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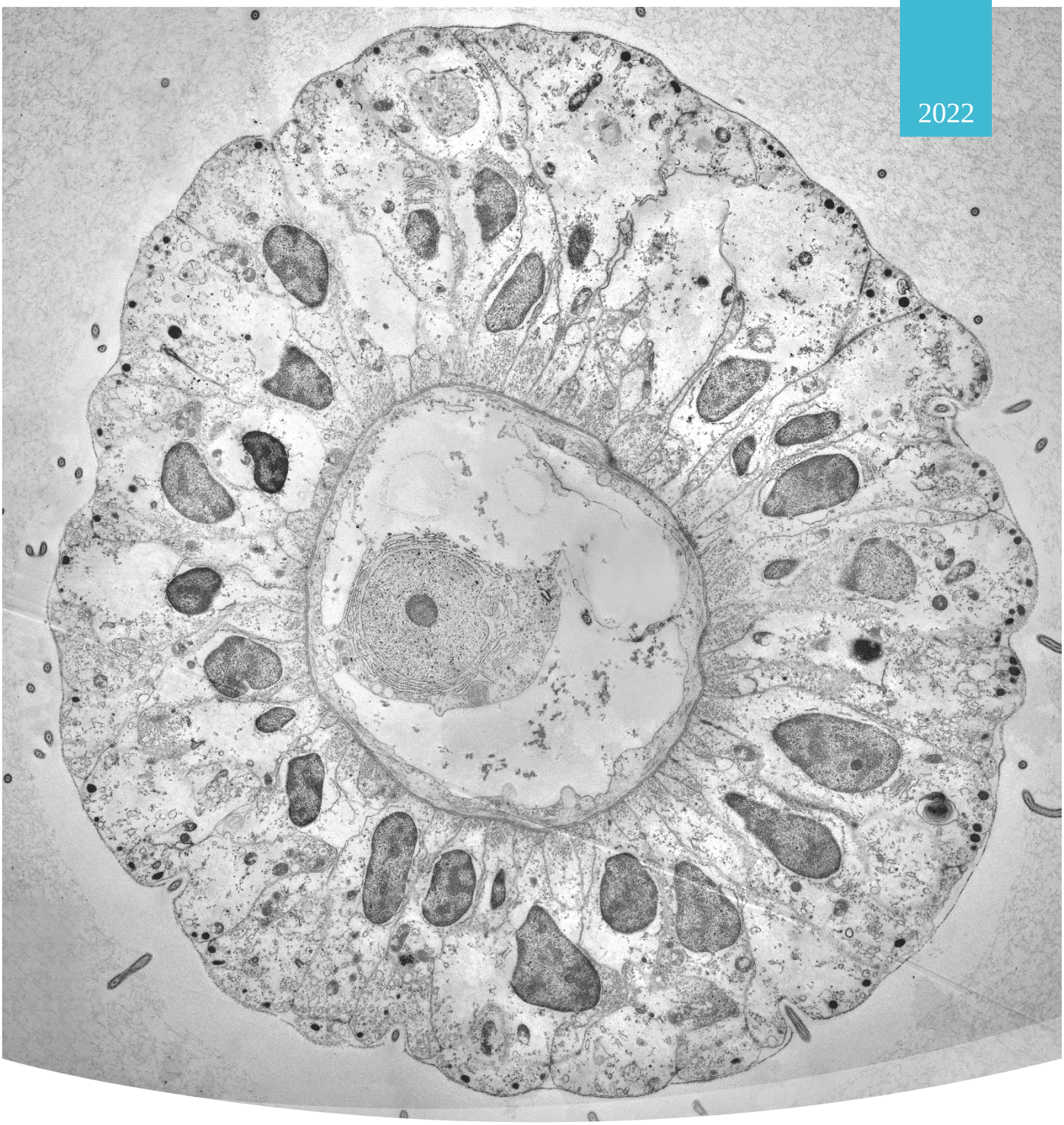

Morphological and molecular analysis of the meiofaunal cnidarian *Halammohydra* Remane, 1927 (Hydrozoa)

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Morphological and molecular analysis of the meiofaunal cnidarian *Halammohydra* Remane, 1927 (Hydrozoa)

Cumulative Dissertation

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I. RESEARCH REPORT

II. STUDIES

I. RESEARCH REPORT

1. General Introduction

1.1 Meiofauna and their traits

Marine sediments are a habitat to a very special group of organisms called meiofauna. It is a size defined group and contains representatives of almost all invertebrate taxa. Some taxa are even exclusively found here, such as Gnathostomulida, Kinoryncha, Loricifera and Micrognathozoa (Cerca et al. 2018; Fenchel 1978; Giere 2009). The size of the organisms is standardized to the mesh width of sieves. Animals should pass a sieve with a mesh size of 1000 µm (or 500 µm) and retain in 63 µm sieve (or 44 µm, depending on the source, Giere 2009; Schmidt-Rhaesa 2020). They mainly live in the interstitial system of marine sediments, which is the water-filled space between the sediment particles (Schmidt-Rhaesa 2020).

In order to live in this special environment, meiofaunal organisms show distinct adaptations. The most prominent one is the small size. Furthermore, they have elongated bodies and sometimes reduced appendages. Many organisms have special adhesive structures to temporarily adhere to any surface. All this helps to move in between the sand grains. Reproduction is adapted as well. They have only a low number of oocytes at once and the development is almost always direct, a planktonic larva is lacking (Fenchel 1978; Giere 2009; Schmidt-Rhaesa 2020).

Meiofaunal animals are described as sedentary because of the combination of these characters, especially the small body size, the adhesive organs and the lacking of a planktonic larva, thus the ability to distribute over a large scale of distance is thought to be restricted (Giere 2009). In contrast to that are findings of some amphi-oceanic or even cosmopolitan species (e.g., Bik et al. 2010; Boeckner et al. 2009; Cerca et al. 2018; Faurby et al. 2011; Fontaneto 2019; Guil 2011; Hagerman & Rieger 1981; Schmidt & Westheide 2000; Worsaae et al. 2019b). This contradiction is called the "meiofauna-paradox". Reinvestigations of some species, morphologically and with modern molecular methods, revealed overlooked characters in some cases but also a high cryptic diversity (e.g., Fontaneto et al. 2009; Jörger et al. 2012; Leasi et al. 2016; Tessens et al. 2021; Todaro et al. 1996; Worsaae et al. 2019a). Especially molecular methods provide important information for the identification of meiofauna with limited morphological characters (Cerca et al. 2018; Fontaneto et al. 2015). Additionally, there are dispersal hypotheses, which might explain the wide distribution of other species, such as stepping stones, occasional rafts or continental plate drift (Giere 2009; Sterrer 1973; von Soosten et al. 1998; Westheide 1991).

All this shows that there are many open questions regarding the meiofauna, which should be studied in more detail. Most studies are done on the major groups with wide occurrence, such as nematodes or copepods, but studies of smaller groups with more restricted habitat preferences are lacking. This is especially true for meiofaunal cnidarians. Less than 30 species of cnidarians are meiofaunal and the majority are hydrozoans (Schmidt-Rhaesa et al. 2020). There is only one genus representing staurozoans (*Stylocoronella*) and none of the groups Anthozoa and Cubozoa (Kikinger & Salvini-Plawen 1995). Most genera (seven) are polyps of the group Hydroidolina (Hydrozoa). One of them is *Protohydra leuckarti* Greeff, 1870 and it was the first described meiofaunal cnidarian. This polyp has no tentacles

and can change its body shape drastically, which are perfect adaptations to the interstitial system (Greeff 1870). During following years many more meiofaunal cnidarians were discovered and not only polyps found their way into the sediment. Four genera of medusa, all belonging to Trachylina (Hydrozoa), are part of the meiofauna as well: *Halammohydra* Remane, 1927 (see 1.2), *Otohydra* Swedmark & Teissier, 1958 (see 1.3), *Armorhydra* Swedmark & Teissier, 1958 and the most recent one *Marsipohydra* Sanamyan & Sanamyan, 2012 (see 1.3).

1.2 The genus *Halammohydra*

The largest genus of meiofaunal cnidarian, with nine species described so far, is *Halammohydra* Remane, 1927. Most of them were described from Europe (six) and three from India (Schmidt-Rhaesa et al. 2020, table 1). *Halammohydra* consists of a gastric tube of about 300 to 500 µm in length with a terminal mouth opening (fig. 1A). Aborally, it decreases its diameter and forms a neck, which is then connected to a special structure called aboral cone. It is thought to be the remnant of the reduced umbrella (Clausen 1967; Remane 1927). Two whorls of tentacles and one whorl of statocysts insert into the aboral cone alternating to each other. At the tip of the aboral cone, a specialized adhesive organ is located (fig. 1B). It is used to adhere temporarily to surfaces and a common feature of the meiofaunal life style (Giere 2009). The whole body of *Halammohydra* is covered in cilia, which are used for locomotion. When gliding, the animal is oriented with the aboral pole in front and tentacles of both rings are directed orally (Remane 1927). Most species have isorhizas and two size classes of stenoteles. There are slight differences in some species described, such as missing one of the size classes (e.g. *H. intermedia* and *H. vermiformis*, Clausen 1967; Swedmark & Teissier 1957b) or having euryteles instead of stenoteles (e.g. *H. adherens* and *H. coronata* Clausen 1967; Swedmark & Teissier 1967).

Halammohydra has a separate sex and a direct development without a polyp stage (Remane 1927). Only a few studies contain information about the early development and only in detail on the species *Halammohydra schulzei* and *H. vermiformis* (Clausen 1971; Swedmark 1957; Swedmark & Teissier 1950, 1957b, a, 1967; Werner 1964). The gonads are located on one side of the gastric tube in the endoderm (Ehlers 1993). It was described, that the spermatozoa and oocytes enter into the gastric lumen with a rupture of the gastrodermis and are released into the water via the mouth opening (Clausen 1971; Ehlers 1993; Swedmark & Teissier 1966). Oocytes are shed individually and stuck to sediment particles until the embryonal development is finished (Swedmark & Teissier 1957b, a; Werner 1964). One of the first characters to be developed is the adhesive organ by an invagination of the ectoderm. The resulting urn shaped structure consists of mucus-secreting gland cells (Swedmark & Teissier 1966).

table 1: All nine so far known species of *Halammohydra* with first author(s) and type – locality.

species	author	type - locality
H. schulzei	Remane, 1927	Germany, Helgoland
H. octopodides	Remane, 1927	Germany, Kieler Bucht
H. vermiformis	Swedmark & Teissier, 1957	France, Roscoff
H. adherens	Swedmark & Teissier, 1959	France, Roscoff
H. intermedia	Clausen, 1967	Norway, Espegrend
H. coronata	Clausen, 1967	Germany, Helgoland
H. chauhani	Rao, 1975	India
H. andamanensis	Rao, 1978	India, Rangat Bay
H. sagarensis	Rao, 1978	India, Sagar Island

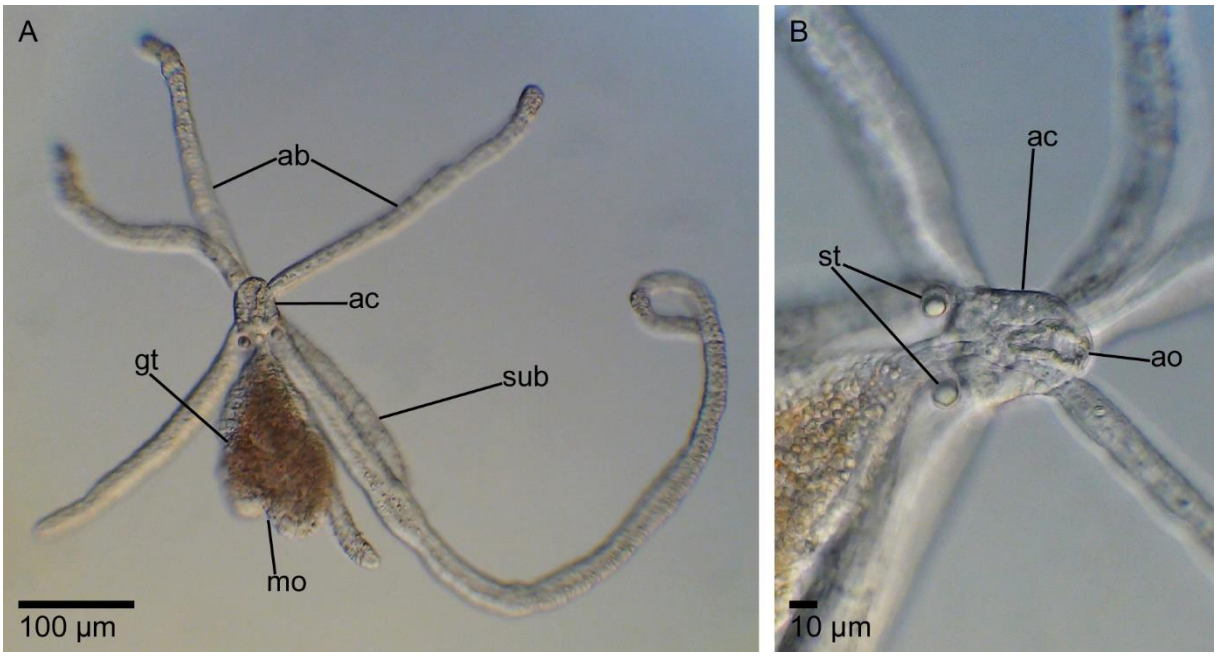

fig. 1: Habitus of *Halammohydra vermiformis*. **(A)** Overview of the entire animal, showing the gastric tube (gt) with mouth opening (mo) connected to the aboral cone (ac) and two whorls of tentacles, aboral (ab) and subaboral (sub). **(B)** Magnification of the aboral cone (ac). Two statocysts (st) and the adhesive organ (ao) are visible.

Simultaneously, primary tentacles form. After that, the development of first nematocyst and cilia happens and the larva starts to move. With the addition of one pair of tentacles in the aboral whorl and four tentacles in the subaboral whorl, the larva detaches from the sediment particle and swims with its cilia, as the adults (Swedmark & Teissier 1950, 1957b, a, 1966). There is a slight difference in the order of the developing tentacles in the two investigated species resulting in *H. schulzei* detaching from the particle with eight tentacles and *H. vermiformis* with seven (Swedmark & Teissier 1957a). Further steps of the development happen almost simultaneously: the mouth opens, first statocysts develop and additional tentacles emerge by division of the primary ones, at least for *H. schulzei* (Swedmark & Teissier 1950). *Halammohydra vermiformis* already reached its final tentacle number at this stage (Swedmark & Teissier 1957b). It was found, that some individuals can be sexual mature before reaching the total number of tentacles (Swedmark & Teissier 1966).

Most studies investigated the general habitus of *Halammohydra*. Only a few had a closer look in the cell structure. The earliest work is by Remane (1927). He did a detailed histological work of the whole body of *H. schulzei* and *H. octopodides* and described all the different cell types, especially in the gastrodermis. His study was followed by Swedmark & Teissier (1967) with a histological comparison of the aboral cone of *H. adherens* and *H. schulzei*. It shows a highly muscular area around the adhesive organ in the aboral cone in *H. adherens*. In addition to that, this part is more separated from the rest of the aboral cone, compared to *H. schulzei*. This structure is possibly the reason for the highly adhesive behavior of *H. adherens*. Ultrastructural studies were only done on specific parts of the body, such as the nematocysts (Clausen 1991) and on microsporidia in the adhesive organ of *H. intermedia* (Clausen 2000) or the ultrastructure of the male gonad of *H. schulzei* (Ehlers 1993). There is one immunohistochemical study about the nervous system in *H. octopodides* (Polte & Schmidt-Rhaesa 2011). It shows a distinct nerve ring in the aboral cone innervating the tentacles with some short and one long neurite and a plexus surrounding the mouth opening. It is connected to the nerve ring with a few longitudinal neuritis.

All these studies together give a fundamental overview, but are missing some important structures, such as the distribution of myofibrils, the female gonad and its oocytes or the organization of the aboral cone. Additionally, more information is needed about different species, to understand the species-specific structures and behaviors.

1.2.1 Place within the cnidarian tree

When Remane discovered *Halammohydra* in 1927, he placed this genus into Trachylinae because of the structure of the statocysts, the lacking of a polyp stage and similarities in the habitus. Due to the fully ciliated body, the location of the gonads and the similarities to the larval stages, he put them further into Narcomedusae but in an isolated position, creating Halammohydridae. Later, with the discovery of another fully ciliated meiofaunal cnidarian named *Otohydra vagans* Swedmark & Teissier, 1958 the order Actinulida was created. The name Actinulida originated in the similarities to the actinula larva (Swedmark & Teissier 1966). Since there are some important differences of *Halammohydra* and *Otohydra* (see 1.3), they were placed in the families Halammohydridae and Otohydridae (Swedmark & Teissier 1966). The exact position of *Halammohydra* was not known, until Collins et al. (2008) conducted a molecular study, with sequences of an unidentified specimen of *Halammohydra*. It resulted in the placement into Trachylinae and an origin within the family Rhopalonematidae (Trachymedusae, fig. 2). These are the only sequences available so far and there, none for *Otohydra*.

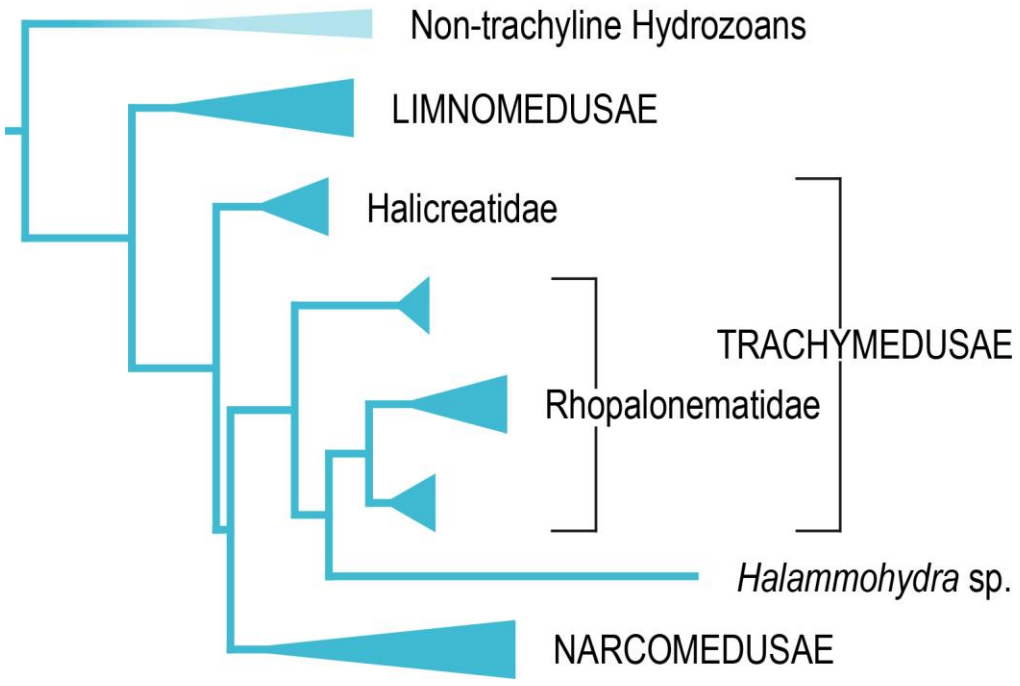

fig. 2: Schematic of the Hydrozoan phylogenetic tree. *Halammohydra* sp. shows an origin in the family Rhopalonematidae. Modified after Collins et al. 2008.

1.2.2 Geographical distribution

The first discovery of *Halammohydra* was made in the Baltic Sea and on the German island of Helgoland (Remane 1927). Specimens were found in medium to coarse sand but also fine sand and even shelly gravel. Most records are from subtidal locations, but reports from the intertidal are known as well. *Halammohydra* was mainly found in Europe and India. In Europe, most records are from Germany (Clausen 1967; Ehlers 1993; Polte & Schmidt-Rhaesa 2011; Remane 1927; Schmidt 1969; Schulz 1952) and France (d'Hondt 1968; Renaud-Debyser 1964; Swedmark & Teissier 1957b, 1959, 1967; Teissier 1950). Five of the six European species were found in the surrounding waters of Helgoland in Germany and four were recorded from Roscoff, France (fig. 3). This is a comparably high amount, taking the low abundance and patchy appearance into account.

Other European records (fig. 3) with few findings are from Sweden (Boaden 1960; Dahl 1953), United Kingdom (Boaden 1961, 1963, 1966; Gray 1971; Harvey & Wells 1961; Moore 1979) and Norway (Clausen 1963, 1967, 1991, 2000, 2004), and single records from the Netherlands (Wolff et al. 1974), Adriatic Sea (Salvini-Plawen 1991) and Spain (Martínez et al. 2019; Martínez et al. 2009). Most records outside of Europe (fig. 3) are from India (Altaff et al. 2005; Janakiraman et al. 2016; Mohan & Dhivya 2010; Nagabhushanam 1972; Rao 1975, 1978, 1993; Rao & Ganapati 1965, 1966; Rao & Misra 1980; Salvini-Plawen & Rao 1973; Sugumaran et al. 2009; Sugumaran & Padmasai 2019; Varadharajan & Soundarapandian 2013) and a few are from Western Atlantic and Caribbean Sea (Bush & Zinn 1970; Calder & Kirkendale 2005; Garraffoni et al. 2017; Hochberg et al. 2014; Jörger et al. 2014; Kånneby et al. 2014).

Due to the history of meiofaunal research, the majority of those records originate from Europe and were made in the vicinity of marine stations. This likely does not reflect the true geographical distribution of the genus *Halammohydra* or the respective species.

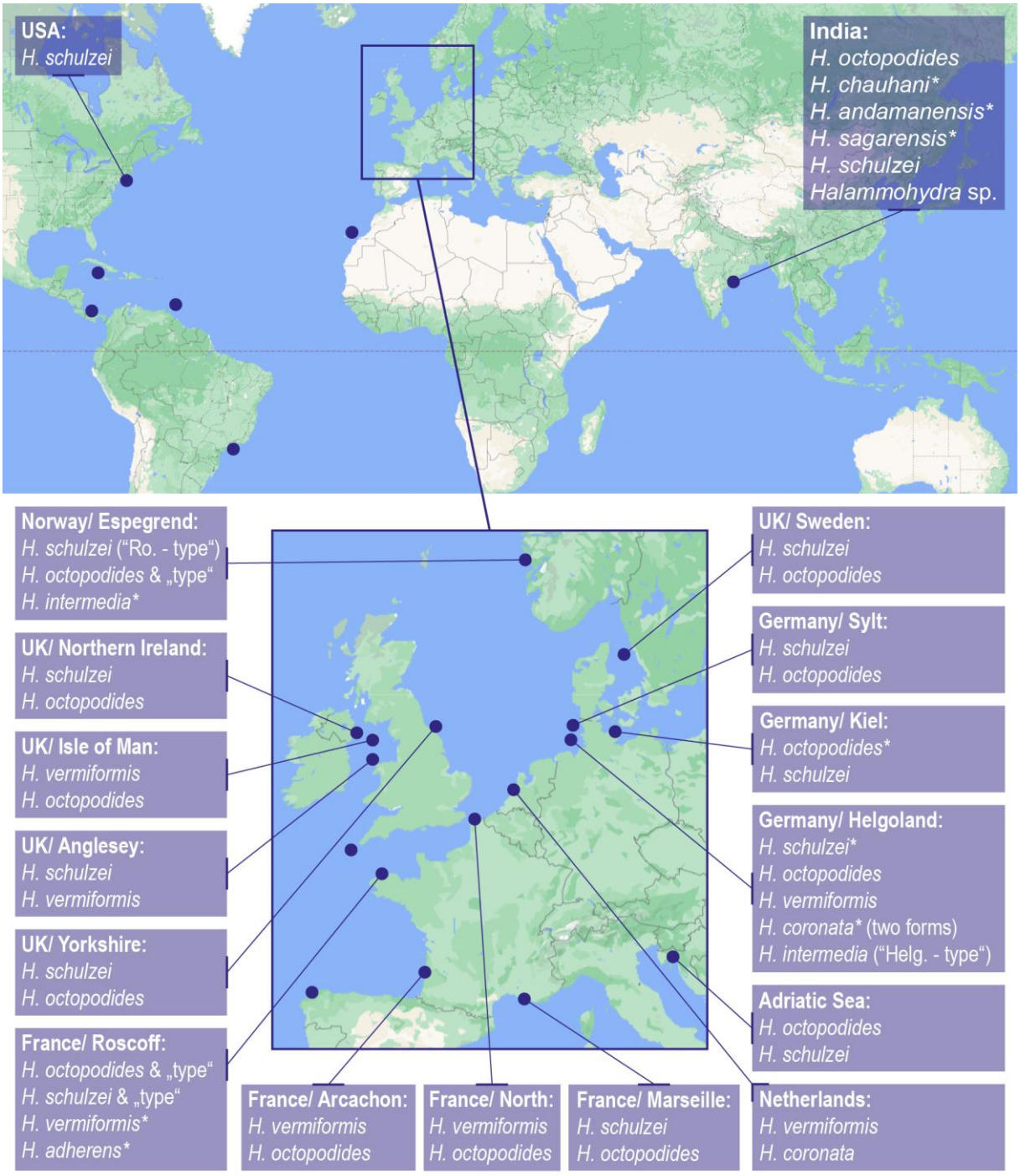

fig. 3: Reported locations of Halammohydra around the world with magnification of Europe. Dots without a connected square are unidentified specimens. Type-localities of every species are marked with asterisk (*). Map source: Google maps.

1.2.3 Species identification and its problems

For the identification of *Halammohydra* on a species level, certain characters are important. First the general size and the shape of the gastric tube can be used. For example, *H. vermiformis* has most of the times an elongated or a completely round gastric tube in contrary to other species, which are mostly ovoid. An important structure is the aboral cone. It contains a lot of criteria for identification, such as the general shape or the shape and depth of the adhesive organ.

The tentacles are the most important but, unfortunately, the most variable structures. Some species, such as *H. coronata* or *H. vermiformis* were recorded with a small range of tentacle number (see Clausen 1967; Swedmark & Teissier 1957b), whereas other species, such as *H. schulzei* or *H. octopodides* were recorded with a wide range of tentacle numbers within one species (see Remane 1927; Swedmark 1957). These numbers even overlap between species, which complicates the identification (fig. 4). In some cases, the difference in tentacle numbers (in combination with other characters) led to the description of "variants" or "types" of different species. This is the case for *H. schulzei*, *H. octopodides* and *H. intermedia* (Clausen 1967; Swedmark 1957). Thus, this feature alone cannot be used for identification and has to be used in combination with others, such as the shape and length of the tentacles. For example, *H. vermiformis* and *H. octopodides* both have one about two times longer tentacles in the subaboral whorl as a clear character (Remane 1927; Swedmark & Teissier 1957b). Other important features are the tentacle bases (no structure, thickening, club shaped), but this character can be very subjective, since sometimes a thickening of the bases can be highly pronounced or very subtle. The direction of the tentacles in each whorl can give some information as well. Species, such as *H. vermiformis* or *H. coronata* have a visible separation between both whorls (compare fig. 1 & 5). The aboral tentacles are directed aborally and the subaboral ones are directed orally. This is especially visible, when the animals glide.

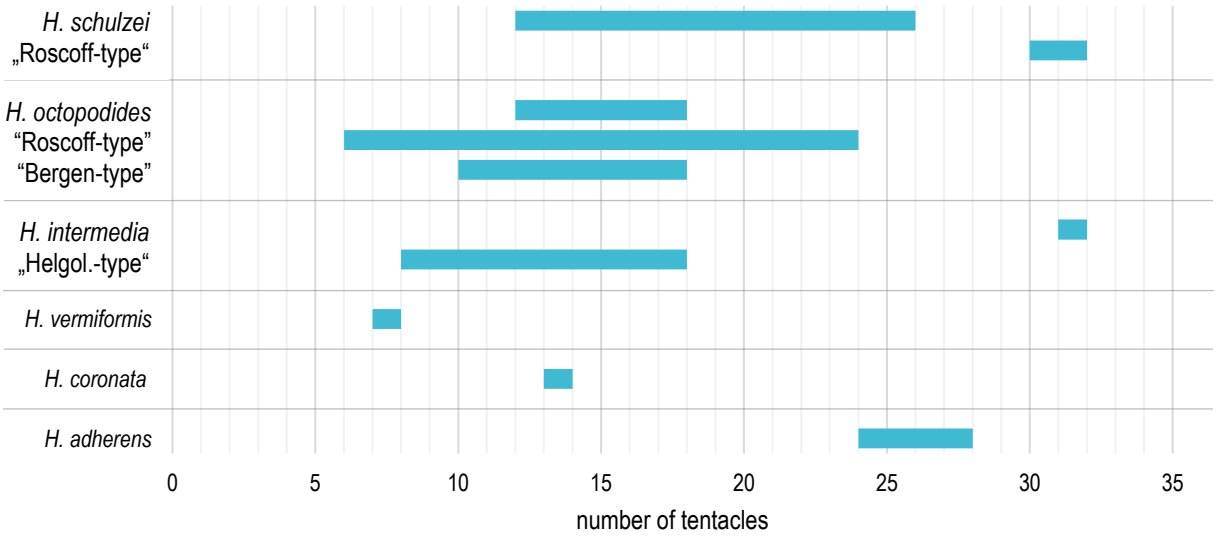

fig. 4: Summary of reported total tentacle numbers of the six European species of *Halammohydra* and their "variants/ types".

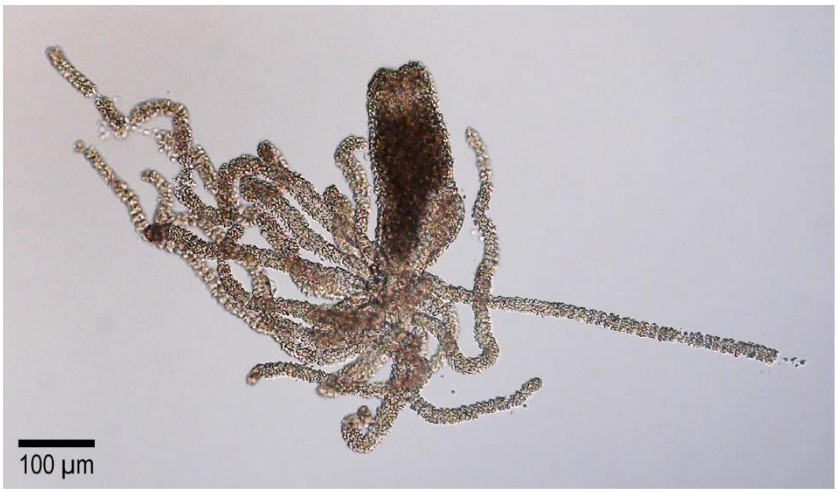

fig. 5: Light microscopy image of *Halammohydra* sp. adhering to the slide. Important characters for identification, such as the shape of the aboral cone or the tentacle bases are obscured.

The cnidome differs only slightly between species and is only useful in specific cases, such as the elongated euryteles in *H. adherens* (Swedmark & Teissier 1967). Different species can also show differences in the behavior. *Halammohydra schulzei* and *H. adherens* for example, were described to be very adhesive (Remane 1927; Swedmark & Teissier 1967), whereas *H. coronata* or *H. vermiformis* are found to be more free-swimming and less adhesive (Clausen 1967; Remane 1927). The adhesive behavior can help in the identification but also totally obscure important features (fig. 5).

One of the main problems in these character is the high variability within one species. This is especially problematic, when a species is only described based on a low number of specimen, which is the case for *H. coronata*. It was described on only three specimen, thus the whole range of the characters might not be documented (Clausen 1967). Additionally, *Halammohydra* is highly contractible, which can obscure important characters. All of this can complicate the morphological identification on a species level and since there are no species-specific sequences available so far, an unambiguous identification remains challenging.

1.3 The genus *Otohydra*

In 1958, Swedmark & Teissier discovered another fully ciliated meiofaunal medusa in a subtidal station in Roscoff (France, shelly gravel). *Otohydra vagans* Swedmark & Teissier, 1958 has a size of about 500 µm and an ovoid body with on ring of tentacles (fig. 6A), surrounding the hypostome with the mouth opening (fig. 6B). At the bases of the tentacles, statocysts are located. Most specimen have 12 to 16 tentacles with 8 to 12 statocysts. The maximum recorded was 24 tentacles with 12 statocysts (Swedmark & Teissier 1958). It is described to swim constantly with the aboral pole in front using the cilia. Nematocysts of the stenotele type are present on the whole body, but most abundant on the tentacles (fig. 6C, Swedmark & Teissier 1958). There is information about a second species with the name *Otohydra tremulans* Lacassagne, 1973, but it is unaccepted[1].

[1] This information is only visible on World Register of Marine Species (WoRMS) and based on a record in "van der Land, J.; Vervoort, W.; Cairns, S.D.; Schuchert, P. (2001). Hydrozoa, ***in***: Costello, M.J. *et al.* (Ed.) (2001). *European register of marine species: a check-list of the marine species in Europe and a bibliography of guides to their identification. Collection Patrimoines Naturels,* 50: pp. 112-120". The original description of "Lacassagne, M., 1973. Biologie des Hydrozoaires mésopsammiques. Proc. II. Meiofauna Conference, York" is unavailable, thus there is no information about the identification characters of the place of record.

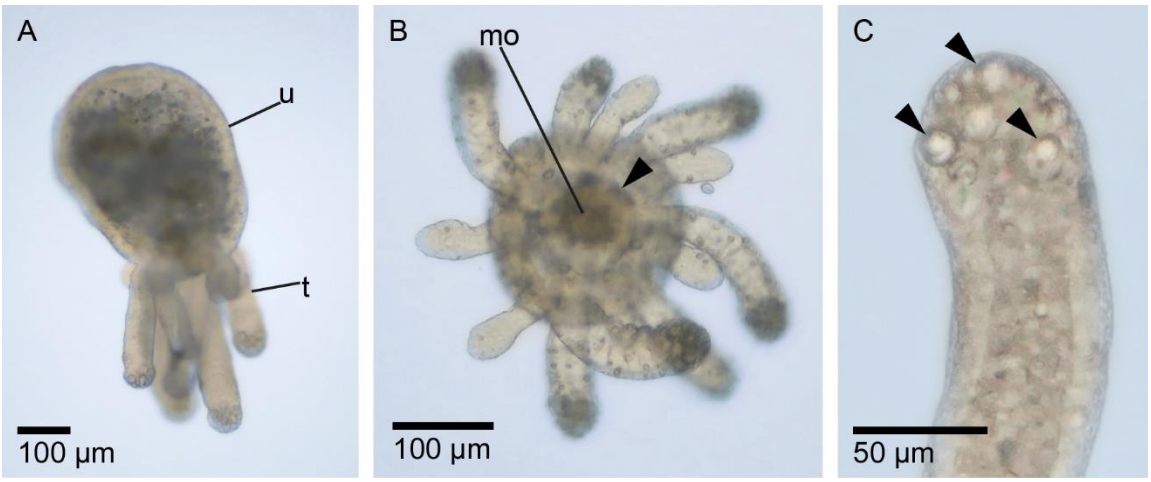

fig.6: Light microscopy images of *Otohydra* sp. **(A)** lateral view a specimen showing the umbrella (u) and tentacles (t). **(B)** Top-down view of a specimen with a visible ring of the retractable hypostome (arrowhead) and the mouth opening in the center (mo). **(C)** Magnification of the tip of one tentacle showing the concentration of large nematocysts (arrowheads).

Otohydra vagans is hermaphroditic and has a direct development without a polyp stage. It has two gonads, a male and a female one. Maturation, fertilization and embryonal development take place in an incubation cavity, which is located between endoderm and ectoderm (Swedmark & Teissier 1958). It can contain two to three embryos of different developmental stages. When the juveniles are born, they have 8 tentacles and 4 statocysts (Swedmark & Teissier 1958, 1959, 1966).

There are only a hand full of records of *Otohydra* so far. The first finding was made in Roscoff in France (Swedmark & Teissier 1958, 1959), followed by single findings from Rovigno in Croatia (Salvini-Plawen 1966), Ria de Ferrol in Spain (Besteiro & Urgorri 1988), Otsuchi Bay in Japan (Takashima 2001) and a doubtful finding on the Canary Islands (Martínez et al. 2019). All localities were subtidal and in coarse sand or shelly gravel.

Due to the similar statocysts, a direct development and the cilia covering the whole body of *Halammohydra* and *Otohydra*, both were grouped together into Actinulida in the two families Halammohydridae and Otohydridae (Swedmark & Teissier 1966). Nevertheless, there are major differences between both genera. *Otohydra* does not have a special adhesive organ or an equivalent to a nerve ring, as in *Halammohydra.* The general body shape resembles a medusa with an umbrella, it has only one whorl of tentacles and the mouth opening with the hypostome is located in between them. Additionally, *Otohydra* is hermaphroditic, whereas *Halammohydra* has a separate sex (Swedmark & Teissier 1958, 1959, 1966).

In 2012, Sanamyan & Sanamyan discovered *Marsipohydra pacifica* in East Kamchatka (subtidal, gravel). It has similar characters than *Otohydra*, hence it was placed into the family Otohydridae. As in *Otohydra* and *Halammohydra*, the whole body is covered in cilia, but the general body structure is closer to *Otohydra*, with an umbrella, one whorl of tentacles, a manubrium in the center of the tentacles and a direct development with releasing fully formed medusae (Sanamyan & Sanamyan 2012). The main differences of *Marsipohydra pacifica* are the presence of two types of tentacles (short with adhesive enlargement at their ends and long and tapering), a separate sex with dimorphism and an external brood pouch (Sanamyan & Sanamyan 2012).

The relationship of all three genera is not confirmed with molecular data, since there are no sequences of *Otohydra* or *Marsipohydra* available so far.

1.4 Methodology

For the investigation of the different species of *Halammohydra*, specimens needed to be collected, documented (see 1.4.1) and sequenced (see 1.4.2). A detailed study of the cell structure was done with transmission electron microscope (TEM, see 1.4.3). In the following, an overview of the methods used and their reasoning is shown.

1.4.1 Field work and documentation

Specimens of *Halammohydra* were mainly found in fine to coarse sand and even shelly gravel. There are no records for finer sediment. Hence, samples were taken at locations with fitting sediment properties. Additionally, localities of the literature were used to choose appropriate sampling sites. Most species are recorded from Helgoland (Germany) and Roscoff (France), thus the sampling took place there. Additionally, there is a known reliable location at the south tip of the German island Sylt. A fourth field trip was conducted in Bergen in Norway. Unfortunately, the recorded locations did not contain the correct sediment and no specimens were found there.

In the context of different projects, specimens were found on the Azores (Portugal) and Tenerife (Spain). Furthermore, fixed material of previous field trips was provided by Katrine Worsaae (University of Copenhagen, Marine Biology). These specimens originate not exclusively from Europe, but also from the West Atlantic. A detailed description of all sampling sites can be found in *Study III*.

The best way to take subtidal samples is to use a van-veen-grab or a dredge. At intertidal locations, the sediment can be collected with a shovel. This was the case for one location on Helgoland and on Sylt. One of the most common methods to extract meiofauna from the sediment is the anesthesia-decantation method (Giere 2009; Jörger et al. 2021). For this, the sediment was stored for one to two days with about 2 cm water covering the surface, to create an anoxic environment in the deeper layers. The animals in the sediment relocate closer to the surface, which reduces the amount of sediment to process. Small portions of the sample were mixed with a magnesium-chloride ($MgCl_2$) solution corresponding to the salinity of the sampled locations. $MgCl_2$ relaxes the animals and by stirring of the sample, the animals suspend in the liquid part of the solution. This property was used to separate the animals from the sediment by decantation of the liquid part into a 63 µm sieve. After transferring the sample into a petri dish and reviving the animals with seawater, it was inspected using a stereo microscope. This extraction method is very suitable for soft-bodied meiofauna, since it is gentle and very effective (Giere 2009). It can be repeated with the same sample, to extract most of the animals. Additionally, it is comparably easy to conduct in different laboratories, since only the $MgCl_2$ and a few supplies are needed.

To identify *Halammohydra* on a species level, the specimens had to be inspected under a compound microscope. This can be stressful for the animals and was visible by the contraction of the body and the adhesion to the slides. The specimens could be relaxed to a certain amount with the $MgCl_2$-solution. Since specimens of *Halammohydra* are soft-bodied animals, a digital documentation of all the characters was necessary. When the animals are fixed, they lose many of the important features, turn white. A reinvestigation under the compound microscope is difficult and can result in destroying of the animal. Images and videos are an important method to preserve the identification characters for later.

Most specimens were fixed individually in 98 % ethanol, to have a direct link of the documented morphology and the sequences (see 1.4.2). Some well-preserved specimens were fixed in Trumps solution (combination of sodium cacodylate buffer, formalin, and glutaraldehyde) for morphological methods (see 1.4.3). Few specimens, especially the ones with destructions, were squeezed with a slide and used for the analysis of the nematocysts. For this, the nematocysts had to fire, to document all parts of the cysts and identify them.

1.4.2 Molecular work

Specimens fixed in ethanol were prepared for sequencing in the home institute (Leibniz Institute for the Analysis of Biodiversity Change). The DNA was extracted by first digesting every specimen and then purifying the sample with magnetic beads. This method is time efficient and easy to conduct on a higher number of samples. Since the animals are tiny and extracted individually, the amount of resulting sample was low and needed a precise planning in further steps.

The easiest method for processing a high amount of animals is Sanger sequencing of single genes. For this, two mitochondrial (16S and CO1) and one nuclear gene (18S) were chosen. 16S and CO1 are barcoding genes and commonly used for species identification, hence many reference sequences are available (Fontaneto et al. 2015). Another commonly used gene is 18S. It is a slow evolving gene (Hillis & Dixon 1991) and does not always resolve on a species level (Fontaneto et al. 2015; Tang et al. 2012) but can help to find deeper phylogenetic differences. Using three genes as markers is a good approach to, first, generate useful information to fill databases for future studies, and, second, to compare the resulting phylogenetic trees and find possible differences, since every gene evolved differently (Fontaneto et al. 2015). With this, the credibility of the results can be checked. Further details about DNA extraction, the complete polymerase-chain-reaction (PCR) protocol and the used primers can be found in *Study III*. Resulting PCR products were sent to Macrogen Europe B. V. and sequenced because there is no infrastructure for that in the LIB.

Sequences were analyzed individually and in a concatenated supermatrix. By this procedure, differences between the genes can be recognized and taken into account in the final interpretation. Additionally, two phylogenetic analyses were done to compare the topologies and node support values (Bayesian Inference and Maximum Likelihood). This is commonly done, since different analyses can result in different outcomes, thus a combined interpretation is needed. The same datasets were used for three species delimitation tests (Automatic Barcode Gap Discovery/ ABGD, Generalized Mixed Yule Coalescent/ GMYC and Poisson-Tree-Process/bPTP) with the same reasoning. Lastly, the genetic divergence or k2p values, were calculated. These values can give further information on the differences within or between groups. All preparation steps of the sequences and settings for the phylogenetic analyses and species delimitation tests can be found in *Study III*.

1.4.3 Ultrastructural imaging

For transmission electron microscopy (TEM), specimens were fixed in Trumps solution with glutaraldehyde, since it is recommended for soft-bodied fauna (Giere 2009). Advantages of this fixative are its durability, because specimens can be stored at room temperature for a long period of time, and its easy handling. It can be used directly, without changing of the buffer and is well suited for fixing samples in the field (Giere 2009). In the home institute, specimens were postfixed with osmium tetroxide (1 %, in sodium-cocadylate buffer) and embedded in LR White resin following an established modified protocol by McDonald (1984) and Purschke et al. (1991). This protocol was used several times before with good results, hence it was used here again. A combined method of semi thin (0.5 µm) and ultra-thin (70 nm) sections was chosen for time-efficient processing of the specimens. Additionally, this allows overviewing and detailed imaging of cell structures, helping understand the internal organization and changes in the different tissues.

Ultra-thin sections were contrasted with lead citrate and uranyl acetate, whereas semi thin sections were stained with toluidine blue. Both were digitized, using the TEM with a corresponding program and a compound microscope which can scan slides automatically. This helped with further analyzing the structures (*Study II*).

1.5. Aims of this thesis

The genus *Halammohydra* is, in comparison to other meiofaunal organisms, understudied, mainly due to the challenges in the morphological identification and the lower abundance. In the aspect of biodiversity, it is important to know the occurrence of not only the most represented groups, but also the smaller ones with certain habitat preferences, preferably on a species level. This can help to reveal patterns of speciation and distribution. For this, a reevaluation of the already described species and an investigation of new character combinations, thus possibly new species is needed. Since the morphological identification is challenging (see 1.2.3), molecular methods are required as an additional feature. An integrative approach might help dealing with uncertainties, especially in the cases of the described "variants" or "types" for some species.

