RUNNING TITLE: Life cycle of *Hydroides elegans*

Developmental staging of the complete life cycle of the model marine tubeworm *Hydroides elegans*

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ABSTRACT

Background: The marine tube worm model *Hydroides elegans* is an emerging system for probing the molecular foundations of bacterial influences on development. However, a complete description of the life cycle from fertilization through sexual maturity remains scattered in the literature, and lacks standardization. **Results:** Here we present a unified staging scheme detailing the major morphological changes that occur during the entire life cycle of the animal. These data represent the most complete record of the entire life cycle, and serve as a foundation for connecting molecular changes with morphology. **Conclusions:** These descriptions and associated staging scheme are especially timely as this system gains traction within research communities. As a frontrunning animal model for studying how bacteria stimulate development, characterizing major developmental events like metamorphosis in greater resolution is essential for future investigation of the molecular mechanisms in bacteria and in the worm host that are driving these changes.

INTRODUCTION

The biofouling marine tube worm, *Hydroides elegans*, is an indirect developing polychaete with significance as a model organism for questions in developmental biology and the evolution of microbial symbioses. The role of microbes in mediating animal development is of increasing interest. This is in part due to the diversity of organisms that participate in host-microbe relationships, as well as the diversity of developmental outcomes that arise from interactions with bacteria. The transition to multicellularity[1] as well as switches between asexual to sexual reproductive strategies[2] in choanoflagellates, maturation of the gut in zebrafish[3] and mammals[4], organogenesis in the Hawaiian bobtail squid[5], and initiation of metamorphosis in marine invertebrates[6,7] including *Hydroides*[8,9] are just some of the varied ways in which microbial interactions influence eukaryotic development.

Simple model systems like *Hydroides* are highly tractable in a laboratory setting. They release thousands of eggs and sperm at a time, and spawning is non-lethal. Fertilization occurs externally, and the embryos/larvae will rapidly develop in a simple dish of sea water. Metamorphosis of *Hydroides* larvae will occur at five days, and is catalyzed by contact with a heterogeneous biofilm. In the lab, metamorphosis can be recapitulated by contact with axenic biofilms composed of a single stimulatory bacterial strain[8,10,11]. *Hydroides* is beginning to reveal more on the diversity of developmental strategies and body plans in the *Spiralia*, and enables characterization of the pathways through which eukaryotic organisms sense and respond to an expansive array of microbial products that induce major developmental transitions.

Despite its utility as a developmental model[12-17], characterizations of the growth and development of *Hydroides* remains fragmented, and the community has yet to establish a standardized staging scheme that spans the entirety of development. Staging schemes have become a commonplace tool across all major developmental model systems including frog[18], chick[19], zebrafish[20], and sea urchin[21-23], and are fundamental for the development and utility of non-traditional model organisms[24-28]. Staging schemes provide a unified narrative of development under tightly controlled environmental conditions, and offer accuracy and consistency in describing the progression of key developmental events. This is especially, important within the Spiralia which have highly conserved cell divisions and fates early in development, yet give rise to an incredible diversity of body plans[14,29,30]. To this end, we provide here a complete staging of the life cycle of *Hydroides* (Figure 1) from fertilization through

sexual maturation, as well as offer perspectives on how this model can be leveraged to advance our understanding of diverse and fundamental biological questions, and whose applications can serve as solutions to challenging modern biological questions.

NATURAL HISTORY OF HYDROIDES

Adult *Hydroides* are protandrous hermaphrodites, and are found in intertidal to sublittoral zones throughout the global tropical and subtropical oceans[31,32]. They are part of a larger group of tube-building annelids in the family Serpulidae. These animals are filter feeders, extending branching appendages, collectively called the branchial crown, out from the opening of their tubes to collect particles from the water. In addition, they have a special funnel-shaped structure called an operculum that closes off the opening to the tube when the animal retracts inside. As one of the most prolific biomineralizing annelids, this species has garnered much attention as a biofouling organism that deposits its calcareous tubes on ship hulls and other submerged surfaces in harbors and marinas[11,32,33]. They are highly resilient, tolerating variable temperatures (15-30°C) and salinities (15-37 PSU)[13], and able to colonize surfaces treated with different antifouling agents[34,35]. However, despite being seen as "pests" in harbors and marinas, these animals are valuable research models. They, along with other members in the genus, have been utilized for biological research since the species was first described over 100 years ago[36], with increasing interest over the last few decades. This surge in interest in *Hydroides* centers around antifouling and the biphasic life history of this animal which utilizes microbial intervention to complete development[8].

