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MESOZOA

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Phylum Dicyemida

Phylum Orthonectida

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Chapter 14

Mesozoa (Phylum Dicyemida and Phylum Orthonectida)

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Introduction

Unseen by the naked eye, the renal appendages (synonymous with kidneys, renal sacs, and renal organs) of benthic cephalopods, including squid, octopus, and cuttlefish (Figure 1), contain thousands of tiny worm-like organisms known as dicyemid mesozoans (Furuya and Tsuneki, 2003; Furuya et al., 2004; Finn et al., 2005). They can colonize 1 or both renal appendages at high numerical densities (Figure 2) and although simple in body structure, their life cycle is complex and not fully characterized. They are not known to infect any other marine organism, although the occurrence of a dispersal, free-swimming embryo form has led to questions as to whether an intermediate host or dormant stage exists.

Over 100 species have been formally described based on morphological characteristics, with documentation from the western and northeastern Pacific Ocean, northern Indian Ocean, Mediterranean Sea, northwestern and eastern Atlantic Ocean, Gulf of Mexico, Antarctic Ocean, and Southern Ocean. Only recently have molecular genetic analyses been applied to this group in an attempt to validate new species descriptions based on classical taxonomic methods and shed light on the unknown position in the Tree of Life for the enigmatic group of organisms.

Taxonomic Classification

Both the dicyemids and orthonectids (a group which parasitize a number of marine invertebrate phyla) have long been considered a class within the phylum Mesozoa (see Stunkard, 1972; Hochberg, 1983; McConnaughey, 1983a; 1983b).

Figure 1. The only known hosts of dicyemid mesozoans including A) squid (*Sepioteuthis australis*, southern calamari), B) octopus (*Octopus kaurna*, southern sand octopus), and C) cuttlefish (*Sepia apama*, giant Australian cuttlefish). Source: S. Catalano. License: CC BY-NC-SA 4.0.

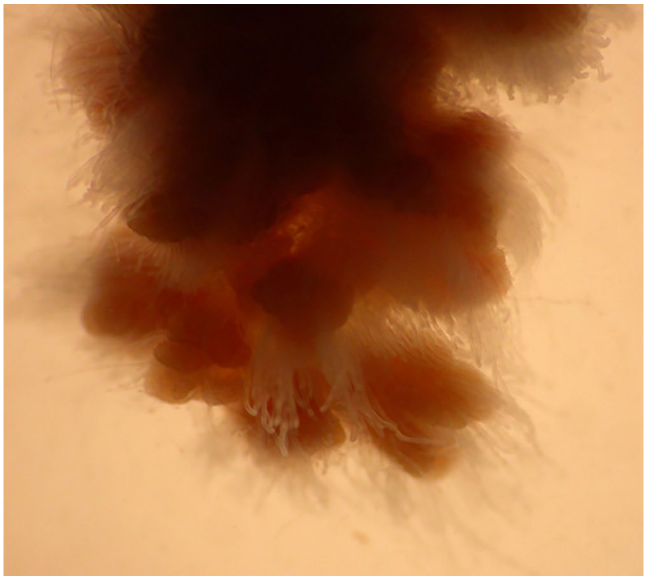


Figure 2. Hundreds of dicyemid mesozoans attached to the renal appendage (in red) of the giant Australian cuttlefish (*Sepia apama*). Each white strand represents 1 individual adult dicyemid. Source: S. Catalano. License: CC BY-NC-SA 4.0.

However, due to distinct differences between these 2 groups in terms of morphology and life cycle stages, it is now accepted to treat each group as separate phyla, phylum **Dicyemida** and phylum **Orthonectida**. The phylum **Dicyemida** contains 3 families, Dicyemidae Van Beneden 1882, Conocyemidae Stunkard 1937, and Kantharellidae Czaker 1994, although the validity of the Kantharellidae is questionable and uncertain due to the single species from this family being inadequately described (Furuya et al., 2007). Nine genera are recognized within the 3 families:

Family Dicyemidae
Dicyema von Kölliker, 1849
Dicyemennea Whitman, 1883
Dicyemodeca (Wheeler, 1897) Bogolopova, 1957
Pseudicyema Nouvel, 1933
Pleodicyema Nouvel, 1961
Dodecadicyema Kalavati & Narasimhamurti, 1980

Family Conocyemidae
Conocyema Van Beneden, 1882
Microcyema Van Beneden, 1882

Family Kantharellidae
Kantharella Czaker, 1994

The largest number of described species are in *Dicyema*, followed by *Dicyemennea*, with the other genera being monotypic or containing a small number of species (Catalano, 2012). Catalano (2012) provides a comprehensive list of the 112 species described up until 2012, however, an additional 12 species were described up until 2019 (Catalano, 2013a; 2013b; Catalano and Furuya, 2013; Castellanos-Martínez et al., 2016).

