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## Liquid uptake in eusocial Vespidae (Hymenoptera)

The morphology and function of the mouthparts and the preoral cavity


verfasst von / submitted by

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## Abstract

The mouthparts of Vespidae evolved to efficiently forage various solid and liquid foods, such as animal proteins, carbohydrate rich fluids and water as well as woody fibres for nest construction. Before liquids are swallowed and transported into the crop, particles are filtered out. This study examines the morphology and function of the mouthparts and alimentary tract, focussing on this filtration process. The feeding organs and preoral cavity are studied using µCT, 3D reconstructions of semithin sections, SEM and feeding experiments with glass beads in workers of *Vespula germanica*. To test for filtration, barium sulfate has been used as a fluid contrast agent, a method rarely used in the entomology. The results indicate that particles bigger than 212 µm are filtered by the mouthparts. The cibarium bears interlinked microtrichia arranged in rows oriented towards the infrabuccal pocket. Together with the cuticle structures of the epipharynx and the hypopharynx, this enables an additional filtration of particles bigger than 152 µm. This could be the maximum size of particles, which are able to safely pass the narrow wasp waist. For the first time a detailed illustration of the multi-layered filtration system formed by the mouthparts and the preoral cavity and its function is presented.

**Keywords**

mouthparts, preoral cavity, liquid uptake, filtration, Vespidae, Hymenoptera, µCT, SEM

## Zusammenfassung

Die Mundwerkzeuge von Vespidae sind angepasst, um effektiv sowohl feste und flüssige Nahrungssressourcen, wie tierische Proteine, kohlehydratreiche Flüssigkeiten und Wasser, als auch Pflanzenfasern für den Nestbau zu sammeln. Bevor Flüssigkeiten geschluckt und in den Kropf transportiert werden, werden Partikel herausgefiltert. Diese Studie untersucht die Morphologie und Funktion der Mundwerkzeuge und des Verdauungstraktes in Hinblick auf diesen Filtrationsprozess. Die Mundwerkzeuge und die preorale Mundhöhle wurden mithilfe von µCT, 3D Rekonstruktion von Semidünnschnitten, Rasterelektronenmikroskopie und Fütterungsexperimenten mit *Vespula germanica* unter der Zuhilfenahme von Glaskugeln untersucht. Um auf Filtration zu testen, wurde Bariumsulfat als flüssiges Kontrastmittel eingesetzt, eine Methode, die in der Entomologie sehr selten zum Einsatz kommt. Die Resultate zeigen, dass Partikeln, die eine Größe von 212 µm übersteigen von den Mundwerkzeugen filtriert werden. Das Cibarium ist ausgekleidet mit verschränkten und in Reihen angeordneten Mikrotrichien, die in die Richtung der Infrabukkaltasche ausgerichtet sind. Zusammen mit den kutikulären Strukturen des Epi- und Hypopharynx, erlauben diese eine weitere Filtration von Partikel größer als 152 µm. Das könnte die maximale Partikelgröße sein, die sicher die enge Wespentaille passieren kann. Zum ersten Mal wird eine detaillierte Illustration des mehrphasigen Filtrationssystems präsentiert, welches durch die Mundwerkzeuge und die Preoralhöhle gebildet wird.

**Schlüsselwörter**

Mundwerkzeuge, Preoralhöhle, Flüssigkeitsaufnahme, Filtration, Vespidae, Hymenoptera, µCT, REM

## Introduction

Fluid feeding as the primary food source evolved several times independently in many flower visiting insects (Krenn et al., 2005). For the fluid uptake the mouthparts are usually formed as a proboscis, specialised for the extraction of concealed nectar (Krenn et al., 2005). Hymenoptera evolved a labio-maxillary complex, which functions either as a proboscis or licking organ and retained mandibles used for biting tasks (Krenn et al., 2005; Mauss et al., 2019; Vilhelmsen, 1996). Social Vespidae, comprising of Vespinae and Polistine, show diverse foraging activities (e.g. collection of woody fibres, carbohydrate rich fluids, water, hunting and scavenging of animal proteins) (Edwards, 1980). These foraging activities are primarily fulfilled with their mouthparts, which need to be both versatile but also efficient for liquid uptake (Edwards, 1980; Matsuura & Yamane, 1990; Weyrauch, 1939). With a closer look at the morphology related with this liquid food uptake by adult Vespinae and Polistinae species, I want to investigate how they are able to effectively take up fluids, even when challenged with particles suspended in the liquid.

### Foraging

Social Vespidae collect mainly four different types of resources: animal proteins, woody fibres, water and carbohydrate rich fluids (Duncan, 1939; Edwards, 1980; Richter, 2000; Spradbery, 1973).

Sugary fluids e.g. tree sap, honeydew, nectar and ripe fruits are important ressources for adult Vespidae for their daily acitivity, needed for the development of a colony and are collected from the sources in species or genus dependent quantities (Matsuura & Yamane, 1990; Richter, 2000). Similar to water, the carbohydrate rich fluids are stored in the crop and are later shared amongst other adults and larvae by trophalaxis (Edwards, 1980). As fluids from various resources are taken up, impurities and particles may occur which Vespidae have to be able to deal with.

Water is simillary collected as it is needed for nest building and cooling as well as providing hydration for larvae (Edwards, 1980; Richter, 2000). It is collected at various water sites, swallowed and stored in the crop for transportation (Richter, 2000).

Woody fibres are the main source of the nesting material of Vespinae and Polistinae which are collected from trees and other wooden structures in species dependent stages of weathering (Edwards, 1980; Weyrauch, 1939). To retrieve the fibres the chosen spot is initially impregnated with saliva, then the paper fibres are cut using the mandibles in a backward motion, aligned with the grain (Edwards, 1980; Spradbery, 1973; Weyrauch,

1939). The cut fibres are rolled to a clot, transported to the nest were they are masticated both by the use of their prolegs and palpi as well as wetted using saliva to form a paper like substance (Edwards, 1980; Spradbery, 1973; Weyrauch, 1939). This substance is then applied in stripes or a pillar and moulded in a second step using the mandibles. (Mauss et al., 2019; Spradbery, 1973)

Proteins are actively collected mainly for their larvae in form of insects and dead carcasses (Richter, 2000). Although Vespinae and Polistinae are opportunistic generalists (Richter, 2000), a specific range of prey items is usually hunted which differs both between species and individuals of the same nest (Kasper et al., 2004; Matsuura & Yamane, 1990; Richter, 2000). The prey is attacked, overwhelmed and killed with the use of the mandibles (Edwards, 1980; Richter, 2000). Afterwards the thorax is usually cut out, malaxated and carried to the nest (Matsuura & Yamane, 1990; Richter, 2000). The time point of malaxation seems to be varying and may be influenced by the prey and the possibility of transport of the prey to the nest (Edwards, 1980; Greene, 1991; Matsuura & Yamane, 1990; Richter, 2000). In the nest the meat is additionaly malaxated to a species specific piece size before the meat-bolus is positioned on the mouthparts of the larvae, in a size appropriate for the larvae (Edwards, 1980; Greene, 1991; Spradbery, 1973). Liquids which are accessible during malaxation are taken up by the adults and are a protein source for adults (Edwards, 1980; Greene, 1991). The other main protein source for adults are droplets of salivary fluid, received from the larvae via a trophallaxis, in this case fluid is exchanged between wasp and kin (Edwards, 1980; Greene, 1991; Mauss et al., 2019; Spradbery, 1973).

## Mouthparts

The anatomy and morphology of wasp mouthparts are well studied (Duncan, 1939; Kirmayer, 1909). Vespidae and Polisitinae have stout and heavily sclerotized mandibles with a four-toothed cutting edge (Duncan, 1939; Mauss et al., 2019; Silveira & Dos Santos Junior, 2011). The mandible is primarily used for cutting and transport tasks, and a sexual dimorphism has been recognised in the musculature of the mandibles, as only femals are foraging (Baraneket al., 2018; Spradbery, 1973).

The labio-maxillary complex, an autapomorphy of Hymenoptera, consists of the maxilla and labium, which are hinged together with the labio-maxillary jugum, a sclerite of the submentum. (Duncan, 1939; Seifert, 1995; Vilhelmsen, 1996). The maxillae consist of a slender cardo, which broadens distally where the flat, triangular stipes connects (Baranek et al., 2018; Duncan, 1939; Mauss et al., 2019). The six-segmented maxillary palpus attaches distally on the stipes as do the small lacinia and the lateral galea, which are frontal to the

palpus (Duncan, 1939). Both the lacinia and galea are less sclerotized than the cardo and stipes but bear numerous long setae and bristle shaped sensilla (Baranek et al., 2018; Duncan, 1939).

The labium can be divided into the submentum, mentum and prementum which form the posterior base of the labium (Duncan, 1939). The ventral component of the labium, the ligula, is composed of the unpaired lobular shaped glossa and paired paraglossae, which are laterally positioned to the glossa (Duncan, 1939; Kirmayer, 1909). Both, glossa and paraglossa are equipped with specialised hook shaped microtrichia, the spatulae, which are important for liquid transport (Baranek et al., 2018; Duncan, 1939). Additional four acrosomal buttons can be found on posterior side of the tips, two on the glossa and one each paraglossa, equipped with sensilla basiconica, possibly with chemosensory function (Baranek et al., 2018; Duncan, 1939; Seifert, 1995; Zacharuk, 1985).

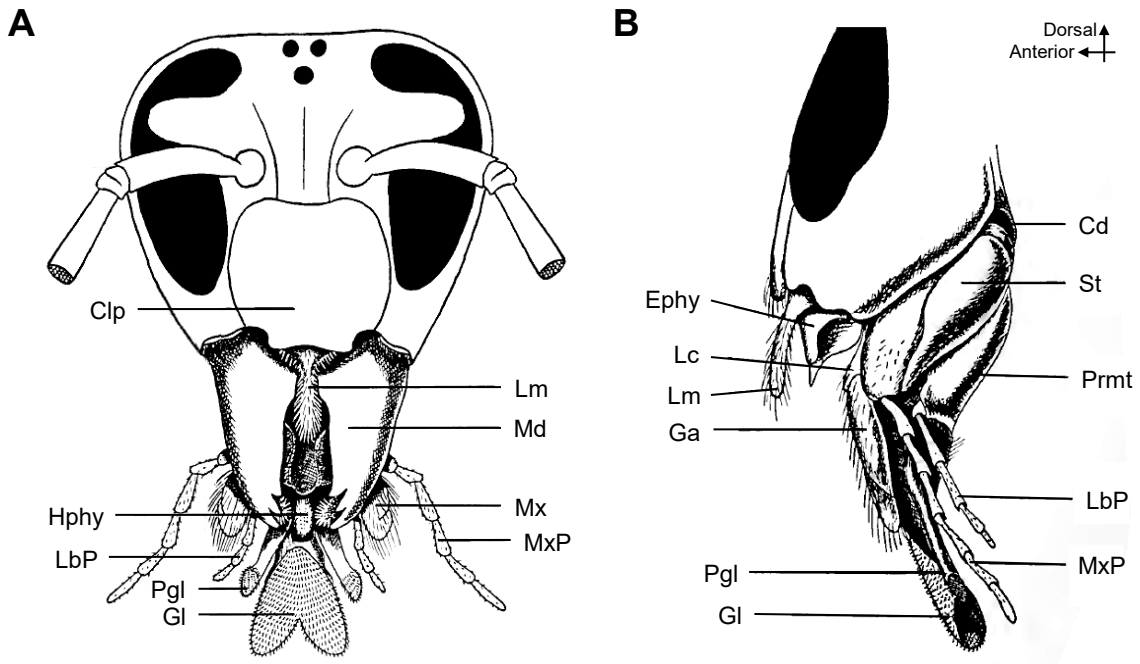


Figure 1: Head and mouthparts of *Vespula vulgaris* (Linnaeus, 1758) in frontal and lateral view, based on the drawings of Kirmayer (1909); A: frontal view B: lateral view with mandibles removed; Abbreviations: lp: Clypeus, Cd: Cardo, Ephy: Epipharynx, Ga: Galea, Gl: Glossa, Hphy: Hypopharynx, LbP: Labial palpus, Lc: Lacinia, Lm: Labrum, Md: Mandibel, Mx: Maxilla, MxP: Maxillary palpus, Pgl: Paraglossa, Prmt: Prementum, St: Stipes.

