The *Sclerolinum contortum* symbiosis from hydrocarbon seeps in the Gulf of Mexico

Verfasserin

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angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer. nat.)

Wien im Juli 2012

Studienkennzahl lt. Studienblatt: A 091 439
Dissertationsgebiet lt. Studienblatt: Zoologie
Betreuerin / Betreuer: Univ. Prof. Dr. Monika Bright
Twenty years from now you will be more disappointed by things you didn’t do than by the ones you did do.

So throw off the bowlines.

Sail away from the safe harbour.

Catch the trade winds in your sails.


MARK TWAIN
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Introduction

The slender two tentacled tubeworms of the small, little known genus *Sclerolinum* belong together with vestimentiferans, *Osedax* and frenulates to the monophyletic polychaete family Siboglinidae Caullery, 1914 (McHugh, 1997; Rouse and Fauchald, 1997; Rouse et al., 2004). These unique polychaetes evolved through symbiosis, “the living together of unlike organisms” (De Bary, 1879). Adult siboglinids rely completely on their endosymbiotic bacteria for nutrition, as they reduce their functioning digestive system during their ontogeny and develop a new, highly vascularized organ, termed the trophosome, to accommodate and nourish the symbionts (Cavanaugh et al., 1981; Southward, 1982; Katz et al., 2011).

Although the symbiont-housing organ is the most conspicuous unifying character of Siboglinidae, the trophosomes of the various siboglinid taxa differ in developmental origin, microanatomy and cell cycle dynamics. The visceral mesodermal trophosome of vestimentiferans (Bright and Sorgo, 2003; Nussbaumer et al., 2006) is organized in countless interconnecting lobules and provided with an complex blood vascular system (van der Land and Norrevang, 1977; Gardiner and Jones, 1993; Malakhov et al., 1996; Bright and Sorgo, 2003). A cell cycle is directed from the center to the periphery within each lobule (Pflugfelder et al., 2009). In *Osedax* females the trophosome derives from the somatic mesoderm and a cell cycle is performed from posterior to anterior within the organ (Katz et al., 2011). Less is known about the symbiont-containing organs of frenulates and *Sclerolinum*, both described as two-layered organs, whereby the inner layer is composed of bacteriocytes and the outer one is the coelomic lining (Southward, 1982). The frenulate trophosome develops from the endoderm of the gut (Callsen-Cencic and Flügel, 1995), whereas the origin of the *Sclerolinum* trophosome is still unknown.

Each host generation gets infected by the free living presumptive symbiont *de novo*. At least three distinct clades of *Gammaproteobacteria* thrive in symbiosis with Siboglinidae suggesting a specific host-symbiont association at higher taxonomic level in Siboglinidae (Thornhill et al., 2008). Vestimentiferans, *Sclerolinum* and frenulates harbor chemolithoautotrophic endosymbionts.
that oxidize reduced compounds, in most cases sulfide, to gain energy used for carbon fixation through the Calvin-Benson cycle to sustain their own growth (Felbeck, 1981; Felbeck et al., 1981; Southward et al., 1981; Lösekann et al., 2008) and to provide energy to the host by release of organic carbon (Felbeck and Jarchow, 1998; Bright et al., 2000) and digestion (Southward, 1982; Bosch and Grassè, 1984b, a). Osedax’s symbionts are heterotrophic Oceanospirillales (Goffredi et al., 2005).

Siboglinids live in a variety of reducing marine habitats with access to oxygenated seawater like hydrothermal vents, hydrocarbon seeps, whale and wood falls. In general, Sclerolinum species either dwell on decaying organic materials like sunken wood or in sulfidic sediments (Smirnov 2000). However, Sclerolinum contortum Smirnov, 2000 is reported from the arctic, cold seep Haakon Mosby Mud Volcano, cold seeps of the Storegga Slide in the Norwegian Sea (Lazar et al. 2010), and from an hydrothermal vent field of the Arctic Mid-Ocean Ridge (Pedersen et al. 2010). Such reducing marine environments are rich in hydrogen sulfide, an important energy source for chemoautotrophic bacteria but highly toxic to aerobic organisms (National Research Council, 1979). Siboglinid hosts accommodating sulfide-oxidizing bacteria even depend on this toxic substance to provide their symbionts, and at the same time they have to protect the symbiosis from sulfide poisoning. It is obvious that sulfide detoxification mechanisms are crucial to aerobic organisms colonizing sulfidic habitats. Thiotrophic bacteria converting toxic sulfide into non-toxic sulfur compounds could serve as a means of sulfide detoxification and may have been an initial evolutionary force for the establishment of siboglinid symbiosis enabling the host to inhabit such extreme environments.

The small genus Sclerolinum with seven described species (Southward, 1961; Webb, 1964; Southward, 1972; Ivanov and Selivanova, 1992; Smirnov, 2000) challenged the scientists in terms of its phylogenetic position due to its morphological peculiarities since its discovery. Their body is divided into a miniscule cephalic lobe, a forepart equipped with two tentacles, a long trunk region, and a segmented opisthosoma (Southward et al., 2005). Their body organization neither fit to the first known frenulate species, united in the phylum Pogonophora (Johansson, 1937, 1939), nor to
the later discovered vestimentiferans (Webb, 1969). *Sclerolinum*, first placed within frenulates (Southward, 1961), constitutes now the monogeneric taxon Monilifera with equal rank to frenulates and vestimentiferans (Ivanov, 1991). Recent molecular, morphological, and combined studies supported *Sclerolinum* as the sister group to vestimentiferans (Halanych et al., 2001; Rouse, 2001; Rousset et al., 2004; Zrzavý et al., 2009) and *Osedax* as sister group to *Sclerolinum* plus vestimentiferans and frenulates as the most basal group (Rouse et al., 2004; Hilário et al., 2011). However, the internal anatomy of *Sclerolinum* is still unknown and several characters like the existence of a metameric trunk region or an internal diaphragm are still in discussion. Due to this lack of knowledge homologizations of body regions between *Sclerolinum* and other siboglinid taxa are questionable.

The main intention of this thesis was to gain knowledge of the *Sclerolinum* symbiosis. *Sclerolinum* is a small and neglected genus, but nevertheless it is essential in elucidating several aspects of siboglinid evolution. Emphasis was put on the identification of the host and the symbiont species; the organization of the *Sclerolinum* trophosome to elucidate the evolutionary origin of the siboglinid trophosomes; on the characterization of the symbiotic bacteria with focus on the ultrastructure, metabolism, and a possible sulfide detoxifying function with respect to the establishment of the *Sclerolinum* symbiosis; and the *Sclerolinum* body organization to determine if it resembles the vestimentiferan organization as inferred by recent molecular and morphological studies. The investigated *Sclerolinum* specimens were recovered from hydrocarbon seeps in the Gulf of Mexico.

The establishment of an obligate endosymbiosis and the subsequent reduction of the digestive system must have played revolutionary roles during siboglinid evolution. Consequently, the question of the origin and the evolution of the trophosome are key questions in understanding the phylogeny of Siboglinidae. Using electron and light microscopy we investigated the organization of the *Sclerolinum contortum* symbiont-housing organ. The main topics of the first manuscript are the origin and microanatomy of the *Sclerolinum* trophosome in order to determine whether it resembles the complex vestimentiferan trophosome derived from the visceral mesoderm as suggested by the relationship between *Sclerolinum* and Vestimentifera. Further, it deals with the question how
homeostasis is balanced in the *S. contortum* symbiont housing organ and the evolution of the siboglinid trophosome.

The posterior trophosome of *Sclerolinum contortum* is full of giant crystals located between the symbionts. The second manuscript focuses on the identification and formation process of these crystals by applying high-pressure freezing and freeze substitution for light microscopy, transmission electron microscopy, scanning electron microscopy, Raman microspectroscopy, and energy dispersive x-ray analysis. It raises the question if these symbionts not only serve as food source but also protect their hosts from harmful effects of hydrogen sulfide by oxidizing it. Information on the symbiont phylogeny and symbiont metabolism is given as well.

The main aim of the third manuscript was to investigate the anatomy of *Sclerolinum contortum* by applying light and electron microscopy in order to provide comparative data of one representative of this important genus for subsequent comparisons between the body organizations of different siboglinid taxa. This resulted in detailed descriptions of the microanatomy of most of the organ systems. Additionally, precise information on the external morphology of *S. contortum* is given and the host phylogeny is elucidated.

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Organization and microanatomy of the *Sclerolinum contortum* trophosome (Polychaeta, Siboglinidae)

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**Publication status:** published 2011 in *Biological Bulletin*, 220(2), 140-153

**Personal contributions of Irmgard Eichinger:**

- collected the material during the cruise to the Gulf of Mexico in 2007
- fixed and embedded the samples for TEM and LM
- produced the ultrathin sections
- performed LM and TEM
- performed morphometric and statistical analysis
- wrote the manuscript
Organization and Microanatomy of the Sclerolinum contortum Trophosome (Polychaeta, Siboglinidae)

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Abstract. The trophosome—an organ especially evolved to accommodate symbiotic bacteria—is a key character of the polychaete family Siboglinidae. Astonishingly, the trophosomes vary in organization and origin between the different siboglinid taxa. The trophosome of the small genus Sclerolinum was nearly unknown until now. Here we investigated the trophosome of S. contortum from the Gulf of Mexico, using light and electron microscopy. We show that this organ derives from the visceral mesoderm and propose that the trophosome of the sister clade Vestimentifera and Sclerolinum is a homologous character. Like that of juvenile vestimentifersans, the trophosome of Sclerolinum trophosome is simply organized. This study reveals that the Sclerolinum trophosome exhibits two regions that differ in the organization of host tissue and the size and shape of the symbionts. We suggest that a specific cell cycle within the symbiont-housing organ is directed along the longitudinal body axis, with a region of proliferation anteriorly and a region of degradation posteriorly. Using Raman microspectroscopy we demonstrate that the endosymbionts of S. contortum from the Gulf of Mexico contain sulfur vesicles, and we argue for a chemosynthetic sulfur-oxidizing metabolism.

Introduction

Symbioses between multicellular organisms and microbes are ubiquitous. Many of these associations are beneficial for the partners, and some of them are so intimate that, through living together, the hosts' organization and morphology have been altered considerably to accommodate a living space for their symbionts (Douglas, 2010). Most obviously, in many intracellular endosymbioses specific symbiont-housing organs have developed, such as the light organ in bobtail squids, the root nodules in legumes, the bacteriomes in insects, and the trophosome in siboglinid tubeworms. Almost every cell type and organ in animals can become infected, and symbioses are known to have evolved in various ecto-, endo-, and mesodermal tissues; for example, the gill epidermis in various bivalves, the endodermal gut epithelium of the tsetse fly, the mesodermal fat body in cockroaches, or the ovaries in fruit flies (Bright and Bulgheresi, 2010). Although our knowledge of the diversity of symbioses has continuously increased, our understanding of why Putative symbionts infect certain specific tissues but are restricted from others is still in its infancy.

In the small polychaete family Siboglinidae Caullerery, 1914 (McHag, 1997; Rouse and Fauchald, 1997; Rouse et al., 2004), consisting of Vestimentifera, Sclerolinum, Oseus, and Fremlula, all representatives—with the exception of Oseus dwarf males (Rouse et al., 2004)—apparently have a symbiont-housing organ, called a trophosome, originating from different germ lines.

Concomitantly, the entire digestive tract, including mouth and anus, degenerates during development. In vestimentifersans, Sclerolinum, and fremlulae, endosymbionts are chemolithoautotrophic Gamma proteobacteria that oxidize reduced compounds, in most cases sulfide, to gain energy for carbon fixation to sustain their own growth (Felbeck, 1981; Felbeck et al., 1981; Southward et al., 1981; lösekan et al., 2008). These symbionts provide energy to the host by translocation of organic carbon through release (Felbeck and Jarchow, 1998; Bright et al., 2000) and diges-
tion (Southward, 1982; Bosch and Grassé, 1984a, b). Oesaxax’s symbionts are heterotrophic Gammmaproteobacteria (Goffredi et al., 2005). Nutritional interactions have not yet been studied in detail, and ideas on who is feeding whom are controversial (Goffredi et al., 2005; Goffredi et al., 2007).

Initially the trophosome had been described as a mesodermal tissue in frenulates (Ivanov, 1963; Southward et al., 1981) and vestimentiferans (van der Land and Norrevang, 1975, 1977). The occurrence of bacteria in the trophosomal tissue was discovered only in 1981 (Cavanaugh et al.). Southward (1982) investigated the ultrastructure of the trophosomes of several frenulate species and of Sclerolinum brattstromi. In frenulates the trophosome is restricted to the postanal region of the trunk. Although a structural variation of the frenulate trophosome seems to exist, in general this organ consists of two epithelia and blood spaces sandwiched between the basal matrix of the epithelia, whereby the inner one is composed of bacterioocytes and the outer one is the coelomic lining. Because the embryonic origin was not studied in detail, Southward did not solve the question of whether the central lumen of the bacterial cylinder seen in some species or restricted to small spaces filled with cilia in one species could be interpreted as a coelom or as the lumen of the gut. The trophosome of S. brattstromi was described as a solid core of bacterioocytes surrounded by blood space and epithelium.

Developmental studies on vestimentiferan and frenulate larvae have identified a provisory gut in the larvae. The occurrence of bacteria in the gut epithelium favored the idea of an endodermal origin of the trophosome in vestimentiferans (Jones and Gardiner, 1988; Southward, 1988) and in frenulates (Calfesen-Cencic and Flügel, 1995). In contrast, a combined study with ultrastructural methods and fluorescence in situ hybridization using symbiont-specific probes revealed the visceral mesodermal nature of the vestimentiferan trophosome (Nussbaumer et al., 2006).

The symbiont-containing organ of vestimentiferans is a complex multilobed structure with a sophisticated blood vascular system occupying the whole trunk region (van der Land and Norrevang, 1977; Gardiner and Jones, 1993; Malakhov et al., 1996; Bright and Sorgo, 2003). Each lobe consists of an apolar tissue of bacterioocytes encased by an aposymbiotic coelothel, is traversed by an axial efferent blood vessel, and is supplied with ramifying peripheral afferent blood vessels. The peripheral vessels originating from the central blood vessel extend between the bacterioocytes as blood lacunae. The axial blood vessels branch off the dorsal blood vessel. In accordance with the direction of blood flow from the anterior to the posterior in the ventral vessel and then from the posterior to the anterior in the dorsal vessel as in polychaetes in general (Fransen, 1988), blood flows from the periphery of the trophosome, passing between the bacterioocytes into the axial vessels.

In Oesaxax the trophosome—developed only in females (Rouse et al., 2004)—is formed by bacterioocytes and non-symbiotic cells lying between the body wall muscle layer and a peritoneum in the ovicase and ovisac region; thus it is derived from the somatic mesoderm (Katz et al., 2011).

In contrast to well-studied vestimentiferans and spectacular “whale-bone-eating” Oesaxax, the small genus Sclerolinum, with only seven described species (Southward, 1961, 1972; Webb, 1964b; Ivanov and Selivanova, 1992; Smirnov, 2000), is little known. These species are slender two-tentacled tube worms with a body divided into a forepart, a long trunk, and a segmented opisthosome. Like all other sabellids, Sclerolinum species live in reducing marine habitats with access to oxygenated seawater. Some thrive on decaying organic material such as rotten wood or ropes; others, like Sclerolinum contaminum, which was described from an arctic mud volcano (Smirnov, 2000), dwell buried in siltic mud. S. contaminum has recently even been reported to inhabit a hydrothermal vent field at the Arctic Mid-Ocean Ridge (Pedersen et al., 2010).

