MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Infection of giant tubeworm skin by Endoriftia symbiont and Mollicutes"

verfasst von / submitted by

Regina Pechotsch BSc

angestrebter akademischer Grad/in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2016 / Vienna, 2016

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 833
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Master Ecology and Ecosystems
Betreut von / Supervisor:	Univ.-Prof. Dr. Monika Bright
Mitbetreut von / Co-Supervisor:	

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

ABSTRACT

After the discovery of hydrothermal vents in the deep sea, the symbiosis in Vestimentiferan tubeworms has been intensively investigated. The giant tubeworm *Riftia pachyptila*, which inhabits the East Pacific Ocean, is one well-known member of this group. *Riftia* is a gutless and mouthless worm and relies for its nutrition on a chemolithoautotrophic, sulfur-oxidizing gamma proteobacteria, *Candidatus* Endoriftia persephone. This endosymbiont inhabits host cells in a special organ known as the trophosome. It enters the host via horizontal transmission anew each host generation through the developing tube and skin when the tubeworm larvae settle. Further infection is terminated in juveniles by apoptosis of epidermis, muscles and mesodermal tissue. In adult tubeworms the symbiont is restricted to the trophosome and leaves its host only upon worm death. A longer transmission mode or the release of Endoriftia in live animals has not been shown yet. Beyond endosymbionts detected in the tube, Endoriftia was found only in the trophosome; the skin was considered to be free of bacteria. Two previous diploma theses changed these findings. Endoriftia was detected in the skin of adult individuals and in juveniles of *Riftia* from two different sites by denaturing gradient gel electrophoresis (DGGE) and fluorescence *in situ* hybridization (FISH). Additionally, another bacterium belonging to the class of Mollicutes was recorded in the skin by DGGE. Mollicutes, which is host dependent, is known for its pathogenic role in humans and as a beneficial partner in insects. Based on these new insights, a longer transmission mode and a release of symbionts or a migration into and out of the tubeworm was hypothesized. To determine the transmission timeframe and to include or exclude an infection by a new symbiotic bacterium besides Endoriftia, this study focuses on determining and localizing Endoriftia and Mollicutes in the skin of adult *Riftia pachyptila*. FISH was used to localize the symbiont and Mollicutes in skin samples from two different sampling sites. Beyond its localization in the trophosome, Endoriftia was found extracellularly in huge aggregations stuck to the outside of the skin, scattered in the coelom, and a few intracellularly inside the pyriform glands between the muscles of the skin. These results indicate a contamination during the separating process of the different tissues on the research vessel, in which the symbionts from the trophosome stuck to skin tissues and led to positive results in the above-mentioned diploma theses. The results also show that a few symbionts survive the apoptosis process inside the skin. No Mollicutes was detected inside or outside the skin of *Riftia*. This again suggests a contamination event during the lab work in the DGGE experiments of the diploma studies. Thus, the present study confirms a dual partnership between Endoriftia and *Riftia* after an initial infection in the larval stage of the host.

Keywords: giant tubeworm, chemolithoautotrophic endosymbiont, Mollicutes, fluorescence *in situ* hybridization

INTRODUCTION

The deep sea has been a mystery for a long time. It was believed, that no life was possible below several hundred meters' depth. In 1869 the biologist Wyville Thomson has disproved this belief, when he had detected life in 4600 meters' depth. Some year later the famous "Challenger"-expedition under the direction of Wyville Thomson has taken place. On this journey of discovery the deep-sea fauna has been investigated at many different locations of the Worlds oceans and animals and also deep-sea volcanoes has been discovered (Linklater, 1972; Thomson, 1873). Nearly hundred years later, in 1977, deep-sea hydrothermal vents and their particular fauna have been detected on the Galapagos Rift in the East Pacific (Corliss et al., 1979; Desbruyères & Segonzac, 1997). Deep-sea hydrothermal vents are located at back-arc basins, mid-ocean ridges and at some seamounts (Van Dover et al., 2002). Due to volcanic activity and tectonic events the hydrothermal vent environment exhibits a chronical instability (Desbruyères et al., 2006). High toxic fluid flow changes in fluid composition, temperatures from ambient deep-sea water ranging from 2°C to 4°C to more than 300°C to 400°C, minimal pH values of 4,4, and high pressures of more than 250 bar determine the life of the hydrothermal vent community (Elderfield & Schultz, 1996; Kelley et al., 2002; Le Bris et al., 2006; Von Damm, 2000). Organisms had adapted to all these extreme environmental conditions and had found a way to survive in the darkness of the deep-sea. As no photosynthesis is possible in the dark, scientists found out, that deep-sea hydrothermal vent ecosystems are based on microbial chemoautotrophic production (Ruby et al., 1981; Van Dover, 2000). Hydrogen sulfide, methane or hydrogen, which are spilling out of the hydrothermal vents (Corliss et al., 1979; Edmond et al., 1979; Lilley & Gordon, 1979) are the main energy source of chemosynthesis and generate carbon compounds (Cavanaugh et al., 1981; Jannasch & Wirsen, 1979; Ruby et al., 1981). Chemolithotrophic microorganisms, which are mainly located in vent effluents are the primary producers in the deep-sea environment (Van Dover et al., 2002). They use chemical inorganic compounds, e.g. like sulphide from hot vents, as an electronic donor, and oxygen as an electron acceptor to generate energy and fix afterwards inorganic carbon to produce organic carbon (Dubilier et al., 2008; Van Dover et al., 2002). These microbes are then filtered or grazed by invertebrates (e.g. barnacles and limpets) (Van Dover et al., 2002). Scientists discovered, that many invertebrates (e.g., vestimentiferan tubeworms, bivalve mollusks, provannid gastropods, and bresiliid shrimp) live in chemosynthetic symbioses with chemoautotrophic microorganisms as either epi- or endosymbionts (Cavanaugh et al., 2006; Dubilier et al., 2008; Van Dover et al., 2002). Chemosynthetic symbioses are not only found at hydrothermal vents, they occur as well in shallow-water sediments, continental slope sediments, cold seeps and whale and

wood falls (Bright et al., 2014; Dattagupta et al., 2009; Dubilier et al., 2008; Duperron et al., 2013; Ott et al., 2014; Petersen et al., 2016; Thurber et al., 2011). Generally, symbiosis is the association of two or more species, that live together independent of the effects on the fitness of each partner (de Bary, 1879). Three different types of symbioses are distinguished – commensalism, mutualism and parasitism (Van Beneden, 1875). In the commensalistic relationship, one partner benefits while for the other partner the interaction is regarded neutral (Leung & Poulin, 2008; Lewin, 1982). In contrast, the mutualistic relationship is considered as a form, where both partners benefit from this kind of symbiosis and in parasitic interactions one partner benefits and the other one is harmed (Leung & Poulin, 2008; Lewin, 1982).

The first host and most famous marine invertebrate, in which a chemoautotrophic symbiosis was discovered, was the giant tubeworm *Riftia pachyptila* (Polychaeta, Siboglinidae) (short Riftia) (Cavanaugh et al., 1981; H. Felbeck, 1981; M. L. Jones, 1981). Before the discovery of this animal in 1977 at the Galapagos Rift, only phototrophic and heterotrophic symbioses were known (Dubilier et al., 2008). *Riftia* is a vestimentiferan tubeworm and lives attached to cooled lava around hydrothermal vents alongside the East Pacific Rise (21°N-31°S), the Guaymas Basin, and at the Galapagos Spreading Center in 2500 meters depth (Desbruyères et al., 2006; Jones, 1981). The vestimentiferan exists at these sites in areas with a strong diffuse vent flow and in an environment with a changing temperature and chemical composition (Haymon et al., 1993; Hessler & Smitey, 1983; Hessler et al., 1985; Sarrazin et al., 1997; Shank et al., 1998). The fluids of these vents contain sulphide, methane, hydrogen, carbon dioxide, silicate and sometimes ferrous iron (Bright & Lallier, 2010). *Riftia* is called the giant tubeworm because its chitinous tube can reach a length up to 2 meters (Jones, 1981). With a growth rate of 85 cm/year it is the fastest growing invertebrate currently known (Lutz et al., 1994).

Riftia lives in an endosymbiosis with a chemolithoautotrophic sulfur-oxidizing gammaprotoebacterium called *Candidatus* Endorifitia persephone (short Endoriftia) (Robidart et al., 2008), which colonizes its host from the free-living population in the surrounding water of hydrothermal vents (Harmer et al., 2008; Nussbaumer et al., 2006). The 16S rRNA of Endoriftia was also found in two co-occurring vestimentiferan tubeworm species *Tevnia jerichonana* (Jones, 1985) and *Oasisia alvinae* (Jones, 1985) at the East Pacific Rise (Corliss et al., 1979) and in *Ridgeia piscesae* (Jones, 1985) at the Explorer Ridge, the Juan de Fuca Ridge and Gorda Ridge (Bright & Lallier, 2010).

Endoriftia is a chemolithoautotrophic bacterial symbiont, which indicates a special development of chemosynthesis named thiotrophy (Bright & Lallier, 2010). Reduced sulphur like sulphide is used as an electron donor and oxygen acts as an electron acceptor (Bright & Lallier, 2010; Dubilier et al., 2008). Under anoxic conditions nitrate

functions as an alternative electron acceptor instead of oxygen (Gardebrecht et al., 2012; Hentschel & Felbeck, 1993; Pospesel et al., 1998). The metagenome of the endosymbiont was published in 2008 (Robidart et al., 2008). The genome contains not only genes for sulfur oxidation and carbon fixation, it also encodes genes for another carbon fixation pathway, the reductive tricarboxylic acid (reverse TCA or rTCA) cycle (Bright & Lallier, 2010; Klose et al., 2016; Robidart et al., 2008). Moreover genes for chemoreception and motility were found, supposing that the free-living community of Endoriftia utilizes chemotaxis to meet their particular host (Bright & Lallier, 2010; Robidart et al., 2008). According to Gardebrecht et al. (2012), genes expressing fimbriae, pili or the flagellum were suggested to induce the connection of the symbiont to the hosts surface during the infection act. Additionally, genes for ABC transporters were detected indicating a heterotrophic metabolism that is useful for a life outside the host in the surrounding water (Robidart et al., 2008).

Riftia lives in a mutualistic relationship with its endosymbiont Endoriftia (Bright & Lallier, 2010; Klose et al., 2016) and is one of many invertebrates, that lives in a mutualistic association hosting chemoautotrophic bacteria as either epi- or endosymbionts (Van Dover et al., 2002; Vrijenhoek, 2010).

The host takes up sulphide, oxygen and carbon dioxide from the surrounding environment by its gas exchange organ, the plume, binds sulphide and oxygen to a special form of hemoglobin (Arp et al., 1987). Riftia has three different extracellular hemoglobins, two dissolved ones in the vascular blood (V1 and V2) and one hemoglobin in the coelomic fluid (Zal et al., 1998). These hemoglobins bind oxygen and sulfide at two different sites reversibly and concurrent (Arp et al., 1987; Arp & Childress, 1983; Zal et al., 1998). The link of sulfide does not interrupt the simultaneous oxygen binding (Arp et al., 1987; Childress et al., 1991; Childress et al., 1984; Zal et al., 1998). Furthermore theses specific hemoglobins link sulfide with a higher affinity and therefore protect the animal against sulfide toxicity (Powell & Somero, 1986; Powell & Somero, 1983; Zal et al., 1998). The extracellular hemoglobins transports all these inorganic nutrients via the bloodstream to the inside of the bacteriocytes, where the symbiont lives in a special organ called trophosome (Bright & Lallier, 2010; Childress et al., 1984; Childress & Fisher, 1992). The symbiont oxidizes sulphide to sulphate and energy is gained in the form of ATP (H. Felbeck, 1981). This energy is then used for the Carbon-Benson-Bassham (CBB) and the reverse tricarbocylic acid (TCA) cycle to generate organic carbon in the form of succinate and malate (Bosch & Grassé, 1984; Felbeck & Jarchow, 1998). These C4 sugars are then released to the host (Lutz et al., 1994; Stewart & Cavanaugh, 2005). Additionally, *Riftia* digests some of the symbionts (Bosch & Grassé, 1984; Bright et al., 2000).

The body of adult individuals of *Riftia* is encircled by a chitinous, cylindrical and flexible white tube, which is 2-3 mm thick and closed at the rear end (Gaill & Hunt, 1986; Gardiner & Jones, 1993). The anterior end of the animal consists of an obturaculum with an branchial plume extending from the tube and of a muscular vestimentum, following the obturaculum, which is the head of the animal with a heart, a brain and the excretory organ enclosed in it (Bright & Lallier, 2010; Gardiner & Jones, 1993). The trunk region is the biggest area of the animal. A collagenous cuticle with pores, which are connected to pyriform glands, and an epidermis secreting the cuticle, cover the trunk part outside (Bright & Lallier, 2010). The pyriform glands produce the chitinous tube of *Riftia*. A layer of circular and longitudinal muscles encircles inside a coelom filled with coelomic fluid and the trophosome (Bright & Lallier, 2010). The cuticle, epidermis, circular and longitudinal muscles and the pyriform glands represent the skin of the animal (Bright & Lallier, 2010). The trophosome, which forms 16% of the wet weight of adult tubeworms, consists of many lobules and is surrounded by blood vessels (Bright & Lallier, 2010; Childress et al., 1984). The symbionts are living inside the trophosome. Gonads are extended alongside the trunk as well (Bright & Lallier, 2010). At the posterior end of the body is the opisthosome, which forms a short multiple segmented region (Bright & Lallier, 2010).

An important topic in symbiosis is how hosts acquire their symbionts and how they are transferred between each new host generation. Symbiont transmission ensures the continuity of symbiosis through host generations (Bright & Bulgheresi, 2010). Two transmission modes are distinguished: horizontally and vertically transmission. Horizontally transmission describes a mode, where a free-living symbiont is transferred from the environment into the host, e.g. *Aliivibrio fischeri* in the bobtail squid *Euprymna scolopes*, rhizobia in the root nodules of soybeans (Bright & Bulgheresi, 2010). By contrast, vertically transmission is a form of transfer, where the symbiont is transferred, often through the female germ line, from the parents to the offspring, e.g. the earthworm and its extracellular endosymbiont *Verminephrobacter eiseniae* or the bryozoan *Bugula neritina* and the extracellular endosymbiont *Candidatus* Endobugola sertula (Bright & Bulgheresi, 2010). Mixed types of transfers are also possible like the nematode *Steinernema carpocapsae*, which harbors the symbiont *Xenorhabdus nematophila* (Bright & Bulgheresi, 2010).

The time of symbiont infection is different in the transmission modes. In horizontally transmission, the infection often takes place during the post-settlement larvae in tubeworms, or juveniles in bivalves and squid (Bright & Bulgheresi, 2010). Vertically transmitted symbionts are taken up by e.g. some sponges, the gutless oligochaetes, bivalves and bryozoans in developing or fertilized eggs (Bright & Bulgheresi, 2010). The site of infection is usually not the conclusive destination of residence for the symbionts.

They migrate until they reach their final location. The symbionts have different options in different hosts to reach the final symbiont housing organ (Bright & Bulgheresi, 2010). *Aliivibrio fischeri* migrates in its host, the bobtail squid, by flagellum-mediated chemotaxis from the squid ducts to its final destination the deep crypts of the nascent light organ (Bright & Bulgheresi, 2010). Endoriftia enters its host horizontally through the developing tube and skin and migrates from the epidermis of the skin to its end location the trophosome of *Riftia* (Bright & Lallier, 2010). The symbiont can migrate either extracellularly (e.g. squid ducts), intercellurlarly (like rhizobia getting in the cracks of soybeans), via transcellular tunnels (e.g. rhizobia initiating root hair curling) or intercellurlarly and intracellularly (e.g. tubeworms) (Bright & Bulgheresi, 2010).

Symbiotic bacteria either live ectosymbiotically outside or endosymbiotically inside of the host. Ectosymbionts are located on the mouthparts and gill chamber of the vent shrimp *Rimicaris* for example or on the dorsal surface of the Polychaete *Alvinella* (Dubilier et al., 2008). Endosymbionts exist in the gills of gastropod snails or in the host cells in the trophosome of *Riftia* (Dubilier et al., 2008). Additionally symbionts can be determined more precisely concerning the host cells: extracellular symbionts are located outside of host cells, intercellular means between host cells, which is basically extracellular and intracellular ones reside inside of host cells (Bright & Bulgheresi, 2010). Thiotrophic ectosymbiotic bacteria of the stilbonematinae *Laxus oneistus* live for example extracellularly stuck to the skin of the animal (Ott et al., 1995). In contrast, the endosymbiont *Verminephrobacter eiseniae* occurs extracellular in the lumen of the nephridial ampullae of the earthworm *Eisenia foetida* (Bright & Bulgheresi, 2010). The endosymbiont Endoriftia exists on the other hand intracellular in the host cells of the trophosome of *Riftia* (Nussbaumer et al., 2006).

Vertically transmitted symbionts do not exist in a free living state and are therefore not selected from the environment (Bright & Bulgheresi, 2010; Bright & Lallier, 2010). All those vertically transmitted symbiotic bacteria are obligate symbionts (Bright & Bulgheresi, 2010). They possess a low strain variability and consequently lack exchange of genetic material (Bright & Bulgheresi, 2010; Dubilier et al., 2008). The life of symbionts, which are horizontally transmitted, is facultative (Bright & Lallier, 2010). These symbionts occur in a free-living state in the environment and are also associated with the host (Bright & Lallier, 2010). Sometimes the free-living population is increased by symbionts, which are release from its host (Bright & Bulgheresi, 2010; Bright & Lallier, 2010). Horizontally transmitted symbionts are often genetically more diverse because the environmentally gained symbionts came usually from a genetically diverse and large free-living pool (Bright & Bulgheresi, 2010; Dubilier et al., 2008; Vrijenhoek, 2010).

