Ultrastructure, Formation and Evolution of Chaetae in Annelids

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for my parents

L. Doğan Tılıç and Helga Rittersberger-Tılıç

and my little brother Toprak

"He smiles a lot. But I think there might be worms inside him making him smile."

— **Stephen King** [The Strand]

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Summary

Annelid systematics has been quite unstable over the past century. Especially, with the introduction of molecular techniques to unravel phylogenetic relationships, previously established morphology-based phylogenetic trees were completely changed. This challenges earlier ideas on homology and autapomorphies. Annelid worms bear chaetae. These are chitinous extracellular structures that have an incredible structural diversity, but show significant constancy within species and supraspecific taxa. Each chaeta of an annelid is formed within an ectodermal invagination, and the modulation of the apical microvilli pattern of the basalmost cell of this invagination determines the structure of the chaeta. This process appears to be conservative enough that certain chaetae – once evolved – can be passed on to descendants and, thus, become characteristic for ceratain taxa. Therefore, structural and developmental uniformity or discrepancy of chaetae should be carrying a significant phylogenetic signal and can be used to test homology hypotheses.

This thesis presents a series of studies that employ chaetal characters such as chaetogenesis and chaetal arrangement to test homology hypotheses and help us understand how annelid chaetae, which are critically important in the systematics of the group, evolved.

One of the main aspects of this work is the evolution of hooked chaetae in annelids. These chaetae resemble tiny anchors and are mainly associated with tube-borrowing sedentary worms. A series of previously published papers in fact utilized the comparative study of chaetogeneses as a basis to establish a sound hypothesis on the homology of this type of chaeta and proposed a common ancestry of hook bearing taxa. In light of recent molecular phylogenies this

assumption no longer holds true. In order to understand and explain the structural diversity and unity amongst the hooked chaetae of annelids the existing information on chaetal formation of hooked chaetae is extended and further taxa were included to provide a complete picture of the diversity; now covering all the major branches of the annelid tree. The formation of hooded hooks in errant eunicid species *Lumbrineris tetraura* and *Lumbrineris (Scoletoma) fragilis* differs significantly from the superficially similar looking hooded hooks of Capitellidae and Spionidae. This indicates an independent evolution of the hooded hooks, which is consistent with inferences based on phylogenetic analyses. The study on the chaetation and the formation of hooked chaetae in maldanids, revealed striking similarities to their well established sister-taxon Arenicolidae and supports a homology of hooks at least for these sister-groups. In another study the chaetae of the sabellariid *Sabellaria alveolata* were investigated. The results of this investigation showed remarkable differences in chaetal formation to any other hooked chaetae studied so far, arguing against their homology and demonstrating that hooked chaetae formation is not as uniform as previously assumed.

Chaetal arrangement and the position of the chaetal formative site is utilized to test the homology of chaetae in Echiura to the chaetae in remaining annelids. Echiuran worms have an extremely derived morphology and their position within annelids is established only recently. The study of chaetation in *Thalassema thalassemum* and *Echiurus echiurus* revealed that a ventral pair of chaetae evolved in the stem lineage of Echiura by transforming neuropodial cappillary chaetae and shows that the hemi-circles of anal chaetae evolved once within Echiura as a derived condition.

Furthermore, a more systematic approach was employed in another study to test hypotheses on the evoloution and radiation of chaetal types within a monophyletic taxon (Eunicida). The general hypothesis based on ancestral state reconstructions is that early

annelids only possessed simple capillary chaetae. The results of the study on eunicid chaetae shows that chaetae diversify within a monophyletic taxon but also provides explanations for cases in which chaetation or chaetal diversity is secondarily lost.

The series of studies and results presented herein show that chaetal structure and development, together with their topological arrangement provide a valuable set of characters that can be used as an instrument for testing homology hypotheses. Contrary to common belief that morphology is a rather descriptive discipline, this thesis demonstrates that morphology can be hypothesis driven. Especially when the interpretation of morphological characters can be corroborated with well-supported and robust phylogenies a better understanding of structural diversity and evolution can be achieved.

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Introduction

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1.1 Annelid phylogeny

Annelida, commonly reffered to as segmented worms or ringed worms, is a large phylum comprising over 21 000 recognized species (Weigert et al. 2014). An annelids body can be subdived into three regions; (1) a presegmental "head region" consisting of the prostomium and peristomium, (2) the bulk of the body consisting of serially repated segments and (3) the pygidium, the posterior end of the animal. Each segment consists of two pairs of chaetae, two coelomic cavities, one pair of nephridia, and one pair of nerve centers (ganglia). Out of this basic bauplan a remarkable diversity of body forms can be constructed (Rouse and Pleijel 2001) (Fig. 1.1). Annelid worms can be found in a huge variety of ecological niches worldwide, ranging from deep-sea sediments and hydrothermal vents to the soil in our gardens. They play a central role in all benthic and soil ecosystems. Some species, like the medicinal leech (*Hirudo medicinalis* Linnaeus, 1758) (Fig. 1.1A) or the Samoan delicacy the Palolo worm (*Palola viridis* Gray, 1840) even play a significant role in human society. Despite their ecological relevance our knowledge on the evolution and adaptation of this fascinating taxon is limited.

Annelids are morphologically extreamly divers (Fig. 1.1). With the advent of molecular approaches morphologically even more divergent taxa are being included into Annelida. Echiura (Fig. 1.1H), Sipuncula (Fig 1.1F), Pogonophora and Vestimentifera are currently well supported as annelid subtaxa (Kojima et al. 1993; Bartolomaeus 1995; McHugh 1997; Struck et al. 2007; Dunn et al. 2008; Hejnol et al. 2009; Dordel et al. 2010; Struck et al. 2011; Golombek et al. 2013; Kvist and Siddall 2013; Weigert et al. 2014). Further taxa like Myzostomida and Diurodilida are being placed within annelids with more and more

support (Bleidorn et al. 2007; Bleidorn et al. 2009; Hartmann et al. 2012; Helm et al. 2012; Golombek et al. 2013; Laumer et al. 2015). The inclusion of these groups, indicates a much higher evolutionary variability and do not conform the traditional synapomorphies of annelids, i.e. segmentation and chitinous chaetae (Rouse and Pleijel 2001).

Figure 1.1 Annelid diversity **A** *Lysidice punctata* Grube, 1855 (Eunicida) **B** *Marphysa bellii* (Audouin & Milne Edwards, 1833) (Eunicida) **C** *Eulalia tripunctata* McIntosh, 1874 (Phyllodocida) **D** *Nereis (Alitta) virens* M Sars, 1835 (Phyllodocida) **E** *Hirudo medicinalis* Linnaeus, 1758 (Clitellata) **F** *Sipunculus nudus* Linnaeus, 1766 (Sipuncula) **G** *Sabellaria alveolata* (Linnaeus, 1767) (Sabellida) **H** *Bonellia viridis* Rolando,1821 (Echiura) **I** *Cirriformia tentaculata* (Montagu, 1808) (Terebellida) **J** *Chaetopterus variopedatus* (Renier, 1804) (Chaetopteridae). A, B and D after McIntosh 1910; C after McIntosh 1908; G after McIntosh 1922; I and J after McIntosh 1915; E, F and H after Kirby 1889.

Annelid systematics has been quite unstable over the past century. Traditionally, annelids have been divided into two major groups "Errantia" and "Sedentaria" (de Quatrefages 1866). However, Dales (1963; 1977) rejected this grouping, argueing that these only reflected the mode of living (Errantia: vagile worms, Sedentaria: sessile worms) rather than a phylogenetic relationship.

Rouse and Fauchald's (1997) cladistic analysis is the largest and the most taxonomically inclusive morphology based annelid phylogeny to date. Their study included data on 80 accepted families of marine polychaete annelids, as well as non-polychaete taxa, such as Euarthropoda, Onychophora, and Clitellata and their allies (Aeolosomatidae and Potamodrilidae). They proposed a monophyletic Polychaeta, consisting of two major clades, Scolecida and Palpata; the latter divided into Aciculata and Canalipalpata (Fig. 1.2A).

More recent molecular phylogenies, however, do not support the phylogeny based on morphological characters. In fact, of all the major metazoan taxa, Annelida has shown among the highest discordance between morphology-based and molecularly-inferred phylogenies (Andrade et al. 2015). Struck et al.'s (2011) phylogenomic analysis of annelid phylogeny and Weigert et al.'s (2014) phylogeny using transcriptomics, resulted in the resurrection of the old taxonomic names Errantia and Sedentaria. More recent and more inclusive studies (Andrade et al. 2015; Struck et al. 2015) conducted by completely independent data-sets seem to support the same tree topology (Fig. 1.2B).

Considering this disparity resulting from morphology-based classifications that differ from phylogenies based on Sanger sequencing and phylogenomic analyses, we need to re-think and re-analyse previously established homology hypotheses in light of recent molecular phylogenies of annelida.

Figure 1.2. Changes in annelid phylogeny. **A** Phylogenetic tree of Annelida based on morphological characters after Rouse and Fauchald (1997). **B** Phylogenetic tree of Annelida based on molecular data after Weigert et al. (2014) →

A

Phyllodocida
Eunicida
Amphinomida
Spinther
Nerillidae
Aberranta
ACICULATA

Terebelliformia
Cirratuliformia
Sabellida
Oweniidiae, Siboglinidae,
Sabellariidae, Serpulidae
Spionida
Chaetopteridae, Magelona
Polygordiidae
Protodrilida
CANALIPALPATA

PALPATA

POLYCHAETA

Arenicolidae
Maldanidae
Capitellidae
Orbiniidae
Opheliidae
SCOLECIDA

Psammodrilidae
Clitellata
Echiura

B

Clitellata
Terebelliformia
Arenicolidae - Maldanidae
Opheliidae
Echiurida - Capitellidae
Spionidae - Sabellariidae
Serpulidae - Sabellidae
Cirratuliformia - Siboglinidae
Orbiniidae
SEDENTARIA

Phyllodocida
Eunicida
ERRANTIA

ANNELIDA

Sipuncula
Amphinomidae
Chaetopteridae
Magelonidae
Oweniidae

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1.2 Chaetae and Chaetal arrangement

Chaeta are arranged in one dorsal and one ventral group on either side of each segment. They are involved in locomotion, defense, anchoring, food collection, drilling into hard substrata, to mention a few of their tasks in annelids. Chaetae, are the chitinous bristles of annelid worms and can occur in a variety of shapes and arrangements in different taxa (Fig. 1.3). This makes chaetae one of the most important diagnostic characters when identifying annelid species (Fauchald 1977; Schroeder 1984). Chaetal features are a *conditio sine qua non* in any annelid identification key, which is due to their high constancy in species and supraspecific taxa.
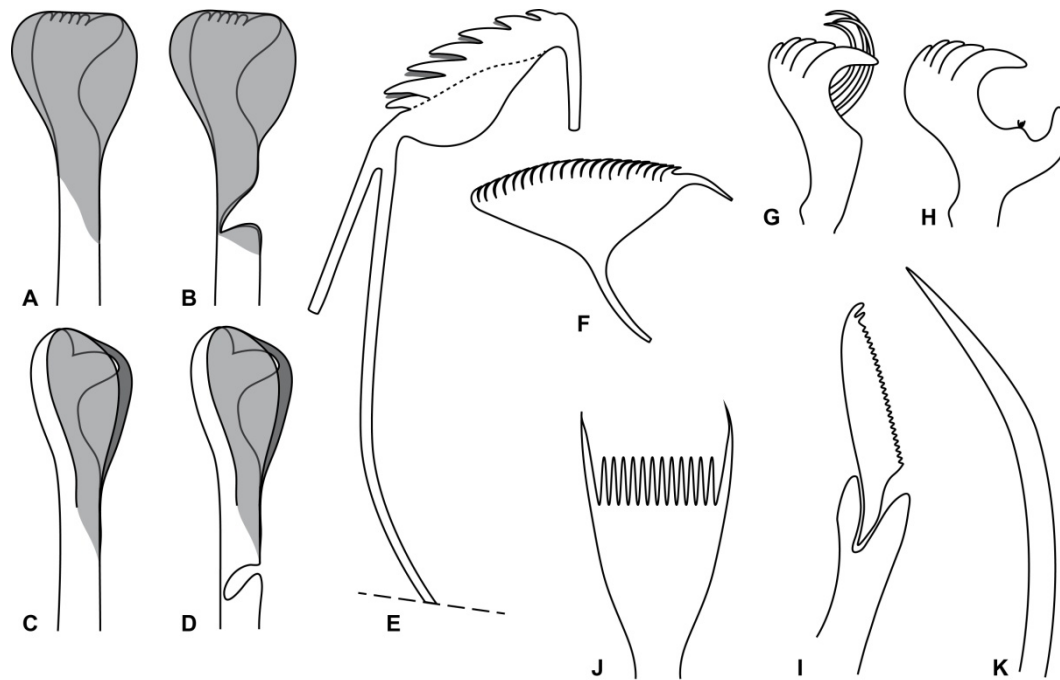
Figure 1.3. Diversity of haetal types. **A-B** Lumbrinerid-type hooded hooks **C-D** Eunicid hooded hooks. **E** Sabellariid uncinus. **F** Chaetopterid uncinus. **G** Maldanidan bearded hooked chaeta. **H** avicular uncinus. **I** Compound capillary chaeta. **K** Simple capillary chaeta.

The plesiomorphic condition in annelids is that they posses a dorsal and a ventral group of chaetae on each segment. These chaetae often form a row, whereby formation of new chaetae is restricted to one edge, i.e., the ventral edge in notopodia and the dorsal edge in neuropodia. Since, chaetae are in direct contact with the animals

environment they are susceptible to deterioration and wear out in time. Older chaetae, therefore get constanly replaced by newly developing chaetae. In a chaetal row often a degenerative site on the opposite end of the formative site can be observed (Fig. 1.4).

formation ──────────────▶ degeneration
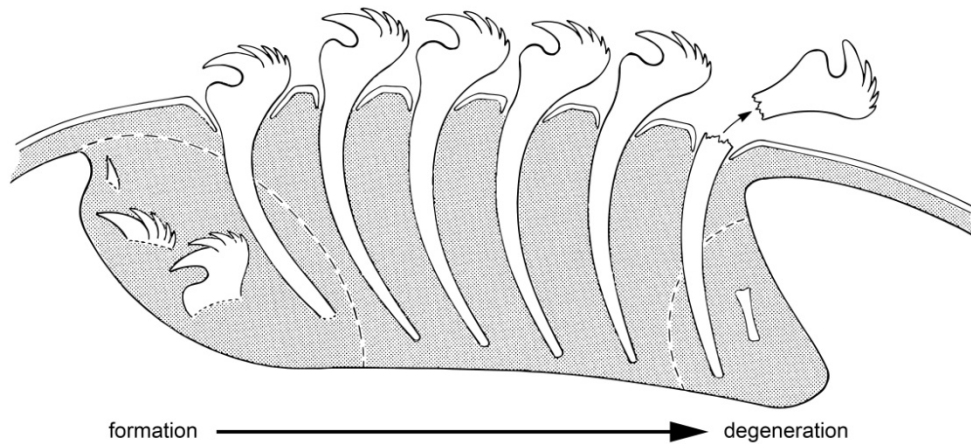
Figure 1.4. Arrangement of hooked chaetae in a row with a clearly distinct formative site at one end of the row and a site of chaetal degeneration on the other end. after Bartolomaeus 1998

There are also certain exceptions to the general pattern of chaetal arrangement. Like the inversion of the formative site of the neuropodial row of hooked chaetae to dorsal positon in Arenicolidae (Bartolomaeus and Meyer 1997). This also seems to be the case in Maldanids and is considered a synapomorphy of these two taxa and provides further support for their close relationship.

This shows that chaetal arrangement can provide a useful source of information for systematics and that comparative studies of chaetal arrangement can help understand transformations and deviations from the plesiomorphic condition in annelids.

The formative site of oweniid neuropodial chaetae is hard to compare to other taxa. Oweniid neuropodial chaetae do not occur in rows but in patches and new chaetae are added to the patch along the entire caudal edge of this patch (Meyer and Bartolomaeus 1996). Due to the dorsal formative site in the chaetal rows of the newly metamorphosed *Owenia fusiformis* specimens, the patches of adult animals have been interpreted as a derived condition (Meyer and

Bartolomaeus 1996; Hausen 2005).
Certain magelonid species and
most probably chaetopterids also do
not have a restricted formative site
(Hausen 2005). These irregularities
in the position of the formative site
have also been regarded as a
derived condition. This idea,
however, needs reconsideration due
to the position of these taxa in
recent annelid phylogenies.

1.3 Chaetogenesis

Aside from being a valuable
source for taxonomists, chaetae
have also been the focus of many
studies regarding functional
ecology (Woodin and Merz 1987;
Merz and Edwards 1998; Merz and
Woodin 2000; Pernet 2000; Merz
2015) and last but not least, the
cellular mechanisms behind the
highly dynamic chaetal formation
process described by Bouligand
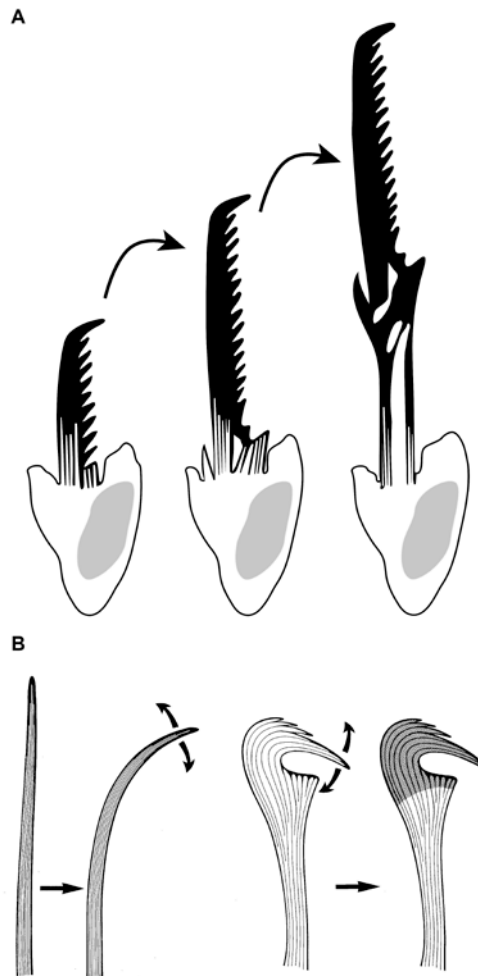
Figure 1.5. A Schematic representation of chaetogenesis in *Nereis vexillosa.* after O'Clair and Cloney (1974). **B** Flexibility of a capillary chaeta and restricted elasticity in hooked chaetae by intercalation of electron dense material (grey shading). After Bartolomaeus 1998

(1967) and O'Clair & Cloney (1974) (Fig. 1.5A) have fascinated many scientists (Warren 2015) and provide an intriguing field of study.

Chaetae are formed within an ectodermal follicle. This follicle comprises a small number of cells; the basalmost chaetoblast and a few follicle cells (Bouligand 1967; Specht and Westheide 1988; Hausen 2005) . Each chaetoblast has an array of apical microvilli that grow and shrink during chaetogenesis. The controlled modification of different groups of microvilli on the apical portion of a chaetoblast as well as

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regulation of N-acetylglucosamine secretion and extracellular polymerization gives a chaeta its final shape (Hausen 2005, Souza et al. 2011, Ogawa et al. 2011, Koide et al. 2015). The main component of a chaeta is ß-chitin, which is cross-linked by proteins, but in some chaetae inorganic components, like calcium, magnesium and iron, can also be incorporated, most probably for an increased hardening and fortification of a chaeta (George and Southward 1973; Bartolomaeus 1992).

A chaeta grows at its basis and is pushed upwards during its development. The final structure of a chaeta is composed of hollow chitin tubes; the canals inside which are merely the empty spaces of the chaetoblast's microvilli that have retracted after the final polymerization of chitin. This typical structure of a chaeta can be observed with light microscopy and has been described early on (Lippert and Gentil 1963; Bouligand 1966, 1967; Scherf 1970; George and Southward 1973; Gustus and Cloney 1973; O'Clair and Cloney 1974). The internal structure of a chaeta, reminiscent of a glas fiber stick, has great influence on its flexibility and stiffnes (Kryvi and Sørvig 1990; Merz and Woodin 1991). Alternating between microvillar channels that are fully filled with electron-dense chaetal material and hollow channels can result in different mechanical properties of different parts within one chatae; i.e. the rigid rostrum and the flexible shaft of hooked chaetae (Fig. 1.5B).

The process of chaetal formation is an elaborate and highly complex interplay of cellular instruments, not unlike a cellular 3D printer, with the microvilli of the chaetoblast acting as the printing heads, assembling the complex final structure of a chaeta through the selective addition of material in time and space (Warren 2015). A variety of factors such as; (1) the number and diameter of microvilli, (2) the merging and separation of different groups of microvilli of a single chaetoblast, (3) fusion of microvilli, (4) bending of microvilli and (5) the spatio-temporal modification of these factors are involved in the

formation process of a chaeta. Furthermore, polymerization of ß-chitin along the microvillar surface needs to be in perfect harmony with the remaining dynamics of chaetogenesis. All these processes must be higly regulated and genetic programming is hereby a necessity to warrant the constancy of chaetal arrangement and structure that can be observed within annelid species and supraspecific taxa. This pattern appears to be conservative enough that certain chaetae, once evolved, can be passed on to descendants and, thus, become also characteristic for supraspecific taxa. Therefore, structural and developmental uniformity or discrepancy of chaetae should be carrying a significant phylogenetic signal and can be used to test homology hypotheses.

1.4 Hooked chaetae

Many sedentary annelids possess so called hooked chaetae. Due to the resemblance of these hooks to tiny anchors and their association with a tube-dwelling benthic life-style, it has been argued that they are primarily used to resist removal of worms from their tubes (Woodin and Merz 1987).

With the introduction of transmission electron microscopy into the study of chaetal formation it became possible to fully reconstruct chaetogenesis through the investigation of different developmental stages. A range of studies investigating the formation process of hooked chaetae and uncini in "sedentary" polychaetes (Arenicolidae: Bartolomaeus and Meyer 1997; Psammodrilidae: Meyer and Bartolomaeus 1997; Oweniidae: Meyer and Bartolomaeus 1996 Pectinariidae; Bartolomaeus 1995; Terebellidae: Bartolomaeus 1998; Serpulidae, Sabellidae: Bartolomaeus 2002) showed a uniform chaetogenesis in all of the studied taxa, which led to a homology hypothesis of hooked chaetae.

Formation of a hooked chaeta always starts with the preformation of the rostrum (sensu Holthe 1986) by a dense group of microvilli. This is then followed by a change in the orientation of the

chaetoblast surface, bending the microvilli and accordingly leading to the characteristic strong curvature of the rostrum. Microvilli with larger diameters arise adrostrally and form the capitial teeth. Here, only one microvillus functions as template of one tooth. The rostrum is thus always composed of many channels, whereas each apical tooth is composed of one. While the capitial teeth are being formed, a large number of microvilli preform the subrostral process. Finally, all microvilli align in order to form the manubrium (shaft). Chitin is released continuously to elongate the manubrium.

Eventhough this general pattern of chaetogenesis is identical in many annelids, the hooked chaetae of certain taxa miss a rostrum. For example, a rostrum is missing in the pectinariids *Pectinaria koreni* and *P. auricoma* (Hausen 2005) and the serpulid *Spirorbis spirorbis* (Bartolomaeus 1995), in the abdominal uncini of the sabellid *Fabricia sabella* (Bartolomaeus 2002) and in the hooks of the oweniid *Owenia fusiformis* (Meyer & Bartolomaeus 1996). When a rostrum is absent, chaetogenesis starts with the formation of the capitium, but is otherwise identical to hooked chaetae with a rostrum. The missing rostrum in the above mentioned taxa has therefore been interpreted as a reduction and not as an indication of convergence (Hausen 2005).

According to the new molecular phylogenies of Annelida Oweniidae and Chaetopteridae always represent the basal most branching taxa and form a grade (Weigert 2014, Struck et al. 2015 and Andrade et al. 2015). In light of new phylogenies the view on the homology of hooked chaetae must probably be changed. All these taxa bear hooked chaetae, uncini or hooded hooks. The previous assumption that all hook bearing taxa shared a common ancestor no longer holds true, which means that hooked chaetae possibly evolved earlier than expected and that there are possible hidden convergencies in the previously established homology of hooked chaetae.

1.5 Aims and contents of this study

As mentioned above, introducing phylogenomics and molecular approaches into phylogeny inference shook and re-shaped the annelid tree severely. The results contradicted earlier ideas on homology and autapomorphies. The main purpose of this study is to bring a new perspective and insight into chaetal characters; like chaetogenesis and the topological arrangement of chaetae. The following series of investigations and publications demonstrate the significance and relevance of comparative studies on chaetal formation. The existing information on chaetal formation of hooked chaetae is extended and further taxa were included to provide a complete picture of the diversity, covering all the major branches of the annelid tree. The results of these studies were then put into a greater context by using the new annelid phylogeny as a backbone.

Morphology is a discipline that is generally believed to be rather descriptive than hypothesis driven. With this thesis and the studies presented in the following, I aim to alter this belief by testing different hypotheses on evolutionary transformations of morphological structures, i.e. the chaetae of annelids. These hypotheses are based on recent molecular phylogenies and comparative morphology.

The work presented in the following chapters can be divided in three categories; comparetive investigations of (1) chaetal formation, (2) chaetal arrangement, more specifically the position of the formative site and (3) distribution and evolution of different chaetal types. Chapters 2, 3 and 4 present studies in which a comparative analysis of chaetogeneses is used as an instrument to test the expected homology/convergence of a certain chaetal type. In chapter 5, chaetal topology and the position of the chaetal formative site is used as a character to test the homology of echiurid chaetae. Chaetal arrangement is investigated for additional taxa in Chapter 7. In chapter 6 a systematic approach is employed to test hypotheses on the evoloution of chaetal type diversity within a monophyletic taxon (Eunicida).

PUBLICATIONS

The contents of the following chapters have been published or are in the process of being published in a variety of scientific journals. They are presented in the following as the author's version and the publication information for every individual article is provided in the beginning of each chapter.

Chaetal Arrangement and Chaetogenesis of Hooded Hooks in *Lumbrineris* (*Scoletoma*) *fragilis* and *Lumbrineris tetraura* (Eunicida, Annelida)

2

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Abstract

Since the structure and arrangement of chaetae are highly specific for annelid species and higher taxonomic entities, we assume that rather conservative information guarantees formation of specific chaetae. Each chaeta of an annelid is formed within an ectodermal invagination, and the modulation of the apical microvilli pattern of the basalmost cell of this invagination determines the structure of the chaeta. Any hypothesis of the homology of chaetae could thus be tested by examining the process of chaetal formation. Investigations into the ultrastructure and formation of hooded hooks in different capitellids and spionids revealed that these chaetae can be homologized. The hood of each of their hooded hooks is formed by elongation of two rings of microvilli peripheral to the chaetal *anlage*, which give rise to the inner and outer layer of the hood. The hood layers are well separated and surround an empty space. Superficially similar hooded hooks are described for certain Eunicida. Presently available cladistic analyses

suggest that the hooded hooks of eunicidans evolved independently of those in Capitellidae and Spionidae. Compared to the latter two families, we therefore expected to find differences in chaetogenesis of the hooded hooks in the eunicids *Lumbrineris* (*Scoletoma*) *fragilis* and *Lumbrineris tetraura* (Lumbrineridae). This was the case. In these eunicidans, the hood was formed by the bisected apical wall of the chaetoblast right after the mid-apical section of the chaeta had been sunk deeply into the chaetoblast during its formation. The apical wall generated a brush of microvilli that preformed the hood. Because the microvilli of the hood showed some accelerated differentiation, they soon merged with those of the slowly growing setal shaft to form the broad manubrium of the hooded hook in lumbrinerids. Our study confirms the predicted differences in chaetogenesis of the superficially similar hooded hooks of capitellids and spionids compared to those of eunicids.

2.1 Introduction

Chaetae of Annelida are chitinous extracellular structures that are formed within an ectodermal pouch or chaetal follicle (Bouligand 1967; Schroeder 1984; Specht & Westheide 1988; Hausen 2005). The chaetal follicle consists of a few follicle cells and a single basal chaetoblast. The pattern of the apical microvilli of the chaetoblast is modified constantly, while N-acetyl-glucosamine is released between the bases of the microvilli. This material polymerises extracellularly, forming the chaeta; thus, the definitive structure of the chaeta is primarily caused by changes in the pattern of chaetoblast microvilli, i.e. by dynamic microvilli (O'Clair and Cloney 1974). Chaetae are considered an autapomorphy of annelids, and reconstructions of the ancestral annelid pattern (bauplan) concur that early annelids bore simple capillary chaetae (Struck 2011; Struck et al. 2011). The dynamic developmental mode of chaetae allows for the formation of a plethora of different chaetal types, and these different chaetae are of

immense functional importance, e.g., in defense, locomotion, burrowing, and anchoring of the body to the tube (Merz and Woodin 2006). Arrangement and structure of the chaetae are so conserved in annelid species and supraspecific taxa that chaetation has become an important source of diagnostic characters for taxonomists. Since the dynamic structure, orientation, and number of microvilli of the chaetoblast determine chaetal structure, these factors must be strictly regulated to guarantee that chaetae are identical within an individual, a population or a species. Regulation of these factors appears to be conservative enough that certain chaetae, once evolved, can be passed on to descendants and thus become characteristic of supraspecific taxa. Studies into chaetogenesis of uncini in certain "sedentary" polychaetes revealed that the structure of these chaetae results from a uniform process of chaetogenesis (Arenicolidae and Maldanidae: Bartolomaeus & Meyer 1997; Bobin 1944; Tilic, unpubl. data; Psammodrilidae and Oweniidae: Meyer & Bartolomaeus 1996; Pectinariidae; Terebellidae, Serpulidae, Sabellidae: Bartolomaeus 1995, 1998, 2002); thus, chaetogenesis in these cases seems to contain some phylogenetic signal. Studies of chaetogenesis also support the hypothesis of homology between uncini and the hooded hooks of capitellids and spionids (Hausen 2001, 2005; Bartolomaeus et al. 2005). In members of these two families, hooded hooks are found in the abdominal chaetigers. These hooded hooks resemble uncini whose apical portion is enclosed by a hood, consisting of an outer and an inner lamella. Studies of the formation of the hood supports the assumption of homology of spionid and capitellid hooded hooks (Hausen and Bartolomaeus 1998; Hausen 2005).

Similar hooded hooks, however, have also been described in Lumbrineridae, a taxon that clearly belongs to a clade called the Errantia or Aciculata, which consists of at least Eunicida and Phyllodocida (Rouse & Fauchald 1997; Struck et al. 2014; Weigert et al. 2014). Both in a molecular phylogenetic analysis of Eunicidae

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(Zanol et al. 2010) and in a recent revision of the taxonomy of Eunicidae based on morphological characters (Zanol et al. 2013), lumbrinerids are considered basally branching Eunicida and therefore taken as outgroups for unravelling the phylogeny of the Eunicidae. On the light microscopical level the hooded hooks of Lumbrineridae appear to be similar to those of Capitellidae and certain Spionidae, since they also consist of a large spine that is surmounted by smaller ones. The main axis of the larger spine is perpendicular to the manubrium, and a large hood surrounds the spines (Hilbig 1989). The structural similarities between the hooded hooks of Capitellidae and Spionidae to those of Lumbrineridae are either the result of convergent evolution, or the result of an early origin during annelid evolution, with subsequent loss or modification in many different lineages. Since the latter scenario appears to be unlikely, we expected striking differences in the mode of chaetogenesis that reflect the assumed convergent evolution of hooded hooks in Lumbrineridae on the one hand, and Capitellidae and Spionidae on the other. Recognisable differences in mode of chaetogenesis would also support the value of chaetogenesis as a phylogenetic character. In order to test this idea we analysed chaetogenesis in *Lumbrineris* (*Scoletoma*) *fragilis* Müller 1766 and *Lumbrineris tetraura* Schmarda 1861. Details of chaetal arrangement and the position of the growth zone were used to corroborate the hypothesis of a convergent evolution of hooded hooks in Lumbrinereidae, Spionidae and Capitellidae.

2.2 Methods

2.2.1 Transmission and scanning electron microscopy

Specimens of *Lumbrineris tetraura* and *Lumbrineris fragilis* were collected from sandy areas underneath and between smaller stones at the rocky shore close to Plozevet and in Concarneau (Brittany, France). For fixation for transmission electron microscopy (TEM), we used 2.5% glutaraldehyde buffered in 0.1 M sodium

cacodylate at room temperature for 1 h. Ruthenium red was added to the fixative, and the animals were dissected prior to fixation. After having been rinsed in the same buffer, the animals were postfixed in 1% OsO_4 in 0.1 M sodium cacodylate for 1 h, dehydrated in an acetone series, and embedded in Araldite. The chaetal follicle and the formative sites of six chaetigers of a young individual of *L. tetraura* were sectioned into a complete series of silver-interference coloured sections, stained with uranyl acetate and lead citrate, and analyzed using Zeiss EM 10B and Hitachi H500 transmission electron microscopes. The course of formation of the hooded hooks was reconstructed from electron microscopical data from different formative sites of *L. tetraura* using Fiji (1.45b) (Schindelin et al. 2012) / TrakEM (Cardona et al. 2012) for cell identification, and Amira (4.0) for 3D-visualization.

For scanning electron microscopy (SEM), specimens of L. tetraura and *L. fragilis* were fixed in Bouin's fluid, dehydrated in an alcohol series (during this step, the animals were sonicated to remove debris and sand particles from the chaetae), and critical point dried with CO_2 in a Balzers critical point dryer. After dehydration they were sputter-coated with gold (Balzers Sputter Coater) and examined using Novoscan and Hitachi scanning electron microscopes.

2.2.2 Confocal laser scanning microscopy

Specimens used for confocal laser scanning microscopy (CLSM) were fixed in 4% paraformaldehyde. The chaetigers were dissected to separate single parapodia or a single segment. Isolated parapodia and segments were permeabilized in four 5-min changes of phosphate buffered saline (PBS) with 0.1% Triton X-100 (Fisher Scientific). The parapodia were then stained overnight at 4°C with TRITC-phalloidin at a dilution of 1:100. After staining, parapodia were rinsed in three quick changes and subsequently in two 10-min changes of PBS with 0.1% Triton, and one 10 min rinse in PBS without Triton. Smaller parapodia were mounted on poly-l-lysine coated coverslips, and larger

ones were directly placed in hollow-ground slides. The samples were quickly dehydrated in isopropanol (2 min each in 70%, 85%, 95%, 100%, 100%), cleared in three 15-min changes of Murray Clear, and mounted in Murray Clear. Slide preparations were sealed with nail polish.

2.2.3 Light microcopy, histology, and 3D reconstruction

Chaetae were isolated from pieces of living specimens of *L. tetraura* by incubation in 5% NaOH for 2-3 h. The chaetae were rinsed in distilled water, mounted on slides, and examined using Nomarski differential interference contrast with an Olympus BX-51 microscope. Their structure was digitally recorded with an Olympus camera (Olympus cc12).

For reconstruction of the chaetal sac, specimens of both species were relaxed for 1.5-2 h in a 1:1 mixture of 7% $MgCl_2$ and seawater. They were subsequently fixed in 1.25% glutaraldehyde buffered in 0.05M phosphate buffer with 0.3M NaCl for 1.5-2 h. Afterwards, the specimens were postfixed in 1% OsO_4 in a 0.05M phosphate buffer. Before embedding, the specimens were dehydrated via an ascending acetone series. They were then transferred via propylene oxide to Araldite. The specimens were fragmented into smaller pieces within the resin. Polymerization was started with benzyldimethylamin (BDMA). Serial 1µm sections were prepared using a diamond knife (Diatome Histo Jumbo) on a Leica Ultrarcut S ultramicrotome, following the method described by Blumer et al. (2002). The sections were stained with toluidine blue (1% toluidine, 1% sodium-tetraborate, and 20% saccharose) and mounted in Araldite. The semi-thin sections were analyzed with an Olympus BX-51 and photographed with an Olympus camera (Olympus cc12) equipped with the dot slide system (2.2 Olympus, Hamburg). The images were aligned with IMOD (Boulder Laboratories: Kremer et al. 1996) and IMOD-align

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(http://www.evolution.uni-bonn.de/mitarbeiter/bquast/software). The software 3ds max 13.0 was used for 3D modeling of the chaetae. The histological images were imported as surface materials (discrete) and the chaetae were modeled using standard cylindrical or conic objects. When necessary these were modified as NURBS (Non-uniform rational B-Splines)-surfaces. The outline of the chaetal follicle was created using NURBS-curves on selected section planes.

2.2.4 Data repository and voucher material

To allow data transparency, all of the aligned serial sections used for 3D modeling are freely accessible in the morphological database, MorphDBase (Grobe & Vogt 2009). Series of semi-thin sections for *L.tetraura* can be found at

https://www.morphdbase.de?E_Tilic_20131009-M-10.1, and for *L. fragilis* at https://www.morphdbase.de?E_Tilic_20131009-M-12.1 and https://www.morphdbase.de?E_Tilic_20131009-M-11.1. Aligned TEM sections showing chaetogenesis are can be found at

https://www.morphdbase.de?E_Tilic_20131009-M-13.1,

https://www.morphdbase.de?E_Tilic_20131009-M-14.1, and

https://www.morphdbase.de?E_Tilic_20131009-M-15.1.

Conspecific individuals of both species studied were collected at the same sampling site and deposited at the Zoological Museum of the University of Göttingen as voucher material.

2.3 Results

2.3.1 Parapodial structure and the arrangement of chaetae

Each parapodium has a small and rounded prechaetal lobe, and a larger postchaetal lobe. Macroscopically, the parapodia of both species studied are uniramous, but series of semi-thin sections and subsequent 3D- reconstruction of the chaetal sac of *Lumbrineris*

fragilis (Fig. 2.1A-G) and *Lumbrineris tetraura* (Fig. 2.2A-G) revealed that their notopodia are extremely vestigial: a dorsal group of small chaetae, embedded inside the parapodia in both species (Figs. 2.1G; 2.2G), originate from their own chaetal sac, independent of that of the neuropodia. Due to their position and their origin we regard them as notopodial chaetae which have been internalized. Although the notopodial chaetae were much smaller and thinner than the neuropodial chaetae, they could be seen by confocal laser scanning microscopy (CLSM: Fig. 2.3A-C). Since only the neuropodial chaetae protrude to the outside of the body, the following description refers to the neuropodial chaetae.

In *L. fragilis* the neuropodial chaetation was consistent throughout the body; all chaetigers only bore ~4 hooded hooks. In *L. tetraura,* anterior and posterior parapodia differed in this respect. Anterior parapodia bore seamed capillary chaetae as well as hooded hooks, whereas hooded hooks were the only chaetae in posterior parapodia (Fig. 2.3B,C). In both species, the chaetae arose from a small rim that was transverse to the longitudinal body axis (Fig. 2.3D,E). Internally the structure of the chaetal sac was influenced by the insertion of parapodial and chaetal muscles (Fig. 2.3A-C). The outline of the chaetal sac thus became extremely irregular and folded a few micrometers below the parapodial surface, so that groups of chaetae appeared to be isolated within the parapodium (Figs. 2.1C; 2.2C-E). Nevertheless, all chaetae of one parapodium originated from a single chaetal sac; within each chaetal sac, hooded hooks, capillary chaetae, and acicula were aligned in a single dorso-ventrally oriented row, sometimes in a zig-zag pattern. The aciculae did not protrude from the cuticle, were inserted much more deeply in the parapodia than the remaining chaetae, and were always located posterior to the row of capillary chaetae and hooded hooks (Figs. 2.1A,B; 2.2A,B; 2.3). In both species, new chaetae were formed continuously in each chaetiger, irrespective of the position and thus of the age of the parapodium.
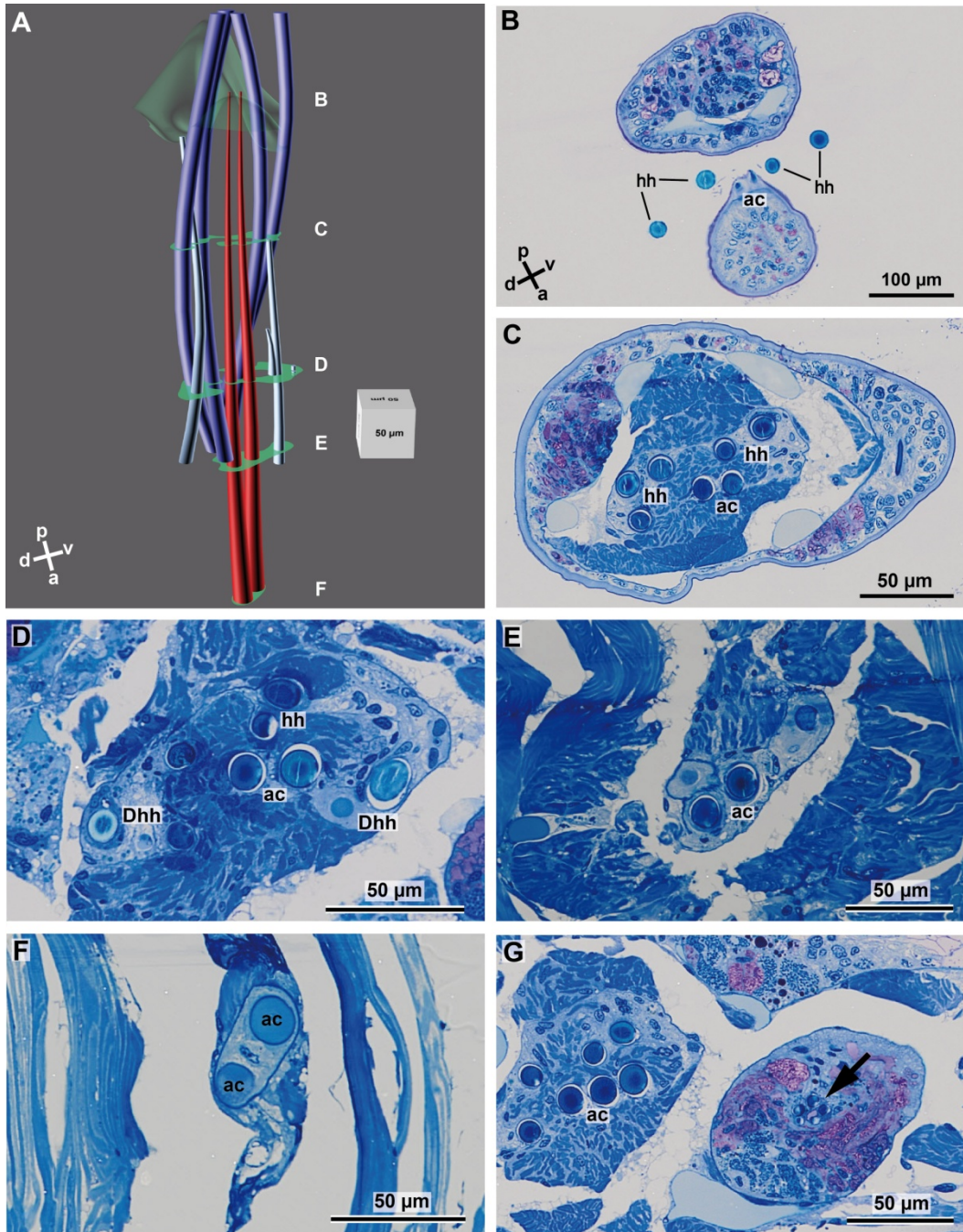
Figure 2.1 *Lumbrineris fragilis.* **A** 3D reconstruction of the chaetal arrangement. **B-F** Aligned semithin sections of the chaetal follicle used to generate the 3D reconstruction. Corresponding section planes are marked in **A**. **G** Semithin section of the parapodium. The black arrow indicates the rudimentary notopodial chaetal elements. *a* anterior, *ac* acicula, *d* dorsal, *Dhh* developing hooded hooks, *hh* hooded hooks, *p* posterior, *v* ventral.

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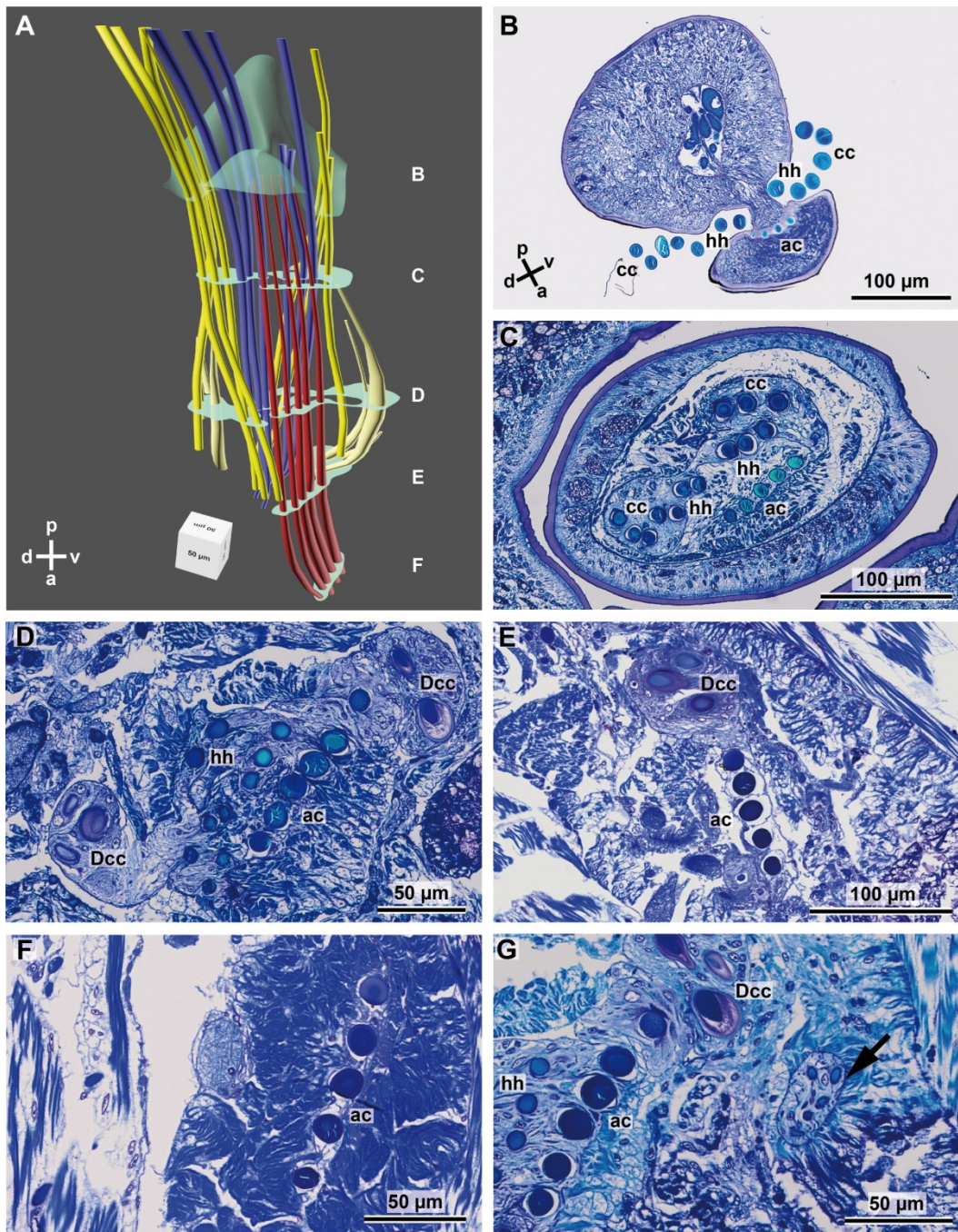
Figure 2.2 *Lumbrineris tetraura.* **A** 3D reconstruction of the chaetal arrangement. **B-F** Aligned semithin sections of the chaetal follicle used to generate the 3D reconstruction. Corresponding section planes are marked in **A**. **G** Semithin section of the parapo parapodium. The black arrow indicates the rudimentary notopodial chaetal elements. *a* anterior, *ac* acicula, *cc* capillary chaetae, *d* dorsal, *Dcc* developing capillary chaetae, *hh* hooded hooks, *p* posterior, *v* ventral.

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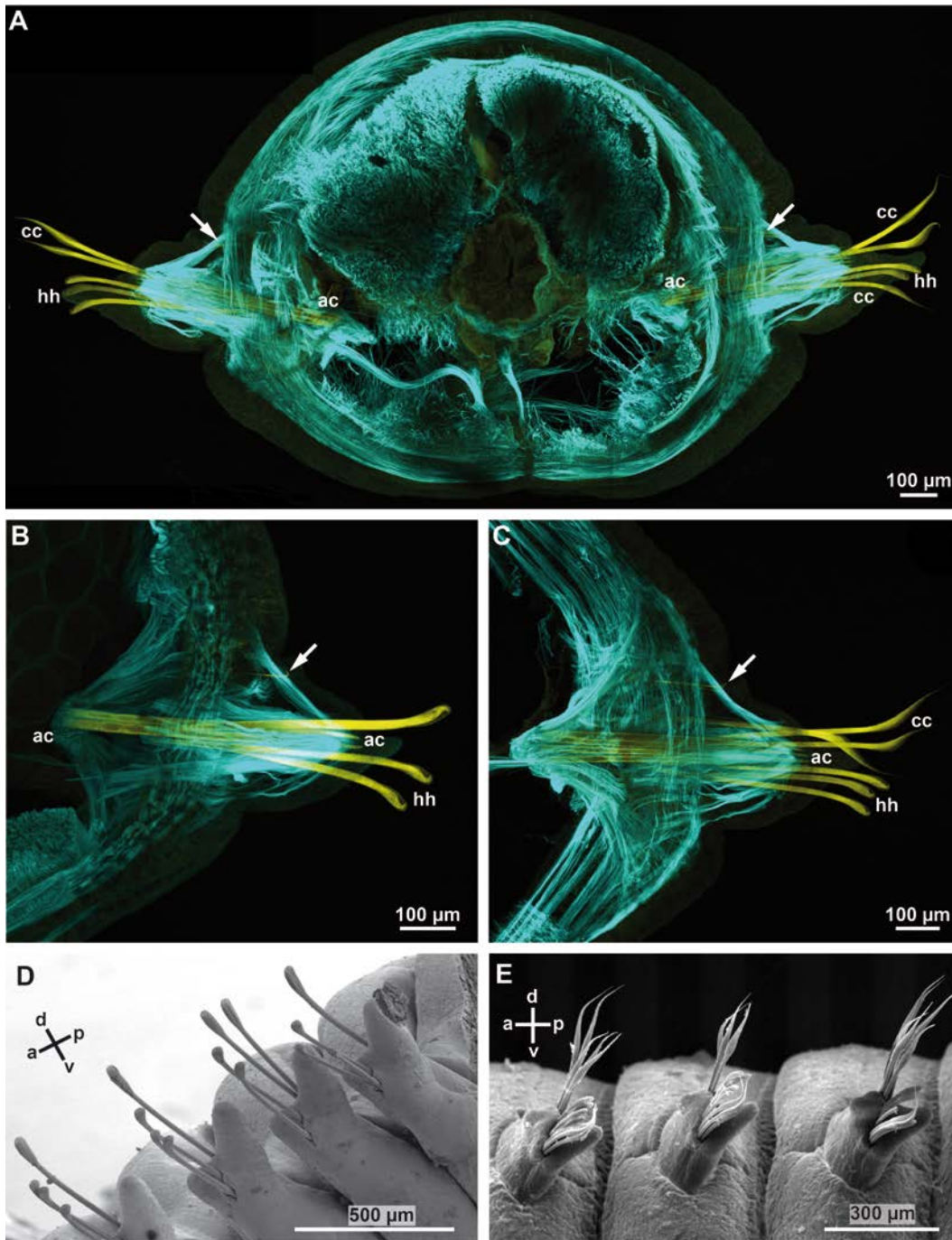
Figure 2.3 *Lumbrineris tetraura.* **A** Confocal z-projection of a phalloidin-stained cross section of an anterior segment. The white arrows indicate the rudimentary notopodial chaetal elements. **B** Confocal z-projection of a phalloidin-stained posterior parapodium without capillary chaetae. The white arrow indicates the rudimentary notopodial chaetal elements. **C** Confocal z-projection of a phalloidin-stained anterior parapodium with capillary chaetae. Chaetae are autofluorescent. The white arrow indicates the rudimentary notopodial chaetal elements. **D** Posterior chaetigers with hooded hooks only. **E** Anterior parapodia bearing capillary chaetae. *a* anterior, *ac* aciculae, *cc* capillary chaetae, *d* dorsal, *hh* hooded hooks, *p* posterior, *v* ventral.

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Each chaetal sac contained two formative sites. In *L. fragilis*, newly forming hooded hooks are shown in the 3D-reconstruction of chaetae in a frontal segment (Fig. 2.1A). In a 3D-reconstruction of a corresponding segment, *L. tetraura* only shows newly forming capillary chaetae, due to the heterogenous chaetation of the anterior segments (Fig. 2.2A). The number of acicula gradually increased during the chaetogenesis (Fig. 2.4A-C). In *L. tetraura* the anteriormost segments sometimes contained up to five acicula, while in *L. fragilis* there were maximally two acicula in one parapodium (Figs. 2.1A; 2.2A).

