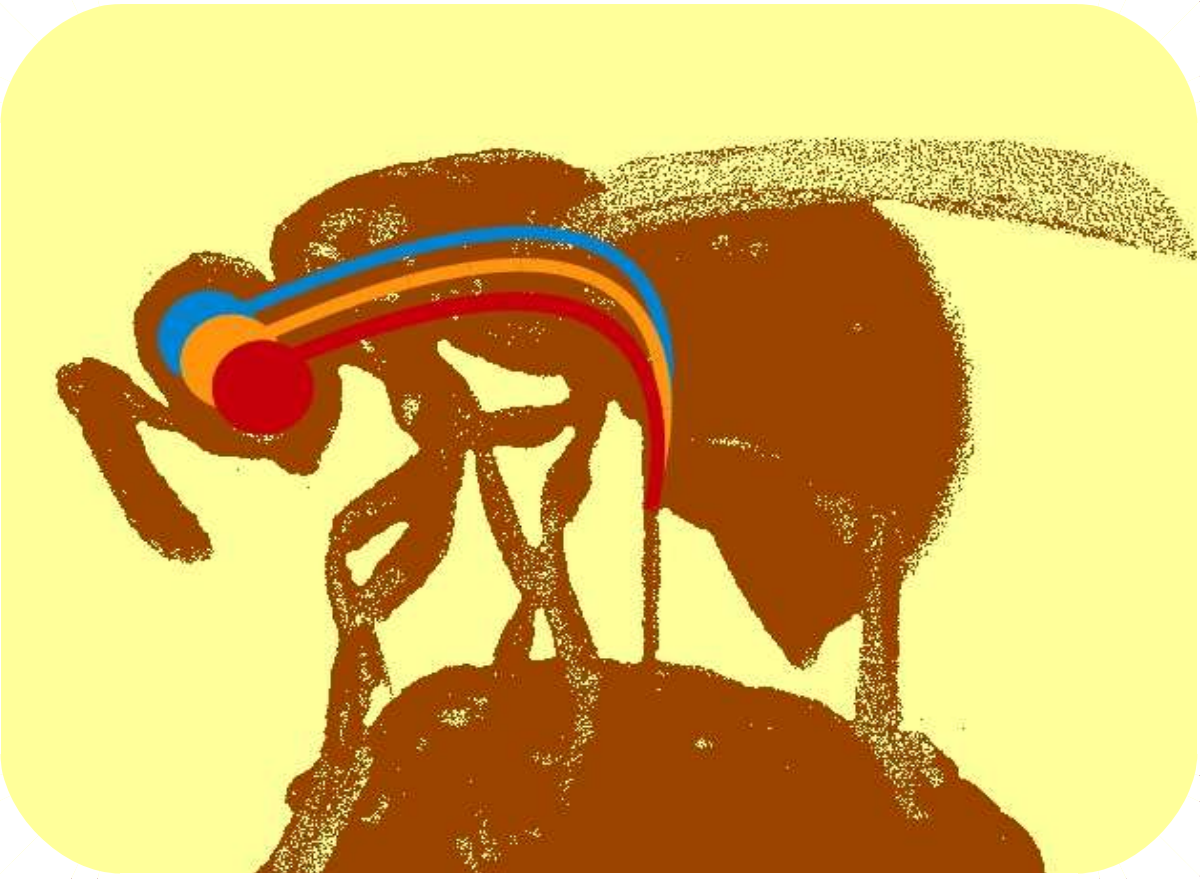
Wageningen University
Laboratory of Entomology

Brains insi(ght)de

Tracing structures and neurons in the brain of parasitic wasps



Alexander Haverkamp

WAGENINGEN UNIVERSITY
LABORATORY OF ENTOMOLOGY

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Tracing structures and neurons in the brains of parasitic wasps

No: 011.05
Name: Alexander Haverkamp
Study program: Plant Science
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1st Examiner; Prof. Dr. Marcel Dicke
2nd Examiner: Dr. Hans M. Smid

Preface

In the summer of 2009 I had the unique opportunity to participate in an applied research project studying the population dynamics and behaviour of the spruce web-spinning sawfly (*Cephalsia abietis*) in the Bavarian forest. During these observations I was amazed, but also puzzled by the their fixed behaviour patterns, forcing these tiny wasps to try the same strategies over and over. At the same time I was introduced to a current debate driving neurobiologists, psychologists as well as philosophers : leading neuroscientists, questioned our freedom of mind by proposing a biological determination of human behaviour. Quickly I was caught by the apparent parallels between my own dilettante observations on insects and these fundamental philosophical questions. Finally, the wish to extend my knowledge on the biological bases of behaviour combined with my love for insects became a major driving force to start my master at Wageningen University and for selecting a master thesis topic. At this point a would already like to thank Hans Smid and Katja Hoedjes for offering such an exciting thesis topic. For me personally, this thesis, was a chance to discover the biological realization of behaviour on the back of some of the most fascinating creatures.

Abstract

To unravel the evolution of the brain, across-species comparisons of neuronal tissues and behavioural traits offer exciting opportunities. In this report it was aimed to acquire information on the neurobiology of the parasitic wasp *Nasonia vitripennis* to provide a basis for further inter-species comparisons. Within this wider topic, a specific focus was on the neuronal pathway of ovipositing related, appetitive learning.

Through electrophysiological recordings on the receptor cells of the ovipositor one sensillum was found which yielded, promising results for further studies on host discrimination at a receptor cell level.

On the brain level, this study investigated the innervations and projections of reward-sensitive neurons as well as their interaction with punishment- sensitive neurons. These investigations were based on immuno-chemical labellings and yielded interesting results for both neuron types. Among the reward-sensitive neurons one prominent projection was found emerging from the suboesophageal ganglion and reaching towards the mushroom bodies in an 'umbrella-like' pattern. The main innervations of the reward-sensitive neurons were found in the Kenyon cells at the ventral-medial side of the mushroom body calyces. In contrast to this, the punishment-sensitive neurons did not innervate the Kenyon cells or calyces, but the pedunculi of the mushroom bodies. An overlap between punishment- sensitive neurons and reward- sensitive neurons was only found in the central body complex and the lateral horn. To provide a basis for further studies on these neurons, an activity dependent labelling technique was adapted, which yielded promising first results.

Besides the direct investigation of the reward pathways this study also aimed to create a general overview over the brain morphology of *N. vitripennis* by creating virtual reconstructions of five individual female brains. Within these reconstructions, eight paired and six unpaired neuropils were consistently labelled and their average volumes and surface areas were measured. Additionally one brain, which most closely resembled the average brain, was chosen as a preliminary standard brain. These three-dimensional reconstructions of the *Nasonia* brain not only provided a starting point for inter-species comparisons, but will also aid understanding of neuron projections and innervations.

Finally, the results acquired on neuron innervations and on the brain morphology of *Nasonia* were compared with information on other insect brains with a special focus on the honey bee. The comparison, between *Nasonia* and the honey bee revealed interesting differences in the innervation and projection patterns of the reward-sensitive neurons. These neurons strongly innervate the calyces in the honey bee, whereas only weak innervations were found in *Nasonia*. In contrast no major differences were found with regards to the punishment-sensitive neurons.

A comparison of brain morphology with the brains of three other insect species revealed relatively large antennal lobes and relatively small optic lobes in the *Nasonia* brain, which appeared to be correlated with the general behavioural ecology of *Nasonia.* Additionally, a more detailed comparison was made between the calyx shape of *Nasonia* and the shape of the honey bee calyx. These revealed a considerably less elaborated calyx in *Nasonia* although the for Euhymenopterans typical double structure of the lobes was clearly visible.

Combining the results outlined above, the following overall conclusions were derived in comparison with the honeybee:

The less elaborated shape of the calyces in the *Nasonia* brain is positively correlated with smaller optic lobes as well as less intensive innervations of reward- sensitive neurons, but negatively correlated with a relatively large volume of the antennal lobes.

On the basis of these correlations it is argued, that appetive, but not aversive, optical learning has driven the evolution of more elaborated calyces, whereas olfactory conditioning also utilises the antennal lobes.

Furthermore, different neuronal mechanisms for appetitive and aversive learning are proposed based on the different innervation and projection sides of these neurons in the *Nasonia* brain.

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1. General introduction

While searching for nectar and pollen, a bee faces a rather dynamic environment which changes not only over the season, but also with daytime and weather. Despite this, the bee can not only find a suitable flower in the first place but will also find its way back to the same flower again, even after several days and over many kilometres (Gould and Gould, 1988). Some parasitic wasps, however might even surpass the astonishing achievements of the bee. These wasps are capable of finding their tiny hosts on the downside of a leaf or even inside of a bird nest, which might by itself already be hidden in a tree cavity. Furthermore, these wasps are not only able to find their hosts, but some of them are even capable of monitoring egg clutches, which will only in future become suitable hosts (van Nouhuys and Kaartine, 2008). To do so these wasps do not only learn to associate the presence of hosts with certain odours but also to form spatial memory of the host position (Vet *et al.*, 1995, van Nouhuys and Kaartine, 2008). These achievements of bees and wasps, but also of other insects, appear already quite impressive, if we would image them as a task for our own mind. Hence, they become even more astonishing when considering the minute brains of these insects. The brain of the bee contains approximately 950,000 neurons; still it is able to remember at least 700 flower odours (Witthöft, 1967; Schoonhoven, *et al.* 2005), an achievement that might even challenge the 100 billion neurons of a human brain (Carlson, 2010).

1.1 Two parasitoids and their behavioural ecology

Besides the well-known honeybee, parasitic wasps, such as *Nasonia vitripennis* (Fig. 1) and *Cotesia glomerata* (Fig. 2), provide maybe less known, but not less exciting examples of insect learning in a complex environment. Both wasp species are gregarious endoparasitoids, meaning that they place several eggs inside of their hosts. In the case of *N. vitripennis*, the wasp uses cocoons of nearly all fly species of the families *Calliphoridae* and *Muscidae* that regularly occur in bird nests (Peters and Abraham, 2010). *C. glomerata* parasitizes the larvae of the cabbage with butterflies *Pieris brassicae* and *Pieris rapae* (Feltwell, 1982). During their search for a suitable host, the females of both wasp species face the challenge that the hosts, both fly pupae as well as caterpillars, have evolved to be as undetectable as possible (Schoonhoven *et. al.*, 2005). To overcome these difficulties the wasps developed the ability to learn to associate certain odours with the presence of their hosts (Vet and Dicke, 1992).

Fig. 1: *Nasonia vitripennis*

Fig. 2: *Cotesia glomerata*

1.2 Basis paradigms of learning.

Wasps can acquire associations between odour and host finding by classical or Pavlovian conditioning (Hoedjes *et al*, 2011). This learning paradigm basically predicts that after combining a neutral stimulus with a meaningful, unconditioned stimulus (US), the neutral odour will be learned and finally become a newly conditioned stimulus (CS), which then gives rise to the behaviour that was previously triggered by the US (Kupferman, 1991). In the case of *C. glomerata* and *N. vitripennis* the neutral odour would be any odour present on the plant or in the bird nest when the wasp encounters its host. The US would then be experienced through the encountering of a host, followed by an oviposition experience. Subsequently, the previously neutral odour that was present in the nest would be learned and become the novel CS. Remarkably: the wasps remember these associations for several days and sometimes even after a single learning trial (Smid *et al.* 2006).

1.3 Spatial learning and abstract thinking

Even though the high learning rates of wasps and bees appear impressive, they still represent rather simple forms of memory, since they only require a connection between one stimulus and an second stimulus or between a stimulus and a certain behaviour (Giurfa, 2003). However, if the general foraging behaviour of a bee or a wasp is considered it quickly becomes evident that such a straight forward connection between two different stimuli will not always be sufficient to solve complex searching tasks (van Nouhuys and Kaartinen, 2008). However, for many hymenopterans abilities for complex spatial learning and pattern recognition have been shown (Tinbergen, 1972; Menzel *et al*. 2005, Fukushi and Wehner, 2004). A classical example for these more sophisticated forms of learning can be found in the elegant experiments by Tinbergen (1972). Digger wasps collect prey and store it in an underground nest in which the wasp later on lays its eggs. Tinbergen (1972) placed a ring of pin cones around the nest of the wasp and observed that the wasp was always searching within this circle, even when

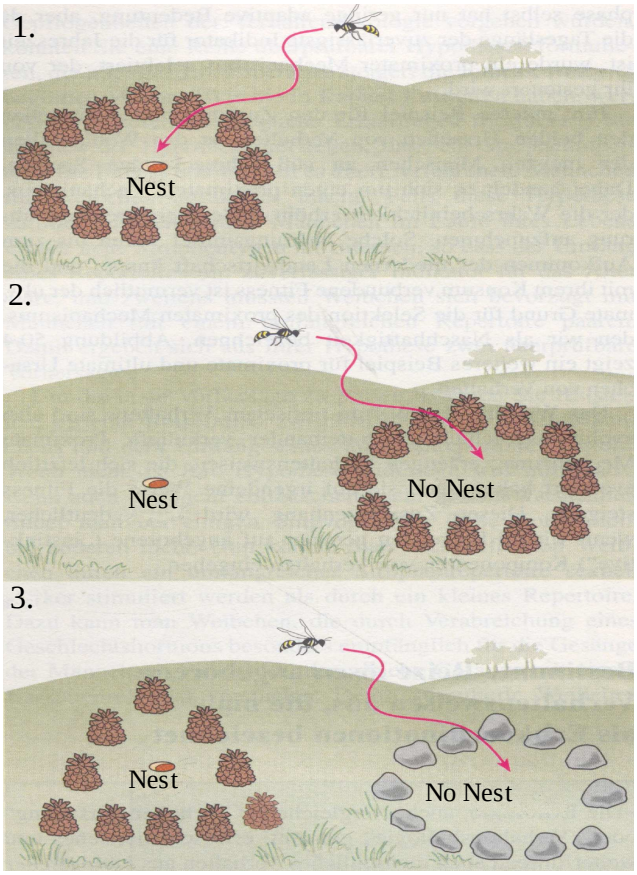

Fig. 3: Spatial learning and image abstraction, in homing digger wasps, further explanation see text
(adapted from Campbell, 2000)

Tinbergen moved the pin cones away from the original position. However, when the pin cones surrounding the nest were restructured into a triangle and a ring of stones was placed next to it, the wasp would search within the circle of stones (Fig. 3). By these simple experiments Tinbergen (1972) not only demonstrated that digger wasps were able to learn spatial landmarks, but also that they were able to abstract visual input into geometrical figures.

Summarizing, the finding on the host and food searching behaviour of wasps and bees, it appears that both olfactory conditioning as was as spatial learning are utilized (Hoedjes et al 2011, van Nouhuys and Kaartinen, 2008, Timbergen, 1972). Nevertheless, one might expect that different species use these to forms of learning to very different degrees.

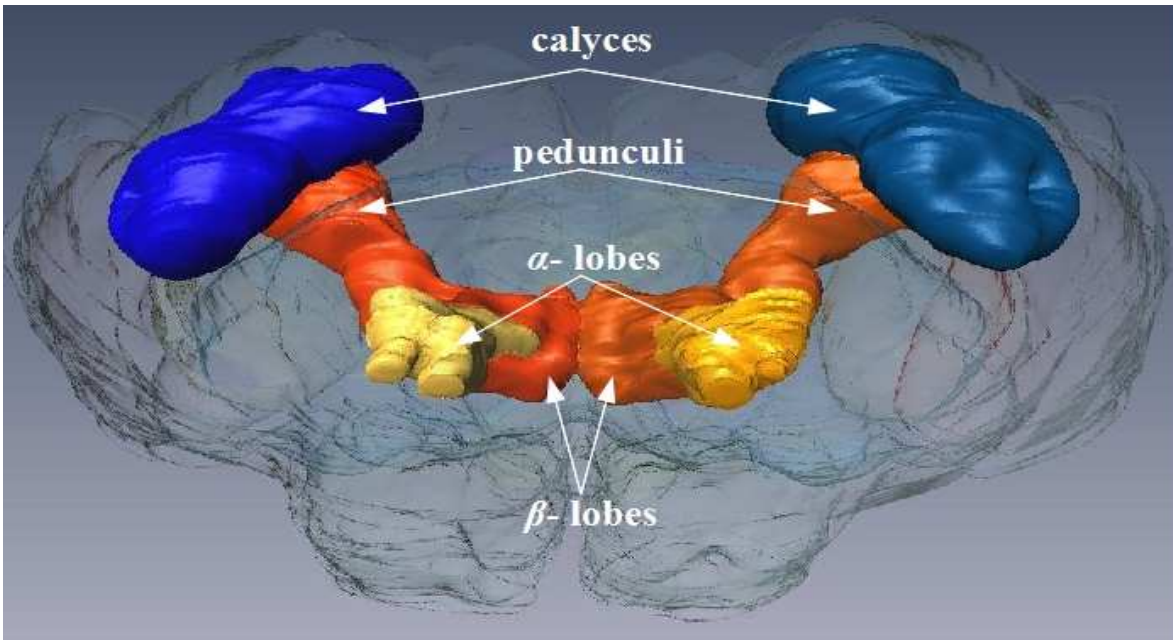

Fig. 4: Frontal view on a 3D reconstruction of the *Nasonia vitripennis* mushroom bodies. The outline of the entire brain is shown in transparent

1.4 The neuronal substrates of learning

For mammals, it has been demonstrated, that spatial memory is encoded in specific cells, so called 'place' cells within the hippocampus of the rat brain (O'Keefe and Speakman, 1987). The Mushroom bodies (MBs) are assumed to be the neural equivalent of the hippocampus in the insect brain (Hourcade *et al.*, 2010). The MB are formed by a group of extremely densely packed neurons called Kenyon cells, the neurits of the Kenyon cells form the different structures within the MB: the axons build up the pedunculi, the α- lobes and the *β*- lobes, whereas the dendrites of the Kenyon cells form the calyces (Mobbs, 1982) (Fig. 4). All neuropils of the MB receive input from the primary neuropils as well as from the reward- sensitive and punishment- sensitive neurons (Mobbs, 1982). However, in contrast to the other three neuropils the calyces show different sub-compartments for olfactory input than for visual information (Farris, 2008). This characteristic property pinpoints the

calyx as an important brain part for the integration of different stimuli and recent studies have also argued that these neuropils may provide the neural substrate for spatial learning (Farris and Schulmeister, 2011), assuming functional similarities between Kenyon cells and the 'place'- cells know from the rats. However, such a mechanism for spatial learning appears to be extremely costly in terms of neuron investment and it can be expected that this mechanism would be under a high evolutionary pressure (Chittka and Niven, 2009). Interestingly, olfactory conditioning appears to be rather independent of the calyces and to rely more on the antennal lobes (AL) (Malun *et al*. 2002). These apparent links between certain types of learning and certain neuropils offer wide opportunities for studies comparing both brains structures as well as the behavioural ecology of different species.

1.5 Has spatial learning driven calyx evolution?

Social Hymenopterans such as the honeybee and ants posses relatively enlarged and elaborated calyces, which had led to the conclusion that these neuropils must be related to social behaviour (Durjardin, 1850; Gronenberg and Riveros, 2009). However, in a recent survey Farris and Schulmeister (2011) analysed the calyces of 24 non- social and three social hymenopteran species, but no differences related to social behaviour were found. Besides social behaviour this study also focused on the optic input to the calyces. Interestingly, they found that the calyx shape and the relative volume was strongly enlarged in those species where optic input into the calyces was found. From this it was concluded that optic input, but not social behaviour was important for the evolution of elaborated calyces. Previous studies have pointed at the role of the calyces during more complex learning tasks (Devaud, 2007; Strausfeld *et al*., 2009). With reference to these studies Farris and Schulmeister (2011) argued that not only input of optical information into the calyces, but even more importantly, spatial learning has driven the evolution of more elaborated calyces. However, no study so far has investigated the correlation of learning related neuron innervations and calyx shape and volume.

1.6 Dopamine and octopamine trigger memory formation.

Simple learning patterns are based on the connection of the US with a neutral stimulus, which will then become the CS (Kupferman, 1991). At the neural level the US is represented by the reward- or punishment sensitive neurons whereas the neutral stimulus is delivered by neurons of the sensory pathways (Kandel, 2006). In insects, reward-sensitive neurons express the neuromodulator octopamine, whereas punishment-sensitive neurons express the neuromodulator dopamine (Schwaerzel *et al.* 2003). In a landmarking study Hammer (1993) revealed one octopaminergic neuron in the honeybee, termed ventral medial unpaired maxillary neuron 1 (VUMmx1), which,

when activated directly induced memory formation in a simple conditioning trail. VUMmx1, mainly innervates the antennal lobes (AL), the lateral horn (LH) and especially the MB calyces. Similar to VUMmx1, Aso *et al.* (2010) found one dopaminergic neuron, which was able to induce aversive memory in the fruit fly *Drosophila melanogaster* when activated. Due to this close connection between memory formation and octpaminergic and dopaminergic neurons, strong innervations of these neurons can be taken as a rather safe predictors for memory related processes at the innervated brain parts. Similarly, it can also be expected that the shape and the volume of a certain brain region influences the learning pattern of an insect (Rein *et al*. 2002). Unfortunately, no study so far has directly related the innervations of reward- and punishment- sensitive neurons to neuropil shape and volume as well as to the behavioural ecology of an insect. In the study presented over the following chapters, it was aimed to partly fill this gap, by using images of single neurons as well as three-dimensional neuropil reconstructions.

1.7 Standardising a brain

One particular reason why correlations between neuropil shape and innervation patterns of neurons are still sparse could be due to difficulties in grasping the general shape of a brain or a neuropil. However over the last years, more sophisticated computation tools as well as advancements in immuno-labelling enabled the generation of standardized, three-dimensional (3D) brain reconstructions (Rein *et al.* 2002; Brandt *et al.* 2005). Such virtual reconstructions allow the averaging of neuropil and brain shapes over several individual brains and thereby generate standardized models, free of individual shape differences (Rein *et al.* 2002). At the same time, virtual, standard brains also allow the combination of studies made on different individual brains (Rybak *et al.* 2010). Especially the tracing of single neurons is often performed by a different technique than the labelling of neuropils (Kurylas *et al.* 2008), which further hampers studies relating neuron innervations to neuropil shape. In these cases, virtual brain reconstructions can provide a general platform for combining information on neuropils and on single neurons (Brandt *et al.,* 2005). Unfortunately, standardised brains are so far only available for a few well-studied model species, which belong to very distant insect genera (el Jundi *et al* 2009). This limitation has so far hampered the use of virtual reconstructions for comparative studies and the availability of more standardized brains of more closely related species is required to overcome this struggle. Nevertheless virtual brains enable the combinations of studies on neuron innervations as well as the shape and volume of neuropils. Through this virtual insect brains offer great potentials for new insight into brain evolution and function, using a comparative approach (el Jundi *et al* 2009).

1.8 Aims and outline

One aim of this study was to provide a 3D virtual, standardised brain of the wasp *Nasonia vitripennis.* At the same time, it was also intended to describe the pathways of the rewarding stimulus from the receptor cells on the ovipositor on to the higher-order neuropils in the brain. In addition, it was also aimed to give an overview of punishment-sensitive neurons in the brain of *Nasonia vitripennis.*

This combination of a 3D virtual brain and detailed information on neuronal innervations enable a direct approach towards the question how different forms of learning might have influenced the shape and volume of different neuropils, such as the antennal lobes and the mushroom body calyces. Using additional information from Brandt *et al* (2005) on the honeybee brain, a comparison between *Nasonia* and the bee will be performed to further investigate the factors, that may have driven the evolution of the antennal lobes and the calyces. This analysis not only takes the relative volumes of the primary olfactory and optical neuropils into account, but will also consider the innervation patterns of reward- and punishment- sensitive neurons and will thereby allow a first conclusion on how different forms of learning have influenced the shape of a brain.

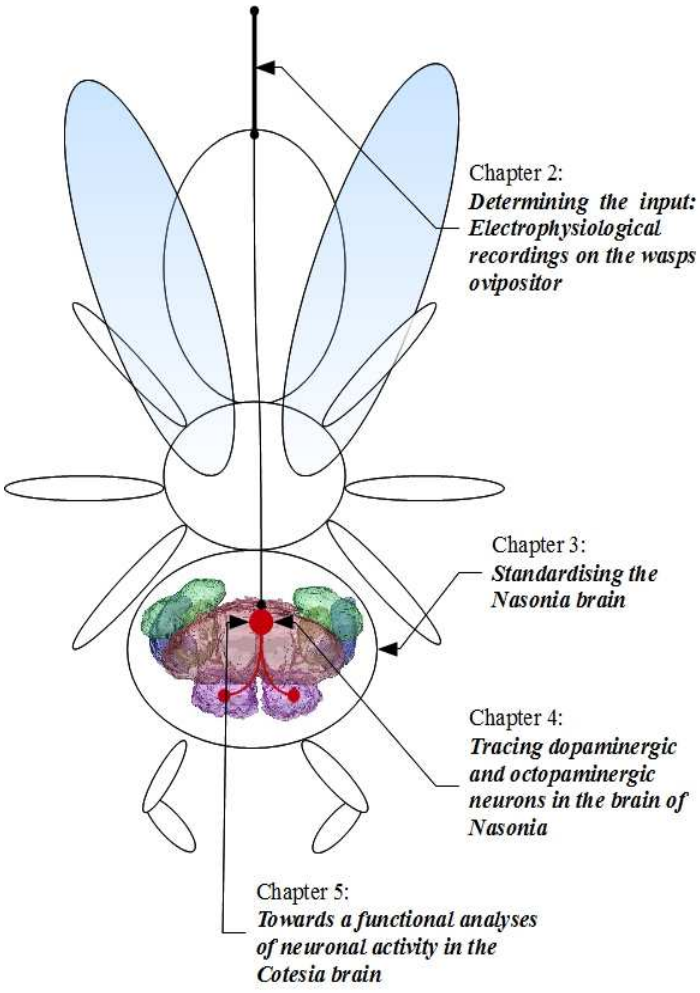

Fig. 5: Schematic outline

2. Determining the input: Electrophysiological recordings on the wasp's ovipositor

Abstract

Perception of the environment provides the input for all higher cognitive processes. In this chapter an attempt was made to investigate the host-perception by the taste cells on the ovipositor of parasitoids and thereby to provide a basis for further studies on memory formation in these wasps. Problems in handling the host haemolymph as well as difficulties in localising the ovipositor taste sensilla (OTS) are addressed. A practical method was found to stimulate these OTS by using capillaries filled with the host haemolymph and coated with phenylthiourea. This coating prevented haemolymph oxidation and considerably improved recordings of electrophyisiological signals produced by the OTS. Furthermore, even though no fully reliable method for locating the single OTS was developed, the assumed position of a single basiconic sensillum was shown to yield a reasonable quality of recordings. Finally, these findings are supported by preliminary results for the OTS of *Cotesia glomerata.*

2.1 Introduction

Learning rate in parasitic wasps has recently been found to be strongly dependent on the quality of the reward (Collatz *et al*. 2006; Kruidhof *in preparation*). In addition to this, several species of parasitoids have demonstrated the ability to assess the quality of a host even on the basis of single molecules in the host haemolymph (Gauthier *et al.*, 2004). However, the mechanism by which a host and therefore a reward is perceived and subsequently stored into memory remains still largely unknown (van Lenteren *et al.*, 2007; Giurfa, 2007).