DEVELOPMENT

Unlike the gametes of other marine invertebrates, for example sea urchins, there is no obvious morphological changes to the eggs of *Hydroides* upon fertilization (Figure 1, zygote). These worms are part of the Spiralian clade that represents an exceptionally diverse array of body plans that all arise from a stereotypical early cleavage pattern. The early cleavages of *Hydroides* are equal[14] and thus, unlike other members of the Spiralia and even other Annelids[14,29,30], the "D" quadrant of the embryo is not morphologically distinct from the other regions at the 4-cell stage (Figure 2, E4). The third cleavage, yielding an 8-cell embryo (Figure 2. E8), is the first division during which the spiral pattern becomes apparent. This division is a

sinistral (left-handed) twist, as the blastomeres on the animal side of the embryo rotate counter-clockwise relative to the vegetal blastomeres[14]. The 8 cells of the embryo complete the next cleavage cycle concurrently, yielding the 16-cell stage (Figure 2, E16). This is in contrast to the blastomere divisions leading from 16- to 32-cell stage (Figure 2, E32), and the 32- to 64-cell stage (Figure 2, E64) transition which do not occur synchronously[14].

The formation of the gut via invagination at the vegetal pole[16,37] begins shortly after the completed cleavages of the 64-cell stage embryo, now called a blastula (Figure 3, B). The embryos also become ciliated and hatch to begin their free-swimming stages, forming a characteristic apical tuft typical of many marine larvae, along with a thick ciliary band that will eventually form the prototroch[38]. The blastopore partially closes along the ventral midline forming the mouth, and the developing gut tube traverses the blastocoel (Figure 3, EG-LG), bending towards the aboral side of the animal, eventually fusing to form the anus[15,16,37,38]. The complete gut is tripartite, having a differentiated fore-, mid-, and hindgut. Feeding of the trocophore larva (Figure 3, T) can then begin, and the beating of the plush ciliary band forming the prototroch, as well as the opposing accessory band, the metatroch, helps to direct food particles towards the mouth.

Over the course of several days, the trocophore larvae feeds. Two eye spots form in sequence, and the posterior portion of the body elongates[12]. Three segments arise nearly simultaneously on the posterior portion of the body, each segment develops chaetal sacs and parapodia[12]. The larva is now a segmented nectochaete (Figure 3, S), and continues to feed, though no additional segments are added to the body until after metamorphosis. Upon reaching competency (Figure 3, C) at 5 days post-fertilization, the larva has three complete body segments and is characterized by sharper definition between the head and trunk of the body. The competent larva is then ready to settle on a substrate and undergo metamorphosis. Metamorphosis is induced by contact with heterogeneous multispecies or monospecies biofilms[7,8,10,39]. Settlement is characterized by a slowing of swimming speed, and probing of a surface. If suitable, the larva attaches (Figure 4, M1) itself to a surface and begins to secrete a primary proteinaceous tube[40] (Figure 4, M2, dashed line) and shed the prototroch cilia (Figure 4, M3, *) which are no longer needed for swimming. Major tissue remodeling occurs as the anterior region of the larva's body changes shape to form a collar (Figure 4, M4), food groove cells between the prototroch and metatroch are shed (Figure 4, M5), and cilia from the metatroch are

lost (Figure 4, M5) as lobes extended laterally (Fig 4, M6-M7) on the head of the animal and a mineralized secondary tube is deposited.

Post-metamorphic juveniles (Figure 4, J-1d and J-1w) continue to elaborate the anterior portion of the body, forming an elaborate branchial crown for feeding (Figure 4, J-1w), as well as the operculum in 10-14 days. They continue to deposit their calcareous tubes and within a week of metamorphosis grow to several millimeters in length. Under ideal culturing conditions with ample food and clean, well oxygenated water, they have a generation time of approximately three to four weeks[11]. In the wild, they settle gregariously, forming dense communities in a matter of weeks, and can be cultured as single tubes in the lab to isolate individual sexually mature adults (Figure 4, A).

