PhD-Thesis
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Evolution and structure of neuromuscular systems in spiralian meiofauna

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Manuscript II: Detailed reconstruction of the nervous and muscular system of Lobatocerebridae with an evaluation of its annelid affinity. Kerbl A., Bekkouche N., Sterrer W., and Worsaae K. (published)

Manuscript III: Detailed reconstruction of the musculature in Limnognathia maerski (Micrognathozoa) and comparison with other Gnathifera. Bekkouche N., Kristensen R. M., Hejnol A., Sørensen M. V., and Worsaae, K. (published)

Manuscript IV: Nervous system and ciliary structures of Micrognathozoa (Gnathifera) – evolutionary insight from an early branch in Spiralia. Bekkouche N., and Worsaae K. (submitted)

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Abstract

Abstract

Spiralia is a vast clade of Metazoa comprising large and well-known organisms, e.g., Annelida and Mollusca, but also many microscopic animals such as Gastrotricha or Gnathifera (including, Rotifera) of the often overlooked meiofauna. To date, the phylogeny and morphology of Spiralia have been difficult to resolve and understand. The present thesis focuses on spiralian meiofauna to i) reconstruct the phylogeny of this clade using transcriptomics and place enigmatic meiofaunal taxa and ii) resolve the morphology of three important taxa, mainly employing confocal laser scanning microscopy and immunohistochemistry: the spiralian incertae sedis, Lobatocerebrum, the recently described monospecific phylum Micrognathozoa (Gnathifera), and an early branching Gastrotricha, Diuronotus aspetos.

The new spiralian phylogeny reveals with high support that the deepest branches of Spiralia consist of meiofaunal representatives, that Gnathifera is the sister group of remaining Spiralia, that Gastrotricha+Platyhelminthes branches off next and that Lobatocerebrum is an Annelida. The morphological surveys of the musculature, nervous system, glands, and ciliation on three phylogenetically distinct taxa yield more insight into their evolution: Lobatocerebrum is an aberrant annelid showing only few common traits with Annelida, yet, our detailed studies unravel putative resemblances of muscular, nervous and glandular system to previous findings in annelids. Micrognathozoa shows more resemblances with Rotifera than Gnathostomulida (these three taxa together forming Gnathifera). Furthermore, we could infer possible plesiomorphic states of Gnathifera such as the paired ventro-lateral nerve chords (shared with many Spiralia) as well as recover putative Gnathifera apomorphies such as the pharyngeal ganglion; all adding new information on the evolution of this group. Diuronotus aspetos shows a unique combination of muscular traits not easily traceable, but in contrast the nervous system traits can be compared in high details, hereby bridging to other Chaetonotida (Gastrotricha). Moreover, we describe new gastrotrich characters such as the ciliary pattern or a system of pharyngeal canals of possible importance for future comparative approaches.

These different studies show that information on rare and phylogenetically isolated animals with their unique combination of neural and muscular characters are necessary to understand the evolution of Spiralia. Also, several organ systems should be considered for systematic comparisons, here emphasized with ciliary and glandular systems in Micrognathozoa, Gastrotricha and Lobatocerebrum showing potential phylogenetic information.
**Resumé**


De betydeligste resultater af den nye Spiralia fylogeni er, at de dybeste grene af Spiralia består af meiofaunale repræsentanter, at Gnathifera er søster gruppe af de resterende Spiralia, og at Lobatocerebrum er placeret i Annelida. De morfologiske undersøgelser af muskulatur, nervesystemer, kirtler, og ciliation på de tre fylogenetisk adskilte taksa giver yderligere indsigt i deres udvikling: Lobatocerebrum er en afvigende annelid, og viser kun få fælles træk med Annelida. Micrognathozoa viser flere ligheder med Rotifera end Gnathostomulida (disse tre taksa danner tilsammen Gnathifera). Desuden kunne vi udlede mulige plesiomorfiske tilstande i Gnathifera, såsom de parrede ventrolaterale nervefibre (delt med mange Spiralia) eller tilstedevarseln af et svælgganglie, hvilket tilføjer ny information om evolutionen af denne gruppe. Diuronotus aspetos viser en enestående kombination af gastrotrich træk, især kan nervesystemet nemt sammenlignes med andre Chaetonotida (Gastrotricha), desuden beskriver vi nye karakterer såsom de ciliære mønstre, og et system af svælgkanaler der har mulig betydning for fremtidige komparative studier.

Disse studier viser, at sjældne og fylogenetisk isolerede dyr, med deres unikke kombination af neurale og muskulære træk, er nødvendige for at forstå udviklingen af Spiralia. Ydermere, bør hvert organsystem tages i betragtning ved systematiske sammenligninger, da ciliemønstre og kirtelsystemer i Micrognathozoa, Gastrotricha og Lobatocerebrum viser potentiel fylogenetisk information. Endelig, er denne afhandling med til at opklare en række manglende viden om nogle centrale meiofaunale taksa, tilførende sammenligneligt materiale til yderligere forskning.
I) Background and justification of the study

In the past few years, our picture and understanding of the phylogenetic relationships of animals have been greatly changed and improved thanks to the advances in large-scale molecular phylogenies, e.g. (Edgecombe et al. 2011, Dunn et al. 2014, Telford et al. 2015, Halanych 2016). Yet, the evolution of specific organs systems is still far from being understood and explaining their evolution between all the subgroups of animals is still challenging. Although evolutionary developmental biology (evo-devo) e.g. (Arendt et al. 2008, Manuel 2009, Lauri et al. 2014, Marlow et al. 2014, Hejnol and Martin-Duran 2015) and descriptive morphology e.g. (Schmidt-Rhaesa 2007, Brusca et al. 2016, Schmidt-Rhaesa et al. 2016) have done great advances in describing and understanding the body patterning and the organization of specific organs systems of many taxa, the overall picture of how these structures are related is still unclear (Hejnol and Lowe 2015).

One of the main questions still left is the size and complexity of the first Bilateria. During the last decades, studies on complex model organisms (e.g. mouse, zebrafish, fruit fly) showed that the genetic underlying mechanisms patterning complex organs and their arrangement are similar, and thus thought to be inherited from a common ancestor, e.g. (Prud'homme et al. 2003, Arendt et al. 2008). However, many groups of animals of smaller size are still unstudied when it comes to evo-devo, phylogenetic sampling or morphology (Hejnol et al. 2015). This inequality in the study of different groups of animals can lead to a bias in the reconstruction of animal evolution, and these gaps need to be filled.

Meiofauna, or meiobenthos, consists of animals passing through a 1mm sieve and retained by a 42µm sieve (Higgins and Thiel 1988). This very practical and arbitrary definition with limited zoological information includes taxonomically and ecologically diverse animals, e.g. exclusively meiofaunal Gastrotricha; marine, freshwater or inland like Rotifera; sessile animals such as urochordates; non-vermiform forms like Cnidarians. Therefore, many animals belong to meiofauna, and – due to the difficulty to collect and manipulate them – they are still understudied. However, their size is not proportional to their evolutionary relevance, and many groups with a crucial phylogenetic position belong to meiofauna (Rundell and Leander 2010). Spiralia, one of the three largest groups of Bilateria next to Ecdysozoa (e.g. insects, nematodes) and Deuterostomia (e.g. vertebrates, echinoderms) counts several primitively meiofaunal clades,
such as the Gastrotricha and the three Gnathifera taxa: Gnathostomulida, Micrognathozoa (including one species: Limnognathia maerski Kristensen and Funch, 2000 and Rotifera. Additionally, phylogenies of Platyhelminthes indicated a meiofaunal origin of this group comprising many secondarily large sized animals (Egger et al. 2015, Laumer et al. 2015) (but see Ax, 1956, showing that this was long suspected). Gnathifera, Gastrotricha and Platyhelminthes together form the disputed group “Platyzoa” (Cavalier-Smith 1998), a taxon found by molecular phylogenies but with no morphological justification, and many authors have questioned its relevance e.g. (Zrzavý 2003, Dunn et al. 2008, Giribet 2008). Furthermore, two other genera of special interest in this thesis, Diurodrilus and Lobatocerebrum, so far had a disputed position within Spiralia (Rieger 1980, Jenner and Littlewood 2008, Worsaae and Rouse 2008). Even though they have originally been supposed to belong to Annelida, further studies have questioned their annelid affinities. In this context, understanding the phylogenetic position of these groups as well as describing their morphology is necessary.

II) Aims

The purpose of this work is to describe the morphology of some of these animals to enhance our knowledge on animal evolution as well as to integrate them in a phylogenetic context, therefore focusing on:

-A large transcriptomic data set which was analyzed in order to position Diurodrilus, Lobatocerebrum and Micrognathozoa within the Spiralian phylogeny, as well as to resolve the platyzoan relationships (Laumer et al. 2015), and assess the importance of meiofauna in the evolution of this group (manuscript I).

-Lobatocerebrum, a former incertae sedis not studied for more than 30 years since its discovery (Rieger 1980, Rieger 1981), and the description of its muscular, nervous and glandular system in order to better understand its annelids affinities (Kerbl et al. 2015) (manuscript II).

-the recently described Micrognathozoa (Kristensen et al. 2000) and still unknown internal anatomy with study of its musculature (Bekkouche et al. 2014) (manuscript III), nervous system and ciliation (manuscript IV), aiming to shed new light on the evolution of Gnathifera, as it is the sister group of other Spiralia.
the gastrotrich key taxon *Diuronotus*, and its detailed morphology in order to have a better understanding of the evolution and diversity of the internal organ systems of Gastrotricha with the study of an important taxon in the morphologically poorly known Chaetonotida (manuscript V).

While the first manuscript provides the phylogenetic framework of this thesis, the studied organisms show three distinct examples of the diversity of meiofauna within Spiralia: i) *Lobatocerebrum riegeri* Kerbl et al., 2015 shows a case of a highly divergent meiofaunal animal among a well defined group of mainly macrofaunal animals, Annelida), ii) *Limnognathia maerski* is an example of a meiofaunal species so distinct from other groups that it justified (according to some authors) the erection of a supra-specific rank (Kristensen et al. 2000, Giribet et al. 2004), iii) *Diuronotus aspetos* Todaro et al., 2005, one species within the relatively well known meiofaunal group Gastrotricha. These three case studies illustrate our lack of knowledge on spiralian meiofauna and their internal anatomy, and the here presented thesis aims to elucidate the anatomy of each of these taxa in order to evaluate if their morphology can be of comparative relevance at their very different phylogenetic levels. It also aims to offer comprehensive descriptions in order to provide relevant comparative information for further studies on closely related organisms.

**III) Scientific justification**

The introduction of systematic phylogenetics (cf. cladistics) by Willi Hennig in 1966 (Hennig 1966), led to a scientific Khunian revolution (Kuhn 1962) in the domain of systematics and evolutionary biology. This theory did not only initiate deep conceptual changes in the interpretation of the phylogenetic relationship between organisms, but also in the use and interpretation of the characters themselves. Cladistics proposed a method where characters could be discussed and used in a transparent way for phylogenetic reconstruction, contrasting with the previously employed evolutionary systematics. However, soon after e.g. (Field et al. 1988), the field of molecular systematics has undergone a rapid increase until today, and consequently, the discipline of morphology has been in a “crisis”. Indeed, the increasing availability of molecular data seemed to have very quickly outcompete the use of morphological data for phylogenetic reconstruction, sometimes consigning morphology to a simple descriptive discipline, e.g. (Mooi and Gill 2010, Jenner 2011). This replacement however, was not the consequence of theoretical justifications,
but only of technical advances. Therefore morphology has no philosophical reasons to be excluded and should not be forgotten.

In this context, morphology is an important ontological tool. Indeed a mandatory descriptive step/process is necessary in order to define the entities zoologists are discussing in evolution, and the evolutionary interpretation of morphological structures comes in three steps:

- The first step is to understand and describe these structures in a formal way in order to make them comparable with other structures of the same organism and of other organisms.

- The second step is to actually compare these structures with other organisms/taxa and state hypotheses about homology relationships.

- The third step is to interpret the relationship of these structures; are they homologous as supposed in the second step or not?

If these three steps are not necessarily well segregated in the scientific process, the first one corresponds to the field of descriptive morphology, the central point of the present thesis. The two subsequent steps belong to the field of phylogenetic reconstruction and interpretation, but depend directly on the first step.

The recent description of new so-called “phyla” (Ax 1956, Kristensen 1983, Funch and Kristensen 1995, Kristensen et al. 2000, Kristensen 2002) resulted in more questions about the understanding of animal evolution than expected, mainly because both comprehensive molecular and morphological datasets were not available at the time. Although the aim of this thesis is partly to gather and provide new molecular data on these taxa (i.e. transcriptomes), its main goal is to morphologically describe these various lesser known animals to also supplement the morphological dataset. For this purpose, we mostly applied Confocal Laser Scanning Microscopy (CLSM) and widely used fluorescent histochemical stainings to label and investigate the nervous system and the musculature (DAPI, phalloidin, antibodies directed against tyrosinated α-tubulin, acetylated α-tubulin, serotonin and FMRF-amide). Indeed, these two organ systems have been widely studied and included in phylogenetic discussions (see for instance, among other textbooks, “The evolution of organ systems” (Schmidt-Rhaesa 2007), and the “Handbook of Zoology: Gastrotricha and Gnathifera” (Schmidt-Rhaesa 2015), or “Structure and evolution of Invertebrates
nervous systems” (Schmidt-Rhaesa et al. 2016)). It is not to say that these organ systems are always straightforward to compare and homologize between different animals, but that the large available literature, on top of their crucial biological function, make them very suitable organ systems for morphological comparison across phylogenetically diverse animals.

IV) Methods of investigation

A) Collection of material
First of all, animals are collected and fixed for the needed studies. Most material is collected via magnesium chloride narcotization and decantation (Higgins et al. 1988), i.e. the animals in the sediment are anesthetized with isotonic MgCl$_2$, suspended by agitation with the surrounding organic matter, and concentrated. Thereafter, the extract is deposited on a sieve after washing the MgCl$_2$, to allow the animals to crawl through the mesh and get separated from the retained organic matter. Then animals are collected individually and fixed in the appropriate manner (Glutaraldehyde/trialdehyde for electron-microscopy, paraformaldehyde for confocal microscopy, ethanol for molecular analysis, etc.). One of the major limitations of meiofauna studies is the accessibility and difficulty to manipulate animals. Indeed, some animals: i) have remote locations such as Limnognathia maerski and Diuronotus aspetos found in Greenland, ii) have very strict seasonality as Limnognathia maerski found only in summer, iii) have patchy distribution e.g. the fortunate finding of many Diurodrilus subterraneus Remane, 1934 in few spots on a beach in Sweden (Ystad) allowed us to collect sufficient material for transcriptomics (Laumer et al. 2015), or iv) are extremely rare as the finding of nine specimens of Lobatocerebrum riegeri in Israel was only permitted through the joint effort of four people over the course of two weeks (Kerbl et al. 2015). Furthermore, the size of these animals makes them easy to lose and break during manipulation, and difficult to mount for high-magnification microscopy.

B) Confocal Laser Scanning Microscopy (CLSM)
Morphological characters in this thesis are mainly described with the use of CLSM with immunohistochemistry and fluorescent stainings. The main organs targeted will be musculature, nervous system and ciliation (locomotory, sensory, etc.). The size of meiofaunal animals is especially suitable for CLSM (Wanninger 2007, Leasi and Todaro 2008), indeed, the high resolution
of this technique allows us to reconstruct details of the animal at the cellular level. Additionally, entire animals can be mounted and scanned. The transparency and the thinness of these animals also permit the light to go through the animals with low to virtually no loss of signal. The output is a 3D reconstruction of the targeted organ systems, giving an overall picture of the organic arrangement. Data are then interpreted with 3D imaging software.

It is necessary to emphasize that CLSM only reveals the 3D repartition of a specific fluorescence within the animal, which carries several limitations, as for instance i) an overlapping of different fluorochrome fluorescence leading to a limited segregation of the different stainings, ii) the auto-fluorescence of non-targeted structures, iii) the non-specificity of the antibodies iv) the non-extensiveness of some stainings only revealing a subpart of an organ system.

On the other hand, some of these limitations can be used in a positive way. Auto-fluorescence and non-specificity can lead to the recognition and the characterization of additional and unexpected structures, and the non-extensiveness of staining can lead to specific characterization of some structure, as for instance the recognition of very nerve cells.

C) Transmission electron microscopy (TEM)

Before the use of CLSM, TEM was one of the most widely used method of comparative biology of meiofauna and a profusion of work illustrates this statement, e.g. (Harrison and Ruppert 1991, Ahlrichs 1993, Wiedermann 1995, Kristensen et al. 2000). Indeed, thanks to its very high resolution, TEM offers the accessibility of a wide variety of characters that the size and simplicity of meiofauna does not offer at the dimensions of conventional light microscopy. Therefore, a great amount of details has been collected on the ultrastructure of many organs of meiofaunal animals. Unfortunately, these data often neglect the 3D arrangement of large organ systems throughout the body of these animals. This emphasizes how TEM is complementing CLSM, each technique providing information at a specific level. However, it is easy to overlook structures on TEM as illustrated by the examples of the specific pharyngeal cilia in *Limnognathia maerski* (manuscript IV), or of a canal system in the pharynx of *Diuronotus aspetos* (manuscript V), although visible in the previous publications (Ruppert 1991, Kristensen et al. 2000). On the other hand, a clear demarcation between *Lobatocerebrum psammicola* Rieger, 1980 and
*Lobatocerebrum riegeri* due to the difference of the glandular system would not have been possible without TEM.

D) Phylogenetic reconstruction

Two phylogenetic analyses using molecular data have been conducted in this thesis, each with quite different objectives. The first one, a transcriptomic analysis involving hundreds of genes, acquired through transcriptome sequencing, aimed at resolving the interrelation of Spiralia and placing several taxon of zoological importance (Laumer et al. 2015). The second one, more humble, aimed at confirming the morphological placement of a genus into a recently erected family of Gastrotricha (Leasi et al. 2008) via target sequencing and the use of only three loci (manuscript V). However, these two approaches offer an important framework for the interpretation of morphological evolution within the studied groups.

V) Filling the interstitial gaps of the spiralian phylogeny

**Manuscript I: Spiralian Phylogeny Informs the Evolution of Microscopic Lineages**

Laumer, C. E., Bekkouche, N., Kerbl, A., Goetz, F., Neves, R. C., Sørensen, M. V., Kristensen, R. M., Hejnol., Dunn C. W., Giribet G. and Worsaae K.

A) Results and implications of the new phylogeny of Spiralia

So far phylogenomics reconstruction of the spiralian phylogeny with high support was difficult (Giribet 2008, Hejnol et al. 2009, Kocot 2016), hampering the understanding of animal evolution. However, the sampling has previously been heavily biased toward macroscopic animals. The here presented study focused on the placement of several enigmatic taxa, but also on proposing the most comprehensive sampling of microscopic animals ever used in a phylogenomic study of Spiralia. This sampling comprised the three taxa Micrognathozoa, *Lobatocerebrum* and *Diurodrilus* previously unplaced with molecular data. Besides, it also included already previously considered taxa as two representatives of Gnathostomulida, *Diuronotus aspetos* as a key taxon in Gastrotricha, and a representative of Catenulida, the sister group of the remaining
Platyhelminthes (Larsson and Jondelius 2008, Egger et al. 2015, Laumer et al. 2015). The results showed that Gnathifera represent the sister group of other Spiralia, and that Rouphozoa (Platyhelminthes + Gastrotricha) is the sister group to the remaining Spiralia. This confirms the results by Struck et al. in 2014 (Struck et al. 2014) who found a similar topology. However, Struck et al. could not recover the monophyly of Gastrotricha (only when removing Lepidodermella, and Dactylopodola) and their study did not include Micrognathozoa. The stronger support for the topology of our study has important consequences since it points to a meiofaunal origin of Spiralia (Vinther 2015) (Fig. 1) and questions the evolution of many central characters. Indeed, under this topology, it is unclear if the plesiomorphic conditions of Spiralia involve the presence of: a coelom (body cavity), an anus, spiral cleavage in the early developmental stages, or two separated ventrolateral nerve cords (Hejnol et al. 2015, Kocot 2016). Therefore, it is very plausible that the ancestor of Spiralia, and maybe Bilateria, was a small acoelomate animal lacking an anus and having direct development. However, this can only be confirmed with the resolution of i) the placement of Cyclophora, ii) the placement of Chaetognatha, iii) the resolution of the phylogeny of Ecdysozoa, to reconstruct the ancestral meiofaunal or macrofaunal state of this group.
Figure 1: Summary of the phylogenetic tree of Laumer et al. 2015 (Laumer et al. 2015). Mi and Ma show groups with primitively assumed “Microscopic” or “Macroscopic” condition, respectively.

B) Impact of the study on subsequent studies

Adding to these general results, more specific relations have to be mentioned here since they have implications on the other parts of this thesis presented below:

-The phylogenetic position of *Lobatocerebrum* as an annelid and sister group to Sipuncula, another taxon of very peculiar unsegmented annelids. This refutes the idea that *Lobatocerebrum* represents its own group within Spiralia and shows a case study of loss of characters related to miniaturization.

-The sister group relationship between Micrognathozoa and Rotifera as already strongly suggested by some authors (Ahlrichs 1997, Kristensen et al. 2000, De Smet 2002, Wulfken and Ahlrichs 2012),
but not yet confirmed with molecular data (Giribet et al. 2004, Worsaae et al. 2008), reinforcing the monophyly of Gnathifera.

-The sister group relationship between Gastrotricha and Platyhelminthes. Although this relationship is still difficult to interpret and has little implication on the present studies of Gastrotricha, it is worth mentioning that the inclusion of Diuronotus aspetos in this phylogenomic study seems to stabilize the position of Lepidodermella squamata, leading to the recovery of the monophyly of Gastrotricha.

VI) The “resurrection” of Lobatocerebrum: the enigmatic Spiralia is now an enigmatic Annelida

Manuscript II: Detailed reconstruction of the nervous and muscular system of Lobatocerebridae with an evaluation of its annelid affinity. Kerbl A., Bekkouche N., Sterrer W., and Worsaae K.

A) Introduction and studies on Lobatocerebrum

Lobatocerebridae is a family of Annelida described in 1980 (Rieger 1980), originally comprising one species, and now one new described species in the manuscript presented in this study (Kerbl et al. 2015). This family of very long and slender, worm-like, completely ciliated, and very elusive animals has puzzled zoologists for a long time (Rieger 1980, Rieger 1981, Haszprunar et al. 1991, Zrzavý et al. 2001, Zrzavý 2003, Jenner et al. 2008), and could not be previously placed in the Metazoan phylogeny. Despite the cosmopolitan repartition of these animals (Rieger 1980, Kristensen 1983, Kerbl et al. 2015, Laumer et al. 2015), they are so rare and discrete that studies on their morphology have been scarce since their discovery (as shown by the collection of “only” nine specimens by four persons over the course of two weeks (Kerbl et al. 2015)). However, one of the manuscripts of the presented thesis (manuscript I) confirmed, with the use of transcriptomics, the previously suspected inclusion of Lobatocerebrum within Annelida. This phylogenetic placement warranted a re-assessment of the morphology of Lobatocerebrum with modern techniques. Therefore we described the musculature, nervous system and glandular system of Lobatocerebrum with CLSM, complemented with TEM and live observations. The results mostly
confirmed the findings of Rieger, 1980 (Rieger 1980), but CLSM permitted a better three-dimensional understanding of these animals and allowed us to describe the internal anatomy with more details as well as to find previously undescribed structures. The study of the musculature confirmed the inner position of the circular muscles relative to the longitudinal muscles, with the circular muscles actually being “transverse muscular ring complexes”, consisting of individual diagonal fibers originating from one longitudinal muscle and extending to the next one on the transversal section. Several of these muscles are giving together the impression of a continuous circular muscle. Similar muscles, though crossing each other, give a star appearance and are only found in the rostrum. The nervous system investigation confirmed the presence of a prominent lobular brain, a pair of ventro-lateral nerve cords extending along the entire body length, and a pair of subpharyngeal ganglia supplying a pair of commissures. Additionally, details of the brain and anterior nerves were given, and we documented the presence of a previously undescribed unpaired median longitudinal nerve as well as two trunk commissures without associated ganglia. The presence of the median nerve and of additional commissures weakly corroborates an annelid affinity see (Kerbl et al. 2015) for a full review. Finally, a new species of Lobatocerebrum, Lobatocerebrum riegeri, was described due to its different proportions, glandular system and geographical position differing from the previously assessed Lobatocerebrum psammicola. To summarize, the detailed morphological re-description of Lobatocerebrum does not show any unambiguous trait relating it to Annelida, but the combination of characters such as the complex brain with numerous commissures, the median nerve cord and the ganglionated commissures, corroborates, without confirming, its relation with annelids. Finally, the present phylogenomic (Laumer et al. 2015) and morphological (Kerbl et al. 2015) studies indicate that Lobatocerebrum is another aberrant annelid, extending the already long list, e.g. (Zrzavý et al. 2009, Weigert 2016).

**B) Further possible researches around Lobatocerebridae**

Unfortunately, the extremely divergent morphology of Lobatocerebrum does not give many insights on its origin within Annelida. Furthermore, developmental and in-situ hybridization researches on Lobatocerebridae appear so far unrealistic due to the extreme elusiveness of this animal. This suggests that further morphological investigations of Lobatocerebrum psammicola and L. riegeri are unlikely to shed light on its evolution. On the other hand, further researches on the phylogenetic placement of interstitial annelids could lead to a better understanding of the
origin of *Lobatocerebrum*, and of the numerous other interstitial annelids (Westheide 1990). Fortunately, recent studies appear to move toward a better phylogenetic placement of the different interstitial families of annelids (Andrade et al. 2015, Laumer et al. 2015, Struck et al. 2015). One of these studies (Struck et al. 2015) indicates that there might have been two large interstitial radiations in Annelida (not including *Lobatocerebrum*), comprising among others, Protodrilidae and Dinophilidae, respectively. However, these studies do not include e.g. Psammodrilidae, Parergodrilidae, Aelosomatidae and *Hrabeiella*, which are still to place. Furthermore, Problematica still exist around Annelida, namely *Jennaria pulchra* Rieger, 1991, or the parasitic Orthonectida. *Jennaria pulchra* demonstrates a similar case to *Lobatocerebrum* in being an interstitial vermiform animal with no apparent segmentation, and it was suggested to be related to Annelida in its original description (Rieger 1991). Unfortunately, this animal has never been reported after its description despite intensive researches (Worsaae personal communication). Although Rieger (1991) rejected a sister group relationship with Lobatocerebridae, molecular and morphological studies with recent methods could confirm or reject this hypothesis. Ultrastructural studies on Orthonectida have suggested that they may be related to Annelida (Slyusarev and Kristensen 2003), and molecular phylogeny could not reject this hypothesis (Petrov et al. 2010). Interestingly, a recent study by Slyusarev and Starunov (2016) reconstructed details of the musculature of one species of Orthonectida, thereby showing circular muscles inside the longitudinal musculature, with the circular ones seemingly originating from the longitudinal fibres – an intriguing configuration very similar to what is found in *Lobatocerebrum*. Last but not least, more species of Lobatocerebridae are suspected (Rieger 1980, Kristensen 1983), which could potentially offer a broader morphological diversity of the family, and give more elements to understand their evolution. Finally, the resolution of one or several mentioned lacks in the knowledge of aberrant annelids could help to better understand the evolution of *Lobatocerebrum* and divergent annelids. In conclusion, although our current understanding of the morphology of *Lobatocerebrum* is of limited use to unravel annelid evolution, the thorough morphological description provided in this thesis was necessary to give a comparative framework for all possible further research approaches mentioned above.
VII) Micrognathozoa, the third member of Gnathifera

Manuscript III: Detailed reconstruction of the musculature in *Limnognathia maerski* (Micrognathozoa) and comparison with other Gnathifera. Bekkouche N., Kristensen R. M., Hejnol A., Sørensen M. V., and Worsaae, K.

Manuscript IV: Nervous system and ciliary structures of Micrognathozoa (Gnathifera) – evolutionary insight from an early branch in Spiralia. Bekkouche N., and Worsaae K. (submitted)

A) Introduction: the importance of Micrognathozoa

In 1994, Kristensen and Funch found a small ciliated organism bearing jaws in a fresh water pound in Greenland, with all found specimens apparently being female (Kristensen et al. 2000). The presence of complex jaws in this animal allowed the authors to immediately relate this new organism to the well-known rotifers. However, it possessed ventral ciliation and lacked the ciliated corona, contrary to Rotifera. In 1995, Rieger and Tyler (Rieger and Tyler 1995) proposed a sister group relationship between the jawed Gnathostomulida and Rotifera (including Acanthocephala) due to the similar ultrastructure of the jaws consisting of parallel rods with an electrodense core and an electroluscent cortex. At the same time, Ahlrichs in 1995 (Ahlrichs 1995) proposed the name Gnathifera for this clade. The unification of Gnathostomulida and Rotifera has been encouraged by the discovery of the new animal, which was not formally described at this time, but only informally discussed between zoologists. The proposed character unifying Gnathifera was again the ultrastructure of the jaws (Rieger et al. 1995). This close relationship between Rotifera and Gnathostomulida finally permitted to relate together two “aschelminthes” of previously uncertain phylogenetic placement. Indeed, many interrelationships between Rotifera, Gnathostomulida and other taxa were proposed, as for instance, Rotifera + Platyhelminthes (Markevich 1993) or Gnathostomulida + Gastrotricha (Rieger 1976, Zrzavý et al. 2001) or Gnathostomulida + Platyhelminthes (Ax 1956, Ax 1996). Finally, in 2000, the new taxon was formally described as *Limnognathia maerski* Kristensen and Funch, 2000 (Kristensen et al. 2000), belonging to the monospecific class Micrognathozoa within the phylum Gnathifera. Later on, in 2004, Giribet et al.
(Giribet et al. 2004) attempted to place Micrognathozoa in the animal phylogeny using four different genes, but the results had very low support. Additionally, this study introduced Micrognathozoa as its own phylum. Ranks relevance can be discussed (see Giribet (2016) for recent discussions on this matter), but whether Micrognathozoa are a class, a phylum or only one species does not matter much in this discussion. In any case, this ranking emphasizes the interest of zoologist for this singular animal.

The original description of Micrognathozoa (Kristensen et al. 2000) provided numerous details of the jaws anatomy as well as some information on the inner anatomy and the ultrastructure of the animal. However the discussion of this manuscript was more focused on the phylogenetic implication of Limnognathia maerski than on its internal morphology. The complexity of the jaws of Micrognathozoa continued to attract the curiosity of zoologists and the two following morphological works were fully focused on the details of the jaws (De Smet 2002, Sørensen 2003). Interestingly, De Smet found some animals in subantarctic islands (Crozet Island), and the detailed study of the jaws did not show any difference to the Greenlandic animals, leading to the conclusion that Micrognathozoa from Greenland and Crozet Island belong to the same species.

Until the present study, these few works constituted almost the totality of the knowledge we have on Micrognathozoa. Additionally, molecular studies supported the monophyly of Gnathifera, without placing the Micrognathozoa (Witek et al. 2009, Struck et al. 2014). Furthermore, recent phylogenies showed the importance of Gnathifera since they seem to be the sister group to all the other Spiralia (Struck et al. 2014, Laumer et al. 2015). This stresses two important needs: resolving the internal relationships inside the Gnathifera, which today appears to be attained, and acquiring more information on the morphology of different Gnathifera. The very rare information on the internal anatomy of Micrognathozoa and their systematic interest makes them a crucial target of this study.

**B) The musculature of Micrognathozoa** (Bekkouche et al 2014)

The muscular reconstruction of Limnognathia maerski reveals a quite peculiar arrangement difficult to relate to other Gnathifera (Bekkouche et al. 2014). The body wall musculature consists of seven major longitudinal muscles in the trunk and 13 pairs of dorsoventral muscles. This organization in discrete and well separated muscles bundles is more similar to the musculature
found in Rotifera, e.g. (Sørensen 2005, Leasi and Ricci 2010, Leasi et al. 2012), than the one found in Gnathostomulida (Tyler and Hooge 2001, Müller and Sterrer 2004). Additionally, some longitudinal muscles of L. maerski do not extend throughout the entire body, compartmentalizing the body in different regions similar to what is observed is Rotifera, e.g. (Leasi et al. 2010, Leasi et al. 2012). Moreover, the dorso-ventral muscles of L. maerski resemble the dorso-ventral or semi-circular muscles found in many Rotifera. However, there also are differences since the dorso-ventral muscles of L. maerski are positioned inside the longitudinal musculature, while they are positioned outside in Rotifera (Leasi et al. 2010) (this is also the case in Gnathostomulida (Müller et al. 2004))(Fig. 2). It is therefore likely that the dorso-ventral musculature of L. maerski is not homologous to the circular muscles of Gnathostomulida and the dorso-ventral muscles of Rotifera.

The pharyngeal musculature of Limnognathia maerski is complex and consists of six paired and two unpaired muscles, articulating the different sclerites of the trophi with each other. In this respect, it resembles the muscular organization of the mastax of Rotifera, e.g. (Riemann and Ahlrichs 2008, Wulfken et al. 2010), probably constrained by the similarity in the arrangement of the jaw system of L. maerski and Rotifera in contrast to Gnathostomulida (Sørensen et al. 2003, Müller et al. 2004). However, specific homologies between the pharyngeal muscles described in Rotifera and L. maerski are not possible since a consensus has not been reached on the homologies of the different sclerites of L. maerski with the sclerites of Rotifera (Fig. 3). Since only the incus of Rotifera can be homologized with the jaw sclerites of Gnathostomulida and L. maerski, the so called “musculus fulcro ramicus” found in many Rotifera (e.g. (Wilts et al. 2010, Wilts et al. 2012)) is the only muscle which could be homologized with the “caudal muscle” of L. maerski. Furthermore, a ventral pharyngeal muscle is present, forming a muscular plate under the trophi.
and is probably involved in the movement of the entire pharynx. However, no equivalent has been found in other Gnathifera. Interestingly, this muscle is similar to an important pharyngeal muscle found in *Diurodrilus* (Worsaae et al. 2008), with which *L. maerski* has been extensively compared.

Figure 3: Different hypothesis of homology between the jaw sclerites of Micrognathozoa and other Gnathifera according different authors (Kristensen et al. 2000, De Smet 2002, Sørensen 2003, Riemann et al. 2008, Sterrer and Sørensen 2015)

Furthermore, the detailed morphological description of the pharyngeal musculature of *Limnognathia maerski*, together with reports of behavior observed in the living animal permitted assumptions about the jaw movements. Kristensen and Funch (2000) described the existence of fast snapping movement of the main jaws during foraging, and the extrusion of the ventral jaws grasping food, moving independently to the rest of the jaws (also described by De Smet, 2002), which contrasts the movement of the malleus of Rotifera relative to the incus (both being linked e.g. Riemann et al., 2008). In a similar fashion than Riemann et al., 2008, a jaw movement sequence of Micrognathozoa has been inferred (Fig. 4 and (Bekkouche et al. 2015)). This of course has to be confirmed by further behavior studies and high speed imaging, but shows the value of morphological studies in understanding and interpreting how such small and intricate structures can function.
Figure 4: Assumed jaw movement sequence of *Limnognathia maerski* according to behavioral observations and studies on the pharyngeal musculature (Bekkouche et al. 2015). Color coding after Sørensen (Sørensen 2003) interpretation of Fig. 3.

C) Nervous system, ciliation and glandular system of Micrognathozoa (Bekkouche and Worsaae, submitted)

The nervous system of *Limnognathia maerski* is quite simple and consists of an anterior brain, a pair of ventro-lateral nerve cords and a pharyngeal ganglion. Few other structures are described as, for example, an anterior and posterior commissure, a peripheral nervous system and a pair of thin ventro-median nerves. Interestingly, a peripheral nervous system innervating different sensory structures is also found in Rotifera (Hochberg 2006, Fontaneto and De Smet 2015). On the other hand, the ciliation of *L. maerski* shows a previously unsuspected complexity with more than one pair of ciliophores anteriorly and the presence of pharyngeal cilia very similar to the ciliary receptor of Rotifera (Clement and Wurdak 1991). Additionally, CLSM could confirm the presence of anterior and posterior nephridia, and of a multiciliated collecting duct, which is also reported from Rotifera (Ahlrichs 1993, Ahlrichs 1993) but absent in Gnathostomulida (Lammert 1985). Furthermore, a set of two glands, one unpaired and one paired, is found dorsally in the head
opening dorso-apically, and these glands are similar to the retrocerebral organ of Rotifera (Fontaneto et al. 2015). Surprisingly, these results show that – although the nervous system is of limited use to confirm the relationship between Micrognathozoa and Rotifera – some glands and specific ciliary structures are. This study also confirms that the paired ventro-lateral nerve cords and the pharyngeal ganglion are common traits of Gnathifera, the first one being a plesiomorphy (Hejnol et al. 2015), and the second one a synapomorphy of the group.

D) Conclusion, opening and further studies on Gnathifera

Although the muscular and nervous system of Limnognathia maerski show quite superficial, but numerous, resemblances to Rotifera, the ciliary and glandular systems show more convincing shared characters with Rotifera. Together with the ultrastructure of the tegument (Ahlrichs 1997, Kristensen et al. 2000), the organization of the jaws (De Smet 2002, Wulfken et al. 2012), the shared presence of a specific arrangement of the pharyngeal cilia, the structure of the nephridia and the possible retrocerebral organs furthermore support the sister group relationship between Rotifera and Micrognathozoa. However, nervous system investigations on the early branching rotifer Seisonidae (Rotifera) are lacking, and the inner anatomy of Gnathostomulida is still largely unexplored. Indeed, very few studies on their nervous system and musculature are available. Fortunately, our ignorance on the structure and diversity of the nervous system of Gnathostomulida should not last for long since a collaborative ongoing project carried out by Ludwik Gąsiorowski (Gąsiorowski, Bekkouche and Worsaae, unpublished) aims to solve this problem. The forthcoming study investigates the nervous system of several Gnathostomulida by means of CLSM and should shed more light onto the evolution of the nervous system of Gnathifera (Fig. 5). Preliminary results show substantial variation in the number of longitudinal nerves and brain morphology, and also shows e.g. the presence of pharyngeal cilia related to the buccal ganglion (though more scarce than in Rotifera and Micrognathozoa).
Figure 5: Comparison of the anterior nervous system of three species of Gnathostomulida, representing its three main clades. Ventral nervous system in blue, dorsal nervous system in red. Unpublished results from Gąsiorowski et al.

Finally, with the thorough description of the inner anatomy of Micrognathozoa, one point can be raised: the surprising consistency of the morphology of Gnathifera and their seemingly straightforward relationships. Indeed, for this phylogenetic depth, and the morphological simplicity of these animals, such phylogenetic resolution (including the monophyly of subgroups of Gnathostomulida (Sørensen et al. 2006) and Rotifera (Sørensen and Giribet 2006, Wey-Fabrizius et al. 2014, Sielaff et al. 2016)), supported by both molecular data and morphology (Rieger et al. 1995, Ahlrichs 1997, Kristensen et al. 2000, De Smet 2002, Zrzavý 2003, Witek et al. 2009, Wulfken et al. 2012, Struck et al. 2014, Laumer et al. 2015), may represent a unique case in zoology.
VIII) The morphology of *Diuronotus aspetos*, an interesting gastrotrich and its implication in the understanding of gastrotrich evolution


A) Introduction: the Gastrotricha, an understudied, yet important taxon

Gastrotricha are small and ventrally ciliated animals. These “turbellariform worms” have an extensive cuticle covering the cilia, an organization unique in Metazoa (Ruppert 1991, Kieneke et al. 2008). Rather understudied, they are found in most aquatic environments, from any sandy beach, oceanic bottom, freshwater environment or even humid soil. They are divided into two large groups: the often elongated and marine Macrodasyida possessing multiple adhesive glands, pharyngeal pores and an inverted “Y” cross section of the pharyngeal lumen, and the often fresh water and tenpin shaped Chaetonotida, Paucitubulatina, with only two adhesive posterior glands, no pharyngeal pores and a “Y” cross section of the pharynx lumen. A third taxon, *Neodasys*, belongs to Chaetonotida (Multitubulatina), is characterized by multiple adhesive glands and a peculiar adhesive system, and has a disputed phylogenetic position (Rothe et al. 2011, Kieneke and Schmidt-Rhaesa 2015).

Not only are members of Gastrotricha cosmopolitan and often play a very important part of the microscopic fauna, but they also have a disputed phylogenetic position. Originally supposed to be close to rotifers due to their superficial resemblance (Hyman 1951), ultrastructural studies suggested that they could be the sister group to or even nested within Ecdysozoa, the clade of molting animals comprising arthropods and nematodes. This was proposed on the base of three characteristics: the complex multilayered extensive cuticle, the “myoglanduloepithelial” pharynx very similar to the one found in Nematoda (Ruppert 1982), and the circumpharyngeal brain found also in Nematoda and various other Ecdysozoa (see Schmidt-Rhaesa, 2007 and Kieneke et al., 2015, for discussion). Although some morphological evidences pointed to an ecdysozoan
relationship, molecular phylogenies supported a Spiralian relationship supported only by few morphological data. Gastrotricha were then often placed into the disputed “Platyzoa” (Cavalier-Smith 1998, Giribet et al. 2000, Halanych 2004, Hejnol et al. 2009). Subsequently, recent studies on phylogenomics suggested for the first time a quite robust position of Gastrotricha as a sister group of Platyhelminthes in the clade Rouphezoa (Struck et al. 2014, Laumer et al. 2015). As explained above, this puts gastrotrichs forward as a group of high interest for animal evolution. Prior to such conclusions, however, a better understanding of the inner evolution of Gastrotricha is needed. Although knowledge about gastrotrich evolution has notably increased recently with the implementation of CLSM (Hochberg and Litvaitis 2001, Hochberg and Litvaitis 2001, Leasi et al. 2008, Rothe et al. 2011, Rothe et al. 2011), a lot of work is still needed to better understand the variability of the inner anatomy of Gastrotricha. In order to increase this knowledge and further understand the evolution of Gastrotricha, we studied one of the key taxon: Diuronotus aspetos.

B) Results and discussion: the morphology of Diuronotus aspetos

Diuronotus aspetos, a large member of Chaetonotida, has been recently described (2005) (Todaro et al. 2005) and justified the erection of a new genus within Gastrotricha. Its morphological similarities with the rare and enigmatic Musellifer have been recognized from the original description and further confirmed (Balsamo et al. 2010), consequently leading to the erection of a new family, Muselliferidae (Leasi et al. 2008). The study presented in this thesis, as well as previous morphological and molecular investigations (Kieneke et al. 2008, Leasi et al. 2008, Kånneby et al. 2014), suggest that indeed, Muselliferidae belongs to the deep nodes of the phylogenetic tree of Paucitubulatina, emphasizing the importance of this family for understanding the evolution of Gastrotricha. Muselliferidae are especially rare: Musellifer is occasionally reported in very low abundance (Hummon 1969, Kånneby et al. 2014) (sometimes only one (Kånneby et al. 2014)) and Diuronotus is found in few locations (Denmark and Greenland (Todaro et al. 2005), Germany (Kieneke 2015), and informally mentioned in North America (Ruppert 1982)).

First, we placed D. aspetos into the phylogeny of Paucitubulatina, confirming with molecular data that it is the sister group of Musellifer. Additionally, we studied D. aspetos with CLSM, supporting the peculiarity of this gastrotrich. The musculature, for instance, shows only few longitudinal muscles, but numerous pairs of dorso-ventral muscles in the transversal section (up to five), a
configuration not found in other Paucitubulatina (Leasi et al. 2008). We also described the musculature of the furca in detail, showing the presence of circular muscles around the adhesive glands, and some semi-circular muscles in the posterior region of the trunk. These special traits make the specific muscles of *Diuronotus aspetos* difficult to homologize with other Paucitubulatina. The nervous system is also described in great detail, giving valuable information, since so far *Xenotrichula* was the only other Paucitubulatina for which the nervous system was carefully assessed (Rothe et al. 2011). We found the common gastrotrich arrangement of one dumbbell shaped brain and a pair of ventro-lateral nerve cords (Kieneke et al. 2015), but also described some specific nervous structures such as i) additional ventro-median cords ventral to the pharynx, ii) a ventral commissure of the brain shifted anteriorly, associated to a dorsal commissure forming an anterior nerve ring, and the presence of iii) post-pharyngeal and iv) anal ganglia. Interestingly, some of these characters can be homologized with *Xenotrichula* (Rothe et al. 2011), and even specific perikarya of the brain can be compared *Neodasys* (Rothe et al. 2011) and *Xenotrichula*. The pharynx is also comprehensively described, including its nervous system, showing pharyngeal cilia in the pharynx of Paucitubulatina with CLSM for the first time, but also demonstrating the existence of a system of seemingly hollow canals in the pharynx. These canals are of unknown function and have never been described in other Gastrotricha previously. Finally, the ciliary system is studied, showing for the first time the presence of two pairs of protonephridia in Paucitubulatina (Kieneke et al. 2008, Kieneke and Hochberg 2012), and resolving the detailed pattern of the repartition of the ventral multiciliated cells. This detailed study shows the presence of previously undescribed structures (additional muscles, nerves or the pharyngeal canal system), and furthermore demonstrates that the nervous system might be easily comparable across Paucitubulatina. We therefore emphasize that this investigation will serve as a basis for future descriptions in other Paucitubulatina or other Gastrotricha.

