LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

FAKULTÄT FÜR BIOLOGIE

Visual system of basal Chelicerata

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

Vorgelegt von

Dipl.-Biol. Tobias Lehmann

München, 2014

SAMMELN UND FORSCHEN ZUM NUTZEN DER WELT

ZSM

Zoologische Staatssammlung München

Cover: left, *Achelia langi* (Dohrn, 1881); right, *Euscorpius italicus* (Herbst, 1800); photos by the author.

1. Gutachter: Prof. Dr. Roland R. Melzer
2. Gutachter: Prof. Dr. Gerhard Haszprunar
Tag der mündlichen Prüfung: 22. Oktober 2014

Erklärung

Diese Dissertation wurde im Sinne von § 12 der Promotionsordnung von Prof. Dr. Roland R. Melzer betreut. Ich erkläre hiermit, dass die Dissertation nicht einer anderen Prüfungskommission vorgelegt worden ist und dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

_____ _____

Ort, Datum Dipl.-Biol. Tobias Lehmann

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

_____ _____

Ort, Datum Dipl.-Biol. Tobias Lehmann

List of publications

Paper I

Lehmann T, Heß M & Melzer RR (2012). Wiring a Periscope – Ocelli, Retinula Axons, Visual Neuropils and the Ancestrality of Sea Spiders. PLoS ONE 7 (1): 30474. doi:10.1371/journal.pone.0030474.

Paper II

Lehmann T & Melzer RR (2013). Looking like *Limulus*? – Retinula axons and visual neuropils of the median and lateral eyes of scorpions. Frontiers in Zoology 10 (1): 40. doi:10.1186/1742-9994-10-40.

Paper III (under review)

Lehmann T, Heß M, Wanner G & Melzer RR (2014). Dissecting an ancestral neuron network – FIB-SEM based 3D-reconstruction of the visual neuropils in the sea spider *Achelia langi* (Dohrn, 1881) (Pycnogonida). BMC Biology: under review.

Declaration of author's contribution

In this thesis, I present the results from my doctoral research conducted from 2009 until 2014, carried out under the supervision of Prof. Dr. Roland R. Melzer at the Ludwig-Maximilians-Universität München.

Contribution to paper I: Lehmann T, Heß M & Melzer RR (2012)
Tobias Lehmann and Roland R. Melzer conceived and designed the project. Collection of specimens was made by Tobias Lehmann. The experiments were performed by Tobias Lehmann (TEM, Wigglesworth technique, Cobalt fills, and Golgi impregnations) and Martin Heß (3D-Reconstruction). The data were analysed by Tobias Lehmann under the guidance of Roland R. Melzer. Manuscript concept, designing of all figures, and writing was done by Tobias Lehmann under the guidance of Roland R. Melzer.

Contribution to paper II: Lehmann T & Melzer RR (2013)
Tobias Lehmann conceived and designed the project. Collection of specimens was made by Tobias Lehmann. All experiments were performed by Tobias Lehmann (Wigglesworth technique, Cobalt fills, DiI/DiO labelling, and 3D-Reconstruction). The data were analysed by Tobias Lehmann under the guidance of Roland R. Melzer. Manuscript concept, designing of all figures, and writing was done by Tobias Lehmann under the guidance of Roland R. Melzer.

Contribution to paper III (under review): Lehmann T, Heß M, Wanner G & Melzer RR (2014)
Tobias Lehmann and Roland R. Melzer conceived and designed the project. Collection of specimens was made by Tobias Lehmann. The experiments were performed by Tobias Lehmann (FIB-SEM, 3D-Reconstruction, and Golgi impregnations), Gerhard Wanner (FIB-SEM), and Martin Heß (3D-Reconstruction). The data were analysed by Tobias Lehmann under the guidance of Roland R. Melzer and Martin Heß. Manuscript concept, designing of all figures, and writing was done by Tobias Lehmann under the guidance of Roland R. Melzer and Martin Heß.

_____ _____

Ort, Datum Dipl.-Biol. Tobias Lehmann

_____ _____

Ort, Datum Prof. Dr. Roland R. Melzer

Table of contents

1. Summary

The visual systems in basal chelicerates are poorly understood, even though they can provide valuable insights for the understanding of arthropod eye evolution. Moreover, comparable morphological characters are desperately needed to reconstruct the phylogenetic tree of Chelicerata within Arthropoda, especially concerning the respective positions of Pycnogonida (sea spiders) and Scorpiones (scorpions). According to the concepts of neurophylogeny and neural cladistics, characters of the nervous system can provide important evidence about phylogeny. Therefore, in the present thesis the visual systems in sea spiders and scorpions are studied with traditional and modern methods on the three different levels of neuropils, cells, and synapses.

The visual neuropils in sea spiders and scorpions are studied with the traditional methods of Golgi impregnations, cobalt fills, and the Wigglesworth technique that allow comparisons with previous results. The visual neuropils are unequivocally identified by means of projections from the cells of the retina (R-cells) to distinct regions within the brain. The descriptions of the visual neuropils include their number, arrangement, and morphology. In addition, the visual neurons and their synapses in sea spiders are studied with the Focused Ion Beam Scanning Electron Microscope (FIB-SEM) at the highest possible resolution. This cutting-edge method allows describing the morphology and synaptic pattern of involved visual neurons and producing a 3D-reconstruction of the connectome of a visual neuropil.

In pycnogonids (*Achelia langi*, *A. vulgaris*, and *Endeis spinosa*) the R-cells are linked to a first visual neuropil in the lateral protocerebrum, and to a second visual neuropil in the central protocerebrum. The second visual neuropils of both hemispheres contact each other. Additionally, in close vicinity to the second visual neuropils an unpaired neuropil in the brain's midline is found, possibly the arcuate body, which is a prominent association centre of visual information in chelicerates. In scorpions (*Euscorpius italicus* and *E. hadzii*) the photoreceptor cells of the median eye retina are linked to a first visual neuropil, and the arhabdomeric cells to a second neuropil, while some fibres originating in the tritocerebrum additionally connect the arcuate body with the median eyes. R-cells of the lateral eyes are linked to a first and a second visual neuropil as well. Furthermore, the second median and the second lateral eye visual neuropils overlap each other; this means that there is a region with axon terminals from both eye types. FIB-SEM examination of the first visual neuropil in

the sea spider *A. langi* revealed six different neuron types postsynaptic to the R-cells: five types of descending unipolar neurons, and one type of ascending neurons. Mapping of all identifiable chemical synapses indicates that the descending unipolar neurons are postsynaptic to the R-cells, hence are second-order neurons. The ascending neurons are predominantly presynaptic and sometimes postsynaptic to the R-cells, and may play a feedback role.

At the level of neuropils the innervation pattern of the eyes in pycnogonids is similar to that of the median rudimentary eyes in *Limulus*, but it also shares characters with that of the lateral eyes in Tetraconata (Crustacea + Hexapoda). The innervation pattern of the median eyes in scorpions is similar to that of the “normal” median eyes in *Limulus*. At the respective levels of cells and synapses there are striking similarities in morphology and synaptic organization of the different neuron types between the visual system in *Achelia* and the lateral compound eye visual system in Tetraconata, especially in *Drosophila melanogaster*.

The visual system in pycnogonids combines features of the median and lateral eye visual system in other arthropods, which supports the hypothesis that pycnogonid eyes represent a precursory stage in the evolution of median and lateral eyes. Furthermore, the eyes in Xiphosura and Scorpiones indicate close evolutionary relationships, at least of their visual systems.

Zusammenfassung

Das visuelle System basaler Cheliceraten ist bisher wenig untersucht, obwohl es wertvolle Erkenntnisse für das Verständnis der Augenevolution bei Arthropoden liefern kann. Darüber hinaus werden dringend vergleichbare morphologische Merkmale für die Stammbaumrekonstruktion der Chelicerata innerhalb der Arthropoda – vor allem in Bezug auf die Position der Pycnogonida (Asselspinnen) und Scorpiones (Skorpione) – benötigt. Nach den Konzepten der Neurophylogenie und der neuronalen Kladistik können Merkmale des Nervensystems wichtige Erkenntnisse über die Phylogenie liefern. Daraufhin werden nun in der vorliegenden Arbeit die Sehsysteme von Pycnogoniden und Skorpionen sowohl mit traditionellen als auch mit modernen Methoden auf den drei verschiedenen Ebenen der Neuropile, der Zellen und der Synapsen untersucht.

Die visuellen Neuropile der Pycnogoniden und Skorpione werden mit traditionellen Methoden, wie Golgi-Imprägnierungen, Kobalt-Färbungen und Wigglesworth-Färbungen, untersucht, was eine gute Vergleichbarkeit mit früheren Ergebnissen ermöglicht. Die visuellen Neuropile werden mittels der Projektionen der Zellen von der Retina (R-Zellen) in bestimmte Regionen im Gehirn eindeutig identifiziert. Die Beschreibung der visuellen Neuropile beinhaltet deren Zahl, Anordnung und Morphologie. Zusätzlich werden die visuellen Neuronen und ihre Synapsen bei Pycnogoniden mit dem FIB-SEM („Focused Ion Beam Scanning Electron Microscope“) in höchstmöglicher Auflösung untersucht. Diese innovative Methode ermöglicht die Beschreibung der Morphologie und des synaptischen Musters der beteiligten visuellen Neuronen sowie die Erzeugung einer 3D-Rekonstruktion des Konnektoms eines visuellen Neuropils.

Bei Pycnogoniden (*Achelia langi*, *A. vulgaris* und *Endeis spinosa*) sind die R-Zellen mit einem ersten visuellen Neuropil im lateralen Protocerebrum und mit einem zweiten visuellen Neuropil im zentralen Protocerebrum verbunden. Die zweiten visuellen Neuropile beider Hemisphären berühren einander. In unmittelbarer Nähe zu den zweiten visuellen Neuropilen wird außerdem ein unpaariges Neuropil auf der Mittellinie des Gehirns beschrieben, möglicherweise der „arcuate body“, ein wichtiges Assoziationszentrum für visuelle Informationen bei Cheliceraten. Bei den Medianaugen der Skorpione (*Euscorpius italicus* und *E. hadzii*) sind die Photorezeptorzellen der Retina mit einem ersten visuellen Neuropil und die Zellen ohne Rhabdom (arhabdomere Zellen) mit einem zweiten Neuropil verbunden, während einige Fasern mit Ursprung im Tritocerebrum zusätzlich den „arcuate body“ mit den Medianaugen verbinden. Die R-Zellen der Lateralaugen sind ebenfalls mit einem ersten und einem zweiten visuellen Neuropil verbunden. Ferner überlappen die zweiten Median- mit den zweiten Lateralaugen-Neuropilen. Dies bedeutet, dass es einen Bereich mit Axonterminalen von beiden Augentypen gibt. Die FIB-SEM-Analyse des ersten visuellen Neuropils der Asselspinne *A. langi* ergab sechs verschiedene Neuronentypen, die postsynaptisch zu den R-Zellen sind: fünf Typen von absteigenden unipolaren Neuronen und einen Typ von aufsteigenden Neuronen. Die Analyse aller identifizierbaren chemischen Synapsen zeigt, dass die absteigenden unipolaren Neuronen postsynaptisch zu den R-Zellen und daher Neuronen zweiter Ordnung sind. Die aufsteigenden Neuronen sind überwiegend präsynaptisch und manchmal postsynaptisch zu den R-Zellen und spielen daher möglicherweise eine Feedback-Rolle.

Auf der Ebene der Neuropile gleicht das Innervierungsmuster der visuellen Neuropile von Pycnogoniden dem der rudimentären Medianaugen bei *Limulus*, es finden sich aber auch Merkmale, die dem Muster der Lateralaugen bei Tetraconata (Crustacea + Hexapoda) gleichen. Die Innervierung der Medianaugen der Skorpione entspricht dem der "normalen" Medianaugen bei *Limulus*. Auf der Ebene der Zellen beziehungsweise der Synapsen gibt es in der Morphologie der verschiedenen Neuronentypen und deren synaptischer Organisation auffallende Ähnlichkeiten bei *Achelia* und den Lateralaugen bei Tetraconata, vor allem bei der bereits recht genau untersuchten *Drosophila melanogaster*.

Das visuelle System bei Pycnogoniden zeigt somit Merkmale sowohl aus dem Median- als auch aus dem Lateralaugensystem anderer Arthropoden. Dies führt zu der Hypothese, dass die Augen der Pycnogoniden möglicherweise ein Vorläuferstadium in der Evolution der Median- und Lateralaugen darstellen. Darüber hinaus weisen die Augen bei Xiphosura und Skorpiones auf eine enge evolutionäre Verwandtschaft hin, zumindest in Bezug auf ihre visuellen Systeme.

2. General introduction

2.1. Introduction to Chelicerata

Chelicerata and Mandibulata (Myriapoda, Crustacea, and Hexapoda) are the two sister taxa of extant Arthropoda. The name Chelicerata was introduced in 1901 by the Berlin-based zoologist Richard Heymons (Greek *χηλη*, chele, claw; *κερας*, ceras, horn) (Dunlop 2011). The marine origin of this invertebrate group is still witnessed in Pycnogonida (sea spiders) and Xiphosura (horseshoe crabs). The biology of some fossil chelicerate groups, for example the extinct Eurypterida (sea scorpions), is also described as marine. Independently to Mandibulata within Chelicerata the highly successful terrestrial lineage of the megadiverse group of Arachnida (scorpions, spiders, harvestmen, mites and others) evolved.

Altogether more than 100000 chelicerate species have been described so far (Dunlop 2010). This group is characterized by their extreme morphological diversity (Figure 1). For instance one of the smallest arthropods – the gall mite *Eriophyes parvulus* (80 µm) – and the largest arthropod that ever lived on earth – the extinct sea scorpion *Jaekelopterus rhenaniae* (2.5 m) – are chelicerates (Westheide and Rieger 2006; Braddy, Poschmann et al. 2008).

The taxon Chelicerata is defined by their first appendages – the chelicerae – which are eponymous for this taxon. Whereas mandibulates have chewing mouthparts called mandibles, the chelicerae are usually shaped like claws or pincers and are mostly used for grasping and tearing up prey (Dunlop 2011). Besides the chelicerae, there are few other convincing autapomorphies, e.g. the division of the body in two tagmata, the cephalothorax (or prosoma) and the abdomen (or opisthosoma) (Ruppert, Fox et al. 2004; Westheide and Rieger 2006). The traditional apomorphy that the deutocerebrum (the second ganglion of the arthropod brain) and the corresponding segment with its appendage are absent in chelicerates, is challenged. Recent molecular and morphological studies show that chelicerates possess a deutocerebrum very well. It is associated to the chelicerae, which hence are homologous to the first antennae of mandibulates (Telford and Thomas 1998; Mittmann and Scholtz 2003).

The phylogeny of the chelicerate orders has been the subject of argument for over a century. A classical morphology based phylogenetic system after Weygoldt and Paulus (1979) is given in figure 2; here Scorpiones are classified as basal Arachnida and as the sister taxon to all other arachnids (Lipoctena). Another hypothesis suggests two principal lineages

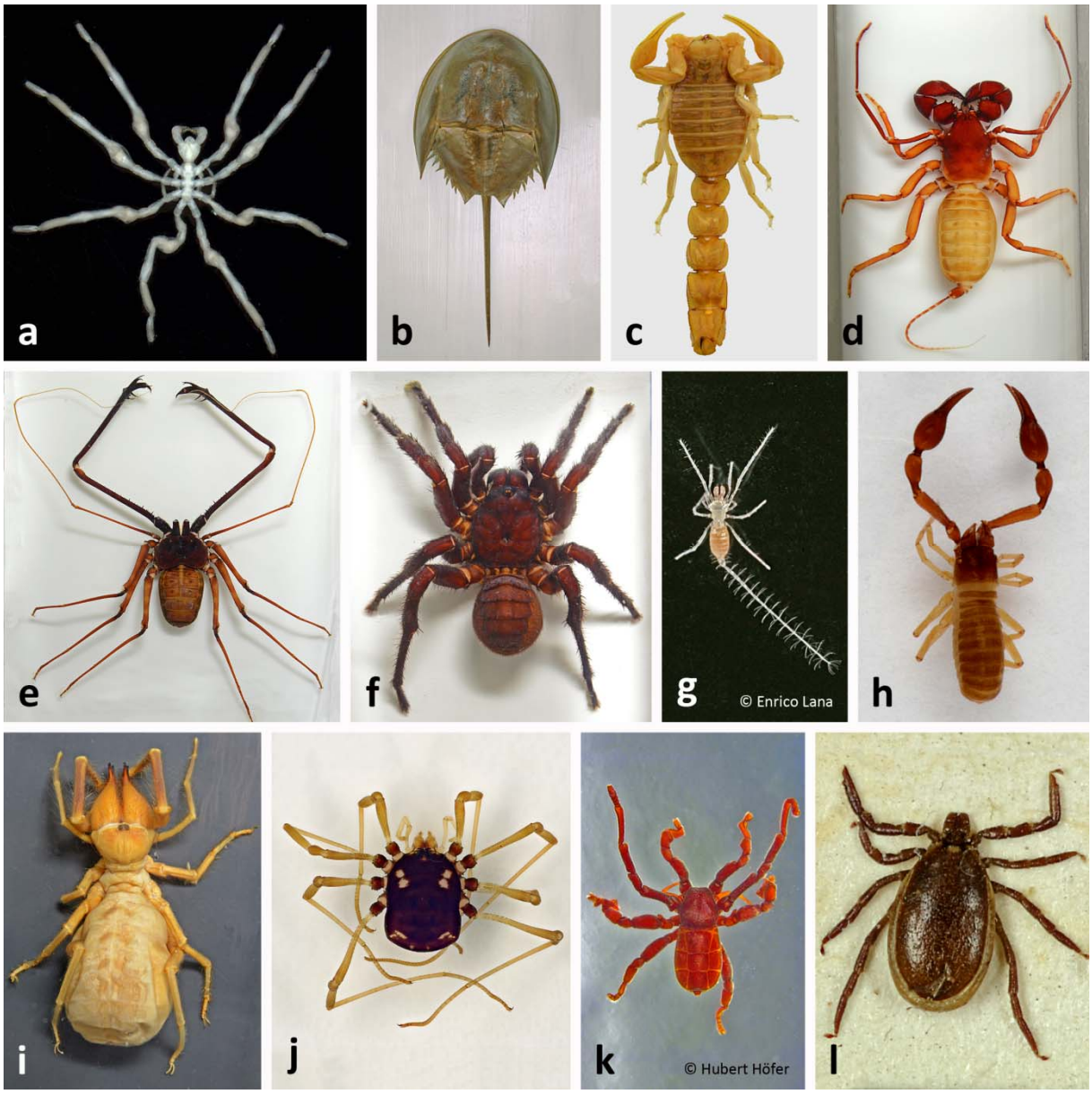


Figure 1. Representatives of the twelve recent Chelicerata orders. Specimens (except g and k) from the Bavarian State Collection of Zoology. **a**, *Callipallene spectrum* (Dohrn, 1881), Pycnogonida. **b**, *Limulus polyphemus* (Linnaeus, 1758), Xiphosura. **c**, *Androctonus australis* (Linnaeus, 1758), Scorpiones. **d**, *Thelyphonus caudatus* (Linnaeus, 1758), Uropygi. **e**, *Phrynichus reniformis* (Linnaeus, 1758), Amblypygi. **f**, *Liphistius malayanus* Abraham, 1923, Araneae. **g**, *Eukoenenia strinatii* Condé, 1977, Palpigradi. **h**, *Neobisium sylvaticum* (Koch, 1835), Pseudoscorpiones. **i**, *Galeodellus caspius* Birula 1890, Solifugae. **j**, *Paranemastoma quadripunctatum* (Perty, 1833), Opiliones. **k**, *Cryptocellus becki* Platnick & Shadab 1977, Ricinulei. **l**, *Ixodes ricinus* (Linnaeus, 1758), Acari. All photos by the author, except g by Enrico Lana and k by Hubert Höfer.

within Arachnida, namely Dromopoda (Scorpiones, Opiliones, Pseudoscorpiones, Solifugae) and Micrura (Uropygi, Amplypygi, Araneae, Palpigradi, Ricinulei, Acari) (Shultz 1990). Recently five arachnid clades were proposed, one of them being the clade Stomothecata comprising Scorpiones and Opiliones, but the relationships between those five clades are

unresolved (Shultz 2007). Combined morphological and molecular or mere molecular studies sometimes support either the one or another tree (Wheeler and Hayashi 1998; Giribet, Edgecombe et al. 2002; Masta, Longhorn et al. 2009). However, the basal position of Pycnogonida and Xiphosura (see also below) is not under consideration in these studies. And lastly some palaeontologists continue a long tradition of placing scorpions even outside Arachnida with Eurypterida (Dunlop and Webster 1999; Braddy, Aldridge et al. 1999). Eurypterida (sea scorpions) are the extinct sister taxon to Xiphosura, forming together the group Merostomata.

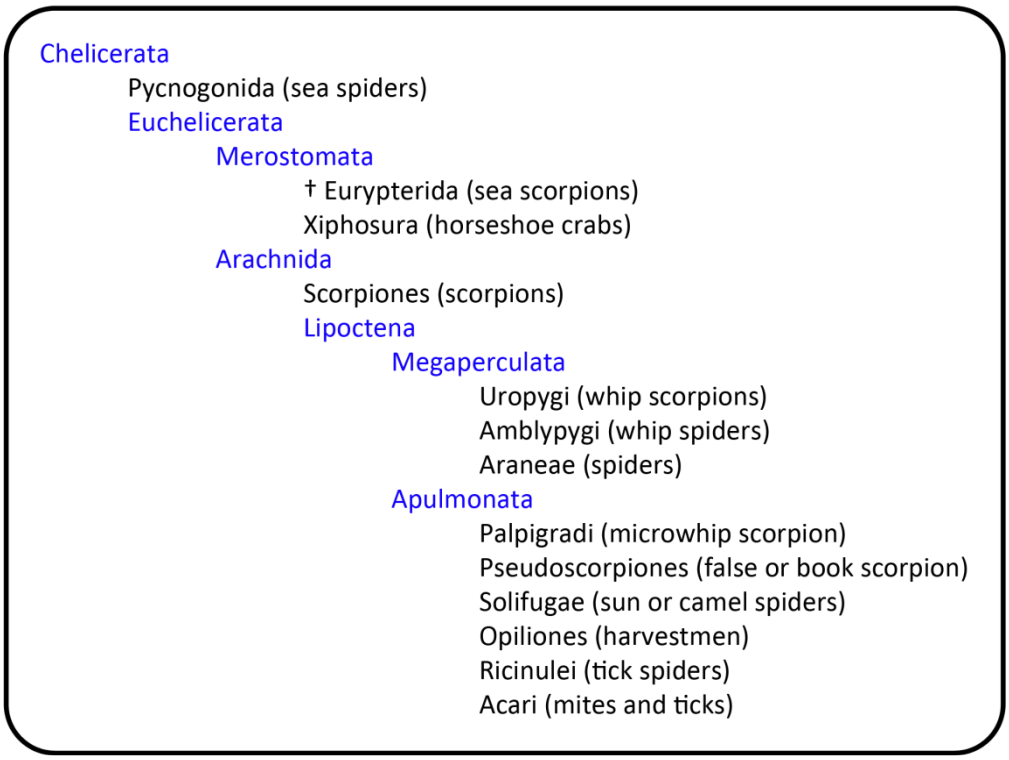


Figure 2. A taxonomic system for Chelicerata after Weygoldt and Paulus (1979)

With the rise of molecular phylogenetics the position of Chelicerata within the arthropod tree has been reorganised in the last years (Meusemann, von Reumont et al. 2010; Regier, Shultz et al. 2010; Rota-Stabelli, Campbell et al. 2011; Giribet and Edgecombe 2012; Legg, Sutton et al. 2013). Three alternative topologies are shown in figure 3. Cambrian fossils and molecular clock data indicate that the main burst of arthropod radiation emerged near the base of the Cambrian, at least 500 million years ago (Jensen 2003; Budd and Telford 2009; Rota-Stabelli, Campbell et al. 2011). The monophyly of Pycnogonida, Euchelicerata, Myriapoda, Tetraconata/Pancrustacea (Crustacea + Hexapoda), and Hexapoda is well supported, by both morphological and molecular results (Giribet and Edgecombe 2012). However convincing morphological features that clearly support one of these three trees

(Figure 3) are missing. The traditionally close relationships between myriapods and hexapods are an artefact of convergent character acquisition during terrestrialisation. From a present-day perspective most probably Chelicerata (including Pycnogonida) is the sister taxon to Mandibulata, which includes the three groups of arthropods with mandibles as mouthparts: Myriapoda, Crustacea, and Hexapoda. Alternative concepts are Paradoxopoda (Myriapoda + Chelicerata) and Cormogonida (Pycnogonida as sister taxon to all other arthropods) that have little morphological support (Giribet, Edgecombe et al. 2001; Mallatt, Garey et al. 2004; Giribet and Edgecombe 2012; Legg, Sutton et al. 2013). The major unresolved phylogenetic problems are the position of Pycnogonida and Myriapoda, the interrelationships of arachnid orders and the details of crustacean paraphyly with respect to Hexapoda.

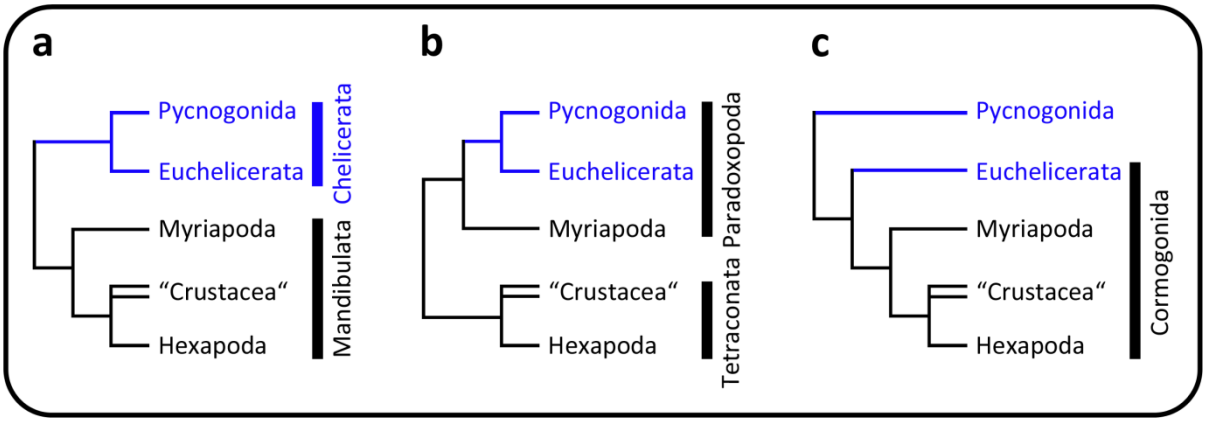


Figure 3. Current hypotheses of interrelationships in Arthropoda. a, traditionally the Chelicerata (Pycnogonida + Euchelicerata) are seen as sister taxon to the Mandibulata (Myriapoda, Crustacea, and Hexapoda). **b**, an alternative hypothesis is that Tetraconata or Pancrustacea (Crustacea and Hexapoda) are sister taxon to Paradoxopoda or Myriochelata (Myriapoda + Chelicerata). **c**, in a third hypothesis even the monophyly of Chelicerata is refused and Pycnogonida are seen as sister taxon to all other arthropods (Cormogonida). After Legg, Sutton et al. (2013).

As one can see pycnogonids and scorpions – the study objects in this thesis – have an exceptional position in the arthropod and chelicerate tree, which needs desperately comparable morphological characters.

2.2. Pycnogonida

The Pycnogonida or sea spiders are exclusively marine invertebrates, numbering about 1300 described species worldwide (Arango and Wheeler 2007). Due to their usually small size, slow-motion movement and cryptic life style these common animals are normally completely unfamiliar to laypersons. Actually if one knows where to look, sea spiders can be found in all oceans from the littoral zone to the deep sea, from cold polar waters to the

warm tropics. While most species are benthic, few are interstitial, some are pelagic and some are commensals or ectoparasites of other invertebrates. Usually they feed on sessile or slow-moving organisms (e.g. bryozoans or anemones) (Arnaud and Bamber 1987).

Sea spiders are normally small animals, littoral species have a leg span of at most a few centimetres, while polar and deepwater species can achieve a leg span of 70 cm. Looking at a sea spider one could think, that the animal has no body and exists only of legs. That's why in the past in the German and Italian literature they were named Pantopoda (Greek *παντο*, just; *πους*, foot) and a popular name is "nobodies". Today Pycnogonida is the valid taxon name for all sea spiders, fossil and recent, while Pantopoda is used for all species with a reduced abdomen (one fossil and all recent species) (Dunlop and Arango 2005).

Some of the oldest arthropod fossils are sea spiders, dating back to the Ordovician and Silurian (Rudkin, Cuggy et al. 2013; Siveter, Sutton et al. 2004), probably even to the Cambrian (Waloszek and Dunlop 2002), indicating that this taxon is highly ancestral.

Due to the fact that convincing synapomorphies with other major arthropod groups are scarce, their affinities are difficult to resolve (see above). Recent neuroanatomical studies of the innervation patterns of the pycnogonid brain have shown that the cheliphores of pycnogonids and the chelicerae in other chelicerates are homologous (Jager, Murienne et al. 2006; Manuel, Jager et al. 2006) and hence are a convincing synapomorphy for Chelicerata (Pycnogonida + Eucheliceratа). In order to find further comparable characters useful for phylogenetic considerations the visual system is studied with traditional and modern techniques in this thesis.

2.3. Scorpiones

Scorpions occur in all continents except Antarctica and are most common in the tropics and subtropics, while some species are also found in temperate climates, e.g. southern Canada, Patagonia, or the European *Euscorpius* species (Ruppert, Fox et al. 2004; Westheide and Rieger 2006). In general public scorpions are known and feared for their venomousness, but only about 30 out of over 1700 species are known to have venom capable of killing a human being (Polis 1990; Chippaux and Goyffon 2008). However, every year more than 3000 stings lead to death, mostly in Mexico (Chippaux and Goyffon 2008). In a lethal dose, death occurs within five to 20 hours by respiratory arrest (Westheide and Rieger 2006).

The affinities of scorpions in the chelicerate tree are unresolved (see above). The oldest unequivocal arachnid fossils are scorpions, dating back to the Silurian, while debate remains regarding whether their earliest representatives were aquatic or terrestrial (Laurie 1900; Dunlop 2010; Garwood and Edgecombe 2011). If scorpions and one or more other arachnid lineages came onto land independently or terrestrialisation occurred only once in Arachnida is also still under debate (Dunlop and Webster 1999; Scholtz and Kamenz 2006; Dunlop 2010). However, scorpions are among the oldest terrestrial animals, and hence the eyes of scorpions are probably offshoots of the oldest eyes adapted to terrestrial life. The visual system of scorpions is studied in this thesis with similar methods as in pycnogonids, to expand the circle of comparable characters and taxa.

2.4. Characters from the nervous system and phylogeny

Characters which can provide insights about taxonomic relatedness can be obtained from morphology, fossils, nucleotide sequences, and others. One argument for using characters of the nervous system to study phylogenetic relationships is that neuropils and pathways are likely to be stable over considerable long periods of geological time and provide powerful indicators of relatedness (Strausfeld 2012). In the early 20th century the Swedish neuroanatomists Nils Holmgren and his pupil Bertil Hanström were among the first authors who recognised the relevance of brain architecture in understanding arthropod phylogeny (Holmgren 1916; Hanström 1926; Hanström 1926; Hanström 1928). On the basis of the innervation of the eyes and the arrangement of the visual neuropils they proposed the two clades Chelicerata and Mandibulata (comprising Hexapoda, Crustacea, and Myriapoda). Furthermore, they supposed the origin of hexapods from crustaceans rather than from myriapods, what was ignored for a long time and is recently recovered with the Tetraconata/Pancrustacea concept by neuroanatomists using new staining methods (e.g. immunohistology), imaging techniques (TEM) and cladistic methods (Dohle 2001; Richter 2002). Since the two Swedish pioneers several nervous system characters, including basic elements of the brain (e.g. the deutocerebrum, see above), configurations of the visual neuropils, and the ultrastructure of the eyes, have played important roles in the debate on arthropod relationships. For this field of research two different approaches – “neurophylogeny” (Paul 1989; Harzsch 2006) and “neural cladistics” (Strausfeld 2012) – were established. The neurophylogenetic approach follows the tradition of Holmgren and

Hanström and tries to resolve relationships on selected features of the nervous system. Neural cladistics in turn tries to combine neuroanatomy with cladistics sensu Hennig (Hennig 1950) and establishes a system of classification in the form of cladograms representing genealogical relationships among taxa on the basis of several apomorphies (derived characters).

A classic example that shows how valuable characters from the nervous system and especially from the visual system are, are the above mentioned researches regarding the Tetraconata/Pancrustacea concept. These characters are the ultrastructure of the eye which gave the group its name (tetrapartite crystalline cone in the ommatidia) (Melzer, Michalke et al. 2000; Dohle 2001; Richter 2002), the brain and optic lobe anatomy (Strausfeld 2009; Strausfeld and Andrew 2011), and similarities in neurogenesis (Ungerer and Scholtz 2008) as a synapomorphy for hexapods, malacostracan and nonmalacostracan crustaceans.

2.5. Nervous systems and eyes in Chelicerata

The nervous system of chelicerates (exceptions in pycnogonids see below) is highly cerebralised and consists of a supraesophageal ganglion (or syncerebrum or brain) dorsal to the esophagus and a subesophageal ganglion (composed of several fused single ganglia), both connected by circumenteric connectives, and abdominal ganglia (Westheide and Rieger 2006; Ruppert, Fox et al. 2004). The brain is composed of three ganglia: proto-, deuto-, and tricocerebrum (Richter, Stein et al. 2013). The protocerebrum contains the visual centres and is connected to the eyes via the optic nerves. Besides the visual neuropils, the corpora pedunculata or mushroom bodies and the arcuate body are prominent association centres. Both neuropils play an important role in processing the visual input (Strausfeld, Hansen et al. 1998; Homberg 2008). The deutocerebrum is responsible for the chelicerae and the tritocerebrum for the pedipalps (Telford and Thomas 1998; Mittmann and Scholtz 2003; Manuel, Jager et al. 2006). The subesophageal ganglion is connected via sensory and motor nerves to all other cephalothoracic appendages. The abdominal ganglia are in the basal horseshoe crabs and scorpions arranged on a ventral nerve cord and in Arachnida these ganglia are incorporated into the subesophageal ganglion. The nervous system of pycnogonids takes a special role. In contrast to other chelicerates their CNS is less cerebralised, the subesophageal ganglion innervates the ovigers, followed by a ventral nerve cord which has a prominent ganglion for each pair of walking leg. The abdominal ganglion is

incorporated into the last walking leg ganglion and innervates the abdomen (Winter 1980; Manuel, Jager et al. 2006).

Usually the main sense organs in Chelicerata – due to their mostly nocturnal lifestyle – are sensory setae. These are often accumulated on the pedipalps or on a pair of specialised walking legs.

As is typical for arthropods in Chelicerata median and lateral eyes occur (Paulus 1979). In the earliest euchelicerate lineages (pycnogonids have only one type of eyes, generally interpreted as median eyes) the lateral eyes are compound eyes, which is preserved in the basal Xiphosura and also found in fossils (e.g. Eurypterida and fossil scorpions) (Westheide and Rieger 2006; Ruppert, Fox et al. 2004). In modern arachnids the compound eyes are reduced, whereas several ommatidia are fused and share a common lens and a single retina. In some scorpion and whip scorpion species there are originally five (for the rest three) pairs of lateral eyes, in whip spiders and spiders there are three pairs, and in other arachnids the number is further reduced or lateral eyes are even absent. The retinula or R-cells can be everse (rhabdom orientated to the light) or inverse (light travels across the axon and the nucleus before reaching the light sensitive rhabdom) (Paulus 1979; Westheide and Rieger 2006).

The original number of four median eyes is retained in pycnogonids (which have no lateral eyes) and is reminiscent in xiphosurans, which have two median eyes plus one fused median rudimentary eye (Heß, Melzer et al. 1996; Battelle 2006). Arachnids have, if any, one pair of median eyes. The R-cells are everse (Paulus 1979; Westheide and Rieger 2006). An exception are pycnogonids were the R-cells are described as (pseudo)inverse with an abnormal sequence of nucleus, rhabdom, and axon (Heß, Melzer et al. 1996).

The knowledge on the arrangement and innervation patterns of the visual neuropils is in most chelicerate orders at the stage of Holmgren (1916) and Hanström (1928). So far just for *Limulus polyphemus* (Xiphosura), *Cupiennius salei* (Araneae), and *Rilaena triangularis* (Opiliones) recent studies exist (Chamberlain and Barlow 1980; Calman, Lauerman et al. 1991; Harzsch, Vilpoux et al. 2006; Battelle 2006; Oberdorfer 1977; Strausfeld, Weltzien et al. 1993; Strausfeld and Barth 1993; Babu and Barth 1984; Breidbach and Wegerhoff 1993). Comparable data on the visual system of pycnogonids and scorpions, which have a key role in the chelicerate evolution as their basalmost and their first terrestrial representatives, are missing.

2.6. Sampling and sourcing of specimens

The sea spiders used in this thesis (*Achelia langi* (Dohrn, 1881), *A. vulgaris* (Costa, 1861), and *Endeis spinosa* (Montagu, 1808)) were collected by the author during several field trips to Rovinj (Croatia), Banyuls-sur-Mer (France), and Roscoff (France) from a variety of habitats in depths between 0 and 35 m. While sampling various techniques were used: hand sampling of specimens under stones while snorkelling or scuba diving (Montoya Bravo, Meltzer et al. 2006), sampling of algae (e.g. *Halopteris scoparia*) that are a habitat for sea spiders while

Figure 4. Sampling of specimens. a, collection of different algae as habitat of sea spiders. **b**, dredging of bryozoan communities. **c**, sorting of sea spiders under a stereo microscope. **d**, *Achelia echinata* Hodge, 1864 under a stereo microscope. **e**, sampling of scorpions under logs and stones. **f**, *Euscorpius italicus* (Herbst, 1800) in natural habitat. All photos by the author, except c and e by Roland Meyer.

snorkelling or scuba diving and dredge collection on epibenthic bryozoan communities (Schüller 1989). In the latter two methods the animals have to be isolated from the algae or the bryozoans under a stereo microscope (Figure 4). As by-product the collection of Mediterranean sea spiders at the Bavarian State Collection of Zoology was considerably enhanced (Lehmann, Heß et al. 2014; Lehmann, Krapp et al. 2014).

The scorpions (*Euscorpius italicus* (Herbst, 1800)) were collected by the author in Rovinj (Croatia) as well; they are frequently found under stones, logs, or bark (Figure 4). Additional material (*Euscorpius hadzii* Di Caporiacco, 1950) was provided by b.t.b.e. Insektenzucht GmbH (Schnürpflingen, Germany).

2.7. Aims of the thesis

Nowadays the morphology of the visual system in arthropods and especially in chelicerates is understudied in science. Except for a few model species, like *Limulus polyphemus* (see review by Battelle (2006)) or some insect species (especially *Drosophila melanogaster,* summary by Strausfeld (2012)), in the majority of the taxa little is known about the innervation of the brain by the R-cells and about the number, arrangement and morphology of the visual neuropils.

Therefore the aim of the present thesis is to get new insights into the visual system of two chelicerate groups – sea spiders and scorpions. As described above these two play a key-role in the understanding of arthropod and chelicerate evolution and phylogeny.

In order to get robust and comparable data of the morphology of the visual system, the R-cells are marked with different tracers (Table 1). With this the visual neuropils are identified unequivocally, and the characters of the neuropils within the protocerebrum are described.

These findings are verified and enhanced with further methods (e.g. Wigglesworth technique; table 1). Additionally, the structure of the eyes in pycnogonids is studied with TEM. Thus, the characters of the visual system in sea spiders and scorpions become comparable with that in other arthropods, especially with that in *Limulus* which is particularly well examined.

With the development of new 3D-EM techniques and increasing computing power, the research field of connectomics arose just recently. The term “connectome” refers to the mapping of all neural connections within an organism's nervous system or a confined part of it. Using this approach in the final part of the present thesis the visual neuropils of a sea

Table 1. Overview of methods used in this thesis.
In this thesis various modern and traditional methods were used in order to get several strains of evidence. Besides with cutting edge techniques, like the FIB-SEM, significant results were also achieved with established techniques, like Golgi impregnations (Golgi 1873; Cajal and Sanchez 1915), Wigglesworth technique (Wigglesworth 1957), or Cobalt fills (Pitman, Tweedle et al. 1972; Altman and Tyrer 1980). These "old fashioned" but still highly diagnostic methods were used to achieve a better comparability of the new results found here with the findings of other researchers partly dating back to the beginning of the 20th century, who also used these methods.