BACTERIA-STIMULATED METAMORPHOSIS of HYDROIDES

Given the remarkable impact that marine biofilms have on the life cycle of *Hydroides*, it comes as no surprise that much attention has been given to characterizing the nature of this host-microbe association during metamorphosis. Metamorphosis is an exciting transition in development that occurs in nearly all animal lineages, and thus the molecular mechanisms driving this change in *Hydroides* are likely to reveal fundamental means of interaction at play between hosts and microbes across the tree of life.

Many different single strains of bacteria induce *Hydroides* metamorphosis[8]. For example, *Pseudoalteromonas luteoviolacea,* is a strong inducer of metamorphosis in *Hydroides*, as are other *Pseudoalteromonas* strains, and *Cellulophaga lytica* from the Bacteroidetes group[41]. Along with the diversity of inductive strains, there are also diverse bacterial products that promote the same developmental endpoints[42]. For example, *P. luteoviolacea* produces a contractile injection system[43] that is related to the contractile tails of bacterial viruses (bacteriophage). These syringe-like structures are assembled into arrays and deliver a protein payload[44], the effector Mif1, to *Hydroides* larvae which then interact with host-derived signaling networks[45] to induce metamorphosis. These phage tail-like arrays are termed MACs, for Metamorphosis Associated Contractile structures. Other bacteria strains which do not possess any genes encoding MACs are also able to stimulate metamorphosis, through different mechanisms[42]. For example, lipopolysaccharide from outer membrane vesicles of some bacteria induce *Hydroides* metamorphosis[41] and other soluble cues likely do as well since cell-free fractions can initiate

metamorphosis. These biochemically unique products that induce metamorphosis from diverse bacteria open up a future of discoveries based on *Hydroides* that are filled with possibilities.

THE FUTURE OF HYDROIDES

As the *Hydroides* model continues to gain traction in the research community, it is clear there are many areas with potential for discovery. This includes comparative evolutionary and developmental biology, genetic tool development, biotechnology, and the discovery of bacteria-derived natural products.

Comparative models in Evolution and Development

Comparative models in evo-devo utilizing *Hydroides* can offer unique perspectives on the establishment of host-microbe symbioses. The complex network of genes in developmental regulatory networks have yet to be integrated with components from microbial symbionts. In the Spiralia, where much of the early development occurs in a stereotypical fashion, this could provide useful insight into how host and microbial systems intersect. In fact, the animal sensing machinery responsible for detecting and responding to bacteria by initiating metamorphosis is not well-resolved for *Hydroides*, or any other animal aside from the highly conserved signaling systems (e.g., PKC and MAPK[9,45-48]). Thus, there exists a conspicuous gap in knowledge that this Annelid model is primed to fill, and which could offer insight into the mechanisms of bacteria-animal communication operating in other animal lineages.

Comparative approaches also allow for the investigation of overlap or repurposing of molecular machinery that drives metamorphosis and facilitates host-microbe interactions. For example, immune genes have rapidly evolved and function primarily to detect and respond to encounters with bacteria. However, repurposing of these components could facilitate interactions with non-pathogenic bacteria and intersect with developmental pathways like those activated during metamorphosis[9,49]. The evolutionary origins of these signaling systems remains unclear, but the streamlined innate immune systems of Annelid models enable further study into these questions without the added complexity that adaptive immunity brings, and are less derived than models like *C. elegans*. The *Hydroides* genome contains more genes in common with humans than *C. elegans* does[9], placing *Hydroides* as a model system with the potential for discoveries relating to human development, health, and disease.

Furthermore, the *Hydroides* model sits squarely at the junction of questions in ecology, evolution, and development. As a broadcast spawning marine invertebrate that has a vast dispersal range, we can gain insight into how animal ecology is influenced by environmental microbes that stimulate metamorphosis. This would be applicable, for example, in reef environments where recruitment and successful settlement of dispersed pelagic larvae is essential for maintaining the habitat. These eco-evo-devo perspectives[50] may contribute to more fundamental principles that drive the generation and evolution of host-microbe symbioses, and also add a new layer of context to ecosystem dynamics and management for vulnerable marine habitats.