Although known for half a century, these tube worms did not attract the attention of many scientists; thus it is not surprising that little information exists. Nevertheless, since its discovery Sclerolinum has been problematic in terms of its phylogenetic position, and its relationship within sabellids has been the subject of much discussion. First Sclerolinum was placed within the frenulate family Polynothidae (Southward, 1961); later a new family Sclerolinidae (Webb, 1964a), and even a new subclass Monilifera, was created that included Sclerolinidae with equal rank to Frenulata and Vestimentifera (Ivanov, 1991). Recently, molecular (Halanych et al., 2001), morphological (Rouse, 2001), and combined rRNA (Roussel et al., 2004) studies congruently revealed Sclerolinum as the sister group to Vestimentifera (but see also Schulze, 2003). In a molecular study, Rouse et al. (2004) corroborated the relationship between Sclerolinum and Vestimentifera, and placed the newly discovered Oesaxax as sister group to Sclerolinum plus Vestimentifera.

It is obvious that the establishment of an endosymbiosis must have played a revolutionary role during sabellid evolution. Consequently, the question of the origin and the evolution of the trophosome are key questions in understanding the phylogeny of Sabellidae. We reinvestigated the trophosome of a Sclerolinum population from cold seeps of the Gulf of Mexico. The host could be identified as Sclerolinum contaminum (Smirnov, 2000) on the basis of the 16S rRNA gene, and only one bacterial 16S rRNA phylogeny was detected within the trophosome (Eichinger, pers. obs.). Using light and electron microscopy, we focused on the origin and organization of the trophosome in order to determine whether it resembles the complex vestimentiferan trophosome derived from the visceral mesoderm (as suggested by the relationship between Sclerolinum and Vesti-
mentiferan), the somatic mesodermal Osedax trophosome, or the endodermal organizes fenulate trophosome. We provide the first evidence that the Sclerolitina trophosome evolved from the visceral mesoderm, and thus we propose that the trophosomes of Vestimentifera and Sclerolitina are homologous structures having evolved in their last common ancestor.

Materials and Methods

Specimens of Sclerolitina contortum were collected from two sites (AC6818, AC601) of the hydrocarbon seeps in the Gulf of Mexico in depths of 2700 m and 2200 m during the “Expedition to the Deep Slope” (chief scientist C. R. Fisher) with the NOAA research vessel Ronald H. Brown in June 2007. The samples were recovered with the ROV Jason during dives J2-282 and J2-283. After recovery, small samples of the trophosome were taken from several individuals and fixed for transmission electron microscopy (TEM). Whole worms about 6 cm in length (one female and one male) were fixed for light microscopy (LM).

Samples for TEM were fixed overnight in a mixture of 1.5% acrolein, 3% glutaraldehyde, 1.5% paraformaldehyde in 0.1 mol l⁻¹ cacodylate buffer with 10% (w/v) sucrose, then rinsed with the same buffer several times, postfixed in 2% OsO₄ in 0.1 mol l⁻¹ cacodylate buffer with 10% (w/v) sucrose for 2 h, rinsed again with the buffer, and dehydrated in a graded ethanol series up to 70%. All these steps were carried out on ice. Dehydration in an ethanol series was continued up to 100%. Infiltration was carried out with a 3:1 followed by a 1:1 100% ethanol and Agar Low Viscosity Resin mixture for 1 h each, followed by 1:3 100% ethanol and Agar Low Viscosity Resin mixture for 1.5 h. After infiltration with pure resin overnight, samples were transferred to fresh resin and hardened at 65 °C for 18 h. Ultrathin sections were cut on a Reichert Ultracut S microtome and placed on Formvar-coated slot-grids, stained with uranyl acetate and lead citrate by hand, and analyzed with a Zeiss EM 902 transmission electron microscope.

For LM, whole specimens were fixed in 4% formaldehyde buffered with 0.1 mol l⁻¹ phosphate-buffered saline (PBS), cut into small pieces, dehydrated in a graded ethanol series, and embedded in Agar Low Viscosity Resin. A series of 1-μm semithin sections was cut on a Reichert Ultracut S microtome, stained with toluidine blue, and examined with a Zeiss Axioscop A1 light microscope.

For Raman microspectroscopy, a suspension of symbiotic bacteria, obtained by squeezing the posterior trophosome in 1× PBS buffer, was dried onto a CaF₄ slide and placed under a confocal LabRAM HR800 Raman microspectrometer (Horiba, Germany) equipped with a 50-mW 532.17-nm laser. Before the samples were analyzed, the system was aligned using a silica reference with a distinct Raman peak at 520 cm⁻¹. Cells for Raman analysis were chosen by morphology in the live-view mode of the LabSpec software, ver. 5.25.15 (Horiba), and were exposed to the laser beam for durations of up to 5 s. To prevent photo-damage of the cells, the laser intensity was dimmed to 10% by an intensity filter. The confocal pinhole was generally set to 250 μm, leading to an axial resolution of about 2.5 μm. However, for some experiments a pinhole of 150 μm was used (axial resolution of about 1.5 μm). Raman spectra were usually acquired between 100 and 600 cm⁻¹ to cover the area with the unique sulfur peaks. Raman spectra were baseline corrected, normalized, and exported to a file format readable by Excel (Microsoft). Additionally, Raman mapping was performed with selected samples, using the setting described above and applying the respective function in the LabSpec software.

For morphometric analyses, micrographs of ultrathin sections of bacteriocytes of the anterior and posterior region of the trophosome from three worms were used. The micrographs had a magnification of 11000×. A square of 37 μm² was placed randomly within the bacteriocytes 10 times for each region and each worm, resulting in 30 squares for each region of the trophosome. Using Image J software, ver. 1.40g, the area of the bacteria expressed as a percentage of the total bacteriocyte area, and the area of the sulfur-vesicles expressed as percentages of the total bacterial and total bacteriocyte areas were calculated. The peribacterial space, defined as the space between the bacterial cell wall and the membrane of the symbiosome, was negligible. The diameters in sections of 475 bacteria of both the anterior and posterior trophosome were measured by using the AnalySIS program, ver. 3.2. SPSS, ver. 16.0, was used to test for differences between the two regions of the trophosome using the Student’s t-test and the Mann-Whitney U-test.

Terminology Used

Bacteriocyte. Host cell containing symbionts (Southward, 1982).

Blood lucana. In this study, used for a blood-filled space between cells of an apolar tissue; intercellular sinus sensu van der Land and Nurwangan, 1977; lucana syngiene sensu Bosch and Grassé, 1984a; blood space sensu de Bergh, 1986; blood capillary sensu Bright and Sorgo, 2003; Maklakov et al., 1996).

Blood vessel. Blood-filled space in extracellular matrix of epithelia; it is lined only by extracellular matrix (Ruppert and Carle, 1983).

Epithelium. Polar tissue: a single or multiple layer of cells with an apical belt-shaped junctional complex, mostly parallel cell polarity, and the extracellular matrix deposited on
apical (cuticles) or basal (basal matrix) surfaces (Rieger, 1986; Rieger and Lombardi, 1987).

Mesenchyme. Apolar tissue: cells with polarities of any orientation, without apical belt-shaped junctional complexes, being submerged in the extracellular matrix (Rieger, 1986).

Myoepithelium. Any epithelium containing myocytes (Rieger and Lombardi, 1987).


Somatic mesoderm. Outer walls of a series of cavities developed from two mesodermal bands during polychaete larval development; it gives rise to the somatic musculature and the somatic coelomic lining that consists of muscle cells or peritoneocytes (Anderson, 1966; Fransen, 1988; Westheide and Rieger, 2007).

Visceral (splanchnic) mesoderm. Medial walls of a series of cavities developed from two mesodermal bands during polychaete larval development; it gives rise to the visceral (splanchnic) coelomic lining, which is the median mesentery suspending the gut and enclosing a dorsal and ventral blood vessel; it may consist of muscle cells or a peritoneum (Anderson, 1966; Fransen, 1988; Westheide and Rieger, 2007).

Results

General organization of the trophosome

The trophosome extended over the entire trunk region between the two longitudinal blood vessels from immediately posterior to the ventral ciliary band of the forepart to the posterior end of the trunk delimited by the septum between trunk and first opisthosomal segment. A central lumen was never detected.

Within the trophosome we distinguished between an anterior and a posterior region due to gradual changes in organization of host tissues, amount of bacteriocytes, and symbionts' sizes and shapes. The anterior region was characterized by some bacteriocytes that were mostly concentrated ventrally around the dorsal blood vessel and embedded within an extensive, nonsymbiotic mesenchyme filling the coelomic cavity. Large intercellular blood lacunae penetrated the mesenchyme and bacteriocytes (Figs. 1A, 2A). In the posterior region the bacteriocytes invaded the coelomic body cavity, replacing the mesenchyme. They were pervaded by intercellular blood lacunae and encased by a nonsymbiotic peritoneum (Figs. 1B, 2B). In both the anterior and posterior trophosome regions the coelomic cavity was restricted to small spaces between the muscle layer of the somatic body wall and, respectively, the mesenchyme or visceral peritoneum. Thus, anteriorly the trophosome was composed of a small bacteriocyte population and an extensive mesenchyme, while posteriorly the trophosome was composed of a large bacteriocyte population and a peripheral peritoneum.

Along the entire trophosome, most of the bacteriocytes were organized as apolar tissue, but a few bacteriocytes were part of the myoepithelium surrounding the dorsal blood vessel (Fig. 3A, B). Basally, a continuous basal matrix (60–90 nm) was secreted by bacteriocytes and myocytes. Apical junctional complexes regularly found between myocytes could not be detected between bacteriocytes and myocytes.
Bacteriocytes and symbionts

The cytoplasm of the bacteriocytes was rich in glycogen and contained some electron-dense, roundish granules. Mitochondria and rough endoplasmic reticulum (rER) were scarce (Fig. 2C, D). The nuclei were mostly oval in the anterior trophosome region but irregularly shaped, extending between the symbionts, in the posterior trophosome region.

Most conspicuously, the symbionts enclosed in symbiosomes were dominantly rod-shaped and loosely distributed within the bacteriocytes’ cytoplasm of the anterior trophosome region (Fig. 2C), but they were coccoid and densely packed in the bacteriocytes of the posterior trophosome region (Fig. 2D). In sections, the rod-shaped symbionts were significantly smaller in diameter (1.11 μm ± 0.22) than the coccii (2.60 μm ± 0.51) (Student’s t-test, \( P = 0.01 \)). Also, the area of the symbions within the bacteriocytes increased significantly, more than 2 times, from anterior (37.01% ± 12.17) to posterior (85.97% ± 6.21), occupying more than three-quarters of the total bacteriocyte area in the posterior trophosome (t-test, \( P = 0.01 \)).

The symbionts’ cell wall consisted of an outer membrane and a cytoplasmic membrane typical for gram-negative bacteria (Claus and Roth, 1964). The bacterial cytoplasm rich in glycogen was moderately electron-dense and contained ribosomes and intracytoplasmic membranes that branched off the cytoplasmic membrane. In a central electron-translucent core were chromatin strands (Fig. 4A, B).
to published values for pure S8 (Trotimov et al., 2009). Similar shifts have been observed by others, if S8 was measured inside microbial cells (Pasteris et al., 2001; Himmel et al., 2009). Raman mapping analysis of the 468-cm⁻¹ band showed that this sulfur signature was confined to relatively large, discrete areas within the symbiont cells that strongly resembled the membrane-bound vesicles observed by electron microscopy (Fig. 5B, C). In electron microscopic studies, these sulfur vesicles were rare anteriorly, but abundant posteriorly. Estimating the proportional area, they took up only 1.64% ± 1.78% of bacteriocytes and 3.5% ± 5.7% of symbionts in the anterior trophosome, but 17.02% ± 7.72% of bacteriocytes and 19.68% ± 8.33% of symbionts in the posterior trophosome (Mann-Whitney U-test, P = 0.01).

Symbionts were mostly encased individually in a symbosome membrane adjacent to the bacterial cell wall (Fig. 4A) unless they were proliferating (Fig. 4B). As such dividing symbionts were frequently found in the anterior trophosome region (Figs. 2C, 3B), division of the symbosome membrane must have followed shortly after bacterial division.

In the posterior trophosome, however, we occasionally observed two to three symbionts in one symbosome, pointing to bacterial division but a lack of symbosome division. This condition was often combined with the presence of a strongly condensed host nucleus. In many such cases the bacterial cell walls started to break down (Fig. 4D).

Although symbionts undergoing lysis were rare anteriorly, degradation was evident posteriorly. Combining static micrographs into potential successional stages, we propose two processes of symbiont degradation: one involves individual symbionts only; the other involves large symbiont aggregations. In both cases degrading bacteria or bacterial aggregations were next to intact symbionts and randomly scattered within the posterior trophosome.

**Figure 3.** Epithelial lining of the dorsal blood vessel. (A) Dorsal mesentery surrounding the dorsal blood vessel consisting of myocytes and bacteriocytes. (B) Bacteriocyte as part of the lining of the dorsal blood vessel. ba, bacteria; bc, bacteriocyte; ib, intravasal body; dm, dorsal mesentery; dv, dorsal blood vessel; mc, myocytes.

The symbionts contained conspicuous membrane-bound, electron-translucent vesicles (Fig. 4A). Raman microspectroscopic analysis, with a spectral resolution of about 1.5 cm⁻¹ on single symbiont cells from the posterior trophosome showed very strong bands at about 468 cm⁻¹ (S-S stretching), 215 cm⁻¹, and 147 cm⁻¹ (both S8 bending), indicating that rhombic S8 sulfur is present (Ward, 1968). The latter two bands have blue-shifted shoulders with about the same height (Fig. 5A). According to Ward (1968), this might be attributed to crystal effects of rhombic sulfur. In addition, weaker bands at 191 cm⁻¹, 238 cm⁻¹, and 438 cm⁻¹ were observed, which are also assignable to S8 sulfur (Fig. 5A) (Trotimov et al., 2009). The measured S8 sulfur bands of the symbiont appear slightly red-shifted compared

1. Individual symbionts: In the first degradation step, we observed electron-dense bacterial cytoplasm containing a core of chromatin strands surrounded by electron-light cytoplasm. This was combined with an undulating outer bacterial membrane irregularly separated from the cytoplasmic membrane (Fig. 6A). Later degradation stages had an extremely condensed dark cytoplasm, and chromatin strands could not be distinguished. The bacteria were deformed, consisted mainly of electron-translucent sulfur vesicles, and broke down into smaller components (Fig. 6B).

2. Whole bacterial aggregations contained in a single symbiosome (Figs 6C–G): After division, intact bacteria initially remained together within one symbiosome (Fig. 6C). Then individual bacteria appeared to fuse (Fig. 6D), losing discernible cell
walls and integrity (Fig. 6E, F) until they became extremely large individual bacterial masses with lengths up to 8.7 μm (Fig. 6G). All these stages were observed in intact bacteriocytes with inconspicuous nuclei, mitochondria, and rER.

Mesenchyme and peritoneum

The mesenchymal cells of the anterior trunk region devoid of bacteria were filled with glycogen and contained mitochondria, rER, and various granules that differed in electron density and size (Fig. 7A). The single-layered epithelium of flattened peritoneal cells surrounded the bacteriocytes, which were interspersed with blood lacunae peripherally in the posterior trunk region (Fig. 7B). The peritoneocytes often extended deep into the trophosome between the bacteriocytes (Fig. 2B). Apically, the cells were joined by junctional complexes facing the trunk coelom. Adapically, the inconspicuous basal matrix of the peritoneum was adjacent to the bacteriocytes and blood lacunae. The peritoneocytes contained many mitochondria, rER, and some electron-dense granules, but lacked bacteria.

Trunk blood vascular system

Over the whole length of the trophosome, intercellular blood lacunae were interconnected with each other and formed a network. Here and there the myoepithelia of the dorsal and the ventral vessel opened to let the blood disperse freely in lacunae between bacteriocytes (Fig. 1A), allowing a blood flow coming from the ventral blood vessel, passing between the bacteriocytes, and flowing back into the dorsal blood vessel.