Proximal of the ligula is the salivarium situated, which includes the opening of the salivary ductus (Duncan, 1939). Dorsally of the salivary orifice is the preoral cavity situated, which is a tubular area ranging between the salivarium and the anatomical mouthopening (Vilhelmsen, 1996). The posterior part is formed by the hypopharynx, a mesially grooved membranous surface area with short setae (Duncan, 1939; Kirmayer, 1909). It is supported

by the ventral parts of the labium (Duncan, 1939). On the lateral boarder of the roughly rectangular shaped hypopharynx runs a rigid sclerite with a comb–like array of setae (Duncan, 1939; Kirmayer, 1909)

Frontally to the mandible, the labrum and the epipharynx are situated, that cover the frontally the preoral cavity (Duncan, 1939). The labrum is largely concealed by the clypeus, and only a narrow elongated process is visible from exteriorly, that is distally well equipped with sensilla trichoidea, possibly with mechanosensory function (Baranek et al., 2018; Duncan, 1939; Zacharuk, 1985). The prominent epipharynx is posteriorly covering the labium by wing-like lobes, which reach into the lateral angles of the preoral cavity (Duncan, 1939). These wings contain a lamina, the epipharyngeal bar, which fits on a similar bar at the lacinia (Duncan, 1939). The epipharynx has additionally a long slender, ventral oriented spine in the midline (Duncan, 1939). At the proximal boarder of the epipharynx a dense comb-like array of setae, the oral pecten, are protracting posterior into the preoral cavity (Duncan, 1939; Kirmayer, 1909). These oral pecten are important for filtration of solid particles and mark the functional mouth opening (Duncan, 1939; Edwards, 1980; Kirmayer, 1909; Vilhelmsen, 1996). Opposing to the oral pecten, on the posterior side of the preoral cavity is a transversal opening to the infrabuccal pocket formed by the hypopharynx (Duncan, 1939; Kirmayer, 1909).

## Organs and structures associated with the mouthparts

The infrabuccal pocket, an invagination of the hypopharynx, and can be found in form of a groove to a more prominent globular shape in many hymenoptera taxa from Symphyta to Apocrita (Duncan, 1939; Gotwald, 1969; Kirmayer, 1909; Krenn, Mauss, & Plant, 2002; Krenn et al., 2005; Snodgrass, 1910; Vilhelmsen, 1996). In Vespidae it is of globular shape with a horizontal opening and can regularly be observed to be filled with solid matters, similar to Formicidae (Duncan, 1939; Edwards, 1980; Gotwald, 1969; Kirmayer, 1909; Wang, Billen, Pan, & He, 2018). The infrabuccal pocket is thought to be in general a storage or digestion organ, allowing the animal to extract nutrients out of solid material (Duncan, 1939; Kirmayer, 1909; Wang et al., 2018). However no muscles have been recorded in Vespidae to attach at this organ, which makes it questionable if and how a controlled movement is possible (Duncan, 1939; Edwards, 1980; Wang et al., 2018). It is not known if glands are interacting with this organ which would help to assume a nutrient extracting function. Additionally it is not fully understood, how Vespidae separate particle into the infrabuccal pocket.

The cibarium, the parts of the preoral cavity dorsal of the functional mouth opening, is formed by the epipharyngeal wall, the hypopharyngeal lobe and the sitophore (Vilhelmsen, 1996;

Zimmermann & Vilhelmsen, 2016). This part of the preoral cavity is fronto-dorsally oriented and after a 90° bend it continues dorso-posterior into the pharynx (Kirmayer, 1909; Porto & Almeida, 2019; Vilhelmsen, 1996). The cibarium is equipped with strong musculature which is helps pumping fluid (Vilhelmsen, 1996). Prior to the bend glandular openings can be found and posterior to the bend lateral sensory organs (Kirmayer, 1909). In general the posterior side of the cibarium is sclerotized and the hypopharyngeal lobe bears dense rows of microtrichia (Duncan, 1939; Kirmayer, 1909; Porto & Almeida, 2019). Although the general morphology is described, the form and function of the microtrichia and other cuticular features is unclear in detail.

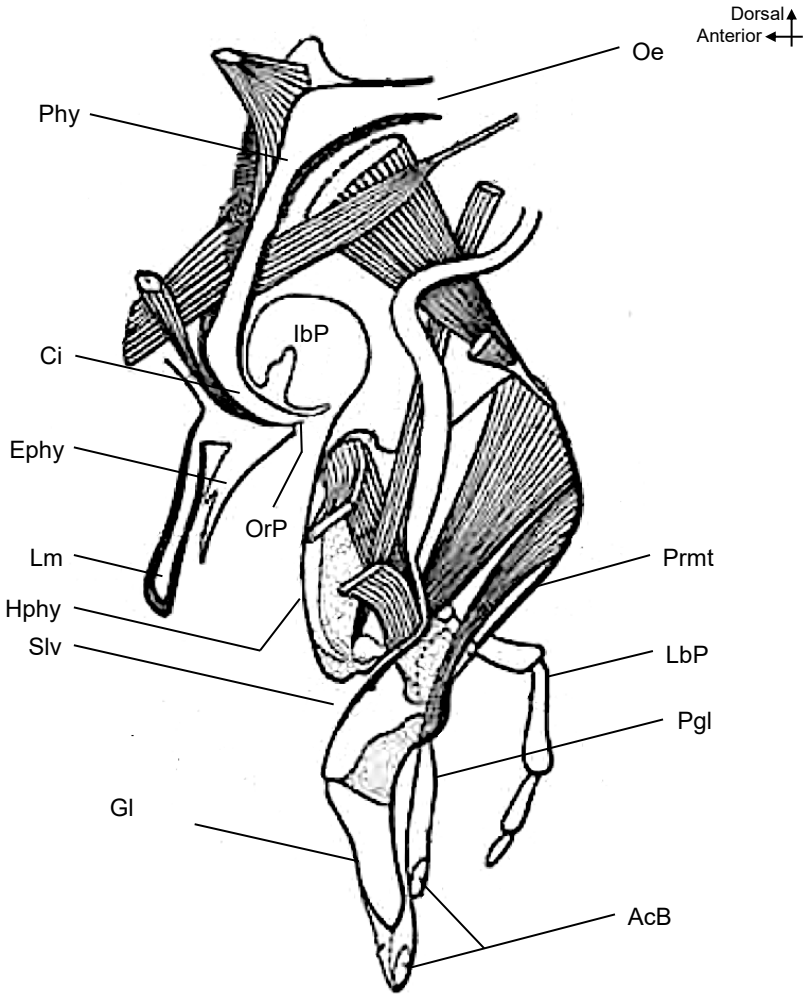


Figure 2: Mouth opening and adjacent organs in sagittal view of *Vespula pensylvanica* (de Saussure, 1857) queen, edited from the schematic drawing of Duncan (1939); Abbreviations: AcB: Acrosomal button, Ci: Cibarium Ephy: Epipharynx, Gl: Glossa, Hphy: Hypopharynx, IbP: Infrabuccal pocket, LbP: Labial palpus, Lm: Labrum, Oe: Oesophagus, OrP: Oral pecten, Pgl: Paraglossa, Phy: Pharynx, Prmt: Prementum, Slv: Salivarium

## Fluid uptake

For the uptake of fluids, Vespinae and Polisitinae use the labio-maxillary complex, epipharynx and hypopharynx in an arrangement termed “Wespenrüssel” – wasps proboscis (Baranek et al., 2018; Duncan, 1939; Kirmayer, 1909; Krenn et al., 2005; Mauss et al., 2019). By protraction of the labio-maxillary complex, the glossa and paraglossa are extended forward and liquids can adhere to it in a tongue-like manner (Baranek et al., 2018). Similar to bees the glossa and paraglossa can then be retracted by muscles in a position that the liquid is transferred from the glossa to the wasp proboscis (Duncan, 1939; Kirmayer, 1909; Snodgrass, 1942).The proboscis itself is formed by the epipharynx and the maxillae which form the frontal part of the proboscis and labium primarily with the hypopharynx for the posterior cover (Duncan, 1939). The frontal elements connect with epipharyngeal bar and the lacinial bar and the posterior connection between the maxillae and the hypopharynx is formed by two rows of pecten, which are situated at the galea and laterally on the hypopharynx, with the hypopharyngeal pecten (Duncan, 1939). On the maxilla, both the galea and the lacina bear further long setae, especially those of the galea project to the opening of the proboscis when formed, however their functions are unknown (Kirmayer, 1909).

As adult Hymenoptera in general can only ingest fluids or semi fluids due to their narrow mouth opening and the aculeate waist (Hunt, 1991; Janet, 1895; Spradbery, 1973), the filtration of these fluids is an important step in the liquid uptake. The infrabuccal pocket usually containes dirt, detritus and other solid particles and expelled as compressed pellets (Duncan, 1939; Janet, 1895; Kirmayer, 1909). The oral pecten or the functional mouth opening in general are expected to play an important role in the filtration process (Duncan, 1939; Edwards, 1980; Eisner & Happ, 1962). In general, the few reports of filtration in Vespidae are vague. It is not known how bigger particles are expelled, as well as the sizes of the particles that are filtrated and collected in the infrabuccal pocket of Vespidae.

## Aims

The aim of this study was to explain how wasps handle liquids and react to particle contamination. In addition the morphology of the preoral region was studied in detail in various species. Feeding experiments were conducted to investigate the size of filtrated particles, the role of the mouthparts during that process and to visualize and measure swallowed particles.

The following main questions were approached:

1. Do eusocial Vespidae filter solid particles of specific sizes from liquids using the mouthparts and the structures in the preoral chamber?
   I hypothesise that particles of different sizes are either filtered with the mouthparts and with the oral pecten.

2. Are morphological adaptations for filtration in the cibarium present and do they differ within eusocial Vespidae?
   I hypothesise that there are species specific differences in the arrangement and size of filtration features.

3. Does the infrabuccal pocket fulfill a storage function in eusocial Vespidae?
   I hypothesise that the infrabuccal pocket has primarily a collection or storage function. Particles that are filtrated at the oral pecten are transported into the infrabuccal pocket.

## Materials & Methods

For this study female worker individuals of three different Vespinae species, *Vespula germanica* (Fabricius, 1793), *Dolichovespula saxonica* (Fabricius, 1793) and *Vespa crabro* Linnaeus, 1758 as well as females of *Polistes dominula* (Christ, 1791) from the subfamily Polistinae are studies for comparison. All specimens have been collected in various locations in Vienna, Lower Austria and Styria in 2018-2020.

Due to the complex and confusing situation of synonyms in the oral region of Hymenoptera, the terms presented by Duncan (1939), Porto & Almeida (2019) and Vilhelmsen (1996) were followed. In the functional-morphological analysis were only rarely differences between the genera present. Therefore a description for all four taxa is presented and differences are specificly addressed.

### Feeding experiment

Females of *Vespula germanica* (n = 62) were caught at the Naschmarkt in Vienna, as there was a high abundance of workers foraging dried fruits at booths. After catching they were transferred to the lab and stored in a regular refrigerator at 5 – 7 °C no longer than 24 hours. After sufficient cooling, they were calm enough to put them into a rolled half of a plastic pipette-head to immobilize the animals completely (Fig. 3B). This gave save access to the head, similarly as it is regularly done with honeybees for proboscis reflex conditioning (Matsumoto et al., 2012). The wasp was then put in close proximity to a small plastic tube, which had been sealed using nail polish (Fig 3A). This served as a presentation table for liquid drops.

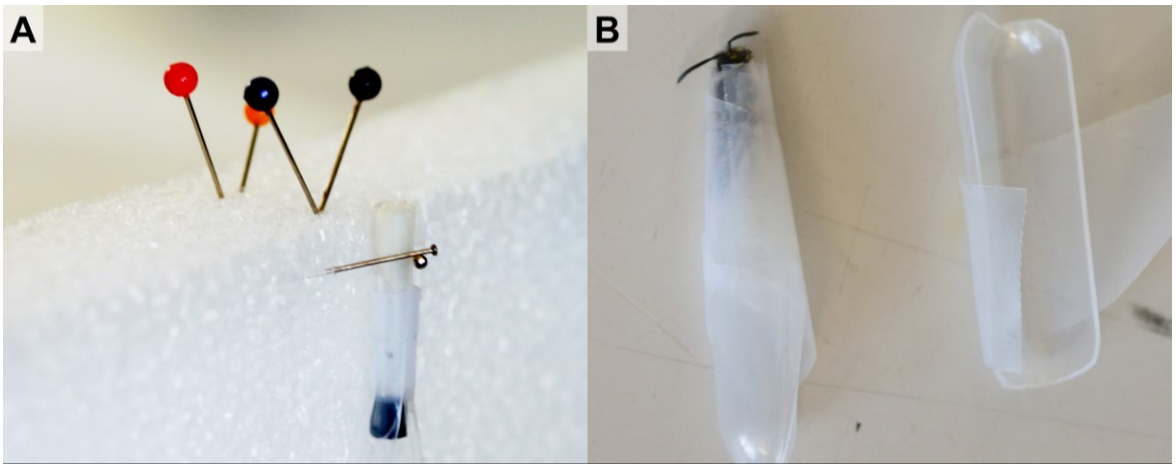


Figure 3: Setup for the feeding experiment; A: Mounting stage in which the immobilzed wasp was mounted with the presentation table in front. B: Immobilization of a single wasp using a cut pipette head.

Different solution and mictures were used:

- A mixures of sugar water with different sized glass beads: 0.5 ml regular granulated sugar which was completely dissolved in 0.5 ml tap water. Additional 0.25 ml glass beads in various sizes and combinations (Tab. 1) (Sigma, glass beads, acid washed: <152 µm G4649, 152-212 µm G-1145, 212-300 µm G-1277, 450-600 µm G-8772) were added.