In addition to the clarification of the species identification, there are many other open questions regarding *Halammohydra*. One of them is the phylogenetic position in the cnidarian tree and the relationship to the other meiofaunal cnidarian *Otohydra*, thus the existence of the group Actinulida. Since there are major morphological differences, this relation is in question and can be answered using molecular methods. Additionally, a detailed investigation of the internal organization and specific structures, such as the female gonad or the aboral cone, is needed to fill the gaps of knowledge and possibly get information about the life style and function of specific structures. All this is presented in the following studies and cited accordingly:

Study I: Tödter L & Schmidt-Rhaesa A (2021) First record of *Halammohydra* (Cnidaria, Hydrozoa) on the Azores. Acoreana Special Volume (11), 97-102.

Study II: Tödter L & Schmidt-Rhaesa A (2022) Ultrastructural organization of *Halammohydra vermiformis* Swedmark & Teissier, 1957 (Cnidaria: Hydrozoa). Zoomorphology, 1-17. doi:https://doi.org/10.1007/s00435-022-00560-w.

Study III: Tödter L, Worsaae K & Schmidt-Rhaesa A (in review) Comparative molecular and morphological species selineation of Halammohydra Remane, 1927 (Hydrozoa) – with description of four new species

Study IV: Tödter L & Schmidt-Rhaesa A (submitted) Morphological and molecular analyses of the meiofaunal cnidarian Otohydra sp. (Hydrozoa, Cnidaria) invalidate the taxon Actinulida

2. Results and discussion

This project combines morphological and molecular methods to investigate the genus *Halammohydra* in detail and tries to answer questions regarding the phylogenetic relationships and function of specific structures. Main results are presented in *Study I* to *IV* and discussed below.

2.1 Challenges during the project

During the data collection, challenges occurred on different levels, which needs to be addressed here. In the field, collecting the animals was sometimes difficult. As other meiofaunal organism, *Halammohydra* has a very patchy appearance, which does not ensure regular success in sampling. For example, on one field trip to Helgoland eight buckets of sediment were collected at a reliable location and only five contained specimens. In the case of the stations of Espegrend in Norway, the sediment changed over time (only mud was found) and the reported locations are not current anymore. Nevertheless, 286 specimens (+16 fixed ones) were found and investigated during this project.

At most locations only a low number of specimens were found. At locations of Helgoland and Roscoff on the other hand, specimens came in comparably high numbers, with about 110 to 120 each. Additionally, it was observed, that sorted specimens died rather fast. Since all specimens had to be documented as detailed as possible, this led to time pressure. It is the best practice to document the animals alive and in a relaxed position, because when they are stressed, they tend to contract the whole body and thus obscure important features (fig.7A). This is one reason why not all characters could be documented. Other times, the characters were not visible because of overlapping structures (fig. 7B), the position of the animal or a specific behavior. For example, some species are very adhesive, so the lateral view of the aboral cone (fig. 7C), which contains information about the shape of the aboral cone and the adhesive organ is not visible. Additionally, this behavior was observed as a stress reaction as well. Relocating the animal was not always possible.

When specimens were investigated using light microscopy, it was tried to identify them directly. This was challenging for most of the cases because of the general problems mentioned in section 1.2.3 and above but also because of the characters itself. Sometimes smooth transitions or intermediate states between two characters were observed, especially in the tentacle bases or the shape of the aboral cone (compare fig. 7D & E). Additionally, about 20 % of the collected specimens were juveniles and could not be identified morphologically or used in the final morphological analysis. An exception to this are juveniles of *H. vermiformis*, because they reach their final number of tentacles very early (Swedmark & Teissier 1957b) and have a special morphology, which is good to identify.

Since the morphological identification was challenging, most specimens were fixed in ethanol for molecular identification. In many cases, it was decided to use uncertain specimens for sequencing to have more information of the other characters in one group. This led to missing information about the cnidome in some species or information about the cnidome without being able to assign it to a species.

In *Otohydra* on the other side, only eight specimens were found in total, which restricted the analysis of the cnidome and ultrastructure to one each. Specimens were also highly fragile after the anesthesia with $MgCl_2$. This was needed, since the animals were constantly swimming in light microscopic investigations. Additionally, some specimen adhered to the slides with the mouth opening and it was impossible to release them without a destruction (fig. 6B). All this resulted in only one intact specimen for ultrastructural investigation, thus this analysis has to be taken with caution. Unfortunately, this specimen was missing statocysts in light microscopy, as well as in ultrastructural images for an unknown reason (fig. 6A). Nevertheless, this specimen was useful to get a good overview of the whole body.

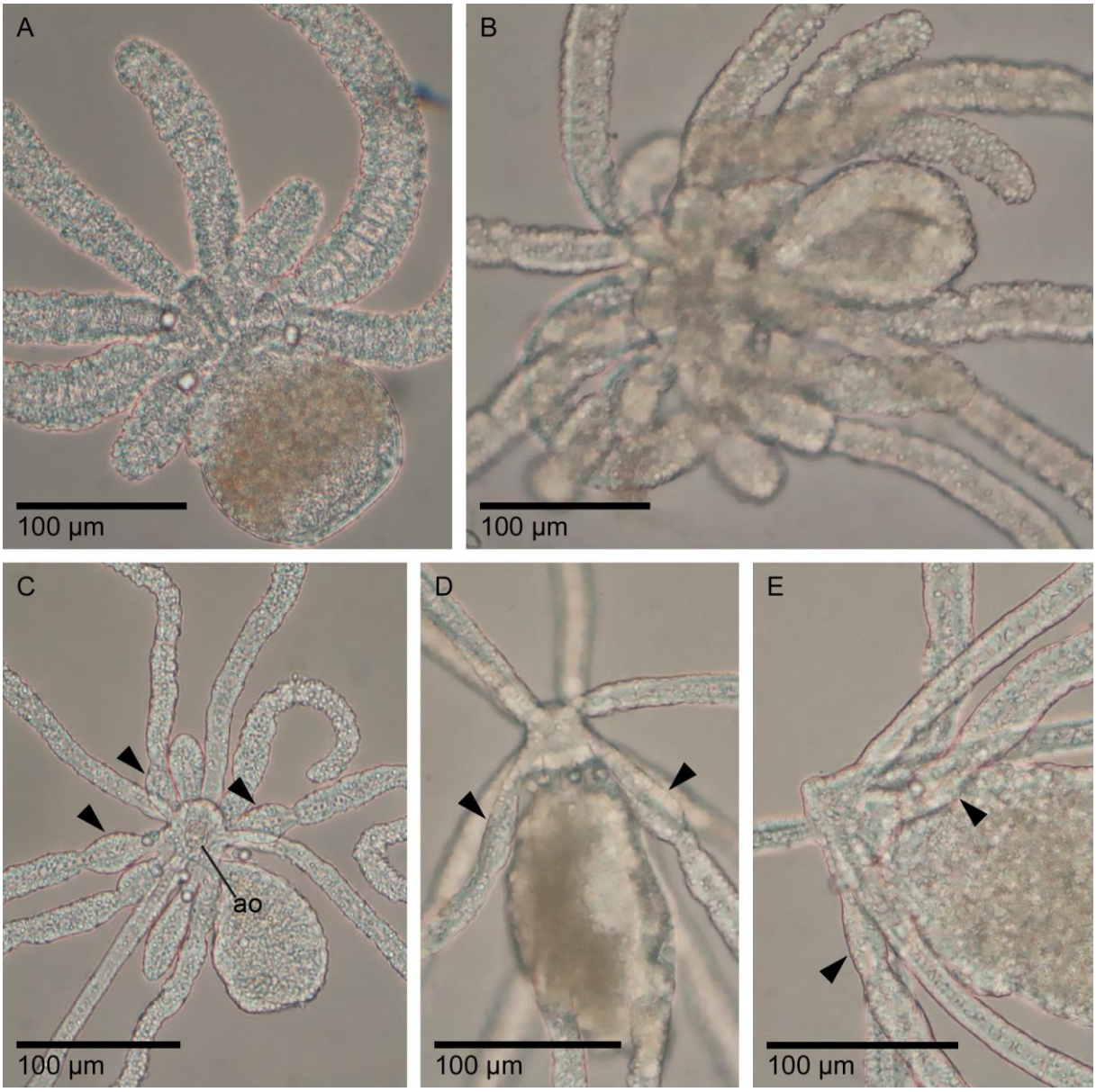

fig. 7: Challenges in the identification of *Halammohydra*. **(A)** *Halammohydra* sp. is contracted, which obscures information about the length of the tentacles, the shape of the tentacle bases and the shape of the aboral cone. **(B)** Tentacles of *Halammohydra* sp. overlap in several regions and obscure structures close to the aboral cone. **(C)** *Halammohydra kerblae* n. sp. adheres to the slide with the adhesive organ (ao). The lateral view of the aboral cone is not visible. This specimen has pronounced bulbs at the bases of the subaboral tentacles (arrowheads) **(D)** Lateral view of *H. kerblae* n. sp. with pronounced club shaped tentacle bases (arrowheads). **(E)** This specimen of *H. octopodides* has less pronounced club shaped tentacle bases.

The combination of semi thin and ultra-thin section to investigate the specimens was a good method to document the whole animal in a reasonable amount of time as well as providing overview (semi thin) and detailed images (ultra-thin). One downside of this method is the possibility of containing important structures only in semi thin sections, which e.g. was the case for the connection of the statocysts to the aboral cone in *Halammohydra*. Additionally, it was not possible to reliably investigate the tentacles, since they were too long. Information about structural changes, such as the density of nematocysts could not be documented. During the preparation of one male specimen of *H. vermiformis* for TEM, one side (the gonadal side) of the gastric tube got destroyed from the middle to the mouth opening. A second male had to be investigated to fill the missing information of this body part. This was time intensive, but comparing intact structures of all specimens (two male and one female) gave similar results, rendering them reliable.

The main challenge in the molecular work was handling the low amount of sample. These animals are tiny and contain a low amount of DNA, which resulted in a sample volume of 20 µl with a mean concentration of about 0.3 ng/µl (0.1 – 2.59 ng/µl). This proved to be especially complicated the beginning of the laboratory work, since different primers and PCR protocols had to be tested to find a fitting one. Gained sequences were mostly of good quality but the alignment of 16S was challenging. The gene contains many indels (insertions/ deletions) and the alignment resulted in many gaps which had to be removed with GBlocks. Nevertheless, both alignments, with and without indels, and in combination with the other genes, showed clear clusters.

Despite all these problems, the combined analysis of light microscopy and molecular methods helped to identify 234 specimens of *Halammohydra* on a species level or clustered them to a group. It was possible to document character variations within species or groups and even describe four new species with this information. For *Otohydra*, it was possible to add information to the sparse literature situation and to reveal molecular data concerning the phylogenetic position within the Hydrozoans.

2.2 Habitus and ultrastructural organization of *Halammohydra* and *Otohydra*

Ultrastructural analyses were done on the species *Halammohydra vermiformis* Swedmark & Teissier, 1957 and *Otohydra* sp., which are presented in *Study II* and *IV*. Both results show a good overview of the organization of the whole body and especially add information to the reproduction system of the two cnidarians.

For the ultrastructural analysis of *Halammohydra*, the abundant *H. vermiformis* was used because this species has a clear body structure with only a few tentacles, which were comparably straight and kept the orientation of aboral tentacles pointing in aboral direction and subaboral tentacles pointing in oral direction after embedding them. In other species, such as *H. schulzei* or *H. adherens* tentacles are numerous and do not have such a clear separation and straight appearance. This might obscure some structures, because they are not as easy to follow, thus *H. vermiformis* was a good choice to investigate the general structure. Additionally, histological and ultrastructural studies were done on *H. schulzei* (Ehlers 1993; Remane 1927; Swedmark & Teissier 1967), *H. octopodides* (Remane 1927), *H. adherens* (Swedmark & Teissier 1967) and *H. intermedia* (Clausen 1991, 2000) before, but not on this species.

The general cell structure of both *Halammohydra* and *Otohydra* with an outer epidermis, an inner gastrodermis and a layer of mesoglea separating them is typical for cnidarians. Both cnidarians are covered fully with cilia, which is a clear adaptation to the interstitial system for gliding in between the sand grains (Giere 2009). Pelagic medusae do not use cilia for locomotion (Werner 1964). Although both genera use cilia to move forward with the aboral pole in front, the behavior of *Halammohydra* and *Otohydra* are quite different (fig. 8). *Otohydra* is constantly gliding and the short tentacles are not

moving or used in the process (Swedmark & Teissier 1958, 1959, *Study IV*, fig. 8A). *Halammohydra* on the other hand, glides more distinctively whilst moving the tentacles. It is not a constant moving, but with breaks and rapid changes of direction (fig. 8B). Additionally, the different organization with a reduced umbrella, results in the tentacles being located on the aboral end, and thus being in front of the gliding direction (Remane 1927, *Study II*, fig. 8). Tentacles are bent in oral direction, whereas species with a visible separation of the tentacle whorls sometimes have a twitching motion in the aboral tentacles, which was observed especially in *H. vermiformis* and *H. coronata*. The function of this is unknown.

Additionally, tentacles are used for food intake as well. It was only documented once during the investigations that a specimen greatly opened its mouth opening and used the tentacles to move the prey in the mouth (*Study II*). This high controllability and movability are possibly a result of the high concentration of myofibrils and neurites in the aboral cone. There is a strong muscle ring present in the aboral cone surrounding the gastrodermis and mesoglea. The elsewhere thin mesoglea is irregular shaped and thicker at this location, because myofibrils need to anchor into it (Haynes et al. 1968), to enable a high movability. At the same location, a prominent nerve ring is situated, which was already shown in immunohistological staining of *H. octopodides* by Polte & Schmidt-Rhaesa (2011). Similar structures, such as the nerve ring, were not observed in *Otohydra*.

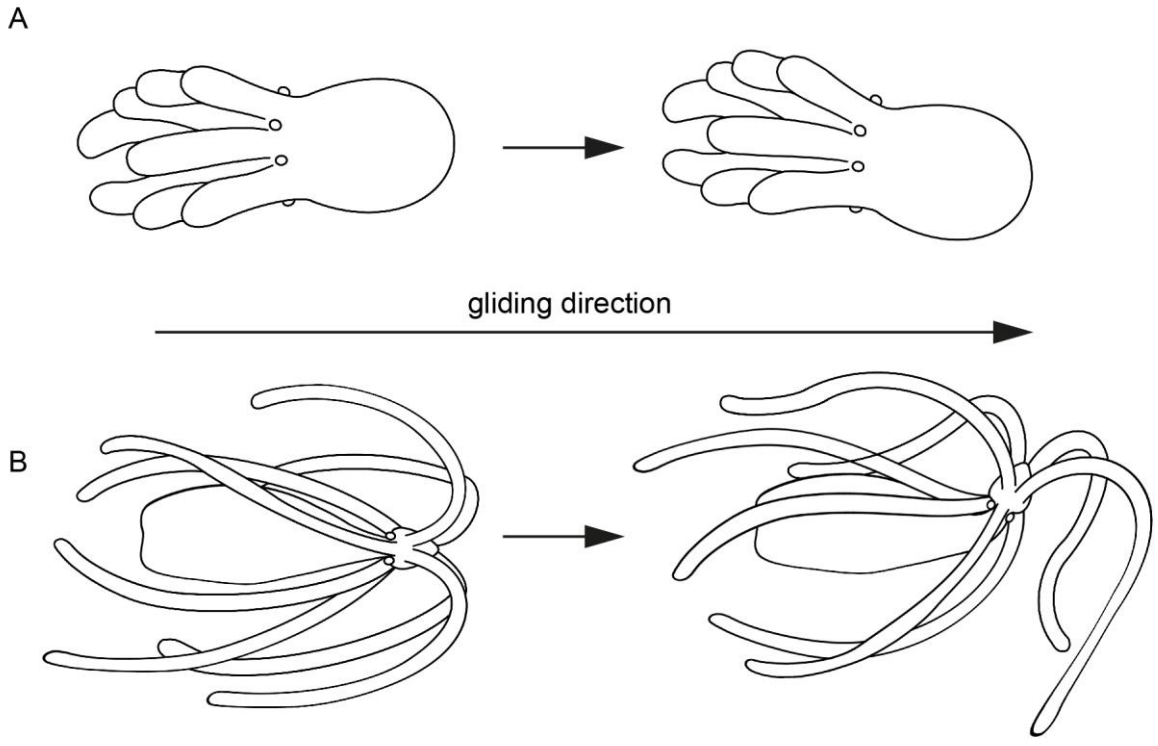

fig. 8: Schematic image of the gliding behavior of *Halammohydra* and *Otohydra*. Both glide with the aboral end in front. **(A)** *Otohydra* glides consistently without moving the tentacles or changing the shape of the body. **(B)** *Halammohydra* can change the direction rapidly by bending the neck. Tentacles are used actively in the process.

A neuronal connection of the nerve ring to the statocysts was not observed. Statocysts are sensory organs, hence a connection it is expected. *Halammohydra* and *Otohydra* have free statocysts with epidermal cells covering the statolith (see Remane 1927; Swedmark & Teissier 1958, 1966). When the medusa changes its spatial orientation, the statocysts moves as well and touch the surrounding sensory cilia, which detect the movement (Singla 1975). In semi and ultra-thin sections of *Halammohydra*, all statocysts lacked the statolith, instead only a vacuole was visible (*Study II*). Since there were no obvious destructions, it is assumed that the statoliths dissolved in one of the preparation steps. In the investigated specimen of *Otohydra* on the other side, no statocysts were detected, hence no detailed comparison can be made. Other specimens had statocysts. The lack of statocysts is just a special occurrence of this specimen (*Study IV*).

Polte & Schmidt-Rhaesa (2011) documented additional neural structures, such as tentacular neurites, a mouth cone plexus or an oral nerve ring in *Halammohydra*. It was not possible to observe these structures with the used methods in *Study II*, but their existence expected, since the mouth opening was observed to move in circular motions and stretched greatly for food intake of bigger prey. Next to the tentacles, the long cilia of the gastrodermis might help with the transportation of the food into the gastral lumen for digestion. Remane (1927) already described the different cell types involved in this. He categorized them as cell type a to h and defined five zones depending on the different concentration of the cell types. *Study II* only documented four cell types and three zones. The mouth opening consists of two cell types, possibly representing zone I (cell type a and b). The lighter cells might be mucous cells. They release mucous to protect the gastrodermis. Darker cells might be secretive cells, which start the first extracellular digestion (Thomas & Edwards 1991). Since this is no histochemical study, this cannot be said with certainty, but the position of the cells and the cilia located close to the surface support this assumption. Remane's second zone was not documented, but cells of the third zone are present.

The majority of the gastrodermis consist of the typical cnidarian digestive cells (Remane 1927; Thomas & Edwards 1991) with gastric zymogen or gland cells scattered in between (Haynes et al. 1968; Remane 1927). The gland cells release enzymes for extracellular digestion of food particles in the lumen, which then can be ingested into the digestive cells (Haynes et al. 1968). This is visible by the coloration of the epithelium, even in light microscopy, and reaches up until into the neck. The resorption of food particles decreases or stops, due to the reduction or absence of the lumen. Cells orient differently and change their shape, due to the reduced diameter and lack of lumen. Remane (1927) defined this as the zone IV with cell type g. The described zone V with cell type h is actually the aboral adhesive organ and not part of the gastrodermis. It results as an invagination of the ectoderm in the early development (Swedmark & Teissier 1957b). In *Otohydra*, the gastrodermis consists of the same typical digestive and gland cells (*Study IV*). Aborally, the digestive cells are huge and filled completely with a vacuole. They are comparable with the zone IV/ cell type g, since both have no central orientation and fewer inclusions because of the lack of the lumen (compare *Study II* and *Study IV*).

The gastrodermis continues as a chordoid rod into the tentacles in both *Halammohydra* and *Otohydra*. In *Halammohydra* these cells are completely filled with a vacuole (*Study II*), which is not as pronounced in *Otohydra* (*Study IV*). Voluminous vacuoles in the tentacles and the gastrodermis are very common within Hydrozoa, having a function of a hydrostatic skeleton (Thomas & Edwards 1991) and being important for the structural integrity.

In *Halammohydra*, the gonadal compartment is located within the gastric tube. Before, the exact position was described to be between the epidermis and the gastrodermis (Remane 1927; Swedmark & Teissier 1966) until Ehlers (1993) investigated the male reproductive system and regarded it as a part of the gastrodermis. The same is assumed in *Study II*, because of the position of the mesoglea. It surrounds both, the gastrodermis and the gonadal compartment, except for the gap on one side of the gastric tube.

Spermatozoa and oocytes were described to be released into the water via the gastrodermis, into the lumen and shed through the mouth opening in the surrounding waters (Clausen 1971; Ehlers 1993; Swedmark & Teissier 1966). *Study II* suggests a different procedure, where spermatozoa and oocytes are not released via the gastric system, but by a rupture of the epidermis. Sections showed a slight indent on the gonadal side of the gastric tube in the male, which is very pronounced in the female and even visible in light microscopy. This indent was documented in several specimens and thus not a random finding. A rupture of the epidermis would explain the destruction of the epithelium and the lack of the mesoglea at this location. Additionally, it is very common among Hydrozoa (Thomas & Edwards 1991). Oocytes of *Halammohydra* stick to sand grains and stay attached until the embryonal development is finished (Swedmark & Teissier 1957b). Observations on the fertilization process are missing.

Next to the external fertilization, internal fertilization is very common among meiofaunal animals as well (Giere 2009), and it was described for *Otohydra* (Swedmark & Teissier 1958). *Study IV* showed the gonad to be located in the gastrodermis as well. Contrary to *Halammohydra*, it is located in the body within the umbrella because of the different anatomy of *Otohydra*. Swedmark & Teissier (1958) documented the gonads at the same position and described an incubation cavity between the ectoderm and the endoderm. This was not observed in *Study IV*, as well as a second gonad. *Otohydra* is hermaphroditic, thus having a male and a female gonad (Swedmark & Teissier 1958, 1959). As in *Halammohydra*, there was a gap in the epidermis and mesoglea on the level of the gonad, which was documented before (Swedmark & Teissier 1958). It is possible, that this specimen already released the juveniles via a rupture of the epidermis; hence, the incubation cavity regressed and is not visible in *Study IV*. The second, aboral and less pronounced gap might be a healing destruction and not in connection with the gonad. All these information have to be taken with caution, since only one specimen was sectioned and investigated with TEM.

The male gonadal compartment and spermatozoa of *Halammohydra* investigated in *Study II* correspond to the structures described in (Ehlers 1993), except for the acrosome and cilia. They were not observed directly in *Study II*. The presence of the cilia was indirectly observed by groups of them between the germs cells. There is no description of the female gonadal compartment of *Halammohydra*, so far. *Study II* shows a possible stratification of maturation from aboral (youngest) to oral (mature), because of different accumulations of the germ cells, immature oocytes and the position of the mature oocyte, which is orally and has a more complex structure than the other two. It consists of two distinct regions, a lighter and a darker structure (with nucleus), and the yolk part. Both are seemingly separated by a membrane, appearing quite unusual at that position. Since there is no nucleus in the yolk compartment, it is unlikely, that it is a composition of two structures being a separate oocyte and a yolk part. Additionally, such a separation is not present in earlier stages. Therefore, the function of the unusual membrane remains unexplained. Similar structures were not reported before (see e.g. Beams & Kessel 1983; Tardent 1984). In the center of the female is a prominent indent. It might be a remnant of a recent rupture to release an oocyte. However, if there is a stratification of maturation, the position of the indent would indicate a release of the second mature oocyte and not the most mature one. Since only one specimen was investigated, more data is needed to clarify if this is a typical process or just a random finding.

Halammohydra has an adhesive organ at the tip of the aboral cone, which consists of large cells packed with secretory vesicles. In the center is a lumen filled with cilia, which stick out of the aboral pore. It is used to temporarily adhere to a surface. This process might be a combination of secretes and myofibrils surrounding the structure. The cells produce adhesive secretes, which are transported to the tip of the aboral cone with the cilia. In addition, a slight sucker effect is possible. It might be released by the contraction of the surrounding myofibrils. *Halammohydra vermiformis* is mainly documented free-swimming and with a less adhesive behavior compared to other species. Hence, the structure of the

adhesive organ potentially differs to more adhesive species, such as *H. schulzei*, in terms of the size or the thickness of the surrounding layer of myofibrils. This was especially documented in *H. adherens* (Swedmark & Teissier 1967). This species is very adhesive, hence the name, and longitudinal sections of the aboral cone revealed numerous myofibrils surrounding the large adhesive organ (Swedmark & Teissier 1967). The different shapes of the adhesive organ might also be important. This specimen of *H. vermiformis* had a conical shape. Other species have cup- (*H. octopodides, H. intermedia, H. adherens*) or pear shaped (*H. schulzei*) adhesive organs, which can reach deeper into the aboral cone. This can result in a thicker layer of secretory cells and potentially be more adhesive.

The adhesive organ was described by some authors to be part of the gastrodermis (Remane 1927) or a cup of gastrodermal cells and mesoglea surrounds it (Swedmark & Teissier 1966). *Study II* did not document a connection of the gastrodermis and the secretory cells of the adhesive organ. A layer of mesoglea was only documented surrounding the structure in the most oral part. The adhesive organ of *H. vermiformis* is, compared to other species, small and less sunken in. For example, in *H. schulzei* and *H. octopodides*, the adhesive organ reaches rather deep into the aboral cone, therefore the suggested cup of gastrodermis and mesoglea surrounding it is possible. Remane (1927) on the other hand, documented this connection but interpreted it as part of the gastrodermis. It is important to investigate the adhesive organ of additional species, to find functional and/ or species-specific differences.

Halammohydra vermiformis is described with two types of nematocysts: stenoteles, which are present on the whole body, and isorhizas, which were found only on the tentacles (Clausen 1967; Swedmark & Teissier 1957b). *Study II* found more or less the same distribution, with heteronemes present on the entire animal. Certain regions have an increased concentration, for example the developing areas for nematocysts or on the tentacles. The found heteronemes are potentially stenoteles, since only two types were described. The second type of nematocysts found is only present on the tentacles and is lacking a shaft, which is a character for a haploneme (Östman 2000). Additionally, supportive rods around the nematocyst were reported before in an investigation of the cnidome in *H. intermedia* and the nematocysts were identified as haplonemes (Clausen 1991). The images do not allow further identification, but it is very likely, that these nematocysts are isorhizas, which is the second type reported for *H. vermiformis*.

For *Otohydra* two types of nematocysts were found in *Study IV*. Swedmark & Teissier (1958) documented only stenoteles, which were especially on the tentacles, with a concentration of large cysts at the tip of them. The same was observed in *Study IV*. Heteronemes with a prominent shaft in the undischarged capsule and a basal dilation in the shaft in the discharged state were documented and are most likely stenoteles (Östman 2000). Two size classes of stenoteles were present, whereas the large cysts were concentrated at the tips of the tentacles. Additionally, a second type was documented, which is potentially an eurytele, because of the shape of the capsule and the distal dilation of the shaft (Östman 2000). The eurytele type and the small size class of the stenoteles were not observed before (Swedmark & Teissier 1958).

There are obvious reasons for *Halammohydra* to be placed into Hydrozoa and further into Trachylinae (Remane 1927; Swedmark & Teissier 1957b): a lack of a polyp stage and the structure of the statocysts are clear characters of Trachylinae. The possession of stenoteles, the direct development and the placement of the gonad (here gonadal compartment) on the manubrium (here gastric tube) hint at a close relationship to Trachymedusae and Narcomedusae (Bouillon & Boero 2000a; Clausen 1967; Marques & Collins 2004; Remane 1927). The study of Collins et al. (2008) then showed a possible origin within Rhopalonematidae (Trachymedusae), which is only supported morphologically by the structure of the statocysts (Bouillon & Boero 2000b; Bouillon et al. 2006, *Study II*). The typical position of the gonad in Hydrozoa is in the epidermis, which differs in *Halammohydra.* It is located in the gastrodermis with

a connection to the epidermis by a gap in the mesoglea. This unusual intermediate stage as well as the position in the gastrodermis is uncommon, but was documented in some hydrozoan species (Bouillon et al. 2004).

2.3 Updated geographical distribution of *Halammohydra*

Nine species of *Halammohydra* were described, mostly from Europe (e.g. Clausen 1967; Remane 1927; Swedmark & Teissier 1957b) and India (e.g. Rao 1978; Rao & Ganapati 1966; Rao & Misra 1980), but this geographical distribution resulted very likely from sampling biases, since most early meiofaunal studies were done in Europe (Giere 2009), causing the high amount of records. Additional findings in the Western Atlantic and Caribbean Sea (e.g. Bush & Zinn 1970; Calder & Kirkendale 2005; Garraffoni et al. 2017, *Study III*) and on the Azores (*Study I* and *III*) suggest a wider geographical distribution. Nonetheless, it is noticeable that single locations in Europe, especially Helgoland and Roscoff, harbor a high number of species compared to other genera of meiofaunal Cnidaria.

During this project, specimens of already known but also completely new locations were analyzed and summarized in *Study III*. Additionally, four new species were described: *H. teissieri* n. sp., *H. swedmarki* n. sp., *H. kerblae* n. sp. and *H. joergerae* n. sp. . The main focus of sampling specimens was on the locations surrounding Helgoland (Germany) and Roscoff (France), due to the high amount of species recorded (e.g. Clausen 1967; Remane 1927; Swedmark 1957). *Study III* added three of the four new described species and the first record of *H. coronata* in Roscoff (fig. 9). Before, *H. coronata* was reported from Helgoland (Clausen 1967) and the Netherlands (Wolff et al. 1974). *Halammohydra teissieri* n. sp. was added as a new record to Helgoland. In total, four species (one new species) and one unidentified group ("Helgoland/ Sylt") were found on Helgoland, and seven species (three new species) and one unidentified group ("Roscoff") were found in Roscoff (see *Study III*, fig. 9)

The dominating species with the highest numbers was *H. vermiformis*. It was described from Roscoff (Swedmark & Teissier 1957b) but the majority was found on Helgoland. Only two morphologically identified specimens were found in Roscoff. On Helgoland, *H. vermiformis* was recorded from the subtidal location at the Youth Hostel (Clausen 1967), but not from the "Dune", as they were found in *Study III*. Remane (1927) documented in his description of *H. octopodides* small individuals with characters of *H. vermiformis* from the Northern Beach of the "Dune", so it is possible, that he already found this species there, but did not identify it. Furthermore, new records of *H. vermiformis* are from Denmark, Sylt and Cuba (fig. 9, *Study III*). On Sylt, only *H. octopodides* (Polte & Schmidt-Rhaesa 2011) and *H. schulzei* (Schmidt 1969) were reported before. Denmark and Cuba are completely new records for *Halammohydra*.

Additional new locations of *Halammohydra* are the Azores, Tenerife and Fernando de Noronha in Brazil (fig. 9). *Study I* assigned the first records on the Azores primarily to *H. schulzei*, but with uncertainty. Phylogenetic analyses show two distinct groups, whereas one is described as the new species *H. joergerae* n. sp. and the other remains unidentified (*Study III*, fig. 9, see 2.4). Specimens from Tenerife and Brazil are not identified because of missing morphological and molecular information (see 2.4). Other species of *Halammohydra* were found at locations where they were recorded before. Interestingly, *H. octopodides* was described on Helgoland (Remane 1927), but only two specimens were found here. The majority was collected in Roscoff.

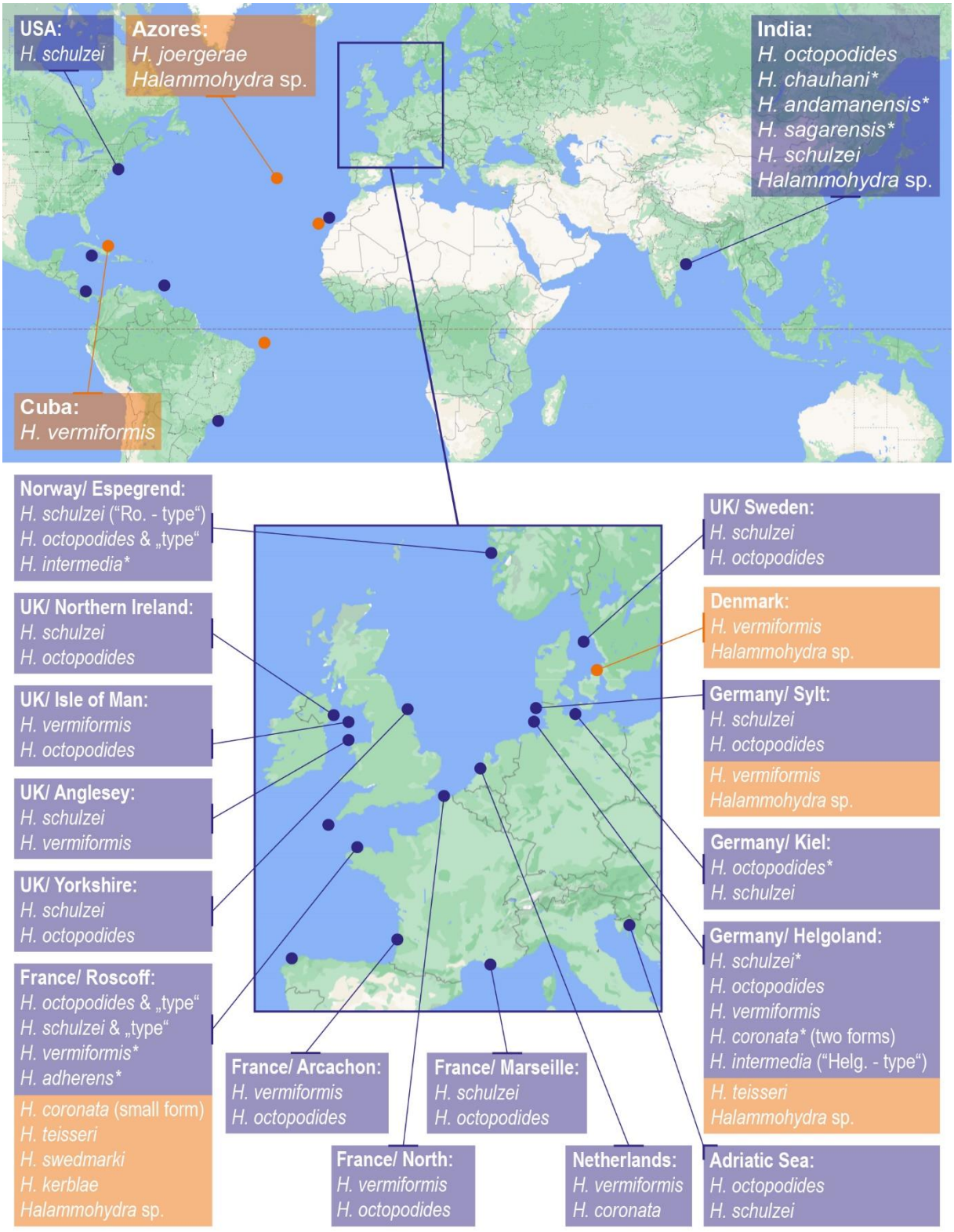

fig. 9: Reported locations and added findings of this study of *Halammohydra* around the world with magnification of Europe. Dots without a connected square are unidentified specimens. Type localities of every species are marked with asterisk (*). Map source: Google maps.

2.4 Morphological and molecular identification of *Halammohydra*

The morphological identification of *Halammohydra* on a species level is challenging, due to different reasons (see 1.2.3), thus molecular data are needed as additional information. *Study III* is the first study investigating species-specific sequences in combination with morphological features. This approach is useful to reinvestigate described species, delimit new species or mOTUs and to document character variation within one species. With this method, it is even possible to assign specimens to a group that display unclear characters because of body damage or contraction and thus could not be identified morphologically.

In the phylogenetic analysis (fig. 10) of *Study III*, three genes (16S, 18S, and CO1) were used to delimit 16 clusters of which seven were assigned to four described species (*H. vermiformis*, *H. octopodides*, *H. coronata* and *H. adherens*). Four clusters were described as new species (*H. teissieri* n. sp., *H. swedmarki* n. sp., *H. kerblae* n. sp. and *H. joergerae* n. sp.), leaving five clusters unidentified ("Helgoland/ Sylt", "Azores", "Roscoff", "Tenerife 1" and "Tenerife 2"). 16S and CO1 produced more detailed results on a species level compared to the 18S gene. ABGD and GMYC of 18S identified only five clusters and grouped the remaining sequences together. Since 18S is a slow evolving gene (Hillis & Dixon 1991) which does not always contain species specific phylogenetic information (Fontaneto et al. 2015; Tang et al. 2012), this was to be expected. Topologies of the trees and species delimitation tests show mainly similar results, with minor differences and the interpretation of the validity of a group was difficult in a few incidences. For example, the group "Tenerife 1" contains three specimens, but only 18S sequences are available. ABGD and GMYC grouped them together, whereas bPTP separated them in three singletons (fig. 10). Having only one gene and differing results in the species delimitation tests complicated the interpretation.

The CO1 gene on the other hand, turned out to be useful to determine between species in this genus. It was debated, if CO1 is a good gene for species delimitation in Cnidaria, since slow evolutionary rates are reported in most Anthozoa (Hellberg 2006; McFadden et al. 2010; Shearer et al. 2008). However, Bucklin et al. (2011) and Ortman et al. (2010) consider it as useful in Medusozoa. In *Halammohydra*, it showed similar evolutionary rates as 16S and the alignment was even easier than in 16S, because of the lack of indels (insertions/ deletions) in CO1 (*Study III*).

Four already described species were identified using the combined methods (*Study III*). The most abundant species was *H. vermiformis*. Since the special morphology has a low variation in characters, it is easy to identify. The most striking characters are the tentacles, with one long tentacle in the subaboral whorl and mainly 7 tentacles in total. Few specimens have 8 tentacles, but it was not recorded higher (Swedmark & Teissier 1957b). Especially specimens from Sylt bearded 8 tentacles and were confused with *H. octopodides* at first, but molecular investigations clearly clustered them to *H. vermiformis*.

Phylogenetic analyses separated specimens of *H. vermiformis* in two clusters (fig. 10, *Study III*). Specimens of both clusters were similar in morphology, differing only slightly in size and the habitat, whereas size differences can be explained by sampling or measuring bias. There is one subtidal cluster with specimens exclusively found at the location "Pier at the Youth Hostel" of Helgoland and a cluster with specimens from different locations. Specimens were mainly from intertidal locations, except the station in Denmark. Here, specimens were collected in 7-9 m depth, but above the halocline. There is a reduced influence of the waves but the lower salinity is a similarity to intertidal locations. One extreme station was found on Sylt, where the animals inhabit the moist sand of the beach at low tide. Variations in salinity are very frequent. This indicates a potentially higher tolerance to the variability of abiotic factors, such as salinity, in one of the sister clusters of *H. vermiformis*.

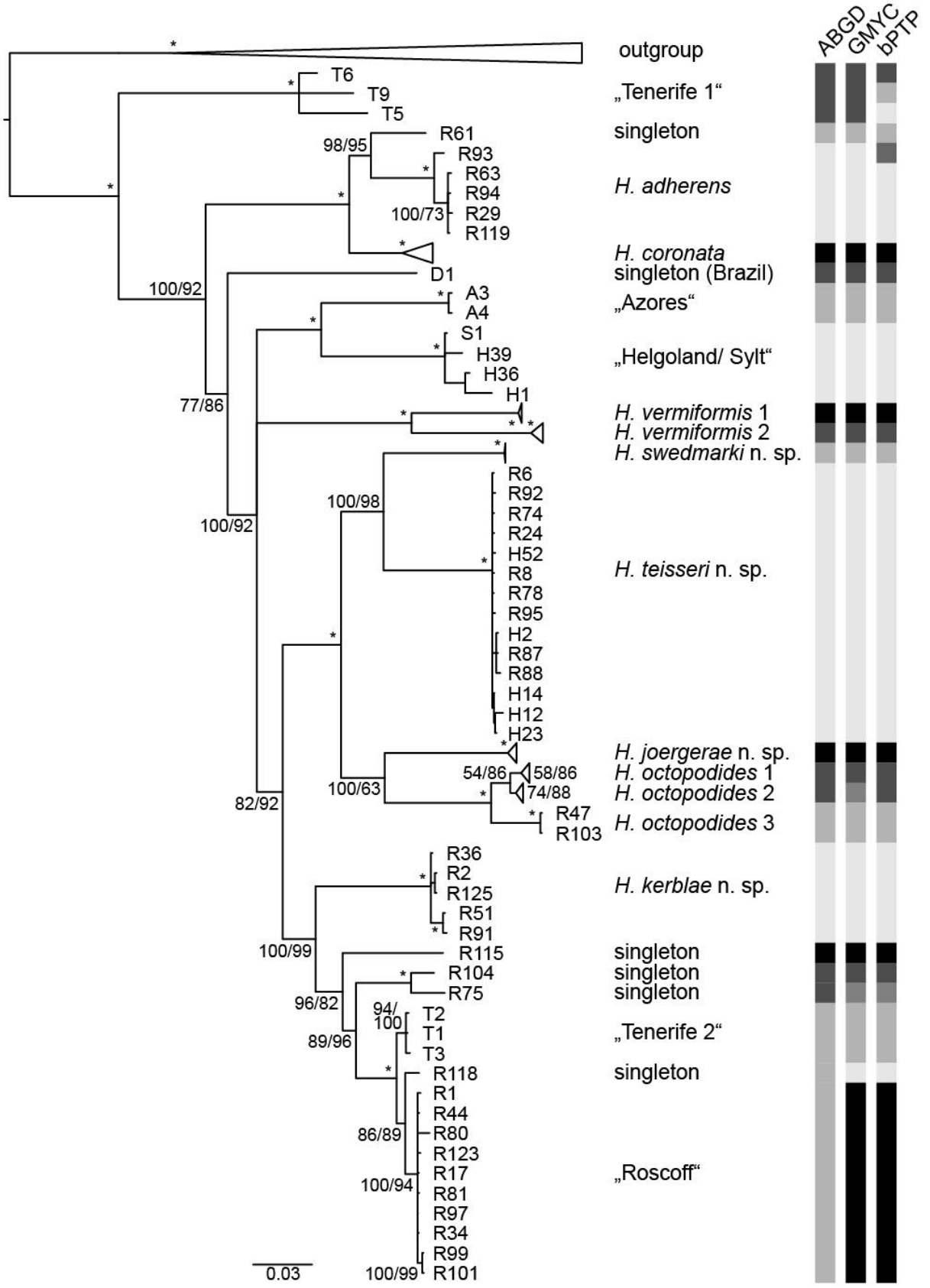

fig. 10: Phylogenetic tree of *Study III* with all three genes concatenated and support values of BI/ML (posterior probability/bootstrap value). Nodes with an * have a support of 100/100. Some clusters are collapsed. Summarized species delimitation of ABGD, GMYC and bPTP results are shown for each cluster.

The second species found was *H. octopodides*, with three clusters without obvious morphological or environmental differences (fig. 10). All specimens show characters of previous records (Clausen 1963, 1967; Remane 1927; Renaud-Debyser 1964; Swedmark 1957; Swedmark & Teissier 1957a) except for a slightly lower total number of tentacles. This can be due to the life stages of the collected specimens. Sequences of *H. octopodides* 1 and 2 were grouped together in species delimitation tests (except GMYC). Together with the low internal node support value, both clusters are very likely one species. The cluster of *H. octopodides* 3 is separated from them in every analysis without obvious reason. Since there are only two specimens in this cluster, the molecular and morphological data is limited, thus we regard all three clusters of *H. octopodides* as one species (fig. 10).

Halammohydra coronata was described with two forms from Helgoland. The smaller form was reported from the station "Pier at the Youth Hostel", whereas the larger form was found at the "Amphioxus"-flat (Clausen 1967). Specimens found in *Study III* are of the smaller form, corresponding to the same location and the described morphological features. In addition to the known characters, some specimens also had one long tentacle in the subaboral whorl. This was not documented before, but the smaller form was described on only three specimens, thus this variable character might not have been present in the described specimens.