Typically for generic and species classification, the number and orientation of cells in each tier of the calotte, the presence or absence of abortive axial cells, the presence or absence of syncytial stages, the size of the adult stages, the number of cells comprising the body, the shape of the calotte, the anterior extension of the axial cell, the presence or absence of verruciform cells, and the structure of the infusoriform larvae are distinguishing morphological characters (Hochberg, 1982; 1983). However, recent molecular analyses have shed a level of doubt on some of these morphological characters, particularly calotte cell counts for genera classification, as the placement of a *Dicyema* species, with 4 metapolar cells in its calotte, grouped within the *Dicyemennea* clade, which is known to have 5 metapolar cells in their calottes (Catalano et al., 2015). Further molecular analyses, which include multiple species from all the known genera alongside additional molecular markers, will be needed to resolve and either validate or dismiss the current level of classification based on morphological traits. New species descriptions should now not only include measurements from all life cycle stages (nematogen, rhombogen, vermiform embryo, and infusoriform embryo) along with line drawings and light micrograph images of distinguishing characters (classical morphological measures, for example, as in Furuya, 2009), but also molecular analyses with the *COI* (*c* oxidase subunit I) gene sequenced as a minimum for inclusion in the preliminary phylogenetic tree for dicyemid mesozoans as presented by Catalano et al. (2015). Recently, Drábková et al. (2022) used several phylogenomic methods to generate a phylogeny that shows a common ancestor of the Dicyemids and the Orthonectida with ancestral Platyhelminthes as the basal group from which the Mesozoa arose.

Morphology

The body plan of a dicyemid mesozoan is very simple, comprising 8 to 40 cells with no body cavities, differentiated organs, tissues, or glands (Suzuki et al., 2010), although the dispersal embryo form, known as the **infusoriform embryo**, is morphologically distinct from the remaining 3 forms. The **vermiform adult**, **vermiform embryo**, and **rhombogen adult** (collectively known as the **vermiform stages**, Figure 3), all contain a central, long **axial cell**, which is where developing embryos are derived (Awata et al., 2006). This cylindrical axial cell is then protected by the presence of ciliated **peripheral cells** that surround the axial cell in a single layer, although at the anterior region, the peripheral cells are modified to form a **calotte**. The calotte serves as the dicyemid's anchor—it is inserted into the convoluted surface of the host renal appendage allowing the parasite to maintain a foothold while the remainder of its body hangs free in the surrounding environment obtaining nutrients, as seen in Figure 2 (Furuya et al., 2003a; 2007). Traditionally the number and arrangement of cells in the top 2 tiers of the calotte (known as the **metapolar** and **propolar cells**) has been used to assign new species into 1 of the 9 described genera (Figure 4). In particular, it has been reported that the dicyemids have 4 propolar cells, but different numbers of metapolar cells: *Dicyema* (4 metapolar cells, propolar cells opposite to metapolar cells), *Pseudicyema* (4 metapolar cells, propolar cells alternate with metapolar cells), *Dicyemennea* (5 metapolar cells), *Dicyemodeca* and *Pleodicyema* (6 metapolar cells), *Dodecadicyema* (6 metapolar cells plus 3 micropolar cells: Small cells which form the anterior tip of the calotte, only found in *Dodecadicyema* species) (Figure 4). The species of the family Conocyemidae are characterized by having no metapolar cells, but either parapolar cells or