Genetics and Tool Development for Host and Microbes

A clear and current bottleneck in the development of *Hydroides* as a model system for host-microbe interactions is a lack of established techniques for genetically manipulating both the *Hydroides* host and the various types of environmental bacteria that stimulate *Hydroides* metamorphosis. There has been recent success in the genetic manipulation of related Annelids such as *Platynereis dumerilii*[51,52] and *Capitella teleta*[53,54] and we are optimistic that *Hydroides* will be amenable to genetic manipulation. Furthermore, improvements in husbandry and long-term culture will further facilitate generation of animal lines in *Hydroides*, enabling *Hydroides* to keep pace with advancements, like CRISPR gene editing, in other marine invertebrate larvae[55-60]. In parallel, emerging synthetic biology tools in established model microbes also hold promise for the manipulation of more diverse marine bacteria that promote *Hydroides* development (CITE – Amanda toolkit when available). This in turn enables detailed molecular study of material metamorphosis-inducing machinery.

Biotechnology and Bacterial Products

Future work leveraging *Hydroides* holds promise for promoting biotechnology in the contexts of evolution, the environment, and human health. For example, insights gained from *Hydroides* to understand bacteria-stimulated metamorphosis could aid in the husbandry of invertebrates in aquaculture[61]. *Hydroides* has been studied intensively as a biofouling organism[11] and research on antifouling technologies could provide significant economic relief for shipping and naval sectors[33]. The products bacteria produce to promote *Hydroides* metamorphosis and the

machinery *Hydroides* uses to sense these products could lead to broader discoveries about mechanisms and tools for host-microbe interactions. For example, we found that gene clusters encoding Contractile Injection Systems originally discovered to promote *Hydroides* metamorphosis are strikingly similar to gene clusters found in *Bacteroidales* bacteria from healthy human microbiomes[62]. Moreover, understanding the payload delivery and targeting mechanism of the MACs produced by *P. luteoviolacea* could enable us to produce new types of therapeutic delivery systems[63].

In the last 10 years, genetics has opened a new window into the products from bacteria that stimulate metamorphosis[43,44]. This, paired with classical chemical approaches, have enabled researchers to interrogate the diverse chemical nature of metamorphosis-inducing metabolites produced by bacteria in the environment. Furthermore, genetics has also opened avenues to investigate the relative contributions or strength of particular microbes or distinct chemical structures in inducing metamorphosis *in vivo*. Understanding the diverse nature of bacterial products that are capable of stimulating metamorphosis in *Hydroides* may also inform on how this process unfolds in other animals and guide our understanding of how symbiotic associations evolve at the chemical level.

Ultimately this emerging compilation of work in the *Hydroides* model system raises interesting questions about the nature of this marine tubeworm-microbe relationship: 1) do other diverse molecular cues for metamorphosis exist?; 2) given the heterogeneity of marine biofilms, what are the respective contributions of individual bacteria within it to metamorphosis, effectively how potent are the various cues, are there combinatorial effects of these products, and are they distinct enough to be differentiated by the animal?; and 3) do these diverse cues actually yield identical developmental outcomes, or does the mechanism of metamorphosis initiation influence the sequence of signaling and morphogenetic events[17], and if so, how?

Looking more broadly, environmental bacteria are becoming increasingly recognized for their significant roles in regulating health and development[6,64]. The utilization of the *Hydroides* – microbe model offers additional opportunities to understand the conservation and diversity of microbial drivers of eukaryotic development and disease states. Additionally, this model enables precise manipulation of host-microbe interactions in the eco-evo-devo landscape that can help determine what factors dictate whether a microbe will interact as a “friend” or “foe”.

EXPERIMENTAL PROCEDURES

Husbandry, Spawning and Fertilization

Adult worms are collected from Quivira Basin in Mission Bay, San Diego CA. Animals are housed in a suspended basket in a recirculating tank at room temperature, supplied with artificial filtered sea water (AFSW) at 35 PSU. Adults are fed *ad libidum* with a monoculture of the marine algae *Isochrysis galbana* (Carolina Biological: Item #153180). To spawn animals, individual adult tubes are selected and placed in separate petri dishes filled with AFSW. Using forceps, the tubes are cracked, revealing the animal inside which releases thousands of eggs or streams of sperm upon disturbance.