The symbiont Endoriftia enters the marine invertebrate *Riftia* after an embryonic and larval development in the pelagic environment by horizontally transmission anew each

host generation through the developing tube and skin of the settling trochophore larva. A trochophore larva is a free-swimming marine planktotrophic polychaete with a prototroch in the anterior body area, a second ciliary band posterior to the prototroch, protonephridien and with no mouth opening or anus (Bright & Lallier, 2010; Hatschek, 1885; Marsh et al., 2001; Rouse, 1999; Salvini-Plawen, 1980). The symbiont migrates from the epidermis of the skin to the visceral mesoderm surrounding the gut (Nussbaumer et al., 2006). The host develops into a juvenile, the gut reduces and the symbiont potentially initiates the development of the trophosome, which develops from a one-lobule into a multilobule stage (Nussbaumer et al., 2006). The host becomes mature and the gut and mouth are absent in adult animals. The endosymbiont lives inside special host cells, called bacteriocytes, in the trophosome in adult individuals of *Riftia*.

Only short initial infection was found in *Riftia* so far and continuing uptake of Endoriftia after this event has never been shown until now (Nussbaumer et al., 2006). As soon as the trophosome is well developed in juveniles, further uptake of symbionts after early infection ceases by massive apoptosis of skin and non-trophosome symbiont-containing tissues (Nussbaumer et al., 2006). In larger juveniles and adults Endoriftia was only found in trophosome and tube, but not in the skin (Nussbaumer et al., 2006). It was thought that no other bacteria are harboring the skin of adult individuals and that this tissue and also all body openings like for example gland openings, channels or genital openings are sterile.

A potentially release of symbionts was not known in the giant tubeworm until a current detection of escaping symbionts upon host death (Klose et al., 2015). Over a short time a huge amount of Endoriftia leaves its dead host and refills the community of the free-living population (Klose et al., 2015). Additionally the monitoring of the turnover of tubeworm clumps after a volcanic event produced evidence for fast colonization, growth and death (Klose et al., 2015). The release of Endoriftia in living individuals of *Riftia* was not observed to date.

However, in two diploma theses Endoriftia was found in the skin of all six investigated samples of adult individuals of *Riftia* by PCR (polymerase chain reaction) and by DGGE (fingerprint method denaturing gradient gel electrophoresis) (Buck, 2013; Scharhauser, 2013). Further, another bacterium belonging to the class of Mollicutes was detected in five of six skin samples (1540-1542, 1556, 1557) of adult individuals by DGGE (see Buck 2013; Scharhauser 2013). Mollicutes are bacteria of gram-positive origin, which are lacking a cell wall (Trachtenberg, 2005). It is a very thin bacterium (0,3 μm) and normally completely nutrient dependent on its host (Durand et al., 2010; Trachtenberg, 2005). The bacteria are well-known pathogens e.g. in humans, or as a beneficial partner in the mesoplasma of insects (Trachtenberg, 2005; Wang et al., 2004). Besides the existence of Mollicutes in humans and insects, these bacteria live as well in sea anemones,

ascidians, abalone, in a hydrothermal shrimp (*Rimicaris exoculata*) and in plants (Duperron et al., 2012; Durand et al., 2010; Razin, 1978; Wang et al., 2004). Additionally, Buck (2013) and Scharhauser (2013) detected Endoriftia extracellularly stuck to cuticle of the epidermis and intracellularly in the epithelium lining the blood vessel in adult specimens of *Riftia* of one animal by fluorescence in-situ hybridization (FISH). The symbiont was also detected in the epidermis of the skin in one sample of a juvenile by FISH (Scharhauser, 2013). Thus, six skin samples of adult individuals of *Riftia* from a cruise in 2011 were investigated with FISH in these two diploma theses and one animal showed positive FISH results (Buck, 2013; Scharhauser, 2013). Further, six specimens of juvenile tubeworms from a cruise in 2010 were analyzed as well with FISH and Endoriftia was found in one of these skin samples (Scharhauser, 2013).

Because of these previous findings the aim of this study was to further investigate in detail the potential occurrence of Endoriftia and Mollicutes in skin of adult individuals of *Riftia*. There is a possibility that a longer transmission mode takes place in the tubeworm than just in the trochophore larva and early juvenile and that there is a continuous infection in juveniles or adult individuals. Alternatively, symbionts may migrate in and out of the host after initial infection or that the host releases Endoriftia also during its life and not only upon host death. A detection of Endoriftia in skin of adult individuals could eventually point to a longer transmission time frame or an earlier release of symbionts before host death. Additionally, the detection of Mollicutes in skin would change our current understanding of this dual-partner symbiosis. Based on these different possibilities my question was whether there is any bacterium in the skin of adult individuals of *Riftia*? Does Endoriftia still enter the adult tubeworm and colonizes the skin of the animal? If the symbiont is in the skin, where is it exactly in this tissue? Do any other bacteria live in the skin of *Riftia*? The null hypothesis for this study was that the skin of adult *Riftia* is free of bacteria. Based on the study of Nussbaumer et al. (2006) we expected the skin of the tubeworm to be sterile in contrast to the results of Buck (2013) and Scharhauser (2013).

FISH was used to detect and localize the symbiont and Mollicutes in skin samples of adult individuals of *Riftia* (Wagner et al., 2003). The same samples of animals were used for this study, which showed positive signals of Endoriftia and Mollicutes in the previous diploma theses. Two different positive controls were applied for the FISH experiments. The first control was the use of one glass slide with trophosome sections. As the symbionts live inside the bacteriocytes in the trophosome, these would be positively labeled in the FISH experiments. According to former studies the skin is free of bacteria (Nussbaumer et al., 2006). Therefore no positively labeled symbiont in the skin could mean that there is no bacteria but it could also represent that the FISH experiment didn't work, because there is no proof for a sterile skin until now. Thus trophosome tissues

were analyzed in every FISH experiment. Another affirmative control was to confirm the Mollicutes probe by Clone-FISH, which was done by Andrea Nussbaumer. This specific probe was successfully used for FISH after an effective insertion of the 16S rRNA of Mollicutes into an *Escherichia coli* bacterium. A total of five adult animals from two different sampling sites (P-Vent North and TICA) were used to answer the questions in this study.

MATERIAL AND METHODS

Sample collection

During the cruise of R/V Atlantis at the East Pacific Rise specimens of adult *Riftia pachyptila* were collected at the vent sites TICA (9°50.404 N, 104°17.495 W) and P-vent North (9°50.2816 N, 104°17.732 W) in 2500 meters depth in October 2011. The sampled tubeworms were dissected (with sterile scissors and forceps) quickly after collection on board into three different tissues (trophosome, skin and tube). Additionally, the animals were separated into upper, middle and lower part of the body (Buck, 2013; Scharhauser, 2013). Finally, the samples of trophosome, skin and tube were cut into small pieces of less than 1 cm^3 of size, fixed with 100% ethanol and stored at 4°C (Scharhauser, 2013) (Tab. 1, Fig. 1).

Tab. 1. Used samples of adult specimens of *Riftia pachyptila*, which were separated into three different tissues (trophosome, skin and tube) and further dissected into three body parts (upper, middle and lower).

| Sample	Sample site	Year	Gender	Size (cm) tube/plume	Tissue layer	Part of animal	Abbreviation
1540	TICA	2011	Female	60/15	Trophosome Skin Tube	Upper/Middle/Lower Upper/Middle/Lower Upper/Middle/Lower	Tru/Trm/Trl Su/Sm/Sl Tu/Tm/Tl
1541	TICA	2011	Male	40/7	Trophosome Skin Tube	Upper/Middle/Lower Upper/Middle/Lower Upper/Middle/Lower	Tru/Trm/Trl Su/Sm/Sl Tu/Tm/Tl
1542	TICA	2011	Female	54/11	Trophosome Skin Tube	Upper/Middle/Lower Upper/Middle/Lower Upper/Middle/Lower	Tru/Trm/Trl Su/Sm/Sl Tu/Tm/Tl
1556	P-Vent North	2011	Female	21/7	Trophosome Skin Tube	Upper/Middle/Lower Upper/Middle/Lower Upper/Middle/Lower	Tru/Trm/Trl Su/Sm/Sl Tu/Tm/Tl
1557	P-Vent North	2011	Female	31,5/7,5	Trophosome Skin Tube	Upper/Middle/Lower Upper/Middle/Lower Upper/Middle/Lower	Tru/Trm/Trl Su/Sm/Sl Tu/Tm/Tl
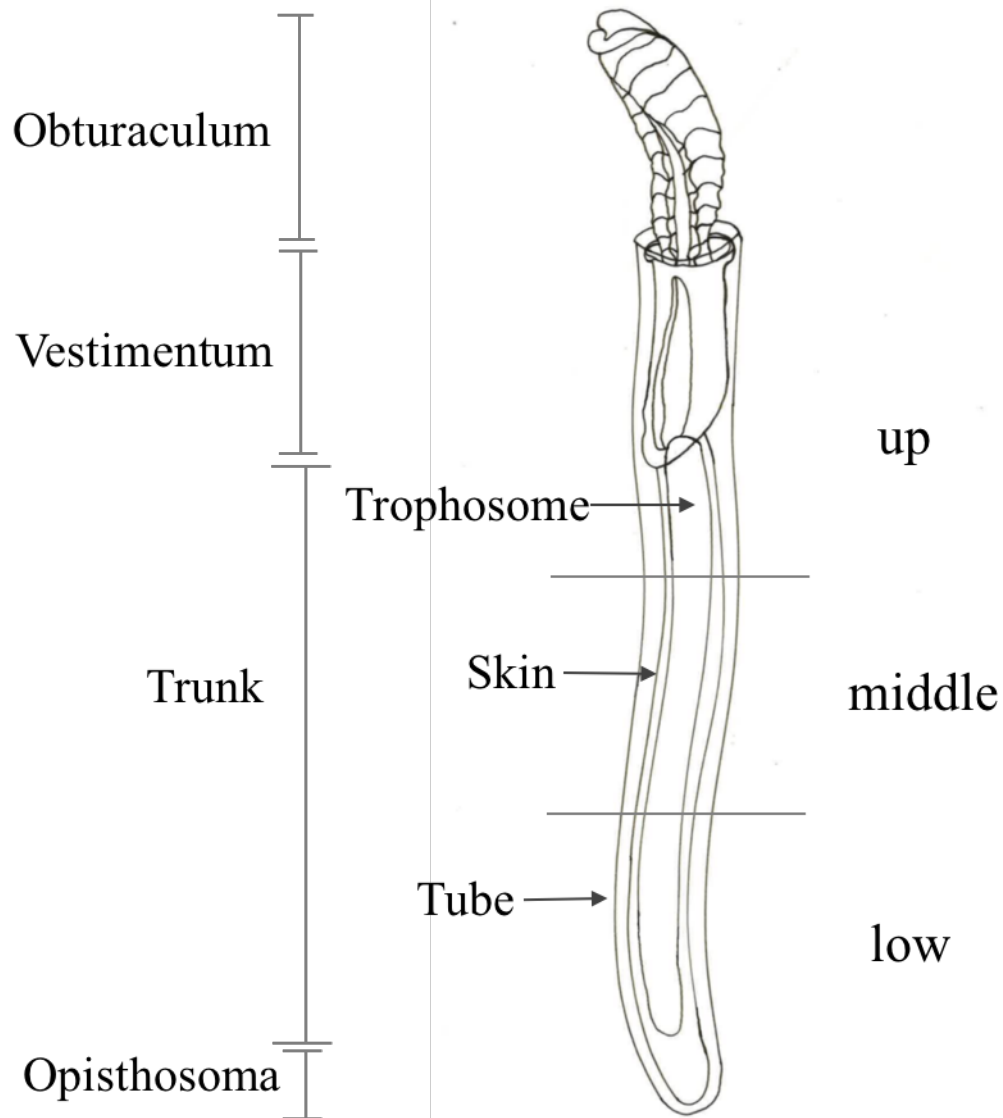
Fig. 1. Illustration of *Riftia pachyptila*. In the left column are the body regions (Obturaculum, Vestimentum, Trunk, Opisthoma), on the left side of the illustration are the tissues (Trophosome, Skin, Tube) and on the right side of the illustration are the areas of sectioning (up, middle, low) described.

Fluorescence *in situ* hybridization (FISH)

Embedding

25 mm^3 large pieces of the small parts (upper, middle and lower part) of trophosome, skin and tube of adult animals were dissected (Scharhauser, 2013). The samples were embedded in LR White low viscosity resin (Nussbaumer et al., 2006; Scharhauser, 2013; Schimak et al., 2012).

Sectioning

The different parts of trophosome, skin and tube (upper, middle and lower part) of five animals (1540-1542, 1556-1557) were sectioned on an ultramicrotome (UltracutE, Reichert-Jung, C. Reichert AG, Vienna, Austria). Semi-thin sections, 1 µm, were sliced with glass knives and put on a glass slide. Eight sections per slide were placed on a slide and dried on a heating plate at a maximum of 32°C to avoid destroying of ribosomes. To prevent slipping of sections on the glass slides, they were covered with a chromalaun-gelatine mixture and dried before using them.

Hybridization and Washing

Skin samples of *Riftia pachyptila* (1540-1542, 1556-1557) were analyzed for symbiont and Mollicutes presence and localization by fluorescence *in situ* hybridization (FISH). For every FISH experiment one slide with eight sections of trophosome was analyzed additionally for symbiont presence as a positive control for FISH.

Different probes in different combinations were used on each slide: symbiont specific probes, RifTO445 (Nussbaumer et al., 2006), to target the 16S rRNA of *Candidatus* Endoriftia persephone, a specific probe, Mol753R (designed by Baranyi, C.), targeting the 16S rRNA of Mollicutes, a general bacterial probe EUB-mix 338 (consisting of EUB338 I, EUB338 II and EUB338 III) for bacterial detection and a nonsense probe NONEUB (Wallner et al., 1993) (Tab. 2). The different combinations of probes labelled with the fluorescent label Cy3, Cy5 or FLUOS were analyzed and compared to achieve best fluorescent signals. The symbiont specific or/and Mollicutes probes were compared with EUB-mix probes for distinct symbiont or Mollicutes detection.

The oven, where the fluorescence *in situ* hybridization took place, was set at a constant temperature of 46°C. After choosing respective slides for the current FISH experiments, the particular sections were marked with liquid blocking PAP pen by drawing circles around the sections. Then a hybridization buffer was prepared. It consisted of a particular amount of MilliQ, 5 M sodium chloride pH 8.0, 1 M Tris/HCl pH 8.0, 10 % sodium dodecyl sulfate (SDS) and of formamide. Humid chambers were prepared, by putting a folded paper tissue into a slide transport box and soaking the tissue with a respective amount

of the hybridization buffer. Afterwards the hybridization buffer and the slide transport boxes were prewarmed in the oven at 46°C. Different combinations of probes of different fluorochromes of CY3, CY5 and FLUOS were used to label bacteria in the sections. 20 µl of the hybridization buffer were mixed with 1,5 µl of each probe and prewarmed at 46°C in the oven. Slides were incubated in proteinase K and afterwards dipped shortly into 20 mM Tris/HCl. The particular probe mixtures were then put on the respective dried sections on the glass slides. Two glass slides each were then placed in one slide transport box and put into the oven for a hybridization of three hours. In two FISH experiments we extended the hybridization time in the oven from three hours to a hybridization over night. The aim was to test, if the hybridization process was more efficient with a longer hybridization time. As the signals of three hours hybridization were stronger, we adapted a hybridization time of three hours for all FISH experiments. After the according hybridization time in the oven, the slides were washed in a preheated washing buffer to remove probes with an unspecific binding (Scharhauser, 2013). The washing buffer consisted of a particular amount of MilliQ, 5 M sodium chloride pH 8.0, 1 M Tris/HCl pH 8.0, 10 % SDS and 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8.0 and had been prewarmed in a water bath to 48°C. The slides were then dipped into MilliQ and dried in the dark. DAPI was either added to the washing buffer or put separately on all sections to stain the sections. For a better overview of the sections and to locate positive signals more precisely in a section, one section per slide was not treated with probes and stained instead with Toluidine blue. Finally, all dried sections were mounted with a particular amount of Citifluor, glass covers were placed on the sections and sealed by a nail enamel. Slides were stored at 4°C until slide analysis.

Tab. 2. Used FISH probes with respective fluorescence labels.

Probe	Sequence of probe	Used Labels	Reference
EUB-mix 338	EUB338 I 5'-GCT GCC TCC CGT AGG AGT-3' EUB 338 II 5'-GCA GCC ACC CGT AGG TGT-3' EUB 338 III 5'-GCT GCC ACC CGT AGG TGT-3'	Cy3/Cy5	(Amann et al., 1990) (Daims et al., 1999) (Daims et al., 1999)
RifTO445	5'-TCC TCA GGC TTT TCT TCC-3'	Cy3/ FLUOS	(Nussbaumer et al., 2006)
Mol753R	5'-TCC TTT CAT GCC TCA ACG-3'	Cy3	(Buck, 2013)
NON338	5'-ACT CCT ACG GGA GGC AGC-3'	Cy3/Cy5/ FLUOS	(Wallner et al., 1993)

Slide analysis

A Zeiss Axio Imager M2 epifluorescence microscope was used for the visual analysis of the sections treated with FISH. The microscope was equipped with an AxioCam MRm and linked to a Windows computer with an axio vision software (Axio Vision 4.8.2). The overlays of the different probe pictures were produced with GIMP (The GIMP Developer team, http://www.gimp.org/). Toluidine blue stained sections were analyzed with a Zeiss Axio Imager A1 epifluorescence microscope equipped with an AxioCam MRc5 and linked to a Windows PC with a Carl Zeiss ZEN analysis software (ZEN 2012, blue edition).

RESULTS

Trophosome

Generally, the trophosome exhibits a clear zonation. In the central of the trophosome are bacteriocytes with rod-shaped bacteria. Host cells with small coccoid bacteria are situated in the median of the trophosome. Cells with big coccoid symbionts reside the periphery, where apoptosis of the bacteriocytes with digestion of the symbionts was shown in former studies (Bright & Lallier, 2010; Pflugfelder et al., 2009) (Fig. 2, Fig. 3, Fig. 4).