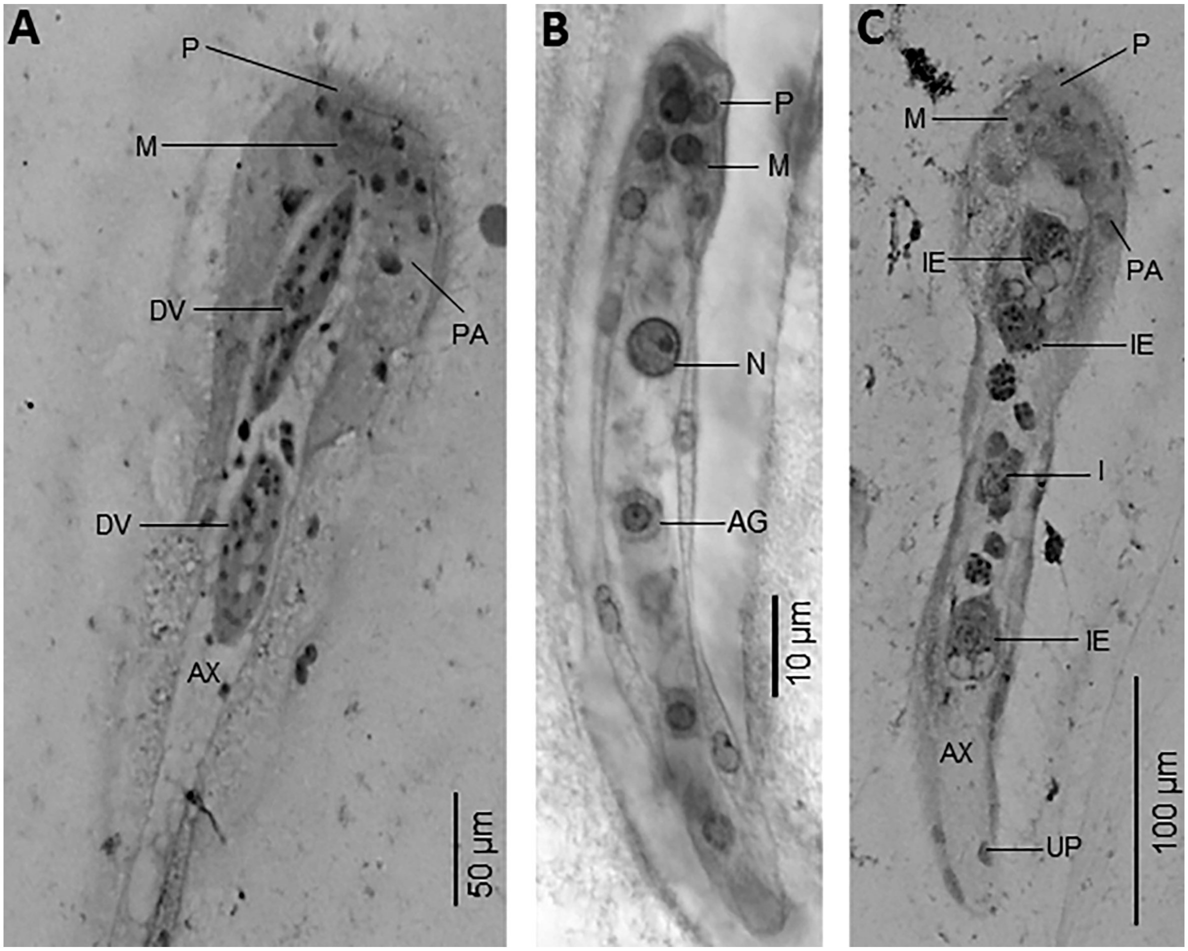


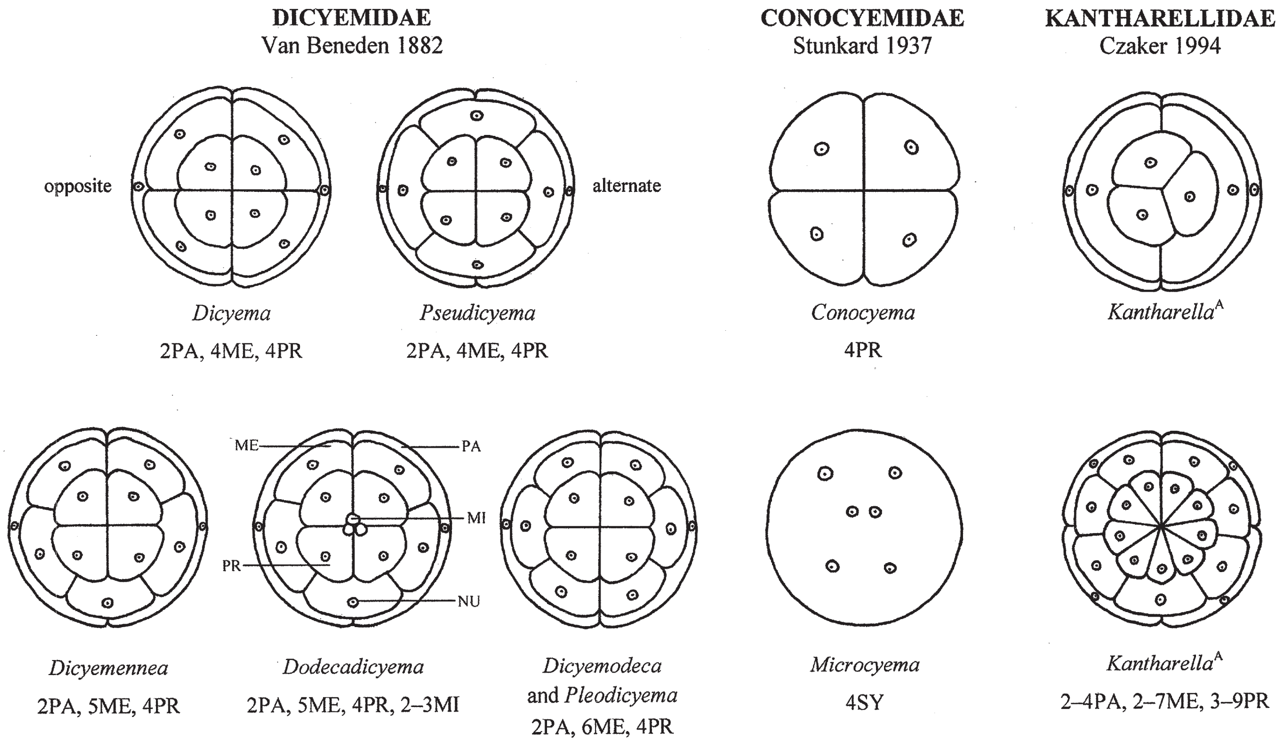
Figure 3. The vermiform stages: A) Adult nematogen (*Dicyema pyjamaceum*) with 2 developing vermiform embryos within the axial cell, B) close up of the vermiform embryo (*Dicyemennea floscephalum*) within the axial cell of a nematogen, C) adult rhombogen (*Dicyema pyjamaceum*) with 3 infusoriform embryos within the axial cell. Abbreviations: AG, agamete; AX, axial cell; DV, developing vermiform embryo; I, infusorigen; IE, infusoriform embryo; M, metapolar cell; N, nucleus; P, propolar cell; PA, parapolar cell; UP, uropolar cell. Adapted from Catalano and Furuya, 2013; Catalano, 2013a. Source: S. Catalano. License: CC BY-NC-SA 4.0.