Eggs are collected with a trimmed plastic pipette tip and washed into fresh FASW to minimize debris left over from tube fracture. Sperm is collected with a pipette in minimal sea water, and then diluted further into a fresh sperm suspension for fertilization. Eggs are diluted to ~500/mL and 4-5 drops of sperm suspension is added. The dish is swirled to mix. Ten minutes after the addition of sperm to the eggs, the eggs are collected and washed into fresh FASW three times to eliminate excess sperm and minimize the risk of polyspermy. Eggs and embryos are monitored over the next several hours to ensure fertilization since no obvious morphological changes occur at fertilization. All cultures of embryos are maintained at a density of 1 larva/ml in an incubator at 25°C, with foil or petri dish covers to prevent evaporation. Larval cultures have water changes daily starting at Day 2, and are fed *I. galbana* daily at a density of $6x10^4$ cells/mL. At 5 days post-fertilization, the majority of larvae have reached competency (there is some asynchrony due to variation in individual larval feeding efficiency) and are transferred to a dish that has a natural biofilm coating the bottom of the dish. The natural biofilm is generated by filling the dish with raw seawater from the worm's natural habitat and allowing that to sit for four to five days. The worms metamorphose over the course of ~12 hours upon interacting with the natural biofilm, and are then either imaged or kept for longer-term culture to adulthood. Metamorphosed worms that are kept for culture to adulthood are fed *I. galbana* every other day *ad libidum*, and water changes are completed on an identical schedule.

Live-cell Imaging of Development

All stages except the adult of *Hydroides elegans* were imaged live on a Zeiss Axio Observer.Z1 using objectives ranging from 5x to 100x oil immersion, all with differential

interference contrast (DIC) optics. Images were captured using the ZEN software suite, and measurements were added using Fiji (National Institutes of Health, Bethesda, Maryland)[16]. Composite DIC images were rendered from Z-stacks of animals using Helicon Focus Pro Unlimited (v6.8.0, Helicon Soft Ltd.). Adult animals were imaged live on a Wild Heerbrugg M5a stereo microscope fitted with a QImaging R3 Retiga CCD camera.

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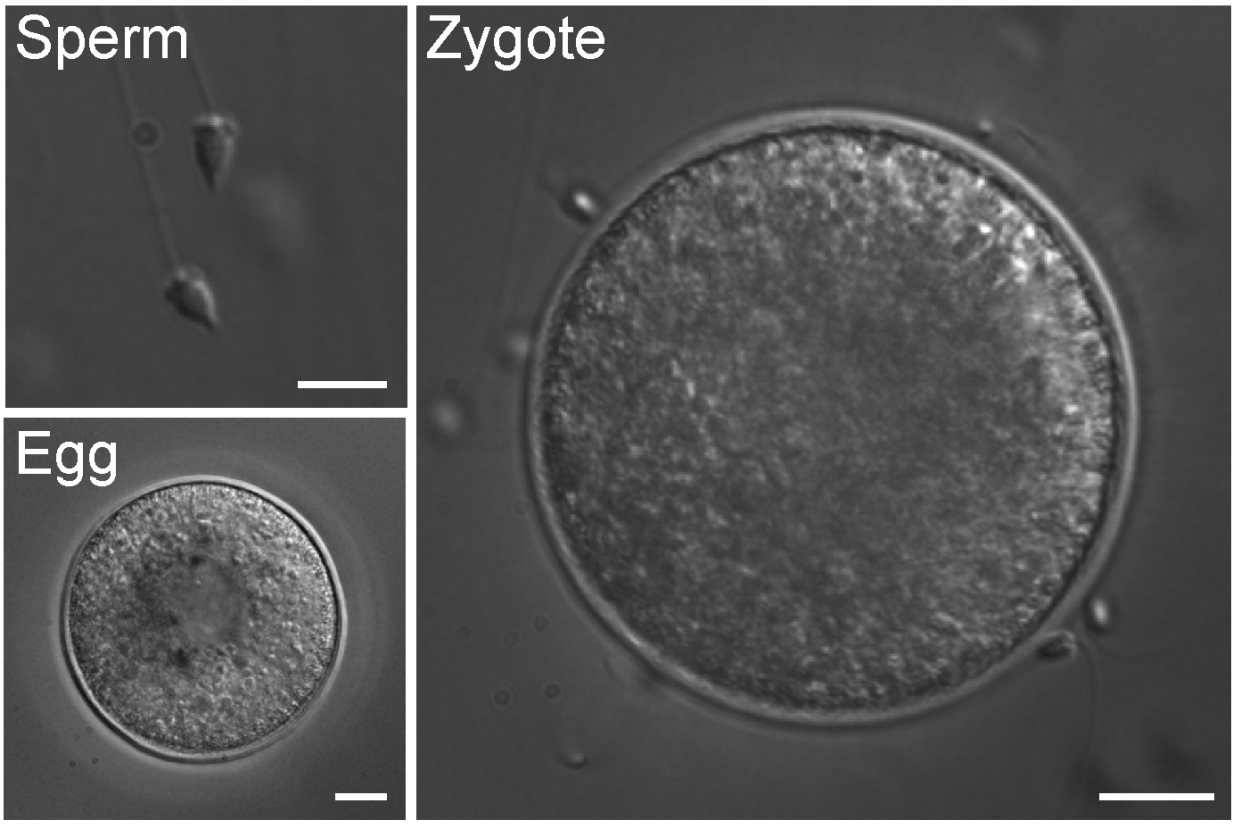