The large form of *H. coronata* was described to be in close relation to *H. adherens* because of the elongated micro- and macroeurytels present instead of stenoteles in both species (Clausen 1967). Since no specimens of the larger form of *H. coronata* was found, this similarity could not be tested. However, phylogenetic analyses revealed a close relationship between the smaller form of *H. coronata* and *H. adherens* and clustered them together as sister groups (fig. 10, *Study III*). Specimens of *H. adherens* were collected mostly at the same location ("Trezen ar Skoden" in Roscoff) and sediment (shelly gravel) as in the literature (Swedmark & Teissier 1959, 1967), but one specimen was also found at the station "Bazin Malvog" in medium sand. Swedmark & Teissier (1967) described *H. adherens* to be very large (about 800 µm) and with 12-14 tentacles in each whorl. This was only documented in one specimen, others were much smaller and had less tentacles. It appears that this species has a higher variability of the identification features than reported. Since Swedmark & Teissier (1967) described this species with micro-and macroeuryteles, the documented elongated nematocysts visible in light microscopy are possibly macroeuryteles. However, this was not confirmed with a detailed analysis of the nematocysts by squeezing them. Interestingly, no other group showed this type of nematocysts in light microscopy. Only the larger form of *H. coronata* was described with this type of nematocysts but unfortunately, no specimens were found. This raises the question, whether this larger form is an individual species and potentially intermediate between *H. coronata* and *H. adherens* or actually *H. adherens*.

Two of the six European species (*H. intermedia* and *H. schulzei*) were not found in *Study III*. *Halammohydra intermedia* is an intermediate between *H. schulzei* and *H. octopodides* and was reported from Norway and Helgoland (Clausen 1967). No specimens with characters of this species were found. *Halammohydra schulzei* on the other side is a species which was reported from many different locations (fig. 9): Helgoland (Clausen 1967; Remane 1927), Sylt (Schmidt 1969), Western Baltic Sea (Schulz 1952), Roscoff (Swedmark 1957; Swedmark & Teissier 1957b, a; Teissier 1950), Marseille (Swedmark 1957), United Kingdom (Boaden 1961), Norway (Clausen 1963, 1967) and one record from the Western Atlantic (Bush & Zinn 1970). This amount of records gives the impression of a wide geographical distribution compared to other species, but it is not confirmed in *Study III*. The group with characters closest to *H. schulzei* is "Roscoff", with an exception of the number of tentacles and statocysts. *Halammohydra schulzei* was described with 14 to 24 (Remane 1927; Swedmark 1957) and up to 26 tentacles (Swedmark 1957; Swedmark & Teissier 1957b) and 12 statocysts (Remane 1927). The number of "Roscoff" is slightly lower with 10 to 18 tentacles and 5 to 7 statocysts. Additionally, there is no information about the tentacle length within one or between both whorls. Specimens of "Roscoff" have

longer subaboral tentacles, which are of unequal length. This character is not visible in the few available images in the literature. It is not sure, if this character is absent in the described specimens of *H. schulzei* or not described and thus absent because of the choice of pictures.

A second group with character combinations similar to *H. schulzei* is "Azores"; hence, they were preliminary assigned to this species (*Study I*). Molecular analyses on the other hand, did not place "Roscoff" and "Azores" in close relation and complicate the identification (fig. 10, *Study III*). The first description of *H. schulzei* was from Helgoland (Remane 1927), thus a closer look into the group "Helgoland/ Sylt" is needed. It is the sister group of "Azores", but important features, such as the pronounced bulb at the tentacle bases in the subaboral whorl are lacking and some specimens have a low number of tentacles compared to *H. schulzei*. There are too many differences to reliably identify this group as *H. schulzei*. Additionally, the geographical distance to the Azores does not support an identification as well, but it is not excluded. It is surprising that no specimens of *H. schulzei* were found at the location of description on Helgoland (Remane 1927). There are records from Roscoff (Swedmark 1957; Swedmark & Teissier 1957b, a; Teissier 1950), but with the uncertainties in the identifications, more data is needed to reliably assign one of the groups in question to *H. schulzei*.

For *H. schulzei, H. octopodides* and *H. intermedia* "types" or "variants" with slightly different characters were described (Clausen 1967; Swedmark 1957). Specimens with those character combinations were not found in *Study III*. Hence, the existence of them could not be tested, but since there are more groups found than species described, it is very likely, that the "variants" are separate species as well.

Next to the four known species, four clusters were described as new species, which is supported by the node values and species delimitation tests (fig. 10): *H. teissieri* n. sp., *H. swedmarki* n. sp., *H. kerblae* n. sp. and *H. joergerae* n. sp. . *Halammohydra teissieri* n. sp. has similarities to *H. schulzei,* except for the lack of the thickening at the tentacle bases and the longer aboral tentacles. *Halammohydra swedmarki* n. sp. on the other side has no similarities to a known species. The prominent feature of this species are the longer tentacles compared to other species. *Halammohydra kerblae* n. sp. is very similar to *H. coronata*. Major differences are the presence of a thickening at the tentacle bases and the lack of a long tentacle in the subaboral whorl. The last new species is *H. joergerae* n. sp. . It has no similarities to any described species and the geographical distance supports the new description. Additionally, there is a singleton from Brazil, which is supported by all analyses to be a separate species. Since it is a singleton and there are no detailed morphological data (fixed material), it is not described as a new species.

The remaining clusters and singletons have a lower support in node value or species delimitation tests or the morphological data are lacking (fig.1). Hence, they are not described as new species. At least two different species occur on Tenerife. "Tenerife 2" is closely positioned to "Roscoff", but most species delimitation tests separate them in two groups. Morphologically, there are differences as well, such as the lack of the thickening in the tentacle bases in "Tenerife 2". Additionally, there is a wide geographical distance between both groups, which would support two separate species. Unfortunately, there is not enough data to characterize them reliably. The singleton R118 from "Trezen ar Skoden" is positioned between both groups. Species delimitation tests (except ABGD) do not group them together, but there is no useful morphological data available. Hence, no further conclusions can be made. "Tenerife 1" contains three specimens but only 18S sequences are available. bPTP of these sequences even separated all three specimens in three groups, thus it is not possible to say, if it is one species or three different ones. The position of "Tenerife 1" as a sister group to all other species of *Halammohydra* is interesting and needs to be investigated further.

Positioned between *H. kerblae* n. sp. and the clade of "Tenerife 2" and "Roscoff" are three singletons, which have similarities in morphological characters to the latter clade, except for the lacking thickening

at the tentacle bases of the subaboral tentacles. Additionally, R104 and R75 are juveniles, thus the morphological information is not useful. All three specimen provide not enough reliable data to describe new species.

2.4.1 *Otohydra* and the taxon Actinulida

In *Study IV*, eight specimens of *Otohydra* were found. Since they were found at the exact same location and sediment type as *Otohydra vagans* Swedmark & Teissier, 1958 was described, it is very likely that these specimens represent the same species. Although the number of tentacles and statocysts is slightly lower or overlap the numbers of *O. vagans* (12-16 tentacles and 8-12 statocysts), it is possible to be the same species, since the numbers in a young life stage are lower. Juveniles have up to 8 tentacles and 4 statocysts, resembling an adult (Swedmark & Teissier 1958). The numbers then increase. Additionally, no specimen at all and especially with lower tentacle numbers was documented with an embryo.

Study IV is the first study investigating *Otohydra* with molecular methods. Gained sequences suggest an origin within Rhopalonematidae (Trachymedusae), as it was suggested for *Halammohydra* (Collins et al. 2008), but both are not in a direct sister relation (fig. 11). Sequences of *Halammohydra* are in a sister relation to a group of rhopalonematids with *Aglantha digitale* O. F. Müller, 1776, *Pantachogon haeckeli* Maas, 1893, *Rhopalonema velatum* Gegenbaur, 1857 and others, which correspond to the results of Collins et al. (2008), forming a sister clade to the group containing sequences of *Otohydra* (fig. 11). This indicates, that the group Actinulida does not exist, which is supported by the morphological differences as well.

Contrary to the phylogenetic study of Collins et al. (2008), *Study IV* added a representative of the group Ptychogastriidae, which is nested within Rhopalonematidae. *Otohydra* was clustered to *Ptychogastria polaris* Allman, 1878 (Ptychogastriidae), hence it is not clear if *Otohydydra* belongs to Ptychogastriidae or Rhopalonematidae. Only the free ecto-endodermal statocysts and the simple gonads are characters similar to the bentho-pelagic medusa of Ptychogastriidae (Galea et al. 2016). A missing link between both might be the recently discovered meiofaunal medusa *Marsipohydra pacifica* Sanamyan & Sanamyan, 2012. This species is thought to be in a close relation to *Otohydra* and thus belongs to the family Otohydridae (Sanamyan & Sanamyan 2012). It has characters close to *Otohydra*, but also great differences, such as having two types of tentacles, a filiform and an adhesive type, and being gonophoric, which are characters of Ptychogastriidae (Galea et al. 2016). On the other hand, *M. pacifica* has an eight lobed umbrella and a different position of the gonads which differs to *Otohydra* and Ptychogastriidae. In *Otohydra*, the gonads are located in the umbrella, in Ptychogastriidae they are on the manubrium and in *M. pacifica* four male testis or one female brood pouch are attached to the manubrium (Galea et al. 2016; Sanamyan & Sanamyan 2012). Since there are no sequences available of *M. pacifica*, the relationship between the group Rhoplaonematiidae and the two species *O. vagans* and *M. pacifica* is not fully resolved yet.

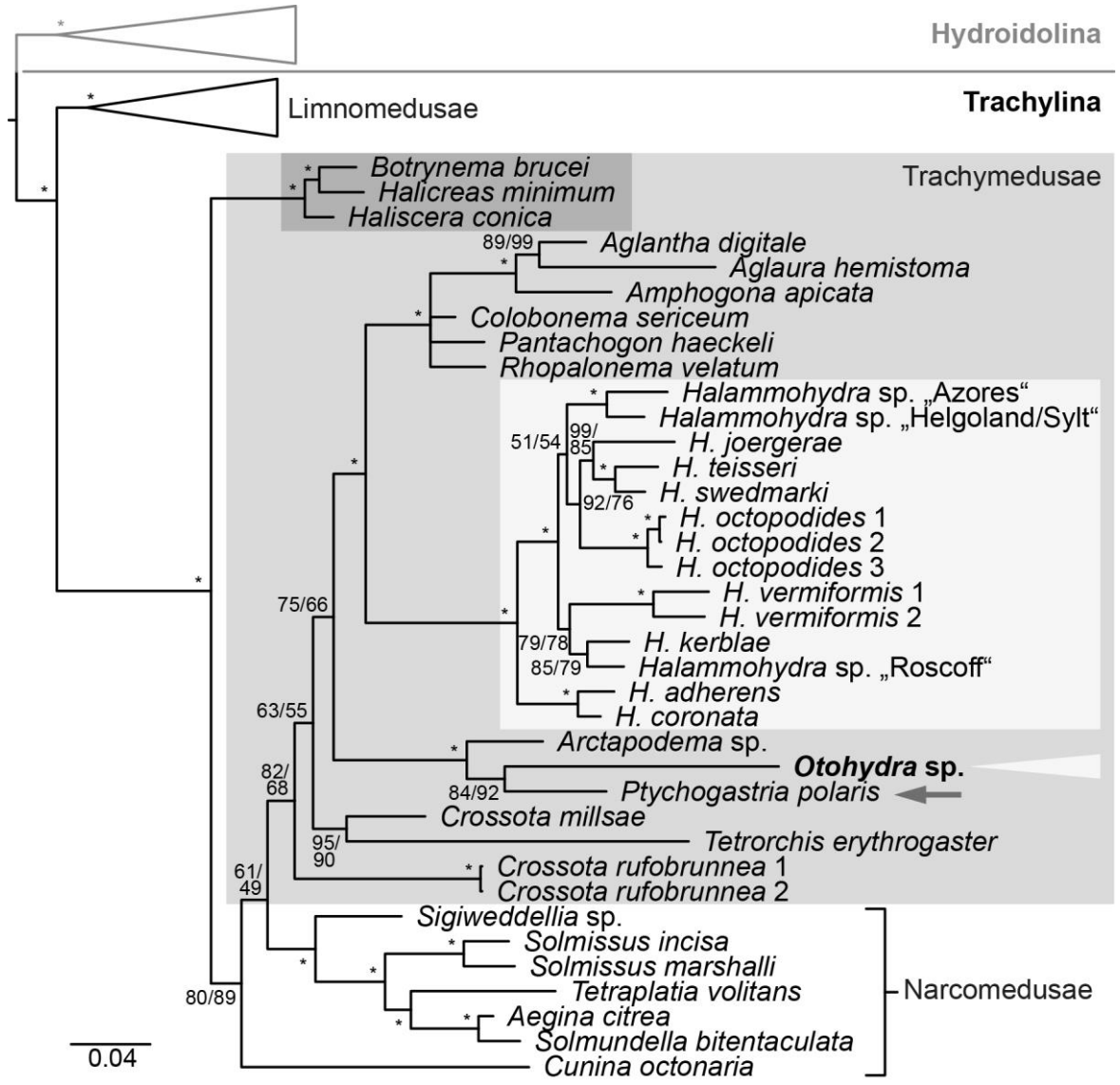

fig. 11: Phylogenetic tree of *Study IV* with the concatenated dataset (16S and 18S) with support values of BI/ML (posterior probability/ bootstrap value). Node values with an * have a support > 95/95. The group Trachymedusae contains representatives of Halicreatidae (dark grey rectangle), Ptychogastriidae (arrow), Halammohydra (light grey rectangle) and Rhopalonematidae (remaining representatives in the medium grey rectangle).

2.5 Conclusion

The majority of meiofaunal studies focus on bigger groups and neglects smaller ones, which are necessary too for the investigation of the biodiversity of one habitat. This project answers questions regarding the meiofaunal cnidarian *Halammohydra* and, to some extent, it is thought to be closely related genus *Otohydra*. This close relation is incorrect based on phylogenetic analyses (*Study III* & *IV*), rendering the group Actinulida is invalid. *Halammohydra* has its origin within Rhopalonematidae of Trachymedusae (Collins et al. 2008, *Study IV*), whereas the exact assignment of *Otohydra* is not determined yet and vary between Rhopalonematidae and Ptychogastriidae (both Trachymedusae). For resolving this, molecular information of *Marsipohydra pacifica* might be interesting (*Study IV*). Nonetheless, the separate position of *Otohydra* and *Halammohydra* revealed an additional transition to the meiofaunal way of life within Cnidaria.

This project (*Study III*) helped to reveal the different variability of character within one species as well as overlapping features. Although, the morphological identification remains difficult, molecular data are useful to distinct between species and showed a higher species diversity in Europe than previously assumed, with four newly described species and potentially more. Additionally, unexpected findings on the Azores and Tenerife indicate a higher geographical distribution of *Halammohydra* than previously reported (*Study I* & *III*). Lastly, gained sequences contribute to the molecular database by providing species-specific sequences of three genes, which will be useful for future studies (*Study III*).

Next to the molecular investigation, the detailed observations on the cell structure with TEM helped to understand the general organization of the whole body of *Halammohydra* and *Otohydra* (*Study II* & *IV*). Especially, information concerning the gonadal compartment/ gonad of both and the organization of the aboral cone with adhesive organ of *Halammohydra* were needed and more such studies should be done with the aboral cone of different species of *Halammohydra* to reveal the exact process of adhering and help correlate morphology and behavioral differences and thus environmental preferences (*Study II).*

3. Abstract

Marine sediments inhabit many microscopic organisms with perfect adaptations to their environment, such as the small body size, adhesive structures or the lack of a planktonic larva. These so-called meiofauna are thought to not be able to distribute over a large scale of distance because of this, however some species are amphi-oceanic and even cosmopolitan. Many reinvestigations found morphological or molecular differences, but not for every species. There are several hypotheses brought forward to explain this "meiofauna-paradox", but investigations of all groups are needed to find the underlying processes. Most studies are done on large groups, such as nematodes or copepods. Smaller groups with special habitat preferences are lacking. Especially the few meiofaunal cnidarians are understudied. The largest group of them is the highly modified medusa *Halammohydra* Remane, 1927 with nine species described so far, but the species identification is rather difficult. Together with *Otohydra* Swedmark & Teissier, 1958 they build the taxon Actinulida. This relationship is doubtful because of the many morphological differences and has to be investigated molecular. Additionally, detailed information are needed of specific structures, such as the reproductive system of both and the adhesive organ of *Halammohydra* to find species specific structures as well as structures for the classification within the cnidarian tree.

This project investigates specimens of *Halammohydra* from different locations mainly in Europe and a few specimens of *Otohydra* from Roscoff in France. They were extracted from the sediment with the anesthesia-decantation method and fixed for molecular and ultrastructural investigations. The detailed investigation of the cell structure of *H. vermiformis* and *Otohydra* sp. with semi and ultra-thin sections helps to understand the general organization and adds information to the knowledge about both, especially concerning the gonadal compartment/ gonad of both and the aboral cone with adhesive organ of *Halammohydra*. For both it is proposed that the spermatozoa and oocytes (*Halammohydra*)/ juveniles (*Otohydra*) are released into the water via a rupture of the epidermis, due to the structural changes in the tissues. Additionally, detailed information about the female gonadal compartment of *Halammohydra* are documented for the first time.

Every specimen of *Halammohydra* was documented with a camera to find species-specific characters for identification, which were used in combination with single gene sequencing (16S, 18S and CO1) and species delimitation tests (ABGD, GMYC and bPTP). This integrative approach helps with verification of describes species, adds character information as well as finds new species. Additionally, it fills the database with sequences, which are needed for future studies. Phylogenetic analyses (Bayesian Interference and Maximum Likelihood) delimit 16 clusters of which seven are assigned to four known species (*H. vermiformis, H. octopodides, H. coronata* and *H. adherens*), four are describes as new species (*H. teissieri* n. sp., *H. swedmarki* n. sp., *H. kerblae* n. sp. and *H. joergerae* n. sp.) and five remain unidentified ("Helgoland/ Sylt", "Azores", "Roscoff", "Tenerife 1" and "Tenerife 2"). These results show a higher diversity and distribution as previously expected. In addition, the morphological documentation shows different variabilities of characters within the species and overlapping between different species.

Molecular analysis of *Otohydra* specimens reveals no close relation to *Halammohydra*, thus invalidate the taxon Actinulida. They are positioned within Rhopalonematidae (Trachymedusae), as it is the case for *Halammohydra*, but clusters close to a species of Ptychogastriidae. If *Otohydra* belongs to Rhopalonematidae or Ptychogastriidae is not resolved yet, due to the slightly lower support value and the morphological differences to Ptychogastriidae. To answer this, the recently found meiofaunal cnidarian *Marsipohydra pacifica* Sanamyan & Sanamyan, 2012 might help, since it is positioned close to *Otohydra* and has some characters of Ptychogastriidae. Molecular information are needed. Nonetheless, these results show another independent transition to the meiofauna way of life within Cnidaria.

4. Zusammenfassung

Marine Sedimente beherbergen viele mikroskopische Organismen, die perfekt an ihre Umgebung angepasst sind, zum Beispiel durch die kleine Körpergröße, Klebestrukturen oder das Fehlen einer planktonischen Larve. Diese sogenannte Meiofauna scheint sich deswegen nicht weit verbreiten zu können, trotzdem sind einige Arten amphi-ozeanisch oder sogar kosmopolitisch. Viele erneute Untersuchungen dieser Arten zeigten morphologische oder molekulare Unterschiede, aber nicht bei allen. Es gibt einige Hypothesen, die versuchen dieses „Meiofauna Paradox“ zu erklären, aber Untersuchungen aller Gruppen werden benötigt um die individuellen Prozesse zu finden. Die meisten Studien wurden an großen Gruppen, wie Nematoden oder Copepoden, gemacht, während kleinere Gruppen mit speziellen Habitat-Präferenzen fehlen.

Besonders die wenigen Cnidaria in der Meiofauna wurden wenig untersucht. Die größte Gruppe ist die modifizierte Meduse *Halammohydra* Remane, 1927, mit neun beschriebenen Arten, dessen Bestimmung teilweise schwierig ist. Zusammen mit *Otohydra* Swedmark & Teissier, 1958 bilden sie das Taxon Actinulida. Diese Verwandtschaft ist fraglich aufgrund der morphologischen Unterschiede und muss molekular untersucht werden. Außerdem werden detaillierte Informationen über spezielle Strukturen benötigt, wie zum Beispiel das Fortpflanzungssystem beider oder das Adhäsivorgan von *Halammohydra*, um artspezifische Strukturen oder Strukturen für die Klassifizierung innerhalb der Cnidaria zu finden.

Dieses Projekt untersucht Individuen von *Halammohydra* von unterschiedlichen Orten hauptsächlich in Europa und wenige Individuen von *Otohydra* von Roscoff in Frankreich. Sie wurden mithilfe der „anesthesia-decantation“- Methode aus dem Sediment extrahiert und für molekulare und morphologisch Untersuchungen fixiert. Die detaillierte Untersuchung der Zellstruktur von *H. vermiformis* und *Otohydra* sp. mit Semi- und Ultradünnschnitten hilft die generelle Organisation zu verstehen und fügt Informationen hinzu, besonders über die Gonade beider oder der aboralen Kappe mit dem Adhäsivorgan von *Halammohydra*. Für beide wird für die Freisetzung der Spermatozoa und Oozyten (*Halammohydra*)/ Juvenilen (*Otohydra*) eine Ruptur der Epidermis vorgeschlagen, aufgrund der strukturellen Änderungen des Gewebes. Außerdem wurden die Zellstruktur der weiblichen Gonade von *Halammohydra* das erste Mal dokumentiert.

Jedes Individuum von *Halammohydra* wurde mit der Kamera dokumentiert, um artspezifische Merkmale für die Identifizierung zu finden, die dann in Kombination mit Einzelgensequenzierung (16S, 18S und CO1) und „species delimitation tests“ (ABGD, GMYC und bPTP) analysiert wurden. Dieser integrative Ansatz hilft mit der Überprüfung der beschriebenen Arten, fügt Informationen über Merkmale hinzu und findet neue Arten. Außerdem füllt es die Datenbank mit Sequenzen, die notwendig für zukünftige Projekte sind. Phylogenetische Analysen (Bayesian Interference und Maximum Likelihood) unterscheiden 16 Cluster, von denen sieben zu vier beschriebenen Arten zugeordnet (*H. vermiformis, H. octopodides, H. coronata* und *H. adherens*), vier als neue Arten beschrieben werden (*H. teissieri* n. sp., *H. swedmarki* n. sp., *H. kerblae* n. sp. und *H. joergerae* n. sp.) und fünf unbestimmt bleiben (“Helgoland/ Sylt”, “Azores”, “Roscoff”, “Tenerife 1” und “Tenerife 2”). Diese Ergebnisse zeigen eine höhere Diversität und Verbreitung als vorher angenommen. Außerdem zeigt die morphologische Dokumentation unterschiedliche Variabilität der Merkmale innerhalb einer Art und Überlappungen zwischen Arten.

Die molekulare Analyse von *Otohydra* Individuen zeigt keine nahe Verwandtschaft mit *Halammohydra*, also ist das Taxon Actinulida ungültig. Sie befinden sich innerhalb der Rhopalonematidae (Trachymedusae), genauso, wie *Halammohydra*, aber gruppieren sich mit einer Art von Ptychogastriidae. Ob *Otohydra* zu Rhopalonematidae oder Ptychogastriidae gehört ist unklar, aufgrund

der leicht niedrigeren „node support“ Werte und den morphologischen Unterschieden zu Ptychogastriidae. Bei einer Antwort kann die kürzlich gefundene Meduse *Marsipohydra pacifica* Sanamyan & Sanamyan, 2012 helfen, da sie zu *Otohydra* gruppiert wird und einige Übereinstimmungen mit den Merkmalen der Ptychogastriidae hat. Dafür werden molekulare Informationen benötigt. Nichtdestotrotz zeigen diese Ergebnisse eine weiter unabhängige Entwicklung zum Meiofauna-Leben innerhalb der Cnidaria.

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7. Declaration of oath / Eidesstattliche Versicherung

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids.

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

_____

Signature/ Unterschrift

II. STUDIES

8. Statements of authorship

Study I

Tödter L & Schmidt-Rhaesa A (2021) First record of *Halammohydra* (Cnidaria, Hydrozoa) on the Azores. Acoreana Special Volume (11), 97-102.

Own contribution:

- Documentation of the individuals in the field
- Analyzing the characters
- Drafting the manuscript including all figures

Original research: 70%
Figures and illustrations: 100 %
Text: 80 %

Study II

Tödter L & Schmidt-Rhaesa A (2022) Ultrastructural organization of *Halammohydra vermiformis* Swedmark & Teissier, 1957 (Cnidaria: Hydrozoa). Zoomorphology, 1-17. doi:https://doi.org/10.1007/s00435-022-00560-w.

Own contribution:

- Collecting and documentation of the individuals
- Identification of the species
- Digitalizing and analyzing semi and ultra-thin sections
- Drafting the manuscript including all figures

Original research: 90%
Figures and illustrations: 100 %
Text: 80 %

Study III

Tödter L, Worsaae K & Schmidt-Rhaesa A (in review) Comparative molecular and morphological species selineation of *Halammohydra* Remane, 1927 (Hydrozoa) – with description of four new species

Own contribution:

- Collecting and documentation of the individuals
- Identification of the species
- Analyzing character
- Conducting and analyzing the molecular work
- Drafting the manuscript including all figures

Original research: 80%
Figures and illustrations: 100 %
Text: 75 %

Study IV

Tödter L & Schmidt-Rhaesa A (submitted) Morphological and molecular analyses of the meiofaunal cnidarian *Otohydra* sp. (Hydrozoa, Cnidaria) invalidate the taxon Actinulida

Own contribution:

- Documentation of the individuals
- Conduction and analyzing the molecular work
- Digitalizing and analyzing semi and ultra-thin sections
- Drafting the manuscript including all figures

Original research: 90%
Figures and illustrations: 100 %
Text: 80 %

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Statement zur Autorenbeteiligung

Frau Lenke Tödter hat in ihrer Dissertationsschrift die Beteiligung der Autoren an den einzelnen Veröffentlichungen dargelegt. Ich bestätige hiermit, dass die genannten Werte der tatsächlichen Arbeitsverteilung entsprechen.

Prof. Dr. Andreas Schmidt-Rhaesa

9. List of studies

Study I: published paper, p. V-X

Tödter L & Schmidt-Rhaesa A (2021) First record of *Halammohydra* (Cnidaria, Hydrozoa) on the Azores. Acoreana Special Volume (11), 97-102.

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Study II: published paper, p. XI-XXVII

Tödter L & Schmidt-Rhaesa A (2022) Ultrastructural organization of *Halammohydra vermiformis* Swedmark & Teissier, 1957 (Cnidaria: Hydrozoa). Zoomorphology, 1-17. doi:https://doi.org/10.1007/s00435-022-00560-w.

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Study III: manuscript, submitted to Organisms Diversity & Evolution, 21. July 2022, p. XXVIII-LX

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AÇOREANA, 2021, Suplemento 11: 97-102

FIRST RECORD OF *HALAMMOHYDRA* (CNIDARIA, HYDROZOA) ON THE AZORES

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ABSTRACT

One of the most diverse interstitial cnidarian genera is *Halammohydra* Remane, 1927. The hydrozoan of the order Actinulida was recorded before in Europe (i.e., Germany, Norway, France), India and the West Atlantic. This is the first record of *Halammohydra* from the Azorean islands. Specimens were collected at different stations from sandy sediment during the summer school "Exploring the marine meiofauna of the Azores – from discovery to scientific publication" (15.07 – 24.07.2019) and extracted from sediment samples by the anesthesia – decantation method. All individuals had a combination of features of the two very similar species *H. schulzei* and *H. intermedia*. Due to the general lack of one long tentacle in the subaboral whorl, specimens were identified as *H. schulzei*. The high variation and different combination of characters give reason to question the difference of both species. This needs to be investigated in further molecular studies.

RESUMO

Um dos géneros de cnidários intersticiais mais diversificados é *Halammohydra* Remane, 1927 O hidrozoário da ordem Actinulida foi antes registado na Europa (i.e. Alemanha, Noruega, França), Índia e Atlântico Oeste. Este é o primeiro registo de *Halammohydra* das ilhas dos Açores. Os exemplares foram recolhidos em diferentes estações de sedimento arenoso durante a escola de verão "Explorando a meiofauna marinha dos Açores – da descoberta à publicação científica" (15.07 – 24.07.2019) e extraídos de amostras de sedimentos por anestesia – método de decantação. Todos os indivíduos possuíam uma combinação de características das duas espécies semelhantes, *H. schulzei* e *H. intermedia*. Devido à ausência generalizada de um longo tentáculo na volta subaboral, os exemplares foram identificados como *H. schulzei*. A elevada variação e a diferente combinação de caracteres dão força à questão da diferença de ambas as espécies. Tal necessita de ser investigado em estudos moleculares futuros.

INTRODUCTION

The interstitial system is a habitat for representatives of almost all taxa. Groups like Nematoda or Copepoda occur in high numbers and diversity of species. One of the less common groups are the Cnidaria. With nine species, the genus *Halammohydra* Remane, 1927 of the hydrozoan order Actinulida is the most diverse genus among interstitial cnidarians (Schmidt-Rhaesa *et al.*, 2020). It was first discovered by Remane (1927), who also provided a very detailed general description. *Halammohydra* is a medusa with a reduced umbrella. Its body is divided in two distinct parts, the gastric tube (manubrium) and the aboral cone with adhesive organ, tentacles and statocysts. The gastric tube is of an ovoid or elongated shape and ends with a mouth opening distally. Resorbed food results in a brown to yellow coloration of the gastrodermis in the gastric tube of living animals. The gonad is located on one side of the gastric tube. It can be comparably large and shift the gastric lumen to the other side, thus resulting in a slightly shifted mouth opening. The aboral end of

the gastric tube connects to the aboral cone via a neck. Attached to the cone are three whorls of appendages alternating to each other. On the oral side, there is one whorl of statocysts (often called lithostyle statocysts), followed by two whorls of tentacles. These tentacle whorls are named the aboral and the subaboral whorl. The number of tentacles in each whorl and of the statocysts is an important identification criterion. It can be an equal (i.e., *H. schulzei* Remane, 1927 or *H. octopodides* Remane 1927 (Remane, 1927) or a different (i.e., *H. vermiformis* Swedmark & Teissier, 1957 or *H. coronata* Clausen, 1967) (Swedmark & Teissier, 1957a; Clausen, 1967) number in each whorl. Additionally, some species, like *H. intermedia* Clausen, 1967 or *H. vermiformis*, have a very distinct and long tentacle in the subaboral whorl, which is usually thicker than the others (Swedmark & Teissier, 1957a; Clausen, 1967). Juvenile individuals develop primary tentacles of the aboral whorl first and then of the subaboral whorl. After that, tentacles in each whorl duplicate to a species-specific number (Swedmark & Teissier, 1950). Therefore, only adult animals can be used for identification. Another criterion is the general shape and the base of the tentacles. *Halammohydra schulzei*, for example, has a strong thickening at the base of the subaboral tentacles and a less thick or absent structure at the aboral tentacle bases (Remane, 1927). The very similar species *H. intermedia* does not have a strong thickening at the subaboral tentacle bases, but a more club shaped structure. In this species, aboral tentacles are usually shorter than subaboral ones too (Clausen, 1967). At the aboral end of the cone is a special structure for temporary adhesion to sand grains, which is very useful in the interstitial system. The shape of this organ can be an identification character as well. *Halammohydra schulzei* has a pear shaped adhesive organ, in contrast to *H. intermedia*, in which it is cup shaped (Clausen, 1967). Morphological variation within one species occurs frequently, which can make the identification difficult and may, in some cases, even question species delimitation. With a size of about 400 µm in most species, *Halammohydra* prefers coarse sand but it can be found in fine sand as well. Most records are from Europe (i.e., Remane, 1927; Swedmark & Teissier, 1950; 1957a; Dahl, 1953; Boaden, 1961; Clausen, 1967; Schmidt, 1969; Wolff *et al.*, 1974; Martínez *et al.*, 2019) and India (i.e., Rao & Misra, 1980; Rao, 1993; Sugumaran & Padmasai, 2019) and a few from the West Atlantic (i.e., Bush & Zinn, 1970; Calder & Kirkendale, 2005; Jörger *et al.*, 2014). This is the first record of the genus *Halammohydra* of the Azorean islands.

MATERIAL AND METHODS

In association with the summer school "Exploring the marine meiofauna of the Azores – from discovery to scientific publication" (15.07 – 24.07.2019) in Ponta Delgada, specimens of *Halammohydra* were found by different participants at the stations 16a, 47, 49, 50, 52 and 53. For further information about the stations and sampling techniques, see Jörger *et al.*, 2021. Animals were extracted by anaesthetization with $MgCl_2$-seawater and decantation through a sieve with the mesh size of 63 µm. Samples were transferred to a petri dish and scanned for meiofaunal animals. Specimens were documented with a camera (UCMOS 05100 KPA; 5.1 MP 1/2.5" APTINA CMOS SENSOR), identified morphologically (when possible) and fixed in 96 % ethanol individually, for subsequent molecular studies.

RESULTS

Thirty eight specimens of *Halammohydra* were found at stations 16a, 47, 49, 50, 52, and 53, most of them at station 49 (Table 1). Sixteen specimens were juveniles and thus could not be identified with certainty. The following descriptions refer to the 22 adult specimens. The general habitus with an ovoid gastric tube and the aboral cone sticking to the slide is shown in Figure 1A. The documented animal was very adhesive and even with anaesthetization of magnesium chloride, it did not release from the glass. This was noticed in several individuals. The mean size of the gastric tube for all adult specimens was 344 ± 103 µm in length and 167 ± 42 µm in width. The aboral cone measured 59 ± 13 µm in length and 58 ± 17 µm in width. The adhesive organ was about half the depth of the aboral cone and had a cup shaped form, when visible (Figure 1E). A pear shaped adhesive organ was not recorded. Most specimens had 12 tentacles in total, with six tentacles in each whorl. Two specimens had 10 (5+5) tentacles and the highest number was 18 (9+9) tentacles. Some individuals were adults with a fully developed gonad and had very short tentacles, as in Figure 1A. There was a thickening at the base of the subaboral tentacles, which ends in a deep indentation in the tentacle (Figure 1C), this structure is absent in the aboral tentacles (Figure 1B), but this was not noted for all individuals. Some specimens had a less intensive club shaped tentacle base (Figure 1D), which tapers towards the cone. Other individuals had no such structure in the subaboral whorl (Figure 1E). Tentacles of the aboral whorl of all individuals were roughly the same length and longer or the same length as the subaboral ones. Subaboral tentacles were of different length, but none of them was notably longer. Except for the basal structure, the general shape of the tentacles of the different whorls was either similar (e.g. Figure 1A and E) or completely different (e.g. Figure 1D). Specimens with different tentacle shape had a regular and slender form in the aboral tentacles and an irregular and thicker form in the subaboral tentacles. The number of statocysts was in most specimens the same as the number of tentacles per whorl, their size was about 10 µm. Sometimes not all statocysts were visible due to overlying tissues. In general, the specimens moved calmly and tended to adhere to sand grains. Finding them needed a calm hand and searching strategy, because they were easily disturbed by slight shakes, resulting in a tightly coiled animal. After some time of no disturbance, the animals started to spread their tentacles and scan the environment again.

DISCUSSION

This is the first record of *Halammohydra* from the Azorean islands. Specimens were found in stations with sandy sediment. In the literature, they

TABLE 1: Number of juveniles and adult found at the different stations. Most of the specimens were found at station 49.

Station	**16a**	**47**	**49**	**50**	**52**	**53**
juvenile	2	0	12	2	0	0
adult	0	2	17	0	2	1

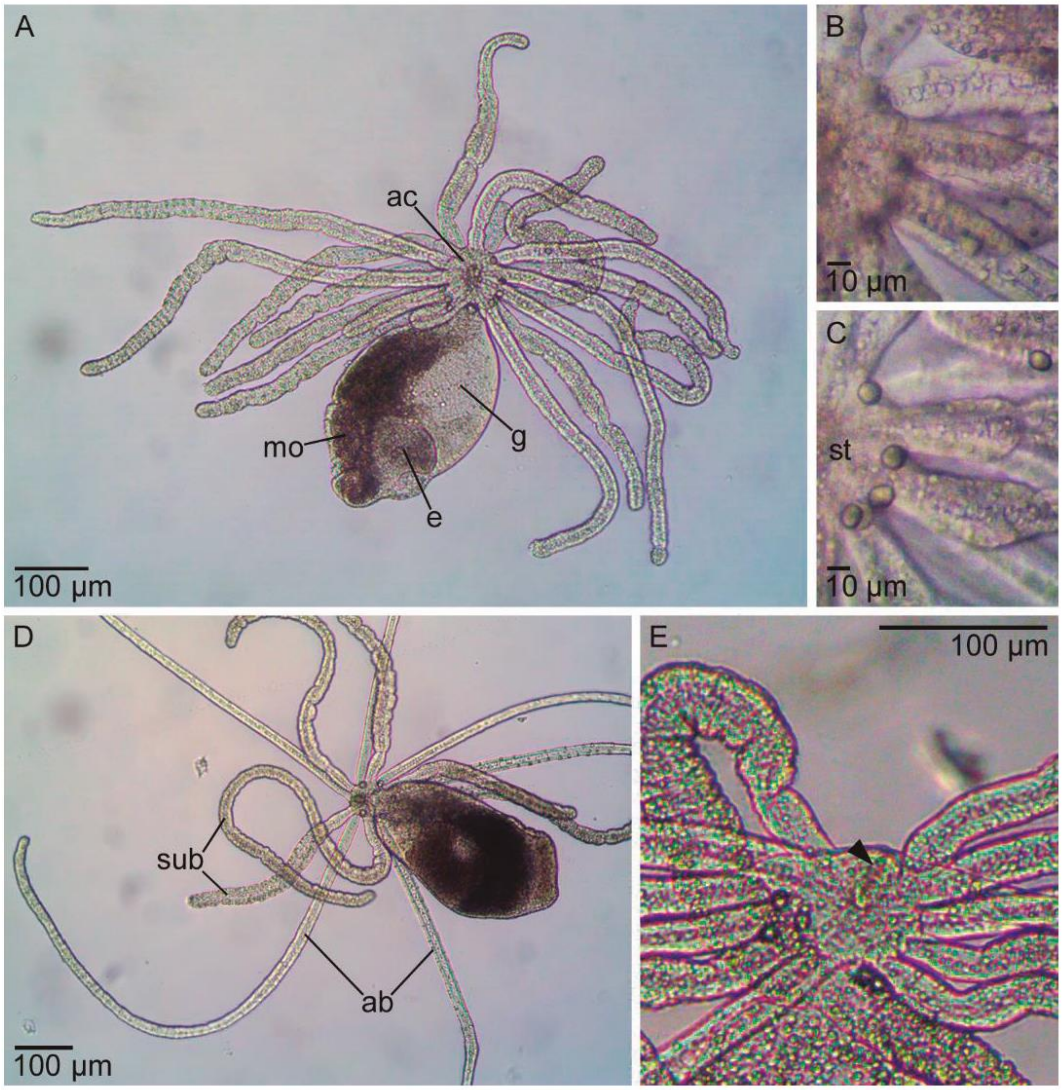

FIGURE 1: Habitus of *H. schulzei* and magnification of the tentacle bases and the aboral cone. **A**, specimen sticking to the slide with its adhesive organ positioned in the aboral cone (**ac**). In the gastric tube a huge gonad (**g**) with an egg (**e**) is visible. The mouth opening (**mo**) is shifted to one side. Magnification of the tentacle base of the (**B**) aboral and (**C**) subaboral tentacles of the individual in **A**. Statocysts with statoliths (**st**) are visible. (**D**) Habitus of another specimen, with clear difference in structure of the tentacle whorls. Aboral tentacles (**ab**) were of slender form, with no thickening at the base. Subaboral tentacles (**sub**) were of thicker irregular shape and with a club shaped tentacle base. (**E**) Magnification of the aboral cone with a visible adhesive organ (arrowhead).

were mainly found in coarse sand but there are some records of specimens found in fine sediment (i.e., Remane, 1927; Clausen, 1967; Rao & Misra, 1980). Finding *Halammohydra* can be challenging, because they are not as numerous as other taxa and due to their tendency to coil together

when disturbed, they are easily overlooked. This may be the reason why there are comparably few published records. In combination with identification problems due to a high morphological variability, only little information about the species distribution is available. Especially the species *H. schulzei*, *H. octopodides* and *H. intermedia* can have a very similar morphology. Due to the strong adhesion documented, *H. octopodides* can be excluded. This species is less adhesive and swims more actively (Remane, 1927; Clausen, 1967), in contrast to *H. schulzei* and *H. intermedia*. As many of the investigated specimens adhered to the slide during examination, the shape of the aboral cone and the adhesive organ was almost impossible to observe. Both shapes are important to distinguish *H. schulzei* from *H. intermedia* (Clausen, 1967). When the adhesive organ was visible, it was more cup shaped and thus a feature of *H. intermedia*. The number of tentacles is of limited value as identification criterion. For distinguishing between *H. schulzei* and *H. intermedia*, it is not useful, because they are in the same range. Additionally, sexual maturity can be reached before all tentacles are developed (Swedmark & Teissier, 1957b), which makes it difficult to identify the final number. Some individuals showed a strong thickening at the bases of the subaboral tentacles, as documented for *H. schulzei*, but this was highly variable. One of the most distinctive characters is the single very long tentacle in the subaboral whorl of *H. intermedia* (Remane, 1927; Clausen, 1967), which was not documented in any of the specimens. Additionally, aboral tentacles were always longer or at least the same length as the subaboral ones. This is contrary to the description of *H. intermedia*, where the aboral tentacles are shorter.

In summary, the specimens from the Acores Islands show a mixture of characters between those reported for *H. schulzei* and *H. intermedia*. The lack of conspicuous characters like the presence of one long tentacle (as in *H. intermedia*) supports an identification as *H. schulzei*. However, our results raise general questions whether *H. schulzei* and *H. intermedia* are two distinct species or whether there is an overlap in the morphological variability of both species. Molecular data (currently under way) may help to solve this question.

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ORIGINAL PAPER

Ultrastructural organization of *Halammohydra vermiformis* Swedmark & Teissier, 1957 (Cnidaria: Hydrozoa)

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Abstract
Within meiofauna, cnidarians are represented by only a few species most of which are in the genus *Halammohydra* Remane, 1927. It represents highly modified medusae. Information about this group is limited, which complicates its placement in the Cnidarian tree and the relationship to another meiofaunal cnidarian, *Otohydra* Swedmark & Teissier, 1958. This needs to be clarified with molecular, but also with morphological methods. In this study, the internal organization of *H. vermiformis* Swedmark & Teissier, 1957 from Sylt and Helgoland (Germany) was examined using transmission electron microscopy (TEM). The ultrastructure of both sexes is documented in this study, i.e. the gastric tube including gonadal compartment, aboral cone, statocysts and tentacles. It is proposed that spermatozoa and oocytes are not released into the water through the gastrodermis, but by rupture of the epidermis, because of structural changes in the epidermis. In both, male and female, there is an indent in the gastric tube and a gap of the mesoglea at the same position. Additionally, we describe the complex structure of the aboral cone with the specialized adhesive organ as well as the accumulation of myofibrils and neurites in the orally directed part of the cone, which indicates high controllability and ability to move in this region.

Keywords Meiofauna · Interstitial · Trachylinae · Transmission electron microscopy · Adhesive organ

Introduction

Among the diverse meiofaunal animals in marine sediments, there are few representatives of the phylum Cnidaria and they are dominated by Hydrozoans (Schmidt-Rhaesa et al. 2020). There is only one Staurozoan genus (*Stylocoronella*) and no representatives of Anthozoa and Cubozoa are known so far (Kikinger and Salvini-Plawen 1995). Within Hydrozoa, meiofaunal cnidarians can represent either the polyp or the medusa stage. Seven genera are attributable to the polyp stage and they are classified among the Hydroidolina. The first described meiofaunal cnidarian was *Protohydra leuckarti* Greeff, 1870, a polyp without tentacles and with the ability to drastically change body shape (Greeff 1870). The reduction of tentacles and the flexibility of the body are perfect adaptations to the interstitial system. With intensified investigations of sediment in the following years, more cnidarian representatives were discovered. Interestingly, not only polyps found their way into the sediment, but medusae, too. Presumably, medusa-derived taxa are found in four genera, all belonging to the subclass Trachylinae. The best-investigated group and the genus with the most known species (nine) is *Halammohydra* Remane, 1927. It was first discovered by Remane in 1927 in the Baltic Sea and on Helgoland in Germany, and then found in different locations in Europe, mainly Germany (e.g. Clausen 1967; Polte and Schmidt-Rhaesa 2011; Schmidt 1969; Swedmark and Teissier 1957), France (e.g. Swedmark and Teissier 1957, 1967; Teissier 1950), Norway (e.g. Clausen 1963, 2000) and United Kingdom (e.g. Boaden 1961, 1963). Other European locations are Sweden (Boaden 1960; Dahl 1953), Ireland (Boaden 1966), the Netherlands (Wolff et al. 1974), Italy (Salvini-Plawen 1991), Spain (Martínez et al. 2009; 2019) and Portugal (Tödter and Schmidt-Rhaesa 2021). Outside of Europe, *Halammohydra* was reported particularly from India (e.g. Rao 1978; Rao and Ganapati 1966; Salvini-Plawen and Rao 1973; Sugumaran and Padmasai 2019; Sugumaran et al.