Serial semi-thin sections of the posteriormost segments in *L. tetraura* allowed a better insight into chaetogenesis, since the specimen studied still had an active growth zone, so that the course of chaetal formation could be followed by studying the developing segments anterior to the growth zone (Fig. 2.4A-E). Both neuropodial and notopodial chaetae could be seen from the youngest segment onward, although chaetogenesis in notopodia appeared to be a little bit delayed. Chaetogenesis always started with the formation of an acicula inside a small basiepidermal chaetal sac. During further development, the chaetal sac extended into deeper tissue layers and enlarged while additional chaetae were formed inside. This was especially evident in neuropodia, while in the notopodia the chaetal sac remained small and the tiny acicula therein remained the only chaeta for a long time. Later, a few additional small chaetae were added. In neuropodia, the acicula increased rapidly in diameter and the first hooded hooks started to develop inside the chaetal sac (Fig. 2.4A,B). Their formation took place dorsally and ventrally to the acicula, so that each chaetal sac contained two formative sites. The dorsal formative site was always next to the acicula, and the ventral one clearly offset at the ventral edge of the chaetal sac. Chaetogenesis alternated between both sites and finally gave rise to a row of chaetae. During this process different stages of chaetogenesis could be found in a single chaetal sac (Figs. 2.1A; 2.2A; 2.4C,D). Depending on the position of the formative site the

oldest chaetae were found in different positions relative to the aciculae. Those formed by the dorsal formative site were located at the dorsal edge; those formed by the ventral formative site were found next to the aciculae and thus in a central position in each row of chaetae. In general the diameter of each acicula was much larger than that of the hooded hooks (Fig. 2.5A,B). The posterior ten chaetal sacs did contain a single acicula, but further anteriorly a chaetal sac could contain up to four aciculae (Fig. 2.2A).

2.3.2 Ultrastructure of hooded hooks

The hooded hooks possessed a large spine or main tooth that was almost perpendicular to the main axis of the manubrium or shaft. Light microscopy showed several small canals inside the main tooth and their characteristic bending towards the manubrium (Fig. 2.5A). Several smaller spines or teeth surmounted the main tooth adrostrally; they were aligned in a single row (Fig. 2.5C,D). A large hood surrounded these apical structures; its larger portion was rostral, and its smaller portion was adrostral (Fig. 2.5C,E). A small apical slit allowed a view of the tip of the main tooth and some of the smaller teeth by SEM. Rostrally this slit ended beneath the main tooth, whereas it was much deeper on the adrostral side. The hood was not empty; it was filled with several irregularly arranged rods (Fig. 2.5E) that were embedded in an amorphous matrix. Each rod consisted of an electron-dense wall and an electron-lucent core. Towards the periphery their arrangement was more regular. Here several rows of electron-dense rods formed the outer surface (layer) and the inner surface (layer next to the shaft) of the hood. Since they extended beyond the outer and inner surface, the hood was covered by densely arranged knobs (Fig. 2.5C, F). The rods become less regularly arranged towards the centre of the hood. During preparation for SEM, the inner matrix shrank, so that the space between the rods appeared empty (Fig. 2.5E). The inner layer of the hood also consisted of densely arranged electron-

dense rods. This inner layer was isolated from the manubrium just below the rostrum, and merged with the outer layer on either side of the rostro-adrostral axis of the chaeta. With Nomarski interference contrast optics the margin of the inner layer of the hood was clearly visible beneath the rostrum (Fig. 2.5A). Hartmann-Schröder (1996; p. 268) misinterpreted this margin as subdistal tooth.
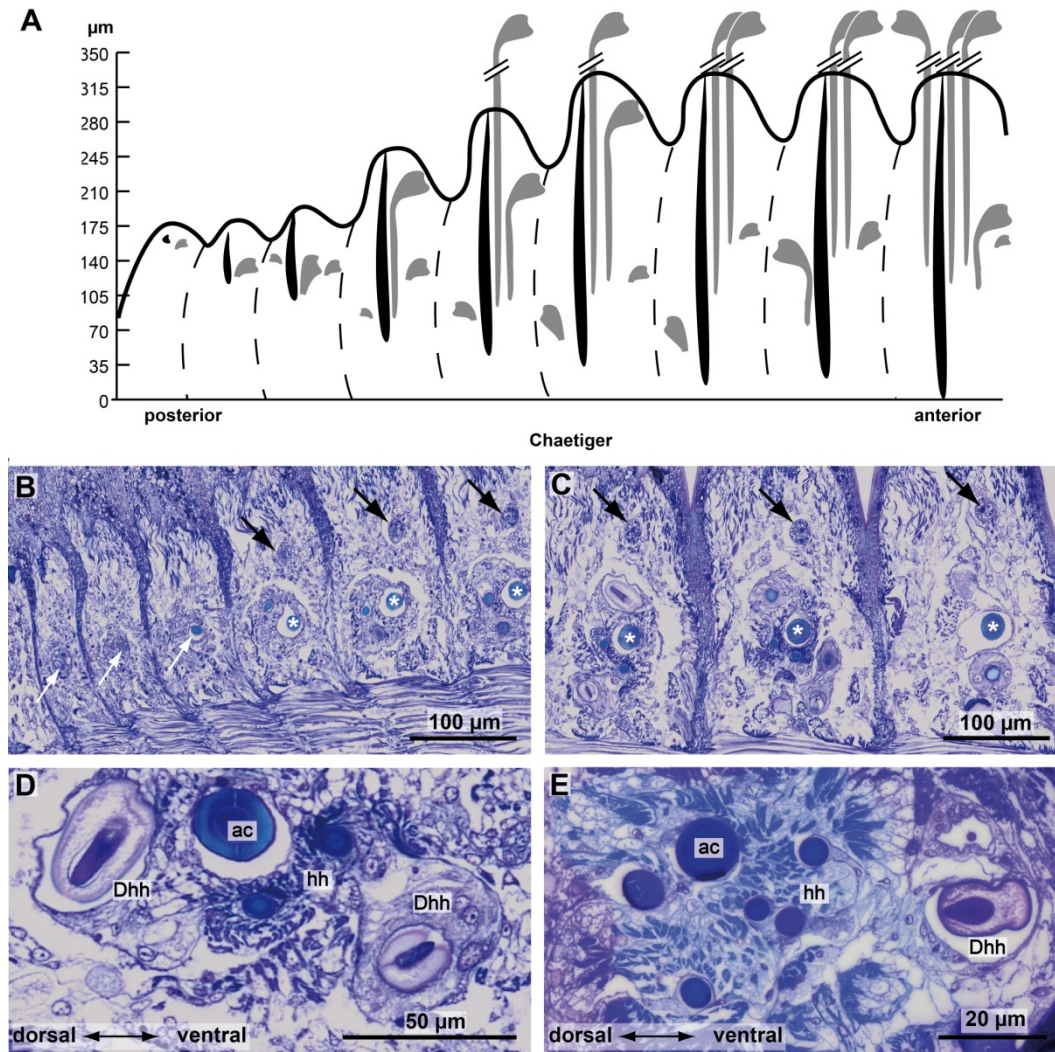
Figure 2.4 *Lumbrineris tetraura.* **A** Graphical illustration of chaetal development in the posteriormost chaetigers. Aciculae are shown in black and hooded hooks in grey. **B-C** Semithin sections showing developing hooded hooks and aciculae in the posterior segments. White arrows and the asterisks mark the aciculae, and black arrows indicate rudimentary notopodial chaetae. **D-E** Semithin sections showing developing hooded hooks at higher magnification. *ac* aciculae, *Dhh* developing hooded hooks, *hh* hooded hooks.

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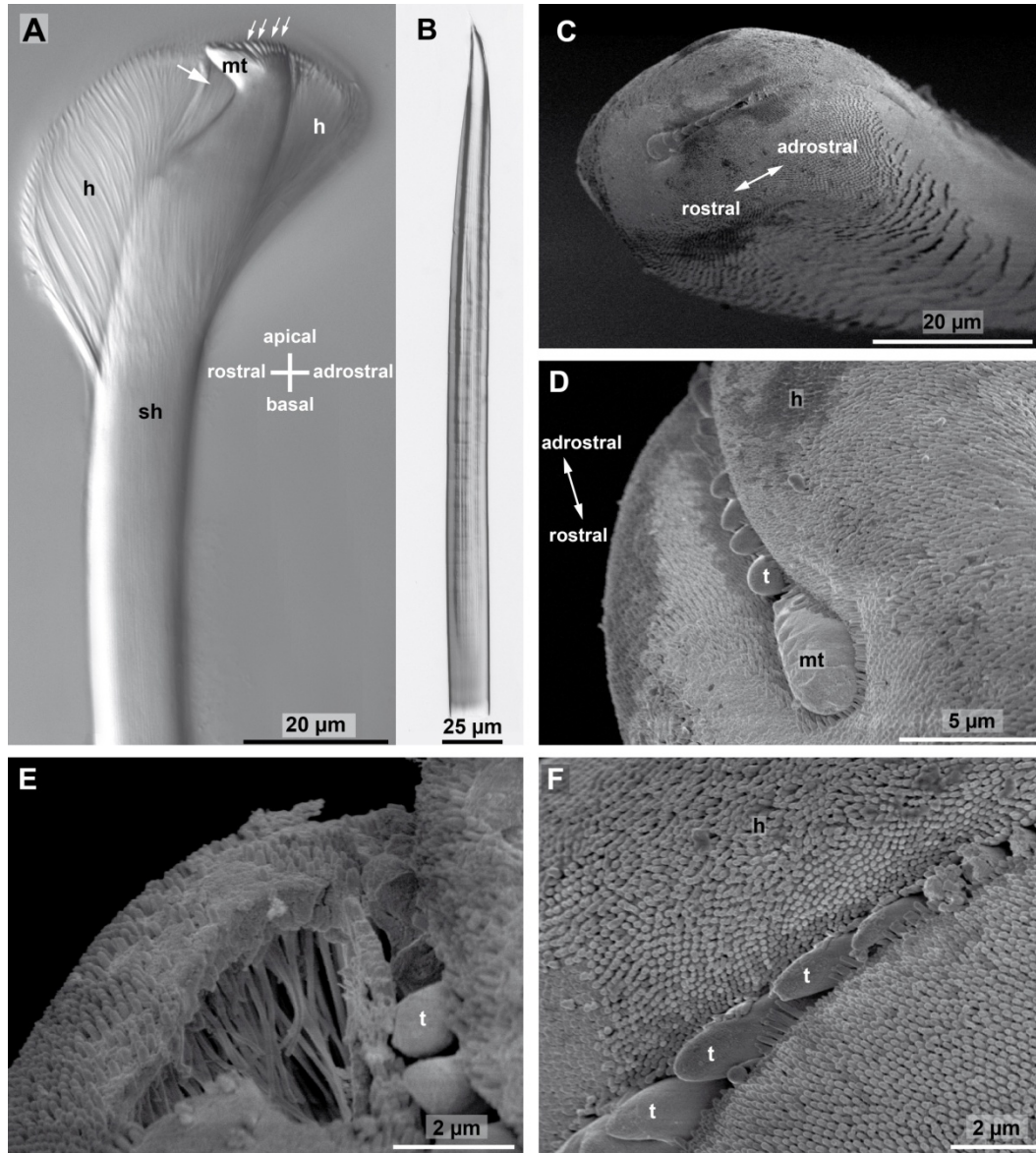
Figure 2.5 *Lumbrineris tetraura*. **A**. Apical portion of a hooded hook with hood (h), main tooth (mt), smaller teeth (arrows), and shaft (sh). The large arrow marks the inner margin of the hood. **B**. An acicula. **C**. Tip of a hooded hook with small spines surrounded by the large hood. **D**. Spines are aligned in a row and consist of a large tooth and smaller teeth (t). **E**. Partly opened hood. Note the numerous internal rod-like elements. **F**. The internal rod-like elements pierce the surface of the hood to form a dense layer of knob-like elements. *h* hood, *mt* main tooth, *sh* shaft, *t* smaller teeth.

2.3.3 Chaetogenesis

Chaetogenesis is a continuous process. Due to our methods (TEM of fixed material), this process was inferred from a series of different stages. Three of them are illustrated in this paper, one showing the formation of the teeth (Fig. 2.6A-D), the second showing the beginning of the formation of the hood (Fig. 2.6E-H), and a third showing completion of the hood (Fig. 2.7A-D). For comparative reasons formation of an acicula (Fig. 2.7E) and a reconstruction of lumbrinerid hooded hook chaetogenesis (Fig. 2.8A-F) are shown as separate figures.

Chaetogenesis of hooded hooks can be divided into two steps, because the formation of the hood is spatially and temporarily separated from the development of the remaining apical section of the chaeta. In the following description chaetogenesis of the apical teeth and the adjacent part of the manubrium will, thus, be separated from the formation of the hood. In terms of topology we therefore will distinguish an inner *anlage* that gives rise to the spines and apical manubrium from an outer *anlage* that gives rise to the hood.

Formation of hooded hooks occurred within an ectodermal invagination that was continuous with the exterior and filled with some extracellular material. This compartment was surrounded by five prospective follicle cells; a single large cell was at the base of this cavity. All cells were interconnected by adluminal adhaerens junctions that linked an inner subapical net of actin filaments (Fig. 2.6A). Chaetogenesis started when a few small, ellipsoid clusters of microvilli appeared on the surface of the chaetoblast. These microvilli extended into the extracellular compartment surrounded by the follicle cells; each cluster of microvilli was isolated from the adjacent one by protrusions of the first basalmost follicle cells. While chaetal material that was released between the bases of the microvilli polymerised, additional clusters of microvilli were generated that were composed of a lesser number of microvilli than the previous ones. These were added to the existing clusters along a rostro-adrostral gradient, so that the

oldest clusters were situated rostrally. During this process, additional microvilli arose peripherally to each cluster and enlarged it until it consisted of 2-5 rows of 9-11 microvilli. Each cluster gave rise to a single apical spine. Since the initially formed clusters were always larger than the subsequently formed ones, the apical spines decreased in size towards the adrostral edge of the chaetal *anlage* (Fig. 2.6B). After the final number of apical spines was achieved, addition of further microvilli reduced the space between the clusters and led to a merger of all microvilli (Fig. 2.6C,D). When formation of the apical spines was completed, all spines were aligned along the rostro-adrostral axis of the *anlage*. By that time microvilli had begun to withdraw from the spines, and the canals left by them were refilled by electron-dense material. Formation of the inner *anlage*, which would become the apical-most hooked part of the chaeta, was now complete. Additional electron-dense material released from vesicles of the first two follicle cells formed an enamel that covered the irregular surface of the chaeta (Figs. 2.6C, 2.7D). This material was produced inside Golgi stacks. The first two follicle cells and the chaetoblast interdigitated such that a protrusion of the follicle cells deeply extended into the chaetoblast and surrounded the inner *anlage* (Figs. 2.6C,D, 2.8A,D).

During this initial phase of chaetogenesis the inner *anlage* continuously sank into the chaetoblast. First, second, and third follicle cells that enwrapped the inner *anlage* during formation followed this movement and formed a small cytoplasmic ring that surrounded the *anlage* and separated it from the chaetoblast (Fig. 2.6C,G). This ring was connected to the follicle cells with small rostral and adrostral cytoplasmic bridges that pierced the chaetoblast (Fig. 2.8B,E). The chaetoblast thus formed lateral walls on either side of the rostro-adrostral axis of the inner *anlage*; the walls surmounted the inner *anlage*. The chaetoblast now started to generate large clusters of microvilli on top of these lateral walls, and initiated formation of the outer *anlage* that would become the hood (Figs. 2.6E, 2.7D). Since

these microvilli extended beyond the inner *anlage*, chaetal material released between the bases of the newly formed microvilli formed a cap-like structure that extended beyond the inner *anlage* on either side of its rostro-adrostral axis (Fig. 2.8B,E). Both cap-like structures remained adrostrally separated by the bridge-like connection between the cytoplasmic ring and perikarya of the first, second, and third follicle cells (Fig. 2.6F). The rostral gap was caused by the position of the third follicle cell in a similar manner (Fig. 2.6E). In fully differentiated hooded hooks, thus, an apical slit indicated the former position of the follicle cells (Fig. 2.5C). At this stage, an inner cluster of microvilli forming the inner *anlage* and two groups of outer microvilli forming the outer *anlage* could be discriminated (Figs. 2.7C, 2.8E,F). During further development, additional outer microvilli were formed to broaden the outer *anlage* of the hood. Finally, both microvilli clusters fused on the rostral side, forming a horseshoe-shaped patch of microvilli (Fig. 2.7A,B). The adrostral cleft was caused by the position of the first and the third follicle cell (Fig. 2.7B). Compared to the inner *anlage*, release and polymerisation of chaetal material was accelerated in the outer *anlage*. This led to structural differences between hood and shaft in the fully differentiated hooded hook. The latter was more compact and stained much more electron-densely than the hood, where electron-dense fibres, which marked the former position of the microvilli, appeared loosely connected by electron-lucent material (Fig. 2.7A,B,D). Merging of microvilli of the inner *anlage* reduced its diameter and initiated formation of the chaetal shaft or manubrium (Fig. 2.8C,F). When by differential deposition of chaetal material the outer and inner group of microvilli were finally on the same level, the first and second follicle cells began to retract (Fig. 2.7B). Thus, a large gap remained between the hood and the hooked part, which is characteristic for fully differentiated hooded hooks.

Figure 2.6 *Lumbrineris tetraura*. Chaetogenesis of hooded hooks. **A-D** Serial sections showing formation of the teeth preceding formation of the hood. Note the interaction of follicle cells 1 and 2 (*FC1, FC2*) with the chaetoblast (*CB*), which influences the prospective structure of the chaeta. Large arrows mark adhaerens junctions; small arrows label bundles of actin filaments. **A** Electron-dense tips of small teeth (*t*) and main tooth (*mt*). Note several canals inside each, filled with electron-dense material. **B**. The teeth are preformed by several microvilli and are empty after the withdrawal of the microvilli. **C** Microvilli (*mv*) fuse to preform the shaft. **D** The chaetoblast interdigitates with the follicle cells. Distances between serial sections are **A-B**, 1.7 μm; **B-C**, 2.2 μm; and **C-D**, 2 μm. **E-H** Serial sections showing formation of the hood. Note interactions between the chaetoblast and various follicle cells (*FC1-FC4*). Morphogenetic changes of cells are inferred from a high density of actin filaments (*af*).

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← **E** The hood is preformed by microvilli (*mv*) of two latero-apical lobes of the chaetoblast. The central part of the chaeta has already been completed. **F** Main tooth (*mt*) indicates re-orientation of the anlage. **G** *FC1* forms a cytoplasmic ring around the chaetal anlage. **H** Microvilli preforming the shaft sunk deeply into chaetoblast. An asterisk indicates the coelom. Distances between serial sections are **E-F**, 3.5 µm; **F-G**, 7.4 µm; and **G-H**, 2.6 µm. *af* actin filaments, *CB* chaetoblast, *ECM* extracellular matrix, *FC* follicle cell, *mt* main tooth, *mv*, microvilli, *n* nucleus, *t* small teeth.

The microvilli of the outer *anlage* were now on the same level as those of the inner *anlage*, and surrounded them (Fig. 2.7C). Both merged during further development, although both could still be discerned for a while since by their microvilli differed in diameter. Deposition of chaetal material between the bases of the microvilli and their continuous withdrawal proceeded, and the manubrium became longer. No significant modification of the microvilli pattern occurred in this phase of chaetogenesis. During elongation the newly formed seta was pushed towards the surface, and finally became visible externally. Formation of aciculae was much simpler. The basal chaetoblast continuously added microvilli to a central core group of microvilli. While chaetal material was continuously released between the bases of the microvilli, the diameter of the aciculum enlarged.
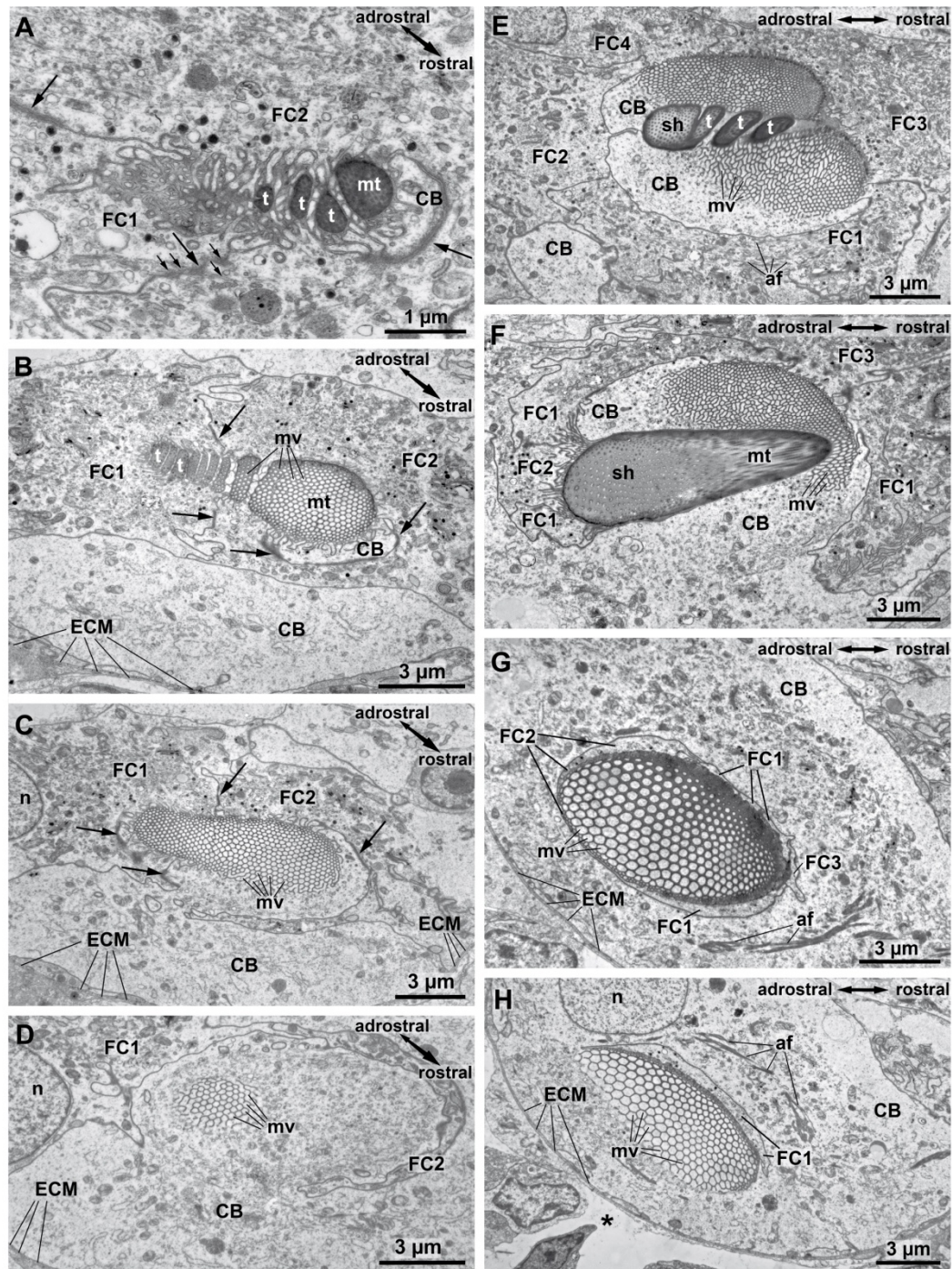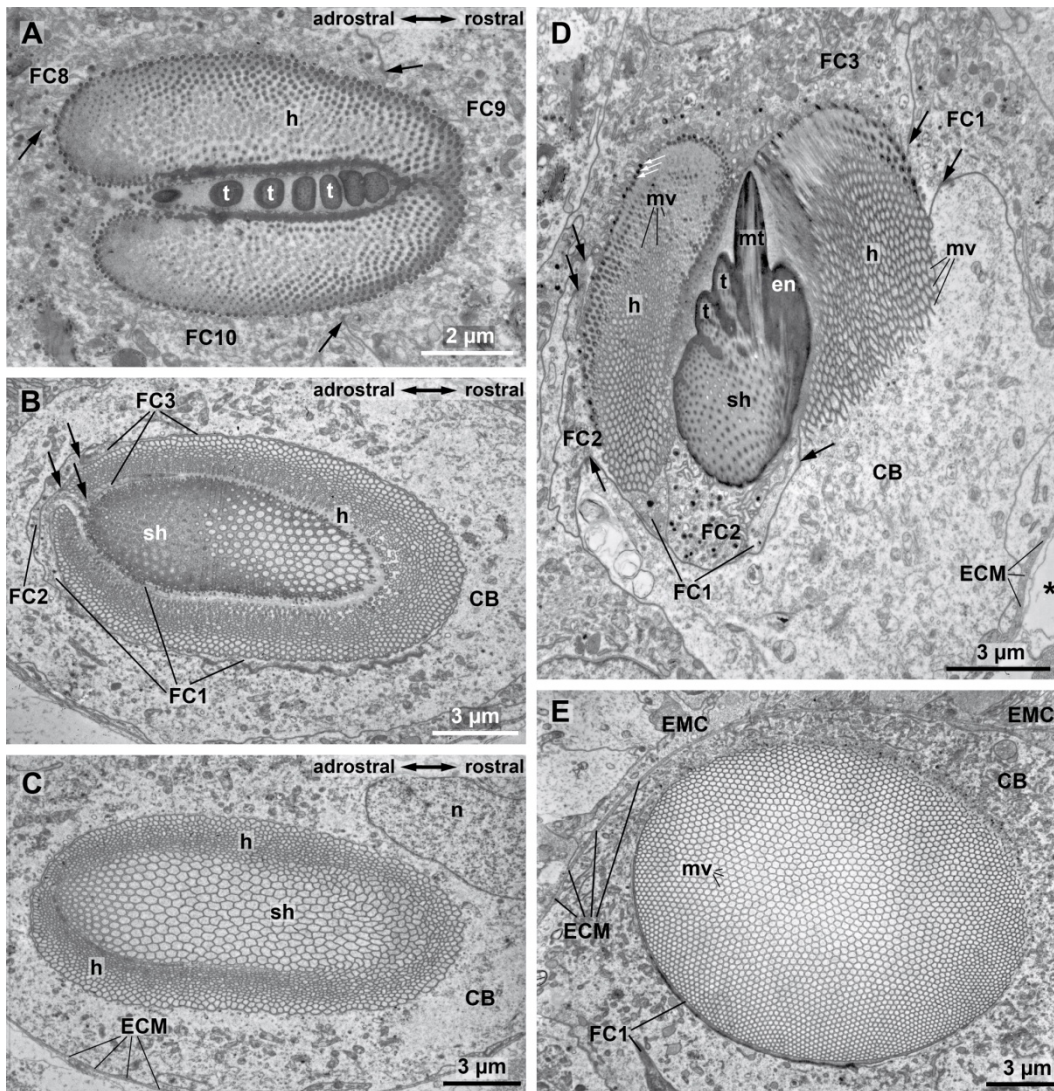
Figure 2.7 *Lumbrineris tetraura*. Chaetogenesis of hooded hooks. **A-C** Serial sections showing formation of the hood. **A** Completed hood with central teeth (*t*). Note the electron bright filling between the electron-grey chitinous rods and their dark filling. Large arrows mark adhaerens junctions. **B** Protrusion of follicle cells (*FC*) preform a gap between hood and shaft (*sh*) of the chaeta. Entire anlage is surrounded by chaetoblast (*CB*). Large arrows mark adhaerens junctions. **C** Merger of microvilli preforming the shaft and the hood (*h*). Distances between serial sections are **A-B**, 15 µm; and **B-C**, 3 µm. **D** Semisagittal setion of hooded hook *anlage*. Follicle and chaetoblast interact during formation of shaft (*sh*) and hood (*h*). White arrows mark the canals filled with electron dense material. **E** Transverse section of aciculum-forming chaetoblast. *CB* chaetoblast, *ECM* extracellular matrix, *EMC* epitheliomuscular cells of coelomic lining, *en* enamel, *FC* follicle cell, *h* hood, *n* nucleus, *sh* shaft, *t* teeth
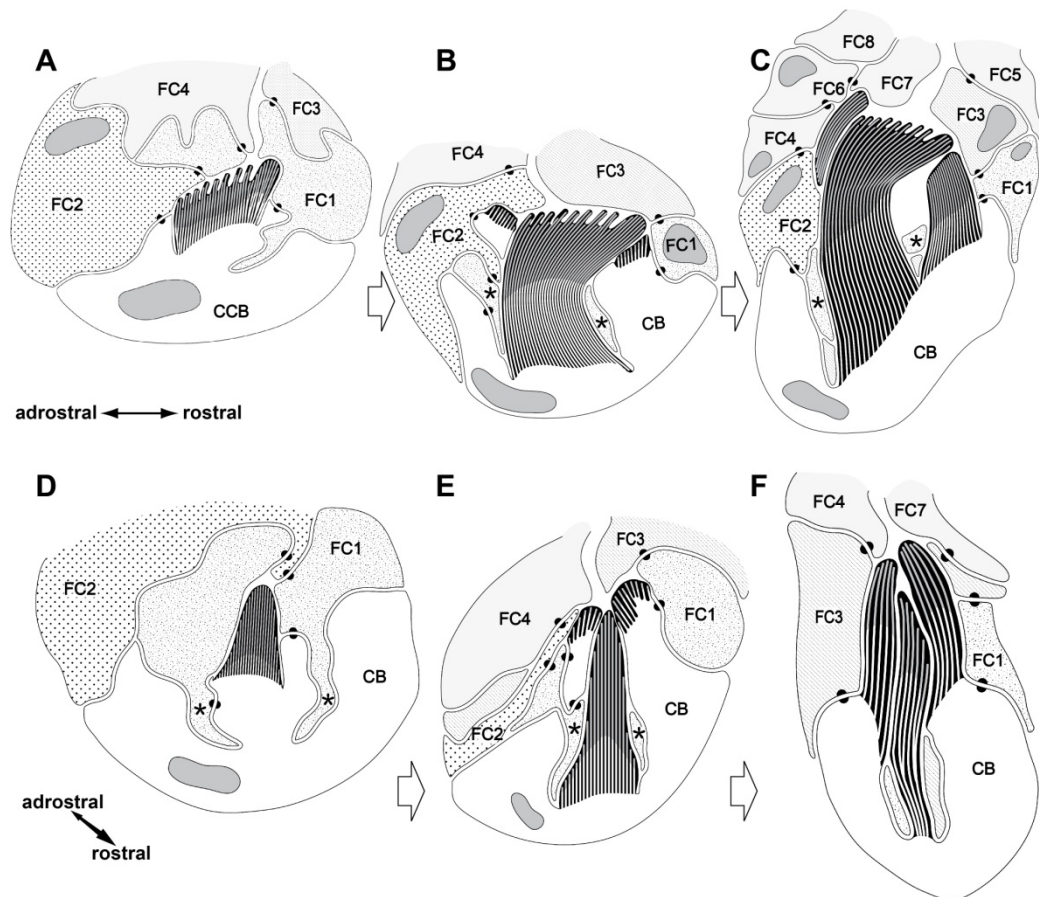
Figure 2.8 Schematic representation of chaetogenesis as series of sagittal (A-C) and transverse (D-F) sections of subsequent representative stages of the *anlage* to illustrate the interaction between the chaetoblast and the follicle cells. The follicle cells are numbered along the baso-apical axis of the anlage. **A**,**D**. Earliest stage with apical teeth. **B**,**E**. Formation of the hood by lateral lobes of the chaetoblast. **C**,**F**. Final stage of hood formation. Asterisks mark the cytoplasmic ring around the chaetal *anlage*. CB, chaetoblast; FC, follicle cell.

The small tip and the rapid increase in diameter can be seen in isolated aciculae (Fig. 2.5B). When the final diameter was reached, no further change in the microvilli pattern of the chaetoblast occurred; the diameter of the chaeta remained constant for its entire length. Electron-dense vesicles released their contents into the small gap between chaetoblast and chaetal *anlage* to form an electron-dense enamel around the acicula (Fig. 2.7E).

2.4 Discussion

In Eunicida the notopodia are either partially or completely reduced (Tilic and Bartolomaeus 2014). In both of the analyzed species of *Lumbrineris*, chaetal elements of the rudimentary notopodia were still present. In *Eunice torquata* Quatrefages 1866 the notopodium is reduced to a dorsal cirrus, which still contains chaetal elements. In *Lysidice ninetta* Audouin & Milne-Edwards 1833, however no reduced chaetal elements of the rudimentary notopodia are present (Tilic and Bartolomaeus 2014). All species of the Eunicida possess at least hooded hooks in addition to seamed capillary chaeta and all additional kinds of chaetae have evolved within this group (Tilic and Bartolomaeus 2014).

Hooded hooks are also described from spionid, magelonid and capitellid polychaetes (Hausen 2005). The hooded hooks in species of all three groups are basically arranged in double rows, but always possess a single formative site within a parapodium. This formative site is always located at the ventral edge of the notopodial chaetal sac and at the dorsal edge of the neuropodial chaetal sac (Hausen & Bartolomaeus 1998 and literature therein). In both lumbrinerid species studied here, hooded hooks and capillary chaetae are aligned in a single row, which may show a zig-zag-pattern. Each row and each chaetal sac contains a ventral and a dorsal formative site. To find out whether this pattern is characteristic for all Eunicida will be part of our ongoing studies into chaetogenesis in this group.

Hooded hooks are described from all lumbrinerid species (Carrera-Parra 2006). It is remarkable that in *L. tetraura* and *L. fragilis* there are two formative sites in each chaetal sac. Though the process of chaetogenesis has not been described in other lumbrinerids, the process described here for *L. tetraura* and *L. fragilis* is assumed to be characteristic of Lumbrineridae. Provided this assumption is true, the similarities between lumbrinerid hooded hooks and those of spionids, magelonids, and capitellids (Hausen 2005) result from different processes (Table 1.1). After summarizing the course of hooded

hook chaetogenesis in both groups as well as that of hooked chaetae and uncini in related taxa, we will return to this point.

2.4.1 Hooded hooks, hooked chaetae, and uncini in other annelids

In a series of papers it was argued that the course of chaetogenesis substantiates the hypothesis of a homology of hooked chaetae and uncini in arenicolids, maldanids, terebellids and sabellids and hooded hooks in spionids, magelonids and capitellids (summarized in Hausen 2005). In arenicolids, maldanids, terebellids, and sabellids the uncini or hooked chaetae consist of a large spine or rostrum (main fang, main tooth) that is surmounted by several smaller spines (teeth) that together comprise the capitium (Hausen 2005). The main axis of the shaft or manubrium is always perpendicular to that of the rostrum. Below the rostrum the manubrium is enlarged to form a subrostral process. In hooded hooks of capitellids and spionids this apical section is enwrapped by a hood consisting of two chitinous lamellae. Uncini, hooked chaetae, and hooded hooks are aligned in a single or double row within a chaetal sac. In all species of these two taxa studied, chaetogenesis occurs in a single formative site in each sac. Chaetogenesis of uncini and hooked chaetae always starts with a group of microvilli that are a template for the rostrum. Continuous release of N-acetyl-glucosomine at the base of the microvilli forms the rostrum. Each of the subsequently formed larger microvilli is a template for a tooth of the capitium. During formation of these larger microvilli, the axis of the chaetoblast shifts so that the spines of the capitium surmount the rostrum after uncini formation has been completed. When the axis of the chaetoblast is shifting, additional microvilli are generated below the rostrum; these fuse with those that formed the rostrum and capitium. Merging of microvilli generates the shaft of the chaeta. In capitellids, magelonids, and certain spionids this structure is altered by an apical hood enwrapping the rostrum, capitium and the

apical portion of the uncini (Hausen 2005). These chaetae are traditionally called hooded hooks. Microvilli which appear on the surface of the chaetoblast after the axis of the chaetoblast begins to shift are the template for the hood. Since the main tooth is perpendicular to these microvilli, they form an adrostral, horseshoe-shaped group so that a rostral pore (Spionidae) or slit (Capitellidae) remains after the hood is completed. During formation the microvilli split into two layers, an inner and an outer one. These layers are templates for the inner and the outer lamella of the hood that surrounds rostrum and capitium in *Capitella capitata* (Fabricius 1780) (Capitellidae) and the bifid tip of the *Scolelepis squamata* Müller 1806 (Spionidae). Merging of the inner layer and the manubrium precedes fusion of the outer layer and the shaft. Due to developmental correspondences a general homology between uncini and hooded hooks of capitellid species has been proposed (Hausen & Bartolomaeus 1998; Schweigkofler et al. 1998; Bartolomaeus et al. 2005; Hausen 2005). Subsequent studies into the chaetogenesis of additional spionids and magelonids showed some differences, but largely confirmed this pattern (Hausen 2001; Hausen 2005) (Table 1.1).

Table 1.1 Structure and chaetogenesis of hooded hooks in capitellid, spionid, and lumbrinerid species

	Lumbrineris species (Lumbrineridae, Eunicida)	*Capitella capitata* (Capitellidae)	*Scolelepis squamata* (Spionidae, Spionida)
Hooded hook substructures			
main tooth	+	+	+
smaller teeth	surmount main tooth	surmount main tooth	surmount main tooth
smaller teeth number	at leat 10	more than 10	1
smaller teeth position	single adrostal row	several adrostal rows	adrostal to main tooth
shaft	+	+	+
hood	peripherally condensed rods	inner and outer lamella	inner and outer lamella
hood compartment	discontinuously filled with chitinous rods	electron-lucent (empty) with a few fibrils	electron-lucent (empty)
hood surface	electron-dense knobs	electron-dense knobs	electron-dense knobs
hood opening	adrostal slit	rostral slit	rostral pore
Hooded hook formation			
main tooth	group of microvilli	group of microvilli	group of microvilli
smaller teeth	group of microvilli	single microvillus each	group of microvilli
shaft	group of microvilli	group of microvilli	group of microvilli
hood	2 lateral groups of microvilli	several crescent rows of microvilli	2 crescent rows of microvilli
hood compartment	not present	between rows of microvilli	between rows of microvilli
fusion with shaft	entire hood	inner and outer hood lamella sequentially	inner and outer hood lamella sequentially

2.5 Conclusions

There are several structural and developmental differences between the hooded hooks in the lumbrinerid species studied and those of spionids and capitellids. These concern the formation of the apical portion of the chaetae and the formation of the hood (summarized in Table 1.1). While in *C. capitata* and certain spionids only the

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rostralmost tooth of the hooded hook, the rostrum, is preformed by a group of microvilli, those teeth that surmount the rostrum are preformed by a single large microvillus each. In *L. fragilis* and *L. tetraura*, all teeth of the hooded hook are formed at the same time; there is no delay in their appearance. Their hood is formed after the apical portion of the hooded hook has been completed and it is formed by a homogeneous group of microvilli that arise from that part of the chaetoblast which extends beyond the apical inner part of the chaeta. There is no outer and inner lamella formed from different groups of microvilli as in spionids and capitellids. An adrostral slit in the hood is caused by follicle cells during the course of formation, while the rostral cleft or pore in capitellid and spionids is caused by the position of the rostrum. Formation of the hood then is accelerated relative to the remaining chaeta so that both inner apical portion and hood finally merge and form the manubrium in lumbrinerids. Merging of the inner hood and the outer hood in spionids, magelonids and capitellids, however, is sequential (Hausen & Bartolomaeus 1998; Schweigkofler et al. 1998; Hausen 2005). These differences argue for a convergent evolution of hooded hooks in the lumbrinereids and the other taxa mentioned.

Differences between similar structures either result from transformations of an ancestral structure, or from convergent evolution. Differences alone, thus, do not decisively indicate convergent evolution; the latter must be the most parsimonious explanation in the context of a phylogenetic tree. According to morphological data, Eunicida, Phyllodocida, and Amphinomida form a monophyletic taxon called Errantia or Aciculata, characterized by internal chaetae (aciculae) and antennae among other traits (Rouse & Fauchald 1997; Bartolomaeus 1998). A taxon Errantia consisting of Phyllodicida, Eunicida, Amphinomida and Orbiniidae is monophyletic in recent molecular analyses by (Struck 2011; Struck et al. 2011, 2014). Weigert et al. (2014) could confirm the monophyly of Errantia, if Amphinomida

were excluded. Hooded hooks are absent in species of Phyllodocida, Orbiniida, and Amphinomida, but present in species of the Eunicida. Provided that the mode of formation of hooded hooks of *L. fragilis* and *L. tetraura* is characteristic of all lumbrinerids and also for eunicids, the most recent tree in Weigert et al. (2014) as well as the trees of Rouse & Fauchald (1997) and Struck et al. (2011, 2014) clearly favour the assumption that hooded hooks in Eunicida are not homologous to those in spionids and capitellids. According to these trees, it is more likely that hooded hooks evolved at least twice in annelids, corroborating our conclusion that the differing course of chaetogenesis of lumbrinerid hooded hooks reflects convergent evolution with eunicidan hooded hooks.

2.6 Acknowledgements

Phylogenetic significance of chaetal arrangement and chaetogenesis in Maldanidae (Annelida)

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Abstract

Chaetae are important structures to facilitate locomotion in annelids. Being at the interface between the organisms and its environment, chaetae are supposed to underlie strong functional constraints to optimize the relation between structure and function. As such chaetae are potentially susceptible for convergent evolution. On the other hand, chaetae gained enormous taxonomic importance due to their conservative structure in species and supraspecific taxa which reasonably can only be explained by strong evolutionary constrains that conserve their structure. In this paper, we study the chaetation and chaetogenesis in two species of Maldanidae, *Clymenura clypeata* Saint-Joseph 1894 and *Johnstonia clymenoides* Quatrefages 1866 to unravel conservative traits in their structure and development. In a literature survey across maldanids, we address questions on the ontogenetic variation, on homology and on the phylogenetic significance especially of the bearded hooked neurochaetae. We provide evidence that functionally constraint ontogenetic variation overlies historically (phylogenetically) constraint expression of structural information and can show that within maldanids a variety of different

chaetal types must be homologous due to their ontogenetic continuity. Furthermore, we use chaetation and chaetal characters to discuss the subgroup relationships within Maldanomorpha in the light of recent cladistics analyses based on morphological and molecular data. This study shows that functional considerations need to use phylogenies as backbone.

3.1 Introduction

Chaetae are chitinous epidermal structures that are one of the most important diagnostic characters to identify annelid species (Fauchald 1977; Schroeder 1984). This is due to the constancy of chaetal arrangement and structure within annelid species and supraspecific taxa. Chaetae are extracellular structures that are formed within an ectodermal pouch, called chaetal follicle (Bouligand 1967; Schroeder 1984; Specht and Westheide 1988; Hausen 2005). It consists of a few follicle cells and a basal chaetoblast. During chaetogenesis, the apical microvilli pattern of the chaetoblast is constantly modified, while N-acetylglucosamine is released between the bases of the microvilli (Hausen 2005; Souza et al. 2011; Ogawa et al. 2011; Koide et al. 2015). Extracellular polymerization of this material finalizes the formation of chaetae. Hence, the definitive structure of the chaetae is primarily caused by changes in the microvilli pattern, i.e., by dynamic microvilli (O'Clair and Cloney 1974). Since structure, orientation and number of microvilli and modulation of these three factors during chaetogenesis determine the chaetal structure, chaetogenesis must underlie strict process regulation. This is necessary to guarantee that chaetae are identical within an individual, a population or a species. Regulation of this pattern appears to be conservative enough that certain chaetae, once evolved, can be passed on to descendants and, thus, become also characteristic for supraspecific taxa.

Hooked chaetae in annelids are present in a larger number of annelid taxa, such as Sabellidae, Siboglinidae, Terebellida, Arenicolidae and Maldanidae (Fauchald 1977; Rouse and Pleijel 2001). These chaetae consist of a main tooth or rostrum sensu Holthe (1986), several smaller teeth surmounting the rostrum and comprising the capitium sensu Holthe (1986), and a shaft or manubrium. Hooked chaetae are generally aligned in a single or a double row. After being formed on one end of the row, the chaetae appear on the surface, come into function, become worn out and finally degenerate at the opposite end of the row. Rows of hooked chaetae are therefore flanked by a formative side and a site of chaetolysis (Pilgrim 1977; Hausen 2005). The formative site generally contains several developing chaetae, making them an ideal subject to study chaetogenesis. In a number of studies, we analyzed the formation and potential phylogenetic significance of hooked chaetae (Bartolomaeus 1995a, 1998, 2002; Bartolomaeus and Meyer 1997; Meyer and Bartolomaeus 1996, 1997).

The identity of chaetal types across annelid taxa leads to assume that strong historical (evolutionary) constraints influence the presence or absence of certain types of chaeta. Chaetae and chaetogenesis therefore should contain a strong phylogenetic signal (Hausen 2005; Tilic et al. 2014, 2015). On the other hand, chaetae arise from the annelid body surface and thus are at the interface between the environment and the animal. Therefore, environmental constraints are also expected to influence the absence or presence of certain types of chaetae. This aspect has repeatedly been addressed by Merz and Woodin (2000, 2006) for the hooked chaeta of certain "sedentary" polychaetes (Woodin and Merz 1987; Merz 2015). Due to the resemblance of hooked chaetae to tiny anchors, Woodin and Merz (1987) argue that hooked chaetae underlie strong environmental constraints as polychaetes with such chaetae live in tubes internally lined by a mucus layer (Merz 2015). If, however, the hooked chaetae evolved repeatedly as an answer to ecological constraints, one would

expect differences in their structure and formation that can be interpreted as indicators for convergent evolution. The degree of identity of chaetae in different species, therefore, should be a first indicator for the robustness of the hypothesis on their primary homology.

In this paper, we investigate the ultrastructure and formation of chaetae in *Clymenura clypeata* Saint-Joseph 1894 and *Johnstonia clymenoides* Quatrefages 1866 to test chaetal arrangement, chaetae and chaetogenesis as a character source for unraveling phylogenetic relationships within the Maldanidae and the position of this group within Annelida. The recent cladistic analysis of De Assis and Christoffersen (2011) will be used to particularly analyse three aspects in more detail, (1) the influence of the structure of the chaetal sac on the chaetal arrangement, (2) the influence of ontogenetic variation in hooked chaeta on homology hypotheses and (3) the significance of chaetogenesis on phylogeny hypotheses with respect to evolutionary and functional constraints.

3.2 Methods

3.2.1 Animals

Clymenura clypeata (Staint Joseph 1864) (Euclymeninae, Maldanidae) was collected during low tide at two different localities at the Atlantic coast in France, i.e., on a sandy beach at Cap Ferret in the Bay of Arcachon (France) in September 1994 and on the sand bar in the Bay of Poldouhan close to Concarneau (Brittany, France) in September 1995 and April 2012. *Johnstonia clymenoides* Quatrefages 1866 (Euclymeninae, Maldanidae) was found in muddy sediments between stones at low tide close to Plozevet (Brittany, France) in September 1995. In April 2013, they were collected from a comparable habitat at Le Cabellou close to Concarneau. The animals were fixed for light and electron microscopical studies. All animals were adults, most of them with genital products inside the coelomic cavity.

3.2.2 Transmission and scanning electron microscopy

Specimens used for transmission electron microscopy (TEM) were fixed in 2.5% glutaraldehyde buffered in 1 M sodium cacodylate at room temperature for 1 h. Later, the specimens were postfixed in 1% OsO_4 – 1M sodium cacodylate buffer. The specimens were then dehydrated through an ascending acetone series and then transferred via propylene oxide to Araldite. When necessary, the specimens were fragmented into smaller pieces within the resin. Polymerization was started with BDMA (benzyldimethylamine).

Chaetal follicle and formative sites of three different individuals of each species were sectioned into a complete series of silver-interference-colored ultrathin sections (70–75 nm), using diamond knives on a Reichert Ultracut E microtome. These were placed on formvar-covered, single-slot copper grids and stained with uranyl acetate and lead citrate in an automated TEM stainer (QG-3100, Boeckeler Instruments). The sections were examined using Zeiss EM 10B and Zeiss Libra 120 kV transmission electron microscopes. The chaetal formation was reconstructed using the information gathered from serial ultrathin sections of three formative sites and two series of semithin sections of *C. clypeata*, one series of ultrathin sections in *J. clymenoides* (Fig. 3.9). The coverage of different stages of chaetogenesis of hooked chaetae was dense enough to allow insights into the dynamics of the entire process that will be described in the following.

For scanning electron microscopy (SEM), *C. clypeata* and *J. clymenoides* were fixed in Bouin's fluid and dehydrated in an alcohol series, and the specimens were kept in a 5 % phosphotungstic acid solution for an hour to increase the heavy metal content in the tissue. They were critical point dried with CO2 in a critical point dryer (CPD 030, Bal-TEC) and sputtered with gold (SCD 005, BAL-TEC). The specimens were examined in Novoscan 30 (Zeiss) and Leitz AMR 1000 scanning electron microscopes (SEM). During dehydration, the animals were sonified to remove debris and sand particles from the chaetae.

3.2.3 Confocal laser scanning microscopy

The specimens used for confocal laser scanning microscopy (CLSM) were fixed in 4 % paraformaldehyde in seawater. The chaetigers were dissected to separate single parapodia or single segments. Isolated parapodia were permeabilized in four 5-min changes in 0.01 M PBS (phosphate buffered with saline, pH 7.4) with 0.1 % Triton X-100 (Fisher Scientific). The parapodia were then stained overnight in 4 °C with TRITC phalloidin at a dilution of 1:100. After staining, parapodia were rinsed in three quick changes, subsequently in two 10-min changes in PBS with 0.1 % Triton and one 10-min rinse in PBS without Triton. The samples were quickly dehydrated in isopropanol (2 min 70 %, 2 min 85 %, 2 min 95 %, 2 min 100 %, 2 min 100 %), cleared in three 15-min changes in Murray's clear and mounted in hollow-ground slides with Murray Clear.

3.2.4 Light microscopy, histology and 3D reconstruction

The 3D reconstructions of the chaetae and the chaetal sac were modeled using serial semi-thin sections of 1 μm thickness as a reference. Specimens used for semi-thin sectioning were fixed in 1.25 % glutaraldehyde buffered in 0.05 M phosphate buffer with 0.3 M NaCl for 1.5–2 h. The animals were postfixed in 1 % OsO_4 in 0.05 M phosphate buffer, dehydrated in an acetone series and embedded in araldite. The serial sections were prepared using a diamond knife (Diatome Histo Jumbo) on a Leica Ultracut S ultramicrotome, following the method described by Blumer et al. (2002). The sections were stained with toluidine blue (1 % toluidine, 1 % sodium tetraborate and 20 % saccharose) and mounted with araldite. The semi-thin sections were analyzed with an Olympus microscope (BX-51) and photographed with an Olympus camera (Olympus cc12), equipped with the dot slide system (2.2 Olympus, Hamburg). The images were aligned

with IMOD (Boulder Laboratories, Kremer et al. 1996) and IMOD-align (http://www.evolution.uni-bonn.de/mitarbeiter/bquast/software).

The software 3ds max 13.0 was utilized for the 3D modeling of the chaetae. The histological images were imported as surface materials, and the chaetae were modeled using standard cylindrical or conic objects. When necessary, these were modified as NURBS surfaces.

Chaetae of paraformaldehyde fixed material (see paraformaldehyde fixation for CLSM) were isolated from the surrounding tissue by incubation in 5 % NaOH for 2–3 h in room temperature. The chaetae were rinsed in distilled water, mounted on microscopic slides, examined using Nomarski differential interference contrast with an Olympus BX-51 microscope and photographed with an Olympus Camera (Olympus CC12).

3.2.5 Data repository and voucher material

Voucher specimen collected from the same sampling side is deposited in the collection of the Institute of Evolutionary Biology and Ecology (IEZ), University of Bonn. To allow data transparency, all of the aligned serial sections used for 3D modeling are freely accessible in the morphological database, MorphDBase: https://www.morphdbase.de (Grobe and Vogt 2009).

Johnstonia clymenoides—Aligned serial semi-thin sections of the parapodium.
Direct link: www.morphdbase.de/?E_Tilic_20150220-M-26.1
Clymenura clypeata—Part 1: Aligned serial semi-thin sections of the parapodium.
Direct link: www.morphdbase.de/?E_Tilic_20150220-M-25.1
Clymenura clypeata—Part 2: Aligned serial semi-thin sections of the parapodium.
Direct link: www.morphdbase.de/?E_Tilic_20150220-M-24.1

Clymenura clypeata—Aligned serial semi-thin sections of the neuropodial formative site 1.

Direct link: www.morphdbase.de/?E_Tilic_20150220-M-23.1

Clymenura clypeata—Aligned serial semi-thin sections of the neuropodial formative site 2.

Direct link: www.morphdbase.de/?E_Tilic_20150220-M-22.1

3.3 Results

3.3.1 Structure of the parapodia

In both species, the parapodia are small extensions of the body wall, with distinctly different neuropodia and notopodia (Fig. 3.1a–c). While the notopodia are conical dorsolateral protrusions of the body wall with a bundle or double row of capillary chaetae, the neuropodia consist of an oval, slightly elevated epidermal field called torus, containing numerous torus-specific gland cells. A rim transverse to the anterior–posterior axis of the animal is located in the center of the torus and gives rise to a single row of hooked chaetae.