Method	Purpose	Microscope
3D-reconstruction in Amira of semithin or cobalt filled section series	Reconstruction of nerve fibre pathways and of arrangement of neuropils	Light microscope
Wigglesworth technique	Arrangement and structure of neuropils	
Golgi impregnations	Detailed image of individual nerve cells	
Cobalt fills	Detailed image of individual nerve cells or of nerve bundles; identification of retinula cell terminals and of visual neuropils innervated by R-cells	
DiI/DiO labelling	Detailed image of individual nerve bundles, identification of retinula cell terminals and of visual neuropils innervated by R-cells	Confocal and fluorescence microscope
Ultrathin sectioning	Ultrastructure of eyes and of neuropils	TEM
3D-reconstruction in Amira of FIB-SEM image series	Connectome of the visual system at different levels: - neuropil arrangement - populations of distinct neurons - synaptic pattern	FIB-SEM

spider are studied with the FIB-SEM (Focused Ion Beam Scanning Electron Microscope) in the highest possible degree of detail (Table 1). The visual system of arthropods is a connectomics classic, but so far knowledge is restricted to *Drosophila* as model organism, and hindered by limited methodologies, which allow us to analyse only sections of a neuropil.

Therefore in this thesis a connectome of a non-model organism, but with high relevance for neurophylogeny or neural cladistics since pycnogonids are one of the most ancestral

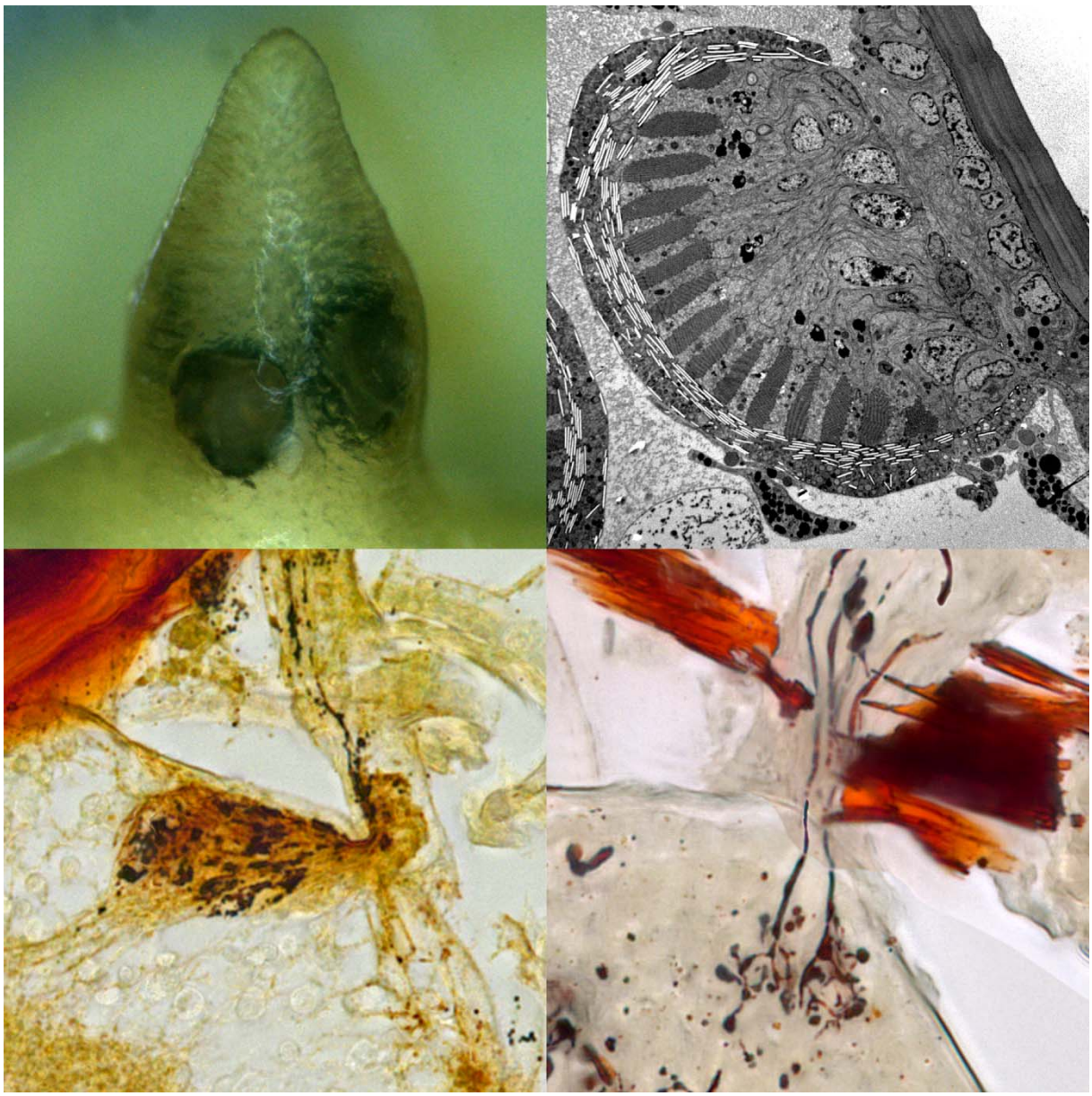
arthropods, is presented. For the first time in invertebrates the FIB-SEM, that overcomes most of the previous technical limitations, is used to study the synaptic connectivity. The advantages of this cutting-edge method are that the generation of a 3D-image-stack is fast and without loss, the images are perfectly aligned with a z-resolution of 15 nm (TEM approx. 70 nm), while the x-y-resolution and contrast compared to TEM are only slightly reduced. After image acquisition, these are aligned, manually segmented, and surface rendered in the computer software Amira. In three image stacks of different resolution, each of several hundred sections, the visual neuropils in the sea spider *Achelia langi* are analysed in order to (1) study the arrangement of the visual nerve fibres and neuropils, (2) identify and characterise neurons postsynaptic to the R-cells, and (3) learn about the synaptic pattern of these cells.

The connectome in this ancestral form must be a precursor of the more advanced systems of the model organisms. With this in view the characters become comparable with other arthropod linages, especially with the lamina (first visual neuropil) of the compound eyes of *Drosophila melanogaster*, the only other arthropod visual system studied so far in such great detail.

In this thesis the variety of characters are studied and described free from homology assumptions in the first place and only thereafter the results are compared with other arthropod taxa in order to detect potential homologies. By means of these homologies conclusions about the phylogeny are possible. Furthermore, aspects of the ground pattern of the visual system of chelicerates and arthropods are elaborated.

3. Paper I

Lehmann T, Heß M & Melzer RR (2012). Wiring a Periscope – Ocelli, Retinula Axons, Visual Neuropils and the Ancestrality of Sea Spiders. PLoS ONE 7 (1): 30474.



OPEN ACCESS Freely available online

PLoS one

Wiring a Periscope – Ocelli, Retinula Axons, Visual Neuropils and the Ancestrality of Sea Spiders

Tobias Lehmann[1,2]*, Martin Heß[2], Roland R. Melzer[1]

1 Bavarian State Collection of Zoology, Munich, Germany, **2** Department Biology I, Ludwig-Maximilians-Universität München, Planegg-Martinsried, Germany

Abstract

The Pycnogonida or sea spiders are cryptic, eight-legged arthropods with four median ocelli in a 'periscope' or eye tubercle. In older attempts at reconstructing phylogeny they were Arthropoda incertae sedis, but recent molecular trees placed them as the sister group either to all other euchelicerates or even to all euarthropods. Thus, pycnogonids are among the oldest extant arthropods and hold a key position for the understanding of arthropod evolution. This has stimulated studies of new sets of characters conductive to cladistic analyses, e.g. of the chelifores and of the hox gene expression pattern. In contrast knowledge of the architecture of the visual system is cursory. A few studies have analysed the ocelli and the uncommon "pseudoinverted" retinula cells. Moreover, analyses of visual neuropils are still at the stage of Hanström's early comprehensive works. We have therefore used various techniques to analyse the visual fibre pathways and the structure of their interrelated neuropils in several species. We found that pycnogonid ocelli are innervated to first and second visual neuropils in close vicinity to an unpaired midline neuropil, i.e. possibly the arcuate body, in a way very similar to ancestral euarthropods like *Euperipatoides rowelli* (Onychophora) and *Limulus polyphemus* (Xiphosura). This supports the ancestrality of pycnogonids and sheds light on what eyes in the pycnogonid ground plan might have 'looked' like. Recently it was suggested that arthropod eyes originated from simple ocelli similar to larval eyes. Hence, pycnogonid eyes would be one of the early offshoots among the wealth of more sophisticated arthropod eyes.

Citation: Lehmann T, Heß M, Melzer RR (2012) Wiring a Periscope – Ocelli, Retinula Axons, Visual Neuropils and the Ancestrality of Sea Spiders. PLoS ONE 7(1): e30474. doi:10.1371/journal.pone.0030474

Editor: Eric James Warrant, Lund University, Sweden

Received October 17, 2011; **Accepted** December 19, 2011; **Published** January 18, 2012

Funding: This study was supported by a Graduiertenstipendium (BayEFG) from the Universität Bayern e.V. to T. Lehmann, and by the Deutsche Forschungsgemeinschaft (DFG Me 2683/6-1). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: lehmann@zsm.mwn.de

Introduction

Sets of neuroanatomical characters have contributed important arguments to the discussion about the phylogenetic position of Pycnogonida. Lately, studies of the first head segment [1] in Pycnogonida and of its appendages, the chelifores [2],[3],[4] have shown that the innervation of the protocerebrum is promising in this respect. In arthropods the protocerebrum's sensory parts are primarily responsible for the visual system. Due to its phylogenetic relevance the latter is well studied [5], as exemplified by the Tetraconata concept (Crustacea + Insecta), in which the structure of the eyes is eponymous [6],[7]. In many arthropods both lateral and median eyes occur, pycnogonids possess only a periscope-like ocular tubercle with four ocelli generally interpreted as median eyes, whereas classical lateral eyes are absent. The visual system of sea spiders is sparsely examined, which is surprising considering their key role as basal chelicerates/arthropods. The eyes of littoral species – which are also used for this study – exhibit an optimum light sensitivity of between 530–545 nm, similar to many marine invertebrates which occupy a comparable habitat [8]. Probably their most important function is to orientate the animal to the incident light [8]. The quadruple of median ocelli in sea spiders seem to represent an ancestral character state of median eyes in Arthropoda and/or Euarthropoda [5], and correspond well to what might be precursors of nauplius eyes and median eyes in other arthropods. Remarkably, only few taxa have been studied in detail with light [9],[10] and electron microscope [11],[12], revealing some features typical of median eyes, i.e. that they are pigment cup ocelli with latticed rhabdom, surrounding pigment layers, and cuticular lens. Conversely, the structure of the retinula or R-cells that could be described as "pseudoinverted", and the presence of a tapetum lucidum (guanine multilayer reflector) might be derived conditions. This very uncommon retinula cell architecture shows more similarities to the lateral eyes of spiders than to 'normal' median eyes [12]. Notably, our knowledge of the visual neuropils connected to the eyes is also cursory at this time. Hanström's [13] classical study suggested some putative visual neuropils and their fibre connections based on classical histology (with a few addenda contributed by Winter [14]), but they have never been identified using unequivocal markers or tracers. Deeper knowledge of, e.g., R-cell projections and visual neuropil architecture is missing, hence there is no stable basis on which to compare visual system features among pycnogonids and to those of their putative arthropod outgroups. In Chelicerata other than Pycnogonida, the visual systems of *Limulus polyphemus* [15],[16],[17] and *Cupiennius salei* [18],[19], which are important model organisms in the field of visual neuroscience, are especially well studied. In scorpions the only study of the visual neuropils is that of Hanström [13].

In the present study we therefore use a multiple-method (3D semithin serial reconstruction, transmission EM, Wigglesworth stains, cobalt backfills, Golgi technique) and multiple-species

(*Achelia langi*, *A. vulgaris*, *Endeis spinosa*) approach. The visual neuropils are identified, and their basic architecture is analysed along with the termination sites of retinula cell axons, revealing basic features of the visual system generally studied in Arthropoda to allow comparison with other arthropod lineages.

Results

The visual system of the studied pycnogonids is composed of (from distal to proximal): four ocelli in a periscope-like eye tubercle (Fig. 1a); several nerve fibres projecting from the eyes proximal to the dorsal protocerebrum (Fig. 1b); a dorsolateral thickening where the nerve fibres from the two eyes of one hemisphere concentrate without forming synaptic varicosities before entering the protocerebrum (Fig. 1b, 2b, 3b); and two successive distinct visual neuropils prepossessed by R-cell axons and terminals in each brain hemisphere where the retinula cells terminate (Fig. 3). Furthermore, there is an unpaired midline neuropil in the central protocerebrum located underneath the second visual neuropils.

Transmission EM of *Achelia vulgaris* confirms the "pseudoinverted" structure of the ocelli also for this species (Figs. 1c, d). Each of the four ocelli is connected to the brain via several nerve fibres originating in a consorted manner in the form of a dorsoventral row from the inner side of the ocelli (Fig. 1b). These fibres are composed of a few axons from neighbouring retinula cells and hence represent one part of the retina, i.e. one sector of the visual

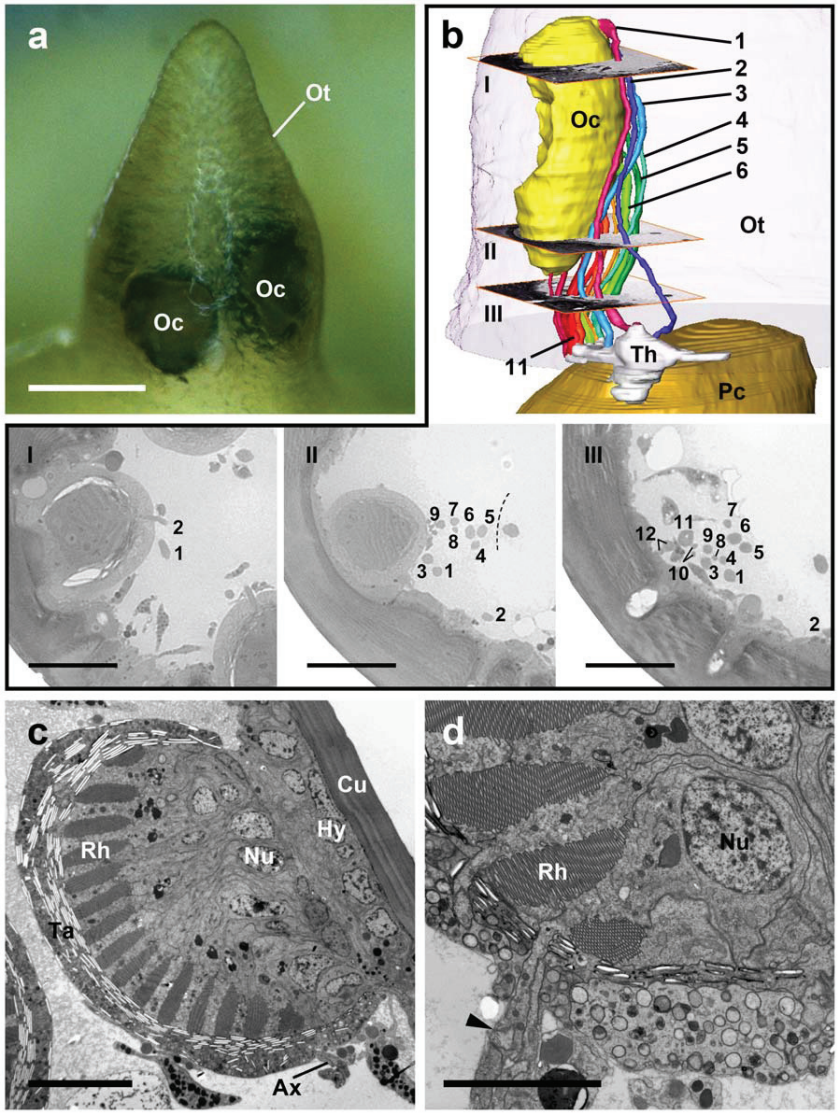


Figure 1. Periscope-like ocular tubercle with ocelli and nerve fibres to the protocerebrum. a, Light microscopic picture of the ocular tubercle (Ot) in *Endeis spinosa* showing two of the four ocelli (Oc). Bar 100 μm. **b**, 3D semithin serial reconstruction of nerve fibres projecting from left rostral ocellus to dorsolateral thickening distal to first neuropil (*Endeis spinosa*). Note retained relative positions of nerve fibres representing subsets of retinula cells (indicated by numbers). I–III: Three selected planes (Richardson staining; for position, see rendering at top right), showing profiles of groups of photoreceptor nerve fibres, originating from neighbouring r-cells, indicated by numbers. I, Frontal section from top quarter of eye. II, Frontal section from bottom quarter of eye. III, Frontal section through loose strand of nerve fibres just below eye. Bars 25 μm. Oc, ocelli; Ot, ocular tubercle; Pc, protocerebrum; Th, thickening. **c**, Transmission EM of a single ocellus in *Achelia vulgaris* showing the arrangement of the retinula cells. Ax, axon; Cu, cuticle; Hy, hypodermis; Nu, nucleus; Rh, rhabdom; Ta, tapetum. Bar 5 μm. **d**, Transmission EM of a retinula cell with a sequence from outside to inside of nucleus (Nu), rhabdom (Rh), and axon (arrowhead) demonstrating their "pseudoinverted" structure (*Achelia vulgaris*). Bar 10 μm.
doi:10.1371/journal.pone.0030474.g001

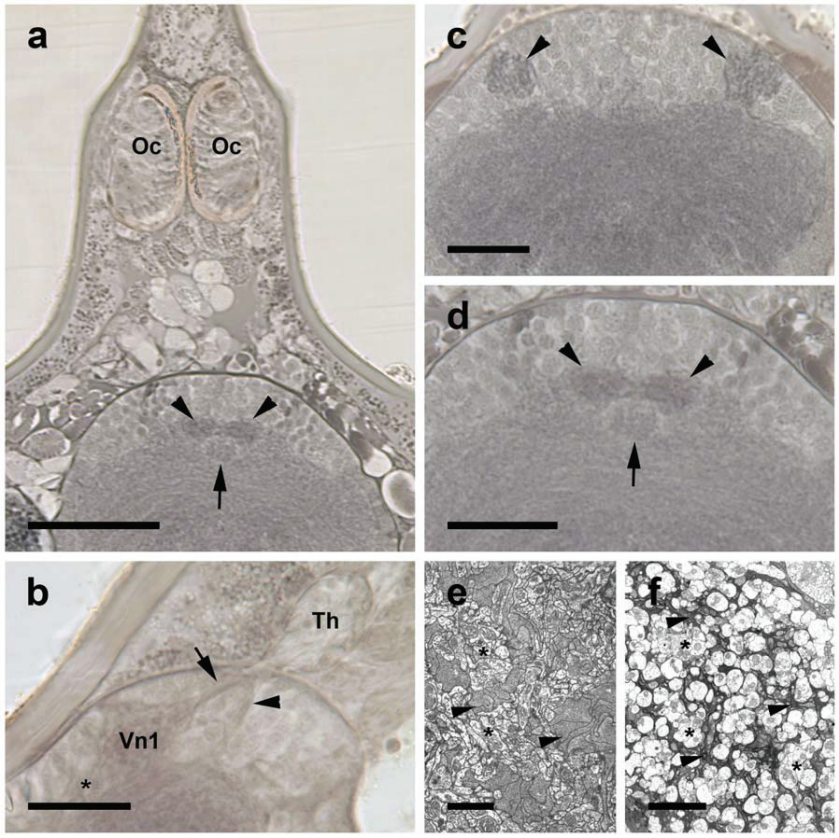


Figure 2. Anatomy of the visual neuropils (a–d, Wigglesworth stains, *Achelia vulgaris*; e, f TEM, *Endeis spinosa*). Note dark stain of sensory neuropils after application of Wigglesworth's technique. **a**, Eye tubercle with two ocelli (Oc) and protocerebrum with left and right second visual neuropils (arrowheads) and arcuate body (arrow), transversal section. Bar 50 μm. **b**, Thickening (Th) distal to protocerebral cell body rind, first visual neuropil (Vn1), bifurcation of visual tract into a subset of fibres projecting to first (arrow) and second neuropil (arrowhead), respectively, and tract connecting first visual neuropil with protocerebrum (asterisk), sagittal section. Bar 25 μm. **c**, First visual neuropils (arrowheads) dorsolaterally in rostral part of protocerebrum, transversal section. Bar 25 μm. **d**, Second visual neuropils (arrowheads) deeper in protocerebrum in a more rostral and central position, and arcuate body (arrow), transversal section. Bar 25 μm. **e**, **f**, Frontal section of distal (**e**) and (**f**) of proximal region of first visual neuropil showing arrangement of retinula axon terminals (arrowheads) and dendrites and cell bodies of visual second order neurons (asterisks). Note that in distal region (**e**) retinula axons are broad; in proximal region (**f**) they are narrow. Bars 5 μm.
doi:10.1371/journal.pone.0030474.g002

environment. The fibres join successively, and finally the nerves of the two left and accordingly the two right ocelli combine in a thickening dorsolaterally on each brain hemisphere just before they enter the protocerebrum (Figs. 1b, 2b, 3b–d). The 3D-reconstruction of *Endeis spinosa* shows a primitive form of retinotopic projection arrangement of these nerve fibres, since they maintain the same order – from top to bottom of each ocellus – as they enter the thickening before the brain (Fig. 1b), i.e. nerve fibres originating from the dorsalmost eye portion enter the thickening caudally, and the ventralmost fibre projects to its rostral part. In this thickening all nerve fibres from the eyes are bundled and a re-assortment of the single axons takes place (Fig. 3b), but a typical neuropil architecture caused by fine dendritic arborisations and axon collaterals was not detected. Cobalt backfills via the ocelli in *Achelia langi*, *A. vulgaris* and *Endeis spinosa* reveal two distinct retinula axon target regions in each hemisphere of the protocerebrum, a first and a second visual neuropil (Fig. 3). The first neuropil is located dorsolaterally in the rostral part of the protocerebrum as an oval-shaped region laterally embedded in the cell body rind of the brain (Fig. 2b, c). The second neuropil lies deeper, under the cell body rind and in a more rostral and central position in the protocerebrum. The second neuropils of both brain hemispheres contact each other in the brain's midline, and are dumbbell-shaped when seen together (Fig. 2a, d).

After entering the brain the fibre bundle is split; one part of the axons has its terminals in the first visual neuropil (Fig. 3a, b, c, f), the other part passes the first one and terminates in the second neuropil (Fig. 3a, d, e, g). This division is also observed by TEM and Wigglesworth stains in *Achelia vulgaris* (Fig. 2b). With the latter method, the first and second visual neuropil can be recognised as dark-stained areas, as is typical for Wigglesworth-stained sensory neuropils (Fig. 2). In addition, a tract originating from the first neuropil has been identified that projects basally into the protocerebrum (Figs. 2b; 3c, f). Axially beneath the left and right second visual neuropil lies a roundish, unpaired midline neuropil, also somewhat darker-stained, which can be identified as the arcuate body (Fig. 2a, d).

Transmission EM of the first visual neuropil reveals several clusters of cells with high electron density, identified as retinula axon terminals, surrounded by cells with low electron density, identified as second order neurons, with synapses between these neurons (Fig. 2e, f). In the distal region of the visual neuropil these cells fill a large part of the neuropil, in the proximal region they taper off (Fig. 2e, f). At least some of the second order neurons likely project deeper into the protocerebrum – via the tract shown in Figures 2b and 3c, f – hence are visual interneurons.

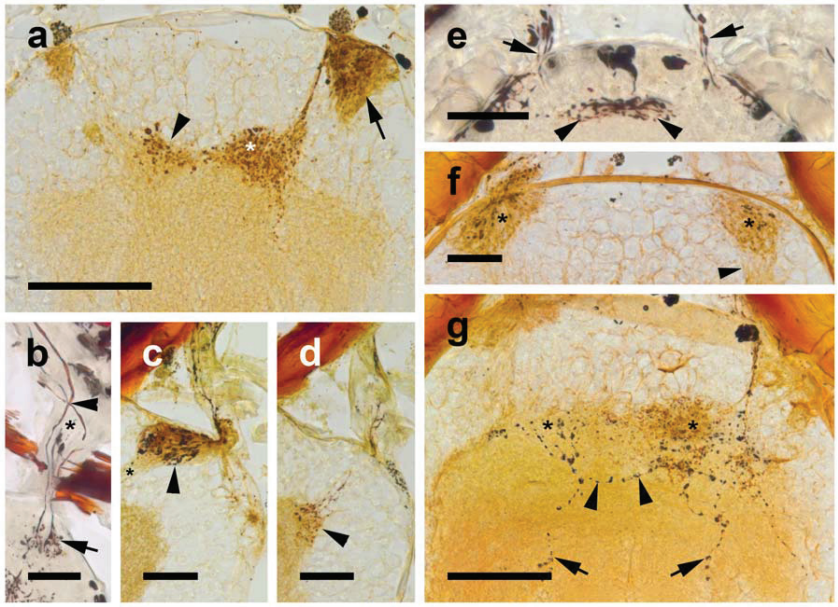


Figure 3. Neuroanatomy of the visual neuropils revealed with cobalt backfills (a, c, d, f, g) and Golgi technique (b, e). **a**, **b**, *Achelia langi*; **c**–**e**, *Achelia vulgaris*; **f**, **g**, *Endeis spinosa*. In **a** and **g** cobalt backfills of two sections are combined. **a**, First (arrow) and second (asterisk) visual neuropil identified with cobalt backfills, transversal section. Note dense arrangement of cobalt filled profiles in both neuropil pairs. Arrowhead points to a few axons of the right retinula cells that send axon collaterals to the contralateral, left neuropil. Bar 50 μm. **b**, Retinula axons projecting from dorsal through dorsolateral thickening (asterisk) into first visual neuropil (arrow) where they form short collaterals and synaptic varicosities; note re-assortment of single axons (arrowhead), transversal section. Bar 25 μm. **c**, **d**, Retinula axon terminals in first (**c**) and second (**d**) visual neuropil, with synaptic varicosities in both neuropils (arrowheads); in c a tract connects first visual neuropil with protocerebrum (asterisk); sagittal sections. Bars 25 μm. **e**, Retinula axons (arrows) and second visual neuropils (arrowheads), transversal section. Bar 25 μm. **f**, **g**, Cobalt backfills of retinula axons terminating in first (**e**) and second (**f**) visual neuropils (asterisks); in **f** a tract connects first visual neuropil with protocerebrum (arrowhead); in **g** a fibre connecting ipsi- and contralateral second neuropil is seen (arrowheads), note a single fibre per brain hemisphere that travels through second visual neuropil and terminates deeper in protocerebrum (arrows); transversal sections. Bar 50 μm.
doi:10.1371/journal.pone.0030474.g003

Golgi-impregnated brains of *Achelia langi* and *A. vulgaris* show that in this neuropil the terminals are branched and have synaptic varicosities (Fig. 3b). In the second visual neuropil, cobalt backfills identify only varicosities with certainty, whereas branching is suggested only (Fig. 3a, d, e, g). Axons of the right and left second visual neuropils contact each other medially (Fig. 3e, g); a few axons of the right retinula cells also terminate in the contralateral left neuropil, and vice versa (Fig. 3a). This is supported by cobalt backfills in which retinula cells of only one hemisphere are stained, but terminals that also end in the contralateral neuropil can be identified. Furthermore, one single axon per brain hemisphere travels through the second visual neuropil and terminates even deeper in the brain (Fig. 3g), with varicosities all over its extension.

Discussion

Our studies confirm that the brain area described by Hanström [13] as "*Sehmasse*" is a genuine visual neuropil. This neuropil was also found by Winter [14] ("*Seemasse 1*"). In addition he suggested the presence of a second visual neuropil ("*Seemasse 2*") postero-ventrally adjacent to the first neuropil, but this one was not stained by our cobalt backfills, though a tract projecting to this region is identifiable in our stains. If present, this neuropil would therefore not be a target of visual fibres, but of visual interneurons.

Conversely, the brain area interpreted by Winter [14] as the calyx of the mushroom body corresponds in position and shape exactly to the second visual neuropil that we identified with cobalt backfills. How can this contradictory result be explained? Winter described the mushroom body without going into detail; his observations were based on classical histology only. He named a region under the cell body rind as paired "Corpora pedunculata", which equates to the calyx of mushroom bodies [20], with ventrally adjacent "Stielelementen", which equate to the pedunculus of mushroom bodies [20]. In the meantime Strausfeld *et al.* [21] described a different brain area as the mushroom body. In this interpretation the mushroom body lobes were characterised – like those of onychophorans – as horseshoe-shaped, and as confluent across the midline of the protocerebrum, but a primitive nature was suggested. This indicates that Winter might have misinterpreted the mushroom body. This view is also supported by the present findings, since the mushroom bodies in arthropods are generally not innervated by median eye retinula axons [21], and the neuropil in question is unequivocally identified here as a visual neuropil.

Furthermore, we possibly localised the arcuate body in a position of the protocerebrum different from the one suggested by Hanström or Winter ("*Zentralkörper*" [13],[14]), i.e. right beneath the second visual neuropils, a region not specified by those authors. In the chelicerates only one unpaired midline neuropil in the protocerebrum is known, the arcuate body [22]. It has a dorso-posterior position in the brain's midline and is closely related to the visual system [22],[23]. The same features are found here for pycnogonids, although this neuropil is not as complex as in other chelicerates or onychophorans but rather small. Thus, this neuropil may be the arcuate body of pycnogonids, but more research about this issue will have to be done.

Thus, our study leads to a new interpretation of the visual system as well as of the general architecture of the pycnogonid protocerebrum. The visual system comprises three main elements:

(1) a thickening where the retinotopic nerve fibres from the median eyes are docking and re-assorted; (2) a first and (3) a second visual neuropil, each targeted by subsets of the retinula axon terminals; and (4) the second visual neuropil is located in close vicinity to an unpaired midline neuropil, possibly the arcuate body. Furthermore, there are projections to the contralateral second neuropil and fibres projecting to centres located deeper in the protocerebrum. These highly specific features allow a detailed comparison with the situation found in other arthropods.

In Tetraconata or Pancrustacea one finds only a single median ocellar nerve with terminals of the ocellar photoreceptor in the dorso-median protocerebrum (e.g. *Balanus nubilus* [24] and *Schistocerca gregaria* [25]). In Myriapoda median eyes are absent. In Chelicerata and Onychophora the projections of the median eye nerves differ fundamentally from those in Mandibulata – and are similar to the pycnogonid condition found here – in having a paired nerve that connects the eyes with the brain. In derived taxa such as the spider *Cupiennius* [18] there is only one target region of the retinula axon terminals of the median eyes (principal eyes or anterior median eyes): the first anterior median eye neuropil, dorso-lateral in each brain hemisphere. A similar situation is found in scorpions [13], and it differs from our findings on pycnogonids. Conversely, in *Limulus* [16] two target regions of the median eyes in each brain hemisphere exist: the two ocelli are indeed only innervated to the ocellar ganglion, but the fused rudimentary median eye is innervated to the ocellar ganglion and simultaneously to a region near the central body, as also shown here for sea spiders. In Onychophora (*Euperipatoides rowelli*) [26],[27], one of the putative sister taxa of Euarthropoda, the presence of photoreceptor terminals in a first visual neuropil, which lies directly beneath the eye, was suggested [26],[27]. From this first neuropil, an optic tract projects further and then bifurcates as in pycnogonids. Its ventral branch extends to a second visual neuropil near the mushroom body calyces, while the dorsal branch gives rise to another second visual neuropil, which flanks the arcuate body laterally. Thus, comparing the median eye visual system of pycnogonids to that of other (pan)arthropods, the similarities are greatest to xiphosurans and onychophorans, intermediate to spiders and scorpions, and lowest to mandibulate arthropods.

The dorso-posterior position of the pycnogonid arcuate body is also in accordance with that in other chelicerates and in onychophorans (see review by Homberg [22]), but in *Limulus* and arachnids it is more or less horseshoe-shaped, and in Onychophora it is subdivided in lamina posterior and lamina anterior. In these taxa the arcuate body is associated with the visual system, in *Limulus* and arachnids actually with the median eyes [22]. The close vicinity to the second visual neuropils leads one to assume that in pycnogonids the arcuate body is also associated with the visual system.

The similarities between Pycnogonida and Onychophora and Xiphosura, the two taxa with the greatest accordance, are that all three taxa have (1) a paired nerve that connects the eyes with the brain; (2) two visual neuropils within the brain connected to (median) eyes; and (3) that one of the visual neuropils lies in direct vicinity to an unpaired midline neuropil, i.e. arcuate body. But there are also differences to these two taxa; in *Limulus* only the axons of the fused rudimentary median eye has these two target regions (the axons of the two other median eyes all end in the ocellar ganglion), and these retinula axons have some branches both in the ocellar ganglion and in the region near the central body. In pycnogonids the retinula axons have branches only in the first or second visual neuropil, and never in both neuropils simultaneously. In onychophorans there are three visual neuropils: one first visual neuropil beneath the eye, and two second neuropils within the brain; in pycnogonids only two genuine neuropils containing R-cell axon terminals and the distal thickening are found. However, bifurcation of visual tracts is found only in Onychophora and Pycnogonida. In onychophorans it has not been analysed whether the photoreceptor axons terminate in the first visual neuropil only or also in the second neuropils. This would be valuable information for further comparisons.

Features that might be unique to sea spiders, as they have not been found in other arthropods, are that some of the terminals of retinula axons end in the contralateral second visual neuropil, and that fibres project to deeper areas of the protocerebrum.

The sets of characters studied here for pycnogonids and those of other arthropods are summarised in the data matrix given in Table 1 and in Figure 4. The visual system in sea spiders shows far more similarities to those in basal xiphosurans and even in an arthropod outgroup – oynchophorans – than to those in derived chelicerates like scorpions and spiders (Table 1, Fig. 4). This represents another argument for placement of the sea spiders at the base of the Chelicerata or even Euarthropoda, as suggested by recent molecular trees [28], [29].

The fact that the visual system of pycnogonids shows more similarities to the fused rudimentary median eye of *Limulus* than to the 'normal' median eye, is of special interest. If arthropod eyes originated from simple ocelli similar to larval eyes [30], pycnogonid eyes could be one of their early offshoots, which date

Table 1. Data matrix with pycnogonid eye features (this study) compared to median eyes of other arthropods (citations for exemplary taxa used are given above).

Feature	Onychophora *Euperipatoides rowelli*	Pycnogonida *Achelia spp., Endeis spinosa*	Xiphosura* *Limulus polyphemus*	Arachnida *Cupiennius salei*	Crustacea *Balanus nubilus*	Hexapoda *Schistocerca gregaria*
A	0	0	0	0	1	1
B	0	0	0	0	1	1
C	-	0	0	1	1	1
D	0	0	1	1	1	1
E	-	0	0	1	1	1

A, eye nerves paired and arranged in bilateral symmetry (0) or unpaired (1); B, visual neuropils paired and arranged in bilateral symmetry (0) or unpaired ocellar centre (1); C, number of visual neuropils innervated by R-cell axons greater than one (0) or equal to one (1); D, bifurcation of subsets of visual fibres targeting two different neuropils present (0) or absent (1); E, second visual neuropil with visual fibre terminals in close vicinity to arcuate body present (0) or absent (1). Due to absence of median eyes, Myriapoda are omitted; "-" indicates that the feature has not been studied.
*characters of fused rudimentary median eye.
doi:10.1371/journal.pone.0030474.t001

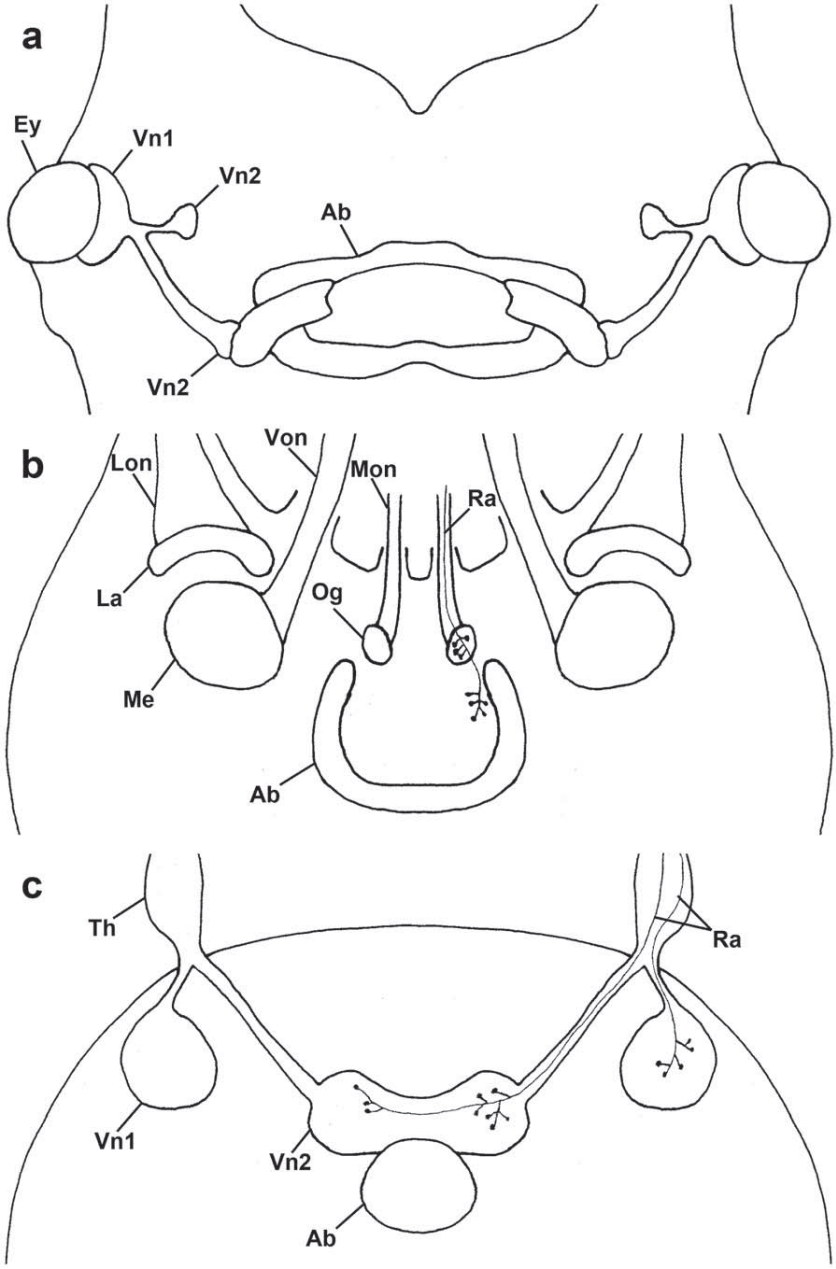


Figure 4. Comparison of visual systems of (a) Onychophora (*Euperipatoides rowelli*), (b) Xiphosura (*Limulus polyphemus*), and (c) Pycnogonida (*Achelia* spp., *Endeis spinosa*). Ab, arcuate body; Ey, eye; La, lamina; Lon, lateral optic nerve; Me, medulla; Mon, median optic nerve; Og, ocellar ganglion; Ra, retinula axon; Th, thickening; Von, ventral optic nerve; Vn, visual neuropil. **a**, Visual pathways from the eyes are shown, with first and second optic neuropils indicated. After Strausfeld *et al.*[*27*]. **b**, Terminals of median rudimentary photoreceptor have some branches in ocellar ganglion, then continue and terminate near central body. After Calman *et al.*[16]. **c**, Summary of the situation found in the three pycnogonid species studied here.
doi:10.1371/journal.pone.0030474.g004

back at least 500 Myr to the Cambrian [31], and be older than the appearance of distinct lateral and median eyes.

Materials and Methods

Specimen collection

The specimens of *Achelia langi*, *A. vulgaris* and *Endeis spinosa* were collected during field trips in 2009 and 2010 to Rovinj (Croatia), Isola del Giglio (Italy), and Roscoff (France).

3D-Reconstruction

Eye tubercle (prepared as for TEM) was cut into a most complete semithin cross-section series (1 µm) using a HistoJumbo diamond knife on a RMC-MTXL ultramicrotome. The slices were mounted on glass slides, stained with methylene blue (after Richardson *et al.* [32]), coverslipped and photographed with a conventional light microscope (40x, NA 0.95). The images were contrast enhanced in Photoshop and then aligned, segmented and rendered in Amira.

TEM

After dissection of abdomen, legs and proboscis the animals were fixed in 4% glutardialdehyde in 0.1 M cacodylate buffer at 4°C, postosmicated and embedded in epoxy resin. Ultra-thin sections of 70–100 nm thickness were made with a diamond knife on an RMC-MTXL ultramicrotome. The sections were stained with uranyl actetate and lead citrate, and inspected in an FEI Morgagni transmission EM at 80 kV.

