than the olfactory sensilla.

In this study, the sensilla coeloconica (Sco) with a terminal tip pore were observed at the flagellum of the antenna (Figure: 7B and C) and it also helps to generalize the idea that these sensilla may have an olfactory function. These sensilla were previously described as chemosensory sensilla based on the electron microscopic morphological data of the larval antenna of *L. depressa* (Gaino & Rebora, 1999) and based on the chemosensory coding using the electrophysiological recording in its olfactory receptor neurons in Drosophila (Yao et al., 2005). Those sensilla were also suggested as a chemo-, hygro-, and thermosensory sensilla (Altner et al., 1981). These types of sensilla arrangement of Sco were also observed in other nepomorphan taxa (Nepidae and Belostomatidae) (Nowińska & Brożek, 2019). Moreover, within the group of aquatic bugs of Nepomorpha, some species (e.g., *Ochterus marginatus, Aphelocheirus aestivalis)* possess the olfactory sensilla basiconica. (Nowińska & Brożek, 2020). On the proboscis of *N. glauca,* the trichoid sensilla subtype1 (TRS1) with the terminal pore, which is described as a contact chemoreceptive sensilla, the gustatory papilla sensillum (PAS) and the chemo sensitive peg in pit (PIP) has been found (Figure: 8) as previously described by Brozek (Brozek, 2013).

The presence of a very few tip-pore chemosensilla (Figure: 7B and C) and no putative olfactory sensilla with wall-pore on the cuticle of the antennae, in this study, suggests that the olfaction may not be the main sensory modality in this semi-aquatic bug *N. glauca.* rather, the presence of big optic lobes (Figure: 9) and the numerous mechanoreceptive sensilla e.g., leaf like sensilla (SL) (Figure: 5A), sensilla trichodea (ST), and sensilla chaetica (Sch) on the antennae (Figure: 7) of *Notonecta glauca* indicates that they depend mainly on vision and vibrational cues in foraging (e.g., Freund & Olmstead, 2000). On the other hand, a behavioral study on the Notonectidae species showed that to avoid predation by Belostomatidae, the large predatory aquatic hemipteran, the Notonecta species use optical and chemical cues, or both (Ferzoco et al., 2019).

4.2. Brain organization of *Notonecta glauca*

The optic lobes (OL) are the center for visual information processing of the brain, composed of lamina, medulla and lobula complex (Ito et al., 2014). All these neuropils are also present in the *N. glauca*'s OL (Figure: 9). Both male and female *Notonecta glauca* have relatively large OL (75 % of central brain volume) (Table 7) compared to relatively smaller AL (0.3 % of central brain volume) (Table 7), in contrast, the relatively OL (22% of the whole brain) and larger AL (14.5% of the whole brain) have been described recently in terrestrial hemipteran mirid bug *Apolygus lucorum* (Xie et al. 2019).

The OL and the small AL in aquatic bug *N. glauca* might be due to the lifestyle of water adapted Hemiptera and its biology. The large OL indicated that, the visual sensation may play an eminent role in its life rather than olfaction. The large complex eyes, and thus the vision of the predatory bug *N. glauca* play also a major role to avoid predation and execution foraging (Freund & Olmstead, 2000). Hanström hypothesized that the Geocorisae (land-living Hemiptera) generally possess a larger AL and smaller visual center compared to the Hydrocoraisae (water living) which have relatively few glomeruli in AL, but well-defined visual centers (Hanström, 1928).

Like other insects, AMMC and AL of *N. glauca* are adjacent to the neuropils in the deutocerebrum located between the proper central brain, or PL, and the tritocerebrum (Figure: 15). The AMMC is the primary information processing center for processing the mechanical stimuli, such as vibration, gravity, wind direction and balancing of the body during flight (Kamikouchi et al., 2009). In this study, the AMMC of *N. glauca* found to receive terminal arborization from the antennal sensory neurons (SNs) (Figure: 15). This projection of antennal SNs in the AMMC (Figure: 15) might originate from the mechanosensilla that are located on the scape and pedicel of the antennae (Figure: 7) which corresponds to the arrangements in other insects such as dragonfly, jumping plant lice psyllid, and the hemipteran bugs (Homberg et al., 1989; Kristoffersen et al., 2008; Rebora et al., 2013; Xie et al., 2016).

A mushroom body (MB) with lobes and peduncles has been observed throughout this study (Figure: 10), which is similar in shape according to an earlier report for aquatic bugs

(Strausfeld et al., 2009). The relative size of MB is rather small (0.35% and 0.25% relative to the central brain volume in female and male respectively) (Table 7) in *N. glauca* compared to other terrestrial Hemiptera e.g., MB of *Apolygus lucorum* occupies 4% of their whole brain volume (Xie et al., 2019). In fruit fly *Drosophila melanogaster* and in the honeybee, *Apis mellifera* MB takes up 3 % and 21 %, respectively, of total brain volume (reviewed in Rybak, 2013).