Notopodial and neuropodial chaetae arise from a chaetal sac that is continuous with the epidermis and is surrounded by ECM (Fig. 3.5f–h). Each chaetal sac is composed of a number of chaetal follicles that are arranged in single row, so that the neuropodial chaetal sac reminds a fin or a blade that extends into the coelom. The chaetal follicle consists of a basal chaetoblast and a number of follicle cells (Fig. 3.5h). Both, chaetoblast and follicle cells are epithelial cells that rest on the ECM ensheathing the chaetal sac. Each chaetal follicle contains a single chaeta (Fig. 3.5f); neither ECM nor any muscles separate individual follicles from each other. The follicle cells are sequentially arranged along the vertical axis of the chaeta. Those of the upper third of the follicle are not firmly attached to the chaeta, whereas the chaetoblast and basalmost follicle cells contain bundles of intermediate filaments that fix the chaeta in the follicle. These filaments often form strong bundles inside the cells.
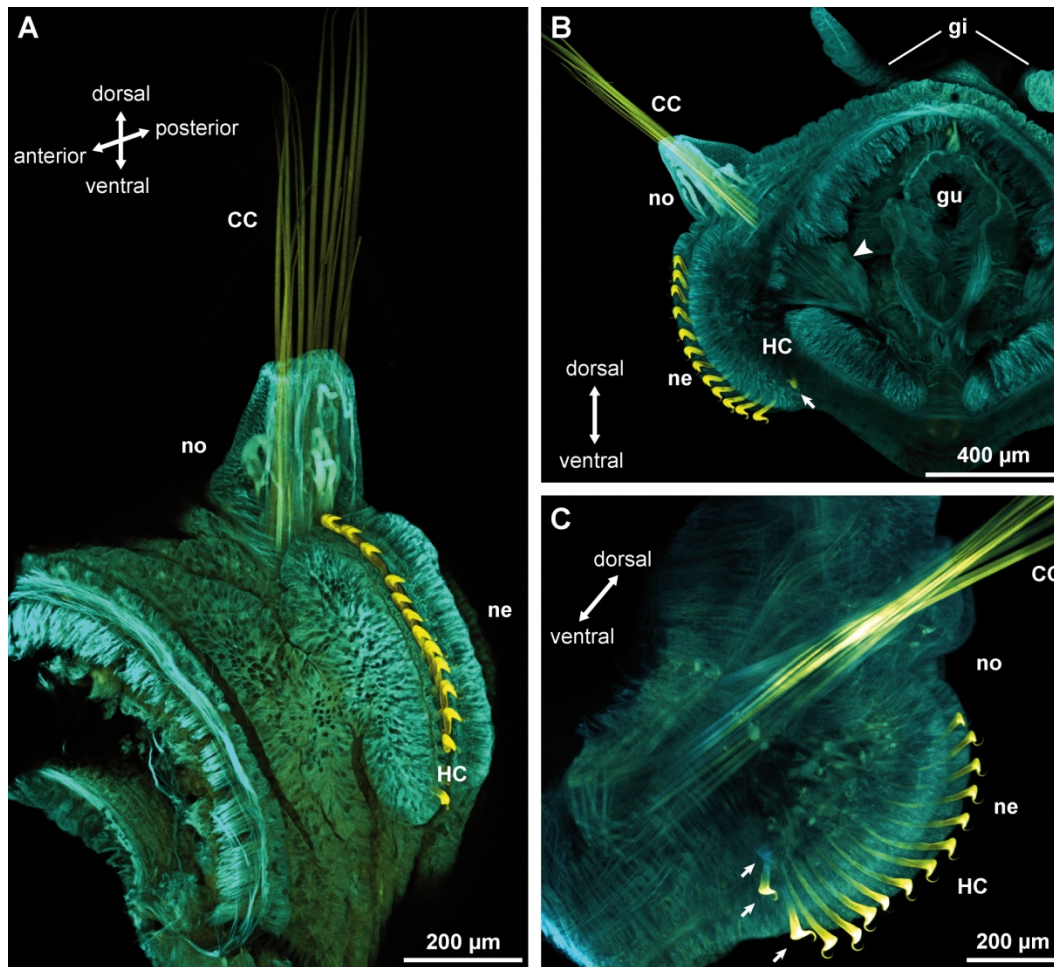
Figure 3.1 Confocal z-projections of phalloidin-stained preparations *Johnstonia clymenoides* (a, b) and *Clymenura clypeata* (c). a, b Notopodium (no) with capillary chaetae (CC) is conical protrusions of the body wall; neuropodium (ne) with transversally aligned hooked chaetae (HC) is slight elevations of the body wall. c Ventral row of chaetae extends deeper into the body of the animal and is bent at its base. Arrows newly formed hooked chaetae. Note their rotation during their course of formation. Arrow head chaetal musculature of notochaetae; gu gut; gi digitiform gills of the posterior segments; yellow auto fluorescence; cyan phalloidin.

Chaetae are continuously replaced in both species and are formed in a special pouch at the ventral edge of the blade-like neuropodial chaetal sac. In notopodia, new chaetae are formed in the center of the chaetal sac and are added in an alternating matter to each side to form a row of chaetae. The chaetal sac thus can be seen as consisting of two blades that are connected by the formative site. The notopodial chaetal sac is folded, so that both blades face each other.
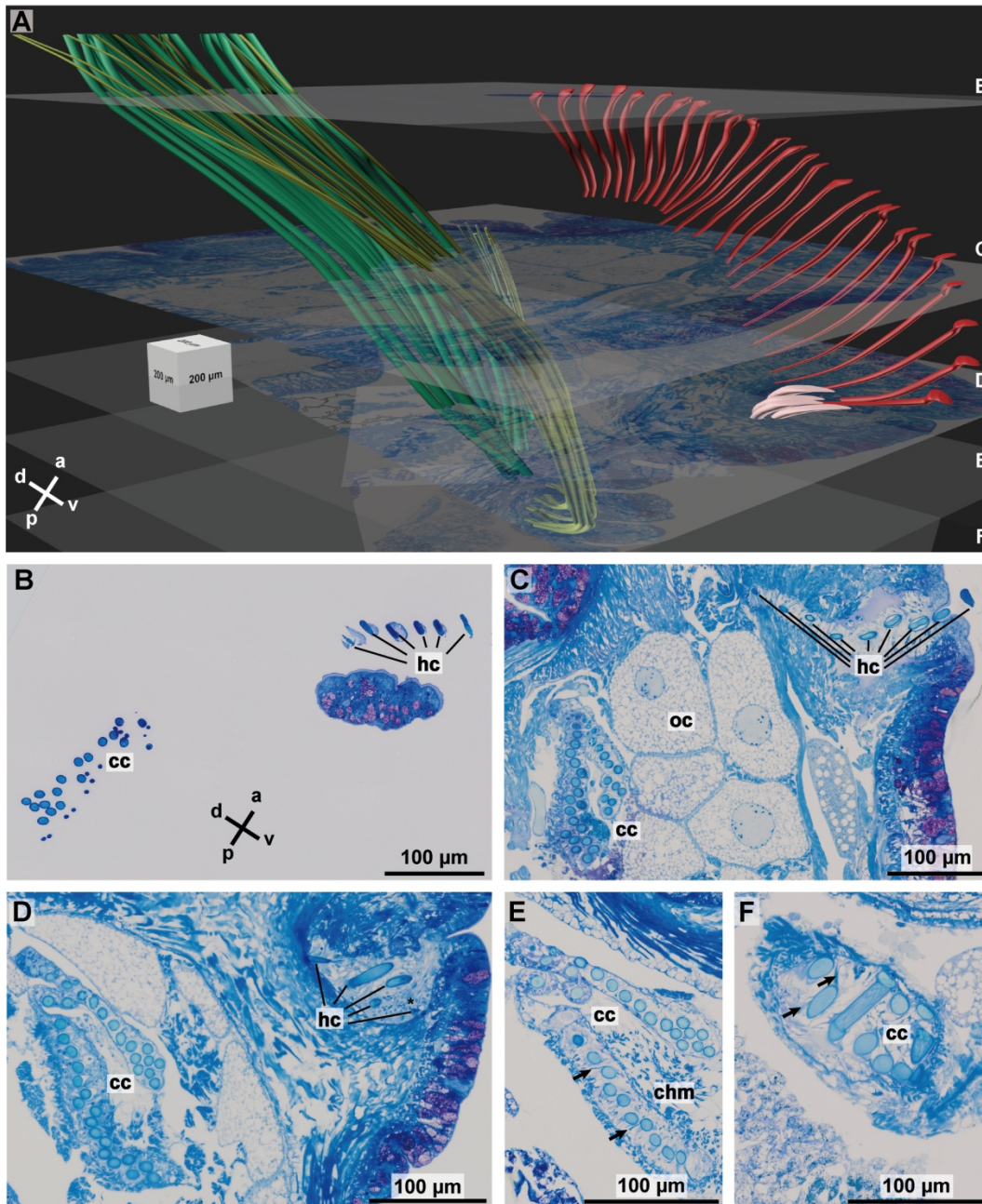
Figure 3.2 *Clymenura clypeata.* a 3D reconstruction of the chaetal arrangement in a posterior segment. Red neuropodial hooked chaetae; yellow ventral row of capillaries, green dorsal row of capillaries; pink developing hooks; light green and light yellow developing notochaetae. Orientation scheme: a anterior, d dorsal, v ventral; p posterior. b– f Aligned semi-thin sections of the chaetal follicles used to generate the 3D reconstruction.
(direct link: www.morphdbase.de/?E_Tilic_20150220-M-24.1;
direct link: www.morphdbase.de/?E_Tilic_20150220-M-25.1).
Corresponding section planes are marked in (a); cc capillary chaetae, hc hooked chaetae, chm chaetal musculature, arrows intermediate filaments, *formative site. Distances between serial sections are b, c 562 µm; c, d 364 µm; d, e 269 µm; e, f 296 µm

The formative site is always located posteriorly, and both halves always point anteriorly. The chaetal sac, therefore, seems to be bifurcated in horizontal sections (Figs. 3.2a, d, e, 3.4c).

In the neuropodia of both species, an anterior and a posterior series of interconnected radial muscles adhere to the ECM of the neuropodial chaetal sac. Each muscle originates on the level of the basalmost follicle cells, crosses the coelomic cavity and adheres on the subepidermal basal lamina. The common ECM that enwraps the chaetal sac and the interconnection of the radial muscles guarantees coordinated action of all or at least groups of chaeta within the follicle, but impedes any independent movement of the chaeta, like rotating around their axis. In the notopodia, the chaetal muscles are basically identical. Radial muscle fibers originating at the basis of the chaetal sac cross the coelom and extend to the outer body wall, giving the chaetae an arrangement similar to an arrow pulled back in a bow (Fig. 3.1b). Upon contraction, these muscles shorten and push the chaetae out of the body surface. Additional radial muscles (protractors) are located between both halves of the notopodial chaetal sac allow to independently move both rows of chaetae, at least to a certain extend (Fig. 3.2e). In fixed animals, one row of capillary chaetae generally appears to be shorter than the other, because it is situated deeper in the body (Figs. 3.1c, 3.2a, 3.3a). This situation is caused by differential contraction of the radial muscles and the structure of the chaetal sac.
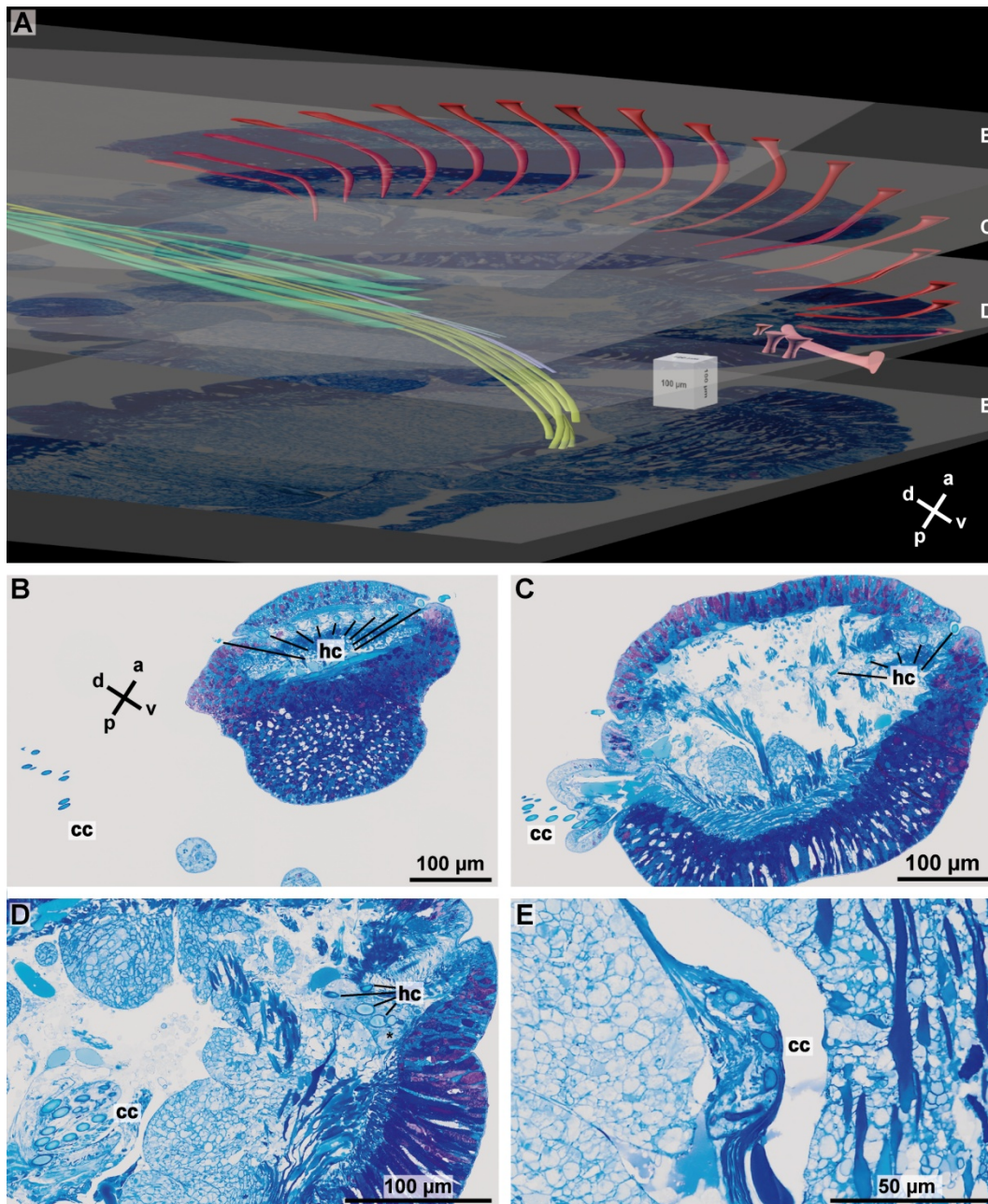
Figure 3.3: *Johnstonia clymenoides*. a 3D reconstruction of the chaetal arrangement in an anterior segment. Red neuropodial hooked chaetae; yellow ventral row of capillaries, green dorsal row of capillaries; pink developing hooks; purple developing notochaetae. Orientation scheme: a anterior, d dorsal, v ventral, p posterior. b–e Aligned semi-thin sections of the chaetal follicles used to generate the 3D reconstruction (direct link: www.morphdbase.de/?E_Tilic_20150220-M-26.1).

Corresponding section planes are marked in (a). cc capillary chaetae, hc hooked chaetae, *formative site. Distances between serial sections are b, c 103 µm; c, d 198 µm; d, e 230 µm

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3.3.2 Notochaetae and chaetal sac

In both species studied, the number of capillary chaetae decreases from anterior to posterior segments. Longer chaetae form an anterior row of chaetae, while the posterior row consists of smaller chaeta. Both rows are transverse to the anterior–posterior body axis until segment 10. From segment 13 onward, both rows are parallel to the anterior– posterior body axis with the longer chaetae forming a dorsal row and the smaller ones forming a ventral row. The transition between both conditions occurs constantly in segments 11 and 12 in both species (Fig. 3.4). When analyzing stacks of horizontal serial sections of the chaetal sac from the animal's surface down to its base, one can see that the differences in chaetal arrangement result from the changes in the structure of the chaetal sac.
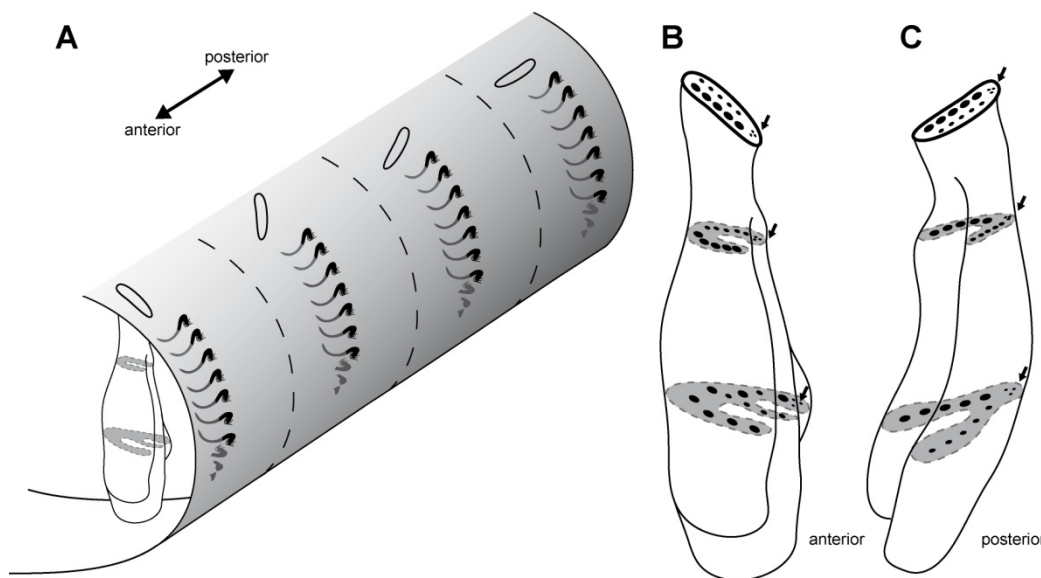
Figure 3.4 a Scheme of stepwise alteration of the notopodial chaetal sac and chaetal arrangement along the antero-posterior body axis between chaetiger 10 and chaetiger 13 in *J. clymenoides* and *C. clypeata*. b Scheme of an involute anterior chaetal sac. c Scheme of an untwisted posterior chaetal sac. Arrows mark site of chaetal formation

As mentioned above, the chaetal sac is bipartite with two anteriorly directed blades connected by a single posterior formative site. This structure can be seen in all posterior segments. In anterior

segments, however, the chaetal sac is involute, whereby the ventral blade is coiled in by the dorsal blade and the formative site (Fig. 3.4b). In these segments, the chaetal sac is reminiscent of an onionskin layering (Fig. 3.3d) and will be called involute chaetal sac. Its formative site is posterior–ventral to the tip of the ventral branch which again is posterior to the tip of the dorsal branch (Fig. 3.4b). During its further course toward the epidermis, the anterio-dorsal part of the chaetal sac rotates counterclockwise through 90° to become the anterior margin of the subepidermal section of the chaetal sac. The chaeta are now transversal to the body axis: those emanating from the dorsal branch form the anterior and those arising from the ventral branch form the posterior row of notochaetae (Fig. 3.4b). The latter do not extend as far above the body surface as those of the anterior row, because the ventral branch of the chaetoblast sits deeper in the animal (Figs. 3.2f, 3.3e). Since all notochaetae taper toward their tip, the posterior row of chaetae always appears to consist of much shorter and smaller chaetae in fixed animals.

3.3.3 Neurochaetae

Except for the first four segments, all hooked chaetae possess a beard; they are formed at the ventral edge of the transverse rim (Figs. 3.1b, c, 3.2a, 3.3a). The hooked chaetae of both species possess several substructures with a specific arrangement. Each hooked chaetae consists of a rostrum, smaller teeth that surmount the rostrum and make up the capitium and a long, curved manubrium (Fig. 3.5e). In both species, the rostrum is curved and bent toward the manubrial axis (Fig. 3.5b, c). This angle is more obtuse in the hooked chaeta of the anterior three chaetigers of *Clynemura clypeata*; rostrum and manubrium are aligned in the acicular spines of the first chaetiger. In both species, the capitium consists of a single row of large teeth slightly offset from the midline of the rostrum (Fig. 3.5a, c). The teeth surmount each other, but decrease in diameter and length so that they

are staggered along the rostro-adrostral axis of the hooked chaetae (Fig. 3.5a). While the longitudinal axis of the largest teeth more or less parallels that of the rostrum, it gradually rotates from tooth to tooth. Smaller teeth on either side of this median row increase in size toward the adrostral side of the capitium until they are as large as the last tooth of the median row. Rostrum and capitium form the tip of the sigmoid manubrium (Fig. 3.5e). During its course from the tip to the base, the manubrium initially runs rostrally (with respect to the chaeta) for onefourth of its length, forms a knee, runs parallel to the transverse axis of the animal, enlarges in diameter to form a ring-like part and then continuously decreases in diameter toward its base. The last one-third of the chaeta is curved adrostrally. A group of long and thin protrusions emerge from the subrostro-lateral section of the manubrium and surround the tip of the rostrum. These protrusions overtop the rostrum and are curved adrostrally, thus resembling the upper canines of the Sulawesi babirusa or pig deer. The entire structure is termed beard (Fig. 3.5a). In all segments following segment two, chaetae are continuously formed in a ventro-posterior pouch of the chaetal sac located slightly lateral to the transverse axis of the neuropodial rim (Figs. 3.2d, 3.3d, 3.5f–h). This pouch marks the formative site of the neuropodium.

3.3.4 Chaetogenesis of hooked chaetae

Each formative site contains several (10–12) developing hooked chaetae at different stages of chaetogenesis. Within each follicle, the apical microvilli of the chaetoblast form the template for the chaeta which consists of chitin released by the chaetoblast and the follicle cells (Figs. 3.6, 3.7, 3.8). Since follicles are continuously generated at the ventral edge of the neuropodial rim, the young follicles are pushed dorsally and take a characteristic course within the ventrolateral chaetal pouch of the chaetal sac (Fig. 3.5f, g).
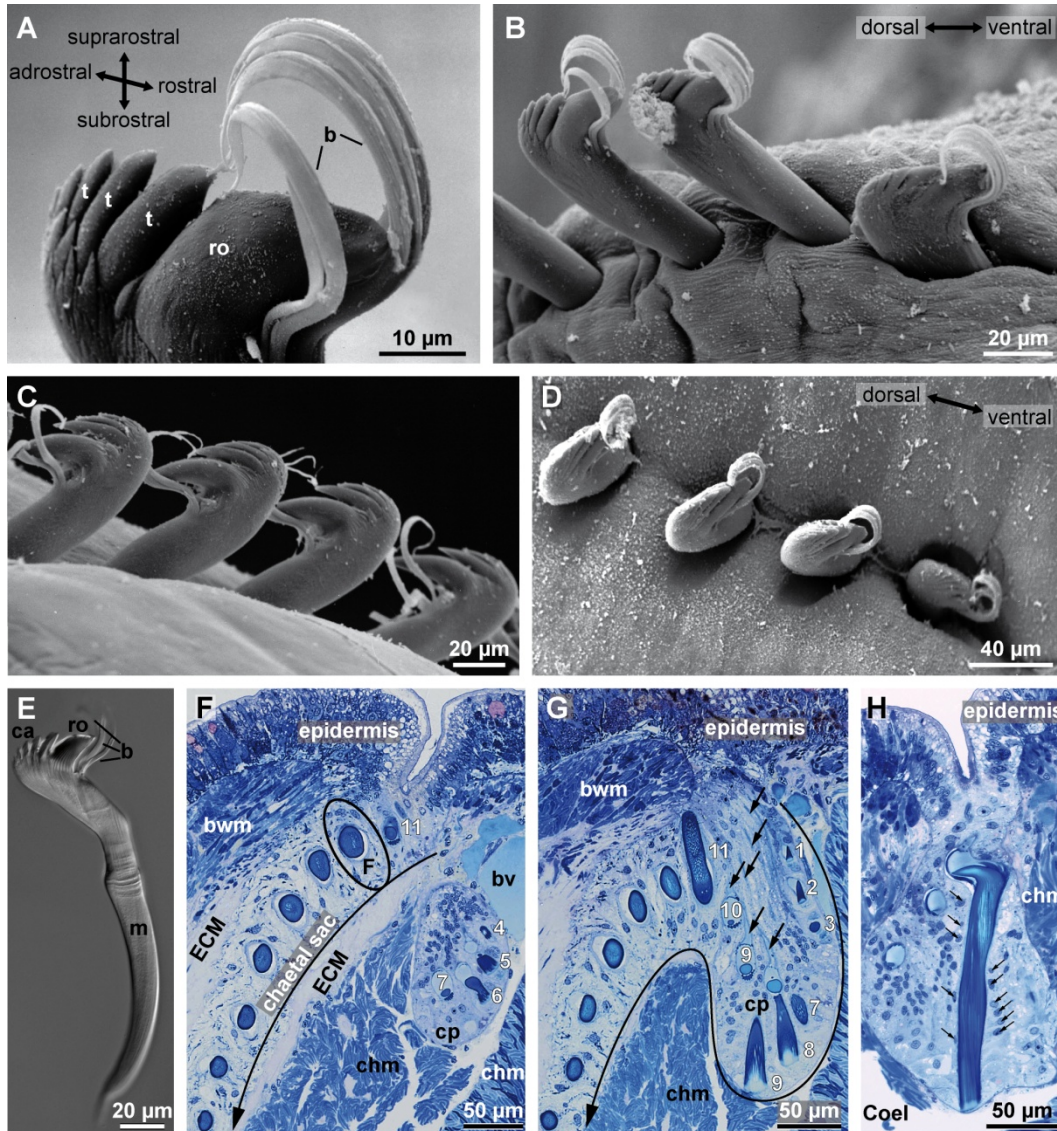
Figure 3.5: Hooked chaeta and their formation. a, b *Clymenura clypeata*. a Apical portion of a hooked chaeta with rostrum (ro), teeth of the capitium (t) and barbules (b). b Ventral edge of the neuropodial rim with a newly formed chaeta. c–e Johnstonia clymenoides. c Row of hooked chaetae. d Ventral edge of the neuropodial rim with a newly formed chaeta. e Normarsky contrast microscopy image of a hooked chaeta. Note the parallel lines inside the chaetal pouch at the ventral edge of the neuropodial rim, wherein new bearded hooked chaetae are formed.
(Direct link: www.morphdbase.de/?E_Tilic_20150220-M-23.1; Direct link: www.morphdbase.de/?E_Tilic_20150220-M-22.1). f, g Series of the chaetal pouch with numbered stages of chaetal formation, distance between (f) and (g):32 lm; arched large arrows propagation of chaetae within the neuropodial rim; large arrows mark intrafollicular compartment. h Almost completed chaeta, small arrows nuclei of follicle cells. ep epidermis, bv blood vessel, bwm body wall musculature, chm chaetal musculature, cp chaetal pouch, ECM extracellular matrix, F chaetal follicle (encircled)

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While forming the chaeta, the follicle sinks deeper into the tissue, is pushed posteriorly by newly formed follicles, turns anteriorly again and is finally aligned with the older follicles of the chaetal sac. The cellular interaction cause the chaetoblast to initially shift its apico-basal axis and then to twist around this axis. Continuous release of chitin consolidates every cytokinetic process as chaetal structure, so that positional changes during chaetogenesis as wells as modification of the microvilli pattern are reflected by structure of the completed chaeta (Fig. 3.5e).

Figure 3.6 Early chaetogenesis of hooked chaetae in *Clymenura clypeata* (a) and *Johnstonia clymenoides* (b–d). Formation of rostrum (ro) and teeth (t) of capitium. a A cluster of microvilli is the template for the rostrum. Chaetoblast (CB) and follicle cell one (FC1) and two (FC2) of the anlage rest on retroperitoneal face of the extracellular matrix (ECM). b Electron-dense material covers the microvilli. Notecytoplasmic actin filament system (af) interacting with actin filaments of microvilli. c–d Cytoplasmic actin filaments (white stars) are shaping the interaction between follicle cell 1—chaetoblast. c Strong single microvilli (mvt) are templates for suprarostral teeth of capitium. d Electron-dense material covers microvilli preforming the capitial teeth. Arrows adherens junctions, n nucleus

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Formation of the rostrum and capitium

The earliest stage is found in a young follicle that consists of more than five cells. These surround a small compartment which is connected to the epidermal surface, but plugged by some electron-dense material. All cells are epithelial, and only the apex of chaetoblast bears microvilli (Fig. 3.6a). The number of microvilli increases by peripheral addition of further microvilli. Since the microvilli also increase in diameter, the basal section of the compartment becomes wider. The lateral walls of the chaetoblast that surround the central microvilli grow upward, while the group of microvilli sinks deeper into the chaetoblast (Fig. 3.6b). The microvilli contain actin filaments that extend into the cell soma to interact with actin filaments that adhere to the adherens junctions and thus are rectangular to those originating in the microvilli (Fig. 3.6b, c). The actin filaments are so dense in this region that they cause a greyish staining of the cytoplasm directly underneath the microvilli. The first follicle cell also contains a dense meshwork of actin filaments that originate from the adherens junctions and are located in that part of the cells which face the developing chaeta (Fig. 3.6c, d). The chaetoblast and the adjacent first follicle cell contain vesicles with electron-dense material, which in some sections can be seen fusing with the cell membrane between the microvilli (Figs. 3.7, 3.8c). Release of this vesicle contents coincides with the appearance of an electron-dense cap that covers the bundle of microvilli and forms the tip of the rostrum of the hooked chaeta (Figs. 3.6c, 3.7a). During further differentiation, additional microvilli are generated on the surface of the chaetoblast, and the electron-dense cap increases in diameter and length. The length of the microvilli is constant during chaetogenesis, so that, while chitin is added to elongate the chaeta, small canals remain where the microvilli had been before (Fig. 3.7a). These canals are filled by electron-dark material. Depending on their position, the diameter of the central microvilli that

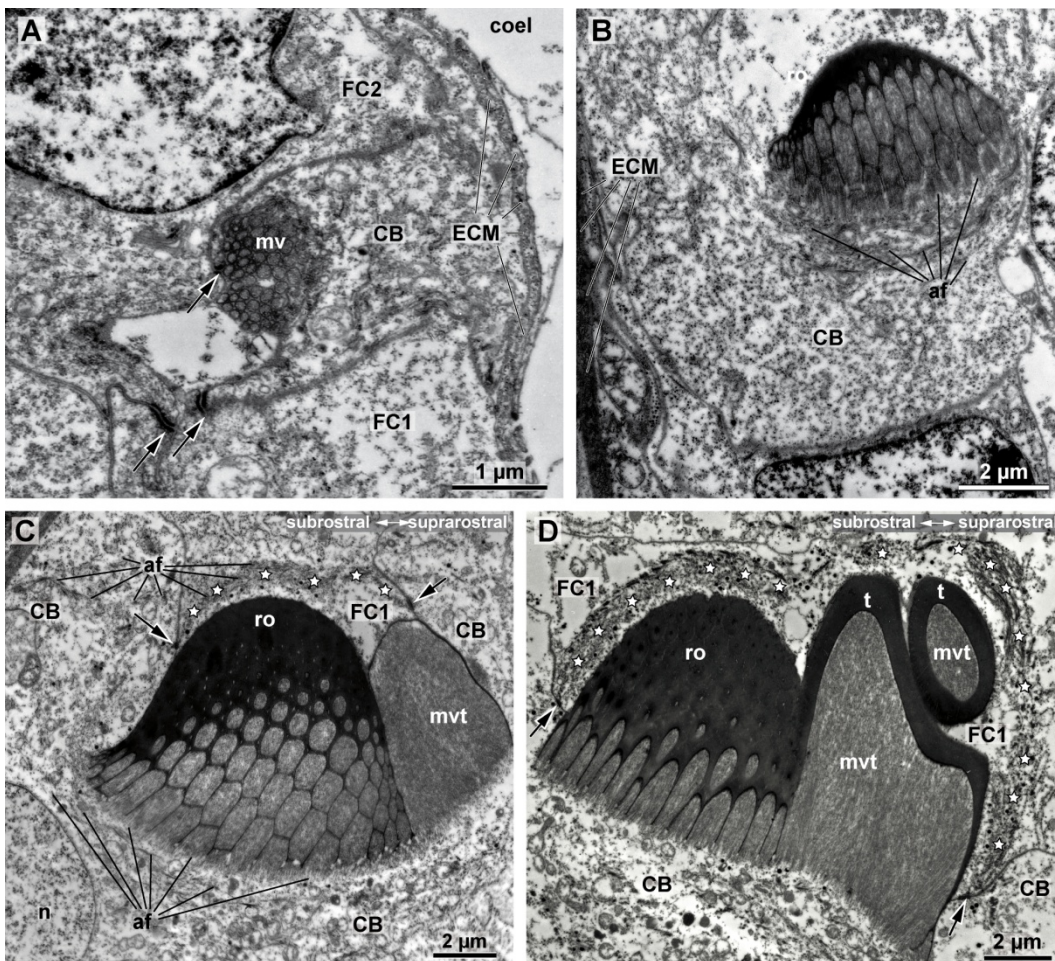form the template for the rostrum ranges between 0.45 and 0.9 micrometer (n = 29, $\bar{x}$ = 0.68, s = 0.13; Fig. 3.7a).
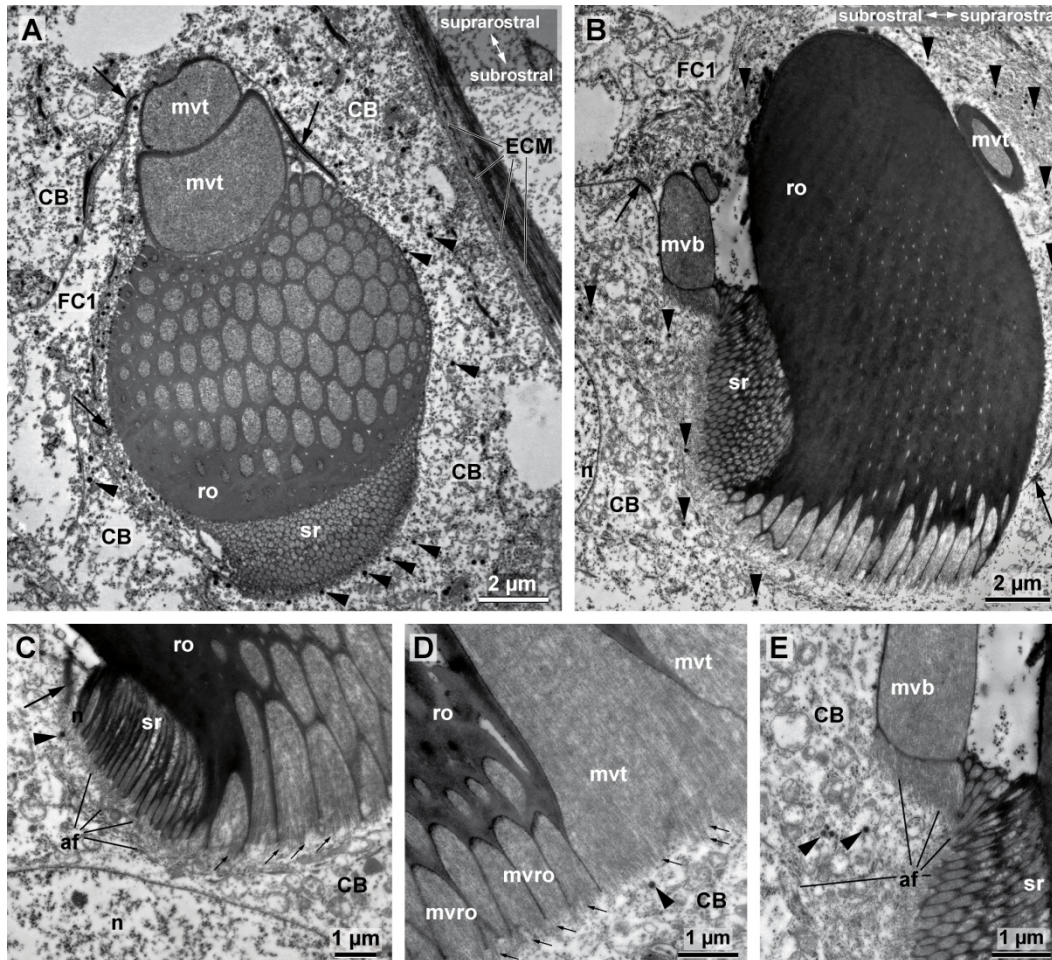
Figure 3.7 Chaetogenesis of hooked chaetae in *Clymenura clypeata* (a) and *Johnstonia clymenoides* (b–e). Formation of subrostral process (sr) and barbules. a Transverse section of the developing rostrum (ro) with strong microvilli (mvt) forming the template of the capital teeth and small microvilli preforming the subrostral process. b Later stage, sagittal section. Thick microvilli (mvb) preforming the barbules are rectangular to the small microvilli of the developing subrostral process (sr). c Microvilli preforming rostrum and subrostral process, note the size difference and actin (small arrows) filament distribution. d Size difference between microvilli preforming capital teeth (mvt) and rostrum (mvro). e Higher magnification of B. Note differing orientation of the actin filaments (small arrows). large arrows adherens junctions; arrow heads vesicles with electron-dense material; af actin filaments; CB chaetoblast; FC1 follicle cell 1; FC2 follicle cell 2; n nucleus

Microvilli on the prospective concave side of the rostrum are smaller and curved; they initiate the characteristic curvature of the rostrum. On the prospective convex side, large conical microvilli are formed (Figs. 3.7b, d). These microvilli measure up to 5.2 µm in diameter at their base and are the templates for the teeth of the capitium. These microvilli are sequentially formed; the largest one is next to the developing rostrum, and the diameter of the following ones decreases (Fig. 3.9a).

Cytokinetic interaction

During this initial phase of chaetogenesis, the nucleus of the chaetoblast that initially was located next to the apical microvilli shifts laterally, and the distance between the base of the microvilli and the base of the chaetoblast decreases (Fig. 3.7b). Thus, the chaetoblast almost completely surrounds the chaetal anlage when formation of the rostrum and the capitium is nearly completed (Fig. 3.7a). While the nucleus is displaced, the entrance angle of the actin filaments into the microvilli changes gradually by 40°–45° relative to their original orientation. These changes are along with a slight rotation of the chaetoblast that causes some asymmetrical structure of the microvilli cluster that preforms the rostrum (Figs. 3.6c, 3.7b). The dynamic changes on the surface of the chaetoblast and of its position relative to neighboring follicles cause that the microvilli appear to be continuously withdrawn from these deposits. Combined with the mentioned asymmetrical distribution of the unequally sized microvilli peripheral to the developing rostrum, these initial changes in the shape and structure of the chaetoblast initiate the curvature characteristic of the hooked chaetae in maldanid species (Figs. 3.8b, 3.9b). Since the angle between the tip of the rostrum and the microvilli that form its template gradually changed and the strong microvilli serving as template for the teeth of the capitium appeared on the convex side of the rostrum with temporal delay, the teeth of the capitium are not parallel to the rostrum (Fig. 3.8a, b).

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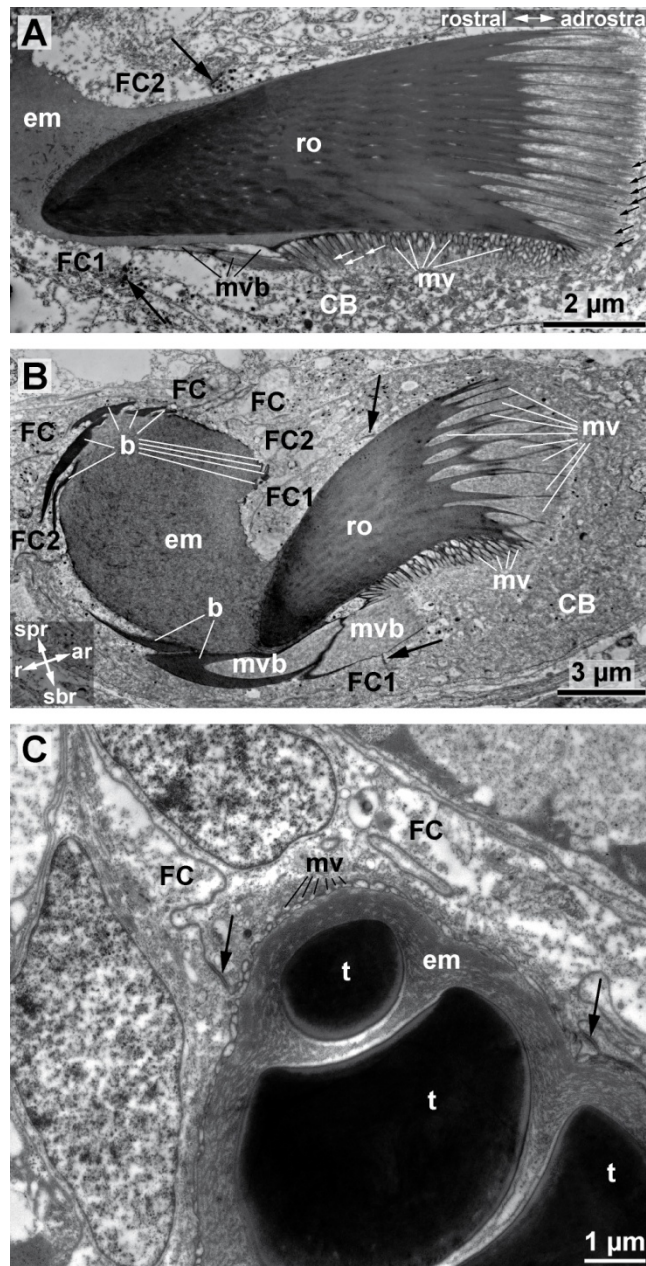
Figure 3.8 Parasagittal sections of developing hooked chaetae in Johnstonia clymenoides (a, c) and *Clymenura clypeata* (b); capitial teeth outside the plane of sectioning in (a) and (b). a Newly formed rostrum is surrounded by extracellular material (em) that is lined by the apices of the follicle cells. Note rostral microvilli (mvb) that become template of the barbules. b Formation of barbules (b) is almost complete; their specific structure is caused by developmental timing and position. Orientation scheme: spr suprarostral, sbr subrostral, ad adrostral, r rostral. c Completed teeth (t) of capitium. After withdrawal of the microvilli the remaining compartments are refilled and the teeth are solid. Inset Fusion of an electron-densely filled vesicle (arrow) with the submicrovillar cell membrane. CB chaetoblast; follicle cell (first two are numbered); mv microvillus; small arrows actin filament bundles, large arrows adherens junctions

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Formation of the subrostrum and the beard

After formation of rostrum and capitium, the anlage is asymmetric with respect to the tip of the rostrum, which is almost completely enclosed by the chaetoblast. At this time, an additional series of 1 to 2μm-long microvilli appears next to the prospective concave face of the rostrum (Figs. 3.7c, e, 3.8a, b). They are almost identical in diameter (n = 12, $\bar{x}$ = 0.18, s = 0.1) and are the template for the 1 (Figs. 3.7c, e, 3.8a, b). These microvilli are rectangular to the small subrostral ones and are templates for the barbules of the beard. They elongate and extend into the extracellular compartment that houses the developing chaeta (Fig. 3.8b). While growing, the microvilli increase in size and finally measure up to 1.6 μm in diameter. Since the space within the extracellular compartment is restricted by the developing chaeta, the microvilli are forced to expand lateral of the rostrum to its convex side, where they are bent subrostrally. Electron-dense material is deposited on the surface of these microvilli, which then are retracted. The electron-dense, hair-like and curled structures are the subrostal beard characteristic for hooked chaetae in maldanid species. Since the small subrostral series of microvilli never released electron-dense vesicles nor did it become covered by electron-dense material, a gap between the hairs of the beard and the rostrum remains.

Formation of the manubrium

After the beard has been formed, all microvilli converge to a single cluster by differential growth, which is reduced in diameter primarily by fusion of microvilli. Due to the initial changes in the orientation of the main axis of the chaetoblast, the rostrum bends relative to this cluster by more than 90° (Fig. 3.9c). This cluster is the template for the manubrium. The chaetoblast and the follicle cells continuously release the electron-dense material from tiny vesicles. The vesicles of the chaetoblast fuse with the cell membrane between the bases of the microvilli and elongate the manubrium (Fig. 3.8c,

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inset), whereas those released by the follicle cells form the compact and strengthened peripheral wall or cortex of the hooked chaeta, often termed enamel. Fusion of adjacent microvilli or peripheral addition of microvilli shapes the structures of the manubrium. The developing hooked chaeta now grows toward the ventral edge of the neuropodial rim. Adding chitin to the base elongates the chaeta. Where microvilli had been before, canals remain inside the manubrium (Fig. 3.9d). These canals appear to be empty, in contrast to those inside rostrum, capitium and barbules that are filled by electrondense material (Figs. 3.6a, 3.7d, 3.8c). Finally, the chaeta extends to the exterior. The formation is now complete, and intermediate filaments appear inside the follicle cells and the chaetoblast. Hemidesmosomes connect them to the chaeta and to the perifollicular ECM. The chaetoblast surrounds the chaetal base; the microvilli that determined the structure of the chaeta remain in the chaetal base.
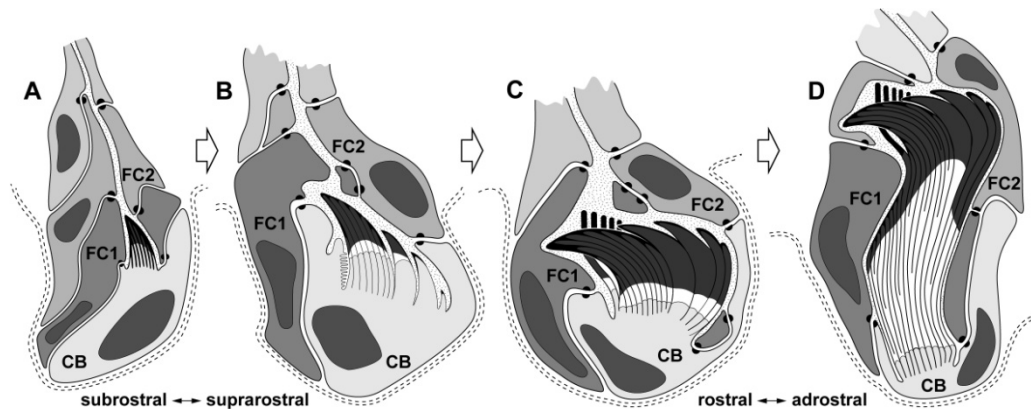
Figure 3.9 Reconstruction of bearded hook chaetogenesis in *Johnstonia clymenoides* and *Clymenura clypeata* as series of longitudinal sections. a Formation of the rostrum. b Formation of the teeth of the capitium. Note subrostral microvilli and microvilli preforming the barbules (asterisk). c Completion of the apical section of the chaeta. d Formation of the manubrium. Dashed double line extracellular matrix

With this final step, the formation of the hooked chaetae is completed and the new chaeta is aligned with the previously formed ones. Young chaetae are always added at ventral edge of the neuropodial rim, while old chaetae must be lost at the dorsal edge of the neuropodium. Here, degenerating chaeta were actually seen inside

follicles filled with lysosomes and showing all signs of apoptosis (data not shown). This observation corroborates Pilgrim (1977: 293), who described degeneration on the dorsal edge of the neuropodial rim in Clymenella torquata (Leidy 1855). Hooked chaetae do not exist during the entire life span of the maldanid after once having been formed. Chaetae must therefore be highly dynamic structures, characterized by continuous formation and replacement.
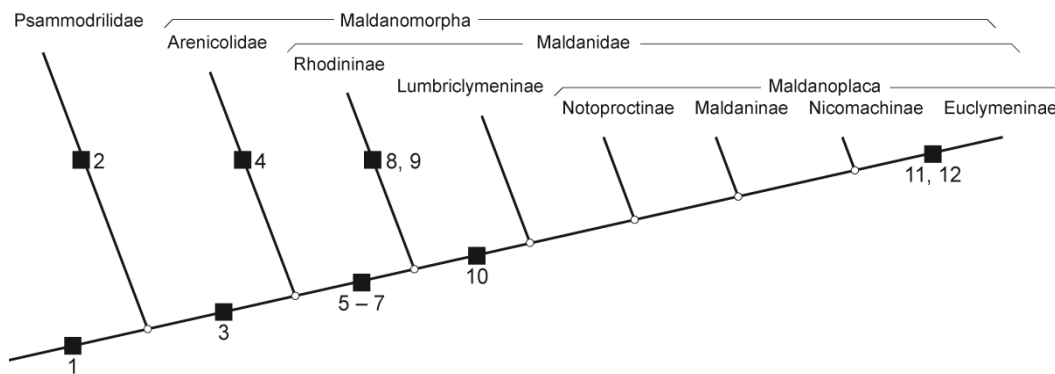
Figure 3.10 Maldanomorph relationships redrawn from de Assis and Christofferson (2011) with potential autapomorphic changes in chaetation mapped on the branches; Capitellidae are outgroup. 1 bearded hooked chaetae (Meyer and Bartolomaeus 1997; Bartolomaeus and Meyer 1997; this paper); 2 aciculae in cirri of chaetiger 1–6 (Bartolomaeus 1995a); 3 ventral position of formative site in neuropodial rim (Fig. 3.12e); 4 neurochaetae in adults are acicular spines with capitium (Bartolomaeus and Meyer 1997); 5 notopodial chaetal sac involute (Pilgrim 1977; this paper), 6 posterio-ventral position of formative site in notopodia (Pilgrim 1977; this paper), 7 hooked chaetae in adults are transformed in chaetiger one to four (Arwidsson 1906); 8 neurochaeta are absent in chaetiger 1–4 in adults; 9 from chaetiger 5 onward neurochaetae are a double row of avicular uncini (Fig. 3.11h–k; Arwidsson 1906; de Assis and Christoffersen 2011); 10 single row of large rostro-median capital teeth (Fig. 3.11m); 11 chaetal sac straight from chaetiger 13 onward in adults (Figs. 3.3d, 3.4); 12 notochaetal double rows longitudinal from chaetiger 13 onward in adults (Pilgrim 1977; Hausen and Bleidorn 2006)

3.4 Discussion

Maldanidae is a monophyletic group of annelids based on several autapomorphies like a dorsally located, keel-shaped prostomium with lateral nuchal grooves, fusion of pro- and peristomium whereby the latter forms lips, post-prostomial rings and pre-anal segments without

chaetae (De Assis and Christoffersen 2011). Elongate median chaetigers and pronounced intersegmental borders between most segments led to the vernacular name "bamboo worms" for the entire group. Within Maldanidae, Rhodininae are sister group of the remaining Maldanidae that consist of Lumbriclymenidae and Maldanoplaca (De Assis and Christoffersen 2011). The latter contains the monophyletic taxa Notoproctinae, Maldanidae, Nicomachinae and Euclymeninae (Fig. 3.10). Both studied species, *C. clypeata* and *Johnstonia clymenoides*, fall into the latter group.

3.4.1 Notochaetae in Maldanidae

The chaetae of the notopodium (notochaetae) are generally aligned in a transverse double row; only a fewspecies possess a single rowin the first or the first three chaetigers (Meyer and Westheide (1997) for *Boguea panwaensis* Meyer and Westheide 1997; Bleidorn and Hausen (2007) for *Axiothella cirrosa* Bleidorn and Hausen 2007). Each transverse double row consists of longer anterior and shorter posterior chaeta, an observation already mentioned by Arwidsson (1906). In all species of Euclymeninae, however, the double row of notochaetae is parallel to the antero-posterior body axis from chaetiger 13 onward in the posterior segments; stepwise transition between both conditions can always be seen in chaetiger 11 and 12 (Pilgrim 1977; Hausen and Bleidorn 2006; De Assis and Christoffersen 2011). Our study shows that re-orientation in notochaetal arrangement in the mid-body region is caused by the altered structure of the notochaetal sac. This sac is involute and consists of two curved anterior blades that enclose their posterior–ventrally located merger wherein new chaetae are formed. Except for the posterior segments in euclymenin species, such an involute chaetal sac is characteristic for all maldanid species. A straight notochaetal sac characteristic for the posterior segments of euclymenin species consists of two parallel anterior blades that merge posteriorly to form the formative site. The correlation between chaetal

sac structure and position of the notopodial double row is indirectly supported by older studies. Given that chaetae are continuously replaced (Pilgrim 1977), new chaetae will be added to the posterior margin of each notopodium in posterior segments of euclymenin species, but to the ventral margin of the notopodia in the remaining maldanid species. When seen from the exterior, new chaetae can easily be recognized by their shortness (Hausen 2005). Arwidsson (1906) mentions such smaller chaetae at the ventral margin of the notochaetal sac in some segments of Rhodine gracilior Tauber 1970, Hausen and Bleidorn (2006) and Bleidorn and Hausen (2007) described this for further maldanid species, and Pilgrim (1977) described a ventral formative site in anterior notopodia and a posterior formative site in posterior notopodia of euclymenin species.We assume that a straight chaetal sac giving rise to notopodial double rows parallel to the anterior–posterior body axis must have evolved in the stem lineage of the Euclymeninae (Fig. 3.10: characters 11 and 12), while an involute chaetal sac and transverse notopodial double rows represent the primary condition in Maldanidae.

3.4.2 Diversity and homology of neurochaetae in Maldanidae

There are tremendous differences in the shape of the neuropodial chaeta in Maldanidae which at first glance could argue against homology (Fig. 3.11). Chaetiger 1 merely bears a single acicular spine (Fig. 3.11a). In some species, this spine may have a capitium surmounting an upright rostrum (Fig. 3.11c; Arwidsson 1906; Imajima and Shiraki 1982; Green 1997; Bleidorn and Hausen 2007; Kongsrud and Rapp 2012). Chaetigers 2 and 3 possess a much lower number of chaetae than the following ones (Arwidsson 1906; Pilgrim 1977; Green 1997), sometimes only two or three. In fully grown adults, these are also acicular spines (Fig. 3.11b), but in younger individuals they are sometimes beardless hooked chaeta (Arwidsson 1906; Pilgrim

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1977). From chaetiger 4 onward, most species of the Maldanidae possess a single row of neuropodial hooked chaetae with a beard composed of barbules that arise subrostrally to clasp the rostrum (Fig. 3.11g). In some species, however, a row of beardless hooked chaetae has been described in the fourth to sixth chaetiger for some species. Moreover, depending on their position within the row, they differ in structure (Fig. 3.11d–f). Arwidsson (1906) already addressed these differences and reported transitional stages ("Übergangsstadien") between acicular and hooked chaeta, especially in chaetigers 4 and 5. Sometimes a beard could be found in the ventrally located chaeta, whereas the dorsally located one lacked barbules. His thorough description of the neurochaetal structure indicates that within a row, acicular spines or beardless hooked chaetae may precede hooked chaetae with barbules. Pilgrim (1977) and Wolf (1983) explicitly mentioned that shape and size of hooked chaetae differ within a neurochaetal row and refer these differences to age-dependent changes in chaetal formation. Given that the formative site is ventral, modified hooked chaeta (Fig. 3.11a–f) had been formed earlier than the bearded hooked chaetae, so that the structure of neurochaetae is altered during life history. We therefore assume that acicular spines, beard-less and bearded hooked neurochaetae are homologous within Maldanidae (Fig. 3.11).