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2009), but also from Panama (Calder and Kirkendale 2005), Brazil (Garraffoni et al. 2017; Jörger et al. 2014) and the Caribbean (Hochberg et al. 2014; Kånneby et al. 2014).

The morphology of all *Halammohydra* species is simple, yet displays special adaptations to the interstitial system. It is thought to be derived from a medusa that has lost its umbrella (Remane 1927). The entire body is ciliated and the gastric tube is thus likely homologous to the manubrium of a hydromedusa. It is connected to the aboral cone (reduced umbrella) via a neck. Two whorls of tentacles and one whorl of statocysts are attached to the aboral cone and there is an adhesive organ for temporary fixation at the tip of it. Due to its flexible body and the cilia, the animal is perfectly adapted for moving in the interstitium. Similar to other meiofaunal groups, *Halammohydra* has a direct development without a pelagic larva. The absence of a polyp stage and the morphology of statocysts led Remane (1927) to place *Halammohydra* among the Trachylinae, and further into Narcomedusae because of similarities between their larval stages. With the discovery of *Otohydra* Swedmark & Teissier, 1958, another fully ciliated meiofaunal medusa with direct development, the order Actinulida with the two families Halammohydridae and Otohydridae was erected (Swedmark and Teissier 1958, 1959). For a long time, the exact position within the cnidarian tree was not known for sure, until Collins et al. (2008) presented a phylogenetic study of Trachylinae, with a special focus on the placement of *Halammohydra*. Before the publication of Collins et al., no molecular data were available for this genus. The results confirmed their placement in Trachylinae and a closer relationship to the family Rhopalonematidae (Trachymedusae). There are no sequences available for *Otohydra*, so the validity of the taxon Actinulida cannot be investigated. The organization of the entire body of *Halammohydra* is described by histology (Remane 1927; Swedmark and Teissier 1967) and immunohistochemistry (Polte and Schmidt-Rhaesa 2011), and specific structures by ultrastructure (Clausen 1991, 2000; Ehlers 1993). Clausen did an ultrastructural study on nematocysts in the entire body (Clausen 1991) and on microsporidia in the adhesive organ of *Halammohydra intermedia* Clausen, 1967 (Clausen 2000), and Ehlers investigated the male gonad ultrastucturally, which he defined as a gonadal compartment in the gastrodermis (Ehlers 1993). These are the only ultrastructural studies. Therefore, there is a need for a more detailed examination to do potential phylogenetic considerations.

This insufficient morphological knowledge needs to be updated and summarized to answer general questions about the systematic position of the group and function of certain body structures. Especially a closer look at the adhesive organ and its function, as well as the female reproduction system is needed, as there is presently only one study on the male gonadal compartment (Ehlers 1993). All this information combined is essential to validate the systematic position within the Trachylinae and can help to clarify the relation to the genus *Otohydra*. In the present work, we present an ultrastructural study of the entire body of *Halammohydra vermiformis* Swedmark & Teissier, 1957. It can be identified by its conical adhesive cone and the 7 (3 aboral and 4 subaboral) and sometimes 8 (4 and 4) tentacles. Aboral tentacles are slender and shorter than subaboral ones, whereas subaboral tentacles are unequal in length. They have a variety of body shapes from elongated to round and a cnidom consisting of stenoteles and isorhizas. With this study, we add information to the published findings, especially on details of the gonadal structure and the aboral cone.

Material and methods

Specimens of *Halammohydra vermiformis* were collected in the south of Sylt (Hörnum, 54°45.352 N, 8°17.666 E, two males) and on Helgoland in Germany (at the sandy island called "Dune", 54°11.394 N, 7°54.723 E, one female). In Hörnum, sediment samples of coarse sand were taken with a shovel from the beach at low tide and processed at the Wadden Sea Station in List (Alfred Wegener Institute, AWI). At Helgoland, sediment samples were collected in the shallow subtidal in 50 cm water depth at the North shore of the island "Dune" and processed at the Biological Institute Helgoland (AWI). Samples were stored in a cool environment and covered by a few centimeters of water, not longer than 1 week.

For extraction, a 7% magnesium chloride solution was used to anesthetize the animals. A small portion of the sand was mixed with this solution and incubated for 10–15 min with occasional stirring. The supernatant of the sample was decanted into a sieve with a 63 µm mesh size and the animals were collected under a stereomicroscope. Individuals of *Halammohydra vermiformis* were examined further under the compound microscope with higher magnification (Leica DM2500) and documented by photos and videos with a handycam (Sony). Numerous specimens were investigated within the frame of another project and of the 46 specimens of *H. vermiformis,* three mature specimens were selected for transmission electron microscope (TEM) investigation. Two males and one female were relaxed with magnesium chloride and fixed in Trumps (combination of sodium cacodylate buffer, formalin, and glutaraldehyde).

Specimens were postfixed with osmium tetroxide (1%, in sodium-cacodylate buffer) and embedded in LR White resin following a modified protocol by McDonald (1984) and Purschke et al. (1991). A combination of semi-thin (0.5 µm) and ultrathin (70 nm) sections was used to section through the entire animals. Ultrathin sections were contrasted with lead citrate and uranyl acetate and investigated using a Zeiss EM902A TEM. Digital photos were taken. Semi-thin

sections were stained with toluidine blue and investigated with light microscopy. Slides were scanned automatically with a light microscope (LEICA DM6000B) and corresponding software (LEICA MetaMorph 1.5.0). Acquired images were adjusted using ImageJ (version 1.52a), Adobe Photoshop and Adobe Illustrator. During the preparation process, one male was accidentally destroyed from the center of the gastric tube to the mouth opening.

Results

Habitus of *Halammohydra vermiformis*

Specimens of *Halammohydra vermiformis* are composed of an elongated gastric tube or manubrium and an aboral cone, bearing one whorl of statocysts and two whorls of tentacles (aboral and subaboral, Fig. 1A–D). On one end of the gastric tube is the mouth opening with the short and slightly tapering proboscis (Fig. 1E) and long cilia sticking out of the opening (Fig. 1F). In living animals, the short proboscis makes irregular circular movements. Aborally, the gastric tube is connected to the cone via a neck, characterized by its smaller width (Fig. 1D). The gastrodermis is of darker color and extends from right above the proboscis to the neck. In the neck, the yellow to brown color slowly fades into transparency, which is similar to the rest of the body. The lighter structure in the gastric tube is the gonadal compartment. Due to its extensive volume, it shifts the gastrodermis and thus the mouth opening to one side. Depending on the state of contraction, the neck is rather long and thin and connects to the conical-shaped aboral cone (Fig. 1D). In addition, the transition from the gastric tube into the neck is gradual and only visible by the narrowing of the overall diameter. On the oral end of the cone, one whorl of four statocysts is attached, followed by two whorls (subaboral and aboral) of four tentacles each (as in the investigated males, in total eight tentacles) or three aboral and four subaboral tentacles (as in the investigated female, seven tentacles in total). Tentacles of both rings alternate in position to each other. The adhesive organ has a shape of an inverted cone and is located at the tip of the aboral cone (Fig. 1D). All tentacles are of the same slender shape, without any indents along their length or conspicuous diameter change at the bases. Tentacles of the aboral whorl are of the same length and visibly shorter than the majority of the subaboral whorl (Fig. 1C). One tentacle of the subaboral whorl is at least two times longer than the others (Fig. 1B). The entire body is covered with cilia, which are used for

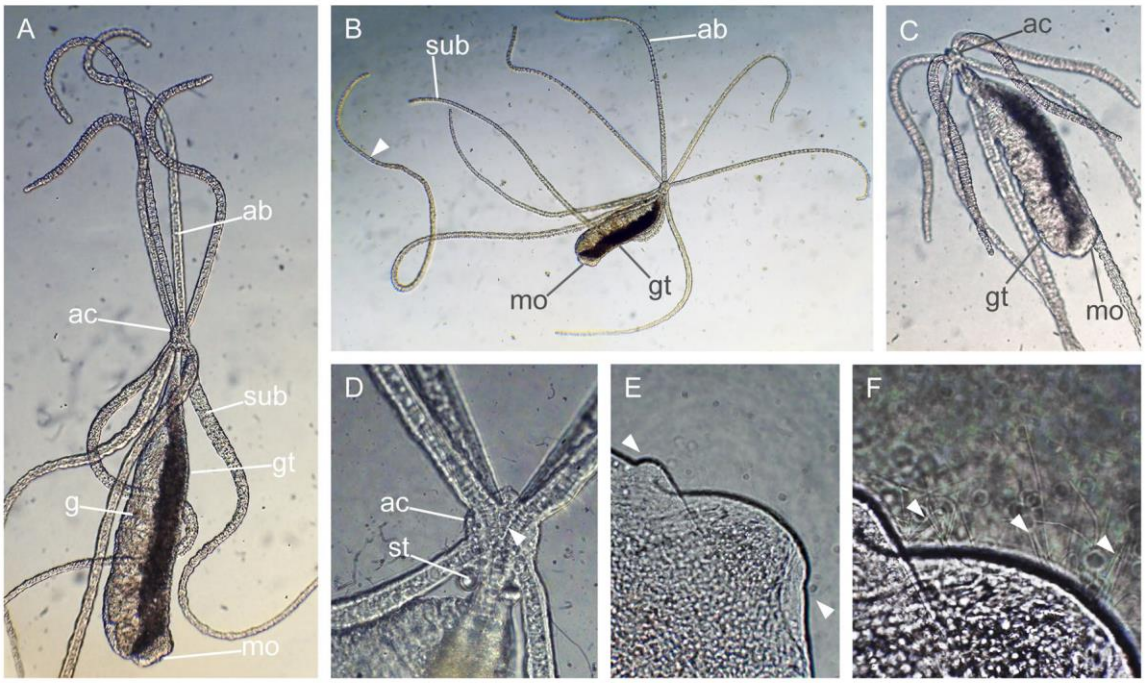


Fig. 1 Light microscopy images of the habitus of *Halammohydra vermiformis* (male). **A** It has an elongated gastric tube (gt) with a lighter-colored gonadal compartment (g) and a terminal mouth opening (mo) connected to an aboral cone (ac) via a neck. Two whorls of tentacles, aboral (ab) and subaboral (sub) are connected to it. **B** One tentacle of the subaboral whorl is about two times longer (arrowhead). **C** When gliding, all tentacles show in the oral direction. **D** Magnification of the aboral cone, showing the statocysts (st) and the adhesive organ (arrowhead). **E** Magnification of the mouth opening with its small proboscis (arrowheads). **F** Further magnification showing the long mouth cilia (arrowheads) sticking out of the mouth

gliding. For this, all tentacles are directed orally and the animal glides with the aboral cone in front (Fig. 1C). All investigated specimens were moving very actively and rarely adhering to sediment particles or the petri dish.

The gastric tube

Epidermis and mesoglea

The general ultrastructure of specimens of *H. vermiformis* consists of an epidermis and a gastrodermis separated by a layer of extracellular matrix called mesoglea (Fig. 2A). A

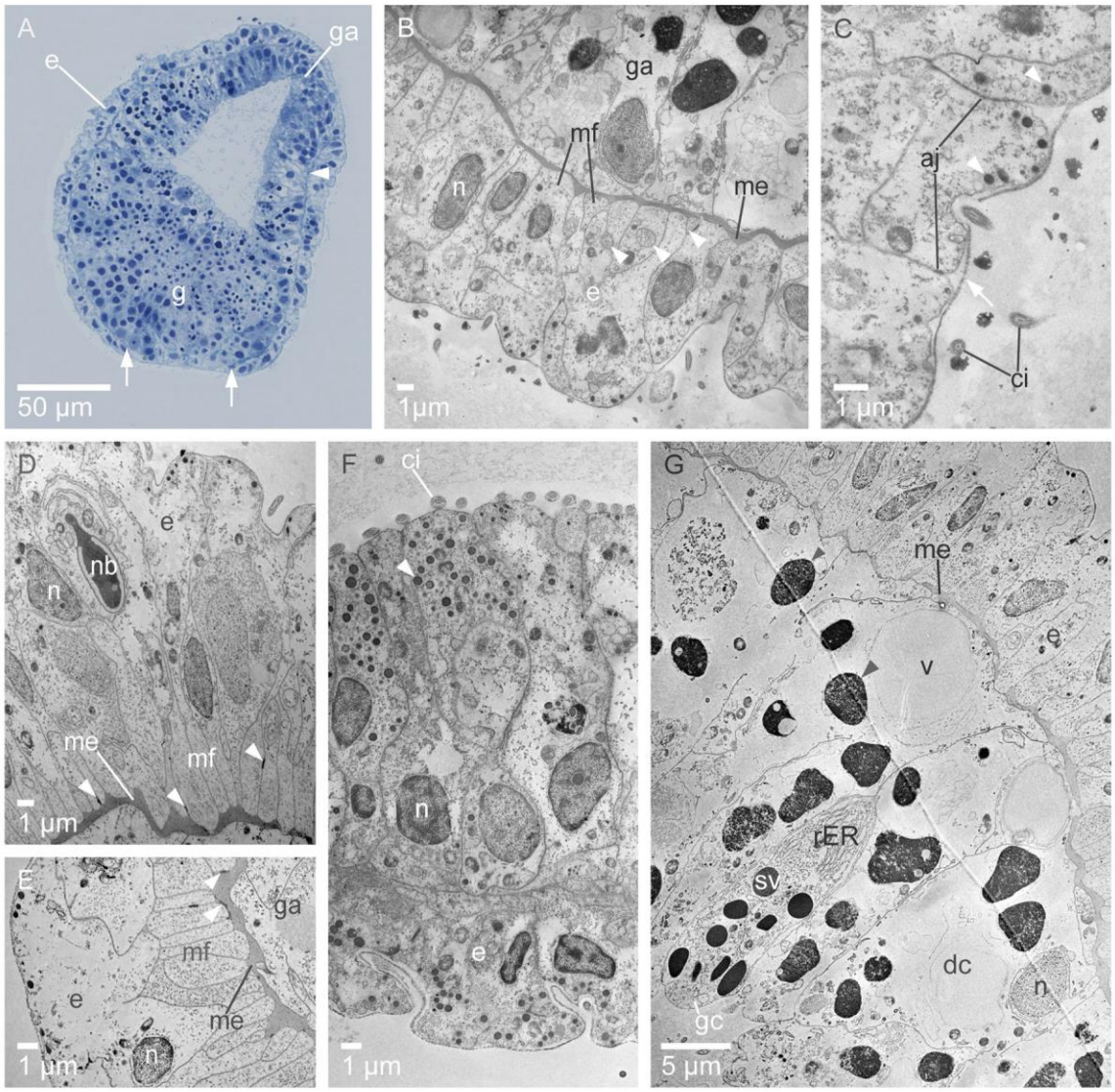


Fig. 2 Semi-thin section (**A**) and ultrastructural images (**B–G**) of the gastrodermis and epidermis in the gastric tube of *Halammohydra vermiformis*. **A** Overview of a section through the gastric tube close to the mouth opening showing the gastrodermis (ga) and gonadal compartment (g) surrounded by an epidermis (e), separated by a thin layer of mesoglea (arrowhead), which seems to vanish on one side (arrows). **B** Epidermal cells have more or less central nuclei (n) and basal myofibrils (mf) close to the mesoglea (me), which are especially concentrated in the basal cell sections (arrowheads). **C** Magnification of the apical part of the epithelium, with apical electron-dense vesicles (arrowheads), adhesive junctions (aj), cilia (ci) and a thin irregular-shaped electron-dense layer covering the epidermis (arrow). **D** Aboral part of the gastric tube with several nematoblasts (nb) and electron-dense structures (arrowheads) connecting basal extensions. **E** In the neck, strong connections of the epidermis to the mesoglea are indicated by electron-dense spots (arrowheads). **F** Orally located gastrodermal cells (of **A**) have basal nuclei (n) and cilia (ci) close to the surface. Some cells contain apical vesicles (arrowhead). **G** Aborally, the gastrodermis consists of lighter digestive cells (dc) with big electron lucent (v) and smaller electron-dense vesicles (arrowhead), and club-shaped gland cells (gc) with round secretory vesicles (sv) and cisternae of rough endoplasmic reticulum (rER)

single layer of epitheliomuscular cells (EMCs) surrounds the entire body (Fig. 2B). This cell type has a more or less columnar shape in cross section and is attached basally to the mesoglea. In some areas, cells connect to the mesoglea along almost their entire width. Other areas have a higher amount of cell sections basally. Apically, most cells have a slightly increased width forming the outer surface. Cell nuclei are mainly in a central position and show uncondensed chromatin with a slightly visible nucleolus. Electron dense, round vesicles (Fig. 2C) with a diameter of roughly 0.3 µm occur close to the apical membrane and the entire outer surface is covered by an irregular-shaped electron-dense layer. Apically, electron-dense sections of the membranes connecting each cell are adherence junctions (Fig. 2C). The entire body is covered with cilia; in all observed cases, the cells were monociliary. In the basal part of the cells, clusters of longitudinally oriented myofibrils and more cellular compartments are present per section than apically (Fig. 2B, D, E), which shows basally branching cells, that interdigitate to a high degree. The resulting basal compartments are mostly filled with myofibrils. In general, the mesoglea is thin and homogeneous. In the oral part of the gastric tube, it has a thickness of about 0.3 µm with a few variations (Fig. 2B). Aborally, the thickness increases up to 0.9 µm with high variation (Fig. 2E). It has a more irregular shape and strong connections to basal myofibrils of the EMCs, indicated by electron-dense spots, likely hemidesmosomes, at the boundary between the layers (Fig. 2D, E).

Throughout the body, the epidermis undergoes slight changes in the shape of cells, the density of muscle fibers, and the extent of basal branching. Orally, the thickness of the epithelium varies between 8 and 15 µm. The cells are rather wide and extend mostly throughout the entire height of the epithelium (Fig. 2B). Only a few basal extensions interrupt this pattern and the density of the myofibrils is low. The thickness of the epithelium changes only slightly in the aboral direction. There is a slight increase of thickness in an area orally, close to the mouth opening, and a stronger increase aborally (up to 29 µm, Fig. 2D), right below the transition into the neck. These two locations are areas of nematocyst development, which can be recognized by several inserted cells and developing nematocysts (= nematoblasts). The aboral region of nematocyst development is larger and only on one side of the body (the gastrodermal side). Only a few nematocysts were documented in other parts of the epidermis. Further in the aboral direction, the number of basal cell compartments and the density of the myofibrils increases. In contrast to the epidermis of the mouth opening, basal myofibrils are more concentrated. Additionally, the mesoglea is less homogenous and varies in thickness. The basal region of the epithelium consists of several slender cells and some of them extend apically (Fig. 2D). Others are only located basally. In the neck region (Fig. 2E), the basal extensions dominate half of the epidermis and are filled completely with muscle fibers. Electron dense thickened regions of the membrane, putative adherens junctions, are present between the basal cell compartments (Fig. 2D, E). The thickness of the muscular basal part of the epithelium increases from about 2.3 µm (mouth opening) to 7.5 µm (neck) with some variations. The general thickness of the epithelium decreases from up to 29 µm, at the aboral end of the gastric tube, to 11 to 13.5 µm in the neck.

Gastrodermis

Mesoglea separates the epidermis from the gastrodermis and mostly also the gonadal compartment (Fig. 2A). The gastrodermis is an epithelium of mostly columnar cells, which extend from the gastral lumen to the mesoglea or gonadal compartment. There are different types of cells visible throughout the gastric tube, which are described from oral to aboral in the following.

At the mouth opening, columnar cells are filled completely with several small inclusions, apical electron-dense vesicles and a basal nucleus (Fig. 2A, F), and apically cilia insert into the cell, which sticks out of the mouth opening (Fig. 1F). Another cell type is of the same size, but with fewer inclusions and no apical vesicles (Fig. 2F). The regions with these two cell types overlap partly (Fig. 2A, F) and are limited to the area of the mouth opening. Both cell types form an epithelium with an almost smooth surface to the lumen and cilia run parallel to the cell surface (Fig. 2F). The main cell type of the gastric tube is a digestive type (Fig. 2G). Digestive cells extend from the gastral lumen to the mesoglea with varying width, where the widest part is basal. The cytoplasm is clear without many inclusions and some large vesicles are present (Fig. 2G). Apically, the cell is filled with several small inclusions and throughout the cell, there are large electron-dense irregular shaped vesicles (Fig. 2G). In between the digestive cells, there are gland cells, less in abundance than the digestive cells and not spanning the entire height of the epithelium (Fig. 2G). Gland cells are filled with many small inclusions and larger round secretory vesicles. These vesicles are electron dense and have a regular shape with a smooth surface. Basally, the cell is filled with cisternae of the rough endoplasmic reticulum.

The entire gastric tube of the living animal is very flexible and changes the overall diameter and the diameter of the lumen frequently. In the aboral direction, however, the lumen decreases until it reaches the neck, where only some cilia fit into the lumen until it closes completely. The digestive cell type continues into the oral part of the neck, where a different cell type and orientation are present. Cells, which were directed to the center of the gastric tube are now without orientation. Additionally, there are no large electron-dense

irregular-shaped vesicles and gland cells are lacking completely (Fig. 5B).

Gonadal compartment—male

The other tissue present in the gastric tube is the gonadal compartment which takes up a large amount of space in the gastric tube (Figs. 1A, 3A). It extends from right above the mouth opening to under the transition into the neck and shifts the entire gastric system to one side. Like the gastrodermis, it is over most of its extent separated from the epidermis by a thin layer of mesoglea (Fig. 3B). Gastrodermis and gonadal compartment are not separated by mesoglea. On the gonadal side of the gastric tube, the mesoglea ends abruptly on both sides of the gonadal compartment (Figs. 2A, 3C) and germ cells bulge towards the epidermis and restrict it to a thin layer. This was noticed in many sections throughout the gastric tube, extending from the start of the gonadal compartment at the mouth opening until the transition into the neck, where the mesoglea is completely closed again. In semi-thin sections of another specimen is a visible indent in a short section of the gastric tube at the exact position of the lacking mesoglea (Fig. 3A). The same was observed in the female specimen (Fig. 4E, E';

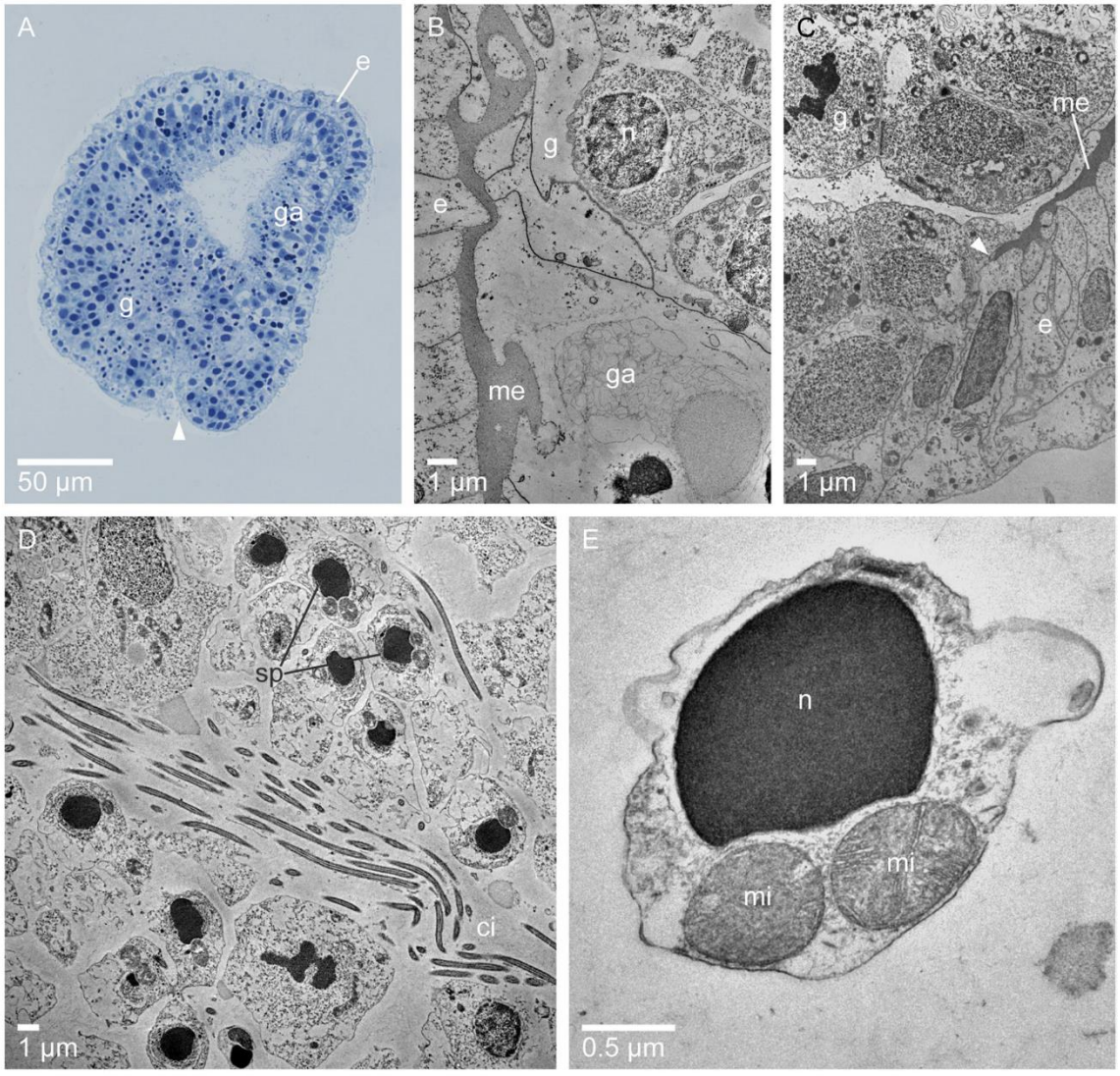


Fig. 3 Semi-thin (**A**) and ultrastructural images (**B–E**) of a male gonadal compartment of *Halammohydra vermiformis*. **A** Cross-section of the center of the gastric tube with the gonadal compartment (g), gastrodermis (ga) and epidermis (e). There is an indent on the gonadal side (arrowhead). **B** Image of all tissues in the gastric tube, the mesoglea (me) separating epidermis (e) from the gonadal compartment (g) and gastrodermis (ga) and a nucleus (n) of a germ cell. **C** The mesoglea (me) ends abruptly on both sides of the indent (arrowhead). **D** In between germ cells, groups of cilia (ci) and developing spermatozoa (sp) are visible. **E** Magnification of a spermatozoon with a nucleus (n) of condensed chromatin and two mitochondria (mi). The cilium is not in the plain of this section

see below). Unfortunately, the ultrastructurally investigated individual was destroyed in the central region of the gastric tube to the mouth, so there is no ultrastructural image of this region in a male specimen.

Cells of the male gonadal compartment are loosely connected with no prominent electron-dense cell connections (Fig. 3B–D). Some cells are in close contact with adjacent cells (Fig. 3C), while others have small (Fig. 3B) to large (Fig. 3D) gaps separating them. The cells themselves are filled completely with electron-dense structures and a large nucleus, which shows uncondensed chromatin and a prominent nucleolus. In the extracellular area, there are several groups of cilia visible (Fig. 3D). Throughout the entire gonadal compartment, no specialized areas were documented. Cells with uncondensed nuclei are next to patches of cilia and between them are different spermatogenesis stages. The putatively mature spermatozoon is round-headed; it has a nucleus with condensed chromatin and below this two mitochondria, which are round and almost equally sized (Fig. 3E). The attachment of cilia or basal bodies, as well as the apical vesicles, were not observed in the sections investigated.

Gonadal compartment—female

The female gonadal compartment starts one-third of the gastric tube above the mouth opening and extends up to the transition into the neck (Fig. 4A). It varies in shape depending on the volume of the oocytes in different areas of the gonadal compartment. The specimen used for closer examination had three voluminous oocytes, with two smaller ones (aboral) and one bigger one (oral), which are even visible in light microscopy (Fig. 4A). Aborally, smaller and immature germ cells are located and create a thickening on one side of the gastric tube (Fig. 4B). It increases in oral direction and is separated from the epidermis by mesoglea (Fig. 4C). As in the male specimens, there is an abrupt ending of the mesoglea on both sides of the gonadal compartment (Fig. 4C'), but there is always a thin layer of epidermal cells separating the gonadal compartment from the outer environment. The germ cells themselves are densely packed and have a nucleus with a prominent nucleolus. In the oral direction, two oocytes are located. Both have the same structure with a large portion of yolk and a central nucleus (Fig. 4D). The oocytes take up almost half of the gonadal compartment, which also is filled with additional germ cells. In this section, the gastrodermis is shifted to one side and takes up one-third of the gastric tube. Ultrastructural images show, that the yolk is filled up with darker structures, many electron-dense and some less dense vesicles (Fig. 4D'). The nucleus has a very prominent nucleolus, as the other germ cells. In the center of the body, there is an indent into the side of the gonadal compartment (Fig. 4A, E). It is more distinctly visible than in the male (compare to Fig. 3A). Ultrastructural images show a complete deformation of the epidermis and a loss of the single-layered character (Fig. 4E'). The cells have different shapes and some single germ cells intrude into the epidermis. This indent is clearly visible in light microscopy as well and was documented in other specimens too (Fig. 4A).

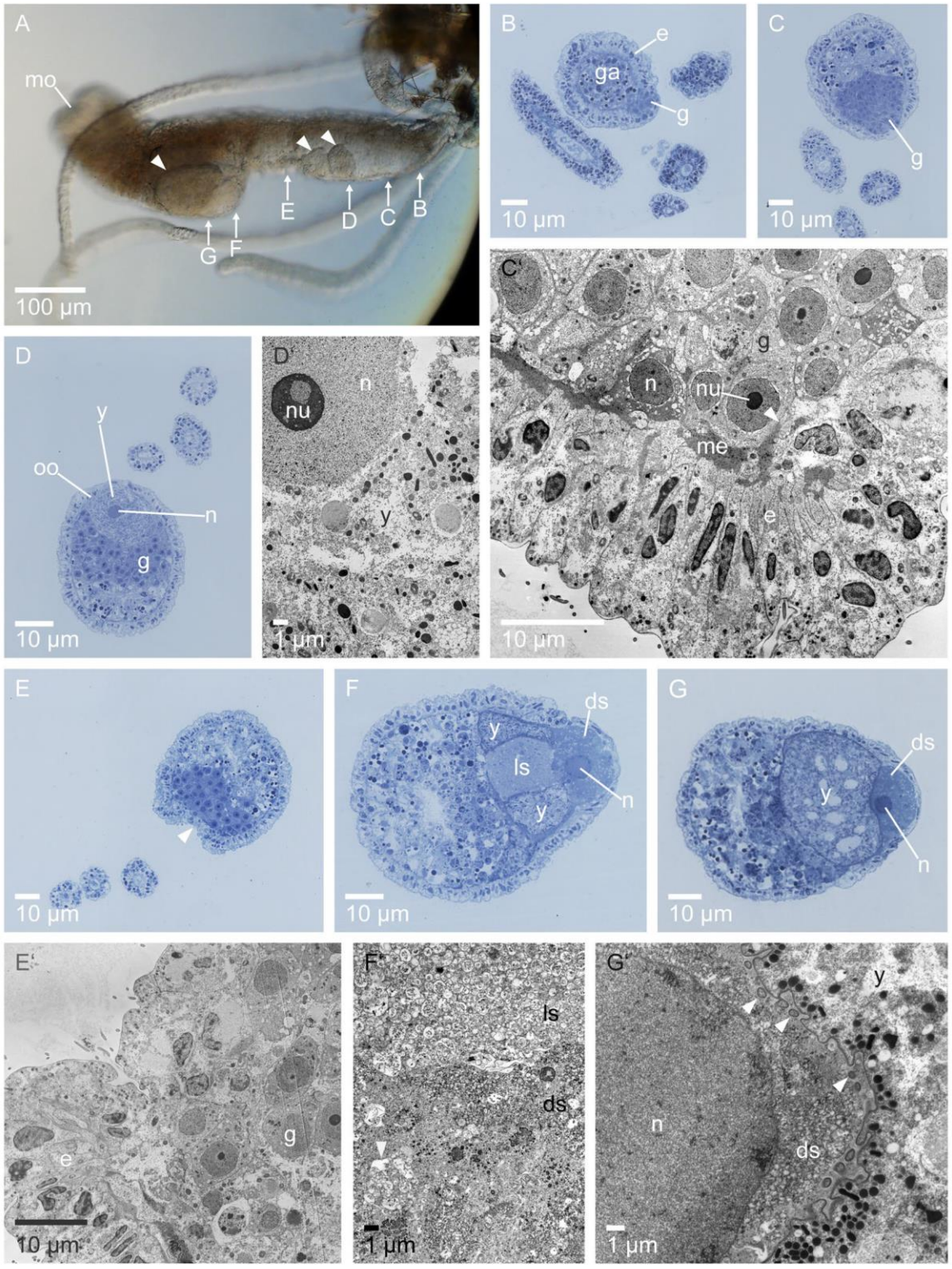
The third and largest oocyte consists of three substructures and the nucleus. Aborally, the oocyte has the yolk wrapping around an electron-lucent structure (here called lighter structure), which is connected to an electron-dense structure (here called darker structure) on one side. The darker structure encloses the nucleus (Fig. 4F). There is no clear separation visible between the lighter and the darker structure (Fig. 4F'). The lighter structure is completely filled with vesicles and smooth endoplasmic reticulum. In contrast, the darker structure consists of many granules and occasional groups of vesicles and smooth endoplasmic reticulum. Orally, the lighter structure vanishes and the yolk takes up most of the space (Fig. 4G). It is separated from the darker structure and all other cells by a plasma membrane, which also produces extensions reaching into the darker structure (Fig. 4G'). The darker structure with the nucleus does not proceed in the oral direction. There was no nucleus found in the yolk. This third oocyte takes up almost the entire gonadal compartment. There are only a few undeveloped germ cells left on the gastrodermal side.

The aboral cone

Statocysts and tentacles

The transition from the gastric tube into the neck is gradual, so there is no clear definition of the start of the neck. Only the decrease in diameter indicates the beginning of the neck region (Fig. 5A), which then transitions into the aboral cone. The aboral cone starts with the connection of four statocysts alternating with the subaboral tentacles (Fig. 5C). Statocysts are connected via a thin stalk of epidermal cells (Fig. 5E). The statocysts themselves consist of a few epidermal cells and a vacuole (Fig. 5E, F). There was no statolith visible, as well as, no destruction of tissue was documented in the semi- or ultra-thin sections. A dark line, likely mesoglea, runs within the connection of the statocysts to the aboral cone, but it is only visible in the semi-thin section (Fig. 5E). Next to the connection to the aboral cone, cilia with a strongly developed rootlet are located (Fig. 5F). In the living animal, the statocysts show a pulsating movement.

Aboral tentacles insert alternating to subaboral ones and thus have the same orientation as the statocysts. All tentacles are solid, made up of epidermis surrounding a chordoid rod of inner cells. Both layers are separated by a thin and uniform mesoglea. The central cells are



voluminous and elongated, per cross section the rod is composed of only one cell (Fig. 5G). These cells have almost no inclusions, but contain a single vacuole, displacing the cytoplasm to the outer edge and around the nucleus (Fig. 5G), which is evenly electron-lucent colored and has a prominent nucleolus. It is surrounded by cisternae of rough endoplasmic reticulum and a few smaller vesicles. In the periphery, close to the mesoglea, few myofibrils with circular orientation are present. Nuclei of the epidermal cells are located in the center. Tentacles are covered with cilia.

◂**Fig. 4** Female gonadal compartment of *Halammohydra vermiformis* with oocytes from aboral to oral. **A** Light-microscopy image of the female gastric tube with mouth opening (mo). Arrowheads indicate three oocytes in the gonadal compartment. Arrows indicate the position of the sections shown in **B–G**. **B–G** Semi-thin sections of the gastric tube. **C'–G'** Ultrastructural images according to **C–G**. **B** Aboral part of the gastric tube with epidermis (e), gastrodermis (ga) and a thickening due to the gonadal compartment (g), (**C**) which increases in size in oral direction. **C'** As in the male, the mesoglea (me) ends abruptly (arrowhead), but the germ cells are closely packed with a big uniform nucleus (n) and prominent nucleolus (nu). **D** Both aborally located, smaller oocytes (oo) have the same structure of a prominent nucleus (n) surrounded by yolk (y). **D'** The yolk is filled completely with different types of vesicles. **E** In the center of the gastric tube, there is a strong indentation (arrowhead). **E'** In this area, the epidermis loses its well-ordered character and there is no clean separation of epidermal (e) and germ cells (g). **F** The orally located oocyte has three different areas and a nucleus. The yolk (y) is enclosing a lighter structure (ls) orally, which is connected to a darker structure (ds) surrounding the nucleus (n). **F'** Magnification of the lighter and darker structure with groups of smooth endoplasmic reticulum (arrowhead, as in the lighter structure). There is no membrane separating both structures visible. **G** In the oral direction, the lighter structure disappears. **G'** An uneven membrane with extensions (arrowheads) separates the yolk from other structures

Muscle and nerve ring

Longitudinally oriented myofibrils are located in the basal part of the epidermis. In the gastric tube, the area filled by myofibrils is thin orally (Fig. 2B) and increases in the aboral direction until they fill almost all basal cell sections (Fig. 2D), which continues and intensifies in the aboral cone (Fig. 5A–D) and results in a pronounced ring structure. In the orally directed part of the aboral cone, the emerging ring is uneven and has a thickness of about 5–12 µm. The wide apical cells displace the slender basal cells, which have a flame-like shape (Fig. 2E). This continues aborally until the slender cells almost dominate the epithelium (Fig. 5B). The ring becomes more pronounced and even in thickness until it reaches its maximum of 10.5 to 13.4 µm when the gastrodermis has its minimum of 10.5 µm and subaboral tentacles connect to the aboral cone (Fig. 5C, D). Throughout this process, the layer of mesoglea thickens slightly and gets more irregular. Some parts of it extend in between the basal epidermal cells and there are strong cell connections visible as electron-dense spots (Fig. 5B). Aborally, the muscle ring regresses and the basal cells containing muscle fibers get wider (Fig. 6A, B). The layer of mesoglea evens out.

This is the area where the nerve ring emerges (Fig. 6A–D). Several small cell sections accumulate circular around the regressing muscle ring and the gastrodermis. These cell sections are neurites and vary in size and shape and show no nucleus. Throughout this ring, there are several small, electron-dense structures, which are dense cored synapses (Fig. 6D). These round vesicles contain an electron-dense spot and are distributed in the entire ring with varying densities. Even in the gastrodermis, a cluster of these structures was documented (Fig. 6C). Throughout the body, no such accumulation was noticed.

When the nerve ring regresses, the myofibrils emerge again but enclose the inner tentacle tissue, which is now in contact with the gastrodermis (Fig. 6E). A layer of mesoglea separates both tissue types. Occasionally, the core cells of adjacent tentacles have contact with each other (Fig. 6F) and with the gastrodermis (Fig. 6G) by very small connection sites. Surrounding myofibrils run into the tentacles (Fig. 7A) and the concentration decreases around the adhesive organ (Fig. 7B).

Adhesive organ

The adhesive organ begins in the center of the aboral cone, where the inner tentacle tissue of both whorls connects to the gastrodermis (Fig. 7A). Orally it is diamond-shaped because of the contact with the inner tentacle tissues and aborally it is round with 20 µm in diameter (Fig. 7B). It consists of a few cells with poorly visible cell borders. The cells are mainly filled with electron-dense round vesicles with smooth surfaces. In the center of the adhesive organ is a lumen (Fig. 7A–C), which increases in diameter in the aboral direction. The lumen is completely filled with cilia, which insert into the cells apically and stick out of the aboral pore creating the aboral cilia tuft (Fig. 7C). The orally directed part of the adhesive organ is enclosed with mesoglea, which is not visible in the aborally directed part anymore. Instead, there is a structure of plasma membranes creating a pronounced boarder separating cells of the adhesive organ from other epidermal cells around it (Fig. 7D). Some myofibrils are visible as lines around the adhesive organ.

Cnidome

Throughout the entire body, different densities of nematocysts are visible. All nematocysts and developmental stages were found in the epidermis and none in the gastrodermis. Additionally, three specialized sections of developing nematocysts were documented. The first and smallest is located on the gastric tube in the oral direction. This location contains only a low density of developing nematocysts. The second location on the gastric tube is in the aboral direction, close to the transition into the neck on the gastrodermal side. Here, the epidermis is noticeably thicker than in the rest of the gastric tube (Fig. 2D). The third and largest location is at the basal part of the tentacles (Fig. 8A). In this region, the density of developing nematocysts is highest and there is a slight thickening of the epidermis compared to other locations in the tentacle, but this thickening does not result in a prominent bulb at the base when observed in light microscopy (Fig. 1A–D).

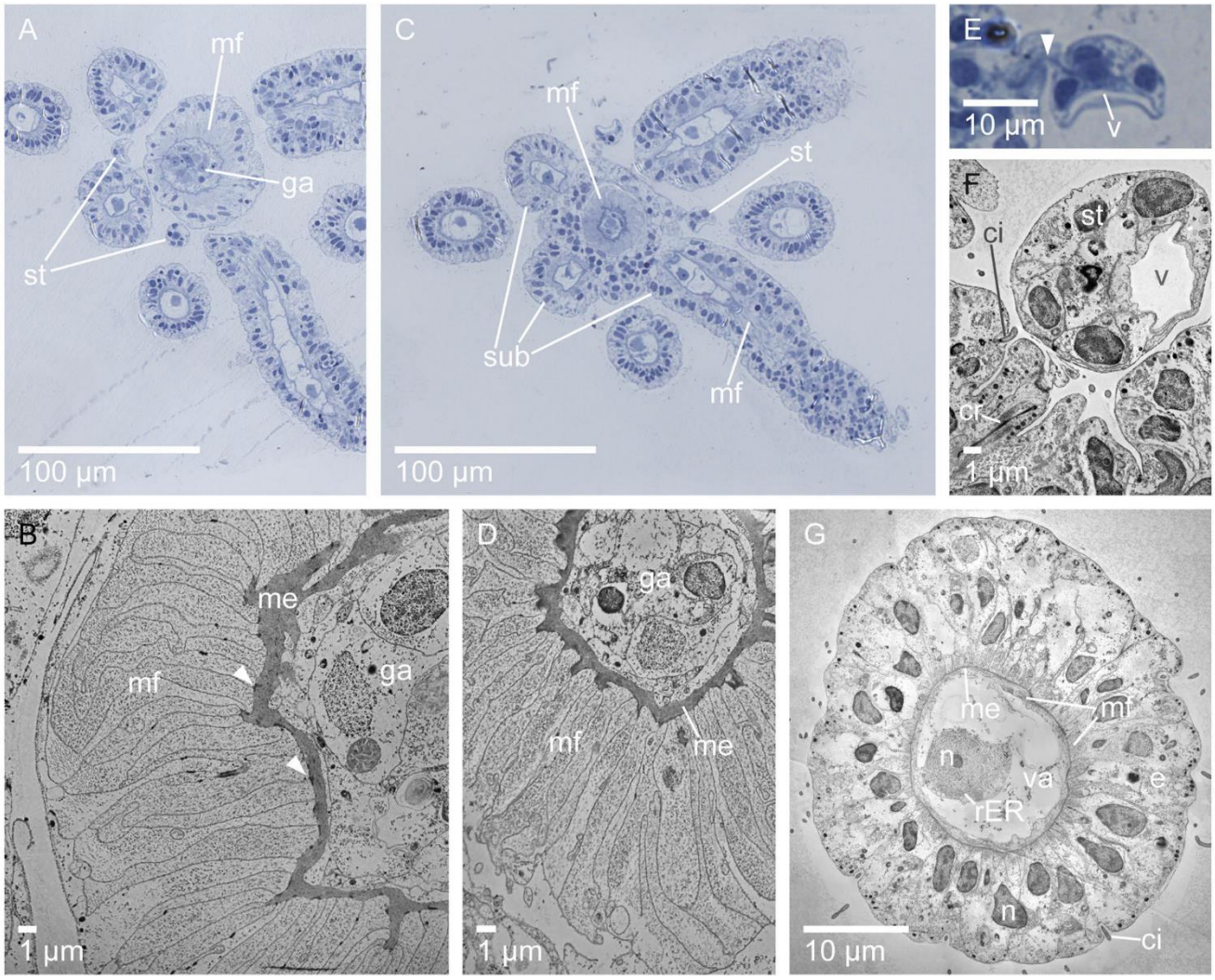


Fig. 5 Semi-thin section (**A**, **C**, **E**) and ultrastructural images (**B**, **D**, **F**, **G**) of the orally directed part of the aboral cone, statocysts and tentacle of *Halammohydra vermiformis*. **A** At the transition of the neck into the aboral cone the gastrodermis (ga) has a closed lumen and myofibrils (mf) create a light ring structure. First statocysts (st) are visible. **B** Ultrastructure of **A** showing the ring structure of the myofibrils (mf), the gastrodermis (ga) and the irregular-shaped mesoglea (me) with several electron-dense spots (arrowheads). **C** Orally directed part of the aboral cone with a strong ring of myofibrils (mf) surrounding the gastrodermis; statocysts (st) and subaboral tentacles (sub) connecting to the cone. **D** Ultrastructure of **C** showing a strong muscle ring. **E** Statocysts are connected to the cone via a thin link of epidermal cells. There is a dark line running through the connection (arrowhead). The position of the statolith is indicated by an empty vacuole (v). **F** Ultrastructure of a statocyst (st) with its vacuole (v), a cilium (ci) and cilia rootlet (cr) at its connection to the cone. **G** Transverse section of a tentacle with an outer epidermis (e) with central nuclei (n) and basal myofibrils (mf) and an inner tentacle tissue filled almost completely with a vacuole (va). At the outer edge, myofibrils (mf) are visible and in the center, a nucleus (n) surrounded by a rough endoplasmic reticulum (rER) is located. Tentacles are covered in cilia (ci)

Different developmental stages occur next to each other in these regions. Early stages of the nematoblast, the cell developing into the nematocyst, contains a voluminous nucleus with uncondensed chromatin and a prominent nucleolus, a well-developed rough endoplasmic reticulum and the developing nematocyst (Fig. 8B). The nematocyst has an electron-lucent capsular wall and a dense matrix. Throughout the cytoplasm of the cell, sections of the external tube coiling are located (Fig. 8B, C). In a longitudinal section, the division in the capsular region, determined by the capsular wall, and the external tube is clearly visible (Fig. 8C). In a later stage, there is no external tube, the composition of the matrix is granular and the prominent shaft of the heteroneme is visible (Fig. 8D). The nematocyst now fills the entire cell but the capsular wall is still wide and the nucleus has a crescent shape, touching the capsule (Fig. 8E).