Osmium-Ethyl Gallate procedure (modified after Wigglesworth [33])

After dissection of abdomen, legs and proboscis the animals were fixed in 3% glutardialdehyde in 0.1 M cacodylate buffer at 4 °C. After postosmication the animals were stained for 48 hours at 4 °C in a saturated ethyl gallate solution, dehydrated, kept overnight in methyl benzoate, embedded and sectioned (5–8 µm).

Cobalt backfills (modified after Altman & Tyrer [34])

$CoCl_2$ crystals were inserted in one or two ocelli with a tungsten needle. After diffusion times between 1 and 5 hours, cobalt was precipitated with $(NH_4)_2S$ solution. Animals were fixed in AAF (ethanol, glacial acetic acid, formaldehyde), silver intensified, embedded, and sectioned (10–12 µm).

Golgi technique

Abdomen, legs and proboscis were dissected and the cuticle regions surrounding the central nervous system were perforated in order to increase the chances for staining the desired areas. The preparations were submitted to two cycles of the Golgi-Colonnier method [35], embedded and sectioned (10–20 µm).

Terminology

All neuroanatomical terms and definitions were adopted from Richter *et al.* [20].

Acknowledgments

We thank Stefan Friedrich, Heidemarie Gensler and Eva Lodde for expert technical assistance and Martin Spies for polishing the English. We would like to express our gratitude to two anonymous reviewers whose comments have helped a lot to improve the manuscript.

Author Contributions

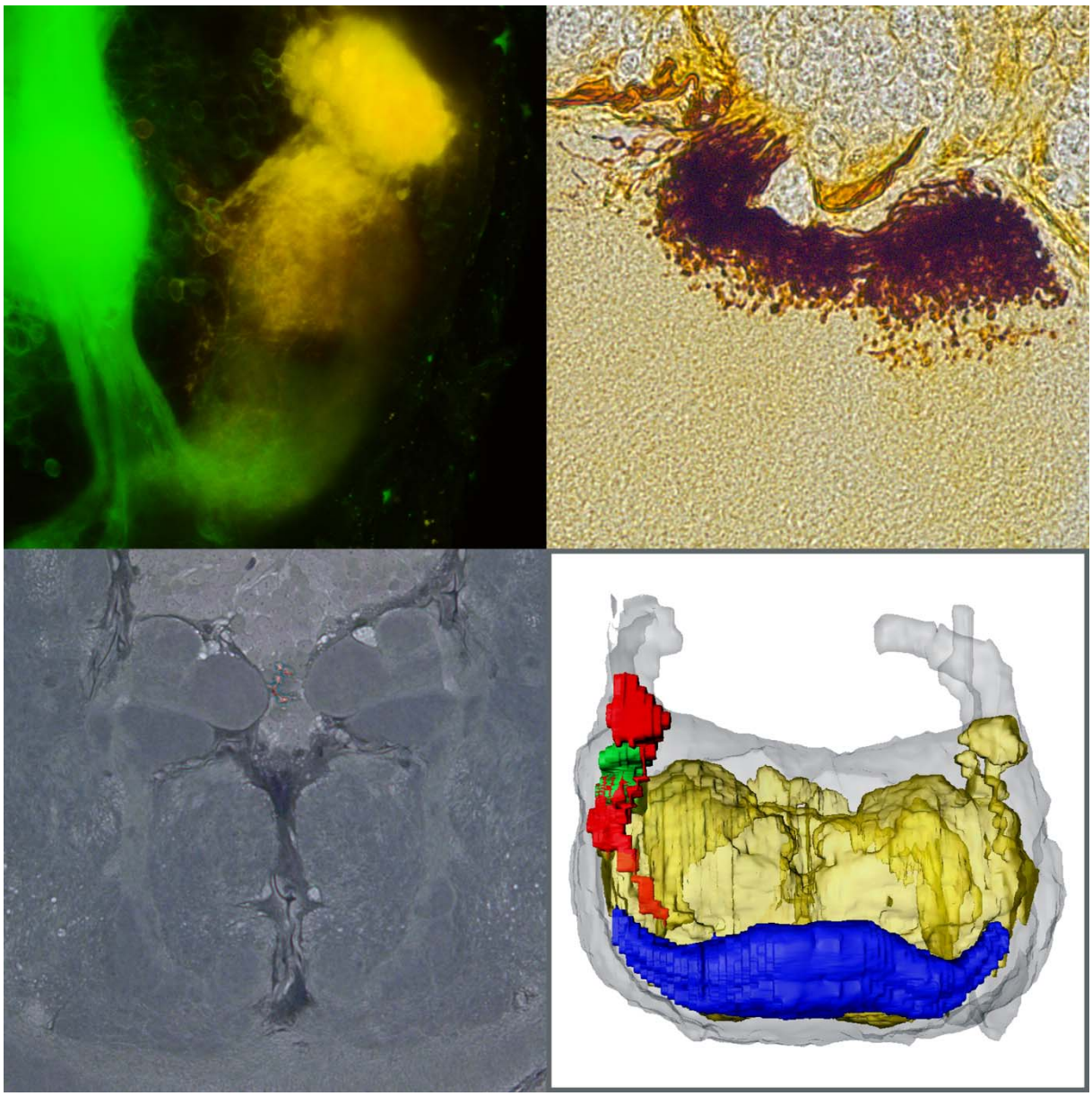
Conceived and designed the experiments: TL MH RRM. Performed the experiments: TL MH RRM. Analyzed the data: TL MH RRM. Contributed reagents/materials/analysis tools: TL MH RRM. Wrote the paper: TL RRM.

References

1. Manuel M, Jager M, Murienne J, Clabaut C, Le Guyader H (2006) Hox genes in sea spiders (Pycnogonida) and the homology of arthropod head segments. Dev Genes Evol 216: 481–491.
2. Maxmen A, Browne WE, Martindale MQ, Giribet G (2005) Neuroanatomy of sea spiders implies an appendicular origin of the protocerebral segment. Nature 437: 1144–1148.
3. Brenneis G, Ungerer P, Scholtz G (2008) The chelifores of sea spiders (Arthropoda, Pycnogonida) are the appendages of the deutocerebral segment. Evolution & Development 10: 717–724.
4. Jager M, Murienne J, Clabaut C, Deutsch J, Le Guyader H, et al. (2006) Homology of arthropod anterior appendages revealed by Hox gene expression in a sea spider. Nature 441: 506–508.
5. Paulus HF (1979) Eye structure and the monophyly of the Arthropoda. In: Gupta AP, ed. Arthropod phylogeny. New York, Cincinnati, Atlanta, Dallas, San Francisco, London, Toronto, Melbourne: Van Nostrand Reinhold Company. pp 299–383.
6. Melzer RR, Diersch R, Nicastro D, Smola U (1997) Compound eye evolution: Highly conserved retinula and cone cell patterns indicate a common origin of the insect and crustacean ommatidium. Naturwissenschaften 84: 542–544.
7. Dohle W (2001) Are the insects terrestrial crustaceans? A discussion of some new facts and arguments and the proposal of the proper name 'Tetraconata' for the monophyletic unit Crustacea plus Hexapoda. Annales De La Societe Entomologique De France 37: 85–103.
8. Jarvis JH, King PE (1979) The response of the Pycnogonids Nymphon gracile leach and Pycnogonum littorale (Strom) to light stimuli of different wavelength and intensity. Marine Behaviour and Physiology 6: 307–317.
9. Sokolow I (1911) The structure of the pantopod eyes. Zeitschrift Fur Wissenschaftliche Zoologie 98: 339–380.
10. Helfer H, Schlottke E (1935) Pantopoda. Bronn S Kl U Ordn Tierreichs 5: 1–314.
11. Jarvis JH, King P (1973) Ultrastructure of the Photoreceptors in the Pycnogonid species, Nymphon gracile (Leach), and Pycnogonum littorale (Strom). Marine and Freshwater Behaviour and Physiology 2: 1–13.
12. Hess M, Melzer RR, Smola U (1996) The eyes of a nobody, Anoplodactylus petiolatus (Pantopoda; Anoplodactylidae). Helgolander Meeresuntersuchungen 50: 25–36.
13. Hanström B (1928) Vergleichende Anatomie des Nervensystems der wirbellosen Tiere: unter Berücksichtigung seiner Funktion. Berlin: J. Springer. 628 p.
14. Winter G (1980) Beiträge zur Morphologie und Embryologie des vorderen Körperabschnitts (Cephalosoma) der Pantopoda Gerstaecker, 1863: Entstehung und Struktur des Zentralnervensystems. Zeitschrift für Zoologische Systematik und Evolutionsforschung 18: 27–61.
15. Chamberlain SC, Barlow RB (1980) Neuroanatomy of the visual afferents in the horseshoe crab (Limulus polyphemus). Journal of Comparative Neurology 192: 387–400.
16. Calman BG, Lauerman MA, Andrews AW, Schmidt M, Battelle BA (1991) Central projections of Limulus photoreceptor cells revealed by a photoreceptor-specific monoclonal antibody. J Comp Neurol 313: 553–562.
17. Harzsch S, Vilpoux K, Blackburn DC, Platchetzki D, Brown NL, et al. (2006) Evolution of arthropod visual systems: development of the eyes and central visual pathways in the horseshoe crab Limulus polyphemus Linnaeus, 1758 (Chelicerata, Xiphosura). Dev Dyn 235: 2641–2655.
18. Strausfeld NJ, Weltzien P, Barth FG (1993) Two visual systems in one brain: neuropils serving the principal eyes of the spider Cupiennius salei. J Comp Neurol 328: 63–75.
19. Strausfeld NJ, Barth FG (1993) Two visual systems in one brain: neuropils serving the secondary eyes of the spider Cupiennius salei. J Comp Neurol 328: 43–62.
20. Richter S, Loesel R, Purschke G, Schmidt-Rhaesa A, Scholtz G, et al. (2010) Invertebrate neurophylogeny: suggested terms and definitions for a neuroanatomical glossary. Front Zool 7: 29.
21. Strausfeld NJ, Hansen L, Li Y, Gomez RS, Ito K (1998) Evolution, discovery, and interpretations of arthropod mushroom bodies. Learn Mem 5: 11–37.
22. Homberg U (2008) Evolution of the central complex in the arthropod brain with respect to the visual system. Arthropod Structure & Development 37: 347–362.
23. Strausfeld NJ, Strausfeld CM, Loesel R, Rowell D, Stowe S (2006) Arthropod phylogeny: onychophoran brain organization suggests an archaic relationship with a chelicerate stem lineage. Proc Biol Sci 273: 1857–1866.
24. Hudspeth AJ, Stuart AE (1977) Morphology and responses to light of the somata, axons, and terminal regions of individual photoreceptors of the giant barnacle. J Physiol 272: 1–23.
25. Goodman LJ, Patterson JA, Mobbs PG (1975) The projection of ocellar neurons within the brain of the locust, Schistocerca gregaria. Cell Tissue Res 157: 467–492.
26. Mayer G (2006) Structure and development of onychophoran eyes: what is the ancestral visual organ in arthropods? Arthropod Struct Dev 35: 231–245.
27. Strausfeld NJ, Strausfeld CM, Stowe S, Rowell D, Loesel R (2006) The organization and evolutionary implications of neuropils and their neurons in the brain of the onychophoran Euperipatoides rowelli. Arthropod Struct Dev 35: 169–196.
28. Giribet G, Edgecombe GD, Wheeler WC (2001) Arthropod phylogeny based on eight molecular loci and morphology. Nature 413: 157–161.
29. Regier JC, Shultz JW, Zwick A, Hussey A, Ball B, et al. (2010) Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. Nature 463: 1079–1083.
30. Melzer RR (2009) Persisting stemma neuropils in Chaoborus crystallinus (Diptera: Chaoboridae): development and evolution of a bipartite visual system. J Morphol 270: 1524–1530.
31. Siveter DJ, Sutton MD, Briggs DE (2004) A Silurian sea spider. Nature 431: 978–980.
32. Richardson KC, Jarett L, Finke EH (1960) Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol 35: 313–323.
33. Wigglesworth VB (1957) The use of osmium in the fixation and staining of tissues. Proc R Soc Lond B Biol Sci 147: 185–199.
34. Altman JS, Tyrer NM (1980) Filling selected neurons with cobalt through cut nerves. In: Strausfeld NJ, Miller TA, eds. Neuroanatomical Techniques: Insect Nervous System. New York: Springer-Verlag. 496 p.
35. Colonnier M (1964) The Tangential Organization of the Visual Cortex. J Anat 98: 327–344.

4. Paper II

Lehmann T & Melzer RR (2013). Looking like *Limulus*? – Retinula axons and visual neuropils of the median and lateral eyes of scorpions. Frontiers in Zoology 10 (1): 40.



FRONTIERS IN ZOOLOGY

RESEARCH Open Access

Looking like *Limulus*? – Retinula axons and visual neuropils of the median and lateral eyes of scorpions

Tobias Lehmann[1,2*] and Roland R Melzer[1,2,3]

Abstract

Background: Despite ongoing interest in the neurophysiology of visual systems in scorpions, aspects of their neuroanatomy have received little attention. Lately sets of neuroanatomical characters have contributed important arguments to the discussion of arthropod ground patterns and phylogeny. In various attempts to reconstruct phylogeny (from morphological, morphological + molecular, or molecular data) scorpions were placed either as basalmost Arachnida, or within Arachnida with changing sister-group relationships, or grouped with the extinct Eurypterida and Xiphosura inside the Merostomata. Thus, the position of scorpions is a key to understanding chelicerate evolution. To shed more light on this, the present study for the first time combines various techniques (Cobalt fills, DiI / DiO labelling, osmium-ethyl gallate procedure, and AMIRA 3D-reconstruction) to explore central projections and visual neuropils of median and lateral eyes in *Euscorpius italicus* (Herbst, 1800) and *E. hadzii* Di Caporiacco, 1950.

Results: Scorpion median eye retinula cells are linked to a first and a second visual neuropil, while some fibres additionally connect the median eyes with the arcuate body. The lateral eye retinula cells are linked to a first and a second visual neuropil as well, with the second neuropil being partly shared by projections from both eyes.

Conclusions: Comparing these results to previous studies on the visual systems of scorpions and other chelicerates, we found striking similarities to the innervation pattern in *Limulus polyphemus* for both median and lateral eyes. This supports from a visual system point of view at least a phylogenetically basal position of Scorpiones in Arachnida, or even a close relationship to Xiphosura. In addition, we propose a ground pattern for the central projections of chelicerate median eyes.

Keywords: Chelicerata, Scorpiones, Visual system, Central projections, Phylogeny

Introduction

Scorpions have two classes of eyes: one pair of large elevated eyes in the middle of the carapace commonly referred to as median eyes, and two to five pairs of small eyes along the anterior, lateral margin of the carapace, commonly referred to as lateral eyes [1]. In both types, the eye is composed of a cuticular lens, photoreceptor cells, arhabdomeric cells, efferent neurosecretory fibres, and pigment cells. However, there are characteristic differences in ultrastructure: in the lateral eyes the focusing lens and the vitreous body are lacking, and the rhabdomeres of all retinula cells form a contiguous rhabdom; median eyes, on the other hand, possess a focusing lens and a vitreous body, and the rhabdomeres of 4-6 retinula cells form separated star-shaped rhabdoms [2-4]. Additionally a pair of minute accessory lateral eyes have been demonstrated in prenymphs and nymphs of *Parabuthus transvaalicus* at the posterior end of the lateral eye row, and separated from these by a cuticular ridge [5]. These eyes are composed of photoreceptor cells, arhabdomeric cells and efferent neurosecretory fibres, but a cuticular lens and pigment granules are absent.

* Correspondence: lehmann@zsm.mwn.de
[1]SNSB – Bavarian State Collection of Zoology, Münchhausenstraße 21, Munich 81247, Germany
[2]Department Biology II, Ludwig-Maximilians-Universität München, Großhaderner Straße 2, Planegg-Martinsried 82152, Germany
Full list of author information is available at the end of the article

The median eyes are the scorpion's main eyes, allow good image processing with relatively high acuity and good spatial discrimination, and exhibit a distinct circadian sensitivity rhythm [3]. In lateral eyes, due to the construction of the dioptric apparatus as well as the anatomy of the retina, the visual acuity is reduced. They have been suggested to function mainly as extremely sensitive light detectors, e.g. for *Zeitgeber* stimuli to synchronize a circadian clock [3,4]. The neurobiology of this circadian clock is well known for the North African desert scorpion, *Androctonus australis* (see summary by Fleissner [6]).

So far, the visual systems of scorpions have been studied mainly in a neurophysiological context, whereas their morphological features are undescribed on a level that would allow phylogenetic comparisons [7-9]. Holmgren [7] suggested a series of four visual neuropils ("Seemasse 1–4"), with the median eyes linked to the first and the lateral eyes to the second neuropil. Holmgren's pupil, Hanström [8], identified the same neuropils, but distinguished between median and lateral eye neuropils and suggested that the median eyes are linked to one neuropil and the lateral eyes to three subsequent neuropils, while some fibre bundles project from the median eye neuropil to the third lateral eye neuropil. Fleissner [9] reported that the photoreceptor cell axons of the median eyes terminate within a first neuropil ("lamina"), while the axons of the arhabdomeric cells terminate in a second neuropil ("medulla"); the retinula cell axon terminals of the lateral eyes were not defined.

Lately the structure and development of various nervous systems have played important roles in debates concerning arthropod evolution and phylogeny. For this field of research two different approaches – "neurophylogeny" [10,11] and "neural cladistics" [12] – were established.

In Chelicerata other than Scorpiones, especially well studied visual systems are that of the xiphosuran *Limulus polyphemus* [13-16], which is an important, well investigated species in the field of visual neuroscience, and those of several Araneae [17-20] (*Salticus scenicus*, *Habrocestum pulex*, and *Cupiennius salei*). Recent investigations addressed visual systems in Pycnogonida [21] *(Achelia langi*, *A. vulgaris*, and *Endeis spinosa*), the sister taxon to Euchelicerata or even to Euarthropoda, and in Onychophora [22,23] (*Euperipatoides rowelli*, *Epiperipatus biolleyi*, and *Metaperipatus blainvillei*), a putative arthropod outgroup.

The phylogenetic position of Scorpiones was discussed in various ways over the last one hundred years: Analysis based on morphological data either saw Scorpiones as highly ancestral Arachnida and as the sister taxon to Lipoctena (= all other arachnids) [24], or grouped Scorpiones together with Opiliones, Pseudoscorpiones, and Solifugae, to form the arachnid subgroup of Dromopoda [25]. Recently five arachnid clades were proposed, one of them being the clade Stomothecata comprising Scorpiones and Opiliones, but the relationships between those 5 clades is unresolved [26]. Combined morphological and molecular analyses support Dromopoda [27,28]. Also in molecular studies, the phylogenetic position of scorpions is interpreted in different ways [29,30]. And lastly some palaeontologists continue a long tradition of placing scorpions outside Arachnida with Eurypterida [31,32]. Eurypterida (the sea scorpions) is the extinct sister taxon to Xiphosura, with which it forms the group Merostomata.

To make the visual system in scorpions accessible for phylogenetic comparison with those in other chelicerates, the present study employs several independent approaches (3D serial reconstruction, Cobalt fills, DiI / DiO labelling, Wigglesworth stains). In the scorpion species *Euscorpius italicus* (Herbst, 1800) and *E. hadzii* Di Caporiacco, 1950, the visual neuropils of the median and lateral eyes are identified with Cobalt fills and DiI / DiO labelling, and their general architecture is studied along with the termination sites of retinula cell axons. Additionally the main neuropils of the protocerebrum are described by means of osmium-ethyl gallate procedure and AMIRA 3D-reconstruction. This reveals features of the visual system generally studied in Chelicerata, to allow comparisons with other lineages.

Results

General layout of the visual system

The visual system in the studied scorpion species, *Euscorpius italicus* and *E. hadzii*, is composed of two median eyes located medially on top of the cephalothorax, and two pairs of lateral eyes located along the front corners of the cephalothorax. Nerve fibres project from the median and lateral eyes proximally to the dorso-lateral protocerebrum. The two median eyes supply two distinct, successive visual neuropils as targets of the R-cell axons; few fibres additionally connect the median eyes with the arcuate body (Figure 1). The first neuropil is located dorso-anteriorly in the lateral part of the protocerebrum, as an oval-shaped region laterally embedded in the cell body rind of the brain (Figure 1A). The second neuropil lies deeper, under the cell body rind and in a more ventral and lateral position in the protocerebrum (Figure 1B–F).

The two lateral eyes also supply two distinct, successive visual neuropils as targets of the R-cell axons (Figure 2). The second visual neuropils of the median and lateral eyes overlap each other; this means that some R-cell axons of the median and lateral eyes end in a shared region of the second visual neuropil (Figures 3, 4). The first neuropil is located in the lateral and anterior part of the protocerebrum, 50–100 μm ventrally underneath the

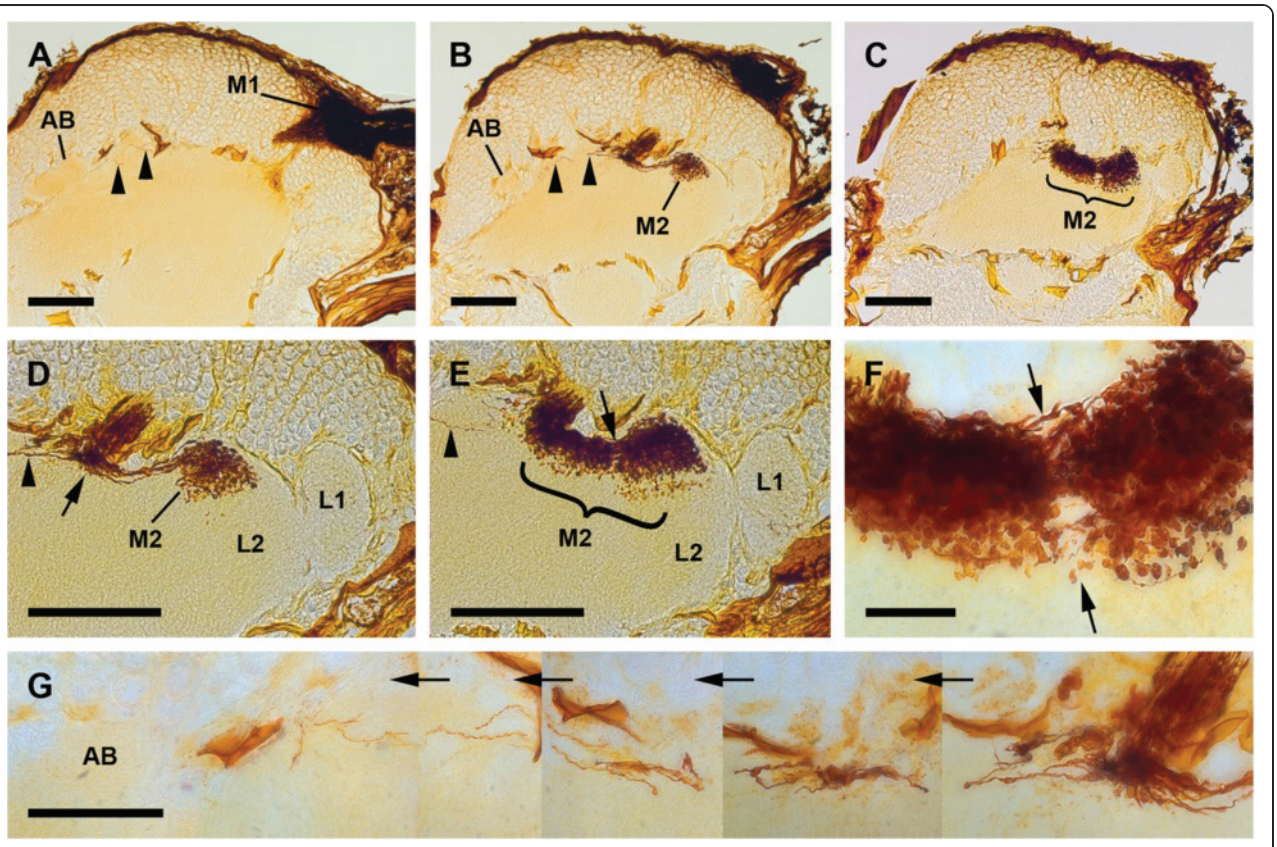


Figure 1 Cobalt fills via median eyes, sagittal sections. A, first median eye visual neuropil posteriorly in dorso-lateral protocerebrum. Note dense arrangement of Cobalt-filled profiles. Arrowheads point to axons extending to arcuate body. Bar 100 μm. **B**, bifurcation of fibres projecting from first to second median eye neuropil. Arrowheads point to axons extending to arcuate body. Bar 100 μm. **C**, second median eye visual neuropil under cell body rind, divided by an annulus into posterior and anterior subunit. Bar 100 μm. **D**, detail of bifurcation, varicosities in anterior subunit of second median eye visual neuropil. Arrowhead points to axons extending to arcuate body. Bar 100 μm. **E**, detail of second median eye visual neuropil, showing varicosities in both subunits. Arrowhead points to axons extending to arcuate body. Bar 100 μm. **F**, detail of annulus (arrows). Bar 25 μm. **G**, combination of five successive sections to demonstrate path of Cobalt-filled axons connecting median eyes with arcuate body via bifurcation seen in B and D. Bar 50 μm. AB, arcuate body; L1, first lateral eye visual neuropil; L2, second lateral eye visual neuropil; M1, first median eye visual neuropil; M2, second median eye visual neuropil.

first visual neuropil of the median eyes. It is oval, and laterally embedded in the cell body rind of the brain (Figure 2A–C). The second neuropil lies posterior to the first neuropil and is also oval (Figure 2D, E).

The visual neuropils are unequivocally identified with Cobalt fills and DiI / DiO labelling (Figures 1, 2, 3 and 4), and can also be recognised with osmium-ethyl-gallate staining (Figure 5), as dark-stained areas, as is typical for dense neuropils such as sensory neuropils. The third target region, i.e. that of the median eyes in the vicinity of the arcuate body, is also identified with both, Cobalt fills and DiI / DiO labelling (Figures 1, 4).

Furthermore, the arcuate body occupies a superficial, dorso-posterior position in the brain; its shape is slightly bent anteriorly (Figure 5F). The mushroom bodies are located parallel to the midline on each side of the protocerebrum (Figure 5D, E). Both neuropils can be recognised with osmium-ethyl-gallate staining.

Cobalt fills and DiI labelling via median eyes

Both methods of staining via the median eyes reveal two distinct retinula axon target regions in each hemisphere of the protocerebrum, a first and a second visual neuropil (Figures 1, 4A–C). Furthermore, fibres attributed to visual neurons connect the median eyes with the arcuate body (Figures 1, 4A–C).

Cobalt fills: Immediately after entering the brain the retinula axons build synaptic varicosities all over their extension within the neuropil (Figure 1A). After the first neuropil the retinula axons project ventro-posteriorly in a tract through the cell body rind deeper in the protocerebrum (Figure 1A, B). In this tract no synaptic varicosities appear. After passing through the cell body rind the axons diverge in two directions (Figure 1B, D). The larger parts of the axons first make a U-turn, then project anteriorly towards the visual neuropils of the lateral eyes (see below), while a few axons run further

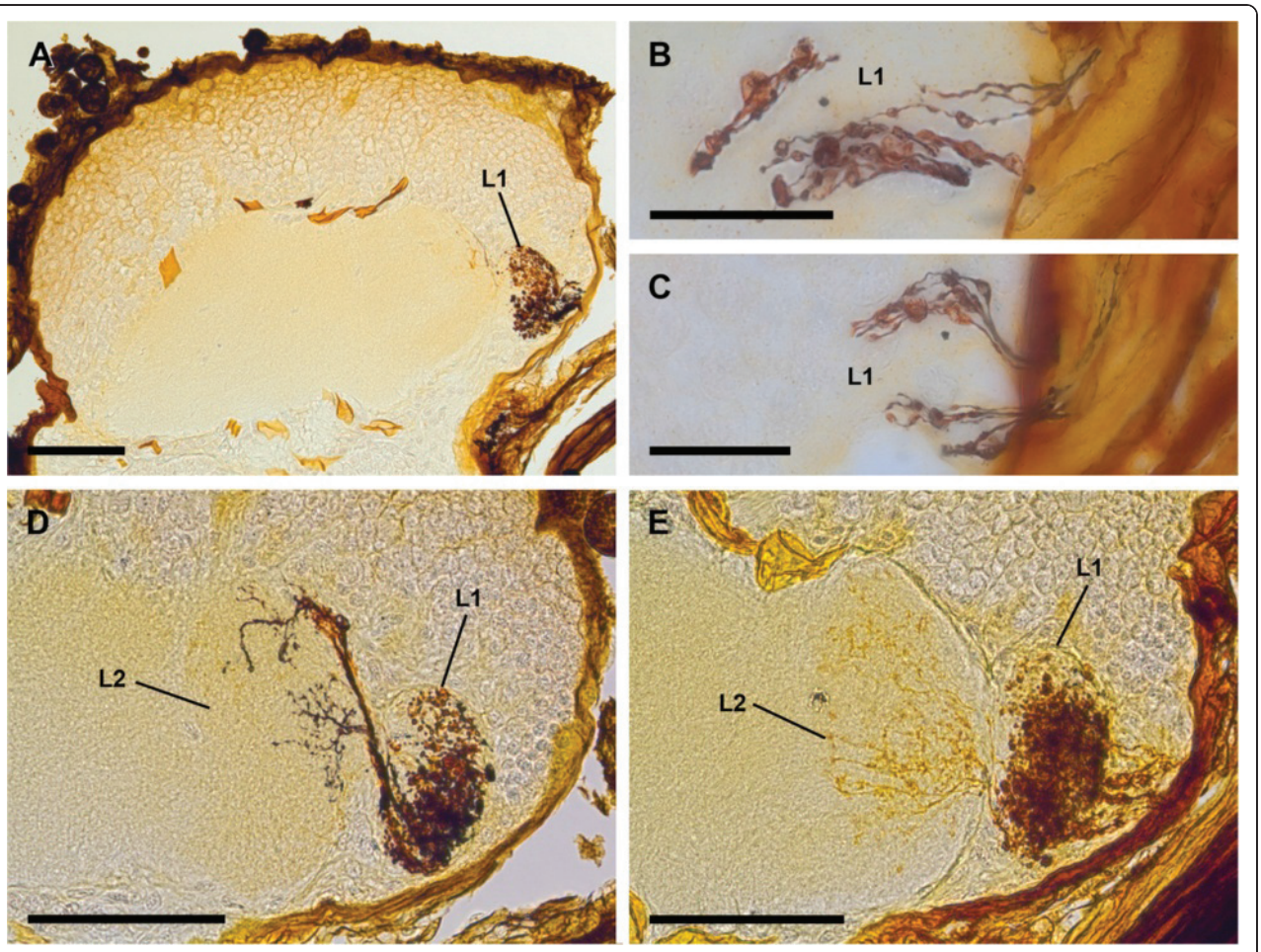


Figure 2 Cobalt fills via lateral eyes, sagittal sections. A, first lateral eye visual neuropil posteriorly in ventro-lateral protocerebrum. Note dense arrangement of Cobalt-filled profiles. Bar 100 μm. **B**, **C**, details of Cobalt-filled retinula axons with varicosities at entrances to first lateral eye visual neuropil. Bars 25 μm. **D**, **E**, Cobalt fills of retinula axons terminating in first and second lateral eye visual neuropils. Note that some fibres seem to cross between first and second visual neuropils. Bars 100 μm. L1, first lateral eye visual neuropil; L2, second lateral eye visual neuropil.

posteriorly to the vicinity of the arcuate body – which lies dorso-posteriorly in the protocerebrum – without entering the arcuate body directly (Figure 1A, B, D, E, G). These posterior-running fibres connecting the median eyes with the arcuate body were observed in a few specimens only. Immediately after the bifurcation, about half a dozen fibres with few synaptic varicosities are visible, but only one or two fibres are Cobalt-filled as far as the vicinity of the arcuate body. This might have resulted from experimental diffusion times (1–4 h) too short for such a long distance (approx. 300 μm). The anteriorly running fibres end in the second visual neuropil (Figure 1B–F). This neuropil lies underneath the cell body rind and is split in two subunits, an anterior and a posterior one, divided by an annulus (Figure 1E, F). Synaptic varicosities occur in both subunits. The anterior subunit lies in the dorsal part of the second visual neuropil of the lateral eyes (see below).

DiI labelling: The same target regions identified with Cobalt fills could be labelled with DiI (Figure 4A–C). After the first neuropil the retinula axons project ventro-posteriorly and diverge in two directions. The larger parts of the axons project to the second neuropil, while few fibres attributed to visual neurons run further posteriorly to the arcuate body. The morphology of the second visual neuropil is very similar to that visible in the Cobalt fills (Figure 4B). Again the neuropil is composed of two subunits divided by an annulus. However, the two subunits extend more ventrally; in the Cobalt fills, synaptic varicosities of the anterior subunit can be found only in the dorsal part of the second visual neuropil of the lateral eyes, while with DiI labelling synaptic varicosities can be found throughout this neuropil. This may be a result of the long diffusion time and hence of transcellular labelling. The fibres running posteriorly towards the arcuate body can be identified with DiI labelling as well. Synaptic varicosities after the bifurcation are

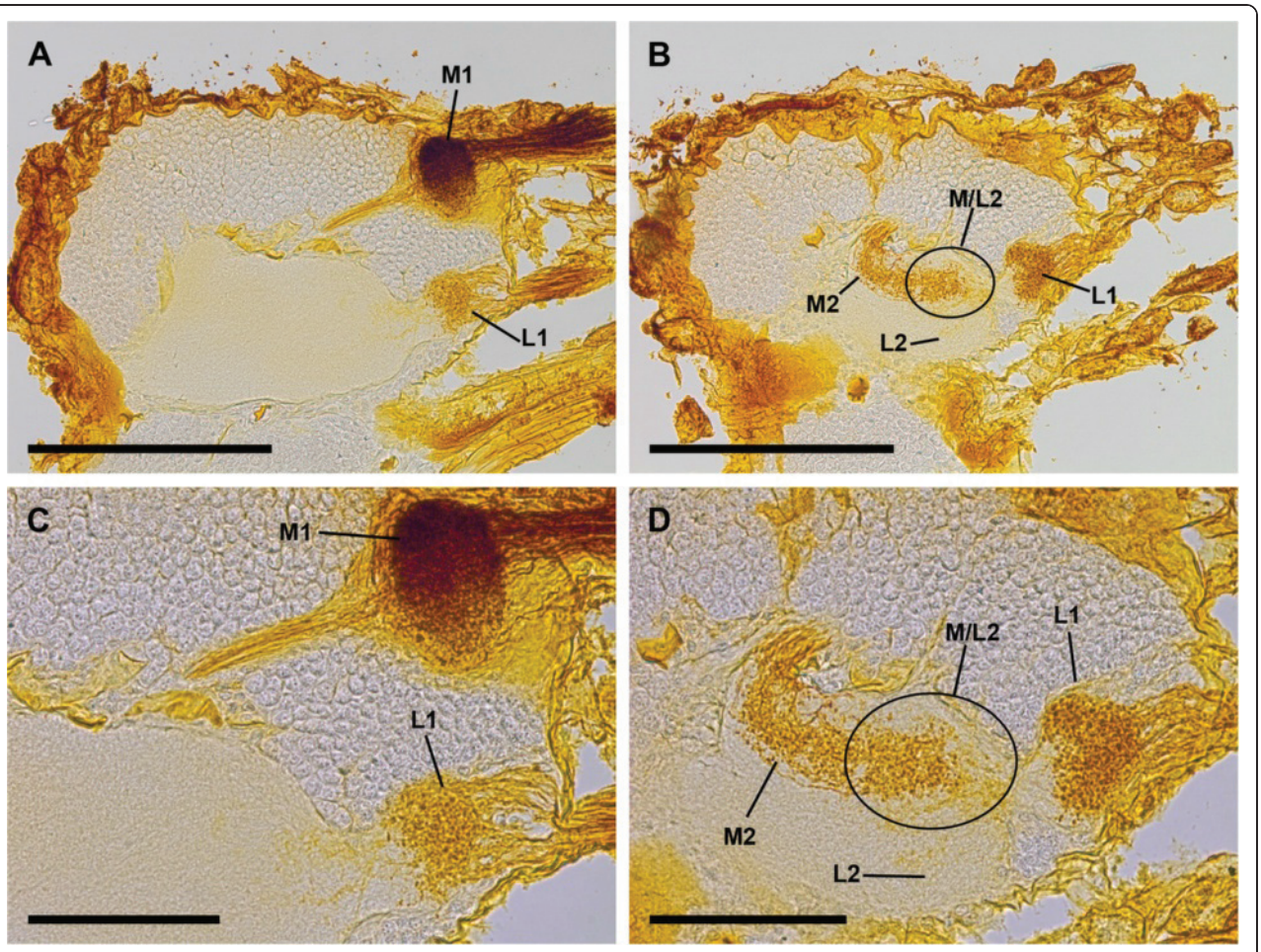


Figure 3 Cobalt fills simultaneously via median and lateral eyes, sagittal sections. A, first median and lateral eye visual neuropils, located posteriorly in lateral protocerebrum. Both neuropils with Cobalt-filled retinula axons. Bar 250 µm. **B**, second visual neuropils of median and lateral eyes. Besides regions with only Cobalt-filled R-cell axons of median or lateral eyes, encircled region with Cobalt-filled R-cell axons of both median and lateral eyes. Bar 250 µm. **C**, detail of first median and lateral eye visual neuropils. Note tract through cell body rind projecting to second median eye visual neuropil. First varicosities appear posterior to first lateral eye visual neuropil, indicating second lateral eye visual neuropil. Bar 100 µm. **D**, detail of second visual neuropils of median and lateral eyes. One can distinguish between lateral and median eye fills, lateral fills brighter. Besides regions with only Cobalt-filled R-cell axons of median or lateral eyes, encircled region with Cobalt-filled R-cell axons of both median and lateral eyes. Bar 100 µm. L1, first lateral eye visual neuropil; L2, second lateral eye visual neuropil; M1, first median eye visual neuropil; M2, second median eye visual neuropil; M/L2, region of L2 or M2 with Cobalt-filled R-cell axons of both median and lateral eyes.

better recognisable than in the Cobalt fills. Furthermore, the axons are labelled all the way through the arcuate body, the pale labelling resulting from the fact that only few axons are present in this area (Figure 4A).

Cobalt fills via lateral eyes

Cobalt fills via the lateral eyes also reveal two distinct retinula axon target regions in each hemisphere of the protocerebrum, a first and a second visual neuropil (Figure 2).

After entering the first visual neuropil the retinula axons build synaptic varicosities all over their extensions (Figure 2A–C). A chiasma between the first and second visual neuropils is not positively identified in any of the chosen section planes (sagittal, frontal or transversal), but fibres that seem to cross between first and second visual neuropil are observed (Figure 2D, E).

In the second visual neuropil the retinula axon terminals are branched and have synaptic varicosities (Figure 2D, E). In the dorsal region of this neuropil terminals of the retinula axons of the median eyes are observed in preparations in which retinula axons of both median and lateral eyes are Cobalt-filled (Figure 3B, D) (see below).