In this study fluorescence *in situ* hybridization (FISH) showed positive signals of EUBmix and symbiont specific probes for the symbionts in the bacteriocytes of all trophosome sections of adult *Riftia pachyptila*. EUBmix 338 labels of CY3 and CY5 lighted up very well in all analyzed sections. Also a double labeled symbiont specific probe (RifTO455) in CY3 showed clear signals. Only the RifTO445 probe labelled with the fluorochrome FLUOS showed patchy signals for Endoriftia. Decreasing the formamide concentration of the hybridization buffer led to explicit signals of RifTO445 FLUOS (Fig. 5, Fig. 6).

No positive signal of the Mollicutes specific probe (Mol735R) was detected by FISH in any of the trophosome sections.
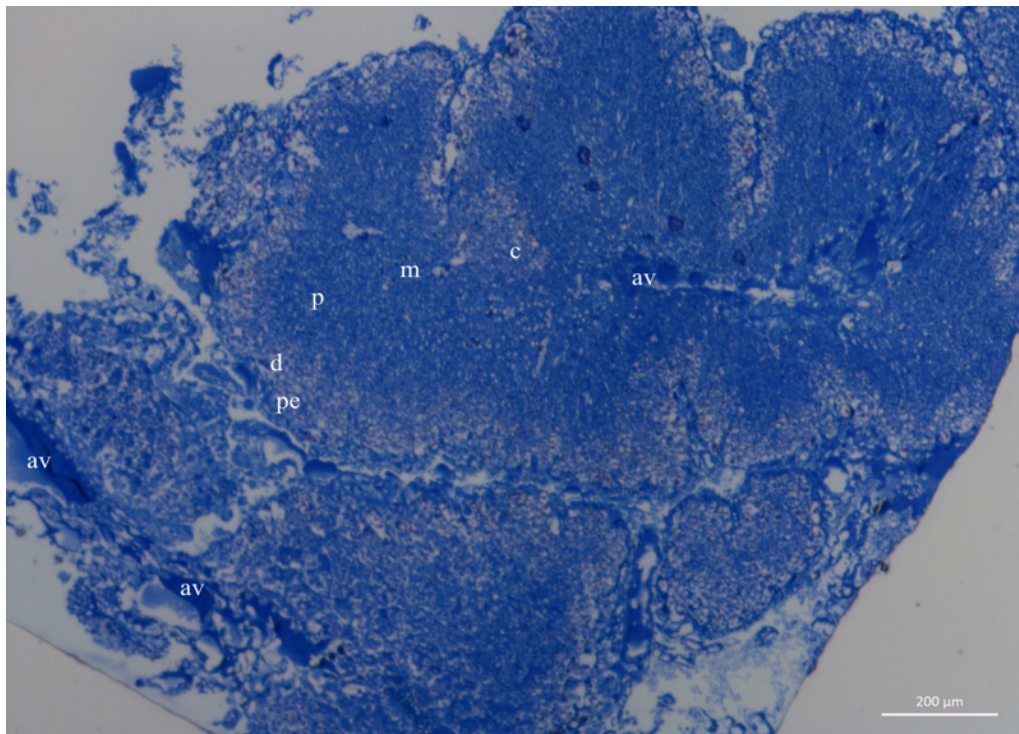

Fig. 2. Light microscope image of one trophosome lobule (1540 Tm) stained with Toluidine blue. Explicit zonation of trophosome lobule in axial blood vessel (av), central (c), median (m), peripheral (p), some degrading bacteriocytes (d) and peritoneum (pe).
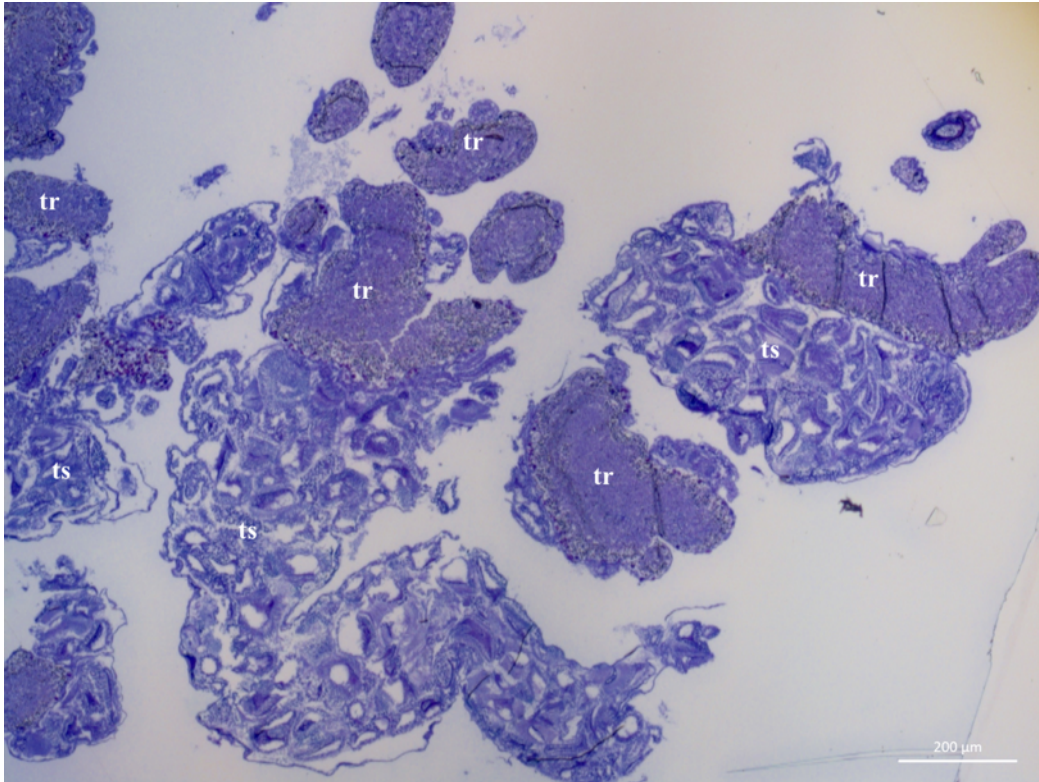
Fig. 3. Light microscope picture of male gonads in a trophosome section (1541 Tm) stained with Toluidine blue. Several stages of testis (ts) and trophosome (tr) tissues are visible here.
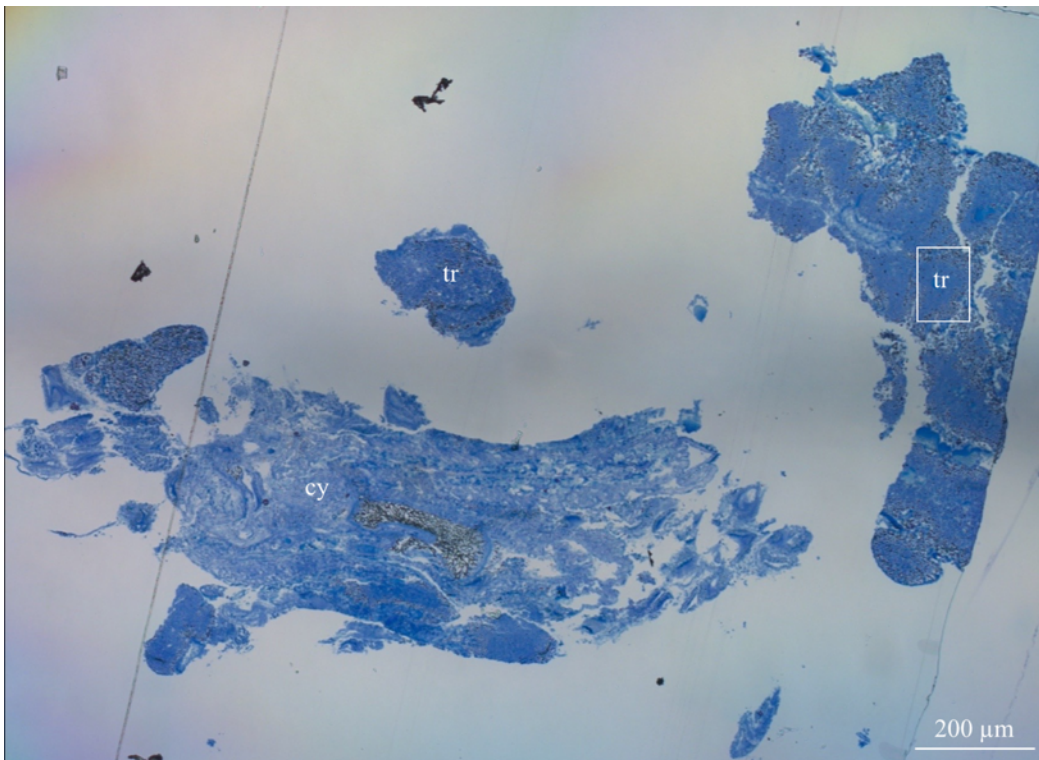

Fig. 4. Light microscope picture of female gonads in a trophosome section (1556 Trl) stained with Toluidine blue. A cytophore (cy) and trophosome (tr) tissues are visible here. The square indicates the part of the trophosome, where the signals of Endoriftia were found (Fig. 5).
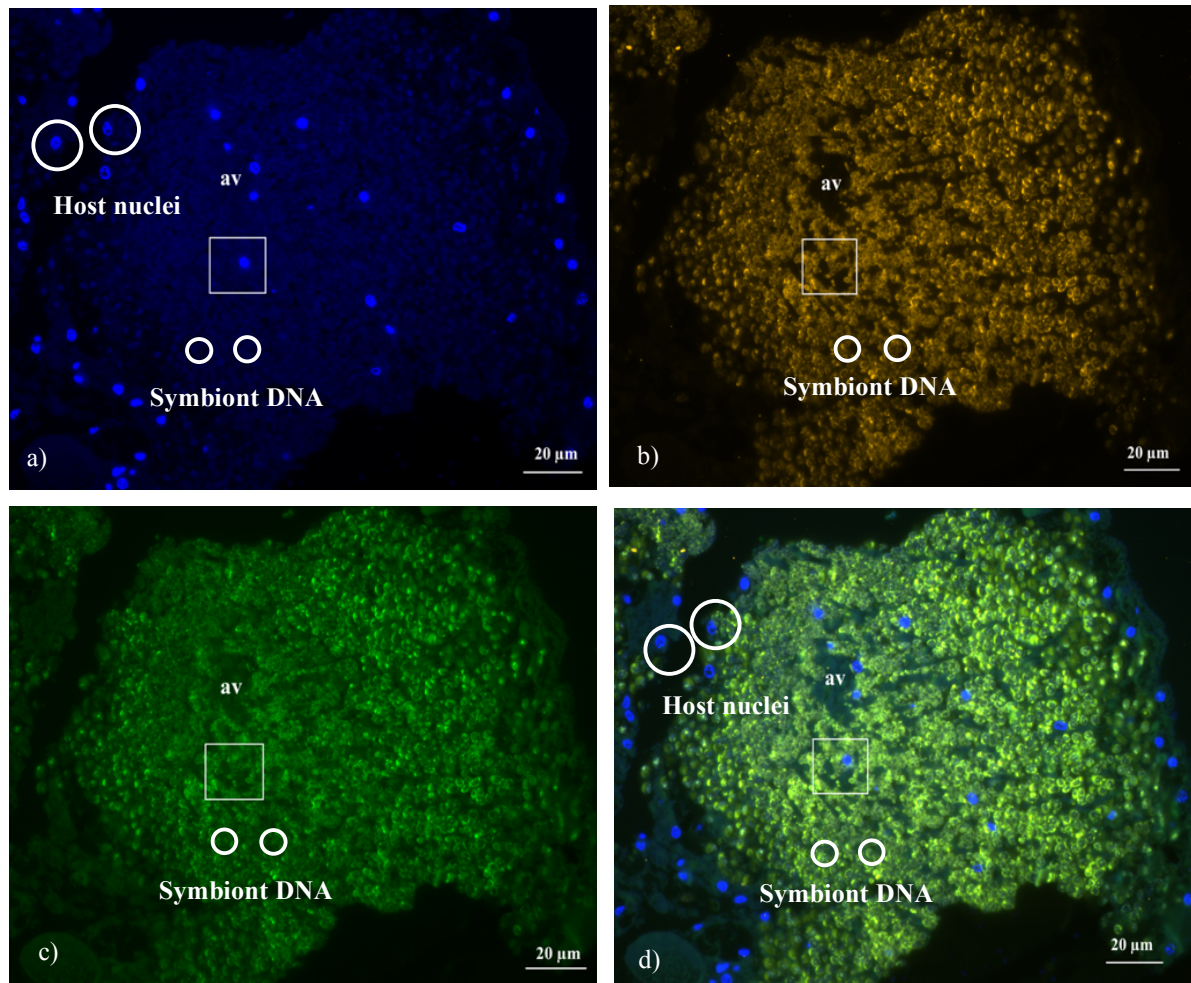
Fig. 5. Epifluorescence image of trophosome sections of *Riftia pachyptila* (1556 Trl) showing symbionts intracellular in the bacteriocytes. DAPI stained DNA (a), EUBmix 338 in CY3 (b), RifTO445 in FLUOS (c) and an overlay of the symbiont specific probe, of EUBmix 338 and DAPI (d). Host nuclei are big rings and dots and the symbionts are smaller rings and dots. The square highlights in each picture the area, which has been enhanced for the representation of one part of the trophosome for Figure 6.
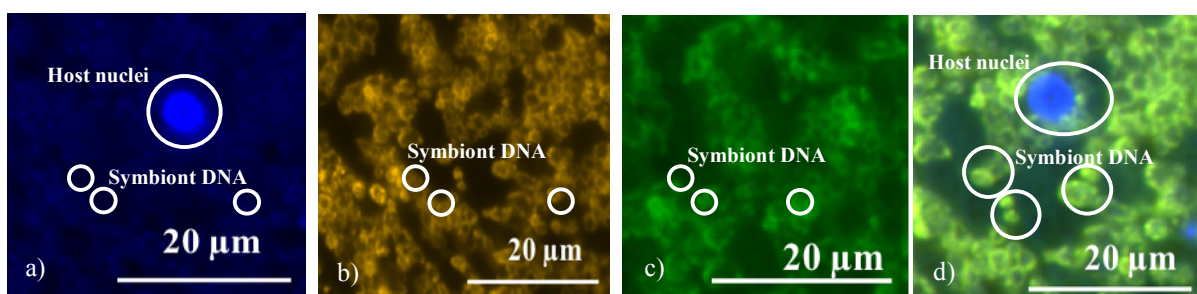

Fig. 6. Enhanced epifluorescence image of one part of trophosome section of Riftia pachyptila (1556 Trl) showing symbionts intracellular in the bacteriocytes and host nuclei. DAPI stained DNA (a), EUBmix 338 in CY3 (b), RifTO445 in FLUOS (c) and an overlay of the symbiont specific probe, of EUBmix 338 and DAPI (d). Host nuclei are big rings and dots and the symbionts are smaller rings and dots.

Skin

The skin of *Riftia pachyptila* consists from the outside to the inside of a cuticle, an epidermis, circular muscles, longitudinal muscles and pyriform glands between the longitudinal muscles (Fig. 7). The whole skin was analyzed with fluorescence *in situ* hybridization (FISH) for this study.

800 skin sections of five animals (1540-1542, 1556-1557) of *Riftia* and approximately 160 skin sections per animal were investigated with FISH. One section per analyzed slide was stained with Toluidine blue for a better overview of the current FISH treated sections (Fig. 7). All analyzed skin samples of all five individuals of *Riftia pachyptila* exhibited positive signals of Endoriftia outside of the skin (Tab. 3). The symbionts stuck in huge aggregations to the cuticle of the epidermis (Fig. 8). Additionally, a few samples of a total of three animals (1540, 1556-1557) showed positive signals of the endosymbiont inside the skin (Tab. 3). These signals were a few and mainly found extracellularly in the coelom stuck to the extensions of the longitudinal muscles (Fig. 11, Fig. 12, Fig. 14, Fig. 15) and in the pyriform glands (Fig. 17, Fig. 18). The symbionts in the pyriform glands were located inside and also outside of the cells (Fig. 17, Fig. 18).

In one animal some parts of the mesentery were found in the skin sections. These sections exhibited high densities of Endoriftia extracellularly between the cells of an undefined tissue adjacent to the mesentery and the longitudinal muscles (Fig. 20, Fig. 21).

Mollicutes wasn't detected in any of the analyzed skin samples with fluorescence *in situ* hybridization (FISH).

Tab. 3. Sampled animals of *Riftia pachyptila*, which showed positive signals of Endoriftia in and outside the skin. The location of the signal of Endoriftia in the equivalent skin samples is marked with a plus in the respective location of the skin in the table.