Figure 1. Gametes and fertilization in *Hydroides elegans*. Sperm, scale = 5µm. Unfertilized egg, scale = 10µm. Zygote at fertilization, scale = 10µm.

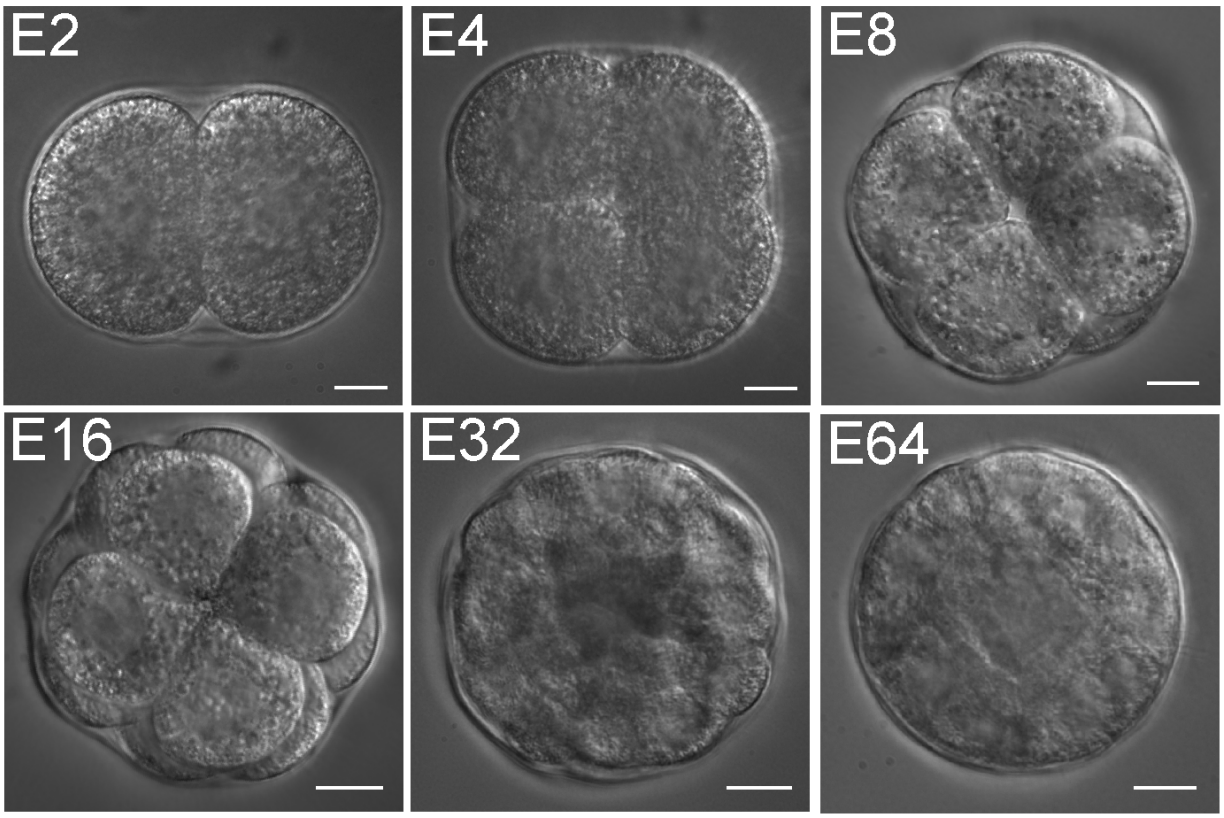

Figure 2. Cleavage stages in *Hydroides elegans*. All panels depicted are in an animal view of the embryo where applicable. Scale for all panels = 10μm. Panels E2-E4 and E32-E64 are single focal planes. Panels E8-E16 are composite images from multiple focal planes. **E2)** 2-cell embryo. **E4)** 4-cell embryo. **E8)** 8-cell embryo. **E16)** 16-cell embryo. The vegetal blastomeres are not in view. **E32)** 32-cell embryo. **E64)** 64-cell embryo.