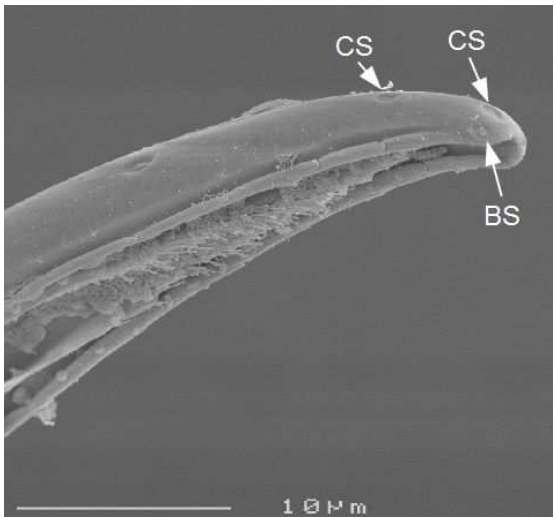

Fig. 6: Scanning electron microscope picture of the *C. glomerata* ovipositor. Two main types of sensilla were recognized at the tip: campaniform sensilla (CS), which are most likely mechanosenoric and basiconic sensilla (BS), which contain most likely chemoreceptors and mechanoreceptors (adapted from Smid, unpublished)

Perception of the environment always starts by the transformation of chemical or physical cues into a series of electrical impulses by a receptor neuron in the peripheral nervous system. Subsequently, this information is transmitted to the central nervous system where it will finally be processed and stored (Davis, 2005). For taste receptor cells, environmental cues mainly consist of dissolved chemicals, which are detected by specific proteins in the membrane of the receptor cell. The specificity of a taste neuron is determined by the number of protein types expressed in this membrane

and taste neurons can vary from very broad to highly specific (Montell, 2009). The receptor cells themselves are hosted as groups in taste hairs, so called sensilla on the surface of the insect cuticle. Such sensilla have also been shown on the ovipositor of several parasitic wasps including *C. glomerata* (Fig. 6) (Smid, unpublished). For *C. glomerata* two main types of these sensilla can be recognised in the more frontal part of the ovipositor: campaiform sensilla and basiconic sensilla. The first group most likely contains mechanoreceptors, whereas the basiconic sensilla might house mechanoreceptors as well as taste receptors (Dweck *et al.* 2008). The main functions of these basiconic ovipositor taste sensilla (OTS) is assumed to be the perception of molecules in the host haemolymph, which might give some information about the host quality to the wasp (Larocca *et al.* 2007). However, hardly anything is known about the properties of the OTS and their receptor cells (Larocca *et al.* 2007; van Lenteren *et al.* 2007). Still, it might be assumed that the specificity of these cells corresponds with the foraging behaviour of the wasps, with specialist cells for the perception of the optimal host (Gauthier *et al.*, 2004).

However, proof for such a hypothesis will require more detailed research on the OTS and their response towards certain host cues (van Lenteren *et al.* 2007). A first step might be the identification of genes coding for the gustatory binding protein, which determine the specificity of an OTS. The recent identification of these genes in the *Nasonia vitripennis* genome may provide valuable insight into the specificity of the OTS receptor cells and their importance to the foraging behaviour and reward learning of parasitic wasps (Robertson *et al.* 2010). However, electrophysiological studies, will then be required to validate these genetic findings and to investigate their consequences for the specificity of OTS on a cellular level (Montell, 2009).

Despite their importance for studies on host perception in parasitoids, only a single study on electrophysiological recordings from OTS has been published so far, which is mainly due to the technical difficulties of such studies (van Lenteren *et al*, 2007). It is, therefore, the aim of this report to outline potential solutions for the technical difficulties associated with recording OTS activity and to provide a basis for further studies on the OTS, which might in turn lead to new insights about the host acceptance and host discrimination in parasitic wasps.

2.2 Material and Methods

2.2.1 Insects

In this study the response of *C. glomerata* wasps towards *Pieris rapae* and *Pieris brassicae* caterpillars was investigated. The next paragraphs describe the rearing of both wasps and caterpillars.

2.2.1.1 Wasps.

C. glomerata mated females, between two to four days old were used in all the experiments outlined in this chapter. The wasps originate from wild populations in The Netherlands and have been reared at the Laboratory of Entomology, Wageningen, The Netherlands, for several generations. The wasp population was maintained on *P. brassicae* under standardised conditions of 20-22°C, with 50-70 % relative humidity and a photoperiod of L16:D8. After emergence, wasps of both sexes are housed together in cages of 40 x 40 x 30 cm to ensure mating. Water and honey was supplied at regular intervals. For all experiments, wasps were collected and sexed according to morphological characteristics.

2.2.1.2 Caterpillars

Fourth instar larvae of *P. brassicae* and *P. rapae* were used in the experiments described in the following section. These caterpillars were also taken from the rearing of the Laboratory of Entomology, Wageningen, The Netherlands. These populations are maintained on Brussel sprouts gemmifera (*Brassica oleracea*) temperature regulated between 20 and 22°C, a relative humidity of 50-70% and a photoperiod of L16:D8.

2.2.2 Electrophysiology

Single sensillum recordings on the wasp ovipositor were performed according to a protocol adapted from van Lenteren *et al.* (2007). To asses the sensitivity of the experimental set-up the following four stimulants were used to excite the ovipositor sensilla: 1) haemolymph of *P. brassicae,* 2) haemolymph of *P. rapae,* 3) 1 molar KCl- solution and 4) distilled water. The general functionality of the system was tested by recording from the lateral sensilla styloconia, located on the mouth parts of *P. brassicae* caterpillars. These sensilla were stimulated by a 0.1 % sucrose solution.

2.2.2.1 Preparation of the ovipositor and caterpillar heads

In order to find the most ideal way of recording from the wasp's ovipositor, two approaches were performed. The first method involved the removal of the unpaired valve from the wasp's abdomen. The valve was then placed to one third into a glass capillary filled with an Ringer solution (9 g* l^{-1} NaCl, 2 g* l^{-1} KCL, 1.34 g* l^{-1} CaCl, 5 g* l^{-1} Polyvinylpyrrolidone) specifically adapted to

electrophysiological recordings (EAG- Ringer). This solution was to keep the tissue in proper condition and allow a high conductivity. The alternative method involved making an incision across the middle of wasp's abdomen and to mount the part with the ovipositor on a capillary filled with EAG- Ringer solution. The mounting of the ovipositor was performed in such a way that the tip of the capillary prevented any further movement of the ovipositor. Subsequently, the two cuticle pockets of the ovipositor were removed to allow access to the three main valves. The caterpillar heads used as the control samples, where cut off and immediately placed on a bended silver wire attached to the indifferent electrode.

2.2.2.2 Collection of caterpillar haemolymph

To collect a sufficient amount of haemolymph, the caterpillar was carefully punctured with a tungsten needle. The resulting bleeding of haemolymph was collected with a phenylthiourea (PTU) coated capillary (Ø 1.5 mm, with filament). The capillaries were prepared 24h before the experiment. In order to achieve a homogeneous coating the capillaries were filled with 98.6 % ethanol saturated with PTU. Subsequently, ethanol was evaporated by storing the capillaries overnight in a desiccator. The volume of the haemolymph used for stimulating the OTS was determined by transferring 2 µl of the haemolymph with a Hamilton syringe to a second coated capillary, which was then instantly placed over a silver wire connected to the recording electrode.

2.2.2.3 Electrophysiological recordings.

The basic recording set-up consisted of a DTP42 amplifier in combination with an IDAC-4 interface (both Syntech, Hilversum, The Netherlands) and an inverted Olympus IX51 microscope (Fig. 7). Within this set-up the ovipositor was mounted on the indifferent electrode. Contact was made with the ovipositor by moving the recording electrode with the stimulants towards the expected position of the sensilla, using an electronic micromanipulator (Eppendorf Micromanipulator 5170, Eppendorf-Netheler-Hinz, Hamburg, Germany). Various locations around the tip of the ovipositor, where the basiconic sensilla were suspected were tested until a clear signal was obtained. All further recordings were taken from this location on the ovipositor. The haemolymph of *P. brassicae, P. rapae,* 1 M KCl solution or distilled water were used as stimulants for the sensilla of the wasp's ovipositor. To test the caterpillar sensilla, a 0.1 % sucrose solution was applied. For each of the four stimulants, which were applied to the OTS as well as for the recordings from the caterpillar sensillum, a series of 10 repetitions with a recording time of 10 seconds each was made. After each treatment the sensilla were cleaned by carefully moving them against a piece of filter paper. Electrophysiological signals picked up by the recording electrode were subsequently transmitted to a personal computer at a sampling rate of 12,000 bits per sec.

During sampling recordings were filtered between 200 and 3000 Hz. and after sampling a second filtering above 2000 Hz. was applied. AutoSpike software version 3.9 (Syntech, Hilversum, The Netherlands) was used both for recording and automatic action potential detection.

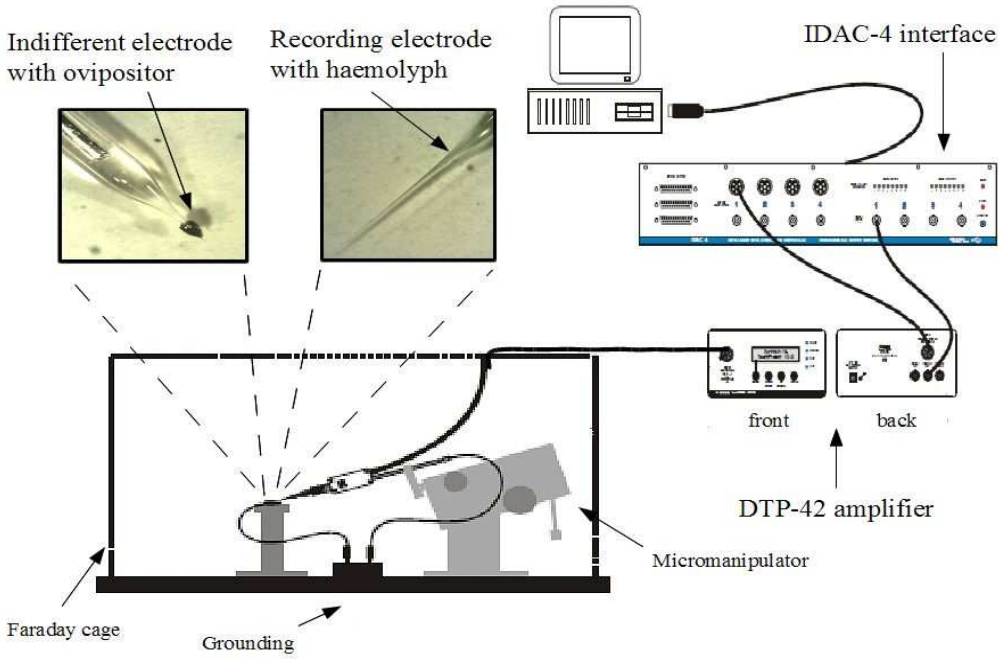

Fig. 7: Basic experimental set-up for electrophysiological recordings (adapted from Syntech 2002)

2.3 Results

2.3.1 Recordings from caterpillar taste sensilla

First, the experimental set-up was validated by recording from taste sensilla of *P. brassicae* caterpillars. An example recording of this is given in Fig. 8, showing a clear train of spikes with a slight decrease in frequency over time. These recordings mainly resemble a typical spike pattern, that would be expected from measurements on these taste sensilla and thereby validate the principal functionality of the experimental set-up.

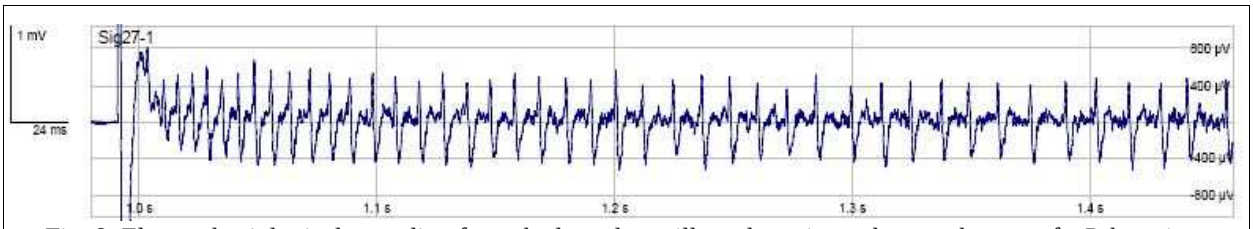

Fig. 8: Electrophysiological recording from the lateral sensilla styloconia on the mouth parts of a P. brassicae caterpillar after stimulation with a 0.1 % sucrose solution.

2.3.2 Handling of the host haemolymph

Subsequently, it was aimed to optimise the quality of recordings from the OTS. It was found, that coating the capillaries with PTU strongly decreased the oxidation of the caterpillar haemolymph, which was used to stimulate the sensilla. This methodological adaptation improved both the handling of the haemolymph as well as the quality of the recordings.

2.3.3 Localization of the ovipositor taste sensilla

In addition to the difficulties with the host haemolymph, the limiting factor for recording from the OTS-recordings is localisation. In the experiments presented here a spot on the tip of the ovipositor was identified, which yielded adequate and reasonably repeatable results of a satisfactory quality (Fig. 9) From comparisons with scanning electron microscope pictures, it was concluded that a single basiconic sensillum is positioned at this spot on the ovipositor surface (Fig. 6).

2.3.4 Recordings from the ovipositor taste sensilla

In spite of the findings outlined above only a single pair of recordings from the tip sensillum of a *C. glomerata* ovipositor showed a sufficient quality (Fig. 9). One of these two recordings was taken after stimulating the sensillum with haemolymph of *P. brassicae*, leading to 2198 spikes * 10 seconds^{-1}, whereas the second recording was made by stimulating the sensillum with haemolymph of *P. rapae*, causing 1936 spikes * 10 seconds^{-1}. Stimulation with 1 molar KCL or distilled water did not lead to any clear recording. A closer examination of the recordings done with the two caterpillar haemolymphs revealed that the stimulation with haemolymph of *P. brassicae* did not only cause more spikes to occur within 10 seconds, it also caused a different recording pattern than the

stimulation with haemolymph of *P. rapae*. In both cases action potentials occurred in series of distinct clusters (Fig. 9 c,d). However the pattern of these spike clusters differs slightly between both treatments. When stimulated with haemolymph of *P. brassicae* the clusters most often consists of a small spike of about 100 µV, followed by a larger spike of 200 to 250 µV. However, in those clusters, which were recorded after stimulation with haemolymph of *P. rapae*, the smaller spike was missing and only a larger second spike occurred. In addition to these differences in the pattern of these spike clusters, the cluster also appeared with a different frequency. The clusters found after stimulating with haemolymph of *P. brassicae* showed a frequency of 6 clusters per 0.1 second, whereas for the cluster occurring after stimulating with haemolymph of *P. rapae* a frequency of 10 clusters per 0.1 sec was found. However, since stimulation with *P. brassicae* showed more action potentials within a cluster, this still led to the higher overall action potential frequency mentioned above. These variations in pattern and frequency of spike clusters, may also give indications for a difference in the number of activated neurons, as will be discussed in the next section.

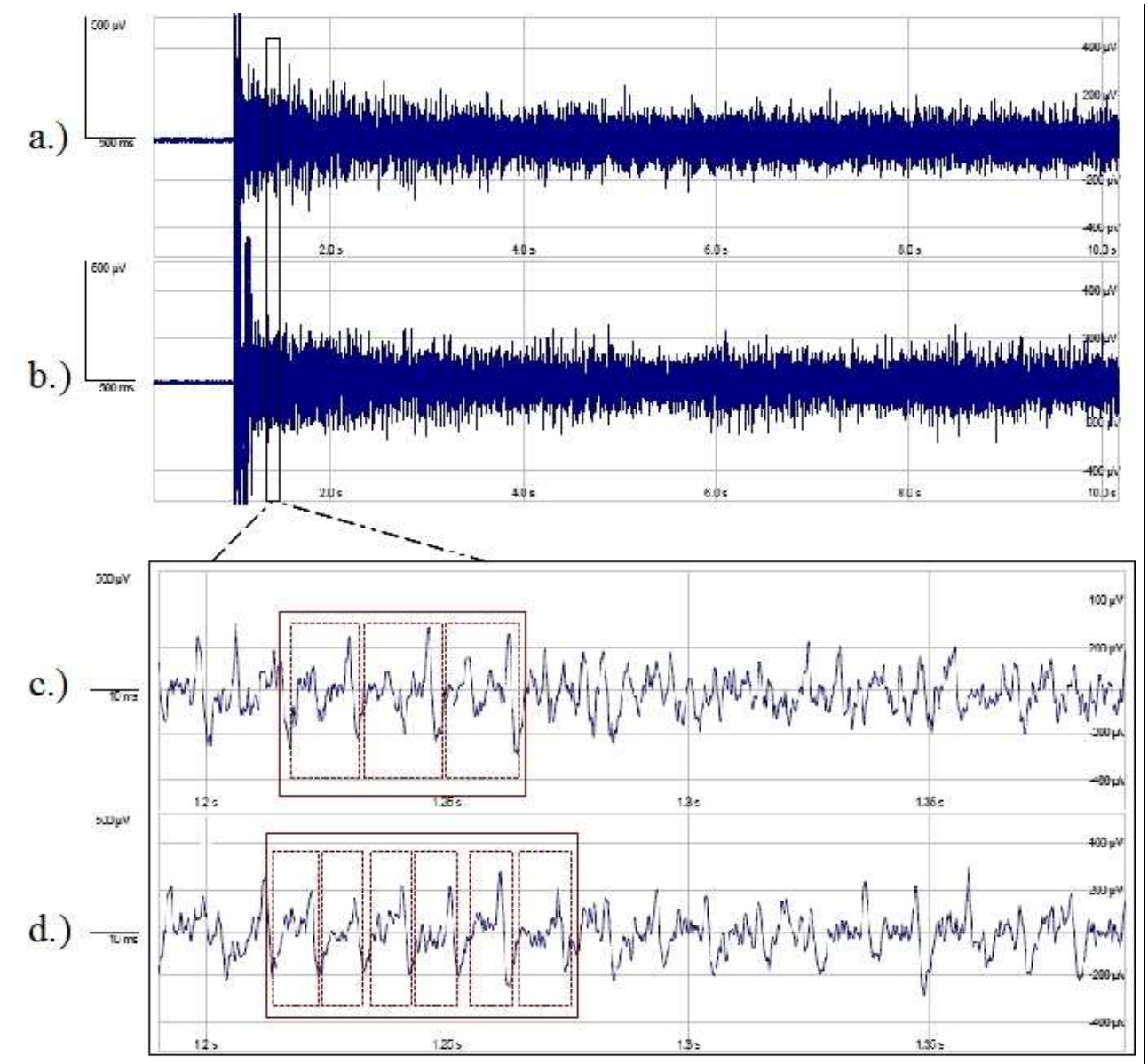

Fig. 9: Single sensillum recordings from the ovipositor of *C. glomerata* after stimulation with haemolymph of *P. brassicae* (a, c) or *P.rapae* (b, d). Continuous red frames indicate trains of action potential clusters (dashed red frames) .

2.4 Discussion

Recordings from the OTS suffer from two major difficulties: the oxidation of the host haemolymph and the small size of the OTS. Oxidation might strongly alter the chemical composition of the host haemolymph and might therefore also change the response of the receptor cells. Furthermore, oxidation leads to an aggregation of proteins, which often cause a blockage of the capillary containing the recording electrode. To overcome these problems a coating of PTU was tested here. This coating prevented the aggregation of proteins and considerably improved the handling of the haemolymph as well as the overall quality of the recordings. PTU is commonly used in physiological studies and is therefore thought not to interfere with the recordings (Cherqui *et al.* 1996). In contrast to this, it was only partly possible to overcome the limitations due to the small size of the OTS with the current experimental set-up. As a first step, a higher maginfication in the microscope might help to improve the handling of the preparation. However, even with a higher magnification the exact positioning of the OTS might still cause some difficulties due to the curved shape of the ovipositor. Additionally the high surface density and the dark colour of the ovipositor surface hamper the visualization of the OTS even further and it can be assumed that the localization of the OTS will remain a major constraint for these studies. Nevertheless, a rather reliable recording position was found at the assumed location of the basiconic sensilla at the tip of the ovipositor (Fig. 6). This finding is largely in line with results form other parasitoids, which indicates that these sensilla might play a role in the perception of the host haemolymph (van Lenteren *et al.* 2007).

At the receptor level, a reward can principally be received by two different mechanisms: a) different receptor cells are activated by different cues at the same time or b) the same receptor neurons are activated at a different frequency (Schoonhoven *et al.* 2005). The preliminary findings presented in this report, would indicate that it is the first mode of action, which plays a role in host discrimination and reward perception for *C. glomerata.* This assumption is based on the differences in spike patterns found in the electrophysiological recordings, which indicate the activation of different receptor cells by *P. brassicae* and *P. rapae*. Under this assumption, the observed difference in spike numbers would then not be due to the same neurons firing at a different rate, but rather due to different neurons with a different firing rate. Unfortunately, nothing is known about the receptor cell within the OTS of *C. glomerata* and results from other parasitic wasp species have led to very different conclusions with regards to the abilities of the OTS to discriminate hosts with different reward qualities (Rajendram and Hagen, 1976; Gauthier *et al.*, 2004)

In some cases, such as the generalist egg parasitoid *Trichogramma* it has been reported that the wasps discriminated only between distilled water and a saline solution, (Rajendram and Hagen, 1976) whereas in other cases very specific responses towards single molecules have been reported

for specialist parasitoids (Gauthier *et al.*, 2004). Due to this it seems reasonable to expect a similar role of the receptor cells in the OTS for host specialisation in parasitoids, as was found for taste receptor cells in herbivorous insects (Schoonhoven *et al*. 2005). For these insects it has been shown that specialist feeders normally also possess highly specialist taste cells, which respond towards compounds characteristic for their host plants (Schoonhoven and Dethier, 1966). Such a theory would also support the hypothesis of specialist receptor cells in *C. glomerata*. Even though *C. glomerata* is not a true specialist on *P. brassicae* it still displays a high preference for this host under natural conditions (Geervliet, 1997). However, even if host- specific receptors are assumed in *C. glomerata* it would still remain to be investigated by which mechanism these cells would influence the formation of LTM. A particular set of neurons in the suboesophageal ganglion (SOG) was shown in multiple insect species to transmit information about sucrose perception directly on to the mushroom bodies and other brain parts involved in memory formation (Schröter *et al*. 2007). It has been assumed that these specific inter-neurons are only activated by a sub-set of receptor cells, whereas the larger part of taste information is directly processed in the SOG (Vosshall and Stocker, 2007). However, further investigation will be needed to test this hypothesis and to show which taste information are indeed transmitted on to higher brain parts.

As an overall conclusion, it can be stated, that even though electrophysiological studies on OTS will face certain technical constraints, such as the difficult localization of the sensilla, they might still reveal fruitful results concerning the evolution of host specialization in parasitoids as well as on the importance of reward perception during memory formation.

3. Creating a Nasonia standard brain

Abstract

The shape and volume of a certain brain area can often be directly related to its functions and properties. In this chapter, three-dimensional (3D) reconstructions of five individual female *Nasonia vitripennis* brains were created in order to provide a first view on the brain morphology of this wasp. Eight paired and six unpaired neuropils were consistently labelled and their average surface area and volume was measured. Additionally, one brain was selected as a template brain representing the average neuropil shape. A comparison of the relative neuropil volumes with three other virtual insect brain reconstructions revealed relatively large antennal lobes and relatively small optic lobes in *Nasonia*. Moreover, a comparison of neuropil shapes between *Nasonia* and the honeybee, showed considerably less elaborated calyces in *Nasonia*. Finally a correlation between olfactory and optic neuropil volume and calyx shape was argued in relation to current findings concurring calyx evolution.

3.1 Introduction

The idea, to relate cognitive processes to biological structures in the brain is at the core of physiological psychology (Carlson, 2010). The first studies on invertebrate neuroscience were already driven by this assumption and when Felix Dujardin discovered the honeybee mushroom bodies (MB) in 1850. He soon hypothesised that this must be the 'organ of intelligence'. At the present stage Dujardin's hypothesis still holds true and research has gone even further in pinpointing cognitive functions on to morphological structures in the insect brain (Menzel, 2001).

3.1.1 'The organ of intelligence'

Since the first description by Dujardin the mushroom bodies are among the best studied neuropil clusters in the insect brain. They are commonly divided into three main compartments, the calyces, pedunculi and lobes (Strausfeld, 2002) (Fig. 4). However a further division in to sub-compartments reveals major difference between insect orders. In the fruit fly for example no further sub-compartments of the calyces are found (Ann-Shyn *et al*. 2011) whereas the lobes can be further divided into five distinct structures: the α- and α'- lobes, the β- and β'-lobes and the γ-lobes. In contrast to this, the calyces of the honeybee can be divided into six different sub-compartments, three in the lateral calyx (basal ring, collar and lip) and three in the medial calyx (basal ring, collar and lip) (Mobbs, 1982). The lobes of the bee, however, can only be divided into two to three sub-compartments, the medial lobe (or β- lobe), the ventral lobe (or α- lobe) and a γ-lobe, which is so strongly fused to the ventral lobe, that it is often not considered independently (Strausfeld, 2002; Brandt *et al*. 2005).

3.1.2 Division of labour within the mushroom bodies and the antennal lobes

Interestingly, different forms of memory formation and cognition were shown to occupy different compartments within the MB (Giurfa, 2003). The lobes were found to receive input from all sensory systems and show a strong learning related plasticity (Grünewald, 1999) and in *Drosophila* it has even become possible to assign different memory stages to the different sub-compartments of the lobes (Krash *et al.* 2007). In contrast to the lobes, the different sub-compartments of the honeybee calyx receive sensory system-specific input: the lip and the basal ring for example receive mainly olfactory information whereas the collar appears to be specific to visual input (Mobbs, 1982). These properties make the calyces a likely candidate for providing the neural substrate of sensory integration and more sophisticated forms of learning (Giurfa, 2003).

The MB are not the only brain structure involved in memory formation. For olfactory conditioning the antennal lobes (AL) were shown to play a major role and studies using pharmacological manipulation of the MB even suggest that the AL alone might be sufficient for this form of learning (Malun *et al.* 2002). From these studies a spatial separation might be suggested, in which the AL would be responsible for olfactory learning and the calyces would provide more complex forms of cognition whereas the lobes might still play a role in both processes (Devaud *et al.* 2007). Although this hypothesis may be appealing, experimental prove is currently still lacking behind. Inter- species comparisons, may bring new insights into these questions, by allowing broader correlations between brain structures and behavioural patterns of different insect species (Chittka and Niven, 2009).