a **syncytial cell**: *Conocyema* (4 parapolar cells) and *Microcyema* (syncytial cell: A single cell with 6 nuclei which is only found in *Microcyema* species) (Figure 4). The species of the Kantharellidae are different from the other families because there is no cell constancy, with 3 to 9 propolar cells, 2 to 7 metapolar cells, and 2 to 4 parapolar cells (Czaker, 1994) (Figure 4). Nonetheless, whether calotte cell counts represent an accurate way of distinguishing dicyemid genera remains in question, with molecular analyses shedding some doubt on this classical level of classification (see Catalano et al., 2015).

Unlike the vermiform stages, the infusoriform embryo is not long and cylindrical, but rather small and circular (Figure 5). The infusoriform embryo is characterized by 4 large internal **urn cells**, each containing a **germinal cell** which is thought to give rise to the next generation, as well as 2 large **apical cells** at the anterior region and beating **cilia** surrounding the body (Furuya and Tsuneki, 2003). One of the most interesting characters of the infusoriform embryo are the **refringent bodies**, which are contained within the large apical cells. These refringent bodies are composed of a chemical with a high specific gravity that accounts for more than one-third of the body weight of the infusoriform embryo, namely, **magnesium salt of inositol hexaphosphate** (Lapan and Morowitz, 1972; Lapan, 1975). This dense chemical provides the infusoriform embryo with negative buoyancy, which is suggested to allow the embryo to remain close to the sea floor to encounter and infect a new host.

Life Cycle

In contrast to their simple morphology, the life cycle of dicyemid mesozoans involves 2 stages of development



^{A} = Alternative views of calotte arrangement for *Kantharella* with the minimum (top) and maximum (bottom) number of PA, ME and PR drawn.

Figure 4. Schematic drawing of anterior end views of calottes showing the arrangement and number of cells characteristic of different genera. Abbreviations: ME, metapolar cell; MI, micropolar cell; PA, parapolar cell; PR, propolar cell; SY, syncytial cell. Adapted from Catalano, 2012. License: CC BY-NC-SA 4.0.