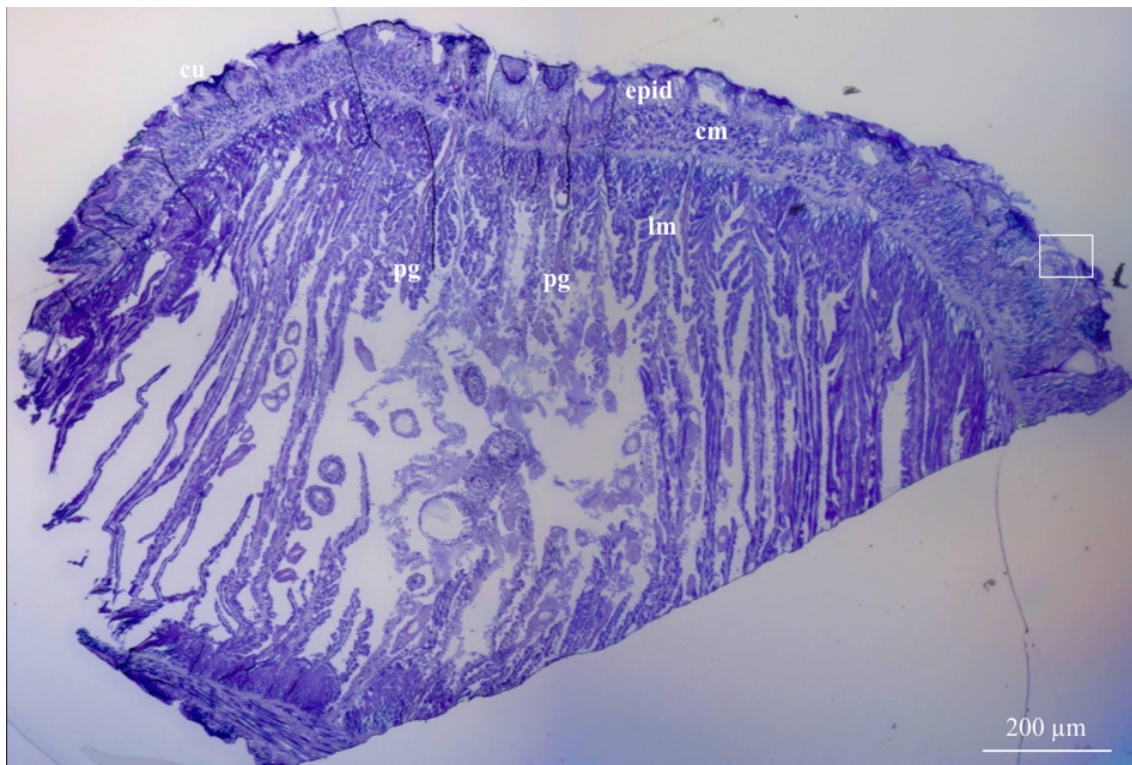| Sample	Part of animal	Coelom	Pyriform glands	Undefined tissue	Stuck to cuticle
1540	Su				+
1540	Sm				+
1540	Sl	+		+	+
1541	Su				+
1541	Sm				+
1541	Sl				+
1542	Sl				+
1556	Su				+
1556	Sm	+	+		+
1556	Sl				+
1557	Su				+
1557	Sm	+			+
1557	Sl				+

Fig. 7. Light microscope image of a skin section (1541 Sl) stained with Toluidine blue. The skin of *Riftia pachyptila* consists from the outside to the inside of a cuticle (cu), an epidermis (epid), circular muscles (cm), longitudinal muscles (lm) and pyriform glands (pg) between the longitudinal muscles. The square in this image marks the cuticle, where signals were found stuck to the cuticle (Fig. 8).
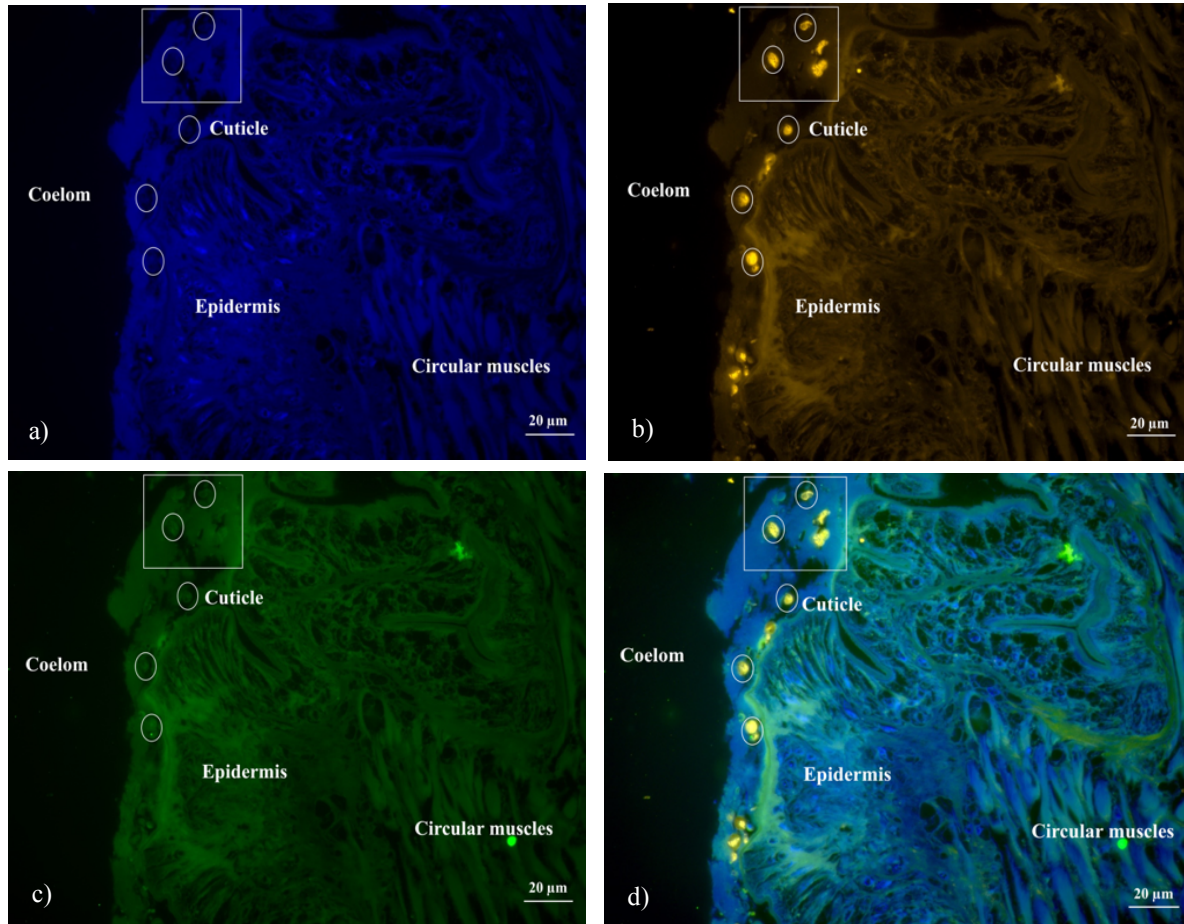
Fig. 8. Epifluorescence image of skin sections of *Riftia pachyptila* (1541 SI) showing symbionts extracellular stuck to the cuticle of the epidermis. DAPI stained DNA (a), EUBmix 338 in CY3 (b), RifTO445 in FLUOS (c) and an overlay of the symbiont specific probe, of EUBmix 338 and DAPI (d). Circles reveal some of the signals of Endoriftia with the different probes/DAPI. The square indicates in each picture one area with extracellular signals. For further details of the area see Figure 9.
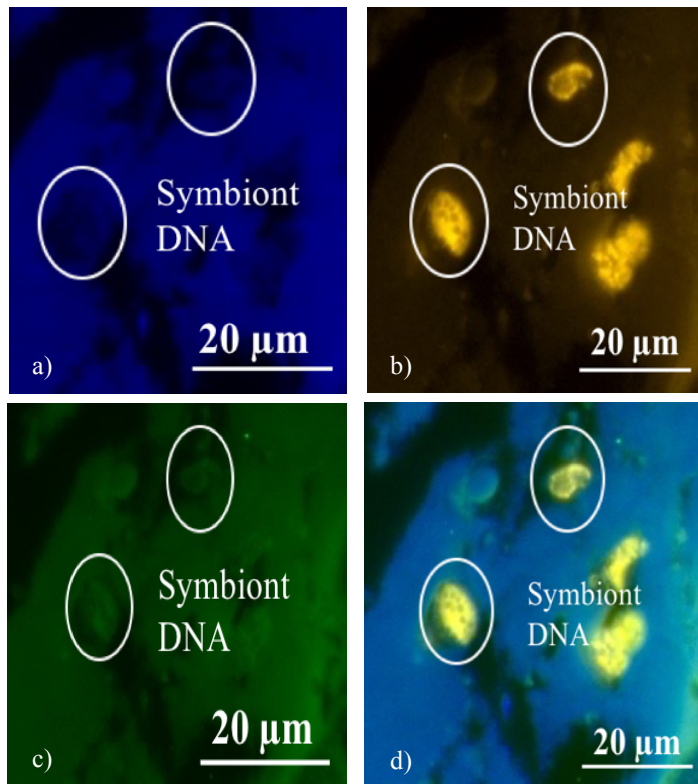
Fig. 9. Enhanced epifluorescence image of skin sections of *Riftia pachyptila* (1541 SI) showing symbionts extracellular stuck to the cuticle of the epidermis. DAPI stained DNA (a), EUBmix 338 in CY3 (b), RifTO445 in FLUOS (c) and an overlay of the symbiont specific probe, of EUBmix 338 and DAPI (d). Circles reveal extracellular signals of Endoriftia with the different probes/DAPI staining.
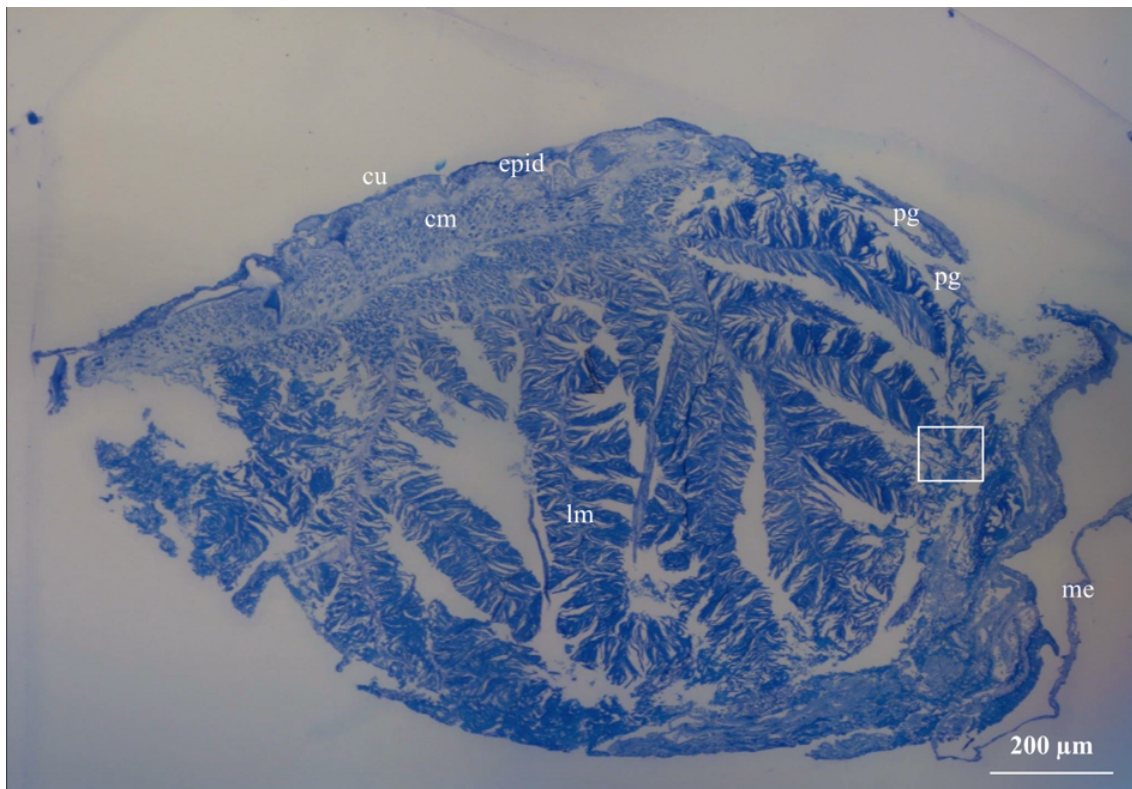
Fig. 10. Light microscope image of a skin section (1540 SI) of *Riftia pachyptila* stained with Toluidine blue. The skin consists of a cuticle (cu), an epidermis (epid), circular muscles (cm), longitudinal muscles (lm) and pyriform (pg). The skin of this specimen contained some parts of mesentery (me). The square in this image marks the extensions of longitudinal muscles, where signals were found (Fig. 11).
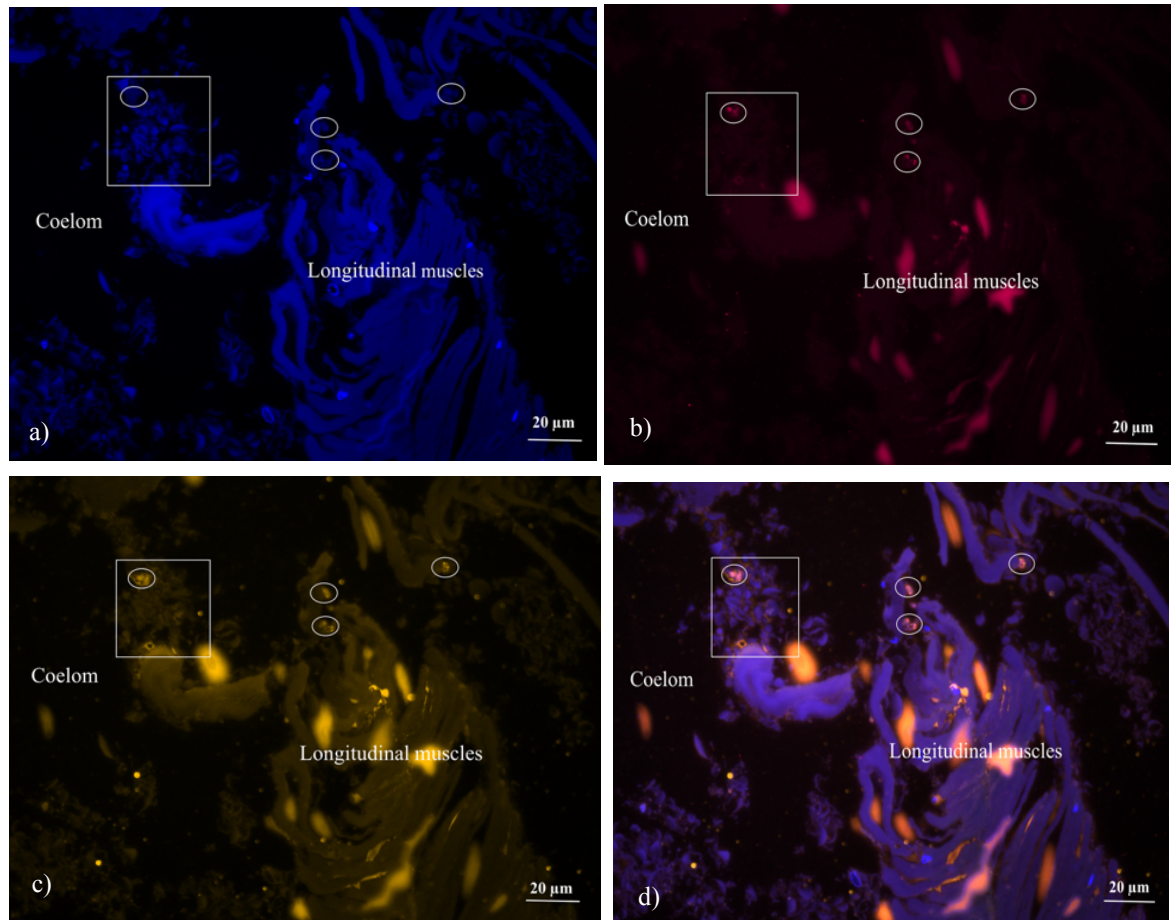
Fig. 11. Epifluorescence image of skin sections of *Riftia pachyptila* (1540 SI) showing symbionts extracellular in the extensions of the longitudinal muscles. DAPI stained DNA (a), EUBmix 338 in CY5 (b), RifTO445 double labeled in CY3 (c) and an overlay of the symbiont specific probe, of EUBmix 338 and DAPI (d). Circles reveal extracellular signals of Endoriftia with the different probes/DAPI staining. The square indicates in each picture one area with extracellular signals. For further details of this area see Figure 12.
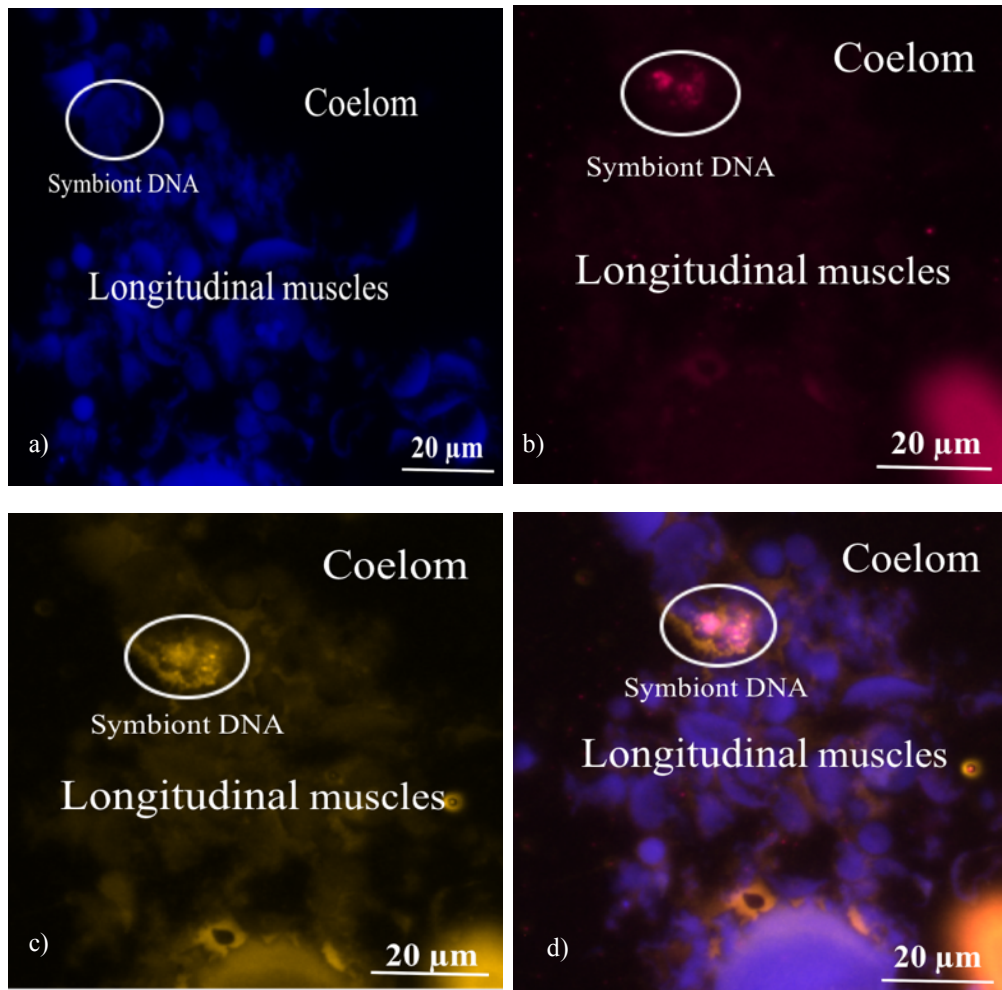
Fig. 12. Enhanced epifluorescence image of skin sections of Riftia pachyptila (1540 SI) showing symbionts extracellular in the extensions of the longitudinal muscles. DAPI stained DNA (a), EUBmix 338 in CY5 (b), RifTO445 double labeled in CY3 (c) and an overlay of the symbiont specific probe, of EUBmix 338 and DAPI (d). Circles reveal extracellular signals of Endoriftia with the different probes/DAPI staining.
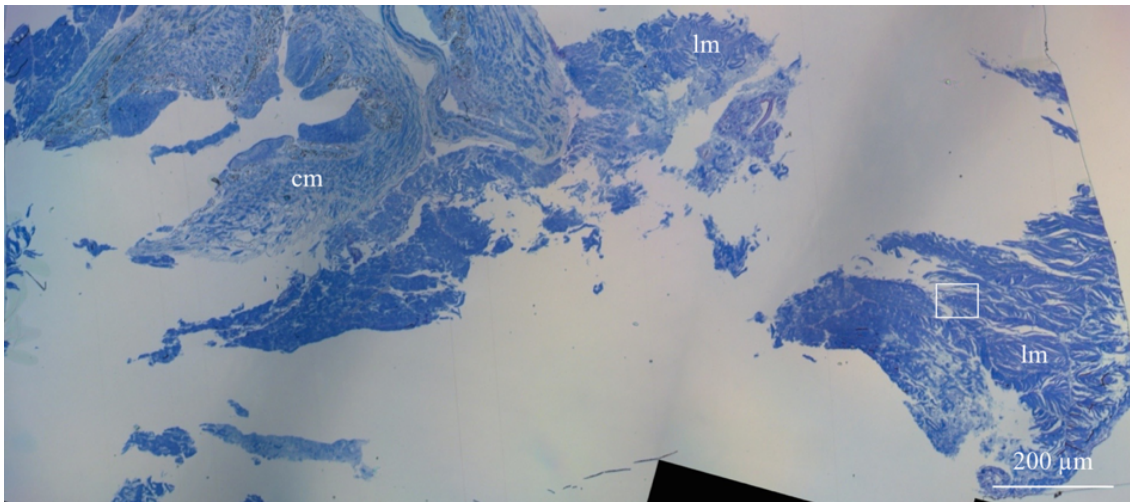
Fig. 13. Light microscope image of a skin section (1557 Sm) of *Riftia pachyptila* stained with Toluidine blue. Circular muscles (cm) and longitudinal muscles (lm) were beveled in this skin section. The square in this image marks the extensions of longitudinal muscles, where signals were found (Fig. 14).