The neuropodial chaetae in Rhodininae differ from those of the remaining Maldanidae (Fig. 3.11). Adult Rodininae, which contain all species of *Rhodine*, *Boguea* and *Boguella*, generally lack neurochaetae in chaetigers one to four, which must be regarded as an autapomorphy of Rhodininae (Fig. 3.10: character 8). Wolf (1983) mentions that each chaetiger of late larval *Boguea enigmatica* Hartman 1945 bears neuropodial hooked chaeta which gets lost in the first four chaetigers later during ontogenesis. Meyer and Westheide (1997) also mentioned a single hooked chaeta in chaetiger two to four in juvenile *Boguea panwaensis*, which are absent in adults. These ontogenetic changes in

the structure of neurochaetae are in accordance with the age-dependent structural differences in neurochaeta mentioned above. Therefore, we expect hooked neurochaetae in late larval stages or juveniles of *Rhodine* species. From chaetiger five onward, a double row of neuropodial chaetae is found in all members of the Rhodininae, which must be an autapomorphy of the group, since all species of Arenicolidae, Psammodrilidae and Capitellidae as well as the remaining species of Maldanidae possess a single row of neuropodial chaetae (Fig. 3.10: character 9; see also de Assis and Christoffersen 2011). The neuropodial chaetae differ in shape and have often been termed avicular (Wolf 1983), manubrioavicular (de Assis and Christoffersen 2011), Rhodine-type or terebellid-type uncini (Wolf 1983; Fig. 3.11h–k). In general, these chaetae resemble hooked chaeta, since their rostrum is surmounted by a number of smaller teeth. The subrostral process, however, is elongated and bears a small process underneath the tip of the rostrum and a larger, ledge-like process facing the rostrum in Rhodine species (Arwidsson 1906), (Fig. 3.11e). In Boguea species, the latter process has the shape of a shoulder, but forms a small spine in *Boguella ornata* Hartman and Fauchald 1971 (Wolf 1983). Older drawings indicate some hair-like structure originating from the small subrostral process (Fig. 3.11i, k). (Arwidsson 1906) and Meyer and Westheide (1997) provide evidence that hairs originate from the subrostral process of in *Boguea panwaensis*. We assume that these hairs have been overlooked in older studies, due to their delicate structure. Their position, however, indicates that the hairs represent a modified beard characteristic for hooked chaetae in Arenicolidae, Psammodrilidae and non-rhodinin Maldanidae. In contrast to De Assis and Christoffersen (2011: character 23), we hypothesize avicular, manubrioavicular, Rhodine-type and terebellid-type chaetae as homologous with the bearded hooked chaetae and their derivatives in other maldanids (Fig. 3.11). This hypothesis would be falsified, if these hairs were formed in a different manner than the

barbules. Neuropodial double rows of alternatingly orientated chaetae with an elongated subrostral process giving rise to small hair-like barbules and ending in a ledge-like tip must be an autapomorphy of the Rhodininae (Fig. 3.10: characters 8 and 9).

The hooked chaetae in species of Arenicolida and Rhodoninae possess a capitium consisting of three or more crescent rows of small teeth that surmount the rostrum (Fig. 3.11l), whereas the remainingmaldanid species uniformly possess three to seven large teeth that surmount the rostrumas amedio-rostral row (Fig. 3.11m). With respect to the phylogeny hypothesis of De Assis and Christoffersen (2011), the single rostro-median row of capital teeth must be autapomorphic for Lumbriclymeninae + Maldanoplaca (Fig. 3.10: character 10).

Our survey provides evidence that structurally different neurochaetae in Maldanidae result from ontogenetic modulations of chaetogenesis, so that caution is needed when neurochaetae of different maldanid species are compared. Differences do not necessarily result from evolutionary transformation, and they may rather be caused by the differing age of the studied specimen. For unraveling in-group relationships of Maldanidae, the phylogenetic signal of these chaetae becomes noisier, the larger the studied individuals differ in age. For this group, it is extremely important to compare the same semaphoronts.

Figure 3.11 Ontogenetic homologs of bearded hooked chaetae. a *Lumbriclymene minor* Arwidsson 1906, chaetiger 1. b *Nicomache minor* Arwidsson 1906 chaetiger 3. c *Notoproctus oculatus* Arwidsson 1906, chaetiger 1. d–f *Nicomache quadrispinata* Arwidsson 1906, chaetiger 5s (d) and first (e) in left row, chaetiger 6 (f); g *Petaloproctus borealis* Arwidsson 1906, chaetiger 11; h *Rhodine gracilior* Tauber 1879, chaetiger 7, posterior row; i–k *Boguella ornata* chaetiger 9, post-row (i), ant row (k), l *Boguea enigmatica*, avicular uncinus, suprarostral view, m *Johnstonia clymenoides*, bearded hooked chaeta, suprarostal view. (a–i redrawn from Arwidsson 1906, k–m redrawn from Wolf 1983). Arrows direction of ontogenetic transformation →
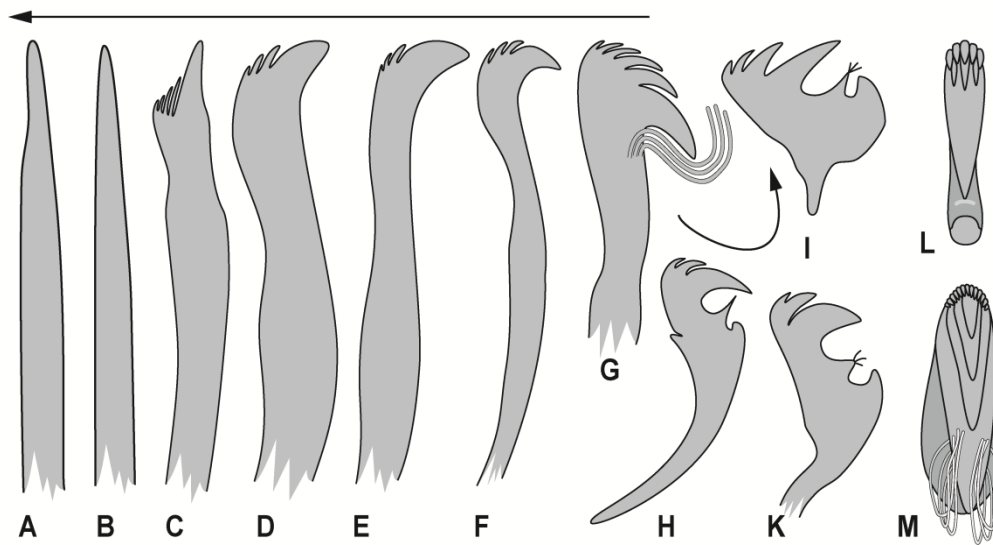
3.4.3 Phylogenetic significance of hooked chaetae

A beard is also found in the hooked chaetae of Psammodrilida and newly settled *Arenicola marina* (Fig. 12b, c) (Bartolomaeus 1995a; Bartolomaeus and Meyer 1997; Meyer and Bartolomaeus 1997). There is strong evidence from chaetogenesis that hooked chaetae with a beard are homologous, although they are expressed in different semaphoronts (newly settled individuals of one species and adults of another), since the hooked chaetae of adult arenicolids lack a beard. In contrast to hooked chaetae, the rostro-adrostral axis is not perpendicular to the manubrium, so that the hooked chaetae of adult arenicolid species are spine-like with an adrostral capitium (Bartolomaeus and Meyer 1997). The structure of the hooked chaetae alters during ontogenesis. Since the formative site is located ventrally in all arenicolid species, both kinds of chaetae can sometimes be found within a single row of hooked chaetae, with the ontogenetically older bearded hooked chaetae being located dorsally and the spine-like ones sitting ventrally within the row (Fig. 3.12d, e).

De Assis and Christoffersen (2011) choose Capitellidae, Arenicolidae and Psammodrilidae as outgroup in their cladistic

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analysis of the Maldanidae. This study confirms Arenicolidae and Maldanidae as sister groups, since both possess a ventral formative site in neuropodia (Pilgrim 1977; Wolf 1983; Bartolomaeus and Meyer 1997) (Figs. 3.10: character 3; 3.12d, e). This monophyletic group has been termed Maldanomorpha and has been confirmed in several phylogenetic analyses based on molecular data as well (Bleidorn et al. 2003; Rousset et al. 2007; Struck et al. 2007). Maldanomorpha are regarded as sister group of Psammodrilidae (Meyer and Bartolomaeus 1997;de Assis and Christoffersen 2011). All three possess hooked chaetae with barbules, albeit these structures may get lost or alter during ontogenesis in different lineages as described above in Maldanidae and by Bartolomaeus and Meyer in Arenicolidae (1997; Fig. 3.10: character 1).
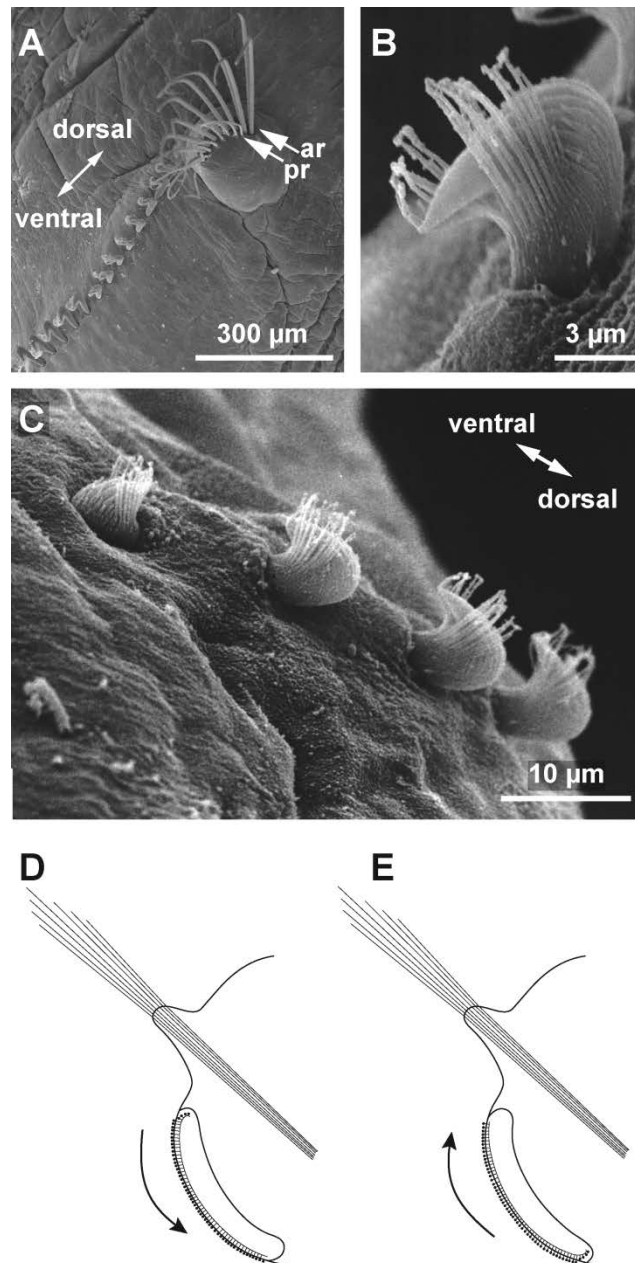
Figure 3.12 Chaetation in *Johnstonia clymenoides* (Eucymenidae, Maldanidae) (a) and newly settled *Arenicola marina* (Linnaeus 1758) (Arenicolidae) (b, c). d, e Position of the formative site. a Notochaetae are arranged in a double row; note differing orientation of neurochaetae within a single row. b Hooked chaeta with beard. c Like in maldanids the formative site is ventral and the new chaetae twist around their longitudinal axis prior to being aligned in the neuropodial row. d The formative site is located dorsally in the neuropodium rim in the Maldanomorpha outgroups and the neurochaetae progress downward (arrow) during their life span. e In Maldanomorpha, the position of the formative site is inverted and the neurochaetae are moved dorsally (arrow) while being used

3.4.4 Evolutionary versus functional constrains for hooked chaetae

Timing, positional relation of substructures and the way, in which the substructures are formed, substantiate the hypothesis of a homology of hooked chaetae (Bartolomaeus 1998; Hausen 2005).

1. The rostrum is always the first structure that is formed during chaetogenesis; its template is always a cluster of several microvilli. Chitin released from the chaetoblast and the follicle cells coats the microvilli which then are passively retracted from the tip of the rostrum upon adding chitin released in the intermicrovillar gap to elongate the developing chaeta.

2. When the rostrum has attained a certain length, additional microvilli appear at the surface of the chaetoblast. These are always formed on the prospective suprarostral side of the rostrum; they are always wider in diameter than those that preformed the rostrum. These microvilli each preform a single tooth of the capitium. They are also coated with chitin and retracted from the capitium.

3. The further the microvilli are offset from the rostrum, the later they appear on the surface of the chaetoblast. This delay results in a staggered arrangement of the capital teeth. Since the chaetoblast is shifting its apico-basal axis, the capital teeth show a differing curvature with those next to the rostrum having almost the same bending and those distant to the rostrum being almost parallel to the longitudinal axis of the manubrium.

4. While the capital teeth are being formed, large numbers of small microvilli are formed subrostrally. They preform the subrostral process and initiate the formation of the manubrium.

5. The microvilli that preformed the rostrum and the capital teeth become aligned with those preforming the subrostral process and start to merge. The final step of chaetogenesis, the formation of the manubrium, is now initiated.

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6. Continuous release of chitin elongates manubrium; the compartments left during retraction of the microvilli are filled by electron-dense material. This refilling of the compartment reinforces the tip of the hooked chaeta, so that the rostrum becomes inflexible and cannot be moved relative to the manubrium. Such a structure may well act as anchor.

Since formation of hooked chaeta is identical in several annelid groups, we proposed a common ancestry of all those annelids having such chaetae (Bartolomaeus 1998; Hausen 2005 and literature herein). This view has been criticized based on experiments evidencing the function of hooked chaetae as anchors and arguing that identical functional constraints inevitably result in similar or identical morphological structures (Woodin and Merz 1987; Merz and Woodin 2000, 2006). Such a view assumes that functional constraints imposed upon an organism by the environment are stronger than historical (evolutionary) constraints the inherited structural and genetic information imposes on the animal and demands inferring phylogenies only from those structures that have no obvious function. If this were true, it should be almost impossible to use morphological and molecular data for phylogeny inference, because every structure and every molecule underlie functional constraints forced on a biological being by the environmental conditions it lives in. Such a pure functional approach has been criticized by Fitzhugh (1991) who underlined and exemplified the need to test the influence of functional constraints on the evolution with phylogenies.

Merz and Woodin (2000, 2006) assume a strict identity of structure, position and orientation of hooked chaeta according to given functional constrains. This is some oversimplification with respect to temporal changes in the structure of hooked chaetae during ontogenesis as exemplified here for maldanids and previously for arenicolids (Bartolomaeus and Meyer 1997) and to spatial changes. At least in maldanid species not all hooked chaetae within a row point

into the same direction (Pilgrim 1977: 292; Woodin and Merz 1987: Fig. 1; Hausen and Bleidorn 2006: Fig. 3.1g; Fig. 3.12a). This observation is explained by differential rotation of the individual chaeta within the chaetal sac (Woodin and Merz 1987: 430). The structure of the chaetal muscles of the species studied here, however, does not allow this. A complete layer of transversal muscles adheres to the apical section of the chaetal sac; loosely interdigitating bundles of transversal muscle are restricted to its mid-basal section. Differential contraction of these muscle merely causes that parts of the chaetal sac are withdrawn, in no way isolated chaetal follicles of the chaetal sac can be twisted or even moved without influencing the neighboring chaeta, keeping in mind that each chaeta is firmly attached to the intermediate filament system of the follicle cells—and that the intermediate filaments are not contractile.

The functional studies of Woodin and Merz (1987) and Merz and Woodin (2000, 2006) were nevertheless important, since they provided experimental evidence for the function of a specific type of chaetae, the hooked chaetae and uncini. It would, however, be interesting to know whether or not functional changes in tube construction or mucous lining are along with the mentioned ontogenetic changes in neurochaetae. The studies of Woodin and Merz (1987) and Merz and Woodin (2000, 2006) allow correlating a specific type of chaetae with the life history of certain annelids and provide an explanation other than inherited information for the existence of a certain type of chaeta. We want to exemplify this for the hooded hooks of certain species of Eunicida and with respect to recent annelid phylogenies based on molecular data.

Within Eunicida, species of Lumbrineridae possess hooded hooks that are similar to those of capitellid and spionid hooded hooks, implying possible homology. Studies of chaetogenesis, however, showed developmental differences especially in the way the capitial teeth and the hood are formed during chaetogenesis (Tilic et al. 2014). Although

these differences could potentially have resulted from evolutionary transformation of amode of chaetogenesis found in species of Capitellidae or Spionidae, recent molecular phylogenies (Weigert et al. 2014) forced the differences to be regarded as resulting from convergent evolution, presumably due to similar functional constraints similar to those Merz and Woodin (2006) hypothesized for hooked chaetae.

Such structural and developmental differences have thus far not been recorded for hooked chaetae. Particularly, their uniform chaetogenesis has been used as main argument for their homology (Hausen and Bleidorn 2006 and literature herein). This view must be changed in the light of recent molecular analyses according to which Owenidae represent the most basally branching annelid taxon (Weigert et al. 2014). Oweniid species possess patches of hooked chaeta that each lacks a rostrum, but otherwise is identically formed like hooked chaeta (Meyer and Bartolomaeus 1996). The lack of a rostrum could as well be indicative for convergent evolution of the oweniid hooked chaetae and those having a rostrum. On the other hand, the rostrum is also reduced within Pectinariidae (Bartolomaeus 1995b), a subgroup of Terebellida. Terebellida primarily possess hooked chaetae with a rostrum (Bartolomaeus 1998; de Matos Nogueira et al. 2013). Despite well-substantiated homology hypotheses on chaetae, however, recent molecular phylogenies show that these chaetae can completely be altered in certain lineages leaving no trace of the ancestral design. The sister group relationship between Echiura and Capitellidae is since long well established (Bleidorn et al. 2003; Rousset et al. 2007; Struck et al. 2007), and a common ancestry of both and that of Terebellida, Arenicolidae, Clitellata and Opheliida is supported by phylogenomic data (Struck et al. 2011; Weigert et al. 2014). Provided hooked chaetae and hooded hooks are homologous, they are replaced by a pair of large ventral chaeta in Echiura (Tilic et al. 2015), a very few dorsal and

ventral spine-like chaeta in Clitellata or simple capillary chaetae in Opheliida.

Knowledge on their structure and development, however, helps where the hypothesis of their homology matches molecular analyses as shown here for maldanids and arenicolids. At least for these sister groups, it is more parsimonious to assume inherited information along with partial transformation instead of repeated evolution due to identical functional constraints.

3.5 Acknowledgments

Structure, function and cell dynamics during chaetogenesis of abdominal uncini in *Sabellaria alveolata* (Sabellariidae, Annelida)

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Abstract

Background Dynamic apical microvilli of a single cell, called chaetoblast, inside an ectodermal invagination form the template of annelid chaetae. Changes in the pattern of microvilli are frozen in time by release of chitin so that the structure of the definitive chaeta reflects its formation. Cellular interactions during chaetogenesis also influence the structure of the chaeta. Analysing chaetogenesis allows for testing hypotheses on the homology of certain chaetal types. We used this approach to test whether or not the unusual uncini in Sabellaria alveolata are homologous to similar looking uncini in other annelid taxa.

Results Our study reveals unexpected details of sabellariid uncini that mechanically reinforce the neuropodia to allow using them as paddles. The final structure of the chaeta is caused by pulses of microvilli formation and dynamic interaction between the chaetoblast and adjoining follicle cells. Cell dynamics during chaetogenesis of the uncini in Sabellaria alveolata exceeds by far previous studies on the formation of this type of chaetae. Despite superficial similarity of uncini in sabellariids and other annelids, differences in structure and

details of formation do not support the homology of this type of chaetae.

Conclusion Chaetogenesis of sabellariid uncini involves unexpected microvilli and cell dynamics and provides evidence that interaction of cells play a larger role in chaetogenesis than previously expected. In addition to their function as anchors, uncini of Sabellariidae stabilize the paddle-shaped notopodia, since each uncinus possesses a long, thin rod that extends deeply into the notopodium. The rods of all uncini of one row form a bundle inside the notopodium that additionally serves as the attachment site of muscles and thus have a similar function to the inner chaeta (acicula) of errant polychaetes (Aciculata).

4.1 Background

Chaetae are chitinous extracellular structures that are important diagnostic characters in Annelida (Fauchald 1977; Schroeder 1984). Chaetae are formed within an ectodermal invagination, the chaetal follicle, which consists of a terminal chaetoblast and a few follicle cells (Bouligand 1967; Specht and Westheide 1988; Hausen 2005). Each chaetoblast has an array of apical microvilli that are modified in time and space while chitin polymerizes alongside the microvilli. Controlled modification of the microvilli pattern, thus, gives a chaeta its final shape (Hausen 2005; Ogawa et al. 2011; Souza et al. 2011; Koide et al. 2015). Alterations of spatio-temporal patterns allow forming a plethora of different chaetal types that can range from highly complex compound hooked chaetae to simple capillaries. Chaetogenesis is such an elaborate interplay of cellular instruments that genetic programming and regulation is a necessity to warrant the constancy of chaetal arrangement and structure within annelid species and supraspecific taxa (Tilic et al. 2014; Tilic et al. 2015a). Given that genetic information is underlying chaetogenesis, we assume any hypothesis on the homology of chaetae

can be tested as identical formation processes are expected for structurally similar chaetae.

Hooked chaetae and uncini possess several small apical teeth giving the chaetae a sawor rasp-shaped appearance when viewed from above. These teeth may or may not surmount a single large tooth. Small apical teeth and, if present, the main tooth are curved relative to the shaft which represents the main axis of the chaeta. Uncini and hooked chaetae are discriminated by the length of the shaft, although its length is an imprecise character that varies intraand supraspecifically (Bartolomaeus 2002). Studies into chaetogenesis of the hooked chaetae and uncini of certain sedentary polychaetes revealed that the structure of these chaetae actually results from a uniform formation process (Sabellidae and Serpulidae (Bartolomaeus 1995; Bartolomaeus 2002;); Arenicolidae (Bobin 1944; Bartolomaeus and Meyer 1997); Maldanidae (Tilic et al. 2015a); Psammodrilida (Meyer and Bartolomaeus 1997), Terebellida (Bartolomaeus 1995; Bartolomaeus 1998); Oweniidae (Meyer and Bartolomaeus 1996); Siboglinidae (Schulze 2001)). One of the major conclusions these studies arrived at says that substructures and course of formation support the homology hypothesis for hooked chaetae and uncini, at least for the studied taxa (Bartolomaeus et al. 2005). Thus far not included into these comparative studies were Sabellariidae, which possess uncini that are aligned in a transverse row at the outer rim of the abdominal notopodia. At least on the light microscopic level the sabellariid uncini do not seem to differ from uncini of the other taxa studied so far.

In this paper we investigate the ultrastructure and chaetogenesis of abdominal uncini in *Sabellaria alveolata* (Sabellariidae). Assuming all uncini are homologous one would expect convincing similarities in chaetal ultastructure and formation. Although for epistemological reasons we never can offer proof for non-homology, recognizable differences in mode of chaetogenesis would not

support the homology of sabellaridan uncini to those of the other hook bearing Sedentaria and allow alternative hypotheses for the position of the Sabellariidae.

4.2 Material and Methods

4.2.1 Animals

Sabellaria alveolata was collected in March 2013 in the rocky intertidal of Concarneau (Brittany, France). Here, *S. alveolata* occurs in dense colonies in sheltered rock crevices, building distinctive hard tubes from the sediment. The tubes were removed from the rocks with the help of a spatula and the animals were fixed in the field immediately after being removed from their tube.

4.2.2 Light microcopy (LM), histology and 3D reconstruction

The specimen of *Sabellaria alveolata* used for the serial semi-thin sections and the 3D reconstruction was fixed in 1.25 % glutaraldehyde buffered in 0.05 M phosphate buffer with 0.3 M NaCl for 1.5 − 2 hours. The fixed animals were stored in the same buffer until they were postfixed in 1% OsO_4 for 45 minutes. The specimens were dehydrated in an acetone series right after the postfixation, transferred in propylene oxide and embedded in araldite. If necessary the specimens were sectioned into smaller pieces within the resin. Polymerization was started with BDMA (Benzyldimethylamin). Series of one micrometer sections were cut with diamond knife (Diatome Histo Jumbo) on a Leica Ultrarcut S ultramicrotome, following the method described by Blumer et al. (2002). The sections were stained with toluidine blue (1% toluidine, 1% sodium-tetraborate and 20% saccharose) and covered with a cover slip mounted with araldite. The semi-thin sections were analyzed with an Olympus microscope (BX-51) and photographed with an Olympus camera (Olympus cc12), equipped

with the dot slide system (2.2 Olympus, Hamburg). The images were aligned with IMOD (Boulder laboratories, (Kremer et al. 1996)) and IMOD-align:(http://www.evolution.uni-bonn.de/mitarbeiter/bquast/software).

3D modeling of the chaetae was executed using the software 3ds max 13. Histological images were imported as surface materials (discreet) and the chaetae were modeled using standard cylindrical objects. When necessary these were modified as NURBS (Nonuniform rational B-Splines)-surfaces. The outline of the neuropodial torus was created using another NURBS surface.

Using the same method a second 3D model was constructed with the aligned TEM-images of the formative site. Here all of the studied developmental stages were modeled in order to visualize their topological position within the formative site.

Single chaetae that were analyzed using a confocal laser scanning microscope and using Nomarsky differential interference contrast under an Olympus BX-51 microscope were isolated from pieces of PFA (1h in 4% paraformaldehyde) fixed specimens of *Sabellaria alveolata* by incubation in 5% NaOH for 4-5 hours. The chaetae were rinsed in distilled water, mounted on microscopical slides and examined.

4.2.3 Confocal laser scanning microscopy (CLSM)

The specimens used for confocal laser scanning microscopy were fixed in 4% paraformaldehyde for 1 hour and later stored in 0.1 M PBS (phosphate buffered with saline) containing 0.01% NaN_3. The chaetigers were dissected to separate single parapodia. Isolated parapodia and segments were permeabilized in four 5-min changes of PBS with 0.1% Triton X-100 (Fisher Scientific). The samples were then stained overnight in 4 ºC with TRITC phalloidin at a dilution of 1:100. After staining, parapodia were rinsed in three quick changes and subsequently in two 10-min changes of PBS with 0.1% Triton and one

10 min rinse in PBS without Triton. Stained and rinsed samples were dehydrated in isopropanol (2 min 70%, 2 min 85%, 2 min 95%, 2 min 100%, 2 min 100%) and cleared in three 15-min changes of Murray Clear. Once they were placed in hollow-ground slides they were mounted in Murray Clear, and sealed with nail polish.

The upper layers of musculature were partially removed from the confocal z stack, digitally, using Photoshop CS6 to allow viewing the chaetae within the torus. The entire CLSM image stack is available for download (link provided under data repository).

4.2.4 Electron microscopy (TEM, SEM)

Specimens used for transmission electron microscopy were fixed using the same fixation method described above for semi-thin sectioning (1.25 % glutaraldehyde buffered in 0.05 M phosphate buffer with 0.3 M NaCl for 1.5 – 2 hours, postfixation with 1% OsO_4 for 45 minutes). These specimens were also embedded in araldite and sectioned into a complete series of silver-interference coloured (70–75 nm) sections using a diamond knife (Diatome Histo Jumbo) on a Leica Ultrarcut S ultramicrotome. The serial section ribbons were placed on formvar-covered, single-slot copper grids and stained with uranyl acetate and lead citrate in an automated TEM stainer (QG-3100, Boeckeler Instruments). The sections were examined using a Zeiss Libra 120 kV transmission electron microscope.

The chaetal formation was reconstructed using the information gathered from serial ultrathin sections and series of semithin sections of *S. alveolata*. The coverage of different stages of chaetogenesis was, with 14 consecutive developmental stages, dense enough to allow insights into the dynamics of the entire process that will be described in the following. The entire aligned stacks of ultra-thin and semi-thin sections are available for download (links provided under data repository).

For scanning electron microscopy (SEM) *Sabellaria alveolata was* fixed in Bouin's fluid, dehydrated in an alcohol series and dried with CO_2 in a critical point dryer (BALZERS). After dehydration the samples were sputtered with gold (BALZERS Sputter coater) and examined with a XL 30 SFEG (Philips Electron Optics) scanning electron microscope. During dehydration the animals were sonified to remove debris and sand particles from the chaetae.

4.3 Results

4.3.1 Parapodial structure and chaetal arrangement

The body of *Sabellaria alveolata* is divided into four regions that are characteristic for Sabellariidae; the thorax, parathorax, abdomen and the cauda. Chaetal elements in the thorax and parathorax comprises of opercular paleae, oar-shaped notochaetae and capillary chaetae. The abdomen of *S. alveolata* forms the largest part of the animal's body and bears segmental biramous parapodia with notopodial uncini and neuropodial capillaries. The cauda has the appearance of an unsegmented tube and is achaetous. Aciculae are absent in all segments.

The abdominal notopodia are paddle-like appendages on either side of the animal's body (Fig.4.1). Those of the first few abdominal segments are broad and large, towards the posterior end they become narrower and elongate. Paired dorsal branchiae appear on the parathoracic segments and in the first 15-20 abdominal segments. They become gradually smaller along the antero-posterior axis and disappear completely in the posterior segments of the abdomen. The uncini are located at the apical margin where they are aligned in a single transverse row. Each chaeta arises from a chaetal follicle and all follicles are aligned within a single chaetal sac without being separated by an extracellular matrix.
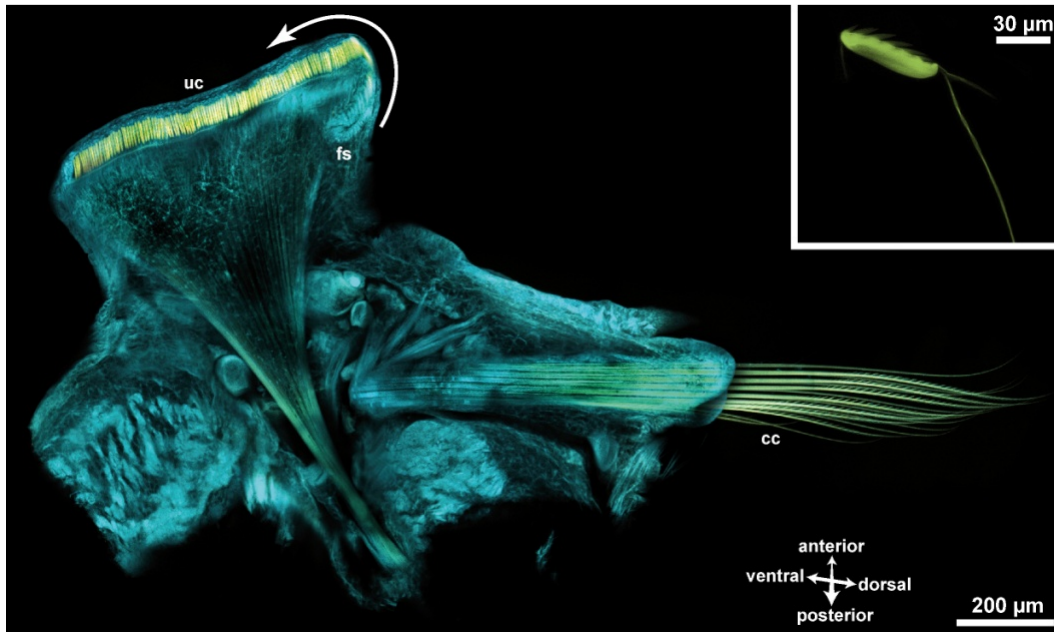
Figure 4.1 Confocal *z*-projection of a phalloidin stained preparation of a single abdominal parapodium of *Sabellaria alveolata. cyan* phalloidin*, yellow* chaetal autofluorescens *uc* uncini, *fs* formative site, *arrow* marks the direction of chaetal development *cc* capillary chaetae, *inset* detail image of an isolated uncinus.

Small, needle-shaped rods originate from the rostral and adrostal portion of each uncinus and extend into the notopodium. Apically these rods are aligned in a row, but the deeper they reach into the notopodium the more they form a bundle (Figs. 4.1; 4.2A). Each rod is surrounded by a follicle cell. The follicle cells of all rods comprise the inner end of the chaetal sac and rest on a common extra-cellular matrix (ecm). Follicle cells and ecm connect the bundle of rods to the parapodial musculature (see cLSM stack) in such a way that only the entire bundle can be moved, but not an individual chaeta. The formative site of the uncini is located at the ventral edge of the chaetal row and contains numerous developing chaetae (Figs 4.1; 4.2A,B; 4.4A), so that chaetogenesis could be inferred in detail from an ultrastructural analysis of a series of different stages.

The neuropodia of the abdomen only possess capillary chaetae that are either simple or pinnate (Fig. 4.1, 4.2D). Neuropodial capillary chaetae are long and also reach deeply into the parapodium. Their overall position is right-angled to the bundle of rods of the notopodial

hooks (Fig. 4.1). The basis of the neuropodial chaetal sac is connected with a network of radial chaetal muscles to the outer body wall, giving the chaetae the characteristic arrangement similar to an arrow pulled back in a bow (Fig. 4.1). Upon contraction, these muscles shorten and push the chaetae out of the body surface.
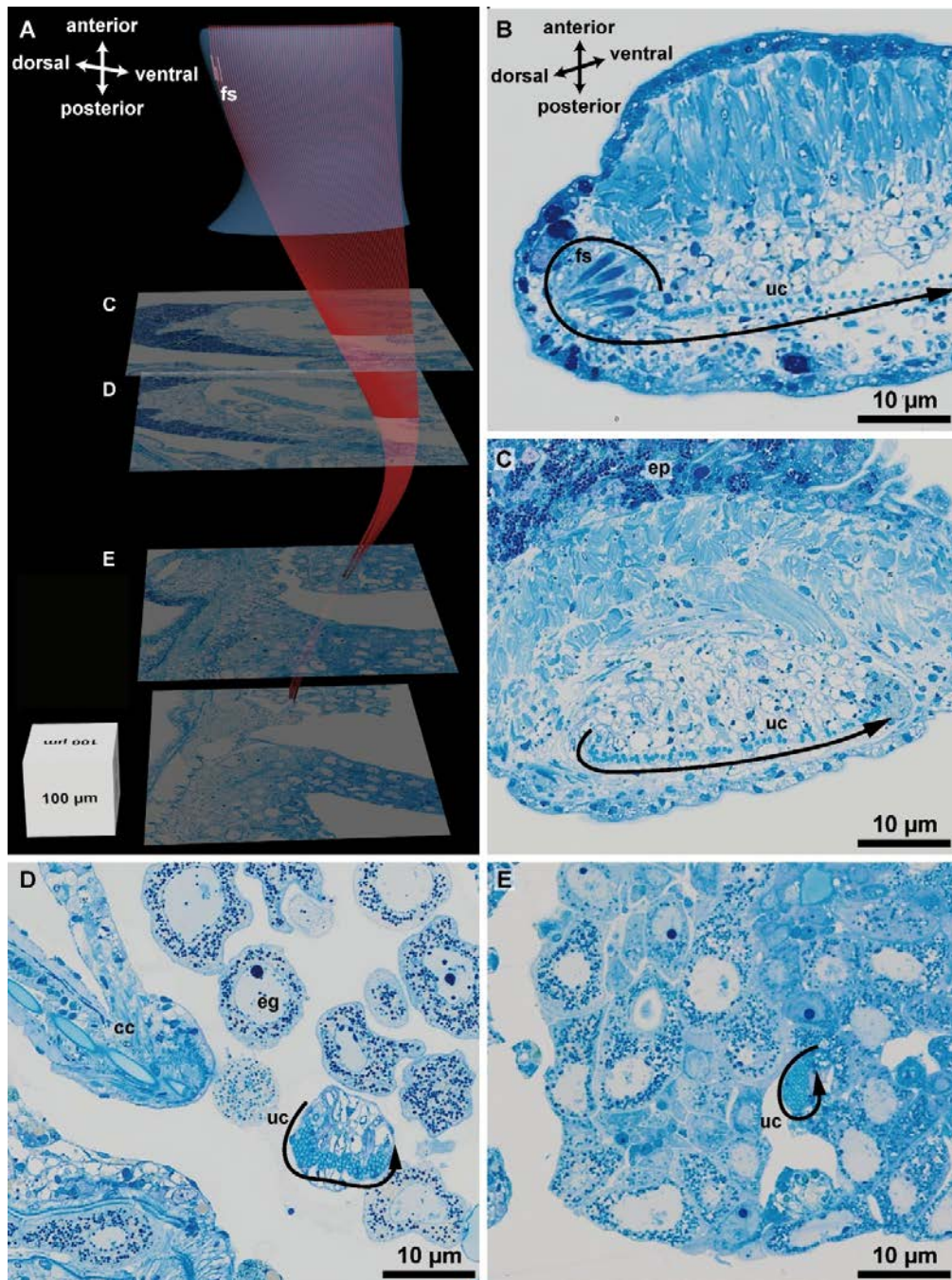
Figure 4.2 A. 3D model of the chaetal arrangement inside an abdominal torus. **B-E.** Aligned semi-thin sections used to construct the 3D model. Corresponding section planes are marked in A. The *arrow* indicates the direction of chaetal formation. *fs* formative site, *uc* uncini, *cc* capillary chaetae.

4.3.2 Structure of the uncini

Uncini in *Sabellaria alveolata* have a complex structure. The apical portion of a chaeta consists of a single median tooth followed by 5 to 6 pairs of teeth (Fig. 4.3D,E). The single small median tooth, called rostrum here, marks the rostral face of the uncinus; the size of the paired teeth decreases along a rostral-adrostral gradient, so that the adrostral pair of teeth is smaller than the rostral ones. All teeth and the rostrum originate from a blade-like shaft toward which they bend by 40°. Light microscopy shows that the shaft is composed of two different parts, separated by a fine rostro-adrostral refracting seam (Fig. 4.3D,E). The portion above this refracting seam directly underlies the teeth. It is small and dense and will be called "base" in this paper. The portion of the shaft below the refracting line is keel-shaped and bright and will be called "socket". This socket has a length of ±45µm from rostral to adrostral. Under Nomarsky contrast small, densely-packed vertical lines that originate in the teeth proceed into the base to end at the refracting seam. In the socket several lines can be seen running longitudinally and almost parallel to the refracting line. The main axes of both, vertical and longitudinal lines, form an angle of ±40° (Fig. 4.3D). The teeth, the small underlying base and a tiny portion of the rostral rod are the only externally visible structures in SEM preparations (Fig. 4.3A,B). As mentioned above, each uncinus possesses two vertical rods, a shorter adrostral one and a bipartite rostral one. Soon after its origin the rostral rod splits into a short anterior and a long posterior rod. While the shorter (anterior) rostral rod is almost as long as the adrostral rod, the longer (posterior) rostral rod extends up to 1.5 mm deep into the notopodium. The posterior rostral rod is almost 80 times longer than the entire apical portion (shaft plus teeth; ±20µm) (Fig. 4.1, 3E). All anterior rods of the notopodial uncini form the above described intranotopodial fiber bundle that serves as the attachment site of notopodial muscles. Both rostral rods have a similar diameter (±1.5 µm) like the adrostral rod.
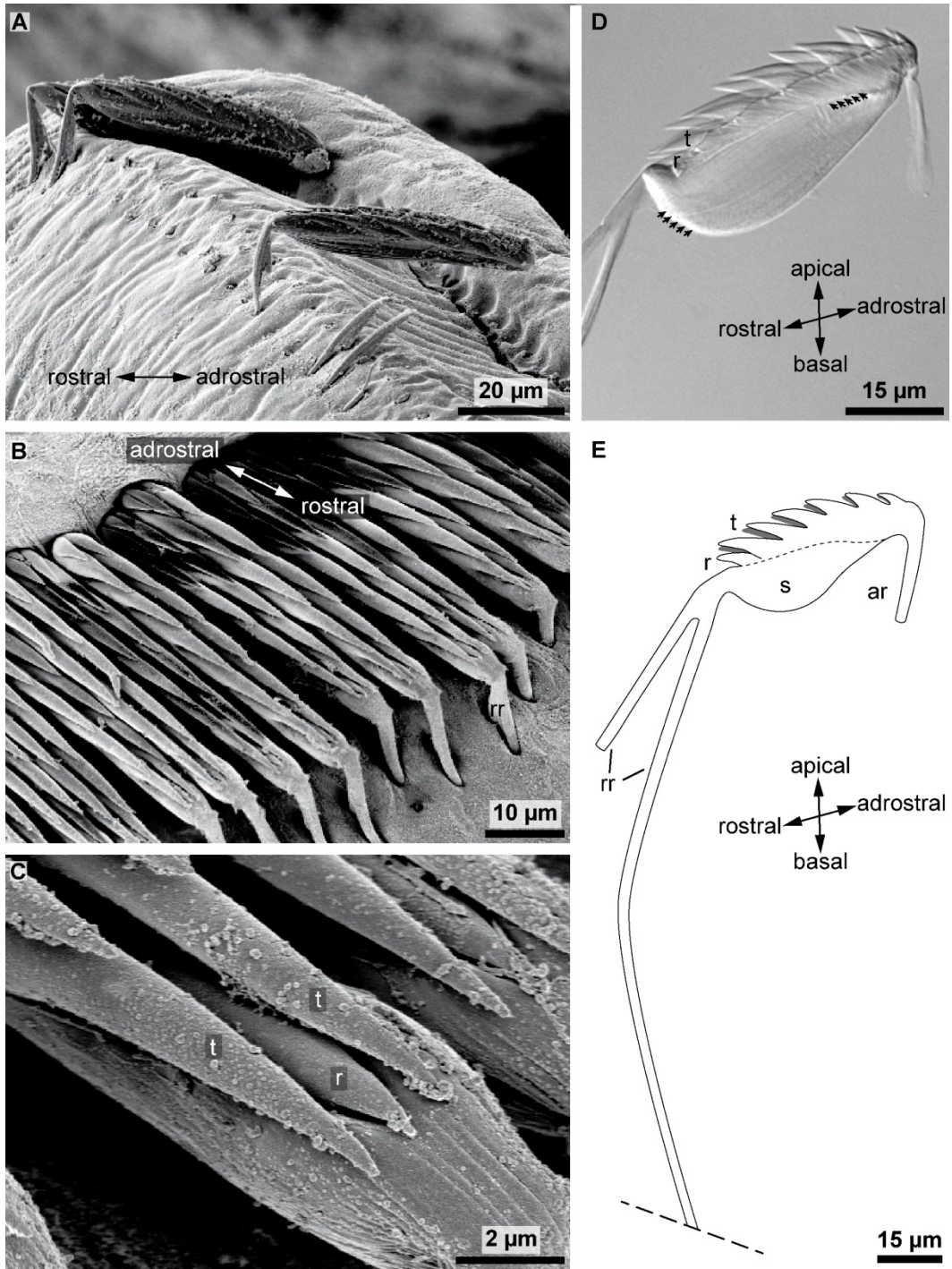
Figure 4.3 A. SEM image of detached abdominal uncini. **B.** SEM image of the row of uncini. **C.** SEM image showing the rostral portion of an uncinus in detail. **D.** Micrograph showing the apical portion of an abdominal hooked chaeta. *arrows* mark the direction of the internal canals. **E.** Schematic drawing of an abdominal uncinus, in scale. *dotted line* illustrates the refracting seam at merger of the chaetal socket and base, *rr* rostral rod, *ar* adrostral rod, *r* rostrum, *t* tooth, *s* socket

4.3.3 Chaetogenesis

Chaetogenesis occurs continuously within the formative site and the TEM study of fixed material allows inferring the entire process of chaetal formation from different developmental stages within a single formative site of the chaetal sac (Figs. 4.4–4.7). Uncini are formed within an ectodermal invagination (chaetal follicle) consisting of, the chaetoblast and at least five follicle cells. All cells are epithelial, interconnected by adluminal adhaerens junctions and rest on a common matrix that surrounds the chaetal sac. All cells surround a small compartment, the chaetal compartment, and bear several short microvilli that reach into the compartment. This compartment narrows to become a small canal that extends towards the epidermis where it opens to the exterior by a small pore. During chaetogenesis the chaeta is secreted into the chaetal compartment. The basalmost four cells are actively involved in chaetogenesis, i.e. the chaetoblast at the base of the chaetal follicle and three adjacent follicle cells. The fourth and fifth follicle cells form a ring that surrounds the chaetal compartment and the proximal section of the canal. Each of these cells possesses a subapically located diplosome (Figs. 4.4D, 4.5A,B,E). In young follicles one of both diplosomes may contact the apical cell membrane, but never induces a cilium (Fig. 4.5A). The microvilli of the chaetoblast and the first two follicle cells are set denser and are longer than those of the remaining follicle cells; the microvilli of the chaetoplast are slightly larger in diameter than those of the follicle cells (Fig. 4.5B). The latter form the template of each substructure of the chaeta. Continuous polymerization of chitin between the bases of the microvilli enlarges the developing chaeta. Given that the microvilli have a constant length, sooner or later the developing chaeta will exceed the microvilli in length and electron-lucent canals will remain inside the chaeta where microvilli had once been. These canals may or may not be filled up secondarily by electron-dense material.
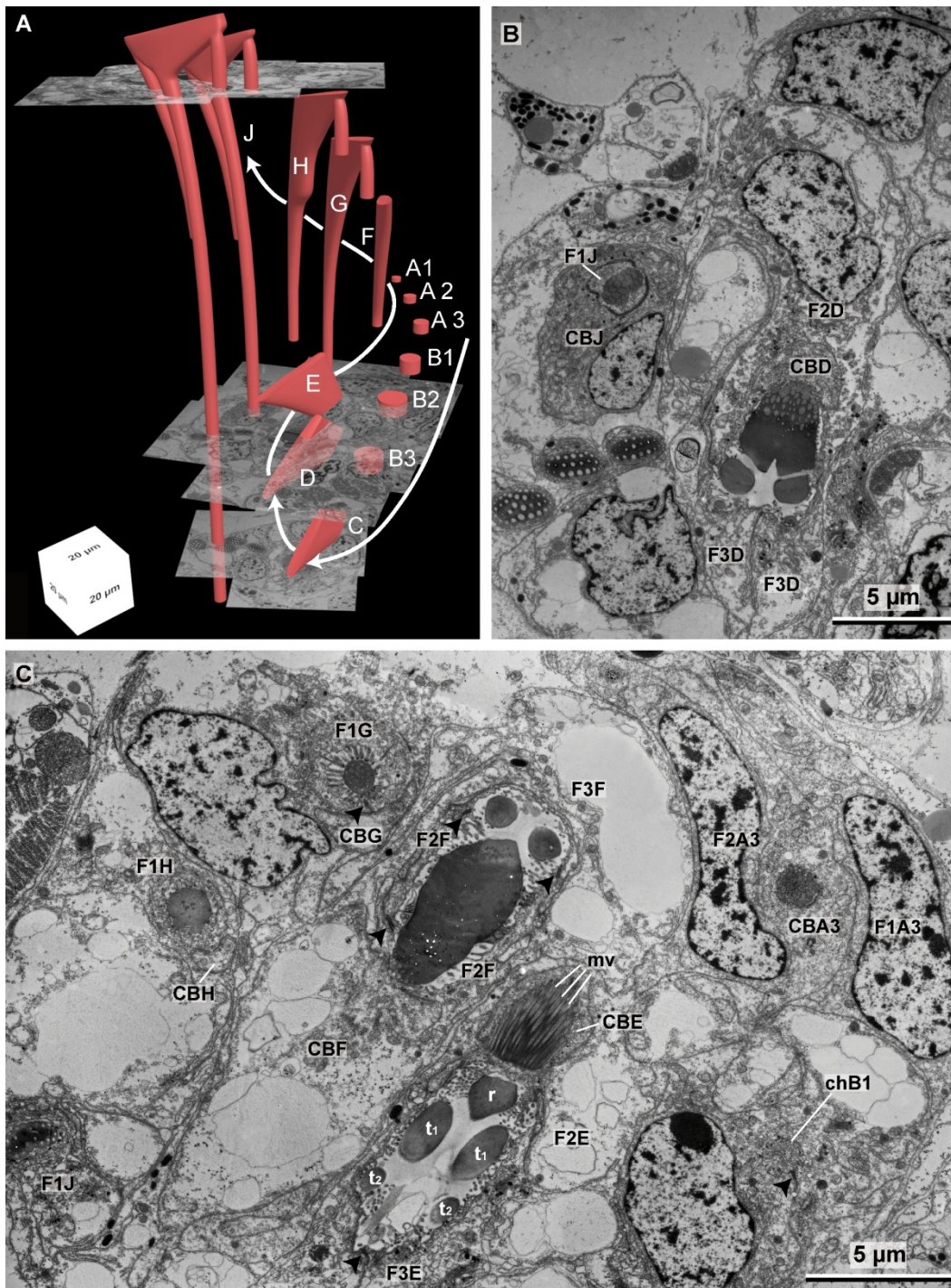
Figure 4.4 A. 3D model of the chaetal formative site, reconstructed using the aligned serial ultra-thin sections. Consequent developmental stages of uncini are labeled from A1–J. This numbering is employed all through the images when referring to these specific developmental stages. **B.** TEM image of the formative site showing the formation of the long rostral rod in J and the formation of the socket in D. **C.** TEM image of the formative site showing the formation of the adrostral rod in H and G, the formation of the socket in E and F, and the formation of the rostrum in A3. Note the chaetal canal of B1 (*chB1*) in the lower right. *F1–F3* follicle cells, *CB* chaetoblast, *r* rostrum, *t* tooth, *arrow heads* mark the adluminal adhaerens junctions.
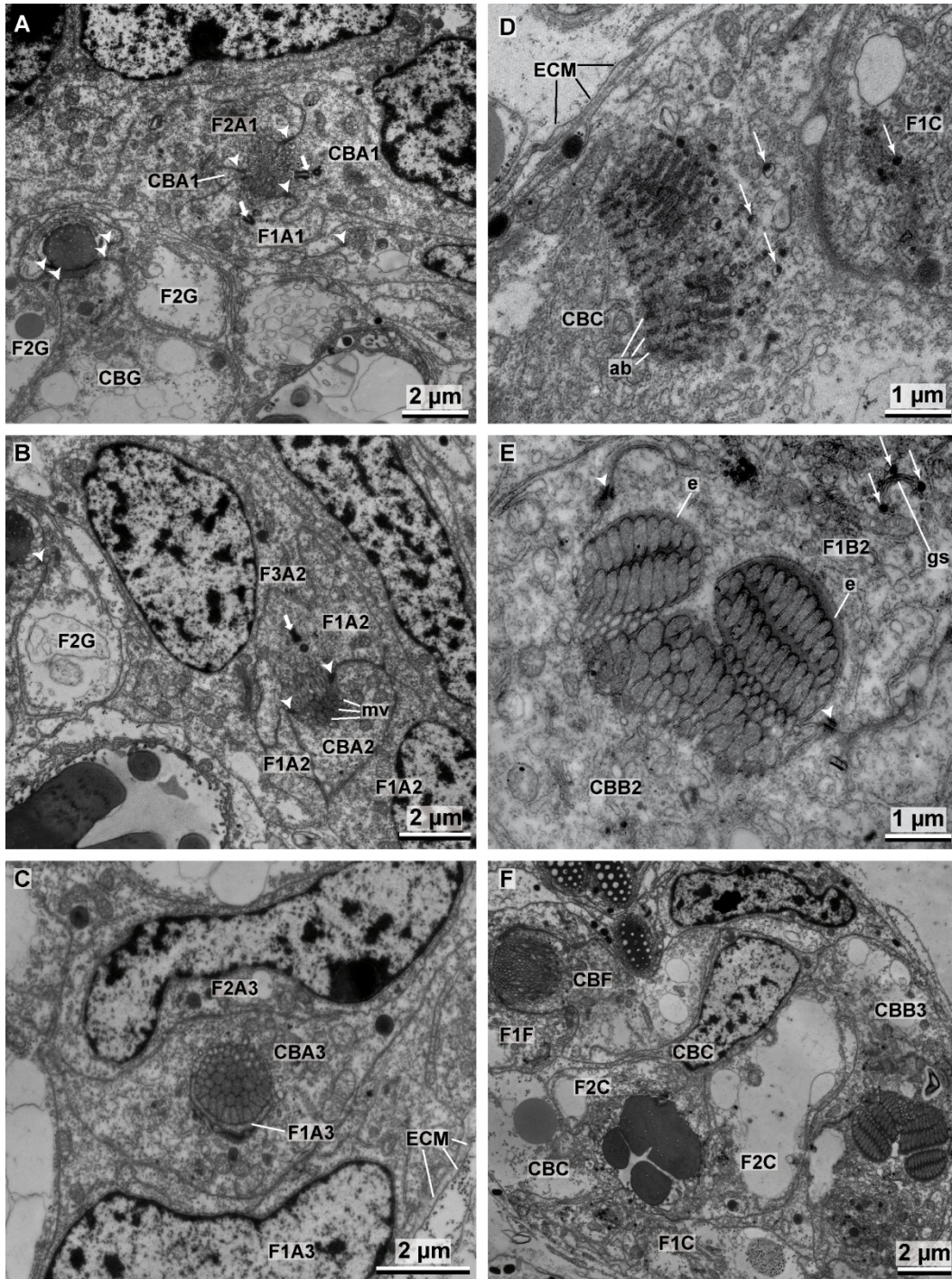
Figure 4.5 A.–C. TEM images of A1–3 showing the initial stage of chaetogenesis and the formation of a rostrum. **D.** Production of chaetal material and the subsequent transportation to the chaetal anlage via vesicles. **E.** Formation of the adrostral teeth in B2. **F.** TEM image of the formative site showing the formation of teeth in B3 with multiple rows of microvilli, older teeth in C with almost completely filled canals and the adrostral portion of the chaeta in F. *F1–F3* follicle cells, *CB* chaetoblast, *arrow heads* mark the adluminal adhaerens junctions, *short arrows* mark centrioles, l*ong arrows* mark vesicles containing electron-dense chaetal material, *ECM* extra-cellular matrix, *ab* actin bundles, *mv* microvilli, *e* enamel, *gs* golgi stack.
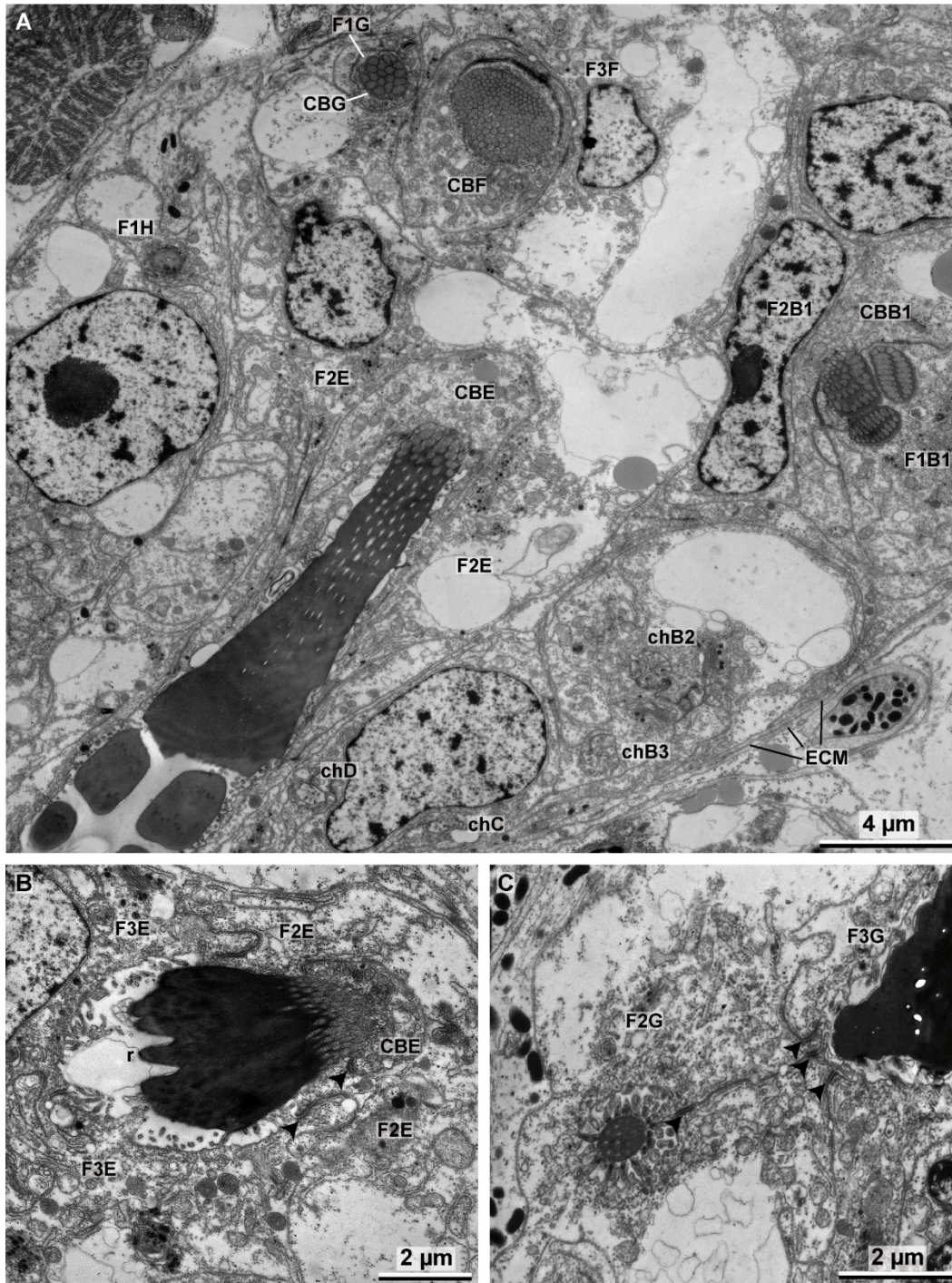
Figure 4.6 A. TEM image of the formative site showing the formation of the short rostral rod in G, the formation of the subrostral portion of the socket in F, the formation of the socket in E and the formation of teeth in B1. Note the canals (*chB2–D*) that connect inferior developmental stages to the outer surface. **B.** Formation of the rostral part of the socket in E. **C.** Adrostral rod of G surmounted by F2. *F1–F3* follicle cells, *CB* chaetoblast, *arrow heads* mark the adluminal adhaerens junctions, *ECM* extra-cellular matrix, *r* rostrum
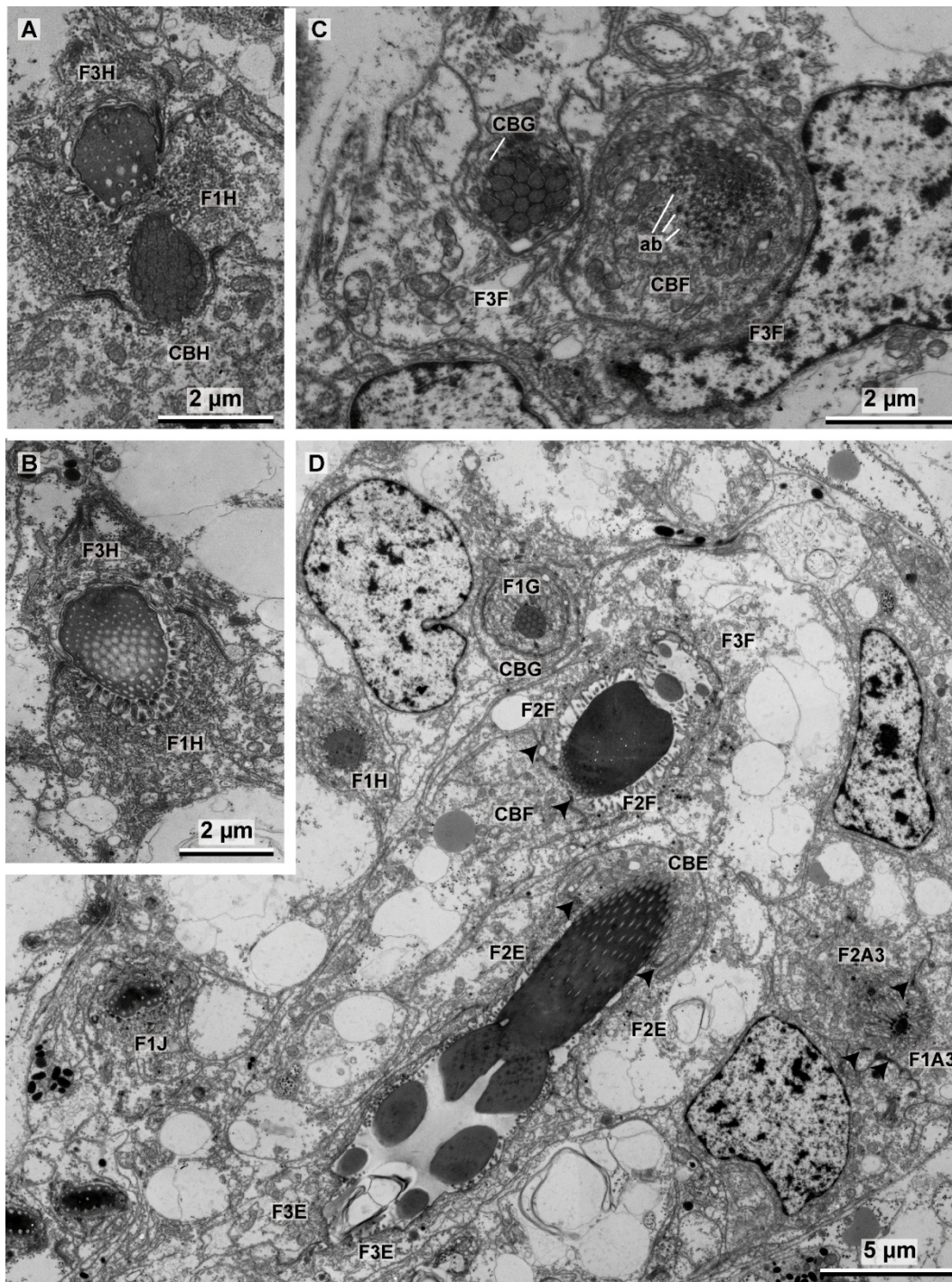
Figure 4.7 A.-B. TEM images showing the merger of the long and short rostral rod in H. Note the newly developing long rod and the fully differentiated short rod in A. **C.** Formation of the short rostral rod in G and the formation of the subrostral process in F, note the bundles of actin filaments that are located under the microvilli. **D.** TEM image of the formative site showing the the fully differentiated long rostral rod in J, developing long rod of H, formation of the short rostral rod in G, formation of the socket in F-E, and the tip of the rosrum in A3. *F1–F3* follicle cells, *CB* chaetoblast, *arrow heads* mark the adluminal adhaerens junctions, *ab* actin bundles.