**C) Conclusion: opening and further researches on Gastrotricha**

This study on *Diuronotus aspetos* shows the necessity to study even (apparently) minor characters (such as ciliary patterns and glands) to understand the evolution and diversity of Gastrotricha. It also shows that extensive descriptive studies such as conducted in the presented thesis and in e.g. (Wiedermann 1995, Rothe et al. 2011, Rothe et al. 2011, Todaro et al. 2015), are mandatory to facilitate a comparative and evolutionary database. To illustrate that, two examples of future
researches on Gastrotricha either inspired by, or broadening, the present study on *D. aspetos* are presented here.

i) On the evolution of the ventral ciliary pattern in Gastrotricha

The presented study on *Diuronotus aspetos*, together with the investigation of ciliation patterns of *Limnognathia maerski* (manuscript IV) indicate the value of CLSM for detailed descriptions of the ciliary system of meiofaunal animal. This led to the description of fine and unexpected details such as the presence of pharyngeal cilia in *D. aspetos*, and the mosaic-pattern of ventral multiciliated cells in *L. maerski*. This potential new type of characters could possibly be of systematic importance and we hope that it will be more exploited in the future. During the course of this thesis, acetylated α-tubulin immunoreactivity have been studied in a range of gastrotrichs, especially Thaumastodermatidae, which are known to possess multiciliated locomotory cells (Todaro et al. 2011). Together with DAPI, these data showed that variability exists between the ciliary pattern of different members of Thaumastodermatidae (Fig. 6) and we hope that further analysis will reveal the systematic relevance of this character.

Figure 6: Comparative representation of the ventral ciliation of various Thaumastodermatidae based on literature (Hummon 2011, Araujo et al. 2014) and own CLSM interpretation, displaying variation in the precise pattern of multiciliated cells. Interpretation from Eleonor Sharples.
ii) On the fine evolution of the nervous system of Gastrotricha

A range of nervous systems of various members of Gastrotricha was analyzed during the course of this thesis, which could unfortunately not be integrated in the presented work. However, several interesting observations could be made. Among those, Synapsin-I-like immunoreactivity showed the presence of an anterior nerve ring (Fig. 7) consisting of the already described ventral commissure of the brain, always associated to a dorso-anterior brain commissure, with the entire ring usually being isolated from the main neuropil of the brain in several Gastrotricha (Schmidt-Rhaesa 2007). Interestingly, an anterior nerve ring, also showing Synapsin-I-like immunoreactivity, was found in the brain of Diuronotus aspetos (manuscript V). These observations showed that the addition of Synapsin-I-like immunoreactivity aids the frequent recovering of the dorsal and ventral brain commissure, which might be a shared character of all Gastrotricha. Additionally, more variation of the brain and nerve cord was found, as illustrated in the Fig. 7, with serotonin-like immunoreactivity among Gastrotricha. If the general morphology of the nervous system of Gastrotricha seems to be conserved, we hope and expect that the variation of the “minor” nerves will be of systematic importance, and will shed new light on the evolution of the nervous system within this group.
Figure 7: Schematic drawing of the anterior nervous system of two species of Macrodasyida interpreted from CLSM: *Paradasys subterraneus* on the left and *Acanthodasys* sp. on the right. Serotonin-like immunoreactivity in red and synapsin-I-like immunoreactivity in green. Note the association of the ventral commissure with a dorsal commissure, forming an anterior nerve ring, set anteriorly and apart from the brain neuropil.

**IX) Conclusion**

The reconstruction of the morphology of the animals studied in this thesis shows that careful investigation of meiofaunal animals is necessary to give a comprehensible framework for understanding Spiralian evolution. Furthermore, the inclusion of several organ systems as presented in this thesis revealed the potential of exhaustive studies as compared to investigations of only a specific subset of organ systems. For instance, some new possible homologies between Rotifera and Micrognathozoa concern glands and ciliation patterns, organ systems rarely considered when it involves this phylogenetic depth (but see (Rieger 1976, Rieger 1981)). Yet, nervous system shows its potential for comparative studies as the assessment of the pharyngeal ganglion as a shared character of Gnathifera. Also, *Diuronotus* offers further illustration of the need of comprehensive descriptions: the nervous system appears to show some degrees of
conservation, making it easy to compare, with *Xenotrichula* (Rothe et al. 2011) or *Neodasys* (Rothe et al. 2011), which contrasts findings of the muscular system, more difficult to compare (but see Leasi et al. 2008). Finally, the present work offers the phylogenetic placement and morphological description of *Lobatocerebrum* and Micrognathozoa, also giving new insights within Gastrotricha, filling a previously important knowledge gap in the incredibly diverse and still poorly understood Spiralia.

X) References

- Slyusarev, G.S. and Starunov, V.V. (2016). The structure of the muscular and nervous systems of the female *Neodasy s chaetonotoideus* (Gastrotricha: Paucitubulatina) by means of immunohistochemistry (IHC) and TEM.
Articles and manuscripts


Manuscript II: Detailed reconstruction of the nervous and muscular system of Lobatocerebridae with an evaluation of its annelid affinity. Kerbl A., Bekkouche N., Sterrer W., and Worsaae K. (published)

Manuscript III: Detailed reconstruction of the musculature in Limnognathia maerski (Micrognathozoa) and comparison with other Gnathifera. Bekkouche N., Kristensen R. M., Hejnol A., Sørensen M. V., and Worsaae, K. (published)

Manuscript IV: Nervous system and ciliary structures of Micrognathozoa (Gnathifera) – evolutionary insight from an early branch in Spiralia. Bekkouche N., and Worsaae K. (submitted)

Manuscript I:

**Spiralian Phylogeny Informs the Evolution of Microscopic Lineages**

Laumer, C. E., Bekkouche, N., Kerbl, A., Goetz, F., Neves, R. C., Sørensen, M. V., Kristensen, R. M., Hejnol., Dunn C. W., Giribet G. and Worsaae K.

Current Biology, Volume 25, issue 15, Pages 2000-2006
Spiralian Phylogeny Informs the Evolution of Microscopic Lineages

**Graphical Abstract**

**Highlights**

- *Diurodrilus* and *Lobatocerebrum*, two problematic meiofauna, are miniaturized annelids.

- Micrognathozoa, the newest-described animal phylum, is the sister group of Rotifera.

- Bayesian mixture models recover strong support for deep spiralian relationships.

- Two clades comprising Platyzoa form separate early branches in Spiralia.

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**In Brief**

Laumer et al. reconstruct the phylogeny of Spiralia, the animal group including molluscs, annelids, flatworms, and many microscopic worms. The new tree suggests that some previously unsampled, interstitial Problematica originated through miniaturization from large-bodied ancestors but also implies a primarily interstitial origin for many lineages.

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Spiralian Phylogeny Informs the Evolution of Microscopic Lineages

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SUMMARY

Despite rapid advances in the study of metazoan evolutionary history [1], phylogenomic analyses have so far neglected a number of microscopic lineages that possess a unique combination of characters and are thus informative for our understanding of morphological evolution. Chief among these lineages are the recently described animal groups Micrognathozoa and Loricifera, as well as the two interstitial “Problematica” Diurodrilus and Lobatocerebrum [2]. These genera show a certain resemblance to Annelida in their cuticle and gut [3, 4]; however, both lack primary annelid characters such as segmentation and chaetae [5]. Moreover, they show unique features such as an inverted body-wall musculature or a novel pharyngeal organ. This and their ciliated epidermis have led some to propose relationships with other microscopic spiralian, namely Platyzelminthes, Gastrotricha, and in the case of relationships with other microscopic spiralians, namely their ciliated epidermis have led some to propose re-

RESULTS

Understanding metazoan evolutionary history requires resolving the phylogenetic positions of not only the major animal groups but also of more obscure lineages showing unique character combinations. Examples of such important “Problematica” are Lobatocerebromorpha [3, 12, 13], Diurodrilida [4, 5], Micrognathozoa [6, 7], and Loricifera [14], representing among the smallest animals ever discovered, which have received phylum-level affiliations or remain of uncertain position within Protostomia. We present here the first nuclear protein-coding data from representatives of all four clades, incorporating these and other new and published protein-coding surveys into a 402-ortholog, 90-taxon supermatrix comprising all free-living lineages of Spiralia (Table S1). Phylogenetic analyses of this matrix were performed using maximum likelihood (ML; Figures 1 and S1), with partitioned analyses of the full-size matrix (Figure 1A) and unpartitioned analyses of two submatrices constructed to investigate putative long-branch attraction (LBA) artifacts (Figures 1B and 1C). To further control for other potential systematic artifacts, we undertook analyses using Bayesian inference (BI) under a site-heterogeneous mixture model (CAT + GTR + I4; [15]), using a matrix groomed of unstable taxa and sites showing evidence of compositional non-stationarity (Figure 2). Bayesian analyses of the complete matrix were also performed (Figure S2).

The ML and BI analyses differ, at least superficially, in the topology they present for deep spiralian interrelationships. Our ML trees from partitioned analyses of the full matrix (Figure 1A) and from analyses of a slow-evolving subset of the full matrix (Figure 1B) are nearly identical and recapitulate results found in previous large-scale ML investigations of spiralian phylogeny [10, 11], e.g., monophyly of Trochozoa, Polyzoa, and Polyzoa [1, 15]. In contrast, analyses of a fast-evolving subset (Figure 1C) of this matrix do not recover the monophyly of Polyzoa, Polyzoa, or even Ecdysozoa. In general, however, few relevant clades
find strong support in any ML analysis, with even several un-controversially monophyletic taxa (e.g., Annelida, Gastrotricha) failing to see strong support (Figure 1). In contrast, the BI analyses under a site-heterogeneous model (CAT + GTR + \( \Gamma^4 \)) find strong support for many spiralian clades, including all those that are also supported in the ML analyses, but also for Spiralia, Gnathifera, and Lophotrochozoa, among others (Figure 2). Thus, while the ML trees and BI consensus phylograms topologically differ, there is no evidence of strongly supported incongruence between ML and BI. Most importantly, BI places both Diurodrilus and Lobatocerebrum as deeply nested members of Annelida (as does ML, although with lesser support). Finally, BI also finds strong support for the non-monophyly of "Platyzoa," with Gnathifera forming the earliest-diverging branch (Figures 2 and S2). Platyzoan non-monophyly is also recovered under ML in our fastest-evolving matrix subset (Figure 1C), but support for basal relationships is poor in this analysis.

The BI analyses of the trimmed (Figure 2) and untrimmed (Figure S2) matrices differ in only few respects. Platyhelminthes + Gastrotricha (called Rouphozoa in [11]) and Lophotrochozoa (in the sense of its original definition by [16] and not the looser common usage introduced by [17]) are supported in the trimmed matrix, but not in the untrimmed matrix. Mixture model inference on both matrices, in sharp contrast to our ML analyses, also recovers the monophyly of the lophophorate phyla with high support, with Phoronida (here as in [18]) forming the sister group of Bryozoa. Mollusca was recovered as the sister group to the other Lophotrochozoa (in marked contrast to recent studies [11, 18]), albeit with weak support in the complete matrix (Figure S2).

Indeed, the only strongly supported deep topological difference observed between analyses of the trimmed versus complete matrix concerns the position of Nemertea, which forms the sister taxon of Annelida in the untrimmed matrix (Figure S2), or of the lophophorate clade in the trimmed matrix (Figure 2). Remarkably, in the complete matrix, we see no support for the hypothesis previously suggested by both molecules and morphology [18–20] of a sister-group relationship between Cyclopedia and Entoprocta (the latter being instead recovered as sister group to Bryozoa; [21]); here, Cyclopedia falls, but with low posterior probability (pp; pp = 0.5), as the sister group of Lophotrochozoa (Figure S2), a result perhaps related to the poor sequencing depth of this transcriptome.

Within Ecdysozoa, we find strong support under BI analysis of the untrimmed matrix (Figure S2) only for Onychophora + Arthropoda and Tardigrada + Nematoda, as found in a recent study focused on Ecdysozoa [22]. However, in the trimmed matrix (Figure 2), support (pp = 0.98) also emerges for a scenario in which the meiofaunal Lorificera fall together with our other scalidophoran representative, Priapulida, as the sister group to other members of Ecdysozoa. Although evidence for Scalidophora itself is poor (pp = 0.78), and we lack a representative of Kinorhyncha, this is the first time molecular data have recovered a clade of Lorificera + Priapulida, two taxa that share many common morphological traits [23].

**DISCUSSION**

**Diurodrilus and Lobatocerebrum Are Miniaturized Annelids**

The deeply nested positions of Diurodrilus and Lobatocerebrum within Annelida suggest independent miniaturizations of these lineages from an indirect-developing, macrofaunal annelid ancestor. Diurodrilus has traditionally been considered a member of Archiannelida [4, 24], a taxon of morphologically simple
interstitial annelids originally considered “ancestral” to the other annelid taxa [25, 26], other members of which have recently been shown to be non-monophyletic and derived from macrofaunal ancestors [27, 28]. However, for Diurodrilus, several authors have also proposed a relationship outside of Annelida, specifically to the recently discovered Micrognathozoa, with which...
they share, e.g., characteristic mid-ventral trunk ciliophores and a ventral muscular plate of the pharynx [5–7]. Equally complicated is the case of Lobatocerebromorpha, originally described as “a turbelliariform member of the annelid line of evolution” [3], i.e., an intermediate between Platyhelminthes and Annelida—a position maintained by Rieger [12] and Hazprunar et al. [2] (who erected for it the phylum Lobatocerebromorpha), which we aimed to test here.

None of these hypotheses are supported in the present study. The precise position in which we recovered Diurodrilus within Annelida—as sister taxon to the macrofaunal Orbinidae—has also been supported by ML analysis of mitogenomic data (although curiously, orbinids appear more distant in gene order analyses) [29]. Remarkably, in previous rRNA-based phylogenetic studies orbinids have been recovered as relatives of Parergodrilidae, another meiofaunal annelid lineage [30]. However, Diurodrilus shows with its apomorphic pharyngeal organ, adhesive toes, and ventral ciliophores no close resemblance to any known orbinid, adult, larval or juvenile [29, 31]. Indeed, it represents the most “reduced” annelid to date, both sexes being of microscopic size and lacking all common annelid traits such as segmentation, coelomic cavities, chaetae, and nuchal organ [26]. With respect to Lobatocerebromorpha, we find it strongly supported as the sister group of Sipuncula [32], constituting an intriguing clade of unsegmented annelids; however, there are no other obvious synapomorphies for the two groups.

Lobatocerebromorpha and Diurodrilus share gross anatomical characteristics with many interstitial annelids, most prominent among these being an acoelomate or pseudocoelomate condition (with coincident protonephridia and absence of a vascular system). This organization may be related to small body size and can arise homoplastically as the consequence of diverse processes, such as an enlarged peritoneal lining and/or endoderm, or lack of cavity formation within the mesoderm [26, 33–35]. These different manifestations of an acoelomate condition, as well as the apparent independent origin of Lobatocerebromorpha, Diurodrilus, and most other interstitial annelid families [26, 28, 31] indicate that their miniaturizations do not follow a predictable pattern. Accordingly, it cannot easily be explained by the popular theory of progenesis [31], especially considering their lack of specific resemblance to larval or juvenile stages of macrofaunal relatives (e.g., Orbinidae). Regardless of the mechanism of their reduction, however, our recovered placement of Diurodrilus and Lobatocerebromorpha within Annelida contributes to the morphological disparity of this taxon, together with the recent positioning of other aberrant annelids such as Sipuncula, Echiura, Myzostomida, and Pogonophora [27].

**Micrognathozoa Is Sister Group to Rotifera within Gnathifera**

All our analyses supported monophyletic Gnathifera—a clade composed of protostomes with a special type of cuticular jaws—with Micrognathozoa as the sister group of Rotifera, both constituting the sister group of Gnathostomulida (Figures 1 and 2). Despite the microscopic size and understudied biology of most gnathiferan lineages (e.g., male micrognathozoans having not been observed), this topology has been supported previously with morphological data [6, 36, 37], albeit not using conventional molecular markers [38]. The main synapomorphies of Rotifera + Micrognathozoa have been uncovered in ultrastructural studies of the epidermis [39] and of the jaw apparatus composed of rod-like structures [37], with Rotifera + Micrognathozoa having some common supporting musculature [7].

**“Platyzoa” Is Likely a Systematic Artifact**

Our mixture model analyses reject the monophyly of Platyzoa [8], a grouping of mainly interstitial taxa whose only shared characteristics, such as minute size (excepting some secondarily large Platyhelminthes and the acanthocephalan Rotifera; [40]), direct development, external ciliation, and an acoelomate or pseudocoelomate condition, are features also found in many other animals. The poorly supported division between Platyzoa/Polyzoa and Trochozoa, which we recover only under ML (Figure 1), neatly correlates (with the exceptions of Diurodrilus and Lobatocerebromorpha) with a division between fast-evolving and slow-evolving spiralian, suggesting the possibility of an LBA artifact [11]. Further, even though under both phylogenetic methods the problematic Diurodrilus and Lobatocerebromorpha are recovered as deeply nested annelids, the positions of these taxa within Annelida differ between reconstruction methods, with ML (Figure 1) placing these fast-evolving lineages in close proximity, consistent also with an LBA effect. It is remarkable that even the use of a statistically well-justified partitioning scheme, as provided by the PartitionFinder algorithm [41], groups the fast-evolving interstitial taxa into a clade (Figure 1). Only under the CAT + GTR + Τ’ four mixture model do we recover non-monophyly of this long-branched assemblage, consistent with previous observations that such flexible models better fit the substitution-pattern heterogeneity characteristic of such large matrices, thereby rendering them more robust to model misspecification and subsequent LBA [42]. Apparently the relevant substitution process heterogeneities in such data may be occurring not between genes but between sites within genes (at, e.g., the domain level; [43]).

Interestingly, a similar resolution of “Platyzoa” as non-monophyletic has also been proposed in another recent study [11], also using RNA sequencing libraries as a source of phylogenetic evidence (several of which we reanalyze here with distinct assembly and orthology assignment algorithms). However, in this study, such a topology only emerged under consideration of specific gene and taxon subsets, and even then, no single analysis offered strong resampling support for all newly introduced clades (i.e., “Rouphozoza” and “Platytrochozoa”). Indeed, choosing to exclude specific data subsets may at times prove positively misleading: for instance, ML analysis of our fastest-evolving submatrix recovers a topology (albeit with low support) similar to our BI analyses (Figure 1C). This may thus be seen as an argument in favor of a “total evidence” approach to phylogenetics even at this scale of inference; although fast-evolving sites and genes may indeed mislead simple reconstruction methods, they may also retain valuable phylogenetic signal [44].

**Was the Spiralian Ancestor a Microscopic, Acoelomate, Direct-Developing Worm?**

The colonization of the interstices of marine sediments is among the most successful modes of life employed by metazoans, with nearly every major animal clade having at least some interstitial representatives and some being known exclusively from this habitat [45–47]. Animals that have adapted to such lifestyles,
sometimes known as meiofauna, bear a common set of characteristics, being generally of microscopic size, direct developing, with limited reproductive output and lifespan, and showing, relative to larger metazoa, a simplified, often acelomate body design. Phylogenetic discussions regarding such meiofauna, including the members of “Platyzoa” [34], interstitial Annelida [3, 31], and other taxa such as the acelomorph flatworms [48], have centered on the question of whether these morphologically “simple” taxa have originated via miniaturization from a macrofaunal ancestor, or have instead inherited their simple morphology from ancestors with similarly microscopic adults.

In this contribution, we aimed to address these themes within the major metazoan clade Spiralia, by resolving the interrelationships between the meiofaunal and macrofaunal members of this clade, including genome and transcriptome sampling of a range of previously sparsely sampled (Gnathostomulida) or unsampled microscopic taxa (Catenulida, Micrognathozoa, Chaetognotoidea, Lobotocerebridae, Diurodrilidae). Under a phylogenetic mixture model (Figure 2), we find uniformly strong support for a topology in which a monophyletic Gnathifera forms the sister group to all other spiralians, with the remaining members of Spiralia split between a clade of, on the one hand, Platyzeminthes and Gastrotricha, and on the other, Lophotrochozoa. A parsimonious reading of this topology posits the common features of these interstitial worms as plesiomorphies, implying an interstitial, direct developing, unsegmented, acelomate or pseudocelomate condition for the spiralian ancestor. This further implies multiple independent origins of, e.g., segmentation, coelomic cavities, planktotrophic larvae, and other morphological structures across Bilateria.

However, under the topology recovered here, only two separate reductions in body size (miniaturizations) and transitions to an acelomate condition—perhaps, though not necessarily, via progenesis—are required to derive Gnathifera and Rouphezoa from a macrofaunal, coelomate spiralian ancestor. If miniaturized taxa such as Lobotocerebrum and Diurodrilus have separate origins within Annelida, might not Gnathifera and Rouphezoa, clades that evince rather distinct manifestations of the acelomate condition [17], therefore also be the remaining survivors of two ancient miniaturization events [13, 48, 49]? The principle of parsimony casts doubt on this scenario, as it posits the existence and independent extinction of two separate macrofaunal lineages related to both branches of “Platyzoa,” a suggestion for which there is no fossil evidence, despite the widespread availability of exceptionally preserved Cambrian fossils of most other soft-bodied macrofaunal bilaterian lineages. This being recognized, there are continued arguments from comparative developmental genetic studies (reviewed by [50]) for homology across Bilateria in traits seemingly specific to macrofaunal animals, most recently extending to larval apical organs [51], a complex, triplicate forebrain [52], and collarlike midline supportive structures [53]. Unfortunately, the interpretation of such studies remains biased by the absence of data on the expression and function of developmental genes during the embryogenesis of gnathiferae, platyzeminthes, and gastrotrichs.

Comparisons to outgroup taxa are critical to understanding the nature of the ancestor of Spiralia and earlier branches (Protostomia, and Bilateria). Ecdysozoa, one of two possible outgroups to Spiralia [1], encompasses substantial body plan diversity, and the relationships within this clade remain incompletely understood. However, it is possibly suggestive in this context that in this analysis as well as others [22], the members of Scalidophora, a clade of primarily interstitial, largely acelomate or pseudocelomate animals, are supported as sister taxon to other ecdysozoans. The precise placement of two other extant vermiform taxa—the enigmatic chaetognaths, representing a likely distinct branch of protostomes in their own right [54], and the acelomorph flatworms (with or without Xenoturbella), representing either early-branching bilaterians or deuterostomes of uncertain precise placement [1]—may also provide some additional signal required to test the homology of the traits common to the “platyzoan” taxa. With the continued availability of genomic and genome-informed datasets from representatives of problematic taxa such as those presented here, we are approaching a clearer picture of the relationships, limits, and shared derived characteristics of not only these microscopic groups but also the most familiar branches of the metazoan tree. The evidence presented here has yielded the first well-resolved spiralian phylogeny inclusive of all free-living groups and hence provides clear hypotheses for future investigations to test, not least among which is the supposition that the ancestor of Spiralia was most probably a meiofaunal animal, as this is the predominant lifestyle of the two earliest-branching lineages within this diverse clade.

**ACCESSION NUMBERS**

See Table S1 for a full list of SRA accession numbers for previously unreported data.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.06.068.

**AUTHOR CONTRIBUTIONS**


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REFERENCES


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Spiralian Phylogeny Informs the Evolution of Microscopic Lineages

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**Figure S1** – Partitioned maximum likelihood phylogram schematized in Figure 1, with full terminal taxon names given. Support values represent a proportion of 100 bootstrap replicates.
Figure S2 – Bayesian inference of spiralian phylogeny from PhyloBayes-MPI v1.4e analysis of the untrimmed matrix (90 taxa, 79,954 amino acids, 57.57% missing data). Nodal support values represent posterior probability.
Table S1 – Summary statistics describing genome and transcriptome assemblies and availability from the 90 taxa used in this study. Newly sequenced species are labeled in bold. Statistics were calculated with scripts provided within Trinity r20140413 or using the fastq-stats program in the ea-utils package. Species with a ‘??’ in read-level cells were provided to us as assemblies only. Peptide counts are for isoform-filtered peptides.
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Supplemental Experimental Procedures
Peptide predictions used for grouping into orthologous sets were derived from a mixture of publically available gene models from well-annotated genome assemblies, and transcriptome assemblies derived from mainly Illumina, 454, and in a few cases Sanger, cDNA sequencing projects accessioned in NCBI’s SRA or EST databases; 13 Illumina cDNA libraries were also newly sequenced for this project. Illumina reads were quality-controlled while maintaining parity using Trimmomatic v0.32 [S1], trimming to a minimum Phred33 score of 20 (in a 4-bp sliding window), and removing all reads with a post-trimmed length of <36 bp; relevant adapter sequences (including SMART cDNA amplification adapters, in the case of libraries produced using this method; [S2]) were removed. 454 libraries were trimmed to a minimum Phred score of 30, and reads with post-trimmed length <30 were removed, using the fastq_quality_trimmer tool from the FASTX toolkit. Illumina and 454 cDNA libraries (following [S3]) were both assembled using the Trinity RNA-seq de novo assembler, r20140413 [S4]. Sanger EST libraries were processed using SeqClean and TGICL-2.1, as described in [S5]. Libraries were screened for metazoan-origin contamination by screening the de novo assemblies against the SILVA 18S rRNA database using BLASTn at an e-value of 1e-100. All transcriptome assemblies were redundancy-reduced using cd-hit-est at c=0.95, and likely ORFs were predicted using TransDecoder r20131117; the longest peptide per retained Trinity subcomponent (i.e. putative unigene) was then selected with a custom Python script (choose_longest_v3.py; [S5]). Further details of data source, library preparation, and several key summary statistics describing properties of raw sequence data, finished assemblies, and predicted peptides, are described in Table S1.
Predicted peptides were grouped into putative orthologous clusters with a single peptide per species using the OMA-standalone algorithm, v0.99x [S6]. We retained all OMA groups with 6 or more members, of which there were 17,066, and performed multiple sequence alignment on this set using the L-INS-i algorithm from MAFFT v7.149 [S7], quantifying alignment errors using ZORRO [S8], and trimming columns assigned an alignment uncertainty score of <0.5 [S5]. From these aligned, sequence-masked orthogroups, we selected 402 orthologs to use for phylogenetic analysis using the matrix reduction (MARE) tool v0.1.2rc, with d=1 and t=1000, to select for an information-dense matrix without the loss of any taxa [S9]. This yielded a matrix of 79,954 AA with a completeness of 42.43%. From this matrix we also prepared a trimmed matrix using BMGE-1.11 [S10], with ‘-g 1 -n BLOSUM30 -s FAST’, so as to retain gappy regions and trim entropic sequences using the least stringent substitution matrix possible, while removing sites that show evidence of non-stationarity: this yielded 72,243 AA with a completeness of 41.83%. *Symbion americanus* and *Barentsia elegans* were deleted from this matrix after inspection of a preliminary PhyloBayes run showed these taxa to be highly unstable during MCMC.

Maximum likelihood phylogenetic inference was performed in parallel on the Harvard FAS Division of Science Odyssey 2 research cluster using ExaML v3.0.0 [S11], with 100 bootstrap replicates calculated manually to measure nodal support, as described in the RAxML-Light manual. For the tree presented in Figure 1A, likelihood was calculated under a partitioning scheme selected by PartitionFinderProtein v1.1.1 [S12], calculating likelihoods with the provided RAxML binary and using with heuristic clustering (‘-rcluster-percent 10’). This selected 62 partitions (beginning from a 402-partition per-ortholog scheme), most of which were assigned the PROTGAMMALG model. Unfortunately BMGE does not take into account the
boundaries between partitions while trimming, so only the untrimmed matrix was considered for partitioned maximum likelihood inference. For the submatrices analyzed in Figures 1B and 1C, we used TIGER v1.2 to rank sites by relative evolutionary rate [S13], writing the scaled rates to an output file using the ‘-rl’ command. We then used a custom python script to parse these rates, defining for the variable sites (those with a rate value less than 1.0) the first and third quartiles, and using the PyCogent library [S14] to retain new submatrices composed of the upper and lower three fastest quartiles, respectively. These matrices were then analyzed under the LG4M+F substitution matrix in ExaML v3.0.0 [S11]. Nodal support from the 100 bootstrap replicates was summarized onto the best-found tree from ExaML using the sumtrees.py program of DendroPy [S15].

Bayesian mixture model inference under the CAT+GTR model was conducted in PhyloBayes-MPI v1.4e [S16], removing constant sites (“-dc”) and running four independent chains each for, in the case of the untrimmed matrix, a minimum of 14,000 generations (maximum 21,784), or in the case of the trimmed matrix, a minimum of 16,000 generations (maximum 21,056). Runs were considered to have converged adequately when the maximum proportion of bipartition differences dropped below <0.3 for at least 3 pairs of chains. The posterior summaries interpreted here were generated from a single pair of chains per matrix, with a burn-in of 5000 generations from the complete matrix (maxdiff= 0.179) and of 3000 generations from the trimmed matrix (maxdiff= 0.143).

Supplemental References


Manuscript II:

Detailed reconstruction of the nervous and muscular system of Lobatocerebridae with an evaluation of its annelid affinity

Kerbl A., Bekkouche N., Sterrer W., and Worsaae K.

BMC Evolutionary Biology, Volume 15, issue 227
Detailed reconstruction of the nervous and muscular system of Lobatocerebridae with an evaluation of its annelid affinity

Alexandra Kerbl1, Nicolas Bekkouche1, Wolfgang Sterrer2 and Katrine Worsaae1*

Abstract

Background: The microscopic worm group Lobatocerebridae has been regarded a ‘problematicum’, with the systematic relationship being highly debated until a recent phylogenomic study placed them within annelids (Curr Biol 25: 2000-2006, 2015). To date, a morphological comparison with other spiralian taxa lacks detailed information on the nervous and muscular system, which is here presented for Lobatocerebrum riegeri n. sp. based on immunohistochemistry and confocal laser scanning microscopy, supported by TEM and live observations.

Results: The musculature is organized as a grid of longitudinal muscles and transverse muscular ring complexes in the trunk. The rostrum is supplied by longitudinal muscles and only a few transverse muscles. The intraepidermal central nervous system consists of a big, multi-lobed brain, nine major nerve bundles extending anteriorly into the rostrum and two lateral and one median cord extending posteriorly to the anus, connected by five commissures. The glandular epidermis has at least three types of mucus secreting glands and one type of adhesive unicellular glands.

Conclusions: No exclusive “annelid characters” could be found in the neuromuscular system of Lobatocerebridae, except for perhaps the mid-ventral nerve. However, none of the observed structures disputes its position within this group. The neuromuscular and glandular system of L. riegeri n. sp. shows similarities to those of meiofaunal annelids such as Dinophilidae and Protodrilidae, yet likewise to Gnathostomulida and catenulid Platyhelminthes, all living in the restrictive interstitial environment among sand grains. It therefore suggests an extreme evolutionary plasticity of annelid nervous and muscular architecture, previously regarded as highly conservative organ systems throughout metazoan evolution.

Keywords: Nervous system, Musculature, Glandular system, Meiofauna, Annelida, Spiralia, CLSM, Immunohistochemistry, Ultrastructure

Background

Although phylogenomic studies have increased our knowledge of metazoan phylogeny significantly [1–4], a few ‘Problematica’ [5, 6] remain unplaced. Chief among those is the interstitial family Lobatocerebridae, which a recent phylogenetic study based on transcriptomic data positioned within Annelida, as sister group to Sipuncula, albeit with moderate support [7]. This enigmatic group of microscopic, thread-like, fully ciliated animals with glandular epidermis, living interstitially between sand grains in the subtidal sandy sea-floor, was described as its own family, Lobatocerebridae, with one species, Lobatocerebrum psammicola [8]. The morphological data available have never indicated a relationship to Sipuncula, although affinities to Annelida as well as to Platyhelminthes have been debated [8, 9]. Due to the ambiguity of the morphological features pointed out by Rieger [8–11], this group was suggested to be its own phylum Lobatocerebrorhorma in 1991, alongside annelids, platyhelminthes, molluscs and other spiralian [6, 12]; a status now denied by the recent phylogenomic analyses [7].

Lobatocerebrum psammicola was described from the shallow waters off the Coast of North Carolina, USA, based on TEM and LM section series [8–11]. The same
articles mention two additional undescribed species from the deep waters off North Carolina and from Eilat, Israel, respectively [8–10]. Additional specimens have been recorded by various authors from marine localities in the Atlantic (for example in Denmark [13], Gran Canaria (Spain) and Elba (Italy, W. Sterrer unpublished), and the Atlantic coast of Panama [7]), but the detailed morphology or taxonomy of these animals (besides L. psammicola) has never been investigated. Lobatocerebridae are found in subtidal marine habitats with coarse sand mixed with fine silt, but with limited organic and terrestrial matter. Although found at shallow depths, they are never abundant, and may be mistaken for platyhelminthes, juvenile nemerteans or gnathostomulids, which might explain their understudied nature and lack of additional records. Due to the inaccessibility of material, the explicit descriptions given by R. Rieger in his series of articles [8–11] have remained the only source for systematic and evolutionary discussions for decades [5, 6, 12, 14].

Lobatocerebridae have been described by Rieger [8–10] as having a thin, elongated body with circular cross section and complete ciliation. The epidermis is furthermore interspersed with a high number of unicellular glands. The ventral mouth opening is located one-third of the length from the tip (delineating the rostrum from the trunk), the dorsal male gonopore is positioned two-thirds of the length from the tip, followed by one to several lateral openings of the seminal receptacles in the posterior end of the body and the subterminal dorsal anus. The most prominent and also eponymous character of the animal is the large, multi-lobed brain, which is located anterior to the mouth opening, nearly taking up the entire cross section of the animal. The intraepidermal, ventral nervous system is reported to consist of two lateral nerve cords and two postpharyngeal commissures. The body wall musculature was described as outer longitudinal and inner circular muscles. The animals are simultaneous hermaphrodites [8–10]. Still, none of these morphological characteristics have made a clear classification into or next to one of the existing nominal phyla possible at the end of the 20th century since the identification of common traits has been ambiguous. However, especially Annelida, Gastrotricha, Gnathostomulida, Mollusca, Nemertea, and Platyhelminthes have been discussed as most likely relatives [6, 8, 11, 12]. Details of the epidermis and other characters were examined by Rieger [8–11] with ultrathin (40–70 nm) sections and transmission electron microscopy (TEM), providing information of great ultrastructural detail. However, a detailed cohesive analysis of several organ systems throughout the entire body, including the complete nervous and muscular system mapped with immunostaining and confocal microscopy is still warranted. This will not only enhance our understanding of their morphology but also facilitate a comparison with morphological data on other interstitial groups gathered within the last two decades [15–17].

Both muscular and nervous systems have been assumed to represent rather conserved organ systems when it comes to their general architecture [18]. Annelids, however, have been found to be highly diverse in their morphological characters, and the ancestral states of musculature [19, 20] and nervous system [21] are still debated. The muscular layout in Lobatocerebridae has been described as internal circular and external longitudinal muscles [8, 10], which contradicts the arrangement found in the majority of annelids [22, 23]. However, cases are known where external circular muscles are reduced [24, 25] and several other muscle sets such as transverse, dorsoventral or bracing muscles have been proposed to functionally represent the circular muscles [22]. Nervous system organization has been suggested to be of high systematic importance, revealing synapomorphies of larger clades within e.g., Crustacea [26], which may be undetectable within other organ systems [21, 27, 28]. However, the nervous system in Annelida varies between being intraepidermal to subepidermal [29], in the number of commissures in the brain (2–4, [29]), the number of circumesophageal commissures (1–2, [29]), the number and arrangement of ventral nerve cords (1–7, medio- to lateroventral [15, 21, 29]) and the number and arrangement of commissures in the ventral nervous system (regularly and mid-segmental to irregularly spread along the entire ventral nervous system [15, 21, 29]).

Based on the previously available information [8, 10] none of the few characteristics of the musculature or nervous system of Lobatocerebridae could be ascribed to annelids only, since they also show similarities to the pattern described especially from interstitial Gnathostomulida, Platyhelminthes, and Mollusca [6, 8–10]. Lobatocerebridae belongs to the meiofauna (animals between 2 mm and 0.06 mm in size [16]), together with exclusively microscopic lineages such as Gastrotricha, Acoelomorpha, Rotifera, Gnathostomulida, Platychelminthes (except for parasitic forms), Tardigrada, Loricifera, Kinorhyncha, as well as miniaturized forms of macrofaunal lineages such as Annelida, Mollusca and Crustacea [16, 30, 31]. The apparent lack of distinct morphological synapomorphies with other clades, the presence of many autapomorphies, and the inaccessibility of material are the main reasons for why the phylogenetic positioning of these interstitial lineages has been so challenging; and why we only most recently have obtained more information on their evolution [7, 32, 33]. Interstitial fauna (living in the interstices between sand grains) all have a microscopic diameter size and most forms are also categorized as meiofaunal. Besides their small size, these interstitial animals often display simple-looking,
worm-like, highly ciliated and glandular, acoelomate bodies with no or few appendages; traits that generally seem to be favored in their confined interstitial environment [16, 34–36]. Several of these seemingly shared traits of interstitial fauna may either have originated as convergent adaptations to their restrictive environment and size, or reflect the recently proposed ancestral meiofaunal condition of Spiralia [7]. Hence, new detailed anatomical investigations of Lobatocerebridae should be evaluated in comparison not only with Annelida, discussing heritage and character evolution, but also with other relevant interstitial metazoans, in order to uncover possible convergent anatomical adaptations to the interstitial space-restricted environment.

The present study will evaluate the recent molecular placement of Lobatocerebridae within Annelida [7], in the light of detailed morphological investigation of nervous, muscular and glandular system with state-of-the-art immunohistochemistry in combination with confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM). Hereby, we attempt to unravel and discuss possible resemblances with relevant interstitial spiralian, and whether these common traits may represent annelid synapomorphies, annelid or spiralian plesiomorphies, or convergent adaptations to the space restricted interstitial environment. Furthermore, with the description of Lobatocerebrum riegeri n. sp., we are adding another species to this enigmatic, otherwise monotypic group.

**Results**

Specimens of Lobatocerebrum riegeri n. sp. overall resemble the body plan described by Rieger [8] for Lobatocerebrum psammicola. More details of the nervous, muscular and glandular systems could be detected in this study, as described in the following (Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9).

**Musculature**

Examined in live and preserved specimens in LM; with phalloidin staining in CLSM and ultrathin sections in TEM; Figs. 1, 2.

**Body wall**

**Longitudinal musculature** As observed by Rieger [8, 10], all muscles of Lobatocerebridae are smooth muscles (confirmed by both CLSM and TEM); no striated musculature was detected in the present study. The longitudinal musculature is organized in six pairs of loose bundles, extending from the rostral tip to the posteroiend most end of the body (Figs. 1, 2a–h). Five pairs of these, the dorsal (dlm), dorsolateral (dllm), two pairs of lateral (llm) and one pair of ventrolateral muscle bundles (vllm), lie dorsal to the two prominent ventral nerve cords, whereas the ventral longitudinal muscles are located ventral to those (Fig. 1a–g).

Each of these muscle bundles consists of three to five muscle fibres (Fig. 2a–f) and has a diameter of 1.2–2.4 μm (measurements based on: number of specimens (n = 3), region of body (r) = 1–4, measurements (m) = 5), deeply embedded into the epidermal cells distal to the transverse muscular ring complexes (see below, tmr). The twelve bundles are regularly distributed along the entire body length (spaced 7.2–10.1 μm apart, n = 3, r = 1–4, m = 5, Figs. 1b–g, 2a–e), except around the mouth opening, where the ventralmost pair (vlm) is shifted closer to the adjacent ventrolateral pair (vllm). The male gonopore or the openings of the seminal receptacles do not cause any similar distortions. All twelve longitudinal muscle bundles extend to the posterior end of the body, inserting subterminally around the anus. While the dorsal, dorsolateral and lateral muscles insert directly, the ventrolateral and ventral bundles first trace the epidermis to the terminal end, before bending antero-dorsally and inserting subterminally around the anus (Fig. 2c).

**Transverse muscular ring complexes** Transverse muscular ring complexes (tmr) are distributed in a regular pattern (spaced 14.5–16.9 μm apart) from the pharynx to the ovary (Fig. 2a), and spaced 6.8–8.9 μm apart posterior of the ovary to the sixth sphincter (n = 3, r = 2, 3, m = 5, Figs. 1, 2b–c). They have previously been misidentified as internal circular musculature [8]. This study, however, could detect that each muscular ring is formed by a series of individual transverse muscle fibres (diameter 0.8–1.3 μm, n = 3, r = 2, 3, m = 5); each of them only spanning the distance between one to three longitudinal bundles (7.6–35.7 μm, n = 3, r = 2, 3, m = 5, Fig. 2j). Up to nine individual transverse fibres are found to constitute one transverse muscular ring complex between all 12 longitudinal muscles (Fig. 2i–j).

Transverse muscles do not form transverse muscular ring complexes in the rostrum, but instead appear as contralateral fibres between longitudinal muscle bundles of opposite sides of the body, hereby creating a star-like structure of individual fibres (star-shaped muscles, ssm, diameter of individual fibres 0.5–1.1 μm, length 10.5–45.2 μm, n = 3, r = 1, m = 5, Fig. 2d, g–h). Their abundance is highest close to the rostral tip (spaced 2.4–5.7 μm apart, n = 2, r = 1, m = 5), where the ducts of the posterior frontal glands are ramifying, and farther separated towards the middle region of the rostrum (spaced 10.3–20.6 μm apart, n = 2, r = 1, m = 5, Fig. 2g–h). The glandular ducts are not muscularized and no closing or constricting mechanism could be detected in this or previous studies [8, 9, 11]. The transverse muscles might therefore be involved in regulating the flow of
Fig. 1 (See legend on next page.)
secretion, in addition to enhancing the flexibility of the rostral tip as observed by behavioral observations (Additional file 1).

**Additional minor body muscles** Specific musculature is formed around the brain, emerging from the ventral and ventrolateral muscles around the pharynx and extending towards the anterior. The lateral pair of these muscles extends lateroventral to the brain, where the fibres branch off around or into the frontal lobe complex (Figs. 1b, 2e–f). The median pair extends to the caudal lobes, where they branch off into more individual fibres and lead to the major, minor and lateral caudal lobes (Figs. 1b, 2e). Due to the intricate network hereby formed around the anterior and posterior regions of the brain, we suggest these muscles to be a supportive structure for the brain, which is probably necessary due to a lack of other structures securing its position in the rostrum.

**Intestinal musculature**

**Pharynx** Although lacking a ventral or axial muscle bulb as found in most annelids, the pharynx is still the most prominent muscular structure in the body, showing five sphincter muscles as already defined by Rieger [8] in addition to the longitudinal body and gut musculature. The first four sphincter muscles of the pharynx surround the mouth opening and mouth cavity (sph1-sph4, adapted from Rieger’s sph0-3 [8]), while the fifth sphincter constricts the digestive tract in the transversal plane, as a short esophagus delineating the pharynx from the midgut (sph5, Figs. 1b, 2a–j). Sphincters 1–4 consist of two to three fibres each (diameter 0.7–1.6 μm), which are always external to the longitudinal muscles of the digestive tract (Fig. 2j). The fifth sphincter (sph5), however, consists of up to eight thin, serially aligned, muscle fibres (diameter 1.2–1.5 μm, n = 3, r = 2, m = 5). It marks the border to the midgut through an elongated constriction to a diameter of 4.5–4.98 μm when relaxed (n = 3, r = 2, m = 5, Figs. 1b, 2a, j). Additionally, the individual fibres are interwoven with the longitudinal gut muscles, rather than being located externally of these (Fig. 2j).

**Digestive tract** The intestinal musculature consists of 12 to 16 individual longitudinal fibres (lmds, diameter 0.66–0.74 μm, n = 3, r = 2, 3, 4, m = 5) arranged in equal distance from each other (spaced 1.5–3.1 μm apart, n = 3, r = 2, 3, 4, m = 5), and therefore resembling the muscular pattern of the body wall musculature (Fig. 2a, i). The circular muscles of the digestive system (cmds), however, are arranged external to the longitudinal muscles of the gut (Fig. 2a, j), as is typical for gut musculature. These true circular muscles (as compared to the transverse muscular ring complexes) are very thin (diameter 0.5–0.6 μm, spaced 3.1–5.8 μm apart, n = 3, r = 2, 3, 4, m = 5) and most consistent in the pharyngeal region anterior and posterior to the fifth sphincter. In the posterior part of the body the longitudinal muscle fibres are embraced by the sixth, last sphincter, which consists of two short circular fibres (diameter 1.35–2.1 μm, n = 3, r = 4, m = 3) and constricts the digestive tract to 6.4–6.6 μm when relaxed (n = 3, r = 4, m = 4, Fig. 2c). The longitudinal muscles of the digestive system fuse with the longitudinal muscles posterior to this constriction (Fig. 2c).