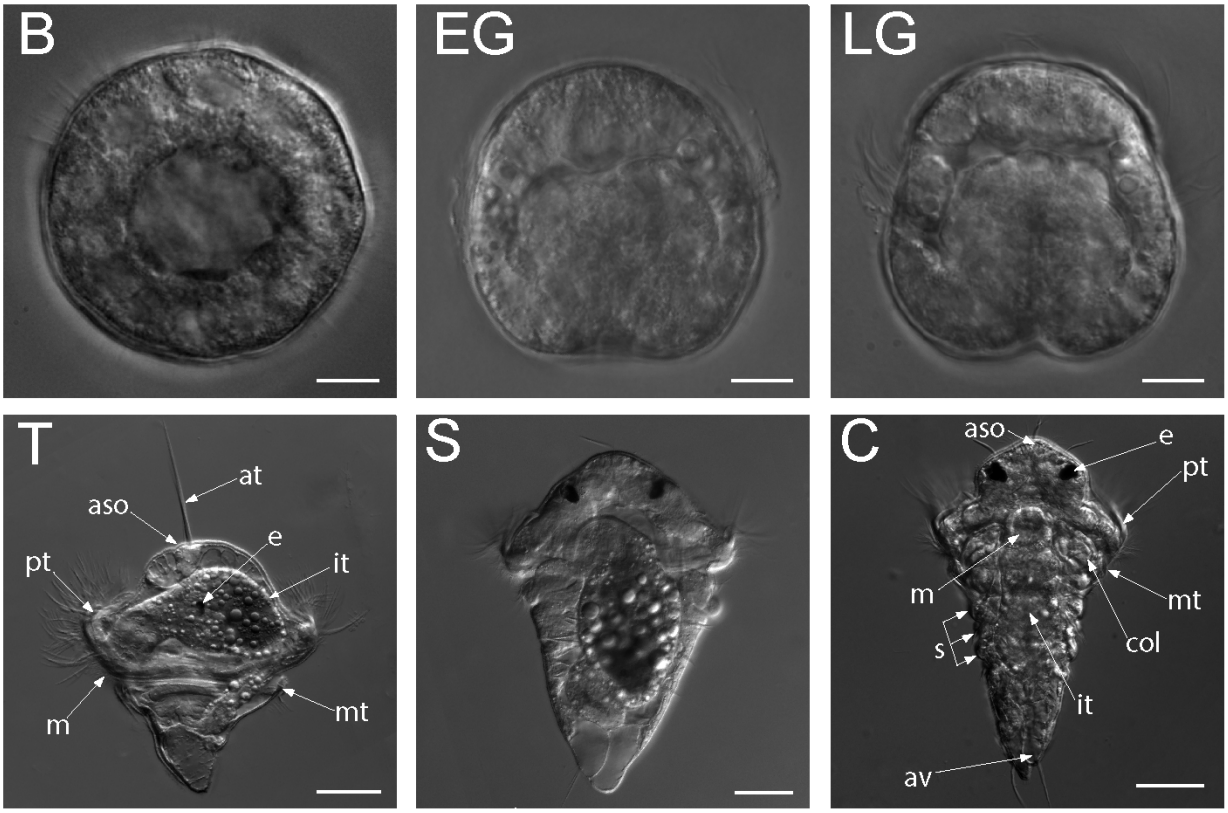

Figure 3. Gastrulation and larval stages in *Hydroides elegans*. Panels B, EG, and LG, scale = 10µm. Panels T, S, and C, scale = 50µm. Panels T, S, and C are composite images from multiple focal planes. **B)** Ciliated blastula. **EG)** early gastrula. **LG)** Late gastrula, hatched. **T)** Feeding trocophore larva in a lateral view. **S)** Segmented nectochaete. **C)** Metamorphically competent larva. Abbreviations: at = apical tuft, aso = apical sensory organ, av = anal vacuole, col = collar, e = eye spot, it = intestinal tract, m = mouth, mt = metatroch, pt = prototroch, s = segments with chaetae.