Discussion

The results of this study point to a sulfur oxidizing endosymbiont of *Sclerolinum contortum* from the Gulf of Mexico. We demonstrate that the bacteria are housed in a trophosome with a visceral mesodermal developmental origin, thus confirming the close relationship between the sister taxa *Vestimentifera* and *Sclerolinum*. The trophosome is an unlobed organ showing organizational similarities to the trophosomes of juvenile vestimentiferans. Many facts indicate a cell cycle exhibited along the longitudinal body
axis from anterior to posterior within the symbiont-containing organ.

Metabolism of the symbionts

Sclerolinum consortum from the Gulf of Mexico most probably harbors an endosymbiont with chemosynthetic sulfur-oxidizing metabolism, as indicated by the presence of sulfur S8 in the bacterial vesicles. Only a limited number of organisms deposit sulfur internally, all of them belonging to the Proteobacteria. Such intracellular sulfur vesicles stored as an intermediate during the oxidation of sulfide and thiosulfate are typical for Chromatiaceae, the purple sulfur bacteria (Brenner et al., 2005; Dahl and Prange, 2006; Imhoff, 2006), the family to which most of the thiosulfotrophic endosymbionts belong (Dubillier et al., 2008). Some of them, such as the endosymbionts of the vestimentiferan Riftia pachyptila, have sulfur vesicles (Pflügfelder et al., 2005). The endosymbionts of a S. consortum population from an arctic cold seep at the Haakon Mosby Mud Volcano have characteristic functional genes for autotrophy and sulfur oxidation (Lösekann et al., 2008). Whether the S. consortum population from the Gulf of Mexico harbors the same bacterial strain remains to be determined.

The conspicuous sulfur vesicles are concentrated mostly in the symbionts of the posterior region. This gradient could be interpreted as a result of an excess in the supply of sulfide relative to oxygen, as suggested by Vetter (1985) for clam endosymbionts of the genera Lucina, Lucinoma, and Calyptogena. Uptake of oxygen and sulfide occurs through the plume in the vent vestimentiferan Riftia pachyptila (Arp et al., 1985), whereas sulfide uptake in the seep vestimentiferan Lamellibrachia lymesi is through the porewater of the sediment across the tube and the skin directly (Julian et al., 1999). The mode of sulfide uptake in Sclerolinum consortum is not known, but this species also inhabits cold seeps and the animals penetrate vertically deep into the sulfide sediment. S. consortum has the same two kinds of hemoglobin as vestimentiferans (Meunier et al., 2010) with the ability to bind oxygen and sulfide simultaneously and reversibly (Arp and Childress, 1983; Arp et al., 1987). In S. consortum the two well-vascularized tentacles (L. Eichinger, pers. obs.) are the most appropriate site of oxygen uptake. As in all polychaetes, the direction of blood flow is from anterior to posterior in the ventral blood vessel and in the opposite direction in the dorsal blood vessel (Gardiner, 1992). The amount of oxygen within the ventral vessel remains the same along the longitudinal body axis as blood leaks from the ventral vessel through the trophosome to the dorsal vessel. Since the bacterial mass increases from anterior to posterior in the trophosome of S. consortum,
the demand for oxygen to oxidize sulfide to sulfate increases as well. We reason that the posterior symbionts are limited in oxygen, and therefore they oxidize sulfide only partially, depositing sulfur as an intermediate storage product.

Origin of the bacteriocytes

The trophosome of Sclerolinum contortum is clearly derived from the visceral mesoderm. Most of the trophosome is an apolar tissue strand composed of bacteriocytes and
mesenchyme anteriorly and of bacteriocytes surrounded by a peripheral peritoneum posteriorly. Some of the bacteriocytes, however, are epithelially organized and contribute to the myoepithelium of the dorsal blood vessel, especially in areas where the vessel is open and blood flows freely between lacuniae and blood vessel. Thus, in these areas such bacteriocytes together with myocytes build the myoepithelial lining of the dorsal blood vessel, and both cell types share the same basal matrix. Therefore, we interpret the bacteriocytes as being derived from the visceral mesoderm, with the epithelially organized bacteriocytes acting as stem cells for a proliferating apolar tissue. The polarity of the epithelium of the dorsal blood vessel with its basal portion lining the lumen is opposite to that of the gut epithelium with the basal portion facing the trunk coelom. Consequently, this opposite polarity makes an endodermal origin of the bacteriocytes nearly impossible, as discussed in Bright and Sargo (2003) for the vestimentiferan trophosome. The lack of apical-junctional complexes between myocytes and bacteriocytes might be explained as an adaptation to the demands of the bacteriocytes and endosymbionts for freely circulating blood in an intercellular network of blood lacunae.

**Bacteriocyte and bacterial cell cycle**

Ultrastructural and morphometric investigations indicate a specific cell cycle in the trophosome of *Sclerolinum consortium*. We identified a clear gradient from anterior to posterior, with small, dividing, rod-shaped symbionts in the anterior region and large coccoid symbionts and bacterial degradation characterizing the posterior region. The idea of bacteriocytes dividing anteriorly and acting as stem cells is supported by the fact that the endosymbionts in the bacteriocytes are commonly found dividing anteriorly and their diameter increases two-and-a-half times from anterior to posterior, but the area they occupy within the bacteriocytes increases only slightly. We interpreted the strongly condensed nuclei of some randomly distributed bacteriocytes in the posterior trophosome as an indication of cell death. Although cell cycle studies with specific antibodies for DNA synthesis, mitosis, and cell death are lacking, we define the anterior trophosome as a region of proliferation of symbionts and bacteriocytes, and the posterior trophosome as a region of degradation of symbionts and, to a minor degree, of host cells. Figure 8 illustrates the proposed cell cycle of the symbionts of *S. consortium* with a dividing bacterial stem population in the anterior trophosome and different kinds of bacterial degradation in the posterior trophosome.

**Comparison of siboglinid trophosomes**

This study reveals a homologous origin of the trophosomes of the sister taxa Vestimentifera and *Sclerolinum*. Already in 1977, van der Land and Nollevang regarded the trophosome of the vestimentiferan tubeworm *Lamellibrachia* as a visceral mesodermal tissue because trophosomal cells often line the outside of blood vessels. An ultrastructural study revealed that the epithelium of the axial blood vessels traversing each trophosomal lobule is composed of myocytes, bacteriocytes, and non-bacteriocytes in *Riftia pachyptila* (Bright and Sargo, 2003)—a situation very similar to the epithelium of the dorsal blood vessel of *Sclerolinum consortium*. But not only the general organization in adults points to a mesodermal origin. In addition, after vestimentiferans develop from small sessile stages into larvae, putative symbionts from the surrounding environment penetrate the larval skin and migrate toward the visceral mesoderm of the dorsal mesentery, initiating the develop-
Figure 8. Bacterial cell cycle exhibited within the Sclerolaimus consortum trophosome. In the anterior trophosome, dividing rod-shaped bacteria act as a bacterial stem population. By division of the anterior bacteriocytes, part of the host population is pushed toward the posterior, and symbionts contained in the host cells gradually grow to large size. Posteriorly, the symbionts degrade individually (a) or as large bacterial aggregations (b). Other symbionts remain in a single symbiosome after division, as they are often found in bacteriocytes with a condensed nucleus. Whether division of the symbiosome membrane takes place after bacterial division could not be followed (3).

ment of the trophosome between the dorsal blood vessel and the gut (Nusbaum et al., 2006). Taking into account the detailed morphological studies of the adult trophosome now available for both taxa, we propose that the trophosome of the sister taxa Vestimentifera and Sclerolaimus is a synapomorphic character that evolved from the visceral mesoderm in their last common ancestor.

In comparison to an adult, multi-lobed vestimentiferan trophosome, the unlobed trophosome of Sclerolaimus consortum is astonishingly simple organized. This difference might be explained by the extremely different diameters of vestimentiferans and Sclerolaimus and thus to different amounts of bacteriocytes and symbionts, leading to dissimilar demands of chemicals for sulfide oxidation and carbon fixation to be delivered by the blood. For large vestimentiferans, this demand might only be met by developing a multi-lobed organ and thus increasing the number of afferent, peripheral blood vessels; for small Sclerolaimus, blood supply through relatively simple openings directly from the ventral blood vessel might be sufficient.

The general organization of the anterior trophosome of the slender S. consortum is very similar to that of the trophosome of small vestimentiferan juveniles, with bacteriocytes situated next to undifferentiated mesenchymal cells (Supplemental fig. 3 in Nusbaum et al., 2006). The posterior trophosome, however, is more similar to the one-lobule-stage trophosome of large vestimentiferan juveniles, composed of an apolar tissue of bacteriocytes filling the coelomic cavity and surrounded by an aposymbiotic peritoneum (Nusbaum et al., 2006; Bright and Lallier, 2010) (Fig. 9E). Even the efferent blood vascular system of the one-lobule stage in these large vestimentiferan juveniles, which have an axial blood vessel as a direct extension of the dorsal blood vessel, looks very similar to comparable areas in S. consortum, where the dorsal blood vessel opens into blood lacunae in the unlobed trophosome. Nevertheless, the Sclerolaimus trophosome has a simpler blood vascular system because it lacks the afferent peripheral blood vessels that are already developed in the one-lobule stage of the trophosome in vestimentiferans and further elaborated in the multi-lobule stage of the adults.

It is important for homeostasis with balanced cell renewal and degradation to be sustained in an organ. Additionally, in a symbiont-containing organ, growth of the bacterial population has to be controlled, and the cell cycle of the host cells has to be synchronized and fine-tuned with the bacterial cell cycle. We reason that the well-known cell cycle in vestimentiferans, which is directed from the center to the periphery of each lobule, differs from that directed from anterior to posterior in Sclerolaimus. In the vestimentiferan adult trophosome, bacteriocytes and symbionts pass through a synchronized, specific cell cycle with terminal differentiation directed from the center to the periphery of each lobule of the trophosome. Cell division occurs in the central region and massive degradation in the periphery, as is evident from different ultrastucture (Bosch and Grassé,
Evolution of siboglinid trophosomes

The main intention of this study was to elucidate the evolutionary origin of the siboglinid trophosomes. For the sister clade Vestimentiferan and Sclerolinum, we propose a homologous visceral mesodermal origin. Nevertheless, the situation gets more complicated when all four siboglinid taxa are considered, as a variety of trophosomes seem to exist. In Oseadax the bacteriocytes belong to the somatic mesoderm (Katz et al., 2011). In the species-rich fumulates a visceral mesodermal (Ivanov, 1963), somatic mesodermal (Southward et al., 1981), or endodermal (Calsen-Cencic and Flügel, 1995) origin of the bacteriocytes have been proposed. This leaves, in our opinion, two possible scenarios for the evolution of the trophosome: (1) the trophosome developed once, in the last common stem species of Siboglinidae, from an unspecialized condition of a symbionts-hosting organ established within several different layers of host tissue; or (2) different trophosomes evolved several times independently, de novo, as analogous organs at least in fumulates, Oseadax, and vestimentiferans + Sclerolinum (Katz et al., 2011). Consequently, either the reduction of the digestive system happened once or independently several times. Today information on Oseadax and Sclerolinum trophosomes is still limited, especially concerning their development. Within the fumulates, published data on the adult organization are even controversial, and the only detailed developmental study (Calsen-Cencic and Flügel, 1995) also lacks proof of whether the symbionts enter through the mouth and the trophosome develops from the gut, or microbes are digested in the gut and symbionts enter through the skin in a process like that in vestimentiferans. Developmental studies using combined methods would be useful to trace the origin of the different kinds of trophosomes and to elucidate the enigma of the evolution of the siboglinid trophosome.

Acknowledgements

This study was financially supported by the Austrian Science Foundation P20282-B17 and the Initiativekolloq Symbiotic Interactions of the University of Vienna. We thank C. R. Fisher for inviting us on the cruise “Expedition to the Deep Slope,” the captain and crew of the NOAA Ship Ron Brown, and the crew of the ROV Jason for their expertise and assistance. Special thanks for support from M. Wagner (head of the Core Facility Raman Microscopy, University of Vienna) and D. Gruber and G. Spitzer (Core Facility for Cell Imaging and Ultrastructure Research).

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Manuscript 2

Morphology, microanatomy and sequence data of *Sclerolinum contortum* (Siboglindae, Polychaeta) of the Gulf of Mexico

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Publication status: submitted to Organisms Diversity & Evolution

Personal contributions of Irmgard Eichinger:

collected the material during the cruise to the Golf of Mexico in 2007
fixed and embedded the samples for TEM and LM
produced the ultrathin sections
performed LM and TEM
performed morphometric measurements and statistical analysis
wrote the manuscript
Abstract

*Sclerolinum* is a small genus of Siboglinidae (Polychaeta), living in an obligate mutualistic association with thiotrophic bacteria as adults. Its taxonomic position based on morphology has been controversial, however molecular data point to a sister taxa relationship with vestimentiferans. 16S rRNA gene sequencing and comparative morphology revealed that the studied population from deep-sea hydrocarbon seeps of the Gulf of Mexico belongs to *Sclerolinum contortum* known from the Arctic Sea. Since no anatomical and microanatomical studies have been published yet, we conducted such a study on *S. contortum* using serial sectioning and light and transmission electron microscopy. We show that the *Sclerolinum* body, divided into a head, trunk, and opisthosoma, is very similar to that of the vestimentiferans and therefore we propose that the body regions are homologous in both taxa. Some morphological characters of *Sclerolinum*, the frenulum and the epidermal papillae of the anterior trunk region, are evaluated as plesiomorphic conditions of Siboglinidae.
Introduction

*Sclerolinum* is a small genus of slender marine tubeworms, which together with vestimentiferans, *Osedax* and frenulates forms the monophyletic polychaete family Siboglinidae Caullery, 1914 (McHugh 1997; Rouse and Fauchald 1997; Rouse et al. 2004). They are renowned for their symbiotic lifestyle in a variety of reducing habitats since all adult representatives - except of *Osedax* dwarf males (Rouse et al. 2004) - rely completely on endosymbiotic bacteria. They reduce their digestive system during ontogeny and develop a new organ, called the trophosome, to accommodate and provide their chemoautotrophic (Felbeck 1981; Felbeck et al. 1981; Lösekann et al. 2008; Southward et al. 1981) or heterotrophic symbionts (Goffredi et al. 2007; Goffredi et al. 2005).

*Sclerolinum* species occur in a wide range of deep-sea environments between less than 500 and about 2000 m depth. Most of the seven described species (Ivanov and Selivanova 1992; Smirnov 2000; Southward 1961, 1972; Webb 1964b) either dwell on decaying organic materials like sunken wood or in sulfidic sediments (Smirnov 2000). *Sclerolinum contortum* Smirnov, 2000 is reported from the arctic, cold seep Haakon Mosby Mud Volcano, cold seeps of the Storegga Slide in the Norwegian Sea (Lazar et al. 2010), and from an hydrothermal vent field of the Arctic Mid-Ocean Ridge (Pedersen et al. 2010). Other not yet described species were found at cold seeps in the Sea of Okhotsk (Sahling et al. 2003) and in hydrothermal vent sediments of Antarctica (Sahling et al. 2005).

The genus *Sclerolinum* has challenged scientists since its discovery. Its organization neither fit well to the known frenulates nor to the later discovered vestimentiferans. Discussed as the most basal (Southward 1999; Webb 1964a) or a more derived siboglinid (Ivanov 1994; Southward 1993), its classification switched from genus to subclass rank. Southward (1961) described the first *Sclerolinum* species and established the genus *Sclerolinum* within the frenulate family Polybrachiidae. Later, considering the peculiarities of *Sclerolinum* a new family Sclerolinidae (Webb 1964a) and even a new monogeneric subclass Monilifera (Ivanov 1991) was created with equal rank to frenulates and vestimentiferans. Today, *Sclerolinum* is considered the sister group to vestimentiferans due to molecular and morphological data (Halanych et al. 2001; Hilário et al. 2011; Meunier et
Osedax is placed as sister group to Sclerolinum plus vestimentiferans and frenulates is the most basal group (Hilário et al. 2011; Rouse et al. 2004), however the position of Osedax is ambiguous in the study of Zrzavý (2009).