Table 1: Mixures of various glass bead sizes in sugar water

| Category | Glass bead size (µm) | Number of Experiments |
|---|---|---|
| S | <152 | n = 19 |
| M | 152-212 | n = 10 |
| L | 212-300 | n = 13 |
| XL | 450-600 | n = 5 |
| SM | <152 + 152-212 | n = 10 |
| ALL | <152 + 152-212 + 212-300 + 450-600 | n = 5 |

- Mixure of concentrated sugar water and barium sulfate (BS). Bariums sulfate solution is regularly used as a contrast agent in humans for x-ray and CT scans (A.J. Megibow & M.A. Bosniak, 1980) and very rarely in insects (Davey & Treherne, 1963). 2% barium sulfate solution diluted in sugar water have been administered.
- Combination of both solution S and BS (SBS: 2% barium sulfate in saturated sugar water solution with 0.25 ml glass beads < 152 µm was added).

Drops of solution were presented on a presentation table. The wasp could take up the fluid mixures ad libido from 2-4 drops. Afterwards they were either put immediately into a fixative, formaldehyde-acetic acid alcohol (FAA 10:3:1) to fixate them or were stored 30-60 mins in the refrigerator before putting them into the FAA. Due to handling issues after the first 10 specimens $CO_2$ was administered before the fixation as a sedative. After fixation for several days the specimens were transferred to 70% ethanol in which they were stored.

The feeding experiment were filmed with Nikon D5300 and D500 (Nikon Corporation, Minato, Tokyo, Japan) cameras and a Sigma 105 mm F/2.8 EX DG Macro OS HSM (Sigma Corporation, Kawasaki, Japan). In some cases a AF-S NIKKOR 50 mm 1:1.4G (Nikon Corporation) has been used with fixed open aperture reversed on the Sigma lens. The Nikon cameras were used in video mode. Single frames were processed using Lightroom 6.1 and Photoshop CS (Adobe Inc., San Jose, USA)

## Scanning electron microscopy

To assess the morphology of Vespinae and Polistinae scanning electron microscopy was used. Images of the heads and mouthparts of all four species were taken to search for genera specific structures. Two different dissections were used to view the internal organs.

1. The head was removed from the body and the antennas were cut off. Then a mid-sagittal cut was performed using a stiff razorblade.
2. The head was removed and a sagittal cut close to the mandibular joint was performed. The musculature and cuticle structures like the tentorium were removed, until it was possible to extract the pharynx with the gnathal pouch plus hypopharynx and epipharynx. The dorsal and ventral parts were separated carefully with the use of tweezers, dissection needles and a microscissor, resulting in two to three parts.

The SEM work was performed at the Core Facility Cell Imaging and Ultrastructure Research (CIUS), University of Vienna - member of the Vienna Life-Science Instruments (VLSI). After dissection the mouthparts were dehydrated with increasing ethanol concentrations (80% 30 min, 90% 45 min, 96% 1h, 3x 100% 1h) on a shaker. The parts were subsequently critical point dried (CPD). For CPD a Leica CPD 300 (Leica Biosystems, Wetzlar, Germany) with the program: 50%, Auto, $CO_2$ In (Slow, 2 min Delay), Exhange (Speed 2, Cycles 30), Gas Out (Head med., Speed slow 100%) was used. After CPD the specimen parts were mounted on aluminium stubs using carbon tabs and silver paste. The stubs were coated with gold with a JEOL JFC-2300HR (Jeol ltd. Tokyo, Japan) for 120 seconds. The SEM Analysis has been performed with a FEI (Philips) XL 30 ESEM (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) using different angles, spot sizes and voltages. Cuticular sizes were measured from the resulting images.

## Micro x-ray and micro computer tomography (µCT) imaging

X-ray images were used to evaluate the the outcome of the feeding experiment. These X-ray images had been photographed from various angles, by rotation of the animals at the longitudinal axis, similar to a µCT. The wasps were mounted upright in a plastic tube. This has been done “wet” as the animals were take out of 70% ethanol, and the liquid adhering on the surface was not removed, to ensured that the animals didn’t dry out during the imaging. A X Radia Micro XCT (University of Vienna, Department of Evolutionary Biology) and a Skyscan 1272 (Bruker, Kontich Belgien, University of Vienna, Department of Evolutionary Biology) were used for the x-rays, both are originally designed for µCT.

The X-ray images were evaluated manually and the position of glass beads was noted in there categories:

(1) None: No glass beads visible inside the head or abdomen. If glass beads were located on the mouthparts it also was counted as not inside the head.
(2) Inside the intrabuccal pocket: glass beads visible inside the head as globular shadows, in the intrabuccal pocket. The mandibular joint was used determine if s glass bead is inside or outside the head, as it is from the lateral view in a close position to the mouth opening.
(3) Abdomen: glass beads were visible inside the anterior abdomen, were the crop is located. To exclude glass beads on the exterior of the abdomen different angles and were checked.

If an animal had taken up glass beads into the head and into abdomen, it was noted as both Inside the infrabuccal pocket and inside the abdomen.

To support the evaluation of the maximum size of glass particles, which can be filtered with the mouthpart, a Chi² contingency table was used with glass beads present in the head (n = 6) and not present inside the Head (n = 17) as columns and the glass beads sizes (Solution 1 S - ALL) as rows. The Chi² statistics have been calculated using a Fisher’s exact and a Cramer’s V using PAST, Version 4.06b (Hammer, Harper, & Ryan, 2001)

For 3D analysis of cuticular elements µCTs of *Vespula germanica* were used. The Worker were fixed in FAA or Bouin solution (saturated picric acid, formaldehyde ,glacial acetic acid 15:5:1) and stored in 70% ethanol until scanning. To enhance the contrast the individuals were dehydrated with an ascending ethanol series and stained with 1% iodine solution for at least 1 day (Metscher, 2009). The X Radia Micro XCT was primarily used for the µCT scans. For the dataset of Figure 11 B & C the Skyscan 1272 was used. The scans were segmented in AMIRA (Version 6.1, Thermo Fisher Scientific, Waltham, Massachusetts), with the aid of

Biomedisa (Lösel et al., 2020). The surface have been calculated using AMIRA and after a reduction and smoothing, they were transferred to Dragonfly for the final rendering.

## Serial semithin section

Worker of *Vespula germanica* were collected from the Naschmarkt, Vienna and fixed in FAA. The head of *Vespula germanica* was cut, dehydrated with an ascending ethanolseries and infiltrated under vacuum conditions to ensure even infiltration by Agar low viscosity resin (Agar Scientific, Essex, UK). The serial sections were cut on the microtome (Leica EM UC6, Wetzlar Germany) with a diamond knife (Histo Jumbo Diatome) at a thickness of 1 µm. Serial cross section were cut from the base of the antenna towards the mouthparts, stopping at the mandibular joint. The sections were stained with a mixture of 1% azure II and 1% methylene blue in an aqueous 1% borax solution at 60–70 °C for 10 sec (Pernstich, Krenn, & Pass, 2003). The cuts were microphotographed with a Nikon Eclipse E800 (Minato, Japan) and further processed by desaturation, white balance, contrast enhanced by CLAHE, sharpening, and resizing using Fiji (Schindelin et al., 2019) and Adobe Photoshop CS (Adobe Inc., San Jose, USA).

The pictures were aligned and pre segmented using AMIRA. Further segmentation was done with the help of Biomedisa (Lösel et al., 2020). The surface has been calculated with AMIRA and rendered in Dragonfly software, Version 2020.2 for Windows. (Object Research Systems (ORS) Inc, Montreal, Canada, 2020; software available at http://www.theobjects.com/dragonfly.)

## Light microscopy

For visualisation of the maxillae stack images were taken with a Nikon SMZ 25 (Evolutionary biology, University of Vienna) and Wild Heerbrug M420 (from Author). The stacks calculated with NIS-Elements (Nikon Corporation) and Lighroom + Photoshop (Adobe Inc.).

## Results

### Feeding and filtration

Feeding *Vespula germanica* workers with sugar water and various substances is an effective method to study fluid–feeding and the filtrating function of Vespidae mouthparts. The individuals ingested the sugar water across all various treatments. To take up fluids the worker extended the labio-maxillary complex and either wetted the glossa with the fluid and retracted it into the wasp proboscis, or directly put the wasp proboscis into the liquid to retrieve fluids. During this process bigger glass beads were prevented from entering (Fig. 4B). Smaller particles were collected inside the mouthparts and disgorged after the wasp has stopped the liquid uptake (Fig. 4A).


Figure 4: Feeding experiment using glass beads with *Vespula germanica*. Single frames from Videos; A: *Vespula germanica* feeding on sugar water with <152 - 600 µm glass beads. Small glass beads enter the proboscis, are collected and ejected after the liquid uptake (black arrow), bigger particles are prevented from entering the proboscis (white arrow). B: *Vespula germanica* fed with 212 – 300 µm glass particles, which prevented from entering the proboscis (white arrow)

The X-ray studied *Vespula germanica*, which had been fed with glass particles, had clearly visible round dark shadows visible in their body. The position could be well determined by the X-ray images as the cuticle was also well visible (Fig. 5). A highly significant difference in the present and absence of glass beads in both infrabuccal pocket ($\chi^2(5) = 51{,}516$, $p < 0{,}001$, $\varphi c \approx 0{,}912$) and crop ($\chi^2(5) = 24{,}428$, $p < 0{,}001$, $\varphi c \approx 0{,}633$) with a high association is present (Tab. 2). In all wasps that were fed particles smaller than 152 µm (S), particles were found in the infrabuccal pocket, however only in 58,8% of these were also glass beads detected in the crop (Tab. 2).

Whenever glass beads bigger than 152 µm were fed, none were found in the crop (Tab. 2). In the size range of 152 - 212 µm only 6 out of 10 times the glass particles were found in the infrabuccal pocket, while in all cases with even bigger no particles were in the infrabuccal pocket (Tab. 2). If the glass particles were combined as in treatment SM and ALL the results were similar to those of treatement S with glass beads < 152 µm (Tab. 2).

Table 2: Feeding experiment using *Vespula germanica* worker. Different sized glass beads have been fed with sugar water (30%) resulting in different in the presence (Yes) and absence (No) of glass beads in the infrabuccal pocket (IbP) and crop.

| **Glass beads** | | **Number of worker** | **Glass beads in IbP** | | **Glass beads in Crop** | |
|---|---|---|---|---|---|---|
| **(µm)** | | **(N )** | **Yes** | **No** | **Yes** | **No** |
| < 152 | (S) | 19 | 19 | 0 | 12 | 7 |
| 152 – 212 | (M) | 10 | 6 | 4 | 0 | 10 |
| 212 – 300 | (L) | 13 | 0 | 13 | 0 | 13 |
| 450 – 600 | (XL) | 5 | 0 | 5 | 0 | 5 |
| < 152 - 212 | (SM) | 10 | 10 | 0 | 4 | 5 |
| < 152 - 600 | (ALL) | 5 | 5 | 0 | 3 | 2 |
| | | | $\chi^2(5) = 51.516$, *p < 0.001*, $\varphi c \approx 0.912$ | | $\chi^2(5) = 24.428$, *p < 0.001*, $\varphi c \approx 0.633$ | |

*Vespula germanica* which have been fixed shortly after feeding barium sulfate (BS), had already destinctly visible shadows from the barium sulfate. It adheared at least temporarily to the cuticle of the digestive tract or stained it and therefore increases the visibility of the alimentary canal. Barium sulfate reached not only the crop but also passed into the midgut, especially when waiting for a few minutes after barium sulfate feeding before fixation (Fig 6 A & B).