3.1.3 Virtual insect brains

To conduct profound inter- and intra-species comparisons standardized reconstructions, which average the volumes and shapes of brain neuropils are required (Rein *et al.* 2002). In the last decade several virtual standard brains have been developed for insects from different genera (Rein *et al*. 2002; Brandt *et al*. 2005; Kurylas *et al.* 2008, el Jundi *et al.* 2009 and Ann-Shyn *et al.* 2011). These brains provide a common framework to compare neuropils in brains of different species and it might be expected that these brain models will provide even more detailed insights into the role of the MB and AL in different forms of learning. However, species for which virtual brain models are available all belong to different insect genera and comparative studies have often been hampered by morphological differences unrelated to cognitive functions (el Jundi *et al*., 2009)

In this study the basis for creating an *N. vitripennis* standard brain will be provided. This will not only allow a comparison of insect species of different orders, but will also allow a detailed comparison of brain structures between two hymenoptera species: *N. vitripenni*s and *Apis mellifera.*

3.2 Material and Methods

3.2.1 Rearing of N. vitripennis wasps

Nasonia wasps of the strain AsymC used in the experiments described below originated from two different populations, kindly provided by the University of Amsterdam and the University of Groningen and were subsequently reared at the Laboratory of Entomology, Wageningen (all three in The Netherlands). Wasps were maintained on calliphorid fly pupae obtained from a commercial rearing. For ovipositing two day old female (20) and male (4) wasps were put together with 10 fly cocoons for three days. After this period the adult wasps were removed and the parasitized cocoons were kept in an incubator for 14 days at 25°C with a relative humidity of 50-70% and a photo-period of L16:D8. After hatching, wasps were transferred to a new vial with a thin layer of agar to provide sufficient liquid. Honey was provided to the wasps *ad libitum*.

3.2.2 Neuropil labelling

To visualize neuropils in in the brain of *N. vitripennis* a labelling protocol adapted from Brandt *et al* (2005) was applied. The individual steps are outlined below.

3.2.2.1 Fixation and dissection of wasp brains

Females were individually caught in glass vials and sedated on ice for 2 minutes. Subsequently, the wasps were decapitated and heads were immersed in 4 % formaldehyde diluted in 50 % methanol in order to fixate the brain tissue. The mouthparts including mandibles were removed for better penetration of the formaldehyde into the brain. The heads were pre-fixated in this way for about 20 minutes, transferred into PBS and the head capsule was completely removed. The brains were again transferred into formaldehyde for 4 hours at room temperature and then stored overnight in PBS at 4°C. All pre-fixation and dissection steps were performed on a cold plate at 3°C.

3.2.2.2 Antibody labelling

The fixated brains were rinsed in PBS for 10 minutes. To clear the brain tissue and to increase the permeability for the following labelling procedure the brains were dehydrated to 100% ethanol by incubating the brains in 70%, 90%, 98% and 100% ethanol for 10 minutes each. Thereafter the brains were immersed in xylene for 2.5 minutes and subsequently rehydrated to PBS using the same dilution steps in reversed order. To increase labelling specificity and to reduce background staining, the brains were washed for 10 minutes in PBS plus 0.5 % Triton X-100 (PBS-T) and transferred into eppendorf-tubes. The brains were blocked in 10 % normal goat serum diluted in PBS-T (NGS-PBS-T) for 1 hour to prevent false- positive binding of the antibody. After this the primary

monoclonal mouse anti- Bruchpilot antibody (nc82) was applied. The antibodies had been produced at the Leiden University Medical centre and was generously provided by Ms. Potikanond. The antibody was used in a concentration of 1:500 diluted in 10% NGS-PBS-T with an incubation time of 24 hours at 4°C. Surplus antibodies were washed of by rinsing the brains for 15, 30, 45 and 60 minutes in PBS-T. After this wash a secondary rabbit anti- mouse antibody was applied in a dilution of 1:200 in 10% NGS-PBS-T (DAKO # 097(102), Glostrup, Denmark). Brains were incubated for 20 hours at 4°C and subsequently again rinsed in PBS-T for 15, 30, 45 and 60 minutes followed by the application of a third antibody. This final goat anti-rabbit antibody had been linked to Alexa Fluor 488 (Invitrogen # 52959A, Eugene, USA) and was used in a concentration of 1:200 diluted in 10% NGS-PBS-T. In addition to this antibody propidium iodide was added in a concentration of 2% to visualise cell nuclei. The incubation time for the antibody and the propidium iodide was set to 4 hours at room temperature. Brains were then washed in several changes of PBS for 15, 30, 45 and 60 minutes. Thereafter the samples were stored overnight in PBS at 4°C. The brains were again dehydrated to 100% ethanol (70%, 90%, 98% and 100% ethanol for 10 minutes each) and immersed in xylene for 2.5 minutes. Finally, the brains were mounted in Depex (Fluka) on microscope slides and analysed by using confocal laser scanning microscopy as described in the next section.

3.2.3 Confocal laser scanning microscopy

All pictures were taken using a Zeiss LSM 510 confocal laser scanning microscope equipped with an argon laser (Wavelength: 458 nm, 488 nm and 514 nm) and two helium- neon lasers (543 nm and 633nm). For the excitation of the Alexa Fluor 488 and the propidium iodide the 488 nm line of the argon laser was used in combination with a long-pass emission filter at 505 nm. All pictures were taken using a 25 x oil- immersion objective (N.A. 0.8). The scanning resolution was set to eight bit and 1024 x 1024 pixel, to increase the sharpness of the pictures every slide was scanned four times and an average picture was taken. Most brains could be scanned entirely with a voxel size of 0.45 x 0.45 x 1.01 µm. However, some samples had to be scanned in an anterior and posterior direction in order to capture the brain depth more accurately. These, stacks from these brains had subsequently been combined using the "Merge" module in of the imaging software Amira 5.3.3 (Visage Imaging GmbH**,** Berlin, Germany). Thereafter all images were down sized to a voxel size of 1 x 1 x 1.01 µm using the "Resample" module of Amira 5.3.3. Through this a total of 20 complete brain images of female *N. vitripennis* wasps were obtained, providing solid bases for the neuropil labelling and reconstruction described in the next section.

3.2.4 Image segmentation, reconstruction and standard brain choice

At the current state, five female brains have been fully labelled and analysed. One brain, however was considered to be an outlier and was excluded from the analyses, as its total volume was substantially larger then the other brains analysed. Out of the four remaining brains, one brain which showed the smallest differentials to the average neuropil volumes and shapes was then selected as a preliminary standard brain.

3.2.4.1 Labelling and segmentation

Since neuropils can not be identified by using their grey values only, the labelling of these brain regions has to be performed semi- automatically, by assigning every voxel to a so called 'label field', which represents a certain anatomical structure. In this study the segmentation editor of the Amira 5.3.3 was used creating label fields for eight paired and six unpaired brain structures. Subsequently, these label fields representing the different neuropils were used for the polygonal surface reconstruction as well as for the morphological analysis. These neuropils were named according to Rein *et al* (2002) and Ann-Shyn *et al*. (2011). Since no colour code currently exists, the neuropile colours were chosen at random, but consistently over all samples.

3.2.4.2 Standard brain selection.

Due to software problems no shape averaging algorithm was applied on to the labelled brains. However, one brain was selected as a representative template by calculating the squared difference between the absolute volume of certain neuropil in one brain and the average volume of that neuropil. Subsequently, the sum of the squared differences of all neuropils was formed. The brain with the smallest squared sum of differences was then taken as the preliminary standard brain.

3.3 Results

In this study, most of the major neuropils were identified and a first overview over their volume, surface area and shape is given (Tab. 1). Additionally, the data generated were compared with results obtained from other insect species in similar studies.

3.3.1 Identification of neuropils and outer cell layer

Over all five brains analysed, eight paired and six unpaired structures were consistently labelled: The apprehensions for these neuropils were given according to Ann-Shyn (2011) with capital letters for the left side and small letters for the right. Out of the optic lobes the medullae (MED and med) and the lobulae (LOB and lob) (Tab. 1; Fig. 10; Fig. 14; Fig. 13; Fig. 17; Fig. 12) were selected. In consistence with Brandt *et al*. (2005) the laminae were left out since their shape was often altered by the dissection. For the antennal lobes (Al and al) , the glomeruli as well as their inter-spaces were selected (Tab. 1; Fig. 10; Fig. 17; Fig. 12). In the central brain the mushroom bodies were the most prominent structures. In this study, the mushroom bodies (MB) were sub- divided into three compartments: 1. calyces (CAL and cal), 2. the pedunculi including *β*-lobes (PE and pe) and 3. the *α*- lobes (*α*- LOB and *α*- lob) (Tab. 1; Fig. 10; Fig. 14; Fig. 13; Fig. 12). The position of the *β*- lobes are indicated in Fig. 10 and Fig. 13. However, they were not labelled separately from the pedunculi to decrease complexity (el Jundi, *et al*, 2009). Behind the *β*- lobes of the MB the central body complex was found. Within this structure, the ellipsoid body (EB) (Tab. 1; Fig. 14), the fan-shaped body (FB) (Tab. 1; Fig. 10; Fig. 14; Fig. 13) and the noduli (NOD and nod) (Tab. 1; Fig. 14) were identified. Again, posterior to the central body complex the protocerebral bridge (PCB) (Tab. 1; Fig. 14; Fig. 13) was brightly visible in most preparations. The lateral horns (LH and lh) (Tab. 1; Fig. 10; Fig. 13; Fig. 12) was found and labelled at the lateral side of the central brain. The identification of this neuropil was firstly based on the antibody labelling described in this chapter, but was confirmed by the octopamine labelling described in the following chapter. These 14 neuropils make about 55% of the total neuropil volume in *N. vitripennis* all other neuropils were assigned to two structures: the protocerebrum (PC) (Tab. 1; Fig. 10; Fig. 14; Fig. 13; Fig. 12) and the suboesophageal ganglion (SOG)(Tab. 1 Fig. 14; Fig. 13; Fig. 17). A borderline between these two structures is difficult to define, since the transition between these two major brain divisions is rather blurred. However, in this study a reliable boarder line was found by visual analysis to separate these two areas. All together the neuropils, made up 62% of the total brain volume. The other 38 % consisted of the outer cell layer. This cell layer covered the brain almost entirely, leaving only a small opening at the posterior side were the ventral nerve cord emerges from the posterior SOG.

Tab. 1: Overview over all labelled neuropils, their apprehensions, surface area and volumes. Data represents means over four adult female brains. Neuropils of the left brain hemisphere are highlighted in grey.

Name	Apprehension	Mean surface area (μm^2)	SD surface area (μm^2)	Mean volume (μm^3)	SD volume (μm^3)	Rel. SD (%)	Rel. volume (%)	Rel. neuropils (%)	SD rel. volume (%)
Antennal lobe left	AL	2.79E+004	3.31E+003	2.71E+005	1.60E+004	5.89	3.78	6.12	0.39
Antennal lobe right	al	2.91E+004	3.57E+003	2.77E+005	2.23E+004	8.06	3.87	6.26	0.58
Calyx left	CAL	2.18E+004	1.87E+003	1.48E+005	1.57E+004	10.6	2.05	3.33	0.16
Calyx right	cal	2.03E+004	1.48E+003	1.45E+005	2.12E+004	14.62	2.01	3.27	0.3
Ellipsoid body	EB	8.56E+003	7.32E+002	2.12E+004	3.05E+003	14.37	0.3	0.48	0.05
Fan-shaped body	FB	9.74E+003	5.98E+002	4.70E+004	2.50E+003	5.32	0.65	1.06	0.03
Lateral horn left	LH	7.41E+003	8.61E+002	4.91E+004	8.06E+003	16.41	0.68	1.1	0.06
Lateral horn right	lh	7.55E+003	4.54E+002	5.10E+004	4.89E+003	9.59	0.71	1.15	0.09
Lobula left	LOB	1.85E+004	1.48E+003	1.91E+005	1.76E+004	9.19	2.64	4.3	0.07
Lobula right	lob	1.82E+004	1.20E+003	1.90E+005	1.09E+004	5.74	2.63	4.27	0.05
Medulla left	MED	3.42E+004	2.14E+003	4.04E+005	2.48E+004	6.14	5.61	9.1	0.16
Medulla right	med	3.36E+004	1.62E+003	3.89E+005	3.70E+004	9.53	5.38	8.74	0.16
Nodulus left	NOD	6.83E+002	2.03E+002	1.51E+003	5.01E+002	33.07	0.02	0.03	0.01
Nodulus right	nod	7.39E+002	2.54E+002	1.68E+003	6.75E+002	40.08	0.02	0.04	0.01
Outer cell layer	CL	6.43E+005	4.42E+004	2.78E+006	3.75E+005	13.5	38.35		2.5
Pedunculus and β-lobe left	PE	1.57E+004	1.24E+003	8.23E+004	1.05E+004	12.77	1.14	1.85	0.11
Pedunculus and β-lobe right	pe	1.58E+004	3.58E+002	8.53E+004	5.19E+003	6.08	1.19	1.92	0.14
Protocerebral bridge	PRB	2.89E+003	6.73E+002	5.84E+003	1.64E+003	28.03	0.08	0.13	0.03
Protocerebrum	PC	1.32E+005	9.13E+003	1.22E+006	1.29E+005	10.57	16.9	27.4	1.5
Suboesophageal ganglion	SOG	8.44E+004	5.46E+003	7.99E+005	2.06E+004	2.57	11.11	18	0.74
α-lobe left	α- LOB	6.78E+003	1.04E+003	3.14E+004	3.98E+003	12.67	0.43	0.71	0.04
α-lobe right	α- lob	6.94E+003	5.94E+002	3.26E+004	1.58E+003	4.85	0.45	0.73	0.03
Total brain	TB	6.43E+005	4.42E+004	7.22E+006	5.35E+005	7.42	100	61.65	0

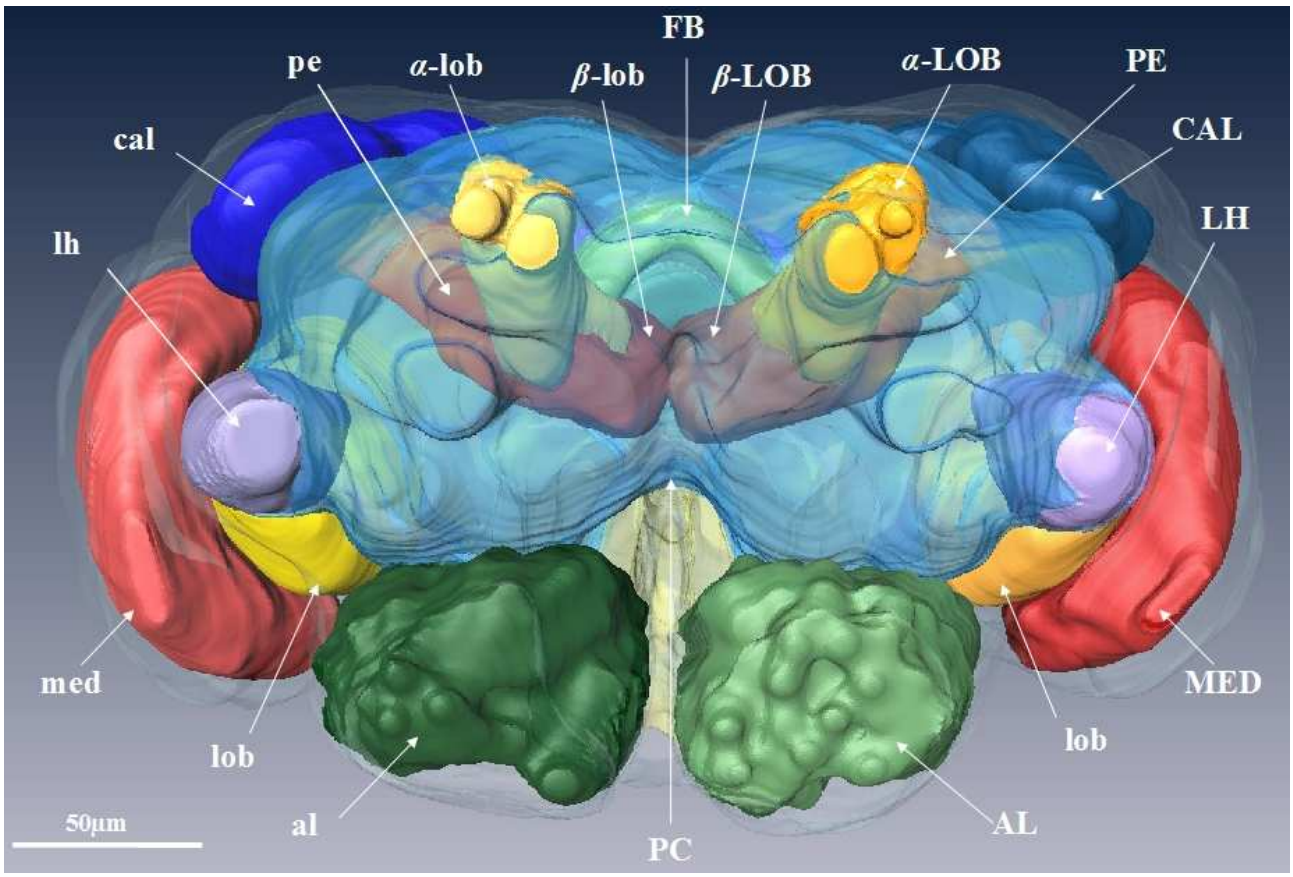

Fig. 10: Frontal view on the 3D reconstruction of neuropils in the brain of *N. vitripennis.*
Full names for all neuropils can be found in Table 1

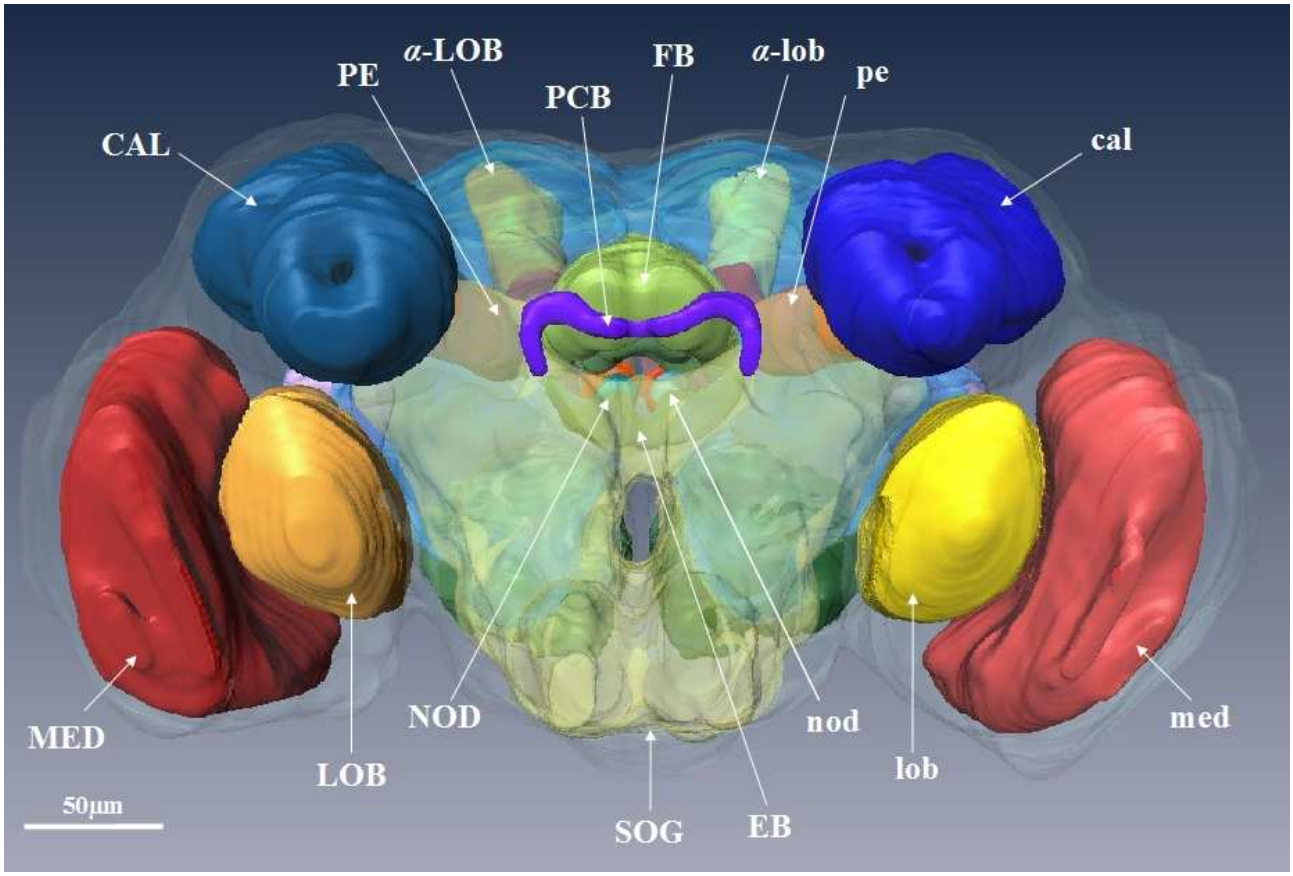

Fig. 11: Caudal view on the 3D reconstruction of neuropils in the brain of *N. vitripennis.*
Full names for all neuropils can be found in Table 1

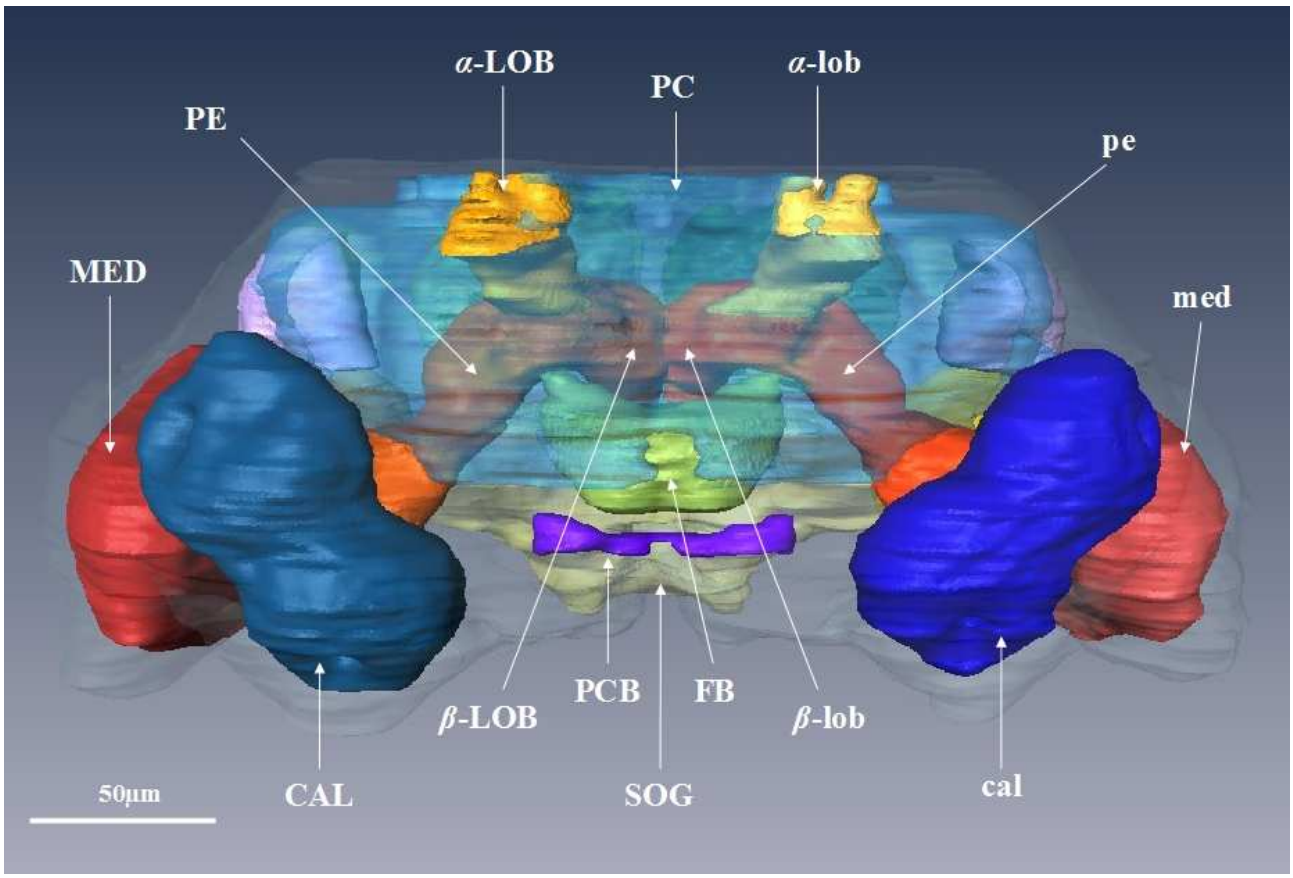

Fig. 12: Top view on the 3D reconstruction of neuropils in the brain of *N. vitripennis.*
Full names for all neuropils can be found in Table 1

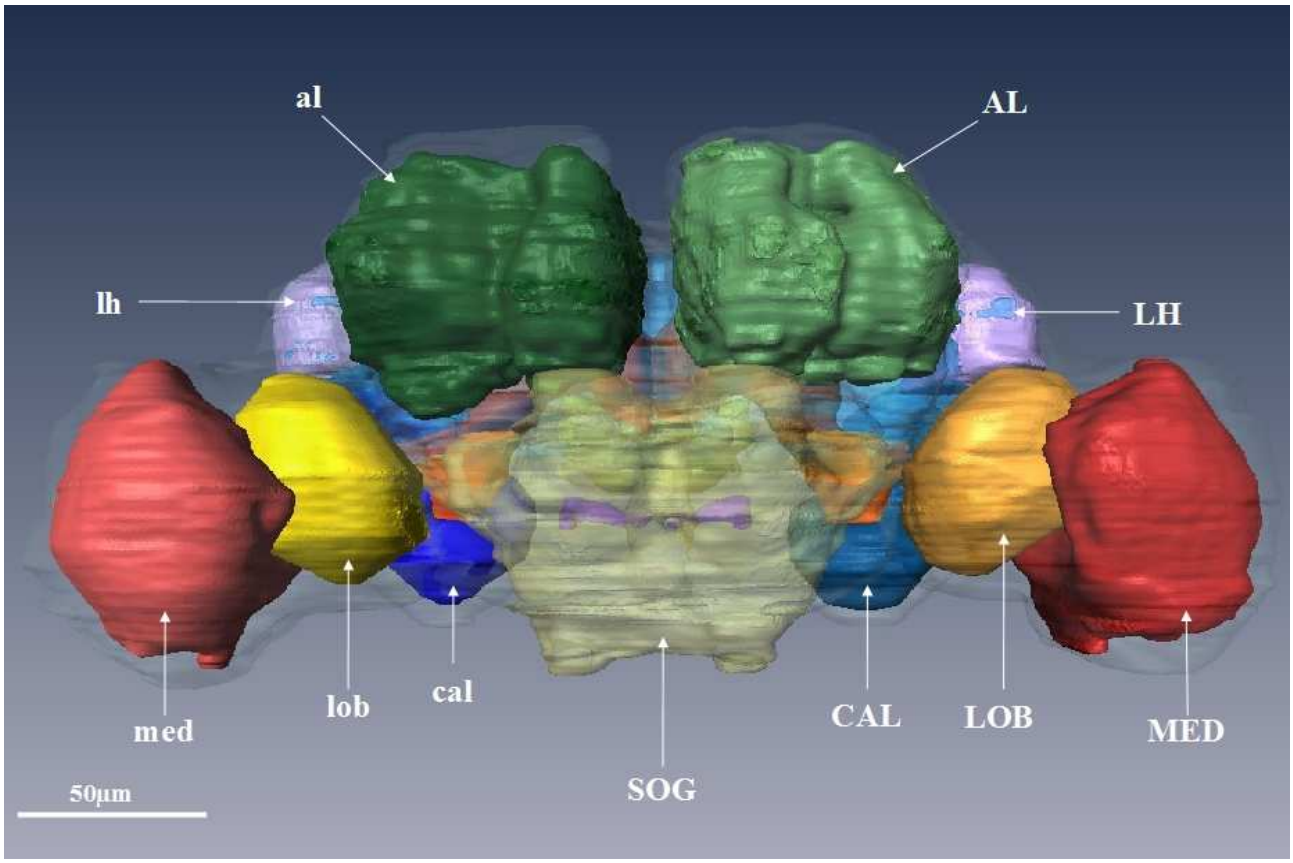

Fig. 13: Bottom view on the 3D reconstruction of neuropils in the brain of *N. vitripennis.*
Full names for all neuropils can be found in Table 1