Siboglinids have a body divided into different regions. In frenulates a cephalic lobe (prostomium *sensu* Rouse 2001), a forepart, a trunk with specialized regions like an anterior metameric region, and an opisthosoma are distinguished (Southward 1993). Osedax females are composed of a crown, a trunk, and an ovisac with roots (Rouse et al. 2004). In Sclerolinum a cephalic lobe, a forepart, a trunk, and an opisthosoma are found (Southward et al. 2005). Vestimentiferan bodies are composed of an obturacular region, a vestimentum, a trunk, and an opisthosoma (Gardiner and Jones 1993).

Vexingly, Sclerolinum lacks a visible external demarcation between the forepart and the trunk. The existence of an internal diaphragm is still in discussion. A muscular diaphragm separating the forepart from the trunk is formed during larval development in frenulates (Bakke 1977; Callsen-Cencic and Flügel 1995; Ivanov 1975). Such a diaphragm is also mentioned in two adult Sclerolinum species (Ivanov 1991; Southward 1961). In addition, Southward (1961) separated an anteriorly-located metameric trunk region from the posterior trunk by the presence of paired lateral ridges occupied by large glands in the former body region. Later, the metameric region was mentioned as dorsally grooved and ventrally ciliated (Southward 1972; Webb 1964b).

Although we lack any knowledge of the internal anatomy of Sclerolinum to date, its body regions have been homologized with those of other siboglinid taxa: the forepart with the frenulate forepart and the vestimentum of vestimentiferans (Hilário et al. 2011; Rouse 2001; Rouse and Pleijel 2001; Rousset et al. 2004; Southward et al. 2005; Webb and Ganga 1980); and the metameric region with the metameric trunk region of frenulates (Southward 1972). The presence or absence of the metameric region has been used as a character for cladistic analyses (Rouse 2001; Rousset et al. 2004; Schulze 2003). The opisthosoma is similarly organized in frenulates, vestimentiferans and Sclerolinum and regarded as homologous region in all three taxa.
We investigated a *Sclerolinum* population from deep-sea hydrocarbon seeps in the Gulf of Mexico (GoM) to improve our knowledge of this highly debated but little studied siboglinid taxon. To identify the host species we used 16S rRNA gene sequencing and comparative sequence analyses. We applied light and electron microscopy for measurements and description of external morphology, but our main intention was to describe the anatomy and microanatomy in order to provide, for the first time, comparative data of one representative of this genus. We found high anatomical similarities between the vestimentiferan and *Sclerolinum* body organization, clearly pointing to a sister taxa relationship, as previously suggested by molecular (Halanych et al. 2001; Rouse et al. 2004), and morphological (Rouse 2001) approaches, as well as combined studies (Rousset et al. 2004; Zrzavý et al. 2009).

**Materials and methods**

During the “Expedition to the Deep Slope” (chief scientist C. R. Fisher) with the NOAA R/V *Ronald H. Brown* in June 2007, *Sclerolinum* aggregations dwelling at the hydrocarbon seeps in the GoM were sampled with cores of 30 cm or 50 cm length at three sites (WR269, AC818, AC601) in depths ranging from 2000 m to 2700 m depth. The samples were recovered with the ROV *Jason* during the dives J2-275, J2-282 and J2-283.

In addition, for genetic studies, reference specimens of *S. contortum* from the Northeast Atlantic were collected during the VICKING cruise with the ROV Victor (dives 275 and 277). Sequencing was performed on one specimen from Storegga Northeast (64°45.27’ N, 4°58.87’E, 745 m depth) and one specimen from Haakon Mosby Mud Volcano (HMMV, 72°00.34’N, 14°58.87’E, 1264 m depth). All samples for subsequent molecular methods were fixed in 100% ethanol and stored at 4°C. Fixation, embedding, cutting, and staining procedure for light and electron microscopy on GoM animals are described in Eichinger et al. (2011).
16S rRNA gene sequencing and phylogenetic analyses of the host

After removal of excess ethanol, total DNA was isolated following a CTAB + PVPP extraction protocol (Doyle and Doyle 1987). The mitochondrial gene coding for the ribosomal 16S RNA was amplified using the primers designed by (Palumbi 1996). The optimal PCR cycling parameters were 1 cycle: 3 min/96°C; 35 cycles: 1 min/96°C, 1.15 min/50°C, 1 min/72°C; 1 cycle: 10 min/72°C. PCR products were visualized after electrophoresis on 1.5% agarose gels containing ethidium bromide under UV-light. Both DNA strands were directly sequenced with BigDye Terminator v. 3.1 (Applied Biosystems), on an ABI 3130 XL automated sequencer. The two mt16S sequence fragments for each species were assembled and edited in CodonCode Aligner (CodonCode) to generate a continuous ca. 500 base pairs fragment. Sequence alignment was performed using Clustal W and checked visually. The phylogenetic tree was generated with MEGA version 5 (Tamura et al. 2011), using a Kimura-2-parameter distance calculation and a neighbor join tree reconstruction. Robustness of tree nodes was tested by bootstrapping the data with 1000 replicates.

Light and electron microscopy

Five complete worms and several worm fragments were fixed for light microscopy and used for the description of the external morphology. One complete female was fixed for light microscopy, cut into small pieces, embedded and cut into a series of 1 µm semithin sections. Several samples of the female and male trunk region, one opisthosoma, and one anterior end of a male were fixed, embedded, cut and stained for transmission electron microscopy. Only the anterior end of the male was treated differently, since it was cut into a series of 1 µm semithin sections alternated with ultra-thin sections.

All sections were made on a Reichert Ultracut S microtome. Semithin sections were viewed with a Zeiss Axio Imager A1 light microscope and ultrathin sections were analyzed with a Zeiss EM 902 transmission electron microscope.
Measurements of taxonomical characters and statistical analyses

Taxonomic characters were measured by using Image J software, ver. 1.40g. Correlations of the length of the worms with body diameters, measured at different body regions, were statistically tested by using Excel (Microsoft) by creating regression plots.

Results

mt16s gene phylogeny of the host

The phylogeny of the Siboglinidae based on the mitochondrial 16S fragment sequenced yielded a very well resolved tree, with strong support for the major clades (Fig. 1). The tree clearly shows a vestimentiferan clade (comprising cold-seep and hydrothermal-vent species) at the base of which Sclerolinum brattstromi and S. contortum form a very highly supported monophyletic group. Ose-dax is basal to these clades, the frenulates are basal to all these species. Based on this marker, the GoM Sclerolinum is indistinguishable from the Northeast Atlantic (HMMV and Storegga) specimens of S. contortum (100% identity with the HMMV sequence). They form a very strongly supported clade, clearly distinct from Sclerolinum brattstromi.

Tube

The tubes were around twice as long as the inhabiting worms (Fig. 2). The maximal tube diameter ranged from 0.35 mm to 0.61mm (n = 5). The tubeworms mostly live buried in mud, only the anterior curled and transparent ends of the tubes extending into the surrounding water. The tips of these anterior ends were frail and easily collapsed (Fig. 3a). The rest of the tubes were more or less straight, yellowish to brownish and firm, except of the posterior parts, which were colorless, extremely thin walled, soft and frayed.
**External morphology**

The body was divided into the cephalic lobe, forepart with two tentacles, trunk, and opisthosoma. The genital openings marked the beginning of the trunk according to Webb and Ganga (1980). This correlated with the beginning of the randomly distributed cuticular plaques of the trunk.

The animals ranged in length from 54 mm to 86 mm, measured from the tip of the cephalic lobe to the end of the opisthosoma, and in diameter from 0.27 mm to 0.45 mm, measured at the first opisthosomal septum \( n = 5 \). These values correlated, while diameters measured at other body regions, for example at the anterior edge of the bridle, did not correlate with the length.

The worms had a very small cephalic lobe with an extension of 68 - 78 µm lacking a separation to the forepart, which was characterized by densely packed glands and a deep narrow dorsal furrow shaped as an upside down Y. The frenulum was located approximately 0.48 mm posterior to the tip of the cephalic lobe and consisted of 12 to 20 roundish to elongated cuticular plaques. These plaques were arranged either as two dense, arched rows extending from dorsal to ventral (Fig. 3b) or in a more scattered manner (Fig. 3c). There was a large variation in size of the frenular plaques from 11 - 27 µm in width and 21 - 85 µm in length. Neither the size nor the number of the frenular plaques correlated with the length of the specimens. A broad densely ciliated ventral field started posterior to the frenulum.

No internal or external separation between the forepart and the trunk were observed. The semithin section series revealed a single dorsal female gonopore at the end of the dorsal furrow, slightly anterior to the end of the ventral ciliated field (Fig. 5a). In the male specimen the dorsal furrow broadened at the posterior end over a short distance forming two ciliated grooves, each ending with a genital papilla (corresponding to the openings of the sperm ducts), slightly posterior to the ventral ciliated field (Fig. 5b). Congruently, the anterior end of the trunk was easily recognizable by scattered, conspicuous, oval plaques situated on top of epidermal papillae. They extended after the end of the dorsal furrow to the first opisthosomal segment and were restricted to the trunk (Fig. 3d).
Only the anterior trunk region had prominent lateral epidermal papillae devoid of plaques, which were the openings of loosely arranged large pyriform glands (Fig. 6a). At the posterior margin of the trunk were one to two rings of uncini forming the girdles.

The opisthosoma consisted of 13 to 16 segments and ranged in length between 1.4 - 1.8 mm. Each segment exhibited an incomplete ring of uncini devoid of chaetae middorsally and mid-ventrally. These rings became more incomplete at the posterior segments (Figs. 3e, f).

The measured five complete worms were not representing the largest specimens in the Sclerolinum aggregations we sampled. The largest found opisthosoma was 2.7 mm long, had a diameter of 0.54 mm at the first septum, and a total number of 19 segments. Assuming the body proportions similar to those of the complete worms studied, this would correspond to the total length of 10 cm.

**General internal organization**

The two tentacles were free of pinnules and ciliated cells. We located two longitudinal blood vessels and a central coelomic cavity, which was occluded by mesenchyme at the base of each tentacle (Figs. 4a, b).

The cephalic lobe was a very short ventral epidermal extension of the forepart containing the nervous center at its base. It had no coelomic cavity, muscle cells, or blood vessels but was invaded by blood lacunae (Fig. 4b). The compact forepart also lacked a visible coelomic cavity. The cavity was occupied by pyriform glands, mesoderm, and the dorsal and ventral blood vessels. The ventral nerve cord bifurcated around the ventral ciliated field (Figs. 4c, d).

The trunk was mostly occupied by the trophosome, the gonads and pyriform glands. The paired coelomic cavities were reduced to small spaces. The dorsal and ventral blood vessels were suspended by the mesenteries (Figs. 6a-c). A single female ovary or paired male testes were connected ventrally to the mesentery. The ventral nerve cord extended over the whole length of the trunk. Several times along the posterior trunk, both the epidermis and the longitudinal muscle layer thickened massively, thus constricting the body cavity. In such areas the cuticular plaques were very dense (Fig. 6d).
The opisthosoma consisted of several segments separated from each other and from the trunk by septa composed of two myoepithelial layers (Fig. 7a). Each segment was partitioned by a median mesentery supporting the dorsal and ventral blood vessel. Conspicuous multicellular epidermal glands reached into the coelomic cavities (Fig. 7b). The ventral nerve flattened and broadened within the opisthosoma and concentrated to a cord at the posterior end of the opisthosoma.

**Epidermal structures**

Apically, the epidermal supportive cells possessed microvilli embedded within a thin cuticle and laid on a basal matrix (Fig. 8a). Multiciliated cells formed a broad ventral ciliated field (Fig. 8b). In males, multiciliated supportive cells were part of the epithelium of the posterior dorsal furrow (Fig. 8c) and constituted the genital grooves extending from the dorsal furrow to the genital papillae (Fig. 5b). Although sensory cells were not specifically sought for, none were noticed.

**Nervous system**

The intraepidermal nervous system consisted of the nervous center located at the base of the cephalic lobe, a main ventral nerve cord extending through the entire length of the body and numerous small nerves. At the transition from the cephalic lobe to the forepart the neurons forming the nervous center were differentiated into a central neuropil surrounded by somata except of the dorsal side (Fig. 4b). All neurites were located basally to the epidermal cells with the basal matrix underlining the nervous cells. Small nerves ran from the tentacles to the nervous center. Neurites originating from the nervous center extended laterally at the beginning of the forepart. A single ventral nerve cord continued from the nervous center (Fig. 4c) and bifurcated around the ventral ciliated field, which was provided with many small nerves (Figs. 4d, 8b), and extended over the whole trunk (Fig. 8d). Within the opisthosoma the nerve broadened, forming a ventral nerve field, which concentrated at the end of the opisthosoma into one narrow cord again. Over the whole length of the nerve cord we found no giant axons.
Epidermal glands

Single gland cells with electron dense granules were distributed in the epidermis over the whole body, but were conspicuously dense on the inner face of the tentacles (Fig. 4a).

Multicellular pyriform glands *sensu* Ivanov (1963) consisted of a duct and a sac-like glandular region. They were composed of multiple secretory cells with microvilli apically facing the glandular lumen. These glands protruded into the interior of the forepart and the trunk. Although the principal composition of these glands was similar, the occurrence, density, and microanatomy varied regionally.

Anterior to the frenulum, these glands were extremely densely packed across the body except midventrally, where the nerve cord was located (Fig. 4c). They were characterized by extensive rough endoplasmic reticulum (rER) and electron-light, amorphous secretion products (Figs. 9a, b). Between posterior the frenulum to the beginning of the ventral ciliary field, they were also densely packed, but full of electron-dark granules, Golgi complex, and rER (Figs. 9c, d). With the beginning of the ventral ciliated field the glands got larger, had a looser dorso-lateral distribution (Fig. 4d) and were composed of glandular cells containing rER, Golgi complex, and different kinds of granules (Fig. 9e). All glands of the forepart had openings lacking epidermal elevations, and they never opened into the dorsal furrow.

The pyriform glands of the trunk were arranged laterally in the anterior trunk region and loosely scattered in the posterior trunk region. Only the anteriorly located glands opened within lateral epidermal papillae (Fig. 6a). All glands of the trunk cytologically resembled the glands of the ciliated region, however some contained intracellular bacteria of unknown identity (Fig. 9f).

Another type of multicellular glands was found in the opisthosoma. Associated with the anterior face of the septa and provided by blood lacunae they reached into the opisthosomal coelomic cavities (Fig. 7b). The long narrow glandular ducts extended along the septa through the muscle layer and the epidermis, and opened to the exterior in a pore. Each multicellular glandular complex had a spacious lumen in the center. The glandular cells connected with apical junctional complexes and showed microvilli apically. The glandular cells contained a large lobed nucleus with a promi-
nent nucleolus, extensive rER often arranged in concentric circles, mitochondria, Golgi complex, and electron-light granules containing electron-dark patches (Figs. 10a-c).

**Mesodermal structures and coelomic cavities**

The body wall musculature was composed of an outer circular and an inner longitudinal muscle layer. These were prominent in the forepart and the trunk (Fig. 8d), but extremely thin in the opisthosoma (Fig. 10a). The few exceptions were the dorsal furrow in the anterior region and the tentacles, which exhibited a single layer of longitudinal myoepithelial cells only (Fig. 8a).

Each tentacle had a central coelom filled by mesenchymal cells at its base (Figs. 4a, b). These mesodermal strands connected to the solid mesoderm of the forepart, which was composed of mesenchymal cells rich in glycogen and interspersed with intercellular blood lacunae and muscle cells of different orientations. There was no visible coelomic cavity within the anterior region (Figs. 4c, d).