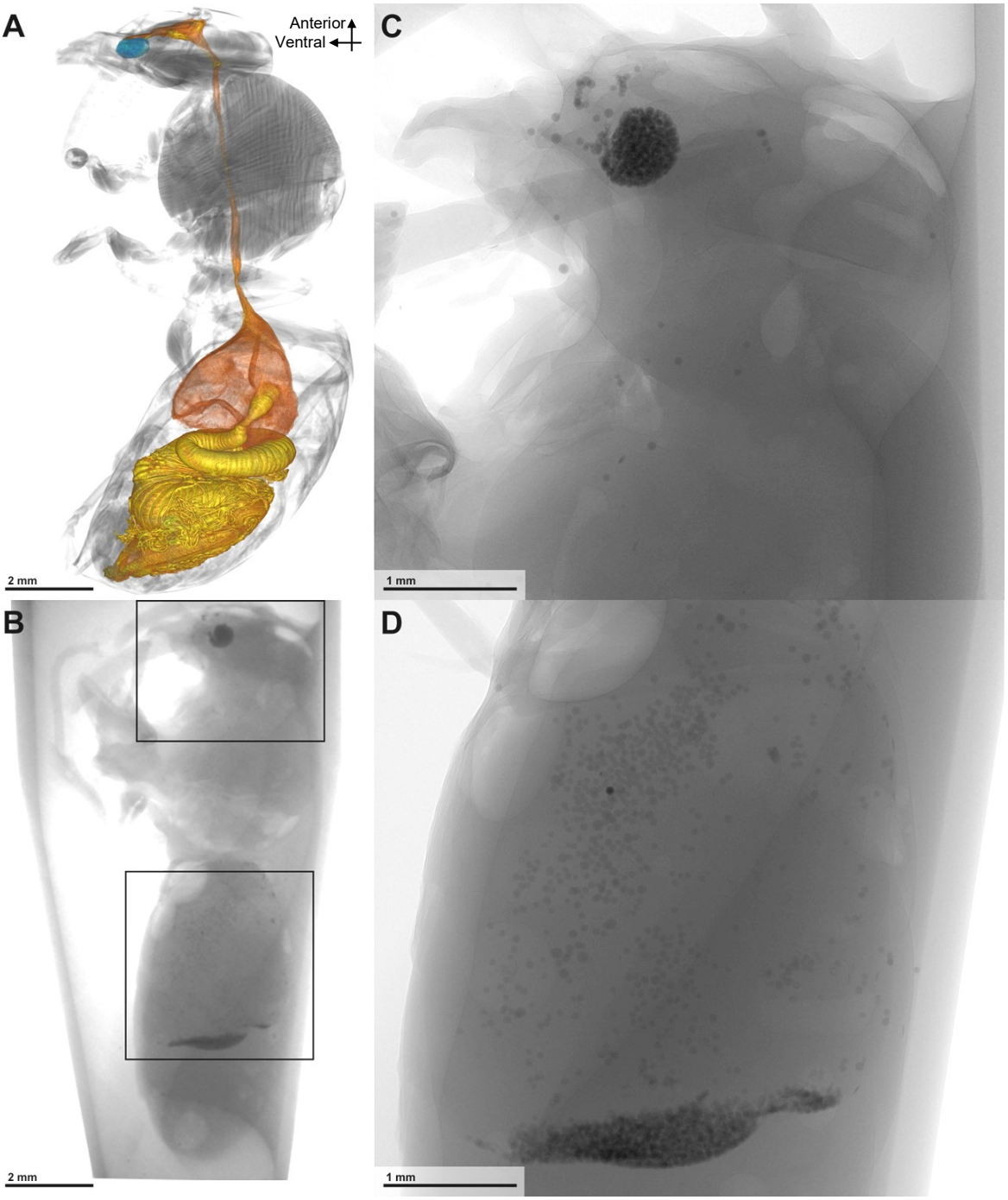


Figure 5: Digestive tract of *Vespula germanica* and positions of glass beads (<152 µm) after feeding. X-ray images, lateral view; A: Digestive tract segmented in orange and the Infrabuccal pocket in blue. B: Glass beads in the infrabuccal pouch and crop (Treatment 1s). C: Details of the head. D: Detail of abdomen.

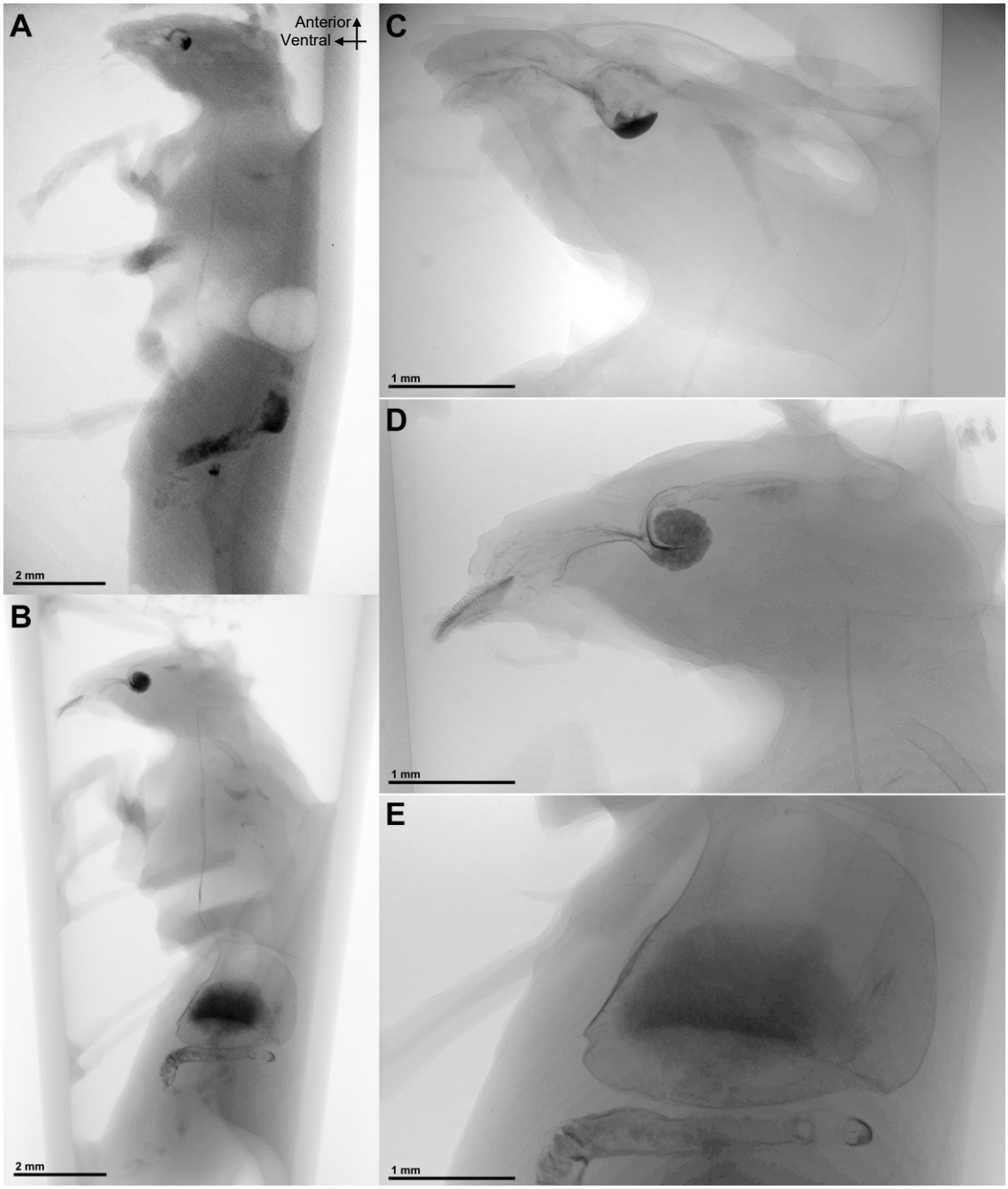


Figure 6: Uptake of barium sulfate and glass beads in a feeding experiment with *Vespula germanica*. X-ray images, lateral view A & B: Overview of *Vespula germanica* after intake of barium sulfate + sugar water (A) and bariumsulfate + glass bead + sugar water (B). C: Head detail of (A), showing smooth shadow of barium sulfate in the infrabuccal pocket. D & E: Detail of the head and digestive tract of (B). Granulate shadows of glass beads are visible in the infrabuccal pocket and primarily smooth shadow of barium sulfate in the crop.

When barium sulfate and glass particles were combined (SBS), a visual identification in the x-ray images of glass beads and liquids was possible. Recognizable by the cloudy darker shadows, is the “liquid” barium sulfate, which largely hadn’t been transported into the intrabuccal pocket, but stored in the crop or even further into the digestive tract. The glass beads, identifyable due to their grainy shadows, were largley collected in the infrabuccal pocket (Fig. 6). No difference in the separation of glass beads and barium sulfate could be detected due to different waiting times, but when the animals had a longer time period (15 - 30 min) after taking up the fluids the barium sulfate was more prominent in the digestive tract posterior the crop.

## Wasp proboscis

The wasp proboscis (“Wespenrüssel”) is formed by the labio-maxillary complex as well as the epi- and hypopharynx. The tips of the galea rests in the elongated position above the salivarium and hypopharynx and enables the glossa to be retracted towards this tip of the tube. The unsclerotized hypopharynx forms a concave longitudinal groove when the proboscis is extended (Fig. 7 B). While the distal part the wasp proboscis is frontally formed by the overlapping maxillae, the proximal part of the wasp proboscis is frontally covered by the epipharynx (Fig. 7 C, 8). The lacinia connects posterior to the epipharynx via the connection of the epipharyngeal bar and lacinial bar (Fig. 9 E, 10 B). The posteriror connection between the maxilla and the hypopharynx is formed by two rows of pecten, the galeal pecten and the hypopharyngeal pecten, as well as the smooth surfaces ventral of the galeal pecten which are pressed against the sclerotized area of the hypopharynx ventrally from the hypopharyngeal pecten (Fig. 9 C & F). Liquid can be taken up by a licking motion of the glossa. The liquid adheres to the spatulae, specialised hook-shaped microtrichia, and can thus be transported to the tip of the proboscis (Fig. 9 D). Alternatively the tip of the wasp proboscis can just be dipped into the liquid. The liquid is then sucked further into the proboscis and enters the preoral cavity formed by the epipharynx and hypopharynx. There liquids pass the oral pecten, marking the functional mouth opening. and further continues into the dorsal part of the preoral cavity, the cibarium (Fig. 8). Both the cibrarium and pharynx are well equipped with musculature, which function as a suction pump. This area is formed by the epipharyngeal wall, the hypopharyngeal lobe and the sitophore in eusocial Vespidae (Fig 7 A & B). The dorso–posterior end of the preoral cavity is marked by the anatomical mouth opening (Fig. 8). The liquid then continues through the pharynx, into the oesophagus to the crop (Fig. 8). There it can be stored, regurgitated or passed through the proventriculus into the gut.

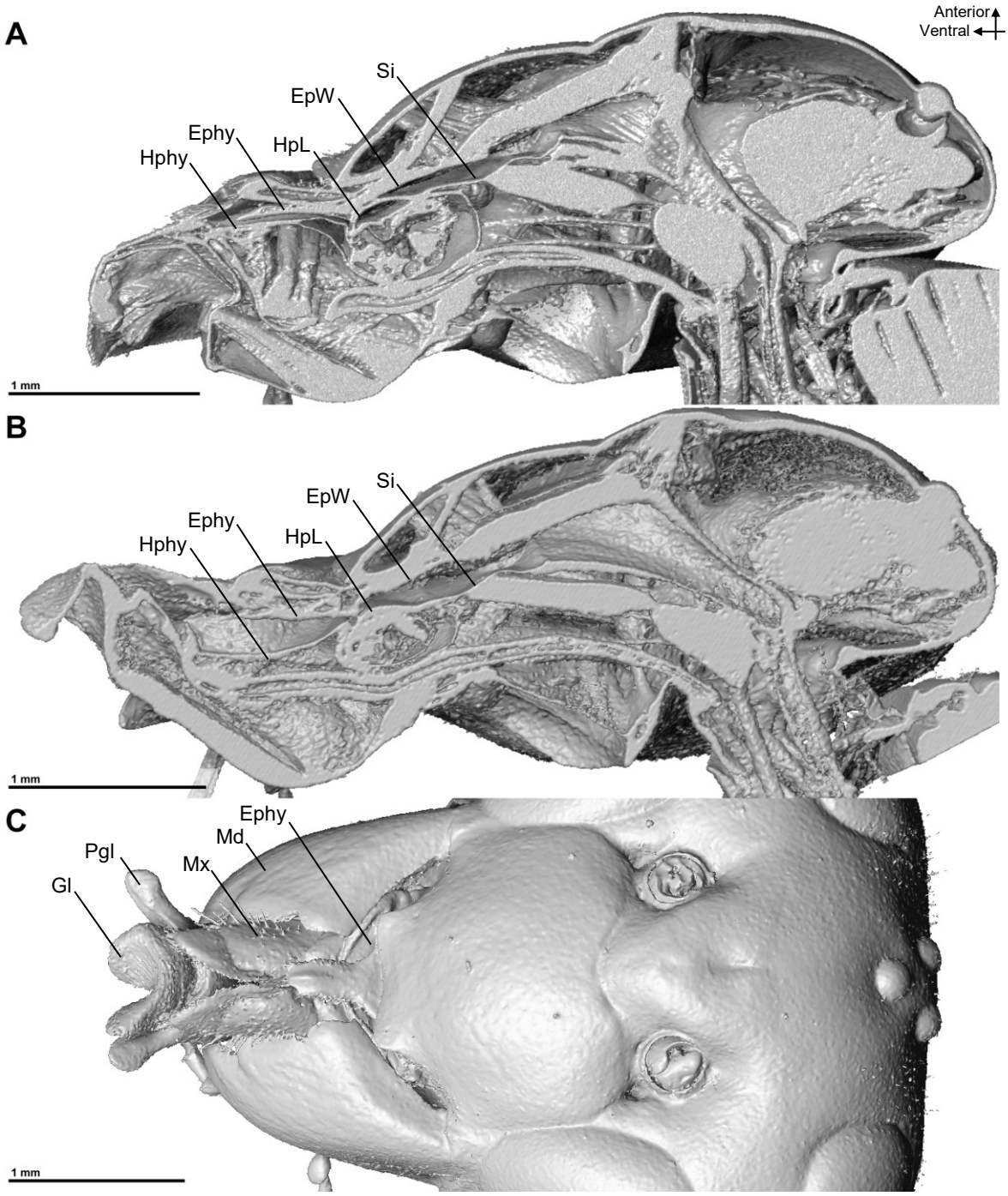


Figure 7: Functional positions of the labio–maxillary complex in *Vespula germanica*. Surface render of a µCT; A: labio-maxillary complex in resting position; sagittal cut. B: labio-maxillary complex in extended position. Hypopharynx concave, increasing volume between hypopharynx and epipharynx; sagittal cut. C: Mouthparts with labio-maxillary complex extended. Glossa extended beyond the mandibles. Maxillae forming the roof of the wasp proboscis ("Wespenrüssel"). frontal view; Abbreviations: Ephy: Epipharynx, EpW: Epipharyngeal wall, Gl: Glossa, Hphy: Hypopharynx, HpL: Hypopharyngeal lobe, Md: Mandibel, Mx: Maxilla, Pgl: Paraglossa, Si: Sitophore