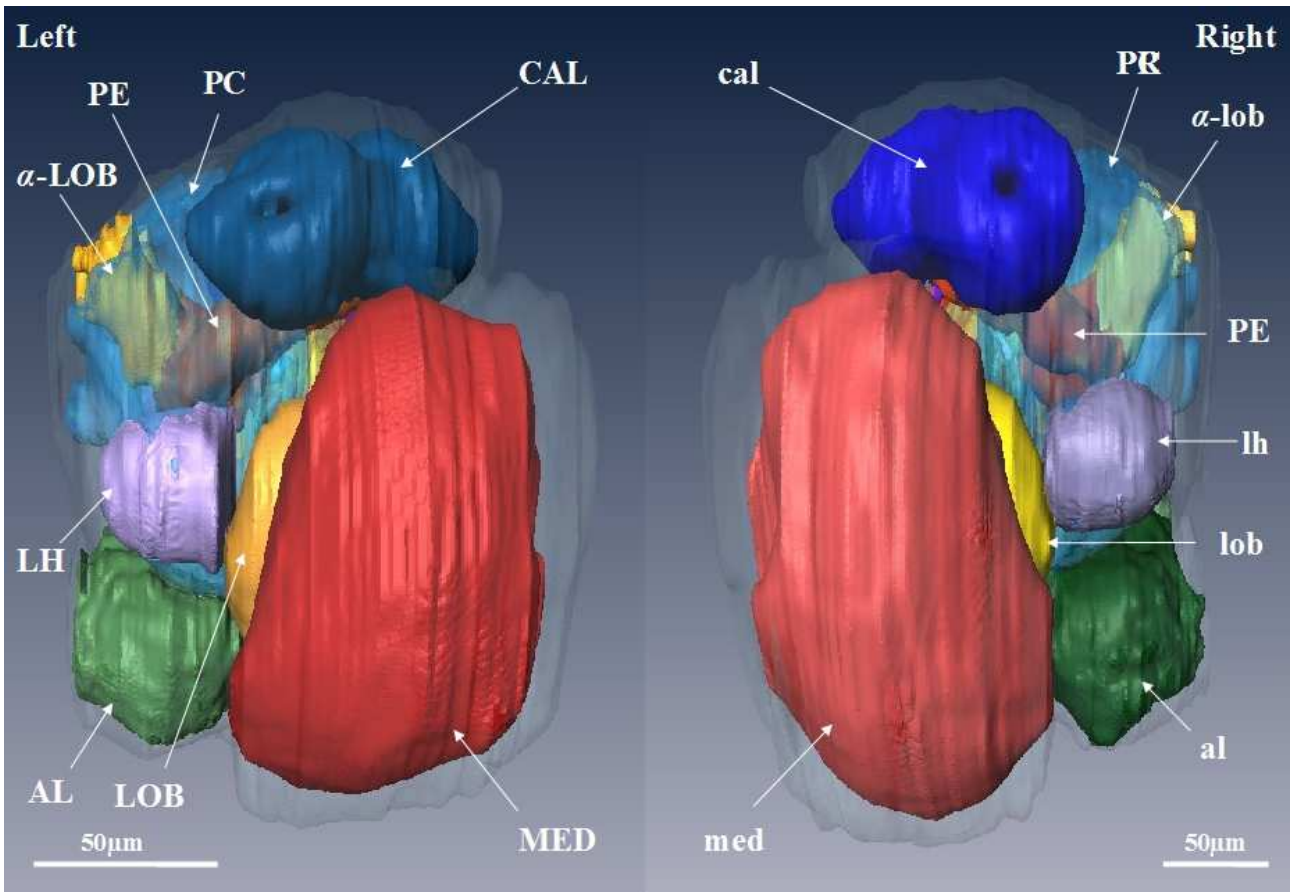

Fig. 14: Side view on the 3D reconstruction of neuropils in the brain of N. vitripennis.
Full names for all neuropils can be found in Table 1

3.3.2 Relative neuropil volume and interspecies comparison

To evaluate the relative neuropil volumes of *N. vitripennis* an inter-species comparison of relative neuropil cluster volumes was conducted. This comparison included data from adult females of *Apis mellifera* (Brandt *et al*. 2005), *Drosophila melanogster* (Rein *et al*. 2002), *Manduca sexta* (el Jundi *et al*. 2009) and the data from this report on *N. vitripennis* (Fig. 15).

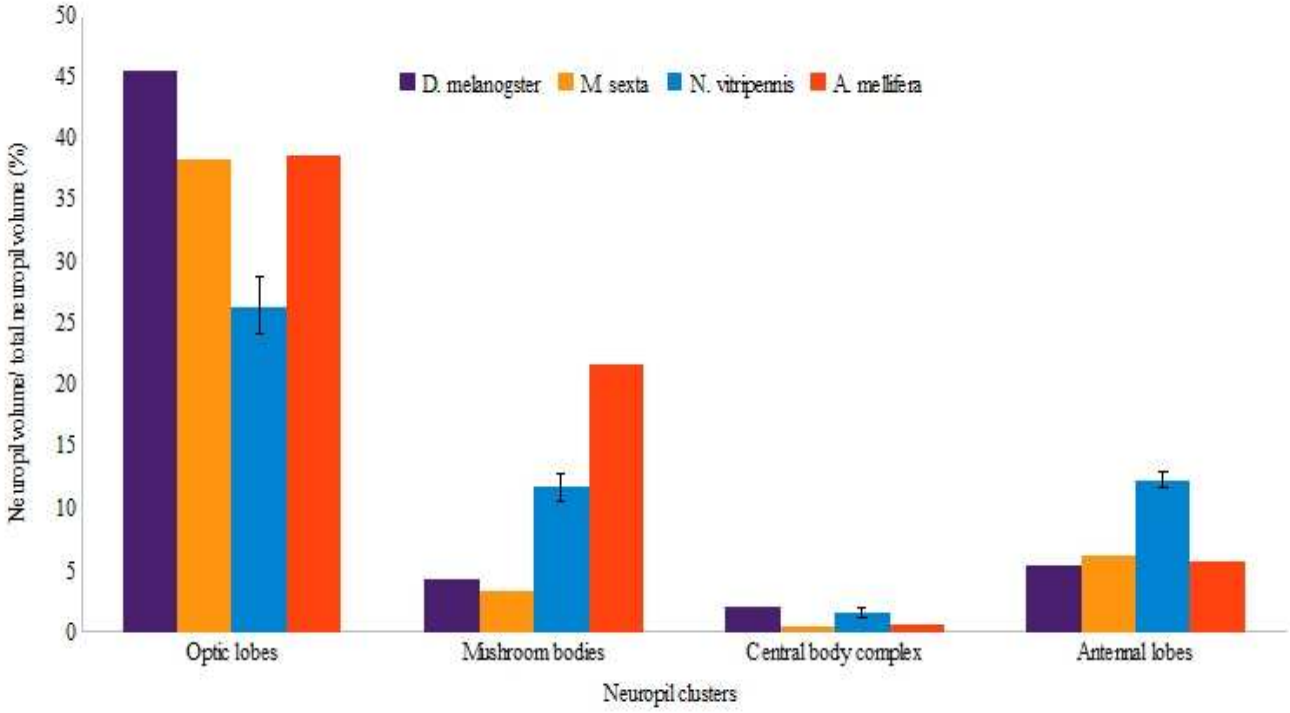

Fig. 15: Comparison of neuropil cluster volume relative to total neuropil volume over four insect species. Graph includes only data from adult females. Error bars for *N. vitripennis* indicate the standard deviation. *Drosophila melanogaster*: Rein et al. (2002), *Manduca sexta*: el Jundi et al. (2009), *Nasonia vitripennis*: this report, *Apis mellifera:* Brandt et al. (2005).

Comparing the relative neuropil volumes within the *N. vitripennis* brain, the medullae were found as the most prominent structures, taking about 18 % of the relative neuropil volume and together with the lobulae the optic lobes form about 26 % of the relative neuropil volume the largest neuropil cluster (Tab. 1). However, in the cross-species comparison the optic lobes of *N. vitripennis* were found to be rather small, being almost of half the size of those optic lobes found in *D. melanogaster* (Fig. 15). In sharp contrast to these results for the optic brain areas the olfactory input regions, namely the AL, were with 12% of the relative volume found to be almost two times as large as those of the other insect species (Fig. 15).

Even more pronounced than these results were the differences between the two hymenoptera species (*A. mellifera* and *N. vitripennis*) and the two other species in terms of MB size. This neuropil cluster consisting of pedunculi, *α*-, and *β*-lobes, accounts for about 16% of the relative neuropil volume in *N. vitripennis* and for about 22% of the relative brain volume in the honeybee. These values are about four and five as times bigger than in *D. melanogaster* and almost five and seven times bigger as in *M. sexta* (Fig. 15). Interestingly, the differences observed between the honeybee and *N. vitripennis* are mainly due to a much higher calyx volume in the bee (Fig. 16). The relative volume of these MB compartments in the honeybee was found to be of about twice the size than the calyces found in *N. vitripennis*, whereas the other compartments were only about 30% smaller in *Nasonia* than in the bee (Fig. 16).

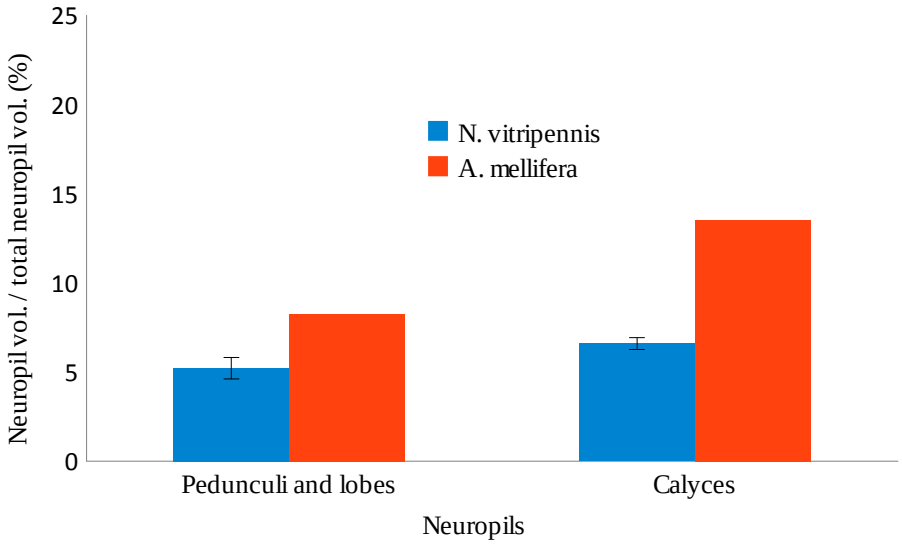

Fig. 16: Comparison of relative volumes of different MB- compartments between *N. vitripennis* and *A. mellifera*. Error bars for *N. vitripennis* indicate standard deviation (*A. mellifera* data from Brandt *et al.* 2005)

Consistent with these differences in volume, also a differences in the general shape and the elaboration of the calyces was observed (Fig. 17). Even though, invaginations and a double structure of the lobes was clearly visible in *Nasonia*, no compartmentalisation in to lip, collar and basal ring such as in the honeybee could be detected (Fig. 17).

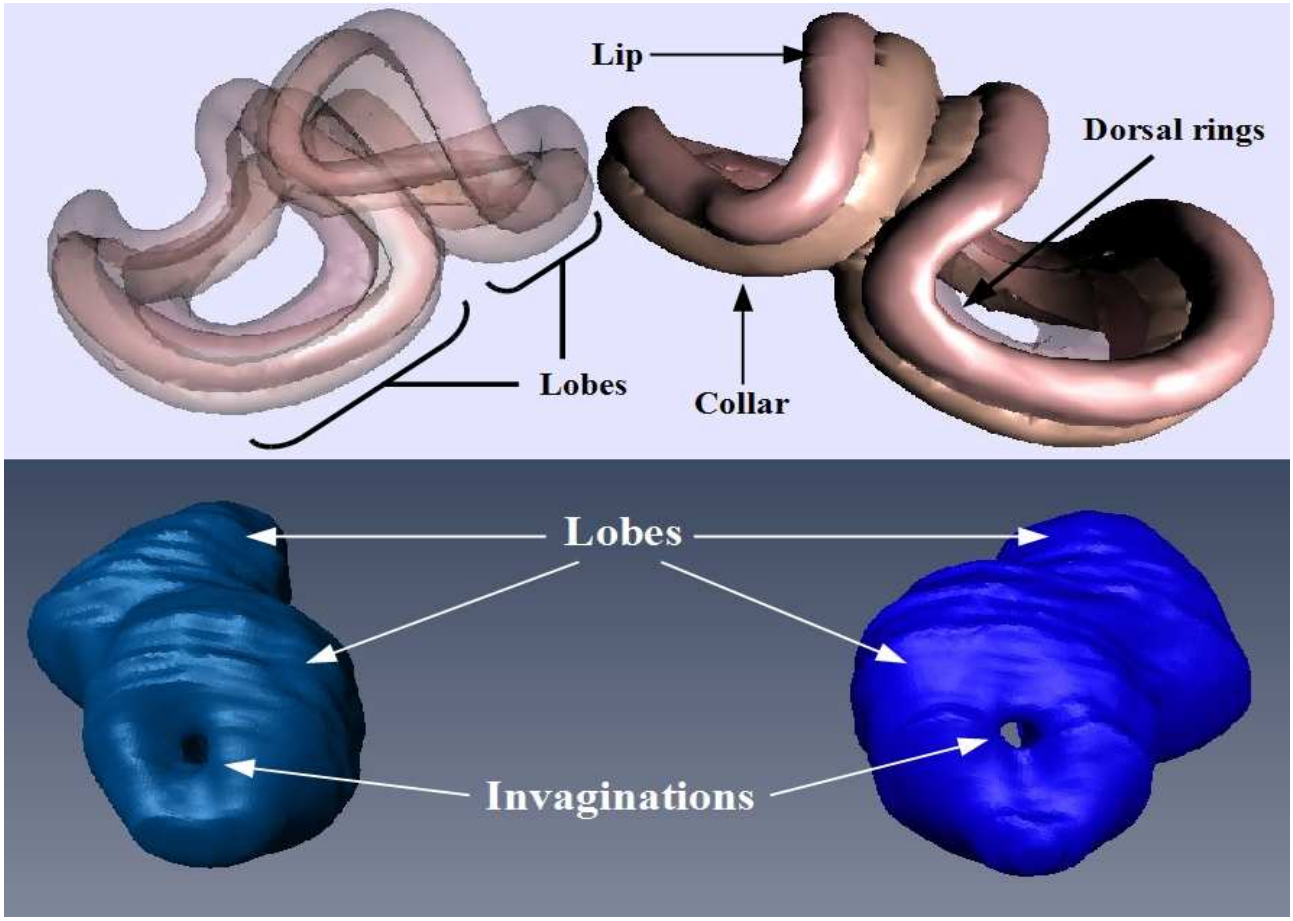

Fig. 17: Calyces of the honeybee (upper picture;adapted from Brand *et al*. 2005) and *N. vitripennis* (lower picture). Even though, two lobes are recognisable in the *Nasonia* calyces no lip, collar or dorsal ring can be distinguished.

The central body complex (CBC) is formed by the ellipsoid body (EB), the fan-shaped body (FB) and the noduli (NOD and nod) and although it takes only a rather small proportion of the relative brain volume in *N. vitripennis* (1.7%), the differences with other insects species were strongly pronounced (Fig. 15). The volume of this cluster was found to be more than twice as big as in the *A. mellifera* and almost four times as big as in *M. sexta*. Only the volume of the fruit fly CBC was with 2.1% found to be even bigger than the one found for *N. vitripennis*.

3.4 Discussion

Determining the neural substrates of perception and cognition has been a key task in insect neurobiology, since the beginnings of this discipline (Dujardin, 1850). Over the last decade computer based 3D reconstructions and standardizations of insect brains have brought a major progress in undertaking this task (Rein *et al*. 2002, Kurylas *et al*. 2008). In this chapter, a template brain of *N. vitripennis* was developed, which will provide the basis for further standardisation of this wasp brain. In this section, the methodological approach and its results will be discussed in comparison to other virtual insect brains.

3.4.1 Brain segmentation

To ensure a high consistency in the definition of neuropil labels and to minimise computational constrains, the image segmentation required compromise between gained information and increasing complexity (Brandt *et al.* 2005). In this study the main interest focused on those regions known to be involved in olfactory memory formation, namely the AL, the LH and the MB (Menzel and Giurfa 2001). For this reason special attention was paid to the labelling of these structures (Fig. 10; Fig. 14; Fig. 13; Fig. 17; Fig. 12):

In the AL, only the glomeruli and their inter-spaces were labelled, whereas the antennal mechano- sensory regions (AMSR) were assigned to the SOG. This differs from the AL definition used by Rein *et al* (2002), in which the AMSR were included in the AL. However, since the glomeruli seem to be of far higher importance for the formation of olfactory memory (Sandoz *et al* 2003) and most other virtual insect brains did not include the AMSR (Brand *et al*., 2005; el Jundi *et al.*, 2009) the definition of the AL taken in this report seems still valid. The quality of the images obtained in this study might further allow a second study on the individual glomeruli, which will provide even deeper insights into the role of AL in olfactory learning (Smid *et al*. 2003).

The LH is strongly fused with the other compartments of the protocerebrum, which made it rather difficult to label this neuropil reliably. For this reason Brand *et al.* (2005) and el Jundi *et al.* (2009) suggested to include the LH into a label comprising the PC and the SOG. In contrast to this Rein *et al.* (2002) and Kurylas *et al*. (2008) did discriminate the LH from the PC. In the study presented here, additional information on the shape of the LH was gained from the labelling of dopaminergic and octopaminergic neurons which both innervate the LH (see chapter 4). With this extra knowledge and because of the importance of the LH for memory formation it was decided to follow the approach of Rein *et al.* (2002) and Kurylas *et al.* (2008) and to discriminate the LH from the PC (Fig. 10; Fig. 17; Fig. 12).

For most of the more complex learning tasks such as non- elementary learning or spatial learning the MB have been found to play a key role (Devaud *et al.* 2007, Farris and Schulmeister, 2011). The MB consist out of three major compartments: the calyces, the pedunculi and the lobes (Mobbs, 1982). However, the further subdivision of these compartments varies largely between species. In honeybees for example, the calyces can be divided in to six sub-compartments (Brandt *et al*. 2005), whereas no subdivision was found for the calyces of *Drosophila* (Ann-Shyn *et al.* 2011). In contrast to this, five different lobes have been shown in the fruit fly (Davis, 2005). However, so far most of the available virtual insect brains did not subdivide the MBs or did only discriminate between calyces and pedunculi plus lobes, only for the honeybee a finer sub- division of the calyces was made (Brandt *et al.* 2005). For the *N. vitripennis* brain a subdivision into calyces, pedunculi plus *β*-lobes and *α*- lobes was made (Fig. 10; Fig. 14; Fig. 13). These compartments were chosen because of there distinct shape. However, it may still be considered to further subdivide the calyces and the pedunculi plus *β*-lobes, depending on the final purpose of the standard brain.

In order to simplify the segmentation process, less well defined neuropils are commonly combined in larger subdivisions of the brain. In this study, these undefined neuropils were either assigned to the PC or the SOG (Fig. 10; Fig. 14; Fig. 13; Fig. 17; Fig. 12). Other studies did not make such a separation, but assigned all undefined neuropils to a larger PC-SOG complex (Brandt *et al*. 2005; el Jundi *et al.* 2009). However, for the *N. vitripennis* brain a reliable boarder line was found, which might not necessarily represent the exact anatomical boundary between PC and SOG, but represents a good compromise between anatomical correctness and sufficient detectability. Furthermore, this subdivision of PC and SOG greatly increased the handling of the template brain and provided meaningful information for the positioning of individual neurons (see chapter 4).

3.4.2 Across-species comparison of neuropil volumes

Specific differences in certain neuropils were often found to be related with specific differences in the behaviour of an insect (Gronenberg and Hölldobler, 1999, Snell- Rood *et al.* 2009). In this study, the template of *N. vitripennis* was compared to three other insect species, for which virtual brains were created using a similar methodology (Fig. 15). Most striking in this comparison were the large differences between *N. vitripennis* and the other species in the optic and antennal lobes. For these brain compartments it was found that the optic lobes were almost half the size of those found in other insects, whereas the AL were almost twice as big as in these species (Fig. 15). A main reason for this might be the fact that the three other insect species are mainly flying species, whereas for *N. vitripennis* walking also plays an important role (Peters, 2006). Arguments for this come from studies on different ant species, where it was found that the walking workers had always

relatively smaller eyes and larger AL than the flying males (Gronenberg and Hölldobler, 1999). Furthermore, *N. vitripennis* has been shown to commonly encounter its hosts in the nests of cave breeding birds (Peters, 2006) and it might be assumed that in such an environment olfactory orientation might be more adaptive than visual information. In addition to this *N. vitripennis* also shows a rather high nocturnal activity (Peters, 2006) which might again favour olfactory over visual perception, just as it was also found for moth and butterflies (Rospars, 1983).

Another brain region which shows rather large differences between the species was the CBC, a neuropil cluster consisting of the FB, the EB and the noduli (Fig. 15). For this neuropil complex the volumes found in *N. vitripennis* and *D. melanogaster* were about three to four times as large as those found in *M. sexta* and *A. mellifera.* Unfortunately, the role of the central body complex in the insect remains largely unknown (Homberg, 2008). It has, however been suggested that the CBC might play a role for the integration of sensory input and motor output as well as in the object recognition and spatial orientation (Homberg, 2008). Interspecies comparisons such as these might give new insights into the function of the CBC and might help to further unravel its role in the insect brain (el Jundi, *et al*. 2009).

Bees and parasitoids are both known for their ability to perform complex learning tasks (Giurfa, 2003; Hoedjes *et al*. 2011). Learning and other higher cognitive processes have often been shown to be correlated with large MB volumes (Chittka and Niven, 2009). It is therefore not surprising that the comparison made here revealed MB volumes in *N. vitripennis* and *A. mellifera* which are four to seven times larger than in the other species (Fig. 15). In comparison to this, the difference between *A. mellifera* and *N. vitripennis* was rather small (with higher values for the honeybee). It is noteworthy that these higher volumes in the bee are mainly due to higher relative volumes of the MB- calyces (Fig. 16). The calyces of social Hymenoptera stand out of all other insect species especially by their elaborated shape and greatly increased relative volume (Mares *et al.* 2005) and it has often been assumed that these increases in elaboration and volume might be due to social behaviour (Gronenberg and Riveros, 2009). However, a recent study also found that within the Hymenoptera large and elaborated calyces are not restricted to the social families of Formicidae and Apidae but occur in all families of the' non-plant' feeding wasps (Euhymenoptera) (Farris and Schulmeister, 2011). Nevertheless, larger differences can still be recognised even within the Euhymenoptera. In this study the brain of *N. vitripennis,* a wasp belonging the super-family of the Chalcidoidae, in which the most simple calyces were found was compared to *A. mellifera* which possesses one of the most sophisticated calyces (Farris and Schulmeister, 2011).

As expected, *Nasonia* was found to possess a much less elaborated calyx structure than the bee. Still a doubling of the lobes and invaginations were visible which indicate a stronger elaboration of the *Nasonia* calyx than Farris and Schulmeister (2011) found in other Chalcidoidae. In contrast to this a relatively smaller ratio between calyx volume and pedunculi plus lobe volume was found in the study presented here than in the study by Farris and Schulmeister (2011) indicating a relatively higher calycs volume both in *Nasonia* and in bees. More importantly, the results presented in chapter 3. also point at a relatively larger difference in calyx volume between *Nasonia* and the bee than found by Farris and Schulmeister (2011). The differences between this study and the study of Farris and Schulmeister (2011) are most likely due to the different Chalcidoidae species used and due to more precise computer reconstructions used for the results presented in this report.

In summary, it can be stated, that our results support the finding of Farris and Schulmeister (2011) that elaborate calyces can also be found outside of the social Hymenoptera families, but that the analyses of Farris and Schulmeister (2011) might have underestimated the differences in relative volumes by not considering the there relative proportion on the entire neuropil volume. These results are particularly interesting since calyces and lobes have been shown to exhibit different functions during memory formation and other cognitive processes (Giurfa, 2003, Chittka and Niven 2009). Here it has been shown that the lobes show learning related plasticity towards a broad range of sensory stimuli whereas the different calyces receive sensory system specific input allowing them to integrate optical and olfactory input (Giurfa, 2003). Given the strong bias in the *N. vitripennis* brain towards olfactory input, it may assumed that this shift is also responsible for the differences in relative calyx volume.

Through this chapter the basis for the development of a *N. vitripennis* standard brain was provided. The results, which were obtained by an interspecies comparison of relative neuropil volumes point at the large potential of neuropil reconstructions for detecting the neural substrates of different cognitive processes and different types of memory formation. Additionally, the data shown in this chapter might provide further insight into the evolution of higher order brain structures such as the mushroom body calyces.

4. Tracing dopaminergic and octopaminergic neurons in the brain of Nasonia

Abstract

Reward-sensitive, octopaminergic, and punishment-sensitive, dopaminergic, neuronal circuits play crucial roles for the formation of memory in the insect brain. In insects, these circuits are often represented by one or a few neurons, which offers interesting opportunities to study their basic function as well as their influence on brain evolution. Unfortunately, information on these two neuron classes are currently limited to a few insect model species, which are only distantly related. In this study it was aimed to visualise both octopaminergic and dopaminergic neurons in the brain of the parasitic wasp *Nasonia vitripennis* and to compare the results to information from other hymenopterans. Among the reward-sensitive neurons one prominent projection was found emerging from the suboesphagael ganglion and reaching towards the Kenyon cells in an 'umbrella like' pattern. For the punishment- sensitivity neurons the most pronounced innervations were found in the pedunculi of the mushroom bodies. Both octopaminergic and dopaminergic neuron innervations overlapped only in the lateral horn and in the central body complex. Especially the results found for the reward-sensitive neurons differed strongly from those found in the honeybee, whereas only minor differences between the bee and *Nasonia* were found with regards to the punishment- related neurons. On the basis of these results, it was hypothesised that, because octopaminergic and dopaminergic neurons innervate different neuropils, they might also show different underlying mechanisms. Additionally, it is proposed that the reward-sensitive neurons in *Nasonia* are functionally different from the once in the honeybee and that these differences are reflected in the learning patterns of both insects as well as in the shape and volume of the mushroom body calyces.