Cobalt fills and DiI / DiO labelling simultaneously via median and lateral eyes

As above the median eyes are directly linked to a first and a second neuropil, and connected to the arcuate

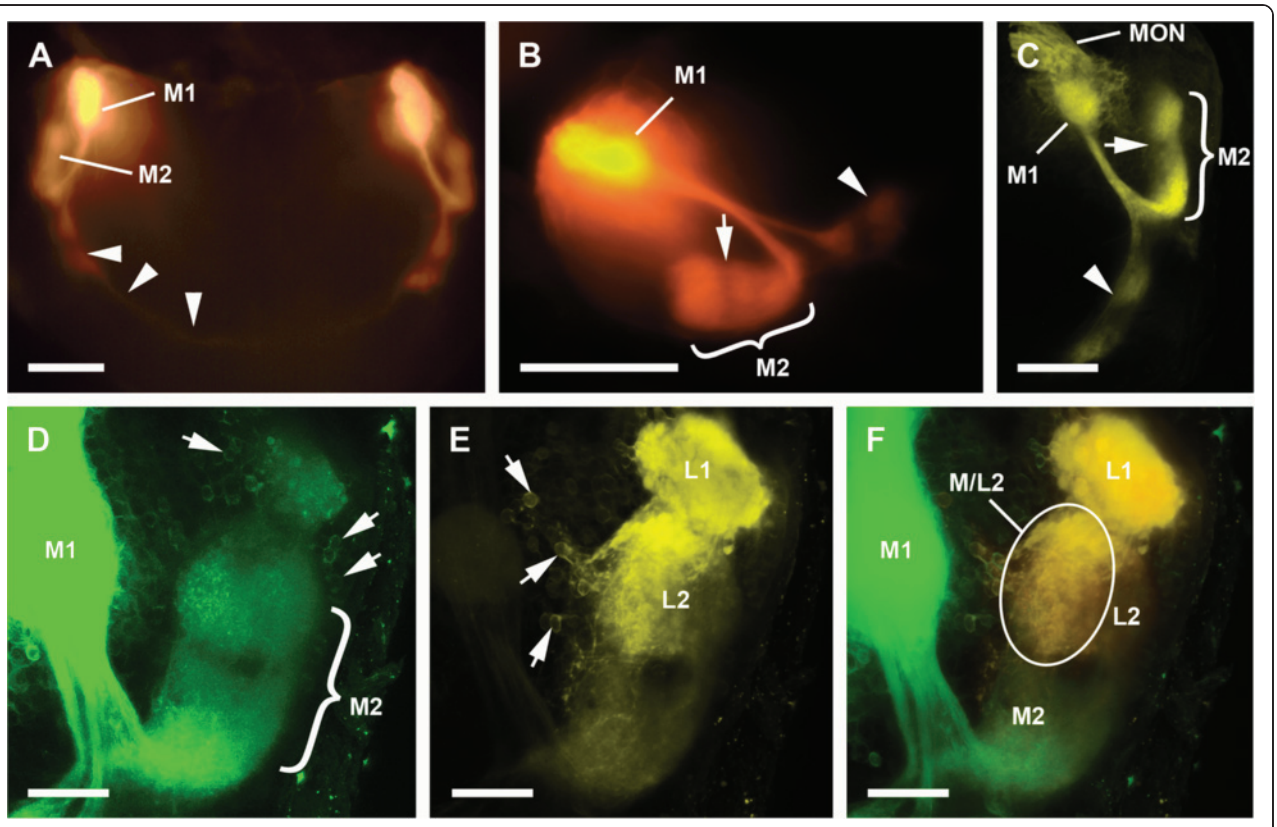


Figure 4 DiI and DiO labelling via median or median and lateral eyes (A, B, fluorescence microscope; C–D, CLSM). A, **B**, DiI-labelled first and second median eye neuropils. Arrowheads point to axons extending to arcuate body with varicosities after bifurcation, few axons terminating within the arcuate body. Note same annulus as seen in Cobalt fills (arrow). **A**, frontal view; **B**, sagittal view. Bars 200 μm. **C**, Specimen as in **A**, **B**, studied with CLSM. Frontal view. Bar 100 μm. **D**–**E**, Combined DiO-labelled median (green) and DiI-labelled lateral (yellow) eye neuropils. Frontal view. Bars 50 μm. **D**, DiO-labelled first and second median eye neuropils (green). Note that DiO-stained cell bodies (arrows) indicate transcellular staining. **E**, DiI-labelled first and second lateral eye neuropils (yellow). Note that DiI-stained cell bodies (arrows) indicate transcellular staining. **F**, Combined image of DiO-labelled median (green) and DiI-labelled lateral (yellow) eye neuropils. Encircled region of second median and lateral eye neuropils with labelled R-cell axons of both median and lateral eyes. L1, first lateral eye visual neuropil; L2, second lateral eye visual neuropil; M1, first median eye visual neuropil; M2, second median eye visual neuropil; M/L2, region of L2 or M2 with labelled R-cell axons of both median and lateral eyes; MON, median eye optic nerve.

body (Figures 1, 3, 4), the lateral eyes are linked to a first and a second neuropil (Figures 2, 3, 4E ,F).

Cobalt fills: The second visual neuropils of median and lateral eyes overlap each other. This means that besides the regions with only Cobalt-filled R-cell axons of median or lateral eyes, there is a region with Cobalt-filled R-cell axons from both median and lateral eyes (Figure 3B, D).

DiI / DiO labelling: The second visual neuropils of median and lateral eyes overlap each other, and the same region with R-cell axons from both median and lateral eyes can be identified with DiI labelling via lateral eyes and with DiO labelling via median eyes (Figure 4E). DiI- and DiO-labelled cell bodies in the cell body rind near the neuropils indicate that transcellular labelling occurred (Figure 4D–E). Hence, in contrast to the Cobalt fills, where no transcellular staining occurred, DiO from the median eyes is identifiable even in the first lateral eye neuropil, and DiI from the lateral eyes even in the posterior subunit of the second median eye neuropil.

Figure 6 shows the summary of the retinula axons and visual neuropils of the median and lateral eyes in *E. italicus.*

Discussion

The present study constitutes another case that a comparison of more recent findings with those of the early 20th century neuroanatomists, Nils Holmgren [7] and Bertil Hanström [8] is worthwhile. The latter authors correctly identified the visual neuropils of scorpions, but misinterpreted the tracts between them. Holmgren described the same neuropils as Hanström, but did not differentiate between median and lateral eye neuropils. Holmgren's first and fourth visual neuropils actually are median eye neuropils, his second and third neuropils are lateral eye neuropils. Hanström made this differentiation,

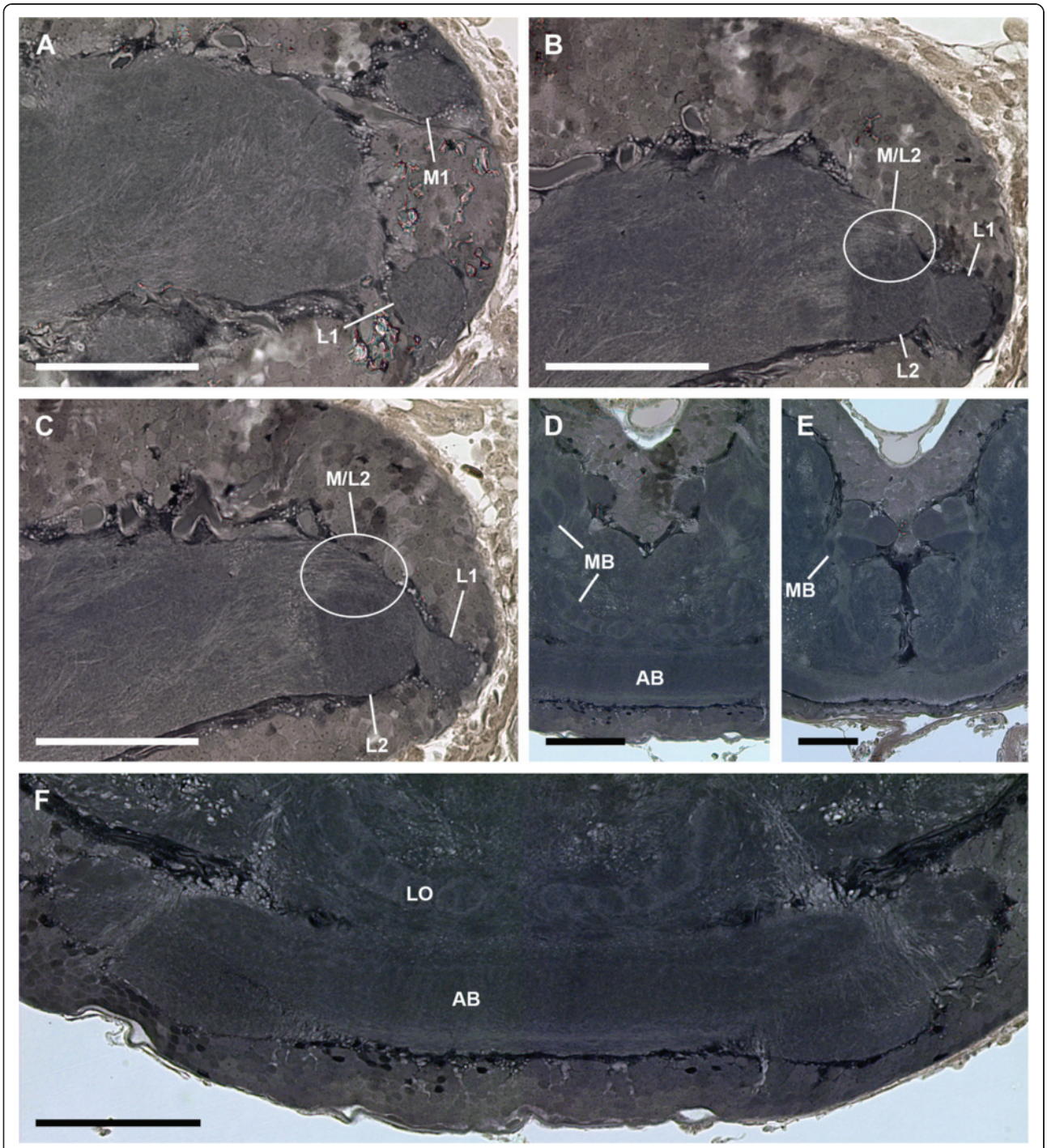


Figure 5 General anatomy of visual neuropils and protocerebrum (Wigglesworth stains). Note dark stain of sensory neuropils after application of Wigglesworth's technique. Bars 100 µm. **A**, first visual neuropils of median and lateral eyes, sagittal section. **B**, **C**, first and second visual neuropils of lateral eyes. Encircled: region where also R-cell axons of median eyes terminate, sagittal sections. **D**, **E**, mushroom bodies located parallel to midline of protocerebrum, frontal section. **F**, arcuate body in dorso-posterior position, frontal section. AB, arcuate body; L1, first lateral eye visual neuropil; L2, second lateral eye visual neuropil; M1, first median eye visual neuropil; M2, second median eye visual neuropil; M/L2, region of L2 or M2 with Cobalt-filled R-cell axons of both median and lateral eyes; MB, mushroom bodies.

but contrary to what he suggested, the median eyes are associated with two subsequent visual neuropils (not only with one), and the lateral eyes are associated with two subsequent visual neuropils (not with three). Hanström described a tract connecting the median eye neuropil with a third lateral eye visual neuropil. In our

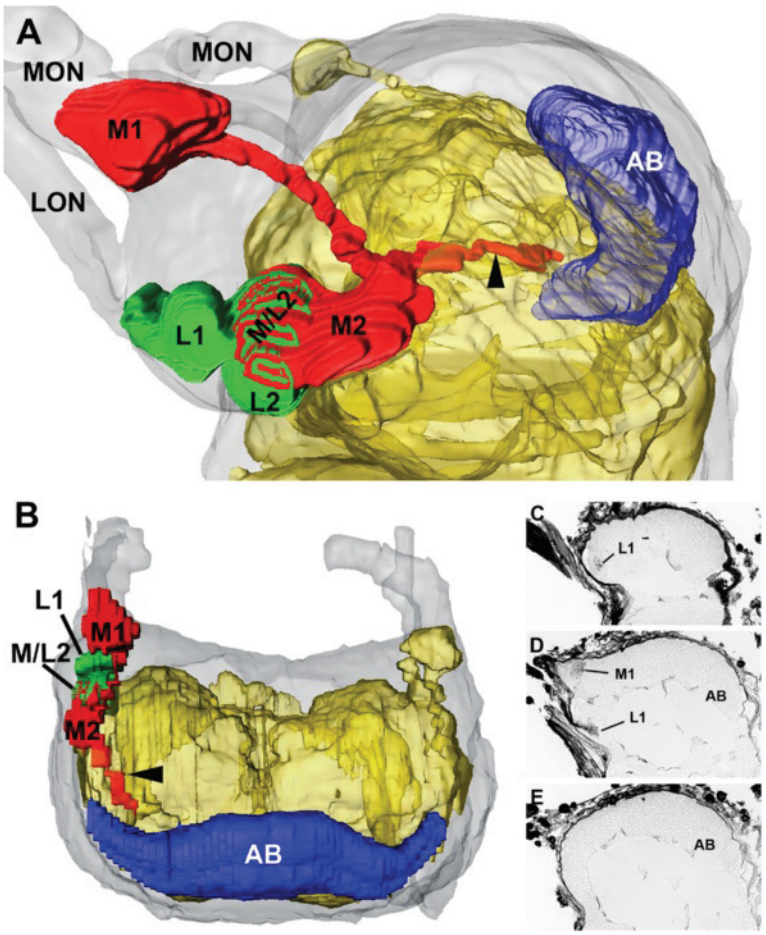


Figure 6 3D serial reconstruction of visual system of left hemisphere in *E. italicus* on the basis of Cobalt fills. A, **B,** 3D reconstruction showing arrangement of neuropils. **A**, lateral view; **B**, frontal view. Grey, protocerebrum; yellow, neuropil; red, median eye neuropils; green, lateral eye neuropils; blue, arcuate body. **C**–**E**, three selected sections (Cobalt fills) showing original data for reconstruction. **C**, parasagittal section showing beginning of first lateral eye visual neuropil. **D**, parasagittal section showing first median and lateral eye visual neuropils, and beginning of arcuate body. **E**, mid-sagittal section without visual neuropils but with arcuate body. AB, arcuate body; LON, lateral eye optic nerve; L1, first lateral eye visual neuropil; L2, second lateral eye visual neuropil; MON, median eye optic nerve; M1, first median eye visual neuropil; M2, second median eye visual neuropil; M/L2, region of L2 or M2 with Cobalt-filled R-cell axons of both median and lateral eyes.

results that tract is the one connecting the first with the second median eye neuropil; Hanström may have been misled by the position of the second median eye neuropil to misinterpret the latter as a third lateral eye neuropil. In addition, a connection between the visual system and the arcuate body was observed by both authors, which can be confirmed here.

Our results show that the median eye retinula cells are linked to a first and a second visual neuropil, while some fibres additionally connect the median eyes with the arcuate body. The lateral eye retinula cells are linked to a first and a second visual neuropil as well. Furthermore, our stainings show that there is a region in which the second median and second lateral eye neuropils overlap each other. One can distinguish three regions (from posterior to anterior): (1) a region with R-cell axon terminals of median eyes only, (2) a region with R-cell axon terminals of both, median and lateral eyes, and (3) a region with R-cell axon terminals of lateral eyes only. This division is particularly evident in the Cobalt fills. In the DiI and DiO labelling transcellular staining occurred. The latter is recognisable by the fact that cell bodies of interneurons are labelled. Hence, the division of these three regions is visible but not as distinct as in the Cobalt fills, where no transcellular staining occurred. There are three alternative ways to describe and name these regions. One may consider this region as one neuropil, as two neuropils overlapping each other, or as three neuropils (one median, one median/lateral, and one lateral eye visual neuropil). We prefer the second alternative and consider this region as two neuropils, one second median eye neuropil and one second lateral eye neuropil, which partly overlap each other. This means that there is a region with R-cell axons of both median

and lateral eyes, but in both second visual neuropils there are also regions with only retinula axon terminals of median or lateral eyes. Moreover, the retinula axon terminals of the lateral eyes are described here for the first time: R-cell axon terminals are found in a first and a second lateral eye neuropil. The crossing fibres we observed probably do not represent a "classical" chiasm as found in Tetraconata [12,33]. A detailed analysis is needed to find out if it might correspond to the chiasm in *Limulus*, which is suggested to be convergent to that in Tetraconata [15,34].

The only other more recent surveys considering the morphology of the visual systems in scorpions were made by Fleissner [9] and Heinrichs and Fleissner [35]. These studies discussed mainly the electrophysiology of the scorpion visual system, and gave only schematic drawings of the approximate locations of the visual neuropils without identifying the latter. However, Fleissner [9] and Heinrichs and Fleissner [35] did report that the different cell types of the median eye retina have different target regions: the photoreceptor cells terminate in a first neuropil (lamina), the arhabdomeric cells in a second neuropil (medulla) [9], while the efferent neurosecretory fibres have their origin/cell body in the tritocerebrum and terminate, while passing through the arcuate body, in the retina of the median eyes [35]. The target region of the photoreceptor cells is located where we found the first median eye neuropil, and the target region of the arhabdomeric cells is where we found the second median eye neuropil; the pathways of the neurosecretory fibres are equal to the fibres we found that connect the median eyes with the arcuate body. Such differentiation of target regions of the different cell types could not be achieved with the methodology chosen for the present study, but will be considered in the discussion below.

Thus our study, while taking the results of Fleissner and Heinrichs into account, leads to a new interpretation of the visual system as well as of the general architecture of the scorpion protocerebrum. The median eyes are associated with two serial neuropils, a first and a second visual neuropil, while some fibres connect the median eyes with the arcuate body. The second visual neuropil is subdivided by an annulus; the posterior subunit contains only retinula axon terminals of the median eyes, while the anterior subunit contains retinula axon terminals of both median and lateral eyes. Furthermore, Fleissner [9] showed that the first neuropil is the target region of the photoreceptor cells, and the second visual neuropil that of the arhabdomeric cells. The morphology of the fibres projecting to the arcuate body is very similar to that of the efferent neurosecretory fibres described by Heinrichs and Fleissner [35]. The authors identified efferent neurosecretory fibres with cell bodies in the tritocerebrum projecting through the arcuate body to the retina of the median eyes. Hence, the fibres projecting to the arcuate body, observed here in Cobalt and DiI stains, are rather retrograde-filled axons projecting from the tritocerebrum through the arcuate body to the retina of the median eyes. Due to the fact that these cells have their cell bodies in the tritocerebrum, they rather "belong" to the brain and are not retinula cells.

The lateral eyes are associated with two serial neuropils, a first and a second visual neuropil. Retinula axon terminals occur in both neuropils, while in the dorsal part of the second visual neuropil retinula axon terminals of both lateral and median eyes are observed.

The slightly bent arcuate body is shown in a superficial, dorso-posterior position in the brain, as is typical for chelicerates [36]. Additionally the mushroom bodies can be observed, located parallel to the midline of the protocerebrum.

These highly specific features described in the present study allow a comparison with the visual systems in other chelicerates and in ancestral arthropods.

Median eyes

In *Limulus* one must distinguish between the paired median eyes and the fused median rudimentary eye (see review by Battelle [16], and Table 1). Chamberlain and Barlow [13] demonstrated by means of Cobalt fills that the median optic nerve, which contains fibres from both, the paired median eyes and the fused median rudimentary eye, is linked to the first median eye neuropil (ocellar ganglion), arcuate body, optic tract, and medulla (which is also the second lateral eye neuropil). Additionally Calman et al. [14] and Battelle [16] showed with antibody staining that the photoreceptor cells of the paired median eyes are linked in each brain hemisphere only to the first median eye neuropil (ocellar ganglion). Moreover, the authors derived the projections of the arhabdomeric cells by subtracting the photoreceptor cell projections from the results of Cobalt fills of the median eye nerve in Chamberlain and Barlow [13]. According to Calman et al. and Battelle the arhabdomeric cells end only in the medulla (second neuropil of the lateral eyes), but if one compares the results of Calman et al. [14] and Battelle [16] with those of Chamberlain and Barlow [13] one can see that Calman et al. and Battelle ignored that numerous collaterals can be found not only in the medulla (second lateral eye neuropil) but also in the optic tract before entering the medulla. Hence, one can see the target region of the arhabdomeric cells as a neuropil of its own that partly overlaps with the medulla (second lateral eye neuropil). This situation is very similar to the situation found here for the median eyes of scorpions: the second visual neuropil as a target of the

Table 1 Distribution of eyes in Onychophora and Chelicerata [1,5,16,22,37]

	Median eyes	Lateral eyes
Onychophora	One pair of eyes (median/lateral affinity unknown)	
Pycnogonida	Four	Absent
Xiphosura	One pair, plus one fused median rudimentary eye	One pair of lateral compound eyes, plus one pair of lateral rudimentary eyes
Scorpiones	One pair	Three to five pairs, plus one pair of nymphal eyes
Araneae	One pair (= principal eyes or anterior median eyes)	Three pairs (= secondary eyes)

arhabdomeric cells partly overlaps with the second lateral eye neuropil as well.

Calman et al. [14] and Battelle [16] also demonstrated with biocytin injection and myosin III immunoreactivity that the fused median rudimentary eye of *Limulus* is linked in each brain hemisphere to the first median eye neuropil (ocellar ganglion) and simultaneously to a region near the arcuate body. This situation is similar to the median eyes of pycnogonids: their eyes are associated with a first visual neuropil and a second visual neuropil in close vicinity to the arcuate body [21]. However, the retinula axons of the fused median rudimentary eye in *Limulus* have some branches in both, the first median eye neuropil and the region near the arcuate body. In pycnogonids the retinula axons have branches only in the first or second visual neuropil, not in both neuropils simultaneously.

In Araneae there is only one target region of the retinula axon terminals of the median eyes (principal eyes or anterior median eyes): the first anterior median eye neuropil, located dorso-laterally in each brain hemisphere [18,19]. Subsequent second-order neurons terminate in a second visual neuropil (medulla); furthermore, a tract that extends into the arcuate body is suggested. Comparing the projections of the median eyes in scorpions with those of the anterior median eyes in Araneae, one finds similarities and differences. The photoreceptor cells project only to a bilaterally paired first visual neuropil. Furthermore, only photoreceptor cells and no arhabdomeric cells are described from the retinae of spiders. Hence, a connection from these cells to a second visual neuropil is missing.

Finally, in Onychophora (*Euperipatoides rowelli*) – one of the suggested sister taxa of Euarthropoda [38,39] whose brain organization is discussed as being similar to that in chelicerates [23,40] – the presence of photoreceptor terminals in a first visual neuropil, which lies directly beneath the eye, is suggested [22]. From this first neuropil, an optic tract projects further and then bifurcates [23]. Its ventral branch extends to a second visual neuropil near the mushroom body calyces, while the dorsal branch gives rise to another second visual neuropil, which flanks the arcuate body laterally. The exact projection of the retinula cells is not identified unequivocally.

Thus, comparing the median eye visual system in scorpions to those in other chelicerates and in onychophorans, there are great similarities to the "normal" median eyes of xiphosurans, and some to the median rudimentary eyes of xiphosurans and median eyes of onychophorans, pycnogonids and spiders. As demonstrated in Lehmann et al. [21], the eyes of pycnogonids and the fused median rudimentary eye of *Limulus*, possibly also the eyes of onychophorans, show striking similarities in their innervation patterns.

The same is true for the median eyes of scorpions and *Limulus*. Both have two distinct, bilaterally paired target regions of the retinula cells: a first neuropil as target for the photoreceptor cells, and a second neuropil, which overlaps with the second neuropil of the lateral eyes, as target for arhabdomeric cells [13,14].

Lateral eyes

Of special interest here are the eyes of *Limulus*, where one must distinguish again between the lateral rudimentary eyes and the lateral compound eyes (see review by Battelle [16], and Table 1). The rudimentary eyes are associated with the same neuropils as the lateral compound eyes, a first (lamina) and a second (medulla) visual neuropil; the second neuropil is also a target region of the arhabdomeric cells of the median eyes (see above) [14]. While the photoreceptor cells of the rudimentary eyes are linked to both, lamina and medulla, the photoreceptor cells of the lateral compound eyes are linked to the lamina only. Moreover, the retinae of the lateral compound eyes contain eccentric cells, which project to the lamina, medulla, optic tract, and to the first neuropil of the median eyes (ocellar ganglion).

Hence, the projections of the lateral eyes of scorpions have some characters in common with the lateral rudimentary eyes of *Limulus*. Like the lateral eyes of scorpions, the rudimentary eyes have projections to a first and a second visual neuropil. In turn, the photoreceptor cells in the lateral compound eye of *Limulus* are linked to the lamina only, while the eccentric cells are linked to the lamina and medulla of the lateral eye, optic tract, and to the first neuropil of the median eyes (ocellar ganglion). Such a connection from the lateral eye to median eye neuropils cannot be observed in the scorpion visual system. The similarity in function and structure between

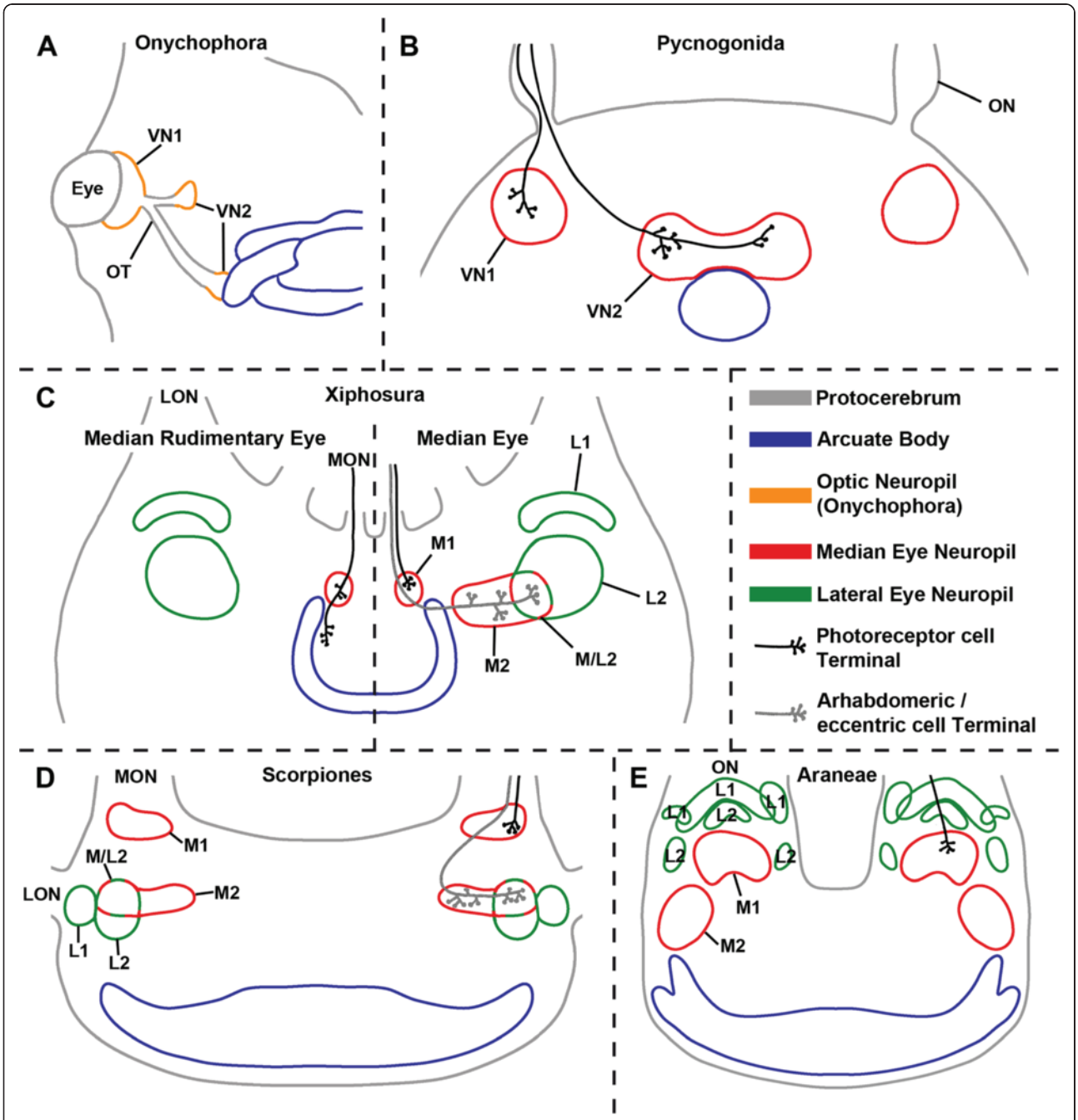


Figure 7 Comparison of median eye visual systems in (A) Onychophora (*Euperipatoides rowelli*), (B) Pycnogonida (*Achelia* spp., *Endeis spinosa*), (C) Xiphosura (*Limulus polyphemus*), (D) Scorpiones (*Euscorpius* spp., *Androctonus australis*), and (E) Araneae (*Cupiennius salei*). **A**, visual pathways from eyes with optic neuropils indicated. After Strausfeld et al. [23]. **B**, retinula cells terminate in first and second visual neuropils. Second visual neuropil in close vicinity to an unpaired midline neuropil, possibly an arcuate body. After Lehmann et al. [21]. **C**, left: terminals of median rudimentary eye have some branches in first median eye neuropil, then continue and terminate near arcuate body; right: median eye photoreceptor cells terminate in first median eye neuropil, arhabdomeric cells in second median eye neuropil, which partly overlaps with second lateral eye neuropil. After Calman et al. [14] and Chamberlain and Barlow [13], second median eye neuropil added (see text). **D**, photoreceptor cells terminate in first median eye neuropil, arhabdomeric cells in second median eye neuropil, which partly overlaps with second lateral eye neuropil. Connection between median eyes to region near arcuate body omitted. According to Heinrichs and Fleissner [35] these fibres belong to neurosecretory cells with origin in the tritocerebrum, hence are cells of the brain rather than retinula cells. **E**, retinula cells terminate in first median eye neuropil. After Strausfeld et al. [18] and Strausfeld and Barth [19]. LON, lateral eye optic nerve; L1, first lateral eye visual neuropil; L2, second lateral eye visual neuropil; MON, median eye optic nerve; M1, first median eye visual neuropil; M2, second median eye visual neuropil; M/L2, region were M1 and L1 overlap; ON, optic nerve; OT, optic tract; VN, visual neuropil.

the eccentric cells and the arhabdomeric cells of scorpions was discussed by Schliwa and Fleissner [3,41]. More research has to be done to distinguish between the exact innervation patterns of the photoreceptor cells and the arhabdomeric cells in the lateral eyes of scorpions.

Conclusions

The large number of characters discussed in this article shows that the central projections of especially the median eyes in Chelicerata provide structures that are extremely useful for discussing aspects of chelicerate ground patterns and phylogenetic relationships. The sets of characters studied here for Scorpiones and those in *Limulus*, Pycnogonida, Onychophora, and Araneae are summarised in Figure 7.

As shown above, the similar innervation patterns of the median and lateral eyes indicate a close relationship concerning the visual system between scorpions and *Limulus*. Other characters supporting this idea are the position and cellular architecture of the accessory lateral eye of scorpions, which corresponds well with that of the lateral rudimentary eye of *Limulus* [5]. Also the functional and structural similarity of the arhabdomeric cells of scorpions with the eccentric cells of *Limulus* lateral eyes must be mentioned [3,41]. Dunlop and Webster [31] discuss further similarities between scorpions and *Limulus*. Besides similar sperm morphology and growth zones, the shared character of star-shaped rhabdoms is mentioned (see also Weygoldt and Paulus [24]). However, the argument of rhabdom morphology must be handled with care: indeed, scorpions and *Limulus* both have star-shaped rhabdoms, but this is only true for the lateral compound eyes of *Limulus* and the median but not the lateral eyes of scorpions. The latter have a net-like rhabdom [3]. Nevertheless, characters of the visual system support the hypothesis of Weygoldt and Paulus [24] that scorpions occupy the basalmost position within Arachnida, or even the idea of palaeontologists that Scorpiones are closely related to Eurypterida [31,32] and hence also to Xiphosura. This, in turn, would question the monophyly of Arachnida, and would mean that scorpions and one or more other arachnid lineages are likely to have come onto land independently [31]. More research concerning the visual systems in Arachnida has to be done, since only few taxa have been investigated, and there are no data on various taxa like Opiliones, Pseudoscorpiones, and Solifugae, which are suggested as sister taxa to Scorpiones by various authors [25-28].

Regarding the basal position of *Limulus* and especially Pycnogonida, it is reasonable to assume that the central projections of the median rudimentary eye in *Limulus* and the four median eyes in Pycnogonida represent the ground pattern for Chelicerata. This ground pattern is characterised by (1) four median eyes, (2) a separated, bilaterally paired nerve that connects the eyes with the brain, (3) a separated, bilaterally paired first visual neuropil with central projections of photoreceptor cells, (4) a second visual neuropil also with central projections of photoreceptor cells, and (5) the second visual neuropil being located in close vicinity to the arcuate body. Derived situations are found in the "normal" median eyes of *Limulus* and in the median eyes of scorpions: in both of these, the photoreceptor cells only project to a separated, bilaterally paired first visual neuropil, while the second type of retinula cells, the arhabdomeric cells, project to a second visual neuropil, which partly overlaps with the second visual neuropil of the lateral eyes. Additionally a third cell type is found in the retina of the median eyes, the efferent neurosecretory fibres, which have their origin/cell body in the brain and terminate in the retina. Another derived situation is found in the median eyes (principal eyes or anterior median eyes) of Araneae, whose photoreceptor cells (as the only cells in the retina projecting to the protocerebrum) simply project to a separated, bilaterally paired first visual neuropil.

Materials and methods

The use of *Euscorpius* spp. in the laboratory doesn't raise any ethical issues and therefore Regional or Local Research Ethics Committee approvals are not required.

Specimen collection

Specimens of *Euscorpius italicus* (Herbst, 1800) (Scorpiones: Euscorpiidae) were collected during field trips to Rovinj (Croatia) in August 2011 and April 2012. Specimens of *Euscorpius hadzii* Di Caporiacco, 1950 were provided by b.t.b.e. Insektenzucht GmbH (Schnürpflingen, Germany).

Cobalt fills

(*Euscorpius italicus*, modified after Altman and Tyrer [42]): $CoCl_2$ crystals were inserted in median, lateral, or median and lateral eyes with a fine tungsten needle (n = 30). After diffusion times between 1 and 4 hours, Cobalt was precipitated with a solution of five drops of $(NH_4)_2S$ in 10 ml H_2O_{dest}. After fixation of the cephalothorax in AAF (85 ml 100% ethanol, 10 ml 37% formaldehyde, 5 ml glacial acetic acid), the brain was dissected and silver intensified: 60 min at 50°C in dark in solution A (10 ml H_2O_{dest}, 3 ml 100% ethanol, 0.5 g gum arabic, and 0.02 g hydroquinone; pH value adjusted to between 2.6 and 3.1 using citric acid), and 15–30 min at 50°C in the dark in solution B (10 ml H_2O_{dest}, 3 ml 100% ethanol, 0.5 g gum arabic, 0.02 g hydroquinone, 0.01 g $AgNO_3$; pH value adjusted to between 2.6 and 3.1 using citric acid). Silver intensification was stopped in an acetic acid solution (50 ml 30% ethanol, 5 g glucose, pH value adjusted to between 2.6 and 3.1 using acetic acid). After dehydration in a graded acetone series, the brain was

embedded in Glycidether 100, and sectioned with a rotary microtome and stainless steel blade in the sagittal, frontal, and transversal planes (14–16 μm).

DiI / DiO labelling

(*Euscorpius hadzii*, after Wohlfrom and Melzer [43]): The cephalothorax was dissected and fixed overnight at 4°C in 4% formaldehyde in 0.1 M PBS. Afterwards specimens were rinsed overnight in 0.1 M PBS, 0.1% NaN_3. Finally, small DiI or DiO crystals (Molecular Probes) were inserted in median or median and lateral eyes with a fine tungsten needle. Diffusion was carried out in darkness on small glass slides enclosed in wet chambers for 17–22 days. To prevent the growth of microorganisms, NaN_3 in PBS was used for moistening. From time to time the specimens were controlled under the microscope. Specimens were studied with a fluorescence microscope and CLSM.

Osmium ethyl gallate procedure

(*Euscorpius italicus*, modified after Wigglesworth [44], Leise and Mulloney [45], and Mizunami et al. [46]): Brains were dissected and fixed in 4% glutardialdehyde in 0.1 M cacodylate buffer at 4°C (n = 7). After postfixation in 2% OsO_4 in 0.1 M cacodylate buffer (3 h at 4°C) animals were stained for 17 hours at 4°C in a saturated ethyl gallate solution, dehydrated in a graded acetone series, embedded in Glycidether 100, and sectioned with a rotary microtome and stainless steel blade in the sagittal, frontal, and transversal planes (5–8 μm).

3D-reconstruction

Brain (prepared as for Cobalt fills) was cut into a complete sagittal series (16 μm). Slices were mounted on glass slides, covered with cover slips, and photographed under a conventional light microscope. Images were contrast-enhanced in Adobe Photoshop, then aligned, segmented and rendered in Amira.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TL conceived the study, carried out the morphological analysis, and drafted the manuscript. RRM conceived and supervised the study and helped writing the paper. All authors read and approved the final manuscript.

Acknowledgements

We thank Eva Lodde and Stefan Friedrich for expert technical assistance; Carolin Schneider, Stefan Friedrich, and Roland Meyer for help with collecting the animals (*E. italicus*); Christian Kronmüller and b.t.b.e. Insektenzucht GmbH (Schnürpflingen, Germany) for providing *E. hadzii* specimens; Martin Heß for his help at the CLSM; and Martin Spies for polishing the English. This study was supported by a Graduiertenstipendium (BayEFG) from the Universität Bayern e.V. to T. Lehmann. We would like to express our gratitude to the anonymous reviewers, whose comments have helped a lot to improve the manuscript.

Author details

[1]SNSB – Bavarian State Collection of Zoology, Münchhausenstraße 21, Munich 81247, Germany. [2]Department Biology II, Ludwig-Maximilians-Universität München, Großhaderner Straße 2, Planegg-Martinsried 82152, Germany. [3]GeoBio-Center at LMU, Richard-Wagner-Str. 10, 80333, Munich, Germany.