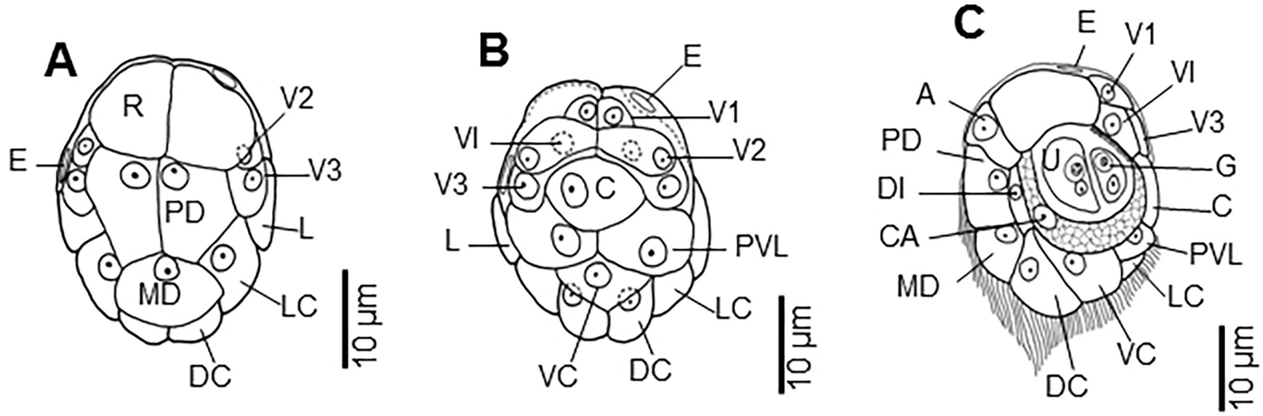


Figure 5. Line drawings of the infusoriform embryo (*Dicyemennea floscephalum*) showing A) dorsal view, cilia omitted, B) ventral view, cilia omitted, and C) sagittal section. Abbreviations: A, apical cell; C, couvercle cell; CA, capsule cell; DC, dorsal caudal cell; DI, dorsal internal cell; E, enveloping cell; G, germinal cell; L, lateral cell; LC, lateral caudal cell; MD, median dorsal cell; PD, paired dorsal cell; PVL, posteroventral lateral cells; R, refringent body; U, urn cell; V1, first ventral cell; V2, second ventral cell; V3, third ventral cell; VC, ventral caudal cell; VI, ventral internal cell. Source: Adapted from Catalano, 2013a. License: CC BY-NC-SA 4.0.

(vermiform and infusoriform) and 2 modes of reproduction (asexual and sexual) (Figure 6) (Furuya et al., 2003b; 2007). The vermiform stages, which are restricted to the renal appendages of the host, comprise the adult nematogen, vermiform embryo, and adult rhombogen. The infusoriform stage, which represents the dispersal stage that escapes from the host via the urine to find and infect a new host, comprises the infusoriform embryo.

While the vermiform stages are similar in terms of morphology, comprising 8–40 cells and a worm-like body shape, the infusoriform embryo is distinct, comprising 37–39 cells and being much smaller in size (typically 32–36 µm in length and 26–28 µm in width) with a rounded body shape (Furuya and Tsuneki, 2003). The vermiform stages are formed asexually from an agamete (axoblasts) whereas the infusoriform embryo develops from a fertilized egg produced around a hermaphroditic gonad called the infusorigen (Figure 7).

Within the axial cell of an adult nematogen, the vermiform embryos grow and develop asexually, with more than 1 embryo common within the single axial cell of the adult. Although the exact mechanism is unknown, the vermiform embryo is then released from the nematogen into the fluid around the renal appendages, finds a free surface for attachment and inserts its anterior calotte into the convoluted surface of the host's renal appendage while the rest of its body hangs free in the surrounding urine acquiring nutrients. As it develops further, it transitions into the adult nematogen and will produce its own vermiform embryo and the cycle continues. A high population density in the kidney, as seen in Figure 2—where an accumulation of a chemical factor in the environment is detected (Lapan and Morowitz, 1972; 1975)—is then thought to trigger a shift from asexual reproduction and increasing numbers within the renal appendage to a sexual mode of reproduction and escaping out of the crowded and highly infected host to find and infect a new, potentially naïve, host. This shift is seen in the form of production of the rhombogen adult in place of the nematogen adult, which contains in its single axial cell, 1 or more infusoriform embryos. These dispersal embryos then escape from the host out into the surrounding environment, ensuring that the species will survive beyond the eventual death of the host (Lapan and Morowitz, 1972).