4.1 Introduction

Neuronal circuits in the mammalian brain often consist of several thousand neurons (Schultz, 1997). In an insect brain the same cognitive process can be achieved by a single neuron (Hammer, 1993). This feature of invertebrate brains provides unique opportunities to study the mechanism and evolution of neuronal pathways on a high resolution while still considering the entire brain environment. (Chittka and Niven, 2009). A clear example for such a research was given by the milestone study of Hammer (1993) showing a single neuron in the bee brain re-sampling the entire positive- reinforcement circuit of the mammalian brain (Chittka and Skorupski, 2011).

4.1.1 The single cell reward system:VUMmx1

In 1993 Martin Hammer showed a single neuron (VUMmx1) in the honeybee brain innervating the antennal lobes (AL) the lateral horns (LH) and especially the mushroom body (MB) calyces. This

neuron was shown to respond to sucrose feeding, but even more importantly to directly induce memory formation when stimulated (Hammer, 1993). This neuron excretes the neuromodualtor octopamine, which was also found to induce memory formation when directly injected into the bee calyces (Hammer and Menzel 1998). Schröter *et al.* (2007) extended the work of Hammer (1993) by testing not only stimulation via sucrose, but also via various other cues, such as light or mechanical stimulation. Through these experiments, ten additional VUM neurons in the honeybee were described, innervating the antennal lobe (AL), the α-lobes of the mushroom bodies (MB) and the SOG itself. However, only one of these neurons showed a similar extensive arborisation pattern of VUMmx1 (Schröter *et al*. 2007).

4.1.2 Octopaminergic neurons and their role in memory formation

Among insects, bees display rather outstanding cognitive abilities (Giurfa, 2007). Studies searching for the neuronal background of this "mental power" are usually drawn to the MB calyces, which appear especially elaborated in the honeybee and other social hymenoptera (Farris and Schulmeister, 2011). However, bees do not only display a highly elaborated calyx structure, with many sub-compartments, but also sophisticated innervations of OA- neurons with in this neuropil. Although, the arborisation patterns of OA- neurons have only been studied in a few species so far, it can be expected that more elaborated calyces may be accompanied by more sophisticated innervation patterns of the OA- neurons in this brain structure (Bräunig and Burrows, 2004; Sinakevitch *et al*., 2005). Insects with less elaborated calyces such as *D. melanogaster* and *Manduca sexta* do not only display a less intensive innervation of this neuropil, but do also show stronger OA- labelling in the MB- lobes (Bush *et al.* 2009, Dacks *et al.* 2005). Interestingly, MB-lobes and calyces have been associated with different forms of memory formation (Giurfa, 2003). These patterns point at a possible role of reward-sensitive neurons during the evolution of more sophisticated calyces and learning abilities in different insect species. However, experimental evidence for this hypothesis is still rare and more detailed studies in more closely related insect species will be required to further investigate the role of OA- neurons in the evolution of learning and the brain structures related to this cognitive process.

4.1.3 Dopamine: octopamine's aversive twin

Just as octopamine was shown to facilitate appetitive learning, dopamine was found to induce the formation of aversive memory (Mercer and Menzel, 1982). Similar to the octopaminergic neuron VUMmx1, also individual dopamine neurons have been shown to induce memory formation when activated artificially (Claridge-Chang, *et al.,* 2009). Moreover, for *Drosophila* even different

individual neurons for different forms of memory formation have been shown (Aso *et al.* 2010). In this elegant study Aso *et al.* (2010) found a pair of neurons in the PPA- cluster which induced short-term memory whereas another pair of neurons in the PPL- cluster facilitated the formation of a more long lasting memory. In contrast to the OA- neurons, however dopaminergic neurons are more widely distributed over the brains of flies and project often only towards a single neuropil (Mao and Davis, 2009). An additional difference towards the OA- neurons can be found in the innervation of the MB. Here, DA- neurons have been shown in several species, such as honey bees, ants but also fruit flies, to rather innervate the pedunculi and the lobes of the MB than the calyces (Blenau *et al.* 1999; Hoyer *et al.* 2005; Claridge-Chang, *et al.*, 2009). However, both dopaminergic as well as octopaminergic neurons were shown to innervate neuropils such as the lateral horn (LH) or the central body complex (Sinakevitch *et al*., 2005; Mao and Davis, 2009). These differences as well as similarities raise interesting questions about the interplay between these two neuromodulators (Giurfa, 2007). Unfortunately, research in different model organisms has so far focused on either dopamine or octopamine, causing major difficulties for studies on the interplay between these two neuron circuits.

In the study presented here, it was aimed to investigate both octopaminergic and dopaminergic neurons in the brain of *N. vitripennis* and to compare this results to finding from other insect species. Through this, it can be expected to obtain new information about the cross talk between these two important neuromodulators. Additionally, it was hoped to gain new insight into the role these neuronal circuits might have played during the evolution of learning and learning-related brain structures, using a comparative approach.

4.2 Material and methods

4.2.1 Rearing of N. vitripennis wasps

All wasps used for the experiments described below originated from the same populations as described under 3.2.1 and were reared under identical conditions.

4.2.2 Neuron labelling

To investigate dopaminergic and octopaminergic neurons in the brain of *N. vitripennis* both neuron classes were labelled independently. A first attempt to label both neuron classes in the same brains by a double labelling procedure will be described.

4.2.2.1 Neuron stimulation

In some cases an ovipositioning experience was given to the wasps to enhance the concentration of neuron-modulators in axon fibres and axon terminals. For octopamine labelling, wasps received an ovipositing experience at specific time intervals: zero minutes, 30 minutes, 2 hours or 24 hours before sedation or not all. Wasps for the labelling of dopamine were tested with an experience immediately or 30 minutes before sedation or without any experience. Wasps that were used for the double labelling always received an ovipositing experience 30 minutes before sedation. In all cases the oviposition experience was given by collecting individual female wasps in glass vials and providing them with one fly cocoon each. Wasps were left in the vials for the time periods indicated above. Females, which showed no ovipositing behaviour within the given time, were excluded from the experiment. After the oviposition time the wasps were sedated by cooling with ice for two minutes, to simplify the decapitation and to prevent oxidisation of the neuromodulators.

4.2.2.2 Tissue fixation and dissection

Due to fast oxidation the fixation of brain tissue is a critical step for the neuron labelling and different fixation protocols were tested in this study. Fixation consisted of a pre-fixation step before dissection and a post-fixation after dissection. Pre-fixation was always done using glutaraldehyde-picric- acetic acid (GPA). However, duration was varied between 10 minutes, 20 minutes and 2 hours. To allow the GPA to penetrate the brain tissue, the mouth parts of the wasps including their mandibles were removed. During, the final dissection brains were carefully taken out of the head capsule. This was performed on a cold plate (3°C) using either phosphate buffer saline (PBS) or GPA as a dissection medium. Subsequently, the brains were again fixed for 2 hours in GPA and afterwards stored overnight at 4 °C in 70% ethanol.

4.2.2.3 Antibody labelling

To increase tissue permeability all brains were dehydrated to 95% ethanol and immersed in heptane for 20 seconds. Afterwards brains were re-hydrated and washed in PBS for 2 hours. In order to reduce potentially oxidized octopamine or dopamine, brains were incubated in 0.5% sodium borohydrate for 20 minutes at room temperature. This step was followed by several washes in PBS and incubated in collagenase (0.5 $mg*ml^{-1}$) for 1 hour to degrade the neurilemma. After this the brains were rinsed in PBS plus 0.5% Triton (PBS-T) and blocked for 1 hour in 10% normal goat serum diluted in PBS-T (NGS-PBS-T). Subsequently, the brains of the octopamine treatment were incubated in rabbit anti-octopamine antibodies (Mobitec # 1003, Göttingen, Germany) diluted 1:500 in NGS-PBS-T. Brains for the dopamine labelling were incubated in mouse anti-dopamine antibodies (Millipore # NMM1701215, Temecula, Canada) also diluted 1:500 in NGS-PBS-T. The samples for the double labelling received both antibodies in the same dilutions. All three treatments were incubated overnight at room temperature. The unbounded antibodies were washed away by rinsing the brains in PBS-T for 3 hours with several changes. Afterwards the brains were treated with a secondary antibody. In case of the octopamine treatment brains were incubated in goat anti-rabbit linked to Alexa Fluor 488 (Invitrogen # 52959A, Eugene, USA) diluted 1:200 in NGS-PBS-T. Brains receiving the dopamine treatments were incubated in rabbit anti-mouse antibodies (DAKO # 097(102),Glostrup, Denmark) diluted 1:200 in NGS-PBS-T. and received a subsequent third antibody: goat- anti rabbit. diluted 1:500 in NGS-PBS-T. For the double labelling brains were incubated in goat-anti rabbit linked to Alexa Fluor 488 and goat anti-mouse linked to Alexa Fluor 633 (Invitrogen # 799233, Paisley, UK) both diluted 1:200 in NGS-PBS-T. Furthermore 1% propidium iodide (Molecular Probes # 35824A, Eugene, USA) was added to all samples. Incubation time was set to 4 hours at room temperature for all treatments. Finally, the brains were rinsed in PBS-T for 2 hours an stored overnight at 4 °C in PBS.

Subsequently, the samples of the octopamine and double labeling treatment were prepared for microscopy by dehydrating 100% ethanol, clearing in xylene and mounting the brains individually in Depex (Fluka) on microscope slides. Brains from the dopamine treatment were washed incubated in goat-anti rabbit linked to Alexa Fluor 488 diluted 1:200 in NGS-PBS-T for 4 hours at room temperature. Afterwards these samples were again washed in PBS-T for 2 hours and stored in PBS overnight. Finally, the dopamine samples were dehydrated, cleared and mounted.

4.2.3 Confocal laser scanning microscopy

All pictures were taken using a Zeiss LSM 510 confocal laser scanning microscope equipped with an argon laser (Wavelength: 458nm, 488nm and 514nm) and two helium- neon lasers (543nm and 633nm). For the excitation of the Alexa Flour 488, which was used in all treatments the 488nm line of the argon laser was used with a band pass emission filter at 505- 530 nm. To make the propidium iodide visible, the same laser was applied with a long-pass emission filter at 585nm. For the double labelling the 488 argon laser was used in combination with the 633 nm helium- neon laser in order to excite the Alexa Flour 633. Overview pictures of the brains were taken using a 25 x oil-immersion objective (N.A. 0.8). For closer investigations of certain brain regions an 40 x oil-immersion objective (N.A. 1.3)was used. Scanning resolution for both overview and close-up pictures was set to eight bit and 1024 x 1024 pixel. Voxel size of the images ranged between 0.14 x 0.14 x 1.01 µm and 0.51 x 0.51 x 1.01 µm. For further analysis the 3D imaging software Amira 5.3 (Visage Imaging GmbH**,** Berlin, Germany) was used. Through semi- automatic segmentation, those voxel corresponding with the cell bodies of the different neurons were assigned to so call 'label fields', which were then manually placed into the standard brain created in chapter 3 (for more detail on the labelling see 3.2.3). Finally all images were ranked by a visual analyses on an ordinal scale from 0 (insufficient) to 3 (excellent). In all pictures the quality of the outer cell layer, the visibility of neuron somata, neuron fibres and axon terminals was ranked.

In total 33 images of the octopamine labelling were taken on seven different dates. For the dopamine labelling 19 images were collected on three dates and 18 brains of the double labelling were acquired on four dates.

4.2.4 Data organization and statistical analyses

To determine the effect of an ovipositing experience on the quality of images taken form brains labelled with octopamine, wasps were given an oviposition an experiences at different intervals before sedation. However, in order to acquire a sufficient number of repetitions, all different time levels as well as different pre-fixation times were combined as one treatment group (n =9) were as the wasps without ovipositing served as a control group (n = 5). All of these wasps were dissected in PBS. To test the effect of different dissection media only wasps with an ovipositing experience were used for the analyses, resulting in one treatment group dissected in GPA (n = 11) and a control group dissected in PBS (n = 9). All brains were pre-fixed in GPA for 10 to 120 minutes.

The statistical programme SPSS 16.0 was used for the statistical analyses of the ranges assigned to the different images. Within this program a one-way ANOVA was applied to test for significant differences between the rankes. Significance levels were set as following: $P \leq 0.0001$ extremely significant (***); $P \leq 0.01$ highly significant (**); $P \leq 0.05$ significant (*).

4.3 Results

Results were obtained for the optimization of the octopamine and dopamine labelling procedure. Several clusters of octopamine and dopamine neurons including their main projections were identified. The double labelling method did not yield any visualization of dopamine neurons and although some octopamine neurons labelled these were not included in the analyses.

4.3.1 Optimization of the labelling procedure

In order to increase the quality of the images and the practicality of the labelling protocol, different adjustments were tested both for the labelling of octopamine and dopamine neurons. Unfortunately, an insufficient number of repetitions was obtained for the dopamine labelling. Due to this no analyses was performed for the dopamine protocol. In contrast to this, the analyses of the octopamine treatments yielded several significant differences

4.3.1.1 Effect of an ovipositing experience on octopamine labelling

Providing an ovipositing experience to the wasps significantly (P = 0.049) increases the visualization of axon terminals. Slightly positive effects of the ovipositing were also seen for the labelling of somata and neuron fibres, although these effects were not significant (Fig. 18).

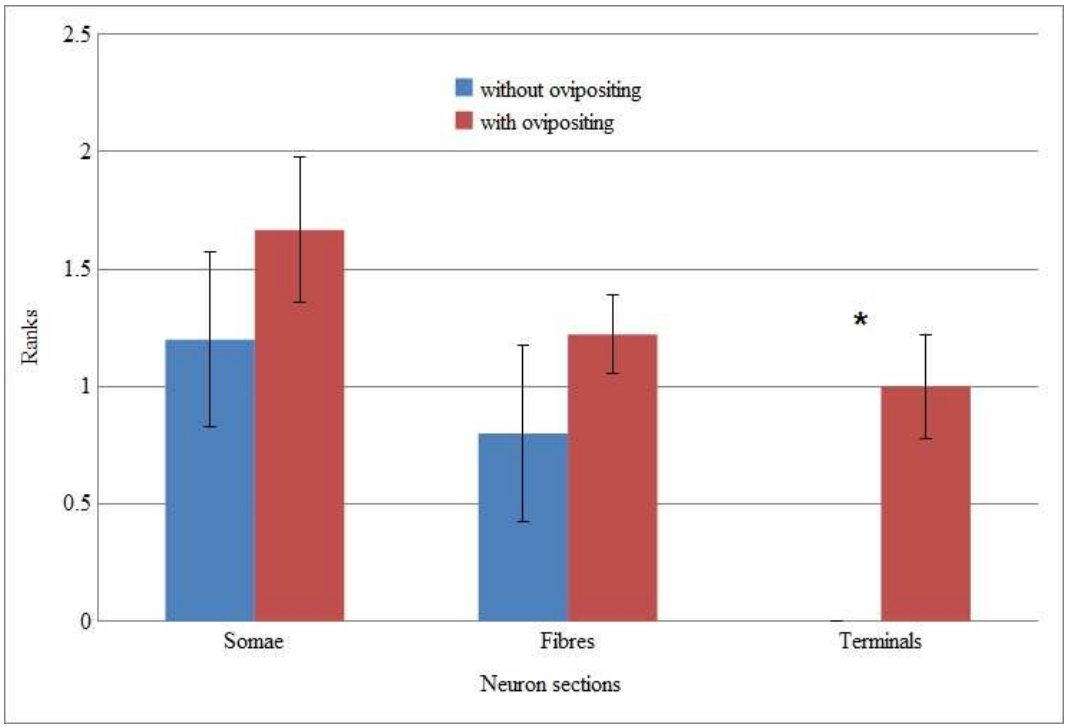

Fig. 18: Effect of an ovipositing experience on the octopamine labelling. Error bars represent the standard error for every group. Asterisk indicates $P \leq 0.05$.

4.3.1.2 Effect of the dissection media on octopamine labelling

Performing the dissections in GPA led to an extremely significant improvement in the visualization of axon terminals ($P < 0.001$) when compared with PBS. Images taken from GPA brains also showed a significantly higher amount of visible fibres ($P = 0.001$). The labelling of the somata was also higher when the brains which were dissected in GPA, however not on a significant level ($P = 0.087$). In contrast to the other categories the quality of the outer cell layer was lower for those brains dissected in GPA. This effect was however also not significant ($P = 0.754$).

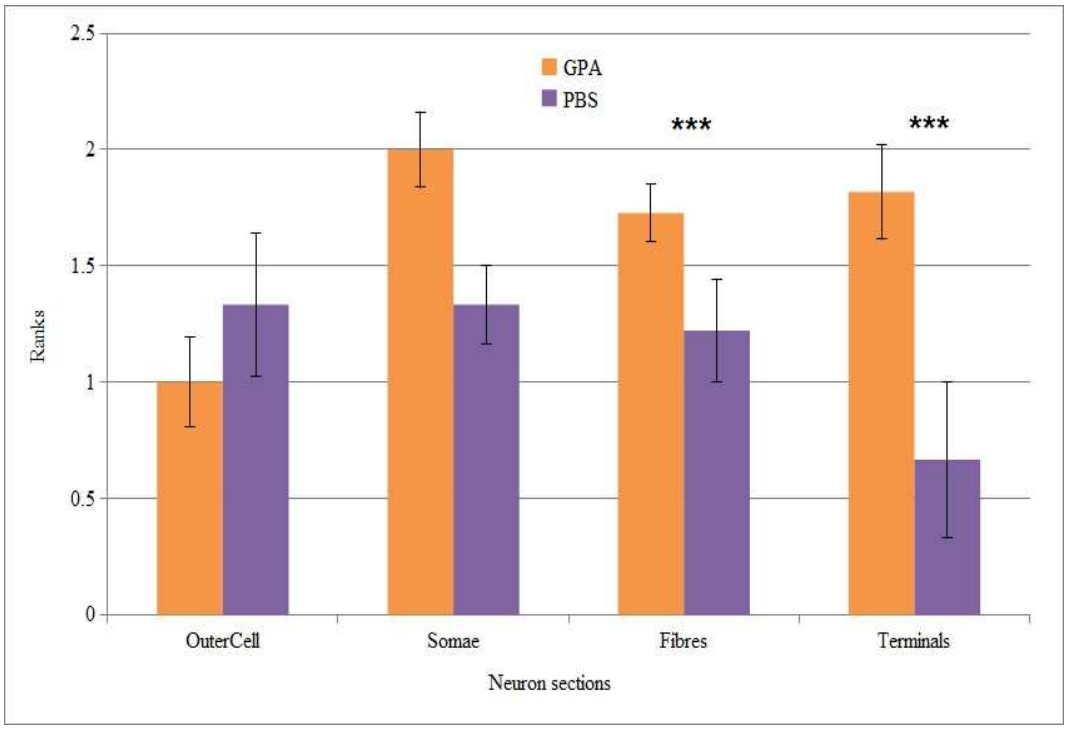

Fig. 19:Effect of the dissection medium on the quality of octopamine labelling. Error bars represent the standard error for every group. Triple asterisks indicates significant differences between treatment $P \leq 0.001$

4.3.2 Visualization of octopaminergic and dopaminergic neurons

A summary of all neurons detected in this study can be found in Table 2. The position of these neurons within the *Nasonia* standard brain, which was described in chapter 3, can be seen in Fig. 20-24. Images of the individual innervations and projections of the different neuron clusters will then be shown in the subsequent paragraphs.

Tab. 2: Overview over octopaminergic and dopaminergic neurons in found in this study

Neuro-modulator	Name	counted somata	Position of the cluster	Axonal projection and side of innervation
Octopamine	OA-MMA	2	anterior-medial to protocerebrum	unknown
Octopamine	OA-MMM	7 to 10	posterior to antennal lobes	Mushroom bodies
Octopamine	OA-VPLa	2	ventral- lateral to SOG (right)	unknown
Octopamine	OA-VPLb	2	ventral- lateral to SOG (left)	unknown
Octopamine	OA-VMM	7	ventral to SOG	oesophageal tract
Dopamine	DA-DLMa	7	ventral-medial to calyx (right)	Mushroom bodies, Lateral horn
Dopamine	DA-DLMb	5	ventral-medial to calyx (left)	Mushroom bodies, Lateral horn
Dopamine	DA-DMPa	2	posterior fan-shaped body (right)	Mushroom bodies, Fanshaped body
Dopamine	DA-DMPb	2	posterior fan-shaped body (left)	Mushroom bodies, Fanshaped body
Dopamine	DA-VLMa	2	ventral- medial to SOG (right)	Suboesophageal ganglion
Dopamine	DA-VLMb	2	ventral- medial to SOG (left)	Suboesophageal ganglion

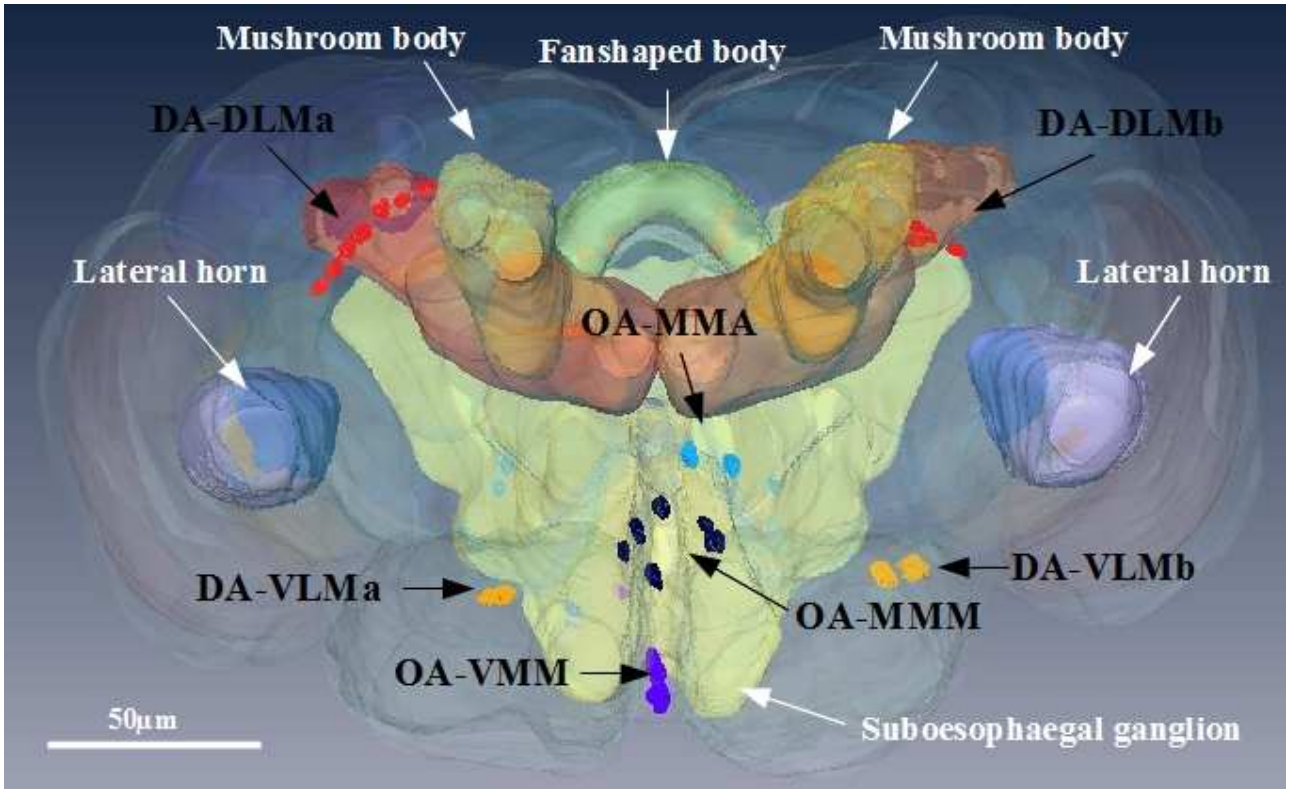

Fig. 20: Frontal view on the detected octopaminergic and dopaminergic neuron clusters incorporated into a *N. vitripennis* template brain showing those neuropils innervated by the neurons. Names of the neurons are given in black, names of neuropils are given in white.

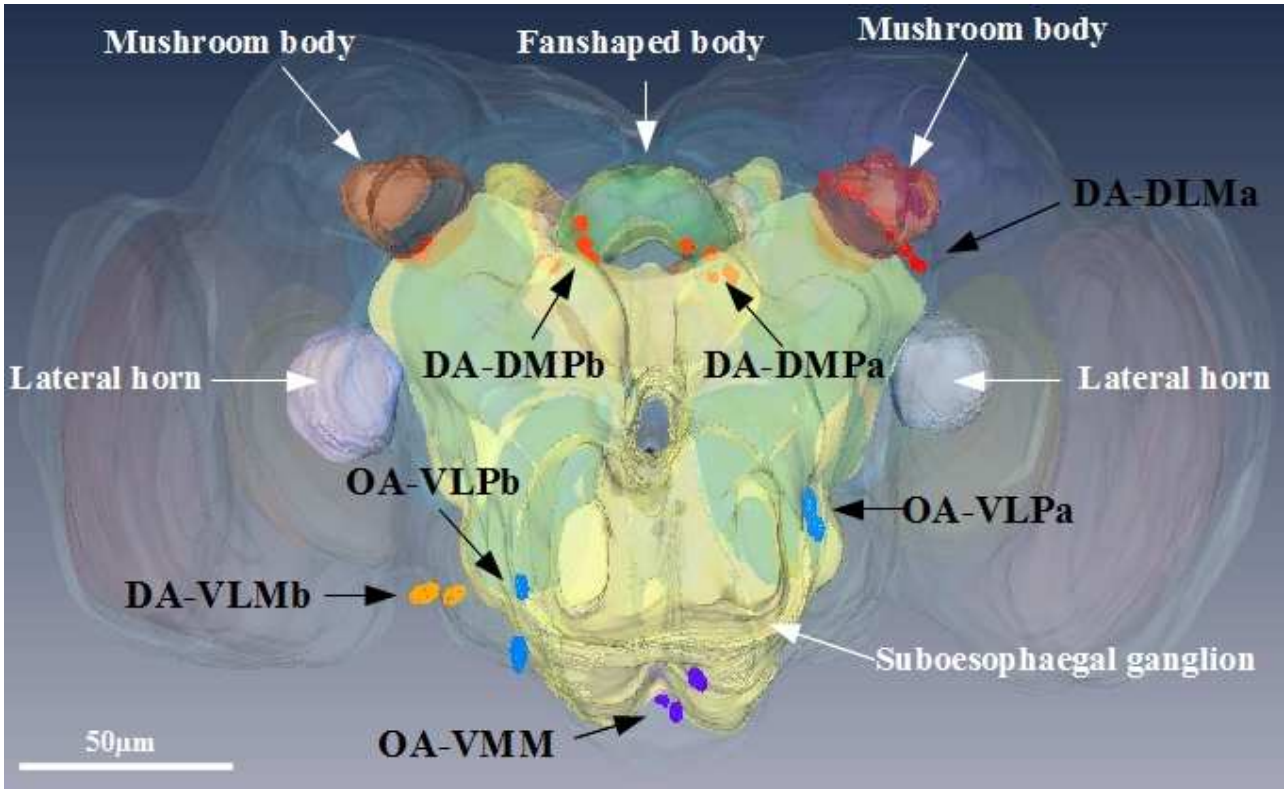

Fig. 21: Caudal view on the detected octopaminergic and dopaminergic neuron clusters incorporated into a *N. vitripennis* template brain showing those neuropils innervated by the neurons. Names of the neurons are given in black, names of neuropils are given in white.