**Nervous system**

*Visualized with acetylated α-tubulin IR, serotonin IR, FMRFamide-like IR, DAPI for cell nuclei and CLSM, Figs. 1, 2, 3, 4, 5, 6, 7.*

The brain in the rostrum of *Lobatocerebrum riegeri* n. sp. is the most conspicuous part of the central nervous system. A series of both anterior rostral and posterior trunk nerve cords emerges from the central neuropil, and some additional nerve bundles are found branching off laterally to the brain (Fig. 4). The brain was described as having one pair of lobes anterior to the neuropil (rostral lobes) and two pairs of lobes (major and minor caudal...
Fig. 2 (See legend on next page.)
lobes [8]) posterior to it. However, this study reveals a more complex system of several sublobes both in the anterior and posterior region (Fig. 4a, b). A total of four main commissures in the ventral nervous system (two posterior to the pharynx, one approximately halfway between the pharynx and the male gonopore, one anterior to the ovary) are recognized. The anterior two commissures, associated with ganglia, connect the two lateral and the median posterior nerve cords with each other (Figs. 1a, c–g, 4a–c, e). The three longitudinal ventro-posterior cords fuse forming a subcerebral commissure. Additionally, peripheral nerves are embedded in the epithelial layer of the animal, forming a grid of longitudinal and semicircular to circular nerves being perpendicular to each other, and being related to the central nervous system.

Acetylated α-tubulin-IR

Central nervous system: Brain The brain of Lobatocerembrum riegeri n. sp. consists of a large neuropil surrounded by impressive multi-lobed groups of perikarya from where longitudinal nerves extend laterally, anteriorly and posteriorly (Figs. 4, 5). The central neuropil comprises several commissures, which seem to be connecting the two main ventral cords in a pattern possibly resembling the annelid dorsal and ventral root of the circumesophageal commissure. The dorsal, median and ventro-anterior commissures are constituted as well defined nervous bundles, consisting of more than 40 nerve fibres. The ventroposterior commissures cannot always be resolved as individual structures, but form a thin sheath of nervous fibres (Figs. 3d, f, 4).

At least three pairs of characteristic large lobes (or ganglia) are arranged around the central neuropil, namely the paired anterior rostral lobes anterior to the neuropil and the pairs of posterior major and minor caudal lobes (respectively lca and lci, Figs. 3a–b, 4b, 5b–d). The major caudal lobes (lobus caudalis major according to Rieger [8], lca) are located mid-ventrally between the minor caudal lobes (lobus caudalis minor according to Rieger [8], lci, Figs. 3a, 4a–b, 5b–d). The minor caudal lobes seem to be subdivided into a lateral and a median sublobe (lcl and lclm, respectively, Fig. 4b). No postcerebral ganglia as described by Rieger [8] have been found, suggesting that either the lateral sub-lobes of the minor caudal lobes or the lateral ganglia, which were found lateral to the central neuropil, have been mistaken for a postcerebral ganglion by Rieger [8]. The rostral lobes (lobus rostralis according to Rieger [8]) appear to be subdivided into one major (lra) and one minor portion (lri) and one lateral sublobe (lrl, Fig. 4a–b).

Although the nervous network of the neuropil is complex and intricate, some major connections could be reconstructed by means of CLSM. Four paired and one unpaired anteriorly directed rostral nerves all originate independently, but adjacent to each other from the anterolateral parts of the neuropil. In addition, several short nerves project out ventrolaterally from the neuropil for 10 to 20 micrometers (lnpp, Figs. 4a–b, 5d). However, no putative specific structure innervated by them could be identified in that region. The four paired and one unpaired rostral nerves anterior to the brain comprise: 1) One pair of ventrolateral anterior nerve cords extending ventro-laterally from the anterior neuropil (avn, Figs. 3a, d–f, 5d) as an anterior extension of the posterior main ventral cords. Each of the ventrolateral anterior cords splits into two thinner bundles to innervate the tip and the sides of the rostrum (avn and avln, respectively, Fig. 4a–b). 2) One pair of dorsolateral nerves splitting up anteriorly (adnc, adlnc, Figs. 4a–b) originating from the lateral neuropil and possibly connected to the nerve stems of the major caudal lobes. 3) One pair of lateral nerve bundles (nlr, Figs. 4a–d) originating dorsomedially at the dorsal root commissure but bending ventrolaterally between the lateral and anterior rostral lobes, where after they condense into a thick bundle continuing ventrolaterally throughout the rostrum until they fan out in the anterior end. 4) One loose pair of nerve bundles (nlr, Figs. 3a, d, 4, 5b–c) originating from the anterolateral
neuropil with minor subbundles (nlri and nlrα, respectively, Fig. 4a–d) leading medioventrally through the major and minor rostral lobes, joining anteriorly of these, and continuing into the anterior part of the rostrum, before spreading out. 5) One unpaired median nerve (mmr) originating middorsally from the dorsal commissure (dc) between the two rostral lobes and extending dorsally through the entire rostrum, until it eventually splits at the tip to innervate the anterior edge (Figs. 4a–d, 5b–c). The function of such a strong innervation of the rostrum is unknown. However, some nerves connect directly to specific cilia, which are stiff and longer than the locomotory cilia and therefore assumed to have sensory function. Many nerves, however, do not seem to connect to any specific epidermal structures and no multicellular sensory organ could be found. Posterior to the neuropil, two pairs of thick dorso-posterior nerve stems extend posteriorly into the major (nlca) and minor caudal lobes (nlci, Fig. 4a–d); again branching into the two median and lateral parts of the minor lobes (nlci and nlcil, respectively, Fig. 4a–d). The nerve stem of each major caudal lobe is composed of nerves originating from the dorsal commissure (which is suggested to resemble the dorsal commissure of the dorsal root) as well as lateral nerves of the neuropil, the latter being seemingly continuous with the rostral dorsolateral nerves. If truly continuous, this may indicate that the dorsolateral nerves are sensory nerves transferring sensory inputs from the rostrum to be processed in the major caudal lobes.

Central nervous system: Ventral cords and commissures

In all specimens investigated, the posterior parts of the ventrolateral nerve cords emerge from the ventrolateral area of the central neuropil and extend to the terminal commissure anterointer to the anus (pc, Figs. 3c, e). They are located dorsolateral to the third (lateral) muscle bundle, although this position varies slightly throughout the body, with the longitudinal muscles sometimes being so deeply embedded within the epidermis that they become more externally positioned than the nerve cords (Fig. 1e–h). The ventrolateral nerve cords consist of three to four times more fibres than the median nerve and measure 3–4 μm in diameter. The longitudinal ventromedial nerve is located intraepidermally, between the two most ventral longitudinal muscle bundles (mnc, Fig. 3g). It is formed by contralateral projections of the ventrolateral nerve at the level of the first commissure, which fuse in the ventral midline with their counterpart at the level of the second commissure. Hereafter, the median nerve continues posteriorly to insert at the terminal commissure. Two projections from the terminal commissure extend for 10–15 μm dorso-posteriorly (pp, Fig. 3c).

Four trunk commissures are connecting the two ventrolateral nerve cords and the median nerve with each other (c1–4, Figs. 1a, c–h, 3b–c, e, 5a). Each commissure apparently consists of as many nerve fibres as the ventro-lateral cords and measures 3–4 μm in diameter. The anteriormost two commissures are located close to each other posterior to the mouth opening, separated by 20–25 μm (c1, c2, Figs. 1a and 3e, g). Since few of the perikarya of the commissures were showing immunoreactivity against serotonin or FMRFamide, only the large ganglia of the first and second commissures could be detected by a few serotonergic cells and DAPI-staining, here showing densely grouped nuclei (Fig. 5a, e). These ganglia are situated dorsoposterior to the commissures and each consists of 30–40 cells (pg1-2, Figs. 3a, b and 5e). The third commissure is located between the pharynx and the male gonopore, approximately 30–50 μm anterior to the gonopore (c3, Fig. 3c). The fourth commissure (c4, Fig. 3c) is located between the testis and ovary.

Single, presumably sensory, cells are sparsely distributed throughout the epidermis of the entire body, but connect to neither the ventral nerve cords nor the peripheral nerves (ss, Fig. 5f). Normally, they consist of one cell with a single cilium often surrounded by a circle of microvilli (Fig. 5f). There is no correlation between a high abundance of these sensoria and specific organs or body regions.

Peripheral nervous system

The peripheral nervous system is embedded in the epidermal cell layer and consists of longitudinal and incomplete circular fibres.
Fig. 4 (See legend on next page.)
These nerves are thinner than the ones of the central nervous system (0.5 µm in diameter) and consist of only very few to individual nerve fibres. The longitudinal peripheral nerves (lpn, Figs. 5h–i) trace the longitudinal muscle bundles throughout the body (Im, Fig. 5i). In the most posterior part of the body, though, they could not be detected with acetylated α-tubulin IR due to the overlaying signal of the central nervous system and the various glands. Their specific origin cannot be assessed, though these thin nerves seem to descend from the central neuropil rather than from the ventrolateral nerve cords.

The incomplete circular nerves (tpn, Fig. 5h) are closely associated with the commissures in the ventral nerve cord, at the level of which they extend from the ventrolateral nerve cords to the dorsal side of the animal. Here, they connect to the longitudinal peripheral nerves exterior to the longitudinal muscle bundles and create a circular connection among these. Additionally and independent of the commissures, one transverse nerve anterior to the pharynx forms an incomplete circle including only lateral and dorsal peripheral longitudinal nerves and three closed rings include all longitudinal peripheral nerves at the level of the seminal receptacles. The latter are set 30–35 µm apart (Fig. 1a). Some additional circular peripheral nerve rings are also found scattered throughout the body. However, they could not be related to any specific structures or reveal a consistent pattern in all specimens investigated.

Tyrosinated tubulin-IR
Immunoreactivity of the tyrosinated tubulin-antibody did not reveal any additional structures adding to the pattern already seen with acetylated α-tubulin-IR. On the contrary, the commissure inside the brain as well as the peripheral nerves could not be revealed using this antibody.

Serotonin-IR
Serotonin-IR was not only labeling nervous structures, but also glands (uni- and multicellular) and stomach content, where the antibodies most likely got retained between particles or in vesicles (Fig. 5e). However, strong labeling of some but not all epidermal cells could be found, with the IR being located in the entire cytosol, but not in the nucleus, which made them therefore resemble serotonergic perikarya (spc, Fig. 5e). Since there was no connection to the nervous system, they could also be specialized gland or epidermal cells with so-far unknown function.

Serotonin-IR also labels all three longitudinal nerves of the ventral nervous system, with one or two strands inside the thick bundles. This pattern is also present in all commissures, but serotonin-IR cannot be detected in any of the peripheral nerves. In the ganglionic pairs associated with the two pharyngeal commissures, four to five perikarya show serotonin-IR, but do not display any specific arrangement inside the ganglion: They seem to be randomly spread between the other cells (spg1-2, Fig. 5e). Additional perikarya with serotonin-IR are found scarcely along the ventral nerve cord.

FMRFamide-like-IR
FMRFamide-like-IR was not consistent between the two specimens investigated. This is mainly due to the rostral glandular structures, which seem to be lying adjacent to the nervous system in Lobatocebrum, and to differences between the studied individuals. Similar to the serotonin - IR described above, the three ventral nerves of the central nervous system, the posterior projection from the terminal commissure (pp, Fig. 5c), as well as the commissures of the central nervous system are revealed using FMRFamide-like-IR (Figs. 3c and 5a). Interestingly, while several nerve fibres in the lateral nerve cords seem to be FMRFamidergic, only one single fibre in the median nerve cord shows this IR, most likely emerging at the level of the pharyngeal commissures. There are no FMRFamidergic perikarya along the ventral nervous system. Only one FMRFamidergic perikaryon in each of the two subpharyngeal ganglia was detected seemingly contributing to the pharyngeal commissure (fpag1-2, Fig. 5a), though its location does not seem to be truly consistent between all specimens investigated.

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Fig. 5 (See legend on next page.)
Possibly as part of the stomatogastric nervous system, two additional pairs of perikarya were revealed dorsal to the mouth and lateral to the pharynx, respectively. Since they are connected ventrally via a thin nerve strand, they seem to constitute the stomatogastric nerve ring described by Rieger ([8], snr, Fig. 5a). Surrounding this structure and disguised by the strong IR of the pharyngeal glands, additional perikarya with very weak FMRFamide-like-IR (sp, Fig. 5a) are found. A further differentiation between the perikarya of the stomatogastric nerve ring and the immune-reactive glands is not possible with any antibody employed in this study.

Though no evidence of the peripheral nervous system could be detected with FMRFamide-like-IR, a FMRFamidergic nerve net is found around the male gonopore. It consists of a thin nerve ring around the male gonopore (nrmg, Fig. 5f) and several individual neurites projecting radially into the ring from their perikarya (mpl, Fig. 5f). Though they are found in all specimens, their number and distribution pattern vary strongly. Additionally, four FMRFamidergic perikarya are distributed scarcely along the spermioduct (spdp, Fig. 5f). No nervous system could be found associated with the ovary or the seminal receptacles.

Glandular structures

*Studied in LM, with acetylated α-tubulin and DAPI staining in CLSM, and in TEM, Figs. 6, 7.* Acetylated α-tubulin-IR of the glandular cell walls [37] proved useful to identify and describe several types of glandular cells in the epidermis.

Epidermal glands

Four types of unicellular epidermal glands were distinguished by acetylated α-tubulin-IR and CLSM: a) ciliated glands; b) smooth flask-shaped glands; c) kidney-shaped gland; and d) unicellular adhesive glands.

Ciliated glands The ciliated gland cells (cg, Figs. 6a–c, 7b) are the largest of the unicellular epidermal glands (diameter 6.9–8.1 µm, length 9.3–11.2 µm, n = 3, r = 1–4, m = 5), distally with a ring formed by shortened stiff cilia around their external opening (sc, diameter 0.6–1.5 µm, n = 3, r = 1–4, m = 5, Fig. 6a, b) and proximally extending into a 30–50 µm long (n = 3, r = 1–4, m = 5), thin tail-region lining the basal membrane. The broad distal region of the gland cells containing the nucleus is located intraepidermally, occasionally alongside the longitudinal muscle bundles, since these are sunken into the epidermal layer (Fig. 6a). The gland cell membranes are lined by twelve to twenty pairwise arranged tubulinergic filaments (tst, n = 3, r = 1–4, m = 5). The cell nucleus has approximately the same size and heterochromatin-content as the nuclei of the surrounding epidermal cells (Fig. 6a–b).

The gland cells are packed with non-electron-dense to weakly-electron-dense vesicles (gv, Fig. 6c). They are found scattered throughout the entire body, though they are most abundant in the posterior region, mainly from the midgut-hindgut-transition towards the posterior end of the body. Although the cellular tail region of the cell may tangent a nerve cord, no close connection or direct nervous innervation of the glands, nor indications of muscular control, were found with CLSM or TEM.

These cells most likely resemble the ‘mucous gland type 1’ in *L. psammica* described by Rieger [8, 10], having a similar characteristic ring of shortened cilia around the opening. This is further corroborated by the similar shape and electron density of the vesicles of these glandular cells [8, 10, 11].
Tubular glands  Tubular gland cells (tg) do not have a cil-
ary ring around their opening, but a continuous lining of
acytated α-tubulin IR in the membrane lining the cell
(diameter 1.5–3.2 μm, length 7.8–8.9 μm, n = 3, r = 1–4,
m = 5, Fig. 6d, e). They are generally characterized by a
slender distal neck-area before the cell widens proximally
(Fig. 6a, d, e). However, a few cells with wide distal open-
ings have been found. A long, thin tail extends from the
basal part of the cell up to 30 μm along the basal lamina,
apparently without connecting to any other structure
(> Fig. 6a). In contrast to the ciliated glands, the smaller
sized tubular gland cells mainly occupy the more distal
part of the epidermal layer, distal to the muscle bundles
(> Fig. 6a). These gland cells are filled with electron-dense,
rod-shaped granules (0.8–1.5 μm in length, 0.2–0.5 μm in
width, n = 3, r = 1–4, m = 5), which are less densely packed
than the vesicles of the adhesive glands (Fig. 6e). They are
highly abundant throughout the entire body (10–15 cells
per 100 μm body length, n = 3, r = 1–4, m = 5), with the
densest distribution in the posterior region of the body.

Kidney-shaped glands  Only one glandular cell type
(kidney-shaped gland cell, ksg) can be distinguished by
the shape of its nucleus: In contrast to all other epider-
mal cell nuclei, nuclei of kidney shaped gland cells are
strictly sickle-shaped (> Fig. 6a, g, h) and their chromatin
denser than the also “deformed” nuclei of ciliated glands
(> Fig. 6c). The cell membrane only contains very few tubu-
linergic elements; yet, dense acetylated α-tubulin-IR
can be detected around the cell opening (diameter 1.1–
1.7 μm, n = 3, r = 1–4, m = 5) and at its base. The overall
appearance of the cell is characteristically kidney-shaped
(diameter 3.3–4.7 μm, length 6.9–7.8 μm, n = 3, r = 4, m =
5, Fig. 6e). Kidney-shaped gland cells are mainly found in
the distal part of the epidermal layer similar to the tubular
gland cells (Fig. 6a). However, the basalmost part of
the cell, which contains the nucleus, can also be found close
to or even internal to the longitudinal muscle bundles
(Fig. 6a, g). These glandular cells are most likely imparting
the greenish speckled appearance of the animals in live
observations (Additional file 1) due to the refractive index
of their content, which consists of non- to weakly-electron
dense and tightly packed vesicles (diameter 0.6–1.2, n = 3,
r = 1–4, m = 5, Fig. 6h). In contrast to the ciliated gland
cells, the vesicles of the kidney-shaped gland cells are less
homogenous in the electron-density of their content, and
denser in their packing, possibly causing the sickle-shape
of the nucleus.

Unicellular adhesive glands  The unicellular adhesive
glands are characterized by a ring of shortened cilia
around the opening, which was suggested to facilitate
mechanical loosening from the substrate instead of a sec-
ond enzymatic gland with releasing function [8, 10] and
therefore morphologically resembles the ciliated glands
though their content and function differ (Fig. 7a, b). Their
secretion is granular, but shows a characteristic structure
with an inner, electron-dense area in a non-electron-dense
oval structure (Fig. 7a, b). Different to the adhesive glands
described in L. psammicola, the glands of L. riegeri n. sp.
do not have linear electron-dense structures in the middle
of the individual granules, but instead linearly arranged
electron-dense dots (Fig. 7a, b). Contrary to the abund-
ance and distribution pattern of the other epidermal
glands cells mentioned above, adhesive gland cells are
restricted to the ventral surface of the body in lower
numbers (1–5 cells per 100 μm ventral body length, n = 3,
r = 1–4, m = 5).

Frontal glands  The main body of the paired posterior frontal glands
(pfg) is found posterior to the brain and anterior to the
pharyngeal region (Figs. 5b, c, 7c). This part of the
glands is difficult to detect with any of the anti-
bodies described above, but can be found combining
the lack of DAPI-signal with overexposed phalloidin-
signal to detect cell membranes and nuclei of volu-
minous cells in a large lobular structure posterior to
the brain lobes (Fig. 7c). The glandular nuclei are
slightly larger than the ones of the brain (diameter 4.3–
5.7 μm × 1.4–2.5 μm, n = 3, r = 1, m = 5). While the gland
body itself is inconspicuous in CLSM, its long ducts,
which are leading ventroanterior of the brain to the tip of
the rostrum, are showing distinct acetylated α-tubulin-IR

(See figure on previous page.)
Fig. 7 (See legend on next page.)
Specific glandular systems in Lobatocerebrum riegeri n. sp. as seen with CLSM and TEM. DAPI in cyan, acetylated α-tubulin in glow or yellow, actin-filaments in green. c, g, h are maximum intensity projections of a subset of the original image stack on various locations of the body, a, b, d–f ultrastructural details of glandular structures. a Sagittal section through the epidermis and an unicellular adhesive gland, b Sagittal section through an unicellular adhesive and a ciliated gland in the epidermis, c brain and portions of the anterior and posterior frontal glands (indicated by white dashed line), d Sagittal section through the anterior tip of the rostrum with ducts of the posterior frontal glands and nerves, e Sagittal section through the mouth opening with glandular cells of the posterior frontal gland and the pharyngeal gland, f Sagittal section through the pharyngeal region with distal parts of the pharyngeal glands, g Distal regions of the ducts of the pharyngeal glands, h Glands around the male gonopore. Abbreviations: afg: anterior frontal gland, ag: adhesive granule, cg: ciliated gland cell, dfg: ducts of the frontal gland, dphg: ducts of the pharyngeal gland, ec: cilia of an epidermis-cell, gg: glandular granules, gv: glandular vesicle, ksg: kidney-shaped gland, lca: major caudal lobe, lr: lateral rostral lobe, lra: major rostral lobe, mg: mouth opening, mgg: male gonopore gland, np: neuropil, pfg: posterior frontal gland, sc: shortened cilium, spd: spermioduct, ss: sickle-shaped nucleus, uag: adhesive gland cell.

Pharyngeal glands

The major glandular structures of the digestive system are the big, multicellular glands of the pharynx, whose products are secreted in the area of the mouth opening (Fig. 7e). 17–18 elongated ducts (diameter 1.8–3.5 μm, length 70–100 μm, n = 3, r = 2, m = 5, Fig. 7g) of posteriorly located glands surround the mouth opening. They are arranged in a denser pattern in its posterior third, while they are more loosely set anteriorly. The main glandular body can be detected posterior to the mouth opening, on the ventral side of the body dorsal to the ventral nerve cords. It is seen as an elongated, bag-like structure filled with spherical, electron dense granules (1.2–1.7 μm, n = 2, r = 2, m = 5) best detected with FMRFamide-like-IR or TEM (Fig. 7e–g). These glands are not epidermal, and their cell bodies are found inside both the longitudinal musculature and transverse muscular ring complexes of the body wall.

Male gonopore glands

Acetylated tubulin-IR was recovered in cells surrounding the dorsal male gonopore. The openings of 16–20 (n = 3, r = 2–3, m = 5) gland cells constituting the complex (Fig. 7h) are connected to the gland bodies via elongated, thin ducts, which are 1.0–1.5 μm in diameter and are all leading to a sunken-in area (14.8–18.3 μm × 6.4–8.2 μm, n = 3, r = 2–3, m = 5, Fig. 7h) around the male gonopore. Approximately half of the cells are densely packed around the anterior end, and the other half around the posterior end, with a small gap between the two portions.

Reproductive system

Studied in LM, with acetylated α-tubulin and DAPI staining in CLSM, Fig. 8.

In all four adult animals investigated, both male and female reproductive organs or gametes could be found, as well as seminal receptacles to store the mating partner’s sperm.

Male gonad

The male gonad is located on the dorsolateral side of the animal, posterior to the third commissure. It is an elongated, thin structure, with the gonopore opening on the dorsal surface of the animal (diameter 1.5–2.7 μm, n = 3, r = 2–3, m = 3, Figs. 7g, 8a). A thin channel (diameter 1.4–1.8 μm, n = 3, r = 2, m = 5) extends posterior to the pore, with a high amount of the long, thin, fibrous sperm stored in the posterior region (Fig. 8a). Where the sperm is produced is unclear; however, the majority of
glands involved in this apparatus are arranged around the gonopore itself, as described above, creating a glandular field (16.2–17.0 μm × 3.5–5.4 μm, n = 3, r = 2, 3, m = 4, Figs. 7a, b, 8a).

**Female gonad**

Up to four eggs, lined up behind each other and increasing in volume posteriorly (Fig. 8b), are the only structures of the female gonad detected with either immunohistochemistry or live observations. The eggs are of irregular shape, reflecting the available space in the body. Although the openings of both seminal receptacles and the male gonad have been found, no obvious opening was detected near the eggs, and they may have to be deposited via rupturing of the epidermis.

**Seminal receptacles**

In the posterior part of the body, the adult animals form one to several seminal receptacles (rs, Fig. 8c, d). These receptacles are thin-walled capsules consisting of few

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**Fig. 8** Reproductive organs in *Lobatocerebrum riegeri* n. sp. as seen with CLSM and transmitted light. DAPI in cyan, acetylated α-tubulin in glow, phalloidin in green. All images are maximum intensity projections of a subset of the original image stack. Orientation is anterior to the left and dorsal side up if not indicated otherwise. **a** Testis with spermioduct and glands around the male gonopore, **b** Ovary, **c–d** Seminal receptacles at the level of the tips of the sperm filaments **c** and with bent sperm filaments **d**. The contours of the receptacles are traced with dashed lines to facilitate orientation. Abbreviations: cg: ciliated gland, e1–2: egg 1–2, llm: lateral longitudinal muscle, lm: longitudinal muscle, mg: male gonopore, mgg: male gonopore glands, n2: nucleus of egg 2, ors1–2: opening of the seminal receptacle 1–2, rs1–2: seminal receptacle 1–2, spd: spermioduct, spf: sperm filaments, t: testis, tg: tubular gland, tmr: transverse muscular ring complex, vlnc: ventral longitudinal nerve cord.
Fig. 9  Lobatocerebrum riegeri n. sp. Anterior is to the left and dorsal to the upper side of the picture in the light micrographs (b–e). a Partly schematized drawing of an adult Lobatocerebrum riegeri n. sp. with the most significant traits emphasized based on light microscopic observation. b anterior part of the rostrum with glandular epidermis and frontal gland ducts, c brain, d, ciliated pharynx and e posterior end of the body with midgut-hindgut-transition in lateral view. Abbreviations: ac: anterior cilia, an: anus, c: cilium, dfg: frontal gland ducts, hg: hindgut, go: glandular opening, ksg: kidney-shaped gland, lca: major caudal lobe, lci: minor caudal lobe, lg: lateral ganglion, lra: major rostral lobe, lrl: lateral rostral lobe, mg: male gonopore, mgg: male gonopore gland, mig: midgut, mo: mouth opening, np: neuropil, ph: pharynx, phg: pharyngeal gland, rs: seminal receptacles, spd: spermiduct, t: testis
cells without any specific immunoreactivity (Fig. 8c, d). Their diameter is 20–30 µm \((n = 3, r = 1, m = 4)\), and the sperm filaments (spf) can be seen inside, bent and curled up (Fig. 7e). The openings of the receptacles (ors, diameter 0.8–1.7 µm, \(n = 3, m = 3\)) are on the ventrolateral side of the body (Fig. 8c).

Motility patterns
Studyed in LM, Additional file 1.

Ciliary locomotion
*Lobatocerebrum riegeri* n. sp. is uniformly ciliated along the entire body and moves mainly by a relatively slow, but steady back and forward ciliary gliding rather than muscular action (Additional file 1). Ciliary mode of locomotion is cost-efficient for minute interstitial animals, yet fast reactions to avoid obstacles are dealt with by contractions of the longitudinal (and to a lesser degree transverse musculature ring complexes) body wall muscles.

Muscular locomotion
Behavioral observations of several specimens revealed different movement patterns of the rostrum and the remaining body: while the posterior part of the body was often found curled up and attached to the substrate, the anterior part did exploratory movements, including contraction along the longitudinal body axis and sweeping of the rostrum from side to side (Additional file 1). This coincides with the lack of transverse muscular ring complexes and presence of star-shaped muscles in the rostrum. During these contractions of the longitudinal muscles, the anterior part of the body appears more wrinkled, also indicating that an elongation or contraction of the longitudinal muscles in the anterior region is not affecting the trunk and posterior part of the body. With all the longitudinal muscles being continuous along the entire body, the stabilizing and immobilizing of the median body during longitudinal contractions may be accomplished by counteracting contractions of the transverse muscular ring complexes in the trunk and posterior part of the body.

The animals also regularly curl up or fold their posterior body in sinuous curves, which may facilitate anchoring the body among sand grains in the substrate. The trunk may also show minor contractions and winding movements occasionally providing a forward movement in a snake-like pattern (Additional file 1). This most likely is due to a combination of muscular and ciliary locomotion.

The posteriormost end of the body can also be active and flexible (performing contractions and elongations as well as bending movements), though this motility is limited to a small region anterior to the anus (10–30 µm, \(n = 3, r = 4, m = 5\)). Occasionally, when the posterior part is curled up or bent, it would act more as an anchor rather than promote forward movement (Additional file 1). *Lobatocerebrum riegeri* n. sp. has never been observed to leave the substrate and swim into the water column.

Movements in the digestive system
Although no feeding behavior could be observed, stomach content was moved continuously in both directions, even when the animal was not moving (Additional file 1). This indicates that the weak musculature of the digestive system, maybe together with the body wall musculature, is responsible for movement of the food through the body. The fifth sphincter here probably plays an important role in sealing the digestive tract and prohibiting food getting expelled through the pharynx and mouth opening again, since no movement of food could be observed in the pharynx anterior to this muscular constriction.

Taxonomy
Phylum Annelida Lamarck, 1809
Family Lobatocerebridae Rieger, 1980
Genus *Lobatocerebrum* Rieger, 1980
Species *Lobatocerebrum riegeri* n. sp.
(Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, Tables 1, 2, 3, 4, Additional file 1)

*Lobatocerebrum sp.* 2 in [8–11], registered in ZooBank (E3DCE97A-7F7A-4799-827A-DF2EA41AE1A5).

Diagnosis
Entirely ciliated *Lobatocerebrum*, unsegmented, hyaline body with glandular epidermis (unicellular, kidney-shaped glands with transparent-green content), 1.08–1.6 mm in length and 0.04–0.06 mm in diameter. Large, lobular brain, with central neuropil displaced 8.22–18.18 U posterior of anterior body edge (relative to total body length). Ventral mouth opening, positioned posterior of the brain, 20.48–34.69U from anterior edge (relative to total body length). Dorsal opening of male gonopore positioned 10-14U posterior to the neuropil (relative to total body length).

Type material
Holotype: one 1.57 mm long mature hermaphrodite (testis, ovary with eggs and seminal receptacles present) (ZMUC-POL-2384), beach in front of the Interuniversity Institute for Marine Sciences (IUI) northwest of Eilat, Israel (N 29° 30.211’ E 34° 55.068), 9 meters deep, coral sand, collected by the authors 20.02.2014.
Paratypes: Two mature and one juvenile specimens (section series, ZMUC-POL-2385, ZMUC-POL-2386, ZMUC-POL-2387), same locality as for holotype, (sampled on 14.02.2014, 16.02.2014 and 18.02.2014); one mature specimen collected by Mike Crezée (section series, ZMUC-POL-2388).
Etymology
The species is named in memory of Reinhard M. Rieger, who discovered and described the first representative of Lobatocerebridae.

Description
Measurements of holotype are given in the text, ranges of all types are given in parentheses; juvenile is not included.

*Lobatocerebrum riegeri* has an elongated, cylindrical, entirely ciliated body, which appears slightly greenish due to the glandular epidermis (Fig. 9a). The total body length is 1.57 mm (varies between 1.08 and 1.6 mm in adults), the body width is 0.04 mm (0.04–0.06 mm, Tables 1, 2). The rostrum is 305 μm (293–368 μm, Fig. 9b); the uniform trunk extends for an additional 1266 μm (710–1336 μm, Table 1). The brain is located dorsally in the rostrum 246 μm (204–266 μm) from the anterior tip, extends for 119 μm (33–44 μm) posteriorly and has an oval, but lobular appearance (two frontal and four posterior lobes embracing the central neuropil visible with LM, Fig. 9c, Tables 1, 2). The mouth opening is 322 μm (330–374 μm) from the anterior tip; extends for 21 μm (20–31 μm, Fig. 9d, Tables 1, 2) and the pharynx is heavily ciliated and supplied with several glands. The transitions from the fore- to the mid-gut 480 μm (450–580 μm) from the anterior tip and from the mid- to the hindgut 820 μm from the anterior tip (800–1300 μm) are marked by a decrease in diameter, sphincter muscles and change in ciliation pattern (strong in fore- and hindgut, weaker in mid-gut). No protonephridia were detected with the techniques applied (adults and juvenile). The male gonopore 593 μm (476–596 μm) from the anterior tip and associated gland cells as well as one testis 758 μm (576–776 μm, Fig. 9a, Tables 1, 2) from the anterior tip are all located dorsally. In mature specimens, big, slightly

| Table 1 Measurements of the specimen of *Lobatocerebrum riegeri* n. sp. investigated in this study and distances of specific structures and organs to the anterior end of the body |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| End of measurement from the anterior tip | Lobatocerebrum riegeri II (CLSM, holotype) | L. riegeri III (CLSM, paratype) | L. riegeri IV (CLSM, paratype) | L. riegeri I (juvenile, CLSM, paratype) | L. riegeri V alive (LM) |
| Total Length [μm] | 1571,9 | 1078 | 1606 | 478 | 1646,6 |
| Total Width [μm] | 40 | 79 | 55 | 66,5 | 51,4 |
| Position of the neuropile | 247 | 196 | 204 | 132 | 250 |
| Position of the brain | Middle of the brain | 246 | 221 | 204 | 137,7 | 266 |
| | Most anterior part | 177 | 179 | 159 | 108 | 215 |
| | Most posterior part | 296 | 251 | 248 | 162 | 304 |
| Position of the mouth | Middle of the mouth | 322 | 374 | 344 | 182,3 | 330 |
| | Most anterior part | 305 | 368 | 293 | 170 | 310 |
| | Most posterior part | 343 | 403 | 375 | 203 | 350 |
| Position of the male gonopore | 593 | 476 | 557 | 596 |
| Position of the testis | Middle of the testis | 758 | 576,5 | 712 | 776 |
| | Most anterior part | 725 | 556,5 | 691 | 741 |
| | Most posterior part | 787 | 596,5 | 732 | 811 |
| Position of the ovary | Middle of the ovary | 1107 | 702,5 | 1053 | 1150 |
| | Most anterior part | 953 | 597,8 | 1036 | 995 |
| | Most posterior part | 1248 | 769,5 | 1070 | 1304 |
| Position of the seminal receptacles | Middle of the receptacles | 1428 | 960,5 | 1350 | 1510 |
| | Most anterior part | 1424,9 | 944,5 | 1340 | 1501 |
| | Most posterior part | 1432,9 | 970 | 1360 | 1519 |

The measurements were taken from both live (n = 1) and fixed and mounted (n = 5) specimens, including one juvenile, as indicated. In the latter, neither the male nor the female gonad could be detected in transmitted light or CLSM-images. Measurements are taken in μm (in case of body length and width) and as μm from the anterior end of the respective animal to a specific point as indicated in the first and second column.
<table>
<thead>
<tr>
<th></th>
<th>Lobatocerebrum psammicola live</th>
<th>L. psammicola fixed</th>
<th>Lobatocerebrum sp. 1</th>
<th>Lobatocerebrum sp. 2</th>
<th>Lobatocerebrum riegeri</th>
<th>L. riegeri conclusions/remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total length [mm]</td>
<td>3.0</td>
<td>2.0–2.2</td>
<td>1.1</td>
<td>1.7</td>
<td>1.57 (1.08–1.6 [0.48])</td>
<td>L. riegeri is shorter than L. psammicola and the other reported specimens</td>
</tr>
<tr>
<td>Total width [mm]</td>
<td>0.11</td>
<td>0.07–0.08</td>
<td>0.06</td>
<td>0.06</td>
<td>0.04 (0.04–0.06 [0.07])</td>
<td>L. riegeri is thinner than the other species and reported specimens, though not relative to the body length</td>
</tr>
<tr>
<td>Relative width</td>
<td>0.036</td>
<td>0.035–0.036</td>
<td>0.055</td>
<td>0.035</td>
<td>0.025 (0.025–0.038 [0.15])</td>
<td>L. riegeri is thinner than the other species and reported specimens, though not relative to the body length</td>
</tr>
<tr>
<td>Position of the neuropile [1–100U]</td>
<td>9</td>
<td>7–12</td>
<td>14</td>
<td>12</td>
<td>18.18 (8.22–18.18 [27.61])</td>
<td>→displaced more posteriorly in L. riegeri than in L. psammicola and the other reported specimens</td>
</tr>
<tr>
<td>Position of the brain [1–100U]</td>
<td>9</td>
<td>7–12</td>
<td>14</td>
<td>12</td>
<td>15.65 (12.7–20.5 [28.8])</td>
<td></td>
</tr>
<tr>
<td>Position of the mouth [1–100U]</td>
<td>14</td>
<td>10–17</td>
<td>20</td>
<td>20</td>
<td>20.48 (20.48–34.69 [38.14])</td>
<td>→displaced more posteriorly in L. riegeri than in L. psammicola, but in the same range as the other reported species</td>
</tr>
<tr>
<td>Position of the male gonopore [1–100U]</td>
<td>38</td>
<td>30–36</td>
<td>No measurements provided</td>
<td>31</td>
<td>37.72 (34.68–44.16)</td>
<td>→ range outside L. sp. 2, but similar to L. psammicola</td>
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<tr>
<td>Position of the testis [1–100U]</td>
<td>47–57</td>
<td>46–56</td>
<td>No measurements provided</td>
<td>35–43</td>
<td>48.21 (44.33–53.48)</td>
<td>→ posterior to L. sp.2, but with the broad range similar to L. psammicola</td>
</tr>
<tr>
<td>Position of the ovary [1–100U]</td>
<td>58–63</td>
<td>48–79</td>
<td>No measurements provided</td>
<td>70.42 (65.17–70.42)</td>
<td>70.42 (65.17–70.42)</td>
<td>→ too broad ranged to be diagnostic</td>
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<tr>
<td>Position of the seminal receptacles [1–100U]</td>
<td>90.5</td>
<td>87–89</td>
<td>No measurements provided</td>
<td>88</td>
<td>90.84 (84.06–90.84)</td>
<td>→ too broad ranged to be diagnostic</td>
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</table>

The measurements of Lobatocerebrum psammicola, L. sp. 1 and L. sp. 2 were taken from [8]. L. riegeri n. sp. (this study) was obtained from this study and translated in the units used by [8] (in 1–100U for the entire body length). For L. riegeri n. sp., all measurements are taken from fixed and mounted specimens in the following order: holotype (range of all adult specimens (juvenile)); L. riegeri n. sp. specimen III was excluded from the range given for body length and width, since it was compressed to a high degree, but was considered for the relative measurements.
Table 3. Compilation of features of the nervous system in representatives of different spiralian groups with previously proposed relationship to Lobatocerebrum riegeri n. sp.

<table>
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<th>ANNELEDA</th>
<th>SIPUNCULA</th>
<th>ORBINIIDAE</th>
<th>MOLLUSCA</th>
<th>SOLENOGASTRES</th>
<th>CAUDOFOVEATA</th>
<th>GASTROPODA</th>
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<td>Lobatocerebrum</td>
<td>Lobatocerebrum</td>
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<td>Intraepithelial</td>
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<tr>
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<td>1 pair</td>
<td>1 pair</td>
<td>2 pairs (+1</td>
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<tr>
<td>longitudinal nerve</td>
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<td>median cord)</td>
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</tr>
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<td>&gt;2</td>
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<td>&gt;2</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>&gt;2</td>
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<tr>
<td>Presence of a</td>
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<td>+</td>
<td>+</td>
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<td>Grid of pairwise</td>
<td>Grid of pairwise</td>
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<td>longitudinal and</td>
<td>arranged</td>
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<td>longitudinal and several</td>
<td>longitudinal and several</td>
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<tr>
<td>Nerve plexus</td>
<td>[57, 73, 74]</td>
<td>[75, 76]</td>
<td>[40, 41]</td>
<td>[40]</td>
<td>[43]</td>
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References: [57, 73, 74, 75, 76, 40, 41, 40, 43]
Table 3: Compilation of features of the nervous system in representatives of different spiralian groups with previously proposed relationship to Lobatocerebridae. Presence of a character is labeled with +, absence with -, numbers and additional informations are given wherever possible. '?' indicates the lack of information in the references mentioned, while reinvestigations from this study (in the case of L. riegeri n. sp.) and assumptions based on additional references are included by putting the assessment in brackets (+) or (−). Only species with previously [8–12] or recently [7] suggested relationship to Lobatocerebridae were considered. Insufficient information in one species was supplemented with closely related species, based on the literature acknowledged in the reference-row.

<table>
<thead>
<tr>
<th></th>
<th>NEMERTEA</th>
<th>GNATHOSTOMULIDA</th>
<th>PLATYHELMINTHES</th>
<th>XENACOELOMORPHA</th>
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<tr>
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<td>ANOPLA</td>
<td>BURSOVAGINOIDEA</td>
<td>FILOSPERMOIDEA</td>
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<td></td>
<td>Cephalothrix linearis</td>
<td>Procephalo-thrix linearis</td>
<td>Lineus viridens</td>
<td>Gnathostomula peregrina</td>
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<td>Location of the ventral nerve cords</td>
<td>Subepidermal</td>
<td>Subepidermal</td>
<td>Subepidermal</td>
<td>Intraepithelial</td>
</tr>
<tr>
<td>Lobular structure</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>? (−)</td>
</tr>
<tr>
<td>Central neuropile</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Number of brain commissures</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>1</td>
</tr>
<tr>
<td>Number of posterior longitudinal nerve cords</td>
<td>1 pair</td>
<td>1 pair + 1 dorsal median + 1 ventral median cord</td>
<td>1 pair</td>
<td>1 pair</td>
</tr>
<tr>
<td>Median posterior nerve cord</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+ (just a short piece)</td>
</tr>
<tr>
<td>Number of rostral longitudinal nerve cords</td>
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<td>Approx. 8 pairs</td>
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<tr>
<td>Total number of ganglia</td>
<td>?</td>
<td>?</td>
<td>1 pair</td>
<td>1 pair</td>
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<td>Nonganglionated posterior commissures</td>
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<td>1</td>
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<td>Presence of a subpharyngeal ganglion</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Subependial plexus, commissural plexus, stomatogastric plexus, proboscidial plexus</td>
<td>5 longitudinal nerves</td>
<td>6 longitudinal nerves</td>
<td>3 dorsal longitudinal nerves</td>
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</tbody>
</table>

References: [42, 48] [42] [77] [78] [17, 79] [17] [46, 80] [81, 82]
Table 4 Compilation of features of the nervous system in representatives of different annelid groups and *Lobatocerebrum riegeri* n. sp

<table>
<thead>
<tr>
<th>ANNELIDA PREVIOUS “PROBLEMATICA”, now ANNELIDA</th>
<th>LOBATOCEREBRIDAЕ</th>
<th>DIURODRILIDAЕ</th>
<th>?</th>
<th>SIPUNCULA</th>
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<tbody>
<tr>
<td><em>Lobatocerebrum riegeri</em> n. sp.</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
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<tr>
<td><em>Lobatocerebrum psammicola</em></td>
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<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
</tr>
<tr>
<td><em>Diurodrilus</em> sp.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Jennaria pulchra</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>Phascolion strombus</em></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Siphonosoma australе</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**BRAIN**

| Location of the ventral nerve cords          | Intraepithelial | Intraepithelial | Intraepithelial | Intraepithelial |
| Lobular structure                            | +               | +              | ?              | ?          |
| Central neuropile                            | +               | +              | +              | +          |
| Number of brain commissures                  | 4              | ?              | 4              | ?          |
| Dorsal root (dorsal/ventral commissure)      | + (+/+)         | ?              | + (+/+)        | ?          |
| Ventral root (dorsal/ventral commissure)     | + (+/+, individual fibres spread out) | ? | + (+/+) | ? |

**NERVE CORDS OF THE CENTRAL NERVOUS SYSTEM**

| Number of posterior longitudinal nerve cords | 1 pair + 1 median cord | 1 pair (+1 median cord?) | 2 pairs | 1 pair | 1 pair (fused during development) |
| Median posterior nerve cord                  | ?                   | -              | -        | -        |
| Number of rostral longitudinal nerve cords  | 2 ventrolateral + < 7 additional, smaller ones | 2 (? | >2 | ? | 0 | 0 |

**GANGLIA AND COMMISSURES ALONG THE VENTRAL NERVE CORD**

| Total number of ganglia | 2 pairs | 2 pairs | 1 (fused pair) | ? | >2 | >2 (during development) |
| Nonganglionated posterior commissures         | >2 | 2 | >2 | 1 | ? | >2 (during development) |
| Presence of a subpharyngeal ganglion          | + | + | + | ? | + | + (during development) |

**STOMATOGASTRIC NERVOUS SYSTEM**

| Stomatogastric nervous system                | + (ring around the pharynx) | + (ring around the pharynx) | + (ring around the esophagus) | + (ring cells in the pharyngeal epithelium) |
| Origin of the stomatogastric nervous system  | Postpharyngeal ganglion     | Postpharyngeal ganglion     | prebuccal ganglion            | + (ring around the esophagus, brain (?) |

**PERIPHERAL NERVOUS SYSTEM**

| Grid of distinct longitudinal and circular nerves | ? | 1 pair of longitudinal nerves, several branches for innervating organs | Some nerves around the pharynx and gut, otherwise not present or not described | Nerve plexus | ? |

**References**

This study [8–11] [61] [9, 83] [57, 73] [58]
Table 4 Compilation of features of the nervous system in representatives of different annelid groups and *Lobatocerebrum riegeri* n. sp

<table>
<thead>
<tr>
<th>ANNELID GROUPS</th>
<th>DINOPHILIDAE</th>
<th>PROTODRILIDAE</th>
<th>PSAMMODRILIDAE</th>
<th>NEREIDIDAE</th>
<th>CAPITELLIDAE</th>
<th>SERPULIDAE</th>
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</thead>
<tbody>
<tr>
<td><em>Dinophilus gyrociliatus</em></td>
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<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
</tr>
<tr>
<td><em>Protodrilus sp.</em></td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
</tr>
<tr>
<td><em>Psammodrilus fauveli</em></td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
</tr>
<tr>
<td><em>Platynereis sp.</em></td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
</tr>
<tr>
<td><em>Pomatoceros lamarckii</em></td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
</tr>
<tr>
<td><em>Spirorbis cf. spirorbis</em></td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BRAIN</th>
<th>Location of the ventral nerve cords</th>
<th>Lobular structure</th>
<th>Central neuropile</th>
<th>Number of brain commissures</th>
<th>Dorsal root (dorsal/ventral commissure)</th>
<th>Ventral root (dorsal/ventral commissure)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraepithelial</td>
<td>Intraepithelial</td>
<td>+</td>
<td>+</td>
<td>+ (+/+</td>
<td>+ (+/+)</td>
</tr>
<tr>
<td></td>
<td>Lobular structure</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+ (+/+</td>
<td>+ (+/+)</td>
</tr>
<tr>
<td></td>
<td>Central neuropile</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+ (+/+</td>
<td>+ (+/+)</td>
</tr>
<tr>
<td></td>
<td>Number of brain commissures</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>4 (+/+)</td>
<td>4 (+/+)</td>
</tr>
<tr>
<td></td>
<td>Dorsal root (dorsal/ventral commissure)</td>
<td>?</td>
<td>+ (+/+)</td>
<td>+ (+/+)</td>
<td>+ (+/+)</td>
<td>+ (+/+)</td>
</tr>
<tr>
<td></td>
<td>Ventral root (dorsal/ventral commissure)</td>
<td>?</td>
<td>+ (+/+)</td>
<td>+ (+/+)</td>
<td>+ (+/+)</td>
<td>+ (+/+)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NERVE CORDS OF THE CENTRAL NERVOUS SYSTEM</th>
<th>Number of posterior longitudinal nerve cords</th>
<th>Median posterior nerve cord</th>
<th>Number of rostral longitudinal nerve cords</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location of the ventral nerve cords</td>
<td>3 pairs + median cord</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Lobular structure</td>
<td>1 pair</td>
<td>-</td>
<td>0 (but innervation of tentacles)</td>
</tr>
<tr>
<td>Central neuropile</td>
<td>+</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Number of brain commissures</td>
<td>+</td>
<td>-</td>
<td>0 (but innervation of tentacles)</td>
</tr>
<tr>
<td>Dorsal root (dorsal/ventral commissure)</td>
<td>+ (+/+)</td>
<td>+</td>
<td>0 (but innervation of tentacles)</td>
</tr>
<tr>
<td>Ventral root (dorsal/ventral commissure)</td>
<td>+ (+/+)</td>
<td>+</td>
<td>0 (but innervation of tentacles)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GANGLIA AND COMMISSURES ALONG THE VENTRAL NERVE CORD</th>
<th>Total number of ganglia</th>
<th>Nonganglionated posterior commissures</th>
<th>Presence of a subpharyngeal ganglion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location of the ventral nerve cords</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>+</td>
</tr>
<tr>
<td>Lobular structure</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>+</td>
</tr>
<tr>
<td>Central neuropile</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>+</td>
</tr>
<tr>
<td>Number of brain commissures</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>+</td>
</tr>
<tr>
<td>Dorsal root (dorsal/ventral commissure)</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>+</td>
</tr>
<tr>
<td>Ventral root (dorsal/ventral commissure)</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>STOMATOgastric NERVOUS SYSTEM</th>
<th>Stomatogastric nervous system</th>
<th>Stomatogastric nervous system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location of the ventral nerve cords</td>
<td>+ (ring around the pharynx)</td>
<td>+ (ring around the esophagus)</td>
</tr>
<tr>
<td>Lobular structure</td>
<td>+ (ring around the esophagus)</td>
<td>+ (ring around the esophagus)</td>
</tr>
<tr>
<td>Central neuropile</td>
<td>+ (ring around the esophagus)</td>
<td>+ (ring around the esophagus)</td>
</tr>
<tr>
<td>Number of brain commissures</td>
<td>+ (ring around the esophagus)</td>
<td>+ (fibre along the gut, ring around the esophagus)</td>
</tr>
<tr>
<td>Dorsal root (dorsal/ventral commissure)</td>
<td>+ (ring around the esophagus)</td>
<td>+ (fibre along the gut, ring around the esophagus)</td>
</tr>
<tr>
<td>Ventral root (dorsal/ventral commissure)</td>
<td>+ (ring around the esophagus)</td>
<td>+ (fibre along the gut, ring around the esophagus)</td>
</tr>
</tbody>
</table>

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Table 4 Compilation of features of the nervous system in representatives of different annelid groups and *Lobatocerebrum riegeri* n. sp.