Fig. 14. Epifluorescence image of skin sections of *Riftia pachyptila* (1557 Sm) showing symbionts extracellular in the extensions of the longitudinal muscles. DAPI stained DNA (a), EUBmix 338 in CY5 (b), RifTO445 in FLUOS (c) and an overlay of the symbiont specific probe, of EUBmix 338 and DAPI (d). Circles reveal extracellular signals of Endoriftia with the different probes/DAPI staining. The square indicates one area with extracellular signals in each picture. For further details of this area see Figure 15.

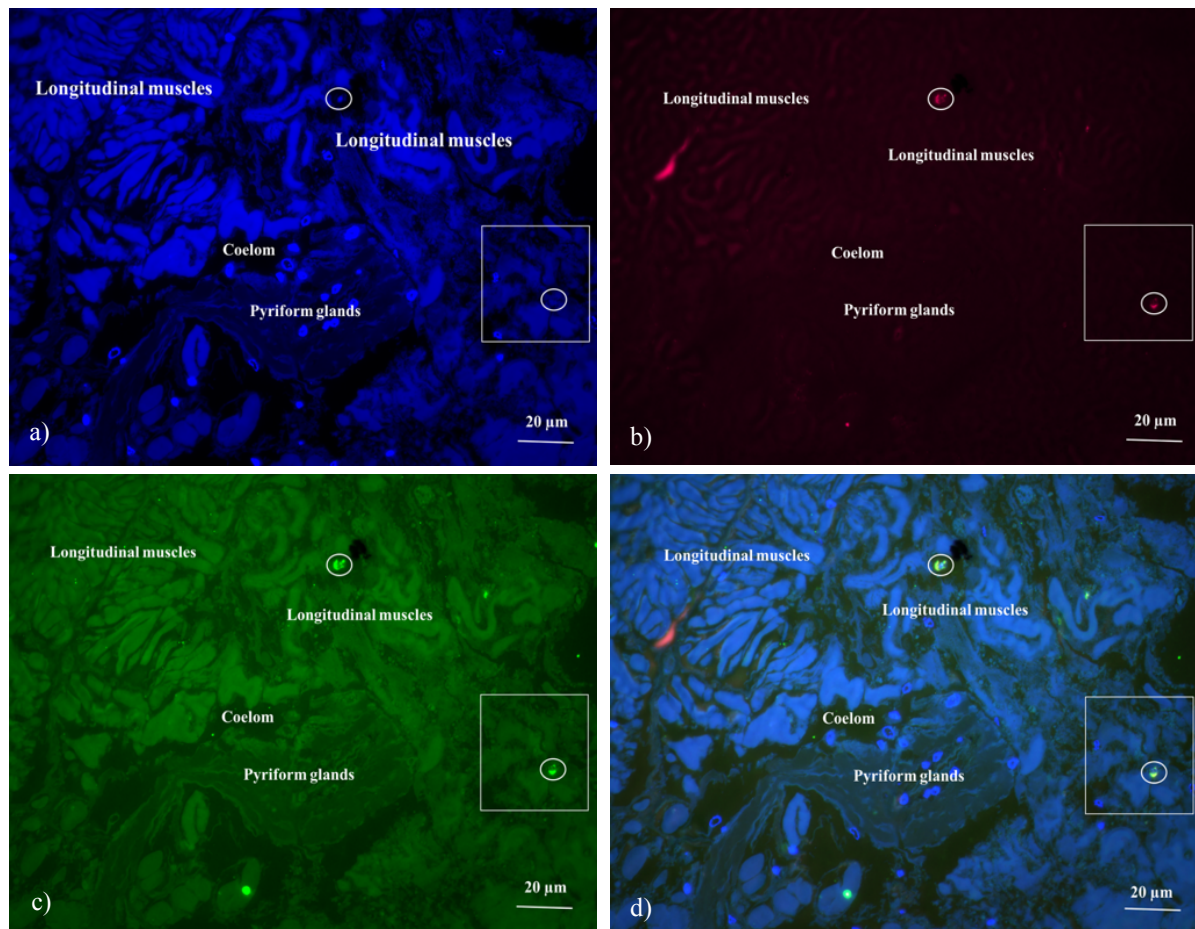Fig. 15. Enhanced epifluorescence image of skin sections of *Riftia pachyptila* (1557 Sm) showing symbionts extracellular in the extensions of the longitudinal muscles. DAPI stained DNA (a), EUBmix 338 in CY5 (b), RifTO445 double labeled in CY3 (c) and an overlay of the symbiont specific probe, of EUBmix 338 and DAPI (d). Circles reveal extracellular signals of Endoriftia with the different probes/DAPI staining and host nuclei.