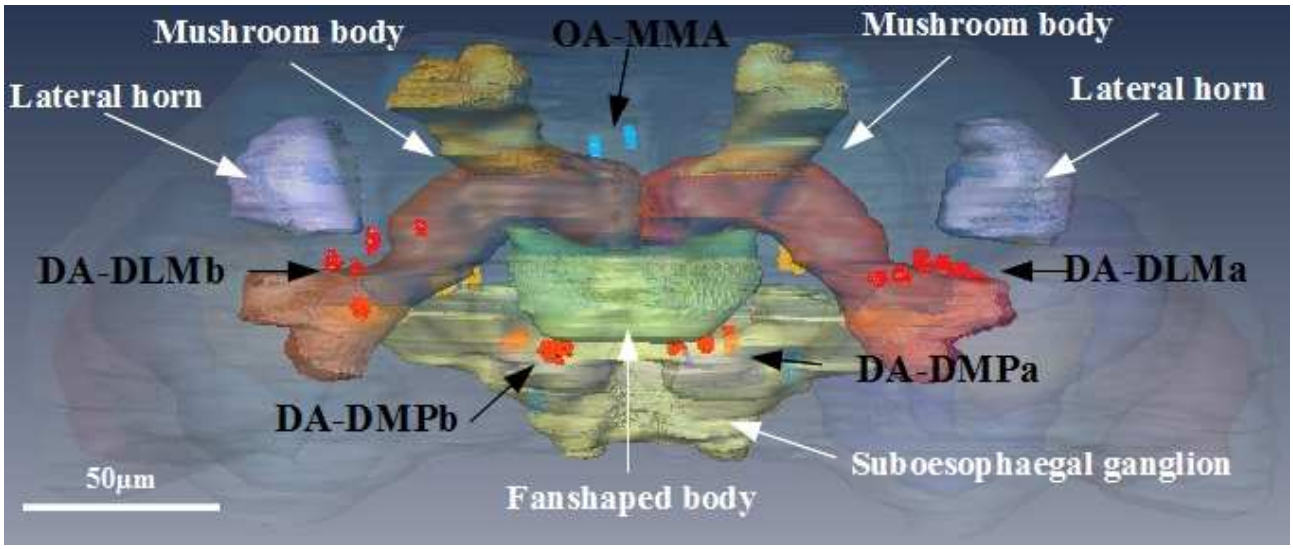

Fig. 22: Top view on the detected octopaminergic and dopaminergic neuron clusters incorporated into a *N. vitripennis* template brain showing those neuropils innervated by the neurons. Names of the neurons are given in black letters, names of neuropils are given in white letters.

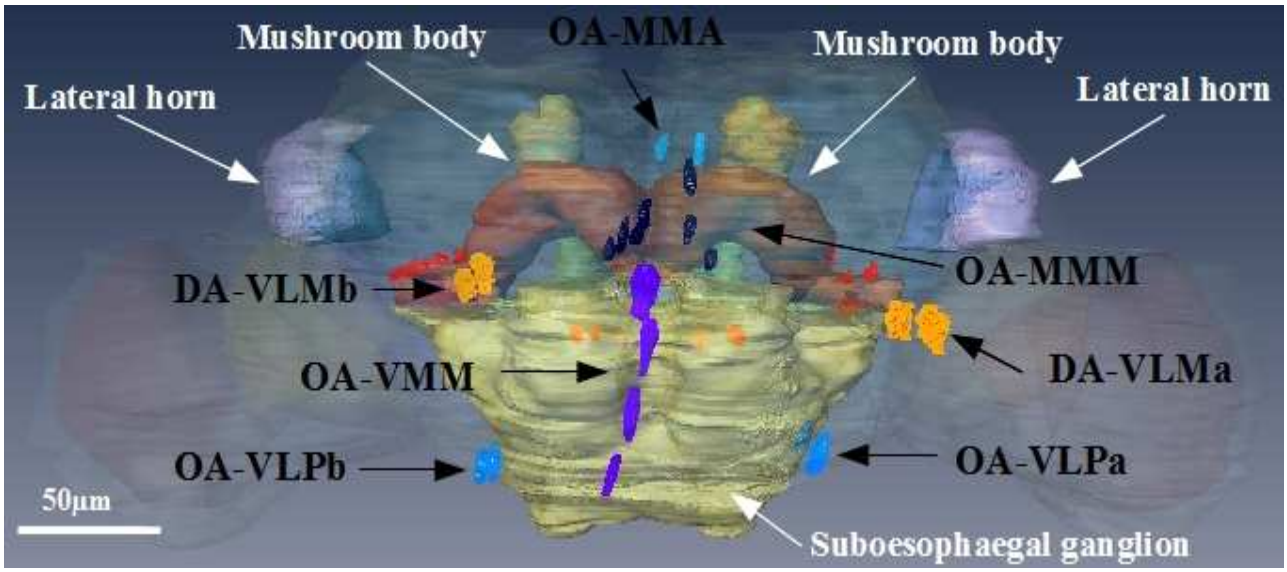

Fig. 23: Ventral view on the detected octopaminergic and dopaminergic neuron clusters incorporated into a *N. vitripennis* template brain showing those neuropils innervated by the neurons. Names of the neurons are given in black, names of neuropils are given in white.

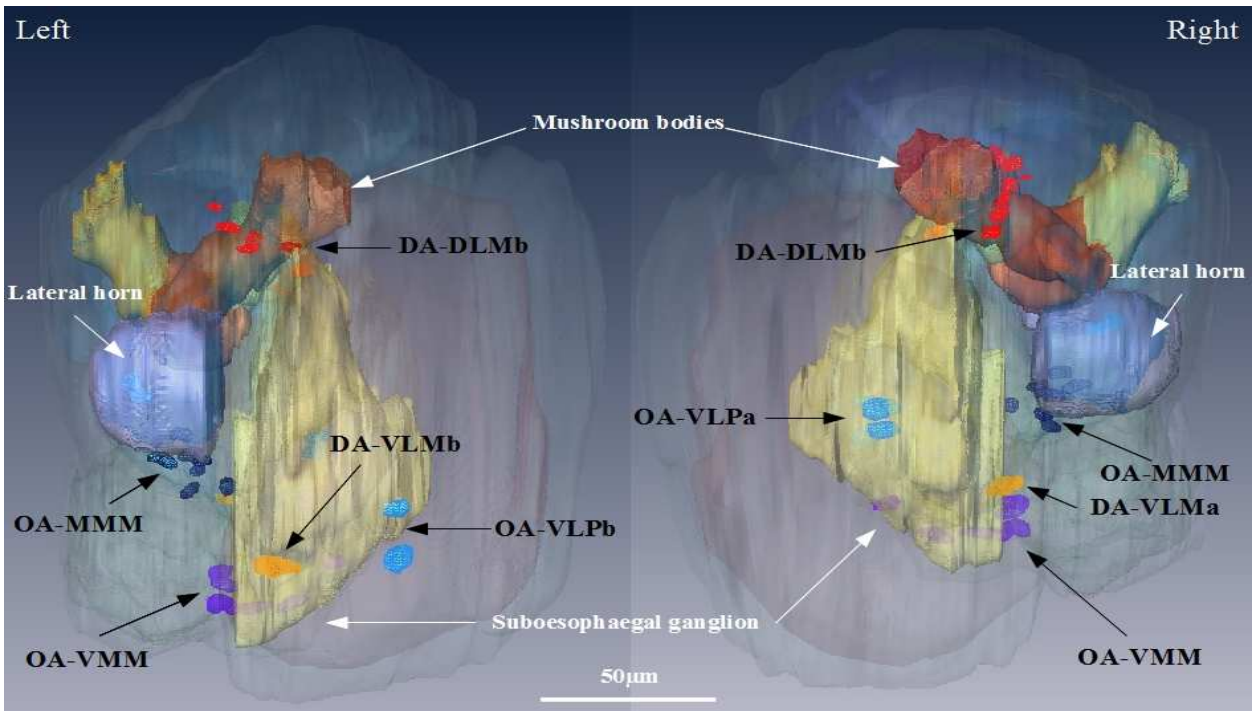

Fig. 24: Lateral views on the detected octopaminergic and dopaminergic neuron clusters incorporated into a *N. vitripennis* template brain showing those neuropils innervated by the neurons. Names of the neurons are given in black, names of neuropils are given in white.

4.3.3 Projections and innervations of octopaminergic neurons

4.3.3.1 Position and Innervations of OA-MMM

The OA-MMM cluster was one of the most prominent group of octopaminergic neurons found in the brain of *N. vitripennis.* It is positioned close to the centre of the brain at the beginning of the oesophageal tract, on the ventral side of the protocerebrum (Fig. 23, Fig. 21, Fig. 22).

Axon fibres leading from the OA-MMM cluster on to the mushroom bodies (MBs) and the calyces were the most pronounced axon fibres found in this study. These fibres were visible in almost all brains labelled for octopamine forming a characteristic umbrella like pattern (Fig. 25).

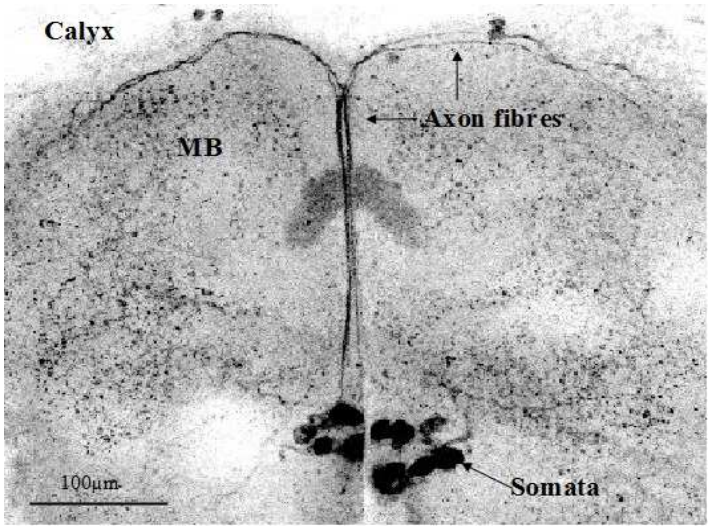

Fig. 25: Octopamine labelling, showing somata and neurites of OA-MMM neurons. Fibres leading from the somata to the MBs and calyces in an umbrella like pattern

Some neurons of the OA-MMM cluster also appeared to send axon fibres in the direction of the lateral horn (LH). The LH was found to be strongly innervated by octopaminergic neurons and the OA-MMM cluster seemed to widely contribute to this innervation.

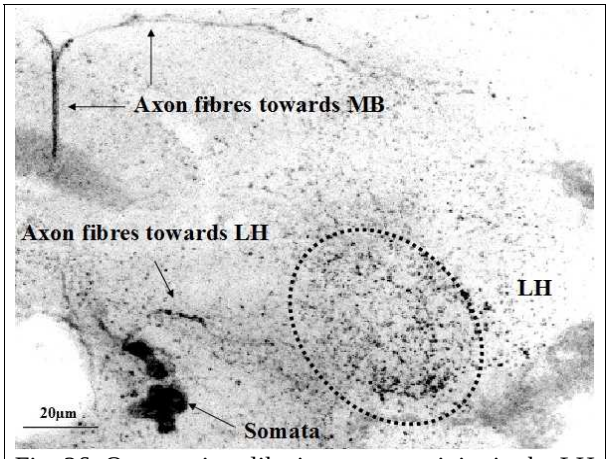

Fig. 26: Octopamine- like immunoreactivity in the LH and OA-MMM neurons. Arrows pointing on axons leading towards the LH and the MB

4.3.3.2 Position and Innervations of OA-VMM

The OA-VMM cluster was the second group of octopaminergic neuron which was reliably labelled in this study. These neurons are a lined along the complete middle line on the ventral side of the suboesophageal ganglion (SOG) (Fig. 23, Fig. 24, Fig. 21, Fig. 22).

The axons of the OA-VMM, were found to mainly project towards the oesophageal tract where they bypass the neurons of the OA-MMM cluster.

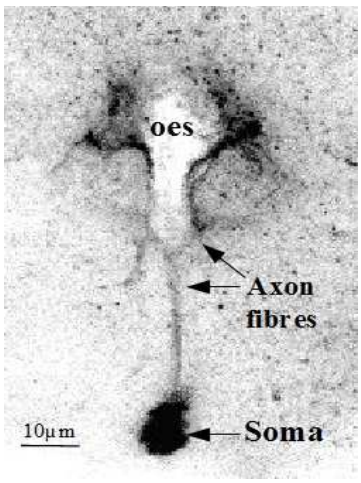

Fig. 27: OA-VMM neuron visualized by octopamine- like immunoreactivty showing the neuron soma and neurites

In combination with the OA-MMM cluster the neurons of the OA-VMM group formed a direct connection between the ventral SOG, the LHs and the MBs.

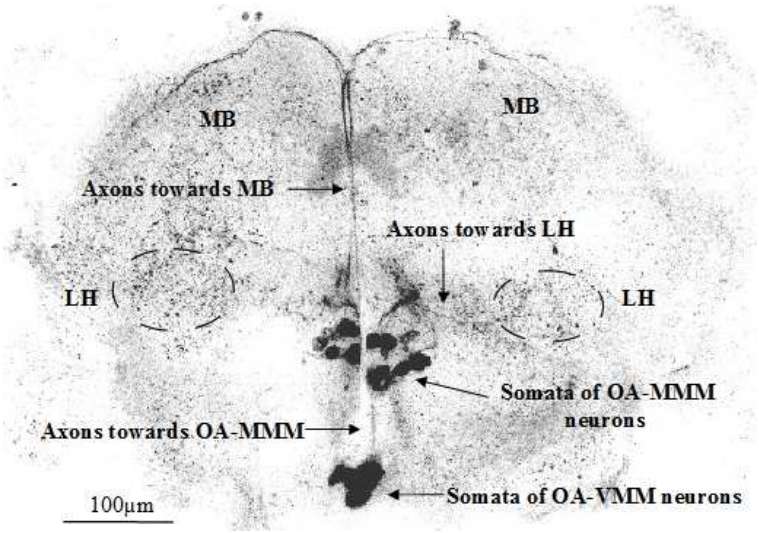

Fig. 28: Overview of octopamine labelling in a *N. vitripennis* brain. Neurons of the OA-VMM cluster as well as from the OA-MMA cluster are shown. forming a bipartite line from the SOG to the MBs and the LHs.

The innervations of the OA-MMM neurons and the OA-VMM neurons were not always visible in the same preparation as the axonal projections. However, in those preparations where innervations were clearly visible, the labelling was mainly concentrated in the central body complex, the LH and especially on the ventral-medial sides of the calyces (Fig. 29). Even though, no direct connection between these innervations and the OA-MMM and OA-VMM was observed, it seems likely that these innervations are connected to these neuron clusters.

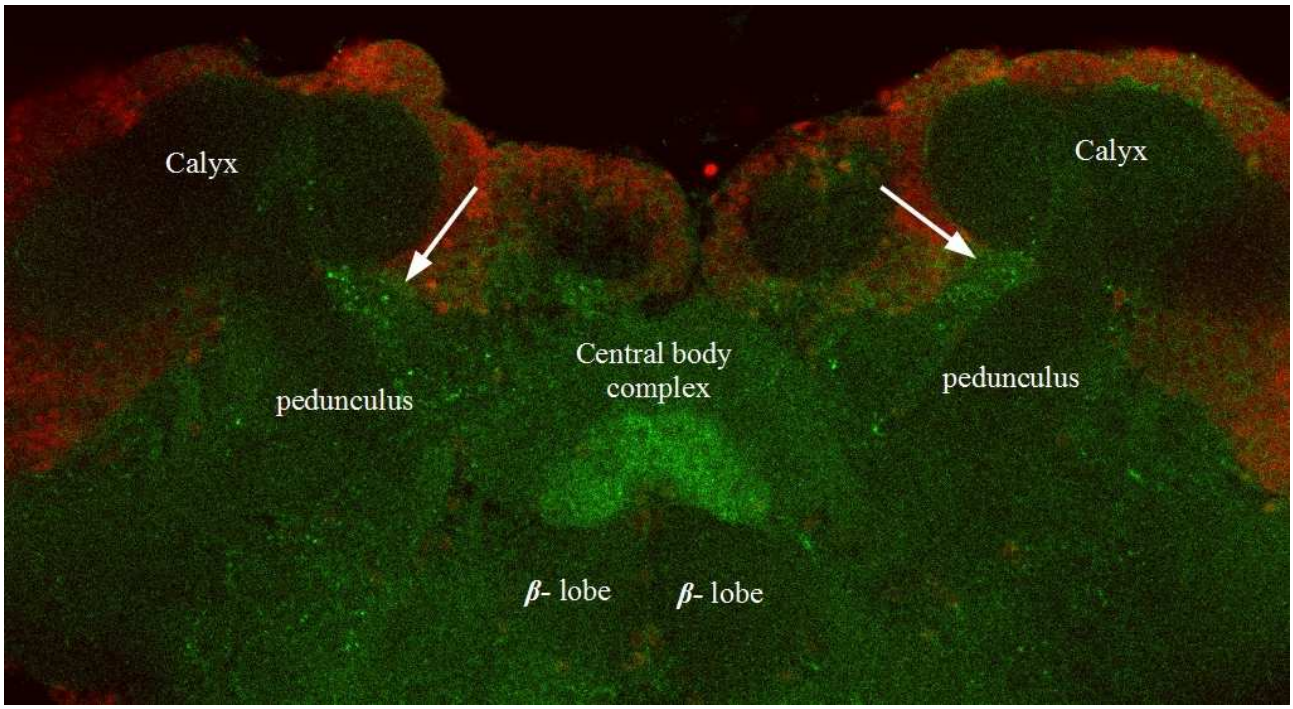

Fig. 29: Octopaminergic innervations in the brain of *N. vitripennis*. Octopamine is labelled in green, Nuclei in red. Arrows point at strong octopaminergic innervations at the ventral-medial site of the calyces.

4.3.3.3 Octopaminergic neurons projecting optic lobes

In several brains fibres exhibiting a strong octopamine like immuno reactivity were found leading from the oesophageal tract towards the optic lobes. However no cell bodies could be explicitly assigned to those fibres (Fig. 30).

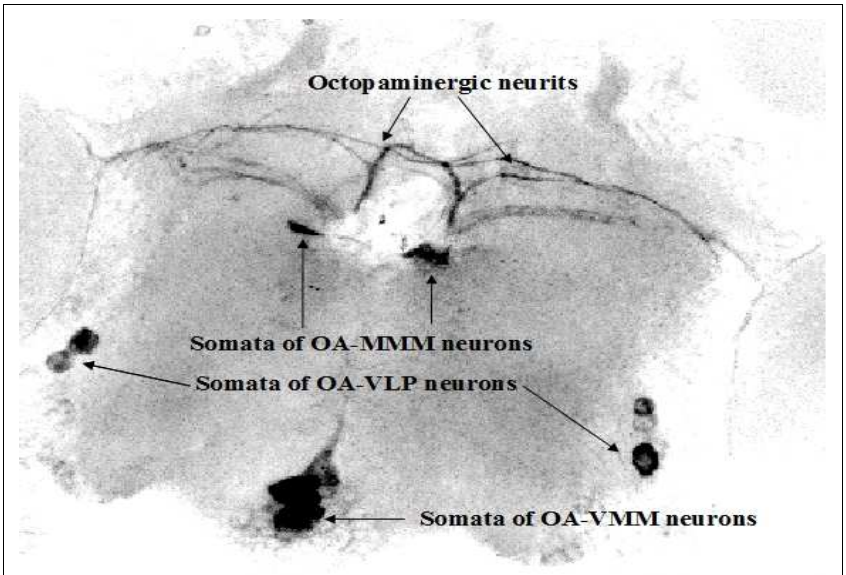

Fig. 30: Octopamine- like immuno reactivity showing neurits extending from the oesophageal tract towards optic lobes. Furthermore cell bodies of OA-MMM, OA-VLP and OA-MMM neuron are visible.

4.3.4 Position and innervations of dopaminergic neurons

4.3.4.1 Position and innervations of DA- DLMa,b

The DA-DLMa and the DA-DLMb cluster are found in the right and left brain hemisphere, respectively. Their somata form characteristic lines along the upper parts of the MB β-lobes, just dorsal to the calyces (Fig. 23; Fig. 20; Fig. 22; for a definition of MB α- and β- lobes see chapter 3).

Unfortunately, no axon fibres could be shown for these neurons. However, strong labelling of dopaminergic terminals was found in the close surrounding of the two clusters suggesting that these neuron do project to those areas. Namely, the LHs, the MB and the fan-shaped body (FB) (Fig. 31; Fig. 32).

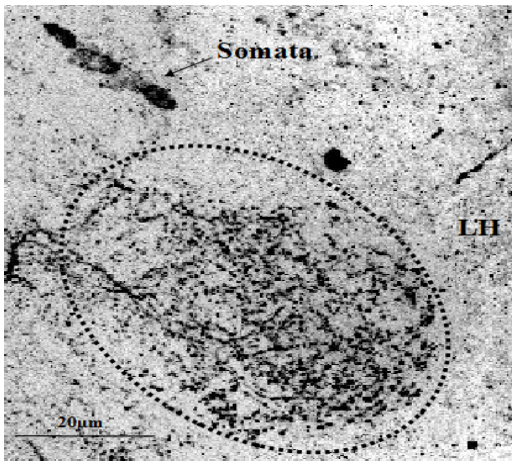

Fig. 31: Dopamine- like immunoreactivity in the LH and the somata of neurons from the DA-DLMb cluster.

4.3.2.3 Position and innervations of DA-DMPa,b

The neurons of the DA-DMP clusters form two groups on the right (DA-DMPa) and on the left side (DA-DMPb) of the neuraxis, just caudal to the FB and dorsal to the SOG (Fig. 24; Fig. 20). As for the DA-DLM clusters no axon fibres were found connected to these neurons. However, strong dopamine innervations were shown in the FB and position of the DA-DMP clusters make them the most likely origin of these innervations (Fig. 32).

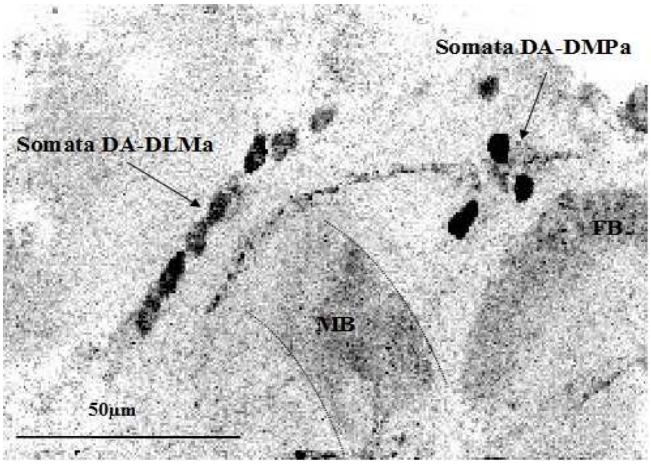

Fig. 32: Dopamine labelling of the DA-DLMa and the DA-DMPa cluster showing the somata of these neurons as well as strong dopaminergic innervations of the MB and the FB.

4.3.2.4 Position and innervations of DA-VLMa,b

The somata of the neurons in the DA-VLMa cluster were regularly found at the lateral SOG in the right brain hemisphere. The DA-VLMb cluster mirrors the position of DA-VLMa neurons in the left hemisphere and was also consistently labelled with anti-dopamine anti-bodies (Fig. 23; Fig. 24; Fig. 21; Fig. 22). Axon fibres of these neurons were clearly visible in most of the dopamine preparations and projected towards the central SOG (Fig. 33).

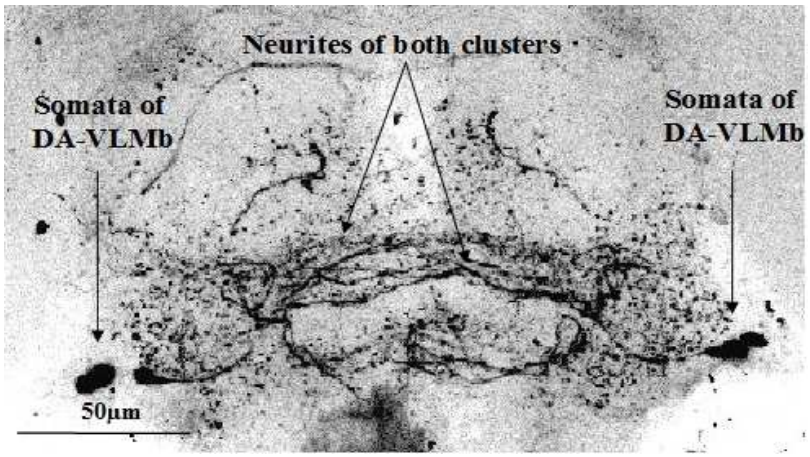

Fig. 33: Dopamine-like immunoreactivty in the medial SOG showing the neurons of the DA-VLMa and DA-VLMb cluster.

4.4 Discussion

In small brains single neurons often resemble the functions, which entire neuronal circuits fulfil in larger brains (Chittka and Niven, 2009). This property offers great opportunities to study the cross-talk between brain areas and for the investigation of neuronal pathways underlining cognitive process (Menzel and Giurfa, 2001). In this section the labelling methods for distinct neuron groups will be discussed and the innervation patterns found in this study will be compared to those of other insect species.

4.4.1 Optimising the octopamine labelling

One aim of this study was the improvement of the immunochemical labellings for octopaminergic neurons, since previous studies on these neurons in parasitoids were often hampered by an insufficient staining of axonal fibres (Bleeker *et al.* 2006). This improvement was achieved by providing an ovipositing experience to the wasps and by dissecting the head capsule directly in GPA. Especially, labelling of the axon terminal was greatly improved by providing an oviposition experience to the wasps before dissection. Several reasons might be assumed for this: One possibility, would be that ovipositing leads to a strong excretion of octopamine which might then lead to a high concentration of octopamine around the terminal buttons before the octopamine diffuses to other brain regions (Carlson 2010). An additional explanation for the increased visualisation might be a temporal activity induced expression of octopamine in the neurons, which might in turn be reflected in an increased labelling of the terminal buttons. Independent, from the mode of action however, these results further confirm the findings of Spivak *et al.* (2003), which show that the excretion and therefore also the labelling of octopamine is highly dependent on the behavioural state of the insect.