Received: 11 December 2012 Accepted: 1 July 2013
Published: 11 July 2013

References

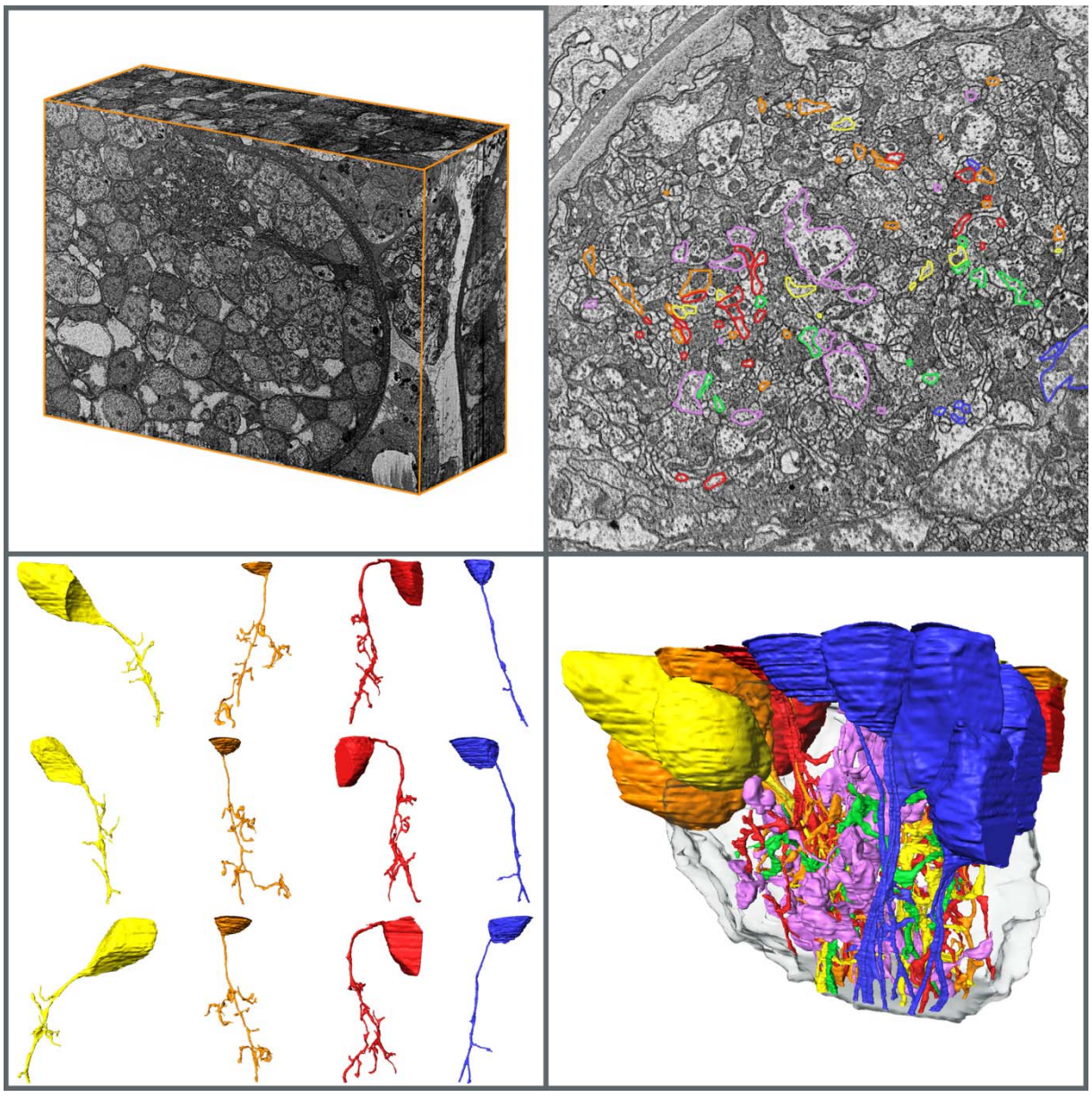
1. Ruppert EE, Fox RS, Barnes RD: *Invertebrate zoology: a functional evolutionary approach.* 7th edition. Belmont: Thomson-Brooks/Cole; 2004.
2. Bedini C: **The fine structure of the eyes of *Euscorpius carpathicus* L. (Arachnida:Scorpiones).** *Arch Ital Biol* 1967, **105:**361–378.
3. Schliwa M, Fleissner G: **The lateral eyes of the scorpion, *Androctonus australis*.** *Cell Tissue Res* 1980, **206:**95–114.
4. Locket A: **Eyes and vision**. In *Scorpion Biology and Research.* Edited by Brownell P, Polis G. Oxford: Oxford University Press; 448.
5. Spreitzer A, Melzer RR: **The nymphal eyes of *Parabuthus transvaalicus* Purcell, 1899 (Buthidae): An accessory lateral eye in a scorpion.** *Zool Anzeiger* 2003, **242:**137–143.
6. Fleissner G: **Neurobiology of a circadian clock in the visual system of scorpions**. In *Neurobiology of Arachnids.* Edited by Barth FG. Berlin, Heidelberg, New York: Springer Verlag; 1986:385.
7. Holmgren N: **Zur vergleichenden Anatomie des Gehirns von Polychaeten, Onychophoren, Arachniden, Crustaceen, Myriapoden und Insekten.** *Kungliga Svenska Vetenskapsakademien Handlingar* 1916, **56:**1–303.
8. Hanström B: *Vergleichende Anatomie des Nervensystems der wirbellosen Tiere: unter Berücksichtigung seiner Funktion.* Berlin: J. Springer; 1928.
9. Fleissner G: **Intracellular Recordings of Light Responses from Spiking and Nonspiking Cells in the Median and Lateral Eyes of the Scorpion.** *Naturwissenschaften* 1985, **72:**46–48.
10. Paul DH: **A neurophylogenist's view of decapod Crustacea.** *Bull Mar Sci* 1989, **45:**487–504.
11. Harzsch S: **Neurophylogeny: Architecture of the nervous system and a fresh view on arthropod phyologeny.** *Integr Comp Biol* 2006, **46:**162–194.
12. Strausfeld NJ: *Arthropod brains : evolution, functional elegance, and historical significance.* Cambridge: Mass.: Harvard University Press; 2012.
13. Chamberlain SC, Barlow RB: **Neuroanatomy of the visual afferents in the horseshoe crab (*Limulus polyphemus*).** *J Comp Neurol* 1980, **192:**387–400.
14. Calman BG, Lauerman MA, Andrews AW, Schmidt M, Battelle BA: **Central projections of *Limulus* photoreceptor cells revealed by a photoreceptor-specific monoclonal antibody.** *J Comp Neurol* 1991, **313:**553–562.
15. Harzsch S, Vilpoux K, Blackburn DC, Platchetzki D, Brown NL, Melzer RR, Kempler KE, Battelle BA: **Evolution of arthropod visual systems: development of the eyes and central visual pathways in the horseshoe crab *Limulus polyphemus* Linnaeus, 1758 (Chelicerata, Xiphosura).** *Dev Dyn* 2006, **235:**2641–2655.
16. Battelle BA: **The eyes of *Limulus polyphemus* (Xiphosura, Chelicerata) and their afferent and efferent projections.** *Arthropod Struct Dev* 2006, **35:**261–274.
17. Oberdorfer MD: **The Neural Organization of the First Optic Ganglion of the Principal Eyes of Jumping Spiders (Salticidae).** *J Comp Neurol* 1977, **174:**95–117.
18. Strausfeld NJ, Weltzien P, Barth FG: **Two visual systems in one brain: neuropils serving the principal eyes of the spider *Cupiennius salei*.** *J Comp Neurol* 1993, **328:**63–75.
19. Strausfeld NJ, Barth FG: **Two visual systems in one brain: neuropils serving the secondary eyes of the spider *Cupiennius salei*.** *J Comp Neurol* 1993, **328:**43–62.
20. Babu KS, Barth FG: **Neuroanatomy of the central nervous system of the wandering spider, *Cupiennius salei* (Arachnida, Araneida).** *Zoomorphology* 1984, **104:**344–359.
21. Lehmann T, Hess M, Melzer RR: **Wiring a Periscope - Ocelli, Retinula Axons, Visual Neuropils and the Ancestrality of Sea Spiders.** *PLoS One* 2012, **7**(1):30474. doi:10.1371/journal.pone.0030474.
22. Mayer G: **Structure and development of onychophoran eyes: what is the ancestral visual organ in arthropods?** *Arthropod Struct Dev* 2006, **35:**231–245.
23. Strausfeld NJ, Strausfeld CM, Stowe S, Rowell D, Loesel R: **The organization and evolutionary implications of neuropils and their neurons in the brain of the onychophoran *Euperipatoides rowelli*.** *Arthropod Struct Dev* 2006, **35:**169–196.

24. Weygoldt P, Paulus HF: **Untersuchungen zur Morphologie, Taxonomie und Phylogenie der Chelicerata. II. Cladogramme und die Entfaltung der Chelicerata.** *Zeitschrift Zool Syst Evol* 1979, **17:**177–200.
25. Shultz JW: **Evolutionary morphology and phylogeny of Arachnida.** *Cladistics* 1990, **6:**1–38.
26. Shultz JW: **A phylogenetic analysis of the arachnid orders based on morphological characters.** *Zool J Linn Soc* 2007, **150:**221–265.
27. Wheeler WC, Hayashi CY: **The phylogeny of the extant chelicerate orders.** *Cladistics* 1998, **14:**173–192.
28. Giribet G, Edgecombe GD, Wheeler WC, Babbitt C: **Phylogeny and systematic position of Opiliones: A combined analysis of chelicerate relationships using morphological and molecular data.** *Cladistics* 2002, **18:**5–70.
29. Masta SE, Longhorn SJ, Boore JL: **Arachnid relationships based on mitochondrial genomes: Asymmetric nucleotide and amino acid bias affects phylogenetic analyses.** *Mol Phylogenet Evol* 2009, **50:**117–128.
30. Regier JC, Shultz JW, Zwick A, Hussey A, Ball B, Wetzer R, Martin JW, Cunningham CW: **Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences.** *Nature* 2010, **463:**1079–1083.
31. Dunlop JA, Webster M: **Fossil evidence, terrestrialization and arachnid phylogeny.** *J Arachnol* 1999, **27:**86–93.
32. Braddy SJ, Aldridge RJ, Gabbott SE, Theron JN: **Lamellate book-gills in a late Ordovician eurypterid from the Soom Shale, South Africa: support for a eurypterid-scorpion clade.** *Lethaia* 1999, **32:**72–74.
33. Strausfeld NJ: **The evolution of crustacean and insect optic lobes and the origins of chiasmata.** *Arthropod Struct Dev* 2005, **34:**235–256.
34. Harzsch S: **The phylogenetic significance of crustacean optic neuropils and chiasmata: a re-examination.** *J Comp Neurol* 2002, **453:**10–21.
35. Heinrichs S, Fleissner G: **Neuronal components of the circadian clock in the scorpion, *Androctonus australis*: Central origin of the efferent neurosecretory elements projecting to the median eyes.** *Cell Tissue Res* 1987, **250:**277–285.
36. Homberg U: **Evolution of the central complex in the arthropod brain with respect to the visual system.** *Arthropod Struct Dev* 2008, **37:**347–362.
37. Westheide W, Rieger R: *Spezielle Zoologie: Teil 1: Einzeller und Wirbellose Tiere.* Heidelberg, Berlin: Spektrum Akademischer Verlag; 2006.
38. Meusemann K, von Reumont BM, Simon S, Roeding F, Strauss S, Kuck P, Ebersberger I, Walzl M, Pass G, Breuers S, *et al*: **A phylogenomic approach to resolve the arthropod tree of life.** *Mol Biol Evol* 2010, **27:**2451–2464.
39. Campbell LI, Rota-Stabelli O, Edgecombe GD, Marchioro T, Longhorn SJ, Telford MJ, Philippe H, Rebecchi L, Peterson KJ, Pisani D: **MicroRNAs and phylogenomics resolve the relationships of Tardigrada and suggest that velvet worms are the sister group of Arthropoda.** *Proc Natl Acad Sci USA* 2011, **108:**15920–15924.
40. Strausfeld NJ, Strausfeld CM, Loesel R, Rowell D, Stowe S: **Arthropod phylogeny: onychophoran brain organization suggests an archaic relationship with a chelicerate stem lineage.** *Proc R Soc B* 2006, **273:**1857–1866.
41. Schliwa M, Fleissner G: **Arhabdomeric Cells of the Median Eye Retina of Scorpions. I. Fine Structural Analysis.** *J Comp Physiol* 1979, **130:**265–270.
42. Altman JS, Tyrer NM: **Filling selected neurons with cobalt through cut nerves**. In *Neuroanatomical Techniques: Insect Nervous System.* Edited by Strausfeld NJ, Miller TA. New York: Springer; 1980:496.
43. Wohlfrom H, Melzer RR: **Development of the sensory system in larvae and pupae of *Chaoborus crystallinus* (DeGeer, 1776; Diptera, Chaoboridae): sensory cells, nerves and ganglia of the tail region.** *Dev Genes Evol* 2001, **211:**124–137.
44. Wigglesworth VB: **The use of osmium in the fixation and staining of tissues.** *Proc Roy Soc London Ser B* 1957, **147:**185–199.
45. Leise EM, Mulloney B: **The Osmium-Ethyl Gallate Procedure is Superior to Silver Impregnations for Mapping Neuronal Pathways.** *Brain Res* 1986, **367:**265–272.
46. Mizunami M, Iwasaki M, Nishikawa M, Okada R: **Modular structures in the mushroom body of the cockroach.** *Neurosci Lett* 1997, **229:**153–156.

doi:10.1186/1742-9994-10-40
Cite this article as: Lehmann and Melzer: Looking like *Limulus*? – Retinula axons and visual neuropils of the median and lateral eyes of scorpions. *Frontiers in Zoology* 2013 **10**:40.

5. Paper III (under review)

Lehmann T, Heß M, Wanner G & Melzer RR (2014). Dissecting an ancestral neuron network – FIB-SEM based 3D-reconstruction of the visual neuropils in the sea spider *Achelia langi* (Dohrn, 1881) (Pycnogonida). BMC Biology: under review.



Dissecting an ancestral neuron network: FIB-SEM-based 3D-reconstruction of the visual neuropils in the sea spider *Achelia langi* (Dohrn, 1881) (Pycnogonida)

Tobias Lehmann[1, 2*], Martin Heß[2, 4], Gerhard Wanner[3], and Roland R. Melzer[1, 2, 4]

Affiliations

[1]Bavarian State Collection of Zoology – SNSB, Münchhausenstraße 21, 81247 Munich, Germany

[2]Ludwig-Maximilians-Universität München, Department Biology II, Großhaderner Straße 2, 82152 Planegg-Martinsried, Germany

[3]Ludwig-Maximilians-Universität München, Department Biology I, Großhaderner Straße 2–4, 82152 Planegg-Martinsried, Germany

[4]GeoBio-Center[LMU], Richard-Wagner-Straße 10, 80333 Munich, Germany

[*]Corresponding author: Tobias Lehmann

Abstract

Background: The research field of connectomics arose just recently with the development of new 3D-EM techniques and increasing computing power. So far, only a few model species (e.g., mouse, the nematode *Caenorhabditis elegans*, and the fruit fly *Drosophila melanogaster*) have been studied using this approach. Here, we present a first attempt to expand this circle to include pycnogonids, which hold a key position for the understanding of arthropod evolution. The visual neuropils in *Achelia langi* are studied using a FIB-SEM crossbeam-workstation, and a 3D serial reconstruction of the connectome is presented.

Results: The two eyes of each hemisphere of the sea spider's eye tubercle are connected to a first and a second visual neuropil. The first visual neuropil is subdivided in two hemineuropils, each responsible for one eye and stratified into three layers. Six different neuron types postsynaptic to the retinula axons are characterized by their morphology: five types of descending unipolar neurons and one type of ascending neurons. These cell types are also identified by Golgi impregnations. Mapping of all identifiable chemical synapses indicates that the descending unipolar neurons are postsynaptic to the R-cells and hence are second-order neurons. The ascending neurons are predominantly presynaptic and sometimes postsynaptic to the R-cells and may play a feedback role.

Conclusions: Comparing these results with the compound eye visual system of crustaceans and insects – the only arthropod visual system studied so far in such detail – we found striking similarities in the morphology and synaptic organization of the different neuron types. Hence, the visual system of pycnogonids shows features of both median and lateral eyes, which supports the idea that the eyes of pycnogonids are highly ancestral.

Keywords

Median eyes, ocelli, lateral eyes, visual system, connectome, Arthropoda, Chelicerata, Pycnogonida

Background

One of the most intriguing questions in vision research is how the neuronal circuitry processes the visual input from the photoreceptors, i.e., the neuronal correlate of the eye and retina's visual architecture. Cell-type-specific wiring rules, the divergence and convergence of information channels and the maintenance of retinotopy are some of the core issues. Here, data acquisition entails the challenge of covering volumes of thousands of cubic micrometers (to enclose entire neurons) with a voxel-resolution of only a few nanometers (to correctly trace membrane profiles and to see synaptic structures). One promising approach is (three-dimensional) reconstruction from serial section TEM, which is nowadays a well-established way of analyzing circuitry of neural networks [1], [2], [3]. However, several hundreds of sections or even more have to be cut without any loss of sections, inspected and photographed with the TEM, resulting in an enormous data volume, which is followed by a complex elastic alignment to compensate inevitable image distortions using an elastic alignment program (e.g., TrakEM2 [4], [5]). Hence, the main criterion in selecting a suitable subject for such a study is a small size. In analyzing nervous systems regarding connectomics, either small animals with a small CNS or a restricted region within the CNS or even within a particular neuropil are possible study subjects to obtain a comprehensive data stack.

Early serial section EM research dealing with arthropod visual systems was performed by Macagno et al. [6] in analyzing the visual system in *Daphnia magna* and later by Meinertzhagen and O'Neil [7] in reconstructing synaptic connections in the lamina cartridges of *Drosophila*. A classic example for the reconstruction of a whole nervous system is the nematode *Caenorhabditis elegans* [8], [9]. An early attempt to use computerized 3D reconstructions to study the axonal wiring of photoreceptor axons is that by Melzer et al. [10] in midges and the scorpion fly. These studies did not have today's computing power at their disposal. In the last few years, personal computers have become capable of handling the enormous data volumes inevitable for 3D reconstructions from serial section TEM. Previous studies using this power have focused on the lamina and medulla in the fruit fly *Drosophila melanogaster* [11], [12], [13].

Furthermore, in recent years, a new generation of 3D-EM tools has been developed [14], [15], [16], which includes Serial Block Face Scanning Electron Microscopy (SBF-SEM or simply SBEM) based either on mechanical sectioning [17], [18] or milling with a focused ion beam

(FIB-SEM, [19], [20]). These methods enhanced the potential of 3D-EM considerably and are applied e.g., on nervous tissue [21], [22], [23] and to display and count synapses in vertebrates [24], [25], [26].

In the present study, we analyze the visual neuropils in the pycnogonid *Achelia langi* with one of these methods, namely FIB-SEM. The advantages of this cutting-edge method are that compared to serial section TEM, the generation of the image-stack is much faster and without loss, the images are perfectly aligned with a z-resolution down to 5 nm (TEM approx. 70 nm), and the x-y-resolution and contrast compared to TEM are only slightly reduced.

The Pycnogonida, or sea spiders, are exclusively marine invertebrates, numbering more than 1300 species worldwide [27]. Although largely unnoticed due to their cryptic life habits and economic insignificance, sea spiders are common benthic animals occurring from the littoral zone to the deep sea, from tropical to polar waters. The phylogenetic position of the Pycnogonida has long been controversial and is still under debate. Today, pycnogonids are placed either within the Chelicerata as sister taxon of the Euchelicerata or as sister taxon of all other Euarthropoda [28], [29]. The fossil record indicates that pycnogonids are indeed a highly ancestral group, with the earliest unequivocal records dating back to the Ordovician and Silur [30], [31]. It has even been hypothesized that Pycnogonida might date back to the Cambrian, i.e., the time of the great appendage arthropods [32]. Studies of the cephalon of pycnogonids [33] and of its appendages, e.g., the cheliphores [34], [35], [36] have shown that the innervation pattern of the protocerebrum contributes important sets of characters to the discussion about the phylogenetic position of sea spiders. For this field of research, comparing the structure and development of nervous systems in a phylogenetic context, two different approaches were established: “neurophylogeny” [37], [38] and “neural cladistics” [39].

The sensory parts of the arthropod protocerebrum are primarily responsible for the visual system. Two different types of eyes are found in arthropods, median and lateral eyes. Pycnogonids possess only a periscope-like ocular tubercle with four ocelli generally interpreted as median eyes, whereas classical lateral eyes are absent. Studies using light [40], [41] and electron microscopy [42], [43] have revealed that these eyes are pigment cup ocelli with a cuticular lens and a latticed rhabdom surrounded by pigment layers, features typical of median eyes. Derived conditions might include the structure of the retinula or R-

cells, described as ‘‘pseudoinverted’’ [43], and the presence of a tapetum lucidum (guanine multilayer reflector). The connection of these R-cells to the brain was lately analyzed with classical and modern neuroanatomical techniques to identify the visual neuropils [44]. Hence, the pycnogonid visual system is composed of a thickening dorsolateral to the protocerebrum where the nerve fibers from the two eyes of one hemisphere concentrate, a bifurcated visual tract, and two successive distinct visual neuropils. This innervation pattern is very similar to that in ancestral euarthropods such as the eyes in *Euperipatoides rowelli* (Onychophora) [45] and the median rudimentary eye in *Limulus polyphemus* (Xiphosura) [46], [47].

The architecture of the visual system of sea spiders is relatively simple compared to that of many other arthropods. Considering the phylogenetic position of pycnogonids as an early offspring of the arthropod tree suggested by both tree reconstruction and the fossil record, one can conclude that the selection of Pycnogonida allows us to understand a visual system in a detailed way due to its simplicity and to learn about early eye evolution in arthropods due to its ancestrality.

In the present study, we take a closer look at the visual neuropils in the pyconogonid *Achelia langi* (Ammotheidae) using the advantages of FIB-SEM. In a low-resolution stack, the arrangement of the visual nerve fibers and neuropils is analyzed. In a second, medium-resolution stack, neurons postsynaptic to the R-cells are 3D reconstructed to gain a more detailed view of the neuroanatomy of the pycnogonid visual system. To utilize two strains of evidence, the morphology of these cells is additionally compared to Golgi-impregnated profiles in *Achelia vulgaris*. Finally, in a third high-resolution stack, the distribution of synapses within these cells is analyzed. These findings reveal features of the visual system generally studied in Arthropoda to allow comparisons with other lineages.

Results

General layout of the visual neuropils in the protocerebrum

In the examined area of the low-resolution FIB stack, the visual tract bifurcates. After entering the protocerebrum, one part of the fibers projects to the first visual neuropil located dorsolaterally in the anterior part of the protocerebrum as an ovoid region laterally embedded in the cell body rind of the brain (Figs. 1, 2). The other part of the fibers projects

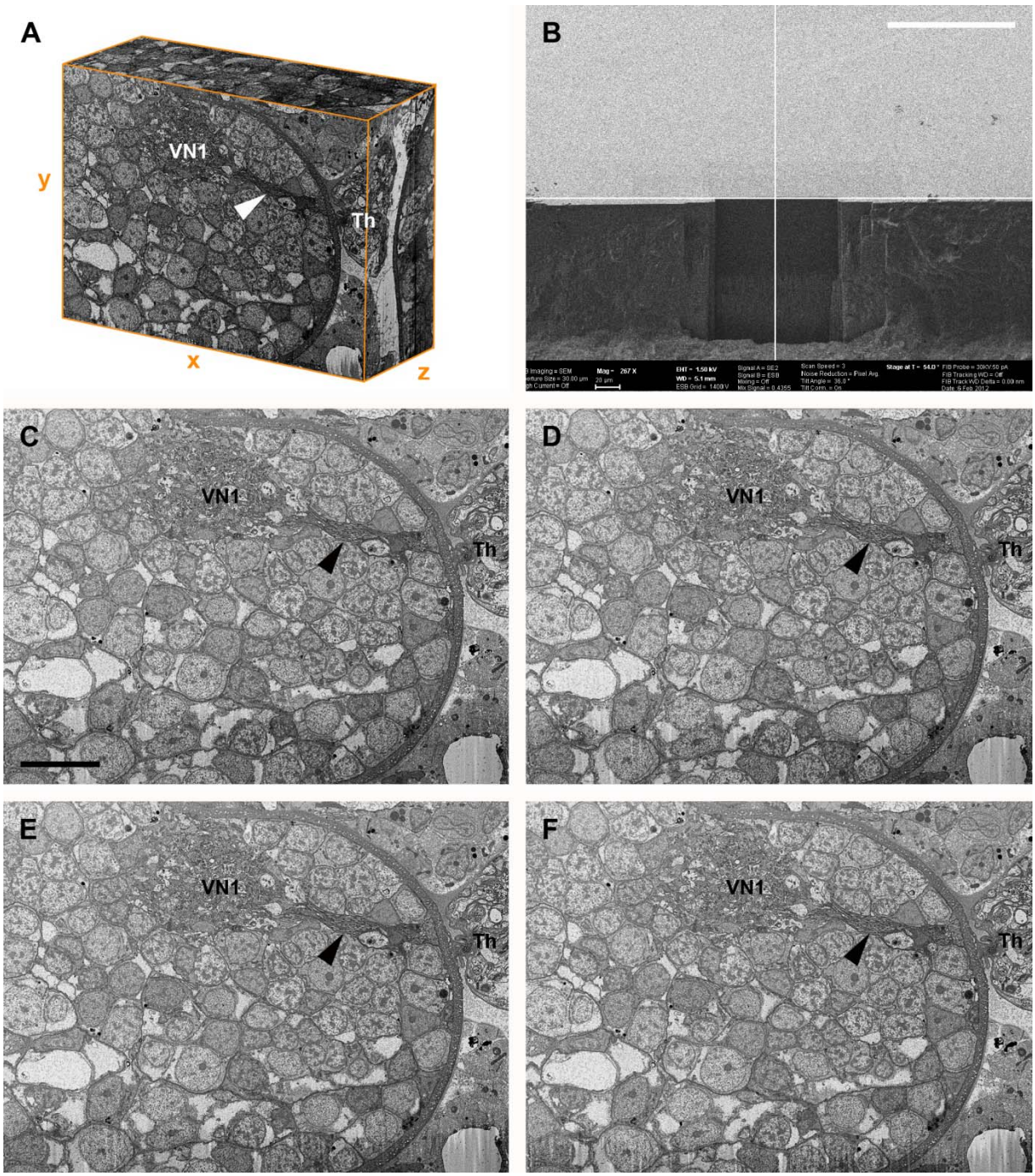


Figure 1: Pycnogonid visual neuropils studied with focused ion beam SEM technique.
A, 3D volume of low-resolution image stack; note sharp xz- and yz-projections due to almost perfect alignment of FIB-SEM. **B**, backscattered electron image of mesa at beginning of milling by FIB-SEM. Bar 100 µm. **C–F**, short consecutive image series at beginning of stack; note minor but visible structural change from slice no. 80 (C) to slice no. 83 (F). Bar 10 µm.
Arrowhead, visual tract projecting through cell body rind; Th, thickening; VN1, visual neuropil 1.

to the second visual neuropil. These fibers likewise bifurcate and enter the second visual neuropil in two portions. This neuropil is located deeper, under the cell body rind and in a more anterior and central position in the protocerebrum (Fig. 2). Both neuropils are in contact with the rest of the neuropils of the protocerebrum. The posterior part of the first

visual neuropil is ventrally connected to the neuropil of the protocerebrum. The second visual neuropil, in turn, is posteriorly not clearly separated from the remaining neuropils.

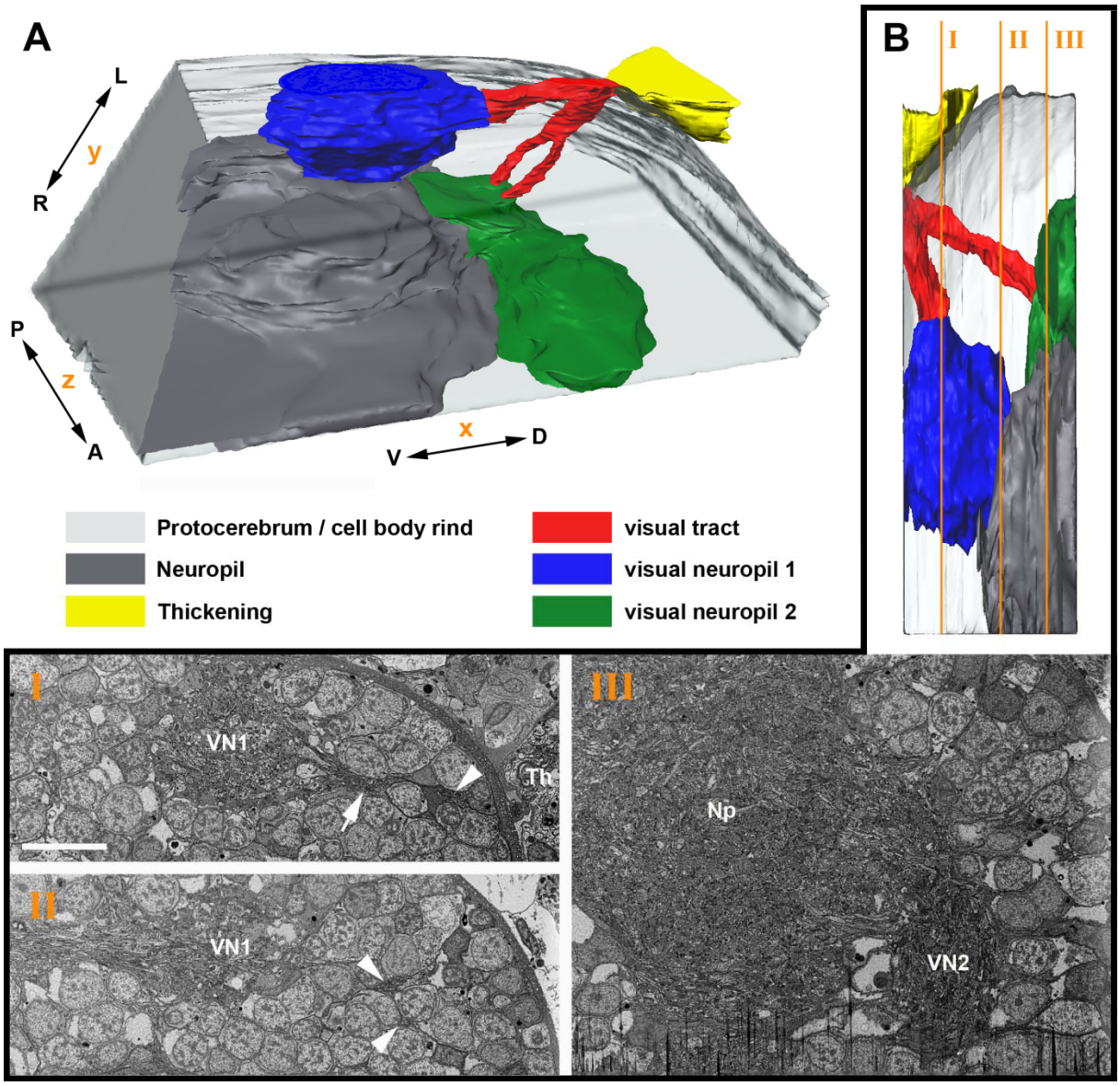


Figure 2: 3D serial reconstruction of visual neuropils of left hemisphere in *A. langi* on basis of low-resolution image stack.

A, 3D reconstruction showing the arrangement and orientation of neuropils; posterior is up, dorsal is right. **B**, three selected sections showing original data for reconstruction; position of sections indicated in 3D reconstruction top right. **I**, medium range of visual neuropil 1 (slice no. 125); note two subsets of visual tract projecting through cell body rind, arrow indicating subset projecting to visual neuropil 1, arrowhead indicating subset projecting to visual neuropil 2. **II**, low range of visual neuropil 1 (slice no. 376); note two subsets of visual tract projecting through cell body rind to visual neuropil 2 (arrowheads). **III**, beginning of visual neuropil 2 (slice no. 640). Bar 10 µm.

A, anterior; D, Dorsal; L, left; Np, neuropil; P, posterior; R, right; Th, thickening; V, ventral; VN1, visual neuropil 1; VN2, visual neuropil 2.

Cell types in the first visual neuropil

In the FIB-SEM (medium-resolution stack) based examination of *A. langi,* a division of the first visual neuropil into two equal subunits or hemineuropils (see also below) was observed (Figs. 3–6). This division appears in the distal third of the neuropil and is apparent throughout the rest of the neuropil. In the FIB-SEM images, the two hemineuropils are characterized by neurites, mostly of small diameters, and are divided primarily by bulky neurites with larger diameters (Figs. 4B, C).

Furthermore, six different types of neurons were reconstructed and classified on the basis of their morphology: five descending cell types (Figs. 3A–E) and one ascending cell type (Fig. 3F). All of these neurons can also be identified by Golgi impregnations (Fig. 3 rightmost). The descending cells are unipolar neurons with cell bodies in the cell body rind above the neuropil, which send a single neurite each into the first visual neuropil. (To keep the Results section free from homology assumptions, the term 'monopolar cells' is intentionally avoided because this term is occupied by the monopolar cells in the compound eye visual system in Pancrustacea; see discussion). Most of the descending neurons can be traced from the cell body all the way through the neuropil to the end of the image stack. Neurons reconstructed without cell bodies can be allocated to their particular cell type on the basis of the morphology of the neurites. A classification of the ascending neurons cannot be made because the cell bodies of these cells are beyond the examined area. However, the cell bodies must be located below the neuropil, whereas the neurites end before the top end of the neuropil. A large section of the ascending cells and all of the descending cells with cell bodies within the examined volume above the neuropil are reconstructed, and some cells are allocated due to their neurite morphology. Individual retinula axons (cells with a high electron density), due to the low contrast of these cells in the FIB-SEM images, and synapses, due to the too-low resolution, cannot be reliably traced in the medium-resolution stack. In the stack having the highest resolution, however, the R-cells and synapses are reconstructed (see below and Fig. 7). The total volume of interest (i.e., neuropil and cell bodies in the examined area) is approximately 4800 μm^3, and the volume of all reconstructed cells is 567 μm^3; hence, the reconstructed cells occupy approximately 12% of the volume.

Descending unipolar neurons (D1–D5)

D1 (Figs. 3A, 4; n=6). The cell bodies in two of the six cells could be reconstructed; the remaining cells were allocated due to their neurite morphology. Cell bodies are found in the

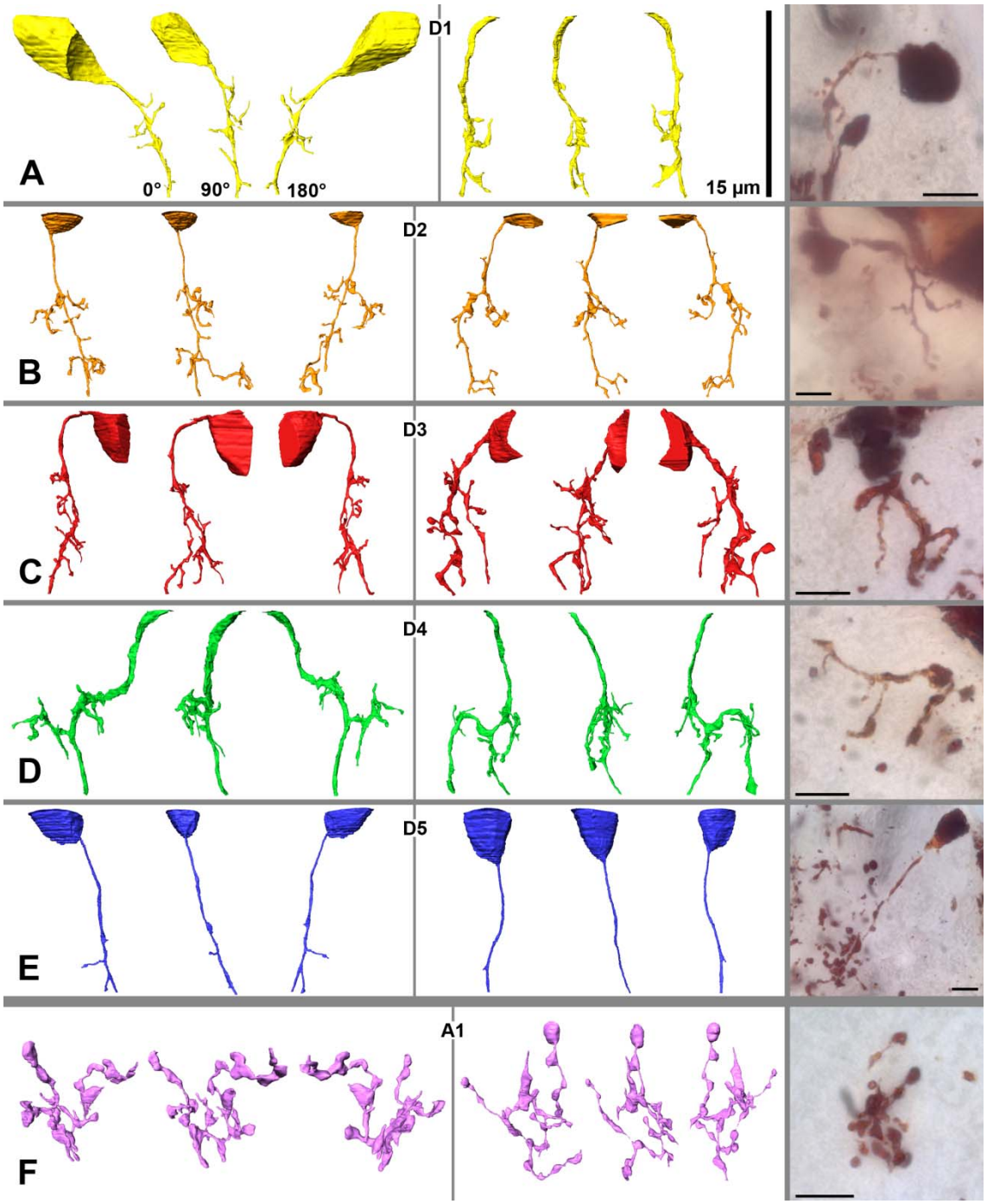


Figure 3: Profiles of six different cell types found in 3D serial reconstruction of visual neuropil 1 of right hemisphere in *A. langi* on basis of medium-resolution image stack and in Golgi-preparations of *A. vulgaris*.

Two representatives of each cell type are shown at three different angles; note additional corresponding profiles of Golgi-impregnated cells on right-hand side. **A,** Descending unipolar neuron 1 (D1), characterized by unbranched neurite with several collaterals. **B,** Descending unipolar neuron 2 (D2), characterized by branched neurite with several collaterals. **C,** Descending unipolar neuron 3 (D3), characterized by bifurcation of neurite with several collaterals. **D,** Descending unipolar neuron 4 (D4), characterized by h-shaped neurite with each branch reaching into one hemisphere; with several collaterals as well. **E,** Descending unipolar neuron 5 (D5), characterized by unbranched neurite without or with just few collaterals. **F,** Ascending unipolar neuron 1 (A1), characterized by neurite with multiple branches, each with several large boutons and thin connectors in between. Each cell spreads throughout wide reaches of both hemineuropils.

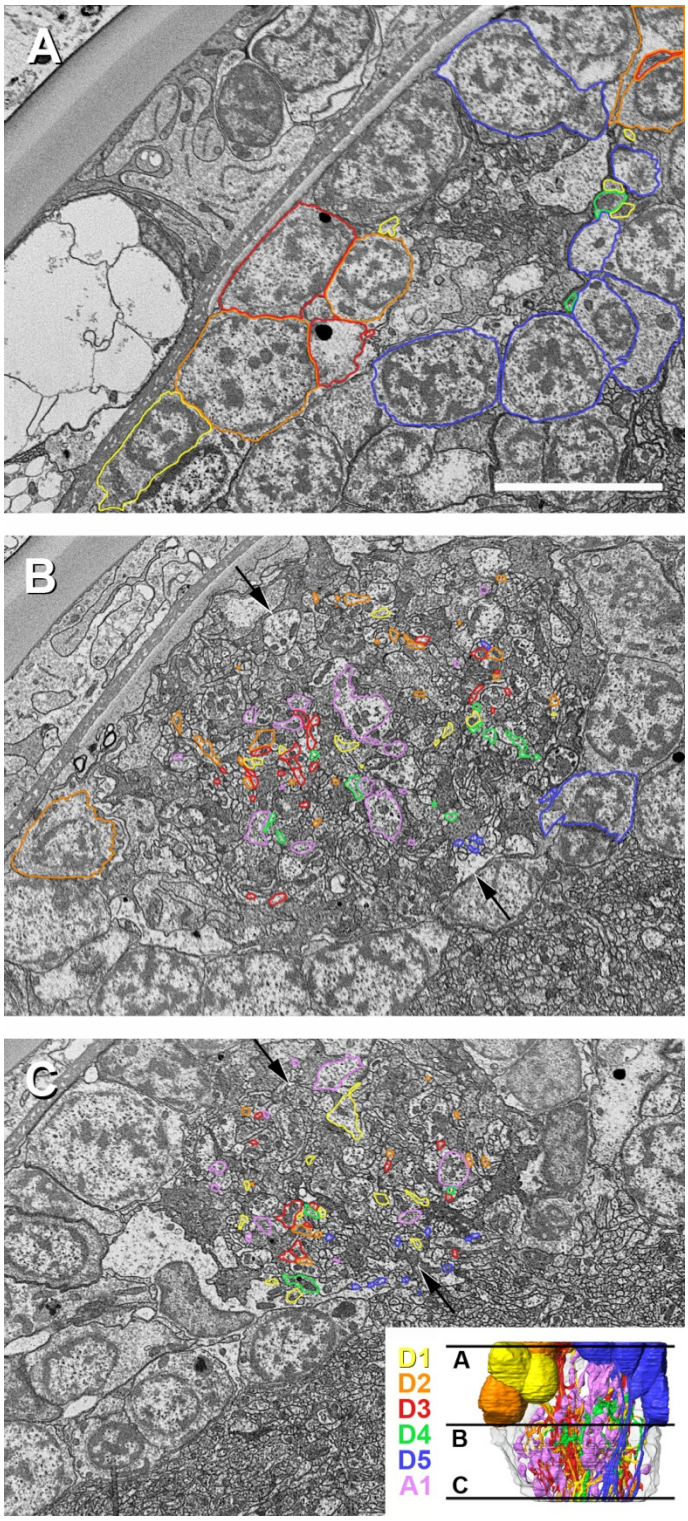


Figure 4: Three selected sections with labeling of different cell types showing original data for reconstruction.
Position of sections indicated in 3D reconstruction bottom right; note cells with high electron density identified as retinula axon terminals surrounded by cells with low electron density identified as postsynaptic neurons. **A,** beginning of visual neuropil 1 (slice no. 23); neuropil surrounded by cell bodies of descending unipolar neurons. Bar 5 µm. **B,** medium range of visual neuropil 1 (slice no. 523); arrows indicate subdivision of neuropil into two hemineuropils. **C**, low range of visual neuropil 1 (slice no. 1017); arrows indicate subdivision of neuropil.

cell body rind above or lateral to the upper third of the neuropil. The neurites are unbranched and slightly curved. All cells can be traced to the end of the image stack. Short collaterals occur in tangential and radial directions throughout the neurite but are accumulated in the medium range of the neuropil. Each cell profile covers only a small area of the neuropil. D1 neurons can be found throughout the neuropil, whereas a single neuron is restricted to only one hemineuropil.

D2 (Figs. 3B, 4; n=5). The cell bodies could be at least partially reconstructed in all cells. They are found in the cell body rind above or lateral to the upper third of the neuropil. The neurites are branched and slightly curved. The branching always occurs in the medium range of the neuropil, and the neurite is divided into a short and a long branch. The long branch of all cells can be traced to the end of the image stack; the short branch ends in the medium range of the neuropil and is radially oriented. Short collaterals occur in tangential and radial directions throughout both branches of the neurite. Each cell profile covers a larger area of the neuropil compared to the D1 cells. D2 neurons can be found throughout the neuropil, whereas a single neuron is restricted to only one hemineuropil.