Fig. 16. Light microscope image of a skin section (1556 Sm) of *Riftia pachyptila* stained with Toluidine blue. Longitudinal muscles (lm) and pyriform glands (pg) were beveled in this skin section. The square in this image marks the pyriform glands, where signals were found (Fig. 17).
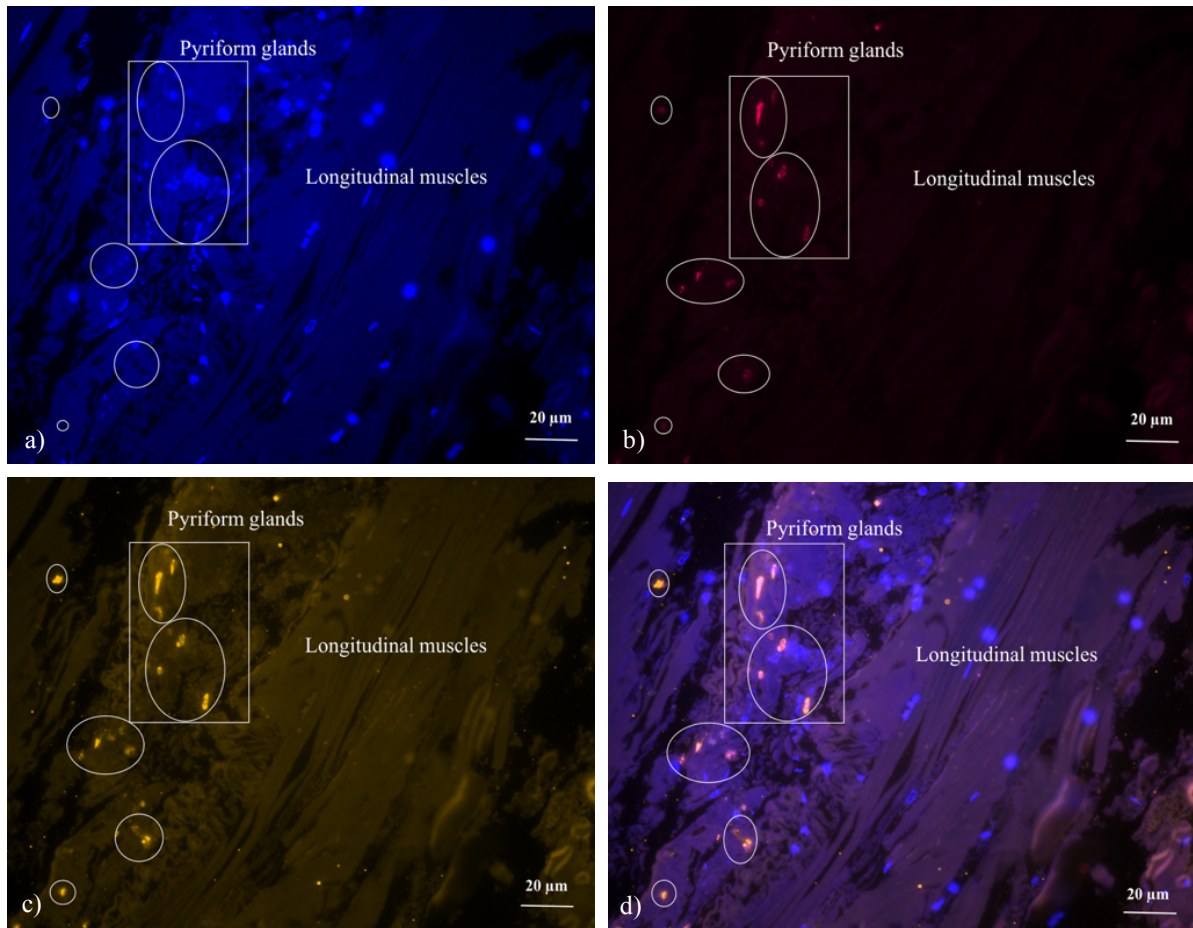
Fig. 17. Epifluorescence image of skin sections of *Riftia pachyptila* (1556 Sm) showing symbionts extracellular and intracellular in the pyriform glands. DAPI stained DNA (a), EUBmix 338 in CY5 (b), RifTO445 double labeled in CY3 (c) and an overlay of the symbiont specific probe, of EUBmix 338 and DAPI (d). Circles reveal extracellular and intracellular signals of Endoriftia with the different probes/DAPI staining. The square indicates in each picture one area with intracellular signals. For further details of this area see Figure 18.
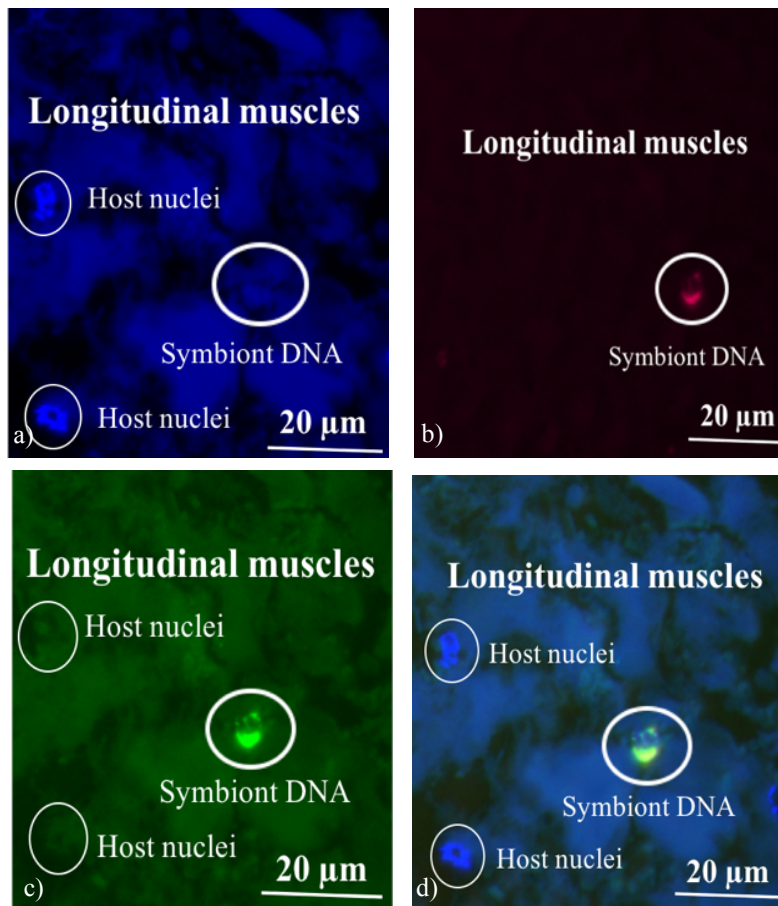
Fig. 18. Enhanced epifluorescence image of skin sections of *Riftia pachyptila* (1557 Sm) showing symbionts intracellular in the pyriform glands. DAPI stained DNA (a), EUBmix 338 in CY5 (b), RifTO445 double labeled in CY3 (c) and an overlay of the symbiont specific probe, of EUBmix 338 and DAPI (d). Circles reveal intracellular signals of Endoriftia with the different probes/DAPI staining and host nuclei.
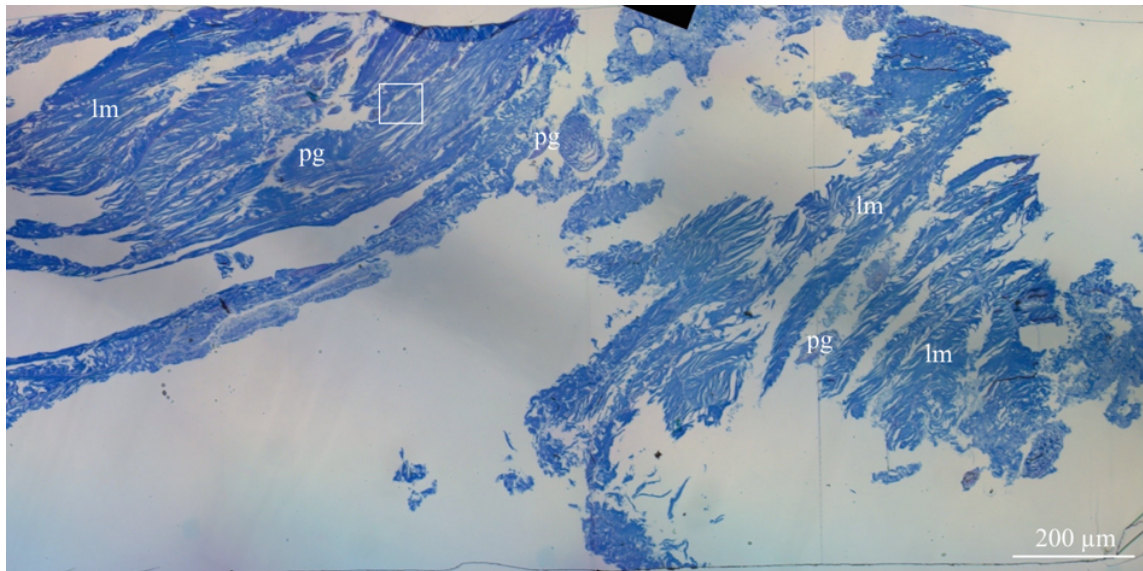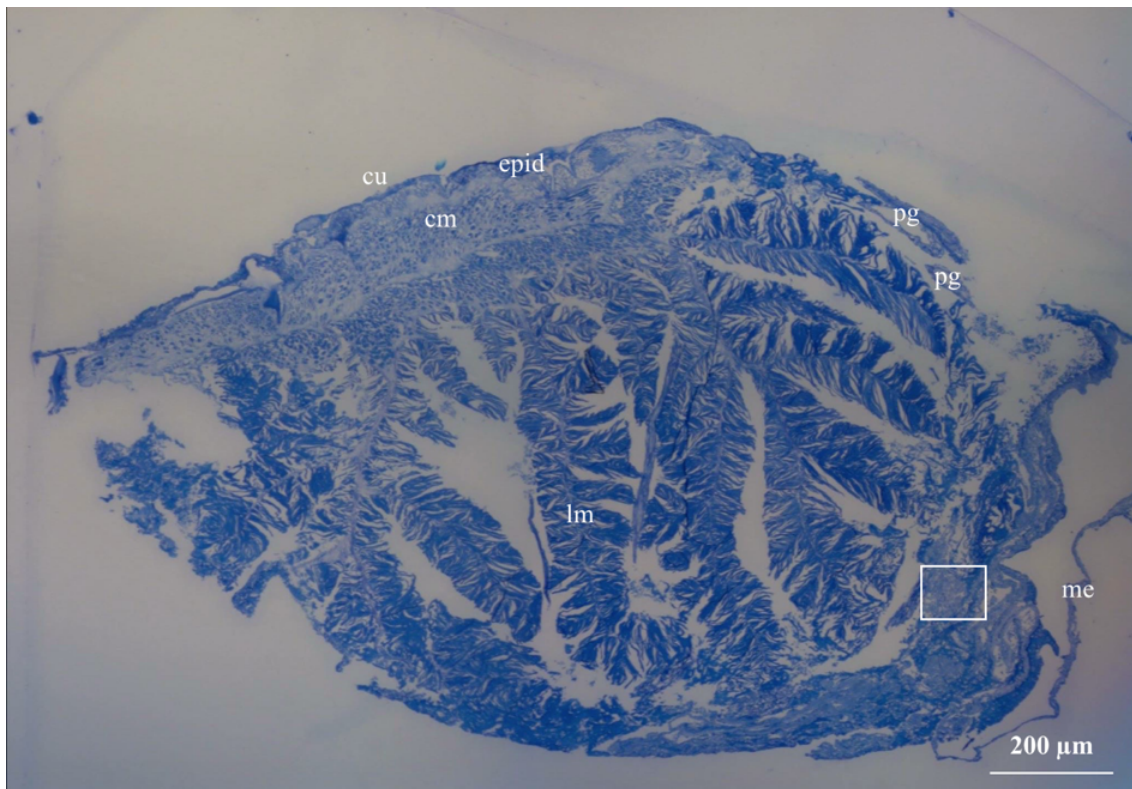
Fig. 19. Light microscope image of a skin section (1540 SI) of *Riftia pachyptila* stained with Toluidine blue. The skin consists of a cuticle (cu), an epidermis (epid), circular muscles (cm), longitudinal muscles (lm) and pyriform (pg). The skin of this specimen contained some parts of mesentery (me). The square in this image marks the undefined area connected with the mesentery, where signals were found (Fig. 20).
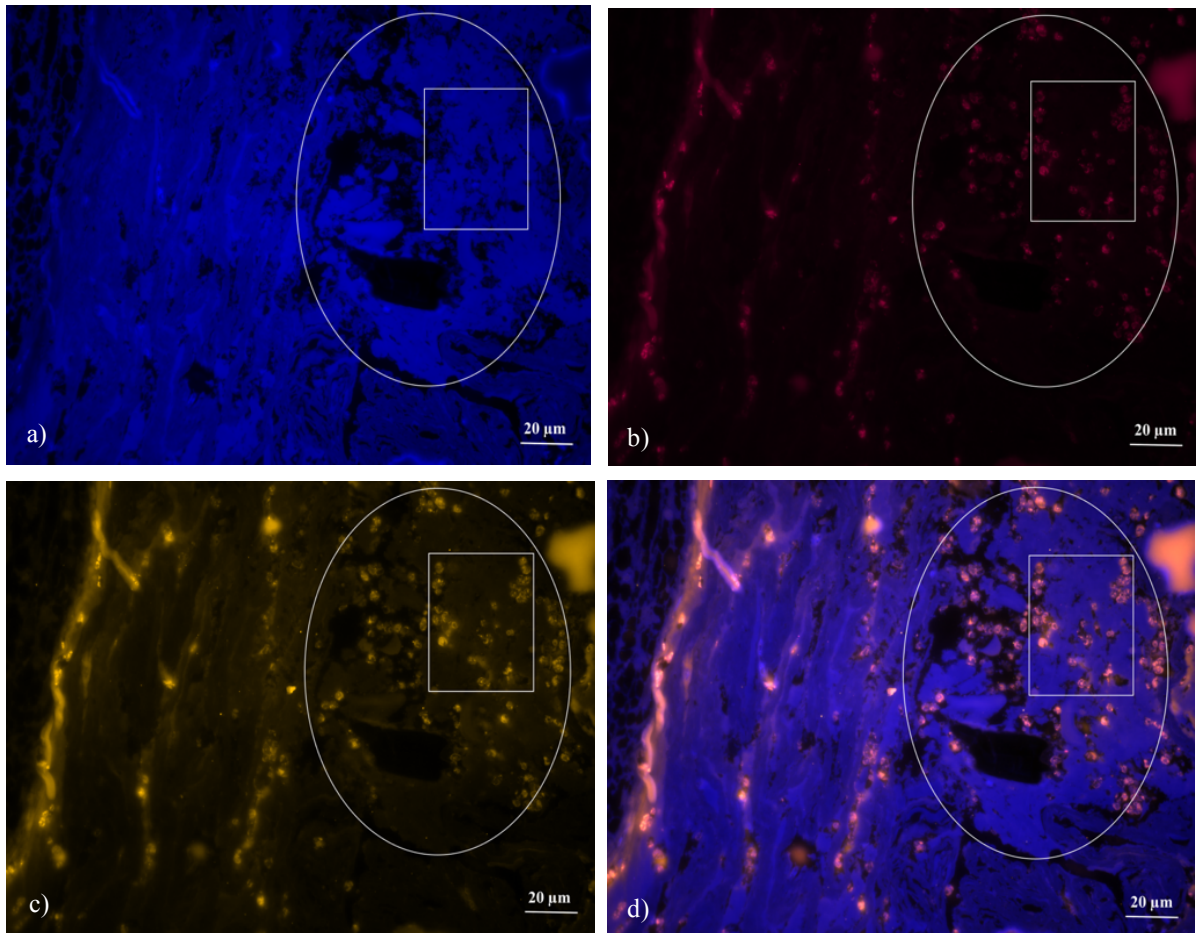
Fig. 20. Epifluorescence image of skin sections of *Riftia pachyptila* (1540 SI) showing symbionts extracellular in an undefined area adjacent to the longitudinal muscles of the skin and the mesentery. DAPI stained DNA (a), EUBmix 338 in CY5 (b), RifTO445 double labeled in CY3 (c) and an overlay of the symbiont specific probe, of EUBmix 338 and DAPI (d). Circles reveal extracellular signals of Endoriftia with the different probes/DAPI staining. The square indicates in each picture one area with extracellular signals. For further details of this area see Figure 21.
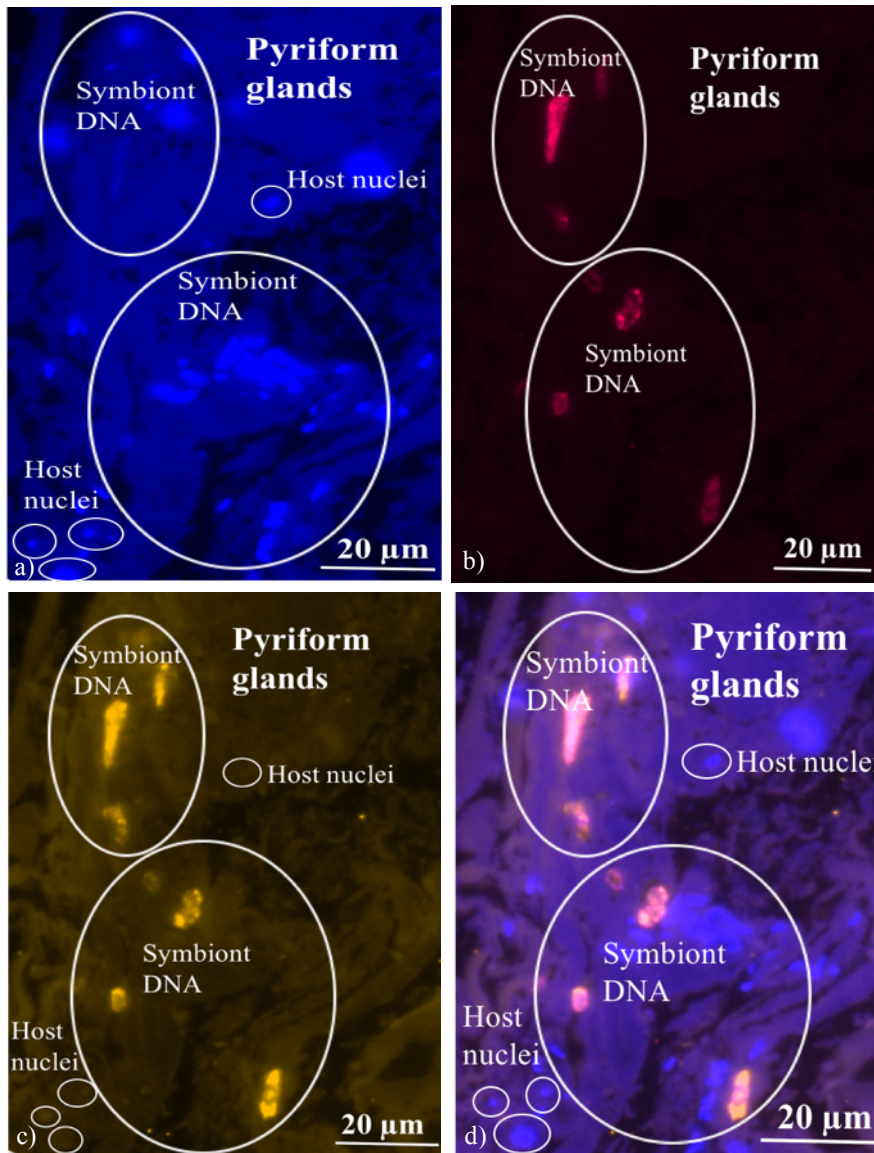
Fig. 21. Enhanced epifluorescence image of skin sections of *Riftia pachyptila* (1540 SI) showing symbionts extracellular in an undefined tissue connected to the mesentery. DAPI stained DNA (a), EUBmix 338 in CY5 (b), RifTO445 double labeled in CY3 (c) and an overlay of the symbiont specific probe, of EUBmix 338 and DAPI (d). Circles reveal extracellular signals of Endoriftia with the different probes/DAPI staining.

DISCUSSION

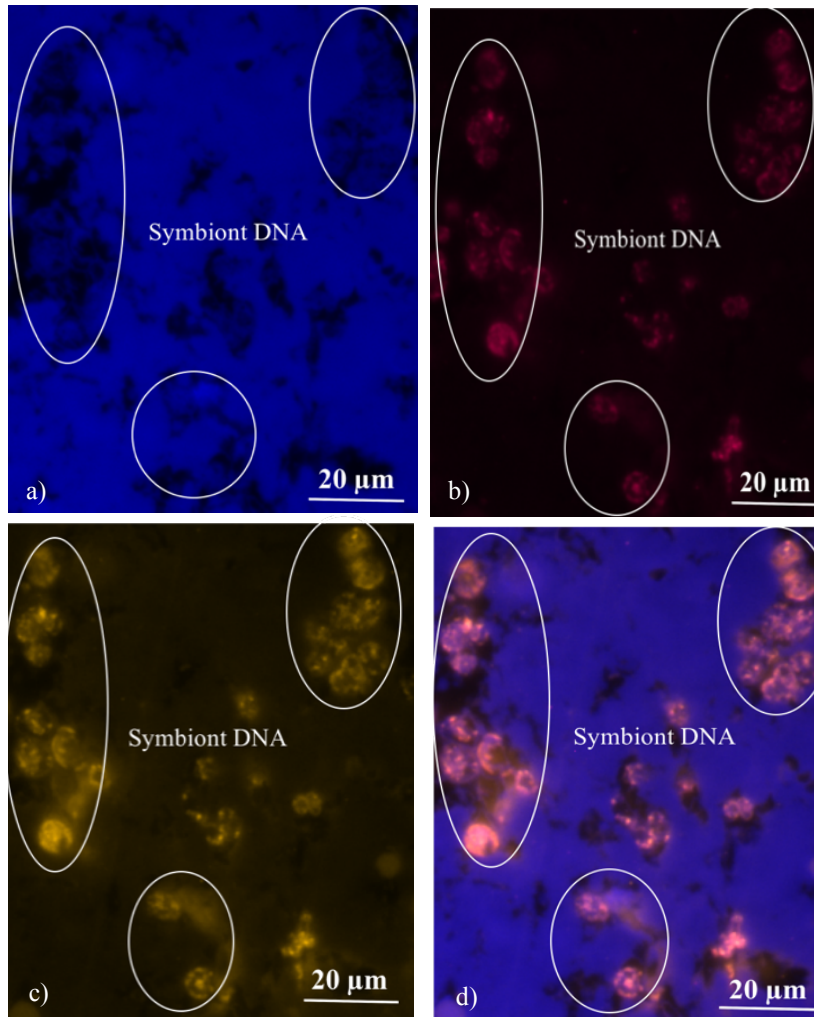**Endoriftia:**

Symbiosis plays an important role in the evolution of life (Nussbaumer et al., 2006). The transmission of symbionts between generations is crucial for the persistence and survival of the host (Nussbaumer et al., 2006). After the detection of Endoriftia in the giant tubeworm and the discovery that the nutrition of the animal is based on these microbes, different transmission modes were supposed (Cavanaugh et al., 1981; Corliss et al., 1979; Felbeck, 1981; Jones, 1981; Jones & Gardiner, 1988; Southward, 1988). An uptake of symbionts with food via the larval mouth and also via the digestive system were significant hypotheses (Jones & Gardiner, 1988; Nussbaumer et al., 2006; Southward, 1988). Nussbaumer et al. (2006) refuted these hypotheses when they determined that the symbiont enters the host through the developing tube and skin of the settled trochophore larva. Furthermore, they detected the symbiont within bacteriocytes in the area of the foregut in the mesodermal tissue and concluded that trophosome development is induced by the mesodermal tissue. A massive apoptosis of host skin and undifferentiated mesodermal tissue was also observed after the symbiont has settled in the trophosome. Contrary to former hypotheses, no symbionts were detected in the gut, no proliferation was found in the midgut and also no participation of symbionts (Nussbaumer et al., 2006). Based on these insights the current model for the chemoautotrophic symbiosis in *Riftia pachyptila* is a horizontal transmission of *Candidatus* Endoriftia persephone into the tubeworm, where a brief initial infection takes place anew in each host generation by the symbiont (Nussbaumer et al., 2006). A free-living population of Endoriftia in the surrounding water acts as inoculum for the host and represents a genetically diverse pool (Bright & Bulgheresi, 2010; Harmer et al., 2008; Polz et al., 2000). After an aposymbiotic phase in the pelagic water, the symbiotic phase of the tubeworm starts with the settlement of the trochophore larva on the basalt rock around hydrothermal vents and with the infection of the mucus of the tube and skin. Only a few symbionts enter and infect the epidermis and then the muscles and the mesodermal tissue, where the symbionts move freely in the cytoplasm and between the cells (Nussbaumer et al., 2006). Endoriftia migrates until the visceral mesoderm, which surrounds the foregut, and is then enclosed in vacuoles of mesodermal cells there (Nussbaumer et al., 2006). These few symbiotic bacteria inside the mesodermal cells probably induce the formation of the trophosome (Nussbaumer et al., 2006). Symbionts live at their final destination inside host cells in the trophosome (Nussbaumer et al., 2006). After the uptake of symbionts, apoptosis in the epidermis, muscles and undifferentiated mesoderm takes place in small juveniles and the symbiont transmission

is terminated (Nussbaumer et al., 2006). Endoriftia has so far been found only in the trophosome in larger juveniles and adults (Nussbaumer et al., 2006). In previous diploma theses the symbiont was detected in skin samples of adult individuals of *Riftia* by DGGE, it was also detected in the epithelium lining the blood vessel in the skin and stuck to the cuticle outside of the skin by FISH (Buck, 2013; Scharhauser, 2013). These new insights raised new questions about a longer transmission mode, a continuous migration of symbionts into or out of *Riftia* or a release of the symbiont by its host. The latter was detected in a current study by Klose et al. (2015) in which an escape of Endoriftia upon host death was observed.

Based on the findings of these diploma theses, I detected Endoriftia outside of the skin stuck to the cuticle in huge aggregations in all investigated adult animals. I also found it extracellularly in the coelom stuck to the extensions of the muscles in three of all examined adult animals with FISH. Endoriftia was detected only once in one animal intracellularly in the pyriform glands inside the skin with FISH. The pyriform glands, which are located between the longitudinal muscles in the skin, contained only a few symbiotic bacteria. Based on these results a continuous migration of symbionts could not be confirmed. Buck (2013) and Scharhauser (2013) concluded in their diploma theses that the finding of Endoriftia in juvenile skin and attached to adult skin was an example for an extended transmission mode with a potential symbiont infection during the whole lifespan of the host. A lifelong transmission occurs for example in legumes, where the symbionts infect their host as long as the organism grows (Gage, 2004). Also in *Osedax*, which, like *Riftia*, belongs to the family of Siboglinidae, the horizontal transmission of several symbionts from the surrounding water occurs multiple times (Salathé & Vrijenhoek, 2012). If Endoriftia continues to migrate in adult *Riftia* (after the short and limited infection by the symbiont in larval stages of the host), then the symbiotic bacterium should also have been localized in the epidermis, the circular muscles or the longitudinal muscles inside the cells. However, the symbiont was not found there using FISH, and was detected only extracellularly on the cuticle of the skin and in the coelom. Accordingly, an extended transmission or a migration of symbionts was excluded.