One of the most abundant types of nematocysts in this individual is the heteroneme (Fig. 8E–H). It occurs on the entire body, but with different densities. On the gastric tube is a minor occurrence, except for the two regions of

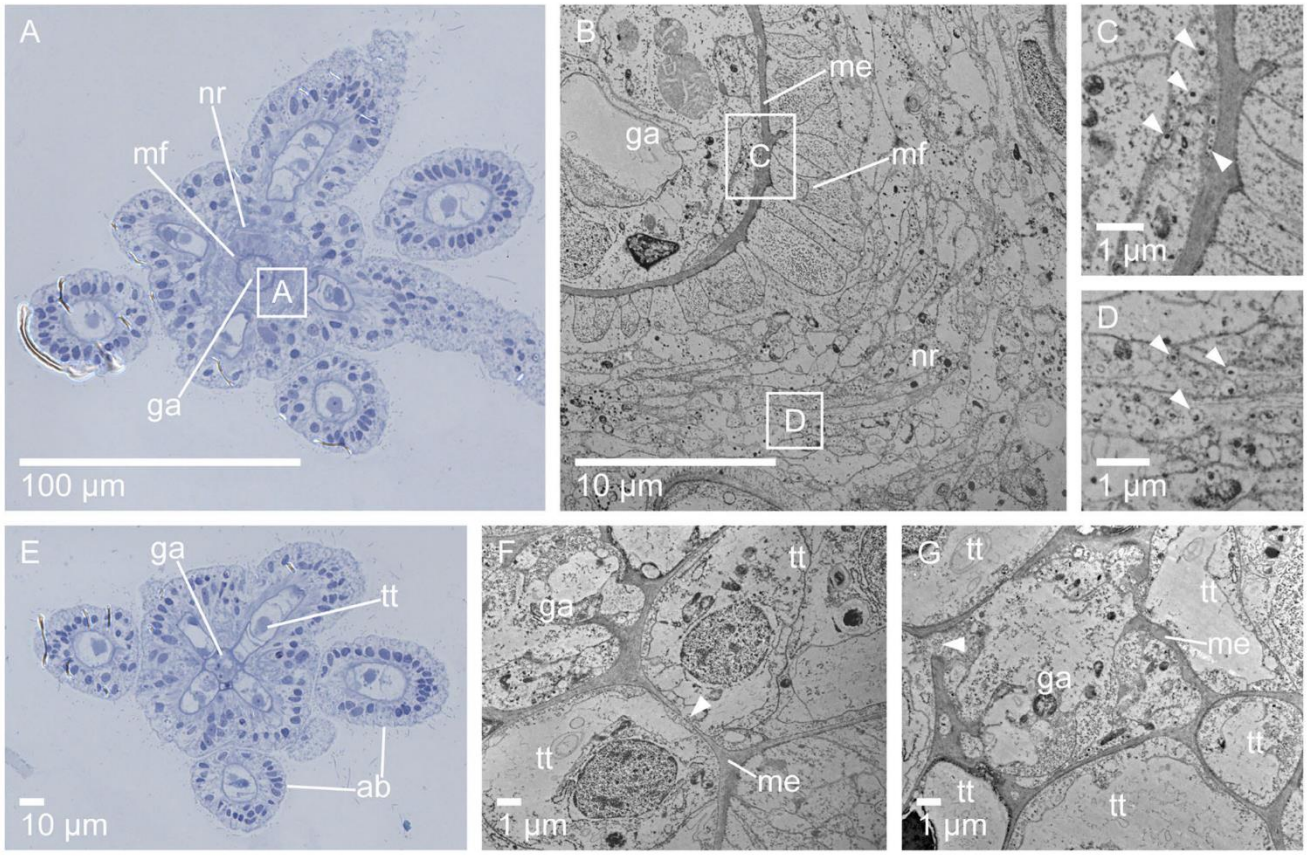


Fig. 6 Semi-thin section (**A**, **E**) and ultrastructural images (**B-D**, **F**, **G**) of the central part of the aboral cone of *Halammohydra vermiformis*. **A** In the center of the aboral cone, the ring of myofibrils (mf) around the gastrodermis (ga) regresses and the nerve ring (nr) emerges. **B** Ultrastructure of **A**. The mesoglea (me) is less irregular and the nerve ring (nr) consists of several small cells. **C**, **D** Magnifications of **B** showing dense cored synapses (**C**) in the gastrodermis (arrowheads) and (**D**) in the nerve ring (arrowheads). **E** Aborally, the inner tentacle tissues (tt) of the subaboral tentacles connects to the gastrodermis (ga). Aboral tentacles (ab) are not connected to the cone here. **F**, **G** Gastrodermis (ga) and inner tentacle tissues (tt) are separated by mesoglea (me) but there are connections between (**F**) the inner tentacle tissues (arrowheads) and (**G**) between tentacle tissue and gastrodermis (arrowhead)

development. On the aboral cone is a higher abundance. Most of the heteronemes are on the tentacles. Here, they occur in higher density but without a specific pattern or concentration of some cysts. Heteronemes have an oval shape with a round basal and a slightly tapering apical part. An operculum is located apically and directed to the outer environment. Its prominent shaft with spines and stylets easily recognizes this type of nematocyst. Even in an immature stage, it is visible (Fig. 8D, E). The shaft itself is enclosed in the inverted sac of the capsular membrane. There are three stylets connected to the shaft. In a cross section of the apical part, the three stylets are visibly enclosed by a folded inverted capsular membrane (Fig. 8H). When the nematocyst is cut in the basal region, no stylets are visible, but a striated pattern on three bulges connected to the inverted capsular membrane (Fig. 8G). The tube itself coils in the capsule and has a three-bladed shape in cross section (Fig. 8E). Besides heteronemes, there is a second type of nematocysts. It has a lower abundance and was only documented on the tentacles (Fig. 8I–K). There is no shaft but a thick tubule enclosed in the capsule. A ring of microtubules, visible as electron-dense circles, surrounds the nematocyst itself (Fig. 8J). In some cells, only a group of microtubules is visible, indicating the capsule in a different section of the cell (Fig. 8K).

Discussion

Halammohydra vermiformis is the most suitable species within its genus to investigate the internal structure due to its high abundance and the low number of tentacles since a higher number might obscure the overall picture. The combination of ultrathin and semi-thin sections is ideal to section specimens in a reasonable time, but it includes the danger to miss ultrastructural data when a structure is accidentally present only in semi-thin sections. In our case, this happened for e.g. the connection of the statocysts to the aboral cone. All observed statocysts lacked a statolith, but as there

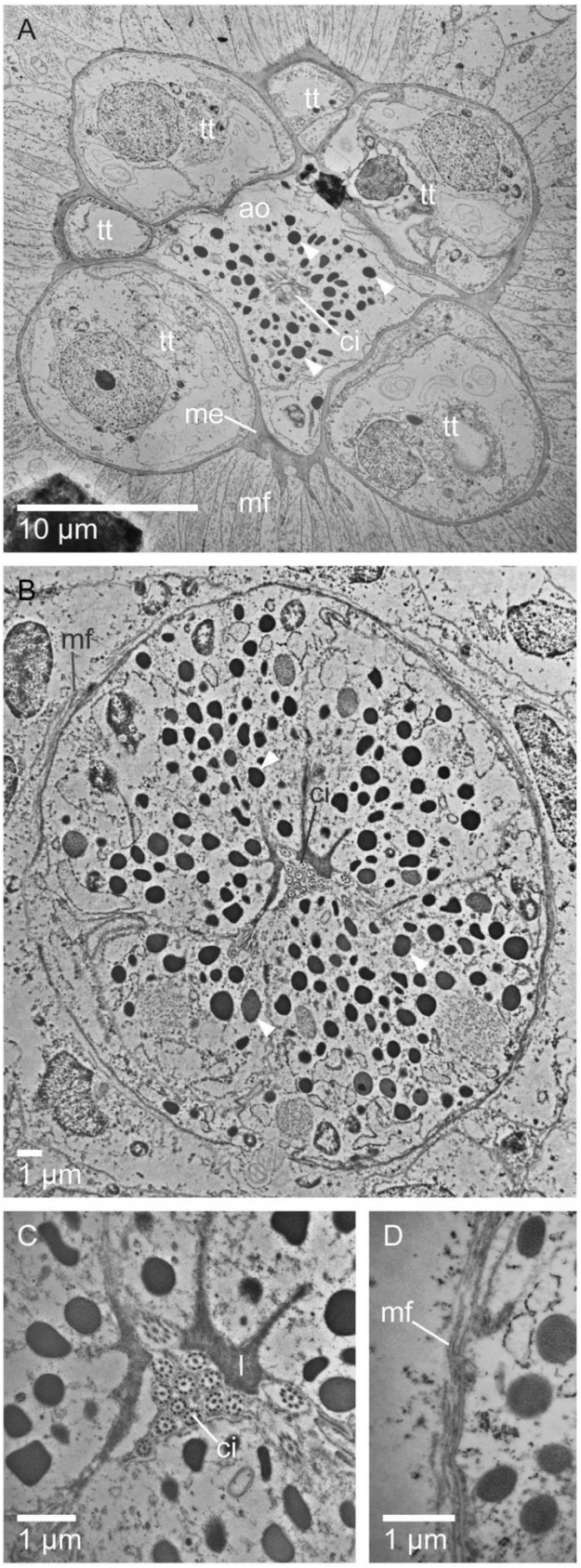


Fig. 7 Ultrastructural images of the adhesive organ (ao) of *Halammohydra vermiformis* from oral to aboral. **A** Orally, the inner tentacle tissues (tt) deform the shape of the adhesive organ (ao). It is filled with electron-dense vesicles (arrowheads). In the center a small lumen with cilia (ci) is visible. All tissues are separated by mesoglea (me) and surrounded by myofibrils (mf). **B** Aborally, the adhesive organ is round in shape and completely filled with electron-dense vesicles (arrowheads). The lumen increases and more cilia (ci) are visible. There is no mesoglea surrounding the structure but a thin layer of myofibrils (mf). **C** Magnification of the lumen (l) filled with cilia (ci). **D** Magnification of the edge with myofibrils (mf)

was no obvious destruction in the tissue of this area, we assume that the statoliths dissolved during the preparation. Additionally, it was difficult to investigate the tentacles as a whole, because they spread out too far to get sections along their entire length. Therefore, structural changes, e.g. densities of nematocysts, could not be followed reliably along the tentacle length.

Hörnum on Sylt is a reliable location for finding specimens of *Halammohydra*. Due to the difficulty to identify species in this genus in general, the exact species composition at this location is not known with certainty. Previously, specimens found in Hörnum were identified as *H. octopodides* Remane, 1927 (Polte and Schmidt-Rhaesa 2011), but our investigations of the morphology identified specimens from this location clearly as *H. vermiformis*.

In general, *H. vermiformis* has the typical structure of a cnidarian, with the outer epidermis, inner gastrodermis and a layer of mesoglea separating them. Cells of the epidermis have cilia, which are used for gliding between the sand grains. This is a clear adaptation to the interstitial system (Giere 2009), as pelagic medusae do not use them for locomotion (Werner 1964). The surfaces of the epidermis are covered by an irregular-shaped electron-dense layer, which is likely the glycocalyx. The chordoid structure of the inner tentacle tissue with voluminous vacuoles is very common among Hydrozoa and functions as a hydrostatic skeleton (Thomas and Edwards 1991). Together with the mesoglea and the musculature formed by epitheliomuscular cells (EMCs) in the tentacle, the hydrostatic skeleton is crucial for the structural integrity and tentacle movement. The mesoglea is mostly thin throughout the body, except for the location of the muscle ring in the aboral neck. It is irregular shaped, due to the tension of the surrounded muscles and its needs to be thicker for the myofibrils to anchor (Haynes et al. 1968), as this is a highly flexible part. Myofibrils of the neck insert into the tentacles and enable a high movability of them.

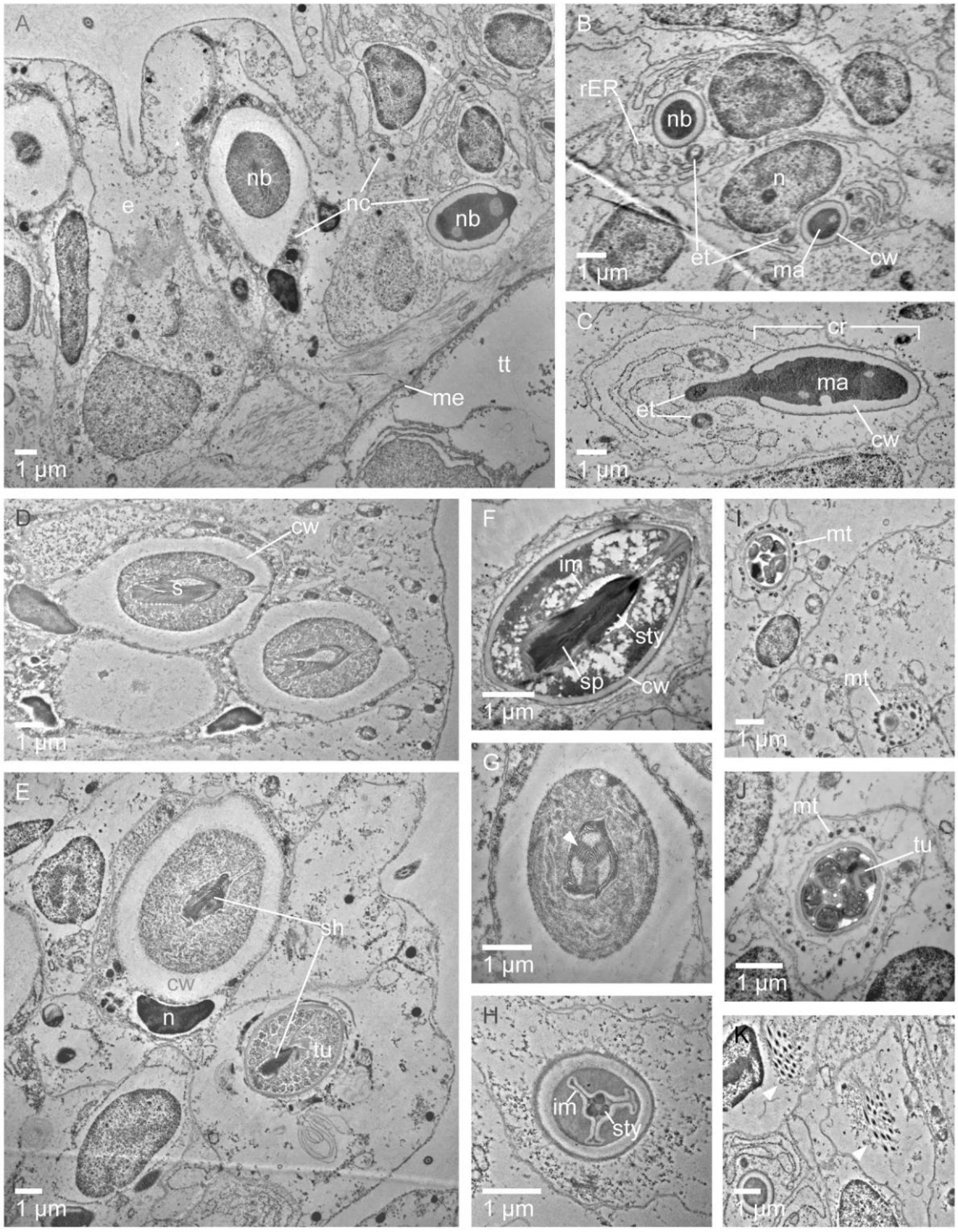
Next to the muscle ring, there is a prominent nerve ring in the aboral cone. This was already shown with immunohistochemical stainings by Polte and Schmidt-Rhaesa (2011) in *H. octopodides*. Apart from this dominant nerve ring, other neural structures documented by Polte and Schmidt-Rhaesa

(2011) such as tentacular neurites, the mouth cone plexus or the oral nerve ring were not observed in this study, mainly because it is impossible with a combined ultrathin/ semithin sectioning to reliably follow the course of fine neurites. A connection of the nerve ring to the statocysts was not observed as well, but since this is a sensory organ, we expect a connection. When the medusa changes its position, the free statocysts move and come in contact with the surrounded sensory cilia to detect the movement (Singla 1975).

A higher concentration of neurites and myofibrils indicates controllability and thus a better ability to move and change shape. This was documented for the mouth opening. It moves in circular motions and can also be stretched greatly for food intake of bigger prey. Moreover, the long cilia of the gastrodermis might help to transport the food into the mouth for further digestion. There are different cell types of the gastrodermis involved in this process, which were already described by Remane (1927) and categorized as cell types a to h. Depending on the different concentrations of the respective cell types, he defined five zones throughout the body. In this study, not all of the described cell types were observed. At the mouth opening, two cell types are present, which might represent zone I (cell type a and b) of Remane's description. The lighter cell type might be mucus cells, which release mucus for protection and the darker cell type might be secretive cells, which initiate the extracellular digestion of food (Thomas and Edwards 1991). This cannot be said with certainty, as this is no histochemical study, but the position of these cells and the unusual placement of the cilia close to the cell surface support this assumption. The second zone of Remane's classification was not documented in this study, but cell types of the third zone are present. One of the major parts of the gastrodermis is the typical cnidarian digestive EMC (Remane 1927; Thomas and Edwards 1991). Scattered in between are the characteristic gastric zymogen or gland cells (Haynes et al. 1968; Remane 1927). They release enzymes into the gastric lumen and initiate extracellular digestion. Predigested food particles can then be ingested into the digestive EMC for further digestion (Haynes et al. 1968). As a result of ingested food, the gastrodermis gains coloration, which is visible even in light microscopy. In the neck, the resorption of food particles reduces or stops due to the small or absent lumen. Furthermore, a different shape and orientation of the cells occurs because of the reduction of the diameter and the loss of the lumen. This corresponds to Remane's zone IV (cell type g, Remane 1927). Remane also described a fifth zone (cell type h), which is actually the aboral adhesive organ. It does not belong to the gastrodermis but is a result of an invagination of the ectoderm early in development (Swedmark and Teissier 1957). The gastrodermis itself persists as a chordoid rod into the tentacles.

Next to the gastrodermis, the gonadal compartment is located in the gastric tube as well. There is only one study regarding the male reproductive system (Ehlers 1993) and none for the female one. The exact position of the gonadal compartment was described before as being between the epidermis and the gastrodermis of the gastric tube (Remane 1927; Swedmark and Teissier 1966), until Ehlers (1993) regarded it to be part of the gastrodermis. The same is assumed here, because mesoglea surrounds both, the gastrodermis and the gonadal compartment, except for the gap, where no mesoglea could be detected. It was described before that the spermatozoa and oocytes are released into the water via the gastrodermis and through the mouth opening (Clausen 1971; Ehlers 1993; Swedmark and Teissier 1966). This study showed a slight indent on the side of the gonadal compartment of the male, which is very pronounced in the female. It is likely that the spermatozoans and oocytes are not released through the gastric system but by a rupture of the epidermis. This would explain the disruption of the single-cell epithelium in the epidermis at this location. Spawning through a rupture of the epidermis is also very common in Hydrozoa (Thomas and Edwards 1991). As a meiofaunal animal, it is essential to have an effective reproduction process. Many meiofaunal organisms have an internal fertilization or stick the oocytes to a sand grain (Giere 2009), which was described for *Halammohydra* (Swedmark and Teissier 1957). It stays attached until the end of the embryonal development. Since observations of fertilization are missing, it remains unclear how this is coordinated.

Structures of the male gonadal compartment and of the spermatozoa are similar to the ones described by Ehlers (1993), with the exception of the acrosome and cilia, as they were not observed directly here. Only the groups of cilia between the germ cells show their presence. The female gonadal compartment of *Halammohydra* has not been described so far. It is possible that there is a stratification of maturation from aboral (youngest) to oral (mature) because of the different positions of the germ cells, immature oocytes and the mature oocyte. The orally located oocyte has a more complex structure than the other two. There are two distinct regions, the lighter and darker structure with nucleus, and the yolk portion. Both are separated by what seems to be a membrane, which appears to be quite unusual in this position. A composition of the mature egg by the oocyte and a separate yolk cell seems unlikely, as the yolk compartment does not include a nucleus and because such a separation is not present in earlier stages of oogenesis. Therefore, the function of this intra-oocyte membrane must remain unexplained. Similar structures have not been reported, to our knowledge (see e.g. Beams and Kessel 1983; Tardent 1984). The prominent indent in the center of the female body might be a leftover of a recent rupture of the epidermis after a release of an oocyte. However, if oocytes are arranged in a



maturity gradient, the position of the indent would indicate that not the most mature oocyte, but the second one was released. This needs further investigation to clarify if this is a typical process or just a random finding.

In the studied individual, the adhesive organ consists of large cells with many secretory vesicles and a central lumen filled with cilia. The temporary adhesion process might be a combination of the production of secretion and action of the myofibrils surrounding the structure. Central cells produce an adhesive secretion and the cilia transport it to the tip of the aboral cone. Additionally, the adhesive organ might have a slight sucker effect for temporary adhesion. Surrounding myofibrils can help to release the individual by contraction. As *H. vermiformis* is mostly seen freely swimming and less

◂**Fig. 8** **A–D** Different developmental stages of nematocysts in *Halammohydra vermiformis*. **A** Longitudinal section of a tentacle within an area of nematocyst development showing the epidermis (e) with nematocysts (nc) in nematoblasts (nb), mesoglea (me) and inner tentacles tissue (tt). **B** Cross section of two nematoblasts with developing nematocysts (nc). The nematocysts have a wide electron-lucent capsular wall (cw) and a dense core matrix (ma). The cell itself contains a large nucleus (n) with the nucleolus, is filled with a prominent rough endoplasmic reticulum (rER) and sections of the external tubes (et) are visible. **C.** Longitudinal section of a nematoblast with developing elongated nematocysts. It is differentiated in a capsular region (cr) and an external tube (et). **D** Developmental stage before maturity with visible spines (sp) and a thick capsular wall (cw). **E–H** Ultrastructural images of heteronemes. **E** Section of a developing and mature heteroneme. In the developing nematocyst, the capsule wall (cw) is still wide and the crescent-shaped nucleus (n) touches the capsule. The shaft (sh) is visible in the center of the nematocysts. In the mature heteroneme, the three-bladed structure of the tubule (tu) is visible. **F** Longitudinal section of a mature heteroneme with spines (sp) and stylets (sty) on the shaft, the inverted capsule membrane (im) and the outer capsule wall (cw). **G** Basal cross section of a developing heteroneme showing a striated pattern on the inverted capsule membrane (arrowhead). **H** Apical cross section of a heteroneme with a folded inverted capsule membrane (im) enclosing the three stylets (sty). **I–K** Second type of nematocyst. **I** Overview of the capsule with a ring of microtubules (mt) surrounding the structure. **J** Magnification of the nematocyst with a thick tubule (tu) and microtubules (mt). **K** In some parts of the epidermis, only the groups of microtubules (arrowheads) are visible

sedentary compared to other species, the adhesive organ might differ from more adhesive species, like *H. schulzei* Remane, 1927, in terms of thickness of the myofibril layer or the overall size and shape of the adhesive organ. In this study's individual, the adhesive organ was conical shaped. Other species have cup- (*H. octopodides, H. intermedia* Clausen, 1967, *H. adherens* Swedmark & Teissier, 1967) or even pear shaped (*H. schulzei*) adhesive organs, which reach deeper into the aboral cone and thus have a thicker layer of secretory cells (Clausen 1967). Some authors described the adhesive organ as part of the gastrodermis (Remane 1927) or with a cup of gastrodermal cells and mesoglea around it (Swedmark and Teissier 1966). This could not be verified here, because we did not observe a connection of the gastrodermis and the cells of the adhesive organ. Moreover, a layer of mesoglea surrounding the structure was only documented on the most oral part. Compared to other species, the adhesive organ of *H. vermiformis* is small and less sunken in the aboral cone. In *H. schulzei* and *H. octopodides*, for example, the adhesive organ is rather deep and therefore the connection with the mesoglea and gastrodermal cells creating a cup is possible. Remane (1927) documented this connection but interpreted it as part of the gastrodermis. These examples show the importance of investigations into the adhesive organ of additional species, to find functional and thus potential species-specific differences.

Halammohydra vermiformis was described to have two types of nematocysts: stenoteles and isorhizas (Clausen 1967; Swedmark and Teissier 1957). Stenoteles were found on the entire body, whereas isorhizas are only present on the tentacles (Clausen 1967). This distribution could more or less be confirmed in this study. Heteronemes were seen on the entire surface of the animal, with increased concentration in certain regions, like the developing areas for nematocysts or on the tentacles. Since only two types of nematocysts were described for this species, the observed heteronemes are potential stenoteles. The second type present was only documented on the tentacles, but the exact type of nematocysts could not be revealed with certainty. The lack of a shaft is a character of a haploneme type (Östman 2000) and the ring of supportive rods was documented in a study about the nematocysts in *H. intermedia* before (Clausen 1991). Further identification cannot be done using our images, but it is very likely, that these nematocysts are isorhizas, as they have been documented for this species.

Within the cnidarian tree, *Halammohydra* was placed into Hydrozoa and Trachylinae very early for obvious reasons (Remane 1927; Swedmark and Teissier 1957): The absence of a polyp stage and the structure of the statocysts are clear characters of Trachylinae. A further placement was problematic for a long time, but due to possession of stenoteles, direct development and placement of the gonad (here referred to as gonadal compartment) on the manubrium (here referred to as gastric tube) favored the relation close to Trachymedusae and Narcomedusae (Bouillon and Boero 2000a; Clausen 1967; Marques and Collins 2004; Remane 1927). A molecular study by Collins et al. (2008) confirmed these suggested relations and revealed a possible origin of *Halammohydra* within Rhopalonematidae (Trachymedusae). Only the structure of the statocysts supports this origin (Bouillon and Boero 2000b; Bouillon et al. 2006). The position of the gonadal compartment differs from the typical Hydrozoa, where it is located in the epidermis. Here it is located in the gastrodermis but has a connection to the epidermis through a gap in the mesoglea. This intermediate stage is unusual. The position in the gastrodermis is uncommon but was documented for other hydrozoan species as well (Bouillon et al. 2004).

With the discovery of *Otohydra*, another meiofaunal cnidarian, the order Actinulida was created for *Otohydra* and *Halammohydra* because of the fully ciliated body, the same type of statocysts and the lack of a planula stage (Swedmark and Teissier 1958, 1966). Despite these similarities, there are great differences as well. Most prominent is the lack of an adhesive organ and the presence of an umbrella in *Otohydra*. Additionally, the genus only has one whorl of tentacles and is hermaphroditic (Clausen 1971). There are no ultrastructural or molecular data available on *Otohydra*, so this has to be examined further to verify the relation to *Halammohydra* and the existence of the order Actinulida.

In summary, the ultrastructural investigation of *H. vermiformis* helped to understand the general organization of this genus and filled some gaps, especially concerning the gonadal compartment. Further studies should be done on the adhesive organ with different species because this might reveal the exact process of adhering and help to better correlate morphology to behavioral differences and thus environmental preferences. Additionally, an ultrastructural and molecular study of *Otohydra* is needed to finally answer the questions regarding the composition and arrangement of genera within the family Actinulida and their placement in the Cnidarian tree.

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Declarations

Conflict of interest This study was funded by Deutsche Forschungsgemeinschaft (DFG) with the grant number SCHM 1278/18-1 and conducted at the University of Hamburg and the Leibniz Institute for the Analysis of Biodiversity Change (LIB) in Hamburg (former CeNak) as part of a PhD project. No approval from research ethics committees was required to accomplish the goals of this study since experimental work was conducted on an unregulated invertebrate species.

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Comparative molecular and morphological species delineation of *Halammohydra* Remanae, 1927 (Hydrozoa) – with description of four new species.

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Abstract

Whereas most Cnidarians are macrofaunal, a few microscopic lineages have evolved and some of them inhabit marine sediments. The meiofaunal genus with the most species is *Halammohydra*, with nine described species. Species are described with high intraspecific variability in e.g., number of tentacles and statocysts and the shape and length of tentacles and body, complicating morphological identification to species level. Additionally, there is not much molecular data available. This study aims to revise already described species with morphological and molecular methods, as well as, to delineate potential new species answering questions about their geographical distribution. For this, specimens were sampled at 16 locations in the Northwest Atlantic and two localities in the East Atlantic, documented with light microscopy and fixed individually for sequencing (16S, 18S and CO1). Herewith morphological characters were linked to a specific sequence, enabling testing of character variation within one molecular phylogenetic group. Phylogenetic analyses were conducted (Bayesian Interference and Maximum Likelihood) in combination with species delimitation tests (ABGD, GMYC and bPTP). Four already described species were identified in the data sets, and all of these were found at multiple localities. Four new species are described. Overall, the combined molecular and morphological data acquisition revealed multiple new species and a high degree of sympatry in *Halammohydra*. This, together with the confirmed excessive intraspecific variation in morphological traits, underlines the necessity of molecular sequencing for taxonomy and species identification of *Halammohydra*.

Keywords

integrative taxonomy, species delimitation, meiofauna, sanger sequencing, Cnidaria, *Halammohydra*

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Introduction

Meiofauna is a highly diverse assemblage of animals across several phyla inhabiting sediments from the intertidal zone to the deep-sea, sharing a microscopic body size (Giere, 2009). Other features, such as elongated body shape, reduction of long appendages or the presence of special adhesive structures help these animals to move between the sand grains in the interstitial environment and even withstand stronger currents. Because of their small body size and general lack of pelagic larvae, the dispersal ability is expected to be rather low (Giere, 2009). Nevertheless, several studies found a broad geographical range for some species, up to a cosmopolitan distribution (e.g., Hagerman & Rieger, 1981; Schmidt & Westheide, 2000; Boeckner et al., 2009; Bik et al., 2010; Faurby et al., 2011; Guil, 2011; Cerca et al., 2018; Fontaneto, 2019; Worsaae et al., 2019a). This contradiction is called the "meiofauna paradox", and several hypotheses have been put forward to its explanation (see e.g., Sterrer, 1973; Westheide, 1991; von Soosten et al., 1998; Giere, 2009). Over the last decades, molecular studies discovered a high amount of cryptic diversity in some meiofaunal animals (e.g., Todaro et al., 1996; Fontaneto et al., 2009; Jörger et al., 2012; Leasi et al., 2016; Worsaae et al., 2019b; Tessens et al., 2021), which resulted in a taxonomic revision and the splitting of previously considered cosmopolites into taxonomic units with more restricted ranges. Population genetic methods may not provide the complete solution to the meiofauna paradox but especially in meiofauna, with somewhat limited identification features, it is of major importance (Fontaneto et al., 2015; Cerca et al., 2018). Hence, more species revisions and, or in combination with, molecular data are needed.

Halammohydra Remane, 1927 is a genus of interstitial cnidarians with nine described species, whose morphological identification is notoriously difficult. Most species are described and recorded in Europe, three were described exclusively from India (Schmidt-Rhaesa et al., 2020). The majority of European records is from Germany (Remane, 1927; Schulz, 1952; Clausen, 1967; Schmidt, 1969; Ehlers, 1993; Polte & Schmidt-Rhaesa, 2011) and France (Teissier, 1950; Swedmark & Teissier, 1957a, 1959; Renaud-Debyser, 1964; Swedmark & Teissier, 1967; d'Hondt, 1968). Other locations are Sweden (Dahl, 1953; Boaden, 1960), United Kingdom (Boaden, 1961; Harvey & Wells, 1961; Boaden, 1963; Boaden, 1966; Gray, 1971; Moore, 1979), Norway (Clausen, 1963, 1967, 1991, 2000, 2004), Netherlands (Wolff et al., 1974), Adriatic Sea (Salvini-Plawen, 1991), Azores (Tödter & Schmidt-Rhaesa, 2021) and Spain

(Martínez et al., 2009; Martínez et al., 2019). Outside of Europe, most records are from India (Rao & Ganapati, 1965, 1966; Nagabhushanam, 1972; Salvini-Plawen & Rao, 1973; Rao, 1975, 1978; Rao & Misra, 1980; Rao, 1993; Altaff et al., 2005; Sugumaran et al., 2009; Mohan & Dhivya, 2010; Varadharajan & Soundarapandian, 2013; Janakiraman et al., 2016; Sugumaran & Padmasai, 2019) and only a few from the Western Atlantic and Caribbean Sea (Bush & Zinn, 1970; Calder & Kirkendale, 2005; Hochberg et al., 2014; Jörger et al., 2014; Kånneby et al., 2014; Garraffoni et al., 2017). *Halammohydra* is exclusively reported from interstitial environments of sandy sediments, ranging from fine to coarse sand or shell gravel.

In the Northwest Atlantic, *Halammohydra* has mainly been sampled in the vicinity of marine biological research stations, especially in Helgoland (Germany), Roscoff (France) and Bergen (Norway). Five of the six European species were recorded around the small island of Helgoland, some of them in sympatry. Taken the relative scarcity of records and species, this diversity of species within localites is surprising. Moreover, the general scarcity of diagnostic morphological traits contradicts the sympatric distribution of several species, indicating physiological rather than morphological specializations.

Halammohydra is a modified medusa with a completely reduced umbrella (Remane, 1927). The main body is a gastric tube, or manubrium, with a mouth opening. On the aboral end, the diameter decreases and forms a neck connecting to an aboral cone, the reduced umbrella. One whorl of statocysts and two whorls of tentacles connect to the aboral cone and the aboral tip bears a special adhesive structure, the so-calledadhesive organ. The whole body is ciliated. Due to its unique morphology, *Halammohydra* has been placed in various positions of the cnidarian phylogenetic tree. Remane (1927) placed it in Trachylinae (Hydrozoa) because of the lack of a pelagic phase and the type of statocysts, and further into Narcomedusae because of similarities to larval stages of other species herein. Swedmark and Teissier (1958) described another fully ciliated medusa in a new monotypic genus *Otohydra*. Remarkably, they chose to erect the empty name Otohydridae for this genus, rather than referring it to Halammohydridae (only containing *Halammohydra*), despite their proposed close relationship of these two families in the group Actinulida (Swedmark & Teissier, 1959). The exact placement of *Halammohydra* remained unclear for a long time, until Collins et al. (2008) conducted a molecular study of Trachylinae including sequences of an unidentified species of *Halammohydra*. This study indicated that *Halammohydra* has an origin within the family Rhopalonematidae in Trachymedusae (Trachylinae) rather than within Narcomedusae (Trachylinae).

No further molecular data are available, especially no species-specific sequences. This is problematic, because the identification of species in this genus is quite difficult. Size and shape of the whole body and the aboral cone, the number and length of the tentacles in each whorl, and the shape of the tentacle bases connecting to the aboral cone are the main diagnostic characters. In some cases, the cnidome is used for identification, too. The assessment of these characters is difficult because of their morphological variability, which is exacerbated by the contractibility of the animals. Moreover, some characters, such as the number of tentacles, have a wide range, which overlaps with other species and thus makes the identification of a single specimen challenging. For example, the total number of tentacles in *Halammohydra schulzei* Remane, 1927 was described as 14 to 26 (Remane, 1927; Swedmark, 1957; Swedmark & Teissier, 1957a) and in *Halammohydra octopodides* Remane, 1927 as 12 to 18 (Remane, 1927; Swedmark, 1957; Swedmark & Teissier, 1957a; Clausen, 1963; Renaud-Debyser, 1964; Rao & Ganapati, 1966; Clausen, 1967). In both these species, the morphological variation in characters such as tentacle number or the cnidome appears to be considerable and led to the description of different types (=varieties) (Swedmark, 1957; Clausen, 1963, 1967).

This study aims to clarify the identification and delimitation of *Halammohydra* species by examining the variability of their morphological characters and combining this with molecular sequences. Species delimitation analyses of material from 18 localities revealed four described and four new species, as well as several unidentified species. Since there are no species-specific sequences so far, the data and species delimitation analyses of this study will also provide a base line for future taxonomic studies of *Halammohydra*.

Material & Methods

Field work and extraction

A total of 302 specimens of *Halammohydra* were collected between 2011 and 2021. Sampling efforts focused on European type localities and locations with previously published findings. Additional specimens were found in context of a summer school on the Azores (Jörger et al., 2021; Tödter & Schmidt-Rhaesa, 2021) (Table 1). Most specimens originated from two locations: 79 specimens from the subtidal station of Helgoland next to the Youth Hostel and 71 from "Basse Plate" in Roscoff. Intertidal samples were collected with a shovel and plastic bags or containers, by removing the upper centimeters of the sediment at several positions for investigation. Subtidal samples were collected in similar manner by scuba diving or research vessels using a Van Veen Grab or dredge.

To extract the animals from the sediment, the anesthesia-decantation-method was used (Higgins & Thiel, 1988). Specimens of *Halammohydra* were sorted using a dissecting scope and investigated in detail alive with a compound microscope (Leica DM2500), documenting morphology and behavior with a mounted camera (Sony Handycam and Canon 6D Mark II with AMScope adapter). Specimens were fixed individually in 100 % ethanol to link photographic documentation and sequencing data. For four of the 18 localities (Denmark, France (Arcachon), Cuba, Brazil) detailed microscopic examination were only conducted on fixed material, limiting the amount of morphological information.

The occurrence of *Halammohydra* was highly patchy. Even in the most reliable localities, there were buckets of sediment with no specimens in it. Wherever any specimens were found, usually there were dozens of specimens. This sometimes complicated a detailed investigation, because specimens did not survive for very long after the extraction from the sample. In vivo investigations were needed to document the behavior and all characters, because the animals tended to contract when dying, which made the investigation of detailed characters difficult.

Specimen identity were analyzed at the laboratory of the Leibniz Institute for the Analysis of Biodiversity Change (LIB) using the images for measuring body size and tentacle lengths with Adobe Photoshop. Morphological and behavioral characters with diagnostic potential were analyzed in combination with the molecular data.

Molecular methods

For the DNA extraction, fixed specimens were digested individually in proteinase K (50 µl mixture of 45 µl Tris HCl with pH 7.5 and 5 µl proteinase K, 20 mg/ml) for 24 hours at 50 °C and purified using magnetic beads (AmpliClean). 100 µl of magnetic beads were added to each sample and incubated for 10 minutes at room temperature. DNA in the sample adhered to the beads, which were separated from

the liquid with a magnet. After discarding the liquid, two washing steps with ethanol followed and 20 µl of water was added to resolve the DNA from the beads. The magnetic beads were discarded.

Three genes were amplified, two mitochondrial (16S and CO1) and one nuclear gene (18S), using polymerase chain reaction (PCR) with primers from the literature (Table 2). Thermo-cycler programs were as follows: 94°C/5 min (94°C/50s, 48°C/50s, 72°C/1 min; 35 cycles), 72°C/5 min for 16S; 94°C/4 min (94°C/20s, 57°C20s, 72°C/1 min 45s; 35 cycles, 72°C/7 min for 18S; and 94°C/5 min (94°C/45s, 45°C/50s, 72°C/60s, 38 cycles, 72°C/5 min for COI. Results of the PCR were checked using gel-electrophoresis. Successfully amplified samples were purified and then sent to Macrogen Europe B. V. (Netherlands) for Sanger sequencing.

Sequence analysis and species delimitation methods

Forward and reverse reads of each gene were quality checked and assembled using MEGA X (Kumar et al., 2018). The resulting sequences were checked with BLASTn to ensure that no contamination happened during the process. Sequences for an outgroup of species within Trachylinae close to *Halammohydra* were downloaded from NCBI GenBank using the study of Collins et al. (2008) as a guideline (see SI 1). All newly sequenced gene fragments are uploaded to GenBank (see SI 2) Alignments for each gene were created using MAFFT (Katoh et al., 2019) with the default settings, checked visually and conserved positions were identified with Gblocks 0.91b (Dereeper et al., 2008) in the default settings (final length without gaps of 16S: 496 bp, 18S: 1562 bp, CO1: 810 bp, concatenated matrix: 2868 bp). The alignments were analyzed individually and in a concatenated supermatrix of all three genes.

Phylogenetic analyses were performed using Bayesian Inference (BI) in MrBayes (Ronquist et al., 2012) and Maximum Likelihood (ML) in IQ tree (Trifinopoulos et al., 2016). For BI, PartitionFinder2 (Lanfear et al., 2016) was used to find the best substitution model for 16S and 18S and the 1st, 2nd and 3rd codon position of CO1 employing the corrected Akaike Information Criterion (AICc) and a greedy search scheme. Results were implemented in the settings for MrBayes and the analysis ran for 15 mill. generations with a burnin of 10 %. ML analyses were done with default settings, an automatic search for the best substitution model and 1000 ultrafast bootstrap repeats. Log files were inspected in Tracer v1.7.1 (Rambaut et al., 2018) for convergence and effective sample size. If these values were insufficient, the number of generations was increased and the analysis was performed again. Resulting tree files were edited with FigTree v1.44 and Adobe Illustrator.

The data sets were also used for species delimitation analyses. Three different approaches were performed to determine molecular operational taxonomic units (mOTUs): Automatic Barcode Gap Discovery (ABGD), Generalized Mixed Yule Coalescent (GMYC), and Poisson-Tree-Process (bPTP). For ABGD analyses (Puillandre et al., 2012), the web-server https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html was used with default settings. GMYC analyses are based on ultrametric trees, which were obtained with Beast v2.6.2 (Bouckaert et al., 2014) using a strict clock, the substitution model GTR and a Monte Carlo Markov chain length of 10 mill., sampling every 1000th generations. Results were checked with Tracer v1.7.1 and consensus trees were built with TreeAnnotator v2.6.2 with a 10 % burnin. The GMYC analyses were done with R (R Core Team, 2013) following Michonneau (2017). The last analysis performed was bPTP (Zhang et al., 2013). Trees obtained by MrBayes were uploaded to the web-server https://species.h-its.org/ptp/, and the analyses ran for 100 000 generations with default settings. Genetic divergence, or k2p values, were obtained with

the program MEGA X. Sequences were grouped according to the cluster in the tree and the species delimitation tests and analyses were done between and within groups.

Results

Halammohydra of the Northwest (and East) Atlantic are investigated from 18 subtidal and intertidal locations with medium to coarse sand or shell gravel (Table 3). This study examines 302 specimens and includes the previously published records of *Halammohydra* from the Azores (Tödter & Schmidt-Rhaesa, 2021) and two here recorded specimens from Cuba and Brazil. *Halammohydra vermiformis* Swedmark & Teissier, 1957 is the most abundant and geographically dispersed species of this study (see Table 3).