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In this study 14 developmental stages of uncini were found in a single formative site that was cut into a series of ultrathin sections, analysed for ultrastructural details and reconstructed. Nine stages are shown in figure in Fig.4.9 and the topological position of these stages within the formative site can be seen in Fig. 4A. Chaetogenesis of uncini in *Sabellaria alveolata* can be divided into three steps: (1) formation of the rostrum, teeth and base (Fig. 4.9A-C), (2) formation of the socket (Fig. 4.9D-F), (3) formation of the rostral and adrostral rods (Fig.4.9F-J).

Formation of rostrum, teeth and base. Chaetogenesis starts when a small cluster of microvilli emerge on the surface of the chaetoblast (Figs. 4.5A-C, 4.9A). These microvilli form the template of the anteriormost tooth, the rostrum, and extend into the chaetal compartment. Chitin polymerizes between the bases of the microvilli and builds the tip of the rostrum. Additional microvilli that appear peripheral to the initial cluster broaden the rostrum. Subsequently, two additional clusters of microvilli are formed adrostrally on either side of the developing rostrum (Fig. 4.5D,E). These are the template of the first pair of teeth. In the same manner five additional pairs of theeth are subsequently added along a rostro-adrostral gradient, so that finally the sixth pair of teeth is situated adrostrally (Figs. 4.4C, 4.9B-C). All teeth and the unpaired rostrum have almost the same size as 2-3 rows, each consisting of 9-12 microvilli, once formed their template. Since the number of microvilli increased towards the base of the teeth, all microvilli finally form a broad and uniform field, which is the template for the base underlying the teeth. Electron-dense material released from vesicles of the first two follicle cells forms an enamel that covers and smoothens the irregular surface of the teeth (Fig. 4.5E). This material is produced inside Golgi stacks and transported to the chaetal surface in vesicles (Fig. 4.5D, E). While more rows of teeth are added and the developing chaeta enlarges, the canals left by the templating microvilli, become more or less completely filled with

electron dense material (Fig. 4.5F). At the end of this first step of chaetogenesis the rostrum, 4 pairs of teeth and the anterior part of the base are formed. The microvilli are completely retracted from the rostral three quarters of the developing chaeta; the canals the microvilli left, are refilled with electron-dense deposits. The chaetoblast merely underlies the adrostral half of the developing chaeta, whereas the rostral half is underlain by the planar apical cell membrane of the second follicle cell. Chaetogenesis is interrupted in this region. The entire anlage is oriented vertically within the chaetal compartment.

Formation of the socket. Once all teeth and the base are formed, the chaetoblast grows towards the rostrum again to underlie the entire base and slightly exceed it rostally. The chaetoblast then forms microvilli that form a homogeneous field These microvilli have a vertical orientation and thus are more or less longitudinal relative to the anlage. They form the template of the socket and chitin polymerizing between the microvilli is added to the base of the anlage. The longitudinal refracting seam visible under Nomarsky contrast between base and socket results from the break in chitin polymerization after teeth and base were formed (Figs. 4.4C, 4.6A,B, 4.7D, 4.9D-F). Re-orientation of the microvilli is also clearly visible under Nomarsky contrast in fully differentiated chaeta as longitudinally arranged lines inside the socket. These lines are actually canals left by microvilli inside the socket during formation (Fig. 4.3D, E). A group of microvilli remains at the apico-adrostral part of the chaeta, while those in the subapical part disappear. Bare cell membrane of the chaetoblast underlies this portion and no chitin is formed (Fig. 4.5F, 4.9E,F). At this time the entire anlage starts to alter its position within the chaetal sac again. Since the microvilli are always vertically oriented they also alter their position relative to the developing chaeta. The subapical group of microvilli forms an adrostral cap while the socket increases in size. Finally the microvilli that

formed the template of the adrostral portion of the socket retract and disappear, except for those microvilli that formed the adrostral cap. The same occurs rostrally, here leaving a large apico-rostral group of microvilli (Fig. 4.9F). Adrostrally the second follicle cell expands into the gap between the developing chaeta and the chaetoblast, so that this part of the developing chaeta is now underlain by the apical cell membrane of the second follicle cell. The subrostral portion of the socket is underlain by the apical cell membrane of the chaetoblast. After the socket has been completed two groups of microvilli remain, a rostral and an adrostral one. The entire anlage now has a horizontal position within the chaetal compartment (Fig.4.9F).

Formation of the rostral and adrostral rods. After the socket is completed the microvilli of the chaetoblast are almost completely reduced, except for a rostal and an adrostral group (Fig. 4.9F). The adrostral group of microvilli elongates and forms the template for the adrostral rod. Chitin polymerization happens rapidly and the adrostral rod elongates, parallel to the apico-basal axis of the uncinus (Fig. 4.9G). The rostral group of microvilli actually consists of two adjacent, but perpendicular patches of microvilli (Figs. 4.6A, 4.7C). They form the template for the rostral rod, which initially is rather massive and oblique to the rostro-adrostral axis of the chaeta. Later the microvilli split into an anterior and a posterior one. The microvilli of the anterior group elongate and become the template for the anterior rostral rod, while the posterior group consists of short microvilli and remains in its original position. Chitin polymerizes rapidly between the microvilli to form the anterior rostral rod. Anterior rostral rod and the adrostral rod are formed simultaneously; one keeps pace with the other during formation (Fig 4.9G). During these initial steps of forming the rods the chaetoblast expands as the anterior rostral rod grows slightly oblique to the apico-basal axis of the developing chaeta. The perikaryon of the chaetoblast is located rostrally and a small adrostal cytoplasmic bridge underneath the socket connects the perikayon to the adrostral

group of microvilli (Fig. 4.4, 4.9G). A small rostral cytoplasmic bridge connects the rostral group of microvilli that is the template of the anterior rostral rod. After both were completed, the microvilli are reduced and the cytoplasmic bridges are withdrawn (Fig. 4.9H). The adrostral cytoplasmic bridge is replaced by the second follicle cell, which already grew between the median portion of the socket and the chaetoblast earlier during chaetogenesis (Fig. 4.9F). The rostral cytoplasmic bridge is replaced by the first follicle cell. During withdrawal the last group of microvilli which remained posterior while the anterior rostral rod was formed, becomes active. Its microvilli elongate and form the template of the posterior rostral rod (Figs 4.4B, 4.9J). While chitin polymerisation elongates the rod, the chaetoblast forms a cup that surrounds the developing posterior rostral rod. The posterior rostral rod increases very rapidly in length and grows parallel to the baso-apical axis of the chaeta (Fig. 4.8). No further modification of the microvilli pattern occurs in this last phase of chaetogenesis. During elongation the newly formed chaeta is pushed towards the surface, and finally becomes visible externally and aligns itself at the ventral edge of the chaetal row. The canals left by the microvilli during growth of the posterior rostral rod are not filled by any material and remain electron-translucent. The same is true for the anterior rostral rod and the adrostral rod. When the formation is complete, intermediate filaments appear inside the follicle cells and the chaetoblast. Hemidesmosomes connect them to the chaeta to mechanically link the chaeta to the perifollicular ecm (Fig. 4.8C). The chaetoblast remains cup-like at the chaetal base and the microvilli that formed the long rostral rod remain inside the basalmost part of the chaeta (Fig. 4.8B,C).
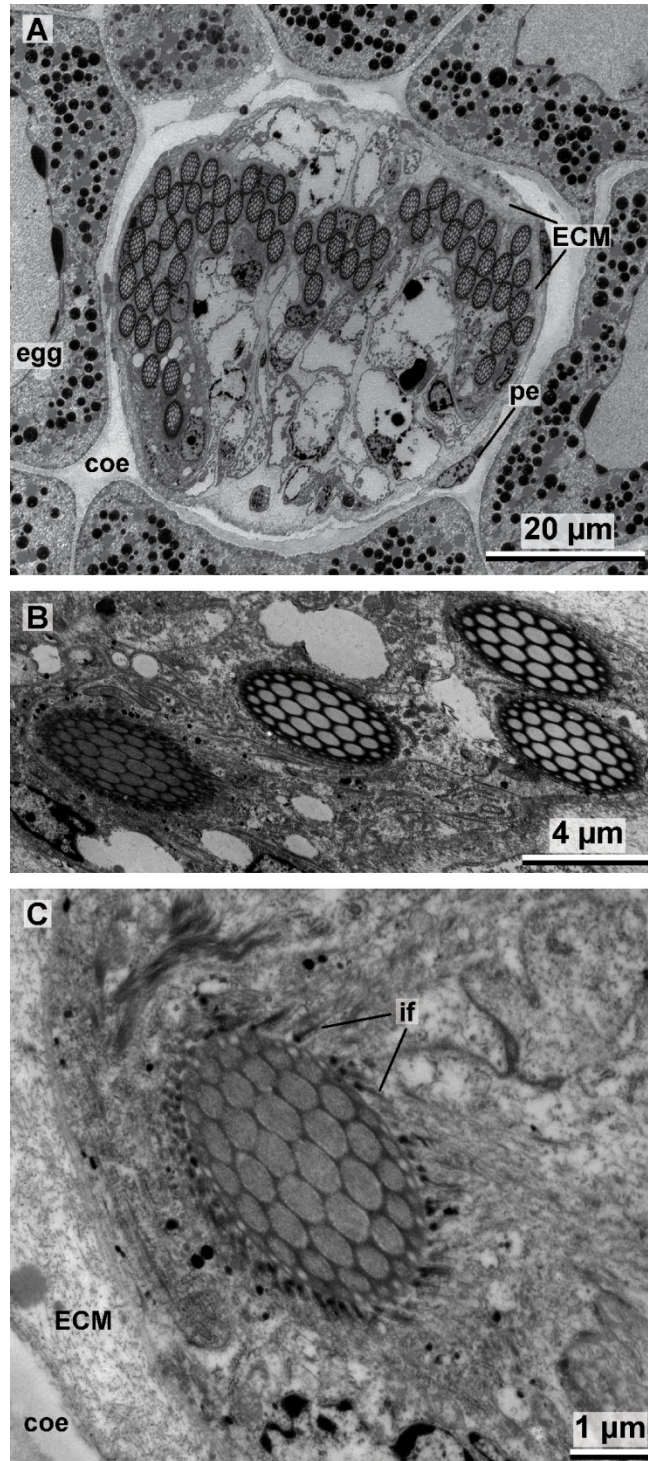
Figure 4.8 A. TEM image of the chaetal bundle showing the arrangement of fully differentiated chaetae. **B.** Canals of the youngest chaetae stilled filled with microvilli in contrast to the hollow canals of older chaetae. **C.** Detail image of the youngest chaetae, note the intermediary filaments (*if*) attached to the chaeta via hemidesmosomes. *coe* coelom, *pe* peritoneum, *ECM* extracellular matrix.
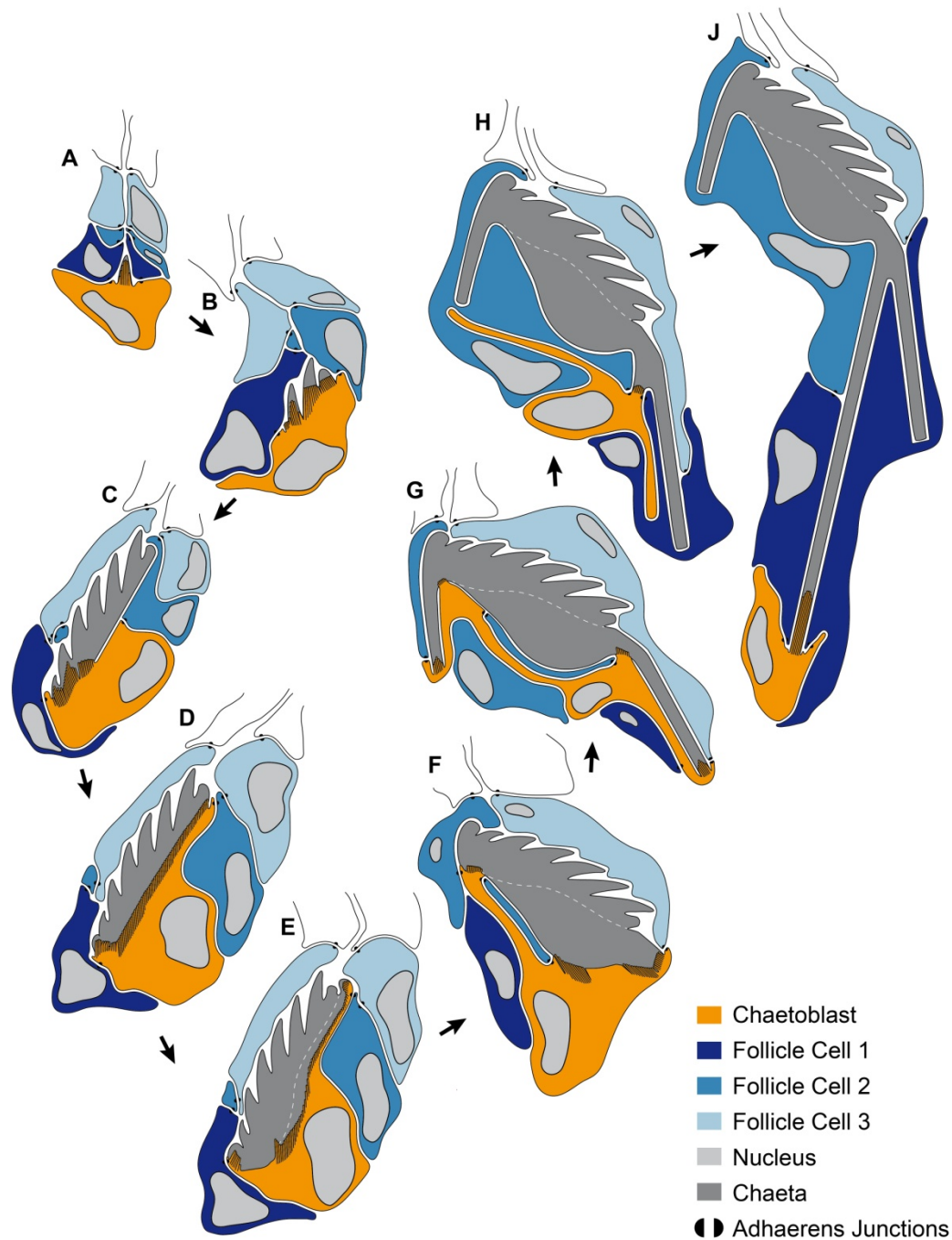
Figure 4.9 Schematic illustration of chaetogenesis and the interaction between the chaetoblast and the follicle cells as a series of sagittal sections of subsequent representative stages of the chaetal formation. Topological position of corresponding development stages are marked in the 3D model in Fig. 4. **A.** Earliest stage of chaetogenesis; formation of the rostrum. **B-C.** formation of the teeth. **D-E.** formation of the chaetal socket. **F-G** Formation of the adrostral rod and the short rostral rod. **H-J** Final step of chaetogenesis; formation of the long rostral rod.

4.4 Discussion

Uncini have repeatedly been described and illustrated for different sabellariid species (Lana and Gruet 1989; Kirtley 1994; Souza dos Santos et al. 2014; Capa et al. 2015), indicating that *Sabellaria alveolata* can be taken as a representative for the entire group. Due to structural similarities of these chaetae across sabellariids we also assume that formation of them is largely identical in sabellariid species. However, the tremendous length of the posterior rostral rod remained thus far largely unnoticed. We suppose that this is caused by its extremely delicate structure, making it difficult to identify the actual extension of this rod without sectioning. Despite missing evidence from other species, we assume that a rostral rod extending deeply into the notopodium is characteristic for all sabellariid species, an assumption that has to be confirmed in subsequent studies. In the following we will discuss our results in terms of function, homology, phylogenetic significance and highlight the cellular dynamics underlying chaetogenesis in *Sabellaria alveolata*.

Function. It has repeatedly been shown that hooked chaetae and uncini correlate with a tubiculous life style and are used to withstand drag forces by interacting with the inner texture of the tube (Merz 2015; Woodin and Merz 1987). Roy (Roy 1974) describes that the sabellariid *Phragmatopoma californica* maintains its position in the center of the tube by extending the notopodia so that they contact the wall. Thereby, the notopodia must be of a certain length to maintain water currents inside the tube for oxygen supply and feces removal. We assume that the uncini serve as anchors to adhere in the visco-elastic wall of the tube (Le Cam et al. 2011). In *Sabellaria alveolata* a second function is related to these chaetae, the structural correlate of which is the rods. The posterior rostral rod is 80 times longer than the shaft and extends deeply into the neuropodium. A rod consists of a few hollow chitin tubes that are enwrapped by an enamel and serve as a rigid, but extremely flexible stick. Since the uncini of *S. alveolata* are alinged in

a transvers row, these rods form a planar array in the tip of the notopodium that thus serves as a broad paddle and allows a maximum contact surface between the row of uncini and tube wall. Deeper inside the notopodium the rods converge to form a bundle that serves as the attachment site for parapodial muscles. The notopodium contains part of the body coelom, which functions as a hydroskeleton. As such, it guarantees stiffness of the notopodium, but does not allow moving it. Since the bundle of rods is highly flexible and serves as an attachment site for transversal muscles, the notopodium can be moved back- and forward. Due to the mechanical properties of the chitinous rods inside, it will always return to its original structure after relocation. The rods thus serve in stability of the notopodium and allow moving it without influencing the shape of the notopodium. Roy (Roy 1974) actually mentions that *Phragmatopoma californica* uses the notopodia to perform rear-to-front motions. These anteriorly directed strokes are used for backward moving when the animal rapidly withdraws into the tube. The structural prerequisite of such a notopodial performance is the internal bundle of rods. In this respect the bundle of rods in sabellariid notopodia has a similar function as the acicula of errant (aciculatan) annelids. The aciculae also function as "skeletal" rods of parapodia to which the parapodial musculature is attached. In certain terebellids (i.e. *Terebella lapidaria*) similar long shafts/basal processes reach deep inside the parapodia (unpublished data). This indicates a convergent evolution of rod like elements inside the parapodia, be it bundles of thin rods like in sabellarids, other chaetal protrusions like in terebellids or large and robust aciculae.

Homology. According to Holthe (Holthe 1986) hooked chaetae (= dentate hooks in Rouse & Plejel (Rouse and Pleijel 2001) consist of a main tooth or rostrum, a capitium surmounting the rostrum and comprises smaller teeth and a manubrium or shaft. Rostrum and teeth of the capitium are curved and bend towards the shaft. Sometimes a subrostral process or distal expansion of the manubrium is found

underneath the rostrum. Such chaetae are known from Sabellida, Terebellida, Oweniidae, adult Arenicolidae (Bartolomaeus 1995; Meyer and Bartolomaeus 1996; Bartolomaeus and Meyer 1997; Bartolomaeus 2002). If the shaft is shorter than the dentate distal section or virtually absent, the hooked chaetae will be called uncini (Sabellida, Terebellida, Chaetopteridae, Sabellariidae) (Rouse and Pleijel 2001). In certain groups the dentate apex (rostrum plus capitium) is partly enveloped by hairlike-protrusions of the subrostum (young Arenicolidae, Maldanidae, Psammodilidae) (Bartolomaeus and Meyer 1997; Meyer and Bartolomaeus 1997; Tilic et al. 2015a) or a hood (Capitellidae, Spionidae, certain Eunicida) (Hausen and Bartolomaeus 1998; Schweigkofler et al. 1998; Tilic et al. 2014). It is no surprise that testing for homology by studying chaetogenesis revealed that hooked chaetae and uncini of certain taxa share identical steps during chaetogenesis (Sabellidae and Serpulidae: (Bartolomaeus 2002) Arenicolidae: (Bartolomaeus and Meyer 1997; Bobin 1944), Maldanidae: (Tilic et al. 2015a), Psammodrilida:(Meyer and Bartolomaeus 1997), Pectinariidae: (Bartolomaeus 1995) , Terebellidae: (Bartolomaeus 1998) , Oweniidae: (Meyer and Bartolomaeus 1996)). In these taxa the rostrum is always the very first structure that develops during genesis and is invariably preformed by a group of mivrovilli. Subsequently, each teeth of the capitium are formed by a large microvillus. Microvilli that served as template for the rostrum and the capitium later merge and form the shaft, which always is perpendicular to the rostrum. These characteristics are found in capitellids hooded hooks, i.e., chaetae, in which a hood surrounds the distal section of the hooked chaeta (Schweigkofler et al. 1998). The identity of the structure and formation patterns of hooked chaetae, uncini and hooded hooks across the above mentioned annelid taxa led to the hypothesis of their homology, which could be substantiated by several corresponding structural and developmental details (Bartolomaeus 1998; Hausen 2005). Other hooked chaetae with a hood

differ from this pattern. In spionid and lumbrinerid species several microvilli and not a single microvillus form the template for the smaller spines that surmount the rostrum (for *Scolelepis squamata* Hausen and Bartolomaeus 1998; for *Prionospio fallax* Hausen 2001, for *Lumbrineris tetraura* Tilic et al. 2014). In addition formation of the hood differs between spionid, capitellid and lumbrinerid species and does not support homology of the hood (Tilic et al. 2014).

Structure and chaetogenesis of uncini in *Sabellaria alveolata* differ significantly from any hooked chaeta described so far, which poses problems on applying the same terminology. Although the unpaired rostral tooth should be termed rostrum as it is the first structure formed during chaetogenesis and preformed by several mivrovilli, further groups of microvilli are the template for the following teeth which thus should not be termed capital teeth. The shaft consists of two sections, the base and socket; both are separated by a refracting line and are pre-formed by microvilli of different orientation. A shaft that is composed of two parts because of controlled spatial and temporal intermissions in the formation processes is thus far unknown. The subcuticular portion of sabellarid uncini consists of an adrostral and a bipartite rostral rod. Similar rod-like processes are known from terebellids (*Nicolea zostericola*; (Bartolomaeus 1998)) and also chaetopterids (Tilic & Bartolomaeus, unpubl. data for *Chaetopterus variopedatus* and *Telepsaphus costarum*). However, formation of these processes differs from the rods in *Sabellaria alveolata*. Two small groups of microvilli, one rostral and one adrostral remain in the mentioned terebellid and chaetopterids species after the microvilli were withdrawn from the shaft after its completion. Polymerization of chitin between the microvilli of both groups then gives rise to both processes, which thus are rather parts of the shaft than additional structures. These differences do not support a homology between the rod in *Sabellaria alveolata* and the rod-like processes of the manubrium in terebellid and chaetopterid species.

Structure and chaetogenesis of uncini in *S. alveolata* thus differ in several aspects from that of other annelids with uncini and hooked chaeta. These differences either result from transformation or convergent evolution. A decision between both alternatives, however, depends on the phylogenetic position of the Sabellariidae.

Phylogenetic implications. Sabellariidae were first described as a subgroup of Sabellida by Lamarck (Lamarck 1818), and later moved to Terebellida by Savigny (Savigny 1822). Levinsen (Levinsen 1883) placed them as a separate suborder, using the name Hermelliformia, which was first coined by Malmgren (Malmgren 1867). Phylogenetic analyses based on morphological data (Rouse and Fauchald 1997; Schulze 2003; Smith 1991) suggest a sister group relationship with Sabellidae. One decisive morphological character in favour of the close relationship to Sabellidae is the so called "chaetal inversion" (for review (Kieselbach and Hausen 2008); (Capa et al. 2012; Capa et al. 2011; Fauchald and Rouse 1997; Kupriyanova and Rouse 2008; Rousset et al. 2004).) Sabellaridae and Sabellidae show a unique chaetal arrangement with abdominal uncini in a notopodial position. This was considered to be an undisputed synapomorphy until Kieselbach and Hausen (Kieselbach and Hausen 2008) provided evidence that the specific chaetal arrangement of Sabellidae and Sabellariidae arose independently (see also (Kieselbach 2012)). Kieselbach and Hausen (Kieselbach and Hausen 2008) also emphasize that the homology of the uncini of sabellids and those of sabellarids is yet to be established. More recent molecular phylogenies of annelids (Struck et al. 2007; Zrzavý et al. 2009; Capa et al. 2011; Capa et al. 2012; Weigert et al. 2014; Struck et al. 2015; Andrade et al. 2015) group them together with Spionida. A sabellariid-spionid sister group relationship had already been suggested by Wilson (Wilson 1929) and later Dales (Dales 1952) and Rouse & Pleijel (Rouse and Pleijel 2001; Rouse and Pleijel 2003). Wilson (Wilson 1929) substantiated this hypothesis with characters of the larval organisation, since both

possess long leval chaetae inserting posterior to the prototroch. Kieselbach (Kieselbach 2012) described a specialized ciliated sensory organ in the prostomium of larval *Sabellaria alveolata* that was thus far only known from Spionida (Hausen 2007). Except for being paired in Spionida this organ shows an identical organization and the same substructures like Spionida, so that this sense organ supports the hypothesis of a sister group relationship of Spionida and Sabellariidae.

The differences of sabellid and sabellariid uncini in terms of substructures and chaetogenesis, however, do not provide evidence for a sister group relationship between both groups. Moreover, the fact that several microvilli and not a single big microvillus form the template for each adrostral teeth is identical in the spionids studied thus far (Hausen and Bartolomaeus 1998; Hausen 2001;) and in *S. alveolata*. The better supported alternative hypothesis of a spionid-sabellariid sister group relationship presently argues against homology of sabellid and sabellariid uncini and for transformations that need to be analysed in subsequent studies.

Cell dynamics In a recent essay Warren (Warren 2015) compared the microvilli of the chaetoblast with the printing head of a 3D-printer, as they ensure assembly of a complex structure by selective addition of material in time and space. Chaetogenesis in *Sabellaria alveolata* illustrates the complexity of this process and provides empirical evidence that in addition to dynamic microvilli cell dynamics influences proper formation of the chaeta. Beside repeated formation of microvilli, the position of the chaetoblast within the formative site and the speed in which chitin polymerizes are important factors shaping the final structure of the sabellariid uncinus. Tilting the axis of the developing chaeta is a prerequisite to form the basal part of the shaft, the socket, as well as the proper orientation of the rods. The chaetoblast itself expands during chaetogenesis, relocates the perikaryon and finally remains as a cup-like structure at the base of the rostral rod. During this final step of chaetogenesis the follicle cell

expands tremendously as it surrounds the entire posterior rostral rod. Since the rod, when completed is 80 times longer than the shaft, the follicle cell expands to a 80 fold of its initial length.

Fixation of a continuous developmental process causes that this process is divided into different stages. The formative site of *S. alveolata* studied in this paper, shows 13 of these stages (Fig. 4.4A). Provided that chaetogenesis is a continuous process, one would expect that the time passed between the stages is always identical, even though it is not exactly known. According to this consideration, the initial phase of chaetogenesis lasts rather long, since we found 6 subsequent stages showing increasing numbers of apical teeth. The remaining steps are rather rapid events, because 6 steps later the entire chaeta is complete, except for the posterior branch of the rostral rod. One step further this structure attained an enormous length. Chitin is produced by the chitin synthases that are located in the cell membrane and has been shown to appear at the bases of microvilli (Peters and Latka 1986; Zimoch and Merzendorfer 2002; Moussian et al. 2015). Provided that chitin synthase is also located in microvillar membrane, one would expect that the longer the microvilli are the higher is the rate of chitin synthesis. Although this remains to be shown experimentally, there is a remarkable correlation between the length of the microvilli and the speed of growth of chaeta in support of this anticipation: the longest microvilli can be found where chaetal elongation occurs rapidly.

4.5 Conclusions

Despite superficially similar to the uncini of Sabellida, Terebellida and other smaller annelid groups the uncini of *S. alveolata* differ in substructures and formation (Table 4.1). These differences concern (1) formation of adrostral teeth by groups of microvilli instead of one large microvillus, (2) bipartition of the shaft and its formation in

temporally separated steps and (3) formation of rostral and adrostral manubrial extensions (4) followed by the formation of an adrostral and a bipartite rostral rod. These differences either result from transformations of an ancestral structure or from convergent evolution. Given that recent molecular and morphological data provide strong support for a sister group relationship between Spionida and Sabellariidae, the unicini in sabellariids on one hand and those of terebellids, sabellids and a few other smaller annelid taxa one the other hand must have evolved convergently. Since Spionidae possess hooded hooks consisting of apically dentate chaeta with a hood and since all apical teeth are pre-formed by groups of microvilli, it is likely that the sabellariid uncini evolved by transforming such dentate chaetae into uncini. Our study also shows that this transformation went along with changing functional demands. In contrast to spionid species, sabellariids live in a reinforced visco-elastic tube to which they are able to firmly adhere, using the uncini as anchors. The specific structure of the notopodium optimizes the contact surface towards the tube wall. In addition the notopodia are used for rapid withdrawal and must be movable. Since they are rather long structures they need some internal reinforcement that acts as attachment site for the transversal muscles. These attachment sites are provided by the rods originating from the uncini, since they form a central, flexible structure comparable to the acicula in aciculate annelids.

Table 4.1 Structure and chaetogenesis of hooked chaetae and uncini in Annelids.

Taxon	species	rostrum/main tooth	adrostral teeth	capitium	shaft bipartite	shaft length	hood	beard	basal extensions	rostrum microvilli	adrostral teeth microvilli	reference
Sabellariidae	*Sabellaria alveolata*, abd	+	+	-	+	s	-	-	r	several	several	this study
Spionida	*Scolelepis squamata*	+	+	-	-	l	+	-	-	several	several	Hausen & Bartolomaeus 1998
	Malacoceros fuliginosus	+	+	-	-	l	+	.	-	several	several	Hausen & Bartolomaeus 1998
	Prionospio fallax	+	+	-	-	l	+	-	-	several	several	Hausen 2001
	Spirorbis spirorbis, abd	-	+	+	-	s	-	-	-	-	single	Bartolomaeus 1995
Sabellida	*Fabricia stellaris*, tho	+	+	+	-	l	-	-	-	several	single	Bartolomaeus 2002
	Fabricia stellaris, abd	-	+	+	-	s	-	-	-	-	single	Bartolomaeus 2002
	Branchiomma bombyx, abd	+	+	+	-	s	-	-	-	several	single	unpubl. data
Terebellida	*Pectinaria koreni*	-	+	+	-	s	-	-	-	-	single	Bartolomaeus 1995
	Pectinaria auricoma	-	+	+	-	s	-	-	-	-	single	Bartolomaeus 1995
	Nicolea zostericola	+	+	+	-	s	-	-	p	several	single	Bartolomaeus 1998
Chaetopteridae	*Telepsaphus costarum*	-	+	+	-	s	-	-	p	-	single	unpubl. data
	Chaetopterus variopedatus	-	+	+	-	s	-	-	p	-	single	unpubl. data
Arenicolidae	*Arenicola marina*, juvenile	+	+	+	-	l	-	+	-	several	single	Bartolomaeus & Meyer 1997
	Arenicola marina	+	+	+	-	l	-	+	-	several	single	Bartolomaeus & Meyer 1997
Maldanidae	*Clymenura clypeata*	+	+	+	-	l	-	+	-	several	single	Tilic et al. 2015
	Johnstonia clymenoides	+	+	+	-	l	-	+	-	several	single	Tilic et al. 2015
Psammodrilidae	*Psammodrilus balanoglossoides*	+	+	+	-	l	-	+	-	several	single	Meyer & Bartolomaeus 1997
Capitellidae	*Capitella capitata*	+	+	+	-	l	+	-	-	several	single	Schweigkofler et al. 1998
Oweniidae	*Owenia fusiformis*	-	+	+	-	l	-	-	-	-	single	Meyer & Bartolomaeus 1996
Lumbrineridae	*Lumbrineris tetraura*	+	+	-	-	l	+	-	-	several	several	Tilic et al. 2014

abd. abdomen, *tho.* thorax, + present, - absent, *s* short, *l* long, *r* rods, *p* processes

4.6 Data Repository

To allow full transparency of the data presented in this study, all of the aligned serial semi-thin and ultra-thin sections and the confocal z-stack of the phalloidin stained parapodium are freely accessible in the morphological database, MorphDBase: www.morphdbase.de (Grobe and Vogt 2009).

Complete series of aligned ultra-thin sections:

Direct link: www.morphdbase.de/?E_Tilic_20151015-M-27.1

Complete series of aligned semi-thin sections:

Part 1– Direct link: www.morphdbase.de/?E_Tilic_20151015-M-29.1

Part 2 – Direct link: www.morphdbase.de/?E_Tilic_20151015-M-28.1

Confocal z-stack of the phalloidin stained parapodium:

Direct link: www.morphdbase.de/?E_Tilic_20151015-M-30.1

4.7 Authors' contribution

ET conducted the experimental work, analyzed the data, drafted the manuscript and prepared the figures. TB significantly contributed to the analysis and interpretation of the data and improved the final manuscript.

4.8 Acknowledgements

Homology and evolution of the chaetae in Echiura (Annelida)

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Abstract

Echiura is traditionally regarded as a small phylum of unsegmented spiralian worms. Molecular analyses, however, provide unquestionable evidence that Echiura are derived annelids that lost segmentation. Like annelids, echiurans possess chaetae, a single ventral pair in all species and one or two additional caudal hemi-circles of chaetae in two subgroups, but their evolutionary origin and affiliation to annelid chaetae are unresolved. Since annelids possess segmental pairs of dorsal (notopodial) and ventral (neuropodial) chaetae that are arranged in a row, the ventral chaetae in Echiura either represent a single or a paired neuropodial group of chaetae, while the caudal circle may represent fused rows of chaetae. In annelids, chaetogenesis is generally restricted to the ventral part of the notopodial chaetal sac and to the dorsal part of the neuropodial chaetal sac. We used the exact position of the chaetal formation site in the echiuran species, *Thalassema thalassemum* (Pallas, 1766) and *Echiurus echiurus* (Pallas, 1767), to test different hypotheses of the evolution of echiurid chaetae. As in annelids, a single chaetoblast is responsible for chaetogenesis in both species. Each chaeta of the ventral pair arises from its own chaetal sac and possesses a lateral formation site, evidencing that the pair of ventral chaetae in Echiura is

homologous to a pair of neuropodia that fused on the ventral side, while the notopodia were reduced. Both caudal hemi-circles of chaetae in *Echiurus echiurus* are composed of several individual chaetal sacs, each with its own formative site. This finding argues against a homology of these hemi-circles of chaetae and annelids' rows of chaetae and leads to the hypothesis that the caudal chaetal rings evolved once within the Echiura by multiplication of ventral chaetae.

5.1 Introduction

Echiura, consisting of 165 exclusively marine species, is a small, but worldwide distributed taxon of unsegmented spiralians (Stephen and Edmonds 1972; Biseswar 2009; Biseswar 2010; Biseswar 2012). Commonly known as "spoon worms", due to their tongue-like extensible proboscis, they occur in benthic habitats and range from the littoral zone to the deep sea (McKenzie and Hughes 1999). Traditionally Echiura was ranked as a phylum, but recent studies, especially molecular, have generated an increasing body of evidence that they actually are derived annelids (McHugh 1997; McHugh 1999; Hessling 2002; Hessling and Westheide 2002; Bleidorn et al. 2003a; Bleidorn et al. 2003b; Hessling 2003; Rousset et al. 2007; Struck et al. 2007; Bourlat et al. 2008; Dunn et al. 2008; Hejnol et al. 2009; Zrzavý et al. 2009; Struck et al. 2011; Weigert et al. 2014) and mostly provide strong support for a sister group relationship between Echiura and Capitellidae. Loss of segmentation in the echiuran stem lineage was substantiated by studies showing serially repeated groups of neurons in the larval nervous system of certain echiuran species (Hessling 2002; Hessling and Westheide 2002; Hessling 2003) as well as three subsequently formed pairs of nephridia (Baltzer 1931; Kato et al. 2011; Lehrke and Bartolomaeus 2011) and confirmed with the recent molecular phylogenies (McHugh 1997; McHugh 1999; Hessling 2002; Hessling and Westheide 2002; Bleidorn et al. 2003a; Bleidorn et al.

2003b; Hessling 2003; Rousset et al. 2007; Struck et al. 2007; Bourlat et al. 2008; Dunn et al. 2008; Hejnol et al. 2009; Zrzavý et al. 2009; Struck et al. 2011; Weigert et al. 2014).

Like annelids, echiuran species possess chitinous chaetae that arise from a chaetal sac, an ectodermal invagination that generally contains several chaetal follicles and is surrounded by the subepidermal extracellular matrix (ECM). In both annelids and echiurans, each chaeta is formed within one chaetal follicle which consists of a basally located chaetoblast and several follicle cells (Orrhage 1971; Spengel 1880; Korn 1982; Hausen 2005). All cells are epithelial, are interconnected by apical adherens junctions ("belt desmosomes"), face the chaeta and rest on the subepidermal ECM. In annelids, the follicle cells of neighboring follicles are not separated by an ECM and directly contact each other (Specht and Westheide 1988; Bartolomaeus 1998; Hausen 2005; Tilic et al. 2014). Despite their structural identity, the positional homology of annelid and echiurid chaetae as well as their evolutionary origin is still unsolved. Annelids possess segmental pairs of dorsal (notopodial) and ventral (neuropodial) chaetal sacs, each giving rise to a single row of chaetae. All echiuran species possess a single pair of ventral chaetae. Species of the Urechidae and Echiurinae additionally possess one or two caudal hemi-circles of chaetae, generally called anal chaetae due to their perianal position. Studies of annelid chaetogenesis have revealed that in this group formation of new chaetae is generally restricted to the ventral part of the notopodial chaetal sac and to the dorsal part of the neuropodial chaetal sac, so that neuro-and notopodial formation sites are adjacent on either body side of each segment (Bartolomaeus 1998; Hausen 2005). This pattern is conserved in all species of the Capitellidae, the presumed sister group of the Echiura (Bleidorn et al. 2003a; Bleidorn et al. 2003b; Struck et al. 2007). These results allow different sets of hypotheses on the evolutionary origin of the echiuran chaetae (Fig. 5.1).
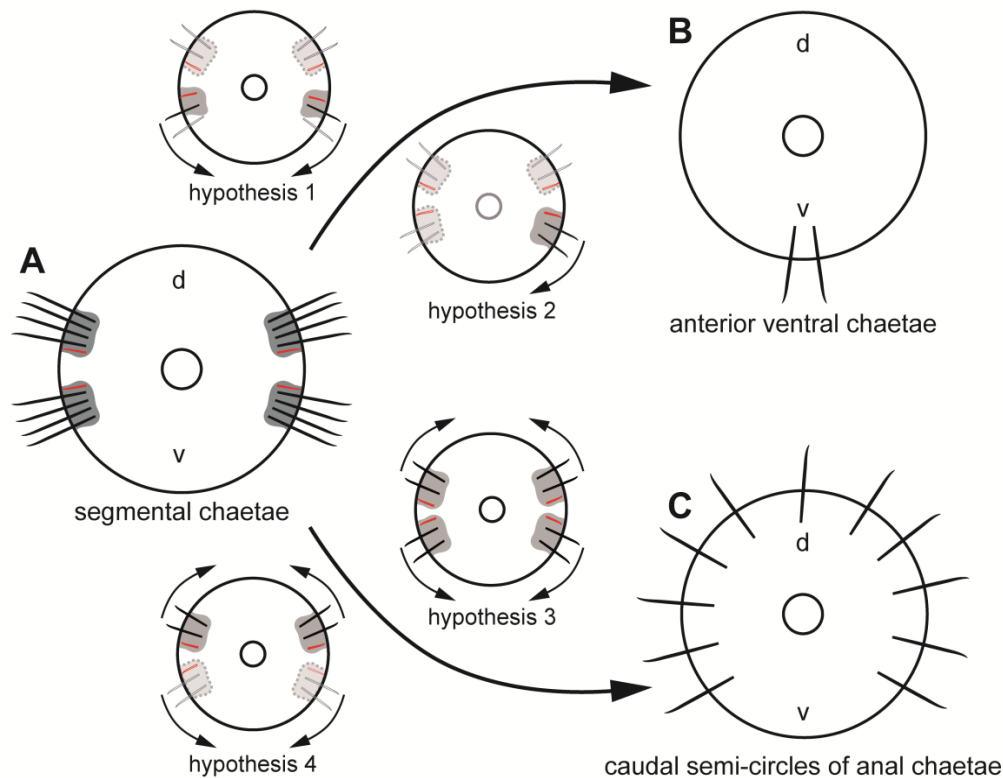
Figure 5.1: Different hypotheses on the evolutionary transformation of annelid and capitellid segmental chaetae (**A**) into echiuran ventral (**B**) and caudal anal chaetae (**C**). Annelid chaetae are formed on the ventral edge of the notopodial chaetal sac and on the dorsal edge of the neuropodial chaetal sac. Developing chaetae are *red*, completed chaetae are *black*, chaetal sac is *grey*. All structures that are reduced according to the different hypothesis are *paler*; presumably reduced chaetal sacs are marked by a *dotted line. Arrows* mark expansion or shifting of chaetal sac. *H1* hypothesis one: notopodial chaetae have been reduced and the neuropodial chaetal sac shifted ventrally. *H2* hypothesis two: notopodial chaetae plus one neuropodial group of chaetae have been reduced and the remaining neuropodial sac shifted ventrally. *H3* hypothesis three: all chaetal sacs expand dorsally and ventrally, respectively. *H4* hypothesis four: neuropodia are reduced and the notopodial chaetae expand dorsally and ventrally.

Origin of the ventral chaetae: (1) The ventral pair of chaetae in Echiura is homologous to a pair of annelid neuropodia. This hypothesis would be supported, if each ventral chaeta arose from its own chaetal sac and if each should possess a lateral formative site. (2) The ventral pair of chaetae in Echiura is homologous to one neuropodium, while the other one is reduced. This hypothesis would be supported, if both ventral chaetae shared a single chaetal sac with a single formative site.

Origin of the caudal hemi-circles of anal chaetae in Echiurinae and Urechidae: (3) The caudal hemi-circle is homologous to dorsally expanded rows of neuropodial and notopodial chaetae of the annelid ancestor. This hypothesis would be supported, if four chaetal sacs, each with a formative site constituted each hemi-circle of anal chaetae. (4) The caudal hemi-circle is homologous to dorsally merged notopodia of the annelid ancestor; the neuropodia are reduced. This hypothesis would be supported, if only one chaetal sac and two formative sites were found in each caudal hemi-circle of anal chaetae. Any other number of formative sites would falsify both hypotheses and corroborate the results of recent molecular phylogenetic studies. Goto et al. (2013) provide convincing evidence for a sister group relationship between Urechidae and Echiurinae and for their placement within the Echiura. Such a position implies that the anal chaetae evolved within the Echiura and leads to the expectation of differences in their mode of formation compared to annelid chaetogenesis.

To test these hypotheses we studied the structure and formation of the ventral chaetae in Thalassema thalassemum (Pallas, 1766) and Echiurus echiurus (Pallas, 1767) and both hemi-circles of anal chaeta in E. echiurus using different histological, ultrastructural and immunohistological methods and 3D reconstructions.

5.2 Material and Methods

5.2.1 Animals

Thalassema thalassemum (Pallas, 1766) (Thalassematidae) was collected from rock crevices in the upper sublittoral at Le Cabellou (Concarneau, Brittany, France) in 2007 and 2013, and kept in sea water tanks until fixation. *Echiurus echiurus* (Pallas, 1767) (Echiurinae) was dredged from the Dogger Bank (North Sea) in November 1992 and from muddy sediments in the German Bight (North Sea) (54°02'N, 008°03'E). The animals were kept in sea water

tanks up to three years until fixation. *Thallasema thallasemum* specimens were relaxed using a solution of 7% MgCl and seawater (1:1) for 1 hour. The ventral chaetae were then dissected out of the relaxed animals and subsequently fixed for confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM). For TEM studies *Echiurus echiurus* specimens were dissected in the fixative. Prior to fixation for histology the specimens were relaxed in a solution of 7% MgCl and seawater (1:1) for 1 hour. In this study six *Echiurus echiurus* specimens were used for preparation, histology and electron-microscopy and twelve *Thalassema thalassemum* specimens were examined histologically, electron-microscopically and by immunostaining.

We neither used endangered species nor were the investigated animals collected in a protected area. All animals were collected with the permission of the local marine biological stations. The Station Biologie Marine, Concarneau, was informed of our collection of *Thallasema thalassemum*. No written permission was necessary. *Echiurus echiurus* was collected subtidally using the research vessel "Uthörn" of the Alfred-Wegener-Institute for Polar and Marine Research. No written permission to collect the animals was necessary.

5.2.2 Transmission electron microscopy (TEM)

For electron microscopy ventral chaetal sacs of *Thalassema thalassemum* were fixed in 2.5 % glutaraldehyde buffered in 0.05 M phosphate buffer with 0.3 M NaCl for 1hour. *Echiurus echiurus* was fixed in 2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate (pH 7.2) at 4°C for 90 min. After having rinsed the specimen several times in the same buffer used for fixation, they were postfixed in 1% OsO_4 buffered in 0.05 M phosphate 0.3 M NaCl saline or in 1% OsO_4 buffered in 0.1 M sodium cacodylate buffer, respectively. All specimens were dehydrated in an ascending acetone series and propylene oxide and subsequently embedded in Araldite. The basal part of a chaetal

follicle and the formative sites were sectioned into a complete series of 70 nm silver-interference coloured ultra-thin sections, stained with uranyl acetate and lead citrate using an automated TEM stainer (QG-3100) and analyzed in a ZEISS Libra-120KV transmission electron microscope.