The trophosome - an organ of visceral mesodermal origin (Eichinger et al. 2011) - extended over the entire trunk. It was composed of a small bacteriocyte population and an extensive mesenchyme anteriorly (Figs. 6a, b) while posteriorly it consisted of a large bacteriocyte population and a peripheral peritoneum (Fig. 6c). Only some small spaces between the body wall muscle layer and the mesenchyme or peritoneum, respectively, were representing the coelomic cavities.

The series of the opisthosomal paired coelomic cavities was partitioned by septa. They were composed of two myoepithelial layers each that inserted into the body wall muscle layer through desmosomes. Circular muscles formed the anterior face, longitudinal muscles the posterior face (Fig. 7a). There were no blood lacunae within the basal matrices between the two muscle layers.

**Blood vascular system**

The blood vascular system consisted of the dorsal and ventral blood vessels, the blood vessels of the tentacles, the blood vessels of the ovary and a network of intercellular blood lacunae.
Each tentacle had two opposing blood vessels situated within the basal matrices between a highly vascularized epidermis and longitudinal myoepithelial cells. Blood lacunae between the epidermis and the muscle layer, and between the epidermal cells, connected the two blood vessels with each other (Figs. 4a, 8a). More proximally the vessels lay between the epidermis and a mesenchyme (Fig. 4a), and at the base of the tentacles the vessels could not be traced.

The lining of the ventral and the dorsal blood vessels was the median mesentery composed of myoepithelial cells except for the dorsal vessel of the trunk region where some bacteriocytes contributed to this epithelium (Eichinger et al. 2011). The two longitudinal blood vessels opened at regular intervals and connected with a network of intercellular blood lacunae located within the mesoderm of the forepart (Fig. 4d), between the mesenchymal cells and/or the bacteriocytes of the trunk region (Fig. 6b), and within the median mesentery of the opisthosoma (Fig. 7b). The dorsal blood vessel was paired at its anterior end (Fig. 4c); most probably representing the branches of the afferent vessels of the tentacles. At the anterior margin of the trunk, within the region of the genital openings, prominent muscles encircled the dorsal vessel (Figs. 5a, b). Within the opisthosoma the dorsal blood vessel ran at a more central position (Fig. 7b).

A distinct cell cord on the ventral side in the dorsal blood vessel formed an intravasal body sensu Schulze (2002) over the whole length of the body except of the opisthosoma. It thickened several times within the trunk blocking the dorsal blood vessel (Fig. 6a).

**Female reproductive system**

A single ovary was located ventrally on the right side of the ventral blood vessel and started approximately after one third of the trunk. At its posterior end it bent, forming a lateral pouch, and passed anteriorly as oviduct running parallel to the ovary (Fig. 11a). The oviduct opened dorsally posterior to the dorsal furrow, slightly anterior to the end of the ventral ciliated field (Fig. 5a). The wall of the oviduct consisted of a ciliated epithelium with apical junctional complexes and a basal matrix surrounded by a thin myoepithelium (Fig. 11b).
Within the ovary the stacked developing eggs increased in size from anterior to posterior. Oocytes of the posterior ovary had a large germinal vesicle containing a prominent nucleolus and were packed with yolk granules and lipid droplets indicating that they were in the vitellogenic phase of the first meiotic prophase. Oocytes were surrounded by flattened follicle cells as well as by blood vessels (Fig. 11c). Blood lacunae were in direct contact with the oocytes even forming small branches ramifying into the oocytes (Fig. 11d). The egg envelope was formed of an extracellular matrix penetrated by oocyte microvilli in regions where the follicle cells were lifted off the oolemma (Fig. 11e). Oval-shaped eggs detected within the anterior oviduct were up to 430 µm in length and 110 µm in diameter (Fig. 11f).

Male reproductive system

In the male specimen, parts of the epidermis of the posterior dorsal furrow were ciliated (Fig. 8c). The furrow broadened at its end forming two ciliated dorsal grooves extending to two genital papillae located dorsally, slightly posterior to the end of the ventral ciliated field (Fig. 5b). These papillae were the openings of paired sperm ducts full of conspicuous filiform spermatozoa. The sperm ducts were connected to the ventral mesentery on either side of the ventral blood vessel (Fig. 12a) and consisted of two opposite orientated epithelia (Fig. 12b).

The spermatozoa were composed of a helical acrosome, an elongated coiled nucleus surrounded by helical mitochondria, a short centriolar region, and a long flagellum with a 9 x 2 + 2 pattern (Figs. 13c-f). Neither spermatophores nor spermatozeugmata could be detected.

Discussion

Biogeography of Sclerolinum contortum and intraspecific morphological plasticity

The molecular phylogeny of the Siboglinidae based on the mitochondrial 16S is in agreement with the phylogeny based on this genetic marker and the nuclear 18S marker (Rouse et al. 2004): Sclerolinum is basal to vestimentiferans, Osedax is basal to this group and frenulates are basal to all Siboglinidae. There is little to no differences in sequences of Sclerolinum GoM and the Northeast-
ern Atlantic specimens of *S. contortum*. Although mitochondrial markers have been shown to have a limited resolution in the cold-seep vestimentiferan genera *Lamellibrachia* and *Escarpa* (Andersen et al. 2004; Miglietta et al. 2010), the lack of differences likely indicates that *S. contortum* has a wide geographic distribution, from the Gulf of Mexico to the Arctic Sea (Lazar et al. 2010; Lösekann et al. 2008; Pedersen et al. 2010; Smirnov 2000).

The most conspicuous character of *S. contortum* is its tube, which is anteriorly highly meandering and posteriorly more or less straight. Additionally, this species is characterized by a dense arrangement of the frenular plaques. The measured specimens from the GoM are larger than the ones from the arctic HMMV. Nevertheless, regardless of the worm’s size, several morphological characters, such as the number and size of frenular plaques, the numbers of opisthosomal segments, and the length of opisthosoma, differ between the two populations (Table 1). In the absence of molecular data, one could have classified the two populations as separate species. Sequence data of more *Sclerolinum* species from various populations would be useful to improve our knowledge on the intra- and interspecific morphological variability and biogeography of this small genus inhabiting diverse reducing environments all over the world.

**The *Sclerolinum* body organization in comparison with vestimentiferan and frenulate body organization**

Studying the adult morphology and microanatomy of *Sclerolinum contortum* GoM and comparing it with the vestimentiferan sister taxon and the basal taxon of Siboglinidae, the frenulates, we propose a hypothesis on the *Sclerolinum* adult body regions as follows: (1) The cephalic lobe and the forepart, containing the nervous center, form the worm’s head, homologous to the vestimentum of vestimentiferans. The tentacles are head appendages. (2) The trunk is homologous to the trunk of vestimentiferans and includes the reproductive system and the trophosome. (3) The opisthosoma is homologous to the opisthosoma of vestimentiferans and frenulates. The terminology is adjusted to polychaetes (Rouse 2001) and vestimentiferans (Bright et al. submitted).
Although the developmental fate of the prostomium and the peristomium, and the formation of the tentacles in *Sclerolinum* are unknown and developmental studies only will ultimately provide proof, we propose that the *Sclerolinum* head is composed of the cephalic lobe and the forepart. It is homologous to the vestimentiferan head, the vestimentum, arising from the prostomium, peristomium and the anterior part of the first chaetiger during larval development (Bright et al. submitted). Further, we interpret the frenulate head as composed of the cephalic lobe and forepart and the muscular diaphragm as the frontier between the head and the trunk (Bright et al. submitted). This frontier is not considered homologous to the frontier between the head and the trunk of *Sclerolinum*. Consequently, we do not consider the *Sclerolinum* head as being equivalent to the frenulate head. This stands in clear contrast to other studies which homologized, partly because of historical considerations, the forepart of *Sclerolinum* with the frenulate forepart and both together with the vestimentiferan vestimentum (Hilário et al. 2011; Rouse 2001; Rouse and Pleijel 2001; Rousset et al. 2004; Southward et al. 2005; Webb and Ganga 1980).

The *Sclerolinum* head extending from the tip of the cephalic lobe to the end of the dorsal furrow, corresponds to the vestimentiferan head, the vestimentum. Support for this homologization comes from cross sections of the forepart of *S. contortum* GoM revealing striking similarities with the vestimentiferan vestimentum. Neither in *Sclerolinum* nor in vestimentiferans a diaphragm separating the head from the trunk exists (Bright et al. submitted). Dorsolateral folds either form a narrow, dorsal furrow in *Sclerolinum* or the more extended vestimental wings of vestimentiferans. In both cases, the epidermis, as well as the somatic musculature, contribute to the dorsal interfolding. A ventral ciliated field bordered by a nerve cord is restricted to the forepart or vestimentum. In vestimentiferans it originates in the neurotroch (Bright et al. submitted). Finally, the glandular arrangement of the head is very similar in both taxa. In *S. contortum* GoM the pyriform glands are densely arranged across the forepart and never open into the dorsal furrow. In vestimentiferans the glands are distributed from the nerve cords to the edges of the vestimental wings (Gardiner and Jones 1993; Malakhov et al. 1996b, 1996a; Webb and Ganga 1980).
In comparison, in frenulates, a diaphragm is formed during development and persists into the adult frenulate (Ivanov 1963; Southward 1993), while such a structure lacks in adults of *Sclerolinum* and vestimentiferans. A dorsal furrow is mentioned in several species of frenulates (Hilário and Cunha 2008; Ivanov 1963; Southward 1961, 1972; Southward 1991). However, this furrow is only a small interfolding of the epidermis alone (Fig 30 in Ivanov 1963) and does not involve the somatic musculature as in *Sclerolinum* and vestimentiferans. The frenulate forepart is free of a ventral ciliated region. During development only the posterior part of the neurotroch present in the metatrochophore remains as ventral ciliated field of the anterior trunk in adult frenulates (Callsen-Cencic and Flügel 1995). The glandular arrangement of the frenulate forepart is variable between species, while it is similar in *S. contortum* GoM and vestimentiferans. Glands seem to be restricted to the region posterior to the frenulum in frenulates. They are arranged throughout the whole region in *Lamellisabella zachsi* (Ivanov 1963), as two separated patches in *Oligobrachia ivanovi* (Southward 1959), or confined to one patch just behind the frenulum in *Siboglinum caulleryi* (Ivanov 1963).

According to Richer et al (2010), the brain is the most prominent anterior condensation of neurons. To distinguish the intraepidermal cluster of neurons in Siboglinidae from subepithelial condensations of neurons in most other polychaetes, we decided to use the term nervous center (see Bright et al. submitted). In *Sclerolinum* the nervous center is located at the base of the cephalic lobe, a miniscule epidermal extension of the forepart, and extending into the anteriormost region of the forepart. The nervous center of vestimentiferans and frenulates develops in the prostomium of the metatrochophore (Bright et al. submitted, Ivanov 1963; Southward 1993). In vestimentiferans, the prostomium merges with the first chaetiger during development and forms the vestimentum of adults, so that the nervous center is located at the anterior margin of the vestimentum in adults. In contrast, in the frenulates the prostomium persists as prominent cephalic lobe in adults and the nervous center is located within the cephalic lobe and the anterior forepart (Ivanov 1963; Southward 1993). Therefore we hypothesize, that the cephalic lobe got gradually reduced during
siboglinid evolution, leading to an incorporation into the vestimentiferan head and consequently, to an inclusion of the nervous center into the anteriormost part of the vestimentum.

All siboglinids exhibit tentacles. However, in vestimentiferans and frenulates they are of different origin. The vestimentiferan tentacles and consequently the obturacular region are differentiations of the first chaetiger (Bright et al. submitted), whereas in the frenulate metatrochophore the coelom of the tentacles originates from the coelom of the prostomium (Ivanov 1975). The Sclerolinum tentacles are hypothesized to have a similar origin to those of vestimentiferans. Support comes from the adult organization of the mesoderm, which is continuous between the tentacles and the anterior region.

The frenulum of Sclerolinum is variable. In most species, a row of plaques, sometimes partially fused (Southward 1961; Webb 1964a), is developed. Only in S. major scattered plaques limited to a small region are reported (Southward 1972). In S. contortum GoM, however, we detected an intraspecific variation in the arrangement of the plaques, either in a row as in most other Sclerolinum species or in a more scattered distribution more similar to vestimentiferans. In the latter, plaques are distributed randomly along the outer surface of the vestimentum (Gardiner and Jones 1993; Southward 1991). The frenulate frenulum is a pair of cuticular crests, which develops from cuticular plaques in Nereilinum murmanicum (Ivanov 1975). Interestingly, the frenula of some species like Unibrachium colombianum (Southward 1972) or Siboglinum meridiale (Ivanov 1963) look like a Sclerolinum frenulum. We suggest that the Sclerolinum frenulum constitutes the plesiomorphic condition of siboglinids from which the frenulate frenulum as well as the scattered cuticular plaques covering the vestimentiferan vestimentum developed during evolution. This is neither in agreement with Ivanov (1994), who regarded the plaques of the vestimentiferans as analogous formations, nor in agreement with Rouse (2001), who interpreted the frenulate frenulum as the plesiomorphic condition. Further, Schulze (2003) scored the cuticular plaques of the Sclerolinum forepart and of the vestimentiferan vestimentum as absent.
The excretory organs are located in the anterior frenulate forepart and vestimentiferan vestimentum (Gardiner and Jones 1993; Ivanov 1963). In spite of careful investigations we could not locate an excretory system in *Sclerolinum*.

(2) Lacking a diaphragm between the forepart and the trunk we define the beginning of the *Sclerolinum* trunk internally with the beginning of paired trunk coelomic cavities, the reproductive system, and the trophosome and externally by the gonopores. This corresponds to the organization of vestimentiferans (Webb and Ganga 1980).

The trunk of *S. contortum* GoM, starting at the level of the gonopores and terminating with the first opisthosomal septum, is homologous to the vestimentiferan trunk, which develops from the posterior part of the first chaetiger (Bright et al. submitted). Evidence comes from comparative adult morphology. Vestimentiferans and *Sclerolinum* have a trunk lacking a ventrally ciliated region, but covered irregularly by small plaque-bearing papillae (Gardiner and Jones 1993; Malakhov et al. 1996a; Webb and Ganga 1980). *Sclerolinum* has girdles of uncini at the end of the trunk (Southward 1972). These might correspond to chaetae found in the first chaetiger of larval (Bright et al. submitted) and juvenile vestimentiferans (Jones and Gardiner 1989; Southward 1988; Southward et al. 2011) but are lost in adults. The *Sclerolinum* and vestimentiferan trophosome extends over the whole length of the trunk and originates from the visceral mesoderm (Bright and Sorgo 2003; Eichinger et al. 2011).

In contrast, the frenulate trunk is anteriorly ciliated (originating the posterior part of the neurotroch according to Callsen-Cencic and Flügel (1995)), exhibits papillae restricted to specialized regions and girdles at mid-trunk position (Ivanov 1963; Webb and Ganga 1980). The trophosome is restricted to the posterior two thirds of the trunk and develops from the endoderm of the gut (Callsen-Cencic and Flügel 1995; Southward 1993).

The metameric trunk region of frenulates is characterized by two rows of large papillae containing pyriform glands. According to Ivanov (1963) these papillae are bulges of the epidermis, that are separated from the general body cavity by a basement membrane and musculature. The glands are confined to these papillae. In contrast, in vestimentiferans small epidermal elevations,
called papillae as well, are covering the trunk irregularly. These papillae are the openings of pyriform glands as well, however, they extend deeply between the muscular tissue (Malakhov et al. 1996a; van der Land and Norrevang 1977). Our investigations clearly revealed that the two lateral rows of epidermal papillae restricted to the anterior trunk of *S. contortum GoM* are simple epidermal elevations as described for vestimentiferans. They are the openings of pyriform glands extending deeply into the body cavity.