Species delimitation tests result in mOTUs. We name mOTUs with single sequences as singletons and groups of sequences as clusters. Some clusters are identified as species in combination with the morphological data (Table 4). This study assigns 234 animals to a species or cluster by morphology and/or obtained DNA sequences, leaving 68 unidentified. Sequences from 161 specimens are used to reconstruct a phylogenetic tree (Fig. 1). Topologies of both phylogenetic analyses (BI/ML) are mostly consistent, only differing in the absence of the polytomy in the ML analysis. The combined analyses of all genes (Fig. 1) show similar results as single gene analyses (XXX dryad link).

Species delimitation tests are conducted on sequences of each gene individually and on the concatenated matrix to find distinct mOTUs (Fig. 1, SI 2). The three gene sequences are not available for each specimen, but most specimens could be assigned to a mOTU with high support. Combined species delimitation analyses result in 16 clusters and six singletons (22 mOTUs). Out of the 16 clusters, seven are assigned to four known species, with two species (*H. vermiformis* and *H. octopodides*, Fig.1) having multiple clusters. Of the remaining nine clusters, four are described here as new species and the remaining five left undescribed due to insufficient information. The results supporting the four known species, four new species and five undescribed clusters are described below.

Due to the smaller variation among the 18S sequences, ABGD and GMYC analyses of 18S tend to find fewer mOTUs, with 10 (ABGD) and five (GMYC) mOTUs. 18S analyses are able to distinguish between “Tenerife 1”, *H. adherens*, *H. coronata*, “Helgoland/ Sylt” and “Azores”. All other sequences of 18S are grouped together in one mOTU. In contrast, bPTP of 18S predicts several mOTUs within “Helgoland/ Sylt”, “Azores”, *H. swedmarki* n. sp., *H. octopodides* 1 and “Tenerife 1” with a total of 26 mOTUs (SI 2)

Mean k2p values of 16S and CO1 sequences within each cluster are noticeably lower than between each cluster, with 0.006 ± 0.002 (0.000 – 0.027 in 16S; 0.000 – 0.017 in CO1) within each cluster and 0.263 ± 0.019 (0.018 – 0.434) in 16S and 0.241 ± 0.015 (0.053 – 0.394) in CO1 between each cluster. There is much less difference in variability between clusters of 18S sequences (mean k2p 0.009 ± 0.001, 0.001 – 0.043). Clusters, which can be identified by ABGD and GMYC, have higher values (mean k2p 0.019 ± 0.009), whereas values for Tenerife 1 are the highest (mean k2p 0.035 ± 0.003). Mean variability within each cluster of 18S sequences is 0.002 ± 0.001 (0.000 – 0.008) (SI 3).

Description of the four known species

Halammohydra vermiformis Swedmark & Teissier, 1957

A total of 105 specimens are identified as *Halammohydra vermiformis* by morphological and molecular methods. Fifteen specimens are from Sylt, 76 from Helgoland (45 from "Pier at the Youth Hostel" and 31 from the "Northern Beach"), two from Roscoff and 12 from field trips lacking detailed morphological examination of live animals (eight from Denmark, three from France, Archachon and one from Cuba; Table 3).

Molecular analyses result in two clusters of this species, *H. vermiformis* 1 and 2. Both clusters have a high number of specimens and support values of 100 in both phylogenetic analyses. Species delimitation tests group them in two clusters with comparably many sequences in each gene (Fig. 1, SI 2). 18S sequences do not resolve both groups in ABGD and GMYC, but do resolve them with a low support in bPTP (SI 2). K2p values between both clusters are 0.187 ± 0.024 (16S), 0.003 ± 0.001 (18S) and 0.207 ± 0.020 (CO1) and thus lower than the mean values of all clusters (SI 3). Both clusters differ in the sampling locality. *H. vermiformis* 1 was sampled exclusively at the subtidal station of Helgoland ("Pier at the Youth Hostel"), *H. vermiformis* 2 at several intertidal locations, except for the ones from "Ellekildehage" (Denmark), which was at seven to nine meters depth but in brackish shallow water.

The clear separation into two clusters recognized in molecular analyses is not reflected in the morphology (Table 4). Both groups have a variety of body shape, ranging from completely round to elongated, with the elongated form dominating (Fig. 2a). *Halammohydra vermiformis* 2 is slightly larger (but with higher variation) with mean gastric tube length of 298 ± 215 µm (n=19) compared to 209 ± 75 µm (n=31) in *H. vermiformis* 1 in length of the gastric tube. Most specimens have a conical aboral cone, with a higher length than width (Fig. 2b). The aboral cone of *H.* vermiformis 2 is slightly larger again (60 ± 12 µm (n=13) in mean length and 46 ± 8 µm (n=10) in mean width), compared to *H. vermiformis* 1 (45 ± 7 µm (n=21) in mean length and 37 ± 9 µm (n=28) in mean width). Whenever it was possible to investigate the adhesive organ, it showed a cup- or diamond-/ inversed cone shape in the aboral cone (Fig. 2b). All specimens have 4 statocysts.

The total number of tentacles is usually 7 (3 aboral + 4 subaboral, Fig. 2a), with the exception of a few animals from Sylt with 8 (4+4) tentacles. No special structure or thickening is documented for the bases of aboral tentacles, and a few specimens have a slight thickening or club-shaped base in the subaboral whorl (Fig. 2b). Aboral tentacles are of slender shape. Subaboral tentacles are slightly thicker or with an irregular surface. Within a whorl, the tentacle length is roughly the same, except for the one long tentacle present in the subaboral whorl. It is about two to three times the length of the other tentacles, has a thicker appearance and sometimes a bulb at the base (Fig. 2a & b). In some cases, it is coiled up. Aboral tentacles are directed aborally and subaboral tentacles orally (Fig. 2a). This was clearly visible while the animals were swimming. In general, specimens observed were less adhesive than other species and mainly found free-swimming with the aboral cone in the direction of movement. Tentacles of the aboral whorl were bent to the oral end while swimming, but the separation of whorls was still visible.

Halammohydra octopodides Remane, 1927

Most of the specimens of *Halammohydra octopodides* are from Roscoff (32 specimens), particularly from the station "Basse Plate" (21 specimens). Two are from the "Pier at the Youth Hostel" of Helgoland (Table 3). Molecular analyses separate this species into three clusters, *H. octopodides* 1, 2 and 3. The two specimens from Helgoland are positioned in the *H. octopodides* 1 clade. Node support values are

low in the separation of *H. octopodides* 1 and 2 but support a distinction from *H. octopodides* 3 (Fig. 1). This separation from *H. octopodides* 3 is documented in ABGD and bPTP as well, except with 18S alone. *H. octopodides* 1 and 2 are grouped together, except in GMYC analyses. It separates *H. octopodides* 1 and 2 and bPTP of 18S groups *H. octopodides* 2 and 3 together with a low support (SI 2). There are no CO1 sequences available for *H. octopodides* 2 and 3. K2p values of 16S between *H. octopodides* 1 and 2 are 0.024 ± 0.005 and thus lower than between *H. octopodides* 1 and 3 (0.072 ± 0.012) or 2 and 3 (0.058 ± 0.011, SI 3).

Specimens from all three groups have a similar morphology (Table 4). The ovoid gastric tube is 175 ± 72 µm (n=24) in length (Fig. 2c). In some specimens, the body is slightly elongated. The aboral cone is triangular or round and 54 ± 10 µm (n=16) in length and 47 ± 6 µm (n=10) in width. Specimens are very adhesive, which complicated the documentation of the adhesive organ. Whenever it was possible to investigate, the adhesive organ was cup-shaped and sometimes reached deep into the aboral cone.

The total number of tentacles varies from 8 to 14, mostly 10, evenly distributed in both whorls. In contrast to *H. vermiformis*, there is no clear separation of the direction of both tentacle whorls. Neither of the tentacle whorls has a visible bulb at the base (Fig. 2c). Some specimens have a slightly club-shaped base in the subaboral whorl. All tentacles are of slender shape, whereas in the subaboral whorl they sometimes have an irregular surface. One tentacle (in four specimens two) of the subaboral whorl are about two times longer than the other tentacles (Fig. 2c). Most of the specimens have no thickening at the base in this long tentacle and the structure is the same as in the subaboral whorl. Tentacles in the subaboral whorl are of unequal length. The number of statocysts is 4 or 5.

Halammohydra coronata Clausen, 1967

Eight specimens of *Halammohydra coronata* are from the “Pier at the Youth Hostel” of Helgoland and three from Roscoff (Table 3). All species delimitation tests and node values of both phylogenetic analyses support the cluster of this species well (Fig. 1, SI 2). The ovoid to elongated gastric tube is 292 ± 96 µm (n= 9) long and connects to a cylindrical and slightly round aboral cone, measuring 56 ± 7 µm (n=4) in length and 46 ± 6 µm (n=3) in width (Fig. 2d). Unfortunately, most specimens adhered to the slide during investigation, which made it impossible to observe the adhesive organ in detail. The number of statocysts is 4 or 5.

In total, specimens have 10 to 15 tentacles, distributed unevenly in the two whorls (Fig. 2d). The number of aboral tentacles is lower, mostly 4, than of subaboral tentacles. When the animals were swimming, this uneven number was best visible, because the tentacles of the aboral and subaboral whorl are held in different directions (Fig. 2d). There is no thickening at the tentacle bases, and the tentacles are slender in both whorls. In some cases, one tentacle is about one and a half to two times longer than the others and it has no bulb at the base. Tentacles in the subaboral whorl have an unequal length (Fig. 2d, Table 4).

In this group, some specimens are clearly identified as *H. coronata*, whereas other are not, due to the lower difference in structure between the tentacles of both whorls, some damage and the adhesion to the slides. The identification of the specimens with unclear characters is based solely on molecular analysis.

Halammohydra adherens Swedmark & Teissier, 1967

All seven animals identified as *Halammohydra adherens* are from Roscoff, six of them from the station “Trezen ar Skoden” (Table 3). Molecularly, this species is closely related to *H. coronata* and GMYC

analysis of 18S even groups these two species into one cluster (Fig. 1). It is important to notice that there is only one sequence of 18S and no CO1 sequence available for *H. adherens*. The cluster of this group is mostly based on 16S sequences. Node support values, ABGD and GMYC imply an affiliation of the specimen R93 from "Bazin Malvog" to this species, but bPTP excludes it (SI 2). K2p values between R93 and *H. adherens* are 0.018 ± 0.006 and between *H. adherens* and *H. coronata* 0.119 ± 0.018 (SI 3).

Specimens of *H. adherens* have an ovoid body shape and are very adhesive (Fig. 3). The structure of the aboral cone and adhesive organ could not be investigated due to this behavior (Table 4). Tentacles of both whorls are not separated and of slender shape. The number of tentacles was 11, 16 or 22 (Fig. 3a). One specimen even has more than 25 tentacles (Fig. 3b). This specimen is exceptionally large, and the amount of tentacles obscured further observation of the aboral cone or the tentacle bases. The other specimens have no special structure at the tentacle bases, and 4 or 5 statocysts are visible. All specimens of this group have elongated nematocysts, which are even observable in lower magnification (Fig. 3c & d). Tentacles of the largest specimen are fully packed with these nematocysts (Fig. 3d), whereas in the smaller ones they are less densely concentrated (Fig. 3c).

Description of the four new species

Halammohydra teissieri n. sp.

Zoobank paper: XXX

Etymology: This species is dedicated to Georges Teissier, who contributed much information about *Halamohydra.*

Material examined: *holotype*: "Pier at the Youth Hostel" on Helgoland, Germany, 16 Sep 2019, subtidal, medium sand, L. Tödter. *paratype*: "Basse Plate" in Roscoff, France, 14 Sep 2020, 21 m depth, medium sand, A. Kerbl and L. Tödter. Images taken under a compound microscope and 16S, 18S and CO1 sequences of the holotype and 16S and 18S sequences of the paratype obtained. Material deposited in Museum of Nature – Zoology in Hamburg under the numbers XXX (holotype) and XXX (paratype).

Description of the Holotype: Ovoid gastric tube with square mouth opening and adhesive behavior. 16 tentacles, evenly distributed in both whorls, aboral tentacles 1.5 times longer than subaboral ones, slender shape, no thickening at the bases and no pronounced long tentacle in subaboral whorl. 8 statocysts.

Remarks: In total, 15 animals from the "Pier at the Youth Hostel" on Helgoland (6 specimens) and four stations in Roscoff ("Basse Plate", "Trezen ar Skoden", "Ognon" and "Bazin Malvog", 9 specimens) are genetically assigned to this species (Table 3). All species delimitation tests, except for ABGD and GMYC of 18S, support this cluster, as well as phylogenetic analyses (Fig. 1). bPTP of CO1 overestimates this cluster creating three mOTUs with high support (SI 2).

Some morphological characters vary within this group compared to the holotype. The body has a length of 172 ± 45 µm (n=9; Fig. 4a). Tentacles vary from 10 to 19 in number, which are evenly distributed in both whorls. The number of statocysts vary from 5 to 8, corresponding to the number of tentacles in each whorl (Table 4). Characters are similar to the ones of *H. schulzei*, but without a pronounced thickening of the tentacle bases, longer tentacles in the aboral whorl, slightly lower tentacle number and different shape of mouth opening.

Halammohydra swedmarki n. sp.

Zoobank paper: XXX

Etymology: This species is dedicated to Bertil Swedmark, who contributed much information about *Halamohydra.*

Material examined: “Chenal l’Ile de Verte” in Roscoff, France, 16 Sep 2020, 0 m depth, at low tide, coarse sand, A. Kerbl and L. Tödter. Images taken under a compound microscope and 16S and CO1 sequences of the holotype obtained. Material deposited in Museum of Nature – Zoology in Hamburg under the number XXX (holotype).

Description of the Holotype: Gastric tube with variable shape, adhesive behavior and a flat aboral cone with cup-shaped adhesive organ (half the depth of aboral cone). 16 tentacles, equally distributed in both whorls, no separation between whorls and slender aboral tentacles about 2 times longer than thicker subaboral ones, both without a thickening at the base and of unequal length. No prominent longer tentacle. 7 statocysts.

Remarks: In Roscoff, four specimens of this group were found at “Chenal l’Ile de Verte” and two at “Basse Plate” (Table 3). Phylogenetic analyses of all four specimens (Fig. 1) and all species delimitation test, except for 18S, support this cluster well (SI 2). Variation of morphological characters is in the number tentacles and statocysts. The number of tentacles varies from 10 to 16, and one specimen even has a minimum of 20 tentacles. Statocysts are either 5 or 7 (Fig. 4b, Table 4). Differences to other species are the aboral tentacles are longer, tentacles of both whorls have unequal lengths and the variable body shapes.

Halammohydra kerblae n. sp.

Zoobank paper: XXX

Etymology: This species is dedicated to Alexandra Kerbl, who was part of the field trip to Roscoff and was a huge help in finding the specimens.

Material examined: *holotype and paratype*: “Basse Plate” in Roscoff, France, 14 Sep 2020, 21 m depth, medium sand, A. Kerbl and L. Tödter. Images taken under a compound microscope and 16S sequence of the holotype and 16S, 18S and CO1 sequences of the paratype obtained. Material deposited in Museum of Nature – Zoology in Hamburg under the numbers XXX (holotype) and XXX (paratype).

Description of the Holotype: Ovoid to slightly elongated gastric tube and trapezoid aboral cone. 12 tentacles, with 4 slender aboral and 8 irregular shaped subaboral ones, club-shaped bases on the subaboral tentacles, no pronounced long tentacles and both whorls pointing in different directions. Statocysts 4.

Remarks: Four specimens of this group are from “Basse Plate” and one from “Ognon” in Roscoff (Table 3). This cluster is well supported by phylogenetic analyses and all species delimitation tests, except for ABGD and GMYC of 18S (Fig. 1, SI 2). Characters of this group varying are the size, the number of tentacles and the statocysts. The gastric tube has a mean length of 159 ± 47 µm (n=3; Fig. 4c) is connected to an aboral cone of trapezoid or conical shape with a mean length of 48 ± 8 µm (n=3; Fig. 4d). Tentacles are 11 or 12 in number, and unevenly distributed between the two whorls: the aboral whorl always has 4 tentacles and the subaboral 7 or 8 (Fig. 4c). Statocysts are 4 to 6 (Table 4). Characters

are similar to the ones of *H. coronata*, but with a pronounced thickenings at the bases of subaboral tentacles and a lacking long tentacle.

Halammohydra joergerae n. sp.

Zoobank paper: XXX

Etymology: This species is dedicated to Katharina Jörger for organizing of the summer school ("Meiozores 2019") on the Azores.

Material examined: *holotype*: "Riberinha" on the island Sao Miguel (Azores), Portugal, 22 Jul 2019, medium coarse sand, K. Jörger and F. Goetz. Images taken under a compound microscope and 18S and CO1 sequences obtained. Material deposited in Museum of Nature – Zoology under the numbers XXX (holotype) and XXX (paratype).

Description of the Holotype: Ovoid gastric tube, square mouth opening, adhesive behavior and cylindrical aboral cone. 12 tentacles, equally distributed in both whorls (6+6), slender tentacles and subaboral ones with unequal lengths and irregular surface. No long tentacles. Statocysts 6.

Remarks: All 13 specimens are from the station "Riberinha" on the Azores (Table 3) and form a cluster, which is well supported by phylogenetic analyses and all species delimitation tests, except for 18S in ABGD and GMYC (Fig. 1, SI 2). In this group, morphological characters varying are the body size, aboral cone, number of tentacles and statocysts and the tentacle bases. The gastric tube measures 348 ± 117 µm (n=5) in length (Fig. 4e). Whenever the aboral cone was visible, it had a conical or cylindrical shape and a cup-shaped adhesive organ reaching in half of the depth of the aboral cone. The tentacle number ranges from 10 to 14, and they are equally distributed in the two whorls. Some specimens have a club-shaped base in the subaboral whorl, but most have no thickening. Statocysts are 5 or 6 (Table 4).

Characters of undescribed groups

"Helgoland/ Sylt"

Nine specimens are assigned to this group morphologically and genetically, eight from the "Pier at the Youth Hostel" of Helgoland and one from Sylt (Table 3). This cluster is supported by both phylogenetic analyses and all species delimitation tests (Fig. 1), except for GMYC of 18S, which groups this cluster together with "Azores". The analysis is based mostly on 18S sequences, since there are only two 16S and one CO1 sequences (SI 2).

Specimens of this cluster have an ovoid, sometimes elongated, gastric tube with a mean length of 227 ± 37 µm (n=6, Fig. 4f). All animals were very adhesive, so the aboral cone could not be investigated. The number of tentacles varies between 9 and 16, evenly distributed in both whorls. Aboral tentacles are slender, whereas subaboral ones are sometimes slightly thicker or with an irregular surface. There is no bulbous base documented in aboral tentacles, but some specimens have a club-shaped or slight thickening in the subaboral tentacles. There is no longer tentacle on the subaboral whorl, and no striking length differences are noticed. The number of statocysts varies between 4 and 8 and is the same as tentacles in the subaboral whorl (Table 4).

"Roscoff"

Animals of this cluster are from the stations "Basse Plate" (10 specimens) and "Banc de Bistarz" (3 specimens) in Roscoff (Table 3). "Roscoff" is well supported by high node values and all species delimitation tests, except for 18S. Contrary to GMYC and bPTP, ABGD grouped "Roscoff" together with the singleton R118 and "Tenerife 2" (SI 2). The support values of the tree clearly separate "Tenerife 2" from the other clusters, as it is visible in the analysis with GMYC and bPTP (Fig. 1).

The ovoid gastric tube has a length of 211 ± 52 µm (n=7), and the animal is very adhesive, which is why the aboral cone could not be investigated (Fig. 4g). Tentacles vary from 10 to 18 in number, are equally distributed between the two whorls. Aboral tentacles have no thickening at the bases, are slender and longer than subaboral ones (Fig. 4g). Subaboral tentacles have a pronounced bulb or club-shaped base, are thicker with an irregular surface and unequal in length (Fig. 4h). There are no noticeably longer tentacles in the subaboral whorl. The number of statocysts range from 5 to 7 (Fig. 4h, Table 4).

"Azores"

Two specimens of the station "Praya dos Moinhos" on the Azores are assigned together (Table 3). Phylogenetic analyses, as well as, species delimitation tests support this cluster (Fig. 1). GMYC of 18S groups it together with "Helgoland/ Sylt", but all other genes not (SI 2).

Specimens have and ovoid gastric tube with 255 or 301 µm in length (Fig. 4i). Only one aboral cone could be investigated, since the animals were very adhesive. It is conical with a length of 41 or 50 µm and the adhesive organ is cup-shaped and reaches half the depth into the aboral cone. Tentacles are 16 (8+8) and 18 (9+9) with slender aboral tentacles and thicker subaboral ones (Fig. 4i). There was no long tentacle in the subaboral whorl, but a thickening at the bases. The number of statocysts is 8 and 9 (Table 4).

"Tenerife 1"

Four animals of the station "Los Abades" on Tenerife group together morphologically and genetically (Table 3). There are only 18S sequences available of "Tenerife 1". ABGD, GMYC and the node values support this group, but bPTP separates all specimens with high support values (Fig. 1, SI 2). They have an elongate gastric tube and are adhesive. It was not possible to investigate the aboral cone and thus the adhesive organ. The number of tentacles is 12 or 14 with 6 or 7 in each whorl. The tentacles are slender with an irregular surface and no thickening at the base. There is no unusually long tentacle documented. The number of statocysts is the same as that of the tentacles in each whorl, 6 or 7 (Table 4).

"Tenerife 2"

Two specimens of the station "Los Abades" and two of the station "Arcos de Playa San Juan" on Tenerife group together morphologically and genetically (Table 3). ABGD groups it together with the singleton R118 (from "Trezen ar Skoden") and "Roscoff", but all other species delimitation tests, as well as the support values of the tree separate them (Fig. 1). There is only one CO1 sequence available and no 18S sequences. In bPTP, this CO1 sequence contradicts the clustering of the 16S sequences (SI 2). There is not much information about the body because the animals were sticking strongly to the

slides. The tentacle number varies between 19 and 21 and is evenly distributed in each whorl. There is no thickening at the base but the tentacles themselves are slightly thicker than in other groups. There is no prominent long tentacle documented and the number of statocysts is 6 or 7 (Table 4).

Singletons

There are six singletons present in the analyses, which are not assigned to any of the clusters. R61 from the station "Basse Plate" in Roscoff is positioned close to *H. adherens* in the tree (Fig. 1). It is an adhesive animal with 11 tentacles and an elongated gastric tube. Tentacles are of slender shape, unequal length and have no thickening at the bases. There are no elongated nematocysts visible in light microscopy, as in *H. adherens*.

The second singleton D1 is placed isolated to other sequences (Fig. 1). This is a fixed specimen from Brazil. Hence, there are no detailed morphological characters. It is a rather small specimen with an ovoid body and 14 tentacles (7+7), where the aboral tentacles are longer and the subaboral are of unequal length. There is no noticeably long tentacle.

Three singletons from Roscoff are placed between *H. kerblae* n. sp. and the cluster of "Tenerife 2" and "Roscoff" (Fig. 1). R115, found at "Basse Plate", has an ovoid body and 15 tentacles of slender shape and no thickening at the base. Tentacles in the subaboral whorl have an irregular surface as well. There are four statocysts and no long tentacle is visible. The other two singletons, R75 ("Bazin Malvog") and R104 ("Trezen ar Skoden"), are placed closer together and ABGD analysis even groups them in one mOTU. Both are juveniles with 12 slender tentacles and no thickening at the bases.

The last singleton R118 from "Trezen ar Skoden" in Roscoff is nested between "Tenerife 2" and "Roscoff" (Fig. 1). Unfortunately, it was very contracted during the investigations, which complicated the detailed documentation. It has an ovoid body with at least 14 tentacles and adheres to the slides.

Topology

Bayesian Interference and Maximum Likelihood analyses result in trees with a similar topology and nodes with mostly high support values (Fig.1). "Tenerife 2" and "Roscoff" create a clade with the singleton R118 nested within it and a sister relation with the clade consisting of the two singletons R104 and R75. Together with the singleton R115 and *H. kerblae* n. sp. it creates a sister clade to the clade of *H. swedmarki* n. sp., *H. teissieri* n. sp., *H. joergerae* n. sp.and the three clusters of *H. octopodides*. Clusters of the latter clade are in a sister relation as well: *H. swedmarki* n. sp. and *H. teissieri* n. sp. are a sister group of *H. joergerae* n. sp. and the monophyletic group of *H. octopodides*, whereas the clusters in each group are in a sister relation as well. This is supported well by the node values, except for the ML value of the second clade (Fig. 1).

All mentioned clusters are part of a polytomy with three clades. The other two clades are of the two sister clades "Helgoland/ Sylt" and "Azores"; and the two clusters of *H. vermiformis*. Close to the base of the tree is a clade containing the singleton R61 and *H. adherens*, creating a sister clade to *H. coronata*. In between this clade and the polytomy, the singleton D1 from Brazil is positioned. "Tenerife 1" is located at the base of the tree.

Discussion

This is the first study investigating species-specific sequences of the interstitial cnidarian *Halammohydra* in combination with morphological characters. The addition of molecular data to the traditional identification helped to verify previously described species and to delimit new species or mOTUs within this genus as well. In addition, it was possible to assign individuals to a group, which could not be identified morphologically because of a damage or an unusual shape due to the contraction of the animal. The integrative approach of this study linked the morphology of every specimen to a specific sequence, which was useful to document the variability of the characters within a mOTU.

To ensure to have all morphological features for the combined analysis, images were taken of every specimen. This was not always easy to achieve. *Halammohydra*, like other meiofaunal animals, has a patchy occurrence, even in reliable locations. If they were found, they came in comparably high numbers at once. This led to time pressure in the detailed investigation because the animals did not survive for too long after the extraction. Additionally, not every character was fully visible due to the behavior (very adhesive or contracted animals) and thus, some information could not be documented. On the other hand, obtaining the genetic material was rather difficult. The animals were very small and did not contain a high amount of DNA, which restricted the number of attempts for the PCR. Nevertheless, it was possible to gain enough information for the identification by combining both methods.

Three genes (16S, 18S and CO1) were used, but only 16S and CO1 gave detailed results on species level. The 18S gene was able to identify five clusters with ABGD and GMYC and grouped the rest of the sequences together. This is a common result for 18S gene, as it is a slow evolving gene (Hillis & Dixon, 1991) and does not always contain phylogenetic information on species level (Tang et al., 2012; Fontaneto et al., 2015). In general, the topology of the trees and the species delimitation test show similar results, differing only in a few parts. The interpretation of the validity of a group became difficult, when there was only the 18S sequence available, e.g. in “Tenerife 1”, or when one species delimitation test shows a different result, e.g. GMYC of 16S separated *H. octopodides* 1 and 2.

The utility of the CO1 gene for species delimitation in Cnidaria has been debated, since there is a slow evolutionary rate in most Anthozoa (Hellberg, 2006; Shearer et al., 2008; McFadden et al., 2010). However, in Medusozoa, CO1 was considered useful on species level (Ortman et al., 2010; Bucklin et al., 2011), but the conducted study by Ortman et al. (2010) contained only few representatives across Cubozoa, Scyphozoa or Hydrozoa (except Siphonophora), whereas groups of Hydrozoa are better represented. In the present study, CO1 was useful to determine between species of *Halammohydra* and showed evolutionary rates similar to 16S sequences. Additionally, the alignment process of CO1 sequences was easier than of 16S sequences, because of the common presence of indels (insertions/deletions) in the latter.

In this study, four already described species were identified morphologically and molecularly. The highest number of specimens was found for *Halammohydra vermiformis*. It was described from Roscoff (Swedmark & Teissier, 1957a), but in our analysis only two morphologically identified individuals were from Roscoff. The majority of specimens was from two locations on Helgoland. Here, at least from the subtidal location at the Youth Hostel, *H. vermiformis* has been found before (Clausen, 1967). There is no record of *H. vermiformis* from the “Dune” of Helgoland so far, but Remane (1927) mentioned in his description of *H. octopodides* small individuals with only 7 tentacles, 3 aboral and 4 subaboral from this location. It is possible, that he already found *H. vermiformis,* but did not identify them as a different species.

Halammohydra vermiformis has a very special morphology with low variation in the characters. This makes it easy to identify. Most specimen have 7 tentacles and few have 8 but not higher (Swedmark & Teissier, 1957b). The latter individuals can be confused with *H. octopodides*, especially if the gastric tube is not as elongated as in other individuals i.e. due to contraction or a variation in shape. *Halammohydra octopodides* mostly has an ovoid to slightly elongated gastric tube. A molecular investigation can differentiate between them with certainty, as it was the case for some individuals from Sylt with eight tentacles. *Halammohydra vermiformis* was not reported from this location before, but *H. octopodides* (Polte & Schmidt-Rhaesa, 2011) and *H. schulzei* (Schmidt, 1969).

Interestingly, there was a separation of morphologically similar individuals of *H. vermiformis* in two clusters in the molecular analysis. The only difference between these clusters is the slight size difference, which can be a result of a sampling or measuring bias, and, much more important, the habitat. One group was found exclusively in the "Pier at the Youth Hostel" of Helgoland, whereas the other group was from several locations. Most of them were in the intertidal, except the station in Denmark, but here the specimens were found above the halocline. At this station, the mechanical influence of the waves is less, but there is a lower salinity, which is also found at times in the intertidal locations, especially at the station of Sylt. Here, animals inhabit the moist sand of the beach at low tide without water covering the sediment and variations in salinity are very frequent. Clusters of *H. vermiformis* are in a sister group relationship, but one group potentially tolerates higher variability in abiotic factors, such as salinity.

Halammohydra octopodides on the other hand is separated into three clusters without an obvious relation to environmental factors. Morphologically, they are similar and fit in the range of descriptions and locations of previous records (Remane, 1927; Swedmark, 1957; Swedmark & Teissier, 1957a; Clausen, 1963; Renaud-Debyser, 1964; Clausen, 1967). Although they were first described on Helgoland (Remane, 1927), only two specimens were found in this study there. The majority was from Roscoff. Species delimitation tests grouped *H. octopodides* 1 and 2 together (except GMYC), which indicates them as one species. The low internal node labels support this as well. *Halammohydra octopodides* 3 is separate in every analysis but there are no morphological characters separating them and they are from the same stations as the animals of group 1 and 2. Additionally, *H. octopodides* 3 contains only two individuals with features falling in the range of morphological characters of *H. octopodides*. However, since we only have limited molecular data and insignificant morphological variation we follow a conservative approach and regard all three clusters as one species. The "Roscoff - type" with a higher number of tentacles and slight differences in the cnidom described by Swedmark (1957) was not found in this study and thus, could not be tested.

For *Halammohydra coronata* a smaller and a larger form was described at Helgoland (Clausen, 1967). Specimens found in this study were of the smaller form, which corresponds to the location and morphological characters described. The larger form was from the "Amphioxus"-flat near Helgoland (Clausen, 1967). One feature not described before was the one long tentacle in the subaboral whorl of some individuals. The description of the smaller form of this species is based on only three specimens, which can be a reason, that not all characters could be documented. There is some variation in this feature, as it only occurred in few animals here. This is the first record of *H. coronata* from Roscoff. Before it was reported only from Helgoland (Clausen, 1967) and the Delta area in the Netherlands (Wolff et al., 1974).

Halammohydra coronata was described to be closely related to *H. adherens* because of the similar cnidome, at least in the larger forms (Clausen, 1967). This similarity could not be tested, since we did not find the larger form here, but the relationship between both species was confirmed in the molecular analyses. Most animals of *H. adherens* were found in shell gravel of the station "Trezen ar Skoden" in

Roscoff, similar to what is reported in the literature (Swedmark & Teissier, 1959, 1967). *H. adherens* was described as being large (about 800 µm), with 12-14 tentacles in each whorl (Swedmark & Teissier, 1967). Such character combination was documented only for one animal in our investigation, assigned to *H. adherens*. Other specimens of *H. adherens* were much smaller and had less tentacles, so there appears to be a higher variability in characters than previously reported. One special feature, compared to other species, is the cnidome. In light microscopy, nematocysts of an elongated shape, possible macroeuryteles, are visible. Swedmark and Teissier (1967) described micro- and macroeurytels with seemingly same shape for this species. However, this cannot be stated with certainty, because no detailed investigation was done, but no other group showed these noticeable nematocysts in light microscopy. This raises the question, if the described larger form of *H. coronata* is actually *H. adherens* or an individual species and a potential intermediate between *H. coronata* and *H. adherens*.

Two species recorded in Europe were not identified in our study or not with certainty. One is *H. intermedia* Clausen 1967, which combines characters of both *H. schulzei* and *H. octopodides* and was described from Norway and also reported from Helgoland (Clausen, 1967). The other is *H. schulzei*, a species with records from many different locations: Helgoland (Remane, 1927; Clausen, 1967), Sylt (Schmidt, 1969), Western Baltic Sea (Schulz, 1952), Roscoff (Teissier, 1950; Swedmark, 1957; Swedmark & Teissier, 1957a, 1957b), Marseille (Swedmark, 1957), United Kingdom (Boaden, 1961), Norway (Clausen, 1963, 1967) and one record from the Western Atlantic (Bush & Zinn, 1970). These records give the impression of a broad distribution and high numbers of specimens for *H. schulzei*, but this could not be confirmed in our investigations. In this study, specimens of "Roscoff" had characters closest to the description, except for the lower number of tentacles and statocysts. *Halammohydra schulzei* was described with a minimum of 14 to 24 (Remane, 1927; Swedmark, 1957) and up to 26 tentacles (Swedmark, 1957; Swedmark & Teissier, 1957b) and 12 statocysts (Remane, 1927). There is no information about the tentacle length within one whorl and between the two whorls in previous records. Aboral tentacles of "Roscoff" were longer than subaboral ones, which were of unequal length. When comparing our observations with the few images available in the literature, these length differences are not visible there. This can be due to the absence of these characters in the described specimens of *H. schulzei* or because of the choice of pictures in the publications.

Another cluster with a similar character combination is "Azores". The characters here observed fall in the range of variation described for *H. schulzei*, thus the specimens were preliminary assigned to this species (Tödter & Schmidt-Rhaesa, 2021). Molecular analyses placed both clusters, "Roscoff" and "Azores", in different positions of the tree, indicating no close relationship and complicating the identification. Since *H. schulzei* was described from Helgoland, a closer look into the cluster "Helgoland/ Sylt" is needed. It is in a sister relation to "Azores" but lacking the pronounced bulb at the tentacle bases in the subaboral whorl and the number of tentacles is too low in some specimens. There are records of *H. schulzei* from Roscoff (Teissier, 1950; Swedmark, 1957; Swedmark & Teissier, 1957a, 1957b), but it is surprising not to find this species at its location of description on Helgoland (Remane, 1927). Hence, the molecular identification of *H. schulzei* remains unclear and needs more data to conclude. Additionally, as for *H. octopodides*, Swedmark (1957) described a "Roscoff - type" for *H. schulzei*, which was not documented in this study and thus could not be tested here.

Besides the identified species, there are four clusters, for which support values and species delimitation tests suggest that they are separate species. These clusters are *H. teissieri* n. sp., *H. swedmarki* n. sp., *H. kerblae* n. sp. and *H. joergerae* n. sp.. They are described as new species here (see results). *Halammohydra teissieri* n. sp. resembles *H. schulzei* in many aspects but is lacking the pronounced thickening at the tentacle bases and aboral tentacles are longer than subaboral ones. *Halammohydra*

swedmarki n. sp. has not many similarities to described species. One striking feature is the length of the tentacles. They appear noticeably longer than in other species. *H. kerblae* n. sp. on the other hand has similarities to *H. coronata*. The differences are the thickening of the tentacle bases and the lacking long tentacle in the subaboral whorl. Specimens of *H. joergerae* n. sp. have different character combinations than the other species and clusters. Additionally, there is one singleton from Brazil in an isolated position of the tree. All analyses support it to be a separate species, but since sequences were only acquired from one specimen and no detailed morphological data could be obtained from the fixed material, no new species is described here.

For the further clusters or singletons, support to separate species is less strong or morphological data are lacking and therefore we do not describe them as new species. In Tenerife at least two species occur. "Tenerife 2" is positioned close to "Roscoff", but two out of three species delimitation tests separate them in two clusters. Additionally, there are some differences in the morphology, like the lacking thickening in the tentacles of the subaboral whorl in "Tenerife 2". Due to the geographical distance, it is possible, that "Tenerife 2" and "Roscoff" are different species, but there is not enough data available to reliable characterize both as new species. The singleton R118 from "Trezen ar Skoden" is nested between them but is not clustered to one of the two groups by species delimitation tests (except ABGD). Since no useful morphological data were documented, no further conclusions can be made. "Tenerife 1" consists of three specimens, but this cluster is only supported by 18S sequences, which does not give reliable results on species level and even bPTP of 18S separated all three specimen, therefore it is not confirmed that the three specimens even belong to the same species. Given the position of "Tenerife 1" as a sister clade to all remaining species of *Halammohydra*, further investigations on these specimens is very interesting.

The three singletons positioned between *H. kerblae* n. sp. and the clade of "Tenerife 2" and "Roscoff" have more morphological similarities to the latter clade than *H. kerblae* n. sp. but are lacking a thickening at the bases of the subaboral tentacles. Additionally, R104 and R75 were juveniles and thus cannot be used for comparison. It is possible, that these are additional species, but there is not enough data to reliably describe new species.

Previously only nine species of *Halammohydra* were described, mostly from Europe (e.g. Remane, 1927; Swedmark & Teissier, 1957a; Clausen, 1967) and India (e.g. Rao & Ganapati, 1966; Rao, 1978; Rao & Misra, 1980), but this is likely a result of a sampling bias. Most of the early meiofaunal research was done in Europe (Giere, 2009), hence the high amount of records. Additional previous findings in the Western Atlantic and the Caribbean Sea (e.g. Bush & Zinn, 1970; Calder & Kirkendale, 2005; Garraffoni et al., 2017) and on the Azores (Tödter & Schmidt-Rhaesa, 2021) suggest a broader distribution than expected. Nonetheless, it is interesting, that Europe, or single localities within, harbor such a high number of different species, especially compared to other genera of meiofaunal Cnidaria. For example, five out of the six species described in Europe were found at Helgoland, while this study adds at least one new species for this location. Another species rich locality is Roscoff. Four species and two "types" were found in Roscoff and this study reveals that there are at least three new species present in the Roscoff area and adds the finding of *H. coronata* as a new record. Our study shows that the species diversity in Europe is distinctly higher than previously assumed, with four new species described from here and several potential new species, for which further investigation is needed.

In conlusion

This study contributes to the molecular database by providing species specific sequences of three genes, which are useful for future biodiversity studies. The combination with morphological investigations

shows the different variability of characters within one species and the sometimes overlapping features between species. Identification of specimen of *Halammohydra* in the field remains difficult for some species, but due to the information about the range of characters, it can be narrowed down to a few species. To ensure a correct identification, molecular methods are needed.

Statements and Declarations

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Data availability All sequences generated and analyzed during the current study are available in GenBank,

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval: No approval of research ethics committees was required to accomplish the goals of this study since experimental work was conducted on an unregulated invertebrate species.

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Table 1 Localities of samples used for this study.