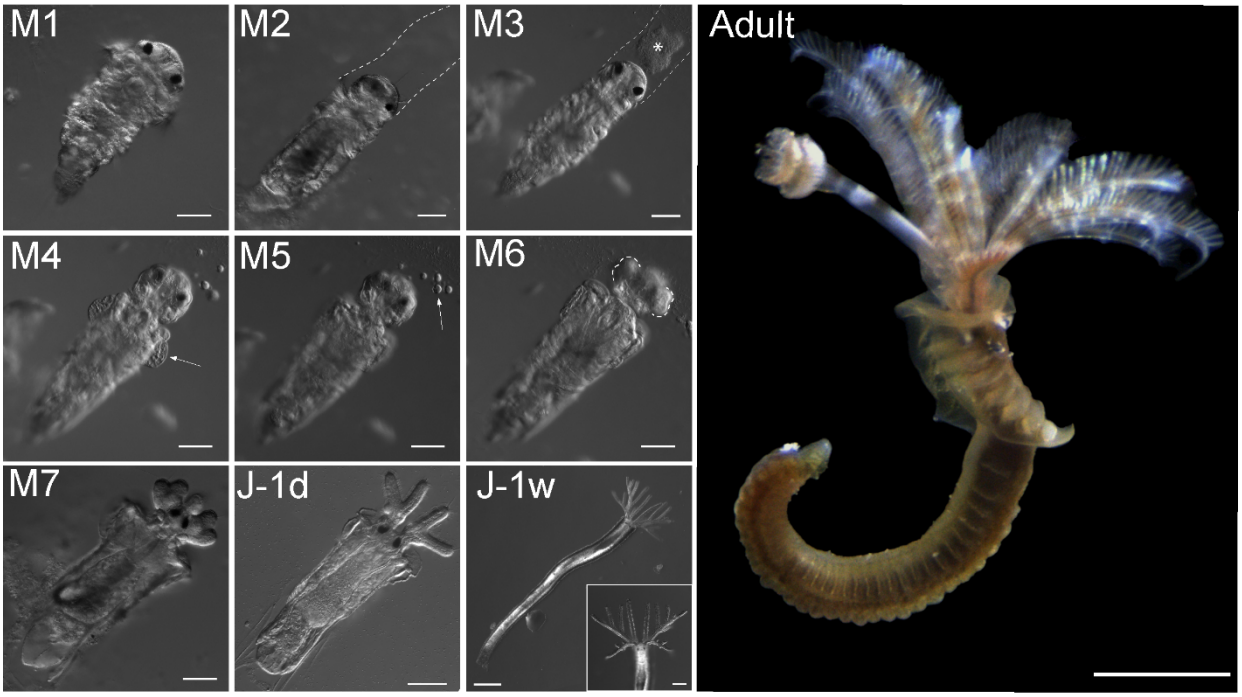

Figure 4. Morphological changes during metamorphosis and sessile life history stages in *Hydroides elegans*. For panels M1 through J-1d, scale = 50µm. **M1)** Attachment to substrate. **M2)** Formation of the primary tube (white dashed line). **M3)** Shedding of prototroch cilia (asterisk). **M4)** Collar (white arrow) eversion. **M5)** Shedding of food groove cells (white arrow). **M6)** Beginning of lobe formation (white dashed line) and loss of metatroch. **M7)** Lateral extension and elaboration of anterior lobes. **J-1d)** Juvenile at 24 hours post-metamorphosis. **J-1w)** Juvenile at 1-week post-metamorphosis, scale = 250µm. Inset of head showing elaboration of the branchial crown, scale = 100µm. **A)** Sexually mature adult dissected out of the calcareous tube, scale = 1mm.