The relatively small calyx-less MB in Notonecta indicates that the olfaction may not be the major sensory modality of this aquatic bug as calyx is one of the most important olfactory processing centers. Despite the absence of calyx in the mushroom body (Figure: 10), the presence of the microglomeruli in the antennal lobe (Figure: 12) of *N. glauca* counteract the previous claim that the secondarily water adapted Hemiptera lost their microglomeruli and calyx in their ALs and MBs respectively (Strausfeld et al., 2009).

The background neuropil staining with the Lucifer Yellow and anti-synapsin antibody staining revealed a gnathal ganglion (GNG) which seems to be a partial fusion of the supraesophageal and suboesophageal ganglion (Figure: 9). This kind of organization has been reported previously in the blood and fluid sucking insects as well as in those insects which are fed on very small particles such as mosquitoes, bugs, moths, butterflies, aphids, bees, and ants (Ignell et al., 2005; Kollmann et al., 2011; Ito et al., 2014; Bressan et al., 2015; Xie et al., 2019). On the other hand, the central brain and GNG are separated in insects that feed on solid food such as cockroaches, locusts, and beetles (Ito et al., 2014; Tang et al., 2014; Xie et al., 2016; Immonen et al., 2017).

4.3. Glomerular antennal lobe of *Notonecta glauca*

The backswimmer *Notonecta undulata* is a so-called anosmic (lacking sense of smell) specimen, another close relative of *Notonecta glauca*, lost their AL glomeruli or even the complete AL during secondary adaptation to the water habitat (reviewed in Strausfeld et al., 1998, 2009; Schachtner et al., 2005). In addition, it was also reported that, within the Heteroptera, the land-living insects possess a larger AL and smaller visual centers compared to those that are aquatic and have small numbers of glomeruli in their AL (Hanström, 1928; Xie et al., 2016). In contrast, in this study, using a diverse set of staining

techniques revealed the presence of small AL with a volume of less than 0.5% (Table 7) as well as presence of a distinct set of glomeruli (Figure: 12), as also reported in literature (Hanström 1928, Pflugfelder 1937). In a recent study, in two terrestrial Homopteran insects, *Hyalesthes obsoletus* Signoret (Homoptera: Cixiidae) and *Scaphoideus titanus* Ball (Homoptera: Cicadomorpha), reported very small antennal lobes, one with a rich set of glomeruli, the other with a reduced AL with a small volume of 0.6 % and 0.1 % respectively relative to central brain volume (Rossi Stacconi et al., 2014), in contrast, the ALs of hemipteran mirid bug *Apolygus lucorum* occupy 14.5% of the whole brain volume with around 75 glomeruli in each AL (Xie et al., 2016; 2019). In this study, around 34 and 30 glomeruli have been identified in the female and male *N. glauca* respectively (Figure: 12A-D) according to ambiguous borders, which might be due to low synaptic density or smaller number of glial cells in the AL (Kristoffersen et al., 2008). A review on primary olfactory brain centers in hexapoda and crustacea revealed that the land-living Hemiptera form glomeruli within their AL that are easily distinguishable while in the small ALs of aquatic insects the glomerular border is hardly identifiable (Schachtner et al., 2005). Glial cells have an important role in the formation of glomeruli (Oland et al., 2008). Barrozo et al. hypothesized that hemipteran insects, in general, possess an unclear AL organization (Barrozo et al., 2009). The hemipteran species differ considerably according to the number of glomeruli, while the mirid bug *Apolygus lucorum*, stink bug *Euschistus hero*s have around 60-80 glomeruli in each AL, around 25-40 glomeruli have been counted in *R. prolixus*, aphid *Acyrthosiphon pisum* (Kristoffersen et al., 2008; Barrozo et al., 2009; Kollmann et al., 2011; Xie et al., 2016) and only 13 in male leafhopper *Scaphoideus titanus* (Rossi Stacconi et al., 2014). The Palaeoptera was reported to have aglomerular antennal lobe (AL) (Rebora et al., 2013; Strausfeld et al., 1998). Although the lifestyle of the aphid psyllid (Homoptera) largely depends on olfactory cues, there was no evidence of well-defined glomeruli reported in AL of *Trioza apicalis*, *Sitobion avenae* (Kristoffersen et al., 2008). This indicates that the formation of well-developed glomeruli might not be necessary for perceiving volatile cues.