<table>
<thead>
<tr>
<th>Origin of the stomatogastric nervous system</th>
<th>Brain (dorso-posterior neuropile)</th>
<th>Buccal ganglion</th>
<th>Brain</th>
<th>Brain</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>PERIPHERAL NERVOUS SYSTEM</td>
<td>Regular grid of longitudinal and circular nerves, nerve plexus dorsal to the ventral nervous system</td>
<td>?</td>
<td>?</td>
<td>Grid of distinct longitudinal and circular nerves</td>
<td>Grid of distinct longitudinal and circular nerves</td>
</tr>
</tbody>
</table>

References: [14, 84, 85], [55, 86], [87, 88], [70, 89, 90], [91], [92], [92]

Details of the brain, the ventral nervous system, the stomatogastric nervous system and the peripheral nervous system are given in an attempt to reveal common features or possible apomorphies in Lobatocerebridae. Presence of a character is labeled with +, absence with -, numbers and additional informations are given wherever possible. ? indicates the lack of information in the references mentioned, while reinvestigations from this study (in the case of *L. riegeri* n. sp.) and assumptions based on additional references are included by putting the assessment in brackets (+) or (−). Insufficient information in one species was supplemented with closely related species, based on the literature acknowledged in the reference-row.
oval but irregular-shaped eggs can be found in the posterior region of the body 1107 μm (702–1107 μm, Tables 1, 2) from the anterior tip. Seminal receptacles, if present (one to three found in the specimens investigated), can be found in the posterior region of the body 1428 μm (960–1428 μm, Table 1) from the anterior tip, opening laterally. The anus opens dorsally 1500 μm (1000–1500 μm, Fig. 9e) from the anterior tip.

Remarks
Lobatocerebrum riegeri is smaller (1.08–1.6 mm in adults compared to 2.0–3.0 mm in adults of L. psammicola) and thinner (0.04–0.06 mm in adults compared to 0.07–0.11 mm in L. psammicola) than the related species [8]. The brain is displaced more posterior (8.22–18.18 U (distance from anterior end to central neuropil relative to total body length) in adults compared to 7-12U in L. psammicola) and the mouth opening is displaced further posterior in the body than in the previously described species (12.7–20.5U in adults compared to 10-17U in L. psammicola). Further distinguishing Lobatocerebrum riegeri from its previously described relative is the fact that it has a different secrete in the unicellular adhesive glands (linearly arranged globular inclusions in the granules in the adhesive glands in L. riegeri as compared to linear, rod-shaped inclusions in L. psammicola). Additionally, the two localities the different species have been found in (North Carolina, USA for L. psammicola and Eilat, Israel for L. riegeri) are far apart from each other and therefore the presence of two species seems to be probable. Further studies also involving molecular data are needed to further support this hypothesis, but are unfortunately not available now.

Discussion
Function and origin of the unique muscular ring complex
The characteristic annelid (and spiralian) muscular arrangement consists of an external circular and internal longitudinal muscle layer [22, 38]. However, the pattern in Lobatocerebraidae differs in having externally positioned longitudinal muscles sunken into the epidermis, and within those inner transverse muscles previously mistakenly interpreted as continuous circular muscles [8]. However, each of these ring complexes resembles a discontinuous muscular network, composed by transverse muscle fragments, which together form serially repeated, discontinuous muscular ring complexes interconnecting the longitudinal muscles. Peristaltic body movements normally caused by contraction of circular muscles where never observed in Lobatocerebrum riegeri; however, the transverse fragments neither seemed to operate independently, but most likely aid to stabilizing the body wall during contraction of the longitudinal fibers. The lack of ring complex muscles in the rostrum on the other hand seems to allow for the high flexibility of the long rostral area in L. riegeri (Fig. 1b, e–g, Additional file 1). A flexibility which otherwise would have been prevented due to their different interconnecting composition compared to regular spiralian circular muscles, located external of the longitudinal muscles, even along the long rostrum of meiofaunal animals such as a the filo/dermoplankton Gnathostomulida [17] and catenulid Platyhelminthes ([39, Table 3). Since a similar muscular solution to both granting flexibility of the rostrum and stabilizing the trunk is not found in other annelids (or sipunculids), the muscular ring complex is considered a unique apomorphy of Lobatocerebraidae.

The paradox of a complex brain in a simple animal
Lobular or compartmentalized, ganglionated brains are commonly found in macroscopic representatives of Spiralia and other metazoan groups (e.g. [18, 40, 41]), but interstitial animals generally do not show such a complex architecture (e.g. [16, 29], Tables 3, 4). However, some interstitial species of nemerteans [42], molluscs (especially in several wormlike gastropods such as Helminthope [43], Rhodope [44], and Pseudovermis [45]) and catenulids [46] also show some compartmentalization of the brain having, for example, visual and olfactory centers (Table 3). An other representative with a ganglionated brain is the enigmatic interstitial “worm” Jennaria pulchra (Figure 3a in [9]), which is described as representing many plesiomorphies of the trochozoan body plan [47] and possibly being an annelid [9]. Different compartments or lobes of the brain are normally related to processing of different sensory stimuli, yet all conspicuous sensory organs such as eyes, sensory appendages or olfactory nuchal organs are lacking in Lobatocerebraidae. Moreover, the indistinct gut content and simple alimentary tract and behavior indicates that Lobatocerebrum sp. is an unselective deposit feeder. Though no sensory structures are found adjacent to, or directly connected to specific regions in the brain, it is still striking how the anterior rostrum is strongly innervated with nerves connected to various parts of the brain. Hence, though unlikely, the glandular secretion or the stimuli of the scattered sensory cells may in fact be processed in a much more organized manner and their signaling complexity exceed our expectations. Nonetheless, the complex lobular architecture of the brain in L. riegeri seems a functional paradox.

Systematic importance of longitudinal nerve configuration
Annelid central nervous systems vary in numbers of main longitudinal nerves, from one ventro-median cord to seven or more ventral nerves (Table 4, [21]). Based upon developmental studies and a broad comparison across Annelida, five ventral cords have been proposed as the ancestral pattern [15], yet this proposed character
The pattern of five nerves is made up of one pair of ventral, one pair of lateroventral and one median cord. The latter is revealed during neurogenesis in several annelids, and has been found in most interstitial annelids, possibly being an annelid apomorphy. However, it is only found elsewhere in Spiralia in a few exceptional cases (and with somewhat different configuration) (Solenogastres [40], some Nemertea [48], Table 3). According to a parsimonious tracing on the latest Spiralian tree [7] one pair of widely separated ventral cords would be the plesiomorphic state of Spiralia (Table 3 and references therein). Likewise, the basi- or intraepidermal position of the nervous system has also been regarded a plesiomorphic trait in Spiralia [49] as well as in Annelida such as now exemplified by the early branching annelid lineage Owe niidae [50–52] opposed to the derived subepidermal position found in many crown group annelids [53, 54]. However, intraepidermal nerve cords have also been found in Siboglinidae (Worsaae K, Rimskaya-Korsakova, NN, Rouse, GW: Neural reconstruction of bone-eating Osedax spp. (Annelida) and evolution of the siboglinid nervous system, submitted) as well as several interstitial annelids [19, 25, 54], showing considerable variance throughout evolution. The intraepidermal position of the paired ventral cords of Lobatocerebrum may hereby not be phylogenetically informative, whereas its additional median cord may be an annelid apomorphy. The two widely separated main nerve cords do not resemble a “typical” annelid pattern, but also do not dispute such a relationship, since such a pattern is also found in several other interstitial annelids such as Dinophilidae [15], Protodrilidae [29, 55], and Nerillidae [56].

Lobatocerebrum – an unsegmented annelid? The ventral nervous system in annelids most commonly consists of longitudinal nerve cords linked by ganglionated, serially arranged commissures, correlated with other serially repeated structures to form segments [49]. However, a clear outer segmentation as well as regularly distributed segmental paired ganglia are lacking in several groups recently assigned to annelids such as Diu odrilidae, Sipuncula, Echiura Siboglinidae and now also demonstrated for Lobatocerebrum riegeri. A similar layout to that of L. riegeri only having two pairs of subpharyngeal ganglia is also found in other spiralian groups (e.g. Gnathostomulida, Catenulida, for more details see Table 3), although the posterior commissures found in L. riegeri (ganglionated and non-ganglionated) are often not described or irregularly distributed (Table 3). Besides the low number of ganglia, there is no correlation of the commissural distribution with that of the few observed nephridia in L. psammicol a [8, 9] nor with any other organ system in L. riegeri, which means that Lobatocerebrum cannot be regarded as segmented at present. This emphasizes, however, that more detailed studies of the developmental pattern in Lobatocerebridae are needed to check for signs of segmentation during ontogeny as found in Echiura and partly in Sipuncula [57, 59].

A grid-like peripheral nervous system supporting a ventralized central nervous system may be a Spiralian plesiomorphy The peripheral nervous system is, especially in spiralian with a ventralized central nervous system, supposed to provide sufficient support and innervation for (sensory) organs in the periphery of the body [49]. Especially sensory cilia and glands are often abundantly distributed in the epidermis of interstitial animals far from the ventral nerve cords and the brain, as can be demonstrated in nearly all spiralian groups [60]. In annelids, the peripheral nervous system is often formed as an irregular grid constituted by longitudinal, oblique and circular nerves [21], relatively similar to those present in L. riegeri, though the pattern here appeared more regular and with the longitudinal nerves projecting directly from the neuropil rather than from the nerve cords. Moreover, the pattern is the general pattern for several spiralian, so it cannot be viewed as a diagnostic trait for annelids (see Tables 3 and 4 for details). Supplemen ting or even replacing this grid, nerve plexi are found around specific organs, most often adjacent to the reproductive organs or the mouth opening in nearly all groups considered for this comparison (see Tables 3 and 4 for details). However, since the grid is generally built from single (or few) fibres, the record of peripheral nervous system architecture especially among interstitial animals is rather incomplete.

Function and origin of the long-necked frontal glands The frontal glands in Lobatocerebrum are among the diagnostic features of this group; the elongated ducts of the prominent glands can neither be found in other annelid groups (with the exception of Diu odrilidae [61]) nor in the majority of other spiralian groups. However, supposedly similar structures are present in catenulid Platyhelminthes (personal observation) and probably also in a few exceptional nemerteans and gnathostomulids
(W. Sterrer, personal observation). The function of these glands is still unclear, though two options seem most likely: i) the secretion of these glands is used to produce a mucus layer to facilitate ciliary gliding; ii) the secretion is used to bind substances (e.g. pheromones or other chemical compounds) from the environment and thereby enhance the animal’s ability to sense the environment and possibly even follow a chemical lead. However, though olfactory organs have been described for many invertebrates [62, 63], with annelids generally having ciliated nuchal organs [64–66], those are rarely glandular or resembling the structure of the frontal glands, why this hypotheses clearly needs further testing.

Origin of meiofaunal characteristics of Lobatocerebridae

Lobatocerebridae has been proposed to originate from a macroscopic, presumably annelid (or annelid-like) ancestor by progenesis (somatic arrest during larval or juvenile development due to early maturation [67]) [8, 9, 11]. This idea was based on its acoelomic condition and the presence of characters also present in annelid or spiralian larvae, such as complete ciliation, an intraepithelial nervous system, protonephridia and a rather simple formation of both musculature and ventral nervous system [8, 9, 11]. No single extant macrofaunal lineage possesses juveniles resembling adult Lobatocerebridae; however, the noted features are also common in other meiofaunal representatives of annelids, molluscs, nemerteans and platyhelminths (see Tables 3 and 4, and references herein for details), where progenesis is often seen as the most plausible pathway along which these interstitial animals have derived from a macroscopic ancestor [67]. Conversely, most of these features are also present in the early branching meiofaunal spiralian lineages (Gnathifera, Platyhelminthes, Gastrotricha) and were, according to the latest Spiralian topology, proposed to resemble spiralian plesiomorphies [7]. So when these traits are found in adult meiofauna they may not necessarily reflect an ancestry from a larval or juvenile stage, but could instead represent plesiomorphic states – or as a third alternative, gradual adaptations (reversals) to the constraints of the space-restricted interstitial environment [11, 16, 30, 31].

Meiofaunal spiralian generally have few nerve cords spaced far apart (rather than midventrally fused/condensed), and possess a body wall musculature spread out as a regular grid (rather than having the longitudinal muscles organized into four or fewer bundles, see Tables 3 and 4 and references therein for details). Besides this pattern possibly being the ancestral spiralian condition, there may exist ‘universal constraints’ on the functionally optimal neuromuscular design when being of microscopic size and with limited cell number, and given the evolutionary toolbox within Spiralia. Hence, the organization of the neuromuscular system may be more directly dependent on e.g., size, ciliary pattern or acoelomatic condition (e.g. as for the mesodermal blood vascular system and protonephridia [11, 68, 69]) in a way we haven’t calculated for. Alternatively (or in addition), the condensation of muscles and nerves into bundles is a pattern often realized during development of annelids and certain spiralian patterns and a combination of traits diagnosing it as an annelid. Most features of the neuromuscular system revealed in L. riegeri by CLSM and TEM are not in themselves diagnostic to annelids and can either likewise be found in other groups or be unique for Lobatocerebridae. While these features on their own cannot reveal significant information about relationships within and between the spiralian groups, the combination of traits such as a nervous system with a complex brain with several commissures, a prominent median nerve cord and several ganglionated commissures, as well as a glandular, multiciliated epidermis and gliainterstitial system [10] together support an affinity to Annelida.

It is not possible to depict neither from the phylogenetic position nor morphological traits whether Lobatocerebridae originated through paedomorphosis or gradual miniaturization from a macrofaunal ancestor as an adaptation to the interstitial environment – or may even have retained plesiomorphic traits. Nonetheless, the lack of specific resemblance to any juvenile annelid relatives indicates a much more complex evolutionary history than what can be explained by a one-step progenetic evolutionary process. Further studies on the development of organ systems such as the musculature and the nervous system...

Conclusion

Although Lobatocerebrum was shown to be an annelid in a recent phylogeny [7], previous studies also suggested similarities to other spiralian groups such as Platyhelminthes, Nemertea, Mollusca and Gnathostomulida [8, 10, 11]. Conducting a detailed study of Lobatocerebrum riegeri with several complementary microscopical techniques revealed details of the musculature, the nervous system and the glandular system and allowed for a detailed description of Lobatocerebrum riegeri next to the previously described L. psammicola. Yet, L. riegeri is very similar to L. psammicola, both representing conservative spiralian patterns and a combination of traits diagnosing it as an annelid. Most features of the neuromuscular system revealed in L. riegeri by CLSM and TEM are not in themselves diagnostic to annelids and can either likewise be found in other groups or be unique for Lobatocerebridae. While these features on their own cannot reveal significant information about relationships within and between the spiralian groups, the combination of traits such as a nervous system with a complex brain with several commissures, a prominent median nerve cord and several ganglionated commissures, as well as a glandular, multiciliated epidermis and gliainterstitial system [10] together support an affinity to Annelida.
may prove useful for accessing the origin of Lobatocerebridae. Nonetheless, this study demonstrates that with Lobatocerebridae being annelids [7], Annelida displays an extreme evolutionary plasticity of the neuromuscular system, which is otherwise regarded as highly conservative throughout metazoan evolution.

**Methods**

**Sampling**

Specimens used for this study were collected in Eilat, Israel, from sand collected from a small (0.5x0.5 m) sand patch between coral blocks at 8.5–9 m depth approximately 100 m southwest of the main pier of the Interuniversity Institute for Marine Sciences (IUI, N 29° 30.211’ E 34° 55.068). Animals were extracted and anesthetized using an isotonic magnesium chloride solution: The upper 2–5 cm of sampled sand was mixed with this solution, and the water with floating particles and anesthetized animals decanted through 63 μm meshes with seawater. Revitalized animals were sorted from the petri dish using dissecting compound microscopes. A total of nine specimens was found, examined and afterwards fixed for the techniques described below as well as for molecular analysis.

**Behavioral studies**

Animals were observed with a dissecting scope in a petri dish prior to being transferred to an object slide in seawater under cover for examination and imaging in a compound microscope with a mounted camera or a video recorder. For later relaxation, a weak MgCl₂-solution was added to the slide. Movies were later analyzed in relation to the morphological studies and interpretation.

**Histology, light microscopy (LM) and transmission electron microscopy (TEM)**

Specimens were carefully anesthetized with isotonic magnesium chloride and afterwards fixed with 2 % glutaraldehyde in 0.1 M osmolarity-adjusted cacodylate buffer over night at room temperature (RT) and afterwards rinsed and stored in 0.1 M cacodylate buffer. The animals were postfixed in 2 % OsO₄ in 0.05 M K₃Fe(CN)₆-solution for 1 h and before embedding in Araldite Epon-812 using standard protocol and polymerization for 20–24 h at 50 °C.

For TEM-analysis, the block was trimmed to the object and sectioned into 40 nm sections using a Leica EM UC7 ultramicrotome (LEICA MICROSYSTEMS, Wetzlar, Germany). Ultrathin section were mounted on Formvar-coated 2x1 mm slot grids, contrasted with 2 % uranyl acetate- and 4 % lead citrate-solution and examined using a JEOL JEM 1010-Transmission Electron Microscope (TEM, JEOL Ltd., Tokyo, Japan) in combination with a digital GATAN OneView camera (GATAN, INC., Pleasanton, CA, United States). The fixation and preparation caused artifacts such as the slight separation of the epidermis from the internal organs of the animal.

**Immunohistochemistry and CLSM**

Specimens were carefully anesthetized with isotonic magnesium chloride and afterwards fixed in 3.7 % paraformaldehyde in phosphate buffered saline (PBS) for 1 to 2 h at RT, followed by several rinses in PBS and storage in PBS with 0.05 % NaN₃. For the investigation of muscular, nervous, glandular and ciliary system quadruple stainings were applied, including F-actin staining (Alexa Fluor 488-labelled phalloidin, INVITROGEN, Carlsbad, USA), DNA-staining (405 nm fluorescent DAPI) and immunostaining (monoclonal mouse anti-acetylated α-tubulin (SIGMA T6793, St. Louis, USA), polyclonal mouse anti-synapsin 1 (3C11 (anti SYNORF1, DEVELOPMENTAL STUDIES HYBRIDOMA BANK, Iowa, USA) and antityrosinated tubulin (SIGMA T9028), polyclonal rabbit anti-serotonin (5-HT; SIGMA S5545) and anti-FMRFamide (IMMUNOSTAR 20091, Hudson, USA)). Prior to adding the primary antibody-mix, the samples were pre-incubated with 0.1 % PBT (PBS + 0.1 % Triton-X, 0.05 % NaN₃, 0.25 % BSA, and 10 % sucrose) for 2 h. Afterwards, samples were incubated for up to 24 h at RT in the primary antibodies mixed 1:1 (in a final 1:200 concentration (or 1:50 for anti-synapsin 1)). Subsequently, specimens were rinsed in 0.1 % PBT three to six times and incubated with the appropriate secondary antibodies conjugated with fluorochromes (also mixed 1:1 in a final concentration of 1:200; goat anti-mouse labeled with CY5 (JACKSON IMMUNO-RESEARCH, West Grove, USA, 115-175-062), goat anti-rabbit labeled with TRITC (SIGMA T5268)) for up to 24 h at RT. This step was followed by several rinses in 0.1 % PBT and post-incubation for 60 min in Alexa Fluor 488-labeled phalloidin (0.33 M in 0.1 % PBT). Thereafter, specimens were rinsed in PBS (without NaN₃) and mounted in Fluoromount-G with DAPI (SOUTHERN BIOTECHNOLOGY ASSOCIATES, Inc., Alabama, USA) or Vectashield with DAPI (VECTOR LABORATORIES, Burlingame, USA).

The mounted specimen were scanned using an Olympus Fluvoview FV-1000 confocal laser scanning microscope (of K. Worsaae, University of Copenhagen, Denmark), with the acquired z-stacks of scans being either projected into 2D-images or analyzed three-dimensionally using IMARIS 7.1 (BITPLANE SCIENTIFIC SOFTWARE, Zürich, Switzerland). This software package was also used to conduct the measurements presented in the following text (n = number of specimens analyzed; r = body region (1 - from the anterior tip to the mouth opening, 2 - from the mouth opening to the male gonopore, 3 - from the male gonopore to the ovary, 4 - from the ovary to the posterior tip of the animal); m = number of measurements per region).
Measurements
All measurements on live animals were taken in Adobe Photoshop after the images were acquired using a standardized scale bar, as was the procedure for measurements taken from TEM-pictures. Measurements from CLSM-image stacks were conducted in Imaris 7.1 using the Measurement-tool in Section-mode. For comparison with the measurements in Rieger [8], distances from the rostral tip to specific organ systems as well as body width and length were calculated in units (U), the entire body length being 100U.

Photoshop and Illustrator
Contrast and brightness of all two-dimensional projections of confocal data and pictures of TEM-sections were adjusted in Adobe Photoshop CC 2015. Schematic drawing as well as plate-assembly was performed in Adobe Illustrator CC 2015.

Additional file
Additional file 1: Motility pattern and details of the adult Lobatocerebrum riegeri n. sp. This movie shows combined clips of alive Lobatocerebrum riegeri n. sp. indicating both morphological characteristics such as the pharynx and brain and motility patterns. (MP4 116178 kb)

References

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Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AK, NB and KW drafted the study. All authors (AK, NB, WS and KW) sampled the animals. AK, NB and KW conducted the laboratory experiments. KW and AK drafted the manuscript. All authors (AK, NB, WS and KW) contributed to the manuscript and approved of the final version.

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Abbreviations
ac: anterior cilia; adnc: anterio-dorsal nerve cord; adlnc: anterior dorso-lateral nerve cord; afg: anterior frontal gland; ag: adhesive granule; amf: anterior point of muscle fusion; an: anus; anc: anterior nerve cord; aven: antero-ventral nerve cord; avc: anterior ventro-lateral nerve cord; bl: basal lamina; br: brain; bsc: brain cell; bsm: brain supporting muscle; c: cilium; c1-4: commissures 1–4; cg: ciliated gland cell; cmd: circular muscle of the digestive system; dns: dorso-anterior commissure of the central neuropil; dfg: frontal gland ducts; dlm: dorso-lateral longitudinal muscle; dfm: dorsal longitudinal muscle; e1–3: egg 1–3; ec: cili of an epidermis-cell; fg1-2: FMRF-amidergic perikarya of the posthyparyngeal ganglia 1 and 2; gp: opening of the frontal glands; ggs: glandular opening; hhgi: head ganglion; kg: kidney-shaped gland cell; ladc: lateral branch of the anterio-dorsal nerve cord; lavc: lateral branch of the anterio-ventral nerve cord; lca: major caudal lobes; lc: minor caudal lobes; lg: lateral ganglion; llm: lateral longitudinal muscle; lms: longitudinal muscle of the digestive system; ln: lateral nerve; lpn: lateral peripheral nerve; lppn: lateral projection of the neuropil; lr: rostral lobe; lra: major rostral lobe; lrl: lateral rostral lobe; mg: male gonopore; mgn: male gonopore gland; mps: penkaryon associated with the male gonopore; mnc: median nerve and connections from the dorsal commissure to the nerves of the major caudal lobes; mrg: midgut; mnc: median nerve cord; mro: mouth opening; mn: median rostral nerve; ne: epidermal nucleus; nlc: nerve of the major caudal lobe; nlc: nerve of the minor caudal lobe; nlr: nerve of the major rostral lobe; nlr: nerve of the lateral rostral lobe; nlr: nerve of the median minor rostral lobe; nlr: nerve of the lateral rostral lobe; np: neuropil; ns: nerve ring around the male gonopore; pcm: projection of the ciliated gland cell; pqf: posterior projection of the tubulinergic strands; sc: shortened cilium; snc: stomatogastric nerve ring; sp: perikarya of the stomatogastric nerve ring; spcs: seorotinerigic cell; spds: sperminoduct; sps: sperm filaments; sp1-4: spinhoer 1–4; ss: sensoria; ss: star-shaped muscle; sns: sickle-shaped nucleus; t: testis; tcn: terminal commissure; tgl: tubuline gland; tm: transverse muscle ring complex; tpp: transverse ring of the peripheral nervous system; tps: tubulinergic sheath; tps: tubulinergic strands; vcn: ventral commissures of the neuropil; vlm: ventrolateral longitudinal muscle; vlm: ventral longitudinal muscle; vlc: ventrolateral longitudinal nerve cord.
Manuscript III:

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Detailed reconstruction of the musculature in *Limnognathia maerski* (Micrognathozoa) and comparison with other Gnathifera

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**Abstract**

**Introduction:** *Limnognathia maerski* is the single species of the recently described taxon, Micrognathozoa. The most conspicuous character of this animal is the complex set of jaws, which resembles an even more intricate version of the trophi of Rotifera and the jaws of Gnathostomulida. Whereas the jaws of *Limnognathia maerski* previously have been subject to close examinations, the related musculature and other organ systems are far less studied. Here we provide a detailed study of the body and jaw musculature of *Limnognathia maerski*, employing confocal laser scanning microscopy of phalloidin stained musculature as well as transmission electron microscopy (TEM).

**Results:** This study reveals a complex body wall musculature, comprising six pairs of main longitudinal muscles and 13 pairs of trunk dorso-ventral muscles. Most longitudinal muscles span the length of the body and some fibers even branch off and continue anteriorly into the head and posteriorly into the abdomen, forming a complex musculature. The musculature of the jaw apparatus shows several pairs of striated muscles largely related to the fibularium and the main jaws. The jaw articulation and function of major and minor muscle pairs are discussed. No circular muscles or intestinal musculature have been found, but some newly discovered muscles may supply the anal opening.

**Conclusions:** The organization in *Limnognathia maerski* of the longitudinal and dorso-ventral muscle bundles in a loose grid is more similar to the organization found in rotifers rather than gnathostomulids. Although the dorso-ventral musculature is probably not homologous to the circular muscles of rotifers, a similar function in body extension is suggested. Additionally, a functional comparison between the jaw musculature of *Limnognathia maerski*, Rotifera and Gnathostomulida, emphasizes the important role of the fibularium in *Limnognathia maerski*, and suggests a closer functional resemblance to the jaw organization in Rotifera.

**Keywords:** CLSM, 3D reconstructions, Jaw apparatus, F-actin, Trophi, Mastax

**Introduction**

*Limnognathia maerski* Kristensen & Funch, 2000, is a minute animal living in fresh water ponds and lakes [1-3]. The animal was discovered in 1994 at Disko Island, Greenland, but not described before 2000, and it has subsequently been reported from the Sub Antarctic Crozet Island [1], in a stream from southern Wales, United Kingdom, and in the river Lambourn (Berkshire), United Kingdom (P. E. Schmid and J.M. Schmid-Araya, personal communication). With a unique combination of characters, it is considered the only member of the recently described Micrognathozoa [2-5], belonging to Gnathifera. However, the phylogenetic relationships within Gnathifera are still debated, and the molecular studies are based on very limited information [5]. So far, the complex jaw apparatus of *L. maerski* has received the main attention in studies, leading to several disputed homology hypotheses for each sclerite of the trophi [1,3,6,7]. However, no detailed studies have addressed the overall morphology of organs systems and further anatomical knowledge on *L. maerski* is warranted in order to compare this unique evolutionary lineage with the other gnathiferan groups, as well as other animals.

*Limnognathia maerski* measures 80-150 μm, possesses a complex set of jaws, a conspicuously arranged ventral ciliation and, so far, only females are known. The
ventrally ciliated head consists of a forehead with ciliary sensory organs and a more posterior part containing the pharyngeal apparatus. The trunk is composed of an accordion-like thorax and a large abdomen with ventral ciliophores and a posterior adhesive pad [3]. In the original description, the overall musculature of *L. maerski* is briefly described. It is composed of several longitudinal and dorso-ventral muscles, minute muscles articulating the dorsal plates and a dense pharyngeal musculature. No circular musculature has been found. However, precise information on the number, configuration and relative size of each set of muscles was not provided. Ultrastructural data provided information on the structure of muscles attachment sites, the absence of myosynctia and myoepithelia, the cross-striated nature of the pharyngeal musculature, and the mainly obliquely striated longitudinal musculature [3].

Following Sorensen [6], the jaws of *L. maerski* are composed of six main elements: i) The median, ventralmost basal plate with posterior stems and anterior flattened and toothed manus, ii) the large and conspicuous ventral fibularium made of different chambers containing cells, extending dorso-laterally, iii) the latero-ventral ventral jaws (pseudophalangia) that articulate posteriorly with the associated accessory sclerites, iv) the medio-dorsal main jaws, each with a posteriorly projecting cauda, surrounded by the fibularium, v) the dorso-lateral dorsal jaws also confined to the fibularium area, vi) and the pharyngeal lamellae, a pair of lamellate structures positioned antero-laterally to the rest of the jaw apparatus. Additionally, Kristensen and Funch [3], describe the lamella orales as a paired structure similar to the lamellae pharyngea, situated dorso-laterally, inside the fibularium. However, the presence of these structures has not been confirmed in any subsequent studies [1,6].

The animal lives in limnic mosses or in the sediment of relatively calm springs and lakes, and was first recognized for its unusual 'ciliate-like' swimming in the water column. It also uses ciliary motion to glide over surfaces. Occasionally, it performs muscular contractions during lateral bending and longitudinal accordion like contractions for directional change, ventral bending while egg laying and dorsal contraction during vomit behavior [3].

Foraging of *L. maerski* involves fine movements of the jaw apparatus as well as larger movements of the head. While feeding, the ventral jaws are protruded and involved in substrate grasping. During the vomit behavior, the forehead is moved upward and backward, and most of the jaw apparatus is protruded through the mouth opening, while it performs fast snapping movements of the jaw elements and forward and backward movements of the main jaws (see reference [3] and Figure 1B). Accessory sclerites and pseudophalangia may move independently of the rest of the jaw apparatus, allowing the ventral jaws to move from a rostro-caudal orientation to a dorso-ventral orientation without moving the other jaws elements [3,6,7].

The body wall musculature differs between the putatively closest micrognathozoan relatives: Gnathostomulida and Rotifera. In Gnathostomulida, the overall musculature consists of numerous circular and diagonal muscles and several bundles of longitudinal muscles (six to nine pairs [8-10]) extending the entire body length, where the superimposition of longitudinal, diagonal and circular muscles forms a dense grid like body wall musculature [9,10]. In the majority of rotifers, most of the longitudinal muscles do not extend through the entire body, but are limited to certain body regions, e.g., coronal retractors in the head or muscles in the posterior part of the trunk, being involved in the contraction of the head and foot, respectively [11-13]. Circular muscles are few and usually incomplete transverse, rather than circular (e.g., [11-15]), although some Gnesiotrocha have complete rings [12,16,17]. Most of the diagonal and transverse muscles are usually absent (e.g., [12,18]), and if present they are only few and/or inconspicuous [14,19]. Splanchnic muscles surrounding the gut are not found in Gnathostomulida [9], whereas they present a very thin musculature in Rotifera. This muscular grid is documented for Seisonidae [11] and Monogononta (e.g., [13,19]) but visceral muscles are not found in Bdelloidea [12,14]. Dorso-ventral musculature has not been described for Gnathostomulida [9], and most of the functionally dorso-ventral muscles in Rotifera are supposedly modified incomplete circular muscles [12,19] meaning “true” dorso-ventral muscles, as reported by Kristensen and Funch [3], seem to be unique for Micrognathozoa.

The jaw musculature also differs between Gnathostomulida and Rotifera, due to the organization of their jaws. In gnathostomulids, the jaw apparatus consists of i) a set of main jaws, and in some taxa ii) an unpaired basal plate [20-22]. In rotifers, the jaw apparatus (trophi) includes 7 main elements: the i) unpaired posteriorly directed fulcrum, ii) paired rami, iii) paired unci, and iv) paired manubria. The fulcrum and rami together form the central element, the incus, whilst the unci and manubria form the mallei (e.g., [23-25]). The rotifer incus has been considered homologous with the gnathostomulid main jaws [21,26]. However, it also has been suggested that some parts of the gnathostomulid articularium (antero-lateral parts of the main jaws) are homologous with the rotifer manubria [27]. The gnathostomulid basal plate is considered autopomorphic for the group, and no homologous counterpart has been identified in the rotiferan trophi. The structural differences in the musculature of gnathostomulid and rotiferan jaw apparatuses clearly relate to the differences in the hard parts and the additional number of rotifer jaw elements. Indeed, most of the musculature supplying the
rotifer trophi consists of relatively small paired muscles connecting the different jaw elements (sclerites), while, in Gnathostomulida, the main jaws are mainly moved together by large muscles attached to the pharynx wall. The movement between jaw elements in Gnathostomulida is consequently achieved by U-shaped muscles (bent transversal muscles) and laterally attached transversal muscles.

Recently, several CLSM studies of phalloidin-stained musculature have been carried out on a great number of microscopic animals, revealing comprehensive information on their overall musculature [9,11,28-30] and also, in the case of gnathiferans, on the musculature of the rotifer mastax [31,32] or gnathostomulid pharynx [26]. Combined with TEM, many details can be inferred on the relative position of muscles and their ultrastructure, but also connections to the other part of the body. In order to compare the general muscular organization as well as jaw musculature of L. maerski with other animals, we here describe its musculature employing F-actin staining and confocal laser microscopy (CLSM) as well as transmission electron microscopy (TEM).

Results
The overall musculature is organised into seven main pairs of longitudinal muscles extending from head to abdomen and 13 oblique dorso-ventral muscles localised in the thoracic and the abdominal part (Figures 2, 3, and 4). No circular muscles are present. The musculature furthermore comprises the dense pharyngeal muscle and the fine anterior forehead muscle. Cross striated muscles are found in the body wall musculature (Figure 1A) as well as in the jaw musculature (Figures 1B,C,D and 5C,D).
Figure 2 CLSM of phalloidin stained muscle system and light microscopy of *Limnognathia maerski*. Anterior end is positioned left on all pictures. **A**: Ventral view, Z-stack of the ventral portion, showing only the muscle system. **B**: Single section showing CLSM of the dorsal muscle system and the contour of the specimen, visualized with transmitted light. **C**: Synapsin2 staining of *L. maerski*, maximum intensity projection of a dorsal substack. Lines show the border of the dorsal cells to which the dorso-ventral muscles attach (illustrated in Figure 4B). advm, anterior dorso-ventral muscles; alm, anterior lateral muscle; cpm, ciliated adhesive pad muscle; fmm, front margin muscle; ldm, lateral dorsal muscle; lvm, lateral ventral muscle; mdm, median-dorsal muscle; mvm, medio-ventral muscle; mn, muscle network; pvm, paramedian ventral muscle; pvm2, posterior lateral muscle; sav1,2, small anterior ventral longitudinal muscles; tdvm, trunk dorso-ventral muscles; vpm, ventral pharyngeal muscles.
Longitudinal musculature

The longitudinal musculature of the trunk consists of seven pairs of main muscles (*three ventral, two lateral, two dorsal*) as well as two short anterior pairs of muscles and two short posterior pairs of muscles.

Ventral muscles

The three ventral main muscles extend the body length aiding the body contraction and extension (Figures 2A, 3 and 4). The longitudinal ventral muscles are implicated in longitudinal contractions and ventral bending.

Figure 3 CLSM of phalloidin stained muscle system of *Limnognathia maerski*. Anterior end is positioned left on all pictures. A, Ventral view of the maximum depth intensity projection. B, lateral view reconstruction of a dorso-ventral Z-stack. Same specimen as Figure 2A,B. C, Dorsal view of the isosurface reconstruction of the muscular system. Same specimen as Figure 2A,B. advm, anterior dorso-ventral muscles; alm, anterior lateral muscle; cpm, ciliated pad muscle; fmm, front margin muscles; ldm, lateral dorsal muscle; lvm, Lateral ventral muscle; mdm, medio-dorsal muscle; mn, muscle network; mvm, medio-ventral muscle; pvm, paramedian ventral muscle; pvm2, posterior lateral muscle; sav1,2, small anterior ventral longitudinal muscles; tdvm, trunk dorso-ventral muscles; tpm, transversal posterior muscle; vpm, ventral pharyngeal muscles.
The paired medio-ventral muscles (mvm, Figures 2A, 3A,C, 4 and 5A,B) consist of two muscle fibres that form bundles originating directly posterior to the ventral pharyngeal muscle and extend along the ventral wall of the gut (mvm: Figure 5A). At its posterior extremity, each medio-ventral muscle separates into two very short muscle fibres that each extend four micrometers before inserting into the epidermis that is anterior of the adhesive ciliated pad (mvm: Figure 4).

Medially, two pairs of small anterior ventral longitudinal muscles (sav1, sav2, Figures 2A, 3A,C and 4) supply the anterior part of the thorax, each originating from the mid-line directly posterior to the ventral pharyngeal muscle. The anteriormost muscle pair (sav1) is bifurcated at both ends: the anterior bifurcation inserts medially just behind the pharynx, while the posterior bifurcation originates in a more lateral region close to the paramedian ventral muscle (sav1: Figure 4). The posteriormost muscle pair (sav2) inserts medially at the level of mvm and extends laterally toward (and originates close to) the paramedian ventral muscle (described below, sav2: Figure 4).

Latero-anterior to the pharynx are three muscles that come together to form the paramedian ventral muscle (pvm, Figures 2A, 3A,B,C, 4 and 5A,B); consequently, the paramedian ventral muscle is trifurcated at its anterior insertion but extends posteriorly as a single muscle bundle. The paramedian ventral muscle follows the course of the trunk and abdomen, where it eventually
bifurcates into two separate bundles. The ipsilateral muscle bundle extends dorsally where it joins the paramedian ventral muscle on the same side of the abdomen, while the contralateral muscle extends to the opposite side of the body and joins the contralateral last dorso-ventral muscle. Thus, each of the last dorso-ventral muscle bundles consists of three separate muscles: a dorso-ventral muscle, an ipsilateral branch of the paramedian ventral muscle and a contralateral branch of the paramedian ventral muscle from the opposite side of the body. (pvm: Figures 2A, 3A, 4 and 5). The paramedian longitudinal muscle follows the outline of the ventral ciliated area and contractions may change the direction during swimming or crawling (pvm: Figure 4).

Each of the two lateral ventral muscles (lvm; Figures 2A, 3A,B,C and 5A,B) inserts anterior of the mouth where they each bifurcate into two smaller branches. Posteriorly, each lateral ventral muscle extends along the trunk and abdomen as a single bundle that eventually bifurcates again. The inner branch joins the paramedian ventral muscle, while the lateral branch inserts in the region of the large posterior gland.

Figure 5 TEM sections of Limnognathia maerski. Muscles highlighted in green. A, transversal section of the trunk. Dorsal side on top. B, close up of figure A, showing the ventral musculature. C, D, coronal section the jaws. The red line shows the symmetry axis of the jaws. The front is on the left. The section in C is more ventral than the section in D. as, accessory sclerite; dm, dorsal muscle; ca, cauda; cm, cauda muscle; fib, fibularium; lfm, lateral fibularium main jaw muscle; lm, pharyngeal lamella muscle; lvm, lateral ventral muscle; mfm, median fibularium main jaw muscle; mj, main jaws; mvm, medio-ventral muscle; pvm, paramedian ventral muscle; tdvm, trunk dorso-ventral muscle; vjm, ventral jaw muscle; vlm, ventral lateral muscle; vpm, ventral pharyngeal muscle.
A pair of ciliated adhesive pad muscles (cpm: Figures 2A, 3A,C and 4), which are present as short longitudinal bands, extend from an anterior zone of the ciliated pad (just posterior of the paramedian ventral muscle mid-line) to a posterior zone of the ciliated pad (cpm: Figure 4). The adhesive ciliated pad muscle is probably involved in the adhesive ciliated pad area contractions. Contraction of the adhesive ciliated pad muscles could contract this area and allow the animal to release from the substratum.

**Lateral muscles**
Two pairs of lateral muscles are present in the trunk. The pair of anterior lateral muscles (alm: Figures 2A, 3B, C and 4B) originates anterior of the mouth, probably bifurcating from the paramedian longitudinal muscle, and continues two thirds into the abdomen, appearing to attach to the lateral epidermal cells. They are positioned at a mid dorso-ventral level. The paired paramedian ventral muscles 2 (pvm2: Figures 2A,B and 3A,B,C) originate ventrally to the paramedian ventral muscles, separating at the mid-thoracic level. Each muscle reaches the dorsal side along the anterior part of the abdomen (pvm2: Figure 2B), extends ventrally at the level of the adhesive ciliated pad and returns at an antero-dorsal position, joining the very posterior dorsal epidermal cells and the paramedian ventral muscle. From this point, both paramedian ventral muscle 2 muscles join close to the mid-line at their posteriormost point, at the level of the last dorsal plate. If an egg is present at the level of the abdomen, one of the posterior lateral muscles is pushed by the egg to the contralateral side to return to the ipsilateral side at the level of the adhesive ciliated pad (pvm2: Figures 2A, 3A,C and 4). This muscle extends along the lateral side of the gut, being probably implicated in dorsal bending of the animal.