Due to these structural differences we do not consider the anterior trunk region of *Sclerolinum* as homologous to the metameric region of frenulates. This is in consensus with Rouse (2001) and Rouse and Pleijel (2001). On the contrary, Schulze stated that the metameric region was the only synapomorphy for frenulates and *Sclerolinum* and placed *Sclerolinum* as sister to frenulates. Also, the combined molecular and morphological analysis of Rousset et al. (2004) scored the metameric region as present in *Sclerolinum* and the tree based on morphological data only shows *Sclerolinum* as basal to frenulates.

The metameric region varies at the species level in frenulates (Ivanov 1963; Southward 1972). Some species, such as *Nereilinum murmanicum* and *Oligobrachia dogieli* (Ivanov 1963), exhibit pyriform glands but lack papillae. We hypothesize that the last common ancestor of Siboglinidae had an anterior trunk region, similar to *Sclerolinum*, provided with two rows of simple epidermal papillae. From this condition the frenulates elaborated metameric papillae restricted to the anterior trunk and arranged in two rows derived, whereas in vestimentiferans the simple papillae became scattered along the whole trunk.

The *Sclerolinum* body lacks any internal partition except of the opisthosomal septa. Instead, there are some massive thickenings of the epidermis and longitudinal muscle layer in combination with highly abundant cuticular plaques in the posterior trunk region, which most probably serve locomotion. Ivanov and Selivanova (1992) may have erroneously referred to such a constriction of the body cavity by describing a septum behind a very long forepart (called mesosoma) of *S. javanicum*. 
(3) Despite some peculiarities, the opisthosoma of *Sclerolinum contortum* GoM is homologous to the vestimentiferan and frenulate opisthosoma. It has a median mesentery, present in vestimentiferans, but missing in adult frenulates (Southward 1975). The opisthosomal septa of *S. contortum* GoM are composed of two myoepithelial layers as described for the vestimentiferan metatrochophore (Bright et al. submitted) and the adult vestimentiferan *Riftia pachyptila* (Jones 1981). The vestimentiferan *Ridgea piscesae* (Southward et al. 2005) and the frenulate *Siboglinum fiordicum* (Southward 1975) have only the posterior epithelia of the septa muscular.

We found one type of multicellular glands in the opisthosoma of *S. contortum* GoM. These glands are connected to the blood vascular system and resemble neither structurally nor cytologically the typical pyriform glands of Siboglinidae. The adult vestimentiferan *R. pachyptila* has two kinds of multicellular opisthosomal glands, one described as short broad, the other one as long and slender. Both differ histologically from the pyriform glands of the rest of the body, but only the long and slender type is associated with blood vessels (Jones 1980). In *R. piscesae* peripheral pyriform glands are distinguished from central glands surrounded by blood lacunae (Southward et al. 2005). However, the vestimentiferan metatrochophore exhibits pyriform glands only in the first and second chaetiger (Bright et al. submitted). Consequently, either the opisthosomal glands linked to the blood vascular system develop later during ontogeny, or they represent modified pyriform glands. Frenulates lack multicellular glands in the opisthosoma (Southward 1975).

The opisthosomal blood vascular system of *Sclerolinum* consisting of one ventral and one dorsal vessel linked by blood lacunae located within the median mesentery is unique within Siboglinidae since in the vestimentiferan *Riftia pachyptila* as well as in the frenulate *Siboglinum fiordicum* the longitudinal blood vessels communicate through septal vessels within the opisthosoma only (Gardiner and Jones 1993; Southward 1975).

Another unique characteristic of the *Sclerolinum* opisthosoma is a broad ventral nerve field. One single nerve cord is described from the vestimentiferan opisthosoma (Jones 1980; Malakhov et al. 1996a) and three separated nerve cords from the opisthosoma of the frenulate *S. fiordicum* (Southward 1975).
Reproductive systems and reproduction

In Sclerolinum contortum GoM as well as in vestimentiferans sex can be differentiated externally by the presence of ciliated grooves extending anteriorly from male gonopores (Gardiner and Jones 1993; Webb 1977). The filiform mature spermatozoa of Sclerolinum are of the modified sperm type sensu Franzén (1956) and correspond with the sperm morphology found in other Siboglinidae, including Osedax species. In all four siboglinid taxa mature sperm are elongated cells with a helical acrosome, a helical nucleus wrapped by mitochondria and a long flagellum (Franzén 1973; Gardiner and Jones 1985, Katz unpubl. Ph D thesis). The spermatozoa of Sclerolinum lie unpackaged within the sperm ducts. Free sperm is also reported from Osedax (Katz unpubl. Ph D thesis), whereas in frenulates sperm is bundled into spermaphores and in vestimentiferans into spermatozeugmata (Ivanov 1963; Jones and Gardiner 1985).

Only the right side of the female reproductive system exists in S. contortum GoM forming a U-shaped system. A bent female reproductive system is the norm in vestimentiferans and frenulates (Hilário et al. 2005; Ivanov 1963; Webb 1977). An asymmetry of the female reproductive system with one side much shorter than the other is reported from the vestimentiferans Lamellibrachia barhami (Webb 1977), Tevnia jerichonana (Gardiner and Jones 1993) and Ridgeia piscesae (Malakhov et al. 1996c), but not in Riftia pachyptila (Gardiner and Jones 1993). No asymmetry is mentioned from frenulate females. On the other hand, females of Osedax have one long gonoduct running along the trunk possibly leading to a single ovary filling the ovisac (Rouse et al. 2004; Rouse et al. 2008).

As far as known, fertilization is internal in frenulates (Southward 1999), Osedax (Rouse et al. 2009; Rouse et al. 2008) and vestimentiferans (Hilário et al. 2005). Female vestimentiferans have a region of sperm storage at the posterior end of the oviducts, and zygotes are released as primary oocytes (Hilário et al. 2005). Although, we could not detect sperm within the pouch of the oviduct by using light microscopy, it probably represents a spermatheca. This would point to an internal insemination in Sclerolinum as well. Nevertheless, investigations on sperm uptake, fertilization, and larval development of Sclerolinum are urgently needed.
Acknowledgements

We would like to thank C. R. Fisher for inviting us to the cruise “Expedition to the Deep Slope”, the captain and crew of the NOAA Ship Ron Brown, the crew of the ROV Jason for their expertise and assistance. Special thanks to D. Gruber and G. Spitzer (Core Facility for Cell Imaging and Ultrastructure Research) for their helpful support. The production of the semithin section series by T. Schwaha is highly acknowledged. This study was financially supported by the Austrian Science Foundation P20282-B17 and the Initiativkolleg ‘Symbiotic Interactions’ of the University of Vienna. Northeast Atlantic specimens of *Sclerolinum contortum* were kindly provided by A. Andersen. They were collected during a program supported by the HERMES project, EC contract no GOCE-CT-2005-511234, funded by the European Commission’s Sixth Framework Program under the priority ‘Sustainable Development, Global Change and Ecosystems’. The phylogeny work has been achieved with the support of the European Community Seventh Framework Programme (FP7/2007-2014) under the HERMIONE project (Hotspot Ecosystem Research and Man’s Impact on European Seas), grant agreement no. 226354.

References


**Figure 1**: Phylogenetic tree of the Siboglinidae, including species of vestimentiferans, *Sclerolinum*, *Osedax*, and frenulates. *Myriochele* sp. (Polychaeta; Oweniidae) was used as an outgroup. The tree was built by neighbor-joining on a Kimura-2-Parameter distance calculated on a 471 bp alignment of a mitochondrial 16S rRNA fragment. Bootstrap values given only when greater than 500 out of 1000 replicates. Accession numbers are given between parentheses for each branch. Location of collection given for *Sclerolinum* only.
Figure 2: Tubes of *Sclerolinum contortum* GoM with characteristic anterior curled and posterior straight part.
Figure 3: General morphology (LM). a Anterior end of the worm within the transparent and collapsed anterior part of the tube. b Forepart with dorsal furrow and frenulum consisting of cuticular plaques arranged in a row. c Frenulum of other specimen consisting of scattered cuticular plaques. d Cuticular plaques of the trunk. e Multisegmented opisthosoma with rings of uncini (double arrowhead). f Uncini of opisthosoma. Abbreviations: cl = cephalic lobe; df = dorsal furrow; te = tentacle; arrowhead = frenular plaque; double arrowhead = uncini
Figure 4: Semithin section series of tentacles and forepart. a Left tentacle at distal position with vascularized epidermis overlaying a single layered myoepithelium (arrowhead) surrounding a central coelomic cavity. Right tentacle at proximal position with mesenchyme filling the coelomic cavity. Each tentacle with two blood vessels (asterisk). b Base of cephalic lobe and of tentacles and beginning of the dorsal furrow; cephalic lobe with the nervous center consisting of central neuropil and peripheral somata; tentacles with mesodermal strands. c Forepart anterior to the frenulum with densely packed pyriform glands, single ventral nerve cord and paired dorsal blood vessel (asterisk). d Forepart posterior to the frenulum with pyriform glands loosely distributed from dorsal to lateral and ventral nerve encasing the ciliated field. Abbreviations: bl = blood lacuna; cc = coelomic cavity; cf = ciliated field; ep = epidermis; df = dorsal furrow; dv = dorsal blood vessel; me = mesoderm; ml = body wall muscle layer; nc = nerve cord; np = neuropil; py = pyriform gland; sg = single gland cell; so = somata; vv = ventral blood vessel
**Figure 5**: Region of genital openings and muscles encircling the dorsal blood vessel (arrowhead) (LM). **a** Transverse section of female immediately posterior to the dorsal furrow showing the opening of a single oviduct (arrow). **b** Transverse section of male at the end of the ciliated grooves showing the genital papillae, the openings of the sperm ducts. Abbreviations: cc = coelomic cavity; cf = ciliated field; cg = ciliated groove; ep = epidermis; gp = genital papilla; ms = mesenchyme; py = pyriform gland; sd =, sperm duct; vv = ventral blood vessel
Figure 6: Semithin section series of anterior trunk region with only a few bacteriocytes embedded within a non-symbiotic mesenchyme a-b and posterior trunk region with massive bacteriocyte tissue c-d. a Lateral pyriform glands opening into epidermal papillae devoid of cuticular plaques; dorsal blood vessel clogged by the intravasal body. b Ventral and dorsal blood vessel connecting to blood lacunae (arrowhead); epidermis with papilla bearing cuticular plaque (double arrowhead). c Posterior end of the trophosome showing bacteriocytes filling the body cavity. d Thickening of epidermis and longitudinal muscle layer in combination with dense arrangement of cuticular plaques (double arrowhead). Abbreviations: bc = bacteriocyte; bl = blood lacuna; cc = coelomic cavity; dv = dorsal blood vessel; ep = epidermis; od = oviduct; iv = intravasal body; lp = lateral papilla; ml = body wall muscle layer; ms = mesenchyme; py = pyriform gland; vv = ventral blood vessel
Figure 7: Semithin section series of the opisthosoma. a Opisthosomal septum consisting of an anterior circular and a posterior longitudinal myoepithelial layer. b Multicellular epidermal glands with prominent nuclei (arrowhead) filling the coelomic cavity of the opisthosoma. Median mesentery (arrow) provided with blood lacunae and suspending the ventral and dorsal blood vessel. Last one at a more median position. Double arrowhead = uncini. Abbreviations: bl = blood lacuna; cc = coelomic cavity; cm = circular muscle layer; dv = dorsal blood vessel; eg = epidermal gland; ep = epidermis; lm = longitudinal muscle layer; vv = ventral blood vessel
Figure 8: Ultrastructure of body wall layer and nervous system. a Body wall of tentacle composed of vascularized epidermis and a single layer of longitudinal muscle cells; in between basal matrix (arrowhead). b Detail of ventral ciliated field of the forepart showing neuropil of one cord of the bifurcated ventral nerve and small nerves (arrow head) innervating the ciliated cells. c Ciliated cells forming part of the dorsal furrow of male specimen. d Ventral nerve cord and body wall layer of the posterior trunk region. Abbreviations: bc = bacteriocyte; bl = blood lacuna; cc = coelomic cavity; cf = ciliated field; cu = cuticle; ep = epidermis; mc = myocyte; ml = body wall muscle layer; mm = median mesentery; np = neuropil; vv ventral blood vessel
Figure 9: Ultrastructure of pyriform glands of forepart a-e and trunk f. a Pyriform gland anterior to the frenulum b with cytoplasm containing amorphous secretion products (asterisk) and rER. c Pyriform gland posterior to the frenulum d with cytoplasm characterized by electron-dark granules (asterisk) and golgi complexes. e Detail of a pyriform glandular cell in the region of the ciliated field with granules (asterisk). f Pyriform gland of the trunk, one glandular cell containing intracellular bacteria (arrow). Abbreviations: bc = bacteriocyte; gl = glandular lumen; gc = golgi complex; mv = microvilli; nu = nucleus; rER = rough endoplasmatic reticulum
Figure 10: Ultrastructure of epidermal multicellular glands of the opisthosoma. a Overview of epidermis, muscle layer with glandular duct (arrowhead) and glandular cell with rER in concentric circles, next to blood lacuna. b Glandular cell with large lobed nucleus and nucleolus. c Detail of glandular epithelium showing apical junctional complex (arrowhead) and cytoplasm full of electron-light granules containing electron-dark patches. Abbreviations: bl = blood lacuna; ch = chaetae; cu = cuticle; gl = glandular lumen; ml = body wall muscle layer; mv = microvilli; ne = nucleolus; nu = nucleus; rER = rough endoplasmic reticulum
Figure 11: Female reproductive system. **a** Semithin transverse section of the single ovary provided with small blood vessels (asterisk), containing oocytes, located between the oviduct and the ventral blood vessel. **b** Ultrastructure of the oviduct composed of an inner ciliated epithelium with apical junctional complexes (arrowhead) and a basal matrix (double arrowhead) surrounded by a myoepithelium. **c** Oocyte in the first meiotic prophase full of yolk granules and lipid droplets surrounded by a small blood vessel, blood lacuna and flattened follicle cells. **d** Oocyte in direct contact with blood lacuna ramifying into the oolemma (arrowhead). **e** Egg envelope consisting of extracellular matrix penetrated by microvilli. **f** Light microscopy of oocyte. Abbreviations: bc = bacteriocyte; bl = blood lacuna; bv = blood vessel; cc = coelomic cavity; ci = cilia; ep = epidermis; fc = follicle cell; ge = germinal vesicle; ld = lipid droplet; mc = myocytes; ml = body wall muscle layer; mm = median mesentery; ms = mesenchyme; mv = microvilli; ne = nucleolus; oc = oocyte; od = oviduct; vv = ventral blood vessel; y = yolk granule
**Figure 12:** Ultrastructure of the male reproductive system. 

**a** Sperm ducts left and right to the ventral blood vessel packed with sperm. 

**b** Epithelium of the sperm duct with apical junctional complexes (arrowhead) surrounded by the coelomic lining (left side); in between basal matrix (double arrowhead). 