4.4. Projection pathway of antennal sensory afferents

This study provides a detailed account of macroscopic and mesoscopic brain features including the three-dimensional reconstruction of major neuropils of the central nervous system of *Notonecta glauca* as well as visualization of the antennal sensory pathways in the brain. The CNS of *N. glauca* is attached to the highly fused ganglia (GNG, proTG) (Figure: 9). This kind of organization pattern has been previously reported in other terrestrial Hemiptera, such as *Apolygus lucorum* (Xie et al., 2016) and blood-sucking bug *Rhodnius prolixus* (Barrozo et al., 2009). The sensory afferents of *N. glauca* enter the brain via the ipsilateral antennal nerve and target the multiple major neuropils including all the ganglia at the ventral nerve cord (Figure: 14) which show that the antennae of *N. glauca* might play multiple sensory functions besides olfaction. This in accordance with the finding of different types of antennal sensilla using scanning electron microscopy that includes the olfactory sensilla, mechanosensory sensilla, and contact chemosensory sensilla (Figure: 7) (Rebora et al., 2013; Xie et al. 2016). To stain all types of sensory neurons that are present in the antenna, the anterograde staining from the base of the most proximal situated scape(us) was performed. The major target area of antennal sensory neurons was found in the AL (Figure: 12, 13A-D), the AMMC, the PL (Figure: 14A and C), and the GNG (Figure: 14B and C), proTG (Figure: 14B) and (PG)(data not shown) which are almost similar to land-living hemiptera *Apolygus lucorum* (Xie et al., 2016) or *Hyalesthes obsoletus* Signoret (Homoptera: Cixiidae) and *Scaphoideus titanus* Ball (Homoptera: Cicadomorpha) (Rossi Stacconi et al., 2014) . The strong staining of the AL glomeruli (Figure: 12) may be originated from the tip-pore sensilla coeloconica present on the flagella of the antenna (Figure: 7) since there was no evidence of a presence of multiporous olfactory sensilla “sensilla basiconica”. This assumption is based on another earlier hypothesis where some researcher assumed that trichoid tip-pore sensilla of spiders used for both gustation and olfaction (Foelix & Chu-wang 1973a; Foelix, 2011; Müller et al., 2020) due to lack of multiporous sensilla. In addition to this, the study of chemosensory coding using electrophysiological recording in olfactory receptor neurons of sensilla coeloconica of fruit fly (Yao et al., 2005) supports the assumption about receiving afferents from the tip-pore coeloconica sensilla by the AL glomeruli.

It might be that the contact chemosensory sensilla chaetica on the antennae (Figure: 7) help to explain the projection in the GNG (Figure: 14B and C) because the sensilla chaetica of the moth *Heliothis virescens* and *Spodoptera littoralis* have been reported to project into GNG (Jørgensen et al., 2006; Popescu et al., 2013). The evidence of few neurons' projections in proTG (Figure: 14B) and PG have been observed in *N. glauca,* and this serves as a demonstration of such a pattern which is found in *Apolygus lucorum* (Xie et al., 2016) and *Rhodnius prolixus* (Barrozo et al., 2009). Their axons provide the arborization in the leg motor center in the thoracic ganglia. Such projection patterns indicate that the antennae play an important role in the insect's locomotion.

A few antenna-protocerebral fibers originate in the AL and leave the deutocerebrum to project into the higher center of the brain e.g., in the medial PL (Figure: 14A and C) bypassing the CB and MB on the dorsal side and terminating in the dorsal PL (Figure: 14A and C). In the dragonfly, honeybee, fruit fly, hemipteran mirid bug and *L. y-signata,* a similar projection pattern has been observed (Kamikouchi et al., 2006; Ai et al., 2007; Mißbach et al., 2011; Rybak, 2012; Rebora et al., 2013; Xie et al., 2016). The role of these projections is still unclear but recently discussed their behavioral thermoregulatory function in grasshopper locusts (Bräunig & Krumpholz, 2013).

Besides, the medial protocerebral lobe receives afferents from Johnston's organ present in the pedicel of the antenna (Kamikouchi et al., 2006) or from mechanosensory campaniform sensilla located on the pedicel (Kamikouchi et al., 2006; Ai et al., 2007; Rebora et al., 2013; Xie et al., 2016). Similar cases were reported in two other hemipteran species *Oncopeltus fasciatus* (Lygaeidae) and *Largus cinctus* (Largidae). These fibers feed-forward trans-synaptically filled PNs that send the processed information to higher brain centers (Dacks et al., 2006; Kelber et al., 2006). This is evidence that the protocerebrum may also serve in mechanosensory processing.