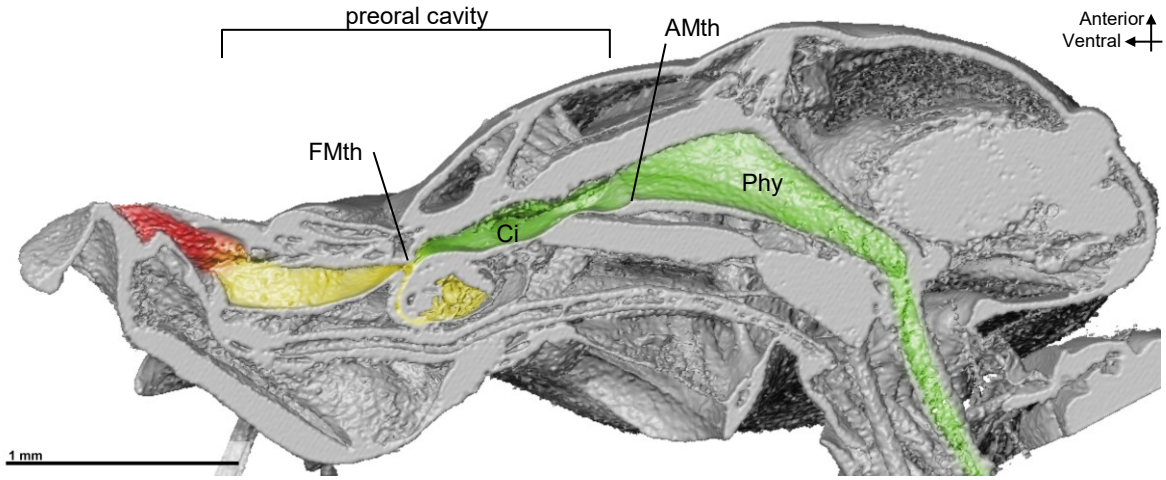


Figure 8: Stages of filtration of the eusocial Vespidae mouthparts and preoral cavity. Surface render of a µCT, with the path of liquid colored. Red: unfiltered liquid, Yellow: Sizes > 212 µm filtered, Green Sizes > 152 µm filtered, sagittal cut; Abbreviations: AMth: Anatomical mouthopening, Ci: Cibarium, FMth: Functional mouthopening, Phy: Pharynx

## Labium

When feeding, the glossa and paraglossa are the first organs, which are in contact with the fluid. The glossa consists of two broad lobes connected in the midline forming a heart-shaped glossa (Fig. 9 B). Lateral of the glossa are the paired paraglossa, on both sides. Both glossa and paraglossa have prominent microtricha arranged in rows. These microtrichia, are spatula shaped, with a slender stoke and a flat head which is pointed towars the mouth opening (Fig. 9 D). The frontal surface of the paraglossa is covered by rows of a single broad cuticle plate, each cuticule plate bearing several spatulae (Fig. 9 D). The ventral two-third of the glossa are covered with these specialised microtrichia, but only the ventral quarter of the paraglossa are covered with spatula (Fig. 9 B, D). The dorsal region of the glossa consist of the anterior lingual plate which is covered with a smooth cuticular surface and ventral oriented setae in the midline. The paraglossa has a similar structure, however the smooth surface extends further dorsally, and the setae on the basal paraglossal sclerite are not only dorsally but also mesally oriented (Fig. 9 B). The glossa of *Dolichovespula saxonica* was noticibly elongated compared with the others species.

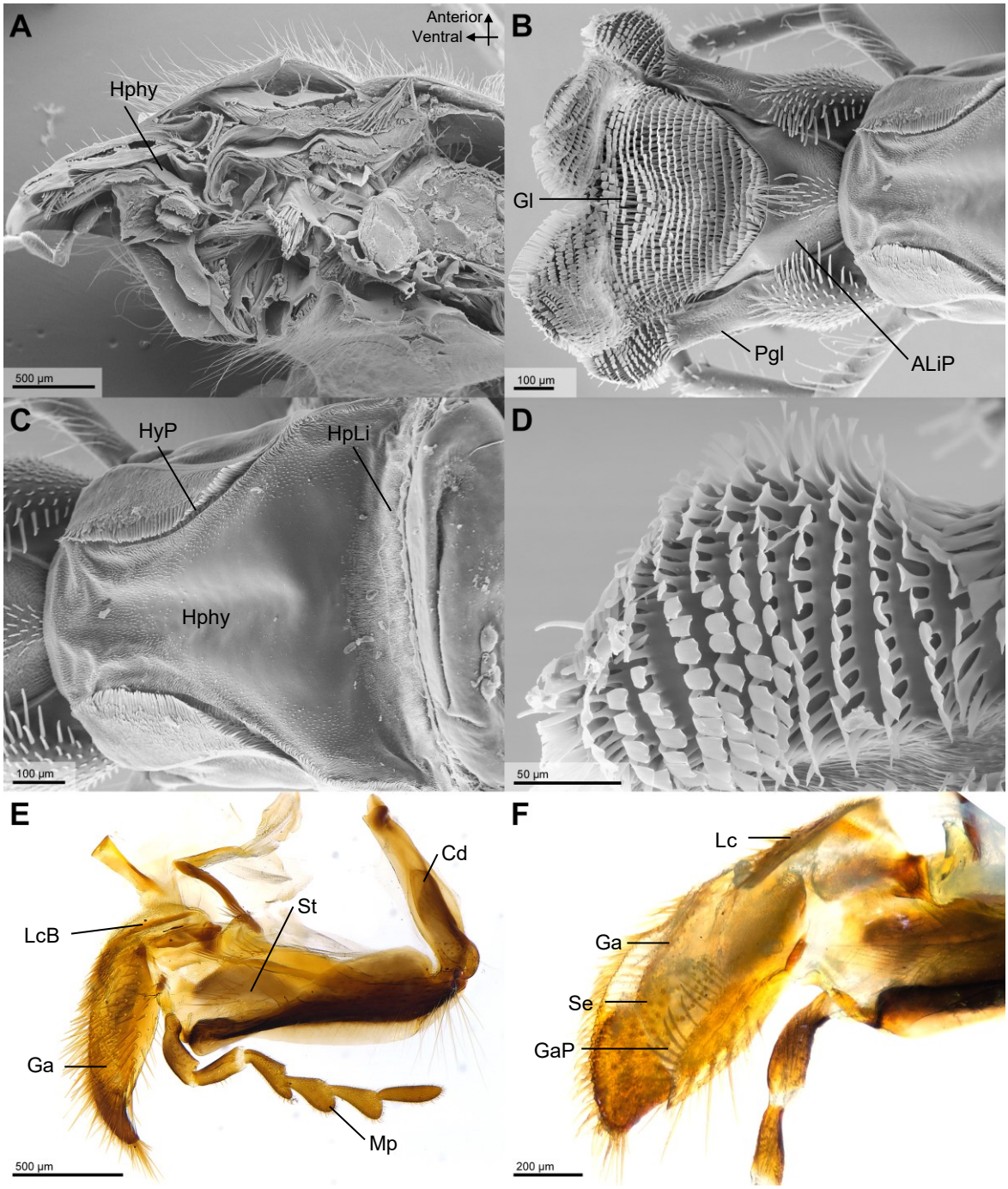


Figure 9: Cuticular surface of the labio-maxillary complex; A: Overview of the preoral cavity. Sagital cut of species in a SEM. B - D cuticula structures related to fluid feeding of the labium and hypopharynx. Frontal view, SEM.E - F: Cuticular strucktures related to fluid feeding of the maxilla; lightmicroscopical image. E: Lateral surface, F: Median surface of the galea, (A – F) *Vespula germanica;* Abbreviations: ALiP: Anterior ligula plate, Cd: Cardo, Ga: Galea, GaP: Galeal pecten, Gl: Glossa, Hphy: Hypopharynx, HpLi: Hypopharyngeal lip, HyP: Hypopharyngeal pecten, IbP: Infrabuccal pocket, LcB: Lacinial Bar, MP: Maxilliar palpus Pgl: Paraglossa, St: Stipes,

## Maxillae

The maxillae is connected to the head via the slender cardo, which is usually posterior oriented. On the distal edge the stipes connects, which is usally positioned in a sharp angle. On the ventral edge close to the distal edge is a six segmented palpus attached. On the distal edge the galea and lacinia are connected (Fig. 9 E). The lacinia is the most anterior small plate with a prominent ridge in the exterior midline, die laciniar bar. The galea is the prominent tip part of the maxillae with strong setae in the exterior side (Fig. 9 E). On the inner side of the galea are short cone shaped sensilla present which are covered by the galeal pecten, which protrude from a ridge posterior to the sensilla (Fig. 9 F). Further posterior of the galeal pecten is a smooth surface (Fig. 9 F).

## Hypopharynx

The hypopharynx is situated between salivarium, at the posterior end of the glossa and the infrabuccal pocket (Fig. 9 A). It forms the ventral boarder of the preoral chamber and is roughly trapezoid shaped (Fig. 9 C). Close to the salivarium, are small dorsal oriented setae present. Lateral of these are two outward bend groves situated which are covered with prominent setae, the hypophyaryngeal pecten. The surface between the hypopharyngeal pecten is loosely covered with microtrichia especially close to the grooves. The area lateral of the hypopharyngeal pecten is scleorized and covered by fine microtrichia. The midline is concave dented which froms a longitudinal groove (Fig. 9 C). At the transition between hypopharynx and infrabuccal pocket is a tranversal lip, which marks the transition zone into the infrabuccal pocket (Fig. 11 B). The cuticle posterior this lip, here termed hypopharnygeal lip, is covered by strong hairlike mictrotrichia, similar to those of the epipharyngeal wall, and hypopharyngeal lobe (Fig. 9 C &11 B).

## Epipharynx

The epipharynx is located posterior to the labrum, covering most of the labrum from inside. It forms the frontal cover of the preoral chamber and extends posterior to the functional mouth opening (Fig. 10 A). The epipharynx is a crescent to half-circular shaped plate with a central spine, oriented anterior. The broad triangula base of the spine reaches posterior and forms the posterior edge of the whole plate. Lateral of the spine are wing-like lobes which slightly bend inwards (Fig. 10 B). On the inner side of this lobes two crescent shaped bars are present, termed epipharyngeal bars (Fig. 10 B).