**c** Longitudinal section through spermatozoa. 

**d** Longitudinal section through the thread-like acrosome attached to the head region. 

**e** Transversal section through the nuclear grooves occupied by mitochondria (arrowhead) and the cilia. 

**f** Longitudinal section through the basal region of the nucleus, the centriolar region (arrowhead) and flagellum. 

Abbreviations: ac = acrosome; fl = flagellum; ml = body wall muscle layer; mm = median mesentery; sd = sperm duct; nu = nucleus; vv = ventral blood vessel
<table>
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<td>n = 18</td>
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<td>regularly bent anteriorly straight posteriorly</td>
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<tr>
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**Table 1**: Comparison between morphological characters of the *Sclerolinum contortum* populations from the Haakon Mosby Mud Volcano (HMMV) and the Gulf of Mexico (GoM) modified from Smirnov 2000. Abbreviations: d = dorsal; l = lateral; v = ventral
Manuscript 3

From small vesicles to giant crystals: symbiont driven sulfur crystal formation in a thiotrophic symbiosis from the deep-sea hydrocarbon seeps

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Publication status: in preparation for submission to in Environmental Microbiology Reports

Personal contributions of Irmgard Eichinger:

designed the study

collected the material during the cruise to the Golf of Mexico in 2007

fixed and embedded the samples for all applied techniques

performed 16S rRNA sequencing, FISH, TEM, LM

wrote the manuscript
Summary

The gutless polychaete *Sclerolinum contortum* inhabiting sulfidic sediments of hydrocarbon seeps in the Gulf of Mexico harbors endosymbiotic bacteria. Inferred from phylogenetic analyses of the 16S rRNA gene the symbionts are presumed to oxidize sulfide to provide energy for the symbiotic association specifically formed at host species level. The symbiotic partners depend on sulfide as an energy source and simultaneously, have to cope with its toxicity. This study reveals countless large sulfur crystals restricted to the posterior trophosome, the symbiont housing organ. These crystals have the same S8 sulfur configuration as the small bacterial sulfur vesicles deposited in the posterior symbionts. We propose that the symbiotic bacteria produce transient, non-toxic sulfur vesicles in response to excess of sulfide relative to oxygen supply, which may be reutilized for oxidation when oxygen becomes available again or may be deposited permanently as non-toxic sulfur crystals. Besides nutritional advantages of living in thiotrophic symbiosis, we emphasize that sulfide detoxification performed by the symbionts may have been an initial evolutionary force for their establishment and continues to remain an important interaction mechanism for their maintenance.
Introduction

Hydrogen sulfide is a rich energy source for chemoautotrophic, sulfide-oxidizing bacteria but it is also highly toxic to aerobic organisms due to its inhibition of the respiratory enzyme cytochrome c oxidase at even nanomolecular concentrations (National Research Council, 1979). Nevertheless, sulfidic marine environments like reduced sediments, hydrothermal vents, hydrocarbon seeps, whale and wood falls, sewage outfalls, and mangrove swamps are inhabited by diverse organisms many of them living in symbiosis with thiotrophic bacteria (Dubilier et al., 2008). Such animal and protist hosts provide their symbionts with reduced sulfur species and oxygen for chemoautotrophy but at the same time need to prevent sulfide poisoning.

Also *Sclerolinum* tubeworms, which form together with vestimentiferans, *Osedax*, and frenulates the symbiotic living monophyletic polychaete taxon Siboglinidae, dwell in reducing marine habitats with access to oxygenated seawater. One species *Sclerolinum contortum* Smirnov, 2000 is reported from arctic cold seeps (Lösekann et al., 2008; Lazar et al., 2010), an arctic hydrothermal vent field (Pedersen et al., 2010), and hydrocarbon seeps of the Gulf of Mexico (Eichinger et al. 2012, submitted).

Adult siboglinids live in obligate symbiosis with intracellular bacteria. At least three distinct clades of *Gammaproteobacteria* thrive in symbiosis with Siboglinidae. Thiotrophic symbionts of vestimentiferans and *S. contortum* are divergent from both the thiotrophic or methanotrophic bacteria associated with frenulates as well as the heterotrophic bacteria of *Osedax* suggesting a specific host-symbiont association at higher taxonomic level (McMullin et al., 2003; Goffredi et al., 2005; Lösekann et al., 2008; Thornhill et al., 2008).

Siboglinids lack a digestive system. They develop a highly vascularized organ, the trophosome, where bacterial symbionts encased by membrane-bound symbiosomes are accommodated within host cells termed bacteriocytes (Cavanaugh et al., 1981; Southward, 1982; Eichinger et al., 2011; Katz et al., 2011). Symbionts of *S. contortum* were suggested to exhibit a cell cycle directed from anterior to posterior within the trophosome located in the worm’s trunk region. Anteriorly, a small proliferating bacterial stem population is housed in a few bacteriocytes. In the posterior tro-
phosome region, however, the bacteriocytes invading the whole body cavity are full of bacteria containing membrane-bound S8 sulfur vesicles. Between these intact symbionts some degrading bacteria are scattered (Eichinger et al., 2011).

In the sister taxon of *Sclerolinum*, the vestimentiferans, carbon dioxide is transported freely dissolved in the blood, whereas oxygen and sulfide bind simultaneously and reversibly to two different kinds of haemoglobins (Arp and Childress, 1983; Arp et al., 1987). These carriers transport and release sulfide to the symbionts while simultaneously suppressing spontaneous oxidation of sulfide and protecting the host tissue and the symbionts from sulfide toxicity by holding free sulfide concentrations low. *S. contortum* has haemoglobins with the same structure (Meunier et al., 2010) but a sulfide binding ability has not been demonstrated yet.

Since the discovery of the symbiotic lifestyle of Siboglinidae main emphasis was put on the nutritional relationship. However, here we investigate the possibility that the symbionts protect their hosts from harmful effects of sulfide by oxidizing it, a crucial initial adaptive value in establishing symbiosis (Vismann, 1990; Vismann, 1991a). In this study, the symbiotic bacteria of *S. contortum* from the hydrocarbon seeps of the Gulf of Mexico were identified based on 16SrRNA gene sequencing, comparative sequence analyses, and specific fluorescence *in situ* hybridization (FISH). By using high-pressure freezing and freeze substitution for light microscopy (LM), transmission electron microscopy (TEM), scanning electron microscopy (SEM), Raman microspectroscopy, and energy dispersive x-ray analysis (EDX), we discovered large sulfur crystals within the trophosome, exclusively restricted to posterior areas of the animals that are *in situ* exposed to sulfide in the sediments. We reason that the endosymbionts play a major role in sulfide detoxification by producing nontoxic sulfur crystals thus enabling the host to colonize this stressful environment.
Results and discussion

Habitat chemistry

We discovered three sites (WR269, AC818, AC601) of hydrocarbon seeps in the Gulf of Mexico in 2006 and 2007, which were densely populated by *Sclerolinum contortum* tubeworms (Eichinger et al. submitted). One core containing tubeworms was recovered from the site WR 269 (26°41.150’N, 91°39.566’W; 1900m depth) in 2006 and used for sulfide and sulfate reduction measurements. Sulfide as well as sulfate reduction activity were below detection limit above the sediment surface, indicating the overlain water was oxygenated. No sulfide was detected in the upper sediment layer of 0-3 cm depth but increased from Σ 300 μmol H₂S (i.e. total species of labile sulfide) measured in 3 cm depth to Σ 5 mmol H₂S in 5 cm depth and fluctuated between 13 and 17 mmol H₂S between 5 and 19 cm depth. Integrated over the upper 13 cm, the resulting sulfate reduction rates were 9.85 mmol m⁻² d⁻¹. This points to anoxic conditions in the sediment since sulfide was present in considerable amounts and sulfate reduction is performed by anaerobic bacteria (Vismann, 1991a).

16S rRNA gene phylogeny of the symbiont

One bacterial phylotype was identified by sequencing the 16S rRNA gene of nine clones obtained after PCR of bacterial DNA isolated from trophosomal tissue of one *Sclerolinum contortum* specimen from site WR 269 sampled in 2007 (Supporting information). The software Pintail (Ashelford et al., 2005) indicated that the obtained sequences were not chimeric. The consensus sequence (Genbank accession number HE614013) was 100% identical to the *S. contortum* endosymbiont from the arctic Haakon Mosby Mud Volcano which is in addition also characterized by the presence of the functional genes cbbM and aprA, indicative for autotrophy and sulfur oxidation (Lösekann et al., 2008).

In phylogenetic analyses performed using ARB (Ludwig et al., 2004), the *S. contortum* symbiont sequences formed a stable clade with the endosymbiont of the vestimentiferan *Escarpa spicata* (98.5% similarity) from a Guaymas Basin vent and an uncultured bacterium (98% similarity) associated with tubes of the vestimentiferan *Lamellibrachia* sp. from cold seeps of the Mediter-
ranean Sea (Fig 1). Similarity to all other organisms in a cluster of gammaproteobacterial symbionts was below 95%. These results point to a wide geographic distribution of the symbiont of *S. contortum* and to a specific association between the host and its symbiont. In comparison, little specificity at host species level exists in the sister taxon vestimentiferans. The larval vestimentiferan host acquires the locally available free-living symbiotic bacterial strain associated with the specific chemosynthetic ecosystem type – seep or vent (Feldman et al., 1997; Di Meo et al., 2000; Nelson and Fisher, 2000; McMullin et al., 2003; Vrijenhoek et al., 2007). Consequently, it seems surprising that the *S. contortum* symbiont did not cluster with symbionts of cold seep vestimentiferans from the Gulf of Mexico, like *Escarpia laminata*, *Seepiophila jonesi* or *Lamellibrachia luymesi*, but instead is most closely affiliated with the endosymbiont found in the vestimentiferan *E. spicata* from a sedimented hydrothermal vent habitat of Guaymas Basin (Di Meo et al., 2000). Interestingly, *S. contortum* is also known from hydrothermal vents (Pedersen et al., 2010), however genetic information on the symbiont is still lacking.

The exclusive presence of bacteria of the isolated 16S rRNA phylotype within the trophosome was confirmed by FISH with specific oligonucleotide probes (Supporting information). Double hybridizations conducted on LR white semithin sections from four animals (WR 269, AC 818) showed a hybridization pattern of the symbiont-specific probe identical to the general probes specific for most *Bacteria* and *Gammaproteobacteria*, respectively. Moreover, the symbiont-specific probe hybridized with all DAPI stained bacteria (Fig 2).

**Deposition of sulfur crystals within the posterior trophosome**

Following techniques were applied to *Sclerolinum contortum* specimens recovered in 2007. Five specimens, 5.4 to 8.6 cm in length, viewed under a dissecting microscope revealed countless, giant crystals even visible through the worm’s tube (Fig. 3A). Two kinds of water insoluble crystals were detected: needle-shaped ones up to 50 µm in length and orthorhombic ones up to 150 µm in length (Fig. 3B, C). LM of whole mounts suggested a restricted distribution of the crystals to the posterior
trophosome. Regions tightly packed with needle-shaped crystals were interspersed with clumps of orthorhombic ones (Fig. 3D).

To preserve the crystals and to confirm their limited presence in the posterior trophosome semithin sections of high-pressure frozen and freeze-substituted samples, infiltrated by Lowicryl HM20 resin, were produced (Supporting information). All detected crystals were restricted to cavities between bacteriocytes filled with bacteria (Fig. 3E).

Two types of elemental analyses were applied to isolated needle-shaped and orthorhombic crystals. EDX spectra of both crystal types were indicative for sulfur (Figure 4). Raman microspectroscopic analysis with a spectral resolution of about 1.5 cm⁻¹ performed according to Eichinger et al. (2011) resulted in spectra indicating that both crystal types consisted of rhombic S8 sulfur (Fig. 5).

**Bacterial driven crystallization process**

Combining static TEM micrographs into a reasonable process of crystal formation we propose that the large elemental sulfur crystals are produced by the symbionts in the posterior trophosome. These bacteria were characterized by discernible cell walls and a moderately electron-dense cytoplasm containing glycogen, chromatin strands and small, membrane-bound, electron-translucent sulfur vesicles (up to 2 µm). The symbionts involved in crystal formation were intact, which was in clear contrast to the condensed and deformed bacteria in degradation (Eichinger et al., 2011). As a first step, the membranes of some bacterial sulfur vesicles disintegrate. Such remnants of sulfur vesicles were seen as diffuse electron-translucent patches within the bacterial cytoplasm (Fig. 6A). As a second step, the symbiont’s cell wall and the symbiosome membrane disintegrate releasing the remnants of the sulfur vesicles into the bacteriocyte’s cytoplasm (Fig. 6B). Here sulfur accumulates, which is visible as conspicuous, electron-translucent areas often with straight edges typical for crystals (Fig 6C). Such areas, interpreted as holes of dissolved crystals, were completely surrounded by intact looking bacteria encased by a cell wall except of the part adjacent to the crystals. Crystallization processes were observed in bacteriocytes with an intact nucleus and cytoplasm rich
in glycogen. Both, sulfur vesicles and sulfur crystals have of the same sulfur S8 configuration supporting the bacterial origin of the crystals. We have no evidence for dissolution of sulfur crystals, indicating a possible accumulation over time. The specimens studied here, were all of similar size. Therefore it was not possible to study whether the number of crystals was correlated with the animal’s size.

**Bacterial driven sulfide detoxification**

*In situ*, these animals inhabit tubes approximately twice as long as their body. The tubes are buried vertically deeply into anoxic sediment with variable amounts of sulfide. Only a small, anterior part of the tube extends above the sediment into oxygenated seawater.

We suggest that oxygen uptake is performed by the two highly vascularized anterior tentacles (Eichinger et al. submitted) and that sulfide uptake happens through the buried part of the tube by diffusion from the sediment porewater, similar to the uptake mechanisms of seep vestimentiferans (Julian et al., 1999). In *Sclerolinum*, as in all polychaetes, blood flows from anterior to posterior in the ventral vessel and in the opposite direction in the dorsal vessel. The amount of oxygen remains the same within the ventral vessel along the longitudinal body axis. The two longitudinal vessels are connected with a network of intercellular blood lacunae and in the trophosome blood flows from the ventral vessel between the bacteriocytes to the dorsal vessel. The *Sclerolinum* trophosome exhibits a gradient from anterior to posterior expressed in a strong increase of the amount of bacteriocytes, the bacterial mass within the bacteriocytes as well as the abundance of the bacterial sulfur vesicles. Eichinger et al. (2011) reasoned that in the anterior trophosome the amount of oxygen is sufficient for host and bacterial metabolism. Whereas in the posterior trophosome, the same amount of oxygen has to meet the metabolic needs of the host as well as of a highly amplified bacterial mass. Additionally, sulfide diffusion into the posterior trophosome might be increased due to deeper sediment layers. Sulfide supply exceeding oxygen supply results in partial oxidation of sulfide and storage of sulfur vesicles by the posterior symbionts. These vesicles can be oxidized if sulfide diffusion or host metabolism is low. However, in the case of constant excess in sulfide the
symbionts are accumulating sulfur. We suggest that bacterial crystal formation is a means to get rid of excess sulfur. Sulfur crystals were also reported from the vestimentiferan *Riftia pachyptila* early on (Jones, 1980; Cavanaugh et al., 1981), but have been neglected since then.

Elemental sulfur combines two advantages in sulfidic environments. It is non-toxic and it does not require oxygen atoms for its formation from sulfide, in contrast to sulfate, the end product of sulfide-oxidation (Powell et al., 1980). Intracellular elemental sulfur vesicles are known from many thiotrophic symbionts such as from the vestimentiferan *Riftia pachyptila* (Pflugfelder et al., 2005), the gutless oligochaete *Inanidrilus leukodermatus* (Krieger et al., 2000), the giant ciliate *Zoothamnium niveum* (Maurin et al., 2010), the gutless platyhelminth *Paracatenula galateia* (Gruber-Vodicka et al., 2011), and several vesicomyid and lucinid clams (Vetter, 1985). They have been proposed to be formed under oxygen limitation as an intermediate energy-storage product that may be utilized when oxygen supply exceeds the rate of sulfide diffusion into the animal (Vetter, 1985). Nevertheless, to date no clear energy-conserving path has been identified linked with direct elemental sulfur oxidation. Rather it has been suggested that elemental sulfur formation represents a reversible side reaction (Nelson and Fisher, 1995).