4.5. The calyx-less mushroom body

The neuropil labelling with the Lucifer Yellow incubation and immunostaining with anti-synapsin antibody revealed a calyx-less MB (Figure: 10). While Strausfeld et al. reported that these calyx-less MBs are mainly found in the aquatic species that lack the AL (Strausfeld et al., 2009); in this current study, the presence of the AL, as well as the glomeruli are found in the brain of aquatic bug *Notonecta glauca* (Figure: 9, 12, and 14A). Neither the MB lobes nor peduncles received any direct input from AL in this study that is also previously reported in aquatic backswimmer *Notonecta* (Strausfeld et al., 2009). A similar calyx-less MB does not receive any input from the antenna in Odonata *Libellula depressa* (Rebora et al., 2013). Despite the absence of well-developed, but nonetheless existing glomeruli (Rebora et al., 2013), the electrophysiological recordings revealed that these dragonflies can perceive the olfactory stimuli (Rebora et al., 2012).

Generally, the afferents from the protocerebral neuropils supply the calyx-less MB lobes. The cockroaches, for example, have visual, tactile, and acoustic modalities borne by the afferents terminating in their MBs medial lobes (Strausfeld et al. 1998). The role in olfaction of this form of a calyx-less mushroom body is highly unlikely (Strausfeld et al. 2009). In contrast, the presences of the olfactory glomeruli in the jumping bristletails Machilidae (Archaeognatha) were reported even though they do not possess MBs in their brain. Moreover, this study showed the presence of glomeruli in the AL (Figure: 12). Thus, it partly falsifies this study's the primary hypothesis where it was assumed that these secondarily water adapted Hemiptera are anosmic (lacking sense of smell) and they do not possess olfactory brain structures, e.g., AL glomeruli and MB calyx. This is also implied by the behavioral study on Notonectidae species which showed that in order to avoid predation by Belostomatidae they use chemical cues (Ferzoco et al., 2019).

4.6. Fiber types in the antenna lobe of *Notonecta glauca*

The AL is divided into at least two functional subdivisions based on different types of fiber (thin and thick fiber) innervation by the antennal afferents (Figure: 13). This type of compartmentalization of the AL is known in other insects, e.g., Orthoptera (Ignell et al., 2001), and Hymenoptera (Nishino et al., 2009) where one compartment bears the olfactory sensory neurons (OSNs) that terminated in glomeruli, and another subdivision with thick fiber might carry thermo- and hygro sensory neurons. Single-sensillum staining of the thermosensory sensilla of cockroach *Periplaneta americana* revealed that most of the stained neurons terminated in the AL (Nishikawa et al. 1995). In this study, some fibers, indeed a third fiber type, i.e., the putative thermosensitive fibers that bypass the AL and projects to the medial PL (Figure: 12A and C). Those afferents in the medial PL may receive thermo sensory input, because such projections in the "superficial ventral inferior protocerebrum" (SVIP) were discussed to possess a thermoregulatory function in locust (Bräunig & Krumpholz, 2013).

5. Outlook

To illuminate the present ambivalence about the state of the olfactory sense in aquatic insects, such as *Notonecta glauca*, this study suggests further behavioral experiments especially in a prey-predator context which may provide conclusive evidence whether they (secondarily water adapted insects) smell or are indeed anosmic as mentioned in previous studies (Strausfeld et al., 2009). Because there is no testimony of wall-pored olfactory sensilla on the studied antennae of *N. glauca*, anterograde staining from a single sensillum of interest, particularly sensilla coeloconica, could be done to identify their putative target areas in the antennal lobe glomeruli. In addition, single sensillum recordings (SSR) in combination with odor stimulation can be used to define the electrophysiological responses to a particular odorants stimulus to determine the sensillum's possible role. Last but not least, the transmission electron microscopic anatomical analysis of the sensillum of interest can also be carried out to find out ultrastructural features (e.g., typical for chemosensory receptors) which are dedicated to olfaction, as similar analysis carried out earlier (Gainett et al., 2017; Müller et al. 2020).

6. Conclusion

Taken together, despite the absence of multiporous olfactory sensilla on the antennae, backfilling staining from the antennae of *N. glauca* revealed an antennal olfactory sensory pathway and their projection pattern in the central nervous system. The antennal lobe has been found as one of the major targets of putative olfactory sensory neurons in an AL glomerular sub zone. Such a projection pattern shows that the antenna of semi-aquatic insects *N. glauca* plays an important role as a multimodal sensory organ including sensing chemicals. The presence of the calyx-less mushroom body and glomerular antennal lobe urges for further investigation to counteract previous claims that suggest that aquatic Hemiptera are anosmic.

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Declaration of Authorship

I, Mohammad Belal Talukder, 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 the 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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