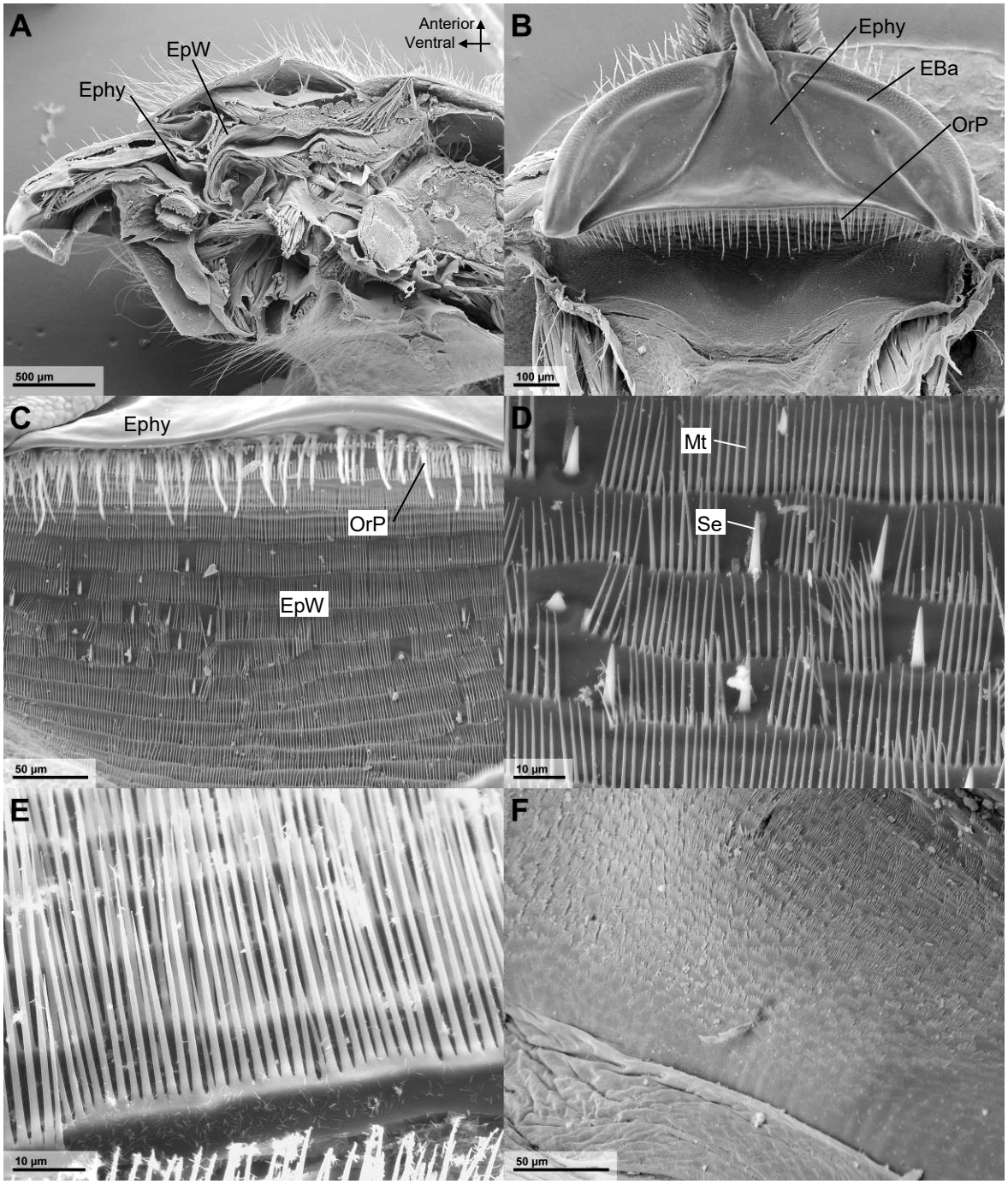


Figure 10: Epipharyngeal structures of Vespinae. SEM; A: Head of *Vespula germanica*, sagittal cut. B: Distal surface structure of the epipharynx. Viewed from posterior in anterior direction. C: Surface structure of the epipharyngeal wall, Viewed from posterior in anterior direction. D & E: Detail of sensilla and microtricha of the epipharyngeal wall. F: Detail of the surface of the epipharyngeal wall opposing the sitophore. (A ,B ,E) *Vespula germanica,* (C ,D) *Dolichovespula saxonica*, (F)*Vespa crabro;* Abbreviations: Ephy: Epipharynx, EBa: Epipharyngeal bar, EWa: Epipharyngeal Wall, Mt: Microtrichia, OrP: Oral pecten, Se: Sensillum,

## Epipharyngeal wall

the epipharyngeal wall rests in conjunction with the dorsal edge of the epipharynx. It expands from the functional mouth opening to the anatomical mouth opening (Fig. 7). The epipharyngeal wall forms the frontal cover of the cibarium that can roughly be separated in two parts. The ventral longitudinal oriented bend, opposing the hypopharyngeal lobe and the more dorsal part of the epipharyngeal wall which is in a dorso-ventral position opposing the sitophore (Fig. 7, 10 A, B, E). The ventral part of the epipharyngeal wall is covered with rows of posterior-ventrally oriented setae and microtrichia (Fig. 10 C). At the first row, that is located at the transition from the epipharynx to the epipharyngeal wall, are socketed setae termed oral pecten. These form the first row of setae with 50-150 µm (n = 6) length and 3-7 µm (n = 4) thickness and mark the functional mouth opening (Fig. 10 C). The oral pecten are easily destinguishable from other microtricha, as they have a disc shaped base with one or two full sized setae attached to each. The oral pecten are pointed in the same direction as the further dorsal microtrichia, posterior-ventrally (Fig. 10 C, 11 B). Dorsal of the oral pecten are rows of long cuticle plates, with numerous microtrichia protruding on the ventral-anterior side of these plates (Fig. 10 E). These microtrichia range between 25 – 50 µm (n = 6) in length and have a thickness of 1 – 2 µm (n = 4), they are oriented ventrally and are bent towards the functional mouth opening. In the first quarter of the pharynx two lateral patches of bristle shaped sensilla are implemented in the microtrichal rows (Fig. 10 D). Two lateral grooves are found at the transition to the longitudinal part of the epipharyngeal wall that form a funnel shaped plate (Fig. 10 B). The frontal grooves reach about two third on each side to the middle line. Here the cuticle structure changes the rows of microtrichia to cuticle plates with much shorter anterior facing spines (Fig. 10 F). The spines of these plates decrease in size from anterior to posterior. The first groove and second are ~100 µm apart. From each of the second groove reaches the middle line, at which the cuticular structure changes again to a smooth surface with a strong sculptural structure (Fig. 10 F).

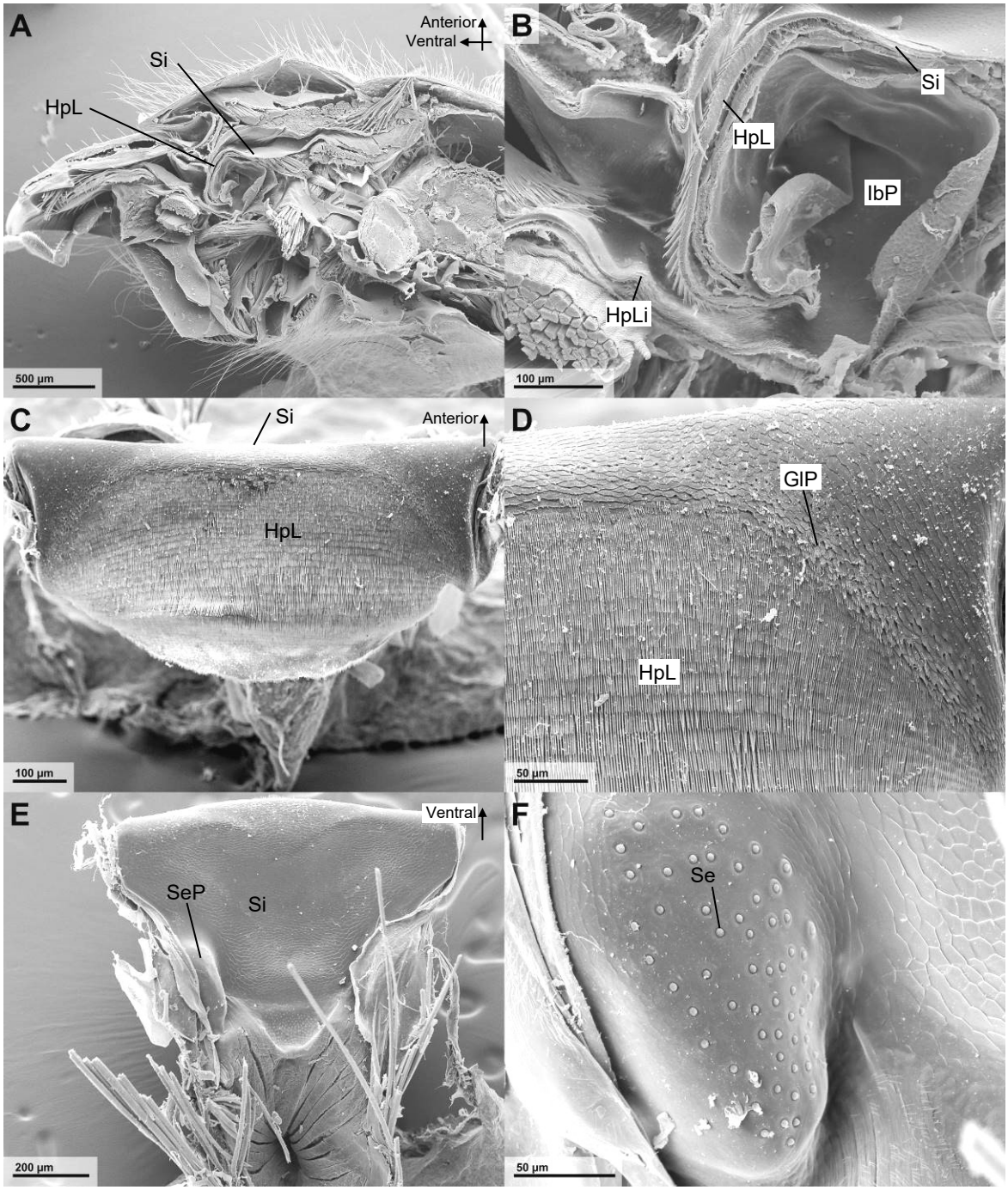


Figure 11: Overview of the hypopharyngeal structure and the sitophore. SEM; A: Head of *Vespula germanica*, sagittal cut. B: Infrabuccal pocket, detail from (A). C & D: surface structure of the hypopharyngeal lobe, frontal view. E: Surface structure of the sitophore and pharynx frontal view. F: Detail of the sensillar patch of (E), (A – F) *Vespula germanica;* Abbreviations: IbP: Infrabuccal pocket, GlP: Glandular Pores, HpL: Hypopharyngeal lobe, HpLi Hypopharyngeal lip, Se: Sensilla, SeP: Sensillar patch, Si: Sitophore

## Infrabuccal pocket

The infrabuccal pocket is a globular-shaped organ, lying posterior-dorsal of the functional mouth opening. It is connected to the hypopharynx and the hypopharyngeal lobe (Fig. 11 A, B). It is a flexible hollow organ 300 - 600 µm in size (n = 4), surrounded by an epithelium. The connection into the infrabuccal pocket froms a transversal slit, which is as wide as the hypopharynx. The connecting duct into the infrabuccal pocket is 200 - 300 µm (n = 4) long in posterior direction (Fig. 11 B). The ventral and dorsal cuticle separate at the end of this tract to form the globular shaped infrabuccal pocket. The cuticle of the duct is from the hypopharyngeal lip to the entrance into the infrabuccal pocket covered with microtricha, which point into the pocket (Fig. 11 B, 12 F). The ventral microtrichia of this duct are shorter, than those on the dorsal side (Fig. 11 B). The microtrichia decrease in length at the entrance into the infrabuccal pocket which has in general a smooth surface (Fig. 11 B)

The infrabuccal pocket is surrounded by a unicellular epithelium. The thickness of this epithelium measures 5 – 35 µm (n = 1), but in the area close to the sitophore and in the lateral corners it is thickend and could have a glandular character, similar to glandular epithelium (Fig. 12 B). No muscles are attached to the infrabuccal pocket. The infrabuccal pocket is usually partially filled. The content had to be removed for surface imaging.

## Cibarium

The posterior cibarium is formed by the hypopharyngeal lobe and the sitophore. The hypopharyngeal lobe extends from the infrabuccal pocket to the sitophore, and the sitophore extends dorso-posteriorly to the functional mouth opening (Fig. 11 A, C & E). The hypopharyngeal lobe is covered by cuticular plates with microtrichia, similar to the epipharyngeal wall (Fig. 11 C). They are orientend the ventro-frontal direction, following the form of the surface and therefore point towards the infrabuccal pocket. Close to the sitophore, the microtrichia are gradually reduced in size (Fig. 11 D). Two lateral patches of glandular pores of the hypopharyngeal gland are present at the dorso-posterior end of the hypopharyngeal lobe (Fig. 11 D). The arrangement of these pores was differing between the species. In the three studied Vespinae this field of pores is located just at anterior edge of the microtricha in a longitudinal shape (Fig. 11 D). In *Polistes dominula* is a gap of smooth cuticle between the field of pores and the edge of the microtricha, and the field of pores had a more round shape.The hypopharyngeal glands are located between the sitophore and the infrabuccal pocket and are connected to the pores via multible excretions tubes (Fig 12 A, B, D, 13).