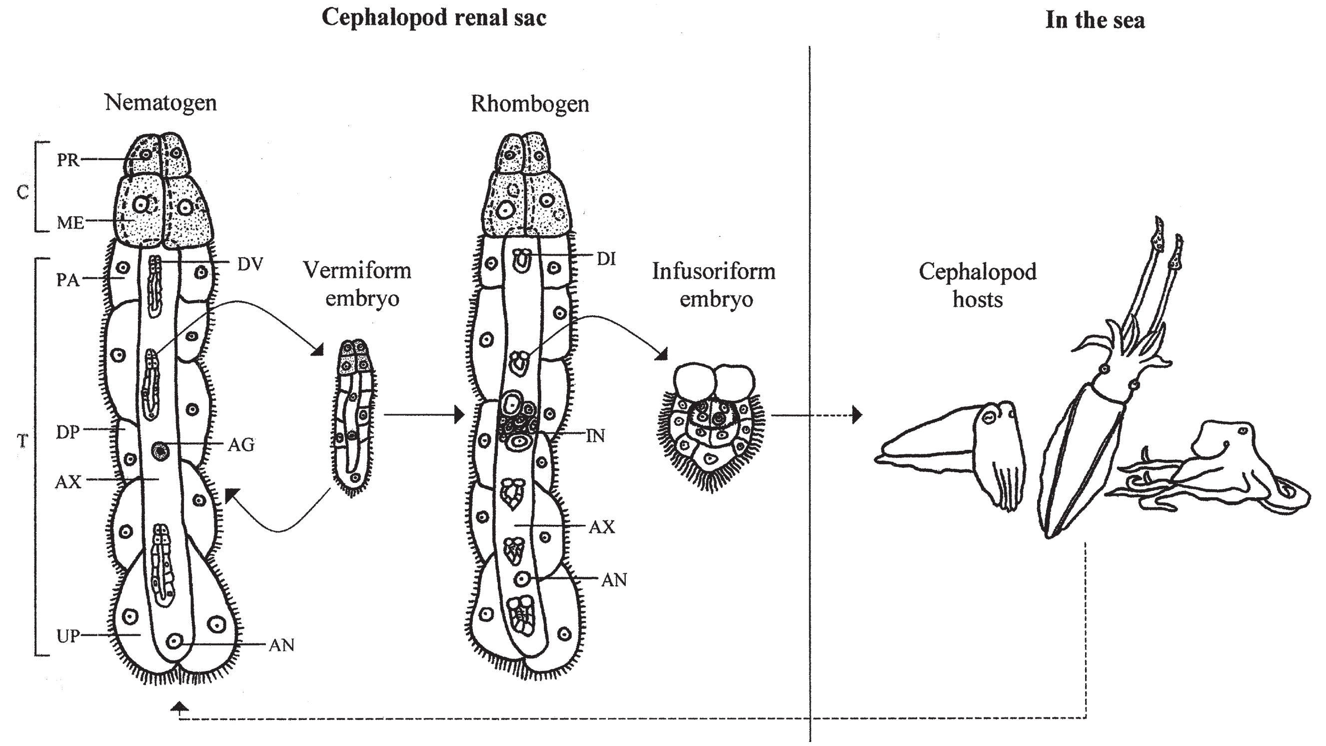


Figure 6. Diagrammatic representation of the morphology and life cycle of dicyemids. The dashed line indicates unknown processes of how the infusoriform embryo finds and infects a new cephalopod in the sea and how it then develops into adult forms. The nematogen, rhombogen, and vermiform embryo represent the asexual vermiform stages; the infusoriform embryo represents the sexual infusoriform stage. Abbreviations: AG, agamete; AN, axial cell nucleus; AX, axial cell; C, calotte; DI, developing infusoriform embryo; DP, diapolar cell; DV, developing vermiform embryo; IN, infusorigen; ME, metapolar cell; PA, parapolar cell; PR, propolar cell; T, trunk cell; UP, uropolar cell. Source: Adapted from Catalano, 2012. License: CC BY-NC-SA 4.0.

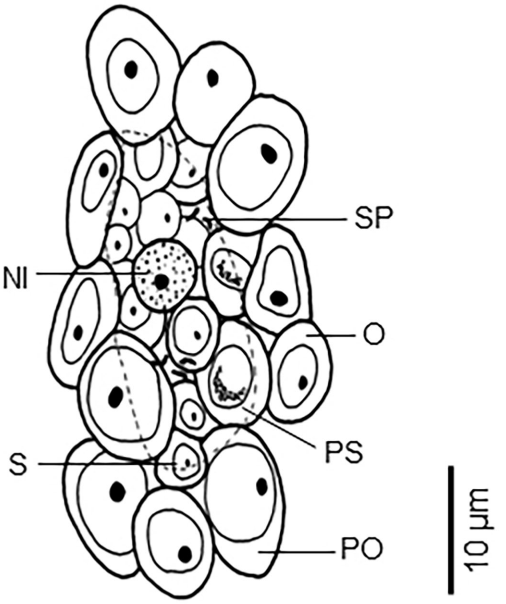


Figure 7. Line drawing of the infusorigen which is located within the axial cell of an adult rhombogen. Abbreviations: NI, nucleus of infusorigen; O, oogonia; PO, primary oocytes; PS, primary spermatocytes; S, spermatogonium; SP, sperm. Source: Adapted from Catalano, 2013a. License: CC BY-NC-SA 4.0.