Locality			Date	GPS Latitude	Longitude	Sediment	Depth (m)	Sampling method
Denmark	Noth Sealand	Ellekildehage	26.08.2011; 30.06.2014; 05.11.2015	56°5’9.6”N	12°31’26.4”E	coarse sand	7-9	mini van veen grab
Germany	Sylt	Beach of Hörnum	20.-29.05.2019	54°45'21.117"N	8°17'39.951"E	coarse sand	beach	with shovel
	Helgoland	Pier at the Youth Hostel	16.-27.09.2019	54°11'18.7908"N	7°53'7.368"E	medium sand	app. 3-5	van veen grab
		“Dune”, Northern beach	10.-15.08.2020	54°11'23.8812"N	7°54'44.5212"E	shelly sand	0.5	with shovel
France	Roscoff	Basse Plate	14.-23.09.2020	48°44'17.5812"N	4°2'25.62"W	medium sand	21	dredging
				48°44'20.58"N	4°2'36.5388"W	medium sand	29	dredging
		Trezen ar Skoden		48°45'34.2612"N	4°5'38.4"W	coarse shell	48	dredging
		Chenal l’Ile de Verte		48°43'44.4"N	3°59'13.2"W	coarse sand	0	at low tide, shovel
		Banc de Bistarz		48°43'57"N	3.59'9.6"W	coarse sand	0	at low tide, shovel
		Ognon		48°44'9.1752"N	4°1'41.0268"W	medium sand	18	dredging
		Bazin Malvog		48°44'10.968"N	4°0'21.924"W	medium sand	7	dredging
	Arcachon	Plage d’Arcachon	30.06.2014	44°39'53.4"N	1°09'48.9"W	coarse sand	0.5	with shovel
Spain	Tenerife	Los Abades	31.-04.09.2021	28°8'25.836"N	16°26'1.14"W	-	19-22	diving
				28°8'24.756"N	16°26'25.944"W	coarse sand	4-5	diving
		Arcos de Playa San Juan		28°10'43.536"N	16°49'0.66"W	coarse sand	20-25	diving
Portugal	Azores (Sao Miguel)	Piscinas Lagoa	15.-24.07.2019	37°44'24.061"N	25°34'29.82"W	sand	app. 8-12	diving, with shovel and plastic containers
		Praia dos Moinhos		37°49'28.048"N	25°26'44.3508"W	sand	app. 3-6	
		Riberinha		37°50'9.8664"N	25°29'2.7996"W	coarse sand	app. 1-4	
				37°50'10.2156"N	25°29'2.1876"W	coarse sand	app. 1-4	
				37°50'11.3784"N	25°29'0.7296"W	coarse sand	app. 1-4	
				37°50'8.2716"N	25°28'56.5032"W	medium sand	app. 2-5	
Cuba	Gibara	Playa Caletones	18.11.2014	21°12'40.6"N	76°14'30.1"W	medium-coarse sand	17-18	diving
Brazil	Fernando de Noronha	Ilha Rata	19.10.2012	3°48'57.7"S	32°23'29.1"W	coarse sand	10-12	diving

Table 2 Primers used for the PCR and their source

	primer		source
16S	SF2	TCGACTGTTTACCAAAAACATA	Allen G. Collins, pers. commun.
	SR2	ACGGAATGAACTCAAATCATGTAAG	
18S	forward	CCGAATTCGTCGACAACCTGGTTGATCCTGCCAGT	(Medlin et al. 1988)
	reverse	CCCGGGATCCAAGCTTGATCCTTCTGCAGGTTCACCTAC	
COI	LCO	GGTCAACAAATCATAAAGATATTGG	(Folmer et al. 1994)
	MedCoir	GGAACTGCTATAATCATAGTTGC	(Ortman et al. 2010)

Table 3 Number of specimens of every species and cluster (identified by morphology and DNA sequences) found at each locality, displayed as total number of specimens/ number of sequenced specimens used in molecular analyses

		NW Atlantic																**E Atlantic**	
		DK	**Germany**			**France**						**Spain**		**Portugal**					
species/ cluster	**total number**	**North Sealand/ Ellekildehage**	**Sylt/ Hörnum**	**Helgoland/ Pier at Youth Hostel**	**Helgoland/ Northern Beach**	**Roscoff/ Basse Plate**	**Roscoff/ Trezen ar Skoden**	**Roscoff/ Chenal l'Ile de Verte**	**Roscoff/ Banc de Bistarz**	**Roscoff/ Ognon**	**Roscoff/ Bazin Malvog**	**Arcachon/ Plage d' Arcachon**	**Tenerife/ Los Abades**	**Tenerife/ Arcos de Playa San**	**Azores/ Piscina Lagoa**	**Azores/ Praya dos Moinhos**	**Azores/ Riberinha**	**Cuba/ Gibara, Playa Caletones**	**Brazil/ Fernando de Noronha**
H. vermiformis	105/59	8/2	15/10	45/28	31/17	1/0	1/0	-	-	-	-	3/2	-	-	-	-	-	1/0	-
H. octopodides	34/21	-	-	2/2	-	21/15	9/3	-	-	1/1	1/0	-	-	-	-	-	-	-	-
H. coronata	11/10	-	-	8/8	-	1/1	1/1	-	-	1/0	-	-	-	-	-	-	-	-	-
H. adherens	7/6	-	-	-	-	-	6/4	-	-	-	1/1	-	-	-	-	-	-	-	-
H. teissieri n. sp.	15/14	-	-	6/5	-	3/3	1/1	-	-	1/1	4/4	-	-	-	-	-	-	-	-
H. swedmarki n. sp.	6/6	-	-	-	-	2/2	-	4/4	-	-	-	-	-	-	-	-	-	-	-
H. kerblae n. sp.	5/5	-	-	-	-	4/4	-	-	-	1/1	-	-	-	-	-	-	-	-	-
H. joergerae n. sp.	13/13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13/13	-	-
"Helgol./ Sylt"	9/4	-	1/1	8/3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
"Roscoff"	13/10	-	-	-	-	10/9	-	-	3/1	-	-	-	-	-	-	-	-	-	-
"Azores"	2/2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2/2	-	-	-
"Tenerife 1"	4/3	-	-	-	-	-	-	-	-	-	-	-	2/2	2/1	-	-	-	-	-
"Tenerife 2"	4/3	-	-	-	-	-	-	-	-	-	-	-	4/3	-	-	-	-	-	-
singletons	6/6	-	-	-	-	2/2	2/2	-	-	-	1/1	-	-	-	-	-	-	-	1/1
unidentified	68/0	3/0	3/0	10/0	-	27/0	5/0	2/0	2/0	-	1/0	-	-	1/0	2/0	-	12/0	-	-
total	**302/161**	**11/2**	**19/11**	**79/46**	**31/17**	**71/36**	**25/11**	**6/4**	**5/1**	**4/3**	**8/6**	**3/2**	**6/5**	**3/1**	**2/0**	**2/2**	**25/13**	**1/0**	**1/1**

Table 4 Summerized characters of species/ clusters of *Halammohydra*. - : missing data, * : data only from one specimen

	tentacles								
	number		**shape**		**thickening at bases**		**length differences**		
species/ cluster	**total**	**per whorl**	**aboral**	**subaboral**	**aboral**	**subaboral**	**one long tentacle**	**within whorl**	**between whorls**
H. vermiformis 1 & 2	7-8	3(4)+4	slender	slender, slightly thicker or irregular surface	no	slight bulb or club-shaped	yes, 2-3 times longer	no	diverse
H. octopodides 1, 2 & 3	8-14	even	slender	slender, some with irregular surface	no	no, some club-shaped	yes, 2 times longer	subaboral unequal	diverse
H. coronata	10-15	uneven, mostly 4 in aboral whorl	slender	slender	no	no	some, 1.5 times longer	subaboral unequal	diverse
H. adherens	11->25	-	slender	slender	no	no	no	-	-
H. teissieri n. sp.	10-19	even	slender	slender	no	no	no	no	aboral tentacles 1.5 times longer
H. swedmarki n. sp.	10-20	even	slender	thicker	no	no	no	both unequal	aboral tentacles 2 times longer
H. kerblae n. sp.	11-12	uneven, 4+7/8	slender	irregular surface	no	bulb or club-shaped, some no	no	no	no
H. joergerae n. sp.	10-14	even	slender	slender, some with irregular surface	no	no, some club-shaped	no	subaboral unequal	diverse
"Helgoland/ Sylt"	9-16	even	slender	slender, slightly thicker or irregular surface	no	sometimes slight bulb or club-shaped	no	no	no
"Roscoff"	10-18	even	slender	thicker or irregular surface	no	bulb or club-shaped	no	subaboral unequal	aboral tentacles longer
"Azores"	16, 18	even	slender	thicker	no	bulb	no	no	no
"Tenerife 1"	12, 14	even	slender, irregular surface	slender, irregular surface	no	no	no	-	-
"Tenerife 2"	19-21	even	slightly thicker	slightly thicker	no	no	no	no	-

continuation of Table 4

species/ cluster	separation of whorls	aboral cone		statocysts	gastric tube	behaviour	other	
		shape	adhesive organ	number	shape			environment
H. vermiformis 1 & 2	yes	conical	diamond shaped/ inversed cone	4	round to elongated	free-swimming		subtidal and intertidal, marine and brackish
H. octopodides 1, 2 & 3	no	conical or round	cub-shaped	4-5	ovoid	adhesive		subtidal
H. coronata	yes	cylindrical or slightly round	-	4-5	ovoid to elongated	free-swimming		subtidal
H. adherens	no	-	-	4-5	ovoid	very adhesive	elongated nematocysts	subtidal
H. teissieri n. sp.	no	-	-	5-8	ovoid	adhesive	tapering or square mouth opening	subtidal
H. swedmarki n. sp.	no	* flat	* cup-shaped	5 or 7	variable	adhesive		subtidal and intertidal
H. kerblae n. sp.	yes	trapezoid or conical	* cup-shaped	4-6	ovoid or slightly elongated	free-swimming		subtidal
H. joergerae n. sp.	no	conical or cylindrical	cup-shaped	5-6	ovoid	adhesive	tapering or square mouth opening	subtidal
"Helgoland/ Sylt"	no	-		4-8	ovoid, sometimes elongated	adhesive		subtidal and intertidal
"Roscoff"	no	-	-	5-7	ovoid	adhesive		subtidal and intertidal
"Azores"	no	* conical	* cup-shaped	8, 9	ovoid	adhesive		subtidal
"Tenerife 1"	no	-	-	6-7	elongated	adhesive		subtidal
"Tenerife 2"	no	-	-	6-7	-	adhesive		subtidal

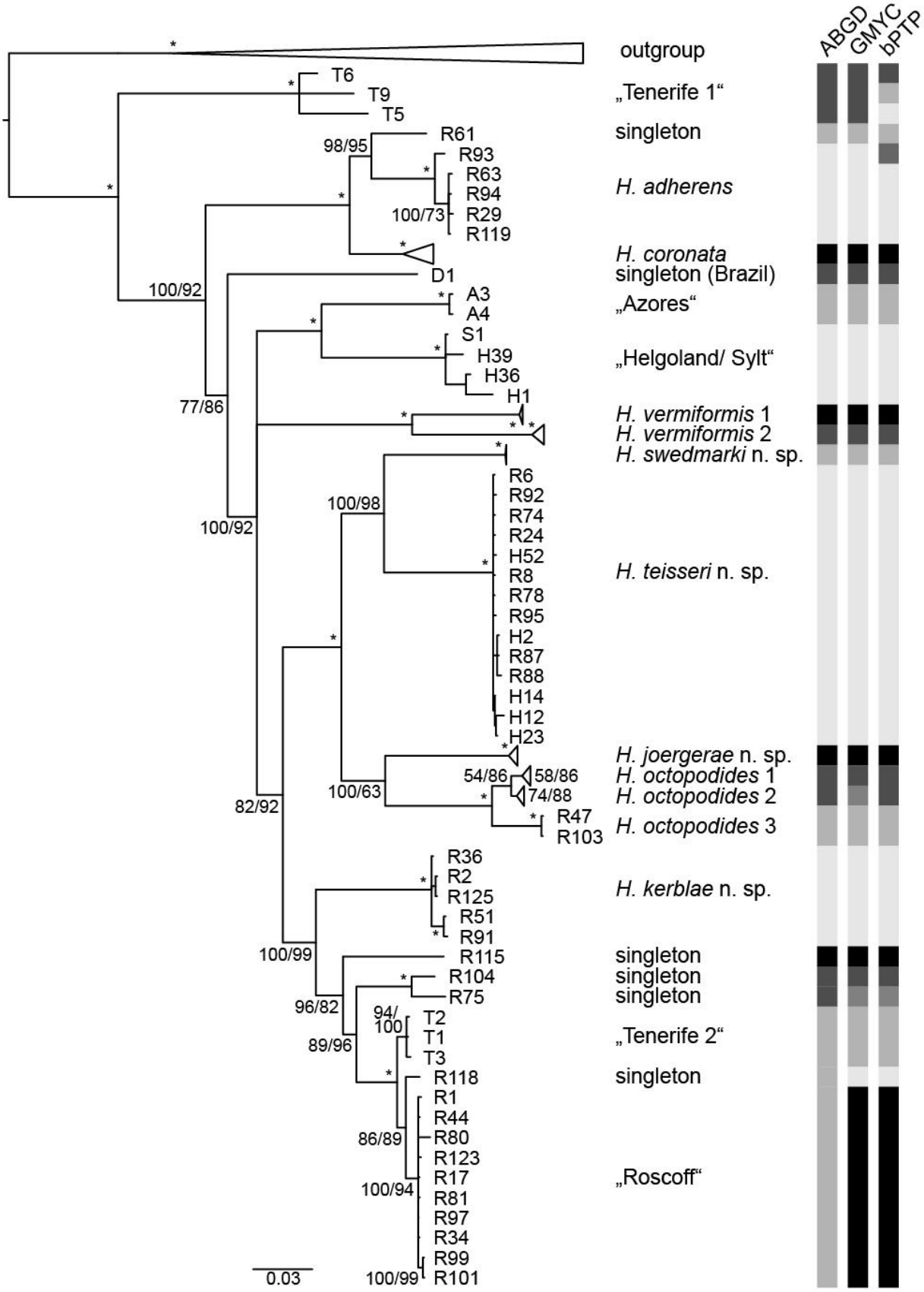

Fig. 1 Phylogenetic tree of all three genes concatenated with support values of BI/ML (posterior probability/bootstrap value). Nodes with an * have a support of 100/100. Some clusters are collapsed. Summarized species delimitation of ABGD, GMYC and bPTP results are shown for each cluster.

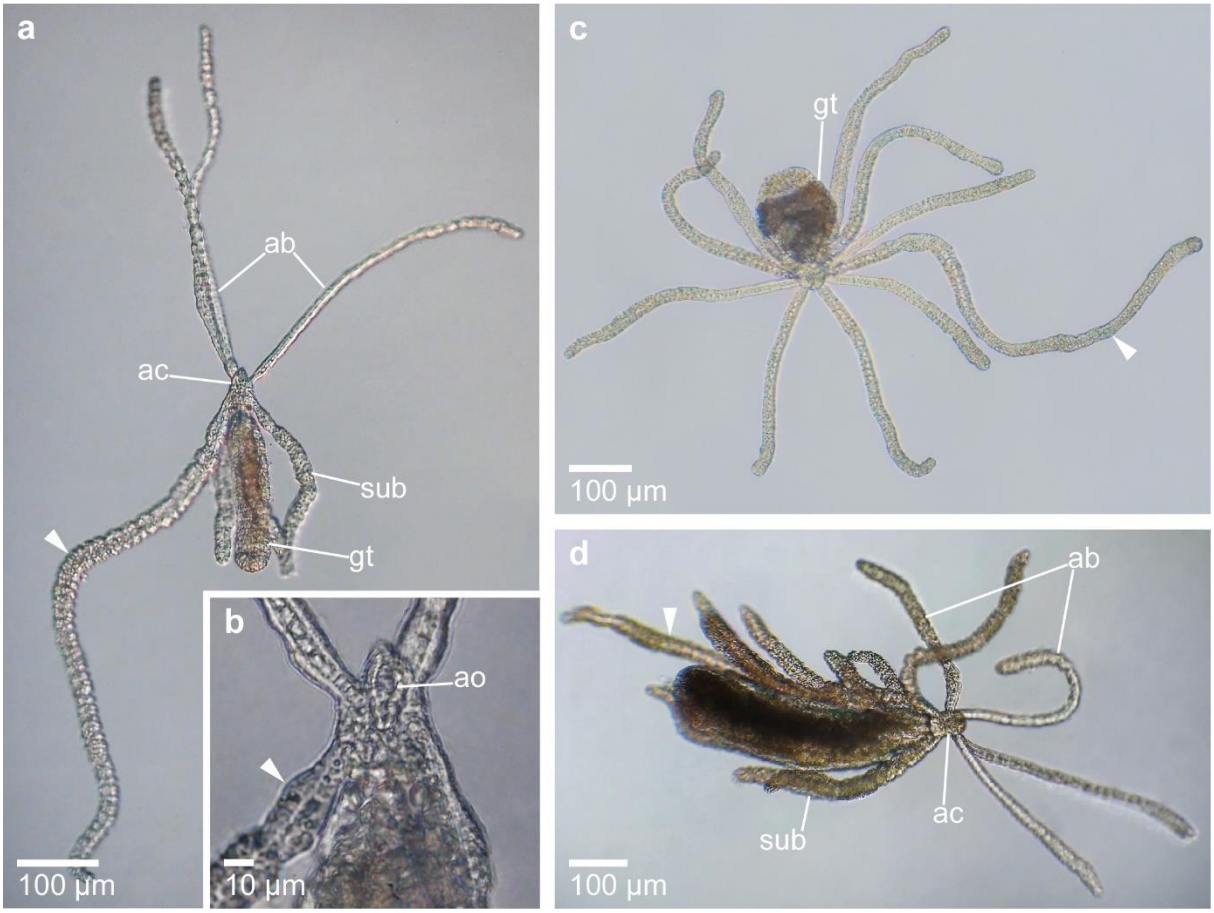

Fig. 2 Light microscopy images of *Halammohydra* species. **a** *H. vermiformis* with 3 times longer tentacle (arrowhead) in the subaboral whorl. **b** Magnification of aboral cone in **a** showing a thickening at the tentacle bases of the subaboral tentacles (arrowhead). **c** *H. octopodides* with 2 times longer tentacle (arrowhead). **d** *H. coronata* with 1.5 times longer tentacle (arrowhead) in the subaboral whorl.

ab= aboral tentacles; ac= aboral cone; ao= adhesive organ; gt= gastric tube; sub= subaboral tentacles

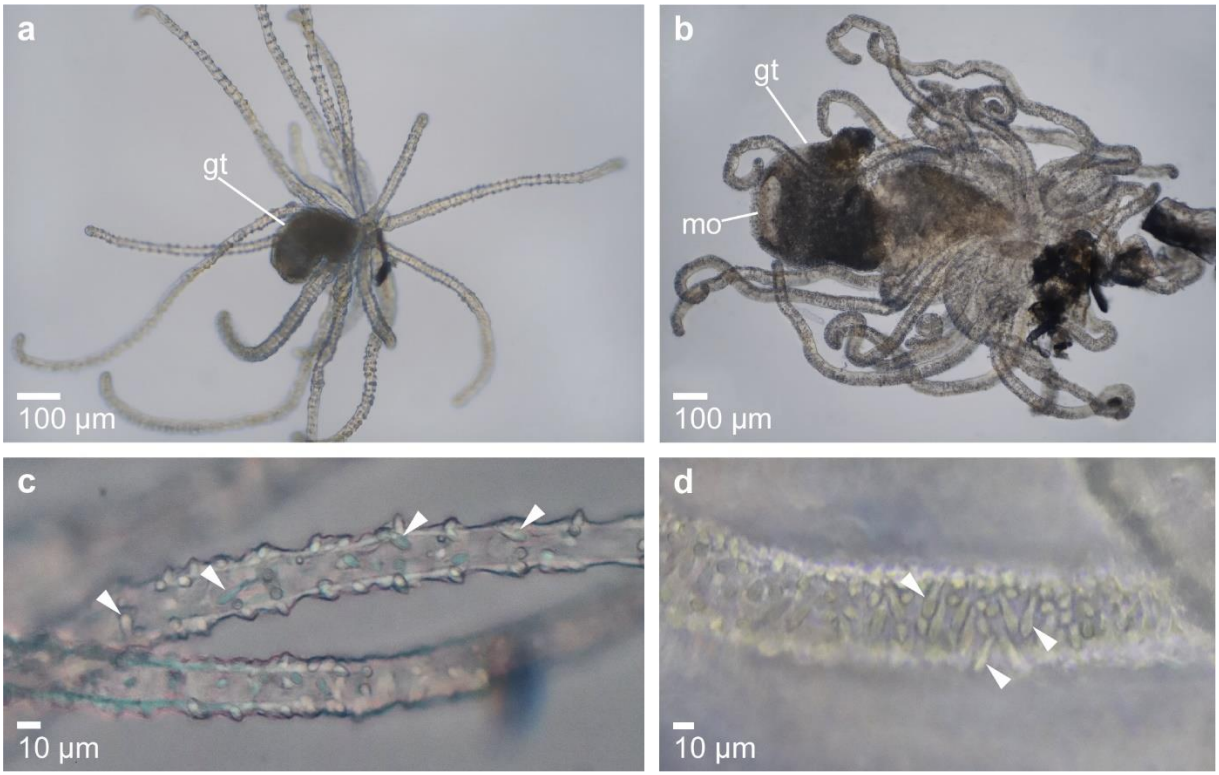

Fig. 3 Light microscopy images of *Halammohydra adherens*. **a** Smaller variant with less tentacles. **b** Larger variant with more tentacles. **c** Magnification of a tentacle of **a** with a few elongated nematocysts (arrowheads). **d** Magnification of tentacle of **c** filled with elongated nematocysts (arrowheads).

gt= gastric tube; mo= mouth opening

Fig. 4 Light microscopy images of *Halammohydra* clusters and new species. **a** *H. teissieri* n. sp. **b** *H. schwedmarki* n. sp. with cup-shaped adhesive organ (arrowhead). **c** *H. kerblae* n. sp. **d** Magnification of aboral cone in **c** showing a club-shaped thickening (arrowhead) at the tentacle bases of subaboral tentacles. **e** *H. joergerae* n. sp **f** "Helgoland/ Sylt" **g** "Roscoff" **h** Magnification of aboral cone in **g** showing a thickening at the tentacle bases of subaboral tentacles. **i** "Azores" (image source Tödter & Schmidt-Rhaesa, 2021)

ab= aboral tentacles; ac= aboral cone; gt= gastric tube; mo= mouth opening; st= statocysts; sub= subaboral tentacles

Morphological and molecular analyses of the meiofaunal cnidarian *Otohydra* sp. (Hydrozoa, Cnidaria) invalidate the taxon Actinulida

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Abstract

Meiofaunal cnidarians are less represented in the interstitial system compared to other groups. The largest genus is *Halammohydra* Remane, 1927, a modified medusa. Together with another medusa of the genus *Otohydra* Swedmark & Teissier, 1958 they classically form the taxon Actinulida. Due to major morphological differences, this relationship is in question and needed to be tested. Specimens of *Otohydra* were acquired from Roscoff in France, extracted from the sediment with the anesthesia-decantation method, investigated in light-microscopy and fixed for molecular and morphological analyses. Two genes (16S and 18S) were sequenced and analyzed in a phylogenetic context. Additionally, the cell structure was investigated with semi and ultra-thin sections. The morphological investigation in general confirms earlier investigations. Additionally, there is a gap in the epidermis on the side with the gonad and it is proposed, that the juvenile is released by a rupture of the epidermis. Molecular analyses reveal no close relationship between *Halammohydra* and *Otohydra*, thus the taxon Actinulida is invalid. Sequences of *Otohydra* are paced within Rhopalonematidae and close to a species of Ptychogastriidae. Whether *Otohydra* belongs to Rhopalonematidae or Ptychogastriidae is not resolved yet and sequences of the recently found *Marsipohydra pacifica* Sanamyan & Sanamyan 2012 might help with the classification.

Keywords

Otohydra, meiofauna, sanger sequencing, transmission electron microscope, Actinulida, Cnidaria

Introduction

Marine sediments harbor a variety of organisms, especially in the microscopic range. This so-called meiofauna is mostly dominated by nematodes and copepods (Giere, 2009). Only a few cnidarian groups are present, which are mainly polyps, but some medusae as well. The genus with the most species is the reduced medusa *Halammohydra* (Schmidt-Rhaesa et al., 2020). It was discovered in 1927 by Remane and placed within the Hydrozoa into Trachylinae (Remane, 1927). With the discovery of another meiofaunal medusae of the genus *Otohydra* Swedmark &Teissier, 1958, the order Actinulida was created, due to similar characters, like the same type of statocysts, the fully ciliated body and the lack of an asexual reproduction (Swedmark & Teissier, 1958, 1959). Additionally, there are similarities in the embryonic development (Swedmark & Teissier, 1959).

But since there are also some major differences between them, both genera are placed in two families within Actinulida, Halammohydridae and Otohydridae (Swedmark & Teissier, 1959). *Halammohydra* has a gastric tube (manubrium), which connects to an aboral cone via a neck. This aboral cone is a reduced umbrella. Therefore, the statocysts and two whorls of tentacles connect to it. It glides in between the sand particles with the aboral pole in front using the cilia covering the whole body. One special feature is the adhesive organ at the tip of the aboral cone. With this, the animal can temporarily adhere to any surface. It has separate sexes and a direct development (Remane, 1927; Tödter & Schmidt-Rhaesa, 2022; Tödter et al., in review).

Contrary to *Halammohydra*, *Otohydra* is hermaphroditic, has no special adhesive organ, only one whorl of tentacles and a more medusa-like appearance with an umbrella and a hypostome. In general, specimens of *Otohydra* are small (500 µm with tentacles), have an ovoid shape and a retractable hypostome with a mouth opening in the center of the tentacle whorl. At the bases of the tentacles, spherical statocysts connect with a thin stalk to the body. Cnidocysts are stenoteles and are especially abundant on the tentacles. *Otohydra* is constantly swimming with the aboral side in front using the cilia (Swedmark & Teissier, 1958). There is only one described species, *Otohydra vagans* Swedmark & Teissier 1958, and one unconfirmed species, *Otohydra tremulans* Lacassagne, 1973 (mentioned on World Register of Marine Species, but the original literature is unavailable). *Otohydra vagans* is described with 12 to 16 tentacles and 8 to 12 statocysts. Large specimens have 24 tentacles and 12 statocysts (Swedmark & Teissier, 1958).

There are only a few records so far. Next to the first finding in Roscoff in France (Swedmark & Teissier, 1958, 1959), there are records from Rovigno in Croatia (Salvini-Plawen, 1966), Ria de Ferrol in Spain (Besteiro & Urgorri, 1988), Otsuchi Bay in Japan (Takashima, 2001) and a doubtful finding on the Canary Islands (Martínez et al., 2019). All locations were subtidal, with either shelly sediment or coarse sand. Except for the first record, not more than two specimens were found per sampling.

In 2012 *Marsipohydra pacifica* Sanamyan & Sanamyan 2012 was discovered in East Kamchatka (Sanamyan & Sanamyan, 2012). It resembles *Otohydra* and thus was placed close to it, into the same family Otohydridae. The validity of the order Actinulida and its relationships are so far not confirmed with molecular data, because sequences of *Otohydra* and *Marsipohydra* are lacking. Up to now, only sequences for *Halammohydra* are available (Collins et al., 2008; Tödter et al., in review). Since there are major differences in the morphology between *Halammohydra* and *Otohydra*, their close relationship may not be true. Hence, molecular sequences of *Otohydra* are needed to prove or disprove the existence of the order Actinulida and, if the existence is disproved, they are needed to find the true phylogenetic position within the hydrozoan tree. This has impact on our understanding of how often the transition to the meiofauna took place within Cnidaria.

For this, the investigation of the structure on a cellular level might be useful as well. Morphological and structural information are important in making conclusions about the relationship to other Hydrozoans. So far, there is only one study with some information about the cellular structure of *Otohydra* (Swedmark & Teissier, 1958). A more detailed reinvestigation with the added knowledge of today in combination with molecular data might help to answer the question about the order Actinulida and the genus *Otohydra*.

In this study, we aim to find the position of *Otohydra* within the hydrozoan tree. Since there are major morphological differences between *Halammohydra* and *Otohydra* we do not suppose a close relationship between both genera. For answering this, we use molecular and morphological methods on *Otohydra* and to reconstruct a phylogenetic tree, as well as, to compare it with the meiofaunal medusa *Halammohydra*.

Material and Methods

Fieldwork

Eight specimens of *Otohydra* sp. were collected at two locations in Roscoff (France), "Basse Plate" (48°44.293 N, 4°02.427 W, 21 m depth, medium coarse sand) and "Trezen ar Skoden" (48°45.571 N, 4°05.640 W, 48 m depth, coarse shell). Sediment samples were collected by dredging from a boat and stored in a cold environment for not more than five days. For the extraction of the animals, the anesthetization-decantation method with 7% magnesium chloride was executed on small subsamples. The liquid part of the sample was poured into a 63 µm sieve and the animals were revived with seawater for the investigation under a stereo-microscope. All specimens were further individually investigated under the compound microscope and documented with a camera (Canon 6D Mark II and AmScope adapter). Since the animals swim constantly, it was tried to anesthetize them with some drops of the $MgCl_2$-solution. This resulted in very fragile specimens and sometimes even in dissolving after too long exposure. Five specimen were fixed in 100 % ethanol for molecular investigations, one in Trumps (combination of sodium cacodylate buffer, formalin, and glutaraldehyde), one was squeezed for the investigation of the cnidome and one got lost during the process.

Molecular work and analysis

In the home institute (Leibniz Institute for the Analysis in Biodiversity Change), specimens in ethanol were digested in proteinase K (50 µm mixture of 45 µl Tris HCl with pH 7.5 and 5 µl proteinase K, 20 mg/ml), purified with magnetic beads (AmpliClean) and amplified (16S, 18S, Table 1).

Thermo-cycler programs were as follows: 94°C/5 min (94°C/50s, 48°C/50s, 72°C/1 min; 35 cycles), 72°C/5 min for 16S and 94°C/4 min (94°C/20s, 57°C20s, 72°C/1 min 45s; 35 cycles, 72°C/7 min for 18S. Sequences were acquired from three specimens, in two specimens this process was unsuccessful. Samples were checked using gel-electrophoresis and sent to Macrogen Europe B. V. (Netherlands) for Sanger sequencing. Gained forward and reverse sequences were checked for quality and assembled in MEGA X (Kumar et al., 2018). The phylogenetic reconstruction was based mainly on the dataset of Collins et al. (2008) and sequences were downloaded from NCBI GenBank. Additionally, some sequences of the result of BLASTn and the dataset for *Halammohydra* of (Tödter et al., in review) were added (Supplementary Material). Alignments were done with MAFFT (Katoh et al., 2019) in the default settings, checked visually and conserved positions were excluded using Gblocks 0.91b (Dereeper et al.,

2008) with default settings. Following analyses were done individually and in a concatenated supermatrix of both genes.

Phylogenetic analyses were conducted in MrBayes (Ronquist et al., 2012) for Bayesian Inference (BI) and IQ tree (Trifinopoulos et al., 2016) for Maximum Likelihood (ML). To find the best substitution model for the BI, PartitionFinder2 (Lanfear et al., 2016) was used with the corrected Akaike Information Criterion (AICc) and a greedy search scheme. In MrBayes, the anaylsis ran for 50 000 000 generations with a burnin of 10 %. In IQ tree, ML analyses were conducted with default settings. Resulting log files were checked using Tracer v1.7.1 (Rambaut et al., 2018) and, if they were insufficient, the analyses were repeated with an increased number of generations. Tree files were edited using FigTree v.1.44 and Adobe Illustrator. Sequences of species of Hydroidolina were used as an outgroup.

Morphological work

The specimen fixed in Trumps was postfixed in osmium tetroxide (1%, in sodium-cacodylate buffer) and embedded in LR White resin following a modified protocol by McDonald (1983) and Purschke et al. (1991). Semi-thin (0.5 µm) and ultrathin (70 nm) sections were done for the entire animal and stained with toluidine blue (semi-thin sections) or contrasted with lead citrate and uranyl acetate (ultrathin sections). Slides of semi-thin sections were scanned with a slide scanner (Olympus Slideview V5200) and the corresponding software (Sideview VS200 ASW). Ultrathin sections were investigated with a Zeiss EM902A transmission electron microscope (TEM) and digital photos were taken. All images were edited using ImageJ (version 1.52a), Adobe Photoshop and Adobe Illustrator.

Results

Phylogenetic reconstruction

Sequences (16S and 18S) of three specimens are acquired and used in the analyses. All three sequences cluster close together and therefore indicate that they belong to the same species. Phylogenetic trees of the single sequences (16S or 18S) show similar results as the concatenated dataset (16S and 18S) of both analyses (BI and ML), thus only the concatenated tree is shown here (Fig. 1). The classical taxa Trachymedusae and Rhopalonematidae are paraphyletic. Sequences of *Otohydra* sp. are placed within "Trachymedusae" close to the species *Ptychogastria polaris* Allman, 1878, which is the only representative of Ptychogastridae in the dataset, and the "rhopalonematid" *Arctapodema* sp. Dall, 1907 (Fig. 1). These three species form a cluster with high support, which is nested within the "Rhopalonematidae". *Ptychogastria polaris* and *Otohydra* sp. form a cluster with a less high support in both phylogenetic analyses. The sequences of *Halammohydra* Remane, 1927 are nested within Rhopalonematidae as well, but not in direct relationship to *Otohydra* sp. sequences. They form a sister group of the cluster containing six "rhopalonematid" species, which then together form a sister group to the cluster containing *Otohydra* sp., *Arctapodema* sp. and *Ptychogastria polaris*.

Morphology

Of the eight specimens found, two are juveniles with 4 short tentacles. The size of the umbrella of the adults ranges from 165-430 µm in length and the whole body (with tentacles) ranges from 292-635 µm. Tentacles have an irregular length (Fig. 2A) and the total number varies from 9 to 14 (except in the juveniles). The number of statocysts is mostly about half the number of tentacles, which is 3 to 7. Nematocysts are present on the whole body, with some small cysts on the umbrella and the tentacles and larger heteronemes concentrate at the tentacle tips (Fig. 2B). The hypostome was not documented

as everted and only visible in a top down view, when the animal adhered to the slide (Fig. 2C). Internal structures could not be documented and there are no visible embryos.

Specimens were found swimming constantly with the aboral pole in front and tentacles trailing behind. When the animals was stressed, it contracted or adhered with the hypostome to a surface (Fig. 2C). In some cases, the adhered specimen was spinning in a circle around the center.

The internal structure of *Otohydra* was studied by TEM in one animal and consists of an epidermis and a gastrodermis separated by a thin layer of mesoglea (Fig. 3, 4 & 5). In several regions, epidermal cells are branchin and interdigitating, which results in multiple cell sections and the impression of a multilayered epidermis. The epidermis is thicker on one side of the animal (Fig. 3 & 4B) throughout the whole body. The surface is covered with cilia and the mesoglea is present between gastrodermis and epidermis in most sections. In the following, the structures will be explained from aboral to oral.

The aboral part of the umbrella consists of a thicker epidermis with several cells. In oral direction the thickness and number of cell sections decreases (Fig. 4B). Basally of the epithel, the number of cell sections is increased and they are filled with myofilaments (Fig. 4C). Cells of the aboral gastrodermis are huge and filled completely with a vacuole which pushes the cytoplasm to the edge of the cells (Fig. 4B, D). In oral direction the amount of electron dense secretory cells filled with secretion vesicles increases and the orientation of the cells in the epithelium is to the center, building a wide lumen (Fig. 4E & F). The surface of the gastrodermis contains many electron dense inclusions and cilia are present in the lumen (Fig. 4F).

In oral direction, the gastrodermis increases in thickness on one side of the gastrovascular lumen (Fig. 5B). In this region, cells of the gastrodermis contain more electron dense inclusions and less vacuoles. Within the gastrodermis a gonad is situated and takes up about half of the epithelium on that side of the lumen. It contains cells of different sizes, some with huge cell nuclei and others with smaller ones, and it is surrounded by an electron dense structure (Fig 5C & D). The gonad touches the mesoglea in some locations but gastrodermal cells surround it in most parts.

There are two gaps in the epidermis and mesoglea on one side of the specimen. One of them is located aborally, less pronounced and with a few lose cells, and the other is located orally on the height and side of the gonad (Fig. 5E). This gap is more pronounced. No clear destruction of tissue is visible at both gaps. Orally, the specimen was cut oblique, thus the structure of the retracted hypostome could not be documented as a whole (Fig. 5F). It has the same structure as the rest of the body, but the cells are narrower and the gastrodermis is almost an epithelium of single cells with many inclusions. Since the hypostome is retracted, its surrounding epidermis touches the epidermis of the umbrella, creating a thin darker stained line (Fig. 5F).

The gastrodermis continues into the tentacles as a rod of cells (Fig. 5F & G). It is surrounded by a layer of mesoglea and the epidermis. There are concentrations of myofilaments in the basal epidermis surrounding the mesoglea and gastrodermis (Fig. 5G). Unfortunately, there are no statocysts visible in this animal, neither in the living animal, nor in the sections, hence they could not be documented.

Two different types of nematocysts are documented in light microscopy and ultra-thin sections (Fig. 6). In ultra-thin sections, several heteronemes with a prominent shaft in the undischarged capsule are documented, as well as some smaller capsules with a slightly asymmetrical shape in longidutinal sections (Fig. 6A). A squeezed specimen shows several smaller capsules and when they are discharged, the shaft has a slight dilation distally (Fig. 6B). The heteronemes are documented with two size classes

and have a basal dilation in the everted capsule (Fig. 6C). Larger capsules are documented in higher numbers in the squeezed specimen.

Discussion

This is the first study investigating *Otohydra* using molecular data and ultrastructural methods. Specimens were collected at "Trezen ar Skoden" and "Basse Plate" in Roscoff, but only the first location is known to the literature (Swedmark & Teissier, 1958), which adds "Basse Plate" with a different sediment type to the locations for *Otohydra*.

Specimens were not highly abundant, which restricted the data collection, since animals were needed for molecular and morphological methods. There were some challenges concerning the light microscopic investigations as well. Specimens were swimming constantly, thus $MgCl_2$ was used to anesthetize them slightly. This resulted in very fragile animals, which are easily destroyed. Additionally, when the animal adhered to the slide with the mouth opening, it was impossible to release them without destroying. Unfortunately, this resulted in only one intact specimen for ultrastructural investigation, which was also lacking statocysts for an unknown reason. There were no destructions documented in this specimen. Nevertheless, the sections were of great quality and gave a good overview of the whole body.

Collected specimens were most likely *Otohydra vagans* Swedmark & Teisser, 1958, since they were found at the exact same locations and sediment conditions as described. The number of tentacles and statocysts is slightly lower or overlap the lower described range of 12-16 tentacles and 8-12 statocysts. This can be due to the young life stage of the specimens, since juveniles have up to 8 tentacles and 4 statocysts and the organization of an adult (Swedmark & Teissier, 1958). The number of tentacles and statocysts increases after that stage. Additionally, no specimen was documented with an embryo.

The ultrastructure of the specimen shows a typical structure of a Hydrozoa with epidermis, mesoglea and gastrodermis consisting of secretory and digestive cells (Thomas & Edwards, 1991). Most of the observed structures confirm the description by Swedmark & Teissier (1958). The investigated individual does not have an incubation cavity, which should be located between the ectoderm and endoderm, but a previously described gap in the mesoglea on the level of the gonad is documented here as well (Swedmark & Teissier, 1958). Additionally, here is a gap in the epidermis. It is possible, that the specimen already gave birth via a rupture of the epidermis and thus the incubation cavity regressed. The aboral gap in the epidermis is not connected to the gonad, so it might be a site of healing. *Otohydra* was described as hermaphroditic with two gonads but in this specimen only one gonad is visible. All these results have to be taken with caution, since only one individual was investigated ultrastructurally.

For *Otohydra* only one type of nematocysts were described before, the stenoteles (Swedmark & Teissier, 1958). This study shows at least two types with two size classes of one type. The documented heteronemes with a prominent shaft and a basal dilation in the discharged state are very likely stenoteles (Östman, 2000). There is no information about different size classes of this type but Swedmark & Teissier (1958) also documented the increase of stenoteles, especially the large ones, at the tip of the tentacles, as it is documented here as well. The second type of nematocysts was not observed before. It is potentially an eurytele, because of the shape of the capsule and the distal dilation of the shaft in the discharged state (Östman, 2000).

This study is the first containing molecular sequences of *Otohydra* and analyzing them in a phylogenetic context. The results suggest an origin within Rhopalonematidae (Trachymedusae) but not in close

relation to *Halammohydra*. Hence, the taxon Actinulida does not exist. The major morphological differences between *Otohydra* and *Halammohydra* support this as well.

The study of Collins et al. (2008) did not contain representatives of the group Ptychogastriidae but one was added in this study. Here, *Otohydra* is clustered close to *Ptychogastria polaris* Allman, 1878, but both are nested within "Rhopalonematidae", thus it is not clear if *Otohydra* belongs to Ptychogastriidae or "Rhopalonematidae". Morphologically, there are no obvious similarities to the bentho-pelagic medusae of Ptychogastriidae, other than the free ecto-endodermal statocysts and the simple gonads (Galea et al., 2016). The only connection might be the recently discovered meiofaunal medusa *Marsipohydra pacifica* Sanamyan & Sanamyan, 2012. This species has characters close to *Otohydra*, such as cilia covering the whole body, no subumbrellar cavity and one ring of tentacles (Sanamyan & Sanamyan, 2012). On the other hand, it has two types of tentacles, a filiform and an adhesive type, and is gonophoric, which are characters of Ptychogastriidae (Galea et al., 2016). There are some major differences as well. *Marsipohydra pacifica* has an eight lobed umbrella, which is not present in *Otohydra* or in Ptychogastriidae. Additionally, the position of the gonads differs. Gonads in *Otohydra* are located in the umbrella, in Ptychogastriidae they sit on the manubrium and in *M. pacifica* four male testis or one female brood pouch are attached to the manubrium (Galea et al., 2016; Sanamyan & Sanamyan, 2012). The relationship of the group Ptychogastriidae and the two species *O. vagans* and *M. pacifica* is not fully resolved yet.

In conclusion, this study invalidates the existence of the taxon Actinulida and shows another independent development of the meiofaunal way of life. Molecular information of *M. pacifica* would be interesting and might help to answer questions regarding the position of *Otohydra*.

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Table and figures

Table 1: Used primers and their source.

	primer		source
16S	SF2	TCGACTGTTTACCAAAAACATA	Allen G. Collins, pers. commun.
	SR2	ACGGAATGAACTCAAATCATGTAAG	
18S	forward	CCGAATTCGTCGACAACCTGGTTGATCCTGCCAGT	Medlin et al. 1988
	reverse	CCCGGGATCCAAGCTTGATCCTTCTGCAGGTTCACCTAC	

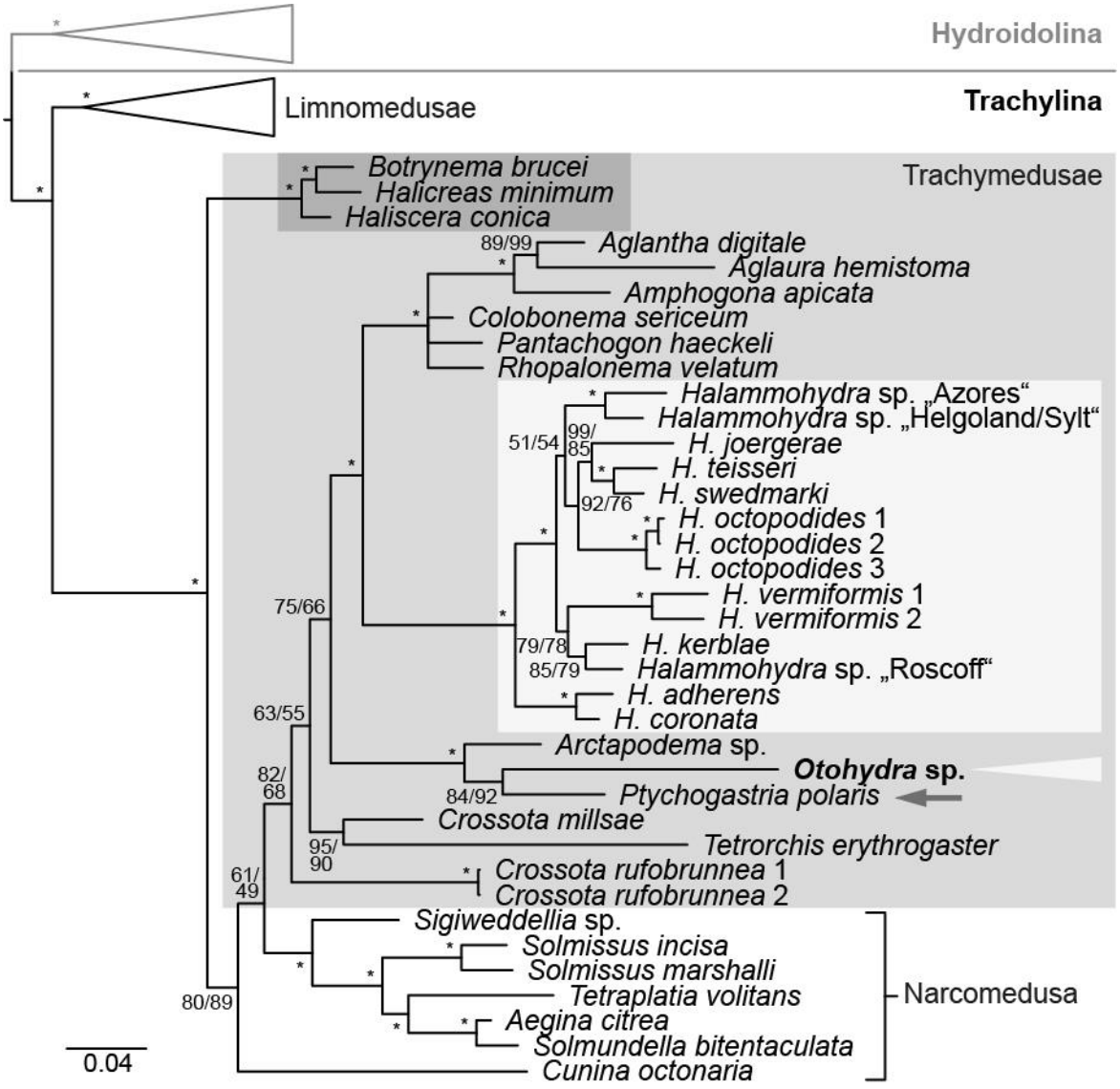


Figure 1: Phylogenetic tree of the concatenated dataset (16S and 18S) with support values of BI/ML (posterior probability/bootstrap value). Nodes with an * have a support > 95/95. The “Trachymedusae” contains representatives of Halicreatidae (dark grey rectangle), Ptychogastriidae (arrow), *Halammohydra* (light grey rectangle) and “Rhopalonematidae” (remaining representatives in the medium grey rectangle

Figure 2: Light microscopy images of the habitus of *Otohydra* sp. specimens. **(A)** Lateral view of the whole animal showing the umbrella (u) and tentacles (t). **(B)** Magnification of one tentacle with a concentration of large nematocysts at the tip (arrowheads). **(C)** Top-down view of the whole animal showing the retractable hypostome (arrowhead) with mouth opening (mo) in the center.