**Dorsal muscles**
Two dorsal pairs of muscles extend through the trunk. The two pairs are close to the midline and extend as two contiguous muscles (Figures 2B, 3A,C and 4).

The medio-dorsal muscle (mdm: Figures 2B, 3A,B,C and 4. dm: 5) originate as a pair of muscles that both insert at the midline in the trunk region (mdm: Figure 4). Each muscle extends antero-laterally for about 10 micrometers before curving back medially and continuing anteriorly as a strictly longitudinal muscle band that inserts dorsal to the pharynx (Figures 2B, 3A,C and 4B).

**Transversal posterior muscles**
Additionally, at the very posterior region, a complex of transversal and dorso-ventral muscles is present (Figures 3C and 4). It is partially formed by the longitudinal muscle extending posteriorly, from the ventral to the dorsal side. Posterior of these muscles, two pairs of dorsal small transversal muscles line each side of the body. It is difficult to determine with certainty if these two pairs are the continuity of the posterior lateral muscle. However, the anteriormost pair of lateral muscles seems to be a continuity of the paramedian ventral muscle (pvm: Figures 2A, 3A,C and 4) while the transversal posterior muscle pair seems to be another set of muscles (tpm: Figures 3A,C and 4). Both pairs of transversal posterior muscles are very dorsal and according their anatomical position could be implicated in a possible anus opening. Along with the posterior longitudinal and dorso-ventral musculature, the complex posterior musculature is probably involved in the oviposition, substrate adherence and, eventually, defecation.

**Dorso-ventral musculature**
The dorso-ventral musculature consists mostly of two sets of muscles: the anterior dorso-ventral muscles and the trunk dorso-ventral muscles (Figure 3C and 4). The posteriormost dorso-ventral complex is the continuation of the paramedian muscle and the paramedian ventral muscle 2 when they fold in the posterior region, and is not serially homologous to the trunk dorso-ventral muscles.

**Anterior muscles**
Five pairs of anterior dorso-ventral muscles (advm: Figures 2B, 3A,B,C and 4) supply the front margin. They appear to support the frontal ciliated sensory region. On each side, the medianmost dorso-ventral head muscle inserts dorsally, at the anterior head margin, close to the mid-line (Figure 2B).

**Trunk muscles**
Thirteen pairs of oblique trunk dorso-ventral muscles (tdvm: Figures 2A,B, 3A-C; 4: 5A,B) supply the thorax and the abdomen. Each trunk dorso-ventral muscle inserts close to the midline on either side of the medio-
ventral muscle, extends laterally dorsal to the paramedian ventral muscle and the lateral ventral muscle, and then curves dorsally to insert on epidermal cells (tdvm: Figures 2A; 4; 5A,B). They join the epidermal cells dorsally, extending along the body sides. They line the gut cells very closely, probably functioning as body-wall musculature as well as gut musculature (tdvm: Figure 5A,B). Five pairs supply the thoracic region and eight supply the abdomen region (tdvm: Figures 2A,B; 3A-C; 4). The penultimate and the last pair of dorso-ventral muscles insert ventrally at the midline where the medio-ventral muscle inserts as well, forming a very muscular zone five micrometres anterior of the adhesive pad. A few micrometres posteriorly, the two paramedian muscles cross transversally, forming with the two last dorso-ventral trunk muscles a triangular set of ventral muscles at the anterior area of the adhesive ciliated pad (tdvm: Figures 2A; 3C; 4).

Forehead musculature
The head musculature is a continuity of the longitudinal body musculature as well as a few specific muscles.

On the frontal margin, the paired frontal margin muscles (fmm: Figures 2A; 3A,C; 4) follow the coronal plan supplying the anterior ciliated region. The median extremity of each muscle is dorsal and bends posteriorly to continue dorsally as two longitudinal median head muscles. At the distal extremities, the front margin muscles are more ventral and supply the frontal ciliated zone. The five pairs of anterior dorso-ventral muscles also supply the frontal ciliated area. The anterior dorso-ventral muscles extend dorsally and quite close to the frontal margin muscle, thus appearing to be in contact with it. In front of the pharynx, dorsally, a cross like complex of small muscles consists of the front margin muscles continuing as a longitudinal median head muscle. The five pairs of anterior dorso-ventral muscles also supply the frontal ciliated area. The anterior dorso-ventral muscles extend dorsally and quite close to the frontal margin muscle, thus appearing to be in contact with it. In front of the pharynx, dorsally, a cross like complex of small muscles consists of the front margin muscles continuing as a longitudinal median head muscle and trifurcates as two lateral small bundles and one median bundle. The bundles of the front margin muscles of each side join the midline with other contralateral front margin muscle (fmm: Figures 3C; 4).

Vento-anteriorly, in front of the mouth opening the continuity of the lateral ventral muscle and the paramedian ventral muscle form a thin muscle network (mn: Figure 2A; 3A,C; 4), probably implicated in some anterior glands or changes of the shape of the head.

Pharynx musculature
The pharynx musculature includes the major ventral pharyngeal muscle and several paired and unpaired muscles articulating the jaws. Jaw muscles have a non-epidermal origin, with each muscle being connected to an epidermal cell associated to a sclerite (Figure 1D). Thus, the musculature of the jaws is probably of mesodermal origin. The function of the musculature is interpreted according to previous studies on feeding behaviour and live observations.

Ventral of the trophi, lining the fibularium, several longitudinal fibres form a large ventral pharyngeal muscle plate (vpm, Figures 2A; 3A; 5C,D; 6A-C) and continues anteriorly as two small lateral muscle fibres. This ventral pharyngeal muscle plate is formed by 8-10 longitudinal cross striated muscle fibres (Figures 1B; 5C, D; 6A,C). The longest median muscle filament presents 8 z-bands (Figure 6A-C). However, even though the ventral pharyngeal muscle plate mostly underlies the fibularium, the ventral pharyngeal muscle is shifted more posteriorly compared to the fibularium. The plate is rounded at the lateral and posterior edges, hereby enveloping the trophi (including the fibularium) laterally and caudally (vpm: Figure 5C,D).

Dorsal to the fibularium, two pairs of muscles extend between the fibularium and the main jaws: one pair of lateral fibularium/main jaw muscles (lfm: Figures 5C; 6D-F), and one pair of median fibularium/main jaw muscles (mfm: Figures 5C; 6D-F). Both of them attach to the fibula caudalis of the fibularium. The lateral fibularium/main jaw muscle originates at the fibula caudalis (of the camera dorsalis 1), and supplies the anterior part of the main jaws. The median fibularium/main jaw muscle originates posterior of the fibula caudalis (of the camera dorsalis 1 and 2), and supplies a less anterior part of the main jaws than the lateral fibularium/main jaw muscle.

One pair of strong caudal muscles lines each cauda of the main jaws (cm: Figures 5D; 6D-F). They are thicker in their posterior parts where they follow the paired caudae of the main jaw. The contraction of this muscle moves the main jaws together.

Two short anterior fibularium/main jaw muscles (afm: Figure 6G-I) attach to the anterior part of the fibula lateralis at the camera lateralis, and link in this way the fibularium with the anterior parts of the main jaws.

Altogether, the anterior fibularium main jaw muscle, the lateral fibularium main jaw muscles, the median fibularium/main jaw muscles and the caudal muscle, are probably responsible for the opening of the main jaws and their previously described backward/forward movements (Kristensen and Funch [3]).

An unpaired very thin striated U-shaped dorsal jaw muscle (djm: Figure 6G-I) attaches at each extremity to the posterior ends of each dorsal jaw.

Lateral to the fibularium, one pair of strong cross striated ventral jaw muscles (vjm: Figures 5C,D; 6G-I) inserts at the posterior part of the accessory sclerite. They extend posterior of the trophi, attaching the sides of the fibularium and inserting posteriorly at the pharynx epithelium.
Anterior to the other parts of the trophi, two strong pharyngeal lamellae muscles (lm: Figures 5C,D; 6G-I) supply the accessory sclerites and the pharyngeal lamellae. The two pharyngeal lamellae muscles are very large and in the continuity of the paramedian ventral muscle and anterior lateral muscle. They enlarge dorso-ventrally at the terminal part. This observation confirms the supposed function of the pharyngeal lamellae (initially lamella oralis) as a supporting structure. This dorso-ventrally enlarged muscle could function in opening and closing the pharyngeal lamellae as a fan, affecting the volume of the pharynx. The ventral jaw muscle is probably functioning together with the pharyngeal lamellae muscle as an antagonist. Indeed, both muscles are connected to the accessory
sclerite. When the pharyngeal lamellae muscles are contracted and the ventral jaw muscles relaxed, the pharyngeal lamellae will open and increase the volume of the pharynx cavity, also probably opening the mouth and allowing ventral jaws extrusion.

**Anti-Synapsin1 immunoreactivity**

Anti-Synapsin 1 immunoreactivity (IR) was tested in ongoing studies of the nervous system (Bekkouche et al. unpublished) and surprisingly yielded a very distinct IR at the borders of the dorsal epidermis cells. This immunoreactivity, which is presented as spots along the borders, resembles the distribution pattern of the unique zip-junctions in *Limnognathia* (equivalents of adherens junctions) (Figure 2C). However this IR interpretation warrants further confirmation. Most importantly, the very distinct cell border signal has been proved useful in the present study for co-localizing the attachment sites of the dorso-ventral muscles. Thereafter it was possible, even in specimens not stained against Synapsin1, to retrieve the borders of the dorsal cells of the epidermis by increasing the brightness of the phalloidin stain (data not shown). The attachment of the last eight trunk dorso-ventral muscles to the dorsal epidermal cells could then be inferred in several specimens (Figure 4B). Furthermore, the synapsin 1 staining clearly shows that *Limnognathia maerski* has cell borders in the epidermis (as opposed to being syncytial) and therefore does not belong to Syndermata (Rotifera and Acanthocephala).

**Discussion**

**Notes on the longitudinal musculature**

In *L. maerski* most of the longitudinal musculature extends the entire body length, or at least the entire trunk, yet some muscles are restricted to certain areas, e.g., the adhesive ciliated pad (cpm: Figures 2A; 3A,C; 4A), the thorax (ldm: Figures 3A,C; 4B), the anterior part of the thorax (sav1,2: Figures 2A; 3A,C; 4A), etc. This repartition of the musculature supports functionally the separation of *L. maerski* into a head, a thorax and an abdomen. Similarly, in rotifers, many longitudinal muscles extend a subpart of the body, aiding the retraction of the foot or the corona [11,12]. Contrarily, most of the longitudinal muscles of Gnathostomulida extend the entire body length [9,10].

**Is the dorso-ventral musculature of *L. maerski* comparable to circular musculature?**

The trunk dorso-ventral musculature of *L. maerski* (tdvm: Figures 2A,B; 3A,B,C; 4; 5A,B) superficially resembles the repeated incomplete circular muscles found in many rotifers. However, as described by Leasi and Ricci [12], “the muscular system of rotifers generally consists of somatic and splanchnic (visceral) fibers. Somatic musculature is composed of two layers: an external layer made of separate circular rings and an internal layer of longitudinal muscles”. *Limnognathia maerski* lacks splanchnic fibers and the somatic musculature is only composed of longitudinal muscles. However, internal of these are found the dorso-ventral muscles. These are serially repeated along the lateral outline of the gut (tdvm: Figure 5A,B). The median position of the trunk dorso-ventral muscles, relative to the two pairs of lateral and paramedian ventral longitudinal muscles, does not conform to the somatic circular muscles found in rotifers, and homology of these muscles is unlikely. However, they can be functionally compared to those of rotifers: with lack of both outer and inner circular musculature, these dorso-ventral muscles may act both as a splanchnic musculature, aiding the movement of the food throughout the digestive system, as well as somatic dorso-ventral musculature, elongating the body during contraction. In rotifers, the incomplete circular muscles act as antagonists of the longitudinal musculature. When these somatic circular muscles contract, the pressure of the body fluids is redistributed and prompts the extension of the body [12]. The same function is assumed in *L. maerski* for the trunk dorso-ventral muscles. It is interesting to note the medio-ventral longitudinal muscles as they seem to extend at the same level as the ventralmost part of the trunk dorso-ventral muscles (tdvm and mvm: Figures 2A; 3A; 4; 5A,B). This suggests that the medio-ventral longitudinal muscles may specifically work as antagonists of the trunk dorso-ventral muscles in the same way as for rotifers.

Giribet et al. [5] propose, among other hypotheses, a relationship between Micrognathozoa and Cycliophora. In Cycliophora, inner dorso-ventral muscles are also present in the Pandora larva and the dwarf male life stages [33-35]. In the dwarf male, several sets of dorso-ventral muscles are present along the entire body length, while in the Pandora larva, only three pairs of dorso-ventral anterior muscles are present in addition to the incomplete circular muscles repeated through the entire body length. It is, though, difficult to establish any functional comparison with *L. maerski* since there is no gut present in these two cycliophoran stages.

Similar to *L. maerski*, dorso-ventral muscles are found internal of the longitudinal muscles in kinorhynchs [36]. Moreover, in the gastrotrich *Draculiciteria*, two sets of dorso-ventral muscles are found: one inside and one outside the longitudinal musculature, each supposed to be derived from splanchnic and somatic circular muscles, respectively [37]. The organization found in kinorhynchs can be compared to the attachment of the trunk dorso-ventral muscles to the epidermal cells containing the dorsal plates in *L. maerski*, even though the two conditions obviously are analogous. Additionally, in both
kinorhynchs and *Draculiciteria*, as well as in rotifers, the contraction of the dorso-ventral musculature is supposed to be involved in the body extension [36,37].

This comparison between small sized pseudocelomate or acelomate animals, leads to the supposition that dorso-ventral muscles play a similar role as circular muscles, aiding the fluid circulation in the body and in *L. maerski*, possibly also changing the shape of the relatively large cells of the endodermis. Thus, the dorso-ventral muscle contractions possibly aid the movement of food particles in the gut, the vomit behavior, and the yet non-observed defecation.

### Functional considerations of the pharynx musculature

Considerations on the jaw musculature of *L. maerski*

Six paired main elements are described in the jaws of *L. maerski*: i) The median basal plates ii) the large ventral fibularia, extending dorso-laterally, iii) the lateral-most ventral jaws, iv) the medio-dorsal main jaws, with posteriorly projecting caudae, v) the dorso-lateral dorsal jaws confined to the fibularium area, vi) and the antero-lateral pharyngeal lamellae [6]. For comparison we refer to the Table 1 that summarizes the various jaw homology hypotheses proposed in the literature between the Rotifera and *L. maerski*. A general consensus appears to exist for the homologies between the articularium and cauda of Gnathostomulida, the ramus and fulcrum of Rotifera and the main jaws and caudae of *L. maerski* [1,3,6,38].

No separate musculature associated to the basal plate in *L. maerski* has been found. Moreover, detailed examination of the ventral view of the SEM images of the jaws of *L. maerski* does not show any clear separation between the basal plates and the fibularium [1,6], suggesting that the basal plate could be an integrated part of the fibularium.

The dorsal jaw muscle apparently only connects the two dorsal jaws and is not attached to the pharyngeal wall. In Sørensen [6], the dorsal jaws are described as caudally attached to the internal side of the fibularia, possibly by a flexible ligament on each side, positioning the jaws in a 90° angle to the main jaws. A contraction of the dorsal jaw muscles would then pull apart the tips of the dorsal jaws, turning the jaws about 45° from their resting position.

The fibularium, as the most conspicuous jaw structure, is involved in the attachment of three out of eight jaw muscles systems, suggesting that the fibularium acts primarily as a supporting structure for the jaws and the pharynx, rather than an element directly implicated in the mastication. This assumption is consistent with the strong ventral pharyngeal muscle underlying the fibularium.

Comparison of the pharyngeal musculature of *L. maerski* with those of other animals

The ventral jaws and accessory sclerites of *L. maerski* make up as a functional unit that has been considered homologous with either the rotifer mallei [3,6] or the rotifer epipharynx [1] (see also Table 1). The ventral jaws can be moved independently and extruded through the mouth opening during foraging while the rest of the jaws are not. In rotifers, the different sclerites are more closely connected through ligaments, and the mallei cannot be fully protruded without also protruding parts of the incus as well (e.g., in *Bryceella stylata* [31] and *Dicranophorus forcipatus* [39]). In *L. maerski* no ligamentous connections exist between the ventral jaws and either the fibularium or main jaws, which allow the ventral jaws to move more independently from the other main elements of the jaw apparatus.

The ventral jaw muscle of *L. maerski* (vjm: Figure 5C, D; 6G-I) can be compared to the musculus circumglandulis of Rotifera. This muscle connects the rami with other parts of the mallei [31,39,40]. Its ventral position, connection with the ramus and conspicuous shape, resembles the ventral pharyngeal muscle (conspicuous muscle made of several bundle) or the ventral jaw muscle (connection and position) in *L. maerski*. However, in rotifers this muscle is assumed to perform the spreading of the rami and eventually also the

### Table 1 Previously proposed homologies of *Limnognathia maerski* jaw parts and Rotifera jaw parts

<table>
<thead>
<tr>
<th>Jaw elements in <em>Limnognathia maerski</em></th>
<th>Proposed homologies with rotifer trophi according to the authors</th>
<th>De Smet [1]</th>
<th>Sørensen [6]</th>
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<tr>
<td>Basal plates</td>
<td>Basal platelet (epipharynx)</td>
<td>Autapomorphy</td>
<td>Autapomorphy</td>
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<td>Ramus</td>
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<td>Accessory sclerites</td>
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<tr>
<td>Main jaws dentarium</td>
<td>Ramus</td>
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<td>Epipharynx</td>
<td>Epipharynx</td>
<td>Pleural rod</td>
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<td>Dorsal jaws</td>
<td>Autapomorphy</td>
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compression of the salivary glands [31], and such functions are not likely for the ventral jaw muscles in *L. maerski*. Hence, no equivalent of the ventral jaw muscle of *L. maerski* is found in Rotifera.

Underlying the fibularium, the conspicuous plate of the ventral pharyngeal muscle is present (vpm, Figures 2A; 3A; 5C,D; 6A–C). Composed of several longitudinal parallel muscles fibers, this structure is found neither in gnathostomulids nor rotifers. In Gnathostomulida though, a pharyngeal capsule is found, but it is formed by circular muscles enveloping the pharynx [27], which is structurally different from *L. maerski*. However, a strikingly similar ventral set of longitudinal muscles, encompassing two fanlike muscles forming a similar bowl, is found in the microscopic worm *Diurodrilus* (*Spiralia incerta sedis*) [30]. In *Diurodrilus*, this pharyngeal bowl also lines the pharynx ventrally, whereas its posterior part extends further dorsally compared to what is apparent in *L. maerski*. In *L. maerski*, the configuration of the muscle plate indicates that it is implicated in the extrusion and sinking movements of the fibularium and possibly causes changes in the volume of the pharyngeal cavity.

Functionally, this muscle could also be similar to the mastax receptor retractor found in the rotifer *Pleurotrecha petromyzon* as well as other rotifers with virgate mastax [40], aiding the total movement of the mastax by changing the shape of the pharynx cavity. However, the rotifer mastax receptor retractor are located dorsal to the jaw, which makes an actual homology with the micrognathozoan ventral pharyngeal muscle unlikely. We assume a similar function of the ventral pharyngeal muscle in *L. maerski*, which when contracting seems to move the entire jaws system, during the so-called vomit behavior. Morphologically, the similarity of the plate-bowl-shaped ventral pharyngeal muscle of *L. maerski* and *Diurodrilus* is striking [30] and not found in Rotifera and Gnathostomulida.

The main jaws represent the central element of the micrognathozoan jaw apparatus, and there is a consensus about homologizing the main jaws with the rotifer incus [1,3,6] (see also Table 1). Two different sets of main jaw muscles connect the main jaws with other sclerites or with the pharyngeal wall. The first set, related to the fibularium, is a “lateral connection” created by the anterior fibularium main jaw muscle, the lateral fibularium main jaw muscle and the median fibularium main jaw muscle. The second one, independent of the fibularium, is a “posterior connection” created by the caudal muscle. In *L. maerski*, the “lateral connection” is the most prominent in the main jaws and it is operated by 3 sets of muscles (anterior fibularium main jaw muscle, lateral fibularium main jaw muscle, median fibularium main jaw muscle, respectively afm, lfm, mfm: Figure 6D–I). In Gnathostomulida, the lateral connection is also dominant, realized by the diductor muscles [9,26] which do not connect to a lateral sclerite but to the dorsal wall of the pharynx. In *L. maerski*, the fibularium has the function of attaching the muscles involved in the lateral connection. Among rotifers, sparse examples of lateral connections can be found. The only muscle having this arrangement is the musculus ramo-manubricus found in *Filinia longiseta* [41] and *Trichoeca rattus* [33], both having very peculiar trophi (respectively malleoramate and asymmetrical virgate). In Rotifera, though, the posterior connection is well documented in the abundant work of the series of confocal and TEM studies by the Ahlrichs Group [31,32,39-41], who refers to this muscle as the musculus fulcro ramicus. Furthermore, Riemann and Ahlrichs, emphasize the wide repartition of this muscle within Rotifera, suggesting the homology of this muscle across the taxon [39]. Then, the cauda muscle of *L. maerski* (cm: Figure 6D–F) could also be homologous to the musculus fulcro ramicus of Rotifera. A difference between those two muscles is that the cauda muscle seems to embed, or at least extend closely the cauda, while the musculus fulcro ramicus is more diagonal in its orientation. Additionally, the cauda muscle goes more posterior and seems to insert in the pharyngeal wall, while the musculus fulcro-ramicus is posteriorly restricted to the fulcrum.

Only muscles functionally implicated in the opening of the main jaws (not in the closing) have been found in *L. maerski*. As proposed for Rotifera and Gnathostomulida, we assume that the kinetic energy release of the cuticular parts provokes a passive closing of the pincer like sclerites in *L. maerski* [26,27,39].

**Conclusions**

Due to its simplicity, the longitudinal musculature of *L. maerski* is only roughly comparable to the musculature of other groups. However, the dorso-ventral musculature shows a functional similarity to the semi-circular muscles of the closely related Rotifera and other meiofaunal animals.

With regards to the pharyngeal musculature, only one specific homology between the cauda muscle of *L. maerski* and the musculus fulcro ramicus of rotifers can be hypothesized. However, the functional and morphological comparisons of the jaw musculature among gnathiforms aid the understanding of how such small complex systems can be moved. Two different “strategies” can be observed in the jaw apparatus of Rotifera versus Gnathostomulida: in rotifers, sclerites are moved by muscles connected to other jaw parts whereas in gnathostomulids the less complex jaws are moved by muscles connected directly to the pharyngeal wall. It is not surprising considering the complexity of the jaws of
L. maerski that the jaw musculature and function are more comparable to that of Rotifera. However, the independence of the ventral jaw of L. maerski relative to the rest of the trophi is an interesting difference between L. maerski and Rotifera. Additionally, the striking similarity between the ventral pharyngeal muscle of Micrognathozoa and the pharyngeal bowl-shaped muscle of Diurodrilus is interesting in relation to the debated close relationship between the jaw-less Diurodrilus and Micrognathozoa [3,30].

Several functional analogies and common patterns could be shown between L. maerski and other Gnathifera or small sized animals, but the systematic value of the musculature of L. maerski still appears quite limited. However, further studies are needed in Gnathifera. De Smet [1] emphasizes the poor knowledge of the epipharynx of Rotifera. For example, Riemann and Ahlrichs [39], in their study on Diernophorus forcipatus cannot assign any clear function to the hypopharyngeal elements. Furthermore, no complete detailed studies of the musculature and function of trophi of the Seisonidae, Bdelloidea (both Rotifera) and Filospermoidea (Gnathostomulida) have been done so far. Nevertheless, a systematic comparison will still be challenging since the trophi of Bdelloidea and Seisonidea are very modified, and the jaws of Filospermoidea have a relatively simple pincer-like structure, such as in Haplognathia.

Material and methods
Collection of specimens
Specimens used for TEM were part of the original material that were collected at the type locality in the Isunngua Spring on Disko Island, West Greenland, 69°43’N 51°56’W, and used for the description of Micrognathozoa [3]. Specimens for CLSM were collected in July-August 2010 and 2013 at the same locality.

Transmission electron microscopy
Specimens were fixed in trialdehyde 8% (after Kalt and Tandler [42] and Lake, [43], without acrolein) and postfixed in 1% osmium-tetroxide with 0.1M sodium cacodylate buffer for 1 hour (h) at 20°C. Specimens were then dehydrated through an ethanol series, transferred to propylene oxide, and embedded in epoxy resin type TAAB 812®. Ultrathin serial sections were stained with uranyl acetate and lead citrate [44]. TEM examinations were performed with a JEOL JEM 100SX transmission electron microscope.

Cytochemistry and CLSM
Specimens of L. maerski were fixed for 2 h at room temperature (or overnight at 4°C) in 2% paraformaldehyde in 0.15M phosphate buffered saline (PBS), pH 7.4, rinsed and stored in PBS plus 0.05% NaN₃. Entire specimens were preincubated two hours in PTA (PBS with 0.5% Triton-X, 0.05% NaN₃, 0.25% bovine serum albumin (BSA) and 5% sucrose) and afterwards incubated for 2h at room temperature in 0.34 μM Alexa fluor 488 phalloidin (Invitrogen, A12379) in PTA and finally mounted in Vectashield® (Vector Laboratories, Burlingame, CA) containing DAPI. For immunostaining against synapsin1, specimens were preincubated two hours in PTA and incubated for 12h at room temperature with antibodies anti synapsin1 raised in Rabbit (ENZO life Sciences, ADI-VAS-SV061-E). Then the specimens were rinsed in PBS, pre-incubated 2h in PTA and incubated 12h at room temperature with the secondary antibody anti-rabbit, conjugated with the fluorophore FITC (SIGMA, prod. num. F0382). Finally the specimens were rinsed in PBS and mounted in Vectashield®. Preparations were analyzed with an Olympus Fluoview FV1000 CLSM or a Leica TCS SP5 CLSM. The specificity of the antibodies was tested by examining specimens where each of the primary and secondary antibodies were omitted.

Image treatment
Z-stacks or parts of them of CLSM files were projected into 2D-images (MIP images – maximum intensity pixel images) and 3D iso-surface reconstructed in Imaris v7 (Bitplane AG, Zürich, Switzerland). Depth coded Z-stack images of F-actin staining are also presented (Leica imaging software), were the depth-gradient follows the area of the spectral light with the uppermost structures appearing red, and the more distant one blue. Free hand drawings and plate setups were done with Adobe Illustrator CS6 and Image modification done with Adobe Photoshop CS6.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AH, KW, MVS, NB, RMK collected the animals. RMK made the transmission electron micrographs. AH, KW, NB stained the specimens for phalloidin and scanned specimens for CLSM. KW, MVS, NB, RMK coordinated and participated in the analysis. KW and NB conceptualized, drafted the manuscript and designed the study. RMK, MVS, AH revised the manuscript. All authors read and approved the final manuscript.

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References


Manuscript IV:

Nervous system and ciliary structures of Micrognathozoa (Gnathifera) – evolutionary insight from an early branch in Spiralia

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Submitted to Royal Society Open Science
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Nervous system and ciliary structures of Micrognathozoa (Gnathifera) – evolutionary insight from an early branch in Spiralia

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Abstract

Recent studies show that Gnathifera, comprising Rotifera, Gnathostomulida and Micrognathozoa, constitute the sister group to the remaining Spiralia (containing, e.g., flatworms, segmented worms and mollusks). Therefore, a better understanding of Gnathifera is central for unravelling the evolution of the highly diverse Spiralia. Here we describe the previously unstudied nervous system and ciliary structures of Micrognathozoa using immunohistochemistry and confocal laser scanning microscopy. The nervous system is simple with a large brain, paired subesophageal ganglia, two trunk commissures, two pairs of ventral longitudinal nerves, and peripheral nerves. The paired ventro-lateral nerve cords are confirmed to be a symplesiomorphy of Gnathifera (possibly even Spiralia), whereas the paired medio-ventral nerves are not previously reported in Gnathifera. A pharyngeal ganglion is described for Micrognathozoa; a complex structure with two apical tufts of ciliary receptors, now shown to be shared by all Gnathifera. The ventral pattern of external ciliophores is redescribed and nephridia with multiciliated collecting tubes similar to those of Rotifera are confirmed. A range of new details from a simple nervous system and complex set of ciliary structures in a microscopic metazoan is hereby unraveled. The many resemblances with Rotifera corroborate their close relationship and shed more light on the evolution of Gnathifera.

Keywords

Limnognathia maerski, meiofauna, neuro-morphology, retrocerebral organ, acetylated α-tubulin, serotonin.

Introduction

Limnognathia maerski Kristensen and Funch, 2000 (Micrognathozoa) [1] is a recently described species belonging to the bilaterian clade “Gnathifera”. Recent phylogenomic studies show that Gnathifera is likely the sister group of all other Spiralia, and therefore is of crucial importance to understand animal evolution [2, 3]. However, studies on the different organ systems of Gnathifera are still warranted. Indeed, this clade is constituted of small, sometimes rare animals, collection of which is difficult and time-consuming, namely Gnathostomulida, Rotifera (= Syndermata, including Acanthocephala) and Micrognathozoa. The deep interrelationships between these three lineages

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is now resolved with both phylogenomics [3] and morphology [1, 4, 5] supporting a sister group relationship between Micrognathozoa and Rotifera, and Gnathostomulida being sister group to this clade. Although rotifers are relatively well studied in many aspects, most of their internal morphology still needs further investigation, as is the case for the internal organization in Gnathostomulida and Micrognathozoa.

Several confocal laser scanning microscopy (CLSM) have been conducted on gnathiferans, but most of them have focused on the musculature, e.g., in rotifers [6-8], one genus of Gnathostomulida [9, 10] and Micrognathozoa [11]. On the other hand, CLSM studies on the nervous system of Gnathifera are quite scarce (e.g., for rotifers [12-15] and for gnathostomulids, [10, 16]), and no studies have yet been carried out on Micrognathozoa. According to previous studies, the nervous system of Gnathostomulida consists of an anterior brain, a buccal ganglion, an anterior and a posterior commissure, and a variable number of longitudinal nerves extending along parts or the entire body length (three paired and two unpaired in Gnathostomula paradoxa Ax, 1956 [10, 17], six pairs in Rastrognathia macrostoma Kristensen & Nørrevang, 1977 [18], and three paired and two unpaired pairs in Pterognathia meixneri Sterrer, 1969 [19, 20]. Most studied rotifers show the presence of a brain, a mastax ganglion, a pair of ventro-lateral nerve cords as well as various head and peripheral nerves innervating the muscles and the sensory organs. However extensive studies of the nervous system of rotifers are rare and most recent publications focused on specific immunoreactivity [e.g. 12-14].

Micrognathozoa were first collected from a cold freshwater spring in 1994 in Greenland [1], and thereafter reported from sub-antarctic islands [21], and the United Kingdom [11]. But specimens from the United Kingdom are extremely rare, and the sub-antarctic islands as well as Greenland are remote localities, making the study of fresh material difficult. These ventrally ciliated meiofaunal animals, measuring up to 150μm in length comprising a head, thorax and abdomen, have very complex jaws, and only females are known so far. The complexity of the jaws (sclerites) has attracted most of the attention, and together with the original description, the sclerite arrangement has been described in details [1, 21, 22], but recently, the musculature of Micrognathozoa was finally resolved [11].
The nervous system was superficially addressed in the original description [1], and therein described as comprising a bilobed brain connected to a pair of ventro-lateral nerve cords with two paired ganglia (in the thorax and in the posterior-most part of the abdomen). Furthermore, the presence of a buccal ganglion is suspected but not confirmed [23]. The ventral ciliation was described as consisting of a dense head ciliation, four pairs of head ciliophores, 18 pairs of ciliophores arranged in two midventral longitudinal rows, and a posterior adhesive ciliary pad. Moreover, two pairs of protonephridia were originally described in the thorax [1] with later discussion on the possible opening of their canal cells into a common collecting tubule [23] and the location of the nephriodiopore remaining unknown.

The nervous system and ciliary patterns of L. maerski (Micrognathozoa) are here described in detail, using confocal laser scanning microscopy and immunohistochemistry, in order to understand the structure and evolution of these different organ systems within Gnathifera, the earliest branching clade in Spiralia.

**Material and method**

**Collection of specimens**

Mosses were collected at the type locality in the Isunngua Spring on Disko Island, West Greenland, 69°43'N 51°56'W 31 July 2013. The mosses were squeezed into a 32μm mesh and the extract thereafter sorted in dissecting scope, picking up the animals alive with a pipette or an Irwin loop.

**Immunohistochemistry and CLSM**

Specimens were anesthetized with 1% magnesium chloride solution added drop by drop until no movements were visible and fixed in 3.7% paraformaldehyde in phosphate buffered saline (PBS) for one to two hours at room temperature (RT), followed by six rinses in PBS and storage in PBS with 0.05% NaN₃. For the investigation of the muscular, nervous, glandular and ciliary system, triple or quadruple staining were applied, including F-actin staining (Alexa Fluor 488-labelled phalloidin, INVITROGEN, Carlsbad, USA), DNA-staining (405nm fluorescent DAPI) and antibodies against neurotransmitters and tubulin (monoclonal mouse anti-acetylated α-tubulin (SIGMA T6793, St. Louis, USA), polyclonal rabbit anti-serotonin (5-HT, SIGMA S5545) and anti-FMFRAmide.
(IMMUNOSTAR 20091, Hudson, USA). Prior to adding the primary antibody-mix, the samples
were pre-incubated with 1% PTA (PBS + 0.1% Triton-X, 0.05% NaN3, 0.25% BSA, and 5% sucrose)
for one hour. Samples were incubated over night at RT in primary antibodies (mixed 1:1 with
glycerol) in a final 1:400 concentration. Subsequently, specimens were rinsed in PBS six times and
incubated with the secondary antibodies conjugated with fluorochromes overnight (mixed 1:1
with glycerol) in a final concentration of 1:400; goat anti-mouse labeled with CYS (JACKSON
IMMUNO-RESEARCH, West Grove, USA, 115-175-062), goat anti-mouse labeled with FITC
(JACKSON IMMUNO-RESEARCH, West Grove, USA, 115-175-062), and goat anti-rabbit labeled with
TRITC (SIGMA T5268) over night at RT. Afterwards they were rinsed in PBS five times and
preincubated for 60 minutes in Alexa Fluor 488-labeled phalloidin (0.33M in 1% PBT). Thereafter,
specimens were rinsed in PBS (without NaN3) and mounted in Fluoromount-G with DAPI
(SOUTHERN BIOTECHNOLOGY ASSOCIATES, Inc., Alabama, USA) or Vectashield with DAPI (VECTOR
LABORATORIES, Burlingame, USA). The specificity of the antibodies was tested by omitting each of
the primary and secondary antibodies.

The mounted specimens were scanned using an Olympus Fluoview FV-1000 confocal laser
scanning microscope (of K. Worsaae, University of Copenhagen, Denmark), with the acquired z-
stacks of scans being either projected into 2D-images or analyzed three-dimensionally using
IMARIS 7.1 (BITPLANE SCIENTIFIC SOFTWARE, Zürich, Switzerland). This software package was also
used to conduct the measurements presented in the following text. Schematic drawings and plate
setup were done with Adobe Illustrator CS6 and image adjustments conducted in Adobe
Photoshop CS6.

Results

Nervous system

The nervous system consists of a large brain occupying most of the forehead, with a dorsal
neuropil, two pairs of major longitudinal nerves connected by paired subpharyngeal ganglia, an
anterior and a posterior commissure, a peripheral nervous system related to the sensory cilia
(sensoria), as well as a pharyngeal ganglion (figures 1 and 2).
The nervous system has been investigated with antibodies directed against acetylated α-tubulin, serotonin and FMRF-amide. The quality and strength of the signal of the immunoreactivity (IR) varied substantially between the different specimens examined, even among freshly collected materiel, with simultaneously fixed and stained specimens. Moreover, in some specimens, for acetylated α-tubulin-like immunoreactivity (LIR) and serotonin-LIR, the signal of the ciliation masks the longitudinal nerves. However, although the acetylated α-tubulin-like-immunoreactive (LIR-reactive) signal revealed more or less details in different specimens, it always supports the same pattern. Not all specimens showed clear serotonin-LIR in the nerves, ganglia and brain. In most specimens, FMRF-amide-LIR only shows a clear pattern in the pharyngeal ganglion and the rest of the signal appears to be unspecific signal.

**Longitudinal nerves**

Two pairs of nerves originate from each ventro-lateral side of the brain neuropil, and the two nerves of each side fuse lateral to the pharynx to form the paired circumesophageal connective (cc, figures 1A,F and 2A,F), extending postero-ventrally to the subpharyngeal ganglia (spg, figures 1A,F and 2A described below). The ventro-lateral nerve cords (vlnc, figures 1A-C,F,H and 2A,H) originate from the subpharyngeal ganglia extending throughout the trunk until they connect in the terminal commissure in the posterior abdomen (pc, figures 1A,B,G and 2E). Posterior to the pharynx, the nerves are interconnected by the anterior commissure (ac, figure A1,F) of the paired subpharyngeal ganglia, the ganglia also supplying the ventro-lateral nerve cords, the circumesophageal connective, and the ventro-median nerve (vmn, figures 1A-C,F and 2A,H). The presence of one to two pairs of perikarya supplying the ventro-lateral nerve cords is suspected, but could not be confirmed with certainty. The ventro-lateral nerve cords are 1.5μm thick and extend along most of the body length, surrounding the adhesive ciliary pad area until the posterior commissure at the posterior margin of the adhesive ciliary pad, where no associated ganglia (clusters of perikarya) could be detected with neither DAPI staining nor the applied neurotransmitters. Co-localization with phalloidin staining shows that the ventro-lateral nerve cords lie adjacent to the paired paramedian ventral muscles (pvm and vlnc, figure 2H, and see [11]) and to the lateral margin of the trunk locomotory ciliation. Thus, it is likely that the ventro-lateral nerve cords innervate either one or both of these systems.
A pair of longitudinal ventro-median nerves (vmn, figures 1A-C,F and 2A,H) extends from the sub-pharyngeal ganglia. They are each about 1µm wide, extend mid-ventrally along the thorax and the anterior part of the abdomen; laterally lining the ventral ciliation, and reaching the anterior edge of the adhesive ciliary pad. Co-localization with phalloidin staining shows that the ventro-median nerve is adjacent to the medio-ventral muscle (mvvm and vmn, figure 2H, and see [11]). We therefore assume that the ventro-median nerves possibly innervate the thoracic median ciliophores (tmc, figure 3A,C,E), the abdominal ciliophores (abc, figure 3A,C,E), and the median longitudinal muscle.

All the nerves described above show acetylated α-tubulin-LIR. Serotonin-LIR is found in the ventro-lateral nerve cords and the median longitudinal nerves as well as in the perikarya of the brain and pharyngeal ganglion (described below). None of the longitudinal nerves show FMRF-amide-LIR.

**Peripheral nerves and sensoria**

Along the lateral sides of the thorax and the abdomen, several pairs of cells show acetylated α-tubulin-LIR, each bearing one sensory cilium (=sensorium) (ss, figures 1A,D,E, 2A and 3B). We assume that as for Rotifera [24], each sensorium is a ciliated nerve cell projecting axons towards the central nervous system; the axons and possibly interneurons constituting the peripheral nervous system (pns, figures 1A,D and 2A). Following the nomenclature of Kristensen and Funch [1] the sensoria are present as three pairs of lateralia (la3-5, figure 3A; la1-2 could not be found), three pairs of dorsalia (do1-3 figure 3A) and two pairs of caudalia (dorsal and ventral, cd1-2, figure 3A). Perikarya (scb, figure 1A,D) of five previously described additional sensoria could not be identified with acetylated α-tubulin-LIR. On each lateral side, he recovered lateralia 3-5 as well as dorsalia 1-3 seem to project axons into one longitudinal dorso-lateral and one lateral neurite bundle, respectively, which meet up in the thorax and together join the circumesophageal connectives, anterior of the subpharyngeal ganglia. An additional branch of these peripheral nerves is found between lateralia 5 and the ventro-lateral nerve cord. Axons of the caudalia possibly connect to the posterior commissure, yet, this could not be ascertained due to the strong acetylated α-tubulin-LIR of the posterior glands.

**Brain**

[https://mc.manuscriptcentral.com/rsos](https://mc.manuscriptcentral.com/rsos)
The compact, undivided brain occupies most of the head (br, figures 1A,E, 2A,B,G and 4H,I). It was visualized with DAPI, acetylated α-tubulin-LIR, serotonin-LIR and FMRF-amide-LIR.

**DAPI**

The brain (br, figures 1A,E, 2A,B,G and 4H,I) consists of very densely packed small perikarya with small nuclei (nuclei diameter 1.5 to 2.5μm, almost indistinguishable from each other) surrounding the neuropil. In the center of the brain, slightly dorsally, is an area free of nuclei (measuring 6-7μm longitudinally and 10-13μm laterally) corresponding to the space occupied by the neuropil. Two auxiliary ganglia (ag, figures 1A and 2A,G) are present postero-lateral to the brain, each consisting of approximately 10 densely packed, small nuclei.

**Acetylated α-tubulin-LIR**

Fine details of the acetylated α-tubulin-LIR were difficult to interpret due to the very diffuse IR, however, few structures could be described: A triangular neuropil is present centrally in the brain (np, figures 1A and 2A,B,F), which seems to comprise two very faint and diffuse anterior and posterior commissures. Each of them supplies a paired nerve extending ventro-posteriorly, the lateralmost nerve supplies the auxiliary ganglion of the brain (ag, figure 1A), where after they fuse into a circumesophageal connective (cc, figures 1A,B,F and 2A,F) lateral to the pharynx. Ventro-posterior of the brain, a pair of short nerves of the mouth ciliation (nmc, figures 1A, and 3D) innervates the paired ciliated tufts at the anterior edge of the mouth (mc, figures 1A, 2A,B and 3A,B,D, see below).

**Serotonin-LIR**

Six pairs of serotonin-LI-reactive perikarya (sb1-6, figure 4G-I) are present around the serotonin-LI-reactive anterior and posterior commissures of the brain neuropil (sacb and spcb, figure 4G-I): one lateral pair (sb1, figure 4G-I) projects neurites into the anterior commissure, and a pair of para-median perikarya (sb2, figure 4G,H) sends neurites into the posterior commissure. Both commissures are connected by an unpaired serotonin-LI-reactive median and a paired serotonin-LI-reactive lateral connective (slcb and smcb, figure 4G-I). Two pairs of serotonin-LI-reactive nerves extend from the posterior commissure: one short pair of serotonin-LI-reactive brain posterior
projections ending blindly (sbpp, figure 4G) and one pair of serotonin-LI-reactive circumesophageal connectives (scc, figure 4G); the latter corresponding to the inner-branch of the acetylated α-tubulin-LI-reactive circumesophageal connective (cc, figures 1A and 2A,F). A cluster of two serotonin-LI-reactive perikarya (sb3-4, figure 4G-I) is present on each side, postero-lateral to the posterior commissure, which sends a pair of anterior projections to join the lateral connective of the brain. Finally, two pairs of perikarya (one large posterior (sb5, figures 4G,H) and one small anterior (sb6, figures 3G,H) supply a pair of serotonergic anterior projections (sbap, figure 3G-I) extending to the anterior margin of the animal.

**FMRF-amide-LIR**

The brain shows a characteristic FMRF-amide-LIR pattern in the neuropil (figure 1E), however, due to the background signal of the anti-FMRF-amide staining, only one anterior pair of dorso-lateral FMRF-amide-LI-reactive brain perikarya could be identified (fbp, figure 1E), which is connected to the neuropil by an FMRF-amide-LI-reactive nerve.

**Subpharyngeal ganglia**

One pair of ventral subpharyngeal ganglia (spg, figures 1A,F and 2A) is present postero-laterally to the pharynx, supplying the circumesophageal connectives, the ventro-lateral nerve cords, the ventro-median nerves, and the anterior commissure. It consists of approximately six to eight nuclei and is only visible with DAPI (no IR with the tested antibodies could be seen).

**Pharyngeal ganglion**

The pharyngeal ganglion is an unpaired cluster of nerve cells, surrounded by the fibularium sclerite, and situated dorso-posteriorly in the pharynx (pg, figures 1A-C,E, 2A,B,G and 4A-F), probably innervating the jaw elements. It shows positive IR for all antibodies tested (directed against acetylated α-tubulin, serotonin and FMRF-amide), revealing a consistent number and location of nuclei (stained with DAPI) in all examined specimens. A dense, filamentous acetylated α-tubulin-LI-reactive net of nerve fibers infiltrates the entire structure and allows the delimitation of the ganglion (atpg, figures 1C,2F and 4A,C), together with the densely packed nuclei. Of the approximately 60 cells identified with DAPI-staining, three paired serotonin-LI-reactive perikarya
are clustered medio-posteriorly in two longitudinal rows, followed by one unpaired serotonergic-
LL-reactive perikaryon (s1-4, figure 4A,B) and four pairs of FMRF-amide-LI-reactive perikarya are
found at the lateral and posterior margins of the pharyngeal ganglion (fp1-4, figure 4A,D,F) as well
as one antero-dorsal pair of perikarya (fp5, figure 4A,E) and a pair of anterior FMRF-amide-LI-
reactive positive spots lacking associated nuclei (fs, figures 4A,F).