This study however, indicates that in *S. contortum* the oxidation of sulfide to elemental sulfur performed by the symbionts additionally serves as sulfide detoxification. Sulfide conversion into different non-toxic sulfur compounds seems to be a widespread phenomenon in sulfidic environments. Sulfide oxidation activity has been demonstrated from *Nereis* species (Vismann, 1990) and the host tissue of the symbiotic clam *Solemya reidi* (Powell and Somero, 1985). The isopode *Saduria entomon* detoxifies sulfide to thiosulfate and sulfite (Vismann, 1991b), the vent crab *Bythograea thermydron* to thiosulfate and sulfate (Vetter et al., 1987). Representatives of Platyhelminthes and Gastrotricha inhabiting oxic-anoxic interfaces in coastal sediment produce elemental sulfur or thiosulfate as the primary end-products (Powell et al., 1980). However, in none of these animals or animal hosts large crystals were described.

Undoubtedly, the sulfur-oxidizing endosymbionts of *S. contortum* are nourishing the gutless host by exploiting the energy contained in sulfide as indicated by bacterial degradation found
in the posterior trophosome (Eichinger et al., 2011). Additionally, we propose that in excess of environmental sulfide relative to oxygen the endosymbionts are controlling sulfide toxicity by converting it to non-toxic elemental sulfur. It is either stored reversibly in vesicles or finally deposited in crystals. One could speculate that at the beginning of the establishment of siboglinid symbiosis the sulfur detoxifying function of the symbiont, protecting the host in its extreme, toxic environment, was a main driving evolutionary force and still remains an important factor for the living together.

**Acknowledgements**

This study was financially supported by the Austrian Science Foundation P20282-B17 and the Initiativkolleg Symbiotic Interactions of the University of Vienna. We would like to thank the captain and crew of the NOAA Ship Ron Brown, the crew of the ROV Jason for their expertise and assistance. Special thanks to M. Wagner (head of the Core Facility Raman Microspectroscopy, University of Vienna), D. Gruber and M. Weidinger (Core Facility for Cell Imaging and Ultrastructure Research) for their helpful support.

**References**


Figure 1 Phylogenetic relationship of the endosymbiont of *S. contortum* from the Gulf of Mexico and other gammaproteobacterial symbionts based on 16S rRNA gene sequences. A consensus tree calculated by the RaxML maximum-likelihood algorithm implemented in ARB is shown. A filter considering only positions which are conserved in at least 50% of all gammaproteobacterial 16S rRNA sequences was used for tree calculations. Maximum parsimony bootstrap values are depicted above the respective branches; only bootstrap values above 90% are shown, GenBank accession numbers are given in parentheses. Alphaproteobacterial 16S rRNA sequences were used as an outgroup. The arrow points to the outgroup, and the bar represents 10% estimated evolutionary distance. The sequence obtained in this study is highlighted in bold. Abbreviations: GoM = Gulf of Mexico; HMMV = Haakon Mosby Mud Volcano.

Figure 2 FISH of cross sections through the posterior trophosome region. Probe Gam42a in Fluos (green) targeting *Gammaproteobacteria* and probe Scon -467 in Cy5 (red) specific for the *S. contortum* endosymbiont were applied simultaneously. DAPI was used as a counter stain (blue). The symbionts appear pale pink due to combined signals. bc = bacteriocyte, ep = epidermis, ml = muscle layer, py = pyriform gland.
Figure 3 Crystals deposited in the *S. conorium* trophosome. A. Whole specimen within the tube viewed under a dissecting microscope containing orthorhombic (arrowhead) and needle-shaped crystals (double arrowhead). B-C. SEM of orthorhombic (B) and needle-shaped crystals (C). D. LM of whole mount of the posterior body region showing regions of densely packed needle-shaped crystals (double arrowhead) interspersed by orthorhombic ones (arrowhead). E. LM of high-pressure frozen and freeze-substituted sample of the posterior trophosome. Crystals are limited to the trophosomal tissue. ep = epidermis, ml = muscle layer, tr = trophosome.
both crystal types consisted of rhombic S8 sulfur (Ward, 1968). Weaker bands at 245 cm⁻¹ and 433 cm⁻¹ also assignable to S8 sulfur (Trofimov et al., 2009) could be observed, while the additional peak at 187 cm⁻¹ visible in the S8 sulfur spectrum was below background level. The main bands indicative for measured S8 sulfur are indicated by vertical dotted lines. The numbers indicate the position of the bands in S8 sulfur in cm⁻¹. The numbers in brackets indicate the wave numbers for the respective sulfur band in both crystal types.

**Figure 4** Representative EDX spectrum of isolated crystal with peaks at 0.14 keV, 0.56 keV, 2.33 keV, and 2.48 keV characteristic for sulfur. A small carbon peak at 0.27 keV is due to carbon coating.

**Figure 5** Raman microspectroscopy of the region between 100 cm⁻¹ and 600 cm⁻¹. (a) Raman spectrum of orthorhombic crystals. (b) Raman spectrum of needle shaped crystals. (c) Elemental S8 sulfur. Both crystal types showed very strong bands at about 470 cm⁻¹ (S-S stretching), 220 cm⁻¹, and 151 cm⁻¹ (both S8 bending), indicating that
Figure 6 Sulfur crystal formation by the symbionts (TEM). A. Symbionts with intact cell wall (arrow), sulfur vesicles and remnants of sulfur vesicles caused by disintegration of vesicle membranes, indicated by double arrowhead. B. Detail of symbiont with partially disintegrated cell wall. End of intact cell wall indicated by arrowhead. Remnant of sulfur vesicle next to sulfur crystal located within the bacteriocyte cytoplasm. C. One sulfur crystal completely surrounded by symbionts. Remnants of sulfur vesicles are located next to the crystal. Bacterial areas adjacent to the crystal are lacking a cell wall. asterisk = remnant of bacterial sulfur vesicle, ba = bacterium, sc, sulfur crystal, sv = sulfur vesicle.
Supporting information

16S rRNA gene sequencing of the symbiont
For purification of bacterial DNA from trophosomal tissue DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was applied. Bacterial 16S rRNA gene was amplified using 34 PCR cycles with primers 616V (Juretschko et al., 1998) and 1492R (Loy et al., 2005) and an annealing temperature of 50°C. PCR products of the desired size were cloned with TOPO TA cloning kit (Invitrogen Life Technologies, Lofer, Austria). Nucleotide sequences of cloned DNA fragments were determined on an ABI 3130 XL genetic analyzer using the BigDye Terminator kit v3.1 (Applied Biosystems, Austria).

Fluorescence in situ hybridizations
Ethanol fixed samples were embedded in LR-White resin after Nussbaumer et al. (2006), semithin-sectioned on a Reichert Ultracut S microtome, mounted on gelatine/chromalaun coated glass slides and dried on a hot plate less than 50°C overnight. Glass slides were subsequently dipped into 50% ethanol, 75% ethanol and 96% ethanol for three minutes each immediately before in situ hybridization with oligonucleotide probes. Hybridizations with an incubation time of 4.5h and subsequent staining with DAPI were carried out as described previously (Nussbaumer et al., 2006). We used universal bacterial probes (EUB338, (Amann et al., 1990) and GAM42a, (Manz et al., 1992)), a symbiont specific probe (Scon-467, (Lösekann et al., 2008)) and a nonsense probe (NON338, (Wallner et al., 1993)) as a negative control. A 10% formamide concentration gave the best signal for the symbiont specific probe and therefore was applied for all probes.

High-pressure freezing and freeze substitution for LM
Samples fixed in 4% formaldehyde buffered with 0.1 mol l⁻¹ phosphate-buffered saline (PBS) were high-pressure frozen using a Leica HPM 100 and stored at -150°C. Freeze-substitution and cryo-embedding in Lowicryl HM20 resin were carried out by using a Leica AFS 2 machine. For freeze-substitution temperature was kept at -90°C for 8 h, raised to -70°C within 2 h, kept at -70°C for 2 h, and raised to -50°C within 4 h. These steps were carried out in 0.1% OsO₄ in 100% acetone, followed by three washing steps with 100% acetone for 1 h each at -50°C. The samples were infiltrated by a 1:1 followed by a 1:2 100% acetone and resin mixture for 1h each. After infiltration with pure resin for 1 h samples were transferred to gelatine capsules filled with fresh resin and polymerised during a 24 h UV exposure at -50°C. Subsequent hardening of the resin at room temperature with UV light was necessary.
References


Discussion

This thesis gives insight into several aspects of the *Sclerolinum contortum* symbiosis from deep-see hydrocarbon seeps in the Gulf of Mexico

*Sclerolinum* is a small genus, but crucial to elucidate the evolution of Siboglinidae. Nevertheless, data on the anatomy of *Sclerolinum* were inconsistent and incomplete resulting in contradictory interpretations of the *Sclerolinum* body organization. For the first time we provide detailed information on the microanatomy of the entire *Sclerolinum* body, which is useful for further phylogenetic studies. This thesis contributes to the discussion about the evolution of the siboglinid trophosomes (Katz et al., 2011). Next, we deal with the question how homeostasis is balanced in the *Sclerolinum* symbiont-housing organ and we provide information on the metabolism of the symbiotic bacteria. Furthermore, we confirm the close relationship between the sister taxa vestimentiferans and *Sclerolinum*, and we propose a hypothesis on the *Sclerolinum* body organization.

The anatomical and ultrastructural studies conducted in this thesis provide a basis for the understanding of the *Sclerolinum* symbiosis as well as for further detailed studies dealing with particular questions and using different kinds of techniques.

Comparative sequence analyses revealed that both, host and symbiont species, are identical to the *Sclerolinum contortum* symbiosis from the arctic Haakon Mosby Mud Volcano indicating high specificity and a broad geographic distribution (Smirnov, 2000; Lösekann et al., 2008; Lazar et al., 2010; Pedersen et al., 2010). These results are discussed in the second and third manuscript.

In particular we were interested in the symbiont-containing organ, a key character of Siboglinidae. The first manuscript characterizes the *Sclerolinum* trophosome as a simple organized organ comparable to trophosomes of juvenile vestimentiferans (Nussbaumer et al., 2006; Bright and Lallier, 2010) supporting the sister taxa relationship between *Sclerolinum* und vestimentiferans. We provide a hypothesis on a cell cycle with terminal differentiation exhibited by the symbi-
otic bacteria and the bacteriocytes along the longitudinal body axis from anterior to posterior. We give evidence that the *Sclerolinum* trophosome derives from the visceral mesoderm and propose that the trophosome of the sister taxa vestimentiferans and *Sclerolinum* is a synapomorphic character that evolved in their last common ancestor. Possible scenarios of the evolution of siboglinid trophosomes are outlined, which are also discussed in Katz et al. (2011).

Not only small intracellular elemental sulfur vesicles produced as intermediate storage products, known from many thiotrophic symbionts (Vetter, 1985; Krieger et al., 2000; Pflugfelder et al., 2005; Maurin et al., 2010; Gruber-Vodicka et al., 2011), were detected, but also extremely large sulfur crystals between the symbionts were discovered. In the second manuscript we propose that in excess of sulfide relative to oxygen, the symbiotic bacteria convert sulfide to non-toxic elemental sulfur, either stored reversibly in vesicles or finally deposited in crystals, in order to control sulfide toxicity. We speculate that this peculiarity of the symbionts was a main driving evolutionary force enabling the host to colonize extreme environments and still remains an important interaction mechanism for the maintenance of the *Sclerolinum* symbiosis.

The overall body organization of *Sclerolinum* corresponds to the vestimentiferan body (Gardiner and Jones, 1993). The third manuscript suggests a division of the *Sclerolinum* body into a head, trunk, and opisthosoma, very similar to vestimentiferans (Bright et al. submitted).

**References**


Summary

*Sclerolinum* is a small genus of slender marine tubeworms, which together with vestimentiferans, *Osedax* and frenulates forms the monophyletic polychaete family Siboglinidae, famous for their symbiotic lifestyle. Siboglinids lack a digestive system as adults, but during their ontogeny they develop a highly vascularized organ, the trophosome, to accommodate bacterial endosymbionts.

A *Sclerolinum* population from deep-sea hydrocarbon seeps in the Gulf of Mexico was investigated to improve our knowledge of this highly debated but little studied siboglinid taxon. This study revealed that both, host and symbiont species, are identical to the *Sclerolinum contortum* symbiosis described from the arctic Haakon Mosby Mud Volcano. These results indicate high symbiont specificity at host species level, a broad geographic distribution of this symbiosis and a sulfide-oxidizing metabolism of the symbiont.

The symbiont housing organ of *Sclerolinum* was nearly unknown until now and an endodermal origin was favored. In this investigation the origin, ultrastructure and organization of the *Sclerolinum* trophosome are described. A visceral mesodermal origin is shown, which indicates that the trophosome of the sister clade vestimentiferans and *Sclerolinum* is a homologous character. A specific cell cycle within the symbiont-housing organ of *S. contortum* directed along the longitudinal body axis from anterior to posterior is described. Within the posterior trophosome small elemental sulfur vesicles produced by the symbionts as well as extremely large sulfur crystals between the bacteria were detected. A symbiont driven sulfide detoxification producing nontoxic crystals and therefore enabling the host to colonize a stressful sulfidic environment is discussed.

The anatomy of *Sclerolinum* was investigated for the first time and resulted in a detailed description of the organ systems. A *Sclerolinum* body organization, divided into a head, trunk, and opisthosoma, very similar to that of the vestimentiferans, is suggested.
Zusammenfassung


Danksagung


Liebe Sigrid, liebe Bettina P. ohne euch hätte ich den Wiedereinstieg in die Wissenschaft nicht geschafft – danke für eure Hilfe, Ratschläge und Tipps!

Sigrid, du hast mich über viele Jahre meiner Dissertationsszeit begleitet, mit vielen Höhen und Tiefen! Es war toll dich als „Projektpartnerin“ zu haben, mit dir Kongresse zu besuchen, zu wissen, dass du da bist, immer mit einem offenen Ohr und viel Sinn für Humor.

Ingrid, Bettina R., Julia – ihr seid die Besten! Es hat immer Spaß gemacht mit euch zu plaudern, Pausen zu machen, zusammen zu sein!

Danke an Frau Prof. Klepal, Daniela Gruber, Frau Dr. Weidinger und Jacqueline Montanaro-Punzengruber für großartige Hilfe und Unterstützung bei allen möglichen Arten von Fragen und Anliegen!

Danke an Thomas Schwaha für die unzähligen Semidünnschnitte!

Ich möchte der gesamten Abteilung für Meeresbiologie danken – es waren schöne Jahre mit euch!

Spezielles Danke schön an Uli und Harald, für die Hilfe im Labor und bei Computerschwierigkeiten!

Patrick, Fiona, Florian ich danke euch von ganzem Herzen für euer Verständnis für doch manch stressige Zeiten! Danke, dass ihr mich oft abgelenkt habt und die Bude toll geschupft habt, wenn ich nicht da war.

Meine lieben Eltern – Danke für eure großzügig Unterstützung und euer Verständnis für mein Interesse an Würmern!
Curriculum Vitae

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1999-2003        Teaching degree in biology for secondary schools

1997-1999        Master thesis at the Department of Marine Biology, University of Vienna

1996-1997        Studies at the University of Marseille, France (Erasmus Fellowship)

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Research Activities


Conferences

2011             2nd International Congress of Invertebrate Morphology, Boston, USA. June (talk)

2010             Symposium on Symbiotic Interactions, Vienna, Austria. November (talk)

2009             6th International Symbiosis Society Congress, Madison, USA. August (poster)
                  4th International Symposium on Chemosynthesis Based Ecosystems-Hydrothermal Vents, Seeps and Other Reducing Habitats, Okinawa, Japan. June (talk)

2008             ChEss workshop Siboglinidae: a model system for the understanding of evolution, adaptive radiation, microbial symbioses, and ecology at extreme environments, Honolulu, Hawaii, USA. October (talk)

                  1st International Congress of Invertebrate Morphology, Copenhagen, Denmark. August (talk)
Publications


in progress


Eichinger I., Hourdez S., Bright M. Morphology, microanatomy and sequence data of Sclerolinum contortum (Siboglinidae, Polychaeta) of the Gulf of Mexico. Submitted to Organisms Diversity & Evolution.

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