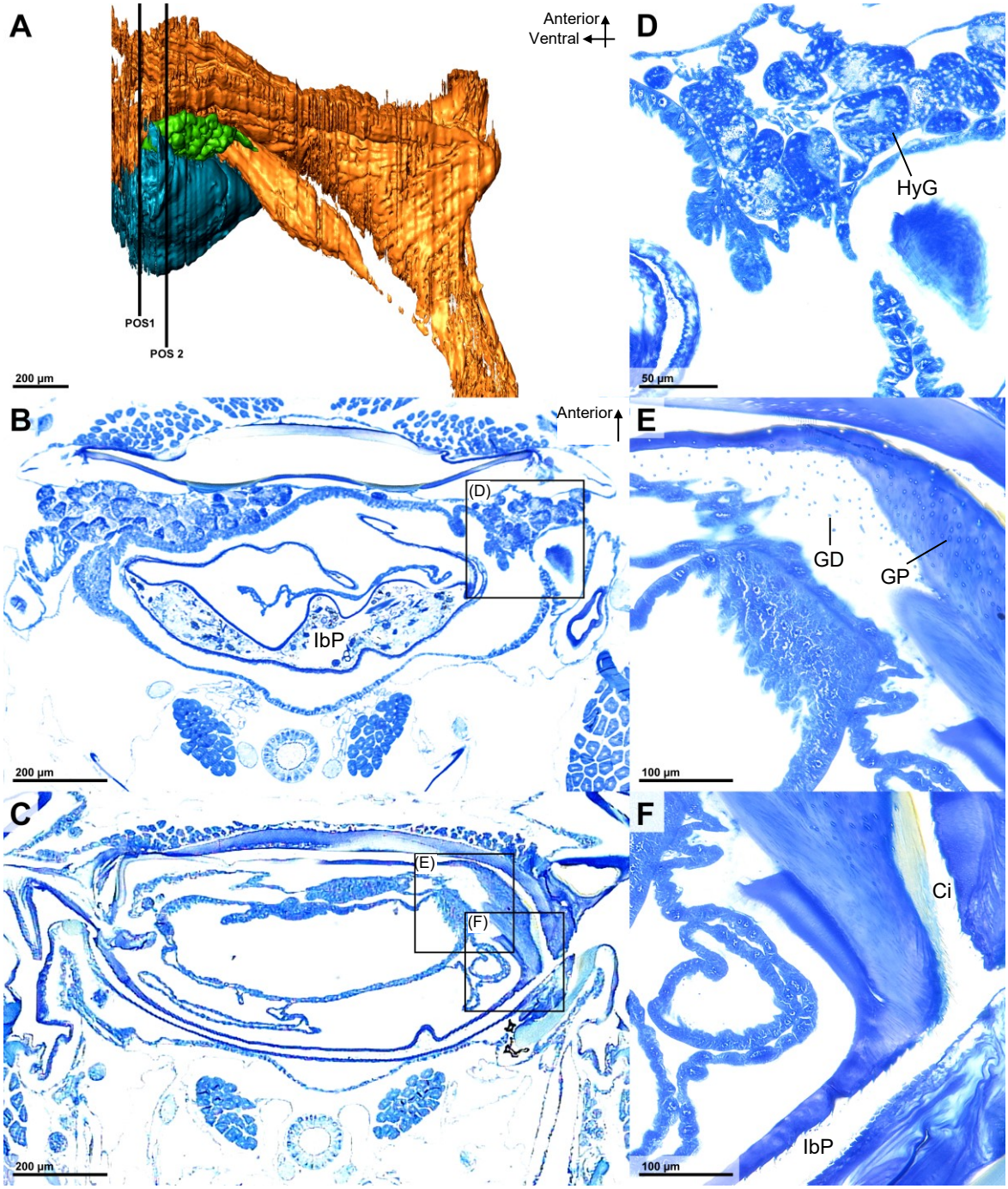


Figure 12: Infrabuccal pocket and hyperpharyngeal gland of *Vespula germanica;* A: Reconstruction of semithin sections showing the hypopharyngeal gland with two position of cross-sections marked. Alimentary canal (orange), infrabuccal pocket (blue), hypopharyngeal glands (green). Lateral view. B & C: Transversal sections of the cibarium and infrabuccal pocket with details indicated by squares. D: Structure of hypopharyngeal gland. E: Gland ducts and extretion pores of the hypopharyngeal gland. F: Microtrichia of the hypopharyngeal wall & epithelial lining of the infrabuccal pocket; Abbreviations: Ci: Cibarium, GD: Glandular duct, GP: Glandular pores, HyG: Hypopharyngeal gland, IbP: Infrabuccal pocket

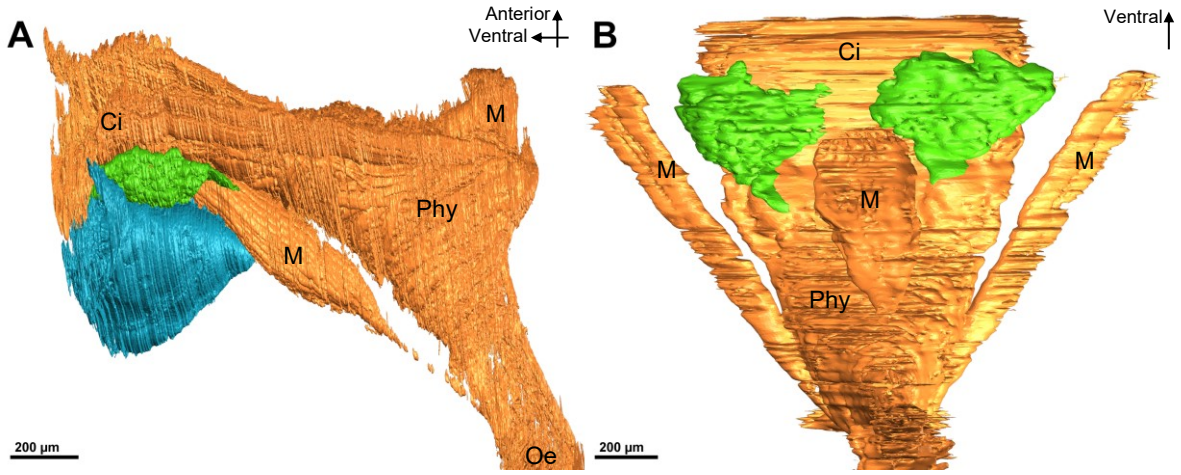


Figure 13: Infrabuccal pocket (blue) and hyperpharyngeal gland (green) of *Vespula germanica.* Alimentary canal and attached muscles marked in orange. Reconstruction of semithin sections; A: Lateral view, B: posterior view, Abbreviations: Ci: Cibarium, M: Muscle, Phy: Pharynx, Oe: Oesophagus

The hypopharyngeal glands consists of two distinct patches of glandular tissue, each roughly 400 µm long and 400 µm wide and 100 µm high (n = 2) (Fig. 12 D, 13). The sitophore lies posterior of the hypopharyngeal lobe and roughly trapezoid in shape (Fig. 11 E). It is covered with smooth cuticular plates. Especially in the posterior area is funnel-shaped present, with spined and hair-like microtrichia (Fig. 11 E). Laterally, there are patches of ~ 100 peg shaped sensilla that are arranged in a distinct elevated area of the sitophore (Fig. 11 F).

## Discussion

The filtration of liquid fluids in Vespidae has been studied extensively for the first time. The filtration of particles has been assumed repeatedly in Vespidae but in contrast to Formicidae, it hasn't been tested yet (Billen & Wilson, 2008; Duncan, 1939; Eisner & Happ, 1962; Glancey et al., 1981; Quinlan & Cherrett, 1978). In this study it could be showen that two separate filtration steps are performed during liquid uptake in Vespidae.

### First filtration step

Liquids are taken up at first either by licking movements of the glossa, using the spatula-shaped microtrichia to which fluids adhere, or by dipping the tip of the wasp proboscis directly into the liquid (Baranek et al., 2018; Duncan, 1939; Kirmayer, 1909; Krenn et al., 2005). The entrance of the wasp proboscis, which is formed by the labio-maxillary complex, is presumably the region where bigger particles are filtered in the first filtration step. When the liquid passes the opening between the galea and hypopharynx, particles bigger than 212 µm are prevented from entering the preoral cavity. Especially on the median side of the galea are pecten and sensilla are present, which could be important for the first filtration process.

The necessary suction force is provided by the cibarium, the cibarial dilator, and the pharynx with the pharyngeal dilator (Duncan, 1939; Snodgrass, 1942). These muscles dilate the pre pharynx or pharynx, which produces the force, which probably sucks liquids in a straight path from the mouthparts toward the suction force producing organs (Duncan, 1939; Snodgrass, 1942). To ensure any spillages of fluids during this process, several sealing structures are present. When the Mouthparts are extended and folded to the "wasp proboscis" the epiharynx connects to the maxilla via two bars, the epipharyngeal bar and the lacinial bar. The maxilla connects to the hypopharynx via rows of pecten, the galeal pecten and the hypopharyngeal pecten but probably also smooth lateral surfaces are pressed together to ensure a tight seal (Duncan, 1939). When the proboscis is extended the hypopharynx is possibly concave depressed, enlarging the volume of the preoral cavity. The cucticular surface of the proboscis probably helps to guide the liquid.

This first filtration step is especially important when rasping fruit flesh or sucking haemolymph of captured prey, as bigger particles are easily present during these tasks and could disrupt the fluid uptake (Edwards, 1980; Matsuura & Yamane, 1990; Richter, 2000; Spradbery, 1973; Weyrauch, 1939).

## Second filtration step

As the liquid is transported though the pre-oral chamber it passes the functional mouth opening, where a second filtration step takes place. Glass beads which have been detected in the infrabuccal pocket were most probable filtered at this position and transported into the pouch. This second filter might be critically important to avoid blockages of the alimentary canal and its narrow sections close to the tentorial bridge the petiolus and a build up of undigestable particles in the crop (Duncan, 1939; Edwards, 1980; Janet, 1895).

Experimental results indicating that even when glass particles could not be reliably filtered, a bigger amount was still prevented from entering. It might be especially important to avoid higher amounts of particles, as these could clump and form bigger sized blockages in the alimentary canal (Eisner & Happ, 1962; Janet, 1895).

The results are similar to the results from Eisner & Happ (1962) who described that particle sizes of 200-300 µm have been expelled by the mouthparts of *Camponotus pennsylvanicus* (De Geer, 1773), Particles smaller than 150 µm were only found in the infrabuccal pocket and smaller than 100 µm were found both in the infrabuccal pocket and the crop. This is very similar to the tested worker of *Vespula germanica* although they are bigger than *Camponotus pennsylvanicus*. It is assumed that the filtration mechanism is the same in wasps and ants.

The smaller *Acromyrmex octaspinosus* (Reich, 1793) and *Solenopsis invicta* Buren, 1972 were found to filter even smaller sizes of 10 and > 0.88 µm, however this could be linked to the bodysize of the animal (Glancey et al., 1981; Quinlan & Cherrett, 1978). Is also quite possible that the different lifestyles, lead to morphological differences, which might explain the differences between various Formicidae (Eisner & Happ, 1962; Glancey et al., 1981; Quinlan & Cherrett, 1978). Also different study methods might lead to different results, as different solid objects were used. It would be interesting to test in future studys, if different lifestyles in Vespidae or Hymenoptera in general can be reflected in filtration capabilities under the same experimental conditions.

The morphological results lead to the following functional interpretation. The epipharyngeal oral pecten can be assumed as the primarily filtration structures, as it has been discussed in the past (Duncan, 1939; Edwards, 1980; Janet, 1895; Kirmayer, 1909). The microtrichia of the epipharyngeal wall and hypopharyngeal lobe are directed against the current of the inflowing fluid, towards the infrabuccal pocket. It is likely that in various positions these hair-like structures are interlinking each other, forming a dense seve, which would assist the oral pecten.

Obviously Vespidae are able to move the hypopharyngeal lobe in dorso – ventral, that would offers an explanation how the filtered particles are transported from the oral pecten into the infrabuccal pocket. By this up and down movement, particles are combed ventrally towards the entrance of the infrabuccal pocket which itself is similarily covered with rows of microtrichia, that are directed further into the infrabucal pouch. If this movement of the hypopharyngeal lobe is continued the orientation of the hairlike microtrichia would push the particles towards the functional mouthopening or further into the infrabuccal pocket.

This would not only allow the animal to gather undigestible material bigger 152 µm outside the alimentary canal and avoid blockages but also keeps the filtrations structures clean and functional during continues feeding. This movement is probably performed during each pumping cycle of the cibarium and pharynx (Vilhelmsen, 1996). It can be assumed that the microtrichia support the filtration of particles similar as discussed for the infrabuccal entrance in Formicidae (Wang et al., 2018). The sensilla present in the epipharyngeal wall might help to control fluid transport and the filtering process.

## Infrabuccal pocket

In both, Formicidae and Vespidae, the pouch is used to gather, compress and glue together the particles prior to be expelled as pellets termed “corpuscules de nettoyage” by Janet (1905) (Duncan, 1939; Gotwald, 1969; Kirmayer, 1909). The cuticula of the infrabuccal pocket in *Ectomomyrmex javanus* (Formicidae) shows distinct surface adaptations within the infrabuccal pocket forming different sized hair (Wang et al., 2018). These hairs are oriented towards the opening of the infrabuccal pocket and the size reduces from the entrance into the pocket (Wang et al., 2018). A similar situation can be observed in eusocial Vespidae.

It has been previously discussed that the infrabuccal pocket in Formicidae is used for filtration and liquids are first transferred into the infrabuccal pocket bevor they are transported further into the cibarium (Hansen et al., 1999; Wang et al., 2018). With the present results in Vespidae, which show a very similar morphological arrangement in the cibarial area, I can’t support this assumption. The arrangement of the microtrichia especially at the epipharyngeal wall rather supports that filtration is performed at the oral pecten. A guidance of the filtered particles away from the functional mouth opening, and a storage of particles inside the infrabuccal pocket is assumed as it has been briefly discussed before in Vespinae (Edwards, 1980) but also in Formicidae (Glancey et al., 1981). As fluids are transported by the suction force from the opening of the wasp proboscis into the pharynx, it seems unlikely that this current is reguided towards the infrabuccal pocket. When wasps were fed with liquid contrast agent, only small quantities of it were found in the infrabuccal pocket and only small portions

of the infrabuccal pocket were stained, while the majority was transported into the posterior alimentary tract. If any filtration would happen actively inside the infrabuccal pocket, I would expect more barium sulfate in the infrabuccal pocket. No musculature could be found that could possibly explain suction into the infrabuccal pocket as well as a expelling. Therefore, it is unlikely that it is used actively for filtration during continues feeding as it has been assumed (Hansen et al., 1999; Wang et al., 2018). This lack of musculature raises the question of how the particles, which are collected in the infrabuccal pocket, are expelled after feeding. Many reports of expelled pellets are known, suggesting that collected particles are compressed and formed into a tight pellet, possibly under the use of a glandular substance (Glancey et al., 1981; Janet, 1895). As no muscles are attached, I assume that this is performed by the use of haemolymph pressure. A combination with a movement or position of the labio-maxillary complex seems also likely as it is an invagination of the hypopharynx and tightly connected to the apical part. This could be performed in combination with increased haemolymphic pressure.