How the pharyngeal ganglion is related to the central nervous system could not be resolved, since
no nerves extending out of the pharyngeal ganglion could be identified. One pair of tufts of
presumably pharyngeal sensory cilia (described below) originate directly from the pharyngeal
ganglion (phc, figures 1A,B,F, 2A,B, 3A,B,D,F and 4A,C). One pair of strongly acetylated α-tubulin-
LL-reactive structures are found postero-laterally to the pharyngeal ganglion, they do not seem to
consist of cilia, and their function are unknown (apo, figures 1B, 2F and 4C).

Ciliation

The ciliation can be separated into five different systems: the external ventral locomotory ciliation,
mouth ciliation, sensory cilia, as well as the internally ciliated nephridia and oviducts.

Locomotory ciliation

Head ciliation

On the head, the ventral ciliation can be divided into a semicircular anterior ciliated field in front
of the mouth opening (acf, figures 2A,B and 3A,D,G) separated by a transverse head groove (hgr,
figures 2A,B and 3A,G) from a horseshoe shaped posterior ciliated field (pcf, figures 2A,B and
3A,G) surrounding the mouth.

Ciliophores

Acetylated α-tubulin-LIR, as well as phalloidin staining proved useful to reconstruct the ventral
ciliary pattern of Limnognathia maerski. The packed cilia of each ciliophore could be differentiated
in optical sections with acetylated α-tubulin-LIR, supported by phalloidin staining, which weakly
marks the ventral cell walls. This showed that instead of one longitudinal row of paired ciliophores
as described in Kristensen and Funch [1], the trunk ciliation consists of a more complex pattern at
the anterior part of the thorax.

At the posterior part of the head and the anterior part of the thorax, the organization of the
ciliophores is the most complicated. All four pairs of head ciliophores described in the original
description of *L. maerski*, which were supposed to be lining the oral plate, could not be found.

However, one pair of head ciliophores (hc, figure 3A,C,E,G) could be found, followed by two pairs
of laterally adjacent ciliophores. These three pairs of ciliophores are likely to correspond to some
of the head ciliophores described by Kristensen and Funch [1]. Three unpaired, transversely
elongated ciliophores (mac, figure 3A,C,E) and two pairs of antero-lateral ciliophores (alc, figure
3A,C) are found posterior to the oral plate. More posteriorly, on the thorax, two paired
longitudinal rows of ciliophores are present: one row of four lateral ciliophores (tlc, figure 3A,C,E)
and one row of five median ciliophores (tmc, figure 2A,C,E). The row of thoracic lateral ciliophores
is in tight contact with the thoracic median row of ciliophores, giving a mosaic appearance,
probably explaining the previous indiscernibility of each row. The cells of the median row are
larger and are adjacent to the midline. The thoracic lateral ciliophores are smaller and each of
them is in contact with two thoracic median ciliophores. At the posterior part of the thorax and
the anterior part of the abdomen, only two longitudinal rows exist, each consisting of six
abdominal ciliophores (abc, figure 2A,C,E), corresponding to the observations of the original
description [1]. On the midline between each median quartet of ciliophores, one small nonciliated
epidermal medio-ventral cell (mvc, figure 2C,D) is present.

Adhesive ciliary pad

The ciliary adhesive pad (acp, figures 1A,G, 2A,B and 3A-C) consists of five pairs of multiciliated
cells: two lateral, two median and one posterior, as described in the original description [1].

Mouth ciliation

In accordance with the original description [1], a mouth ciliation is found most likely involved in
food uptake. However, it only covers the anterior edge of the mouth cavity, comprising paired
laterally elongated tufts of >10 approximately 7\( \mu \)m long cilia (mc, figures 1A, 2A,B and 3A,B,D).
The present CLSM study revealed a conspicuous previously undescribed pharyngeal ciliary tuft in the mouth cavity (phc, figures 1A,B,F, 2A,B, 3A,B,D,F and 4A,C). It extends between the main jaws (mj, figure 3F) and its ciliary roots originate from the pharyngeal ganglion, suggesting that the cilia have sensory function. The cilia are 6-7\mu m long and curved (phc, figures 2B, 3A,B,D,F and 4C), and each of the paired tufts consists of >10 cilia, as also seen in the TEM micrographs shown in Kristensen and Funch, 2000 ([1], figure 23) and Sørensen and Kristensen, 2015 ([25], fig. 3.12.B).

**Nephridia**

Three pairs of acetylated α-tubulin-LI-reactive ventro-lateral longitudinal ciliary structures are found along the thorax and anterior abdomen. The present CLSM data in combination with the TEM data of Sørensen and Kristensen (2015) allow us to reconstruct these structures as an anterior and a posterior pair of protonephridia with an intermediate collecting tube, in accordance with Sørensen and Kristensen [23] but opposing the interpretation of three pairs of nephridia given by Sørensen et al. [26] (fig. 16.13 and 16.15). The present study offers the following more detailed description:

The anterior pair of nephridia originates in the anteriormost thorax, each nephridium comprising two adjacent protonephridial units with two monociliated terminal cells each; all four cilia (8-10\mu m long) joining in one common canal cell (nph1, figures 1B, 2A, 3B,E and fig. 3.15 in Sørensen and Kristensen [23]). The posterior pair of nephridia (nph2, figures 1B, 2A and 3B,E) contains only one unit (contrary to the double units proposed by Sørensen and Kristensen [23], but see fig 3.15B) with two monociliated terminal cells (cilia 7-10\mu m long), possibly originating in the anterior abdomen and extending anteriorly into the posterior thorax, where it meets the collecting tubule. The intermediate collecting tubule (ct, figures 1B, 2A and 3B,E) consists of more than five tightly packed cilia, but the exact number could not be determined. It extends through the second third of the thorax and is 11-13\mu m long. The consistent longer length of the cilia of the collecting tubule and higher cilia density, similar to what is shown in Sørensen and Kristensen [23], are elements allowing us to differentiate these collecting tubules from the actual protonephridia. No associated nephridiopore or additional structure could be found.

**Oviducts**
One pair of acetylated α-tubulin-LI-reactive L-shaped ducts here interpreted as oviducts (od, figures 2A and 3B) is present in the posterior part of the abdomen, but does not consist of cilia. They originate lateral to the midline, posterior to the oocyte, extend 6-7μm postero-medially, terminating in an oovore (ovp, figure 3A, B, C) in the center of the adhesive ciliary pad. Non ciliated oviducts are also reported in Rotifera [27-29], whereas nothing similar has been found in Gnathostomulida [30, 31].

Anterior of the oviduct, a pair of putatively associated 10μm long dorsal accessory cilia (aco, figure 2A, E) is present. Each cilium is adjacent to the oocyte; oriented obliquely, extending from a dorso-median to a ventro-lateral position. Their function is unknown.

**Glands**

A tripartite glandular complex consisting of a central gland (mhg, figure 2A, C, D) and a pair of lateral, elongated glands (ihg, figure 2A, C, D) are found in the dorsal head region of *Limnognathia maerski* (hg, figure 2B). All glands show acetylated α-tubulin-LIR in the cell wall and appear to open dorso-apically on the head. The median gland extends dorso-posteriorly to the level of the pharynx and possesses numerous and densely packed nuclei (mhg, figure 2C). The two lateral glands consist of an elongated longitudinal canal anteriorly (embedding few elongated nuclei (ihg, figure 2C), which extends posterior of the median gland until the dorso-lateral sides of the pharynx (Fig. 2D).

One pair of large glandular cells is found ventro-laterally in the posterior-most abdomen (pgl, figures 1A, 2A, E and 3B); their full configuration was detected through background signal of non-specific fluorescence as well as specific acetylated α-tubulin-LIR. Each cell is 15 to 20μm long, ellipsoid shaped, broadest at its base and narrowing into a neck region, with a 2μm wide opening; the elongated nucleus is positioned at the external side of the cell (npg, figure 2E). The cell wall of the neck region contains numerous, distinct acetylated α-tubulin-LIR, longitudinally striated components; their signal becoming less obvious towards the expanded cellular base. FMRF-amide-LIR and serotonin-LIR is visible in the cell opening (opg, figure 2A, E). Their position corresponds to the “paired openings of unknown function” of Sørensen and Kristensen [23] visible with SEM, which therefore are not nephridiopores as previously suggested.
Discussion

Evolution of ventral cords and associated commissures in Gnathifera

The presence of two ventro-lateral nerve cords in *Limnogastria maerski* was confirmed [1, 23] and their precise configuration explained, unraveling an anterior (with associated subpharyngeal ganglia) and a posterior commissure, as well as two ventro-median nerves branching off from the main ventrolateral cords at the subpharyngeal ganglia; these paired ventro-median nerves are not previously reported in Gnathifera.

In Rotifera, only one pair of longitudinal ventro-lateral nerves has been consistently found with FMRF-amide-LiR, catecholamine-LiR, serotonin-LiR and SCPb-LiR in representatives of both Bdelloidea and Monogononta [12-15, 27, 32, 33]. TEM-investigations by Ahrlich ([34] , fig. 5A) also suggest the presence of at least two longitudinal nerves in the neck region of the early branching Seisonidea. However, antibody-staining only shows a subset of the nervous system and acetylated α-tubulin-LiR has not been tested in these studies. Yet, in a total reconstruction of the nervous system of Monogononta based on light microscopy by Remane [35] (figure 5B), no ventro-median nerves were found even though more delicate nerves were described, such as the peripheral nerves. These have been shown to branch off dorso-laterally from the subpharyngeal ganglia and innervate the sensory organs and dorso-ventral muscles [36], similar to what is here described for *L. maerski*. Though no anterior commissure and ganglia resembling those of *L. maerski* are generally found in Rotifera, similarities can exceptionally be found in the FMRF-amide-Li-reactive and SCPb-Li-reactive perikarya and trunk commissure in *Notommatida copeus* Ehrenberg, 1934 [37] (Monogononta) [13], the FMRF-amide-Li-reactive trunk commissure in *Euchlanis dilatata* (Ehrenberg, 1932) [14, 38], or the so called geniculate ganglion of Monogononta [35] (figure 5B).

In Gnathostomulida, confocal and TEM studies show a more variable number of one to three pairs of longitudinal nerves (plus one dorsal and one median unpaired nerve in *Gnathostomula peregrina* Kirsteuer, 1969 [39] (figure 5C)), of which the paired ventro-lateral nerves form an anterior as well as a posterior commissure in *G. peregrina* [10]. Similar to *L. maerski*, their circumesophageal connectives also originate as two distinct bundles of neurites in *G. peregrina*
[10] (figure 5A,C), further supporting the homology of two ventro-lateral cords in Gnathifera. This character is likely to be shared between most Spiralia [40] suggesting that the ventro-lateral nerve cord of Gnathifera is possibly a symplesiomorphy of this group. However with Gnathostomulida being sister group to the remaining Gnathifera [3] and the only sporadic finding of an anterior commissure and/or subpharyngeal ganglia in both Gnathostomulida and Rotifera, the homology of these specific substructures of the ventral cords remains to be tested. The reported ventro-median nerve in *G. peregrina* is unpaired but two separate medio-ventral strands originating in the anterior trunk are observed in new ongoing studies of other gnathostomulids (Gąsiorowski, Bekkouche and Worsaae unpublished), warranting further analyses of their possible homology to the medio-ventral nerves of *L. maerski*.

**Finding of a synapomorphic pharyngeal ganglion with ciliary receptors in Gnathifera**

The present study confirms the presence of a formerly suspected pharyngeal ganglion in *Limnognathia maerski* [23] with numerous nucleated cells, an observation refuting the suggestion of Gorelick [41], proposing that “Micronagathozoan jaws may also be enervated by anucleate neurons”. In Rotifera, a “mastax ganglion” is suspected but not yet confirmed in Seisonidae [27], and data is scarce on Bdelloidea since only the presence of catecholaminergic nerves related to the mastax suggests its presence in *Rotatoria tardigrada* Ehrenberg, 1832 [32, 38]. However, for Monogononta, this ganglion has shown IR for serotonin, catecholamines and FMRF-amide [13, 14, 32, 42]. Yet, IR, nerves, and perikarya repartition are extremely variable and no detailed comparison with *L. maerski* is possible. In Gnathostomulida, Herlyn and Ehlers [43] reject the presence of a buccal ganglion after failing at finding any correspondent structures in *Gnathostomula paradoxoa*. However, other researches do not support this conclusion and the so-called buccal ganglion, has been described in Filospermoidea with TEM [20] and in Bursovaginoidaea with TEM and CLSM [10, 16, 18, 20]. CLSM studies [10, 16] further show the presence of FMRF-amide-LI-reactive perikarya in the buccal ganglion of *Gnathostomula peregrina*. Although connections between the central nervous system and the pharyngeal ganglion of *L. maerski* have not been found, studies of Gnathostomulida [16, 20] and Rotifera [32], indicate that a pair of nerves originates dorso-laterally from the posterior of the brain, supplying the buccal/mastax ganglion (mgc, Fig. 5B). The present study, as well as the literature, indicates that
homologs to the pharyngeal ganglion of *L. maerski* are found in most Gnathifera (figure 5), thus this character might be a synapomorphy of this group.

The here discovered pharyngeal cilia extending between the main jaws in *Limnognathia maerski* can actually be recovered in previously published transmission electron micrographs such as figs. 23 and 25 in [1]. Intriguingly, sensory cilia with similar position, innervation and configuration are also found in rotifers such as the Bdelloidea (*Philodina roseola* Ehrenberg, 1832 [24, 38] and *Philodina acuticornis odiosa* Milne, 1916 [44, 45]), or Monogononta (*Asplanchna brightwellii* Gosse, 1850 [24, 46]). These cilia likewise protrude between the basal parts of the rami (assumedly homologous to the main jaws of *L. maerski*) and are also anchored at the mastax ganglion (assumedly homologous to the ganglion in *L. maerski*). Additionally, in *Asplanchna brightwellii* the proximal part of the ciliated sensory receptors is well separated into two bundles resembling the paired configuration in *L. maerski* [24]; all supporting their homology and their organization into densely ciliated tufts as a putative synapomorphy of Micrognathozoa and Rotifera. Though so far, data on the early branching rotifer Seisonidae are lacking. In Gnathostomulida, pharyngeal ciliation has never been described, however, scarce cilia are visible in the pharynx of *Gnathostomula paradoxa* ([43] fig. 3), and ongoing investigations indicate the existence of possible homologous short paired ciliary receptors, between the jaws connected to the buccal ganglion in *Gnathostomula paradoxa*, *Austrognathia microconulifera* Farris, 1977 [47] and *Haplognathia* spp. (Gąsiorowski, Bekkouche and Worsaae unpublished). The putative common presence of paired ciliary receptors on the pharyngeal ganglia across Gnathifera thereby further supports the homology of the pharyngeal ganglion (as well as its possibly common sensory function) in Gnathifera.

**Increased resolution of ciliary patterns revealed with high quality CLSM**

Acetylated α-tubulin-LIR as well as phallolidin and DAPI show a more complex pattern of ventral ciliophores than previously described in *Limnognathia maerski* [1]. These results show the relevance of CLSM to resolve spatial patterns in microscopic animals since the collapse of cilia makes difficult the identification of independent cells with light microscopy or scanning electron microscopy. Similar complex anterior ciliary arrangements have been found in the gastrotrichs *Diuronotus aspetos* Todaro, Balsamo & Kristensen, 2005 [48] (Bekkouche and Worsaae, 2005).
unpublished), *Diplodasis rothei* Kienke, Narkus, Hochberg & Schmidt-Rhaesa, 2013 [49] or the microscopic annelids *Diurodrilus* spp. [50]. Interestingly *L. maerski* and *Diurodrilus* have been comprehensively compared [1, 50], and even though phylogenomics recently confirmed that *Diurodrilus* is a distantly related genus of annelids [2, 51], this is another similar character between these two animals. However, though these overall similarity in patterns may reflect homoplasy, the detailed patterns has been shown to be of systematic significance within, e.g., *Diurodrilus* and Gastrotricha [50, 52, 53] and may also potentially be useful for discriminating Micrognathozoa from Greenland versus Antarctica, which was not possible according to jaw morphology [21].

**Nephridial system of Micrognathozoa shows more similarity with Rotifera than Gnathostomulida**

The protonephridial system of *Limnognathia maerski* resembles the one of Rotifera, although only few studies have reconstructed the excretory system of Rotifera in details. However, Ahlrichs provided the complete reconstruction of the protonephridial system of *Paraseison annulatus* (Claus, 1876) [54] (Seisonidae) [34] and *Proales reinhardti* (Ehrenberg, 1934) [37] (Monogononta) [55] from ultrathin section and TEM. Both rotifers possess a terminal syncytium with several multiciliated terminal organs and a capillary canal (resembling the canal cell in *L. maerski*). Furthermore, the terminal syncytium connects to a multiciliated canal region, which shows resemblance to the collecting tubules of *L. maerski*. The main difference in this configuration being the monociliated nature of the terminal organs of *L. maerski* versus the multiciliated organs found in most rotifers [34, 36, 55, 56]. The protonephridial system of Gnathostomulida has been described in detail for *Haplognathia rosea* (Sterrer, 1969) [19] (Filospermoidea) and *Gnathostomula paradoxa* by Lammert [20]. They consist of serially independent organs, each comprising a monociliated terminal cell, a canal cell and a nephridiopore cell; an arrangement found in other animals [57, 58]. Therefore, it can be assumed that the monociliated terminal cells of *L. maerski* is a pleisiomorphic condition shared with Gnathostomulida, whereas the multiciliated collecting tubule supplying the different canal cells is a synapomorphy of *L. maerski* and Rotifera.

**Do Micrognathozoa possess a retrocerebral organ?**

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The tripartite anterior gland of *Limnognathia maerski*, consisting of one unpaired median and a pair of medio-lateral glands opening dorso-apically is very similar in position and size to the retrocerebral organ found in most Rotifera, where they are assumed to play a role in the lubrication of the cilia [24, 27, 36]. If the two organs are homologous, the median gland of *L. maerski* would correspond to the retrocerebral sac, while the lateral glands would correspond to the subcerebral glands more similar to what is found in Bdelloidea [36] (where the retrocerebral sac likewise opens medially and the two subcerebral glands open medio-laterally); hereby indicating that this may be the plesiomorphic condition in Rotifera, and that the retrocerebral organ might be a synapomorphy of Micrognathozoa and Rotifera.

**Conclusion**

This study shows a striking simplicity of the micrognathozoan nervous system, in opposition to the complexity in muscular [11] - and ciliary systems (present study), but it also illustrates the need of CLSM studies together with TEM investigations on meiofaunal animals. Indeed, previous TEM results on Micrognathozoa could not lead to the observation of the second ventro-median pair of longitudinal nerves or the exact details of the ventral ciliation. On the other hand, some conclusions of this paper could not have been possible without previous TEM studies as the identification of the protonephridial unit versus the collecting tubule.

Indeed, many characters described in this study seem to be autapomorphies of Micrognathozoa, such as the presence of a paired ventro-median nerve, or the specific arrangement of ciliophores. On the other hand, some characters constitute putative synapomorphies of Micrognathozoa and Rotifera, such as the peripheral nervous system innervating the sensory structures, the presence of dense tufts of pharyngeal sensory cilia, the organization of the protonephridia and the potential presence of a retrocerebral organ. Furthermore, resolving the morphology of the nervous system of Micrognathozoa allowed us to hypothesize that a ciliated pharyngeal ganglion is a synapomorphy of all Gnathifera and that the presence of two ventro-lateral nerve cords is a symplesiomorphy of Gnathifera, and more generally of Spiralia [40].

Although this study informs on the inner anatomy of Micrognathozoa, many details still warrants further ultrastructural studies such as the protonephridia and the oviducts, or the connection of

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the pharyngeal ganglion to the brain. Additionally, many Gnathifera lack detailed descriptions with
CLSM such as the rotifer group Seisonidae, where only the musculature has been described [7],
and the gnathostomulid groups Filospermoidea and Conophoralia. In the context of the latest
phylogenomic results [2, 3] were Gnathifera has a key phylogenetic position within protostomes
we hope that these issues will soon be addressed.

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Competing interests

We have no competing interests.

Authors’ contributions

NB and KW conceptualized and designed the study, collected the animals, analyzed the data, and
wrote the manuscript. NB gathered most of the immunohistochemical data and made the
illustrations.

References

4687(200010)246:1<1::AID-JMOR1>3.0.CO;2-D).
paraphyly based on phylogenomic data supports a non-coelomate ancestry of spiralia. Molecular Biology
(doi:10.1016/j.cub.2015.06.068).

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**List of abbreviations (for reviewers only)**

| abc | abdominal ciliophores; |
| ac | anterior commissure; |
| acf | anterior ciliated field; |
| aco | accessory cilia of the oviduct; |
| acp | adhesive ciliary pad; |
| ag | auxiliary ganglion; |

https://mc.manuscriptcentral.com/rsos
alc, anterior lateral ciliophores;

apo, acetylated α-tubulin-LI-reactive pharyngeal organ;

atpg, acetylated α-tubulin-LI-reactive pharyngeal ganglion;

bg, buccal ganglion;

br, brain;

cc, circumesophageal connective;

cd1,2, caudalia 1 and 2;

cg, caudal ganglion;

cp, collecting tubule;

dln, dorso-lateral nerve;

dmn, dorso-median nerve;

do1-3, dorsalia 1 to 3;

eg, epipharyngeal ganglion;

egg, egg;

fbp, FMRF-amide-LI-reactive brain perikarya;

fib, fibularium;

fp1-5, FMRF-amide-LI-reactive perikarya of the pharyngeal ganglion;

fs, FMRF-amide-LI-reactive spot of the pharyngeal ganglion;

gg, geniculate ganglion;

gl, gut lumen;

gut, gut;

hc, head ciliophores;

hgr, head groove;

hg, head gland;

jw, jaw;
680  **la3-5**, lateralia 3 to 5;
681  **lhg**, lateral head gland;
682  **lm**, longitudinal muscles;
683  **ln**, lateral nerve;
684  **mac**, median anterior ciliophores;
685  **mc**, mouth ciliation;
686  **mg**, mastax ganglion;
687  **mgc**, mastax ganglion connective;
688  **mhg**, median head gland;
689  **mj**, main jaw;
690  **mo**, mouth opening;
691  **mvc**, medio-ventral aciliated cells;
692  **mvm**, median ventral muscle;
693  **mvn**, main ventral nerve;
694  **ncm**, nerve of the mouth ciliation;
695  **np**, neuropil;
696  **npg**, nuclei of the posterior gland;
697  **nph1-2**, nephridia 1 and 2;
698  **ns**, nervous system;
699  **od**, oviduct;
700  **op**, oral plate;
701  **opg**, opening of the posterior gland;
702  **ovp**, oviporte;
703  **pc**, posterior commissure;
704  **pcf**, posterior ciliated field;
pg, pharyngeal ganglion;

pgl, posterior gland;

phc, pharyngeal cilia;

pns, peripheral nervous system;

pvm, paramedian ventral muscle;

s1-4, serotonin-LI-reactive perikarya of the pharyngeal ganglion;

sbr, serotonin-LI-reactive brain;

sacb, serotonin-LI-reactive anterior commissure of the brain;

sbap, serotonin-LI-reactive brain antero-lateral nerve projection;

sb1-6, serotonin-LI-reactive perikarya of the brain;

sbpp, serotonin-LI-reactive brain posterior projection;

scb, sensorium cell body;

scc, serotonin-LI-reactive circumesophageal connective;

slcb, serotonin-LI-reactive lateral connective of the commissure of the brain;

smcb, serotonin-LI-reactive median connective of the commissure of the brain;

spcb, serotonin-LI-reactive posterior commissure of the brain;

spg, subpharyngeal ganglion;

ss, sensorium;

tlc, trunk lateral ciliophores;

tmc, trunk median ciliophores;

uvmn, unpaired ventro-median nerve;

vg, vesicular ganglion;

vlc, ventral locomotory ciliophores;

vlnc, ventro-lateral nerve cord;

vmn, ventro-median nerve;
730 vn; ventral nerve;
731 vs, visceral nerve;
732
733 **Figure captions**
734
735 **Figure 1:** General nervous system of *Limnognathia maerski*. **A)** Schematic drawing of the nervous system of *L. maerski*. Structures recognized with DAPI in blue, acetylated α-tubulin-LI-reactive nervous system in orange/yellow, and locomotory ciliation in light grey. **B-G)** CLSM maximum intensity projection. Acetylated α-tubulin-LIR color in glow, serotonin-LIR in red, FMRFamide-LIR in purple and DAPI in cyan. **B)** General overview of the nervous system. Note that some deformation occurred during scanning, resulting in an artefactual elongation of the pharyngeal ganglion. **C)** General overview of the serotonin-LI-reactive nervous system. **D)** Details of sensoria and peripheral nervous system. **E)** Overview of the FMRF-amide-LI-reactive brain and pharyngeal ganglion. **F)** Details of the anterior commissure and subpharyngeal ganglion. **G)** Details of the posterior commissure. Anterior end of specimens pointing left on all figures. **ac**, anterior commissure; **acp**, adhesive ciliary pad; **ag**, auxiliary ganglion; **apo**, acetylated α-tubulin-LI-reactive pharyngeal organ; **br**, brain; **cc**, circumesophageal connective; **ct**, collecting tubule; **egg**, egg; **fbp**, FMRF-amide-LI-reactive brain perikarya; **jw**, jaw; **mc**, mouth ciliation; **nmc**, nerve of the mouth ciliation; **np**, neuropil; **nphl-2**, nephridia 1 and 2; **pc**, posterior commissure; **pg**, pharyngeal ganglion **pgl**, posterior gland; **phc**, pharyngeal cilia; **pns**, peripheral nervous system; **sbr**, serotonin-LI-reactive brain; **scb**, sensorium cell body; **spg**, subpharyngeal ganglion; **ss**, sensorium; **vlc**, ventral locomotory ciliophores; **vln**, ventro-lateral nerve cord; **vmn**, ventro-median nerve.

750 **Figure 2:** Profile and details of the nervous system in *Limnognathia maerski*. **A)** Schematic drawing of a lateral view of *L. maerski*. Glandular system in blue, nervous system in orange/yellow and ciliation in green. **B-H)** CLSM maximum intensity projections. Acetylated α-tubulin-LIR in glow, DAPI in cyan, serotonin-LIR in green, and phalloidin in red. **B)** Virtual mid-sagittal section on the midline of the animal. **C-D)** Maximum intensity projection of substacks. **C)** Details of the anterior of the glands of the head. **D)** Details of the posterior of the glands of the head. **E)** Details of the posterior glands. **F)** Details of the acetylated α-tubulin-LI-reactive brain. **G)** Details of the auxiliary ganglion. **H)** Details of the relative position of the longitudinal nerves and musculature. Anterior end of specimens pointing left on all figures. **acf**, anterior ciliated field; **aco**, accessory cilia of the
oviduct; **acp**, adhesive ciliary pad; **ag**, auxiliary ganglion; **apo**, acetylated α-tubulin-LI-reactive pharyngeal organ; **atpg**, acetylated α-tubulin-LI-reactive pharyngeal ganglion; **br**, brain; **cc**, circumesophageal connective; **ct**, collecting tubule; **egg**, egg; **gl**, gut lumen; **gut**, gut; **hg**, head gland; **hgr**, head groove; **jw**, jaw; **lhg**, lateral head gland; **mc**, mouth ciliation; **mhhg**, median head gland; **mo**, mouth opening; **mmv**, median ventral muscle; **np**, neuropil; **npg**, nuclei of the posterior gland; **nph1,2**, nephridia 1 and 2; **od**, oviduct; **op**, oral plate; **opg**, opening of the posterior gland; **pc**, posterior commissure; **pcf**, posterior ciliated field; **pg**, pharyngeal ganglion; **pfl**, posterior gland; **phc**, pharyngeal cilia; **pns**, peripheral nervous system; **pvm**, paramedian ventral muscle; **spg**, subpharyngeal ganglion; **ss**, sensorium; **vlc**, ventral locomotory ciliophores; **vln**, ventro-lateral nerve cord; **vmn**, ventro-median nerve.

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**Figure 4:** Details of the pharyngeal ganglion and the serotonergic brain of *Limnognathia maerski*. **A-G)** Schematic drawings with acetylated α-tubulin-LIR in yellow, FMRF-amide-LIR in purple, DAPI in blue and serotonin-LIR in green. **B-F** and **H-I** CLSM maximum intensity projection with
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Figure 5: Comparison of the nervous system of Gnathifera. Schematic drawing of the dorsal view of the nervous system of three Gnathifera. Different colors represent parts of the nervous system that may be homologous between the different animals, but see the text for a full discussion. Grey structures are parts of the nervous system that cannot be homologized. Anterior end pointing left on all figures. A) Micrognathozoa: Limnognathia maerski, B) Rotifera, Monogononta, modified from Remane 1933 [35], C) Gnathostomulida: Gnathostomula peregrina, modified from Müller and Sterrer, 2004 [10]. ac, anterior commissure; ag, auxiliary ganglion; br, brain; bg, buccal ganglion; cc, circumesophageal connective; cg, caudal ganglion; dln, dorso-lateral nerve; dmn, dorso-median nerve; eg, epipharyngeal ganglion; gg, geniculate ganglion; ln, lateral nerve; mg, mastax ganglion; mgc, mastax ganglion connective; mvn, main ventral nerve; pc, posterior commissure; pg, pharyngeal ganglion, pns, peripheral nervous system; spg, subpharyngeal ganglion; uvmn, unpaired ventro-median nerve; vg, vesicular ganglion; vln, ventro-lateral nerve cord; vmn, ventro-median nerve; vn, ventral nerve; vs, visceral nerve.
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**B-F** and **H,I)** CLSM maximum intensity projection with acetylated α-tubulin-LIR in yellow, FRMF-amide-LIR in purple, DAPI in cyan, and serotonin-LIR in green in **B)** and in glow in **H)** and **I)**.  

**A)** Schematic drawing of the pharyngeal ganglion  

**B)** Details of the serotonergic-LIR of the pharyngeal ganglion  

**C)** Overview of the acetylated α-tubulin-LIR of the pharyngeal ganglion  

**D, E, F)** Successive substacks of the ventral, median and dorsal sections of the pharyngeal ganglion as seen with FMRF-amide-LIR  

**G)** Schematic drawing of the serotonergic brain  

**H)** Details of the serotonin-LI-reactive brain  

**I)** Overview of the serotonin-LI-reactive brain.  

Anterior end of specimens pointing to the top on all figures.  

**apo**, acetylated α-tubulin-LI-reactive pharyngeal organ;  

**atpg**, acetylated α-tubulin-LI-reactive pharyngeal ganglion;  

**br**, brain;  

**fp1-5**, FMRF-amide-LI-reactive perikarya of the pharyngeal ganglion;  

**fs**, FMRF-amide-LI-reactive spot of the pharyngeal ganglion;  

**phc**, pharyngeal cilia;  

**s1-4**, serotonin-LI-reactive perikarya of the pharyngeal ganglion;  

**sacb**, serotonin-LI-reactive anterior commissure of the brain;  

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Abstract:

Background: Diuronotus is one of the latest described genera of Paucitubulatina, constituting one of the three major clades in Gastrotricha. Morphology suggests that Diuronotus is an early branch of Paucitubulatina, making it a key taxon to understand the evolution of this morphologically understudied group. Here we test its phylogenetic position employing molecular data and Bayesian inference, and provide detailed description of the muscular, nervous, and ciliary systems of Diuronotus aspetos, using immunohistochemistry and confocal laser scanning microscopy. Results: We confirm its proposed position within Muselliferidae, and find this family sister group to Xenotrichulidae. The muscular system revealed with F-actin staining shows a simple, though singular, organization of the trunk musculature with a reduction to three pairs of longitudinal muscles and addition of up to five paired longitudinal rows of dorso-ventral muscles versus the six longitudinal and two dorso-ventral pairs, found in most Paucitubulatina. The pharynx is for the first time described in details with acetylated α-tubulin immunoreactivity, including different nerves, two pairs of sensory cilia, paired anterior glands, and a unique canal system of unknown function. The central nervous system revealed with acetylated α-tubulin, serotonin and FMRF-amide-like immunoreactivity is in overall similar to other Gastrotricha, but additionally exposes an anterior nerve ring, several anterior longitudinal nerves, and four ventral commissures (pharyngeal, trunk, pre-anal, and terminal). High-resolution imaging made it possible to trace innervations of ciliary structures and muscles, revealing new functional information of specific nerves. Two pairs of protonephridia are documented, while other Paucitubulatina have one. Moreover, the precise arrangement of multiciliated cells is unraveled, yielding a pattern of possibly systematic importance. Conclusion: several neural structures resemble those found in Xenotrichula (Xenotrichulidae), and may turn out to represent paucitubulatinan or even gastrotrich apomorphies. However, in order to trace the character evolution, detailed morphological studies on additional Paucitubulatina as well as a robust gastrotrich phylogeny are necessary. Yet, the present study offers new inputs on the evolution of organ systems and so far neglected characters in Gastrotricha. Keywords

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Neuromuscular study of early branching *Diuronotus aspetos* (Paucitubulatina) gives insight on the evolution of organs system within Gastrotricha

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Abstract

Background: *Diuronotus* is one of the latest described genera of Paucitubulatina, constituting one of the three major clades in Gastrotricha. Morphology suggests that *Diuronotus* is an early branch of Paucitubulatina, making it a key taxon to understand the evolution of this morphologically understudied group. Here we test its phylogenetic position employing molecular data and Bayesian inference, and provide detailed description of the muscular, nervous, and ciliary systems of *Diuronotus aspetos*, using immunohistochemistry and confocal laser scanning microscopy.

Results: We confirm its proposed position within Muselliferidae, and find this family sister group to Xenotrichulidae. The muscular system revealed with F-actin staining shows a simple, though singular, organization of the trunk musculature with a reduction to three pairs of longitudinal muscles and addition of up to five paired longitudinal rows of dorso-ventral muscles versus the six longitudinal and two dorso-ventral pairs, found in most Paucitubulatina. The pharynx is for the first time described in details with acetylated α-tubulin immunoreactivity, including different nerves, two pairs of sensory cilia, paired anterior glands, and a unique canal system of unknown function. The central nervous system revealed with acetylated α-tubulin, serotonin and FMRF-amide-like immunoreactivity is in overall similar to other Gastrotricha, but additionally exposes an anterior nerve ring, several anterior longitudinal nerves, and four ventral commissures (pharyngeal, trunk, pre-anal, and terminal). High-resolution imaging made it possible to trace innervations of ciliary structures and muscles, revealing new functional information of specific nerves. Two pairs of protonephridia are documented, while other Paucitubulatina have one. Moreover, the precise arrangement of multiciliated cells is unraveled, yielding a pattern of possibly systematic importance.
Conclusion: several neural structures resemble those found in Xenotrichula (Xenotrichulidae), and may turn out to represent paucitubulatinan or even gastrotrich apomorphies. However, in order to trace the character evolution, detailed morphological studies on additional Paucitubulatina as well as a robust gastrotrich phylogeny are necessary. Yet, the present study offers new inputs on the evolution of organ systems and so far neglected characters in Gastrotricha.

Keywords

Neurobiology, meiofauna, Chaetonotida, DNA, phalloidin, Musellifer.

Background

Gastrotricha are small, often sub-millimetric, interstitial worms, ubiquitously found in most aquatic environments with a long debated phylogenetic position [1-3]. They were first considered closely related to various meiofaunal, protostome groups such as rotifers (Trochelminthes [4]), kinorhynchs (Nematorhyncha [5]) or Gnathostomulida (Neotrichozoa [6, 7]). Later, molecular phylogenies placed them within Spiralia with uncertain affinities; within the debated group Platyzoa, comprising Gastrotricha, Platyhelminthes and Gnathifera [1, 8, 9]. Recent phylogenomic studies propose a sister group relationship between Platyhelminthes and Gastrotricha [3, 10]. However, the controversy over the phylogenetic position of Gastrotricha masks other problems existing within the group. Indeed, compared to the diversity and omnipresence of these animals, relatively few phylogenetic and detailed morphological studies have been conducted on this group and the evolution of, e.g., nervous system, muscular system and nephridia is unresolved [11-13]. Also, the diversity is still largely unexplored, exemplified by the recent erection of the family

*Diuronotus* [17] is another recently described gastrotrich genus (2005), comprising two described species: *Diuronotus aspetos* Todaro, Balsamo, and Kristensen, 2005 [17] (Fig. 1) and, *Diuronotus rupperti* Todaro, Balsamo, and Kristensen, 2005 [17], and one undescribed species *Diuronotus* sp. [18-20], transferred from *Halichaetonotus* [17]. They are all found in marine interstitial environments of the North Atlantic; *D. aspetos* from Greenland [17] and Germany [2, 21], *D. rupperti* from Denmark [17] and *Diuronotus* sp. from North Carolina, USA [18]. *Diuronotus* was placed in Muselliferidae (Paucitubulatina, Chaetonitida) next to *Musellifer* [22] with which it shares the presence of a ciliated so-called ‘muzzle’ (or snout) and specific ultrastructural traits of scales and sperm [23].

Gastrotricha are divided into two main taxa: the supposedly monophyletic Macrodasyida and the possibly paraphyletic Chaetonotida, divided further into the Multitubulatina, (consisting of one genus, *Neodasys*, and possessing multiple adhesive glands) and the diverse Paucitubulatina (possessing generally only two adhesive tubes) [24]. Muselliferidae, belonging to Paucitubulatina, is the possible sister group to all remaining Paucitubulatina according to morphological [22, 25] and molecular [26, 27] studies. However, Paps and Riutorts (2012) [28] find an alternative topology with Xenotrichulidae positioned as sister group of the remaining Paucitubulatina, and Muselliferidae being the sister group to Chaetonotidae. Kieneke et al. (2008) [29] find Proichthydiidae as sister group to the remaining Paucitubulatina, and Muselliferidae forming a clade together with Xenotrichulidae sister group to other Paucitubulatina. Nonetheless, these different topologies overall suggest a key position of Muselliferidae within Gastrotricha,
emphasizing the importance of this family for understanding of the evolution of Gastrotricha. Indeed, some features of Muselliferidae, namely the marine habitat and the well-developed hermaphroditism are supposed to be plesiomorphic character traits of Chaetonotida. Yet, detailed morphological studies on this family are still lacking, most likely due to the paucity of these animals and their late discovery [26, 30].

Recently, a series of papers employing confocal laser scanning microscopy (CLSM) described the detailed muscular arrangement of several Paucitubulatina, namely Musellifer [22], Xenotrichulidae [22, 31], Chaetonotidae [22, 32], and Dasydytidae [11, 33], and notably, the helicoidal musculature, proposed to be a gastrotrich synapomorphy [34]. These recent works were used to infer the plesiomorphic arrangement of the musculature of Gastrotricha as constituted by two ventro-lateral longitudinal muscles surrounded by outer circular muscles, and longitudinal splanchnic muscles surrounded by helicoidal and intestinal circular muscles [2]. In Paucitubulatina, the longitudinal muscles appear to be more numerous, and the outer circular muscles, if present, are incomplete and consist of dorso-ventral muscles [2]. These dorso-ventral or semi-circular muscles are found in marine chaetonotids [22], but are often missing or highly reduced in freshwater chaetonotids [11, 22, 33] emphasizing the importance of studying the marine Diuronotus in order to resolve their evolution and contribute to the broader understanding of muscular evolution within Gastrotricha.

To date, only one confocal study on Xenotrichula describes the nervous system of a member of Paucitubulatina in detail [12], while it has been extensively described for Multitubulatina (Neodasys) [13] and in several Macrodasyida with combined immunohistochemistry and CLSM (e.g., [35, 36]), or transmission electron microscopy (TEM) [37]. One of the conclusions of the
Xenotrichula study [13] is the low structural variation of the nervous system within Gastrotricha, always comprising a bilobed brain with a ventral commissure, a pair of anteriorly projecting longitudinal nerves, a pair of ventro-lateral nerve cords along the trunk, and a terminal commissure. These features were also interpreted as ancestral conditions of Gastrotricha in Kieneke and Schmidt-Rhaesa (2015) [2, 12]. Yet, only one Paucitubulatina, Xenotrichula, was considered for this state reconstruction. Moreover, substantial variation exists, such as the presence of an additional ventral nerve in Oregodasys cirratus Rothe & Schmidt-Rhaesa, 2010 [38] and dorsal nerves in Xenodasys riedli (Schöpfer-Sterrer, 1969) [39, 40], or additional trunk commissures in Dactylopodola and Oregodasys cirratus. These studies underline the unexplored diversity of gastrotrich nervous systems, which may especially concern the diverse group of Paucitubulatina, with only one study on the nervous system so far [13] and a total lack of data on Muselliferidae.

Several studies have described the ultrastructure and repartition of protonephridia in Gastrotricha, with a few of them addressing species of Paucitubulatina [41, 42]. Members of Paucitubulatina are suggested to always possess one pair of trunk protonephridia [41], although again, data on Muselliferidae are lacking. Each nephridium was found to encompass two monociliated terminal cells with coaxial cilia, a long canal cell, and a nephridopore cell [2].

In order to enhance our understanding of the evolution of major organ systems within Gastrotricha we acquired new morphological data on Diuronotus aspetos, using CLSM techniques and immunohistochemistry to describe its arrangement of the musculature, nervous system, and ciliation in detail. To assess the previously proposed relationship of D. aspetos within Muselliferidae, we analysed the phylogenetic position within Chaetonotida, using molecular data.
In this phylogenetic context, the morphology of *Diuronotus* and possible homologies are compared and discussed relative to other Chaetonotida, and Gastrotricha in general.

**Material and methods**

**Collecting**

For *Diuronotus aspetos*, the samples were taken with a mini van Veen grab from shallow water (3-6 m water depth) of Flakkerhuk (69°38.63’N 51°51.13’W), Disko Island, West Greenland. All specimens were collected during the Arctic summer in August 2013. Sediment was well-sorted sand of fine to medium grain size. The specimens have been extracted with MgCl₂ narcotization and decantation.

For DNA, specimens of *Xenotrichula* sp. have been sampled in Ystad, Sweden (55°26.28’N 13°55.44’E) in subterranean environment on a beach with fine to medium sized sand, and extracted with MgCl₂ narcotization and decantation. Marine *Aspidiophorus* sp. were sampled from cultures of *Dinophilus gyrociilatus* from Copenhagen University, where they are contaminants and unfortunately of unknown origin.

**Sequence acquisition**

Total genomic DNA was obtained from whole specimens using the Qiagen DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer’s protocol, except for performing the DNA elution in 160 μL of AE buffer in order to increase the final DNA concentration.

Polymerase chain reactions (PCR) using Sanger based markers were prepared to a final volume of 25 μL with 12.5 μL of GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA), 1 μL of
each primer (10 μM concentration), 10-8.5 μL of Milli-Q water (adjusted to amount of DNA template), and 0.5-2 μL of DNA template. Reaction mixtures were heated in a Bio-Rad G1000 Thermal Cycler at 94°C for 3 minutes, followed by 35-40 cycles (primer specific) of 94°C for 30 s, specific primer pair annealing temperatures for 30 s, and an extension at 68°C for 45 s (unless indicated otherwise), and a final extension-phase of 5 min at 72°C. The COI primer set dgLCO1490/dgHCO2198 [43] was run with two cycling steps, both variable in temperature. COI annealing temperatures were 45°C for 45 s and 51°C for 45 s, respectively with extensions of 30 s.

Overlapping fragments of the small 18S rDNA (ca. 1800 bp) were obtained using paired primers corresponding to fragment 1 and 3 of the 18S rDNA [44]: (1) 18S1f/18S5 R (ca. 900 bp) and (3) 18Sa2.0/18S9r (ca. 800 bp) both overlapping. Both primer sets (1) and (3) had annealing temperatures set to 49°C. The 28S primer set used was 28SD3/28SG758 [45, 46] with an annealing temperature of 53°C.

All newly generated sequences were deposited in the GenBank ® database with the following accession numbers NB#####, NB#####, NB#####, NB#####, and NB##### (Table 1).