Furthermore, the results failed to show any escape mechanism of symbionts in living tubeworms. Based on their DGGE and FISH results, Buck (2013) and Scharhauser (2013) supposed that the symbiont is perhaps capable of leaving its host while it is still alive. A mix of lengthy infection and active escape was assumed as well (Scharhauser, 2013). The bobtail squid *Euprymna scolopes* releases its symbiont *Aliivibrio fisheri* daily (Boettcher et al., 1996). Rhizobia is, for example, able to actively leave its host upon the legume's death (Kiers et al., 2003). For *Riftia* an active escape of symbionts upon host death was recently shown (Klose et al., 2015). Endoriftia leaves the dead tubeworm and replenishes the free-living community in the surrounding water (Klose et al., 2015). This

discovery affirms the link between the symbiont population living inside the host and the one present in the surrounding water (Klose et al., 2015). Nonetheless, an escape of Endoriftia from living hosts is currently not known, and the present study also provided no evidence in that direction. Accordingly, the symbionts have been localized in other skin tissues as well such as in the epidermis or muscle cells, but they were exclusively found extracellularly on the cuticle and in the coelom.

Another explanation for finding Endoriftia in skin samples in the above-mentioned diploma studies was that apoptosis was perhaps not completed in each juvenile and that the symbiont is present in skin until the adult age (Buck, 2013). This would help explain the few intracellular signals of Endoriftia in the pyriform glands of the skin in the current study, and it could also account for the intracellular symbiont found by Buck (2013) and Scharhauser (2013) in the epithelium of the blood vessel. Note in this respect that the blood vessels are anatomically not part of the skin. According to Nussbaumer et al. (2006), the pyriform glands produce an extracellular fluid in larvae, forming a mucous film which microbes including the symbionts colonize. Therefore, the few individuals of Endoriftia detected in the glands potentially migrated from this mucous layer into the pyriform glands, which are opened from the skin into the tube, and remained there until the adult stage of the host.

The huge aggregations of Endoriftia that I found extracellularly on the cuticle of the skin of all investigated animals possibly represent a contamination, which supposedly occurred during the dissecting process on the research vessel. The symbionts of the trophosome might have stuck to the skin when the sampled animals were separated into tube, trophosome and skin. According to this interpretation, the abundant symbionts on the cuticle and also the few in the coelom derived from trophosome tissue.

An interesting result of this study was the detection of numerous symbionts located extracellularly in an undefined tissue connected to the mesentery in one adult animal. This would indicate that some symbiotic bacteria normally living in the trophosome reside in this tissue. One interpretation is that Endoriftia extend from the trophosome into the mesentery, which is connected to the trophosome. Alternatively, these bacteria are also merely a contamination from separating tube, skin and trophosome tissue on the research vessel.

The DGGE results of the previous diploma theses, in which Endoriftia was found in skin samples of adult individuals, do not represent evidence for the presence of Endoriftia in skin. This is because this method tests whole tissues and not single cells. FISH detects and localizes specific oligonucleotides and is therefore a more accurate technique to find and specifically locate Endoriftia. Thus, the positive DGGE results of Endoriftia in the skin samples rather reflected those symbiotic bacteria that I detected with FISH outside

of the skin attached to the cuticle and those in the coelom, generating affirmative outcomes in all investigated animals.

Mollicutes:

Another microbe that plays a role in this study is a bacterium belonging to the class of Mollicutes. Although the skin of adult *Riftia* was supposed to be sterile and free of bacteria (Nussbaumer et al., 2006), Buck (2013) and Scharhauser (2013) detected Mollicutes in adult skin samples by DGGE in their diploma theses. The 16S rRNA of this specific bacterium was spotted in 83.3% of all skin samples with a similarity of 94.8% to Mollicutes detected in the gut and gills of the deep-sea polyplacophoran *Leptochiton boucheti* (Buck, 2013; Duperron et al., 2012; Scharhauser, 2013). These results were hypothesized as a possible species-specific infection in *Riftia*, as no bacteria of this class were found in juveniles or in another investigated tubeworm species, *Tevnia jerichonana* (Buck, 2013; Scharhauser, 2013). Scharhauser (2013) assumed a potentially species transmission of Mollicutes by a species associated with *Riftia* or by some type of organism that feeds on the tubeworm.

Mollicutes is a tiny free-living form of life and consists of 4 orders – Acholeplasmatales, Anaeroplasmatales, Entomoplasmatales and Mycoplasmatales (Johansson & Pettersson, 2002). These gram-positive bacteria diverged 65 million years ago, and about 200 species of this class are known (Trachtenberg, 2005). Completely nutrient dependent on their host, they occur extracellularly on mucous films of the urogenital tracts and respiratory systems in humans and in animals such as cows (Rivera-Tapia et al., 2002; Trachtenberg, 2005). Spiroplasma, a genus within the Entomoplasmatales, lives inside insects and plants (Rivera-Tapia et al., 2002; Trachtenberg, 2005). Mollicutes possesses a small set of genes and a low GC content (24-33%) (Rivera-Tapia et al., 2002; Trachtenberg, 2005). *Mycoplasma genitalium* holds the most minor established genome (Trachtenberg, 2005).

Contrary to the findings in the diploma theses, I did not detect any Mollicutes outside or inside the skin by FISH. Although great efforts were made during the lab work to spot this specific bacterium with FISH (e.g. using a double-labelled fluorochrome probe of Mollicutes, which binds to both ends of the 16S rRNA of this bacterium), no Mollicutes was found. These results are open to a number of interpretations.

Buck (2013) and Scharhauser (2013) found Mollicutes only by using DGGE, but not by FISH. Their theses analyzed a few semi-thin skin sections of adult *Riftia* with FISH and did not detect any positive signals. In the present study, the FISH screening with the Mollicutes probe also failed to yield any positive results. One possibility is that no detection of Mollicutes was feasible with FISH because these bacteria are too small in

size (usually 0.2-0.3 µm) (Trachtenberg, 2005). Another possibility is, that the cells of this bacterium are not detectable because of a low ribosome content per cell (Wagner et al., 2003). Therefore, a new investigation with CARD-FISH would be a more accurate technique (Schramm et al., 2002; Wagner et al., 2003). Interestingly, bacteria from the class of Mollicutes were detected in other studies by FISH: *Candidatus* Lumbricincola was found in earthworms (*Lumbricidae*) – a new Mollicutes bacterium in this organism (Nechitaylo et al., 2009).

The most likely explanation for my findings is, that the detection of Mollicutes with DGGE in the previous diploma theses was merely contamination during the lab work. This would explain why I found no Mollicutes inside or outside the skin and also why Buck (2013) and Scharhauser (2013) did not localize this bacterium with FISH. Therefore, Mollicutes is excluded as a second mutualistic bacterium, and Endoriftia remains the only bacterium known to live in a partnership with *Riftia pachyptila*.

CONCLUSION

This study showed that *Riftia pachyptila* lives in a dual partnership with *Candidatus* Endorifitia persephone. Contrary to formerly diploma theses, no Mollicutes was found in or outside the skin of the adult host. Except for the few symbiotic bacteria in the pyriform glands of the skin of only one adult individual, Endoriftia was not detected inside the skin of adult animals. Beyond this one exception, and the fact that no other bacteria (including Endorifitia) were found in the skin of other giant tubeworms, this thiotrophic symbiosis remains limited to the trophosome of adult host individuals after a short initial infection during the early larval stages. For detailed insights into the location of symbionts in the pyriform glands more FISH experiments with other host individuals should be conducted. This would shed light on the meaning of this place of residence of Endoriftia.

REFERENCES

Amann, R. I., Binder, B. J., Olson, R. J., Chrisholm, S. W., Devereux, R., & Stahl, D. A. (1990). Combinations of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.*, *56*(6), 1919–25.

Arp, A. J., & Childress, J. J. (1983). Sulfide binding by the blood of the hydrothermal vent tube worm Riftia pachyptila. *Science*, *219*(4582), 295–297. JOUR.

Arp, A. J., Childress, J. J., & Vetter, R. D. (1987). The sulphide-binding protein in the blood of the vestimentiferan tube-worm, Riftia pachyptila, is the extracellular haemoglobin. *Journal of Experimental Biology*, *128*(1), 139–158. JOUR.

Boettcher, K. J., Ruby, E. G., & McFall-Ngai, M. J. (1996). Bioluminescence in the symbiotic squid Euprymna scolopes is controlled by a daily biological rhythm. *Journal of Comparative Physiology A*, *179*(1), 65–73. JOUR.

Bosch, C., & Grassé, P.-P. (1984). Cycle partiel des bactéries chimioautotrophes symbiotiques et leurs rapports avec les bactériocytes chez Riftia pachyptila Jones (Pogonophore Vestimentifère). I: Le trophosome et les bactériocytes. *Comptes Rendus Des Séances de l'Académie Des Sciences. Série 3, Sciences de La Vie*, *299*(9), 371–376. JOUR.

Bright, M., & Bulgheresi, S. (2010). A complex journey: transmission of microbial symbionts. *Nature Reviews Microbiology*, *8*(3), 218–230. http://doi.org/10.1038/nrmicro2262

Bright, M., Espada-Hinojosa, S., Lagkouvardos, I., & Volland, J. M. (2014). The giant ciliate Zoothamnium niveum and its thiotrophic epibiont Candidatus Thiobios zoothamnicoli: A model system to study interspecies cooperation. *Frontiers in Microbiology*, *5*(APR), 1–13. http://doi.org/10.3389/fmicb.2014.00145

Bright, M., Keckeis, H., & Fisher, C. R. (2000). An autoradiographic examination of carbon fixation, transfer and utilization in the Riftia pachyptila symbiosis. *Marine Biology*, *136*(4), 621–632. JOUR.

Bright, M., & Lallier, F. H. (2010). The Biology of Vestimentiferan Tubeworms. *Oceanography and Marine Biology*, *48*(July 2009), 213–266.

Buck, A. J. (2013). *Diploma thesis "The search for Candidatus Endoriftia persephone in skin and tubes of adult Riftia pachyptila and Tevnia."* University of Vienna.

Cavanaugh, C. M., McKiness, Z. P., Newton, I. L. G. & Stewart, F. J. (2006). *The Prokaryotes - Prokaryotic Biology and Symbiotic Associations*. (F. T. Eugene Rosenberg, Edward F. DeLong, Stephen Lory, Erko Stackebrandt, Ed.). New York: Springer.

Cavanaugh, C. M., Gardiner, S. L., Jones, M. L., Jannasch, H. W., & Waterbury, J. B. (1981). Prokaryotic Cells in the Hydrothermal Vent Tube Worm Riftia pachyptila Jones: Possible Chemoautotrophic Symbionts. *Science*, *213*(4505), 340–342. http://doi.org/10.1126/science.213.4505.340

Childress, J. J., Arp, A. J., & Fisher, C. R. (1984). Metabolic and blood characteristics of the hydrothermal vent tube-worm Riftia pachyptila. *Marine Biology*, *83*(2), 109–124. article. http://doi.org/10.1007/BF00394718

Childress, J. J., & Fisher, C. R. (1992). The biology of hydrothermal vent animals: physiology, biochemistry and autotrophic symbioses. *Oceanography and Marine Biology: An Annual Review*.

Childress, J. J., Fisher, C. R., Favuzzi, J. A., Kochevar, R. E., Sanders, N. K., & Alayse, A.-M. (1991). Sulfide-driven autotrophic balance in the bacterial symbiont-containing hydrothermal vent tubeworm, Riftia pachyptila Jones. *The Biological Bulletin*, *180*(1), 135–153. JOUR.

Corliss, J. B., Dymond, J., Gordon, L. I., Edmond, J. M., von Herzen, R. P., Ballard, R. D., Green, K., Williams, D., Bainbridge, A., Crane, K. & van Andel, T. H. (1979). Submarine Thermal Springs on the Galápagos Rift. *American Association for the Advancement of Science*, *203*(4385), 1073–1083. http://doi.org/10.1017/CBO9781107415324.004

Corliss, J.B., Dymond, J., Gordon, L.I., Edmond, J.M., Herzen, R.P.V., Ballard, R.D., Green, K., Williams, D., Bainbridge, A., Crane, K. & van Andel, T. H. (1979). Submarine thermal springs on the Galapagos rift. *Science, 203*, 1073–1083.

Daims, H., Brühl, A., Amman, R., Schleifer, K.-H., & Wagner, M. (1999). The Domain-specific Probe EUB338 is Insufficient for the Detection of all Bacteria: Development and Evaluation of a more Comprehensive Probe Set. *Syst. Appl. Microbiol. [Internet]*, *22*(3), 434–44.

Dattagupta, S., Schaperdoth, I., Montanari, A., Mariani, S., Kita, N., Valley, J. W., & Macalady, J. L. (2009). A novel symbiosis between chemoautotrophic bacteria and a freshwater cave amphipod. *The ISME Journal*, *3*(8), 935–943. http://doi.org/10.1038/ismej.2009.34

de Bary, A. (1879). *Die Entstehung der Symbiose*. Strassburg: Verlag von Karl, J. Trübner.

Desbruyères, D., Segonzac, M. (1997). *Handbook of deep-sea hydrothermal vent fauna*. Editions Quae.

Desbruyères, D., Segonzac, M., & Bright, M. (2006). Handbook of Deep-Sea Hydrothermal Vent Fauna. *Marine Ecology*, *27*(3), 271–271. http://doi.org/10.1111/j.1439-0485.2006.00107.x

Dubilier, N., Bergin, C., & Lott, C. (2008). Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nature Reviews. Microbiology*, *6*(10), 725–40. http://doi.org/10.1038/nrmicro1992

Duperron, S., Pottier, M. A., Léger, N., Gaudron, S. M., Puillandre, N., Le Prieur, S., Sigwart, J. D., Ravaux, J. & Zbinden, M. (2012). A tale of two chitons: Is habitat specialisation linked to distinct associated bacterial communities? *FEMS Microbiology Ecology*, *83*(3), 552–567. http://doi.org/10.1111/1574-6941.12014

Duperron, S., Gaudron, S. M., Rodrigues, C. F., Cunha, M. R., Decker, C., & Olu, K. (2013). An overview of chemosynthetic symbioses in bivalves from the North Atlantic and Mediterranean Sea. *Biogeosciences*, *10*(5), 3241–3267. http://doi.org/10.5194/bg-10-3241-2013

Durand, L., Zbinden, M., Cueff-Gauchard, V., Duperron, S., Roussel, E. G., Shillito, B., & Cambon-Bonavita, M. A. (2010). Microbial diversity associated with the hydrothermal shrimp Rimicaris exoculata gut and occurrence of a resident microbial community. *FEMS Microbiology Ecology*, *71*(2), 291–303. http://doi.org/10.1111/j.1574-6941.2009.00806.x

Edmond, J. M., Measures, C., McDuff, R. E., Chan, L. H., Collier, R., Grant, B., Gordon, L. I. & Corliss, J. B. (1979). Ridge crest hydrothermal activity and the balances of the major and minor elements in the ocean: The Galapagos data. *Earth and Planetary Science Letters*, *46*(1), 1–18. http://doi.org/10.1016/0012-821X(79)90061-X

Elderfield, H., & Schultz, A. (1996). Mid-Ocean Ridge Hydrothermal Fluxes and the Chemical Composition of the Ocean. *Annual Review of Earth and Planetary Sciences*, *24*, 191–224. http://doi.org/10.1017/CBO9781107415324.004

Felbeck, H. (1981). Chemoautotrophic potential of the hydrothermal vent tube worm Riftia pachyptila JONES (Vestimentifera). *Science*, *213*, 336–338.

Felbeck, H., & Jarchow, J. (1998). Carbon release from purified chemoautotrophic bacterial symbionts of the hydrothermal vent tubeworm Riftia pachyptila. *Physiological and Biochemical Zoology*, *71*(3), 294–302. JOUR.

Gage, D. J. (2004). Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiology and Molecular Biology Reviews*, *68*(2), 280–300. JOUR.

Gaill, F., & Hunt, S. (1986). Tubes of deep-sea hydrothermal vent worms riftia-pachyptila (Vestimentifera) and Alvinella-Pompejana (Annelida). *Marine Ecology Progress Series*, *34*(3), 267–274. JOUR.

Gardebrecht, A., Markert, S., Sievert, S. M., Felbeck, H., Thürmer, A., Albrecht, D., Wollherr, A., Kabisch, J., Le Bris, N., Lehmann, R., Daniel, R., Liesegang, H., Hecker, M. & Schweder, T. (2012). Physiological homogeneity among the endosymbionts of Riftia pachyptila and Tevnia jerichonana revealed by proteogenomics. *The ISME Journal*, *6*(4), 766–76. http://doi.org/10.1038/ismej.2011.137

Gardiner, S. L., & Jones, M. L. (1993). Vestimentifera. In F. W. Harrison & M. E. Rice (Eds.), *Microscopic Anatomy of Invertebrates. Onychophora, Chilopoda and Lesser Protostomata* (p. 371–460.). New York: Wiley-Lyss.

Harmer, T. L., Rotjan, R. D., Nussbaumer, A. D., Bright, M., Ng, A. W., DeChaine, E. G., & Cavanaugh, C. M. (2008). Free-living tube worm endosymbionts found at deep-sea vents. *Applied and Environmental Microbiology*, *74*(12), 3895–3898. http://doi.org/10.1128/AEM.02470-07

Hatschek, B. (1885). Zur Entwicklung des Kopfes von Polygordius. *Arbeiten Aus Dem Zoologischen Institut Zu Wien*, *6*, 109–120.

Haymon, R. M., Fornari, D. J., Von Damm, K. L., Lilley, M. D., Perfit, M. R., Edmond, J. M., & Shanks, W. C., III, Lutz, R. A., Grebmeier, J. M., Carbotte, S., Wright, C. D., McLaughlin, E., Smith, M., Beedle, N. & Olson, E. (1993). Volcanic eruption of the mid-ocean ridge along the East Pacific Rise crest at 9°45-52'N: Direct submersible observations of seafloor phe- nomena associated with an eruption event in April 1991. *Earth Planetary Sci. Lett.*, *119*, 85–101.