The content of the infrabuccal pocket has been described in Vespinae as “dirt” gathered during cleaning. (Duncan, 1939; Kirmayer, 1909) In ants it has been shown that the infrabuccal pocket is not only used to store dirt but also to transport fungi in the Attini and even be lined by a glandular epithelium providing enzymes for digestion (Billen et al., 2013; Billen et al., 2015; Eelen et al., 2006; Mueller et al., 2001; Wang et al., 2018). The epithelial lining could contain glandular cells, similar as reported for Ectomomyrmex javanus Mayr, 1867 or *Monomorium pharaonis* Linnaeus, 1758 (Eelen et al, 2004; Wang et al., 2018). As a clear visual description is lacking, a reliable comparison is not possible. Primarily a storage function of the infrabuccal pocket is assumed to gather particles during the liquid uptake. Resulting in a continuous and efficient fluid feeding process. The function of the glandular excretion are hypothesised to be important to form a pallet that subsequently can be ejected (Eelen et al., 2004).

## Glass beads and barium sulfate

The use of glass beads and barium sulfate in feeding experiments proved to be an efficient and affordable way to stain the alimentary tract or to track particles inside the alimentary tract. The biggest problem of this method is that especially bigger glas beads are sedimenting fast in their solution. To counter this, only single drops were presented, which ensured that the wasps pushed their mouthparts into the suspended glass beads. The recognition of the glass beads inside the body was easy and clear. Even the separation of both glass and barium sulfate was easy in most cases, as the structure of both in X-ray pictures was clearly different. Barium sulfate stained the alimentary canal at least for the investigated time periode, which could be interesting in various applications. If this effect is permanent can't be judged from the results. The animals didn't seem to recognise both substances by chemical senses, as no avoidance behaviour has been observed. Small preliminary tests also showed that a wasp would take up a glass - barium sulfate - sugar water freely if placed in a bigger glas with a presentation table placed in the middle. Overall the testing of filtration properties and functions with glass beads as a substitute for solid objects and the barium sulfate to trace fluids seems to be a easy to use method to study uptake of fluids contaminated by particles.

## Conclusion:

I was able to show that eusocial Vespidae filter solid particles from liquids and do this in two separate steps. The first step occurs at the entrance of the wasp proboscis preventing particles bigger than 212 µm from entering. The second step occurs at the functional mouth opening reliably filtering particles bigger 152 µm, smaller ones were also filtered but not reliably.

Morphological adaptations are present, especially at the second filter with the oral pecten and microtrichia as a filtration system. The microtrichia probably ensure the transport of filtered particles into the infrabuccal pocket, which serves as a receptaculum to store filtered particles.

Therefore the infrabuccal pocket fulfills a storage function, as the fed glass beads where accumulating in the infrabuccal pocket separated from the liquid barium sulfate. The mechanism of emptying is still unclear as no muscles could be found, but a combination of specific movements of the labio–maxillary complex and increased haemolymphic pressure is assumed to empty the infrabuccal pocket.

## References


Baranek, B., Kuba, K., Bauder, J., & Krenn, H. (2018). Mouthpart dimorphism in male and female wasps of *Vespula vulgaris* and *Vespula germanica* (Vespidae, Hymenoptera). *Deutsche Entomologische Zeitschrift*, *65*(1), 65–74. https://doi.org/10.3897/dez.65.23593

Billen, J., Bauweleers, E., Hashim, R., & Ito, F. (2013). Survey of the exocrine system in *Protanilla wallacei* (Hymenoptera, Formicidae). *Arthropod Structure and Development*, *42*(3), 173–183. https://doi.org/10.1016/j.asd.2013.01.001

Billen, J., Mandonx, T., Hashim, R., & Ito, F. (2015). Exocrine glands of the ant *Myrmoteras iriodum*. *Entomological Science*, *18*, 167–173. https://doi.org/10.1111/ens.12107

Billen, J., & Wilson, E. O. (2008). Social insect histology from the nineteenth century: The magnificent pioneer sections of Charles Janet. *Arthropod Structure and Development*, *37*, 163–167. https://doi.org/10.1016/j.asd.2007.07.002

Davey, K. G., & Treherne, J. E. (1963). Studies on crop function in the cockroach (*Periplaneta americana* L.). *Journal of Experimental Biology*, *40*, 763–773. https://doi.org/https://doi.org/10.1242/jeb.40.4.763

Duncan, C. D. (1939). *A Contribution to the biology of North American Vespine Wasps*. Stanford: Stanford University Press.

Edwards, R. (1980). *Social Wasps Their biology and control* (1st ed.). East Grinstead: Rentokil Limited.

Eelen, D., Børgesen, L., & Billen, J. (2006). Functional morphology of the postpharyngeal gland of queens and workers of the ant *Monomorium pharaonis* (L .). *Acta Zoologica*, *87*, 101–111. https://doi.org/https://doi.org/10.1111/j.1463-6395.2006.00212.x

Eelen, D., Børgesen, L. W., & Billen, J. (2004). Morphology of a novel glandular epithelium lining the infrabuccal cavity in the ant *Monomorium pharaonis* (Hymenoptera, Formicidae). *Arthropod Structure and Development*, *33*, 471–475. https://doi.org/10.1016/j.asd.2004.08.001

Eisner, T., & Happ, G. M. (1962). The Infrabuccal Pocket of a Formicine Ant: a Social Filtration Device. *Psyche*, *69*(3), 107–116. https://doi.org/10.1155/1962/25068

Glancey, B. M., Vander Meer, R. K., Glover, A., Lofgren, C. S., & Vinson, S. B. (1981). Filtration of microparticles from liquids ingested by the red imported fire ant *Solenopsis invicta* Buren (*). *Insectes Sociaux*, *28*(4), 395–401. https://doi.org/https://doi.org/10.1007/BF02224196

Gotwald, W. H. J. (1969). *Comparative Morphological Studies of the Ants, with Particular Reference to the Mouthparts (Hymenoptera: Formicidae)*. New York: Cornell University.

Greene, A. (1991). Dolichovespula and Vespula. In K. G. Ross & R. W. Matthews (Eds.), *The social biology of wasps* (pp. 263–305). New York: Cornell University Press.

Hammer, Ø., Harper, D. A. T., & Ryan, P. D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, *4*(1), 1–9.

Hansen, L. D., Spangenberg, W. J., & Gaver, M. M. (1999). The infrabuccal chamber of *Camponotus modoc* (Hymenoptera: Formicidae): ingestion, digestion and survey of bacteria. In W. H. Robinson, F. Rettich, & G. W. Rambo (Eds.), *3rd International Conference on Urban Pests* (pp. 211–219).

Hunt, J. H. (1991). Nourishment and the evolution of the social Vespidae. In K. G. Ross & R. W. Matthews (Eds.), *The social biology of wasps* (pp. 426–450). London: Cornell University Press. https://doi.org/10.1002/14651858.CD003191.pub2

Janet, C. (1895). *Études Sur Les Fourmis, Les Guêpes Et Les Abeilles. 9, Sur "Vespa Crabro" L. : Histoire D'un Nid Depuis Son Origine. Mémoires de la Société Zoologique de France 8*. Paris.

Kasper, M. L., Reeson, A. F., Cooper, S. J. B., Perry, K. D., & Austin, A. D. (2004). Assessment of prey overlap between a native (*Polistes humilis*) and an introduced (*Vespula germanica*) social wasp using morphology and phylogenetic analyses of 16S rDNA. *Molecular Ecology*, *13*, 2037–2048. https://doi.org/10.1111/j.1365-294X.2004.02193.x

Kirmayer, R. (1909). Bau und Entwicklung der Mundteile bei *Vespula vulgaris*. In G. Ruge (Ed.), *Gegenbaurs Morphologisches Jahrbuch* (39 Band, pp. 1–31). Leipzig: Verlag von Wilhelm Engelmann.

Krenn, H. W., Mauss, V., & Plant, J. (2002). Evolution of the suctorial proboscis in pollen wasps (Masarinae, Vespidae). *Arthropod Structure and Development*, *31*, 103–120. https://doi.org/10.1016/s1467-8039(02)00025-7

Krenn, H. W., Plant, J. D., & Szucsich, N. U. (2005). Mouthparts of flower-visiting insects. *Arthropod Structure and Development*, *34*, 1–40. https://doi.org/10.1016/j.asd.2004.10.002

Lösel, P. D., Kamp, T. Van De, Jayme, A., Ershov, A., Faragó, T., Pichler, O., … Heuveline, V. (2020). Introducing Biomedisa as an open-source online platform for biomedical image segmentation. *Nature Communications*, *11*(5577), 1–14. https://doi.org/10.1038/s41467-020-19303-w

Matsumoto, Y., Menzel, R., Sandoz, J., & Giurfa, M. (2012). Revisiting olfactory classical conditioning of the proboscis extension response in honey bees : A step toward standardized procedures. *Journal of Neuroscience Methods*, *211*, 159–167. https://doi.org/10.1016/j.jneumeth.2012.08.018

Matsuura, M., & Yamane, S. (1990). *Biology of the Vespine Wasps*. Berlin: Springer Verlag. https://doi.org/10.1007/978-3-642-75230-8

Mauss, V., Kuba, K., & Krenn, H. W. (2019). Evolution of the Multifunctional Mouthparts of Adult Vespidae. In H. W. Krenn (Ed.), *Insect Mouthparts Form, Function, Development and Performance* (pp. 443–478). Cham, Schwitzerland: Springer Nature Switzerland.

Metscher, B. D. (2009). MicroCT for comparative morphology: Simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues. *BMC Physiology*, *9*(11), 1–14. https://doi.org/10.1186/1472-6793-9-11

Mueller, U. G., Schultz, T. R., Currie, C. R., Adams, R. M. M., & Malloch, D. (2001). The origine od Attini ant-fungus mutualism. *The Quarterly Review of Biology*, *76*(2), 169–197. https://doi.org/https://doi.org/10.1086/393867

Pernstich, A., Krenn, H. W., & Pass, G. (2003). Preparation of serial sections of arthropods using 2, 2-dimethoxypropane dehydration and epoxy resin embedding under vacuum. *Biotechnic & Histochemistry*, *78*(1), 1–5. https://doi.org/https://doi.org/10.1080/10520290312120002

Porto, D. S., & Almeida, E. A. B. (2019). A comparative study of the pharyngeal plate of Apoidea (Hymenoptera: Aculeata), with implications for the understanding of phylogenetic relationships of bees. *Arthropod Structure and Development*, *50*, 64–77. https://doi.org/10.1016/j.asd.2019.04.002

Quinlan, R. J., & Cherrett, J. M. (1978). Studies on the role of the infrabuccal pocket of the leaf-cutting ant *Acromyrmex octospinosus* (Reich) (Hym., Formicidae). *Insectes Sociaux*, *25*(3), 237–245.

Richter, M. R. (2000). Social Wasp (Hymneoptera : Vespidae) Foraging Behavior. *Annual. Review of Entomology*, *45*, 121–150.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., … Cardona, A. (2019). Fiji : an open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682. https://doi.org/10.1038/nmeth.2019

Seifert, G. (1995). *Entomologisches Praktikum* (3rd ed.). Stuttgart: Georg Thieme Verlag.

Silveira, O. T., & Dos Santos Junior, J. N. A. (2011). Comparative morphology of the mandibles of female polistine social wasps (Hymenoptera, Vespidae, Polistinae). *Revista Brasileira de Entomologia*, *55*(4), 479–500. https://doi.org/10.1590/s0085-56262011000400004

Snodgrass, R. E. (1910). The anatomy of the honey bee. *Technical Series*, *18*, 9–151.

Snodgrass, R. E. (1942). The skeleto-muscular mechanisms of the honey bee. *Smithsonian Miscellaneous Collections*, *103*(2), 4–32.

Spradbery, J. P. (1973). *Wasps; an account of the biology and natural history of solitary and social wasps.* Seattle: University of Washington press.

Vilhelmsen, L. (1996). The preoral cavity of lower Hymenoptera (Insecta): comparative morphology and phylogenetic significance. *Zoologica Scripta*, *25*(2), 143–170. https://doi.org/https://doi.org/10.1111/j.1463-6409.1996.tb00156.x

Wang, C., Billen, J., Pan, X., & He, H. (2018). Morphology and ultrastructure of the infrabuccal pocket and its lining epithelium in workers of *Ectomomyrmex javanus* (Hymenoptera: Formicidae). *Micron*, *115*, 50–53. https://doi.org/https://doi.org/10.1016/j.micron.2018.09.001

Weyrauch, W. (1939). Wie ein Wespennest entsteht. *Die Naturwissenschaften*, *4*, 4–57.

Zacharuk, R. E. (1985). Antenna and Sensilla. In G. A. Kerkut & L. I. Gilbert (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology Vol.6* (pp. 1–63). Elmsford, NY: Pergamon Press.

Zimmermann, D., & Vilhelmsen, L. (2016). The sister group of Aculeata (Hymenoptera) - evidence from internal head anatomy, with emphasis on the tentorium. *Arthropod Systematics and Phylogeny*, *74*(2), 195–218.