Phylogenetic analysis

Sequences were cleaned on BioEdit [47], and a consensus has been realized from the reverse and forward sequences. Sequences were blasted on NCBI [48]. In parallel, COI, 18S and 28S of Diuronotus aspetos were found from its transcriptome [3], using Blastall from NCBI. Sangers and transcriptome acquired COI, 18S and 28S genes were aligned and compared, showing low quality and short length of Sangers sequences. Consequently, COI and 28S of the transcriptome were kept, while a consensus of 18S from the transcriptome and the Sangers sequencing was done having an identical overlapping segment. This hybrid approach was possible since specimens used
for the transcriptome and the Sanger sequences came from the same sample. Sequences of *Aspidiophorus* sp. and *Xenotrichula* sp. were added to the dataset. Sequences of other gastrotrichs acquired from GenBank, based on the tree of Kånneby et al. 2012 [49] were added, selecting sequences from each genus (except *Bifidochaetus* [16]) for which sequences were not available at the time of the analysis), and representing the shortest and deepest branches possible. Sequences from Kånneby et al. [26] for *Musellifer*, Kånneby and Todaro [50] for Neogosseidae, and Todaro et al. [51] for the macrodasyidans outgroups have additionally been collected from GenBank. Subsequently, the sequences were aligned gene per gene with Muscle in Seaview [47], checked by hand, and the three genes were concatenated with Sequence Matrix [52]. Finally, this dataset was analyzed with Bayesian inference in MrBayes 3.2.6 [53] under the model GTR+I+Γ. The gamma shape parameter, the substitution rates, the proportion of invariable sites, and the character state frequencies were all unlinked. The dataset was partitioned according to each gene and by codon position for COI and analyzed with 4 MCMC chains for each run, for 30 million generations. Chains were sampled every 1000th generations and the burn-in was set to 25%. Convergence of the two runs as well as analysis quality was ascertained by checking the log likelihood graphs, the average standard deviation of split frequencies, and the model fit with Tracer [54].

**Immunohistochemistry and CLSM**

Specimens were anesthetized with isotonic magnesium chloride and fixed in 3.7% paraformaldehyde in phosphate buffered saline (PBS) for one to two hours at room temperature (RT), followed by six rinses in PBS and storage in PBS with 0.05% NaN₃. Triple or quadruple stainings were applied for the investigation of muscular, nervous, glandular and ciliary systems, including F-actin staining (Alexa Fluor 488-labelled phalloidin, INVITROGEN, Carlsbad, USA), DNA-
staining (405nm fluorescent DAPI) and antibodies against neurotransmitters and tubulinergic
elements (monoclonal mouse anti-acetylated α-tubulin (SIGMA T6793, St. Louis, USA), polyclonal
chicken anti acetylated α-tubulin (SAB3500023-100UG), polyclonal rabbit anti-serotonin (5-HT,
SIGMA S5545) and anti-FMRF-amide (IMMUNOSTAR 20091, Hudson, USA)). Prior to adding the
primary antibody-mix, the samples were pre-incubated with 1% PTA (PBS + 1% Triton-X, 0.05%
NaN3, 0.25% BSA, and 5% sucrose) for one hour. Samples were incubated over night at RT in
primary antibodies mixed 1:1 with glycerol (in a final 1:400 concentration). Subsequently,
specimens were rinsed in PBS six times and incubated with the secondary antibodies conjugated
with fluorochromes over night at RT (mixed 1:1 with glycerol; 1:400 goat anti-mouse labeled with CY5 (JACKSON IMMUNO-RESEARCH, West Grove, USA, 115-175-062), 1:400 goat anti-mouse
labeled with TRITC (JACKSON IMMUNO-RESEARCH, West Grove, USA, 115-175-062), 1:400 goat anti-rabbit labeled with TRITC (SIGMA T5268), and 1:200 goat anti-chicken labeled with Dylight
(JACKSON IMMUNO-RESEARCH, West Grove, USA, 103-495-1550)). They were rinsed in PBS five
times and one time in 1% PTA and pre-incubated for 60 minutes in Alexa Fluor 488-labeled
phalloidin (0.33M in 1% PTA). Thereafter, specimens were rinsed in PBS (without NaN3) and
mounted in Fluoromount-G with DAPI (SOUTHERN BIOTECHNOLOGY ASSOCIATES, Inc., Alabama,
USA) or Vectashield with DAPI (VECTOR LABORATORIES, Burlingame, USA). The specificity of the
antibodies was tested by examining specimens, where either the primary or secondary antibodies
were omitted. Chicken anti acetylated α-tubulin staining did not give satisfying results and is
therefore not shown in this study (Sigma SAB3500023-100UG).

The mounted specimens were scanned using an Olympus Fluoview FV-1000 confocal laser
scanning microscope (of K. Worsaae, University of Copenhagen, Denmark), with the acquired z-
stacks of scans being either projected into 2D-images or analyzed three-dimensionally using
IMARIS 7.1 (BITPLANE SCIENTIFIC SOFTWARE, Zürich, Switzerland). This software package was also used to conduct the measurements presented in the following text according to the conventions introduced by Hummon et al. 1992 [55], i.e. position in the body is given in units (U) as a relative measurements to total body length, measured from anterior to posterior. Schematic hand drawings and plate setup were done with Adobe Illustrator CS6 and image adjustments conducted in Adobe Photoshop CS6.

**Results**

**Phylogeny**

The tree (Fig. 1) shows a fully supported sister group relationship (100% posterior probability (PP)) between the monophyletic Muselliferidae (100% PP) and Xenotrichulidae (100% PP), herein together called “group A”. Within Muselliferidae the genus *Musellifer* (100% PP) (represented only by *Musellifer delamarei* (Renaud-Mornant, 1968) [56] and *Musellifer reichardti* Kanneby, Atherton & Hochberg, 2014) [26]) is found to be sister group to *Diuronotus aspetos*. Group A is found next to “group B”, together constituting the Paucitubulatina (100% PP), with group B showing a monophyletic Dasydytidae (100% PP) and Neogosseidae (100% PP) nested within “Chaetonotidae“, the latter hereby becoming paraphyletic. Supports are high in all nodes of the tree, except for some of the numerous inner nodes of group B.

**Musculature**
The body wall musculature consists of several pairs of longitudinal muscles, numerous dorso-ventral muscles, a thin helicoidal musculature, semi-circular and complete circular muscles, as well as pharyngeal musculature. The pharyngeal musculature is especially dense and has an organization typical of chaetonotid gastrotrichs, as described below in more detail (Figs. 2 and 3).

**Radial muscles**

The pharynx, sensu stricto, is formed by three rows of very dense radial pharyngeal muscles (rpm, Figs. 2D,E,M and 3C,D), and extend to U26 (units are calculated as length from anterior end, relative to total length, see material and methods). The radial muscles are cross-striated and each of them presents three to six Z-discs, which are less numerous anteriorly. The myoepithelial nuclei of the pharynx have a distinctive folded and elongated shape (mn, Fig. 2M). The pattern and repartition of these nuclei seems to be specific and corresponding nuclei could be found in the same position in different specimens.

**Helicoidal muscles**

Hericoidal muscles (hm, Figs. 2N and 3A,B,D,E) are very thin (0.5-1.2μm) and limited to the anterior half of the specimen. It is difficult to confirm the presence of the helicoidal muscles most around the pharynx due to the strong signal of other pharyngeal muscles (dashed lines in Fig. 3C-D). In few locations along the midline of the pharynx very faint diagonal fibers were observed, suggesting that the helicoidal musculature is actually present along the entire pharyngeal region. Distinct helicoidal muscles are found extending from the midgut/pharynx junction at U26 until U42, enveloping the dorsal longitudinal muscle but not the ventral and ventro-lateral longitudinal muscles.
Longitudinal musculature

Three longitudinal muscles span the entire body length: a pair of ventral longitudinal muscles (vlm, Figs. 2 and 3), a pair of ventro-lateral longitudinal muscles (vllm, Figs. 2 and 3), and a pair of dorsal longitudinal muscles (dlm, Figs. 2 and 3). The ventral longitudinal muscle bundle splits several times in a pattern described below for the different body regions.

Pharyngeal region

Several longitudinal muscles are present along the pharynx. Some are limited to the pharyngeal region, while others are the continuity of the body longitudinal muscles mentioned above. Two sets of muscles are strictly limited to the pharyngeal region: a pair of lateral and a pair of dorsal muscles. The lateral pharyngeal longitudinal muscle (lplm, Figs. 2D,E and 3C,D) extends adjacent to the pharynx along its entire length. The pharyngeal dorsal longitudinal muscles (pdlm, Figs. 2D,E,M,O and 3B-D) extend close to the pharyngeal midline along its total length, ventral to the dorsal longitudinal muscles. Moreover, several somatic and splanchnic longitudinal muscles supply the pharyngeal region.

The paired ventral longitudinal muscle (vlm, Figs. 2 and 3) originating in the head, splits along the pharynx into a complex pattern (see Fig. 3B). One of its branches extends more laterally and splits into several sub-branches, supplying the lateral sides of the head.

The paired ventro-lateral longitudinal muscle (vllm, Figs. 2 and 3) lines the pharynx until reaching the head, where it bifurcates at U7, one branch extending ventro-laterally and the other dorso-laterally. Each of them subsequently splits into several minor branches, supplying the lateral sides of the head. These muscles together with the antero-lateral branch of the ventral longitudinal
muscle (vlm), and the head diagonal muscle (hdm, see below) all supply the antero-lateral part of
the head, and besides anchoring the longitudinal muscles for overall body contraction, they may
function separately in contraction of the head (Figs. 2J and 3A,C).

The paired dorsal longitudinal muscle (pdlm) spans the anterior-most extremity of the pharynx.

Trunk region

Three main longitudinal muscles, i.e. ventral, lateral and dorsal, are supplying the trunk. The paths
of the lateral and dorsal longitudinal muscles are relatively straight throughout the body.

However, just posterior to the pharynx, the dorsal longitudinal muscle lines the intestine (Figs. 2F
and 3C,D,E), while it runs closer to the dorsal body wall more posteriorly (Figs. 2G,H and 3F,G). The
ventral longitudinal muscle splits into three muscle bundles at the anterior trunk. Two of these
branches run in parallel mid-ventrally along the trunk (lvlm, mvlm, Figs. 2A,C,F-H,P and 3A,B,F-H),
whereas the third branch extends dorso-laterally and supplies the dorso-lateral sides of the body
until meeting the median-most branch at U86 (dvlm, Figs. 2A,G,L and 3A,B,F).

Posterior region

The median branch of the longitudinal ventral muscle splits posteriorly into two bundles at U86.
One very short (8μm) portion supplies medially the posterior part of the adhesive gland of the
posterior tube, while another longer branch extends into the primary tube, supplying it for
approximately two thirds of its length to U97 (Fig. 3A). Additionally, the lateral branches of the
ventral and lateral longitudinal muscles also extend along the primary tube. The dorsal
longitudinal muscle supplies the anterior third of the secondary tube.

Diagonal muscles
The head diagonal muscle (hdm, Figs. 2J,P and 3A,B,C) forms a V-shape with two medially joined branches. The median part of the muscle is situated in the midline of the body in the posterior region of the head while the two extremities extend to the antero-lateral region of the head.

At the dorso-posterior pharynx, a pair of pharyngeal dorsal diagonal muscles decussate (pddm, Figs. 2P and 3B,D), ventral to the pharyngeal dorsal longitudinal muscle and the dorsal longitudinal muscle. Though their orientation is similar to the helicoidal muscles, their exclusively dorsal extension and their greater width differ significantly from the helicoidal muscles.

Three diagonal muscles are found in the furca, extending from one side to the contralateral one. The two anterior muscles (tmd, Figs. 2A,B,H,I,K and 3A,B,G,H) extend from the midline at U86, halfway to the primary and the secondary tube, respectively. The posterior diagonal muscle (pdm, Figs. 2I,K, and 3A,B,H) extends from U89 laterally into the first third of the secondary tube.

Circular muscles

Pharyngeal circular muscles (pcm 2E,M,O,P and 3A,B,C,D) are present around the pharynx. These muscles are numerous (ca. 110 in one specimen), positioned proximal to each other, and 1-1.50μm thick with increasing diameter towards the posterior region.

Two sphincters are present at each extremity of the pharynx: one anterior pharyngeal sphincter (aps, Figs. 2B,O,P and 3A,B) located just posterior to the mouth, and one posterior pharyngeal sphincter (pps, Figs. 2M,O,P and 3A,B) marking the transition between the intestine and the pharynx. The anterior sphincter is smaller, being 1μm thick and has a diameter of 17μm, while the posterior sphincter is more prominent with 8μm thickness and a diameter of 23μm.
Supplementary circular muscles of the adhesive glands (cmag, Figs. 2H,I,K and 3A,B,G,H) are present in the tubes, forming a muscular layer around the large adhesive glands (ag, Fig. 2K). They thereby supply two cavities - one for each tube. The muscular layer surrounding the primary tube is smaller than the one surrounding the secondary tube, and both structures are connected. This suggests that both tubes are supplied by a single set of glands controlled by muscles. Three adjacent nuclei are found within the layer of circular muscles at the base of the gland (agn, Fig. 2K), near the anus, around U88.

Semi-circular muscles

Ventrally opened semicircular muscles (scm, Figs. 2L and 3A,B,F,G) are present in the posterior part of the specimen, but do not extend into the tubes. They originate ventrally, from each side of the body, and extend to the dorsal side. From there, they project to the contralateral side, external to the longitudinal musculature. They are more numerous in the posterior region, anterior to the furca, where they are separated by 2-5 μm. Semicircular muscles are less numerous and spaced further apart (5-8 μm) in the anterior region of the ovary. They seem to only supply the ovary region: their contraction probably reducing the body diameter and may be involved in the movement/release of eggs.

Dorso-ventral muscles

Numerous thin muscles (1-2 μm in diameter) traverse the entire trunk dorso-ventrally (dvm, Figs. 2A-C,E-H,K,L,N and 3A,B,E-G). These dorso-ventral muscles are spaced approximately 5 μm apart in the region between U18 and U95. In this region, two pairs are found laterally in transverse sections of the pharyngeal region (one external and one more internal pair, dvm, Figs. 2D,E and 3A,B). This number increases more posteriorly in the trunk, where up to five pairs of dorso-ventral
muscles can be detected (dvm, Figs. 2G,K,L, and 3A,B,F). The dorso-ventral muscles extend dorso-ventrally between the different longitudinal muscles and the ciliary bands in various combinations. However, they are never found external to the ventro-lateral longitudinal muscles or between the pair of dorsal longitudinal muscles.

### Nervous system

The nervous system of *Diuronotus aspetos* is described from acetylated α-tubulin-like immunoreactivity (LIR, Figs. 4, 5, 6), serotonin-LIR (Fig. 7) and FMRF-amide-LIR (Fig. 8) (all different LIR of the head region are summarized in Fig. 9). Similar to previously investigated Gastrotricha, the nervous system consists of paired nerve cords, which originate from a bilobed dorsal brain and extend posteriorly. In the following section, previously described and undescribed structures are detailed, such as: i) multiple pairs of longitudinal nerve projections in the head (danp, dlpn, hln, Figs. 6A,B,E,J and 9A,B), ii) paired anterior ventro-median nerves (avmn, Fig. 6A,D and 9B), iii) dorsal nerves posterior to the brain (hdpn, Figs. 6A,H and 9A), iv) paired ventro-lateral nerve cords (vlnc, Figs. 6B-D,F,G,K and 9B), v) paired posterior nerves, projecting into the adhesive tubes (nppt, Fig. 6K), vi) bilobed, dorsal brain with three commissures (a main neuropil and an anterior and dorsal commissure, together forming a nerve ring encircling the pharynx) (np and anr, Figs. 6A-C,E,H-J and 9A,B), vii) two pairs of ganglia along the nerve cord: one anterior and one terminal (pgg and ang, Figs. 6B,F,K and 9B), viii) four ventral trunk commissures (spc, tvc, pac and pco, Figs. 6B,D,G,K and 9B), ix) a pharyngeal nervous system, consisting of three longitudinal nerves (one per pharyngeal row of radial muscles) and supplementary minor nerves (Figs. 4 and 5). Additionally, the serotonin-LIR and FMRF-amide-LIR gave very detailed results, allowing us to collect precise data on the number, position and connection of perikarya (Figs. 7, 8 and 9).
Acetylated α-tubulin-like immunoreactivity (acetylated α-tubulin-LIR)

Acetylated α-tubulin-LIR provides information on most neurites, cilia as well as other portions of cytoskeletons of the cells. However, not all minor neurites of the nervous system are traced and the description focuses on the central nervous system and sensory structures.

Stomatogastric nervous system

The stomatogastric nervous system, confined to the pharynx, consists mainly of three main longitudinal nerves: a dorsal (dpn, Figs. 4A,D-H,J-N and 5A,B,E) and two ventro-lateral nerves (vpn, Figs. 4B,F-H,L-N and 5E), extending basally along the midline of each row of radial muscles (Fig. 4).

The nerves are closely related to three structures: kinocilia, anterior pharyngeal glands and a pharyngeal canal system. The dorsal pharyngeal nerve (dpn, Figs. 4A,D-H,K-O and 5A,B,E) originates at the mouth, where it supplies a buccal nerve ring (bnr, Figs. 4A,B and 5A), encircling the mouth (probably innervating the anterior sphincter (aps, Figs. 2B,O,P and 3A,B) opening and closing the mouth). At U4, two anterior diagonal pharyngeal nerves (adpn Figs. 4A and 5A) originate from the dorsal nerve, extend antero-laterally to the anterior edges of the pharynx and medially join back the dorsal nerve. At U3, one pharyngeal dorso-ventral nerve (pdvn, Figs. 4A,B and 5A) originates from each of the anterior diagonal nerve, and supply ventrally a pharyngeal gland longitudinal nerve (plgn, Figs. 4B and 5C) innervating an anterior pharyngeal gland (apg, Figs. 4A,C,I and 5C) (which opens into the mouth). On the right side of the specimen, a dorso-anterior pharyngeal canal nerve (dpcn, Figs. 4A,D,J and 5A) extends posteriorly from the anterior diagonal nerve, possibly supplying the asymmetric dorsal pharyngeal canal (dpc, Figs. 4A,F,G,L,M and 5E).

At U9 a pharyngeal nerve ring (pnr, Figs. 4A,B,E,K and 5B,D) supplying the ventral and dorsal nerves is present. A pair of paramedian dorsal pharyngeal nerves (dpnp, Figs. 4A,F,L and 5E)
originates from the dorsal nerve at U19 and extends in a parallel fashion on each side, to fuse again with the dorsal nerve at U26 (Figs. 4A,G,M and 5E). The dorsal nerve extends more posteriorly where it innervates a two-celled pharyngeal posterior cluster (ppc, Figs. 4A and 5I) at the posterior margin of the pharynx. Two nerves extend the terminal part of the ventro-lateral pharyngeal sections: the lateral gland longitudinal nerve (plgn, Figs. 4A and 5C), and a median kinocilium longitudinal nerve (plkn, Figs. 4B,C and 5G), with the latter supplying a mouth and a pharyngeal kinocilia, respectively, at U1 and U6 (mk and pk, Figs. 4B-D,J,I and 5C,G). The gland and kinocilium nerves originate at U7 from an elongated ventro-lateral pharyngeal ganglion (vlpg, Figs. 4 and 5H,F), consisting of three nuclei and extending from U7 to U12 (probably integrating the signal collected by the two kinocilia and responsible for the putative terminal gland secretion). The ganglion seems to be furthermore related to the ventro-lateral pharyngeal canal (vlpc, Figs. 4A,B,E-G,K-N and 5F,I) described below. The ventral pharyngeal nerves (vpn, Figs. 4B,F,G,L,M and 5D) supplied by perikarya at U15 and U19 extend from the ganglion, until U28.

Due to the unknown nature of the canal system and its main acetylated α-tubulin-LIR (as well as a weak FMRF-amide-LIR), it is described in this nervous system section. It consists of radially flattened cavities, sometimes asymmetrical, extending longitudinally within the pharynx (Figs. 4A,B,E,F,K,L and 5D,E). Six pharyngeal canals extend the pharynx: i) the unpaired right ventro-anterior pharyngeal canal (avrc, Figs. 4B,E,F,K,L and 5D) extending from U6 to U26; ii-iii) The paired ventro-lateral pharyngeal canals (vlpc, Figs. 4A,B,E-H,K-N and 5F,I), extending from U7 (at the level of the pharyngeal ganglia (vlpg, Figs. 4A and 5H,F)) to U30, and merging dorso-posteriorly; iv-v) the paired ventro-posterior pharyngeal canals extending from U23 for the left one (lpvc, Figs. 4B,G,H,M,N and 5D) and from U26 for the right one (rpvc, Figs. 4B,G,H,M,N and 5D) to U28; vi) the dorsal pharyngeal canal (dpc, Figs. 4A,F,G,L,M and 5E) extending along the right side from U6 to
U15, then reaching the midline and bifurcating in two symmetrical branches, following the paramedian dorsal nerves of the pharynx between U19 and U27. Few nuclei are embedded in the pharyngeal canal system (Fig. 4A,B,G).

Central nervous system

The neuropil (np, Figs. 6A,C,E,H,J and 9A) is 14μm thick and its center is positioned at U15. One 3μm broad nerve extends antero-medially from the neuropil and branches laterally to form a dorsal and a ventral commissure at U12 and U9, respectively, together constituting an anterior nerve ring (anr, Figs. 5D, 6A-C,I,J and 9A,B). At the dorsal section of the anterior nerve ring (anr, Fig. 9), the acetylated α-tubulin-LIR is relatively weak, and the commissure consists of two transverse (anterior FMRF-amide-like-immunoreactive (LI-reactive) and posterior serotonin-LI-reactive) nerves, which eventually fuse dorso-laterally, forming the lateral sections of the anterior nerve ring. One anterior and one posterior indistinct longitudinal nerves (cpn, Figs. 6B,I and 9B) extend from the ventral portion of the anterior nerve ring, innervating two median ciliary patches (described below). The neuropil supplies ventrally a pair of anterior ventro-median nerves (avmn, Figs. 6B,D, and 9B) extending between the anterior nerve ring and the post-pharyngeal ganglion posteriorly (pgg, Figs. 6B,F and 9B). It extends parallel to the pharyngeal median ciliated cell (pmcc, Fig. 10B,G), probably innervating it. Two pairs of dorso-median anterior nerves projections (danp, Figs. 6A,J and 9A) and two pairs of dorso-lateral anterior nerve projections (dlnp, Figs. 6A,J and 9A) extend from the anterior nerve ring and the lateral sides of the neuropil, respectively, projecting anteriorly. One pair of head lateral nerves (hln, Figs. 6A,B,E,J and 9A,B) extends from the lateral sides of the neuropil and bifurcates, posteriorly supplying a cell with a large and diffuse nucleus (possibly a gland cell (lgcb, Figs. 6A,E and 9A)), and anteriorly forming a nerve projection.
Each of these anterior nerve projections probably innervates head sensory organs. Dorso-laterally, the posterior sides of the neuropil supply the ventro-lateral nerve cord (vlnc, Figs. 6B,C,D,F,G,J,K and 9B) of *D. aspetos*, which extends along the entire length of the specimen adjacent to the lateral longitudinal ciliary bands and the ventro-lateral longitudinal muscle, probably innervating these two structures. Two head dorso-posterior nerve (hdpn, Figs. 5E, 6A,H and 9A), extending along the pharynx, eventually supply the post-pharyngeal ganglion. They may innervate the anterior portion of the dorsal longitudinal muscle. A pair of head diagonal nerves (hdn, Figs. 6A,H and 9A) originates dorso-laterally of the neuropil, decussate dorsal to the pharynx at U22, and each extend ventro-laterally to a single perikaryon at U23. Comparison across specimens suggests that the position of these diagonal nerves corresponds to the position of the pharyngeal dorsal diagonal muscle (pddm, Figs. 2P and 3B,D), which it probably innervates. At U27 and U29, two thin nerves originate from the anterior ventro-median nerve, and form together at U28 a sub-pharyngeal commissure (spc, Figs. 6B,D and 9B). At U50, anterior to the testis, a thin trunk ventral commissure (tvc, Fig. 6G) is present. At U84, an anal ganglion (ang, Fig. 6K) of six to eight cells supplies a pre-anal commissure (pac, Fig. 6K). Posterior to the anus, at U87, the two ventro-lateral nerve cords form the posterior commissure (pco, Fig. 6C,K), from which two nerve projections of the primary tube (nppt, Fig. 6C,K) extend.

**Serotonin-like immunoreactivity (serotonin-LIR)**

The nervous system shown by serotonin-LIR consists of a dorsal neuropil, the anterior nerve ring, anterior and posterior projections, the two ventro-lateral nerve cords, one posterior commissure as well as several perikarya.
Three main commissures (an anterior (sacn), a median (smcn) and a posterior (spcn)) in the brain neuropil show serotonin-LIR (Fig. 7A,D,E) as well as an isolated patch postero-median to the neuropil (spp, Fig. 7A,D,E). Three longitudinal nerves showing serotonin-LIR are found in the brain: i) the median-most brain nerve (smbn, Fig. 7A,D), ii) the paramedian brain nerve (spbn, Fig. 7A,D), and iii) the lateral brain nerve (slbn, Fig. 7A,D). Four very thin lateral nerves of the posterior commissure of the neuropil (slpn, Fig. 7A,D) form complex connections with the other nerves of the brain as well as to the dorso-median perikaryon (sdmp, Fig. 7A,C,D,E). A postero-lateral nerve node (spln, Fig. 7A,D,E) is present postero-laterally to the neuropil, being formed by the merging of several nerves, and supplies the ventro-lateral nerve cord (slnc, Fig. 7B,C,F). One dorso-lateral perikaryon (sdlp, Fig. 7A,C,D,E) supplies the postero-lateral nerve node, with a short nerve. The median-most brain nerve extends anteriorly from the posterior of the neuropil until U6 (Fig. 7A).

The lateral brain nerve is short and extends from the postero-lateral nerve node, being supplied by some of the commissures of the neuropil (Fig. 7A). The paramedian brain nerve originates from the postero-lateral nerve node and supplies the serotonin-like-LI-reactive anterior nerve ring (sanr, Fig. 7A-D), subsequently extending more posteriorly as an anterior nerve projection until U4 (Fig. 7A,D). The anterior nerve ring consists dorsally of two and ventrally of one serotonin-LI-reactive nerves (sanr, Fig. 7A,B). The ventro-lateral nerve cord, consisting of two serotonergic-LI-reactive neurites, extends ventrally to supply a serotonin-LI-reactive para-pharyngeal cluster (sppc, Figs. 7B-E and 9B) consisting of three perikarya, and extends to the posterior end forming the posterior commissure. Additionally, single serotonin-LI-reactive perikarya of the post-pharyngeal ganglion and of the anal ganglion are present respectively at U33 and U93 (spog, and spag, Fig. 7B,C,F) as well as nerve projections of the primary tube (snpt, Fig. 7C,F).

**FMRF-amide-like immunoreactivity (FMRF-amide-LIR)**
The FMRF-amide-LI-reactive nervous system consists of the brain neuropil, the anterior nerve ring, the anterior ventro-median nerve, the ventro-lateral nerve cord, the sub-pharyngeal commissure and the posterior commissure. Different parts of the nervous system show varying immunoreactivity intensities, as illustrated in Fig. 8.

The neuropil (fnp, Fig. 8A,C,D,F,G) consists of four connectives: two anterior and two posterior, supplied by several posterior and lateral perikarya. Antero-laterally to the neuropil, one pair of perikarya supplies a very short dorso-lateral anterior nerve projections (fpp, Fig. 8A,D,F,G) (corresponding to the base of the acetylated α-tubulin-LI-reactive projections (dlnp; Figs. 6A,J and 9A)). Additionally, three lateral perikarya of the brain (flpb, Fig. 8A,F,G) and a pair of dorso-posterior clusters of the brain (fdpc, Figs. 8A,F,G and 9A) with three perikarya, are present. Comparisons between differently stained specimens, and use of DAPI, enabled us to infer that the postero-median cell of the FMRF-amide-LI-reactive dorso-posterior cluster corresponds to the serotonin-LI-reactive dorso-posterior perikarya (sdmp, Fig.7; fdpc, Figs. 8A and 9A). Anteriorly, the neuropil supplies the anterior ventro-median nerve (fvmn, Figs. 8B,I and 9B), also supplied by two ventro-lateral perikarya of the brain (fvpb, Fig. 8A,E,I) and a ventral perikaryon of the anterior nerve ring (fvpr, Fig. 8B,I). The nerve ring (fanr, Fig. 8A-D,G,H,J) is supplied by one anterior and one posterior unpaired dorso-median perikarya (fpar, Fig. 8A,D,F,H). Ventro-posterior to the neuropil, a pair of tricellular clusters also supply the ventro-median nerve (fvnc, Fig. 8N,I), which extends further posterior until the two FMRF-amide-LI-reactive perikarya of the post-pharyngeal ganglion (fppg, Fig. 8B,C,K). The paired ventro-lateral nerve cords (flnc, Fig. 8B,C,I,K,L) is supplied by the postero-lateral part of the neuropil and by three anterior perikarya (fapn, Fig. 8B,C,E,I) (two anterior and one posterior, separated by 8μm). The FMRF-amide-LI-reactive sub-pharyngeal commissure (fspc, Fig. 8B,I), ventro-lateral nerve cord, posterior commissure (fpco, Fig. 8C,L), and
nerve projections of the primary tube (fnpt, Fig. 8C,L) follow the description of the acetylated α-
tubulin-LIR.

**Ciliation**

The locomotory ciliation consists of a dense ventro-anterior ciliated area and two thin ciliated bands, which are extending to the posterior part of the specimen at U87 (Fig. 10C). This general pattern supports the original description of Todaro et al. [17], although numerous details can be added. CLSM allowed the identification of individual multiciliated cells and determination of their precise pattern.

Dorsally, the muzzle is covered by two transverse rows of multiciliated cells. The anterior row consists of three pairs of relatively small head dorso-anterior ciliated cells (3μm, hacc, Fig. 10A,E,F), while the posterior row is constituted by a pair of larger head postero-lateral dorsal ciliated cells (hpcc, Fig. CA,E) and a head dorso-median ciliated cell (hmcc, Fig. CA,E) of similar size (6μm). The pattern of the head lateral ciliated cells (hlcc, Fig. 10A,F) could not be resolved in details due to the dorso-ventral mounting of the specimen. However, at least four cells at the dorso-lateral level are present, and probably the same number at the ventro-lateral level.

The ventral head bears 20 multiciliated cells organized in four paired longitudinal rows and one median row, containing 2,2,3,2,2,2,3,2,2 cells (Fig. 10B). Posterior to the head, ventral to the pharynx, from U9, only two adjacent rows of multiciliated cells are present on each side, containing one large pharyngeal median ciliated cell (pmcc, Fig. 10B,G, 45μm long) and three pharyngeal lateral ciliated cells (plcc, Fig. 10B,G,H, 20-35μm long), respectively. Posterior to the
pharynx, only one paired lateral row of cells is present, which extends until the posterior trunk, as
described originally [17].

The two ventro-median ciliary patches at U7 and U11 (acp,pcp, Fig. 10B,H,I, position of patches
measured from the center) are innervated by two short diffuse 5μm wide longitudinal nerves (cpn,
Figs. 6B,I and 7B), joining perpendicularly the anterior nerve ring. Each patch also shows an
acetylated α-tubulin-LI-reactive positive ring around the ciliated area. The divergent morphology
of these anucleate multiciliated cells and their close relation to the nervous system suggest that
they could be sensory structures.

Several sensoria are scattered along the body (ss, Fig. 10D,J), and two pairs of pharyngeal sensory
cilia (mk and pk, Figs. 3A-D,I,J, 4C,G and 10I) are located in the pharyngeal region (see the nervous
system section for further details).

Two pairs of nephridia are found along the body (Fig. 10D): the anterior pair is situated ventro-
laterally and the posterior pair is located dorso-laterally, relatively close to the midline. The
anterior pair of protonephridia (apn, Fig. 10D,J) is situated anterior to the testis at U42 and the
cilia are 20μm long. The posterior pair of protonephridia (ppn, Fig. 10D,K) is situated at U74 with
15μm long cilia. Each nephridium seems to possess two straight coaxial cilia (c, c’, Fig. 10J,K),
thereby resembling the general paucitubulatinian protonephridia with two adjacent monociliated
terminal cells, projecting into a non-ciliated canal cell and ending with a nephridiopore epidermal
cell [2, 41]. The canal cell and the nephridiopore cell have not been stained, why we lack
information on the orientation and opening of protonephridia in D. aspetos.

Discussion
Phylogeny

The present phylogenetic analysis confirm that *Diuronotus aspetos* belongs to the monophyletic family Muselliferidae as proposed previously based on morphology [22, 23, 26]. We furthermore find Xenotrichulidae sister group to Muselliferidae (100% support), opposed to its position next to Group B (“Chaetonotidae” + Dasytydae + Neogosseidae) in Kånneby et al. [26] (69% PP). Moreover, the placement of *D. aspetos* considerably reduces the internal branch length of Muselliferidae, diminishing the possibility of long-branch attraction, with e.g. *Neodasys* ([49] and present study) or *Dactylopodola* [26], and the support of group B is now maximum. Two other interesting points can be noted: Furthermore the sister group relationship between Neogosseidae and Dasydytidae is recovered [50], and the sister group relationship of marine *Aspidiophorus* to the remaining members of the Group B is found again, similarly to Kånneby et al. 2012 [49], but not Kånneby and Todaro 2015 [50].

Musculature

The overall musculature of *Diuronotus aspetos* is relatively simple, consisting of only three pairs of longitudinal muscles in the trunk as well as a unique arrangement of multiple dorso-ventral muscles. The three pairs of longitudinal muscles in *D. aspetos* is, despite its generally larger size, less than what is found in most Paucitubulatina, having at least five pairs of longitudinal muscles that are often distributed as three pairs of splanchnic and three pairs of somatic muscles (*Musellifer, Draculiciteria, Heteroxenotrichula, Xenotrichula, Chaetonotus, Aspidiophorus, and Polymerurus*) [22, 31, 32]. The previously proposed hypothetical ancestral state of musculature in Paucitubulatina [2] shows a split of the dorsal longitudinal muscle (musculus dorsalis) into two branches, not present in *D. aspetos* that instead has a branch of the ventral longitudinal muscle
running dorsally. The more complex branching pattern of the ventral longitudinal muscle might be an adaptation to the large size of *D. aspetos*, compensating for the low number of longitudinal muscle.

The helicoidal musculature encircles the dorsal longitudinal muscles but not the ventral longitudinal muscles or the ventro-lateral longitudinal muscles. The relative position of the dorsal longitudinal muscle indicates a homology to the dorsal splanchnic muscle of other Paucitubulatina. However, its more dorsal position indicate that it support the body wall rather than the digestive tract (see Kieneke and Schmidt-Rhaesa (2015) [2] for further discussion and limitations of this notion), perhaps furthermore compensating for the missing dorso-dermal muscle branch in *D. aspetos* (see[2]). The ventro-lateral longitudinal muscle of *D. aspetos* can be homologized with the somatic ventro-lateral muscle (or musculus lateralis) of other Gastrotricha, and the ventral longitudinal muscle resembles those found in the paucitubulatinan *Muselifer delamerei*, *Xenotrichula intermedia* Remane, 1934 [57] and *Heteroxenotrichula squamosa* Wilke, 1954 [58] [22].

The unique semi-circular muscles of *D. aspetos* may aid the oviposition together with the dorso-ventral muscles, hereby functionally replacing the dorso-dermal longitudinal muscle split enveloping the egg in other Paucitubulatina [22]. They likely act as the posterior complete circular muscles found in the region of the sexual organs of *Neodasys cf. uchidai* Remane, 1961 [59, 60]. Functionally similar circular muscles are also found in the meiofauna gnathiferan *Gnathostomula armata* Riedl, 1971 [61] and *Gnathostomula peregrina* Kirsteuer, 1969 [62] (Gnathostomulida), here arranged in dense pattern around the posterior male organs [63, 64].
The evolution of the dorso-ventral muscles of Chaetonotida as deriving from the circular musculature has been one of the central debates in previous studies [22, 31]. In Macrodasyida and Multitubulatina, the circular musculature consists of splanchnic and somatic elements, the former encircling the intestine and the latter, which derives from splanchnic elements, encircles the ventro-lateral longitudinal muscle on both sides [2, 22, 59]. In Paucitubulatina, the trunk circular muscles are either absent, incomplete or derived as dorso-ventral muscles as in Xenotrichulidae and Musellifer with dorso-ventral orientation [2, 22, 31, 65]. Compared to these arrangements, the configuration is unique in D. aspetos with more than two sets of dorso-ventral muscles in the transverse axis. The median-most dorso-ventral muscles can possibly be homologized with the visceral circular muscles in other gastrotrichs, however, the lateral sets of dorso-ventral muscles present various arrangements relative to the longitudinal muscles throughout the body length, making homologies difficult to assess. Furthermore, dorso-ventral muscles do not seem to be present lateral to the ventro-lateral longitudinal muscle, which would be an arrangement expected from a derived somatic semi-circular muscle such as found in other Paucitubulatina [20, 22]. Consequently, solely the inner-most dorso-ventral muscles of D. aspetos can be homologized with semi-circular muscles of other Paucitubulatina.

The head diagonal muscle of D. aspetos may be homologous to the head semi-circular muscle found in Muselifer delamerei and Dactylopodola baltica (Remane, 1926) [22, 66, 67] showing the same anterior position and shape though a different orientation. The posterior diagonal muscle and the diagonal muscle of the tubes resemble a muscle found in the posterior region of Heteroxenotrichula squamosa (figure 3A, [22]), but no similar muscle exist in Musellifer delamerei, Xenotrichula intermedia or X. punctata Wilke, 1954 [22, 58]. The so called cross-over muscles found in Macrodasyida with a bilobed caudal end has a similar function, being involved in the
movement of the posterior tubes, yet with the lack of presence in other Paucitubulatina and different morphology in *D. aspetos* it is most likely of convergent origin [2, 66, 68].

**Nervous system**

To date, *Xenotrichula intermedia* and *Xenotrichula velox* Remane, 1927 [69] are the only other Paucitubulatina for which the nervous system has been studied with CLSM [13], therefore the present study adds valuable information. On the other hand, the nervous system of *Neodasys* (Multitubulatina was described in details with CLSM) [12, 39], as well as several Macrodasyida [15, 35, 36, 39, 70, 71]. Furthermore *Cephalodasys maximus* Remane, 1926 [67] and *Turbanella cornuta* Remane, 1925 [72] have been described in detail with TEM [37, 73]. This offers a broad, but not comprehensive, bibliographic material to compare the nervous system of *D. aspetos* with other Gastrotricha.

**Stomatogastric nervous system**

Similar to other Chaetonotida [12, 13, 18], one dorso-median and two ventro-lateral longitudinal nerves constitute the overall pharyngeal nervous system of *Diuronotus aspetos*. However, the present study finds several additional structures previously undescribed for chaetonotids such as:

i) five additional symmetric and one asymmetric longitudinal nerves branching off from the main nerves, ii) two previously undescribed commissures (anterior-most buccal nerve ring, middle pharyngeal nerve ring), and iii) a pair of ventro-lateral pharyngeal ganglia (innervating anterior sensory structures).

However, only the pharyngeal nervous system of *Cephalodasys maximus* has been comprehensively described [37] and little is known about the pharyngeal nervous system of
Chaetonotida (but see [12, 13]). Nonetheless, ultrastructural studies by Teuchert (1877) [73] and Ruppert (1982) [18] provide various details of the pharynx in several gastrotrichs, including some details on *Diuronotus* sp.

In Macrodasyida the inverted organization of the pharynx generally offers one ventro-median and two dorso-lateral nerves as well as one additional dorso-median nerve [18]. In *Turbanella cornuta*, an additional asymmetric “thick” ventro-lateral nerve is also present in the pharynx [73], which resembles the one short asymmetric dorso-lateral longitudinal nerve found in *D. aspetos* to a certain degree. *Cephalodasys maximus* presents a pair of ventro-lateral asymmetric nerves (one short, one long) in the pharynx, but they originate more posteriorly from the pharyngeal nerve ring [37]. The different origin contradicts a homology with the asymmetric nerve of *D. aspetos* but show that asymmetry in the pharynx of gastrotrichs might be a frequent phenomenon since these three gastrotrichs are morphologically and phylogenetically diverse (e.g. [15, 29]).

The present study show the presence of two pairs of pharyngeal kinocilia (versus one from TEM observations by Ruppert [18] in *D. aspetos*, absent in other Paucitubulatina, including *Musellifer* [18]. Macrodasyida and *Neodasys* possess multiple triplets of pharyngeal cilia [18], which suggests that they have been dramatically reduced in number in Paucitubulatina, and a single short pair may have either re-appeared in *Diuronotus* or alternatively be overlooked in previous studies on several Paucitubulatina. Ruppert [18] also discusses the presence of discrete glands opening in the mouth of *Chaetonotus* and *Musellifer*, possibly homologous to the anterior pharyngeal glands here described for *D. aspetos*.

Herein is further revealed a presently undescribed pharyngeal canal system within the musculature, occasionally lined by nerves. However, a single transverse TEM micrograph of
Diuronotus sp. by Ruppert (fig. 14, [18]) - proposedly from the level of the ventro-lateral pharyngeal ganglion - reveals a dorso-lateral as well as three ventro-lateral electron-lucent areas, which most likely resemble the canal system. The system may be unique to Diuronotus or Muselliferidae, and its function is unknown.

Central nervous system

The overall morphology of the nervous system of Diuronotus aspetos is similar to other gastrotrichs [2] consisting of a “dumbbell-shaped” dorsal brain with a dorsal neuropil and a pair of ventro-lateral nerves. However, additional nerves and specific perikarya are found in D. aspetos.

Longitudinal nerves

Anterior to the brain neuropil of Diuronotus aspetos, four pairs of dorsal nerve projections are found (acetylated α-tubulin-LI-reactive, in addition to several minor neurites left undescribed), most likely related to the anterior sensoria. Similar nerve projections are described in Neodasys chaetonotoideus Remane, 1927 [12, 74], Cephalodasys maximus [37] and Thaidasys tongiorgii Todaro, Dal Zotto & Leasi, 2015 [15] but due to the scarcity of these descriptions, a closer homology cannot yet be stated. Another two pairs of nerves project antero-ventrally from the brain (serotonin-LI-reactive) in D. aspetos, one of which may be homologous to the commonly found single pair of serotonin-LI-reactive ventral projections in other gastrotrichs (e.g. Neodasys chaetonotoideus, Dactylopodola or Oregodasys cirratus [12, 36, 38]. A similar positioned pair of FMRF-amide-LI-reactive projections is present in Lepidodasys worsae Hochberg and Atherton, 2011 [70] and Xenotrichula [13, 70], and in Oregodasys cirratus these are expressing both FMRF-amide-LIR and serotonin-LIR [38], suggesting that the neurotransmitters of these nerves can vary, and that these nerves are a general character of Gastrotricha (cf. nervous system drawing in [2]).
Another striking character found in *D. aspetos* is the paired anterior ventro-median nerve in the anterior trunk. Short, paired anterior ventro-median nerves are also found in *Thaidasys tongiorgii*, *Turbanella cf. hyalina* Schultze, 1853 [75], and extending the entire body length in *Oregodasys cirratus* [15, 38, 39]. However, the exact connection to other nerves and their extension differs from those of *D. aspetos*. Moreover, studies of the closely related *Neodasys chaetonotoideus* and *Xenotrichula* [12, 13] did not find similar paired anterior ventro-median nerves and we therefore consider the ventro-median nerves in *D. aspetos* a convergence related to the different ciliation of this species.

The paired short dorso-lateral nerves in *D. aspetos* (hdpn, Figs. 6A,H and 9A) are similar in position and extension to the paired dorsal nerves described in the distantly related *C. maximus* [37] as well as the dorsal pharyngeal fibers found in the closely related *Xenotrichula* [13], of which the latter at least seems to be homologous to the dorsal nerves of *D. aspetos*.

**Ganglia and perikarya**

Several immunoreactive perikarya can be compared to other gastrotrichs, mostly *Neodasys* and *Xenotrichula*. However, immunoreactivity of the perikarya is quite variable, and only a fraction of the brain cells are immunoreactive.

Only five pairs of serotonin-LI-reactive perikarya are found in the brain of *Diuronotus aspetos*, situated postero-laterally to the neuropil. They comprise two dorsal pairs of perikarya, supplying the neuropil, and a ventral pair of para-pharyngeal clusters (spgg, Figs. 7B-E and 9B) with three perikarya each, supplying the ventro-lateral nerve cords. The closely related *Xenotrichula* does not possess a serotonin-LI-reactive equivalent to the ventral clusters, but possesses four dorsal pairs of serotonin-LI-reactive perikarya [13], two of which are likely homologous to the two dorsal pairs...
Neodasys chaetonotoideus possesses three dorso-lateral serotonin-LI-reactive perikarya, which have similar positions and connection to the neuropil than the dorsal serotonin-LI-reactive perikarya of D. aspetos. Moreover, N. chaetonotoideus possesses a similar paired cluster of para-pharyngeal serotonin-LI-reactive perikarya, associated to the ventro-lateral nerve cords. This suggests that N. chaetonotoideus and D. aspetos might share some plesiomorphic traits of their serotonin-LI-reactive nervous system, whereas Xenotrichula represents a derived condition. In the so far investigated Macrodasyida, the serotonin-LI-reactive brain is generally simpler than in Chaetonotida, comprising only one dorsal commissure and one pair of dorso-lateral perikarya [15, 38, 71] (sometimes two [76]), although additional serotonin-LI-reactive perikarya can be found in Dactylopodola [36], and Paradasys subterraneus Remane, 1934 [57].