Since octopamine is quickly oxidised by enzymatic activity in the brain (Bleeker *et al* 2006), a more rapid application of the fixation medium was tested in order to prevent the degradation of octopamine. This trial led to a highly- and extremely significant improvement in the visualisation of neurites and terminal buttons, respectively (Fig. 19). Interestingly, the dissection in the fixation medium as used here yielded an even stronger staining than the injection of GPA into the living animal as used by Bleeker *et al.* (2006) on *Cotesia* wasps. However, GPA has been shown to cause a reactive airways dysfunction syndrome when inhaled at high concentrations (Kern, 1991) and a dissection of the head capsule in GPA would expose the investigator for several minutes to the chemical. Due to this, GPA should only be used with sufficient protection and ventilation. Another disadvantage of dissecting directly in GPA is the decreased flexibility of the brain tissue leading to more damage in the preparations. Considering, the health risks of dissecting in GPA it seems

advisable to conduced further trials to determine the lowest concentration of GPA which still allows high quality images of entire neurons. Additionally, a 'mini- fume hood', which was especially adapted for use in combination with a microscope might help to further decrease possible health risks (Smid, pers. communication).

4.4.2. Octopaminergic neurons in the brain of Nasonia

The neuro- modulator octopamine (OA) has often been shown to play a crucial role in the formation of appetitive memory (Schwaertzel *et al.* 2003). Hence, knowledge on the position and innervation of octopaminergic neurons might provide fruitful insights into the mechanisms and evolution of learning (Menzel and Giurfa, 1998). In this study four main clusters of octopaminergic neurons and there arborisations are shown. These results will here be compared to other Hymenoptera species.

The findings in this report for *N. vitripennis*, reveal striking parallels between the OA-neurons of *Nasonia* and those shown by Bleeker *et al*. (2006) for *Cotesia glomerata* and *C. rubecula.* In all three wasp species two dominant clusters of OA- neurons were shown: one close to the AL in a medial position (OA-MMM, Tab. 2; Fig. 23 and Fig. 21-Fig. 30) and one at a ventral, medial side of the SOG (OA-VMM, Fig. 24; Fig. 21; Fig. 22; Fig. 27; Fig. 28; Tab. 2). Furthermore, Bleeker *et al* (2006) showed several individual neurons in the pars intercerebralis of the two *Cotesia* wasps. Even though the study presented here, did not show any neurons at the exact same position, a similar cluster of neurons was found in a slightly more medial position of the *Nasonia* brain (OA-MMA,Tab. 2; Fig. 23; Fig. 21). In addition to Bleeker *et al.*, (2006) the results found in this chapter show one cluster of OA- neurons at the lateral sides of the SOG (OA-VPL, Tab. 2; Fig. 24- Fig. 22). However, at the present stage it is difficult to conclude whether the absence of these neurons in *Cotesia* is due to variations in the labelling or to real differences between the species.

When compared with the honeybee and other hymenopteran species, again some similarities can be found in the general position of octopaminergic neuron clusters. Just as in *Nasonia* and in *Cotesia* the honeybee also shows one dominate OA- cluster closely posterior to the AL and one major cluster cluster at the lateral side of the SOG (Sinakevitch *et al*. 2005). Also the small group of OA- neurons in the pars intercerebralis had been shown for the honeybee by Sinakevitch *et al*. (2005). Besides this the study of Sinakevitch *et al*. (2005) visualised many additional groups of OA-neurons among which also one cluster similar to the OA-VPL form this study was shown (Sinakevitch *et al*. 2005). In summary of these results, it might be stated that the overall pattern of OA- somata appears to be rather conserved among these four hymenoptera species, whereas several differences can be found in the distribution of the smaller OA- clusters.

However, more striking differences become apparent when the innervation pattern of these neurons are compared between *N. vitripennis* and *A. mellifera.* Even though both species show a strong innervation of the LH (Hammer, 1998, Fig. 26) they differ considerably in the innervation of the MB (Sinakevitch *et al*. 2005, Fig. 25;Fig. 28). In the honeybee octopaminergic fibres almost exclusively innervate the calyces of the MB whereas the lobes and the pedunculi receive almost no input (Sinakevitch *et al*. 2005). In complete contrast to this, *N. vitripennis* showed strong innervations close to the pedunculi and the MB- lobes (Fig. 25;Fig. 28) but only a few innervations in the calyces. Unfortunately, the study of Bleeker *et al.* (2006) did not show octopaminergic fibres, which could directly be compared to the other two species. However, the findings of Bleeker *et al.* (2006) did show strong OA- immunoreactivty in calyces, comparable to those shown for the honeybee by Sinakevitch *et al*. (2005). Interestingly, both the lobes and the calyces were shown to play distinct roles during memory formation (Giurfa, 2003), suggesting slightly different mechanisms of memory formation in *Nasonia* than in the other three species.

Due to these results it might be concluded, that even though the distribution of the OA-somata appears to be rather conserved between the four species, the innervation patterns of these neurons seem to differ at important sites in the MB. Considering the different functions of the different neuropils in the MB, it seems likely that these differences in innervations also facilitate different forms of learning (Giurfa, 2003). Especially the calyces were often shown to be important sites for the integration of optical and olfactory information and to strongly contribute to spatial and non- elementary learning (Farris and Schulmeister, 2011; Devaud, *et al.* 2007). However, the calyces are not required for olfactory conditioning (Malun et al. 2002).

Hence, it might be expected to find a less intensive use spatial- and non- elementary learning in *Nasonia* than in the other species, whereas no difference would be expected with regards to olfactory conditioning.

4.4.3 *Dopamine neurons in the Nasonia brain*

Just as octopamine has been shown to be crucial for the formation of appetive memory, dopamine (DA) was found to play an important role for the formation of aversive memory (Schwaerzel *et al.* 2003) and just as for octopaminergic neurons it was shown that information about the position and innervation of these neurons largely contributes to understanding of memory formation (Claridge-Chang, *et al.* 2009). Through the present study the position and projections of three major dopaminergic neuron clusters were revealed. In this section, theses results will be discussed in comparison to other species, such as the honey bee or the fruit fly.

DA- neurons have so far been best studied in the blowfly and in the fruit fly *D. melanogaster* (Mao and Davis, 2009), but were also described in bees and ants (Blenau *et al*, 1999; Hoyer *et al*., 2005). In the two Dipteran species over a hundred DA neurons have been found, distributed over 13 distinct clusters. This is in contrast to the results shown in this study where only 20 neuron in three clusters (Tab. 2) were found. This discrepancy, may be due to the more advanced detection tools available especially for *Drosophila*. Immunochemical labellings as performed in the study presented here, strongly depend on the amount of the target amine in the brain at the time of fixation. Due to this, rather large variations in the labelling can occur (Bleeker, *et al.* 2006). It is therefore hard to decide whether the differences found in this study are merely due to differences in labelling techniques or if they truly reflect differences between the two insect orders. However, besides these differences in numbers, some of the neurons found in this study do show similarities to those neurons commonly recognised in the flies. Especially, the neurons of the DA- DLM cluster do show a similar position and innervation as the PAL- neurons in brain of the fruit fly (Tab. 2; Fig. 23; Fig. 24; Fig. 22; Fig. 20; Fig. 31; Fig. 32, Mao and Davis, 2009). Both neuron clusters show to be positioned below the calyces and to strongly innervate the MB pedunculi (Fig. 32), Claridge-Chang, *et al.,* 2009), making them important candidates for those neurons which deliver the unconditioned stimulus during aversive learning as it has been shown for these neurons in *Drosophila* (Aso *et al.,* 2010)

Besides the MB pedunculi a strong DA labelling was also found in the LH and in the FB (Fig. 31, Fig. 32). Interestingly, the LH was also been shown to be strongly innervated by OA neurons (Fig. 26) and to play a role during memory formation (Hammer, 1993). Unfortunately, the role of the LH during learning has not yet been elucidated. However the fact that the LH is strongly innervated by both octopaminergic and dopaminergic fibres makes this neuropil an interesting site to investigate the integration of both appetitive and aversive reinforcement. Another neuropil, which was found to receive input from both DA- and OA- neurons was the FB of the central body complex (Fig. 25; Fig. 32). However, just as for the LH, the function of the FB is still largely unknown, even though first evidence suggests a role during motor control and orientation (Homberg, 2008). Such functions of the FB would be in line with the innervation by both DA and OA, since both neuromodulators have been shown to be involved in arousal and motor control (Brembs *et al.* 2007, Mao and Davis, 2009).

As an overall conclusion from the octopaminergic and dopaminergic labelling in *Nasonia,* it can be stated that both labellings were dependent on the behavioural state of the wasps. Still, important neuron clusters were found for both neuromodualtors. For octopamine the number and position of these neurons was similar to those in *Cotesia* and to a lower degree to the honeybee. However, the sites of octopamine release differ between the honeybee and *Cotesia* on the one hand and *Nasonia* on the other hand suggesting different sites of appetitive reinforcement during learning. Additionally, also octopaminergic and dopaminergic neurons differ in their innervations of the *Nasonia* MB suggesting different mechanisms for appetitive and aversive learning.

5. Towards a functional analysis of neuronal activity in the Cotesia brain

Abstract

Currently only a few neurobiological methods are available which provide information on neuronal activity on a single cell level. This chapter outlines the first attempt to develop an activity dependent labelling method for insect brains. This technique is based on *in vivo* agmatine uptake and subsequent immunochemical detection. Agmatine labelling was tested on female *Cotesia glomerata* and *C. rubecula* wasps yielding a promising protocol on how to apply this technique. Furthermore some first results were obtained on neuronal activity shortly after an ovipositing experience. These results are discussed in comparison to studies on fruit flies and honey bees and further opportunities for studies on neuron activity using agmatine labelling are pointed out.

5.1 Introduction

Within cognitive processes, single neurons often play crucial roles (Chittka and Niven, 2009). This feature creates the necessity to address questions on the neuronal mechanisms of cognition on at a single cell level (Menzel and Girufa, 2001). However, most neurobiological methods do not provide any information on the activity of these neurons and those which do, are to date limited to a very small group of organisms such as the fruit fly, *Drosophila melanogaster* (Girufa, 2007). However even in these organisms questions on individual neuron activity can only be addressed by identifying a neuron first and investigating its effect thereafter. These approaches have proven to be laborious, even in the small brain of the fruit fly with only 100,000 neurons (Ann-Shyn *et al* 2011).

5.1.1 Agmatine and its properties

In this study an alternative method was tested, using an approach inverted to the one commonly used for *Drosophila*. This activity dependent labelling makes it possible to visualize all neurons involved in a certain behaviour, which allows direct insights into the function of a certain neuron. The method is based on the resemblance of sodium-ions by agmatine, a small organic cation (Marc, 1999) (Fig. 1). In combination with immunochemical methods agmatine becomes a very suitable reporter of neuron activity and offers interesting opportunities for the investigation of single neurons (Marc *et al.* 2005).

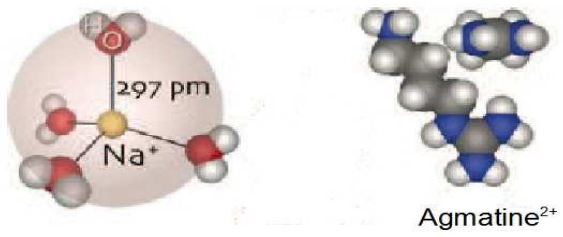

Fig. 34: Agmatine- ion (left) in comparison to Sodium-ions. The graphic illustrates the similarities in size between the two cations (Marc *et al.* 2005)

5.1.2 VUMmx1 and its network integration

The ventral unpaired medial maxillary neuron 1 (VUMmx1) is one of the most frequently mentioned individual neurons in insect neurobiology. Originally discovered by Hammer in 1993 this neuron was shown to substitute for the reward during appetitive learning. Despite its importance many questions about its mode of action and its integration into the neuron network of the brain remain unsolved (Girufa, 2007). The neurons, which provide the input for the VUMmx1 are still largely unknown (Girufa, 2007) and the interaction of VUMmx1 with other neurons with similar properties also remains unclear (Schröter *et al.* 2007). The development of new labelling methods as described here might offer new opportunities to study these questions in several species.

5.1.3 Exploring VUM neurons in Cotesia

C. glomerata and *C. rubecula* are two parasitic wasps, which display, very interesting differences in host learning although closely related (Geervliet *et al.* 1997). *C. glomerata* has demonstrated to be a quick learner, forming memory already after a single oviposition experience. *C. rubecula* in contrast takes about three oviposition experiences, separated by a certain time interval before a long lasting memory is formed (Smid *et al.* 2007). Moreover, in *C. glomerata* it was found that the strength of the memory formation, is dependent on the reward value of the host (Kruidhof *et al.* in preparation). In the study of Kruidhof *et al.* (in preparation) it was shown that the wasp only forms long-term memory after ovipositing in its preferred host, whereas ovipositing in a less optimal host induces long-term memory only after several learning trials. It was often speculated, that the differences in memory formation both between *C. glomerata* and *C. rubecula* as well as between the different rewards for *C. glomerata* are related to variations in VUM neuron activity (Bleeker *et al.* 2006). The development of an activity-dependent labelling method based on agmatine uptake might provide a fruitful technique to investigate these differences in VUM neuron activity. Through this the agmatine labelling might offer new insights into the evolution of learning and into the mechanisms controlling the formation of long term memory.

Despite this wide range of applications, no protocol using agmatine as an activity reporter in insect brains is available so far. In this chapter a first attempt was made to develop such a protocol and to explore the opportunities that such these technique might offer.

5.2 Material and Methods

5.2.1 Insect rearing

C. glomerata and *C. rubecula* wasps were reared and maintained as described in section 2.2.1.1 with the exception that *C. rubecula* wasps were raised on *P. rapae* caterpillars instead of *P.* brassicae. Both caterpillar species (*P. brassicae* and *P. rapae*) were treated as outlined in 2.2.1.2.

5.2.2 Agmatine insertion

To bring the agmatine close to the brain, *Cotesia* wasps were first sedated by cooling on ice for about two minutes. The wasps were then carefully mounted in a specially modified pipette tip, in which the thinner opening of the pipette tip was cut to allow only the head of the wasp to fit through (Fig. 1).

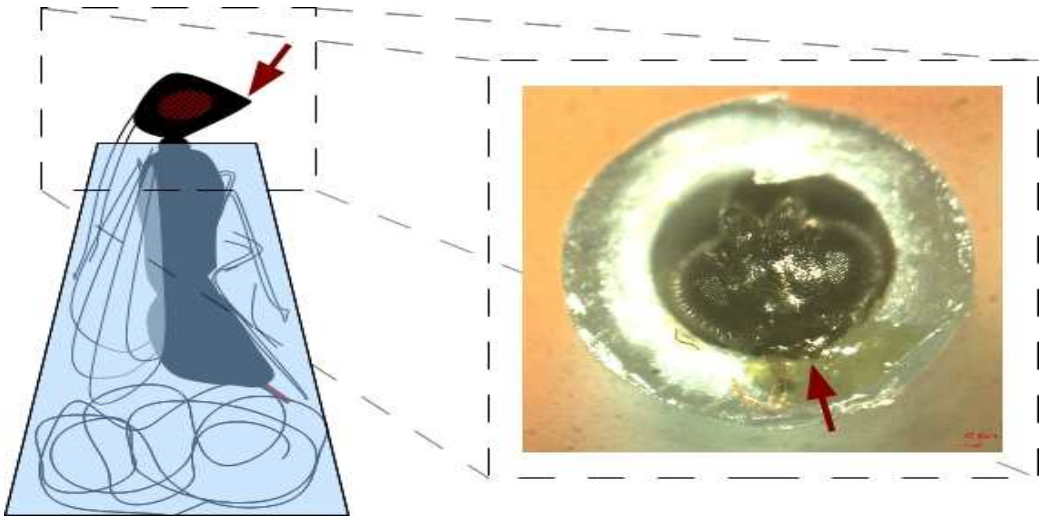

Fig. 35: Schematic drawing of a mounted wasp in side perspective (left side) and microscope picture in top view (right side). Red arrows indicate the point of agmatine insertion. The wasp was trapped in a pipette tip (blue trapezoid in the drawing) and fixed with cotton wool.

Subsequently, the labrum was dissected from the head capsule to gain access to the suboesophageal ganglion (SOG). A crystal of agmatine was then placed into this opening using an ultra thin glass capillary. As timing and not concentration seemed to be more important for the quality of the results, the method above was chosen for its fast and easy handling. However, one disadvantage of this method was the reduced control of the agmatine concentration (see 5.3).

5.2.3 Providing oviposition experience

After inserting the agmatine into the head capsule, wasps were grouped in pairs, one wasp receiving an oviposition experience and the other wasp serving as a control. The wasp assigned to the ovipositing experience was placed in a petri dish (60 mm x 10 mm) together with five first instar *P. brassicae* caterpillars on a piece of leaf (*B. oleracea*; 1 cm x 1 cm). Wasps of the control group in

contrast were placed in an empty petri dish. In all cases control wasps were prepared first and ovipositing treatment wasps second. It was assured that the time gap between the wasps was not more than 30s. Both wasps remained in the petri dish until the first ovipositing of the wasp with the caterpillars was observed and both wasps were immediately sedated on ice thereafter. In cases where wasps showed abnormal behaviour (i.e. no ovipositing observed after five minutes), wasps were discarded. These experiments were repeated three times, each time with slight changes in the protocol. In the first trial *C. rubecula* instead of *C. glomerata* and no control group were used. For the second trial the protocol mentioned above was used, with the exception that wasps were kept for an additional five minutes in an empty glass vial. On the third attempt the protocol was successfully implemented. Based on the results shown in the next section, this should be regard as the standard protocol for further investigations.

A total of 21 brains were collected over three different dates. 13 of these brains were taken from wasps with an ovipositing experience and the other eight were taken from the control group.

5.2.4 Dissection and immunochemistry

In order to prepare the wasp brains for the labelling procedure, the brains were first dissected from the head capsule. This dissection was performed in a fixative of 1 % glutaraldehyde and 2 % formaldehyde in a 0.1 M phosphate buffer. The brains remained in the fixative for four hours and were then stored overnight in PBS at 4 °C. To increase the accessibility of the tissue for the subsequent antibody staining, the brains were dehydrated in 70 % and 90 % ethanol for one minute each and immersed in heptane for 20 seconds (Breidbach, 1990). The brains were then rehydrated and washed four times in phosphate buffer saline (PBS) (Oxoid, Dulbecco "A") for five minutes each. The membranes surrounding the brains were digested by incubating the brains for one hour in collagenase (0.5 mg * ml^{-1} PBS). Subsequently the brains were washed in PBS plus 0.5 % triton (PBS-T) and transferred into eppendorf tubes. Brains were blocked for one hour in 10 % normal goat serum diluted in PBS-T (NGS-PBS-T) to reduce unspecific antibody binding. Rabbit anti-agmatine (Millipore # AB1568-2000T, Temecula, Canada) in a concentration of 1:200 diluted in 10 % NGS-PBS-T was used as a primary antibody. Incubation was performed at room temperature for 24 hours after which brains were washed over three hours in six changes of PBS-T. A secondary goat anti-rabbit antibody linked to Alexa Fluor 488 (Invitrogen # 52959A, Eugene, USA) and diluted 1:100 in NGS-PBS-T was applied to detect the primary antibody. Cell bodies in the outer cell layer were labelled by adding propidium iodide (Molecular Probes # 35824A, Eugene, USA) in a concentration of 1:200. Incubation time for this step was set to 4h at room temperature. Finally brains were dehydrated to 100% ethanol (70%, 90%, 95%, 3 x 100%, 10 minutes each) and cleared in xylol before they were mounted in Depex (Fluka) on microscope slides.

5.2.5 Confocal microscopy

Pictures of all preparations were taken using a Zeiss 510 confocal laser-scanning microscope. Details on microscopy and imaging software can befound in section 4.2.2.4. From the agmatine treatment described here voxel sizes between 0.17 x 0.17 x 1.01 µm and 0.36 x 0.36 x 1.01 µm were obtained.

5.3 Results

5.3.1 Agmatine labelling

Out of the 21 brains used in these experiments four showed a cell specific labelling, resulting in a success rate of 19 %. If however the brains scanned on the 8.11.2010 are left out, for reasons described in the next paragraph, the success rate rises to 40 %. All results are summarised in Tab. 3.

Tab. 3: Summary of agmatine- labelling results over all treatments and all collection dates. Table give the number of wasps or images obtained in the different groups.

Date		Ovipositing experience			Control		
	Total	Total	Specific labelling	Non-specific labelling	Total	Specific labelling	Non-specific labelling
14/10/10	4	4	3	1	/	/	/
08/11/10	11	6	0	6	5	0	5
16/11/10	6	3	0	3	3	1	2

Agmatine insertion resulted in the positive labelling of cell bodies. However this labelling was not in all cases selective, but marked in several brains all cells on the brain surface. This was especially pronounced in the brain scanned on the 8.11.2010 where an additional diffusion time was given after the wasps had received a certain experience. For this collection date no cell specific labelling was found, even though all brains showed a positive staining reaction (Fig. 36) These results indicate a strong correlation

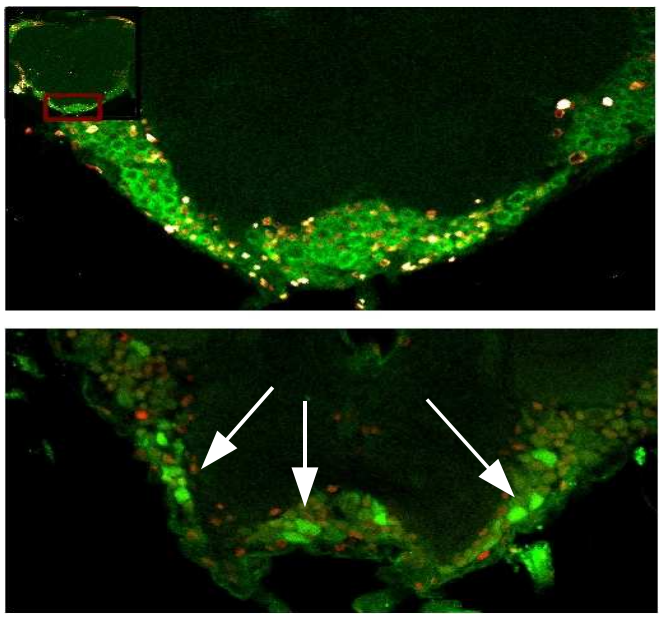

Fig. 36: Non cell specific agmatine labelling (a bow) in comparison cell- specific labelling (below). Arrows indicate the activated cells.

between diffusion time and the number of cells labelled by the agmatine treatment. In contrast to this dependency on the diffusion time, no effect of the agmatine concentration was observed. Even though the amount of agmatine inserted into the head capsule varied between the individual wasps of a certain scanning date, no obvious difference in fluorescence was observed after scanning. However, since agmatine can gain access into all active neurons, the labelling was not restricted towards ovipositing related neurons. Several good pictures of whole neurons were also obtained from one of the brains where the wasp had not received an ovipositing experience (Fig. 37).

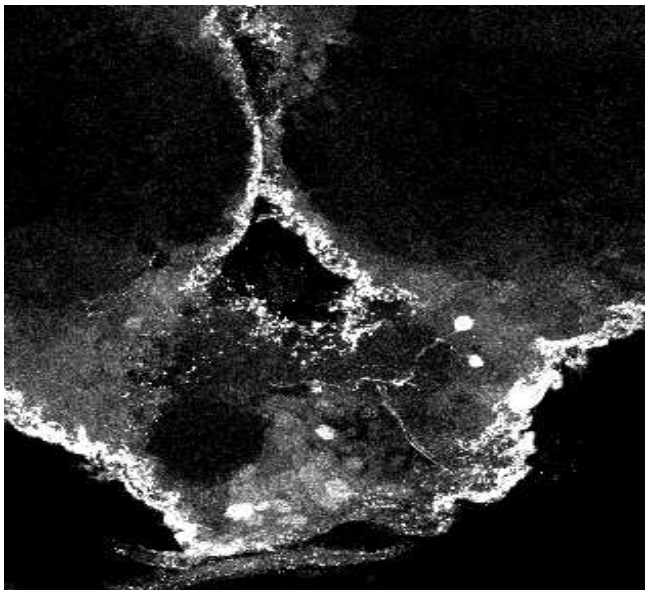

Fig. 37: Selective agmatine labelling of neurons and their neurites in the SOG of a wasp, which had not received an ovipositing experience.

5.3.2 Neuron innervations and similarities

In cases were specific activation of the single neurons was clearly visible, the activity was mostly restricted to neurons surrounding the SOG and to the Kenyon cells, which encompass the calyces of the mushroom bodies. This restricted labelling of the Kenyon cells indicates the activity specificity of the agmatine, even in brain areas widely distant from the insertion side and underlines the advantage of agmatine- labelling over non- specific neuron tracers (Fig. 38).

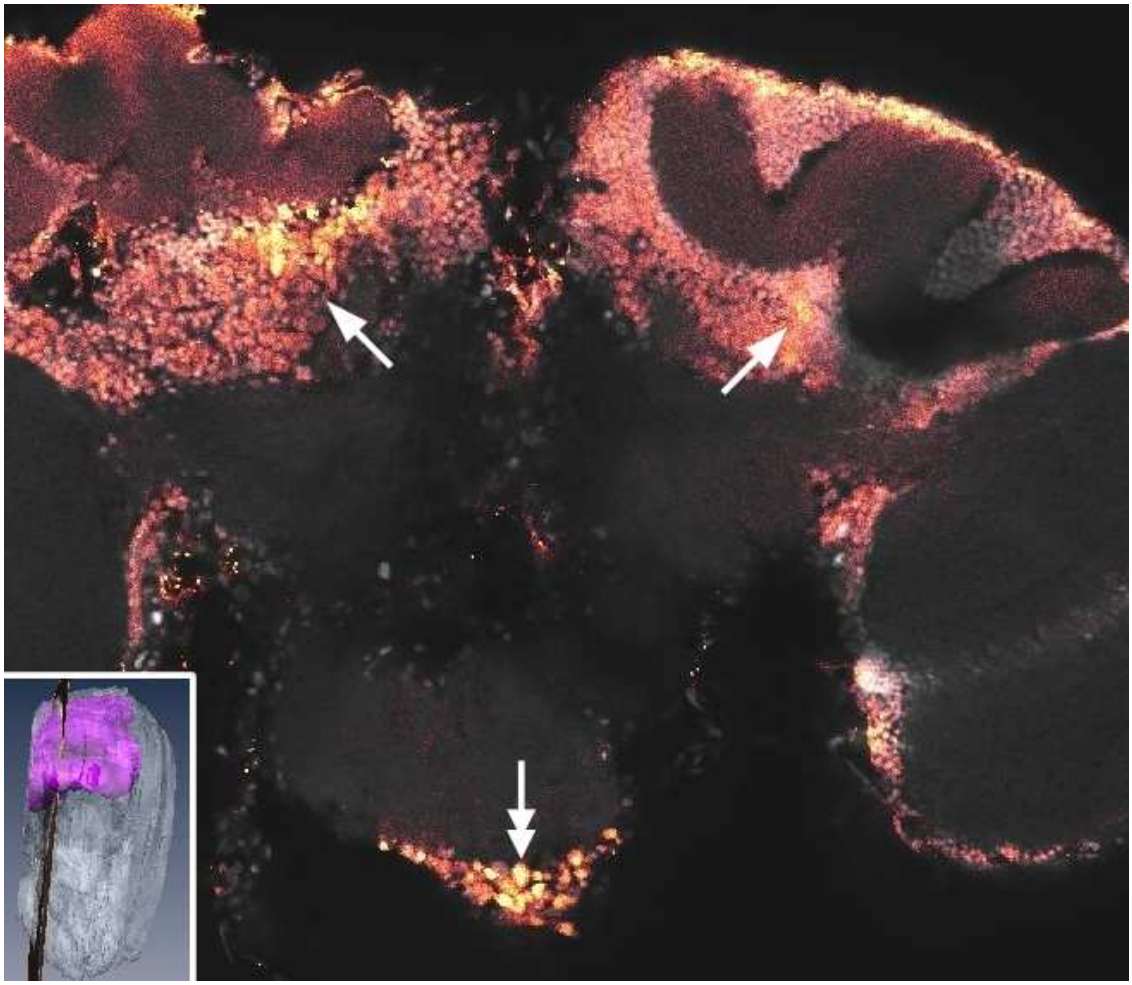

Fig. 38: Selective agmatine labelling of the Kenyon cell (single arrow) and cell in the SOG (double arrow) after the wasp had received an ovipositing experience. Small picture in the lower left corner shows the position of the image in a 3D reconstructions of the brain.