5.2.3 Confocal laser scanning microscopy (CLSM)

Ventral chaetae of Thalassema thalssemum were fixed in 4% paraformaldehyde and subsequently permeabilized in four 5-min changes of PBS (phosphate buffered with saline) with 0.1% Triton X-100 (Fisher Scientific). The preparations were then stained overnight in 4ºC with TRITC phalloidin at a dilution of 1:100 (in 0.3% Triton X-100). After staining, the samples were rinsed in three quick changes and subsequently in two 10-min changes of PBS with 0.1% Triton and one 10-min rinse in PBS without Triton. The chaetae were then directly placed in hollow-ground slides. The samples were quickly dehydrated in isopropanol (2 min 70%, 2 min 85%, 2 min 95%, 2 min 100%, 2 min 100%), cleared in three 15-min changes of Murray Clear, mounted in Murray Clear, and sealed with nail polish.

For the propidium iodide induced staining of the nuclei, paraformaldehyde fixed ventral chaetae of *T. thalassemum* were treated with RNase (1: 100) for 30 min and subsequently stained with propidium iodide (1:40) for another 30 mins. The samples were cleared in an ascending series of glycerol in PBS (15 min 30%, 30 min 60%, 30 min 90%) and mounted in 90% glycerol.

5.2.4 Histology and 3D reconstruction

For semi-thin sections, Araldite-embedded ventral chaetal sacs of *Thalassema thalassmum* were cut into complete series of 0.5 µm sections with a diamond knife (Diatome Histo Jumbo) on a Leica Ultracut S ultramicrotome (Blumer et al. 2002). The sections were stained with toluidine blue (1% toluidine, 1% sodium-tetraborate and

20% saccharose) and mounted with araldite. The sections were then analyzed with an Olympus microscope (BX-51) and photographed with an Olympus camera (Olympus cc12) equipped with the dotSlide virtual slide system (Olympus).

For paraffin histology *Echiurus echiurus* specimens were fixed in Bouin's fixative for 18 h in room temperature and transferred into 70% ethanol. Herein, the animals were sectioned to isolate the caudal section with the hemi-circles of anal chaetae, and the anterior part containing the ventral pair of chaetae. Prior to embedding one specimen was photographed with a Keyence VHX700 digital microscope. The different parts of *Echiurus echiurus* were then completely dehydrated in an ethanol series, followed by incubation in methyl benzoate and butanol. Afterwards each part was pre-incubated in Histoplast (Thermo Scientific, Dreieich, Germany) at 60°C for three days with several medium changes and finally embedded in Paraplast (McCormick Scientific, Richmond, USA). 10 µm thick sections were made using a Reichert-Jung Autocut 2050 microtome (Leica, Wetzlar) and transferred to glass slides coated with albumen-glycerin. Sections were AZAN stained; Malinol (Waldeck, Münster, Germany) was used for mounting the sections.

The histological sections were analyzed with an Olympus microscope (BX-51) and photographed with an Olympus camera (Olympus cc12). The images of the posterior part of *Echiurus echiurus* were aligned with IMOD (Boulder laboratories, (Kremer et al. 1996)) and IMOD-align (http://www.evolution.uni-bonn.de/mitarbeiter/bquast/software). Selected histological images were used as reference for the 3D model generated with the software 3ds max 13.0. The histological images were imported as surface materials and the chaetae were modeled using standard cylindrical or conic objects. These were modified as NURBS-surfaces.

5.2.5 Voucher material and data repository

A conspecific individual sampled form the same field site as the studied animals has been sequenced. The partial 16S sequence has been deposited in NCBI and is available under the accession number GenBank: KM187648.1. Aligned serial semi-thin sections of the ventral chaetae of *Thalassema thalassemum* are freely accessible in the morphological database, MorphDBase (Grobe and Vogt 2009).

Direct link: https://www.morphdbase.de/?E_Tilic_20140929-M-21.1

5.3 Results

5.3.1 Ventral chaetae in *Thalassema thalassemum* and *Echiurus echiurus*

In all specimens studied two large golden chaetae extend from the ventral surface, one on either side of the ventral midline of the body. The tip of the chaeta is curved and bent towards the animal's surface. In relaxed animals only the curved, hook-like section of the chaeta is externally visible (Fig. 5.2A, B). Underneath this hook-like section the diameter of the chaeta initially decreases slightly, then increases rapidly to form a collar and is finally more or less constant until the base of the chaeta (Fig. 5.2 C, D). Below, the collar will be used as a landmark for describing the follicle. Depending on the age and the size of the animal, the ventral chaetae of *Thalassema thalassemum* are up to 3 mm long, those of *Echiurus echiurus* are up to 9 mm long; in any case the length of both chaetae is almost identical.
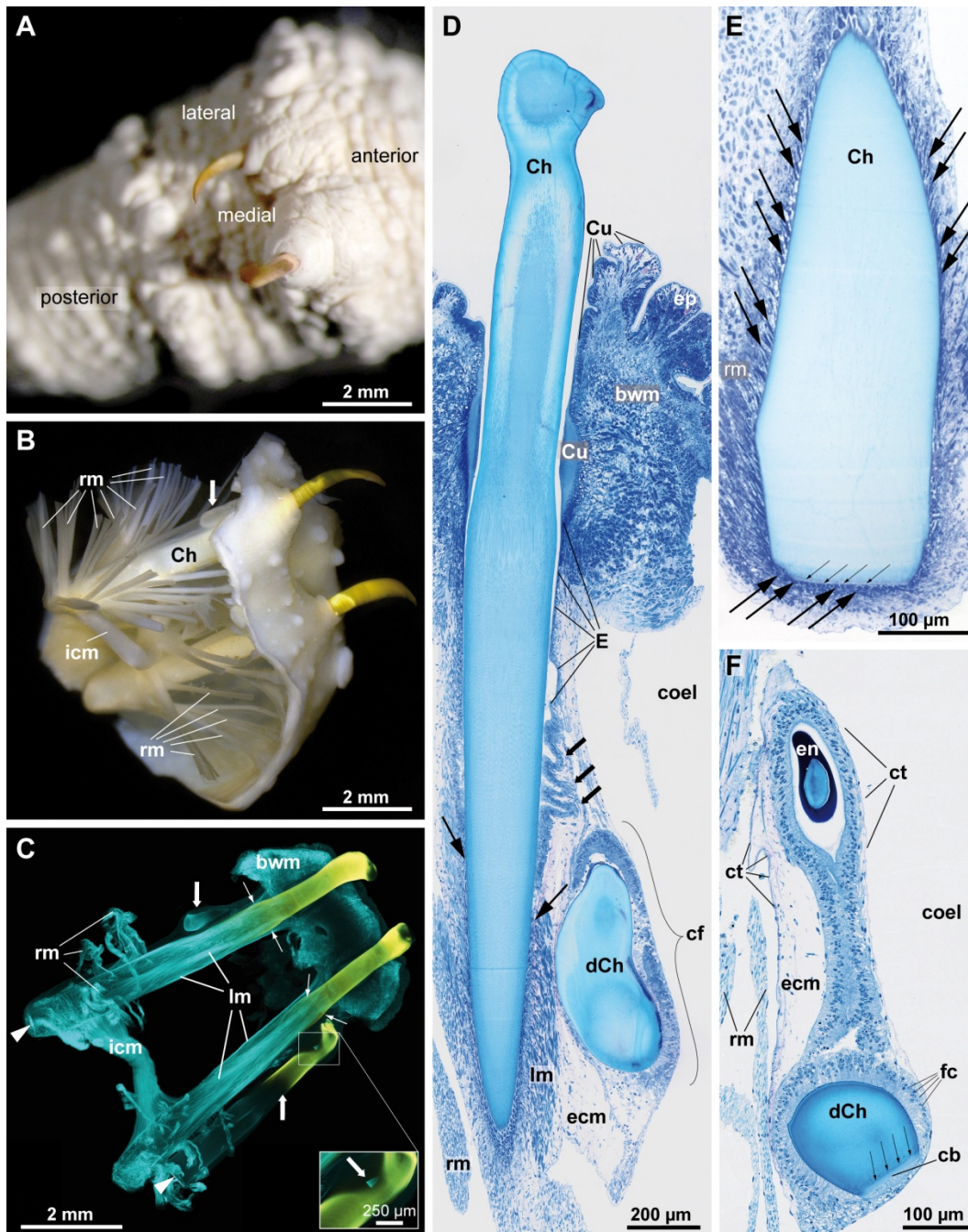
Figure 5.2: Ventral chaetae of *Echiurus echiurus* (**A**, **B**) and *Thalassema thalassemum* (**C-F**). **A**. Extended focus light micrograph of a critical point dried part of a specimen showing the apical hooks of the ventral chaetae. **B**. Extended focus light micrograph, frontal view of a pair of dissected ventral chaetae (*Ch*) showing the chaetal radial muscles (*rm*) and interconnecting muscle (*icm*). Chaetal sacs are delicate and transparent, *arrow* marks a developing chaeta. **C**. Maximum projection of a phalloidin (*cyan*) stained and chitin autofluorescent (*yellow* to *light blue*) dissected pair of ventral chaetae, frontal view, CLSM. Apical microvilli of the chaetoblast marked by *arrow heads*. *Large arrows* mark developing chaetae, *small arrows* mark the collar of the ventral chaetae. **Inset**: early chaetogenesis at higher magnification. **D**.-**F**. Light micrographs of semi-thin (0.5 μm) toluidine blue stained parasagittal sections. **D**. Apical part of ventral chaeta (*Ch*), chaetal sac with follicles (*bold*

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5.3.2 Chaetal sac and chaetal follicles

Each chaeta arises from one chaetal sac that deeply invaginates into the trunk coelom. The chaetal sac is composed of a large, main follicle and a few developing ones and voluminous perifollicular ECM with muscles and is surrounded by a sheath formed by the peritoneum (Fig. 5.2D-F; 5.3A). Muscle bundles originate from the basal section of the chaetal sac and cross the coelomic cavity. A large interconnecting muscle links the chaetal sac of one body side to its counterpart on the other body side and several radial muscles connect the chaetal sac to the body wall (Fig. 5.2B). All muscles are at least partly surrounded by a peritoneum and consist of fiber muscle cells. In addition several smaller muscles run within the chaetal sac, most of them parallel to the main chaeta (Fig. 5.2C-E). The course and orientation of the muscles are similar in both species studied.

In both species studied the largest and fully differentiated chaeta originates from the main, largest follicle of each chaetal sac (Fig. 5.2D; 5.3A). Along its course from apical to basal, its inner lining initially is continuous with the epidermis and thus cuticularized. This cuticle can be seen to the level of the chaetal collar, since it drastically increases in diameter directly above the chaetal collar, decreases there and finally disappears (Fig. 5.2D). Here, the main chaetal follicle forms a series of lateral pouches that increase in size along an apico-basal gradient. The pouches are developing chaetal follicles. The last and most basally located pouch always contains a developing chaeta (Fig.

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5.2D, F; 5.3A). Depending on the size and age of this chaeta, a further developing chaeta may be present in the adjacent follicular pouch (Fig. 5.2C, 5.3A). In all specimens studied, the size of the developing chaetae differed between body sides, indicating that chaetogenesis is not initiated at the same time within the two separate chaetal sacs of an individual.

Each follicle consists of several hundred follicle cells and a basal chaetoblast; all cells are epithelial and rest on the perifollicular matrix (Figs. 5.2E; 5.3A). The size of the nuclei differs between follicle cells and chaetoblast in both species. While the former are ovoid in shape and measure 7.2 ± 0.8 µm (n = 24) in length and 4 ± 0.6 µm (n=20) in diameter, the nucleus of the chaetoblast of fully differentiated chaeta is disc-shaped with a central depression and measures 21-23 µm in width and 8-8.5 µm in diameter (Fig. 5.3B). The metrical data on the nuclei do not differ significantly between both species.

Follicle cells of both species contain strong bundles of intermediate filaments (Fig. 5.4A-C, F, I). Apically, the intermediate filaments extend into branched or unbranched protrusions of the cell surface (Fig. 5.4B, C, I). In the tip of these protrusions the intermediate filaments adhere to hemidesmosomes that connect them to the chaeta (Fig. 5.4A, C, I). In their basal section ventral chaetae of *T. thalassemum* contain shallow ridges so that the outer chaetal surface to which the follicle cells adhere is much larger than in *E. echiurus* which lacks such ridges (Fig. 5.4A, I). Hemidesmosomes on the basal surface of the follicle cell connect the intermediate filaments to the ECM. Opposite to the hemidesmosomes dense plaques of fiber muscle cells adhere to the ECM, so that the follicular intermediate filament system mechanically couples the muscles to the chaeta (Fig. 5.2E).

The chaetoblast is also filled by strong bundles of intermediate filaments that reach into short apical protrusions that extend into the inner of the chitinous tubes of which the chaeta is composed (Fig. 5.4H). Here, hemidesmosmes connect the intermediate filaments of the chaetoblast to the chaeta. The chaetoblast of differentiated chaetae forms a very small cytoplasmic layer underneath the chaeta, contains large amounts of intermediate filaments, a few microvilli and vesicles and the large nucleus (Fig. 5.3B). The chaetoblast is flanked by follicle cells and intensely interdigitates with these, so that the chaetoblast can hardly be discriminated from the latter without using electron microscopy.
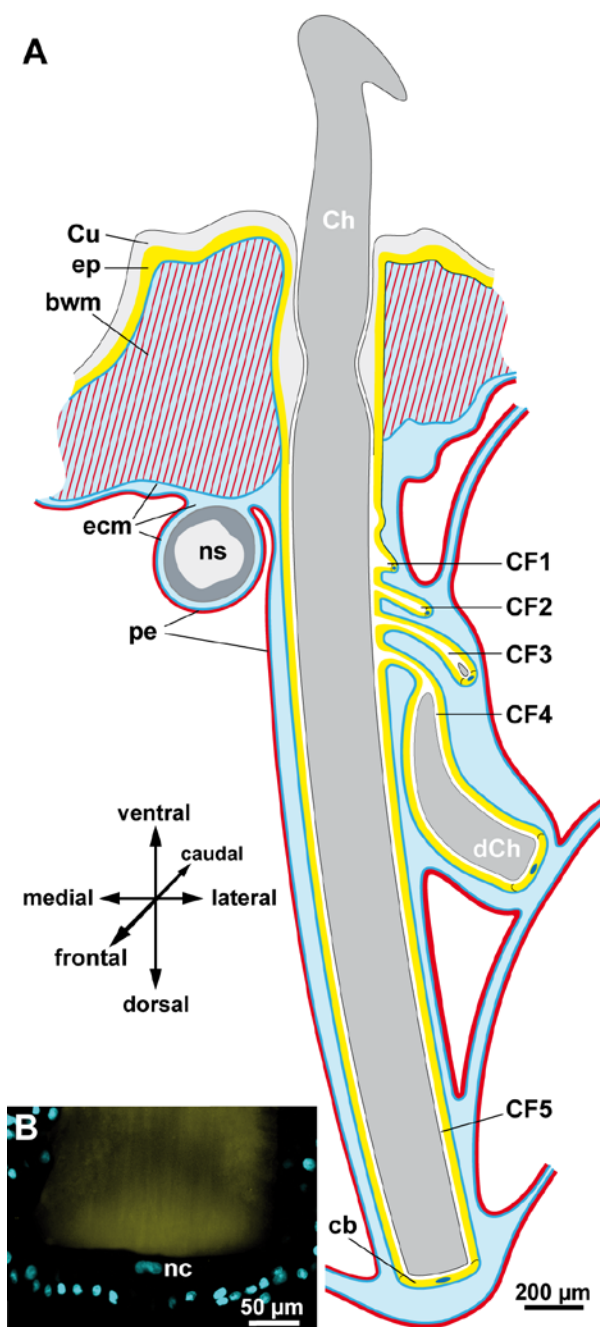
Figure 5.3: Chaeta and chaetogenesis in *Thalassema thalassemum* reconstructed from a stack of semi-thin sections.

A. Chaetal sac with four developing chaetal follicles (*CF1- CF4*) and one completed follicle (*CF5*). Developing chaetae (*dCH*) in *CF3* and *CF4* and complete chaeta (*Ch*) of CF5 are *dark grey*. Chaetal muscles are not shown. **B**. Maximum projection of a CLSM stack of the basal section of CF5 with propidium iodide staining of nuclei (*cyan*) and autofluorescent chaeta (*yellow*). Note large nucleus (*nc*) of chaetoblast. *bwm* body wall muscles, *cb* chaetoblast, *Cu* cuticle, *ecm* extracellular matrix, *ep* epidermis, *ns* ventral nerve cord, *pe* peritoneum.

(**Direct link**: www.morphdbase.de/ ?E_Tilic_20140929-M-21.1)

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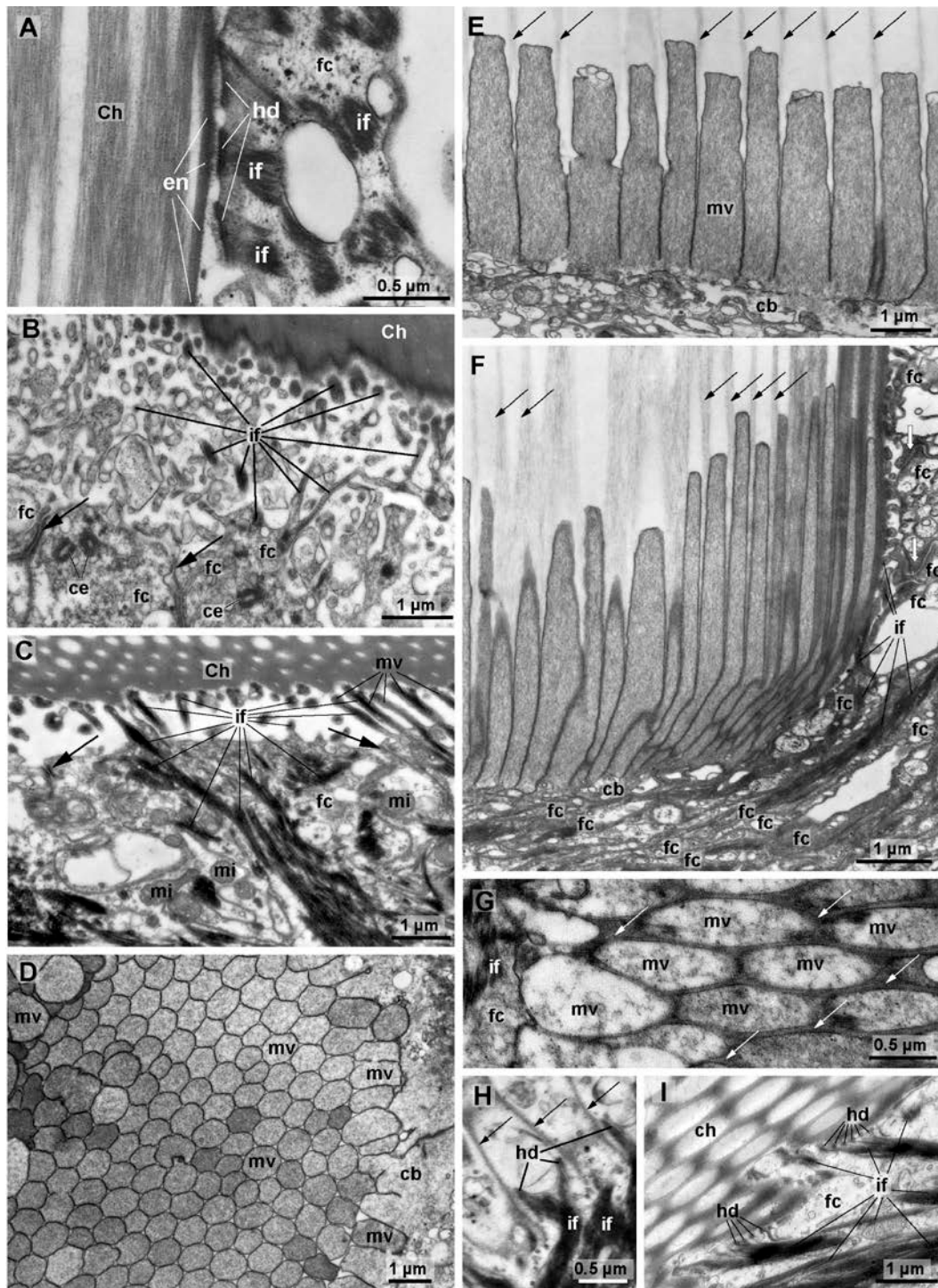
Figure 5.4: Ultrastructure of ventral chaetae of *Echiurus echiurus* (**A**, **D-F**) and *Thalassema thalassemum* (**B**, **C**, **G-I**), TEM. **A-G**. late chaetogenesis, **H-I**. complete chaeta. **A**. Hemidesmosomes (*hd*) connect chaeta (*Ch*) to intermediate filaments (*if*) of follicle cells (*fc*), chaeta is surrounded by an enamel (*en*). **B**, **C**. Intermediate filaments (*if*) inside branched and unbranched microvilli (*mv*) adhere chaeta to follicle cells (*fc*). Apical adherens junctions (*arrows*) interconnect follicle cells, note apical pair of centrioles (*ce*) in follicle cells in B. **D**. Semi-transverse section of microvilli (*mv*) brush border of a chaetoblast (*cb*) during chaetogenesis. **E**, **F**. Parasagittal section of chaetoblast with apical microvilli, *arrows* mark chitin polymerizing between

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← the microvilli. Note densely packed f-actin in microvilli (*mv*). **E**. center of chaetoblast. **F**. lateral part of chaetoblast. Note its tight interdigitation with follicle cells (*fc*), *white arrows* mark adherens junctions. **G**. Semi-transverse section of apical microvilli of the chaetoblast, *white arrows* mark chitin polymerizing between the microvilli (*mv*). **H**. Base of old chaeta, parasagittal section. The apical microvilli have been replaced by protrusions of the chaetoblast that contain intermediate filaments (*if*) which adhere to the chaeta (*arrows*) by hemidesmosomes (*hd*). **I**. Fully differented chaeta with ridges. Hemidesmosomes (*hd*) firmly connect intermediate filaments (*if*) of follicle cells (*fc*) to chaeta. *mi* mitochondria.

5.3.3 Chaetogenesis

Chaetogenesis is a continuous process in both echiurid species during which the number of follicle cells as well as the size of the chaetoblast and its nucleus increases (Fig. 5.3A). Lateral to the main follicle a series of young follicles increasing in size and age can be seen (Figs. 5.2D; 5.3A). The follicle next to the main follicle always contains a developing chaeta (Figs. 5.2C, D; 5.3A). Each consists of a large number of follicle cells and a basal chaetoblast (Fig. 5.2F). As in fully differentiated chaetae follicle cells are joined to the developing chaetae by hemidesmosomes that connect the chaeta to the intercellular intermediate filament system. In *T. thalassemum* these hemidesmosomes are located at the tip of branched apical protrusions, which may be several micrometers long (Fig. 5.4B, C). The follicle cells always contain a subapical pair of centrioles; basal structures, rootlets or any other sign of ciliogenesis are missing (Fig. 5.4B). We therefore assume that the centrioles indicate ongoing mitotic activity of the follicle cells, which is needed to increase the number of follicles during growth of the chaeta. The chaetoblast can easily be recognized by its stout apical microvilli that measure 3.83 ± 0.29 µm (n=15) in length in *E. echiurus* and 6.48 ± 0.52 µm (n=10) in T. thalassemum (Fig. 5.4D-F). Although shorter, the microvilli of the chaetoblast in *E. echiurus* are larger (740 ± 75 nm in diameter (n=28)) than those in *T. thalassemum* (425 nm ± 63 nm in diameter (n=28)). The size of the microvilli is identical throughout each chaetoblast except for the periphery of the chaetoblast, where it decreases in both species (Fig. 5.4D). The

microvilli are densely filled with actin filaments that extend into the chaetoblast where they interconnect and form a subapical meshwork (Fig. 5.4D, E). During chaetogenesis fibrillar electron-grey material, which presumably is chitin, is deposited between the microvilli and elongates the chaeta (Fig. 5.4, E, F). Since the length of the microvilli is more or less fixed, empty tubes remain that the microvilli once filled. These tubes can be seen in any cross section of the chaeta, forming the honey-comb pattern (Fig. 5.4I)

During the further course of chaetogenesis the chaetae are coated by an enamel, which is very thick in the apical section of the chaeta and absent in the basal section (Fig. 5.2F). The source of the material forming this enamel was not seen. We assume that it is released by the follicle cells, because these are the only ones that are next to the apical part of the chaeta. During early chaetogenesis the follicle cells contain no intermediate filaments (Fig. 5.2F). These are formed later during chaetogenesis and increase in number, until they finally dominate the follicle cells and can even be seen at the light microscopical level (Fig. 5.2D, E). Intermediate filaments finally drastically reduce the actin filaments which had been the prevailing filament system during chaetogenesis and restrict it to the periphery of the chaetoblast. Short apical protrusions of the chaetoblast connect the chaeta to the chaetoblast. These processes contain intermediate filaments that adhere to the chaeta by hemidesomosmes (Figs. 5.2C, 5.4H). Whether the microvilli that once preformed the chaeta are transformed into these processes or are replaced by them remains to be shown. When completed the chaeta replaces the old, used chaeta, which seems to be cast off. Shortly before this the old follicle changes its structure. There are lesser intermediate filaments in the follicle cells, their diameter decreases and the stainability with Azan staining changes. Ultrastructural details of these processes and the loss of used chaetae were not observed.

5.3.4 Anal chaetae in *Echiurus echiurus*

Echiurus echiurus possesses anal chaetae that are arranged in two caudal, horse-shoe shaped to hemi-circular rows that surround the anus dorsally; chaetae are absent caudo-ventrally (Fig. 5.5A). Each chaeta is slightly curved with a bent tip that extends beyond the surface of the animal, while the basal section of each chaeta extends deeply into the body cavity of the animals (Fig. 5.5B). In the animal studied histologically, the anal chaetae measure 2.7 ± 0.5 mm (n=14) in length and 277 ± 17 µm (n = 20) in diameter at its base (Fig. 5B, C). Depending on the size and age of the individual, the anal chaetae may be larger or smaller, but always have more or less the same size in one individual. The outer hemi-circle consists of 7 to 8 chaetae, the inner of 6 chaetae (Fig. 5.5A-C). The numbers corroborate the data in Spengel (Spengel 1880).

Each chaeta arises from one chaetal sac which is surrounded by extracellular matrix. Muscles rest on the coelomic face of the matrix, while follicle cells rest on its opposite side and directly face the chaeta. The basal section of each chaetal sac is connected to its neighbors by interconnecting muscles the myofilaments of which are perpendicular to the main axis of the chaetae (Fig. 5.5D). Towards the tip of the chaetae these interconnecting muscles are replaced by radial muscles that originate from the chaetal sac and cross the coelom and adhere to the body wall muscles at the caudal margin of the trunk coelom (Fig. 5.5E-G). There are up to seven of these muscles per chaetae; they seem to form a collar of muscle strands around each of the caudal chaetae (Fig. 5.5B).

Chaetogenesis could be seen in all chaetal sacs of the specimen studied histologically (Fig. 5.5F, G). Developing chaetae are each found inside their own chaetal follicle, where they are surrounded by several follicle cells.
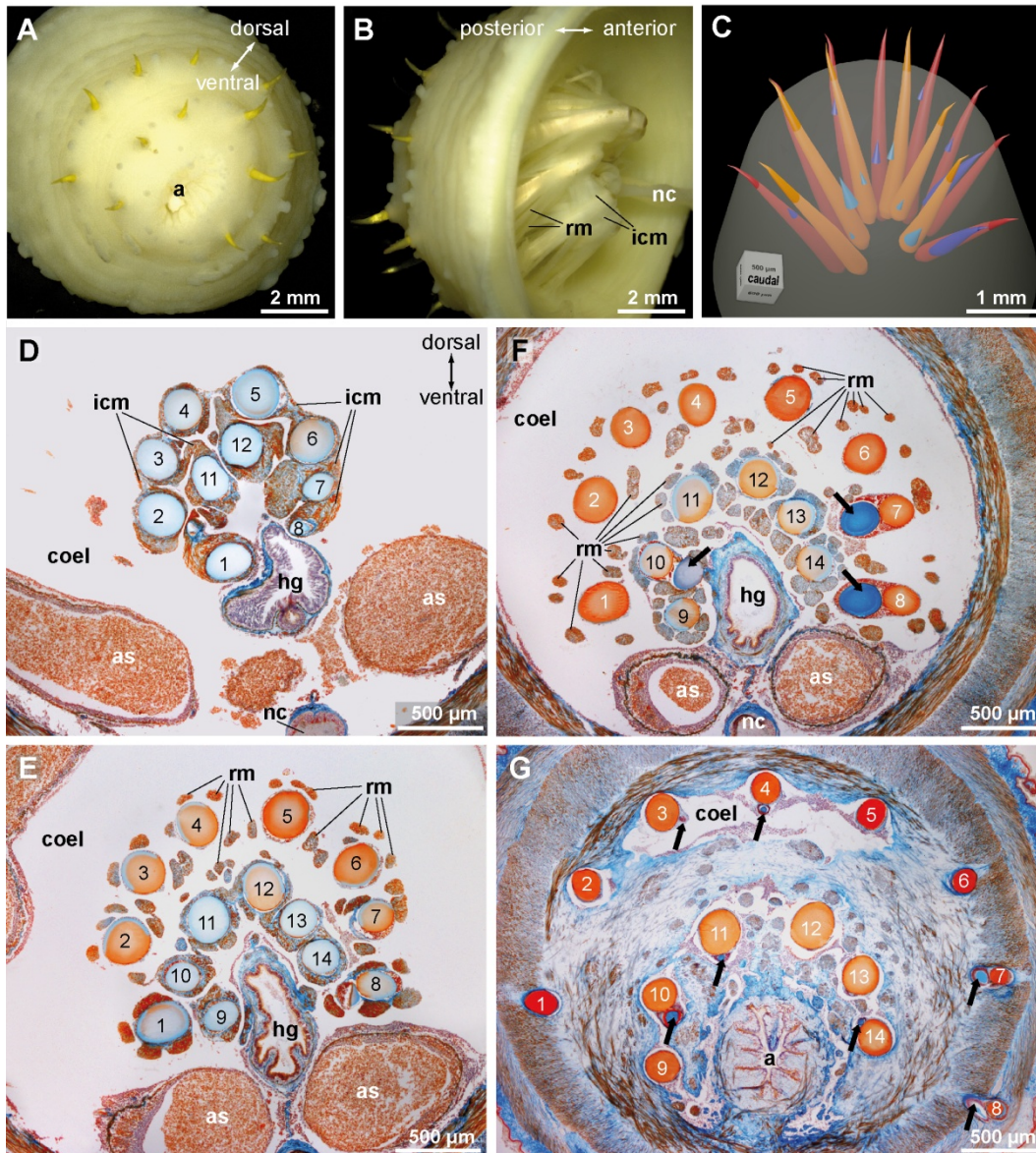
Figure 5.5: *Echiurus echiurus*, anal chaetae. **A**. Extended focus view of the caudal face of the animal, **B**. Extended focus semi-lateral view into the dissected caudal end; Hindgut is removed, nerve cord (*nc*) marks ventral side. Note complex muscular system, interwoven interconnecting muscles (*icm*), and twisted orientation of anal chaetae. **C**. Reconstruction of completed (*orange*) and developing (*bluish*) anal chaetae based on a series, **D**-**G**. Azan stained histological sections from a series of transverse sections from anterior to posterior. The chaetal sacs extend deeply into the coelom (*coel*), the anal chaeta are numbered (outer hemi-circle 1-8, inner hemi-circle 9-14). Distances: **D**-**E** = 600 μm, **E**-**F** = 200μm, **F**-**G** = 600 μm. **D**. Section of the base of the anal chaetae dorsal to hindgut. Note connection of chaetal sacs by interconnecting muscles (*icm*). **E**. Section of the chaetae at the level of the origin of the radial muscles (*rm*). Anal chaetae are grouped in two hemi-circles that are next to each other and partly surround the hindgut (*hg*). **F**. Section close to the posterior end of the trunk body cavity (*coel*). Hemi-circles of anal chaetae are clearly separated and surround three quarters of the hindgut. Note developing chaeta (*arrow*) in the sacs of chaetae 7, 8, and 10.

← **G**. Section of the anal chaeta through the body wall muscles. Hemi-circles of anal chaetae are widely separated. Note tips of developing chaetae (*arrow*) in sacs of chaetae 3, 4, 7, 8, 10, and 11. *a* anus, *as* anal sac.

Within each chaetal sac the developing follicle is adjacent to the chaetal follicle of the fully grown chaeta and not separated from the latter by an ECM. 3D reconstruction revealed that only one developing chaeta is found in each chaetal sac (Fig. 5.5C). This reconstruction also shows that the developing chaeta differ in length and position within each chaetal sac, a finding which indicates that new chaetae are formed individually within each chaetal sac. All these results clearly show that the anal chaetae of *Echiurus echiurus* independently arise from several chaetal sacs that are arranged as a hemi-circular row surrounding the hindgut.

5.4 Discussion

The ultrastructure of echiuran chaetae is similar to those of Annelida (Echiura: Orrhage 1971; Annelida: see Specht and Westheide 1988; Bartolomaeus 1998; Hausen 2005; for review). Fibrillate chitinous material fills the gaps between the tubes which are left by the microvilli of the chaetoblast during chaetogenesis. The chaetoblast is located at the base of an epidermal chaetal follicle and, as long as a chaeta grows, releases chitin into the gap between its microvilli bases that polymerizes to elongate the chaeta. Since the length of the microvilli is more or less fixed, empty tubes remain that the microvilli once filled. These tubes can be seen in any cross section of the chaeta, forming the honey-comb pattern characteristic for annelid chaetae. These tubes may later be filled by some electron-dense material, so that the honey-comb pattern is less clearly visible than in transverse sections of the proximal part of the chaeta.

Like in annelids (O'Clair and Cloney 1974 for *Nereis vexillosa*, Bartolomaeus and Meyer 1997 for Arenicolidae, Tilic et al. 2014 for *Lumbrineris* species) chaetal formation is in accordance with

continuous adjustment of the apical microvilli pattern of the chaetoblast in both echiurid species. In both species modulation merely concerns an increasing number of microvilli during chaetogenesis along with a reorientation of the main axis of the chaetoblast. Increasing number of microvilli in time leads to chaetae with a small tip and a broad base, while reorientation of the chaetoblast's axis causes the hook-like apical section of the ventral chaeta. Such a re-orientation was not seen in the caudal chaetae of *Echiurus echiurus* which lack an arcuate apical section. After the chaeta has been completed the microvilli of the chaetoblast are replaced by protrusions containing intermediate filaments. Such a replacement has also been reported from annelids and marks the end of chaetogenesis (Bartolomaeus 1995).

Chaetoblast and follicle cells are epithelial cells. While the chaetoblast determines the structure of the chaeta in annelid and echiruan species, the follicle cells connect it to the muscular system by an intracellular meshwork of intermediate filaments (Hausen 2005). In addition to these mechanical functions, the follicle cells may produce the enamel that surrounds the chaeta. A very large number of follicle cells lines the chaetal follicle in both echiuran species. It seems likely that their number increases during chaetal formation by mitosis. In both echiuran species developing chaetae are located laterally to the fully formed chaetae within a separate chaetal follicle. The younger the chaetae are, the closer they are located to the animal's surface. During chaetogenesis the young follicle continuously sinks into deeper tissue layers. The position of the formative site as well as spatial re-orientation of the developing chaeta is identical in annelids (Bartolomaeus 1998; Hausen 2005). As in annelids, each chaetal sac consists of a few chaetal follicles, but in contrast to annelids only one chaeta of each sac extends beyond the animal's surface. Each externally visible chaeta thus marks a single chaetal sac.

Presently we have no detailed insight into degeneration of echiuran chaetae, but the fact that chaetae are continuously formed in a chaetal sac provides indirect evidence for replacement of the chaeta. Due to the general course of chaetogenesis we conclude that replacement of chaetae initiates the degeneration of the follicle. In annelids chaetal degeneration has been described in a very few species, although it should be an integral part of each chaetal sac's developmental history (Meyer and Bartolomaeus 1996; Hausen 2005).

To us, however, the most surprising finding was that a single chaetoblast forms the ventral chaetae which may measure more than half a millimeter in diameter. Given that the entire apical surface of the chaetoblast is densely covered by microvilli that are a few micrometers long, the surface of the chaetoblast must be very large. The requirement for the continuous release of chitin suggests that the physiological activity of this cell must be very high. These considerations might explain the hypertrophied nucleus, which according to the low intensity of the propidium iodide staining, relative to the nuclei of the follicle cells, is despite of its size probably not polyploid (Fig. 5.3B).

5.4.1 Comparison and evolutionary significance of chaetae in Echiura

As expected from the assumed position of Echiura as an ingroup of the Annelida (McHugh 1997; Bleidorn et al. 2003a; Struck et al. 2007), the chaetae, the composition of chaetal follicles and chaetal sacs, ultrastructure, and chaetogenesis are similar in these two taxa. Presently, however, we are unable to detect support for a specific sister group relationship to any annelid subgroup, especially not for the Capitellidae, which turn out to be the echiuran sister group when phylogenetically analyzing single genes and phylogenomic data (Bleidorn et al. 2003a; Bleidorn et al. 2003b; Struck et al. 2007).

Capitellid species possess dorsal and ventral pairs of capillary chaetae in the thorax and hooded hooks in the abdomen (Schweigkofler et al. 1998). Each row originates from a chaetal sac and within each sac the formative site is located close to the lateral mid-line of the animal (Fig. 5.6). Chaetogenesis thus is restricted to the ventral edge of the notopodial chaetal sac and to the dorsal edge of the neuropodial chaetal sac. Such a position of the formative site within the different chaetal sacs is assumed to be ancestral in Annelida (Bartolomaeus et al. 2005; Hausen 2005). Although our study does not provide morphological evidence for a Capitellidae-Echiura sister group relationship, our initial hypotheses on the evolution of echiruan chaetae (Fig. 5.1) can be evaluated.

The presence of two separate ventral chaetal sacs, each with a single chaeta extending beyond the animal's surface and a lateral site of chaetal formation, supports our first hypothesis on the transformation of annelid neuropodial chaetae into echiuran ventral chaetae (Fig. 5.6). The second hypothesis that predicted a single ventral sac with a single formative site must therefore be rejected. Provided that Echiura and Capitellidae are sister groups, as supported by molecular data (Bleidorn et al. 2003a; Bleidorn et al. 2003b; Struck et al. 2007), the ventral chaetae of the Echiura must be homologous to the ventral chaetae of Capitellidae. Since the echiuran chaetae do not show any sign of the hood characteristic of the abdominal chaetae of Capitellidae (Schweigkofler et al. 1998), the ventral chaetae should be homologous to thoracic neuropodia, most likely to those of the first chaetiger, because the ventral chaetae in Echiura are the first (and in most species the only) chaetae that are formed during development. We therefore assume that the notopodial chaetae of a common echiurid-annelid ancestor were lost and that the neuropodial chaetae of this segment shifted from a medial to a ventral position (Fig. 5.6).
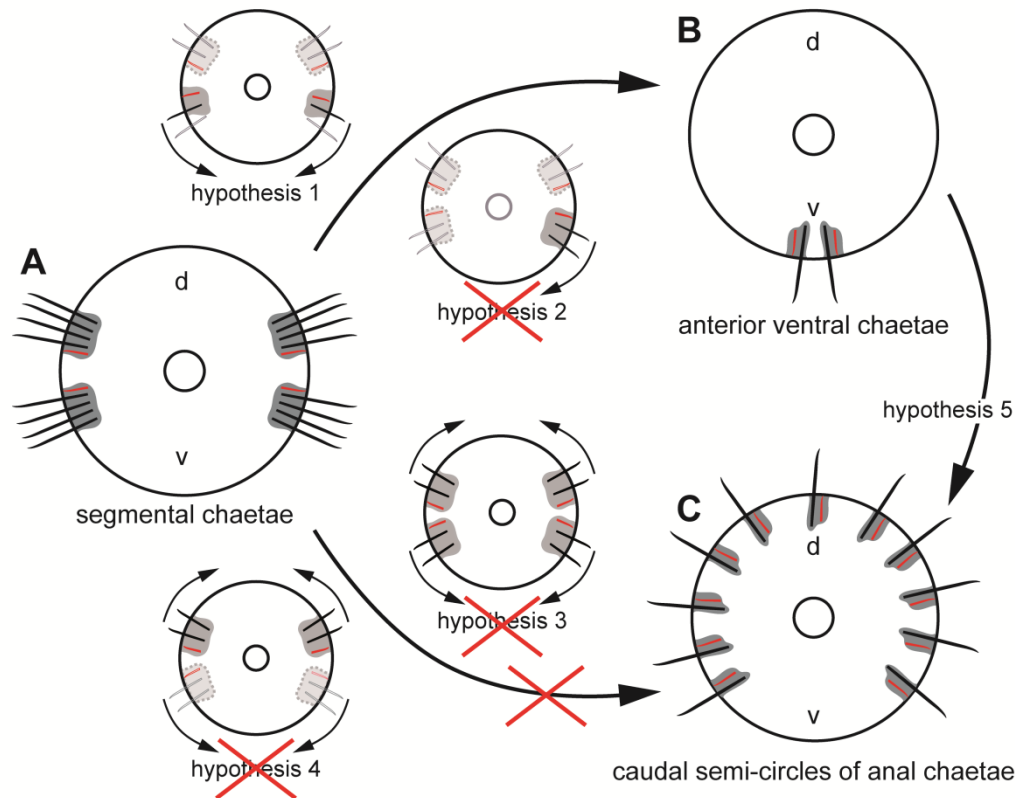
Fig. 5.6: Rejection, support and new hypotheses on the evolutionary transformation of the segmental chaetal pattern characteristic for Capitellidae and most other Annelida (**A**) into that of Echiura (**B**, **C**). The number of the ventral chaetal sacs and position of the growth zone supports the first hypothesis by suggesting that the ventral chaetae evolved from neuropodia by reduction of the number of chaetae and loss of the neuropodia. All other hypotheses are rejected by the results of this paper. A new hypothesis (hypothesis 5) is developed for the evolution of the anal chaetae in Urechidae and Echiurinae, according to which the anal chaetae evolved by multiplication of ventral chaetae.

The presence in *Echiurus echiurus* of one chaetal sac per anal chaeta, each with its own lateral formative site, argues against the homology of the caudal hemi-circles of chaetae to the neuropodia or notopodia of capitellids or any other annelid species. Provided that urechidan and echiurine anal chaetae are formed in an identical manner, hypotheses 3 and 4 are thus rejected. Recent molecular studies actually provide evidence that Echiurinae and Urechidae are sister taxa within the Echiura (Lehrke 2012; Goto et al. 2013). A caudal ring of anal chaetae is found in all urechid species and two hemicircles of such chaetae in all echiurid species (Stephen and Edmonds 1972), such a sister group relationship implies that the

specific arrangement of anal chaetae either represent an autapomorphy of the respective group or that one of these arrangements evolved in their common stem lineage and is plesiomorphic. The direction of evolution remains unknown.

If urechid species also have anal chaetal sacs with one chaeta and lateral formation sites, like the ones described here for Echiurus echiurus, we propose that anal chaetae evolved in a common stem lineage of Echiurinae and Urechidae. Since each of these chaetal sacs is identical to the ventral chaetal sacs, we assume that they evolved by multiplication and rearrangement of the same anlagen that give rise to ventral chaetae (Fig. 5.6). This hypothesis has to be tested by studies of the ontogeny of representatives of Urechidae and Echiurinae. Presently, this hypothesis is mainly supported by the derived position of these sister taxa nested within Echiura (Lehrke 2012; Goto et al. 2013) and the muscular system that is almost identical in ventral and anal chaetae in consisting of interconnecting and radial muscles.

5.5 Acknowledgements

Chaetal type diversity increases during evolution of Eunicida (Annelida)

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Abstract

Annelid chaetae are a superior diagnostic character on species and supraspecific levels, because of their structural variety and taxon specifity. A certain chaetal type, once evolved must be passed on to descendants, to become characteristic for supraspecific taxa. Therefore, one would expect that chaetal diversity increases within a monophyletic group and that additional chaetae types largely result from transformation of plesiomorphic chaetae. In order to test these hypotheses and to explain potential losses of diversity, we take up a systematic approach in this paper and investigate chaetation in Eunicida. As a backbone for our analysis we used a three-gene (COI, 16S, 18S) molecular phylogeny of the studied eunicidan species. This phylogeny largely corresponds to previous assessments of the phylogeny of Eunicida. Presence or absence of chaetal types was coded for each species included into the molecular analysis and transformations for these characters were then estimated using the mK1 likelihood model. Our results show that chaetal type diversity does indeed increase within eunicids and provide possible explanations

for the homology, convergence and loss of chaetal types in eunicidan subtaxa.

6.1 Introduction

Chaetae in annelids have attracted the interest of scientist for a very long time, making them one of the most studied, if not the most studied structures of annelids. This is partly due to the significance of chaetal features when identifying annelids, since chaetal structure and arrangement are highly constant in species and supraspecific taxa. Aside from being a valuable source for taxonomists, chaetae have also been the focus of many studies in functional ecology (Woodin and Merz 1987; Merz and Edwards 1998; Merz and Woodin 2000; Pernet 2000; Merz 2015). Furthermore, the cellular mechanisms behind the chaetal formation process described by Bouligand (1967) and O'Clair and Cloney (1974) have been an intriguing field of study. Chaetae are extracellular, chitinous structures formed within an ectodermal pouch, the so-called chaetal follicle. The basalmost cell within this follicle is the chaetoblast (Bouligand 1967; Schroeder 1984; Specht and Westheide 1988; Bartolomaeus 1998; Hausen 2005). This cell possesses apical microvilli which release N-acetyle-glycosamine into the intermicrovillar extracellular space where it subsequently polymerizes to elongate the chaeta. Pattern, diameter and number of microvilli continuously change during chaetogenesis, so that the structure of the chaeta reflects these temporal changes and are nothing but cell surface dynamics frozen in time (O'Clair and Cloney 1974).

This dynamic formation modality presumably allowed the high diversity of chaetal types to evolve. On the other hand, since chaetal arrangement and structure are highly taxon-specific, chaetogenesis must be under strict regulation. Structure, orientation and number of microvilli of the chaetoblast, and modulation of these factors determine chaetal structure and their regulation must be conservative enough that, once evolved, a certain chaetal type can be passed on to

descendants, becoming characteristic for supraspecific taxa. We therefore assume that once evolved strong functional constraints must be responsible for fixing certain chaetal types within species and supraspecific communities. There is some experimental evidence that such functional constraints actually exist (Merz 2015). If this were true, one would expect that certain chaetal types are positively selected and maintained in a group of descendants and that alteration in functional constraints lead to a partial or complete transformation of chaetae within this group. Chaetae within a monophyletic annelid taxon should show (1) that during evolution, chaetal diversity increases within that group and (2) that additional chaetae types largely result from transformation of chaetae that are present in basally branching taxa. Since there is also evidence that ceratin species secondarily show a rather uniform and simple chaetation (see for instance Aguado et al. (2013) for Chryopetalidae), one also has to ask how chaetal diversity gets lost.

In order to test both hypotheses and to explain potential losses of diversity, we take up a systematic approach in this paper to investigate the distribution of different chaetal types in Eunicida. Eunicida (sensu Fauchald 1977) is a species-rich taxon (over 900 nominal species in 100 genera (Rouse and Pleijel 2001)) of annelids and its monophyly has been established by molecular (Struck et al. 2006) and morphological analyses (Rouse and Fauchald 1997). The characteristic autapomorphy for Eunicida is the cuticular, prominent jaw apparatus composed of multiple elements (Purschke 1987). Eunicida are presently classified into five major subtaxa: Lumbrineridae, Oenonidae, Dorvilleidae, Onuphidae and Eunicidae, as well as two minor groups, the obscure Hartmaniellidae and the symbiotic Histriobdellidae.

Eunicida show a variety of different chaetal types that range from simple capillaries to more complex hooded hooked or compound chaetae. We therefore investigated the chaetae of a range of Eunicida and conducted a thorough survey of chaetal types described in the

literature. As a backbone for our analysis we used a three-gene (mitochondrial COI, 16S rDNA and nuclear 18S rDNA) molecular phylogeny of Eunicida that includes all species we used to analyze chaetal diversity and arguably covers the diversity of the group. We did not include Hartmaniellidae or Histriobdellidae. The latter taxon has lost chaetae and is not relevant in the current paper, while no specimens of Hartmaniellidae were available for analysis or sequencing.

6.2 Material and Methods

6.2.1 Animals

Specimens of *Lumbrineris tetraura* (Schmarda, 1861), *Lumbrineris (Scoletoma) fragilis* (O.F. Müller, 1776), *Eunice* (*Leodice*) *torquata* (Quatrefages, 1866), *Marphysa belli* (Audouin & Milne-Edwards, 1833*)* and *Arabella iricolor* (Montagu, 1804) were collected in the intertidal zone during field trips to Concarneau, France (Brittany). *Ophryotrocha* sp. n. was collected from aquaria of the Scripps Institution of Oceanography. *Diopatra neapolitana* Delle Chiaje, 1841 was extracted from tubes collected in *Zostera* beds during low tide in the bay of Arcachon close to Le Petit Piquey (France) in 1995.

6.2.2 Scanning electron microscopy (SEM)

Specimens used for SEM were fixed in Bouin's fluid. These were dehydrated in an alcohol series, and were kept in a 5% phosphotungsticacid solution for an hour to increase the heavy metal content in the tissue. They were critical point dried with CO_2 in a critical point dryer (Balzers) and sputtered with gold (Balzers Sputter Coater). The specimens were examined in Novoscan and Leitz AMR 1000 scanning electron microscopes. During dehydration the animals were sonicated to remove debris and sand particles from the chaetae.

The *Arabella iricolor* specimen was not treated with phosphotungsticacid and was dehydrated using HMDS, according to the method described by Nation (1983). This specimen was analyzed using a Philips XL30 ESEM.

6.2.3 Confocal laser scanning microscopy (CLSM)

The specimens used for confocal laser scanning microscopy (CLSM) were fixed in 4% paraformaldehyde. In most of the studied species chaetigers were dissected to separate single parapodia or single segments. The specimens were permeabilized in four 5-min changes of PBS (phosphate buffered with saline) with 0.1% Triton X-100 (Fisher Scientific). The parapodia were then stained overnight in 4 ºC with TRITC phalloidin at a dilution of 1:100. After staining, parapodia were rinsed in three quick changes and subsequently in two 10-min changes of PBS with 0.1% Triton and one 10 min rinse in PBS without Triton. Larger samples were dehydrated in isopropanol (2 min 70%, 2 min 85%, 2 min 95%, 2 min 100%, 2 min 100%), cleared in three 15-min changes of Murray Clear, and mounted in hollow-ground slides with Murray Clear. All coverslips were sealed with nail polish. A Leica TCS SPE laser scanning confocal microscope was used for the analysis.

6.2.4 Light microscopy

Chaetae of selected species were isolated by using a 5% NaOH Solution. After the tissue was completely dissolved, chaetae were rinsed and studied under an Olympus microscope. Fixed larvae of *Lumbrineris* sp. and different ontogenic stages of *Ophryotrocha* sp. were investigated as whole-mounts.

6.2.5 Phylogenetic analysis and character evolution reconstruction

Sequences for mitochondrial Cytochrome oxidase subunit I (CO1), 16S rDNA (16S) and 18S rDNA (18S) for fifty eunicid species were included in this study, with *Glycera dibranchiata* (Phyllodocida) used as an outgroup. A complete list of the species and the GenBank sequence accession numbers are presented in Tab.A1.

Sequences of each gene were aligned using MAFFT (Q-INS-i) (Katoh et al. 2002). The three loci were concatenated and analyzed using maximum parsimony (MP) and maximum likelihood analyses (ML). The MP result was obtained via PAUP*4.0b10 (Swofford 2002) with the heuristic search option with 1000 random additions. Clade support was assessed using 1000 jackknife replicates. The ML analysis was completed using RaXML (Stamatakis 2006) and RAxML GUI v. 0.93 (Silvestro and Michalak 2012), with the data partitioned by gene and, for COI, by codon with GTR plus GAMMA used for each of the three gene partitions (Silvestro and Michalak 2012; Stamatakis 2006). Bootstrap support was assessed via 1000 pseudoreplicates using the same model.

The presence or absence of four kinds of chaetae was coded for each terminal: Simple and compound hooded hooks, comb-shaped chaetae and compound capillary chaetae. Additional data on chaetation of those species that are not included into the molecular analysis are added in the results section (Tab. 1). Transformations for these characters were then estimated via the mK1 likelihood model (Lewis 2001) using Mesquite 3.03 (Maddison and Maddison 2015) on the topology (including branchlengths) of the maximum likelihood result.

6.3 Results

6.3.1 Results of the phylogenetic analysis

The maximum parsimony analysis was based on 1599 informative characters in the aligned data of the concatenated three genes (COI, 16S, 18S). There were six most parsimonious trees with a length of 9699 steps (trees not shown). The families of Eunicida were

all recovered as well-supported clades, except for Onuphidae, which was paraphyletic with respect to Eunicidae. The maximum likelihood tree (Fig. 6.4), largely matched the maximum parsimony analysis in topology and support, except for Onuphidae, which it showed to be monophyletic. Lumbrineridae was the sister group to a well-supported clade that comprised the remaining Eunicida. Within this group Oenonidae and Dorvilleidae were sister taxa, though with low support (less than 50%), and they formed the sister group of a taxon consisting of Onuphidae and Eunicidae, which were reciprocally monophyletic. Notably, Onuphidae had bootstrap support of only 67%, while all other family-ranked taxa had bootstrap support of 95% or greater (Fig. 6.4). Our ML results largely correspond to previous assessments of the phylogeny of Eunicida using multiple loci (Struck et al. 2006, Zanol et al. 2010), but ours differs in having Oenonidae and Dorvilleidae as sister taxa rather than a grade. As noted above, this result had low support and needs further assessment and data(next-gen), but is used here for the purposes of assessing chaetal evolution in Eunicida.