Hentschel, U., & Felbeck, H. (1993). Nitrate respiration in the hydrothermal vent worm Riftia pachyptila. *Nature*, *366*, 338–340.

Hessler, R. R., & Smitey, W. M. (1983). The distribution and community structure of megafauna at the Galapagos Rift hydrothermal vents. In L. L. & K. L. J. S. RONA P.A., BOSTRÖM K. (Ed.), *Hydrothermal Processes at Seafloor Spreading Centers* (pp. 735–770). New York: Plenum Press.

Hessler, R. R., Smithey, W. M., & Keller, C. H. (1985). Spatial and temporal variation of giant clams, tube worms and mussels at deep-sea hydrothermal vents. *Bull. Biol. Soc. Wash.*, *6*, 411–428.

Jannasch, H. W., & Wirsen, C. O. (1979). Chemosynthetic Primary Production at East Pacific Sea Floor Spreading Centers. *BioScience*, *29*(10), 592–598. http://doi.org/10.2307/1307765

Johansson, K.-E., & Pettersson, B. (2002). Taxonomy of Mollicutes. In *Molecular biology and pathogenicity of mycoplasmas* (pp. 1–29). CHAP, Springer.

Jones, M. L. (1981). Riftia pachyptila JONES: Observations on the vestimentiferan worm from the Galapagos Rift. *Science*, *213*, 333–336.

Jones, M. L. (1985). On the Vestimentifera, new phylum: six new species and other taxa from hydrothermal vents and elsewhere. *Bull Biol Soc Wash*, *6*, 117–158.

Jones, M. L., & Gardiner, S. L. (1988). Evidence for a transient digestive tract in Vestimentifera. *Proceedings of the Biological Society of Washington*, *101*(2), 423–433. JOUR.

Kelley, D. S., Baross, J. A., & Delaney, J. R. (2002). Volcanoes, fluids, and life at mid-ocean rigde spreading centers. *Annual Review of Earth and Planetary Sciences*, *30*(1), 385–491. http://doi.org/10.1146/annurev.earth.30.091201.141331

Kiers, E. T., Rousseau, R. A., West, S. A., & Denison, R. F. (2003). Host sanctions and the legume–rhizobium mutualism. *Nature*, *425*(6953), 78–81. JOUR.

Klose, J., Aistleitner, K., Horn, M., Krenn, L., Dirsch, V., Zehl, M., & Bright, M. (2016). Trophosome of the deep-sea tubeworm Riftia pachyptila inhibits bacterial growth. *PLoS ONE*, *11*(1), 1–18. http://doi.org/10.1371/journal.pone.0146446

Klose, J., Polz, M. F., Wagner, M., Schimak, M. P., Gollner, S., & Bright, M. (2015). Endosymbionts escape dead hydrothermal vent tubeworms to enrich the free-living population. *Proc Natl Acad Sci U S A*, 1–6. http://doi.org/10.1073/pnas.1501160112

Le Bris, N., Govenar, B., Le Gall, C., & Fisher, C. R. (2006). Variability of physico-chemical conditions in 9°50'N EPR diffuse flow vent habitats. *Marine Chemistry*, *98*(2–4), 167–182. http://doi.org/10.1016/j.marchem.2005.08.008

Leung, T. L. F., & Poulin, R. (2008). Parasitism, commensalism, and mutualism: Exploring the many shades of symbioses. *Vie et Milieu*, *58*(2), 107–115.

Lewin, R. A. (1982). Symbiosis and Parasitism: Definitions and Evaluations. *BioScience*, *32*(4), 254–260. JOUR. http://doi.org/10.2307/1308530

Lilley, M. D., & Gordon, L. I. (1979). Methane, nitrous oxide, carbon monoxide and hydrogen in the hydrothermal vents of the Galapagos Spreading Center. *EOS, Trans., Am. Geophys. Union;(United States)*, *60*(46). JOUR.

Linklater, E. (1972). *The Voyage of the Challenger* (1st ed.). Doubleday.

Lutz, R. A., Shank, T. M., Fornari, D. J., Haymon, R. M., Lilley, M. D., Von Damm, K. L., & Desbruyeres, D. (1994). Rapid growth at deep-sea vents. *Nature*. http://doi.org/10.1038/371663a0

Marsh, A. G., Mullineaux, L. S., Young, C. M., & Manahan, D. T. (2001). Larval dispersal potential of the tubeworm Riftia pachyptila at deep-sea hydrothermal vents. *Nature*, *411*(6833), 77–80. JOUR.

Nechitaylo, T. Y., Timmis, K. N., & Golyshin, P. N. (2009). "Candidatus Lumbricincola", a novel lineage of uncultured Mollicutes from earthworms of family Lumbricidae. *Environmental Microbiology*, *11*(4), 1016–1026. JOUR.

Nussbaumer, A. D., Fisher, C. R., & Bright, M. (2006). Horizontal endosymbiont transmission in hydrothermal vent tubeworms. *Nature*, *441*(7091), 345–348. http://doi.org/10.1038/nature04793

Ott, J. A., Bauer-Nebelsick, M., Novotny, V. (1995). The genus Laxus Cobb, 1894 (Stilbonematinae: Nematoda): description of the two species with ectosymbiotic chemoautotrophic bacteria. *Proceedings of the Biological Society of Washington*, *108*, 508–527.

Ott, J. A., Gruber-Vodicka, H. R., Leisch, N., & Zimmermann, J. (2014). Phylogenetic confirmation of the genus Robbea (Nematoda: Desmodoridae, Stilbonematinae) with the description of three new species. *Systematics and Biodiversity*, *2000*(August 2014), 1–22. http://doi.org/10.1080/14772000.2014.941038

Petersen, J. M., Wentrup, C., Verna, C., Knittel, K. & Dubilier, N. (2012). Origins and Evolutionary Flexibility of Chemosynthetic Symbionts From Deep-Sea Animals. *Biological Bulletin*, *223*(1), 123–137.

Pflugfelder, B., Cary, S. C., & Bright, M. (2009). Dynamics of cell proliferation and apoptosis reflect different life strategies in hydrothermal vent and cold seep vestimentiferan tubeworms. *Cell and Tissue Research*, *337*, 149–65. http://doi.org/10.1007/s00441-009-0811-0

Polz, M. F., Ott, J. A., Bright, M., & Cavanaugh, C. M. (2000). When bacteria hitch a ride. *ASM News*, *66*(9), 531–539. JOUR.

Pospesel, M. A., Hentschel, U., & Felbeck, H. (1998). Determination of nitrate in the blood of the hydrothermal vent tubeworm Riftia pachyptila using a bacterial nitrate reduction assay. *Deep-Sea Research Part I*, *12*(45), 2189–2200. JOUR.

Powell, M. A., & Somero, G. N. (1983). Blood components prevent sulfide poisoning of respiration of the hydrothermal vent tube worm Riftia pachyptila. *Science*, *219*(4582), 297–299. JOUR.

Powell, M. A., & Somero, G. N. (1986). Adaptations to sulfide by hydrothermal vent animals: sites and mechanisms of detoxification and metabolism. *The Biological Bulletin*, *171*(1), 274–290. JOUR.

Razin, S. (1978). The Mycoplasmas. *The Mycoplasmas*, *42*(2), 415–470. http://doi.org/10.1016/B978-0-12-078405-9.50012-4

Rivera-Tapia, J. A., Cedillo-Ramírez, M. L., & Juárez, C. G. (2002). Some biological features of Mollicutes. *Revista Latinoamericana de Microbiologia*, *44*(2), 53–57.

Robidart, J. C., Bench, S. R., Feldman, R. A., Novoradovsky, A., Podell, S. B., Gaasterland, T., Allen, E. E. & Felbeck, H. (2008). Metabolic versatility of the Riftia pachyptila endosymbiont revealed through metagenomics. *Environmental Microbiology*, *10*(3), 727–737. http://doi.org/10.1111/j.1462-2920.2007.01496.x

Rouse, G. W. (1999). Trochophore concepts: ciliary bands and the evolution of larvae in spiralian Metazoa. *Biological Journal of the Linnean Society*, *66*(4), 411–464. JOUR.

Ruby, E. G., Wirsen, C. O., & Jannasch, H. W. (1981). Chemolithotrophic sulfur-oxidizing bacteria from the galapagos rift hydrothermal vents. *Applied and Environmental Microbiology*, *42*(2), 317–324.

Salathé, R. M., & Vrijenhoek, R. C. (2012). Temporal variation and lack of host specificity among bacterial endosymbionts of Osedax bone worms (Polychaeta: Siboglinidae). *BMC Evolutionary Biology*, *12*(1), 1. JOUR.

Salvini-Plawen, L. (1980). Was ist eine Trochophora?-Eine Analyse der Larventypen mariner Protostomier. *Zool Jb Anat*, *103*, 389–423. JOUR.

Sarrazin, J., Robigou, V., Juniper, S. K., & Delaney, J. R. (1997). Biological and geological dynamics over four years on a high-temperature sulfide structure at the Juan de Fuca Ridge hydrothermal observatory. *Mar. Ecol. Progr. Ser.*, *153*, 5–24.

Scharhauser, F. P. (2013). *Diploma thesis "The search for Candidatus Endoriftia persephone."* University of Vienna.

Schimak, M. P., Toenshoff, E. R., & Bright, M. (2012). Simultaneous 16S and 18S rRNA fluorescence in situ hybridization (FISH) on LR White sections demonstrated in Vestimentifera (Siboglinidae) tubeworms. *Acta Histochemica*, *114*(2), 122–130. Retrieved from http://linkinghub.elsevier.com/retrieve/pii/S0065128111000560

Schramm, A., Fuchs, B. M., Nielsen, J. L., Tonolla, M., & Stahl, D. A. (2002). Fluorescence in situ hybridization of 16S rRNA gene clones (Clone-FISH) for probe validation and screening of clone libraries. *Environmental Microbiology*, *4*(11), 713–720. JOUR. http://doi.org/10.1046/j.1462-2920.2002.00364.x

Shank, T. M., Fornari, D. J., Von Damm, K. L., Lilley, M. D., Haymon, R. M., & Lutz, R. A. (1998). Temporal and spatial patterns of biological community development at nascent deep-sea hydrothermal vents (9°50'N, East Pacific Rise). *Deep-Sea Res. II*, *45*, 465–515.

Southward, E. C. (1988). Development of the gut and segmentation of newly settled stages of Ridgeia (Vestimentifera): implications for relationship between Vestimentifera and Pogonophora. *Journal of the Marine Biological Association of the United Kingdom*, *68*(3), 465–487. JOUR.

Stewart, F. J., & Cavanaugh, C. M. (2005). Symbiosis of thioautotrophic bacteria with Riftia pachyptila. In *Molecular basis of symbiosis* (pp. 197–225). CHAP, Berlin, Heidelberg: Springer.

Thomson, W. C. (1873). The Depths of the Sea. An Account of the General Results of the Dredging Cruises of H.M.SS. "Porcupine and Lightning" during the Summers of 1868, 1869, and 1870. *Macmillan, London*. http://doi.org/10.1007/s13398-014-0173-7.2

Thurber, A. R., Jones, W. J., & Schnabel, K. (2011). Dancing for food in the deep sea: Bacterial farming by a new species of Yeti crab. *PLoS ONE*, *6*(11), 1–12. http://doi.org/10.1371/journal.pone.0026243

Trachtenberg, S. (2005). Mollicutes. *Current Biology : CB*, *15*(13), R483-4. http://doi.org/10.1016/j.cub.2005.06.049

Van Beneden, P. J. (1875). Les commensaux et les parasites. *Paris Biblio. Sc. Int.*

Van Dover, C. (2000). *The Ecology of Deep-Sea Hydrothermal Vents*. Princeton University Press.

Van Dover, C. L., German, C. R., Speer, K. G., Parson, L. M., & Vrijenhoek, R. C. (2002). Evolution and Biogeography of Deep-Sea Vent and Seep Invertebrates. *Science*, *295*(2002), 1253–1257. http://doi.org/10.1126/science.1067361

Von Damm, K. L. (2000). Chemistry of hydrothermal vent fluids from 9 degrees-10 degrees N, East Pacific Rise: "Time zero," the immediate posteruptive period. *Journal of Geophysical Research-Solid Earth*, *105*(B5), 11203–11222. http://doi.org/10.1029/1999jb900414

Vrijenhoek, R. C. (2010). Genetics and Evolution of Deep-Sea Chemosynthetic Bacteria and Their Invertebrate Hosts. In *The Vent and Seep Biota* (Vol. 33, pp. 15–49). http://doi.org/10.1007/978-90-481-9572-5

Vrijenhoek, R. C. (2010). Genetics and Evolution of Deep-Sea Chemosynthetic Bacteria and Their Invertebrate Hosts. In S. Kiel (Ed.), *The Vent and Seep Biota: Aspects from Microbes to Ecosystems* (pp. 15–49). inbook, Dordrecht: Springer Netherlands. http://doi.org/10.1007/978-90-481-9572-5_2

Wagner, M., Horn, M., & Daims, H. (2003). Fluorescence in situ hybridisation for the identification and characterisation of prokaryotes. *Current Opinion in Microbiology*, *6*(3), 302–309. http://doi.org/10.1016/S1369-5274(03)00054-7

Wallner, G., Amann, R., & Beisker, W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry [Internet]*, *14*(2), 136–43.

Wang, Y., Stingl, U., Anton-Erxleben, F., Geisler, S., Brune, A., & Zimmer, M. (2004). "Candidatus Hepatoplasma crinochetorum," a new, stalk-forming lineage of Mollicutes colonizing the midgut glands of a terrestrial isopod. *Applied and Environmental Microbiology*, *70*(10), 6166–6172. http://doi.org/10.1128/AEM.70.10.6166-6172.2004

Zal, F., Leize, E., Lallier, F. H., Toulmond, A., Van Dorsselaer, A., & Childress, J. J. (1998). S-Sulfohemoglobin and disulfide exchange: The mechanisms of sulfide binding by Riftia pachyptila hemoglobins. *Proceedings of the National Academy of Sciences*, *95*(15), 8997–9002. http://doi.org/10.1073/pnas.95.15.8997

ZUSAMMENFASSUNG

Nach der Entdeckung von hydrothermalen Quellen in der Tiefsee, sind die Symbiosen in Vestimentifera ausgiebig untersucht worden. Der Riesenröhrenwurm *Riftia pachyptila*, welcher den Ostpazifik bewohnt, ist ein sehr bekanntes Mitglied dieser Gruppe. *Riftia* ist ein darmloser und mundloser Wurm und seine Ernährung beruht auf einem chemolithoautotrophen, schwefeloxidierenden Gammaprotoebakterium, *Candidatus* Endoriftia persephone. Dieser Endosymbiont bewohnt Wirtszellen in einem speziellen Organ bekannt als Trophosom. Es betritt den Wirten über horizontale Übertragung von Neuem in jeder Wirtsgeneration durch die entwickelnde Röhre und Haut, wenn die Röhrenwurmlarve sich niederlässt. Weitere Infektion wird in Juvenilen durch Apoptose von Epidermis, Muskeln und mesodermalen Gewebe beendet. In erwachsenen Röhrenwürmern ist der Symbiont auf das Trophosom begrenzt und verlässt seinen Wirten nur auf Tod des Wurmes. Eine längere Übertragung oder eine Freilassung von Endoriftia in lebenden Tieren wurde bis jetzt nicht gezeigt. Neben der Entdeckung von Endosymbionten in der Röhre, wurde Endoriftia nur im Trophosom gefunden, die Haut wurde als Bakterienfrei betrachtet. Zwei vorangegangene Diplomarbeiten veränderten diese Ergebnisse. Endoriftia wurde in der Haut von erwachsenden Individuen und in Juvenilen von *Riftia* an zwei verschiedenen Standorten mit Denaturierungsgradientengelelektrophorese (DGGE) und Fluoreszenz *in situ* Hybridisierung (FISH) nachgewiesen. Zusätzlich wurde ein weiteres Bakterium zugehörig zu der Klasse der Mollicutes in der Haut mit DGGE verzeichnet. Mollicutes, welches wirtsabhängig ist, ist für seine pathogene Rolle in Menschen und als nützlicher Partner in Insekten bekannt. Basierend auf diesen neuen Einsichten, wurde ein längerer Übertragungsmodus und eine Freilassung von Symbionten oder eine Migration in und aus den Röhrenwürmern angenommen. Um den Übertragungszeitrahmen festzulegen und um eine Infektion von einem neuen symbiotischen Bakterium neben Endoriftia ein- oder auszuschließen, konzentriert sich diese Studie auf die Bestimmung und Lokalisation von Endoriftia und Mollicutes in der Haut von erwachsenen *Riftia pachyptila*. FISH wurde verwendet, um den Symbionten und Mollicutes in Hautproben von zwei verschiedenen Probenahmestellen zu lokalisieren. Neben seiner Lokalisation im Trophosom, wurde Endoriftia in großen Mengen extrazellulär angeheftet an die Hautaußenseite gefunden, vereinzelt im Hohlraum und einige Wenige intrazellulär in den birnenförmigen Drüsen zwischen den Hautmuskeln. Diese Ergebnisse deuten auf eine Kontamination während des Trennungsprozesses der unterschiedlichen Gewebe auf dem Forschungsschiff hin, in welchem die Symbionten des Trophosoms an den Hautstücken hängen blieben und zu positiven Ergebnissen in den zuvor erwähnten Diplomarbeiten führten. Die Resultate zeigen auch, dass wenige Symbionten den

Apoptoseprozess in der Haut überleben. Kein Mollicutes wurde innerhalb oder außerhalb der Haut von *Riftia* entdeckt. Dies weist auch wieder auf eine Kontamination während der Laborarbeit in den DGGE Experimenten von den Diplomarbeiten hin. Demnach bestätigt die aktuelle Studie eine Dual-Partnerschaft zwischen Endoriftia und *Riftia* nach einer Initialinfektion im Larvenstadium des Wirten.