D3 (Figs. 3C, 4; n=6). The cell bodies could be at least partially reconstructed in four cells; the remaining cells were allocated due to neurite morphology. The cell bodies are found in the cell body rind above or lateral to the upper third of the neuropil. The neurites are bifurcated. The bifurcation always occurs in the medium range of the neuropil. Both branches can be traced to the end of the image stack. Short collaterals occur in tangential and radial directions throughout both branches of the neurite. Similarly to the D2 cells, each cell profile covers a larger area of the neuropil compared to the D1 cells. D3 neurons can be found throughout the neuropil, whereas a single neuron is restricted to only one hemineuropil.

D4 (Figs. 3D, 4; n=2). One cell could be reconstructed with only a small portion of the cell body; the other cell was allocated due to the neurite morphology. The cell bodies are found in the cell body rind above the neuropil. The neurites are h-shaped. In the medium range of the neuropil, the neurite is radially oriented and builds two tangential branches, each reaching into one hemineuropil. Short collaterals occur in tangential and radial directions throughout the neurite. All cells can be traced to the end of the image stack. A single D4 neuron occurs in both hemineuropils at once. The cell profiles cover, compared to the other D cells, the largest area of the neuropil because they occur in both hemineuropils.

D5 (Figs. 3E, 4; n=9). The cell bodies could be reconstructed at least partially in all cells. They are found in the cell body rind above or lateral to the upper third of the neuropil. The neurites are unbranched, straight or only slightly curved. Six neurons are without any collaterals and three neurons with just one or two short tangential collaterals. All cells can be traced to the end of the image stack. D5 neurons can be found in the right hemineuropil only. These neurons cross the right hemineuropil at its edge, and in the lower part of the neuropil they can be found in the area that divides the two hemineuropils.

Ascending neurons (A1)

A1 (Figs. 3F, 4; n=6). Cell bodies were not found in the examined area. All reconstructed cells end in the upper third of the neuropil; hence, the neurites could not be traced from the most proximal slice throughout the neuropil to the distal end. The cell bodies of these neurons must therefore be located below the neuropil, meaning that these cells are ascending neurons. The neurites are equipped with multiple branches, each with several large boutons or varicosities and thin connectors in between. These cells have a high-turgor appearance; this means that the boutons have rounded contours. A1 neurons can be found throughout the neuropil; however, branches of A1 neurons accumulate in the area that divides the two hemineuropils. A single neuron occurs in both hemineuropils at once. Each cell profile covers a large area of the neuropil.

Organization of the first visual neuropil

When all neuron types (D1–5, A1) are shown together, no special organization of the neuropil is identifiable (Figs. 5A; 6A). However, by removing the A1 neurons from the 3D reconstruction, a subdivision of the visual neuropil becomes apparent (Figs. 5B; 6B), which is also observed in the FIB-SEM images (see above and Figs. 4B, C). The neuropil is divided into two hemineuropils of equal size. Between the hemineuropils, a border zone exists where less of the D-cells occur. While the D1–4 cells are evenly distributed in both hemineuropils, the D5 cells occur only in the right hemineuropil (Figs. 5B; 6B). When the D5 cells are removed from the reconstruction (Figs. 5C, D; 6C, D) the subdivision becomes more obvious; moreover, a feature of the D4 cell becomes visible: these neurons connect the two hemineuropils. Whereas just a few collaterals of the D1–3 cells reach into the border zone, branches of the D4 cells run through this border and connect both hemineuropils.

When each cell type is shown on its own, their characteristic features become visible (Figs. 5E–J; 6E–J). The D1–3 cells form the main body (apart from the A1 cells) of the visual neuropil (Figs. 5D–G; 6D–G); these cells form the two hemineuropils. Just a few collaterals–but not the main branches–of the D1–3 cells of the two hemineuropil reach into the border zone in between. In contrast to the D1–3 cells, the main branches of the D4 cells cross the border zone and occur in both hemineuropils at once (Figs. 5H; 6H). The D5 cells take a special position; these cells were found in the examined area only in the right hemineuropil (Figs. 5I; 6I). The neurites of the D5 cells run along the posterior edge of this hemineuropil,

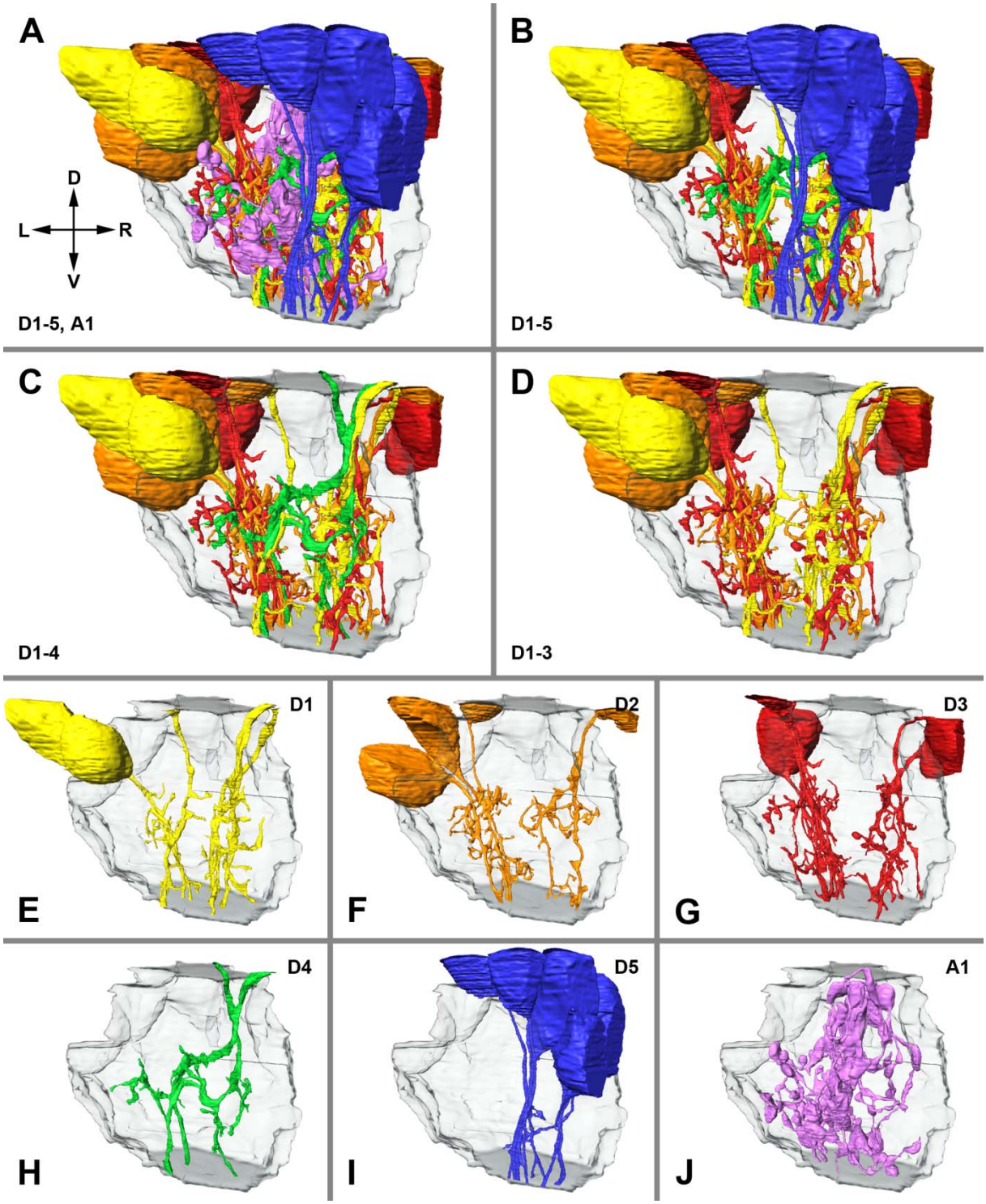


Figure 5: Lateral view of 3D reconstruction of visual neuropil 1.
A, all reconstructed cells of all six neuron types shown; dorsal is up. **B**, A1 neurons omitted thus subdivision of neuropil gets visible; note D5 neurons mainly in right hemineuropil. **C**, A1 and D5 neurons omitted; note D4 neurons occur in both hemineuropils at once. **D**, A1, D4, and D5 neurons omitted; note D1–3 neurons build two hemineuropils. **E–J**, distribution of different cell types separately within neuropil.
D, dorsal; L, left; R, right; V, ventral.

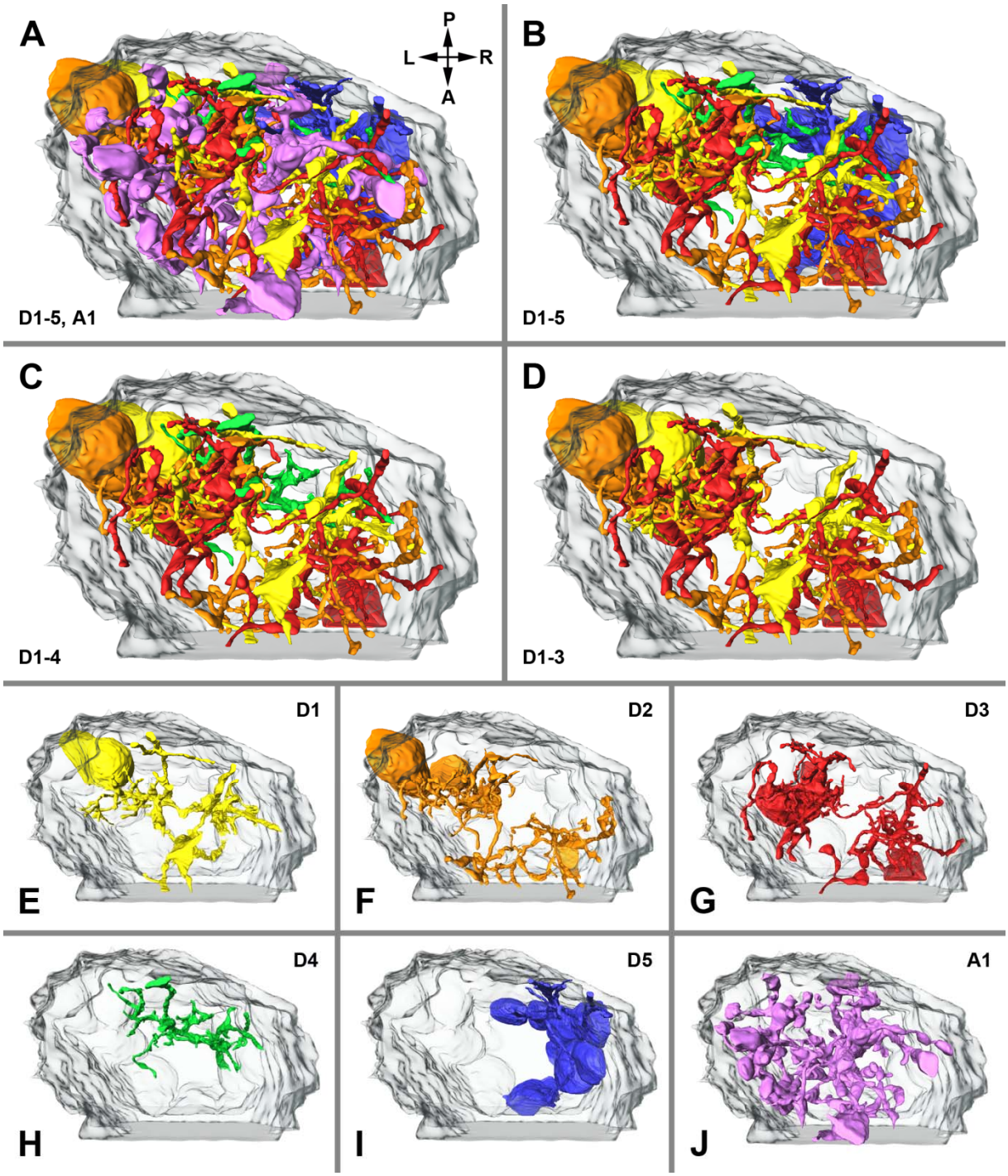


Figure 6: 3D reconstruction of visual neuropil 1 viewed from bottom up.
A, all reconstructed cells of all six neuron types shown; posterior is up. **B**, A1 neurons omitted thus subdivision of neuropil gets visible; note D5 neurons mainly in right hemineuropil. **C**, A1 and D5 neurons omitted; note D4 neurons occur in both hemineuropils at once. **D**, A1, D4, and D5 neurons omitted; note D1–3 neurons build two hemineuropils. **E–J**, distribution of different cell types within separately neuropil.
A, anterior; L, left; P, posterior; R, right.

and in the lower part they are found primarily in the border zone between the hemineuropils.

The A1 cells can be distinguished from the D1–5 cells in morphology and distribution. These cells do not form two hemineuropils; rather, the neurites of these cells are distributed throughout the neuropil and are accumulated in the border zone of the two hemineuropils.

Synaptic organization of the first visual neuropil

In the stack with the highest resolution, R-cells as well as synapses can be reconstructed in addition to descending and ascending neurons (Fig. 7). The stack is located in the medium range of the neuropil. Cells of one hemineuropil were reconstructed in which three different cell types are allocated on the basis of their neurite morphology: R-cells, D-cells, and A-cells. Ultrastructurally, chemical synapses can be recognized by a presynaptic concentration of electron-dense vesicles and electron-dense material in the synaptic cleft accompanied by high membrane density (Figs. 7F, G). However, postsynaptically, no special synaptic structures are found. In the investigated volume, no sign of electric synapses (e.g., gap junctions) could be detected. Altogether, 95 chemical synapses are identified in the studied volume. These are often multiple-contact synapses (dyads, triads, tetrads, etc.). Altogether, approximately 13% of the cells in the hemineuropil are reconstructed (approximately 260 cells counted in the field of interest in the first slice, 33 cells reconstructed). The total volume of interest (area of the examined hemineuropil) is approximately 260 μm^3 and the volume of all cells reconstructed is 34 μm^3; hence, these cells occupy 13% of the volume.

R-cells (Fig. 7C; n= 18): This cell type could not be reconstructed in the medium-resolution stack but could in the high-resolution stack. In the FIB-SEM images, these cells are characterized by high electron density. The morphology of R-cells is similar to that of A-cells: the neurites have multiple branches, each with several large boutons or varicosities and thin connectors in between, with the difference that the R-cells have a low-turgor appearance. This means that the shape of these cells adapts to the shape of the surrounding cells and the boutons have limp contours. Within these cells, an average of 3.3 synapses per cell was found in the reconstructed area; these occur primarily in the boutons. R-cells are predominantly presynaptic to D-cells and sometimes to A-cells. Furthermore, R-cells are frequently postsynaptic to A-cells (Tab. 1). One individual R-cell is presynaptic to several D-cells.

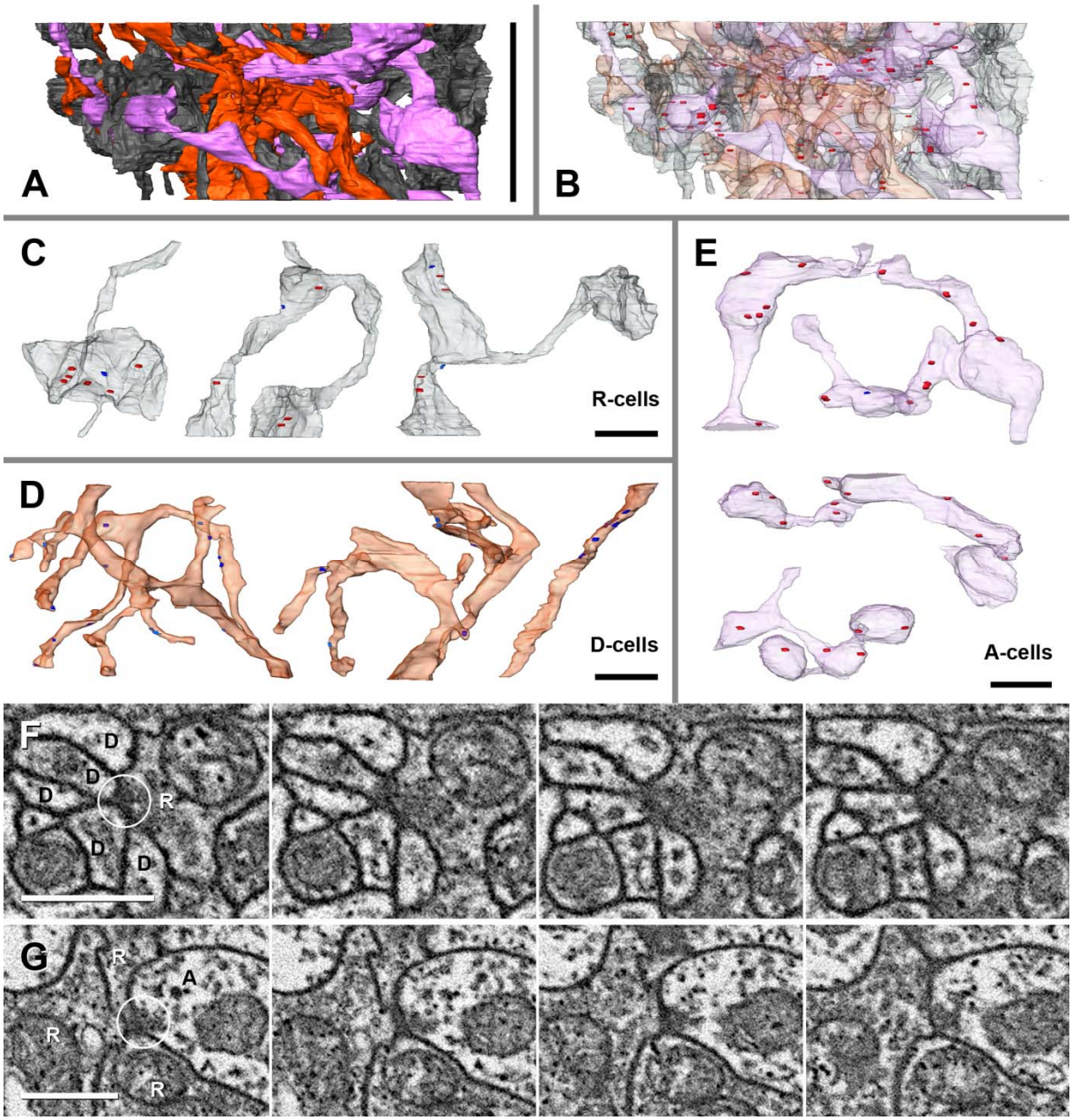


Figure 7: 3D serial reconstruction of medium range of visual neuropil 1 of right hemisphere in *A. langi* based on high resolution image stack.

A, all reconstructed cells of all three neuron types (R-, D-, and A-cells) shown. Bar 3.2 µm (i.e., z-range of the stack). **B**, all reconstructed cells of all three neuron types (R-, D-, and A-cells) shown in transparent and all chemical synapses (presynaptic vesicle clusters) found within these cells indicated in red. **C**, Profiles of three different R-cells; presynaptic sites indicated in red, postsynaptic sites indicated in blue. Bar 1 µm. **D**, Profiles of three different D-cells; postsynaptic sites indicated in blue, no presynaptic sites in these cells. Bar 1 µm. **E**, Profiles of three different A-cells; presynaptic sites indicated in red, postsynaptic sites indicated in blue. Bar 1 µm. **F**, Series of four consecutive FIB-SEM images showing a synapse (encircled) between R- and D-cells (slice no. 26–29): about five D-cells (cells with low electron density) postsynaptic to one R-cell (cell with high electron density). Bar 500 nm. **G**, Series of four consecutive FIB-SEM images showing a synapse (encircled) between A- and R-cells (slice no. 27–30): three R-cells (cells with high electron density) postsynaptic to one A-cell (cell with low electron density). Bar 500 nm.

D-cells (Fig. 7D; n=7): These cells were allocated, due to their neurite morphology, to the D-cells of the medium-resolution stack (Fig. 3). A subdivision into the five different D-cell types cannot be made because only a small portion of the cells on the z-axis were reconstructed. Within these cells, just a few areas with increased vesicle density and other indicators of presynaptic activity were found in the reconstructed area; most cells are without such presynaptic sites. D-cells are predominantly postsynaptic to R-cells and sometimes to A-cells (Tab. 1). One individual D-cell is postsynaptic to several R-cells.

A-cells (Fig. 7E; n=8): These cells were allocated, due to their neurite morphology (high-turgor appearance, boutons with connectors), to the A-cells of the medium-resolution stack (Fig. 3). Within these cells, an average of 8.1 synapses per cell is found in the reconstructed area; these are found primarily in the boutons. A-cells are predominantly presynaptic to R-cells and sometimes to D-cells. Furthermore, A-cells are sometimes postsynaptic to R-cells (Tab. 1).

Table 1: Synaptic pattern of the different cell types in the high-resolution stack.

Presynaptic cells			→ presynaptic to ↓	
R-cells (n=18)	D-Cells (n=7)	A-cells (n=8)		
0	1	43	R-cells	Postsynaptic cells
32	1	8	D-cells	
5	1	0	A-cells	
22	3	14	cells not reconstructed	
3,3	0,9	8,1	synapses/cell (average)*	

* in the reconstructed volume

Discussion

The term ‘connectome’ refers to the mapping of all neural connections within an organism's nervous system or a confined part of it. These "wiring diagrams" can be defined at different levels of scale, corresponding to levels of interest or the spatial resolution of imaging, for example, the microscale, mesoscale and macroscale [48]. A connectome at the macroscale (light microscope level) attempts to resolve different brain regions or neuropils and the pathways in between; these brain maps were established over the last hundred years for

various species. These days with the help of various new techniques and increased computing power, the meso- and microscale (electron microscope) levels come into focus. At the mesoscale level, the morphology of distinct populations of neurons within a processing unit (e.g., a column or a neuropil) are mapped. This level of analysis can be complemented by the microscale level, which involves mapping single neurons and their connectivity patterns (synapses), which according to Sporns et al. [48] will remain infeasible for an entire brain, at least for the near future. Recently, two ambitious scientific research projects, the Human Brain Project (by the European Union) [49], [50] and the BRAIN Initiative (by the United States) [51], [52], were launched to map these connection patterns in the human brain.

At the meso- and microscale levels, the basic architecture of sensory neuropils in both vertebrates (e.g., the visual cortex in the human brain [53]) and invertebrates (e.g., the optic lobes of the compound eyes in insects and crustaceans [54], [39]) is characterized by columns and layers. The vertical columns, for example, in the insect lamina and medulla [11], [12] are composed of repetitive subsets of afferent fibers (e.g., those of the retinula cells) and characteristic postsynaptic neurons (e.g., monopolar cells) that form the basic functional unit of a system (e.g., visual system). Often, these columns are horizontally layered (e.g., strata M1–6 in the medulla).

In the present study, we analyzed the pycnogonid visual neuropil at macro-, meso- and microscale levels to examine the principles that underlie this (simple) visual system and whether they compare to more complicated ones.

In the low-resolution stack, the macroscale observations of Lehmann et al. [44] can be confirmed. After entering the brain, the fiber bundle with the R-cell axons is split; one part of the axons ends in the first visual neuropil, and the other part passes the first visual neuropil and terminates in the second.

At the mesoscale level, aside from the R-cells, six different cell types can be distinguished in the first visual neuropil: five descending and one ascending cell type. The neuron gestalten are identified with two different approaches, providing support that both our 3D-reconstruction and the Golgi-profiles give correct pictures of the neurons.

Three types of descending cells (D1–3) are responsible for the subdivision of the first visual neuropil into two hemineuropils; these cells do not cross the border in between. In contrast, D4 neurons occur in both hemineuropils at once and provide lateral interactions between

the two hemineuropils. The interpretation of the D5 cells is difficult. Here, these cells are found only in the right hemineuropil, which is most likely a sampling artifact, and the D5-cell bodies of the left hemineuropil are beyond the examined volume and hence are not reconstructed. At the microscale level, the D-cells are frequently postsynaptic to the R-cell axons and hence are second-order neurons. One individual R-cell is presynaptic to several D-cells and one individual D-cell is postsynaptic to several R-cells, indicating divergence and convergence. Concerning the synaptic pattern, no reliable separation between the five different D-cells could be made in the high-resolution stack. However, the reconstructed cells vary in the tangential size of the field they cover in a way that is analogous to their appearance in the medium-resolution stack, indicating that the synaptic pattern is similar in all descending cells.

The ascending neurons are higher-order neurons of a wider field throughout both hemineuropils. These cells are commonly presynaptic and sometimes postsynaptic to R-cells and hence play a feedback role in the system.

Furthermore, at the mesoscale level, it is observed that the first visual neuropil is split into two hemineuropils or columns. This is visible in both the SEM images and the 3D reconstructions. The most plausible explanation of this subdivision is that one hemineuropil is linked to the anterior and the other to the posterior eye of the ocular tubercle. Additionally, in the two hemineuropils, at least three different layers of similar thicknesses are observable. In the upper third of the neuropil, the neurites of the unipolar cells enter the neuropil. Here, just few collaterals were found. In the medium range of the neuropil, a number of things happen: most of the collaterals of the unipolar cells are found here, the branching and bifurcation of the D2 and D3 neurons occurs in this region, and finally the D4 neurons build here their tangential branches that reach into the two hemineuropils. Furthermore, in the medium range of the neuropil, which is analyzed at the microscale level in the high-resolution stack, additionally various synapses occur (whether and where synapses occur in the upper and lower ranges of the neuropil remains unclear at present because these regions were not studied at higher resolution). In the lower third of the neuropil, no more branching or bifurcation occurs, but numerous collaterals are found.

This analysis reveals that the R-cells provide the input into the system, primarily on the D-cells. Because the D-cells rarely appear to be presynaptic in the first visual neuropil, these cells most likely synapse and hence integrate information to higher visual centers that were

not identified in this study. These centers could be the second visual neuropil or the arcuate body, which in chelicerates is closely associated with the visual system [55]. The A-cells play a special role in this system, being pre- and postsynaptic to both R- and D-cells. Hence, these cells collect information from the input (R-cells) and the second-order cells (D-cells) but also circulate information back to these cells. Mechanisms such as lateral inhibition, contrast enhancement, and other filter functions could be behind this feedback loop. Furthermore principles of divergence in the R-cells and convergence in the D-cells are found.

A comparison of our findings with that in other arthropods proves to be difficult, as representatives of only a few taxa have been studied in sufficient detail to allow comparison of neuron morphology. Especially for median eye visual systems, just a few Golgi studies are available.

Hanström [56] reported for *Limulus* that neurites with cell bodies around the neuropil enter the median eye neuropil. Some of these neurites end in the arcuate body and some below the arcuate body. Clear statements on the morphology of these cells are lacking, but their position is the same as the descending unipolar cells found here. Strausfeld et al. [57] reported ascending broad field L-cells in the first median eye neuropil of *Cupiennius salei* (Araneae) that spread through a roughly circular area equivalent to several R-cells. By comparison, the ascending cells of *Achelia langi* also spread through wide reaches of both hemineuropils. Quite revealing is the 3D-EM study by Lacalli [58] of the larval nauplius eye center of the copepod *Dactylopusia sp.* Here, the three eyecups of the nauplius eye are connected to the naupliar eye center. This neuropil is subdivided into three cartridges, each receiving R-cell axons from one of the three eyecups. Several second-order unipolar neurons (LR-cells) with cell bodies above the neuropil postsynaptic to the R-cell axons are found. Additionally, higher-order neurons (M- and E-cells) occur in the neuropil. A similar subdivision (2 ocelli, 2 hemineuropils) is found here in the first visual neuropil of *A. langi*. The morphology and synaptic pattern of copepod LR-cells is similar to that of the pycnogonid D-cells, but cells presynaptic to the R-cells, similar to the A-cells in pycnogonids, have not been identified.

The only arthropod visual system studied in great detail so far is that of the lateral compound eyes in some insect and crustacean species, namely 3D-TEM of *Drosophila* [11], [12], [13], [59], Golgi-studies of insects [60], [61], [39], and Golgi-studies of crustaceans [62], [63], [64], [65]. The lamina's (i.e., first visual neuropil's) cell types are best characterized in

the fruit fly *Drosophila melanogaster*, but the principles are similar in other insect species. The R-cells 1–6 provide input from each ommatidium and synapse to the lamina cartridges, the functional units of the lamina, which are composed of approximately 13 cells: the processes of five monopolar cells (L1–5), one or two amacrine cells, as well as three medulla neurons (C2, C3, and T1) and three glial cells. Additionally, two types of long visual fibers from the ommatidium, R7 and R8, pass the lamina and project to the medulla (second visual neuropil) [7]. In contrast, in crustaceans, R-cells 1–7 end in the lamina and R8 in the medulla. Here also, monopolar cells are found with similar characteristics as in insects. However, there is some disagreement about their number and nomenclature [62], [66], [67].

The synaptic organization in the lamina of *Drosophila* is studied and reviewed in detail by Meinertzhagen and O'Neil [7] and by Meinertzhagen and Sorra [11]. In the lamina, the R-cells are predominantly presynaptic to L1–3 and to amacrine cells. The L-cells in turn have only a few presynaptic sites (to R- and other L-cells) in the lamina. The amacrine cells are frequently presynaptic to R- and L-cells and often to T-cells. Finally, of the medulla neurons, only in C-cells few synapses occur, being presynaptic to L-, T-, and amacrine cells; T-cells are free of synapses in the lamina. All of these synapses are often multiple-contact synapses (dyads, triads, and tetrads).

Conclusions

When comparing our results with the characters described in the compound eyes in *Drosophila,* we found striking similarities in the morphology and synaptic pattern of the visual neurons. The situation of the descending unipolar neurons in *Achelia* is similar to the monopolar cells in the compound eyes. Both have their cell bodies above the neuropil, each providing a single neurite that extends through the neuropil. In both, one can distinguish between cells that have collaterals in just one functional unit (i.e., column in *Drosophila* or hemineuropil in *Achelia*; D1–3 in *Achelia* and L1–3 in *Drosophila*) and cells that provide lateral interaction between neighboring columns/hemineuropils (D4 in *Achelia* and L4 in *Drosophila*) and cells without or with very little collaterals in the first visual neuropil that contribute little to the neuropil organization (D5 in *Achelia* and L5 in *Drosophila*). Additionally, the synaptic pattern is similar. The D- and L-cells, respectively, are predominantly postsynaptic to the R-cells, and hence these cells are second-order neurons.

Moreover, in both, these cells are rarely presynaptic to other cells in the particular neuropil. Contrary to these similarities, the morphology of the bifurcated D3 cells in pycnogonids has no counterpart in the compound eye lamina.

Furthermore, the ascending cells that integrate a wider field of the neuropil are found in both systems as well. In *Drosophila* there are three types of ascending cells (amacrine cells and the medulla neurons C and T). In *Achelia,* we found only one not specifically shaped type, but the synaptic pattern of these A-cells resembles the amacrine cells in *Drosophila*. In both species, these cells are frequently presynaptic to R-cells. However, the amacrine cells in *Drosophila* are often also presynaptic to T-cells from the medulla. The medulla has no counterpart in the pycnogonid brain, and hence this cell type and such connections of the ascending neurons are not observed in *Achelia*.

Moreover, the synaptic pattern of the R-cells is the same. In both systems, these cells are predominantly presynaptic to the D- and L-cells, respectively, and frequently to the A- and amacrine cells, respectively, and are postsynaptic to the A- and amacrine cells, again, respectively.

Finally, in both, the synapses between the different cell types are often multiple-contact synapses (dyads, triads, tetrads, or in pycnogonids even more).

Despite this high degree of correspondence, we think it would be premature to use the term homology for the correspondent cell types (D-/L-cells or A-/amacrine-cells) because only a few species have been analyzed at this level.

The pycnogonid visual system stands for a physiologically simple and phylogenetically ancestral one, but we found at least a foreshadowing of the principles of the highly evolved visual systems found in the lateral compound eye of insects and crustaceans. Already, rather than diffusely shaped neurons, distinct neuron types are found that can be characterized by their branching mode, dendrite length, width of the innervated field, and their synaptic pattern. The second-order neurons have a distal cell body and descending neurites that are postsynaptic to terminals of the R-cells. These neurites form functional units (two hemineuropils comparable to the columns in insects and crustaceans), and their branches and collaterals at distinct levels make layers. Additionally, second-order neurons of a wider field are found that connect the hemineuropils, or rather, neighboring columns. And finally, higher-order feedback neurons with ascending neurites and branches that diverge to the wider field of the neuropil, being presynaptic to the R-cells, are found. Additional similarities

with other arthropod median eye neuropils are found in pycnogonids. These are the position of the neuropil and the innervation pattern by the R-cells [44], [68], as well as the subdivision of the neuropil, with each division responsible for one single eye and the presence of unipolar ascending and descending cells.

To put it in a nutshell, the connectome of the first visual neuropil of the pycnogonid *Achelia langi* has a well-organized architecture. It is composed of distinct cell types with characteristic synaptic patterns and already shows principles of the columns and layers design. Additionally, features of both median and lateral eyes are found, which underlines the theory that the eyes of pycnogonids could be older than the appearance of distinct lateral and median eyes.

Material & Methods

Specimen collection

Specimens of *Achelia langi* (Dohrn, 1881) (Ammotheidae) were collected for FIB-SEM during field trips in May 2011 to Rovinj (Croatia). Specimens of *Achelia vulgaris* (Costa, 1861) were collected for the Golgi technique during field trips in 2009 and 2010 to Rovinj. Species were determined following Dohrn [69] and Bamber [70].

FIB/SEM

After dissection of the abdomen, legs, and proboscis in 4% glutardialdehyde in 0.1 M cacodylate buffer at 4°C, the animals were fixed in 4% glutardialdehyde and 1% tannin in 0.1 M cacodylate buffer at 4°C and stored in the fridge at 4°C. After transportation to the lab in Munich, the specimens were osmicated in 1% OsO_4 in 0.1 M cacodylate buffer for 2 h at 4°C. To enhance contrast, specimens were en bloc stained with 4% uranyl acetate for 1 h at room temperature. After dehydration in a graded acetone series, the specimens were embedded in epoxy resin (Glycidether 100; 2d at 60°C and 1d at 90°C).

Low-resolution stack (transversal view): To approach the visual neuropils, the specimen was trimmed transversally with a diamond knife on an RMC-MTXL ultramicrotome until just before the visual neuropils appeared. After trimming of a cuboid-shaped “mesa” containing the pycnogonid brain with a glass knife [71], this mesa was removed from the epoxy block and mounted on an aluminum stub covered with a thin layer of unpolymerized epoxy resin as glue. The transversal block face was now oriented vertically on the stub, allowing

transversal milling of the left neuropils by the FIB. After polymerizing the epoxy resin (1 d at 60°C), the stub was coated with carbon with a Balzers High Vacuum Evaporator BAE 121 to make it conductive.

The sample was milled and imaged with a Zeiss Auriga CrossBeam Workstation (Carl Zeiss Microscopy, Oberkochen, Germany). For slicing, the conditions were as follows: 500 pA milling current of the Ga-emitter; with each step, 10 nm of the epoxy resin was removed with the focused ion beam. SEM images (2048 x 1536 pixels) were recorded from every 3rd slice at 1.5 kV, resulting in a stack of 682 grayscale images (voxel size 32 x 32 x 30 nm; total volume: 65.5 x 49.2 x 20.5 µm).

Medium-resolution stack (frontal view): The specimen was prepared and imaged as for the low-resolution stack, with the only difference being that the specimen was trimmed frontally to allow frontal milling of the left first visual neuropil by the FIB. With a milling rate of 5 nm (every 3rd slice recorded), an image stack with 1031 planes was acquired (voxel size 12 x 12 x 15 nm; total volume: 24.6 x 18.4 x 15.5 µm).

High-resolution stack (frontal view): Same specimen as for the medium-resolution stack. The medium range of the contralateral right first visual neuropil was imaged with FIB-SEM with a milling rate of 5 nm (every 3rd slice recorded, 212 images; voxel size 6 x 6 x 15 nm; total volume: 12.3 x 9.2 x 3.2 µm).

Image editing and 3D reconstruction

The images were contrast-enhanced and sharpened using unsharp masking in Adobe Photoshop® CS5 (Adobe Systems), then aligned, manually segmented, and surface rendered in Amira® 5.2.0 (Visualization Sciences Group).

In the medium-resolution stack, the profiles of a representative ensemble of 34 cells were reconstructed. In the high-resolution stack, the profiles of a representative ensemble of 33 cells were reconstructed and presynaptic sites of 95 chemical synapses are localized on the basis of synaptic vesicles. Care was taken that cells postsynaptic to the reconstructed cells were selectively reconstructed as well.

The interactive supplement figure was created following Ruthensteiner and Heß [72] with updated software.

Golgi technique

The abdomen, legs, and proboscis were dissected and the cuticle regions surrounding the central nervous system were perforated to increase the probability of staining the desired

areas. The preparations were submitted to two cycles of the Golgi-Colonnier method [73], embedded in epoxy resin and sectioned (10–20 µm).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TL and RRM conceived the study. TL and GH performed the experiments. TL and MH analyzed the data. TL, MH, and RRM drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Silvia Dobler, Heidemarie Gensler, Eva Lodde, and Stefan Friedrich for their expert technical assistance and Hannes Geiselbrecht for his encouraging help with Amira. This study was supported by the Deutsche Forschungsgemeinschaft (DFG Me 2683/6–2).

References

1. Hoffpauir BK, Pope BA, Spirou GA: **Serial sectioning and electron microscopy of large tissue volumes for 3D analysis and reconstruction: a case study of the calyx of Held**. *Nature protocols* 2007, **2**(1):9-22.
2. Anderson JR, Jones BW, Watt CB, Shaw MV, Yang J-H, DeMill D, Lauritzen JS, Lin Y, Rapp KD, Mastronarde D: **Exploring the retinal connectome**. *Molecular Vision* 2011, **17**:355-379.
3. Bock DD, Lee W-CA, Kerlin AM, Andermann ML, Hood G, Wetzel AW, Yurgenson S, Soucy ER, Kim HS, Reid RC: **Network anatomy and in vivo physiology of visual cortical neurons**. *Nature* 2011, **471**(7337):177-182.
4. Cardona A, Saalfeld S, Preibisch S, Schmid B, Cheng A, Pulokas J, Tomancak P, Hartenstein V: **An integrated micro-and macroarchitectural analysis of the *Drosophila* brain by computer-assisted serial section electron microscopy**. *PLoS biology* 2010, **8**(10):e1000502.
5. Saalfeld S, Fetter R, Cardona A, Tomancak P: **Elastic volume reconstruction from series of ultra-thin microscopy sections**. *Nature Methods* 2012, **9**(7):717-720.
6. Macagno ER, Lopresti V, Levinthal C: **Structure and development of neuronal connections in isogenic organisms: variations and similarities in the optic system of *Daphnia magna***. *Proceedings of the National Academy of Sciences* 1973, **70**(1):57-61.
7. Meinertzhagen IA, O'Neil SD: **Synaptic organization of columnar elements in the lamina of the wild type in *Drosophila melanogaster***. *J Comp Neurol* 1991, **305**(2):232-263.