6.3.2 Distribution of chaetal types

In Eunicida all chaetae that arise from the body surface and that are externally visible are neuropodial. Notopodial chaetae are normally reduced and if present they do not extend beyond the surface of the animals (Fig. 6.1, 6.2a). In addition to the externally visible chaetae all members of Eunicida possess internalized aciculae. Aciculae are generally large and robust chaetae that are always internal and attached to the parapodial musculature in such a manner that they function as the "skeletal" rods of parapodia.

We indentify six morphologically different types of chaetae in Eunicida. In the literature these are generally specified by adding different adjectives such as limbate, winged, pectinate, spiniger or falciger (Fauchald 1977; Hartman 1968). Although this enhances accuracy in species description and identification, it tends to cause

confusing terminology on higher hierarchical levels, due to a high number of synonymies and uncertain homologies. The main reason is that the attributes are not clearly defined (Fauchald 1977), which poses difficulties in coding prior to redefining them. Often, depending on the authors and timing of the publication, different names have been used to describe the same structure and the same name has been used for different things. To overcome these potential problems, we restrict our analysis to acicula and the six general chaetal types that are easily distinguished: simple and compound capillary chaetae, comb-shaped chaetae, furcate chaetae, simple hooded hooks and compound hooded hooks. Simple capillary chaetae are thin apically tapering cylinders. Compound capillary chaetae are simple capillary chaetae with a joint-like element connecting the apical portion of the chaeta to the shaft. Comb-shaped chaetae are simple capillary chaetae that broaden apically to form a fork- or comb-like end. Furcate chaetae are simple capillary chaetae with two prongs at the apical end. Simple hooded hooks are simple capillary chaetae with a perpendicularly bent rostrum and with one or several smaller adrostral teeth. A large hood surrounds these apical structures. Compound hooded hooks are simple hooded hooks with a joint-like element connecting the manubrium to the apical section of the chaeta. The presence or absence of different chaetal types are mapped on the phylogenetic tree and also presented in Fig. 6.4.

Lumbrineridae. In the adult specimen of *Lumbrineris fragilis* chaetation of neuropodia was uniform throughout the entire body. Each chaetiger only bore ~4 simple hooded hooks. In *Lumbrineris tetraura*, only the posterior parapodia showed such uniformity by bearing simple hooded hooks only. Our SEM studies show that simple and compound hooded hooks of lumbrinerid species are multidentate with a hood fully surmounting the apical portion of the chaetae; only some of the teeth are externally visible (Tilic et al. 2014). The anterior parapodia additionally had simple capillary chaetae. In addition to

these externally visible chaetae, both species had acicula and internalized notopodial chaetae (Fig. 6.1a, b). The number of acicula inside the parapodia increased gradually along the posterior-anterior axis. In adult *L. tetraura*, the anteriormost segments contained up to five aciculae, while in *L. fragilis*, there were maximally two acicula in the anteriormost parapodium. *Ninoe nigripes*, that was also included in the phylogenetic analysis (Fig. 6.4), has simple capillaries and simple hooded hooks (Carrera-Parra 2006).

Hartman (1968) describes the same three chaetal types; acicula, capillary chaetae and simple hooded hooks, for *Lumbrineris zonata*, but also mentions compound hooded hooked chaetae in *Lumbrineris latreilli* and *Lumbrineris zonata* (Tab. 6.1). Hartman (1968) also mentiones acicula, capillary chaetae and simple and compound hooded hooks in further *Lumbrineris* species, like *L. californiensis*, *L. cruzensis*, *L. index*, *L. inflata*, *L. japonica*, *L. linguata*, *L. limicola*, *L. pallida*. In these the hood includes the hinge in compound hooded hooks. Representatives of the latter lumbrinerids were not included in the phylogenetic analysis (Tab. 6.1).

Oenonidae. The studied oenonid species *Arabella iricolor* only bore simple capillary chaetae throughout the entire body (Fig. 6.3b). The parapodial structure is also uniform, with a conical postchaetal lobe and a small dorsal cirrus. The dorsal cirrus actually is a reduced notopodium, since histological sections of *Arabella iricolor* show the numerous vestigial chaetae inside (Fig. 6.2a). Hartman (1968) mentions the simple capillary chaetae and acicula as the only chaetal types for the oenonid species *Arabella geniculata, Arabella mimetica, Arabella semimaculata, Biborin ecbola, Drilonereis falcata, Drilonereis longa, Drilonereis nuda, Labidognathus forcipes, Notocirrus attenuates, Notocirrus californiensis*. The chaetation of the three members of Oenonidae that were included into the molecular analysis can therefore be regarded as representative for the entire group (Tab. 6.1).
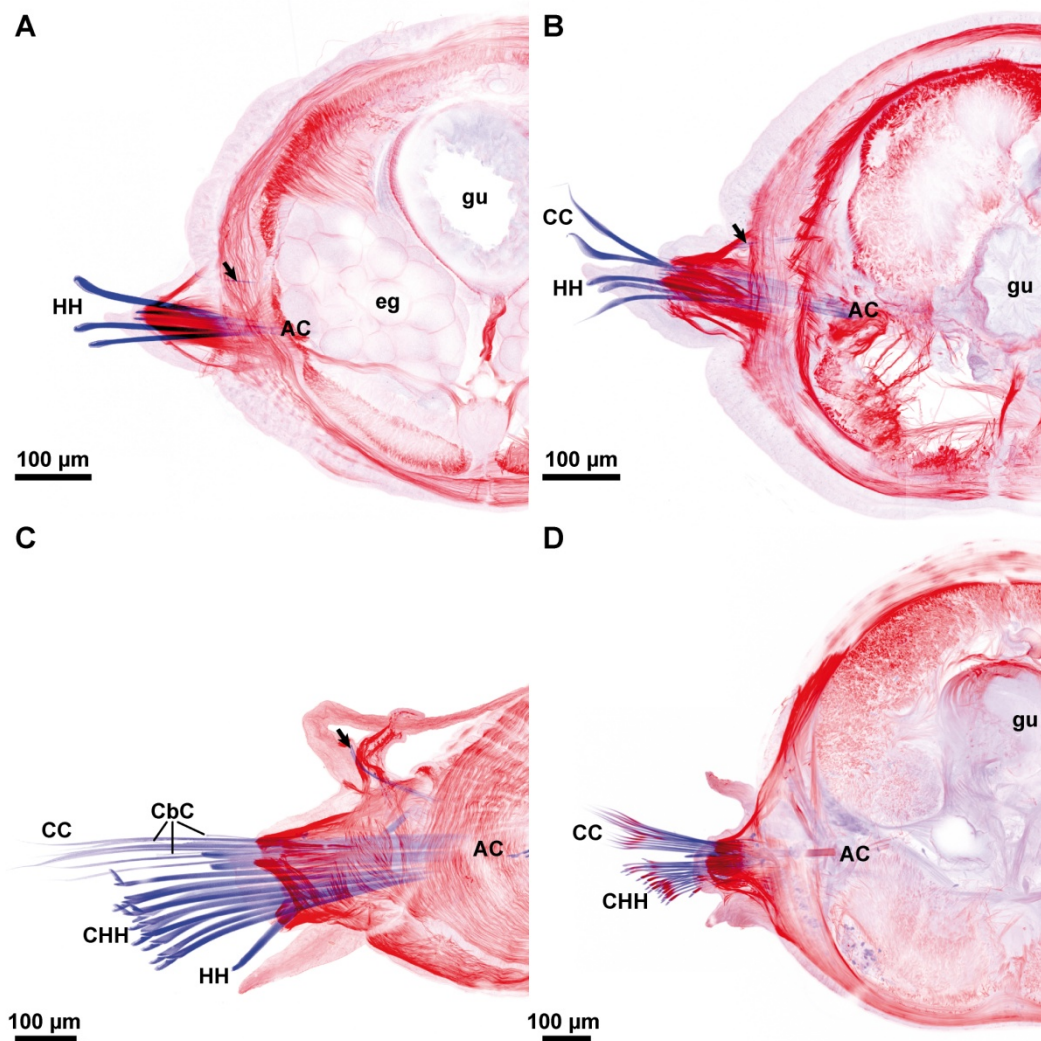
Figure 6.1 Confocal *z*-projections of phalloidin-stained preparations, **a** *Lumbrineris tetraura* **b** *Lumbrineris fragilis* **c** *Eunice torquata* **d** *Lysidice ninetta. red:* phalloidin, *blue:* chaetal autofluorescence, *HH* hooded hooks, *CHH*, compound hooded hooks, *AC* acicula, *CC* capillary chaetae, *CCC* compound capillary chaetae, *CbC* comb-shaped chaetae, *eg* eggs, *gu* gut, *arrow* internalized notopodial chaetae
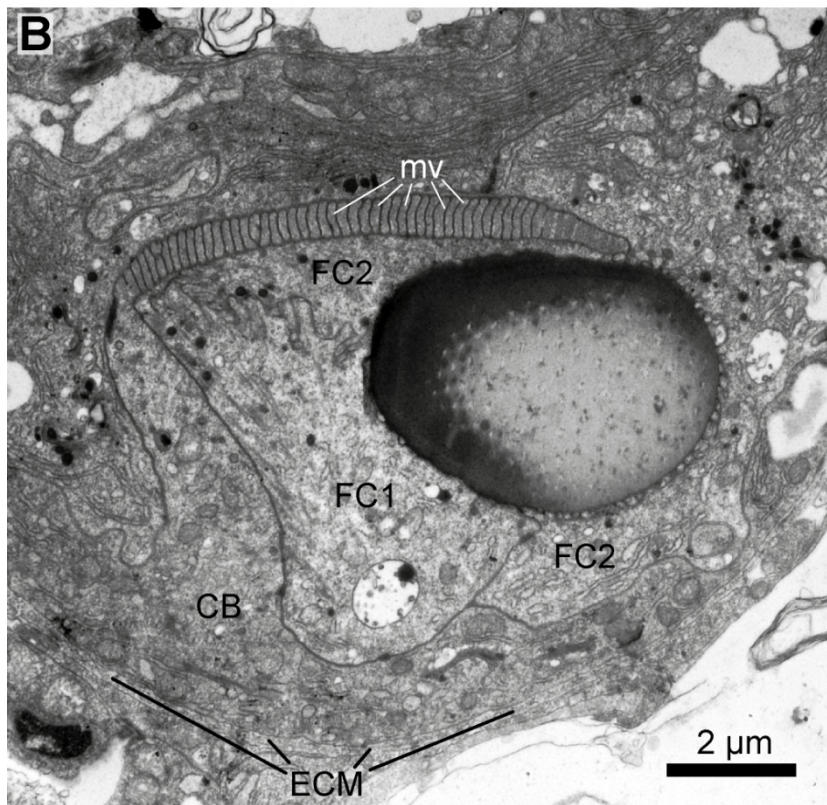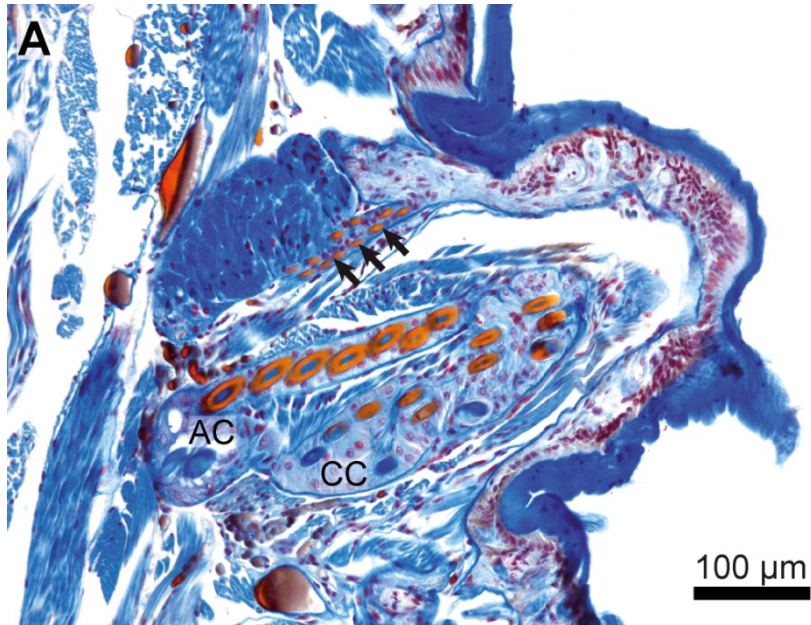
Figure 6.2 a Azan-stained histological section of the parapodium of *Arabella iricolor*. Note the *arrows* pointing the internalized notopodial chaetae, *AC* acicula, *CC* capillary chaetae **b** TEM *Eunice torquata* (Eunicidae) formation of hooded hook. A single row of flat microvilli (*mv*) forms the template of the hood. *CB* chaetoblast, *ECM* extra-cellular matrix, *FC* follicle cell (for TEM-preparation see Tilic 2014).
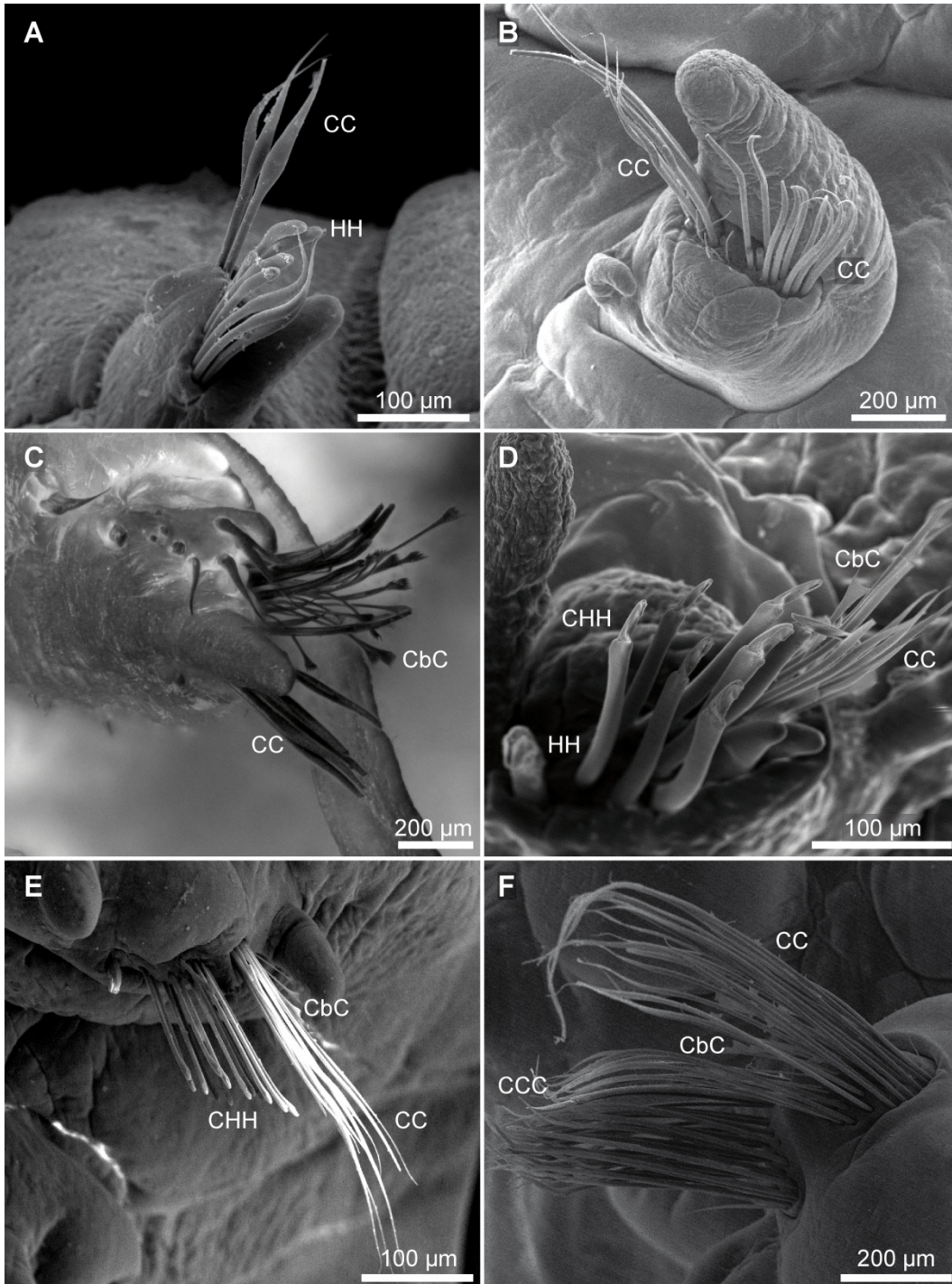
Figure 6.3 SEM micrographs of the parapodia of **a** *Lumbrineris fragilis* **b** *Arabella iricolor* **d** *Eunice torquata* **e** *Lysicidice ninetta* **f** *Marphysa bellii.* Extended focus digital micrograph of *Diopatra neapolitana* **c**, *HH* hooded hooks, *CHH*, compound hooded hooks, *CC* capillary chaetae, *CCC* compound capillary chaetae, *CbC* comb-shaped chaetae

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Dorvilleidae. Except for *Parapodrillus psammophilus*, all dorvilleids included into the molecular study bear simple and compound capillary chaetae. *P. psammodrilus* which groups with two *Ophryotrocha* species in our analysis lacks compound capillary chaetae. The remaining three dorvilleid species we included into our analysis, *Protovillea kefersteinii*, *Schistomeringos rudolphi*, and *Dorvillea erucaeformis*, additionally bear furcate chaetae. This type of chaetae is unique within Eunicida and absent in other eunicidan families (Hartmann-Schröder 1996; Jumars 1974). Furcate chaetae are also present in *Dorvillea australiensis* (Fig. 6.4) and further species, like *Protorvillea gracilis*, *Dorvillea moniloceras*, *Dorvillea atlantica*, and *Dorvillea articulata* (Hartmann-Schröder 1996; Jumars 1974). Vestigial chaetal elements of the notopodia were neither observed in the studied *Ophryotrocha* species nor mentioned in the literature (Tab. 6.1). Other dorvilleid species, namely *Protodorvillea gracilis, Dorvillea moniloceras, Dorvillea atlantica, Dorvillea articulata , Dorvillea erucaeformis* and *Schistomeringos rudolphi* (Hartman 1968; Hartmann-Schröder 1996; Jumars 1974) still possess notopodial chaetal elements inside the dorsal cirrus. Aciculae are present in all dorvilleids.

Onuphidae. Like *Diopatra neapolitana,* all onuphid species included in our analysis possess simple capillary chaetae, simple hooded hooks and comb-shaped chaetae (Fig. 6.3c, 6.4). The hooded hooks are bidentate and the hood only covers the apical portion of the chaetae partially. Hartman (1968) also mentions compound hooded hooks in the onuphid species *Nothria iridescens, Nothria stigmatis, Nothria pallida, Onuphis eremita, Onuphis litoralis, Onuphis parva, Onuphis nebulosa,* and compound capillary chaetae in *Nothria stigmatis, Onuphis nebulosa, Rhamphobranchium langisetum* (Tab. 6.1). In compound hooded hooks, the hinge is basal to the hood. These species were not part of the molecular analysis. Drawings in the literature (Hartman 1968; Paxton 1998) indicate that there are

rudimentary notopodial chaetae internalized in the dorsal cirri of *Onuphis vexillaria, Nothria stigmatis, Onuphis eremita, Onuphis litoralis, Onuphis microcephala, Diopatra dentate, Diopatra aciculata, Diopatra chilienis, Diopatra neopolitana* (Tab. 1). Aciculae are present in all onuphids.

Eunicidae. Eunicidae is the largest eunicidan subtaxon and is represented with 28 species in our analysis. The group displays the highest diversity of chaetal types. Except for furcate chaetae, all described chaetal types are present in Eunicidae. In *Eunice torquata* and *Lysidice ninetta* simple capillary chaetae, simple and compound hooded hooks and comb-shaped chaetae are present (Fig. 6.1c, 6.d). Simple hooded hooks are bidentate and the hood only partially covers the apical portion of the chaetae. In *Eunice torquata* the hood is preformed by a single horseshoe-shaped row of flattened and broad microvilli arising from the apical most portion of the chaetoblast (Fig. 6.2b). In compound hooded hooks, the hinge is basal to the hood. In *Palolo viridis* and *Palolo siciliensis* comb-shaped chaetae are absent (Fauchald 1992). Compound capillary chaetae are present in *Eunice implexa* (Fig. 6.4), *Palolo paloloides* (Fauchald 1992; Hartman 1968) and all *Marphysa* species; and can be seen in the SEM images of *Marphysa bellii* (Fig. 6.3f). Notopodial chaetae are present in *Eunice torquata* nested inside the dorsal cirrus, but are absent in *Lysidice ninetta* and *Marphsa bellii* (Fig. 6.1d, c). Aciculae are present in all eunicids.

Table 6.1 Complete dataset on chaetation of eunicid species included in this study. *HH* hooded hooks, *CC* capillary chaetae, *CbC* comb-shaped chaetae, *CHH* compound hooded hooks *CCC* compound capillary chaetae, *FC* furcate chaeta, *NC* internalized notopodial chaetae.

Higher Taxon	Species	HH	CC	CbC	CHH	CCC	FC	NC	Literature
Lumbrineridae	*Lumbrineris tetraura*	+	+	-	-	-	-	+	Tilic et al. 2014
	Lumbrineris fragilis	+	+	-	-	-	-	+	Tilic et al. 2014
	Lumbrineris acuta	+	+	-	-	-	-	?	Hartman 1968
	Lumbrineris bassi	+	+	-	-	-	-	?	Hartman 1968
	Lumbrineris bicirrata	+	+	-	-	-	-	?	Hartman 1968
	Lumbrineris bifilaris	+	+	-	-	-	-	?	Hartman 1968
	Lumbrineris californiensis	+	+	-	+	-	-	?	Hartman 1968
	Lumbrineris cruzensis	+	+	-	+	-	-	?	Hartman 1968
	Lumbrineris erecta	+	+	-	-	-	-	+	Hartman 1968
	Lumbrineris index	+	+	-	+	-	-	+	Hartman 1968
	Lumbrineris inflata	+	+	-	+	-	-	?	Hartman 1968
	Lumbrineris japonica	+	+	-	+	-	-	?	Hartman 1968
	Lumbrineris latreilli	+	+	-	+	-	-	?	Hartman 1968
	Lumbrineris linguata	+	+	-	+	-	-	?	Hartman 1968
	Lumbrineris limicola	+	+	-	+	-	-	?	Hartman 1968
	Lumbrineris longensis	+	+	-	-	-	-	?	Hartman 1968
	Lumbrineris minima	+	+	-	-	-	-	?	Hartman 1968
	Lumbrineris moorei	+	+	-	-	-	-	?	Hartman 1968
	Lumbrineris pallida	+	+	-	+	-	-	?	Hartman 1968
	Lumbrineris zonata	+	+	-	-	-	-	?	Hartman 1968
	Ninoe fusca	+	+	-	-	-	-	?	Hartman 1968
	Ninoe gemmea	+	+	-	-	-	-	?	Hartman 1968
	Ninoe nigripes	+	+	-	-	-	-	?	Carrera-Parra 2006
Oenonidae	*Arabella iricolor*	-	+	-	-	-	-	+	*this paper*
	Arabella geniculata	-	+	-	-	-	-	?	Hartman 1968
	Arabella mimetica	-	+	-	-	-	-	?	Hartman 1968
	Arabella semimaculata	-	+	-	-	-	-	?	Hartman 1968
	Biborin ecbola	-	+	-	-	-	-	?	Hartman 1968
	Drilonereis falcata	-	+	-	-	-	-	?	Hartman 1968
	Drilonereis longa	-	+	-	-	-	-	?	Hartman 1968
	Dirlonereis nuda	-	+	-	-	-	-	?	Hartman 1968
	Labidognathus forcipes	-	+	-	-	-	-	?	Hartman 1968

Higher Taxon	Species	HH	CC	CbC	CHH	CCC	FC	NC	Literature
	Notocirrus attenuatus	-	+	-	-	-	-	?	Hartman 1968
	Notocirrus californiensis	-	+	-	-	-	-	?	Hartman 1968
Dorvilleidae	*Ophryotrocha sp.*	-	+	-	-	+	-	-	*this paper*
	Ophryotrocha puerilis	-	+	-	-	+	-	-	Hartman 1968
	Parapodrilus psammophilus	-	+	-	-	-	-	-	Hartmann-Schröder et al. 1996
	Protodorvillea gracilis	?	+	-	-	+	+	+	Hartman 1968
	Protodorvillea kefersteinii	-	+	-	-	+	+	?	Jumars 1974
	Dorvillea moniloceras	-	+	-	+	+	+	+	Hartman 1968
	Dorvillea atlantica	-	+	-	+	+	+	+	Hartman 1968
	Dorvillea articulata	-	+	-	+	+	+	+	Hartman 1968
	Dorvillea erucaeformis	-	+	-	-	+	+	?	Hartmann-Schröder et al. 1996
	Schistomeringos rudolphi	-	+	-	+	+	+	+	Jumars 1974
Eunicidae	*Eunice torquata*	+	+	+	+	-	-	+	*this paper*
	Eunice americana	+	+	+	+	-	-	+	Hartman 1968
	Eunice antennata	+	+	+	+	-	-	?	Hartman 1968
	Eunice aphroditois	+	+	?	+	-	-	?	Hartman 1968
	Eunice biannulata	+	+	?	+	-	-	?	Hartman 1968
	Eunice multipectinata	+	+	+	+	-	-	+	Hartman 1968
	Eunice valens	+	+	?	+	-	-	?	Hartman 1968
	Eunice vittata	+	+	?	+	-	-	+	Hartman 1968
	Eunice tenuis	+	+	+	+	-	-	?	Fauchald 1992
	Eunice thomasiana	+	+	+	+	-	-	?	Fauchald 1992
	Eunice antarctica	+	+	+	+	-	-	?	Fauchald 1992
	Eunice norvegica	+	+	+	+	-	-	?	Fauchald 1992
	Eunice filamentosa	+	+	+	+	-	-	+	Fauchald 1992
	Eunice lucei	+	+	+	+	-	-	+	Fauchald 1992
	Eunice rubra	+	+	+	+	-	-	+	Fauchald 1992
	Eunice fucata	+	+	+	+	-	-	?	Fauchald 1992
	Eunice implexa	+	+	+	+	+	-	?	Fauchald 1992
	Eunice cariboea	+	+	+	+	-	-	?	Fauchald 1992
	Eunice amoureuxi	+	+	+	+	-	-	?	Fauchald 1992
	Eunice mutilata	+	+	+	+	-	-	+	Fauchald 1992
	Eunice notata	+	+	+	+	-	-	?	Fauchald 1992
	Eunice harassii	+	+	+	+	-	-	?	Fauchald 1992

Higher Taxon	Species	HH	CC	CbC	CHH	CCC	FC	NC	Literature
	Eunice roussaei	+	+	+	+	-	-	+	Fauchald 1992
	Marphysa belli	+	+	+	+	+	-	-	*this paper*
	Marphysa belli oculata	+	+	+	+	+	-	-	Hartman 1968
	Marphysa conferta	+	+	+	+	+	-	?	Hartman 1968
	Marphysa disjuncta	+	+	+	+	+	-	?	Hartman 1968
	Marphysa mortensi	+	+	?	+	+	-	?	Hartman 1968
	Marphysa sanguinea	+	+	+	+	+	-	?	Hartman 1968
	Marphysa stylobranchiata	+	+	+	+	+	-	?	Hartman 1968
	Marphysa fallax	+	+	+	+	+	-	+	Fauchald 1992
	Palolo viridis	+	+	-	+	-	-	?	Fauchald 1992
	Palolo siciliensis	+	+	-	+	-	-	?	Fauchald 1992
	Palolo paloloides	+	+	+	+	+	-	?	Hartman 1968
	Lysidice ninetta	+	+	+	+	-	-	+	*this paper*
	Nematonereis unicornis	+	+	+	+	-	-	?	Day 2967
	Lysidice collaris	+	+	+	+	-	-	?	Fauchald 1992
Onuphidae	*Diopatra neopolitana*	+	+	+	-	-	-	+	*this paper*
	Diopatra ornata	+	+	-	-	-	-	?	Hartman 1968
	Diopatra splendidissima	+	+	+	-	-	-	?	Hartman 1968
	Diopatra tridentata	+	+	-	-	-	-	?	Hartman 1968
	Diopatra dentata	+	+	+	-	-	-	+	Hartman 1968
	Diopatra aciculata	+	+	+	-	-	-	+	Hartman 1968
	Diopatra chilienis	+	+	+	-	-	-	+	Paxton 1998
	Hyalinoecia juvenalis	+	+	-	-	-	-	?	Hartman 1968
	Hyalinoecia tubicola stricta	+	+	+	-	-	-	?	Hartman 1968
	Nothria conchylega	+	+	+	-	-	-	?	Hartman 1968
	Nothria elegans	+	+	+	-	-	-	?	Hartman 1968
	Nothria geophiliformis	+	+	+	-	-	-	?	Hartman 1968
	Nothria hiatidentata	+	+	+	-	-	-	?	Hartman 1968
	Nothria iridescens	+	+	+	+	-	-	?	Hartman 1968
	Nothria pallida	+	+	+	+	-	-	?	Hartman 1968
	Nothria stigmatis	+	+	+	+	+	-	+	Hartman 1968
	Nothria stigmatis intermedia	+	+	+	+	+	-	+	Hartman 1968
	Onuphis eremita	+	+	+	+	?	-	+	Hartman 1968
	Onuphis litoralis	+	+	+	+	?	-	+	Hartman 1968
	Onuphis microcephala	+	+	+	-	?	-	+	Hartman 1968
	Onuphis nebulosa	+	+	+	+	+	-	?	Hartman 1968

Higher Taxon	Species	HH	CC	CbC	CHH	CCC	FC	NC	Literature
	Onuphis parva	+	+	+	+	?	-	?	Hartman 1968
	Onuphis vexillaria	+	+	+	-	?	-	+	Hartman 1968
	Rhamphobranchium langisetum	+	+	+	-	+	-	?	Hartman 1968
	Onuphis iridescens	+	+	+	-	-	-	?	Fauchald 1982
	Onuphis elegans	+	+	+	-	-	-	?	Fauchald 1982
	Onuphis similis	+	+	+	-	-	-	?	Fauchald 1982
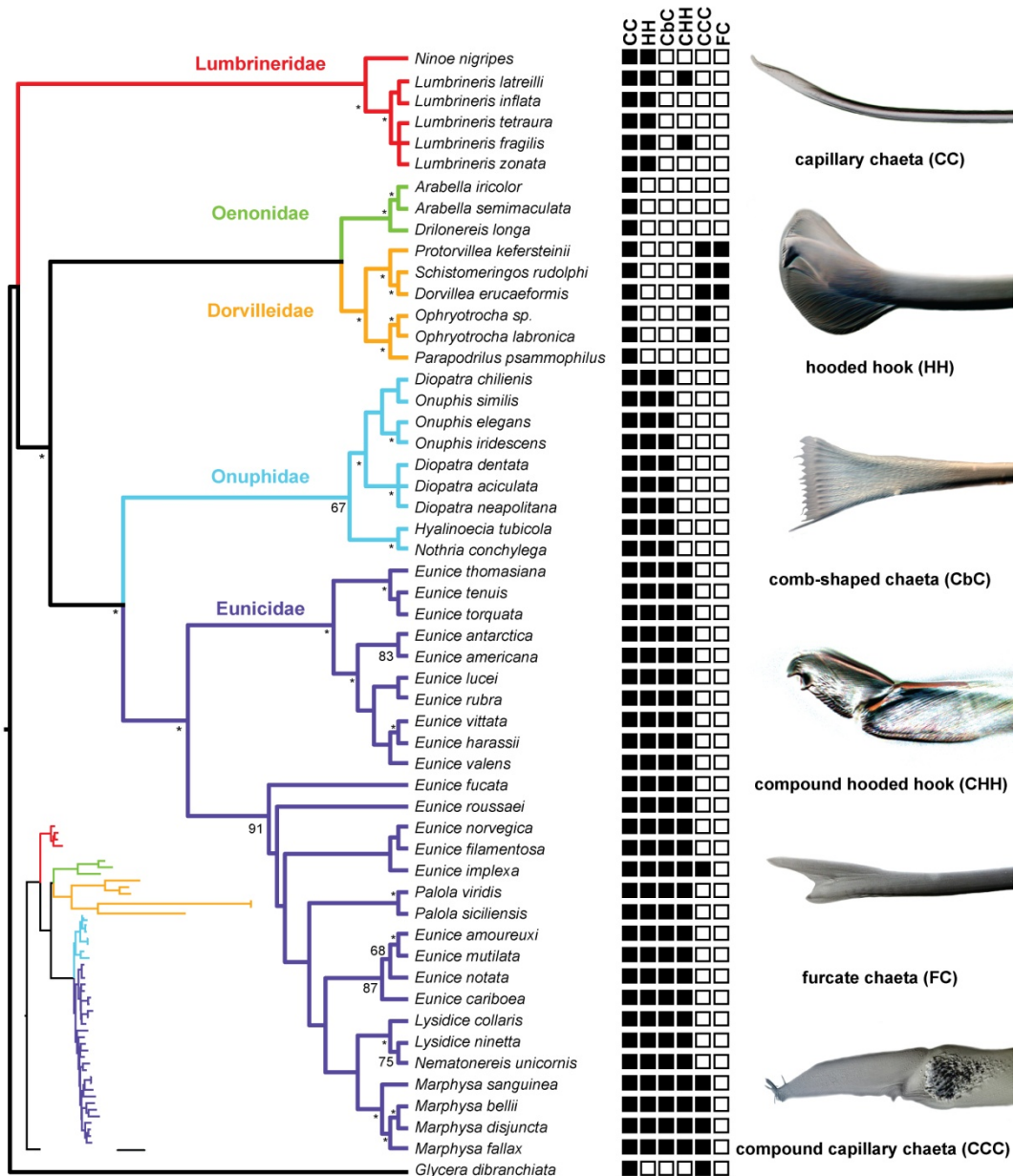
Figure 6.4 Best maximum likelihood (ML) tree of the RAxML analysis of the data set comprising 50 taxa and 3 genes (COI, 16S, 18S). indent: ML tree topology including branchlengths (*scale* 5.0). ***** Bootstrap support = 100. *HH* hooded hooks, *CHH*, compound hooded hooks, *CC* capillary chaetae, *CCC* compound capillary chaetae, *CbC* comb-shaped chaetae, *FC* furcate chaetae. Absence and presence of chaetae types in a species are mapped on to the tree with boxes (*black* present, *white* absent)

6.3.3 Larval chaetae

Larvae of *Lumbrineris sp.* and different ontogenetic stages of *Ophryotrocha sp.* were studied in order to document ontogenetic changes in chaetation. Larval stages of *Lumbrineris sp.* initially only possess simple capillary chaetae (Fig. 6.5b). Hooded hooked chaetae only appear later in the ontogeny. In *Ophryotrocha,* however, all larval stages had the same set of chaetae as adult animals. No variations or changes in chaetation were observed throughout the ontogeny (Fig. 6.5a). Early larval stages of *Diopatra sp.* only bear capillary chaetae as well, indicating that additional chaetal types are added later in the ontogeny (Fig. 6.5c)

6.3.4 Character transformations

Maximum likelihood transformations are shown for simple (Fig. 6a) and compound hooded hooks (Fig. 6.6d), comb shaped chaetae (Fig. 6.6b) and compound capillary chaetae (Fig. 6.6c). Since furcate chaetae only appear within Dorvilleidae, the maximum likelihood transformation suggests that this chaetal type evolved once within dorvilleids. Simple capillaries are present in all Eunicids and the outgroup.

Among Eunicidae, simple hooded hooks are only absent in Oenonidae and Dorvilleidae. The maximum likelihood transformation shows that the last common ancestor of these two taxa had no hooded hooks with a proportional likelihood of 0.86. Hooded hooks are not present in the outgroup and the maximum likelihood transformation shows some ambiguity for the evolution of hooded hooks (Fig. 6.6a). Their absence in the last common ancestor of Eunicida has a low proportional likelihood value of 0.62 and absence in the last common ancestor of all non-lumbrinerid Eunicida has a proportional likelihood value of 0.66. This scenario reflects the most likely possibility that simple hooded hooks evolved independently in Lumbrineridae and

Eunicidae/Onuphidae and that the absence in Dorvilleidae/ Oeononidae is not a loss.

With regards to comb-shaped chaetae, the transformation (Fig. 6.6b) suggests that the plesiomorphic condition for Eunicida is the lack of such chaetae and that they evolved once in the stem lineage for Onuphidae and Eunicidae (proportional likelihood > 0.99). Within Eunicidae these comb-shaped chaetae have been lost in *Palolo viridis* and *Palolo siciliensis* (proportional likelihood > 0.97).

A relatively high proportional likelihood value (prop. likelihood > 0.90) indicates that the absence of compound capillary chaetae is plesiomorphic for Eunicida (Fig. 6.6c). This is shown across most other nodes. The distribution of compound capillaries within Eunicida suggests that these have evolved at least three times within this taxon; once within Dorvilleidae, once in a lineage including *Eunice implexa* and *Palolo paloloides* and once in the *Marphysa* lineage.

The transformation analysis and the distribution of compound hooded hooks suggest that this chaetal type evolved once within Lumbrineridae (Fig. 6.6d) and once for the Eunicidae (prop. likelihood > 0.98).
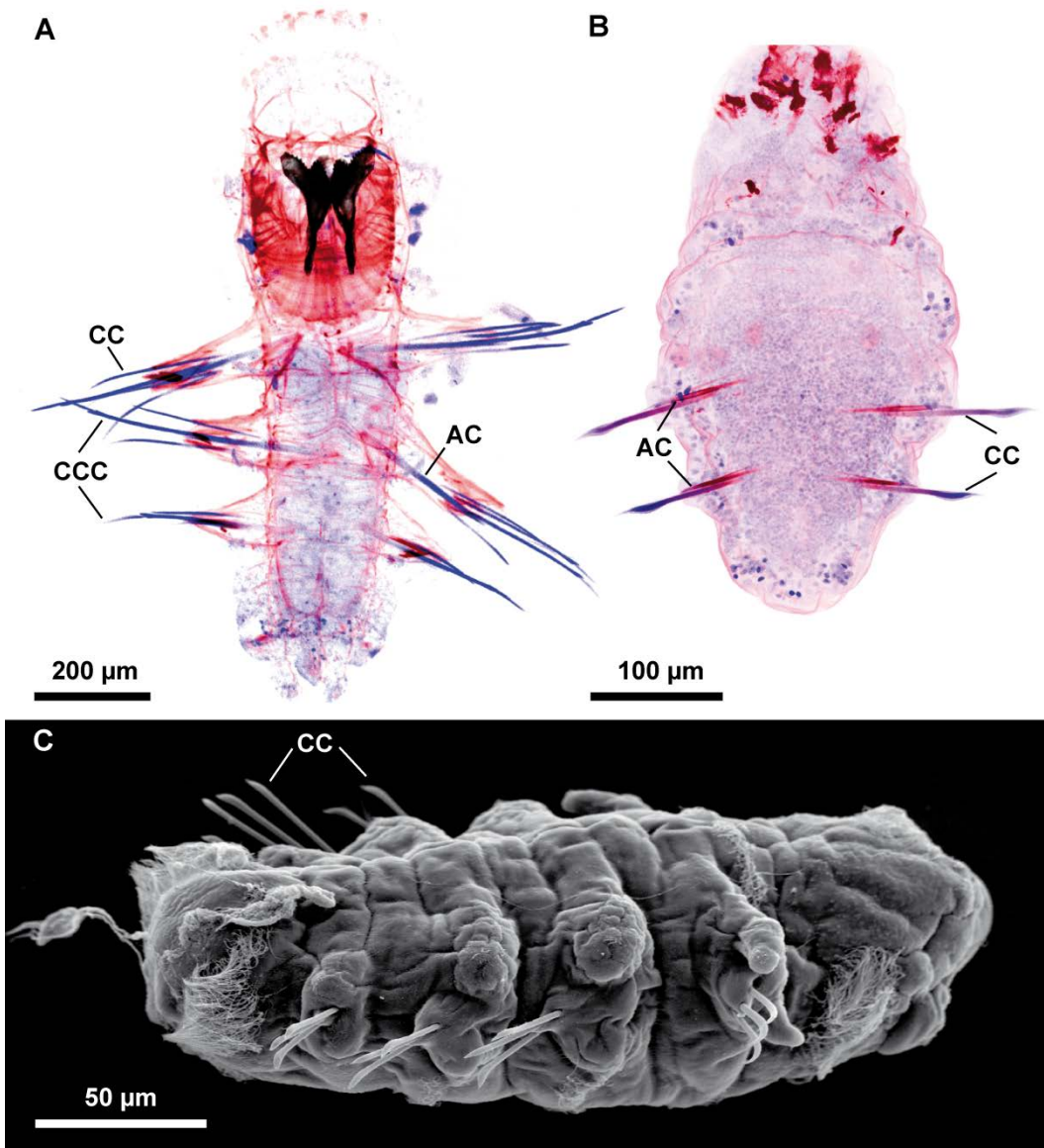
Figure 6.5 Micrographs showing larval chaetae **a** Micrograph of *Ophryotrocha sp.* larva **b** Micrograph of *Lumrineris sp.* larva c SEM Micrograph of *Diopatra sp.* larva. *CC* capillary chaetae, *CCC* compound capillary chaetae
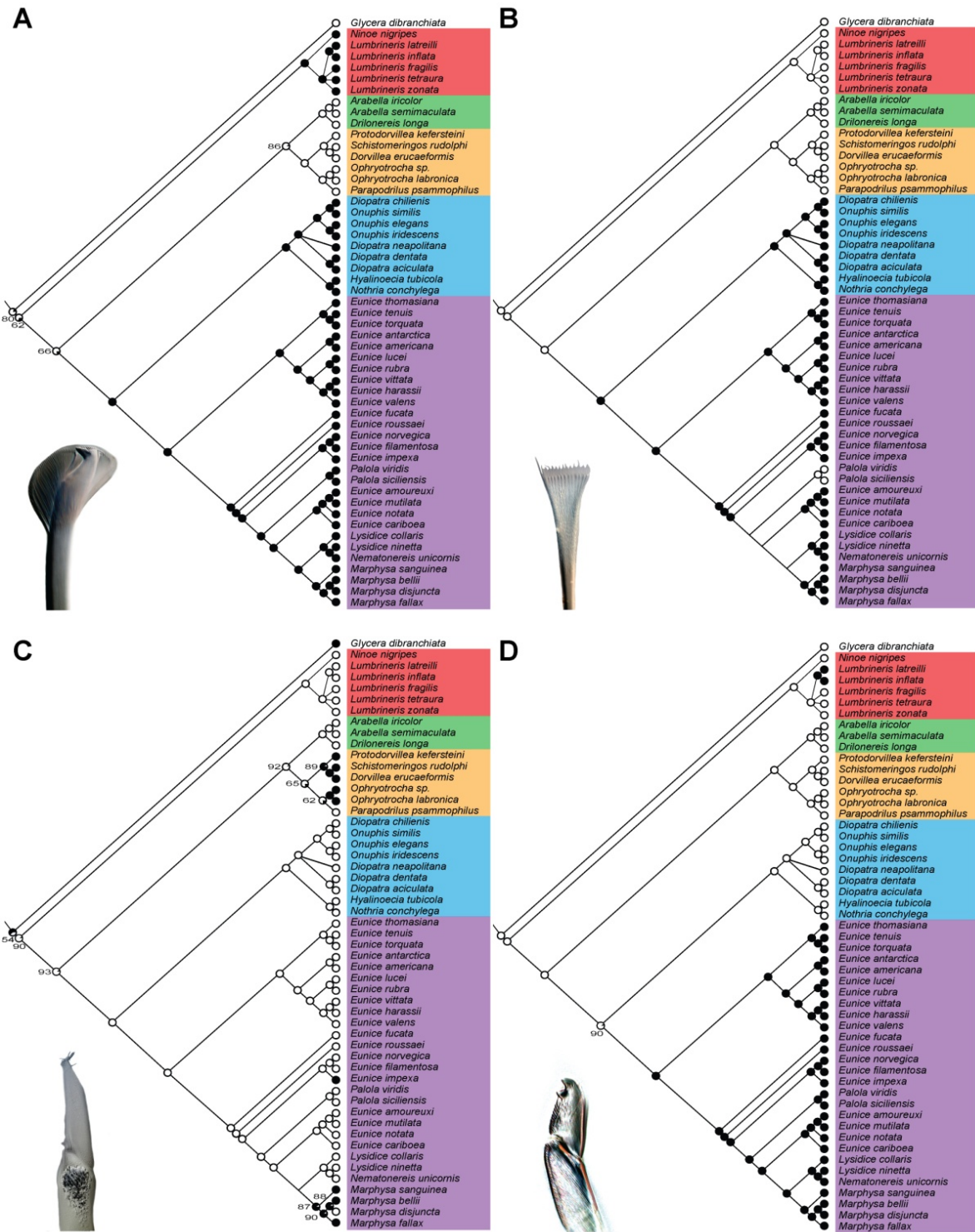
Figure 6.6 Maximum likelihood transformations for the absence and presence of **a** hooded hooks, **b** comb-shaped chaetae, **c** compound capillary chaetae, **d** compound hooded hooks. Scores (for the most likely states) are provided when the estimated proportional likelihood was lower than 95%. *black* present, *white* absent. Colored boxes surrounding the clades as in Fig. 4

6.4 Discussion

6.4.1 Homology of hooded hooks and the evolution of "joints" in hooded hooks

Hooded hooked chaetae are not unique to Eunicida; Capitellida and Spionida also possess such chaetae with a hood-like structure. Based on current phylogenetic hypotheses these would appear to have evolved separately in these two clades (Andrade et al. 2015; Struck et al. 2015) and so are convergent. The formation of the hood differs between them, further supporting their non-homology (Tilic et al. 2014).

Our study shows that hooded hooks within Eunicida differ in their apical structure. Irrespective of being simple or compound, hooded hooks of lumbrinerid species are multidentate, while those of onuphid and eunicid species are bidentate (Fig. 6.7a-d). These differences may either result from transformation (multidentate to bidentate or vice versa and simple to compound or vice versa), so that all eunicidan hooded hooks are homologous (Fig. 6.7) or may indicate convergent evolution. The latter is slightly supported by the proportional likelihood values that indicate primary absence of hooded hooks in the last common ancestor of Eunicida as well as in the last common ancestor of Eunicida excluding Lumbrineridae (Fig. 6.6a). According to these values one could expect that formation of hooded hooks in lumbrinerids differs from that in Eunicidae/ Onuphidae. Our preliminary data indicate that there are actually differences in the formation of the hood. In *Lumbrineris tetraura* multiple rows of microvilli form the template of the hood which thus consists when fully developed of an inner and an outer layer with irregularly arraged chitinous tubes in between (Tilic et al. 2014) while it is a simple sheeth in Eunice torquata, preformed by a single row of modified microvilli (Fig. 6.2b). The question on how often a hinge evolved, however, remains to be solved. Provided the observed differences turn out to be

representative, a hinge evolved at least twice, once within the Lumbrineridae and once in Eunicidae.
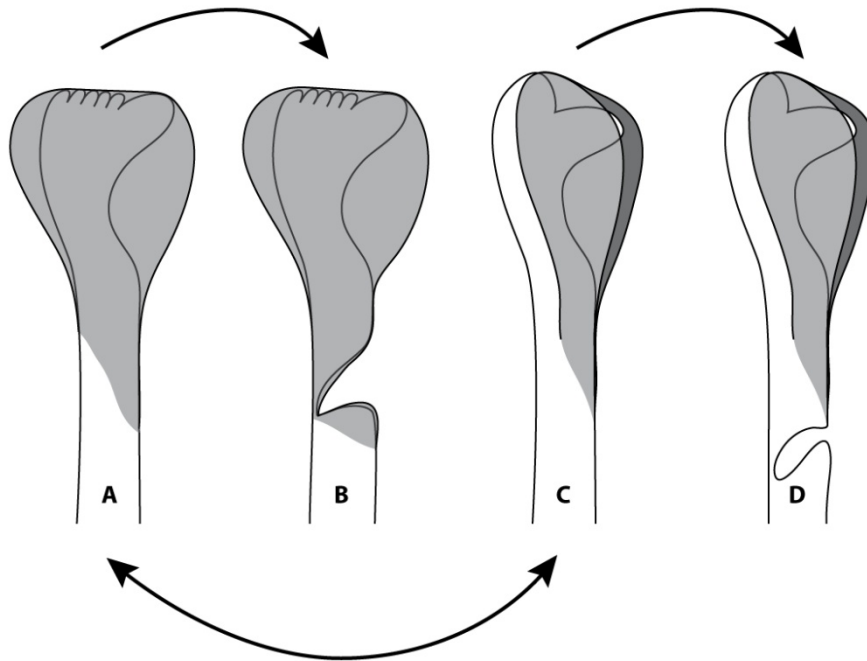
Figure 6.7 Homology hypotheses of hooded hooks in Eunicida. **a-b** multidentate hooded hooks of lumbrinerid species, **c-d** bidentate hooded hooks of onuphid and eunicid species

6.4.2 Comb-shaped chaetae

Comb-shaped chaetae or similar looking forked chaetae are not only present Eunicida, they occur in a variety of other polychaete taxa such as Orbiniidae, Scalibregmatidae, Paraonidae and Nephtyidae (Hausam and Bartolomaeus 2001; Hausen 2005).

Orbiniid forked chaetae are characterized by two tines that fuse towards the base forming the shaft. In between these tines several spines originate. Hausam and Bartolomaeus (2001) described the chaetogenesis of forked chaetae in *Orbinia latreilii* (Orbiniidae). During chaetal formation both tines are formed by two separate bundles of microvilli on the chaetoblast surface. Later, additional microvilli occur at intervals on the inner margins of the tines to form the spines. In contrast to the tines, each spine is formed by a single

large microvillus (Hausam and Bartolomaeus 2001). A study of the ultrastructure and chaetal formation of comb shaped chaetae in Euncidae is currently underway (Tilic and Bartolomaeus, in progress).

In Eunicida, comb-shaped chaetae are restricted to species of Onuphidae and Eunicidae; and are absent in the remaining taxa (Fig. 6.6B). The proportional likelihood value for their presence is 0.99 in the last common ancestor of Eunicidae and Onuphidae, clearly supports their convergent evolution with those seen in other annelids, which are phylogenetically distant (Andrade et al. 2015; Struck et al. 2015).

6.4.3 Evolution of compound chatae

Compound or "jointed" chaetae are found in a variety of polychaete taxa. Jointed chaetae, with a real joint-like structure, consisting of a socket and a ligament, are only known from several families within Phyllodocida (O'Clair and Cloney 1974; Bartolomaeus 1998; Merz and Woodin 2006;). This type of jointed chaetae only has a single ligament (Rouse and Fauchald 1997). Chaetogenesis of these jointed chaetae were studied and described in detail by Gustus and Cloney (1973) and O'Clair and Cloney (1974) in larval and adult *Nereis vexillosa* (Phyllodocida). In contrast to Phyllodocida compound chaetae in eunicidan species do not have a socket and are therefore often also referred to as pseudo-compound chaetae with double ligaments (Rouse and Fauchald 1997; Merz and Woodin 2006;). Compound capillary chaetae can also be found in the paedomorphic meiofaunal taxon Nerillidae (Worsaae 2014). According to the recent phylogenomic analysis by Struck et al. (2015) Nerillidae are placed within Sedentaria in a clade together with Orbiniidae and Parergodrilidae. Other taxa within Sedentaria like Flabelligeridae and Acrocirridae also posses similar chaetal elements, where the chaetal shaft is interrupted by a thinning (Caullery and Mesnil 1898, Hartman and Fauchald 1971, Osborn and Rouse 2011). Merz and Edwards`s (1998) experimental study provides important insights into the possible functionality of

jointed chaetae in *Ophiodromus pugettensis* (Hesionidae: Phyllodocida). Herein they show that flexible joints in chaetae significantly increase the locomotory performance of the worm. Considering this clear functional gain and the distribution of compound chaetae within Eunicida (Fig. 6.6 c, d; Fig. 6.7), as well as in the outgroups, a convergent evolution of joints and similar structures that form a segmented shaft seems plausible.

However, as stated in Rouse and Fauchald (1997) and Merz and Woodin (2006), the details of the morphology of compound chaetae have not been explored thoroughly. Until there is evidence from ultrastructural and developmental data for detailed similarity or even identity of these joints, we assume that they evolved several times independently.

6.4.4 Increasing diversity of chaetal types

Except for a subgroup of Euncidae and in *Ophryotrocha* species, internalized notopodial chaetae are present in all Eunicida. Since notopodial chaetae without a notopodium can hardly be explained, we assume that the notopodium was reduced in the eunicidan stem lineage while the aciculae were kept (Tilic et al. 2014). To our understanding of chaetal function it seems likely that the aciculae are included into a new functional context, so that their presence was positively selected. Studies into the parapodial muscular system and into the locomotion could help analyze the function of these remaining notopodial chaetae.

When we started our study we expected increasing diversity of chaetal types. This assumption could actually be largely confirmed (Fig. 6.8). Without doubt, aciculae and capillary chaetae are present in the last common ancestor. This condition is retained in Oenonidae, whereas in the remaining groups chaetal type diversity increases. Some chaetae are specific for subgroups, like furcate chaetae that are restricted to Dorvilleidae or multidentate hooded hooks that are

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restricted to Lumbrineridae. In the stem lineage of Onuphidae and Eunicidae most likely comb-shaped chaetae and probably bidentate hooded hooks were added to the primary set of chaetae. There is also some evidence that hinges evolved repeatedly in hooded hooks and capillary chaetae.