Besides the visualization of individually activated cell bodies the agmatine labelling yielded several pictures of entire neurons innervating the SOG, showing the neurites as well as the somata of these neurons. One particularly clear scan was taken from the barder region between the mandibular and the maxillary neuromeres of the SOG. The pictures from this scan were compared to previous work on *Drosophila* (Wang *et al.* 2004). The comparison showed a strong resemblance between the neurons found in this report and those neurons which perceive bitter tastes in *Drosophila*. (Fig. 39).

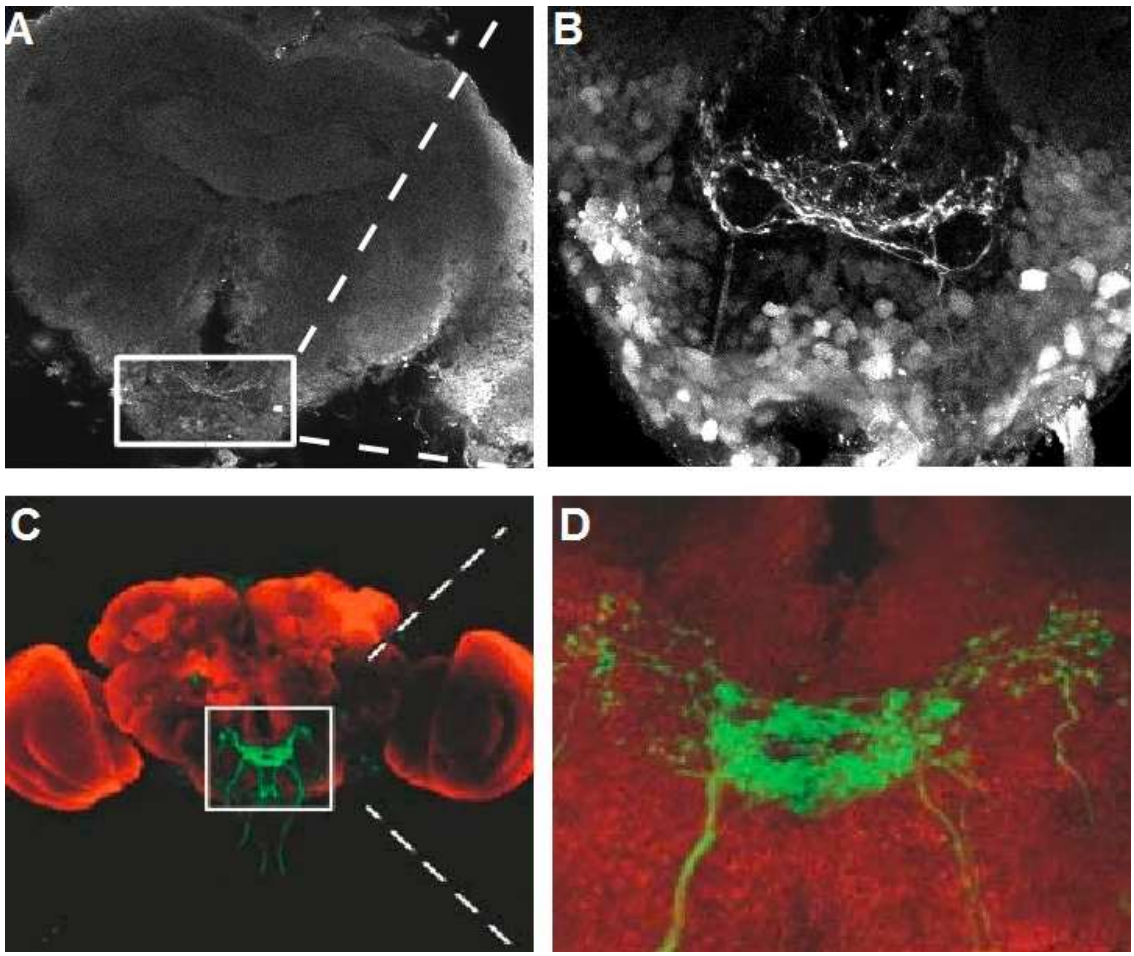

Fig. 39: Overview over the brain of C. rubecula, square outlines the maxillary part of the SOG (A). Neurites in this region were clearly stained by the agmatine insertion (B). In comparison a Drosophila brain (C) and neurites at the same part of the SOG (D) (adapted from Wang *et al*. 2004)

5.4 Discussion

5.4.1 Agmatine labelling

The results presented here suggest that agmatine has a strong potential to be used as a quantitative tool for activity- dependent neuronal labelling. In general agmatine mainly gains access into neurons via sodium- channels which are only permeable when the neuron is exited (Marc, 1999). Due to this property the agmatine will act as a selective marker even at high concentrations, as long as the incubation time is kept short. It has been shown that a short incubation with a high agmatine concentration leads usually to the most clear and selective signals (Marc, 1999). Longer incubation times with a high agmatine concentration might diminish the selectivity of the agmatine labelling for two reasons: 1) agmatine might enter the neurons by non-selective membrane channels (Kalloniatis *et al.* 2004), 2) neurons unrelated to treatment will be active due to a higher input of external information. The results presented in this report are largely in line with these previous findings of Marc *et al.* (2005) and Kalloniatis *et al.* (2004), which demonstrated clear and selective signals when agmatine was inserted in excess with short incubation periods and reduced specificity for staining with longer incubation times. In spite of these encouraging first results, the agmatine labelling holds several pitfalls, which should be taken in to account for further studies. One important characteristic of the agmatine is that it does not mark the voltage activity of a neuron but its channel activity (Marc *et al*. 2005). As a consequence, the agmatine will mark a neuron even in those cases where the outcome of neural integration inhibits the formation of further action potentials (Marc *et al*. 2005). This property of agmatine labelling might hamper studies on signal cascades, but may prove beneficial for studies on neuron networks. However, in case of such a network study it has to be taken into account that the agmatine insertion will mark all active neuron at a certain time point. This has the advantage that neurons without special properties, such as the expression of a rare neuromodulater, can be studied. However it also means that the labelling of a certain neuron after a certain treatment is only a correlation but not a causal proof that this neuron is activated by the treatment and statistical tests will be necessary before final conclusion about the activity of the neuron can be drawn.

Another important consideration, besides the insertion procedure, is that agmatine itself might cause certain behavioural changes. Due to its close resemblance of sodium ions, agmatine can not only pass sodium-channels but can also block the sodium binding site of the the NA-K ATPase (Or *et al.*, 1993). Moreover, it has been shown, that agmatine can act as NO- synthase inhibitor which was found to causes behavioural changes in mice and rats (Feng *et al.* 2002; Krass *et al.* 2010). Even though it seems unlikely that these side effects cause significant changes in the behaviour of the wasps, further experiments should still include a second control group without

agmatine insertion to check for behavioural abnormalities in the wasps with an agmatine treatment.

5.4.2 Neurite labelling

As mentioned above, the agmatine labelling of the neurons found in this study does not prove that these neurons are linked to an ovipositioning experience. Moreover, the innervations of the maxillary part of the SOG found in those wasps which had an oviposition experience rather indicate a connection to the taste receptors on the mouth parts than to the receptors on the ovipositor (Fig. 39). Additionally the fact, that only neurites in the SOG were labelled (Fig. 37) question at least the selectivity of this neurite labelling. It appears possible, that in these cases agmatine has entered the neurons through damaged sites of the tissue and not by selective opening of the sodium- channels. An alternative explanation might as well be that the insertion of the agmatine has stimulated the taste cells of the wasp and this activation has caused the neurite labelling. Support for this explanation can be seen in the fact that even though several neurons are marked, the labelling is still rather restricted. In contrast a much broader labelling would have been expected in case of an agmatine uptake caused by cell damage (Marc *et al.* 2005). Additionally, a comparison with images taken from taste receptor cells in *Drosophila* showed a strong resemblance between the neurites shown in this report and some of the taste receptor cell shown by Wang *et al.* (2004) for *Drosophila*. Interestingly the neurites found in *C. rubecula* do most closely resemble the innervation pattern of those neurons associated with bitter taste in *Drosophila*, indicating that agmatine might have been perceived by the wasp as an bitter or aversive taste. However, more repetitions and different agmatine insertion sites will be necessary to finally determine the selectivity of the neurite labelling. Despite its unclear mode of action, the agmatine labelling of individual neurites in the SOG still provides some first insights into the neuron networks in the SOG and might thereby help to further unravel the neuronal pathway from taste receptor cells to higher brain regions.

5.4.3 Soma labelling

In contrast to the neurite labelling selective labelling of neuron somata was not only present at the insertion side of the agmatine but within the entire cell layer (Fig. 38). Interestingly the labelling was most pronounced in some cells surrounding the SOG and in some of the Kenyon cells. Both of these brain regions have been shown to be of particular importance for memory formation (Menzel and Girufa 2001, Schwaertzl *et al.* 2002). In a landmark study, Hammer (1993) found one particular neuron, which was sufficient to induce appetive learning in the honeybee. This neuron named VUMmx1 originates in the SOG and innervates the antennal lobes the lateral horn the mushroom bodies (MB) and in particular the calyces (Hammer, 1993). Schroeter *et al.* (2007) extended the study of Hammer, finding two more neurons, which express the same neuromodulator as VUMmx1

and also innervate the calyces and the MB. The preliminary results obtained in the study presented in this report, do not directly prove which type of neurons has been activated by the ovipositing experience. Still, an activation of the VUM neurons in the SOG and a subsequent signal transmission to the Kenyon cells around the calyces would offer a coherent explanation for the activation patterns found in this study.

To further investigate this hypothesis, further studies might use a double staining technique against agmatine and against the neuromodulator octopamine, which is produced by the VUM neurons and released at their target sides. Through such studies it would become possible to directly correlate the neuron activity, neuromodulator release and behavioural treatments, offering tremendous perspectives for neuroethological studies.

6. General discussion

Every mind has evolved in relationship with its environment (Pearce, 1997). Within this interplay learning takes a central role, by allowing the animal to benefit from information about its environment from past experiences (Carlson, 2010). In the study presented here it was intended to investigate the brain and memory of the parasitic wasp *Nasonia vitripennis* and to gain a deeper understanding of the interplay between the cognitive traits and the behavioural ecology of this wasp species. The first aim was to create a virtual, three-dimensional reconstruction of the *Nasonia* brain and to trace the pathways of reward- and punishment-sensitive neurons within this brain. The second aim was to investigate how brain areas and neuronal pathways involved in learning are influenced by the behavioural ecology of an animal. This was achieved by comparing the results gained in this study to other virtual insect brains, with a focus on a comparison to the bee brain.

In order to gain more information about the input a brain receives during appetitive learning this report starts by studying the reward pathway on a receptor cell level in chapter 2, the report continues by investigating different neuropils as well as reward and punishment-sensitive neurons in chapter 3 and 4. Finally, chapter 5 extends the work on different neurons by developing new techniques for activity-dependent neuron labelling. The combined results of these chapters enabled the development of a virtual *Nasonia vitripennis* brain as well as the tracing of brain parts involved in memory formation. The development of this virtual brain and the neuron labelling allowed comparisons to be made with other insect species, which have provided significant insights into the function of the MB calyces and the antennal lobes during memory formation.

6.1 Receiving the rewarding stimulus

The receptor neurons on the wasp ovipositor represent one of the first stages in the nervous system at which different hosts and thereby different rewards could be discriminated (Dweck *et al.* 2008). Unfortunately, only one other study apart from the one shown here, investigated the response of ovipositor taste sensilla (OTS) towards different hosts (van Lenteren *et al.* 2007). In the study of van Lenteren *et al.* (2007) the OTS did not show a difference in response towards different hosts. In contrast to this the study presented here in chapter 2 shows one pair of recordings which indicate a difference in response pattern of the OTS towards different hosts (Fig. 9). However, considering the preliminary character of the results obtained in this study and the current technical constraints, further investigations are required before final conclusions can be drawn concerning the response of the receptors towards different hosts. Additionally, host discrimination does most likely not only

involves the OTS but may also includes information from the taste sensilla on the mouth parts and the antenna (Hoedjes *et al*. 2011). Due to this, the reward discrimination should not only be studied at the receptor level but should also investigate reward-sensitive neurons on a brain level.

6.2 Three-dimensional brain reconstructions and their applications

6.2.1 The advantage of virtual reconstructions

When trying to pinpoint the trace of learning and memory in the brain, the mushroom bodies (MB), a prominent cluster of neuropils in the centre of the insect brain, appear to be of crucial importance (Chittka and Niven, 2009). In chapter 3 of this report, 3D reconstructions of five *N. vitripennis* brains were made and the average volume of the neuropils including the mushroom bodies were calculated. The brain, which most closely matched the average volumes, was then selected as a representative standard brain. These 3D reconstructions enable not only a relatively accurate calculation of absolute neuropil volumes, but it also allows a reliable modelling of neuropil shape (Rein *et al.* 2002). Furthermore, a reconstruction of the entire brain as it was done in this study allows a more accurate estimation of the relative neuropil volume. A recent study by Farris and Schulmeister (2011) for example, calculated relative mushroom body volumes of 27 hymenopteran species by dividing the absolute mushroom body volumes by the absolute volumes of the protocerebrum. However, both the MB and the protocerebrum represent higher order neuropils and their function is highly interlinked. It might therefore be expected to find larger MBs in those species which possess a larger protocerebrum. In contrast to this, the approach taken in this study not only takes the protocerebrum volume, but takes the entire neuropil volume (brain volume without the cell layer) into account, when the relative neuropil volumes were calculated. This difference in methodology, most likely led to the more pronounced difference between the honeybee and *Nasonia*, than would have been expected from Farris and Schulmeister (2011). In addition to relative volume, the use of 3D reconstructions, as described in this report, may also allow a more accurate estimation of an average neuropil shape and it can be argued that the difference between the study of Farris and Schulmeister (2011) and the results shown here concurring the calyx shape of the Chalcidoidae are partly due to these differences in methodology.

6.2.2 Correlating brain parts to their input

Both the relative volume of the neuropils as well as the shapes of the mushroom bodies revealed interesting differences when compared to the honeybee standard brain of Brandt *et al.* (2005). The most striking differences in this comparison were found in the shape and the relative volume of the MB calyces (Fig. 16, Fig. 11). *Nasonia* was found to possess a much less elaborated calyx structure than the bee even though a double structure of the lobes and invaginations were visible. Also the relative volume of the calyces was found to be much smaller in *Nasonia* than in the bee. However, in comparison to other, non-Hymenopteran species the relative volume of the *Nasonia* calyces appeared to be relatively larger (Fig. 15). Historically, large MB and especially large calyces were often related to social behaviour, since the calyces appear especially pronounced in social insects (Durjardin, 1850; Gronenberg and Riveros, 2009). However a recent study by Farris and Schulmeister (2011) found elaborated calyces also outside of the social Hymenoptera families indicating that the evolution of elaborated calyces was not primarily driven by the development of social behaviour. The results found in this study therefore support the hypothesis of Farris and Schulmeister (2011) even though they also indicate that their methodological approach may underestimate certain differences in shape and volume between the species.

6.3 Different inputs need different calyx compartments

In most non- Euhymenopteran insects the mushroom bodies receive their main input from the olfactory and gustatory primary neuropils (Gronenberg, 2001), whereas in those insects with a more elaborate MB, the calyx also receives input from the optic lobes (Li and Strausfeld, 1997). Interestingly, in the comparisons made here, between the honeybee and *Nasonia*, the less elaborated calyces in *Nasonia* correlate with smaller relative volumes of the optic lobes (Fig. 15), suggesting a lower input of these primary neuropils into the calyces. This finding is in-line with the hypothesis of Farris and Schulmeister (2011) that the input of the optic lopes into the calyx, but not social behaviour, has driven the compartmentalisation of the calyx. They proposed further that not only the mere input of optical information has caused the elaborate shape of the calyces, but that it was rather the conversion of visual information into spatial memory. However, so far evidence for this hypothesis is still fragmentary, especially because the study of Farris and Schulmeister (2011) did not address the question whether the innervation patterns of reward-sensitive and/ or punishment-sensitive neurons, which are crucial for any type of memory formation, also correlate with a more elaborated calyx.

6.4 Different neuropils for reward and punishment

6.4.1 Neuropils for the reward

The mushroom bodies have often been argued to be the primary centre in the brain for integrating different sensory pathways with reward and punishment sensitive neurons or to use the ethological terms: to integrate the unconditioned and the newly conditioned stimulus (Giurfa, 2007). The unconditioned stimulus is delivered to the MB by punishment and reward-sensitive neurons, which express the neuromodulators dopamine and octopamine, respectively (Schwaerzel *et al.* 2002). Especially in the honeybee but also in other insects the reward-sensitive neurons have been found to strongly innervate all sub-compartments of the MB calyces but especially the lips and the collar (Sinakevitch *et al*. 2005), which are involved in the processing of olfactory and visual information, respectively (Giurfa, 2003). In contrast to this, the results presented in this report indicate that in *Nasonia* only the Kenyon cells on the ventral side of the calyces receive input from the reward-sensitive neurons (Fig. 29) and it may be argued that this rather restricted input-side is correlated with the lower input of the optic lobes into the *Nasonia* calyx. To induce learning, specific to different stimuli, the sites were the optical or olfactory input neurons connect to reward or punishment-sensitive neurons need to be separated (Giurfa, 2003). In the honeybee, the calyces offer such spatially separated sides for optical and olfactory learning. In *Nasonia*, however, the need for an optical learning compartment might be much lower, which would contribute to a less elaborated shape of the calyces.

6.4.2 A neuropil for punishment

In contrast to the reward pathway, less pronounced differences were found when projections and innervations of dopaminergic neurons, which are related to punishment, were compared between the honeybee and *Nasonia*. In both species, the strongest innervations of dopaminergic neurons were found in the upper pedunculi (honeybee: Schürmann *et al*. 1989, *Nasonia*: Fig. 32). Additionally, the dopaminergic labelling was relativity prominent in the lobes of the mushroom bodies. These results were also confirmed by findings in the ant species *Harpegnathos saltator* which demonstrated projection patterns very similar to the one in the honeybee (Hoyer *et al*. 2005), suggesting rather conserved innervation and projection patterns in all three Hymenopteran species. In addition to this, the fact that dopaminergic innervations do only superficially penetrate the calyces, indicates that aversive learning might have different underlying mechanisms than the appetitive learning and might have less strongly contributed to the development of the calyx shape.

6.5 Two different mechanisms of learning?

6.5.1 Mechanisms of aversive learning

From the findings made in this study, it could be proposed that the two neuronal pathways do not only target different brain areas, but that there may also be principal differences between aversive and appetitive learning which in term may have contributed differently to the development of more elaborated calyces. In ants and honeybees the dopaminergic neurons mainly target the pedunculi and the lobes of the mushroom bodies (Schürmann *et al* 1989; Hoyer *et al*. 2005). These neuropils consist of axons arising from the Kenyon cells, the dendrites of the MB-output neurons as well as from axons of the sensory neuropils (Mobbs, 1982, Mauleshagen, 1993). Dopaminergic innervations in the lobes and pedunculi might therefore directly induce learning-related growth of synaptic connections between the Kenyon cell axons, the sensory input neurons and the MB output neurons without passing the information through the somata and the dendrites of the Kenyon cells. Such a circumvention of the Kenyon cell somata and dendrites would allow less cross-talk between different stimuli, since integration of inputs from other neurons could only be integrated at the synapses.

6.5.2 Mechanisms of appetitive learning

Reward- sensitive neurons expressing octopamine, appear to act differently from aversive learning based on dopamine. These neurons mainly target the calyces of the MB (Sinakevitch *et al*. 2005) and it was further shown, that these neurons induce learning related synaptic plasticity in the calyx (Hourcade *et al.* 2010). The calyces are built up from the dendrites of the Kenyon cells, the axons of the reward- sensitive neurons as well as from axons that provide sensory input (Milde, 2004). Hence, in contrast to the punishment-sensitive neurons the reward-sensitive neurons rather target the dendrites of the Kenyon cells instead of the axons. Due to this, the information provided by the reward-sensitive neurons can be integrated by the somata and the dendrites of the Kenyon cells with information from other cells. Such a mechanism would for example allow the integration of visual and olfactory input before the information is passed on to the MB- output cells. This would support the hypothesis of Farris and Schulmeister (2011) suggesting that the MB calyx has evolved the elaborated shape to combine visual and optical information during learning. Furthermore, the results presented here would suggest that this integrative learning mechanism is more prominent for appetive learning than for aversive learning. Hypothesising that appetitive optical learning is dependent on calyx sub-compartments, in which optical input neurons, Kenyon cells and reward-sensitive neurons are brought together, it can be expected to find less optical learning in the smaller and less innervated calyces of *Nasonia.*

However it has also been shown that the calyces are not entirely required for simple appetitive olfactory conditioning, instead this learning task can be performed by the AL alone (Malun *et al*., 2002). Other studies on the AL and olfactory conditioning in the parasitic wasps *Cotesia glomerata* and *Cotesia rubecula* have shown that the these wasps show rather large AL with relatively high numbers of glomeruli (Smid, *et al.* 2003) as well as a high learning rate towards olfactory cues (Hoedjes, *et al.* 2011). Considering these results and the relatively large AL found for *Nasonia* (Fig. 13) it can be predicted that *Nasonia* shows at least similarly high learning rate towards appetitive olfactory cues as the honeybee. This prediction would be in line with the results of Schurmann *et al.* (2009), which show the formation of appetive, olfactory memory in *Nasonia* after a single learning trail.

6.6 The cognitive ecology of Nasonia

6.6.1 Walking in the dark

Nasonia is argued to be a habitat specialist adapted to bird nests built in tree cavities (Peters, 2006). Unfortunately, knowledge on the host searching behaviour of *Nasonia* is still sparse. Nevertheless, their dark and rather small habitat makes it less likely that the short range host searching of *Nasonia* relies heavily on visual information. In studies comparing a nocturnal moth with a day active butterfly relatively large primary neuropils for olfactory input have been found in the moth, whereas the optical neuropils were found to be rather small, in comparison to the butterfly (Rospars, 1983). Similarly, the dark habitat of *Nasonia* might also be reflected in the relatively large volume of the olfactory neuropils and the small volume of the optical neuropils (Fig. 15). Additionally, flying seems to play a less important role in the locomotion of *Nasonia* than walking (Peters, 2006), a fact which might also explain the relativity small volumes of the optic lobes in *Nasonia,* since flight has been shown to be strongly dependent on optical input (Gronenberg and Hölldobler, 1999).

6.6.2 The importance of spatial learning

The brains of different species of day active *Heliconius* butterflies presumably receive similar visual cues, still they strongly differ in calyx volume and shape (Sivinski, 1989). Thus, day time activity alone is less likely to be responsible for a more elaborated calyx shape. Also flight can not be argued to be the main driving force of calyx elaboration, considering the highly elaborate calyces of ant workers which easily surpass the calyces of the flying male ants, both in volume and elaboration (Gronenberg and Hölldobler, 1999). However, visual input might be converted into spatial memory, which has recently been argued to play an important role for the evolution of more elaborated calyces (Farris and Schulmesiter, 2011). For the *Heliconius* butterfly species, mentioned

before, it was found, that those species, which visit their food-plants in a specific order and thus rely on spatial learning, have larger calyces than those species which do not visit their feeding sites in a specific spatial order (Sivinski, 1989). Further evidence that spatial learning might drive calyx elaboration comes from ants and bees. Even though no study directly has investigated the relationship between calyx and spatial learning, the well known capacities of these species for this type of learning (Fukushi and Wehner, 2004, Menzel *et al*. 2005), correlates with their highly sophisticated calyces (Gronenberg, 2001).

From the sparse information on the behavioural ecology of *Nasonia*, it seems less likely that *Nasonia* uses traplining or a central place foraging strategy such as the honeybee. Thus, it can be assumed that spatial learning plays a less important role for the host searching of *Nasonia,* than it does for the foraging of the bee. From this it can then be argued that the less elaborated and relatively smaller calyx in *Nasonia* is a consequence of the small use of optical input for spatial learning .

6.7 New tools are required

To further investigate the neuronal mechanisms underlying different forms of memory formation, the last chapter of this report aimed to develop new techniques for activity dependent neuron labelling. So far, the function of certain neurons was mainly investigated indirectly by using pharmacological tools and immunochemistry. Here, a labelling technique based on agmatine was tested, which would a low a labelling of neurons directly based on their activity (Marc, *et al.* 2005). These preliminary studies already yielded promising visualisations of taste neurons in *C. glomerata.* From these first results it can be expected that agmatine labelling might largely contribute to studies on the integration of sensory neurons and reward- and punishment -sensitive neurons and to provide further prove for the hypothesis, that spatial learning has driven the evolution of more elaborated calyces.

7. Conclusions

Studies on the taste sensilla of a parasitoid ovipositor found one taste sensillum, which showed a different response towards haemolymph from different hosts. Still, further studies will be necessary to overcome the current technical difficulties.

This study proposes the hypothesis that the connection between optical input and reward-sensitive neurons, but not punishment-sensitive neurons, has driven the evolution of an elaborated calyx in hymenoptera, whereas olfactory conditioning also utilises the antennal lobes.

Therefore, a lower importance of optical information processing and spatial learning for the behavioural ecology of *Nasonia* is predicted, when compared with the honeybee. However no differences are expected for olfactory conditioning.

Additionally, different neuronal mechanisms for appetitive and aversive learning are proposed based on the different innervation and projection sides of these neurons in the *Nasonia* brain.

Through all this the high value of comparative studies and the usefulness of three-dimensional brain reconstructions was highlighted.

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