8. White JG, Southgate E, Thomson JN, Brenner S: **The structure of the nervous system of the nematode *Caenorhabditis elegans***. *Philosophical Transactions of the Royal Society of London B, Biological Sciences* 1986, **314**(1165):1-340.
9. Hall DH, Russell RL: **The posterior nervous system of the nematode *Caenorhabditis elegans*: serial reconstruction of identified neurons and complete pattern of synaptic interactions**. *The Journal of neuroscience* 1991, **11**(1):1-22.
10. Melzer RR, Zimmermann T, Smola U: **Modification of dispersal patterns of branched photoreceptor axons and the evolution of neural superposition**. *Cellular and Molecular Life Sciences CMLS* 1997, **53**(3):242-247.
11. Meinertzhagen IA, Sorra KE: **Synaptic organization in the fly's optic lamina: few cells, many synapses and divergent microcircuits**. *Progress in brain research* 2001, **131**:53-69.
12. Takemura SY, Lu Z, Meinertzhagen IA: **Synaptic circuits of the *Drosophila* optic lobe: the input terminals to the medulla**. *J Comp Neurol* 2008, **509**(5):493-513.
13. Takemura SY, Bharioke A, Lu Z, Nern A, Vitaladevuni S, Rivlin PK, Katz WT, Olbris DJ, Plaza SM, Winston P: **A visual motion detection circuit suggested by *Drosophila* connectomics**. *Nature* 2013, **500**(7461):175-181.
14. Helmstaedter M, Briggman KL, Denk W: **3D structural imaging of the brain with photons and electrons**. *Current opinion in neurobiology* 2008, **18**(6):633-641.
15. Müller-Reichert T, Mancuso J, Lich B, McDonald K: **Three-Dimensional Reconstruction Methods for *Caenorhabditis elegans* Ultrastructure**. *Methods in Cell Biology* 2010, **96**:331-361.
16. Briggman KL, Bock DD: **Volume electron microscopy for neuronal circuit reconstruction**. *Current opinion in neurobiology* 2012, **22**(1):154-161.
17. Denk W, Horstmann H: **Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure**. *PLoS biology* 2004, **2**(11):e329.
18. Zankel A, Kraus B, Poelt P, Schaffer M, Ingolic E: **Ultramicrotomy in the ESEM, a versatile method for materials and life sciences**. *Journal of microscopy* 2009, **233**(1):140-148.
19. Gnauck P, Hoffrogge P, Greiser J: **New crossbeam inspection tool combining an ultrahigh-resolution field emission SEM and a high-resolution FIB**. In: *SPIE's 27th Annual International Symposium on Microlithography: 2002*. International Society for Optics and Photonics: 833-840.
20. Principe EL: **How to Use FIB-SEM Data for 3-D Reconstruction**. *R&D Magazine* 2005, **6**:29-30.
21. Briggman KL, Denk W: **Towards neural circuit reconstruction with volume electron microscopy techniques**. *Current opinion in neurobiology* 2006, **16**(5):562-570.
22. Knott G, Marchman H, Wall D, Lich B: **Serial section scanning electron microscopy of adult brain tissue using focused ion beam milling**. *The Journal of neuroscience* 2008, **28**(12):2959-2964.
23. Helmstaedter M, Briggman KL, Denk W: **High-accuracy neurite reconstruction for high-throughput neuroanatomy**. *Nature neuroscience* 2011, **14**(8):1081-1088.
24. Merchan-Perez A, Rodriguez J-R, AlonsoNanclares L, Schertel A, DeFelipe J: **Counting synapses using FIB/SEM microscopy: a true revolution for ultrastructural volume reconstruction**. *Frontiers in neuroanatomy* 2009, **3**:18.
25. Kreshuk A, Straehle CN, Sommer C, Koethe U, Cantoni M, Knott G, Hamprecht FA: **Automated detection and segmentation of synaptic contacts in nearly isotropic serial electron microscopy images**. *Plos One* 2011, **6**(10):e24899.
26. Roncal WG, Kaynig-Fittkau V, Kasthuri N, Berger D, Vogelstein JT, Fernandez LR, Lichtman JW, Vogelstein RJ, Pfister H, Hager GD: **Volumetric Exploitation of Synaptic Information using Context Localization and Evaluation**. *arXiv preprint arXiv:14033724* 2014.
27. Arango CP, Wheeler WC: **Phylogeny of the sea spiders (Arthropoda, Pycnogonida) based on direct optimization of six loci and morphology**. *Cladistics* 2007, **23**(3):255-293.
28. Dunlop JA, Arango CP: **Pycnogonid affinities: a review**. *Journal of Zoological Systematics and Evolutionary Research* 2005, **43**(1):8-21.

29. Regier JC, Shultz JW, Zwick A, Hussey A, Ball B, Wetzer R, Martin JW, Cunningham CW: **Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences**. *Nature* 2010, **463**(7284):1079-1083.
30. Rudkin DM, Cuggy MB, Young GA, Thompson DP: **An Ordovician Pycnogonid (Sea Spider) with Serially Subdivided 'Head' Region**. *Journal of Paleontology* 2013, **87**(3):395-405.
31. Siveter DJ, Sutton MD, Briggs DE: **A Silurian sea spider**. *Nature* 2004, **431**(7011):978-980.
32. Waloszek D, Dunlop JA: **A larval sea spider (Arthropoda: Pycnogonida) from the Upper Cambrian 'Orsten'of Sweden, and the phylogenetic position of pycnogonids**. *Palaeontology* 2002, **45**(3):421-446.
33. Manuel M, Jager M, Murienne J, Clabaut C, Le Guyader H: **Hox genes in sea spiders (Pycnogonida) and the homology of arthropod head segments**. *Development Genes and Evolution* 2006, **216**(7-8):481-491.
34. Maxmen A, Browne WE, Martindale MQ, Giribet G: **Neuroanatomy of sea spiders implies an appendicular origin of the protocerebral segment**. *Nature* 2005, **437**(7062):1144-1148.
35. Brenneis G, Ungerer P, Scholtz G: **The chelifores of sea spiders (Arthropoda, Pycnogonida) are the appendages of the deutocerebral segment**. *Evol Dev* 2008, **10**(6):717-724.
36. Jager M, Murienne J, Clabaut C, Deutsch J, Le Guyader H, Manuel M: **Homology of arthropod anterior appendages revealed by Hox gene expression in a sea spider**. *Nature* 2006, **441**(7092):506-508.
37. Paul DH: **A neurophylogenist's view of decapod Crustacea.** *Bull Mar Sci* 1989, **45**(2):487-504.
38. Harzsch S: **Neurophylogeny: Architecture of the nervous system and a fresh view on arthropod phyologeny**. *Integr Comp Biol* 2006, **46**(2):162-194.
39. Strausfeld NJ: **Arthropod brains: evolution, functional elegance, and historical significance**. Cambridge, Mass.: Harvard University Press; 2012.
40. Sokolow I: **The structure of the pantopod eyes**. *Z Wiss Zool* 1911, **98**(3):339-380.
41. Helfer H, Schlottke E: **Pantopoda**. Leipzig: Akademische Verlagsgesellschaft m.b.H.; 1935.
42. Jarvis JH, King PE: **Ultrastructure of the Photoreceptors in the Pycnogonid species, *Nymphon gracile* (Leach), and *Pycnogonum littorale* (Strom)**. *Marine and Freshwater Behaviour and Physiology* 1973, **2**:1-13.
43. Heß M, Melzer RR, Smola U: **The eyes of a nobody, *Anoplodactylus petiolatus* (Pantopoda; Anoplodactylidae)**. *Helgol Meeresunters* 1996, **50**(1):25-36.
44. Lehmann T, Heß M, Melzer RR: **Wiring a Periscope – Ocelli, Retinula Axons, Visual Neuropils and the Ancestrality of Sea Spiders**. *Plos One* 2012, **7**(1).
45. Strausfeld NJ, Strausfeld CM, Stowe S, Rowell D, Loesel R: **The organization and evolutionary implications of neuropils and their neurons in the brain of the onychophoran *Euperipatoides rowelli***. *Arthropod Struct Dev* 2006, **35**(3):169-196.
46. Calman BG, Lauerman MA, Andrews AW, Schmidt M, Battelle BA: **Central projections of *Limulus* photoreceptor cells revealed by a photoreceptor-specific monoclonal antibody**. *J Comp Neurol* 1991, **313**(4):553-562.
47. Battelle BA: **The eyes of *Limulus polyphemus* (Xiphosura, Chelicerata) and their afferent and efferent projections**. *Arthropod Struct Dev* 2006, **35**(4):261-274.
48. Sporns O, Tononi G, Kötter R: **The human connectome: a structural description of the human brain**. *PLoS computational biology* 2005, **1**(4):e42.
49. Ailamaki A, Alvandpour A, Amunts K, Andreoni W, Ashburner J, Axer M, Baaden M, Badia R: **The Human Brain Project: A Report to the European Commission**. In. Edited by Markram H. Lausanne: The HBP-PS Consortium; 2012: 107.
50. **Human Brain Project: https://www.humanbrainproject.eu/**
51. Alivisatos AP, Chun M, Church GM, Greenspan RJ, Roukes ML, Yuste R: **The brain activity map project and the challenge of functional connectomics**. *Neuron* 2012, **74**(6):970-974.
52. **BRAIN Initiative: http://www.nih.gov/science/brain/**
53. Bear MF, Connors BW, Paradiso MA: **Neuroscience - Exploring the Brain**, 3rd edn: Lippincott Williams & Wilkins; 2007.

54. Bullock TH, Horridge GA: **Structure and function in the nervous systems of invertebrates**. San Francisco, London: W. H. Freeman and Company; 1965.
55. Homberg U: **Evolution of the central complex in the arthropod brain with respect to the visual system**. *Arthropod Struct Dev* 2008, **37**(5):347-362.
56. Hanström B: **Das Nervensystem und die Sinnesorgane von *Limulus polyphemus*.** *Lunds Universitets Årsskrift* 1926, **22**(5):1-79.
57. Strausfeld NJ, Weltzien P, Barth FG: **Two visual systems in one brain: neuropils serving the principal eyes of the spider *Cupiennius salei***. *J Comp Neurol* 1993, **328**(1):63-75.
58. Lacalli TC: **Serial EM analysis of a copepod larval nervous system: Naupliar eye, optic circuitry, and prospects for full CNS reconstruction**. *Arthropod Struct Dev* 2009, **38**(5):361-375.
59. Sprecher SG, Cardona A, Hartenstein V: **The *Drosophila* larval visual system: High-resolution analysis of a simple visual neuropil**. *Developmental Biology* 2011, **358**(1):33-43.
60. Cajal SR, Sanchez D: **Contribucion al conocimiento de los centros nerviosos de los insectos. Parte I: retina y centros opticos.** *Trabajos del Laboratorio de Investigaciones Biológicas de la Universidad de Madrid* 1915, **13**(1):1-168.
61. Fischbach KF, Dittrich APM: **The optic lobe of *Drosophila melanogaster*. I. A Golgi analysis of wild-type structure**. *Cell and Tissue Research* 1989, **258**(3):441-475.
62. Hafner GS: **The neural organization of the lamina ganglionaris in the crayfish: A Golgi and EM study**. *J Comp Neurol* 1973, **152**(3):255-279.
63. Nässel DR: **The organization of the lamina ganglionaris of the prawn, *Pandalus borealis* (Kröyer)**. *Cell and Tissue Research* 1975, **163**(4):445-464.
64. Stowe S, Ribi WA, Sandeman DC: **The organisation of the lamina ganglionaris of the crabs *Scylla serrata* and *Leptograpsus variegatus***. *Cell and Tissue Research* 1977, **178**(4):517-532.
65. Nässel DR, Elofsson R, Odselius R: **Neuronal connectivity patterns in the compound eyes of *Artemia salina* and *Daphnia magna* (Crustacea: Branchiopoda)**. *Cell and Tissue Research* 1978, **190**(3):435-457.
66. Nässel DR: **Types and arrangements of neurons in the crayfish optic lamina**. *Cell and Tissue Research* 1977, **179**(1):45-75.
67. Sztarker J, Strausfeld NJ, Andrew DR, Tomsic D: **Neural organization of first optic neuropils in the littoral crab *Hemigrapsus oregonensis* and the semiterrestrial species *Chasmagnathus granulatus***. *J Comp Neurol* 2009, **513**(2):129-150.
68. Lehmann T, Melzer RR: **Looking like *Limulus*? – Retinula axons and visual neuropils of the median and lateral eyes of scorpions**. *Frontiers in Zoology* 2013, **10**(1):40.
69. Dohrn A: **Die Pantopoden des Golfes von Neapel und der angrenzenden Meeresabschnitte**. *Monographie der Fauna und Flora des Golfes von Neapel* 1881, **3**:1-252.
70. Bamber RN: **Sea-spiders (Pycnogonida) of the north-east Atlantic: Keys and Notes for Identification the Species**, vol. 5. London, Shrewsbury: Linnean Society of London by Field Studies Council; 2010.
71. Hayat MA: **Principles and techniques of electron microscopy: biological applications**: Cambridge University Press; 2000.
72. Ruthensteiner B, Heß M: **Embedding 3D models of biological specimens in PDF publications**. *Microscopy research and technique* 2008, **71**(11):778-786.
73. Colonnier M: **The Tangential Organization of the Visual Cortex**. *Journal of Anatomy* 1964, **98**:327-344.

6. General discussion

The visual system of chelicerates with a focus on pycnogonids and scorpions is studied in this thesis on three different levels of observation. The first and highest level is the level of neuropils. Here the number, arrangement, and morphology of the visual neuropils are observed and discussed. At the second level, individual cells – R-cells, second order and higher order neurons – within a visual neuropil are identified and discussed. Finally, at the third and lowest level synapses of these cells are in the focus of interest. This allows the first comparative analysis of synapses throughout arthropods. The first level is studied in this thesis in both, pycnogonids and scorpions. The second and third level is so far studied in pycnogonids only.

6.1. Level of neuropils

For the description and comparison of the visual systems in arthropods three different types of neuropils are of interest: the visual neuropils of the median eyes, the visual neuropils of the lateral eyes, and the arcuate body. The latter is closely associated with the visual system (Homberg 2008).

In addition, visual neuropils not directly innervated by R-cells are described in literature, e.g. in Araneae (ON2) and especially in the lateral eyes of Tetraconata (e.g. medulla interna, lobula, lobula plate, optic foci). However, with the methods used in this thesis such neuropils could not be identified and in the following mainly the visual neuropils with direct input from the R-cells are described and discussed.

One of the core findings in this thesis is, that with different tracer techniques, histological methods, FIB-SEM and 3D-reconstructions the visual neuropils are unequivocally identified and described in representatives of the two chelicerate groups – Pycnogonida (*Achelia langi*, *A. vulgaris*, and *Endeis spinosa*) and Scorpiones (*Euscorpius italicus*, *E. hadzii*) – for the first time. The sets of characters studied here for the median eyes in Pycnogonida and Scorpiones compared to those of Onychophora, Xiphosura, Araneae, Crustacea, and Hexapoda are summarised in the data matrix given in table 2.

<u>Visual neuropils in Pycnogonida</u>

Pycnogonids have only one type of eyes, generally interpreted as median eyes (Paulus 1979). However, their ultrastructure has some similarities with the lateral eyes of spiders (e.g.

retinula cells with a distal nucleus and a proximal axon, the organization of the tapetum; see also Heß, Melzer et al. (1996) and Lehmann and Melzer (2011)). These four eyes are located on a periscope like ocular tubercle. The two eyes of one hemisphere are connected via retinotopic nerve fibres with a thickening dorso-laterally to the protocerebrum. In this thickening the nerve fibres are re-assorted. After entering the brain, one part of the R-cell axons end in a first and the other part in a second visual neuropil. R-cell axons terminate either in the first or in the second visual neuropil and do not have collaterals in both neuropils at once. The first neuropil is located laterally in the protocerebrum. It is subdivided in two hemineuropils, each responsible for one eye and stratified into three layers. The second neuropil is located in a more central position, in close vicinity to the arcuate body (see below); both neuropil hemispheres contact each other in the brain's midline. Projections from one hemisphere also have collaterals in the contralateral second neuropil.

Median eye visual neuropils in Scorpiones

Scorpions have two typical median eyes located in the middle of the carapace (Paulus 1979). With the tracer methods used here (Cobalt chloride and DiI/DiO) two successive median eye visual neuropils are identified. The first visual neuropil is located in the lateral protocerebrum. The second visual neuropil is also located in the lateral protocerebrum, below the first neuropil. It overlaps with the second lateral eye visual neuropil (see below). Additionally, few fibres connect the median eyes with the arcuate body.

The only other recent studies dealing with the median eye visual neuropils in scorpions (*Androctonus australis*) are those of Fleissner (1985) and Heinrichs and Fleissner (1987). In these electrophysiological studies three different cell types in the median eye visual system were distinguished on the basis of their spiking characteristics: photoreceptor cells, arhabdomeric cells, and efferent neurosecretory cells. To locate the target regions of these cells Cobalt fills and Lucifer Yellow CH stainings were applied in the electrophysiologically identified cells. Fleissner and Heinrichs showed no original data and only a schematic drawing with the approximate location of the neuropils is given. Accordingly the median eye visual neuropil organisation is the same as described here. Furthermore, the authors show that the photoreceptor cells terminate in the first and the arhabdomeric cells in the second median eye neuropil. The neurosecretory cells have their cell body in the tritocerebrum and terminate via the arcuate body in the retina of the median eyes. The morphology of these

cells is very similar to the fibres described here, that connect the median eyes with the arcuate body.

Median eyes in other (pan)arthropods

The specific characters described in the present thesis for pycnogonids and scorpions allow a comparison with the (median eye) visual systems in other chelicerates and (pan)arthropods (including Onychophora; see also table 2).

In doing so, the median eyes of *Limulus polyphemus* (Xiphosura) come into focus. Here one must distinguish between the fused median rudimentary eyes and the median eyes (Battelle 2006). The fused rudimentary median eyes are endoparietal eyes and lie between the median eyes and appear to be a simple cluster of large photoreceptor cells embedded in guanophores. The axons of the photoreceptor cells fuse with each median optic nerve (Battelle 2006; Harzsch, Vilpoux et al. 2006). These axons have collaterals in a first visual neuropil (ocellar ganglion) and proceed further terminating near the arcuate body (Chamberlain and Barlow 1978; Chamberlain and Barlow 1980; Calman, Lauerman et al. 1991; Battelle 2006; Harzsch, Vilpoux et al. 2006). This situation resembles that in pycnogonids where the R-cells also have two target regions, one in close vicinity to the arcuate body. However, in pycnogonids the R-cells terminate either in the first or in the second visual neuropil and not in both neuropils simultaneously as in *Limulus*.

In contrast, the photoreceptor cells of the median eyes of *Limulus* have only one target region, terminating in the first visual neuropil (ocellar ganglion) (Calman, Lauerman et al. 1991; Harzsch, Vilpoux et al. 2006). Furthermore, the arhabdomeric cells have collaterals in the optic tract and terminate in the second lateral eye neuropil (medulla) (Chamberlain and Barlow 1980; Calman, Lauerman et al. 1991; Battelle 2006). These two termination sites of the arhabdomeric cells can also be interpreted as an own second median eye neuropil, which partly overlaps with the second lateral eye neuropil. This situation in turn resembles that in the scorpion brain. Here the photoreceptor cells also terminate in the first median eye neuropil and the arhabdomeric cells in the second median eye neuropil. Furthermore, the second median and lateral eye neuropils overlap each other in both, *Limulus* and *Euscorpius*.

Hence, the innervation pattern of the median rudimentary eyes in *Limulus* is similar to that of the eyes in pycnogonids and the innervation pattern of the median eyes in *Limulus* is similar to that of the median eyes in scorpions (Table 2).

The only other chelicerate visual systems studied so far are those of Araneae (*Cupiennius salei*) (Strausfeld, Weltzien et al. 1993; Strausfeld and Barth 1993) and of Opiliones (*Opilio canestrinii*, *Phalangium opilio*, *Rilaena triangularis*) (Saint-Remy 1890; Holmgren 1916; Hanström 1928; Breidbach and Wegerhoff 1993).

In Opiliones there is some confusion about the number and arrangement of the median eye visual neuropils. Saint-Remy (1890) describes four layers (*couches*), Holmgren (1916) three neuropils, and Hanström (1928) and Breidbach and Wegerhoff (1993) two neuropils, but with a different interpretation of the second neuropil. Hence, there is no consensus in the interpretation of hitherto results. Therefore the R-cell termination sites and thus the visual neuropils have to be identified unequivocally with different tracer methods in the future.

In Araneae the first anterior median eye neuropil is the only target region of R-cells of the median eyes (principal eyes or anterior median eyes) (Strausfeld, Weltzien et al. 1993). It is located laterally in each brain hemisphere. Subsequent second-order neurons terminate in a second visual neuropil (medulla). Furthermore, a tract that extends into the arcuate body is suggested. This situation distinguishes from that in the eyes in pycnogonids and the median rudimentary eyes in *Limulus*, where the R-cells project to two subsequent visual neuropils and resemble those in the median eyes in scorpions and in the median eyes in *Limulus*, where the photoreceptor cells project to a paired first visual neuropil as well. However, only photoreceptor cells and no arhabdomeric cells are described from the retina of the studied spider species. Hence, a connection from these cells to a second visual neuropil – as in scorpions and *Limulus* – is missing.

Outside the Chelicerata the median eye neuropil organisation differs fundamentally. In Myriapoda median eyes and hence corresponding neuropils are not described. According to Harzsch (2006) a consistent ground pattern in Tetraconata (Crustacea + Hexapoda) is missing. However, the R-cells usually terminate in a medially fused neuropil or neuropil-complex located in the dorso-median protocerebrum. In contrast, chelicerates have separated, paired median eye neuropils, usually in the dorso-lateral protocerebrum.

In Crustacea (e.g. *Artemia salina*, *Balanus amphitrite*, *Cherax destructor*) terminals of the photoreceptor cells of the median eyes (restricted to nauplius eyes; the various so-called frontal organs some of which may also have a photoreceptive function are omitted) can be found in a medially fused neuropil in the dorso-median protocerebrum (nauplius eye-centre in Entomostraca). The number of subunits of the neuropil depends on the number of eyes

(Benesch 1969; Sandeman, Sandeman et al. 1990; Harrison and Sandeman 1999; Harzsch, Wildt et al. 2005; Harzsch 2006; Elofsson 2006).

In the basal insect group of Collembola (*Podura aquatica*, *Neanura sp*., *Orchesella villosa*, *Tomocerus vulgaris*, and *T. longicornis*) the R-cell axons of all ocelli target an unpaired ocellar centre in the dorso-median protocerebrum and make synaptic contacts with secondary neurons (Paulus 1972). This innervation pattern is very similar to that in crustaceans (Harzsch, Wildt et al. 2005; Harzsch 2006).

Table 2. Data matrix with sets of characters from median eye visual system. Update of data matrix begun in paper I (Lehmann, Heß et al. 2012) including scorpion features revealed in paper II (Lehmann and Melzer 2013) compared to (median) eye visual system of other (pan)arthropods (citations and studied species for exemplary taxa are given in the text). Matrix restricted to visual neuropils innervated by R-cells (photoreceptor cells and arhabdomeric cells). Due to absence of median eyes, Myriapoda are omitted. "-" indicates that this feature has not been studied or is not applicable. MRE, median rudimentary eye; ME, median eyes.
A, eye nerves paired, separated, and arranged in bilateral symmetry (0) or medially fused eye nerves (1); **B**, visual neuropils paired, separated, and arranged in bilateral symmetry (0) or medially fused visual neuropils (1); **C**, number of visual neuropils innervated by cells from the retina (R-cells = photoreceptor cells and arhabdomeric cells) greater than one (0) or equal to one (1); **D**, number of visual neuropils innervated by photoreceptor cells greater than one (0) or equal to one (1); **E**, visual neuropil with photoreceptor cell terminals in close vicinity to arcuate body present (0) or absent (1); **F**, only one type of eye inserted at anterior body's cuticle (0) or lateral and median eyes present (1); **G**, R-cell axons project to distinct neuropils for each eye (0) or second visual neuropil targeted by axons from both median and lateral eyes.

	Chelicerata						Tetraconata	
Feature	Onychophora	Pycnogonida	Xiphosura (MRE)	Xiphosura (ME)	Scorpiones	Araneae	Crustacea	Hexapoda
A	0	0	0	0	0	0	1	1
B	0	0	0	0	0	0	1	1
C	-	0	0	0	0	1	1	1
D	-	0	0	1	1	1	1	1
E	-	0	0	1	1	1	1	1
F	0	0	1	1	1	1	1	1
G	-	-	0	1	1	0	0	0

Ontogenetic data obtained from cockroaches (*Periplaneta americana*) and locust (*Schistocerca gregaria*) indicate that the primordial R-cell axons of the ocelli terminate in the median protocerebrum close to the protocerebral bridge (part of the central complex) (Mobbs 1976; Toh and Yokohari 1988). However, in adult cockroaches (*Periplaneta americana*) and crickets (*Acheta domesticus*) axons of (newly added) R-cells terminate immediately below the ocelli, in the ocellar plexus, and not in the protocerebrum (Koontz and Edwards 1984; Mizunami 1995; Harzsch 2006).

However, more information on the R-cell connections of the insect and crustacean median eyes to the protocerebrum and on the development of the median eye pathway will be necessary before more detailed comparisons between Tetraconata and Chelicerata can be made (Harzsch, Wildt et al. 2005; Harzsch 2006).

Finally, it is worth looking at the visual system of velvet worms (Onychophora). Velvet worms are suggested as sister taxon of Arthropoda, with which they form the taxon Panarthropoda (Tardigrada + Onychophora + Arthropoda) (Meusemann, von Reumont et al. 2010; Campbell, Rota-Stabelli et al. 2011). Their brain architecture seems to be similar to that in chelicerates (Strausfeld, Strausfeld et al. 2006; Strausfeld, Strausfeld et al. 2006). Velvet worms (*Epiperipatus biolleyi*, *Metaperipatus blainvillei*, and *Euperipatoides rowelli*) have a pair of small rhabdomeric eyes situated near the antennal base consisting of a cornea, a lens and a retina (Eakin and Westfall 1965; Mayer 2006). Furthermore, Mayer (2006) described similarities in morphology and development of onychophoran eyes and median eyes of arthropods.

The presence of photoreceptor terminals in a first visual neuropil, which lies directly beneath the eye, is suggested (Mayer 2006). From this first neuropil, an optic tract projects further and then bifurcates (Strausfeld, Strausfeld et al. 2006). Its dorsal branch gives rise to a visual neuropil, which flanks the arcuate body laterally and a ventral branch extends to another visual neuropil. However, the exact projection of the retinula cells is not identified unequivocally. As in chelicerates the visual neuropils are located in the lateral protocerebrum and the visual system seems to be closely associated with the arcuate body (Homberg 2008). Similar to pycnogonids and *Limulus* subsets of visual fibres bifurcate and target two different neuropils, one in close vicinity to the arcuate body (Table 2).

Lateral eye visual neuropils

Pycnogonids possess only one type of eyes; these are generally seen as median eyes (see above).

In scorpions the lateral eye retinula cells are linked to a first and a second visual neuropil, with R-cell axon terminals in both neuropils. The second neuropil is partly shared by projections from both – median and lateral – eyes (see above).

Here again the visual system of *Limulus* is of special interest. As in median eyes, one must distinguish between two different eye types: the lateral rudimentary eyes and the lateral compound eyes (Calman, Lauerman et al. 1991; Battelle 2006). By comparison the innervation pattern of the lateral eyes of scorpions resembles that in the lateral rudimentary eyes of *Limulus*. Both have R-cell collaterals in a first (lamina) and second (medulla) neuropil. In the lateral compound eyes of *Limulus* in contrast the photoreceptor cells terminate in the lamina only, whereas the eccentric cells of the lateral compound eye retina project to the lamina, medulla, optic tract, and to the ocellar ganglion. This cell type, hence such a connection from the lateral eye to median eye neuropils cannot be observed in the scorpion visual system. The similarity in function and structure between the eccentric cells and the arhabdomeric cells of scorpions was discussed by Schliwa and Fleissner (1979) and (1980).

In Araneae (*Cupiennius salei*) each of the three pairs of secondary eyes terminate in an own paired first lateral eye neuropil (lamina, or ON1) (Strausfeld and Barth 1993). It is the only target region of the R-cells. Furthermore, higher order neurons from the three second visual neuropils (medulla, or ON2; each for one eye) converge at the mushroom body.

And finally, in Tetraconata (various species studied) and Myriapoda (*Lithobius forficatus*) the photoreceptor cell axons of the lateral eyes are split, short fibres are connected to a first visual neuropil (lamina) and long fibres to a second visual neuropil (medulla) (Melzer, Petyko et al. 1996; Harzsch 2006; Strausfeld 2012). In Tetraconata the lateral (compound) eye visual system is connected indirectly with the central complex. In Myriapoda connections from the visual neuropils to a midline neuropil (central complex/body) have not been described because suitable methods to detect such connections were not applied so far (Homberg 2008).

Arcuate body

In the protocerebrum of chelicerates and onychophorans the arcuate body has a unique feature: it is the only unpaired neuropil in the brain's midline (Strausfeld, Strausfeld et al.

2006; Strausfeld, Strausfeld et al. 2006; Loesel, Nässel et al. 2002; Homberg 2008). It occupies a superficial, dorso-posterior position in the brain and its function is closely associated with the median eye visual system. While some investigators have assumed that the arcuate body is homologous with the central complex of mandibulates (Loesel, Nässel et al. 2002; Homberg 2008), others have questioned this view (Strausfeld 1998; Breidbach 1995). The central complex of mandibulates is indirectly connected with the lateral eye visual system.

For pycnogonids the arcuate body is not described unambiguously so far, but a strong candidate is shown here for the first time. Even if it is a rather small neuropil compared to that in other chelicerates, its dorso-posterior position as the only neuropil in the brain's midline is well in accordance with that in other chelicerates and in onychophorans. Especially the close vicinity to the visual neuropils indicates that it is indeed the arcuate body.

The arcuate body of scorpions is shown here as well. It is slightly bent in a superficial, dorso-posterior position in the brain, as is typical for chelicerates.

The advantages of FIB-SEM allows in the following to take a closer look at the first visual neuropil of pycnogonids. The morphology of individual R-cells as well as of higher order neurons along with their synaptic pattern is studied in detail and compared to other arthropods.

6.2. Level of cells

With the help of the cutting-edge method FIB-SEM the connectome of the first visual neuropil in the sea spider *Achelia langi* is reconstructed and six different cell types are characterised. These cell types are also identified with Golgi impregnations. This indicates that both methods give correct pictures of the neuron gestalten.

Along with the R-cells five types of descending unipolar neurons (D1–5) and one type of ascending neurons (A1) are identified. The cell bodies of the descending cells, which send a single neurite each into the first visual neuropil, are located in the cell body rind dorsally to the neuropil. Hence, these cells are unipolar neurons[1]. The cell bodies of the ascending neurons are beyond the examined area, but the soma must be located below the neuropil,

[1] The term 'monopolar cells' is intentionally avoided in order to prevent premature homology assumptions. This term is occupied by the monopolar cells in the compound eye visual system in Tetraconata (see below).

whereas the neurites end before the top end of the neuropil. Hence, a classification in unipolar, bipolar, or multipolar neurons cannot be made for the ascending cells.

The first visual neuropil is subdivided into two hemineuropils (see above), each responsible for one eye. The hemineuropils are the functional units of the neuropil. Three types of the descending cells (D1–3) are responsible for this subdivision. A single D1–3 neuron is restricted to one hemineuropil and does not cross the border in between. In contrast, D4 neurons occur in both hemineuropils simultaneously and provide lateral interactions between the functional units. D5 neurons can be found in the right hemineuropil only; the interpretation of this is difficult. Most likely this is a sampling artefact and the D5-cell bodies of the left hemineuropil are beyond the examined volume and hence are not reconstructed.

The ascending neurons (A1) can be found throughout the neuropil, a single neuron occurs in both hemineuropils at once. Each cell profile covers a large area of the neuropil. However, branches of A1 neurons accumulate in the area that divides the two hemineuropils.

The only arthropod visual system studied so far at this level – where the morphology of individual neurons is considered – is that of the lateral compound eyes in some insect and crustacean species, namely 3D-TEM of *Drosophila* (e.g. Meinertzhagen and Sorra 2001; Takemura, Lu et al. 2008; Takemura, Bharioke et al. 2013), Golgi-studies of insects (e.g Cajal and Sanchez 1915; Fischbach and Dittrich 1989; Strausfeld 2012), and Golgi-studies of crustaceans (e.g. Hafner 1973; Nässel 1975; Nässel 1977; Stowe, Ribi et al. 1977; Nässel, Elofsson et al. 1978). For chelicerates adequate data is missing.

In the fruit fly *Drosophila melanogaster*, the cell types of the first visual neuropil (lamina) are best characterized, but the principles are similar in other insect species, with the exception of neural superposition. The R-cells 1–6 provide input from each ommatidium and synapse to the lamina cartridges. Additionally, two types of long visual fibers from the ommatidium, R7 and R8, pass the lamina and project to the second visual neuropil (medulla). In *Drosophila*, according to the principle of neural superposition, each R1-6 of an ommatidium projects to a different column or cartridge of the lamina. Six R-cells from six different ommatidia send their axons as a group into a single cartridge. In contrast, in most species each R1-6 of an ommatidium projects to the same cartridge (Braitenberg 1967; Kirschfeld 1967; Meinertzhagen and O'Neil 1991).

The lamina cartridges are the functional units of the neuropil. They are composed of approximately 13 cells: five monopolar cells (L1–5), one or two amacrine cells, as well as

three medulla neurons (C2, C3, and T1) and three glial cells (Meinertzhagen and O'Neil 1991).

When comparing the characters in the first lateral compound eye neuropil (lamina) in *Drosophila* with that in the first visual neuropil in *Achelia*, one can find striking similarities in the morphology of the visual neurons. The situation of the monopolar cells is similar to the descending unipolar neurons in *Achelia*. Both have cell bodies dorsally to the neuropil, with a single neurite that extends through the neuropil. The neuropil can be subdivided in functional units, which receive information from one ommatidium or eye (lamina cartridges in *Drosophila* and hemineuropils in *Achelia*). In both, one can distinguish between cells that have collaterals in just one functional unit (L1–3 in *Drosophila* and D1–3 in *Achelia*) and cells that provide lateral interaction between neighboring functional units (D4 in *Achelia* and L4 in *Drosophila*) and cells without or with very few collaterals in the first visual neuropil that contribute little to the neuropil organization (D5 in *Achelia* and L5 in *Drosophila*). Furthermore, the ascending cells that integrate a wider field of the neuropil are found in both systems as well. In *Drosophila* there are three types of ascending cells (amacrine cells and the medulla neurons C and T). In *Achelia* only one not specifically shaped type is found.

The level of cells is complemented in the following by the level of synapses, also studied with FIB-SEM.

6.3. Level of synapses

To date only few methods allow studying the distribution pattern of synapses within a distinct brain area, one of these methods is FIB-SEM. In this thesis the synaptic pattern of the six different cell types in the first visual neuropil in *Achelia langi* is analysed.

The 3D-EM analysis reveals that the R-cells provide the input into the system, being primarily presynaptic to the D-cells. Because the D-cells rarely appear to be presynaptic in the first visual neuropil, these cells most likely synapse and hence integrate information to higher visual centres that were not identified here. These centres could be the second visual neuropil or the arcuate body, which in chelicerates is closely associated with the visual system (see above). The A-cells play a special role in this system, being pre- and postsynaptic to both R- and D-cells. Hence, these cells collect information from the input (R-cells) and the second-order cells (D-cells) but also circulate information back to these cells. Mechanisms

such as lateral inhibition, contrast enhancement, and other filter functions could be behind this feedback loop.

In arthropods there is only one species that is studied at this degree of resolution, again namely *Drosophila melanogaster* (Meinertzhagen and O'Neil 1991; Meinertzhagen and Sorra 2001). Tables 3 and 4 show a high degree of correspondence in the synaptic pattern in *Achelia* and *Drosophila*.

Despite the striking similarity in both cell-morphology and synaptic pattern shown in this thesis in the pycnogonid *Achelia langi* and the hexapod *Drosophila melanogaster*, it would be premature to use the term homology for the correspondent cell types (D-/L-cells or A-/ amacrine cells and medulla neurons C and T) because only a few species have been analysed at this level.

Table 3. Synaptic pattern of the different cell types in *Achelia langi*.
"-" never; "+" sometimes, "++" often, "+++" very often.

Presynaptic cells			→ presynaptic to ↓	
R-cells	D-Cells	A-cells		
-	+	+++	R-cells	Postsynaptic cells
+++	+	++	D-cells	
++	+	-	A-cells	

Table 4. Synaptic pattern of the different cell types in *Drosophila melanogaster*, simplified after Meinertzhagen and Sorra (2001).
"-" never; "+" sometimes, "++" often, "+++" very often.

Presynaptic cells			→ presynaptic to ↓	
R-cells	L-Cells	C-, T- and amacrine cells		
-	+	++	R-cells	Postsynaptic cells
+++	+	++	L-cells	
++	-	-	Amacrine cells	

6.4. Conclusions

There are two alternatives to interpret the findings in this thesis, the first is more or less textbook opinion and the second alternative leads to a new interpretation of eye evolution in Arthropoda.

Hypothesis I: The eyes of Pycnogonida are median eyes

According to textbooks there are two classes of photoreceptor organs in the ground plan of Arthropoda (Paulus 1979; Paulus 2000; Westheide and Rieger 2006; Ruppert, Fox et al. 2004; Harzsch 2006): Median eyes and lateral eyes. Median eyes are situated medially on the head and are innervated to one neuropil in the median protocerebrum that is either bilaterally paired or medially fused (e.g. ocellar ganglia in Xiphosura, nauplius-eye centre in Entomostraca, 2 small spherical neuropils associated with the protocerebral bridge in Malacostraca; ocellar centre in Collembola). All arthropod median eyes are seen as homologous. Lateral eyes are situated laterally on the head and are innervated to two subsequent neuropils in the lateral protocerebrum (mostly named lamina and medulla). Arthropods either have compound lateral eyes (*Limulus*, *Scutigera*, Crustacea, and Hexapoda) or a field of several lateral ocelli (most Chelicerata and Myriapoda).

Regarding the basal position of Pycnogonida and also of *Limulus* and if one adopts the opinion that the eyes of Pycnogonida are median eyes one can assume that the central projections of the four median eyes of Pycnogonida and the median rudimentary eye of *Limulus* represent the ground pattern for median eyes in Chelicerata. This ground pattern is characterised by (1) four median eyes, (2) a separated, bilaterally paired nerve that connects the eyes with the brain, (3) a separated, bilaterally paired first visual neuropil with central projections of photoreceptor cells, (4) a second visual neuropil also with central projections of photoreceptor cells, and (5) the second visual neuropil being located in close vicinity to the arcuate body. Derived situations are found in the “normal” median eyes of *Limulus* and in the median eyes of scorpions: in both the photoreceptor cells only project to a separated, bilaterally paired first visual neuropil, while the second type of retinula cells, the arhabdomeric cells, project to a second visual neuropil, which partly overlaps with the second visual neuropil of the lateral eyes. Another derived situation is found in the median eyes (principal eyes or anterior median eyes) of Araneae, whose photoreceptor cells (as the

only cells in the retina projecting to the protocerebrum) simply project to a separated, bilaterally paired first visual neuropil.

How the situation found in the median eyes of Tetraconata – with one median eye nerve and a single medially fused median eye neuropil in the median protocerebrum as the only target of the retinula cells – fits in this ground pattern is problematic. Weather this is another derived pattern or even convergent remains unclear until a conclusive ground pattern for the projection of median eyes in Tetraconata is described.

Features from hypothesis I support a sister group relationship of Pycnogonida and Euchelicerata within Chelicerata (see also figure 3a, b), with the similar innervation pattern of the median eyes described here as a synapomorphy, different from that in Mandibulata. Furthermore, the eyes of Xiphosura and Scorpiones share many aspects of their brain innervation patterns indicating close evolutionary relationships, at least of their visual systems.

<u>Hypothesis II: The eyes of Pycnogonida are precursors of median and lateral eyes in Arthropoda</u>

In this thesis several features especially from the pycnogonid visual system are discussed. Some of these are similar with that in median eyes of other chelicerates, but surprisingly many features are similar to that in the lateral eyes in arthropods. These are: the photoreceptor cells are connected via short and long axons to two – and not only to one – visual neuropils, the first neuropil is located in the lateral – and not in the median – protocerebrum, the visual systems are closely associated with a modular midline neuropil (arcuate body in Chelicerata and central complex in Tetraconata), and the similar morphology and synaptic pattern of the R-cells, second, and higher order neurons in the first visual neuropil.

Since pycnogonids are one of the most ancestral arthropods and since this ancestral form must be a precursor of the more advanced systems, the findings in this thesis probably question the traditional division in median and lateral eyes. This means that the eyes of pycnogonids maybe offshoots of arthropod eyes how they “looked” like before the division in median and lateral eyes happened. Especially the “rule” that in arthropods median eyes are connected to one paired or fused neuropil in the median protocerebrum and lateral eyes are connected to two paired neuropils (lamina and medulla) in the lateral protocerebrum is questionable.

This probably leads to a new ground pattern of eyes in Arthropoda. This ground pattern is characterised by (1) the presence of both long and short photoreceptor axons, terminating in (2) a paired first visual neuropil in the lateral protocerebrum as target of the short photoreceptor axons and in (3) a paired second visual neuropil in the protocerebrum as target of the long photoreceptor axons, and (4) a close association of the visual system with a modular midline neuropil (arcuate body or central complex). If the similar morphology and synaptic pattern in the first visual neuropil is also part of the ground pattern must be excluded from this discussion since only two species (*Achelia* and *Drosophila*) are studied so far.

This ground pattern is found in the eyes of Pycnogonida, the median and lateral rudimentary eyes in *Limulus*, the lateral eyes of Myriapoda (so far no connection to the central complex/body is proven), and the lateral eyes in Tetraconata, including stemmata or larval eyes (Melzer 2009). Furthermore, Melzer (2009) reviews that stemma-like eyes are found in all arthropod linages including Chelicerata, Myriapoda and Tetraconata and already discusses that these eyes are the precursors of the main lateral eyes. Thus, arthropod eye evolution might have started from small primary eyes from which the main lateral eyes were derived. Probably these small primary eyes are still found in Pycnogonida, which are not only the precursors of the lateral eyes but also of the median eyes in Arthropoda.