Generally the diversity of chaetal types increases during ontogeny of polychaete worms. In *Lumbrineris* and *Diopatra* for example the early larval stages and juveniles only bear capillary chaetae (Fig. 6.5b, c) additional chaetal types like the hooded hooks only appear in later stages. Okuda (1946) describes the development of *Lumbrineris laterilli* with regard to chaetae in great detail. Here, hooded hooks appear only after the 7-chaetiger stage. Increasing of chaetal diversity during development has also been described outside the Eunicida. In *Neanthes sp.* (Phyllodocida) the first two chaetiger stages only bear ordinary jointed homogomph falcigers, from the 3rd chaetiger onward heterogomph falcigers and later heterogomph spinigers appear (Okuda 1946). These observations also provide a framework to explain secondary loss of chaetal diversity. Provided that a species gets fertile at an early developmental stage as a result of progenetic evolution it should retain the simple (larval) chaetation as a result of truncated chaetal diversification. Progenesis, thus could be a proper explanation for secondary loss of chaetal diversity. In Histrobdellidae, which group together with Dorvilleidae (not shown) chaetation is lost completely; most probably as an adaptation to their symbiotic lifestyle. This shows that drastic changes in lifestyle (e.g. symbiotism, parasitism) can also be a further explanation for the loss of chaetation and chaetal diversity.

The distribution of different chaetae within Eunicida and this increasing diversity of chaetal types (Fig. 6.8) support the idea of a radiation and diversification of chaetae within this taxon from a more "simple" set of chaetae, whereby additional chaetal types are added during ontogeny and are not present from the first larval stages

onward. We therefore expect that chaetal diversity is underlain by a gene regulatory network and suggest taking a closer look into it.

Figure 6.8 Maximum likelihood transformations for the number of chaetal types (aciculae and vestigial notopodial chaetae not included) coded as character states. Scores (for the most likely states) are provided in eunicidan lineages when the estimated proportional likelihood was lower than 95%. Colored boxes surrounding the clades as in Fig. 4

6.5 Acknoledgements

Site of Chaetal Formation in Annelids

7.1 Introduction

Arrangement of annelid chaetae provide useful informations for systematics (Hausen 2005) and allows testing homology hypotheses (Tilic et al. 2015b). The general assumption is that the most common ancestor of all annelids possessed a dorsal (notopodial) and a ventral (neuropodial) group of chaetae.

In many taxa within Sedentaria these chaetae are arranged in rows that show continuous turnover since new chaetae are constantly formed and the tips of old chaetae are cast off, while their shaft is resorbed. Each chaetal row thus has a site of chaetal formation and a site of chaetal degeneration (Bobin 1944; Pilgrim 1977; Hausen 2005; Tilic et al. 2015a).

When a transverse row of chaetae is present in the notopodium as well as neuropodium, the formative sites of these rows always lies ventrally in notopodia and dorsally in neuropodia (Hausen and Bartolomaeus 1998; Schweigkofler et al. 1998; Hausen 2001; Hausen 2005). In Arenicolids and Maldanids the formative site of the neuropodial hooked chaetae is located ventrally, which is considered a derived condition and an autapomorphy of Maldanomorpha (Bartolomaeus and Meyer 1997; Bartolomaeus et al. 2005; Tilic et al. 2015a).

All in all, an arrangement in which both formative sites face each other is considered to be the plesiomorphic condition in Annelida. However, Hausen (2005) mentions that there are certain exceptions to this pattern, in Oweniids, Chaetopterids and within Magelonids. All three of these taxa were considered to be derived, and were positioned within Canalipalpata according to the morphological phylogeny of annelids by Rouse and Fauchald (1997). Strikingly exact three taxa

appear at a basal position in the more recent molecular phylogenies of Annelida (Weigert et al. 2014; Andrade et al. 2015; Struck et al. 2015). Considering this, the chaetal arrangement and the location of the formative site was investigated for *Owenia fusiformis* delle Chiaje (Oweniidae), *Chaetopterus variopedatus* (Renier, 1804) and *Spiochaetopterus (Telepsavus) costarum* (Claparède, 1869) Chaetopteridae. Furthermore, the chaetal arrangement of *Lysidice ninneta* (Audouin & H Milne Edwards, 1833) an errant eunicid species was inspected for comparison.

No restricted formative site has been described for Siboglinida. Schulze (2001), was in fact able to locate developmental stages of chaetae on different locations in *Ridgeia piscesae*. Hausen (2005) emphasizes the nescessity to further investigate this taxon to establish whether this is a derived condition or possibly an apomorphy of Siboglinidae. The chaetal arrangement of the chaetae bearing dwarf males of the deep-sea siboglinid "bone-eating" worm *Osedax rubiplumus* is also investigated in the following, to provide further information on chaetation in siboglinids.

7.2 Material and Methods

7.2.1 Semi-thin sectioning and 3D modelling

Animals used for semi-thin sectioning were fixed for TEM preparation (1.25 % glutaraldehyde buffered in 0.05 M phosphate buffer with 0.3 M NaCl for 1.5–2 h. and postfixation in 1 % OsO4 in 0.05 M phosphate buffer). Afterwards they were dehydrated in an acetone series and embedded in araldite. The serial sections were prepared using a diamond knife (Diatome Histo Jumbo) on a Leica Ultracut S ultramicrotome, following the method described by Blumer et al. (2002). The sections were stained with toluidine blue. The semi-thin sections were analyzed with an Olympus microscope (BX-51) and photographed with an Olympus camera (Olympus cc12), equipped with the dot slide system (2.2 Olympus, Hamburg). The images were aligned

with IMOD (Boulder Laboratories, Kremer et al. 1996) and IMOD-align (http://www.evolution.uni-bonn.de/mitarbeiter/bquast/software).

The software 3ds max 13.0 was utilized for the 3D modelling of the chaetae. The histological images were imported as surface materials, and the chaetae were modeled using standard cylindrical or conic objects. When necessary, these were modified as NURBS surfaces.

7.2.2 Light microscopy and CLSM

Specimens used for confocal laser scanning microscopy (CLSM) were fixed in 4% paraformaldehyde. Dissected parapodia and segments were permeabilized in four 5-min changes of phosphate buffered saline (PBS) with 0.1% Triton X-100 (Fisher Scientific). The samples were stained overnight at 4°C with TRITC-phalloidin at a dilution of 1:100. After staining, parapodia were rinsed in three quick changes and subsequently in two 10-min changes of PBS with 0.1% Triton, and one 10 min rinse in PBS without Triton. Smaller parapodia were mounted on poly-l-lysine coated coverslips, and larger ones were directly placed in hollow-ground slides. The samples were quickly dehydrated in isopropanol (2 min each in 70%, 85%, 95%, 100%, 100%), cleared in three 15-min changes of Murray Clear, and mounted in Murray Clear. Slide preparations were sealed with nail polish.

Chaetae were isolated from the surrounding tissue by incubation in 5 % NaOH for 2–3 h in room temperature. The chaetae were rinsed in distilled water, mounted on microscopic slides, examined using Nomarski differential interference contrast with an Olympus BX-51 microscope and photographed with an Olympus Camera (Olympus CC12).

7.2.3 Scanning Electron Microscopy (SEM)

Specimens used for SEM were fixed in Bouin's fluid. These were dehydrated in an alcohol series, and were kept in a 5%

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phosphotungsticacid solution for an hour to increase the heavy metal content in the tissue. They were critical point dried with CO_2 in a critical point dryer (Balzers) and sputtered with gold (Balzers Sputter Coater). The specimens were examined in a Philips XL30 ESEM. During dehydration the animals were sonicated to remove debris and sand particles from the chaetae. The *Osedax rubiplumus* specimen was fixed with GA and was not treated with phosphotungsticacid. It was dehydrated using HMDS, according to the method described by Nation (1983).

7.3 Results

7.3.1 *Lysidice ninetta* (Eunicida)

Lysidice ninetta possesses a variety of chaetal types; such as simple capillary chaetae, simple and compound hooded hooks and comb-shaped chaetae. There is neither a notopodium and nor any rudimentary notopodial chaetal element. These different chaetal types appear to have a separate formative site within the neuropodial chaetal sac (Fig. 7.1A). There is one large aciculum that is located centrally and it does not protrude from the cuticle. It is located deeply inside the parapodium. Sites of chaetal formation are located on either side of the aciculum (Fig. 7.1B). The shape of the chaetal sac is internally extreamly irregular and appears to be influenced by the insertion of parapodial musculature.
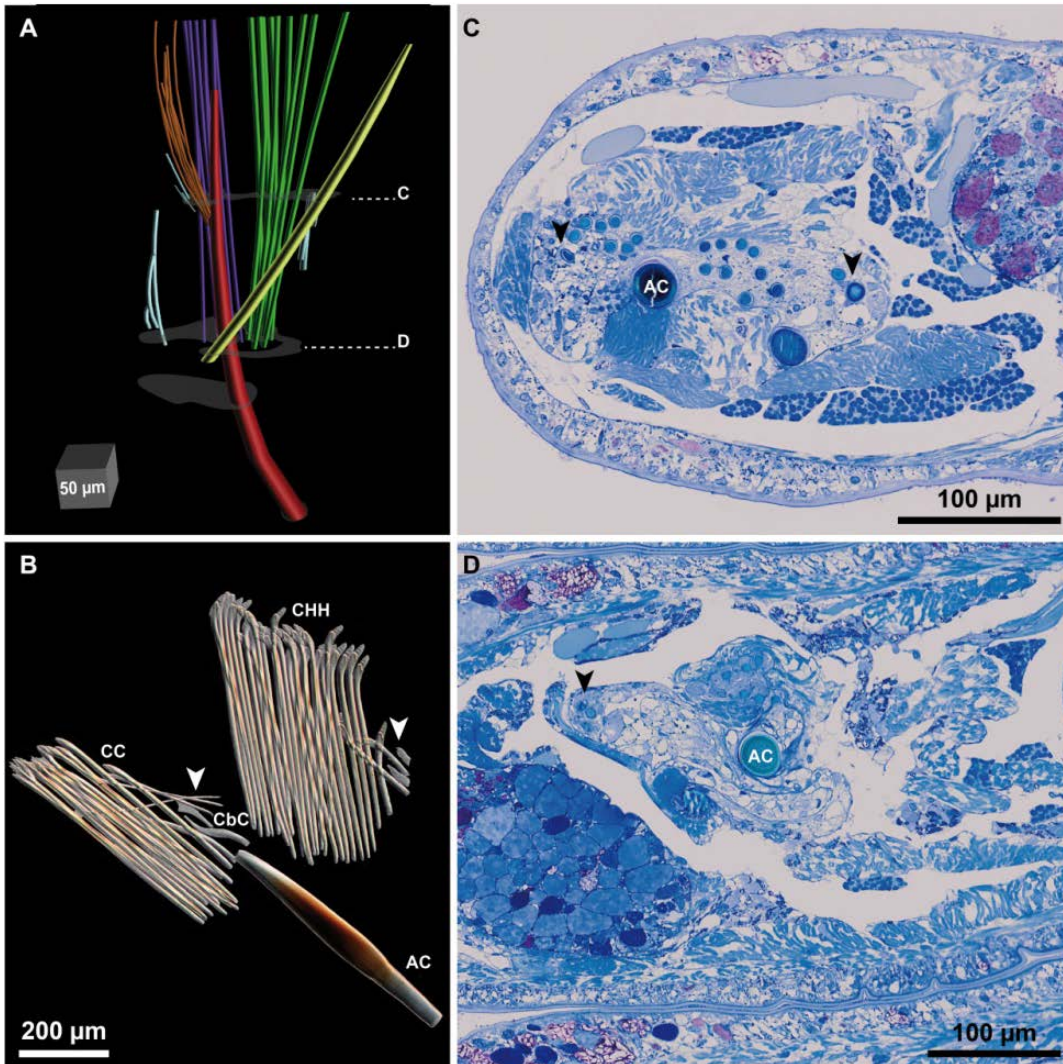
Figure 7.1 Chaetal arrangement in *Lysidice ninetta*. **A** 3D model of chaetal arrangement. Different chaetal types are color coded. The aciculum is red and developing chaetae are light blue. **B** Light microscopy of an isolated chaetal sac. **C-D** Tolouidin stained semithin sections used to generate the 3d model. Their relative positions are marked in the model. *AC* aciculum, *CHH* compound hooded hooks, *CC* capillary chaetae, *CbC* comb shaped chaetae, *arrow heads* mark the formative site

7.3.2 *Owenia fusiformis* (Oweniidae)
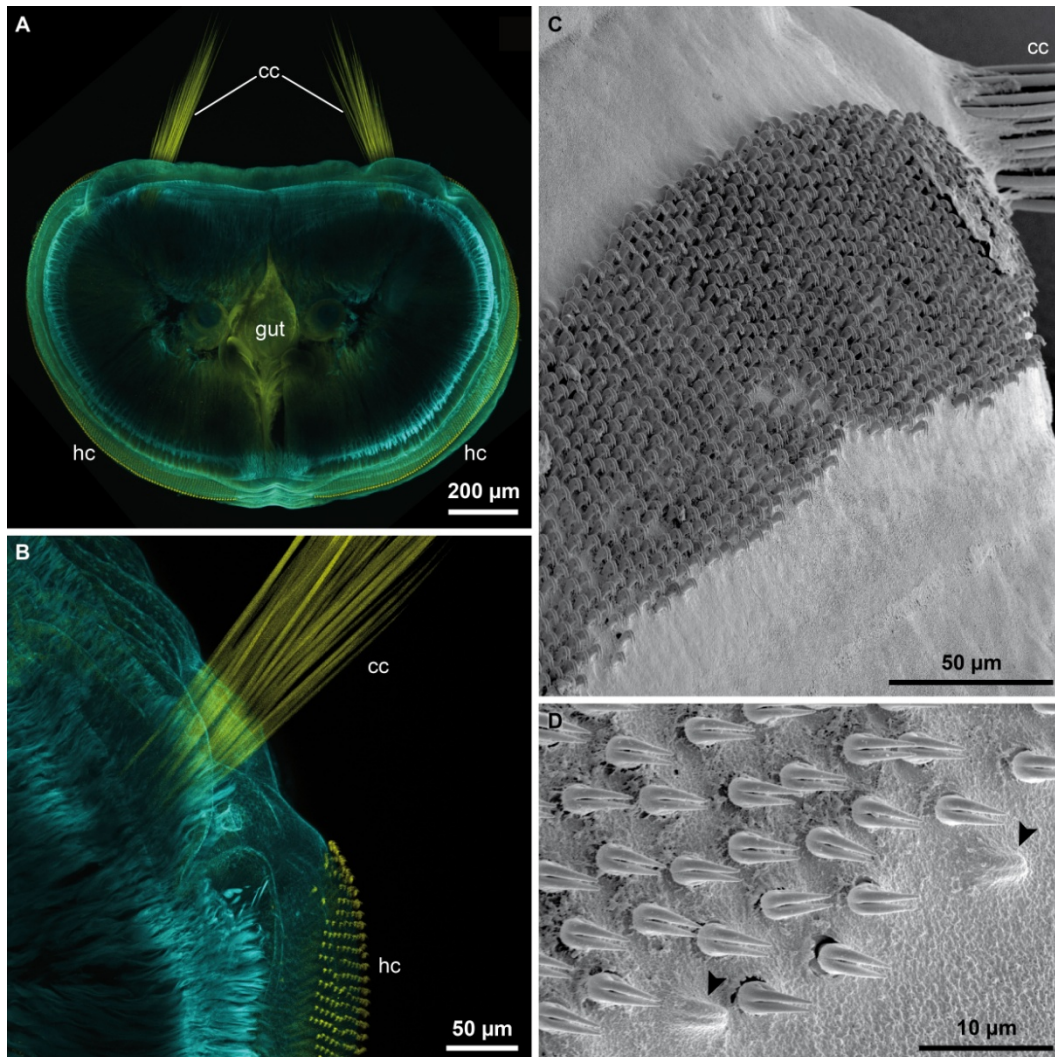
Figure 7.2. Chaetal arrangement in *Owenia fusiformis*. **A-B** Confocal *z*-projections of phalloidin stained preparations. *cyan* phalloidin *yellow* chaetal autofluorescence **C** SEM image showing the patch of hooked chaetae and the bundle of capillary chaetae **D** Developing hooked chaetae (marked with *arrow heads*) piercing through the cuticula. *cc* capillary chaetae, *hc* hooked chaetae.

Neuropodial hooked chaetae of *Owenia fusiformis* are arranged in so-called chaetal patches and the notopodial capillary chaetae are dorsally located and arranged in bundles (Fig. 7.2). Developing chaetae were observed piercing through the animals cuticle along the caudal edge of this patch, without any specific topological restriction (Fig. 7.2D).

7.3.3 Chaetopteridae
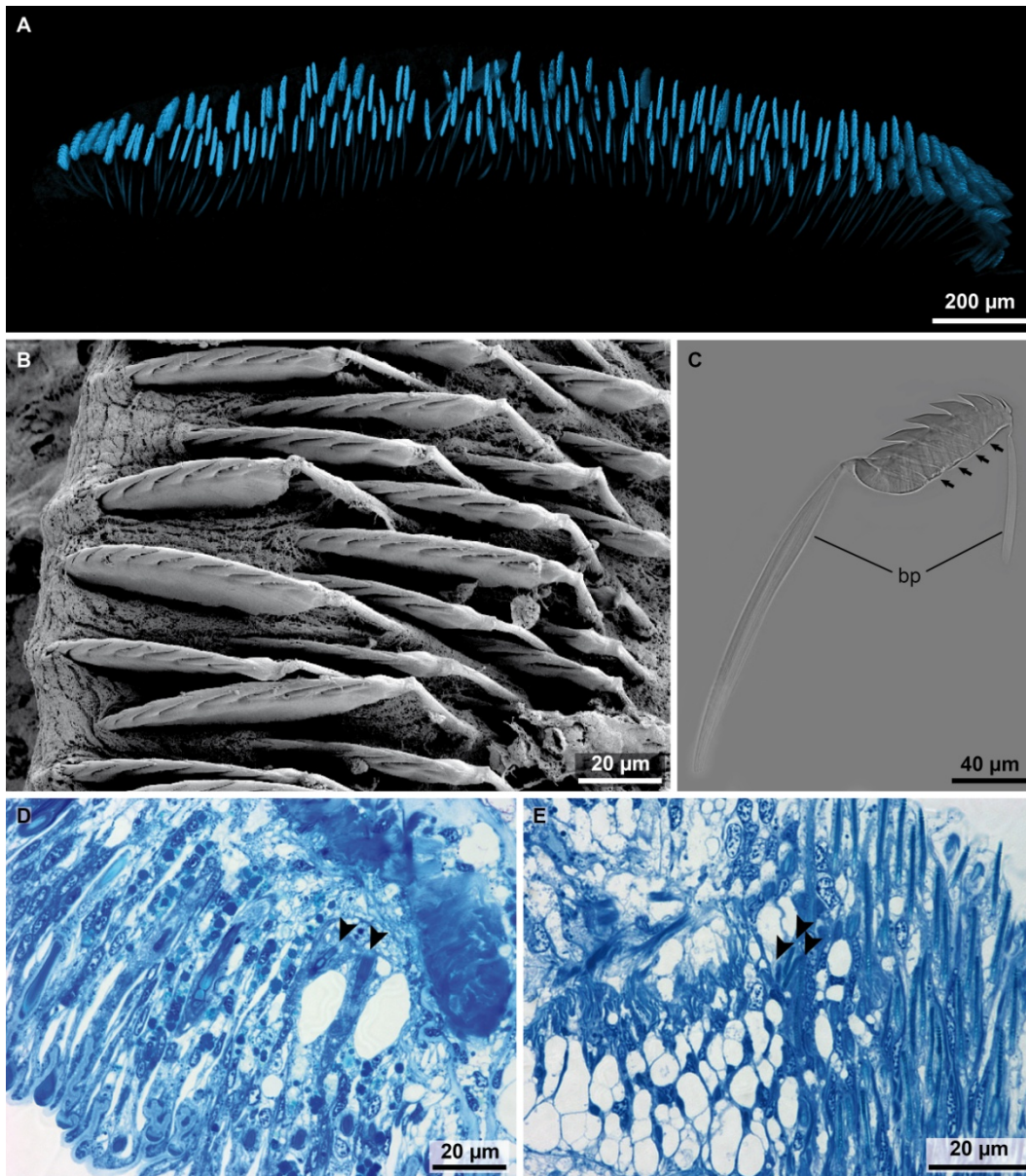
Figure 7.3. Chaetal arrangement in *Chaetopterids*. **A** Confocal *z*-projections (chaetal autofluorescence) of chaetal row in *Chaetopterus variopedatus* **B** SEM image showing the arrangement of uncini **C** Isolated uncinus; *bp* basal processes of the the manubrium, note the canals within the chaeta marked by arrows indicating the formation of each capitial teeth by a single microvillus **D-E** Developing hooked chaetae (marked with *arrow heads*) located in the semi-thin sections. D for *Chaetopterus variopedatus* E for *Spiochaetopterus costrarum.*

The arrangement of uncini in *Chaetopterus variopedatus* is hardly a single row. In fact up to three rows of chaetae were observed, arranged in a zip-like, interlocking manner (Fig.7.3A, B). Each uncinus possesses two manubrial processes that are located basally (Fig. 7.3C). Under Nomarsky contrast the chaetal canals left inside the uncinus by the microvilli of the chaetoblast were visible. Each capitial theeth appears to have one single canal within. The inspection of semi-thin sections *Chaetopterus variopedatus* and *Spiochaetopterus (Telepsavus) costrarum* revealed that new chaetae are being formed anywhere within the rows (Fig. 7.3D, E). Meaning there is no restriction of formation to one edge.

7.3.4 *Osedax rubiplumus* (Siboglinidae)

Figure 7.4. Chaetal arrangement in *Osedax rubiplumus.* **A-B** SEM images showing details of the hooked chaetae **C** 3D Reconstructio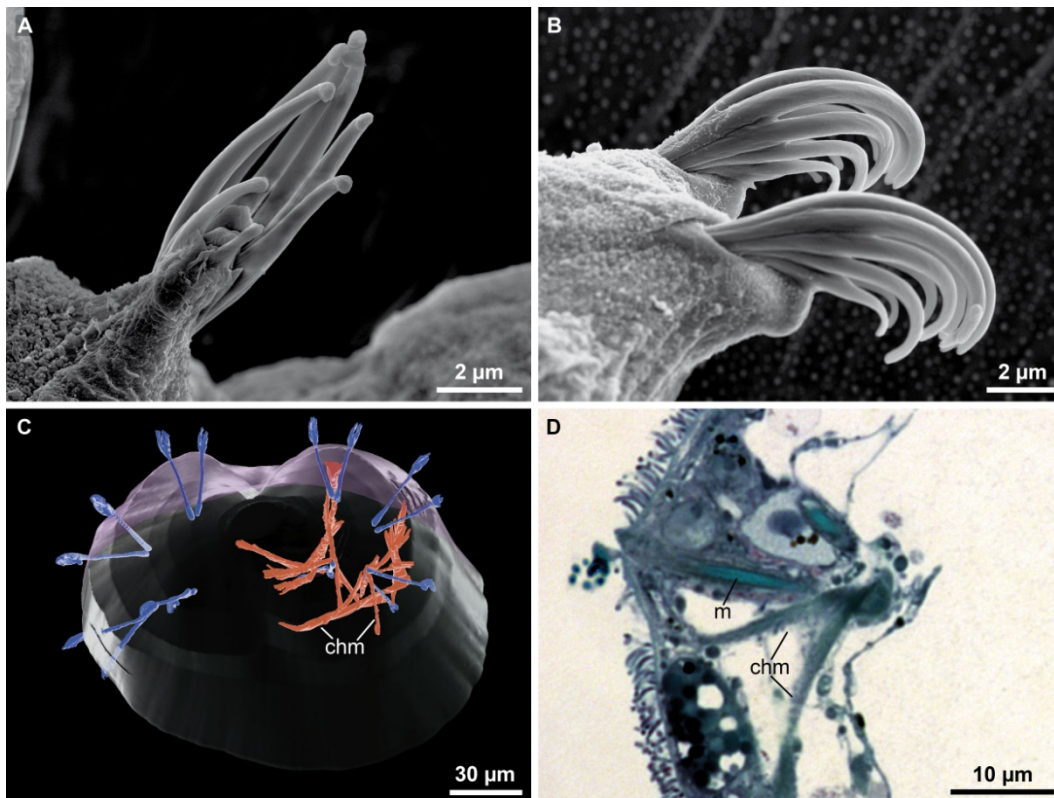n of chaetal arrangement, chaetal musculature (*chm*) is shown for a few chaetae **D** semi-thin section of a chaeta, with a long manubrium (*m*) and the attachement of chaetal musculature (*chm*) to the shaft.

Dwarf males of *Osedax rubiplumus* have a prostomium, a peristomium with a prototroch and two segments. Each of these segments has a ring of eight hooked chaetae with long manubria (±30 µm) (Fig. 7.4C). No developing chaetae were found in the semi-thin sections of the investigated specimen (Fig. 7.4D).

Each chaetal sac is associated with a well developed chaetal musculature. These muscles attach to basis of the chaetal sac and reach the body wall on the other side (Fig. 7.4A).

7.4 Discussion

The chaetal arrangement in the studied chaetopterids confirm the previously mentioned irregularity of the chaetal formative site, since no restriction to an edge of the chaetal row could be found. In fact the chaetae were not aligned in a single row but in a more or less zig-zag shaped pattern.

The chaetation of *Owenia fusiformis,* more specifically the arrangement of hooked chaetae in patches makes a comparison with the rows of chaetae in other taxa hardly possible. However, Meyer and Bartolomaeus (1996) described single rows of chaetae in juvenile stages of *O. fusiformis*, which led to the assumption that the patches of hooked chaetae in adult specimens are modified transverse rows.

Considering that certain magelonids (*Magelona alleni;* see Hausen 2001) also do not have a restricted formative site in their abdominal noto- and neuropodia, a restricted formative site might be a derived condition within annelids. However, the fact that certain magelonids, the juveniles of oweniids do have a restricted formative site still argues against this hypothesis.

Nevertheless, the fact that irregularities and deviations from the more common chaetation pattern occur regularly at these three basally branching taxa is an interesting observation worth mentioning. Further investigations of other species from these taxa could provide

more information that would help reconstruct the plesiomorphic condition in annelids.

Chaetal arrangement and the position of formative sites in the eunicid *Lysidice ninetta* strongly correlates to the chaetal arrangement previously described for *Lumbrineris* species (Tilic et al. 2014). The formation of different chaetal types on both sides of the aciculum and the lack of the notopodium are derived conditions in Eunicida. Further investigations of other aciculate taxa, like Phyllodocida, and Amphinomida, will most-likely provide interesting insights into parapodial evolution in errant taxa.

In the studied siboglinid *Osedax rubiplumus* no chaetal formation was observed. Dwarf males of *Osedax* are highly paedomorphic and inhabit an extreme environment (Worsaae and Rouse 2010). It is nevertheless an important observation, which indicates that not all annelid chaetae need to be replaced during ontogeny. The dwarf males of *Osedax* live in large numbers within the mucous tubes of the females. The majority of the male's resources are invested into spermiogenesis and other processes like chaetogenesis are possibly economized to save costly energy, which is singularly coming from yolk. The arrangement of the chaetal muscles and their sizeable development signify that chaetae are used for a locomotory purpose within the mucous tube of the female worm.

General Discussion

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The series of studies displayed in this thesis explore chaetae, as a character complex to analyze annelid evolution. Tremendous changes in annelid phylogeny that resulted from the introduction of molecular techniques into phylogenetic research, raise special interest to reinvestigate morphological characters and revisit previously established homology hypotheses. The large amount of discrapancies between molecular and morphology based phylogenies compels looking at phenotype evolution research, in order to understand and explain the structural diversity and unity.

Results presented in this thesis demonstrate that comparetive studies on annelid chaetae provide a valuable instrument to test homology hypotheses and help understand recently established sister-group relatioships within annelids, which are often contradicting previous annelid phylogenies based on morphological data. The work presented here can be divided in three categories; comparetive investigations of (1) chaetal formation, (2) chaetal arrangement, more specifically the position of the formative site and (3) distribution and evolution of different chaetal types.

8.1 Chaetogenesis

Formation of annelid chaetae, is a complex and highly active process. A chaeta develops within an ectodermal invagination and the few cells that line this chaetal follicle partake actively in chaetogenesis. Dynamic apical microvilli of the basalmost cell, called chaetoblast, form the template of a chaeta (Bouligand 1967; O'Clair and Cloney 1974). Changes in this microvilli pattern are frozen in time by the release and polymerisation of chitin. Since chaetogenesis appears to be a highly intricate and regulated process, a comperative

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analysis of chaetal formation allows testing homology hypotheses of certain chaetal types. Similar morphogenetic processes support structural homology, wheras significant differences in formation patterns do not provide any support for homology. These differences can be the result of convergent evolution or transformations of existing structures. A conclusion as such, however can only be drawn when the phylogetic position of the taxa is taken into consideration and the condition in the sister-taxa is investigated.

This thesis presents three studies, in which a comparetive analysis of chaetogeneses is used as an approach to test the primary homology hypotheses of certain chaetal types.

The study of lumbrinerid hooded hooks (Chapter 2) revealed significant structural and developmetal differences between the hooded hooks of Lumbrineridae (Eunicida) and the hooded hooks of other taxa such as Capitellidae, Spionidae and Magelonidae. When the position of eunicids in the most recent phylogenetic trees of annelida is considered (Fig. 8.1) this results clearly favours the assumption that hooded hooks in Eunicida are not homologous to those in capitellids, spionids and magellonids and that they evolved at least twice within annelida.

In contrast to Chapter 2, the study on maldanid hooked chaetae (Chapter 3) provides an example where a comparative analysis of chaetogenesis supports the expected homology of a certain chaetal type. The structure and development, matches that of arenicolids. The sister-group relationship of maldanids and arenicolids is well supported by molecular and morphological phylogenies and here, it is more parsimonious to assume inherited information along with partial transformation instead of repeated convergent evolution. Furthermore, the ultrastructure and formation of the neuropodial uncini described for the terebelliform *Nicolea zostericola* in Bartolomaeus (1998) also shows noticeable similarities to maldanid hooks, for example in regard of the subrostral microvilli that form the subrostral process and beard. The uniform ultrastructure and formation again supports the

homology of hooked chaetae in Terebelliformia and Maldanomorpha (Arenicolidae+Maldanidae) (Fig. 8.1).

The study on sabellariid abdominal uncini (Chapter 4) provides a striking example for a case where chaetogenesis and ultrastructure did not support the expected homology of superficially similar chaetae. Cell dynamics and the resulting ultrastructure of chaetal substructures described in this chapter differ drastically from any of the previous studies on hooked chaetae and uncini. A sister group relatioship of sabellids and sabellarids has been previously suggested (Rouse and Fauchald 1997; Schulze 2003; Smith 1991), however more recent molecular phylogenies support a sister goup relatioship with spionids (Struck et al. 2007; Zrzavý et al. 2009; Capa et al. 2011; Capa et al. 2012; Weigert et al. 2014; Andrade et al. 2015; Struck et al. 2015). The differences of sabellid and sabellariid uncini in terms of substructures and chaetogenesis do not provide any evidence for a sister group relationship between both groups. Moreover, the fact that several microvilli and not a single microvillus preforms each adrostral teeth better supports the spionid-sabellariid sister group relationship.

8.2 Chaetal arrangement and the position of the formative site

In addition to chaetogenesis and ultrastructure of individual chaetae, the topology of chaetal arrangement and the position of the chaetal formative site is considered a useful source of information for systematics (Hausen 2005). The general pattern in annelids is; segmental pairs of dorsal (notopodial) and ventral (neuropodial) chaetae. In many sedentary taxa the chaetae form rows where the formative site is restricted to one edge of this row. In most groups the formative site of notopodia lies ventrally and that of neuropodia dorsally, a condition which is considered as plesiomorphic (Hausen 2005).

The study on the ventral chaetae in *Echiurus echiurus* and *Thalassema thalassemum* (Chapter 4), uses the information on the position of the formative site to test their homology to annelid segmental chaetae. Due to their derived morphology and loss of segmentation echiurans were traditionally ranked a phylum. However, their position within Annelida and the sister-group relationship to Capitellidae is meanwhile well established by molecular phylogenies (Bleidorn et al. 2003a, Bleidorn et al. 2003b, Rousset et al. 2007). Capitellid species possess dorsal and ventral pairs of capillary chaetae in the thorax and hooded hooks in the abdomen (Schweigofler and Bartolomaeus 1998). This means that the chaetae of Echiurans must have derived from ancestral rows of chaetae in Annelids and makes them a highly interesting object to study. All echiurans posses a pair of ventral chaetae and certain subtaxa (Urechidae and Echiurinae) posses hemi-circles of anal chaetae. The results of the study showed that each externally visible chaeta of the ventral pair arises from its own chaetal sac and possesses a lateral formation site, evidencing that the pair of ventral chaetae in Echiura is homologous to a pair of neuropodia that fused on the ventral side, while the notopodia were reduced. The chaetae of the anal hemi-circles in *Echiurus echiurus*, however have and individual formative site of their own. This finding argues against a homology of these hemi-circles and annelids' rows of chaetae and lead to the hypothesis that the caudal chaetal rings evolved once within the Echiura by multiplication of ventral chaetae. The position of Urechidae and Echiurinae as sister-taxa within Echiura supports this hypothesis (Lehrke 2012; Goto et al. 2013).

An autapomorphy of Maldanamorpha (Arenicolidae-Maldanidae) is the ventral position of the neuropodial formative site (Bartolomaeus et al. 2005; Bartolomaeus and Meyer 1997). The investigation of hooked chaetae in the maldanids *Clymenura clypeata* and *Johnstonia clymenoides* presented in Chapter 3, corroborates previous findings and clearly shows a ventral formative site of the neuropodial row of hooked

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chaetae. The sister group relationship of maldanids and arrenicolids is well supported and established by morphological as well as molecular phylogenies (Bleidorn et al. 2003; Bartolomaeus et al. 2005; Rousset et al. 2007; Struck et al. 2007). The agreement in chaetal structure and arrangement described in this chapter supports this sister-group relationship.

In Chapter 7 selected annelid taxa are investigated in regard to the position of chaetal formation. Here, the chaetal arrangement in the studied chaetopterids, confirm the previously mentioned irregularity of the chaetal formative site (Hausen 2005) and the chaetation of *Owenia fusiformis,* with the arrangement of hooked chaetae in patches is so aberrant that a comparison with the rows of chaetae in other taxa is hardly possible.

Figure 8.1 summarizes the information on chaetal arrangement and the positon of the formative site all across annelid taxa. This information is mapped on the phylogeny of annelids by Weigert et al. (2014). As mentioned previously the most common chaetal arrangement in Annelids is a vental row of chaetae with a formative site at its dorsal edge and a dorsal row of chaete with a ventral formative site. This seems to hold true. However, there are certain exceptions to this pattern; for example the inversion in Maldanomorpha, which is considered a derived condition and an autapomophy of this taxon. Other irregularities and deviations were observed in chaetopterids, certain magelonids and in adult oweniids. All tree taxa branch basally in the new annelid tree, which possibly indicates that the restriction of chaetal formation at one edge of a chaetal row might not be the plesiomorphic condition in annelids. The fact that juveniles of *Owenia* possess a single formative site with expected topological position could indicate that the restricted formative site in other annelid taxa is a paedomorphic trait. If the investigation of juveniles in chaetopterids and juveniles of maldanids with irregular chaetal arrangements also shows a single, restricted site

of chaetogenesis, paedomorphism becomes a plausible explanation for the chaetal arrangement in the remaining annelid taxa.
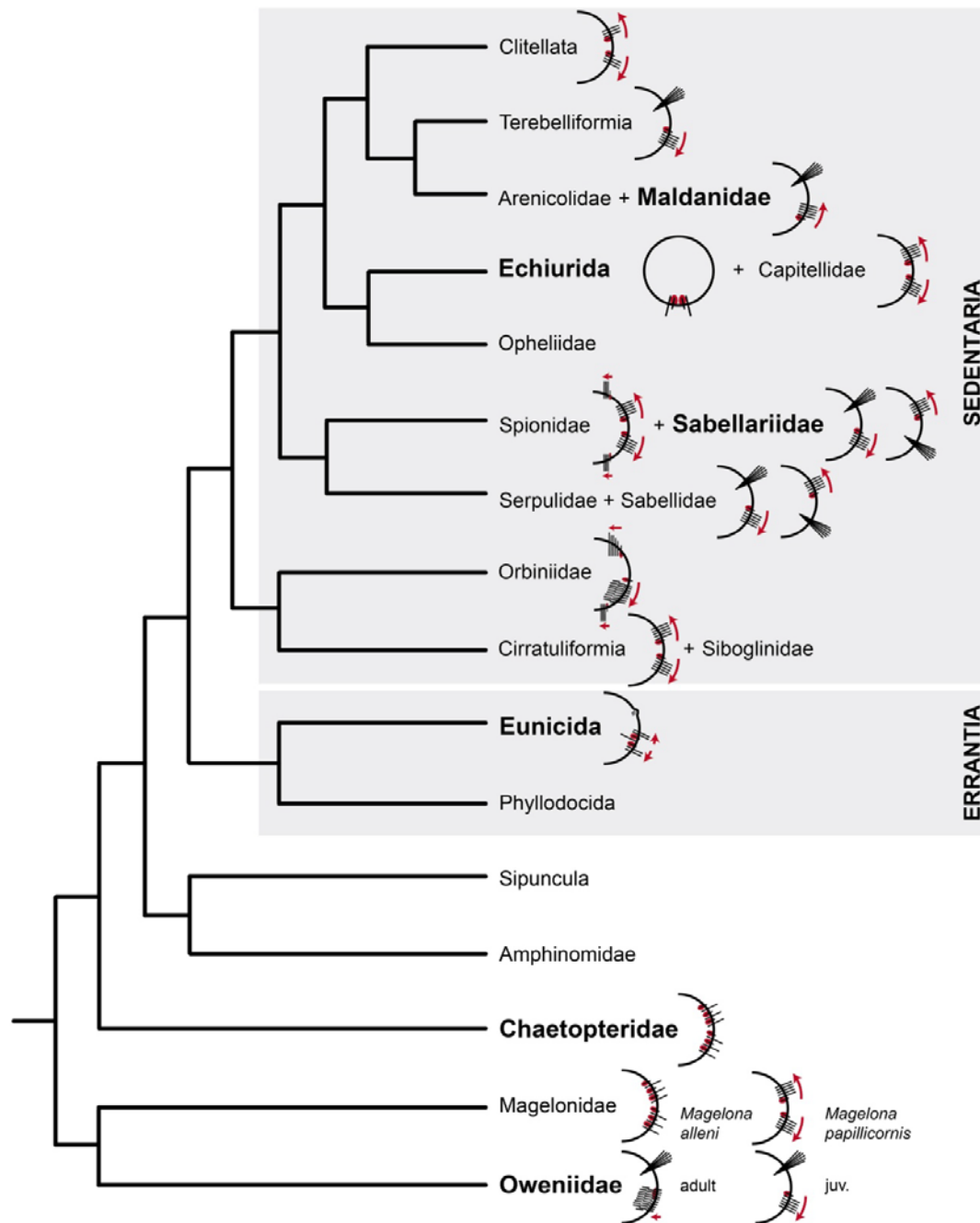
Figure 8.1 Summary of chaetal arrangement and the position o the formative site mapped on the annelid phylogeny by Weigert et al. 2014. Taxa studied in this thesis are in bold. for Terebellida see Bartolomaeus 1995, for Arenicolidae: Bartolomaeus and Meyer 1997, for Maldanidae: Tilic et al. 2015a, for Echiurida: Tilic et al. 2015b, for Capitellidae: Schweigkofler et al. 1998, for Spionidae: Hausen 2001; Hausen and Bartolomaeus 1998, for Sabellaridae: Tilic and Bartolomaeus (subm.), for Sepulidae and Sabellidae: Bartolomaeus 2002; Bartolomaeus 1995, for Orbiniidae: Hoffmann and Hausen 2007, for Cirratuliformia: Hausen 2005, for Eunicida: Tilic et al. 2014, for Magelonidae: Hausen 2001 and for Oweniidae: Meyer and Bartolomaeus 1996.

Chatal arrangement also differs significantly in errant polychaetes, due to the reduction of notopodia (in Eunicida) and the lack of well defined chaetal rows (in Phyllodocida). Investigating the chaetal arrangement and the structure of the chaetal sac in Phyllodocida and Amphinomida could provide further details that will help understand the annelid ground-pattern.

8.3 Evolution of chaetal types

Struck (2011) describes the "ancestral annelid" from ancestral state reconstructions as a vermiform animal bearing "grooved palps, bicellular eyes, nuchal organs and biramous parapodia with lobes of different shape and size bearing both simple and internalized chaetae". The earlist annelid fossils actually also posses biramous parapodia and only simple capillary chaetae (Morris and Peel 2008; Vinther et al. 2011).

Extant annelid taxa have a plethora of different chaetal types showing great structural variety and taxon specifity. A certain chaetal type, once evolved must be passed on to descendants. Hereby, strong functional constrains (Woodin and Merz 1987; Merz and Edwards 1998) are essential for fixing a certain type of chaeta within a lineage of a species and a supraspecific taxon. Therefore, one would expect that chaetal diversity increases within a monophyletic group and that additional chaetae types largely result from transformation of existing chaetae. The study presented in Chapter 6, takes up sytematic aproach to test these hypotheses for Eunicida, a highly divers taxon that is rich in species and chaetal types. The results of this study supports the idea of radiation and diversification within a monophyletic taxon and further provides explanations for the loss of chaetal diversity, such as progenesis and symbiotic lifestyles. During the ontogeny of eunicids, larval stages of many taxa initially only possess capillary chaetae and aciculae, addditional chaetal types are added as the larvae develop. In progenetic eunicids like *Ophryotrocha* only these chaetal types are

present whereas chaetae that are present in the adults of closely related taxa are missing. In Histrobdellidae, the entire chaetation is lost which is most probably as an adaptation to their symbiotic lifestyle.

Furthermore, the results of this study supports the idea that jointed chaetae evolved multiple times within annelids. Jointed chaetae are compound chaetae, where the tip of a chaeta articulates with the shaft. The convergent ecolution of this specific chaetal structure is also in accordance, with Merz and Edwards (1998) observations on the functional gains (increased locomotory performance) of bearing such chaetae.

8.3.1 Evolution and homology of hooked chaetae

Many annelid taxa posses hooked chaetae (Fig. 8.2) and in several of them the formation pattern of the hooks is identical, which led to a homology hypothesis of these hooked chaetae and to a hypothesis of common ancestry of all annelids that bear such chaetae (Bartolomaeus 1998; Hausen 2005 and literature herein). In fact up untill the results presented in this thesis no structural and developmental differences had been recorded for hooked chaetae, which has been the main argument for their homology.

There are two cases presented in this thesis, where the homology of superficially similar hooked chaetae is not supported by identical chaetogeneses. (1) The hooded hooks of lumbrineridan eunicids (chapter 2) that are similar to those of capitellid and spionid hooded hooks but seem to have evolved independantly. (2) The uncini of sabellarids that are not homologous to any uncini described so far. These results demonstrate once again that chaetogenesis is a valuable instrument to test homology hypotheses and show that hook formation is not allways as uniform as previously assumed.

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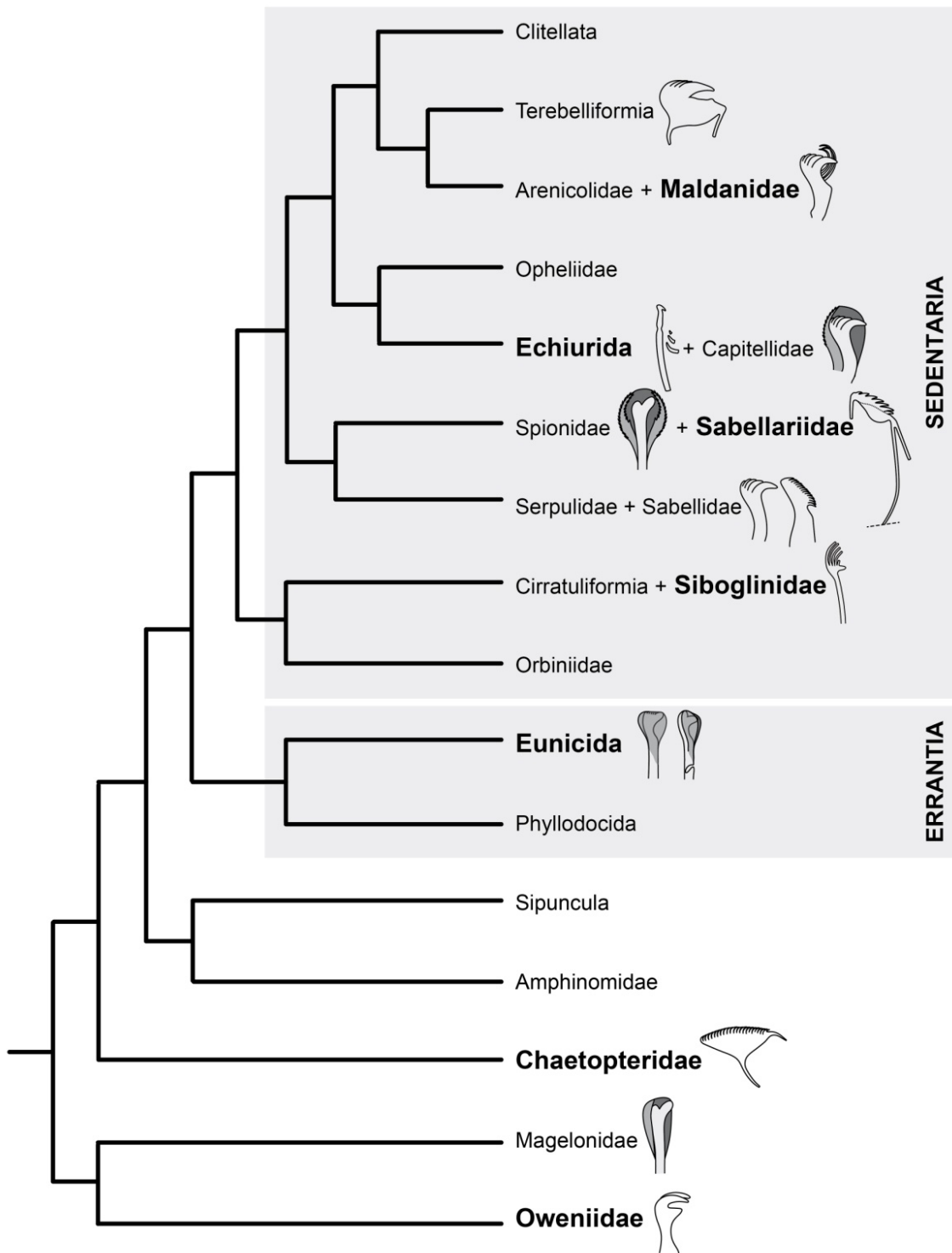
Figure 8.2 Summary of hooked chaetae types mapped on the annelid phylogeny by Weigert et al. 2014. Taxa studied in this thesis are in bold. for Terebellida see Bartolomaeus 1995, for Arenicolidae: Bartolomaeus and Meyer 1997, for Maldanidae: Tilic et al. 2015a, for Echiurida: Tilic et al. 2015b, for Capitellidae: Schweigkofler et al. 1998, for Spionidae: Hausen 2001; Hausen and Bartolomaeus 1998, for Sabellaridae: Tilic and Bartolomaeus (subm.), for Sepulidae and Sabellidae: Bartolomaeus 2002; Bartolomaeus 1995, for Siboglinidae Schulze 2001 , for Eunicida: Tilic et al. 2014, for Magelonidae: Hausen 2001 and for Oweniidae: Meyer and Bartolomaeus 1996

The view that all hooked chaetae are homologous must be changed in the light of recent molecular analyses. According to these Oweniidae and Magelonidae are the most basally branching annelid taxa (Weigert et al. 2014). Oweniid species possess patches of hooked chaeta that each lacks a rostrum, but otherwise develop identically to hooked chaeta (Meyer and Bartolomaeus 1996). In *Owenia fusiformis* both apical teeth of the hook are oriented side by side, however within Oweniids some taxa show a longitudinal disposition of the apical teeth and some even show gradual modifications to a side by side arrangement (Sene-Silva 2002). This would support the assumption that these teeth are homologous to the capital teeth described from other taxa. However, the lack of a rostrum might as well be indicative for convergent evolution of the oweniid hooked chaetae and those having a rostrum. Chaetopterids, another basally branching taxon, also do not have a rostrum. On the other hand, the rostrum is also reduced within Pectinariidae (Bartolomaeus 1995b), a subgroup of Terebellida. Terebellids primarily possess hooked chaetae with a rostrum (Bartolomaeus 1998; de Matos Nogueira et al. 2013).

The sister group relationship between Echiura and Capitellidae is well established (Bleidorn et al. 2003; Rousset et al. 2007; Struck et al. 2007), and a common ancestry of both and that of Terebellida, Arenicolidae, Clitellata and Opheliida is supported by phylogenomic data (Struck et al. 2011; Weigert et al. 2014). Provided hooked chaetae and hooded hooks of sedentaria are in fact homologous, they are replaced by a pair of large ventral chaeta in Echiura (chapter 5), a very few dorsal and ventral spine-like chaetae in Clitellata, simple capillary chaetae in Opheliidae, hooded hooks in Spionidae and unique uncini in Sabellaridae (Chapter 4). When the molecular phylogenies are considered, the distribution of hooked chaetae across these taxa shows that, however well-supported the hypothesis on their homology may be, in certain lineages chaetae can be completely altered, not leaving any trace of the ancestral design.

The study of chaetal structure and development and the interpretation of these data in the light of well-supported and robust phylogenies helps substantiate homologies where the hypothesis matches the molecular phylogeny or helps explain differences either as a result of partial transformation of inherited information or as repeated convergent evolution due to identical functional constraints.

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Appendix

A.1. Supplementary Materials

Table A1 Complete list of sequences used for the phylogenetic analysis with the corresponding GenBank sequence accession numbers.

Higher Taxon	Species	COI	18S	16S
Phyllodocida	*Glycera dibranchiata*	-	AY995208	-
Dorvilleidae	*Dorvillea erucaeformis*	AY838868	AY838846	GQ478122
Dorvilleidae	*Ophryotrocha labronica*	KF305814	AY838855	AF321429
Dorvilleidae	*Ophryotrocha sp.*	-	KT862790	-
Dorvilleidae	*Parapodrilus psammophilus*	-	AF412800	-
Dorvilleidae	*Protodorvillea kefersteini*	KF808171	AF412799	DQ779634
Dorvilleidae	*Schistomeringos rudolphi*	AY598741	AF412804	
Eunicidae	*Eunice americana*	GQ497561	GQ497479	GQ478133
Eunicidae	*Eunice amoureuxi*	GQ497538	GQ497490	GQ478144
Eunicidae	*Eunice antarctica*	GQ497532	GQ497483	GQ478137
Eunicidae	*Eunice cariboea*	GQ497536	GQ497487	GQ478141
Eunicidae	*Eunice filamentosa*	GQ497559	GQ497500	GQ478155
Eunicidae	*Eunice fucata*	-	GQ497489	GQ478143
Eunicidae	*Eunice harassii*	GQ497535	AY525620	GQ478140
Eunicidae	*Eunice impexa*	GQ497546	GQ497501	GQ478156
Eunicidae	*Eunice lucei*	GQ497529	GQ497482	GQ478136
Eunicidae	*Eunice mutilata*	GQ497540	GQ497492	GQ478146
Eunicidae	*Eunice norvegica*	GQ497541	GQ497493	GQ478147
Eunicidae	*Eunice notata*	GQ497544	GQ497497	GQ478152
Eunicidae	*Eunice roussaei*	GQ497543	GQ497495	GQ478149
Eunicidae	*Eunice rubra*	GQ497528	GQ497478	GQ478132
Eunicidae	*Eunice tenuis*	-	AY838850	-
Eunicidae	*Eunice thomasiana*	GQ497563	GQ497499	GQ478154
Eunicidae	*Eunice torquata*	GQ497539	AF123304	GQ478145
Eunicidae	*Eunice valens*	GQ497534	GQ497485	GQ478139
Eunicidae	*Eunice vittata*	-	AF412790	-
Eunicidae	*Lysidice collaris*	GQ497557	GQ497516	GQ478170
Eunicidae	*Lysidice ninetta*	GQ497564	AF412793	GQ478169
Eunicidae	*Marphysa bellii*	-	AF412789	DQ779623
Eunicidae	*Marphysa disjuncta*	GQ497549	GQ497504	GQ478159
Eunicidae	*Marphysa fallax*	-	GQ497505	GQ478160
Eunicidae	*Marphysa sanguinea*	GQ497547	AY525621	GQ478157
Eunicidae	*Nematonereis unicornis*	-	AF412792	GQ478173
Eunicidae	*Palola cf siciliensis*	-	AY176295	GQ478168

Eunicidae	*Palola viridis*	-	GQ497514	GQ478167
Lumbrineridae	*Lumbrineris inflata*	AY366520	GQ497513	AY838832
Lumbrineridae	*Lumbrineris latreilli*	KF815721	AY525622	AY838833
Lumbrineridae	*Lumbrineris zonata*	-	AY525623	HM746713
Lumbrineridae	*Ninoe nigripes*	AY838871	HM746727	AY838837
Oenonidae	*Arabella iricolor*	GU362693	AY995208	HM746709
Oenonidae	*Arabella semimaculata*	AY838866	AY525624	GQ478123
Oenonidae	*Drilonereis longa*	AY838869	AY838844	AY838828
Onuphidae	*Diopatra aciculata*	AY838867	AY838847	AY838826
Onuphidae	*Diopatra chilienis*	-	AY838845	-
Onuphidae	*Diopatra dentata*	GQ497522	AY884162	GQ478129
Onuphidae	*Hyalinoecia tubicola*	JX219843	GQ497475	AY838830
Onuphidae	*Nothria conchylega*	HM473519	AF412794	AF321417
Onuphidae	*Onuphis elegans*	GQ497525	AY838854	GQ478128
Onuphidae	*Onuphis iridescens*	-	HM746729	HM746715
Onuphidae	*Onuphis similis*	-	AY525625	-