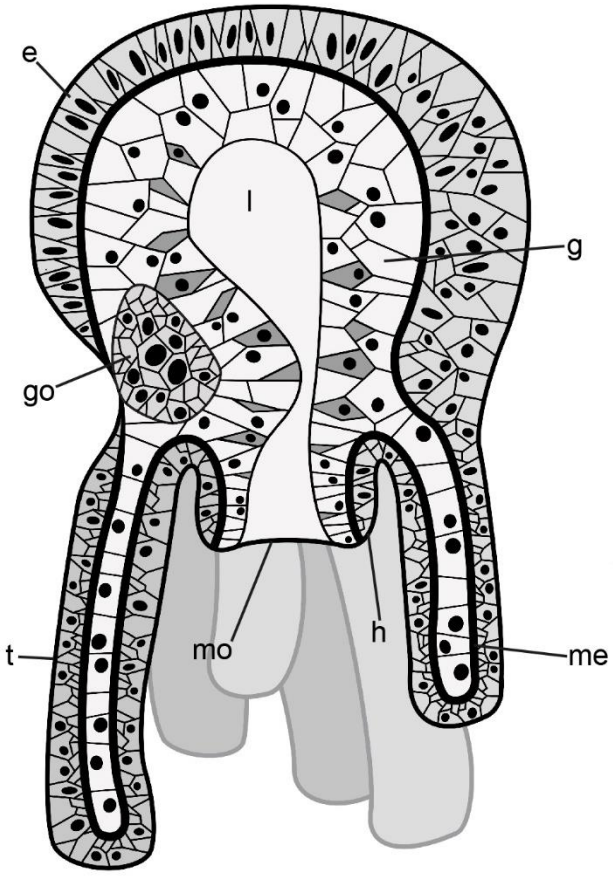
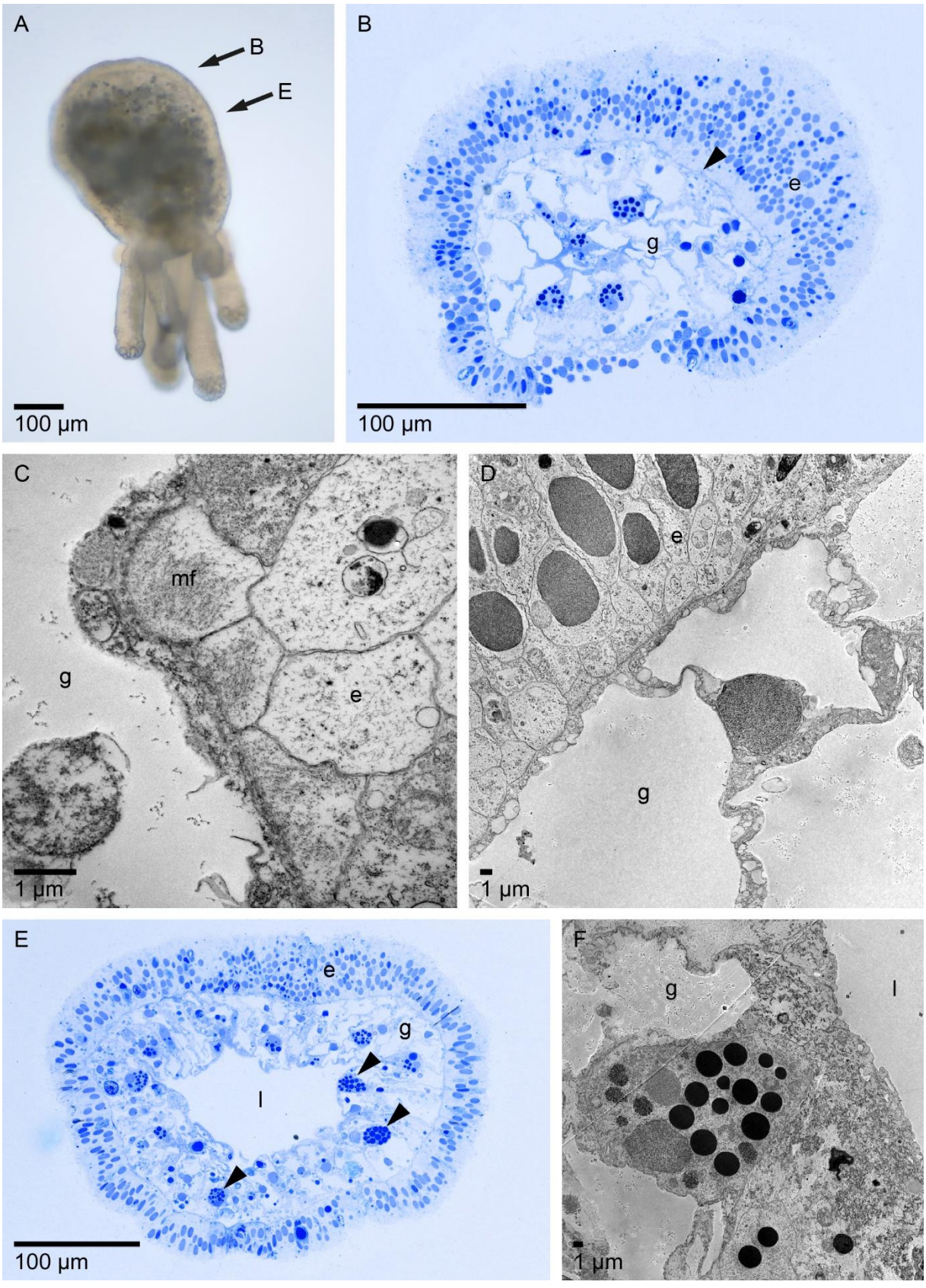


Figure 3: Schematic distribution of cells in longitudinal section of *Otohydra*, based on semi and ultra-thin cross sections. The body is connected to the tentacles (t) with a hypostome (h) and the mouth opening (mo) in the center. Mesoglea (me) seperates epidermis (e) and gastrodermis (g). Black cells in the gastrodermis are secretory cells. The gonad (go) is embedded in the gastrodermis.

Figure 4 (on page LXXII): Semi (B, E) and ultra-thin (C, D, F) sections of the aboral umbrella of *Otohydra* sp. **(A)** Position of sections are marked in the light microscopic image of the specimen with arrows. **(B)** Aboral section of umbrella, showing epidermis (e) and gastrodermis (g) separated by a thin mesoglea (arrowhead). **(C)** Magnification of concentrated myofilaments (mf) in the epidermis. **(D)** Magnification of epidermis (e) and gastrodermis (g). **(E)** Central/ aboral section of umbrella with wide lumen (l) and secretory cells (arrowheads) of the gastrodermis. **(F)** Magnification of a secretory cell.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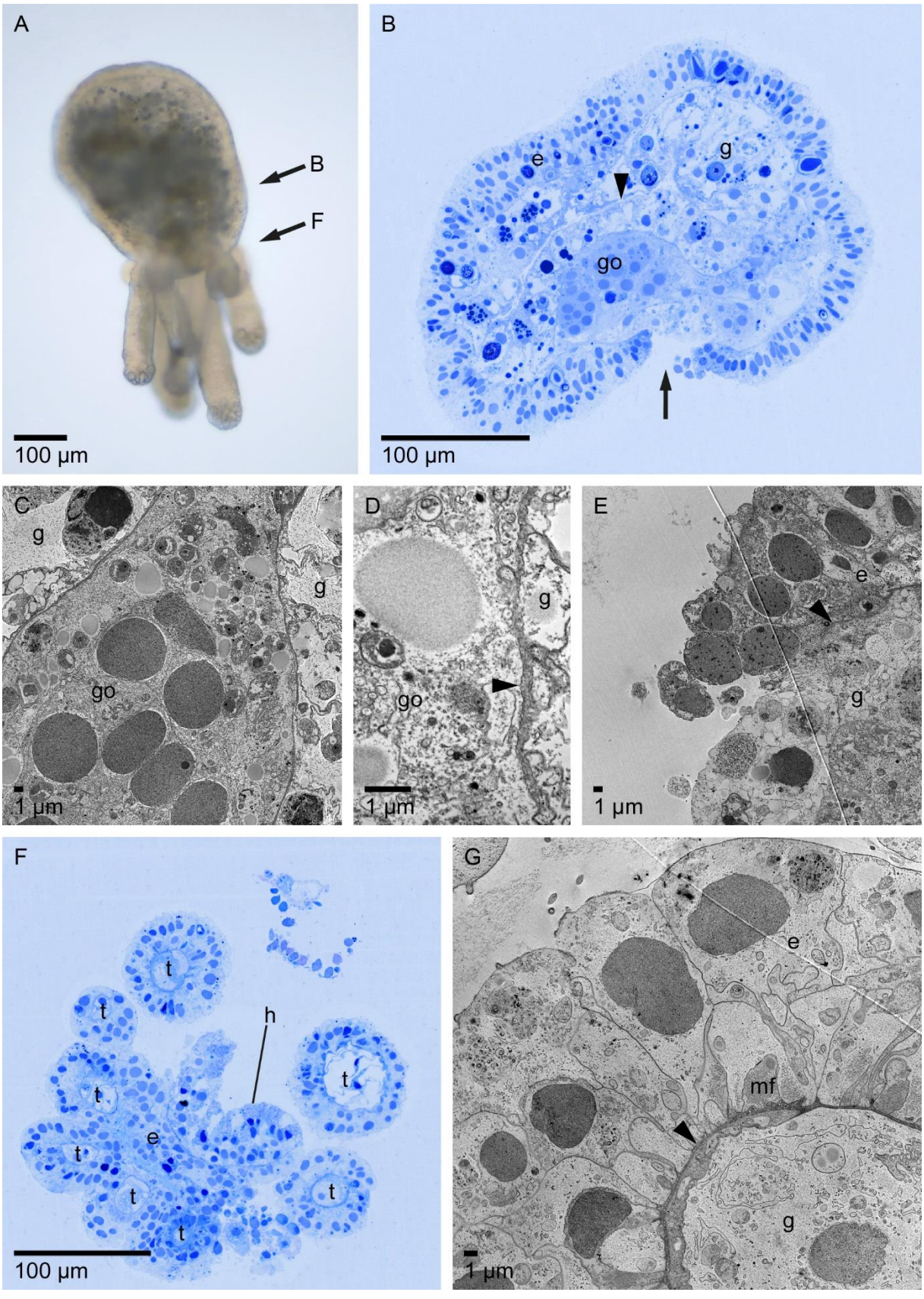
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Figure 5 (on page LXXIII): Semi (B, F) and ultra-thin (C-E, G) sections of the oral umbrella of *Otohydra* sp. **(A)** Position of sections are marked in the light microscopic image of the specimen with arrows. **(B)** Central/ oral section of the umbrella, showing the gonad (go) in the gastrodermis (g) shifting the lumen (arrowhead) to one side and almost closing it. The epidermis (e) surrounds the gastrodermis, except on the side of the gonad (arrow). **(C)** Magnification of the gonad. **(D)** Magnification of the electron dense structure (arrowhead) surrounding the gonad. **(E)** Magnification of the gap in the epidermis. The mesoglea ends on both sides of the gap (arrowhead). **(F)** Oblique section of the mouth opening and half of the hypostome (h). There are several tentacles (t) visible. **(G)** Magnification of a tentacle, with the same structure of epidermis, gastrodermis and mesoglea (arrowhead) separating both. There are several cell sections in the basal epidermis filled with myofilaments (mf).

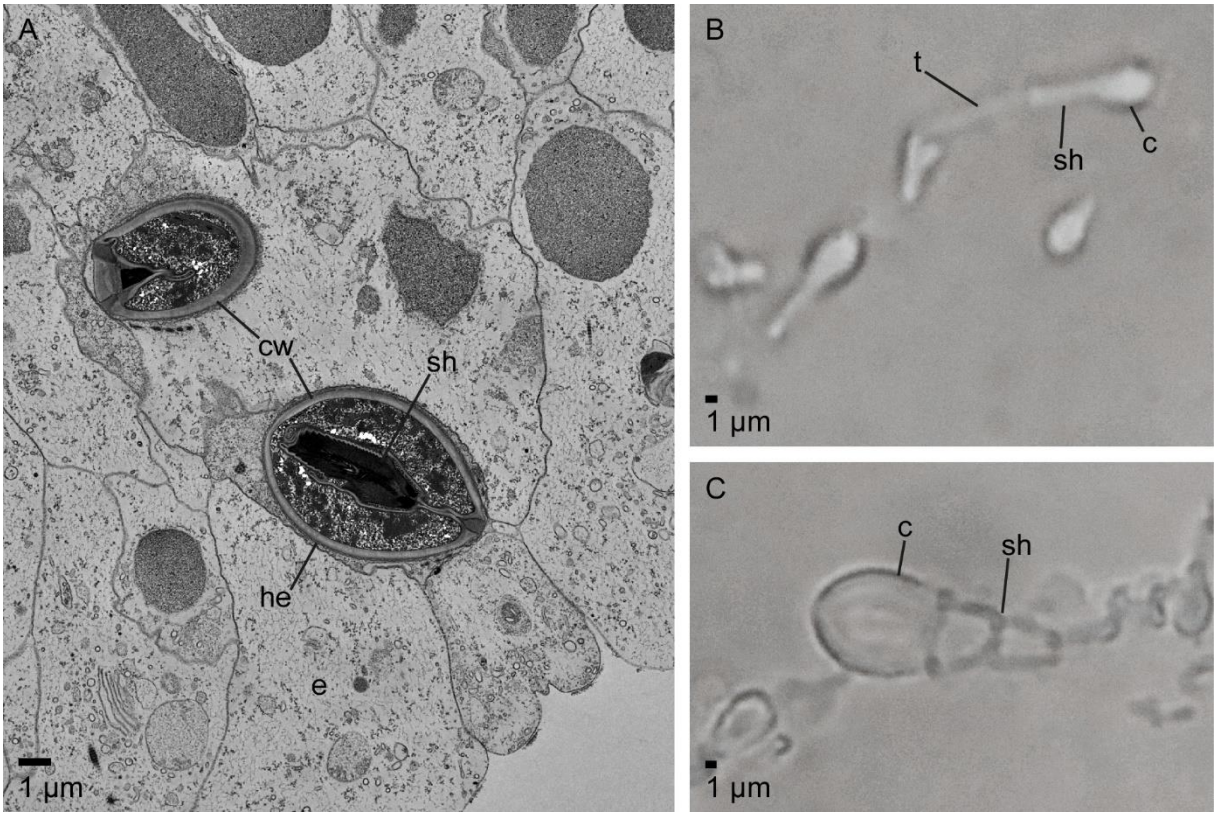

Figure 6: Two types of nematocysts in *Otohydra*. **(A)** Ultra-thin section of two nematocysts in the epidermis (e) with capsule wall (cw) and shaft (sh). The one capsule is a heteroneme (he). **(B)** Light microscopy image of smaller nematocysts of a squeezed specimen, showing the capsule (c) and an everted shaft (sh) with tubule (t). **(C)** Light microscopy image of a larger heteroneme with a capsule (c) and an everted shaft (sh).

10. Appendix

Appendix 1 (*Study III*, SI 1): GenBank numbers of outgroup

Supplementary Information 1: GenBank numbers of the outgroup used in the analyses.

taxa	16S	18S	CO1
Aglantha digitale	EU293985.1	EU247821.1	GQ120073.1
Aglaura hemistoma	EU293984.1	EU247820.1	GQ120074.1
Pantachogon haeckeli	EU293988.1	AF358062.1	GQ120079.1
Rhopalonema velatum	EU293992.1	EU247819.1	GQ120080.1

Appendix 2 (*Study III*, SI 2): Species delimitation tests

Supplementary Information 2: Results of the species delimitation tests conducted on every gene individually and of the concatinated supermatrix in GMYC and bPTP (all), showing the specimen number (Ind#), *GenBank number* (*not included here, because the sequences are not uploaded yet*) and the locality they were found. Resulting mOTUs were color coded. Clusters were named accordingly (species/cluster). Prior maximal distance of ABGD, results of the Likelihood ratio test of GMYC and the support values for every mOTU in bPTP is shown as well.

			species delimitation										
			ABGD			**GMYC**				**bPTP**			
species/cluster	**ind#**	**station**	**16S**	**18S**	**CO1**	**16S**	**18S**	**CO1**	**all**	**16S**	**18S**	**CO1**	**all**
			0,013	**0,003**	**0,003**	***	**	***	***				
Tenerife 1	T5	Los Abades, Punta de la Leproseria									0,999		1
	T6	Los Abades, Punta Realejeros									0,947		1
	T9	Arcos de Playa San Juan									0,947		1
single ind	R61	Roscoff, Basse Plate								1			1

Continuation of SI 2

species/cluster	ind#	station	ABGD			GMYC				bPTP			
			16S	18S	CO1	16S	18S	CO1	all	16S	18S	CO1	all
H. adherens	R29	Roscoff, Drezen ar Skoden								0,814			0,828
	R63	Roscoff, Drezen ar Skoden									0,983		
	R93	Roscoff, Bazin Malvog								0,892			0,992
	R94	Roscoff, Drezen ar Skoden											
	R119	Roscoff, Drezen ar Skoden											
H. coronata	H5	Helgoland, subtidal								0,903	0,674	0,941	0,936
	H8	Helgoland, subtidal											
	H10	Helgoland, subtidal											
	H11	Helgoland, subtidal											
	H18	Helgoland, subtidal											
	H24	Helgoland, subtidal											
	H25	Helgoland, subtidal											
	H27	Helgoland, subtidal											
	R39	Roscoff, Drezen ar Skoden											
	R59	Roscoff, Basse Plate											
Brazil, single ind	D1	Brazil, Fernando de Noronha, Rata channel								1	1	1	1
Azores	A3	Azores, Praia dos Moinhos								0,994	0,957	0,99	0,974
	A4	Azores, Praia dos Moinhos									0,957		
Helgoland/ Sylt	S1	Sylt, Hörnum								0,979	0,378		0,936
	H1	Helgoland, subtidal									0,569	1	0,998
	H36	Helgoland, subtidal									0,414		0,998
	H39	Helgoland, subtidal									0,378		0,936

Continuation of SI 2

species/cluster	ind#	station	ABGD			GMYC				bPTP			
			16S	18S	CO1	16S	18S	CO1	all	16S	18S	CO1	all
H. vermiformis 1	H15	Helgoland, subtidal								0,816		0,943	0,959
	H26	Helgoland, subtidal											
	H31	Helgoland, subtidal											
	H33	Helgoland, subtidal									0,673		
	H34	Helgoland, subtidal											
	H37	Helgoland, subtidal											
	H38	Helgoland, subtidal											
	H43	Helgoland, subtidal											
	H47	Helgoland, subtidal											
	H48	Helgoland, subtidal											
	H50	Helgoland, subtidal											
	H51	Helgoland, subtidal											
	H55	Helgoland, subtidal											
	H56	Helgoland, subtidal											
	H57	Helgoland, subtidal											
	H58	Helgoland, subtidal											
	H61	Helgoland, subtidal											
	H62	Helgoland, subtidal											
	H63	Helgoland, subtidal											
	H64	Helgoland, subtidal											
	H65	Helgoland, subtidal											
	H66	Helgoland, subtidal											
	H67	Helgoland, subtidal											
	H68_1	Helgoland, subtidal											
	H68_2	Helgoland, subtidal											
	H72_1	Helgoland, subtidal											
	H72_2	Helgoland, subtidal											

Continuation of SI 2

species/cluster	ind#	station	ABGD			GMYC				bPTP			
			16S	18S	CO1	16S	18S	CO1	all	16S	18S	CO1	all
H. vermiformis 2	S2	Sylt, Hörnum								0,83	0,647		0,959
	S7	Sylt, Hörnum											
	S8	Sylt, Hörnum										0,915	
	S9	Sylt, Hörnum											
	S11	Sylt, Hörnum											
	S14	Sylt, Hörnum											
	S15	Sylt, Hörnum											
	S16	Sylt, Hörnum											
	S17	Sylt, Hörnum											
	S19	Sylt, Hörnum											
	H2.1	Helgoland, intertidal											
	H2.2	Helgoland, intertidal											
	H2.3	Helgoland, intertidal											
	H2.7	Helgoland, intertidal											
	H2.8	Helgoland, intertidal											
	H2.12	Helgoland, intertidal											
	H2.13	Helgoland, intertidal											
	H2.14	Helgoland, intertidal											
	H2.15	Helgoland, intertidal											
	H2.17	Helgoland, intertidal											
	H2.19	Helgoland, intertidal											
	H2.23	Helgoland, intertidal											
	H2.27	Helgoland, intertidal											
	H2.28	Helgoland, intertidal											
	H2.29	Helgoland, intertidal											
	H2.30	Helgoland, intertidal											

Continuation of SI 2

species/cluster	ind#	station	ABGD			GMYC				bPTP			
			16S	18S	CO1	16S	18S	CO1	all	16S	18S	CO1	all
H. vermiformis 2	H2.31	Helgoland, intertidal											
	H16	Helgoland, subtidal											
	D2	Denmark, Ellekildehage											
	D3_2	France, Archachon											
	D3_3	France, Archachon											
	D6_4	Denmark, Ellekildehage											
H. swedmarki	R71	Roscoff, chenal l'Ile Verte								0,931		0,974	0,968
	R72	Roscoff, chenal l'Ile Verte											
	R73	Roscoff, chenal l'Ile Verte									1		
	R79	Roscoff, chenal l'Ile Verte									1		
H. teisseri	H2	Helgoland, subtidal								0,841			0,948
	H12	Helgoland, subtidal									0,638	0,928	
	H14	Helgoland, subtidal										0,927	
	H23	Helgoland, subtidal											
	H52	Helgoland, subtidal										0,932	
	R6	Roscoff, Basse Plate											
	R8	Roscoff, Basse Plate											
	R24	Roscoff, Drezen ar Skoden											
	R74	Roscoff, Bazin Malvog											
	R78	Roscoff, Bazin Malvog											
	R87	Roscoff, Bazin Malvog											
	R88	Roscoff, Bazin Malvog											
	R92	Roscoff, Ognon											
	R95	Roscoff, Basse Plate											

Continuation of SI 2

species/cluster	ind#	station	ABGD			GMYC				bPTP			
			16S	18S	CO1	16S	18S	CO1	all	16S	18S	CO1	all
H. joergerae	A6	Azores, Riberinha										0,958	0,7
	A11	Azores, Riberinha											
	A14	Azores, Riberinha									0,679		
	A17	Azores, Riberinha											
	A19	Azores, Riberinha											
	A20	Azores, Riberinha										0,983	
	A21	Azores, Riberinha											
	A22	Azores, Riberinha								0,709			
	A23_3	Azores, Riberinha											
	A23_4	Azores, Riberinha											
	A23_5	Azores, Riberinha											
	A9	Azores, Riberinha											
	A23_2	Azores, Riberinha											
H. octopodides 1	H9	Helgoland, subtidal								0,612	0,973	0,948	0,729
	H71	Helgoland, subtidal											
	R12	Roscoff, Basse Plate											
	R19	Roscoff, Basse Plate											
	R20	Roscoff, Basse Plate									0,966		
	R26	Roscoff, Drezen ar Skoden											
	R38	Roscoff, Basse Plate									0,838		
	R46	Roscoff, Basse Plate											
	R52	Roscoff, Basse Plate											
	R60	Roscoff, Basse Plate											
	R77	Roscoff, Ognon									0,922		
	R96	Roscoff, Basse Plate											

Continuation of SI 2

species/cluster	ind#	station	ABGD 16S	ABGD 18S	ABGD CO1	GMYC 16S	GMYC 18S	GMYC CO1	GMYC all	bPTP 16S	bPTP 18S	bPTP CO1	bPTP all
H. octopodides 1	R106	Roscoff, Basse Plate											
	R114	Roscoff, Basse Plate											
	R126	Roscoff, Basse Plate											
H. octopodides 2	R13	Roscoff, Basse Plate									0,495		0,585
	R16	Roscoff, Basse Plate											
	R41	Roscoff, Basse Plate											
	R57	Roscoff, Basse Plate											
	R84	Roscoff, Drezen ar Skoden											
	R111	Roscoff, Drezen ar Skoden											
H. octopodides 3	R47	Roscoff, Basse Plate								0,996			0,998
	R103	Roscoff, Drezen ar Skoden											
H. kerblae	R2	Roscoff, Basse Plate								0,98	0,913	1	0,779
	R36	Roscoff, Basse Plate											
	R51	Roscoff, Basse Plate											
	R91	Roscoff, Ognon											
	R125	Roscoff, Basse Plate											
single ind	R115	Roscoff, Basse Plate								1		1	1
single ind	R75	Roscoff, Bazin Malvog								1	1		1
	R104	Roscoff, Drezen ar Skoden								1			1

Continuation of SI 2

species/cluster	ind#	station	ABGD			GMYC				bPTP			
			16S	18S	CO1	16S	18S	CO1	all	16S	18S	CO1	all
Tenerife 2	T1	Los Abades, Punta de la Leproseria								0,811			0,883
	T2	Los Abades, Punta de la Leproseria											
	T3	Los Abades, Punta de la Leproseria											
single ind	R118	Roscoff, Drezen ar Skoden								0,831			1
Roscoff	R1	Roscoff, Basse Plate								0,755	0,437	0,841	0,831
	R17	Roscoff, Basse Plate											
	R34	Roscoff, Basse Plate											
	R44	Roscoff, Basse Plate											
	R80	Roscoff, Basse Plate										0,994	
	R81	Roscoff, Basse Plate											
	R97	Roscoff, Basse Plate											
	R99	Roscoff, Banc de Bistarz											
	R101	Roscoff, Banc de Bistarz											
	R123	Roscoff, Basse Plate											

Appendix 3 (*Study III*, SI 3): k2p values

Supplementary Information 3.1: Genetic divergence over sequence pairs between groups of 16S sequences.The number of base substitutions per site from averaging over all sequence pairs between groups are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter model [1]. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). This analysis involved 134 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 496 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [2].

	H. joergerae	Azores	outgroup	single_ind D1	*H. vermiformis*_2	*H. coronata*	*H. teisseri*	*H. vermiformis*_1	Helgoland_Sylt	*H. octopodides*_1	Roscoff
H. joergerae		0,0367	0,0626	0,0394	0,0455	0,0331	0,0308	0,0438	0,0337	0,0325	0,0376
Azores	0,2971		0,0591	0,0330	0,0390	0,0293	0,0353	0,0373	0,0232	0,0349	0,0300
outgroup	0,6174	0,5799		0,0484	0,0634	0,0511	0,0488	0,0565	0,0556	0,0491	0,0506
single_ind_D1	0,3315	0,2663	0,5109		0,0387	0,0336	0,0378	0,0465	0,0306	0,0364	0,0315
*H. vermiformis*_2	0,4161	0,3252	0,6629	0,3354		0,0395	0,0427	0,0241	0,0333	0,0440	0,0364
H. coronata	0,2692	0,2409	0,5092	0,2680	0,3817		0,0341	0,0430	0,0303	0,0344	0,0313
H. teisseri	0,2562	0,2872	0,5428	0,3282	0,3527	0,3025		0,0423	0,0310	0,0212	0,0317
*H. vermiformis*_1	0,4055	0,3257	0,6204	0,3814	0,1866	0,3613	0,3849		0,0395	0,0403	0,0353
Helgoland_Sylt	0,2666	0,1439	0,5606	0,2503	0,2898	0,2464	0,2547	0,3440		0,0323	0,0267
*H. octopodides*_1	0,2438	0,3022	0,5160	0,2999	0,3986	0,2940	0,1541	0,3663	0,2708		0,0304
Roscoff	0,3173	0,2184	0,5376	0,2499	0,2982	0,2497	0,2492	0,3029	0,2026	0,2526	
*H. octopodides*_3	0,2694	0,3007	0,5246	0,2830	0,3967	0,2867	0,1797	0,3964	0,2672	0,0717	0,2382
single_ind_R104	0,3400	0,2122	0,5686	0,2266	0,3221	0,2642	0,2466	0,3320	0,2181	0,2708	0,0922
*H. octopodides*_2	0,2528	0,3030	0,5253	0,2971	0,4117	0,2873	0,1674	0,3771	0,2788	0,0238	0,2573
single_ind_R115	0,3163	0,2324	0,6053	0,2452	0,2821	0,2696	0,2461	0,3112	0,2352	0,2631	0,0917
single_ind_R118	0,3230	0,2200	0,5452	0,2362	0,2932	0,2550	0,2531	0,2945	0,2021	0,2412	0,0199
H. adherens	0,3130	0,3025	0,5150	0,2919	0,3807	0,1193	0,3077	0,4339	0,2438	0,3089	0,2417
H. kerblae	0,3149	0,2318	0,5600	0,2469	0,3156	0,2727	0,2424	0,3283	0,2338	0,2449	0,1368
single_ind_61	0,2700	0,2837	0,5147	0,2668	0,3870	0,0939	0,3107	0,3851	0,2724	0,2843	0,2345
H. swedmarki	0,2377	0,2785	0,5191	0,2985	0,3412	0,2931	0,1093	0,3851	0,2574	0,2006	0,2632
single_ind_R75	0,3157	0,2060	0,5636	0,2309	0,3270	0,2458	0,2596	0,3203	0,2152	0,2647	0,0978
single_ind_R93	0,3251	0,3094	0,5061	0,2837	0,3660	0,1171	0,2988	0,4260	0,2394	0,2946	0,2419
Teneriffe_2	0,3251	0,2087	0,5240	0,2308	0,2767	0,2460	0,2339	0,3015	0,1932	0,2513	0,0194

Continuation of SI 3.1

	*H. octopodides*_3	single_ind R104	*H. octopodides*_2	single_ind R115	single_ind R118	*H. adherens*	*H. kerblae*	single_ind R61	*H. swedmarki*	single_ind R75	single_ind R93	Teneriffe_2
H. joergerae	0,0352	0,0375	0,0345	0,0365	0,0384	0,0360	0,0366	0,0329	0,0303	0,0368	0,0378	0,0385
Azores	0,0363	0,0289	0,0372	0,0313	0,0303	0,0370	0,0320	0,0366	0,0360	0,0286	0,0381	0,0293
outgroup	0,0521	0,0547	0,0520	0,0600	0,0522	0,0489	0,0561	0,0496	0,0493	0,0549	0,0479	0,0490
single_ind_D1	0,0363	0,0274	0,0377	0,0306	0,0304	0,0353	0,0324	0,0328	0,0375	0,0278	0,0341	0,0299
*H. vermiformis*_2	0,0474	0,0390	0,0468	0,0354	0,0359	0,0428	0,0388	0,0427	0,0440	0,0389	0,0420	0,0338
H. coronata	0,0333	0,0347	0,0355	0,0338	0,0321	0,0175	0,0342	0,0154	0,0354	0,0330	0,0176	0,0313
H. teisseri	0,0242	0,0325	0,0239	0,0312	0,0324	0,0354	0,0301	0,0358	0,0157	0,0345	0,0345	0,0307
*H. vermiformis*_1	0,0439	0,0371	0,0420	0,0367	0,0373	0,0501	0,0388	0,0465	0,0464	0,0381	0,0487	0,0366
Helgoland_Sylt	0,0315	0,0281	0,0349	0,0285	0,0259	0,0325	0,0307	0,0333	0,0323	0,0292	0,0320	0,0260
*H. octopodides*_1	0,0121	0,0319	0,0049	0,0337	0,0288	0,0361	0,0314	0,0338	0,0272	0,0319	0,0344	0,0305
Roscoff	0,0298	0,0153	0,0322	0,0156	0,0070	0,0303	0,0192	0,0304	0,0325	0,0157	0,0298	0,0057
*H. octopodides*_3		0,0304	0,0114	0,0306	0,0280	0,0347	0,0298	0,0315	0,0303	0,0316	0,0322	0,0296
single_ind_R104	0,2384		0,0331	0,0181	0,0162	0,0339	0,0179	0,0328	0,0343	0,0102	0,0339	0,0160
*H. octopodides*_2	0,0575	0,2660		0,0341	0,0296	0,0381	0,0326	0,0350	0,0296	0,0331	0,0361	0,0318
single_ind_R115	0,2442	0,1158	0,2623		0,0152	0,0318	0,0187	0,0338	0,0333	0,0194	0,0309	0,0150
single_ind_R118	0,2230	0,0922	0,2405	0,0917		0,0315	0,0203	0,0317	0,0333	0,0163	0,0309	0,0062
H. adherens	0,2915	0,2522	0,3090	0,2602	0,2483		0,0337	0,0171	0,0385	0,0372	0,0061	0,0302
H. kerblae	0,2260	0,1361	0,2385	0,1274	0,1462	0,2520		0,0359	0,0346	0,0188	0,0348	0,0188
single_ind_61	0,2601	0,2578	0,2794	0,2608	0,2447	0,1048	0,2497		0,0378	0,0339	0,0168	0,0301
H. swedmarki	0,2174	0,2701	0,2101	0,2539	0,2674	0,3263	0,2643	0,2966		0,0349	0,0373	0,0323
single_ind_R75	0,2390	0,0431	0,2590	0,1385	0,1005	0,2868	0,1393	0,2546	0,2779		0,0371	0,0164
single_ind_R93	0,2735	0,2524	0,2941	0,2478	0,2469	0,0181	0,2605	0,1012	0,3114	0,2918		0,0298
Teneriffe_2	0,2288	0,0899	0,2466	0,0894	0,0201	0,2374	0,1354	0,2282	0,2563	0,0953	0,2361	

Supplementary Information 3.2: Genetic divergence over sequence pairs between groups of 18S sequences. The number of base substitutions per site from averaging over all sequence pairs between groups are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter model [1]. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). This analysis involved 85 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1562 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [2].

	outgroup	*H. joergerae*	*H. octopodides*_1	*H. octopodides*_2	*H. octopodides*_3	*H. teisseri*	*H. swedmarki*	Roscoff	*H. kerblae*	*H. vermiformis*_2	*H. vermiformis*_1	Helgoland_Sylt	single_ind_R75	*H. coronata*	*H. adherens*_R63	Azores	single_ind_D1	Tenerife_1
outgroup		0,00552	0,00564	0,00567	0,00562	0,00569	0,00570	0,00546	0,00550	0,00550	0,00545	0,00550	0,00577	0,00567	0,00564	0,00570	0,00581	0,00565
H. joergerae	0,04216		0,00170	0,00166	0,00160	0,00146	0,00146	0,00179	0,00164	0,00149	0,00145	0,00274	0,00224	0,00305	0,00296	0,00269	0,00341	0,00499
*H. octopodides*_1	0,04449	0,00676		0,00108	0,00097	0,00158	0,00155	0,00183	0,00168	0,00175	0,00177	0,00268	0,00211	0,00329	0,00324	0,00262	0,00339	0,00494
*H. octopodides*_2	0,04456	0,00584	0,00506		0,00050	0,00164	0,00160	0,00181	0,00171	0,00187	0,00182	0,00279	0,00221	0,00327	0,00319	0,00273	0,00361	0,00496
*H. octopodides*_3	0,04304	0,00452	0,00375	0,00129		0,00157	0,00153	0,00178	0,00166	0,00184	0,00181	0,00272	0,00214	0,00316	0,00309	0,00271	0,00355	0,00491
H. teisseri	0,04284	0,00349	0,00628	0,00544	0,00413		0,00089	0,00135	0,00128	0,00146	0,00157	0,00245	0,00188	0,00321	0,00311	0,00253	0,00352	0,00495
H. swedmarki	0,04432	0,00323	0,00610	0,00518	0,00387	0,00154		0,00104	0,00120	0,00145	0,00131	0,00233	0,00163	0,00306	0,00305	0,00235	0,00337	0,00486
Roscoff	0,04403	0,00557	0,00846	0,00754	0,00622	0,00387	0,00232		0,00132	0,00143	0,00139	0,00225	0,00170	0,00295	0,00288	0,00261	0,00347	0,00481
H. kerblae	0,04244	0,00452	0,00740	0,00648	0,00516	0,00283	0,00257	0,00361		0,00139	0,00144	0,00233	0,00186	0,00322	0,00310	0,00248	0,00342	0,00478
*H. vermiformis*_2	0,04252	0,00425	0,00709	0,00752	0,00620	0,00384	0,00359	0,00438	0,00356		0,00116	0,00262	0,00198	0,00289	0,00281	0,00237	0,00362	0,00490
*H. vermiformis*_1	0,04192	0,00323	0,00667	0,00649	0,00517	0,00413	0,00258	0,00361	0,00380	0,00294		0,00263	0,00193	0,00297	0,00307	0,00225	0,00336	0,00484
Helgoland_Sylt	0,04370	0,01093	0,01350	0,01293	0,01158	0,00913	0,00893	0,00998	0,00892	0,01122	0,01152		0,00262	0,00357	0,00331	0,00230	0,00418	0,00492
single_ind_R75	0,04583	0,00781	0,00993	0,00979	0,00846	0,00609	0,00452	0,00557	0,00582	0,00686	0,00577	0,01209		0,00339	0,00320	0,00264	0,00355	0,00488
H. coronata	0,04431	0,01307	0,01699	0,01509	0,01373	0,01465	0,01304	0,01384	0,01437	0,01214	0,01173	0,01958	0,01641		0,00175	0,00315	0,00403	0,00482
*H. adherens*_R63	0,04409	0,01173	0,01682	0,01575	0,01438	0,01331	0,01304	0,01384	0,01303	0,01146	0,01305	0,01556	0,01507	0,00581		0,00334	0,00405	0,00498
Azores	0,04833	0,01176	0,01446	0,01512	0,01375	0,01135	0,00976	0,01214	0,01107	0,01136	0,00957	0,01016	0,01243	0,01707	0,01840		0,00419	0,00484
single_ind_D1	0,05193	0,02057	0,02165	0,02263	0,02123	0,02067	0,01917	0,02162	0,01986	0,02275	0,02013	0,02707	0,02192	0,02818	0,02817	0,02674		0,00522
Tenerife_1	0,04549	0,03370	0,03538	0,03538	0,03390	0,03467	0,03285	0,03319	0,03294	0,03383	0,03223	0,03561	0,03489	0,03091	0,03394	0,03667	0,04345	

Supplementary Information 3.3: Genetic divergence over sequence pairs between groups of CO1 sequences. The number of base substitutions per site from averaging over all sequence pairs between groups are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter model [1]. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). This analysis involved 100 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 810 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [2].

	H. joergerae	Azores	outgroup	single_ind_D1	Helgoland_Sylt	*H. coronata*	*H. teisseri*	*H. vermiformis_1*	*H. vermiformis_2*	*H. octopodides_1*	Roscoff	single_ind_R115	*H. kerblae*	*H. swedmarki*	Tenerife_2
H. joergerae		0,0332	0,0336	0,0235	0,0312	0,0256	0,0250	0,0319	0,0276	0,0198	0,0246	0,0255	0,0312	0,0305	0,0242
Azores	0,3392		0,0323	0,0290	0,0289	0,0286	0,0275	0,0289	0,0299	0,0288	0,0245	0,0285	0,0288	0,0338	0,0256
outgroup	0,4184	0,4034		0,0241	0,0307	0,0303	0,0298	0,0301	0,0302	0,0271	0,0269	0,0267	0,0319	0,0364	0,0239
single_ind_D1	0,2415	0,2727	0,3308		0,0278	0,0257	0,0282	0,0268	0,0288	0,0240	0,0223	0,0249	0,0285	0,0323	0,0227
Helgoland_Sylt	0,3369	0,3037	0,4268	0,3100		0,0291	0,0292	0,0285	0,0290	0,0279	0,0252	0,0253	0,0279	0,0369	0,0257
H. coronata	0,2860	0,2882	0,4022	0,2745	0,3299		0,0250	0,0255	0,0265	0,0251	0,0229	0,0264	0,0281	0,0326	0,0241
H. teisseri	0,2595	0,3109	0,4216	0,2981	0,3063	0,2866		0,0288	0,0276	0,0246	0,0241	0,0221	0,0269	0,0292	0,0237
*H. vermiformis*_1	0,3332	0,3051	0,4064	0,2918	0,3312	0,3001	0,3152		0,0198	0,0249	0,0264	0,0274	0,0314	0,0357	0,0255
*H. vermiformis*_2	0,3035	0,3098	0,4143	0,3171	0,3317	0,3047	0,3066	0,2072		0,0264	0,0244	0,0269	0,0309	0,0328	0,0251
*H. octopodides*_1	0,2090	0,3291	0,3861	0,2595	0,3129	0,2949	0,2843	0,2949	0,2978		0,0213	0,0214	0,0257	0,0262	0,0206
Roscoff	0,2594	0,2376	0,3746	0,2175	0,2942	0,2527	0,2515	0,2753	0,2675	0,2417		0,0173	0,0239	0,0268	0,0084
single_ind_R115	0,2692	0,2947	0,3846	0,2548	0,2945	0,2743	0,2348	0,2965	0,2964	0,2562	0,1807		0,0227	0,0291	0,0169
H. kerblae	0,3311	0,3002	0,4175	0,2898	0,3246	0,3213	0,3024	0,3107	0,3318	0,2908	0,2612	0,2312		0,0317	0,0231
H. swedmarki	0,3244	0,3754	0,4751	0,3327	0,3944	0,3534	0,3298	0,3749	0,3635	0,3022	0,2833	0,2918	0,3151		0,0268
Tenerife_2	0,2552	0,2500	0,3438	0,2134	0,2870	0,2574	0,2405	0,2552	0,2613	0,2168	0,0525	0,1843	0,2405	0,2842	

Supplementary Information 3.4: Genetic divergence of all three genes whithin every cluster with estimates of standard errors. Groups with only one sequence cannot have a divergence, hence n/c. Minimal, maximal and mean values were calculated.

	16S		**18S**		**CO1**	
cluster	**k2p mean**	**standard error**	**k2p mean**	**standard error**	**k2p mean**	**standard error**
Tenerife 1	-	-	0,008	0,002	-	-
single ind_R61	n/c	n/c	-	-	-	-
H. adherens	0,003	0,002	n/c	n/c	-	-
H. coronata	0,010	0,003	0,000	0,000	0,005	0,002
single ind_D1	n/c	n/c	n/c	n/c	n/c	n/c
Azores	0,000	0,000	0,005	0,002	0,000	0,000
Helgoland_Sylt	0,002	0,002	0,002	0,001	n/c	n/c
H. vermiformis 1	0,001	0,001	0,000	0,000	0,001	0,001
H. vermiformis 2	0,001	0,000	0,001	0,000	0,003	0,001
H. swedmarki	0,000	0,000	0,000	0,000	0,001	0,001
H. teisseri	0,027	0,004	0,001	0,000	0,011	0,003
H. joergerae	0,003	0,002	0,000	0,000	0,008	0,002
H. octopodides 1	0,026	0,004	0,005	0,001	0,004	0,001
H. octopodides 2	0,002	0,001	0,003	0,001	-	-
H. octopodides 3	0,000	0,000	n/c	n/c	-	-
H. kerblae	0,009	0,004	0,000	0,000	n/c	n/c
single ind_R115	n/c	n/c	-	-	n/c	n/c
single ind_R75	n/c	n/c	n/c	n/c	-	-
single ind_R104	n/c	n/c	-	-	-	-
Tenerife 2	0,001	0,001	-	-	n/c	n/c
single ind_R118	n/c	n/c	-	-	-	-
Roscoff	0,002	0,001	0,002	0,001	0,017	0,003
MIN	0,000	0,000	0,000	0,000	0,000	0,000
MAX	0,027	0,004	0,008	0,002	0,017	0,003
MEAN	0,006	0,002	0,002	0,001	0,006	0,002

Appendix 4 (*Study IV*, SI): GenBank numbers

Supplementary Information: GenBank numbers of sequences used in the analyses. Sequences of *Halammohydra* are not uploaded yet, thus there are no numbers.

			species	**16S**	**18S**
Hydroidolina					
		Candelabridae	*Candelabrum cocksii*	AY512520	AY920758.1
		Magapiidae	*Fabienna spaerica*	AM183133.1	AY920767.1
		Moerisiidae	*Moerisia* sp.	AY512534	AF358083.1
		Porpitidae	*Porpita* sp.	AY512529	AF358086.1
		Corynidae	*Scrippsia pacifica*	AY512551	AF358091.1
		Laodiceidae	*Melicertissa* sp.	AY512515	AF358075.1
Trachylina					
	Limnomedusae	Oliniasidae	*Aglauropsis aeora*	EU293973	AY920754
			Astrohydra japonica	EU293975	KY077286.1
			Craspedacusta sowerbii	EU293971	AF358057
			Craspedacusta sinensis	AY512507	EU247815
			Limnocnida tanganjicae	EU293972	AY920755
			Maeotias marginata	AY512508	AF358056
			Olindias muelleri	EU293978	AY920753
			Olindias sambaquiensis	EU293977	EU247814
			Geryonia proboscidalis	EU293979	EU247816
			Liriope tetraphylla	AY512510	AF358061
			Liriope tetraphylla	EU293980	AY920756
	Narcomedusa	Aeginidae	*Aegina citrea*	EU293997	AF358058
		Solmundaeginidae	*Solmundella bitentaculata*	EU293998	EU247812
		Cuninidae	*Sigiweddellia* sp.	EU293996	KY007607.1
			Solmissus incisa	EU294002	KY007609.1
			Solmissus marshalli	EU294001	AF358060
			Cunina octonaria	KY007592.1	KY007606.1
		Tetraplatiidae	*Tetraplatia volitans*	EU293999	DQ002501
	Trachymedusae	Halicreatidae	*Botrynema brucei*	EU293982	EU247822
			Haliscera conica	EU293981	EU247825
			Halicreas minimum	EU293983	EU247826
		Ptychogastriidae	*Ptychogastria polaris*	MH407651.1	KY077283.1
		Rhopalonematidae	*Aglantha digitale*	EU293985	EU247821
			Aglaura hemistoma	EU293984	EU247818
			Amphogona apicata	EU293994	MG979355.1
			Crossota rufobrunnea	EU293986	EU247824
			Crossota rufobrunnea	EU293987	EU247823
			Crossota millsae	MH065488.1	MK547165.1
			Pantachogon haeckeli	EU293988	AF358062
			Rhopalonema velatum	EU293992	EU247819
			Tetrorchis erythrogaster	EU293995	KY077285.1
			Arctapodema sp.	MG979383.1	MG979356.1
			Colobonema sericeum	MG979385.1	MG979358.1

Continuation of SI

			species	16S	18S
Trachylina					
	Trachymedusae	Halammohydridae	*Halammohydra vermiformis* 1	-	-
			Halammohydra vermiformis 2	-	-
			Halammohydra octopodides 1	-	-
			Halammohydra octopodides 2	-	-
			Halammohydra octopodides 3	-	-
			Halammohydra coronata	-	-
			Halammohydra adherens	-	-
			Halammohydra teisseri	-	-
			Halammohydra swedmarki	-	-
			Halammohydra kerblae	-	-
			Halammohydra joergerae	-	-
			Halammohydra sp. (Azores)	-	-
			Halammohydra sp. (Helgoland/Sylt)	-	-
			Halammohydra sp. (Roscoff)	-	-