This ground pattern differs from the situation in the median eyes of *Limulus* and scorpions, where the photoreceptor cells are only connected to a paired first visual neuropil and the arhabdomeric cells are connected to a paired second visual neuropil. However, both median eye visual systems have a close association with the arcuate body. In Araneae the median eyes are connected to a paired first visual neuropil only, but via a second visual neuropil (without direct R-cell input) a connection to the arcuate body is described. However, the fact that the “normal” median eyes of *Limulus* are connected to the same first neuropil as the rudimentary median eyes indicates that the situation in the median eyes in Chelicerata is a derived condition of this ground pattern.

From a visual system point of view the hypothesis II indicates that Pycnogonida is placed outside Chelicerata as sister taxon to all other extant arthropods, previously named Cormogonida (see also figure 3c), with the division in median and lateral eyes as autapomorphy of Cormogonida.

6.5. Outlook

These hypotheses and the ground patterns are a preliminary draft, far more research has to be done. Especially the integration in or the separation from this ground pattern of the median eyes in Tetraconata, with a single medially fused neuropil located in the dorso-median protocerebrum, needs further investigation.

As often in comparative morphology more species have to be studied with more methods. First of all this should include the analysis of the visual system and especially of the arcuate body in Pycnogonida with e.g. (immuno)histological methods, including the development, to get more comparable features. As this thesis shows, this animal group holds a key position for the understanding of the evolution of the arthropod visual systems. Furthermore, the study of the innervation pattern of the eyes in more chelicerate taxa should resolve if the similar situation of the median eye visual system in Scorpiones and Xiphosura indicate a sister group relationship. Also research concerning the visual system in Myriapoda, especially in other taxa than Chilopoda, with respect to the central complex/body and a conclusive ground pattern of the median eye visual system in Tetraconata is needed. The innervation pattern of the eyes in Onychophora, as one of the suggested sister taxa of Arthropoda, should provide insights if the eyes of Pycnogonida are phylogenetically even older and have its origin in Onychophora.

Finally, the comparative study begun in this thesis of the morphology of the cells involved in the visual system and of the synaptic pattern of these cells should be extended to the other chelicerate and arthropod taxa.

7. References

Altman JS & Tyrer NM (1980). Filling selected neurons with cobalt through cut nerves. In: Neuroanatomical Techniques: Insect Nervous System. Strausfeld NJ & Miller TA. New York, Springer: 373-402.

Arango CP & Wheeler WC (2007). Phylogeny of the sea spiders (Arthropoda, Pycnogonida) based on direct optimization of six loci and morphology. Cladistics 23(3): 255-293.

Arnaud F & Bamber RN (1987). The Biology of Pycnogonida. Advances in Marine Biology 24: 1-96.

Babu KS & Barth FG (1984). Neuroanatomy of the central nervous system of the wandering spider, *Cupiennius salei* (Arachnida, Araneida). Zoomorphology 104(6): 344-359.

Battelle BA (2006). The eyes of *Limulus polyphemus* (Xiphosura, Chelicerata) and their afferent and efferent projections. Arthropod Structure & Development 35(4): 261-274.

Benesch R (1969). Zur Ontogenie und Morphologie von *Artemia salina*. Zoologische Jahrbücher Anatomie 86(307): 458.

Braddy SJ, Aldridge RJ, et al. (1999). Lamellate book-gills in a late Ordovician eurypterid from the Soom Shale, South Africa: support for a eurypterid-scorpion clade. Lethaia 32(1): 72-74.

Braddy SJ, Poschmann M, et al. (2008). Giant claw reveals the largest ever arthropod. Biology letters 4(1): 106-109.

Braitenberg V (1967). Patterns of projection in the visual system of the fly. I. Retina-lamina projections. Experimental Brain Research 3(3): 271-298.

Breidbach O (1995). Is the evolution of arthropod brains convergent? In: The Nervous Systems of Invertebrates: An Evolutionary and Comparative Approach. Breidbach O & Kutsch W. Basel, Springer: 383-406.

Breidbach O & Wegerhoff R (1993). Neuroanatomy of the central nervous system of the harvestman, *Rilaena triangularis* (HERBST 1799) (Arachnida; Opiliones): principal organization, Gaba-like and serotonin-immunohistochemistry. Zoologischer Anzeiger 230(1-2): 55-81.

Budd GE & Telford MJ (2009). The origin and evolution of arthropods. Nature 457(7231): 812-817.

Cajal SR & Sanchez D (1915). Contribucion al conocimiento de los centros nerviosos de los insectos. Parte I: retina y centros opticos. Trabajos del Laboratorio de Investigaciones Biológicas de la Universidad de Madrid 13(1): 1-168.

Calman BG, Lauerman MA, et al. (1991). Central projections of *Limulus* photoreceptor cells revealed by a photoreceptor-specific monoclonal antibody. Journal of Comparative Neurology 313(4): 553-562.

Campbell LI, Rota-Stabelli O, et al. (2011). MicroRNAs and phylogenomics resolve the relationships of Tardigrada and suggest that velvet worms are the sister group of Arthropoda. Proceedings of the National Academy of Sciences of the United States of America 108(38): 15920-15924.

Chamberlain SC & Barlow RB (1978). Innervation patterns of single optic nerve fibers in the *Limulus* brain: a modification of the cobalt staining technique for cut axons. Vision Research 18(10): 1427-1433.

Chamberlain SC & Barlow RB (1980). Neuroanatomy of the visual afferents in the horseshoe crab (*Limulus polyphemus*). Journal of Comparative Neurology 192(2): 387-400.

Chippaux JP & Goyffon M (2008). Epidemiology of scorpionism: a global appraisal. Acta tropica 107(2): 71-79.

Dohle W (2001). Are the insects terrestrial crustaceans? A discussion of some new facts and arguments and the proposal of the proper name 'Tetraconata' for the monophyletic unit Crustacea plus Hexapoda. Annales de la Societe Entomologique de France 37(1-2): 85-103.

Dunlop JA (2010). Geological history and phylogeny of Chelicerata. Arthropod Structure & Development 39(2): 124-142.

Dunlop JA (2011). Fossil Focus: Chelicerata. Palaeontology Online 1: 1-8.

Dunlop JA & Arango CP (2005). Pycnogonid affinities: a review. Journal of Zoological Systematics and Evolutionary Research 43(1): 8-21.

Dunlop JA & Webster M (1999). Fossil evidence, terrestrialization and arachnid phylogeny. Journal of Arachnology 27(1): 86-93.

Eakin RM & Westfall JA (1965). Fine structure of the eye of *Peripatus* (Onychophora). Zeitschrift für Zellforschung und mikroskopische Anatomie 68(2): 278-300.

Elofsson R (2006). The frontal eyes of crustaceans. Arthropod Structure & Development 35(4): 275-291.

Fischbach KF & Dittrich APM (1989). The optic lobe of *Drosophila melanogaster*. I. A Golgi analysis of wild-type structure. Cell and Tissue Research 258(3): 441-475.

Fleissner G (1985). Intracellular recordings of light responses from spiking and nonspiking cells in the median and lateral eyes of the scorpion. Naturwissenschaften 72(1): 46-48.

Garwood RJ & Edgecombe GD (2011). Early terrestrial animals, evolution, and uncertainty. Evolution: Education and Outreach 4(3): 489-501.

Giribet G & Edgecombe GD (2012). Reevaluating the arthropod tree of life. Annual review of entomology 57: 167-186.

Giribet G, Edgecombe GD, et al. (2001). Arthropod phylogeny based on eight molecular loci and morphology. Nature 413(6852): 157-161.

Giribet G, Edgecombe GD, et al. (2002). Phylogeny and systematic position of Opiliones: A combined analysis of chelicerate relationships using morphological and molecular data. Cladistics 18(1): 5-70.

Golgi C (1873). Sulla struttura della sostanza grigia del cervello. Gazzetta Medica Italiana Lombardia 33: 244-246.

Hafner GS (1973). The neural organization of the lamina ganglionaris in the crayfish: A Golgi and EM study. Journal of Comparative Neurology 152(3): 255-279.

Hanström B (1926). Das Nervensystem und die Sinnesorgane von *Limulus polyphemus*. Lunds Universitets Årsskrift 22(5): 1-79.

Hanström B (1926). Eine genetische Studie über die Augen und Sehzentren von Turbellarien, Anneliden und Arthropoden (Trilobiten, Xiphosuren, Eurypteriden, Arachnoiden, Myriapoden, Crustaceen und Insekten). Kungliga Svenska Vetenskapsakademiens Handlingar 4(1): 1-176.

Hanström B (1928). Vergleichende Anatomie des Nervensystems der wirbellosen Tiere: unter Berücksichtigung seiner Funktion. Berlin, J. Springer: 628pp.

Harrison PJH & Sandeman DC (1999). Morphology of the nervous system of the barnacle cypris larva (*Balanus amphitrite* Darwin) revealed by light and electron microscopy. The Biological Bulletin 197(2): 144-158.

Harzsch S (2006). Neurophylogeny: Architecture of the nervous system and a fresh view on arthropod phyologeny. Integrative and Comparative Biology 46(2): 162-194.

Harzsch S, Vilpoux K, et al. (2006). Evolution of arthropod visual systems: development of the eyes and central visual pathways in the horseshoe crab *Limulus polyphemus* Linnaeus, 1758 (Chelicerata, Xiphosura). Developmental Dynamics 235(10): 2641-2655.

Harzsch S, Wildt M, et al. (2005). Immunohistochemical localization of neurotransmitters in the nervous system of larval *Limulus polyphemus* (Chelicerata, Xiphosura): evidence for a conserved protocerebral architecture in Euarthropoda. Arthropod Structure & Development 34(3): 327-342.

Heinrichs S & Fleissner G (1987). Neuronal components of the circadian clock in the scorpion, *Androctonus australis*: Central origin of the efferent neurosecretory elements projecting to the median eyes. Cell and Tissue Research 250(2): 277-285.

Hennig W (1950). Grundzüge einer Theorie der phylogenetischen Systematik. Berlin, Deutscher Zentralverlag: 370pp.

Heß M, Melzer RR, et al. (1996). The eyes of a nobody, *Anoplodactylus petiolatus* (Pantopoda; Anoplodactylidae). Helgoländer Meeresuntersuchungen 50(1): 25-36.

Holmgren N (1916). Zur vergleichenden Anatomie des Gehirns von Polychaeten, Onychophoren, Arachniden, Crustaceen, Myriapoden und Insekten. Kungliga Svenska Vetenskapsakademien Handlingar 56(1): 1-303.

Homberg U (2008). Evolution of the central complex in the arthropod brain with respect to the visual system. Arthropod Structure & Development 37(5): 347-362.

Jager M, Murienne J, et al. (2006). Homology of arthropod anterior appendages revealed by Hox gene expression in a sea spider. Nature 441(7092): 506-508.

Jensen S (2003). The Proterozoic and earliest Cambrian trace fossil record; patterns, problems and perspectives. Integrative and Comparative Biology 43(1): 219-228.

Kirschfeld K (1967). Die Projektion der optischen Umwelt auf das Raster der Rhabdomere im Komplexauge von *Musca*. Experimental Brain Research 3(3): 248-270.

Koontz MA & Edwards JS (1984). Central projections of first-order ocellar interneurons in two orthopteroid insects *Acheta domesticus* and *Periplaneta americana*. Cell and Tissue Research 236(1): 133-146.

Laurie M (1900). XIX. - On a Silurian Scorpion and some additional Eurypterid Remains from the Pentland Hills. Transactions of the Royal Society of Edinburgh 39(03): 575-590.

Legg DA, Sutton MD, et al. (2013). Arthropod fossil data increase congruence of morphological and molecular phylogenies. Nature Communications 4: 1-7.

Lehmann T, Heß M, et al. (2012). Wiring a Periscope – Ocelli, Retinula Axons, Visual Neuropils and the Ancestrality of Sea Spiders. Plos One 7(1).

Lehmann T, Heß M, et al. (2014). SEM atlas of the Pycnogonida of the Mediterranean Sea: common littoral species. Zoosystematics and Evolution: under review.

Lehmann T, Krapp F, et al. (2014). Pycnogonida - Meeresspinnen. In: Das Mittelmeer: Fauna, Flora, Ökologie – Bd.2/2, Bestimmungsführer. Hofrichter R. Heidelberg, Berlin, Spektrum Akademischer Verlag: in preparation.

Lehmann T & Melzer RR (2011). Retinula axons of Pycnogonida and their terminals in the visual neuropils: Ancestral chelicerate features? Zitteliana Reihe B 30: 32-32.

Lehmann T & Melzer RR (2013). Looking like *Limulus*? – Retinula axons and visual neuropils of the median and lateral eyes of scorpions. Frontiers in Zoology 10(1): 40.

Loesel R, Nässel DR, et al. (2002). Common design in a unique midline neuropil in the brains of arthropods. Arthropod Structure & Development 31(1): 77-91.

Mallatt JM, Garey JR, et al. (2004). Ecdysozoan phylogeny and Bayesian inference: first use of nearly complete 28S and 18S rRNA gene sequences to classify the arthropods and their kin. Molecular Phylogenetics and Evolution 31(1): 178-191.

Manuel M, Jager M, et al. (2006). Hox genes in sea spiders (Pycnogonida) and the homology of arthropod head segments. Development Genes and Evolution 216(7-8): 481-491.

Masta SE, Longhorn SJ, et al. (2009). Arachnid relationships based on mitochondrial genomes: Asymmetric nucleotide and amino acid bias affects phylogenetic analyses. Molecular Phylogenetics and Evolution 50(1): 117-128.

Mayer G (2006). Structure and development of onychophoran eyes: what is the ancestral visual organ in arthropods? Arthropod Structure & Development 35(4): 231-245.

Meinertzhagen IA & O'Neil SD (1991). Synaptic organization of columnar elements in the lamina of the wild type in *Drosophila melanogaster*. Journal of Comparative Neurology 305(2): 232-263.

Meinertzhagen IA & Sorra KE (2001). Synaptic organization in the fly's optic lamina: few cells, many synapses and divergent microcircuits. Progress in brain research 131: 53-69.

Melzer RR (2009). Persisting stemma neuropils in *Chaoborus crystallinus* (Diptera: Chaoboridae): development and evolution of a bipartite visual system. Journal of Morphology 270(12): 1524-1530.

Melzer RR, Michalke C, et al. (2000). Walking on insect paths? Early ommatidial development in the compound eye of the ancestral crustacean, *Triops cancriformis*. Naturwissenschaften 87(7): 308-311.

Melzer RR, Petyko Z, et al. (1996). Photoreceptor axons and optic neuropils in *Lithobius forficatus* (Linnaeus, 1758)(Chilopoda, Lithobiidae). Zoologischer Anzeiger 235(3): 177-182.

Meusemann K, von Reumont BM, et al. (2010). A phylogenomic approach to resolve the arthropod tree of life. Molecular Biology and Evolution 27(11): 2451-2464.

Mittmann B & Scholtz G (2003). Development of the nervous system in the" head" of *Limulus polyphemus* (Chelicerata: Xiphosura): morphological evidence for a correspondence between the segments of the chelicerae and of the (first) antennae of Mandibulata. Development Genes and Evolution 213(1): 9-17.

Mizunami M (1995). Neural organization of ocellar pathways in the cockroach brain. Journal of Comparative Neurology 352(3): 458-468.

Mobbs PG (1976). Development of the locust ocellus. Nature 264: 269 - 271.

Montoya Bravo MF, Meltzer L, et al. (2006). Pycnogonids under infralitoral stones at Cape Savudrija, Northern Adriatic Sea (Pantopoda, Ammotheidae). Spixiana 29(1): 87.

Nässel DR (1975). The organization of the lamina ganglionaris of the prawn, *Pandalus borealis* (Kröyer). Cell and Tissue Research 163(4): 445-464.

Nässel DR (1977). Types and arrangements of neurons in the crayfish optic lamina. Cell and Tissue Research 179(1): 45-75.

Nässel DR, Elofsson R, et al. (1978). Neuronal connectivity patterns in the compound eyes of *Artemia salina* and *Daphnia magna* (Crustacea: Branchiopoda). Cell and Tissue Research 190(3): 435-457.

Oberdorfer MD (1977). The Neural Organization of the First Optic Ganglion of the Principal Eyes of Jumping Spiders (Salticidae). Journal of Comparative Neurology 174(1): 95-117.

Paul DH (1989). A neurophylogenist's view of decapod Crustacea. Bulletin of Marine Science 45(2): 487-504.

Paulus HF (1972). Die Feinstruktur der Stirnaugen einiger Collembolen (Insecta, Entognatha) und ihre Bedeutung für die Stammesgeschichte der Insekten. Zeitschrift für Zoologische Systematik und Evolutionsforschung 10(1): 81-122.

Paulus HF (1979). Eye structure and the monophyly of the Arthropoda. In: Arthropod phylogeny. Gupta AP. New York, Van Nostrand Reinhold Company: 299-383.

Paulus HF (2000). Phylogeny of the Myriapoda–Crustacea–Insecta: a new attempt using photoreceptor structure. Journal of Zoological Systematics and Evolutionary Research 38(3): 189-208.

Pitman RM, Tweedle CD, et al. (1972). Branching of central neurons: intracellular cobalt injection for light and electron microscopy. Science 176(4033): 412-414.

Polis GA (1990). The biology of scorpions. Stanford, Stanford University Press: 587pp.

Regier JC, Shultz JW, et al. (2010). Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. Nature 463(7284): 1079-1083.

Richter S (2002). The Tetraconata concept: hexapod-crustacean relationships and the phylogeny of Crustacea. Organisms Diversity & Evolution 2(3): 217-237.

Richter S, Stein M, et al. (2013). The Arthropod Head. In: Arthropod Biology and Evolution. Minelli A, Boxshall G & Fusco G. Berlin, Heidelberg, Springer-Verlag: 223-240.

Rota-Stabelli O, Campbell L, et al. (2011). A congruent solution to arthropod phylogeny: phylogenomics, microRNAs and morphology support monophyletic Mandibulata. Proceedings of the Royal Society B: Biological Sciences 278(1703): 298-306.

Rudkin DM, Cuggy MB, et al. (2013). An Ordovician Pycnogonid (Sea Spider) with Serially Subdivided 'Head' Region. Journal of Paleontology 87(3): 395-405.

Ruppert EE, Fox RS, et al. (2004). Invertebrate zoology: a functional evolutionary approach. Belmont, Thomson-Brooks/Cole: 963pp.

Saint-Remy GCA (1890). Contribution à l'étude du cerveau chez les arthropodes trachéates. Le grade de docteur es sciences naturelle, Academie de Paris.

Sandeman DC, Sandeman RE, et al. (1990). Extraretinal photoreceptors in the brain of the crayfish *Cherax destructor*. Journal of neurobiology 21(4): 619-629.

Schliwa M & Fleissner G (1979). Arhabdomeric Cells of the Median Eye Retina of Scorpions. I. Fine Structural Analysis. Journal of Comparative Physiology 130(3): 265-270.

Schliwa M & Fleissner G (1980). The lateral eyes of the scorpion, *Androctonus australis*. Cell and Tissue Research 206(1): 95-114.

Scholtz G & Kamenz C (2006). The book lungs of Scorpiones and Tetrapulmonata (Chelicerata, Arachnida): evidence for homology and a single terrestrialisation event of a common arachnid ancestor. Zoology 109(1): 2-13.

Schüller S (1989). Die Pantopodenfauna von Rovinj (Nördliche Adria) und der Jahreszyklus einiger Arten. Bonner Zoologische Beiträge 40: 285-295.

Shultz JW (1990). Evolutionary morphology and phylogeny of Arachnida. Cladistics 6(1): 1-38.

Shultz JW (2007). A phylogenetic analysis of the arachnid orders based on morphological characters. Zoological Journal of the Linnean Society 150(2): 221-265.

Siveter DJ, Sutton MD, et al. (2004). A Silurian sea spider. Nature 431(7011): 978-980.

Stowe S, Ribi WA, et al. (1977). The organisation of the lamina ganglionaris of the crabs *Scylla serrata* and *Leptograpsus variegatus*. Cell and Tissue Research 178(4): 517-532.

Strausfeld NJ (1998). Crustacean–insect relationships: the use of brain characters to derive phylogeny amongst segmented invertebrates. Brain, Behavior and Evolution 52(4-5): 186-206.

Strausfeld NJ (2009). Brain organization and the origin of insects: an assessment. Proceedings of the Royal Society B: Biological Sciences 276(1664): 1929-1937.

Strausfeld NJ (2012). Arthropod brains: evolution, functional elegance, and historical significance. Cambridge, Harvard University Press: 830pp.

Strausfeld NJ & Andrew DR (2011). A new view of insect–crustacean relationships I. Inferences from neural cladistics and comparative neuroanatomy. Arthropod Structure & Development 40(3): 276-288.

Strausfeld NJ & Barth FG (1993). Two visual systems in one brain: neuropils serving the secondary eyes of the spider *Cupiennius salei*. Journal of Comparative Neurology 328(1): 43-62.

Strausfeld NJ, Hansen L, et al. (1998). Evolution, discovery, and interpretations of arthropod mushroom bodies. Learning and Memory 5(1-2): 11-37.

Strausfeld NJ, Strausfeld CM, et al. (2006). Arthropod phylogeny: onychophoran brain organization suggests an archaic relationship with a chelicerate stem lineage. Proceedings of the Royal Society B 273(1596): 1857-1866.

Strausfeld NJ, Strausfeld CM, et al. (2006). The organization and evolutionary implications of neuropils and their neurons in the brain of the onychophoran *Euperipatoides rowelli*. Arthropod Structure & Development 35(3): 169-196.

Strausfeld NJ, Weltzien P, et al. (1993). Two visual systems in one brain: neuropils serving the principal eyes of the spider *Cupiennius salei*. Journal of Comparative Neurology 328(1): 63-75.

Takemura SY, Bharioke A, et al. (2013). A visual motion detection circuit suggested by *Drosophila* connectomics. Nature 500(7461): 175-181.

Takemura SY, Lu Z, et al. (2008). Synaptic circuits of the *Drosophila* optic lobe: the input terminals to the medulla. Journal of Comparative Neurology 509(5): 493-513.

Telford MJ & Thomas RH (1998). Expression of homeobox genes shows chelicerate arthropods retain their deutocerebral segment. Proceedings of the National Academy of Sciences 95(18): 10671-10675.

Toh Y & Yokohari F (1988). Postembryonic development of the dorsal ocellus of the American cockroach. Journal of Comparative Neurology 269(2): 157-167.

Ungerer P & Scholtz G (2008). Filling the gap between identified neuroblasts and neurons in crustaceans adds new support for Tetraconata. Proceedings of the Royal Society B: Biological Sciences 275(1633): 369-376.

Waloszek D & Dunlop JA (2002). A larval sea spider (Arthropoda: Pycnogonida) from the Upper Cambrian 'Orsten'of Sweden, and the phylogenetic position of pycnogonids. Palaeontology 45(3): 421-446.

Westheide W & Rieger R (2006). Spezielle Zoologie: Teil 1: Einzeller und Wirbellose Tiere. Heidelberg, Berlin, Spektrum Akademischer Verlag: 982pp.

Weygoldt P & Paulus HF (1979). Untersuchungen zur Morphologie, Taxonomie und Phylogenie der Chelicerata. II. Cladogramme und die Entfaltung der Chelicerata. Zeitschrift für zoologische Systematik und Evolutionsforschung 17(3): 177-200.

Wheeler WC & Hayashi CY (1998). The phylogeny of the extant chelicerate orders. Cladistics 14(2): 173-192.

Wigglesworth VB (1957). The use of osmium in the fixation and staining of tissues. Proceedings of the Royal Society of London, Series B 147(927): 185-199.

Winter G (1980). Beiträge zur Morphologie und Embryologie des vorderen Körperabschnitts (Cephalosoma) der Pantopoda Gerstaecker, 1863: Entstehung und Struktur des Zentralnervensystems. Zeitschrift für zoologische Systematik und Evolutionsforschung 18(1): 27-61.

8. Danksagung

Ohne die grandiose Betreuung meines Doktorvaters **Prof. Dr. Roland Melzer** wäre diese Arbeit nicht möglich gewesen. Ihm gilt mein größter Dank, für die inspirierenden, lehrreichen und motivierenden Diskussionen, für sein Verständnis und seine Geduld, sowie für die gemeinsamen (Sammel-)Exkursionen ans Mittelmeer.

Besonders danke ich **Stefan Friedrich** für die „expert technical assistance“ in allen Lebenslagen eines Doktoranden.

Meinem Mit-Doktoranden und Freund **Hannes Geiselbrecht** danke ich für die großartige gegenseitige Unterstützung in allen Bereichen während unserer unvergesslichen gemeinsamen Promotionszeit.

Ebenso danke ich **Roland Meyer** für Hilfe, Rat und Tat seit Diplomarbeitszeiten. Auch allen anderen Kolleg[inn]en der Sektion „Arthropoda varia“ – **Ulla Biener-Miller**, **Andrea Weis**, **Dr. Jörg Spelda**, **Dr. Michael Miller**, **Christian Kronmüller**, **Sebastian Swoboda** und **Marc Ritzerfeld** – danke ich für die Mithilfe, die freundschaftliche Arbeitsatmosphäre und die wunderbare Zeit.

Ein herzliches Dankeschön auch an die benachbarte die Sektion „Mollusca”: **Dr. Katharina Jörger**, **Dr. Timea Neusser**, **Bastian Brenzinger**, **Roland Anton** und **PD Dr. Michael Schrödl**. Besonders danken möchte ich dabei **Enrico Schwabe**, der es mir ermöglicht hat mit ihm gemeinsam an der Schiffsexpedition DIVA-Artabria II auf der R/V Hesperides teilzunehmen.

Des Weiteren möchte ich den ZSM- Kolleg[inn]en **Eva Lodde-Bensch**, **Isabella Stöger**, **Horst Schütz**, **Jakob Geck**, **Martin Spies**, **Dr. Jens Bohn**, **Dr. Thomas Knebelsberger** und **Dr. Nico Straube** danken.

Diese Arbeit wäre ohne die Hilfe von Kolleg[inn]en an der Fakultät für Biologie der LMU nicht möglich gewesen. **PD Dr. Martin Heß** danke ich für die Mitautorenschaften und die vielen hilfreichen Anregungen für diese Arbeit, **Heidemarie Gensler** für die Hilfe im Labor und **Prof. Dr. Gerhard Wanner** für die Mitautorenschaft und die Möglichkeit das FIB-SEM zu nutzen.

An dieser Stelle möchte ich auch die **Universität Bayern e.V.** für die finanzielle Unterstützung und das **Institut Ruđer Bošković** in Rovinj/Kroatien dankend erwähnen.

Meiner **Familie** und meinen **Freunden** danke ich für den Rückhalt außerhalb der Welt der Wissenschaft.

Zu guter Letzt möchte ich besonders meiner Frau **Carolin** herzlich danken. Vielen Dank für Alles!!!

9. Appendix

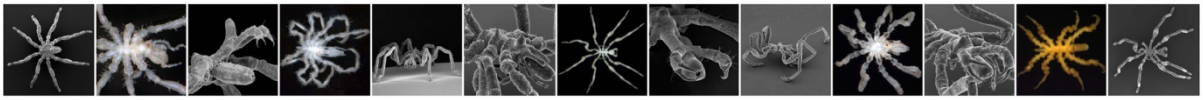
9.1. Relevant Posters

Poster 1 (page 99)
Lehmann T & Melzer RR (2011). Retinula axons of Pycnogonida and their terminals in the visual neuropils: Ancestral chelicerate features?
International Conference on Deep Metazoan Phylogeny - New Data, New Challenges
Ludwig-Maximilians-Universität München (11.-14.10.2011)

Poster 2 (page 100)
Lehmann T & Melzer RR (2012). Retinula axons and visual neuropils of the median and lateral eyes of *Euscorpius italicus* (Herbst, 1800) (Scorpiones: Euscorpiidae)
105. Jahrestagung der Deutschen Zoologischen Gesellschaft (DZG)
Universität Konstanz (21.-24.9.2012)

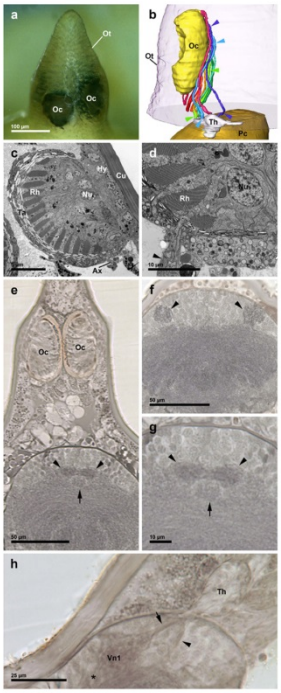
Retinula axons of Pycnogonida and their terminals in the visual neuropils: Ancestral chelicerate features?

Tobias Lehmann and Roland R. Melzer
Zoologische Staatssammlung München
lehmann@zsm.mwn.de, melzer@zsm.mwn.de



INTRODUCTION

The phylogenetic relationships of the Pycnogonida - or sea spiders - within the Arthropoda have been controversial in the last century. Lately sets of neuroanatomical characters[1,2] have contributed important arguments to this discussion. But our knowledge on the visual neuropils connected to the eyes is still at the stage of Hanström's[3] early comprehensive works. In the present study we therefore analyse the visual system of *Achelia langi*, *A. vulgaris*, and *Endeis spinosa* with several neuroanatomical methods: Cobalt backfills, Golgi technique, osmium-ethyl gallate procedure, and TEM.



RESULTS

The visual system of the studied pycnogonids from distal to proximal is composed of:

① Four ocelli in an eye tubercle with a "pseudoinverted" retina (Figs. a-d)

② A dorsolateral thickening where the fibre bundles from the two eyes of each hemisphere concentrate in a primitive type of retinotopy, without forming synaptic varicosities before entering the protocerebrum (Figs. b, h)

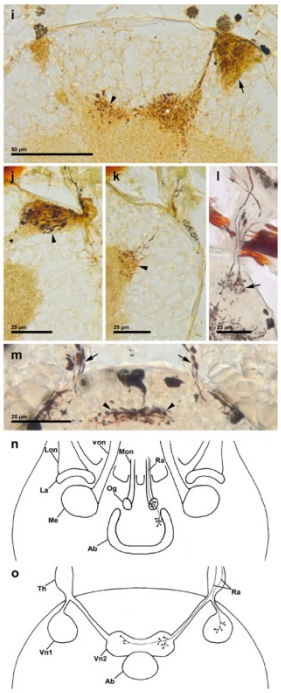
③ Bifurcation of the visual tract (Fig. h)

④ Two successive distinct visual neuropils in each brain hemisphere, a first and a second visual neuropil, both prepossessed by R-cell axons and terminals (Figs. e-m, o)

⑤ A tract originating from the first neuropil, that projects basally into the protocerebrum (Figs. h, j)

⑥ Few retinula cell axons of the right second visual neuropil also terminating in the contralateral left neuropil, and vice versa (Fig.i)

Additionally a roundish, unpaired midline neuropil lies axially beneath left and right second visual neuropil, which can be identified as the arcuate body (Figs. e, g).



Figures | Neuroanatomy of the visual system of *Endeis spinosa* (**a**, **b**), *Achelia vulgaris* (**c-h, j**, **k**, **m**), and *A. langi* (**i**, **l**): TEM (**c**, **d**), Wigglesworth stains (**e-h**), Cobalt backfills (**i-k**), and Golgi technique (**l**, **m**).
Ab, arcuate body; **Ax**, axon; **Cu**, cuticle; **Hy**, hypodermis; **La**, lamina; **Lon**, lateral optic nerve; **Me**, medulla; **Mon**, median optic nerve; **Nu**, nucleus; **Oc**, ocelli; **Og**, ocellar ganglion; **Ot**, ocular tubercle; **Pc**, protocerebrum; **Ra**, retinula axon; **Rh**, rhabdom; **Ta**, tapetum; **Th**, thickening; **Vn**, visual neuropil; **Von**, ventral optic nerve.

DISCUSSION

Our studies confirm that the brain area described by Hanström[3] as "Sehmasse" is a genuine visual neuropil, but additionally we found a second genuine visual neuropil.
Summarised, the visual system comprises three main elements: (1) a thickening where the retinotopic fibre bundles from the median eyes are docking and re-assorted; (2) a first; and (3) a second visual neuropil, each targeted by subsets of the retinula axon terminals. Furthermore an arcuate body in close vicinity to the second visual neuropil is found. These highly specific features allow a detailed comparison with the situation found in other arthropods.
The greatest similarities within the Arthropoda are found between Pycnogonida and Xiphosura[4,5,6]: both taxa have (1) a paired nerve that connects the eyes with the brain; (2) two target regions of the median eye R-cells within the brain; and (3) that one of the target regions lies in direct vicinity to an unpaired midline neuropil, i.e. arcuate body.
The visual system in sea spiders shows therefore far more similarities to those in basal xiphosurans, than to those in derived chelicerates like scorpions and spiders. This represents another argument for placement of the sea spiders at the base of the Chelicerata or even the Euarthropoda, as suggested by recent molecular trees.

ACKNOWLEDGEMENTS
This study was supported by a Graduiertenstipendium (BayEFG) from the Universität Bayern e.V. to T. Lehmann, by the Deutsche Forschungsgemeinschaft (DFG/Me2683/6-1), and by Tunze Aquarientechnik.

REFERENCES

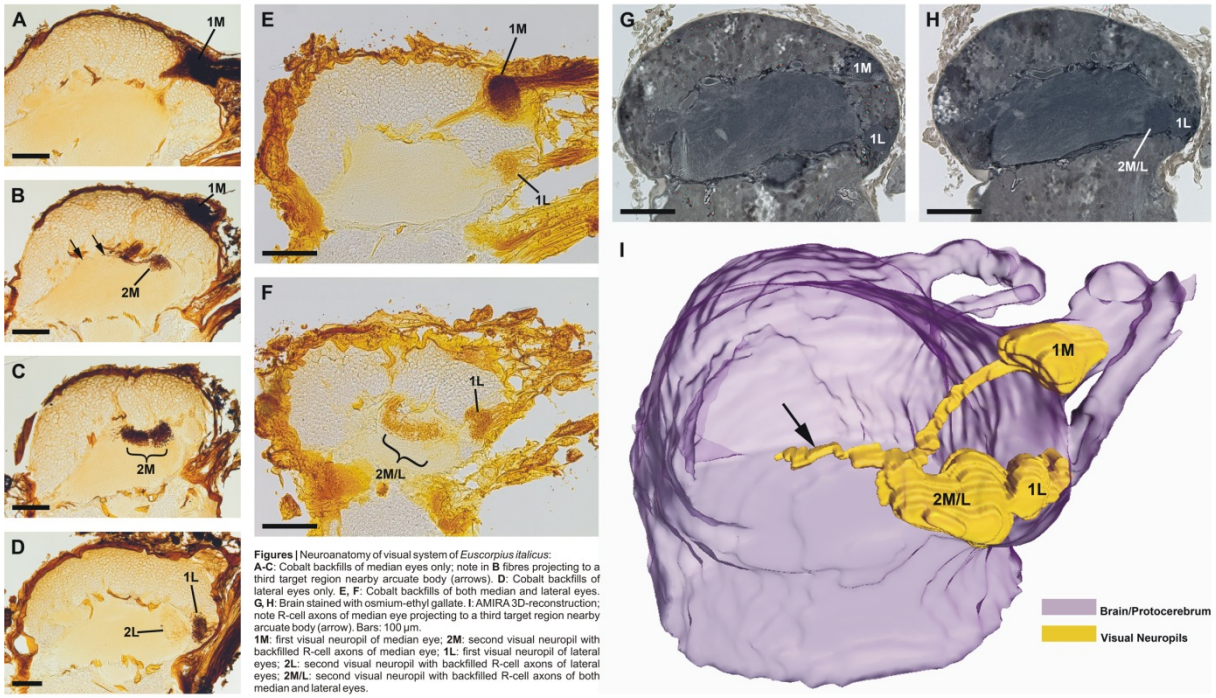
1. Maxmen A, Browne WE, Martindale MQ, Giribet G (2005) Neuroanatomy of sea spiders implies an appendicular origin of the protocerebral segment. Nature 437: 1144-1148.
2. Brenneis G, Ungerer P, Scholtz G (2008) The chelifores of sea spiders (Arthropoda, Pycnogonida) are the appendages of the deutocerebral segment. Evolution & Development 10: 717-724.
3. Hanström B (1928) Vergleichende Anatomie des Nervensystems der wirbellosen Tiere: unter Berücksichtigung seiner Funktion. Springer, Berlin, 628 p.
4. Chamberlain SC, Barlow RB (1980) Neuroanatomy of the visual afferents in the horseshoe crab (Limulus polyphemus). Journal of Comparative Neurology 192: 387-400.
5. Calman BG, Lauerman MA, Andrews AW, Schmidt M, Battelle BA (1991) Central projections of Limulus photoreceptor cells revealed by a photoreceptor-specific monoclonal antibody. Journal of Comparative Neurology 313: 553-562.
6. Harzsch S, Vilpoux K, Blackburn DC, Platchetzki D, Brown NL, et al. (2006) Evolution of arthropod visual systems: development of the eyes and central visual pathways in the horseshoe crab *Limulus polyphemus* (Chelicerata, Xiphosura). Developmental Dynamics 235: 2641-2655.

Retinula axons and visual neuropils of the median and lateral eyes of *Euscorpius italicus* (Herbst, 1800) (Scorpiones: Euscorpiidae)

Tobias Lehmann and Roland R. Melzer
Zoologische Staatssammlung München
lehmann@zsm.mwn.de, melzer@zsm.mwn.de

Lately sets of neuroanatomical characters have contributed important arguments to the discussion about the phylogeny of Arthropoda (Harzsch 2006). Especially the visual system is well studied, which is underlined by the Tetraconata concept (Crustacea + Insecta), where the structure of the eyes is eponymous (Dohle 2001). In contrast the visual system of Scorpiones is studied so far mainly in a neurophysiological context (Fleissner 1985), and its morphological features are yet not described on a level that allows a phylogenetic comparison.

Hence in this study we analyse the morphology of the visual system of *Euscorpius italicus*. The visual neuropils of the median and lateral eyes are identified with Cobalt backfills and the basic structure of the protocerebrum is described by means of osmium-ethyl gallate Procedure and AMIRA 3D-reconstruction.



Figures | Neuroanatomy of visual system of *Euscorpius italicus*:
A-C: Cobalt backfills of median eyes only; note in **B** fibres projecting to a third target region nearby arcuate body (arrows). **D**: Cobalt backfills of lateral eyes only. **E, F**: Cobalt backfills of both median and lateral eyes. **G, H**: Brain stained with osmium-ethyl gallate. **I**: AMIRA 3D-reconstruction; note R-cell axons of median eye projecting to a third target region nearby arcuate body (arrow). Bars: 100 µm.
1M: first visual neuropil of median eye; **2M**: second visual neuropil with backfilled R-cell axons of median eye; **1L**: first visual neuropil of lateral eyes; **2L**: second visual neuropil with backfilled R-cell axons of lateral eyes; **2M/L**: second visual neuropil with backfilled R-cell axons of both median and lateral eyes.

The visual system of *Euscorpius italicus* is composed of:

① Two median ocelli located axially on top of the cephalothorax, and two pairs of lateral ocelli located along the front corners of the cephalothorax.

② Nerve fibres project from the median and lateral eyes, respectively, proximally to the dorso-lateral protocerebrum.

③ The two median eyes supply two successive distinct visual neuropils prepossessed by R-cell axons, at which a few fibres additionally end in a third target region nearby the arcuate body.

④ The two lateral eyes also supply two successive distinct visual neuropils prepossessed by R-cell axons.

⑤ The second visual neuropils of the median and lateral eyes overlap each other, viz. some R-cell axons of the median and lateral eyes end in a shared region.

These findings allow in the future a detailed comparison with the situation found in other Chelicerates like Araneae, Xiphosura, and Pycnogonida (Lehmann *et al.* 2012). Hence the ancestral ground pattern of the visual system of amandibulate Arthropods can be analysed.

References:
Harzsch S (2006) Neurophylogeny: Architecture of the nervous system and a fresh view on arthropod phylogeny. *Integrative and Comparative Biology* 46 (2): 162-194.
Dohle W (2001) Are the insects terrestrial crustaceans? A discussion of some new facts and arguments and the proposal of the proper name 'Tetraconata' for the monophyletic unit Crustacea plus Hexapoda. *Annales De La Societe Entomologique De France* 37: 85-103.
Fleissner G (1985) Intracellular Recordings of Light Responses from Spiking and Nonspiking Cells in the Median and Lateral Eyes of the Scorpion. *Naturwissenschaften* 72: 46-48.
Lehmann T, Heß M, Melzer RR (2012) Wiring a Periscope - Ocelli, Retinula Axons, Visual Neuropils and the Ancestrality of Sea Spiders. *PLoS ONE* 7(1): e30474.

9.2. CD

CD including paper I and II, manuscript of paper III, and the thesis as pdf files.