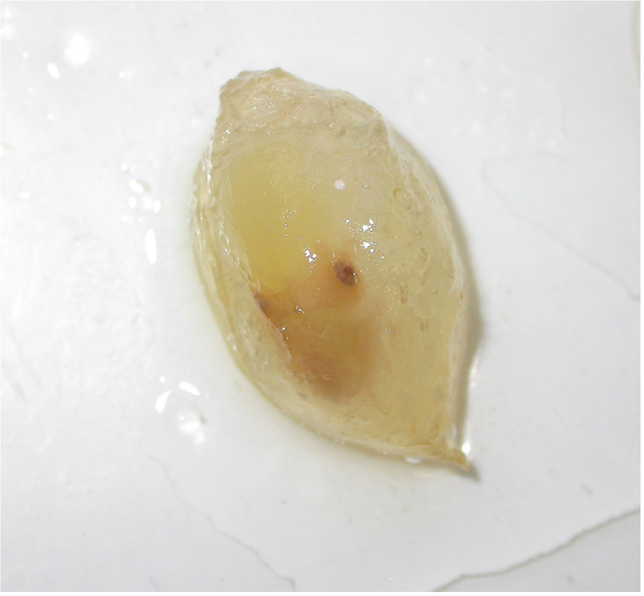


Figure 8. Host eggs have been hypothesized to be the potential stage of new infection for dicyemid mesozoans. Source: S. Catalano. License: CC BY-NC-SA 4.0.

The next stage of the life cycle remains unknown yet quite astonishing given most cephalopods are found to be infected by dicyemid parasites at high intensities. Particularly, it is uncertain how the tiny, infusoriform embryo, with limited swimming capabilities in relation to its host and a short survival time in seawater, then finds a new host, attaches to it or is taken up internally and starts the cycle off again by potentially morphing into the required vermiform stage (adult nematogen) so asexual reproduction can take place and the renal appendages of a new host will then become colonized.

Despite the monstrous challenge that the infusoriform embryo faces of finding and infecting the correct host species with a limited lifespan in a large, fluid, ever-changing environment, dicyemid parasites are still found in almost all benthic cephalopods examined (Catalano et al., 2014). As such, questions have been raised about an intermediate host; however, results from past experimental studies suggest the life cycle is direct (Lapan and Morowitz, 1975). Host eggs were hypothesized to be the potential stage of new infection, as they are abundant and sessile in the environment, allowing a huge number of new individuals to be infected with low energy costs (Figure 8) (Catalano et al., 2013). Additionally, as adult cephalopods have a short lifespan of 1 to 2 years, with mortality common after a single spawning event (Semmens et al., 2007), infection of the host egg stage provides dicyemids with the maximum amount of time for survival. Nonetheless, no dicyemid DNA was recovered from environmental water samples or cuttlefish eggs at the mass breeding aggregation of giant Australian cuttlefish (*Sepia apama* Gray) in South Australian waters (Catalano et al., 2013), leading to the notion that to resolve this unknown in the life cycle, experimental infection is needed. Interestingly, exclusive infection of the asexual stage of the dicyemid (adult nematogen) was found in the left renal appendage of a large giant Australian cuttlefish that had been held in captivity for 2–3 months, recently mated, and naturally died before samples were collected, indicating that dicyemids may persist and continue replicating even after host death (Catalano, 2013b). Furthermore, although the host had died, an immediate priority by the dicyemid was not to disperse, as density increased within the renal appendage with vermiform embryos continuing to produce instead of the dispersal of the infusoriform embryo (Catalano, 2013b). Perhaps a dead host just gets stuck with lingering parasites; or, perhaps, the life cycle of the dicyemid may be more intricate and mysterious than first thought.

Secondary Nematogens

Although not recognized as a regular part of the dicyemid life cycle, an additional form exists, namely the secondary nematogen. This rare form, which in the past has been denied to occur at all (Gersch, 1938), but has been observed by McConnaughey (1951) and Catalano (2013a), is characterized by containing infusorigen and infusoriform embryos together with young vermiform embryos within the axial cell, in essence having features of both adult nematogens and rhombogens (Figure 9). This form is thought to result by accident in the transitional period of development from a nematogen to rhombogen, with persistence of some axoblasts in good condition through the rhombogen period that have been able to resume their activity and produce, once more, viable vermiform embryos (Catalano, 2013a; McConnaughey, 1951). Whether there is then the possibility for reversal back to a full nematogen form, brought about through competition between these 2 modes of reproduction, and the possibility of infusorigens becoming exhausted while axoblasts are still being produced, is unknown. It is also unknown whether the occurrence of secondary nematogens is species-specific (Catalano, 2013a).