The FMRF-amide-LI-reactive perikarya of the brain of D. aspetos are numerous (at least 16 paired and two unpaired of various intensity of the immunoreactivity) and surround the brain neuropil dorsally, ventrally and laterally. Due to the high number and variation of FMRF-amide-LIR of the brain in Gastrotricha, we limit our comparison of D. aspetos to the closely related Xenotrichula [13]. Homologies of the perikarya depend on whether the anterior dorsal and ventral FMRF-amide-LI-reactive commissures in Xenotrichula are homologous to the anterior nerve ring of D. aspetos. If so, the two dorso-median FMRF-amide-LI-reactive perikarya found connected to the anterior dorsal commissure in Xenotrichula, may be homologous to the two dorso-median found in D. aspetos. The additional two described paired lateral and ventral FMRF-amide-LI-reactive perikarya in Xenotrichula are difficult to homologize with those of D. aspetos. However, one pair of undescribed ventral FMRF-amide-LI-reactive perikarya is found laterally on the ventral commissure of Xenotrichula (fig. 4H, [13]) and is possibly homologous to the ventral perikarya of the FMRF-amide-LI-reactive nerve ring in D. aspetos. Finally, one of the cells of the FMRF-amide-LI-
reactive dorso-posterior cluster of the brain of *D. aspetos* may be homologous to the single pair of perikarya found in *Xenotrichula* in the same position.

In a position similar to the post-pharyngeal ganglion of *D. aspetos*, two pairs of FMRF-amide-LI-reactive (no serotonin-LIR) perikarya are described supplying the ventro-lateral nerve cord in *Xenotrichula* [13]. Between these two pairs, two short transverse FMRF-amide-LI-reactive neurites almost constitute a commissure similar to the one of *D. aspetos*, suggesting that the posterior-most pair of perikarya in *Xenotrichula* is homologous to the ganglia found in *D. aspetos*.

An anal pair of serotonin-LI-reactive perikarya contained in the anal ganglion is found in *D. aspetos* as well as a posterior commissure, similar to what is described for *Xenotrichula* and *Neodasys chaetonotoideus* [12, 13]. Yet, no equivalent is found in any Macrodasyida. Herein observations show that the anal ganglion consists of several cells, contrary to other Chaetonotida [77]. Moreover, we describe an additional pre-anal commissure, originating at the anal ganglion, only revealed by acetylated α-tubulin-LIR and hitherto not found in other gastrotrichs.

**Brain commissures**

*Diuronotus aspetos* does show a commissure situated directly ventrally to the main brain neuropil contrary to most Gastrotricha documented, including Chaetonotida (e.g. [12, 13, 15, 39]). This character was central in previous discussions on a possible close relationship between Cycloneuralia and Gastrotricha (e.g. [36, 39, 78, 79]), rejected today (e.g. [3, 80]) since, recent interpretations of the brain of Gastrotricha show that it is not truly circular [80]. In *D. aspetos*, the anterior nerve ring is associated to the brain and its ventral portion resembles the ventral brain commissure of other Gastrotricha, although being more anterior. Furthermore, *Xenotrichula* possesses one ventral FMRF-amide-LI-reactive commissure anterior of the brain [13]. If the FMRF-
amide-LI-reactive anterior commissure of the brain and ventral commissure of *Xenotrichula* are continuous, it can be speculated that *Xenotrichula* also possesses an anterior nerve ring.

**Ventral ciliation**

The main difference from the original description is that the head ventral ciliation forms two medially separated ciliated areas in *Diuronotus aspetos*. Furthermore, a more detailed pattern has been deduced, showing the relevance of CLSM for determining ciliary arrangement [81-83] (but also Kerbl et al., in prep; Bekkouche and Worsaae, in prep, respectively on Dinophilidae (Annelida) and Micrognathozoa). This also opens the way to a new kind of characters in interstitial animals, which could have a systematics value: the pattern of the multi-ciliated cells. Indeed, preliminary results showing variation in the pattern of the ventral multi-ciliated cells of Thaumastodermatidae support this idea (Bekkouche and Worsaae unpublished). Unfortunately, though the description of the general pattern of the ventral ciliation is common in Chaetonotida, details are rare. In few cases, more details were given, for instance for Neogosseidae (with exact description of the ciliary bands [84]). A few studies described ciliary patches in the head of some Chaetonotidae (e.g. [85, 86]), but no exact information about the cells themselves is given, why it is unknown if each patch or band is constituted by one or several cells. This limitation of light and electron microscopy can be overcome by employment of CLSM, but due to the lack of similar studies, we cannot yet comment on the evolution of the fine detailed ciliation pattern of Gastrotricha.

Interestingly, some Paucitubulatina show unpaired ciliary patches on the ventral midline of the head, e.g. *Halichaetonotus atlanticus*, Kisielewski, 1988 [85], *Arenotus strixinoi* Kisielewski, 1987 [86] or *Kijanebalola devestiva* Todaro, Perissinotto & Bownes, 2013 [84], but details are lacking to draw hypothesize any homology with the ventro-median ciliary patches of *D. aspetos*. 
Protonephridial system

Until the present study, all three previously studied Paucitubulatina were known to possess only one pair of protonephridia (Xenotrichula carolinensis stylensis Mock, 1979 [87], Chaetonotus maximus Ehrenberg, 1831 [41, 88] and Polymerurus nodicaudus (Voigt, 1901) [42, 89]. In this context, Diuronotus aspetos is the only Paucitubulatina known to have more than one pair of protonephridia. However, studies on the protonephridial system of Musellifer are needed to confirm if the presence of a single pair of protonephridia has a phylogenetic value or is a due to size dependency. Indeed, the number of pairs of protonephridia in other Gastrotricha is variable and seems to be roughly size dependent (e.g. two pairs for the ca. 250μm long Dactylopodola baltica, and 11 pairs for the ca 1mm long Mesodasys laticaudatus Remane, 1951 [90]) [91].

Conclusion

The present study is the first detailed anatomical description of a member of Muselliferidae, and only the second description of the nervous system within the larger clade Paucitubulitina [13]. The key phylogenetic position of Diuronotus, the surprising new discoveries of the nervous, muscular and ciliary system and several plausible homologies of these structures may be of significant importance for understanding the evolution of organ systems within Gastrotricha. However, as the present study showed, it is necessary to establish the position of Neodasys (as possible sister group to Paucitubulatina), in order to fully trace the evolution of organs systems within Paucitubulatina.

The musculature of D. aspetos presents unique traits for Paucitubulatina such as the reduction of the number of longitudinal muscles, compensated by the splitting of the ventral longitudinal
muscle, or the addition of dorso-ventral muscles in the transversal axis. This, in addition to many
unique minor muscles (e.g. circular muscles of the adhesive glands, pharyngeal diagonal dorsal
muscle) explains why the musculature of \textit{D. aspetos} is difficult to compare to with the previously
studied Gastrotricha. However, the musculature has been shown to be phylogenetically
informative in Paucitubulatina [22] and future studies of additional species may aid to the
evolutionary reconstruction of the \textit{D. aspetos} musculature.

Although the nervous system of \textit{D. aspetos} is in overall similar to other gastrotrichs, it presents
some additional traits such as a pair of anterior ventro-median nerves, the dorso-posterior nerves,
and supplementary commissures, such as the pre-anal commissure. Two ganglia are described
here as well, comprising an anal ganglion, and consisting of several cells in contrast to findings in
other Chaetonotida [77]. These characters, as well as several other (e.g. details of the anterior
nerve ring and immunoreactive perikarya) widen the diversity of nervous system traits in
Gastrotricha, showing that i) many seemingly minor nervous system components are still to be
described in Gastrotricha, and that ii) the nervous system of \textit{D. aspetos} is comparable to, e.g.,
\textit{Xenotrichula}.

Otherwise often overlooked organ systems were described here, such as the pharynx revealing so
far undescribed nerves, a unique system of canals, and the only finding of pharyngeal cilia in
Paucitubulatina (briefly mentioned in Ruppert 1982 [18]). Additionally, investigation of the ventral
ciliation with CLSM reveals detailed of the cellular arrangement refining the previous description
[17]. These findings will hopefully prove to be of potential systematic value within Gastrotricha.

One of the major restrictions of this study was of course the limited number of previously
conducted detailed morphological studies on Gastrotricha, but future investigation on the
morphology of Paucitubulatina as *Musellifer, Draculiciteria*, marine *Aspidiophorus* and freshwater “Chaetonotidae” would vastly improve this picture.

**Declarations**

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We have no competing interests.

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**Authors’ contributions**

NB and KW conceptualized and designed the study, collected the animals, analyzed the data, and wrote the manuscript. NB gathered the immunohistochemical data and made the illustrations.

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**References**


50. Tracer v1.6, Available from http://beast.bio.ed.ac.uk/Tracer


54. Tracer v1.6, Available from http://beast.bio.ed.ac.uk/Tracer


Müller MCM, Sterrer W: Musculature and nervous system of Gnathostomula peregrina (Gnathostomulida) shown by phallolidin labeling, immunohistochemistry, and CLSM, and their phylogenetic significance. Zoomorphology (Berlin) 2004, 123(3):169-177.


**Figure legends**

**Figure 1:** Phylogenetic position of *Diuronotus aspetos* inferred from Bayesian analysis of 18S, 28S, and COI. The analysis includes 58 taxa representing all available genera of Chaetonotida for molecular data on NCBI, and three Macrodasyida as outgroups. Numbers at the nodes represent posterior probabilities in percentages. The picture on the lower left corner is a light micrograph of a live specimen of *Diuronotus aspetos*.

**Figure 2:** CLSM of phalloidin stained muscle of *Diuronotus aspetos*. Anterior of the specimen is pointing left for A,B) and J-P), and dorsal is pointing at the top for D-I). A-N) Muscles in green, nuclei in cyan A) Ventral view of the maximum intensity projection (MIP) of the whole specimen. B) Dorsal MIP of the pharynx. C) Dorsal MIP of the posterior specimen. D- I) CLSM virtual transverse section of various part of the specimen: D) head, E) posterior part of the pharynx, F) anterior of the trunk, G) posterior of the trunk, H) post-anal region of the trunk, I) and furca before bifurcation of the tubes. J) Dorsal MIP of a sub-stack showing details on the head musculature. K)
Dorsal MIP of a sub-stack showing details of the furca separation. L) Ventral MIP of a sub-stack showing details of the semicircular musculature. M) Single section showing details of the inner pharynx. N) Dorsal MIP of a substack showing details of the helicoidal musculature. O) And P), isosurface reconstruction of the pharynx. O) Dorsal view, P) ventral view. ag, adhesive gland; agn, adhesive gland nucleus; aps, anterior pharyngeal sphincter; cmag, circular muscle of the adhesive gland; dlm, dorsal longitudinal muscle; dvlm, dorsal projection of the ventral longitudinal muscle; dvm, dorso-ventral muscle; hdm, head diagonal muscle; hm, helicoidal muscles; lplm, lateral pharyngeal longitudinal muscle; lvlm, Lateral extension of the ventral longitudinal muscle; mn, myocyte nuclei; mwlm, medial projection of the ventral longitudinal muscle; pcm, pharyngeal circular muscle; pddm, pharyngeal dorsal diagonal muscle; pdm, posterior diagonal muscle; pdlm, pharyngeal dorsal longitudinal muscle; ph, pharynx; pps, posterior pharyngeal sphincter; pt, primary tube; rpm, radial pharyngeal muscles; scm, semi-circular muscle; st, secondary tube; tdm, tube diagonal muscle; vlm, ventral longitudinal muscle; vllm, ventro-lateral longitudinal muscle; vlm, ventral longitudinal muscle.

Figure 3: Schematic drawings of the musculature of *Diuronotus aspetos*. Anterior is pointing at the top for A) and B), dorsal is pointing at the top for C-H). A) Ventral view of the musculature, B) dorsal view of the musculature, C-H) cross section of the specimen C) in the head, D) posterior part of the pharynx, E) anterior of the trunk, F) posterior of the trunk, G) post-anal region of the trunk, H) and in the furca before bifurcation of the tubes. Note that in C) and D), the helicoidal pharyngeal musculature is represented in dash lines due to the uncertainty of its presence, and it is not drawn in A) and B). aps, anterior pharyngeal sphincter; cmag, circular muscle of the adhesive gland; dlm, dorsal longitudinal muscle; dvlm, dorsal projection of the ventral longitudinal muscle; dvm, dorso-ventral muscle; hdm, head diagonal muscle; hm, helicoidal muscle; int:
intestine; \textit{lplm}, lateral pharyngeal longitudinal muscle; \textit{lvlm}, Lateral extention of the ventral longitudinal muscle; \textit{mvlm}, medial projection of the ventral longitudinal muscle; \textit{ov}, ovary; \textit{pcm}, pharyngeal circular muscle; \textit{pdm}, posterior diagonal muscle; \textit{pddm}, pharyngeal dorsal diagonal muscle; \textit{pdlm}, pharyngeal dorsal longitudinal muscle; \textit{pps}, posterior pharyngeal sphincter; \textit{rpm}, radial pharyngeal muscles; \textit{scm}, semi-circular muscle; \textit{tdm}, tube diagonal muscle; \textit{vllm}, ventro-lateral longitudinal muscle; \textit{vlm}, ventral longitudinal muscle.

**Figure 4:** Pharyngeal nervous system and canal system of \textit{Diuronotus aspetos}. A,B) Anterior is pointing at the top; C-N dorsal is pointing at the top. A-H) Schematic drawings with nerves in blue and pharyngeal system in yellow, nuclei in grey, glands in green and cilia in red. A) Dorsal section of the pharynx. B) Ventral section of the pharynx. C-H) Successive transverse sections of the pharynx from anterior to posterior. I-N) CLSM virtual transverse sections at the same levels as C-H). Acetylated α-tubulin-LIR in glow and DAPI in cyan. \textit{adpn}, anterior diagonal pharyngeal nerve; \textit{apg}, anterior pharyngeal gland; \textit{avrc}, anterior ventro-median right pharyngeal canal; \textit{bnr}, buccal nerve ring; \textit{dpc}, dorsal pharyngeal canal; \textit{dpcn}, dorso-anterior pharyngeal canal nerve; \textit{dpn}, dorsal pharyngeal nerve; \textit{lpvc}, left posterior ventro-median canal; \textit{mk}, mouth kinocilium; \textit{pdvn}, pharyngeal dorso-ventral nerve; \textit{pk}, posterior pharyngeal kinocilium; \textit{plgn}, pharyngeal longitudinal gland nerve; \textit{plkn}, pharyngeal longitudinal kinocilium nerve; \textit{pmdn}, paramedian dorsal pharyngeal nerves; \textit{pnr}, pharyngeal nerve ring; \textit{ppc}, posterior pharyngeal cluster; \textit{rpvc}, right posterior ventro-median canal; \textit{vlpc}, ventro-lateral pharyngeal canal; \textit{vlpg}, ventro-lateral pharyngeal ganglion; \textit{vpn}, ventral pharyngeal nerve.

**Figure 5:** CLSM of the pharyngeal nervous system and canal system of \textit{Diuronotus aspetos}. A-C,G,I) anterior is pointing at the top. D-F,H) anterior pointing left. CLSM maximum intensity
projection of sub-stacks. Acetylated α-tubulin-LIR in glow, DAPI in cyan. A) Dorso-anterior section of the pharynx. B) Dorso-anterior section of the pharynx, more ventral than B). C) Ventro-anterior section of the pharynx. D) Ventro-posterior section of the pharynx. E) Dorso-posterior section of the pharynx. F) Medio-posterior portion of the pharynx. G) Medio-anterior section of the pharynx. H) Details of the ventro-lateral pharyngeal ganglion. I) Details of the posterior pharyngeal ganglion. adpn, anterior diagonal pharyngeal nerve; anr, anterior nerve ring; apg, anterior pharyngeal gland; avrc, anterior ventro-median right pharyngeal canal; bnr, buccal nerve ring; dpc, dorsal pharyngeal canal; dpcn, dorso-anterior pharyngeal canal nerve; dpn, dorsal pharyngeal nerve; hdpn, head dorso-posterior nerve; lpvc, left posterior ventro-median canal; mk, mouth kinocilium; np, neuropile; pdvn, pharyngeal dorso-ventral nerve; pk, posterior pharyngeal kinocilium; plgn, pharyngeal longitudinal gland nerve; plkn, pharyngeal longitudinal kinocilium nerve; pmdn, paramedian dorsal pharyngeal nerves; pnr, pharyngeal nerve ring; ppc, posterior pharyngeal cluster; rpvc, right posterior ventro-median canal; ss, sensoria; vlpg, ventro-lateral pharyngeal ganglion; vlpc, ventro-lateral pharyngeal canal; vpn, ventral pharyngeal nerve.

Figure 6: Drawing and CLSM of the acetylated α-tubulin-LIR nervous system of Diuronotus aspetos. Anterior pointing left for A-I), and pointing at the top for J) and K). A, B) Schematic drawings of the α-tubulin-LIR of the anterior part of the specimen: nerves in blue, nuclei in grey, and opposite ventral or dorsal nervous system in light grey A) dorsal B) ventral. C) CLSM ventral view of the maximum intensity projection (MIP) of the entire specimen. D-K) CLSM MIP sub-stacks of various parts of the specimen. Acetylated α-tubulin-LIR in glow, and DAPI in cyan in all CLSM pictures. D) Ventro-anterior nervous system. E) Neuropil side F) Ventral, post pharyngeal ganglion. G) Ventral, trunk commissure. H) Dorso-posterior part of the head I) ventro-anterior part of the head J) Dorso-anterior part of the head. K) Ventro posterior terminal part of the specimen. ang,
anal ganglion; anr, anterior nerve ring; avmn, anterior ventro-median nerve; br, brain; cpn, ciliated patch nerves; danp, dorso-median anterior nerve projection; dlnp, dorso-lateral anterior nerve projections; hdpn, head dorso-posterior nerve; hdn, head diagonal nerve; hln, head lateral nerve; lgcb, lateral gland cell of the brain; np, neuropile; nppt, nerve projection of the primary tube; pac, pre-anal commissure; pco, posterior commissure; pgg, post-pharyngeal ganglion; ph, pharynx; spc, sub-pharyngeal commissure; tt, testis; tvc, trunk ventral commissure; vlnc, ventro-lateral nerve cord.

Figure 7: serotonin-LIR nervous system of *Diuronotus aspetos*. The anterior is pointing left for all figures. A, B) Schematic drawings of the serotonin-LIR of the anterior part of the specimen: nerves and perikarya in green, nuclei in grey, and opposite ventral or dorsal nervous system in light grey. A) Dorsal view, B) ventral view. C-F) CLSM images with serotonin-LIR in glow. C) CLSM maximum intensity projection (MIP) of the entire specimen. D) Dorsal MIP of the brain E) CLSM sub-stack MPI showing details of the brain perikarya F) CLSM sub-stack MPI of the ventro-posterior terminal part of the specimen. br, brain; ph, pharynx; sacn, serotonin-LI-reactive anterior commissure of the neuropil; sanr, serotonin-LI-reactive anterior nerve ring; sdlp, serotonin-LI-reactive dorso-lateral perikaryon; sdmp, serotonin-LI-reactive dorso-median perikaryon; slbn, serotonin-LI-reactive lateral brain nerve; slnc, serotonin-LI-reactive ventro-lateral nerve cord; spln, serotonin-LI-reactive lateral nerves of the posterior commissure of the neuropil; spln, serotonin-LI-reactive postero-lateral nerve node; smbnp, serotonin-LI-reactive median-most brain nerve; smcn, serotonin-LI-reactive median commissure of the neuropil; snp, serotonin-LI-reactive neuropil; snpt, serotonin-LI-reactive nerve projection of the primary tube; spag, serotonin-LI-reactive perikarya of the anal ganglion; spbn, serotonin-LI-reactive paramedian brain nerve; spcn, serotonin-LI-reactive posterior commissure of the neuropil; spco, serotonin-LI-reactive posterior
Figure 8: FMRF-amide-LIR nervous system of *Diuronotus aspetos*. Anterior is pointing left for A-G) and I-L) and dorsal pointing at the top for H). A, B) Schematic drawings of the FMRF-amide-LIR of the anterior part of the specimen: nerves in magenta, nuclei in grey, and opposite ventral or dorsal nervous system in light grey. A) Dorsal view, B) ventral view. C) CLSM dorsal view of the maximum intensity projection (MIP) of the entire specimen. D-L) (Except H) CLSM sub-stack MIP of various parts of the specimen. FMRF-amide-LIR in glow, and DAPI in cyan in all CLSM pictures. D) Dorsal view of the whole neuropil. E) Ventral part of the brain. F) And G) different levels of the dorsal part of the neuropil. H) CLSM virtual transverse section of the anterior nerve ring. I) Ventro-anterior part of the head. J) Ventral commissure of the anterior nerve ring. K) Ventral post-pharyngeal ganglia. L) ventro-posterior terminal part of the specimen. Anterior of the specimen on the left for A-G) and I-L) and dorsal on top for H). br, brain; egg, egg; fanr, FMRF-amide-LI-reactive anterior nerve ring; fapn, FMRF-amide-LI-reactive anterior perikarya of the ventro-lateral nerve cord; fdpc, FMRF-amide-LI-reactive dorso-posterior cluster of the brain; flnc, FMRF-amide-LI-reactive ventro-lateral nerve cord; flpb, FMRF-amide-LI-reactive lateral perikarya of the brain; fnp, FMRF-amide-LI-reactive neuropil; fnpt, FMRF-amide-LI-reactive nerve projection of the primary tube; fpar, FMRF-amide-LI-reactive dorso-median perikarya of the anterior nerve ring; fpco, FMRF-amide-LI-reactive posterior commissure; fpp, FMRF-amide-LI-reactive perikarya of the dorso-lateral anterior nerve projections; fppg, FMRF-amide-LI-reactive post-pharyngeal ganglion; fspc, FMRF-amide-LI-reactive sub-pharyngeal commissure; fvmn, FMRF-amide-LI-reactive anterior ventro-median nerve; fvnc, FMRF-amide-LI-reactive anterior ventro-median nerve cluster; fvpb, FMRF-amide-LI-reactive neuropil patch; sspg, serotonin-LI-reactive para-pharyngeal cluster.
ventro-lateral perikarya of the brain; **fvpr**, FMRF-amide-LI-reactive ventral perikarya of the anterior nerve ring; **ph**, pharynx.

**Figure 9:** Schematic drawing of acetylated α-tubulin-LIR, FMRF-amide-LIR and serotonin-LIR nervous system of *Diuronotus aspetos*, showing the correspondences between the different nervous systems. Anterior pointing at the top. Acetylated α-tubulin-LIR nervous system in blue, FMRF-amide-LIR nervous in green, and serotonin-LIR nervous system in green. Cell nuclei in grey and opposite nervous system in light grey. Legends in bold indicate structures showing-LIR for at least two molecules tested. **A)** Dorsal, and **B)** ventral. **anr**, anterior nerve ring; **avmn**, anterior ventro median nerve cord; **br**, brain; **cpn**, ciliated patch nerves; **danp**, dorso-median anterior nerve projection; **dlnp**, dorso-lateral anterior nerve projections; **fdpc**, FMRF-amide-LI-reactive postero-lateral brain cluster; **fvnc**, FMRF-amide-LI-reactive ventro-median nerve cluster; **hdn**, head diagonal nerve; **hln**, head lateral nerve; **hdpn**, head dorso-posterior nerve; **hpdn**, head diagonal nerve; **np**, neuropile; **pgg**, post-pharyngeal ganglion; **ph**, pharynx; **spc**, sub-pharyngeal commissure; **sppc**, serotonin-LI-reactive para-pharyngeal cluster; **vlnc**, ventro-lateral nerve cord.

**Figure 10:** Ciliation of *Diuronotus aspetos*. Anterior pointing at the top for all figures. **A** and **B** drawings of the locomotory ciliation: **A)** dorsal view, **B)** ventral view. **C-K** CLSM maximum intensity projection (MIP) sub-stacks of the acetylated α-tubulin-LIR. **C)** Ventral view of the whole specimen showing the organization of the locomotory ciliation. **D)** Dorsal view of the whole specimen showing parts of the locomotory ciliation and the position of the protonephridia. **E)** And **F)**, dorsal head ciliation. **E)** Is more dorsal than **F)**. **G-I)** Ventral head and pharyngeal ciliation: **G)** is more dorsal than **H)** which is more dorsal than **I)**. **J)** And **K)** details of, respectively, the anterior and the posterior pairs of protonephridia. **acp**, anterior ciliated patch; **apn**, anterior proto-
nephridia; **br**, brain; **c, c’**, cilia of the proto-nephridia; **hacc**, head dorso-anterior ciliated cells; **hlc**, head lateral ciliation; **hlcc**, head lateral ciliated cells; **hmcc**, head dorso-median ciliated cell; **hpcc**, head postero-lateral dorsal ciliated cell; **hvc**, head ventral ciliation; **hvlm**, head ventral lateral-most row of ciliated cells; **hvmm**, head ventral median-most row of ciliated cells; **hvpl**, head ventral para-lateral row of ciliated cells; **hvpm**, head ventral paramedian row of ciliated cells; **mz**, muzzle; **pc**, pharyngeal ciliation; **pcp**, posterior ciliated patch; **ph**, pharynx; **pk**, posterior pharyngeal kinocilium; **plcc**, pharyngeal lateral ciliated cells; **pmcc**, pharyngeal median ciliated cell; **ppn**, posterior proto-nephridia; **ss**, sensoria; **tc**, trunk ciliation; **tcc**, trunk ciliated cells; **tt**, testis.

**Figure abbreviations**

**acp**, anterior ciliated patch;  
**adpn**, anterior diagonal pharyngeal nerve;  
**ag**, adhesive gland;  
**agn**, adhesive gland nucleus;  
**ang**, anal ganglion;  
**anr**, anterior nerve ring;  
**apg**, anterior pharyngeal gland;  
**apn**, anterior proto-nephridia;  
**aps**, anterior pharyngeal sphincter;  
**avmn**, anterior ventro-median nerve;  
**avrc**, anterior ventro-median right pharyngeal canal;  
**bnr**, buccal nerve ring;  
**br**, brain;  
**c, c’**, cilia of the proto-nephridia;  
**cmag**, circular muscle of the adhesive gland;  
**cpn**, ciliated patch nerves;
**danp**, dorso-median anterior nerve projection;

**dlm**: dorsal longitudinal muscle;

**dlnp**, dorso-lateral anterior nerve projections;

**dpc**, dorsal pharyngeal canal;

**dpcn**, dorso-anterior pharyngeal canal nerve;

**dpn**, dorsal pharyngeal nerve;

**dvlm**, dorsal projection of the ventral longitudinal muscle;

**dvm**, dorso-ventral muscle;

**egg**, egg;

**fanr**, FMRF-amide-LI-reactive anterior nerve ring;

**fapn**, FMRF-amide-LI-reactive anterior perikarya of the ventro-lateral nerve cord;

**fdpc**, FMRF-amide-LI-reactive dorso-posterior cluster of the brain;

**flnc**, FMRF-amide-LI-reactive ventro-lateral nerve cord;

**flpb**, FMRF-amide-LI-reactive lateral perikarya of the brain;

**fnp**, FMRF-amide-LI-reactive neuropil;

**fnpt**, FMRF-amide-LI-reactive nerve projection of the primary tube;

**fpar**, FMRF-amide-LI-reactive dorso-median perikarya of the anterior nerve ring;

**fpco**, FMRF-amide-LI-reactive posterior commissure;

**fpp**, FMRF-amide-LI-reactive perikarya of the dorso-lateral anterior nerve projections;

**fppg**, FMRF-amide-LI-reactive post-pharyngeal ganglion;

**fspc**, FMRF-amide-LI-reactive sub-pharyngeal commissure;

**fvmn**, FMRF-amide-LI-reactive anterior ventro-median nerve;

**fvnc**, FMRF-amide-LI-reactive anterior ventro-median nerve cluster;

**fvpb**, FMRF-amide-LI-reactive ventro-lateral perikarya of the brain;

**fvpr**, FMRF-amide-LI-reactive ventral perikarya of the anterior nerve ring;

**hacc**, head dorso-anterior ciliated cells;

**hdm**, head diagonal muscle;
pcm, pharyngeal circular muscle;

pco, posterior commissure;

pcp, posterior ciliated patch;

pddm, pharyngeal dorsal diagonal muscle;

pdm, posterior diagonal muscle;

pdlm, pharyngeal dorsal longitudinal muscle;

pdvn, pharyngeal dorso-ventral nerve;

pgg, post-pharyngeal ganglion;

ph, pharynx;

pk, posterior pharyngeal kinocilium;

plcc, pharyngeal lateral ciliated cells;

plgn, pharyngeal longitudinal gland nerve;

plkn, pharyngeal longitudinal kinocilium nerve;

pmcc, pharyngeal median ciliated cell;

pmdn, paramedian dorsal pharyngeal nerves;

pnr, pharyngeal nerve ring;

ppc, posterior pharyngeal cluster;

ppn, posterior proto-nephridia;

pps, posterior pharyngeal sphincter;

pt, primary tube;

rpm, radial pharyngeal muscles;

rpvc, right posterior ventro-median canal;

sacn, serotonin-LI-reactive anterior commissure of the neuropil;

sanr, serotonin-LI-reactive anterior nerve ring;

scm, semi-circular muscle;

sdlp, serotonin-LI-reactive dorso-lateral perikaryon;

sdmp, serotonin-LI-reactive dorso-median perikaryon;
slbn, serotonin-LI-reactive lateral brain nerve;
slnc, serotonin-LI-reactive ventro-lateral nerve cord;
spc, sub-pharyngeal commissure;
slpn, serotonin-LI-reactive lateral nerves of the posterior commissure of the neuropil;
spag, serotonin-LI-reactive perikarya of the anal ganglion;
smbn, serotonin-LI-reactive median-most brain nerve;
smcn, serotonin-LI-reactive median commissure of the neuropil;
snpt, serotonin-LI-reactive nerve projection of the primary tube;
sppg, serotonin-LI-reactive para-pharyngeal cluster;
ss, sensoria;
st: secondary tube;
tc, trunk ciliation;
tcc, trunk ciliated cells;
tdm: tube diagonal muscle;
tt, testis;
tvc, trunk ventral commissure;
vlm: ventro-lateral longitudinal muscle;
vlm, ventral longitudinal muscle;
vipc, ventro-lateral pharyngeal canal;
vlpq, ventro-lateral pharyngeal ganglion;
vpn, ventral pharyngeal nerve;

Table 1: sequences used for the phylogenetic reconstruction

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Figure 1: Phylogenetic position of *Diuronotus aspetos* inferred from Bayesian analysis of 18S, 28S, and COI. The analysis includes 58 taxa representing all available genera of Chaetonotida for molecular data on NCBI, and three Macrodasyida as outgroups. Numbers at the nodes represent posterior probabilities in percentages. The picture on the lower left corner is a light micrograph of a live specimen of *Diuronotus aspetos*.
**Figure 2: CLSM of phalloidin stained muscle of *Diuronotus aspetos*.** Anterior of the specimen is pointing left for A,B) and J-P), and dorsal is pointing at the top for D-I). A-N) Muscles in green, nuclei in cyan A) Ventral view of the maximum intensity projection (MIP) of the whole specimen. B) Dorsal MIP of the pharynx. C) Dorsal MIP of the posterior specimen. D- I) CLSM virtual transverse section of various part of the specimen: D) head, E) posterior part of the pharynx, F) anterior of the trunk, G) posterior of the trunk, H) post-anal region of the trunk, I) and furca before bifurcation of the tubes. J) Dorsal MIP of a sub-stack showing details on the head musculature. K) Dorsal MIP of a sub-stack showing details of the furca separation. L) Ventral MIP of a sub-stack showing details of the semicircular musculature. M) Single section showing details of the inner pharynx. N) Dorsal MIP of a substack showing details of the helicoidal musculature. O And P), isosurface reconstruction of the pharynx. O) Dorsal view, P) ventral view. ag, adhesive gland; agn, adhesive gland nucleus; aps, anterior pharyngeal sphincter; cmag, circular muscle of the adhesive gland; dlm, dorsal longitudinal muscle; dvlm, dorsal projection of the ventral longitudinal muscle; dvm, dorso-ventral muscle; hdm, head diagonal muscle; hm, helicoidal muscles; lplm, lateral pharyngeal longitudinal muscle; lvlm, Lateral extension of the ventral longitudinal muscle; mn, myocyte nuclei; mvlm, medial projection of the ventral longitudinal muscle; pcm, pharyngeal circular muscle; pdadm, pharyngeal dorsal diagonal muscle; pdm, posterior diagonal muscle; pdlm, pharyngeal dorsal longitudinal muscle; ph, pharynx; pps, posterior pharyngeal sphincter; pt, primary tube; rpm, radial pharyngeal muscles; scm, semi-circular muscle; st, secondary tube; tdm, tube diagonal muscle; vlm, ventral longitudinal muscle; vllm, ventro-lateral longitudinal muscle; vlm, ventral longitudinal muscle.
Figure 2
Figure 3: Schematic drawings of the musculature of Diuronotus aspetos. Anterior is pointing at the top for A) and B), dorsal is pointing at the top for C-H). A) Ventral view of the musculature, B) dorsal view of the musculature, C-H) cross section of the specimen C) in the head, D) posterior part of the pharynx, E) anterior of the trunk, F) posterior of the trunk, G) post-anal region of the trunk, H) and in the furca before bifurcation of the tubes. Note that in C) and D), the helicoidal pharyngeal musculature is represented in dash lines due to the uncertainty of its presence, and it is not drawn in A) and B). aps, anterior pharyngeal sphincter; cmag, circular muscle of the adhesive gland; dlm, dorsal longitudinal muscle; dvlm, dorsal projection of the ventral longitudinal muscle; dvm, dorso-ventral muscle; hdm, head diagonal muscle; hm, helicoidal muscle; int: intestine; lplm, lateral pharyngeal longitudinal muscle; lvlm, Lateral extention of the ventral longitudinal muscle; mvlm, medial projection of the ventral longitudinal muscle; ov, ovary; pcm, pharyngeal circular muscle; pdm, posterior diagonal muscle; pddm, pharyngeal dorsal diagonal muscle; pdlm, pharyngeal dorsal longitudinal muscle; pps, posterior pharyngeal sphincter; rpm, radial pharyngeal muscles; scm, semi-circular muscle; tdm, tube diagonal muscle; vllm, ventro lateral longitudinal muscle; vlm, ventral longitudinal muscle.
**Figure 4:** Pharyngeal nervous system and canal system of *Diuronotus aspetos*.  
A,B) Anterior is pointing at the top; C-N dorsal is pointing at the top. A-H) Schematic drawings with nerves in blue and pharyngeal system in yellow, nuclei in grey, glands in green and cilia in red. A) Dorsal section of the pharynx. B) Ventral section of the pharynx. C-H) Successive transverse sections of the pharynx from anterior to posterior. I-N) CLSM virtual transverse sections at the same levels as C-H). Acetylated α-tubulin-LIR in glow and DAPI in cyan.  
adpn, anterior diagonal pharyngeal nerve;  
apg, anterior pharyngeal gland;  
avrc, anterior ventro-median right pharyngeal canal;  
bnr, buccal nerve ring;  
dpc, dorsal pharyngeal canal;  
dpcn, dorso-anterior pharyngeal canal nerve;  
dpn, dorsal pharyngeal nerve;  
lpvc, left posterior ventro-median canal;  
mk, mouth kinocilium;  
plgn, pharyngeal longitudinal gland nerve;  
plkn, pharyngeal longitudinal kinocilium nerve;  
pmdn, paramedian dorsal pharyngeal nerves;  
pnr, pharyngeal nerve ring;  
ppc, posterior pharyngeal cluster;  
rpvc, right posterior ventro-median canal;  
vlpc, ventro-lateral pharyngeal canal;  
vlpg, ventro-lateral pharyngeal ganglion;  
vpn, ventral pharyngeal nerve.
Figure 5: CLSM of the pharyngeal nervous system and canal system of *Diuronotus aspetos*. A-C,G,I) anterior is pointing at the top. D-F,H) anterior pointing left. CLSM maximum intensity projection of sub-stacks. Acetylated α-tubulin-LIR in glow, DAPI in cyan. A) Dorso-anterior section of the pharynx. B) Dorso-anterior section of the pharynx, more ventral than B). C) Ventro-anterior section of the pharynx. D) Ventro-posterior section of the pharynx. E) Dorso-posterior section of the pharynx. F) Medio-posterior portion of the pharynx. G) Medio-anterior section of the pharynx. H) Details of the ventro-lateral pharyngeal ganglion. I) Details of the posterior pharyngeal ganglion. adpn, anterior diagonal pharyngeal nerve; anr, anterior nerve ring; apg, anterior pharyngeal gland; avrc, anterior ventro-median right pharyngeal canal; bnr, buccal nerve ring; dpc, dorsal pharyngeal canal; dpcn, dorso-anterior pharyngeal canal nerve; dpn, dorsal pharyngeal nerve; hdpn, head dorso-posterior nerve; lpvc, left posterior ventro-median canal; mk, mouth kinocilium; np, neuropile; pdvn, pharyngeal dorso-ventral nerve; pk, posterior pharyngeal kinocilium; plgn, pharyngeal longitudinal gland nerve; plkn, pharyngeal longitudinal kinocilium nerve; pmdn, paramedian dorsal pharyngeal nerves; pnr, pharyngeal nerve ring; ppc, posterior pharyngeal cluster; rpvc, right posterior ventro-median canal; ss, sensoria; vlpg, ventro-lateral pharyngeal ganglion; vlpc, ventro-lateral pharyngeal canal; vpn, ventral pharyngeal nerve.
Figure 6: Drawing and CLSM of the acetylated α-tubulin-LIR nervous system of *Diuronotus aspetos*. Anterior pointing left for A-I), and pointing at the top for J) and K). A, B) Schematic drawings of the α-tubulin-LIR of the anterior part of the specimen: nerves in blue, nuclei in grey, and opposite ventral or dorsal nervous system in light grey A) dorsal B) ventral. C) CLSM ventral view of the maximum intensity projection (MIP) of the entire specimen. D-K) CLSM MIP sub-stacks of various parts of the specimen. Acetylated α-tubulin-LIR in glow, and DAPI in cyan in all CLSM pictures. D) Ventro-anterior nervous system. E) Neuropil side F) Ventral, post pharyngeal ganglion. G) Ventral, trunk commissure. H) Dorso-posterior part of the head I) ventro-anterior part of the head J) Dorso-anterior part of the head. K) Ventro posterior terminal part of the specimen. ang, anal ganglion; anr, anterior nerve ring; avmn, anterior ventro-median nerve; br, brain; cpn, ciliated patch nerves; danp, dorso-median anterior nerve projection; dlnp, dorso-lateral anterior nerve projections; hdpn, head dorso-posterior nerve; hdn, head diagonal nerve; hln, head lateral nerve; lgcb, lateral gland cell of the brain; np, neuropile; nppt, nerve projection of the primary tube; pac, pre-anal commissure; pco, posterior commissure; pgg, post-pharyngeal ganglion; ph, pharynx; spc, sub-pharyngeal commissure; tt, testis; tvc, trunk ventral commissure; vinc, ventro-lateral nerve cord.
Figure 7: serotonin-LIR nervous system of *Diuronotus aspetos*. The anterior is pointing left for all figures. A, B) Schematic drawings of the serotonin-LIR of the anterior part of the specimen: nerves and perikarya in green, nuclei in grey, and opposite ventral or dorsal nervous system in light grey. A) Dorsal view, B) ventral view. C-F) CLSM images with serotonin-LIR in glow. C) CLSM maximum intensity projection (MIP) of the entire specimen. D) Dorsal MIP of the brain E) CLSM sub-stack MPI showing details of the brain perikarya F) CLSM sub-stack MPI of the ventro-posterior terminal part of the specimen. br, brain; ph, pharynx; sacn, serotonin-LI-reactive anterior commissure of the neuropil; sanr, serotonin-LI-reactive anterior nerve ring; sdlp, serotonin-LI-reactive dorso-lateral perikaryon; sdmp, serotonin-LI-reactive dorso-median perikaryon; slbn, serotonin-LI-reactive lateral brain nerve; slnc, serotonin-LI-reactive ventro-lateral nerve cord; slpn, serotonin-LI-reactive lateral nerves of the posterior commissure of the neuropil; spln, serotonin-LI-reactive postero-lateral nerve node; smbn, serotonin-LI-reactive median-most brain nerve; smcn, serotonin-LI-reactive median commissure of the neuropil; snp, serotonin-LI-reactive neuropil; snpt, serotonin-LI-reactive nerve projection of the primary tube; spag, serotonin-LI-reactive perikarya of the anal ganglion; spbn, serotonin-LI-reactive paramedian brain nerve; spcn, serotonin-LI-reactive posterior commissure of the neuropil; spco, serotonin-LI-reactive posterior commissure; spog, serotonin-LI-reactive perikarya of the post-pharyngeal ganglion; spp, serotonin-LI-reactive neuropil patch; sppg, serotonin-LI-reactive para-pharyngeal cluster.
Figure 8: FMRF-amide-LIR nervous system of Diuronotus aspetos. Anterior is pointing left for A-G) and I-L) and dorsal pointing at the top for H). A, B) Schematic drawings of the FMRF-amide-LIR of the anterior part of the specimen: nerves in magenta, nuclei in grey, and opposite ventral or dorsal nervous system in light grey. A) Dorsal view, B) ventral view. C) CLSM dorsal view of the maximum intensity projection (MIP) of the entire specimen. D-L) (Except H) CLSM sub-stack MIP of various parts of the specimen. FMRF-amide-LIR in glow, and DAPI in cyan in all CLSM pictures. D) Dorsal view of the whole neuropil. E) Ventral part of the brain. F) And G) different levels of the dorsal part of the neuropil. H) CLSM virtual transverse section of the anterior nerve ring. I) Ventro-anterior part of the head. J) Ventral commissure of the anterior nerve ring. K) Ventral post-pharyngeal ganglia. L) ventro-posterior terminal part of the specimen. Anterior of the specimen on the left for A-G) and I-L) and dorsal on top for H). br, brain; egg, egg; fanr, FMRF-amide-LI-reactive anterior nerve ring; fapn, FMRF-amide-LI-reactive anterior perikarya of the ventro-lateral nerve cord; fdpc, FMRF-amide-LI-reactive dorso-posterior cluster of the brain; flnc, FMRF-amide-LI-reactive ventro-lateral nerve cord; flpb, FMRF-amide-LI-reactive lateral perikarya of the brain; fnp, FMRF-amide-LI-reactive neuropil; fnpt, FMRF-amide-LI-reactive nerve projection of the primary tube; fpar, FMRF-amide-LI-reactive dorso-median perikarya of the anterior nerve ring; fpco, FMRF-amide-LI-reactive posterior commissure; fpp, FMRF-amide-LI-reactive perikarya of the dorso-lateral anterior nerve projections; fppg, FMRF-amide-LI-reactive post-pharyngeal ganglion; fspc, FMRF-amide-LI-reactive sub-pharyngeal commissure; fvmn, FMRF-amide-LI-reactive anterior ventro-median nerve; fnvc, FMRF-amide-LI-reactive anterior ventro-median nerve cluster; fvpb, FMRF-amide-LI-reactive ventro-lateral perikarya of the brain; fvp, FMRF-amide-LI-reactive ventral perikarya of the anterior nerve ring; ph, pharynx.
Figure 9

A

B

danp
dlnp
br
cpn
anr
dlnp
npbn
hln
np
fvnc
fdpc
sppc
hdn
avmn
hdpn
spc
ph
vlnc
pgg
vlnc
**Figure 10: Ciliation of Diuronotus aspetos.** Anterior pointing at the top for all figures. **A** and **B**) drawings of the locomotory ciliation: **A**) dorsal view, **B**) ventral view. **C-K**) CLSM maximum intensity projection (MIP) sub-stacks of the acetylated α-tubulin-LIR. **C**) Ventral view of the whole specimen showing the organization of the locomotory ciliation. **D**) Dorsal view of the whole specimen showing parts of the locomotory ciliation and the position of the protonephridia. **E**) And **F**) dorsal head ciliation. **E**) Is more dorsal than **F**). **G-I**) Ventral head and pharyngeal ciliation: **G**) is more dorsal than **H**) which is more dorsal than **I**). **J**) And **K**) details of, respectively, the anterior and the posterior pairs of protonephridia. **acp**, anterior ciliated patch; **apn**, anterior proto-nephridia; **br**, brain; **c, c’**, cilia of the proto-nephridia; **hacc**, head dorso-anterior ciliated cells; **hlc**, head lateral ciliation; **hlcc**, head lateral ciliated cells; **hmcc**, head dorso-median ciliated cell; **hpcc**, head postero-lateral dorsal ciliated cell; **hvc**, head ventral ciliation; **hvmm**, head ventral median-most row of ciliated cells; **hvml**, head ventral para-lateral row of ciliated cells; **hvpm**, head ventral paramedian row of ciliated cells; **mz**, muzzle; **pc**, pharyngeal ciliation; **pcp**, posterior ciliated patch; **ph**, pharynx; **pk**, posterior pharyngeal kinocilium; **plcc**, pharyngeal lateral ciliated cells; **pmcc**, pharyngeal median ciliated cell; **ppn**, posterior proto-nephridia; **ss**, sensoria; **tc**, trunk ciliation; **tcc**, trunk ciliated cells; **tt**, testis.
Figure 10