Hosts and Patterns of Infection

The only recorded hosts of dicyemid mesozoans are cephalopods, which include squid, octopus, and cuttlefish (as seen in Figure 1). In general, dicyemid species are highly host-species specific, although typically, 2 or more species are recorded in each host species (Furuya, 1999; 2017). The common octopus, *Octopus vulgaris* Cuvier, has the largest number of dicyemid species recorded from it (11), followed closely by the stubby squid, *Rossia pacifica* Berry, with 9 species (see Catalano, 2012). Hochberg (1990) suggests that *O. vulgaris* and *R. pacifica* may actually represent a host species complex, each with their own distinct dicyemid fauna, and the reported parasites might make up a reciprocal species complex.

When more than 1 dicyemid species do co-occur within a single host individual, generally the calotte shape from each is different, allowing each dicyemid species to colonize a distinct niche or surface of the host renal appendage (Furuya et al., 2003a; Furuya, 2008; Furuya and Tsuneki, 2003). Species of dicyemids that possess similar calotte shapes are rarely found together in a single host individual (Furuya and Tsuneki, 2003). The microhabitat of the renal appendages provides all that the dicyemid requires to complete its life cycle, including a surface for attachment, constant fluid bath, a source of nutrients and a simple exit for release of the dispersal stage (Hochberg, 1982).

In general, the presence of dicyemids is more commonly observed in benthic rather than pelagic cephalopods, which has been related back to the negative buoyancy and sinking ability of the infusoriform embryo. However, other factors, such as host size, age, behavior, and geographic locality likely play a role in the presence of infection, as exceptions

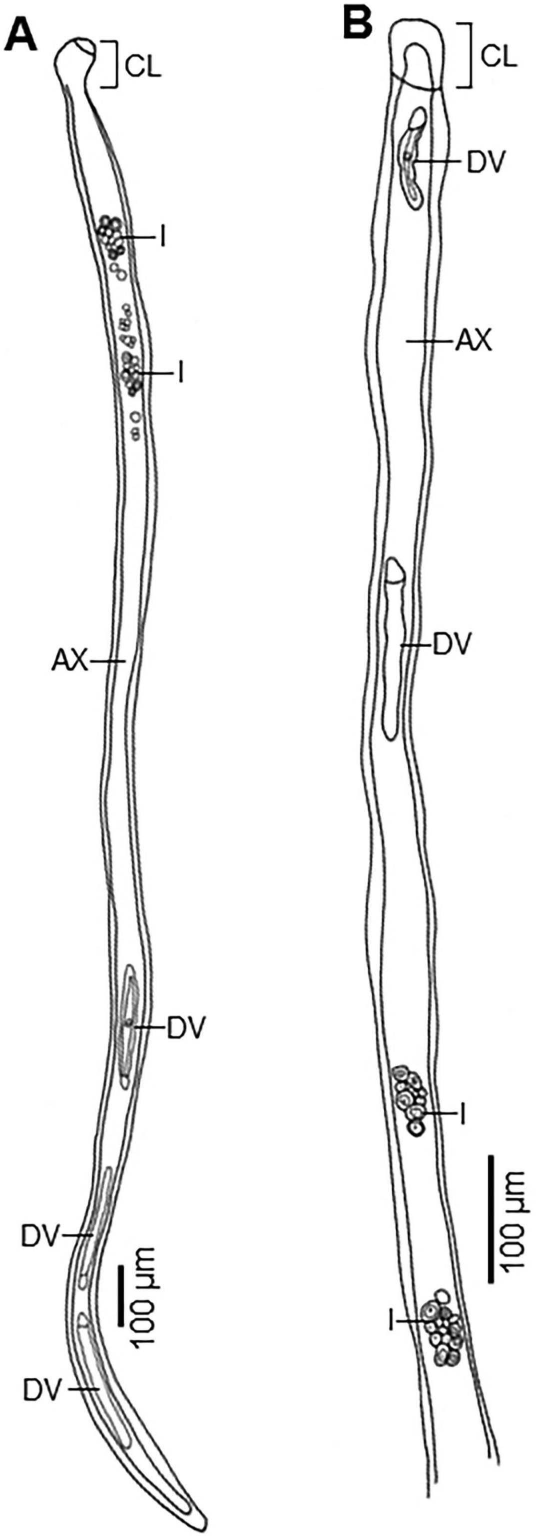


Figure 9. Line drawing of secondary nematogens (*Dicyema furuyi*) from *Sepia papuensis*. Abbreviations: AX, axial cell; CL, calotte; DV, developing vermiform embryo; I, infusorigen. Source: Adapted from Catalano, 2013a. License: CC BY-NC-SA 4.0.

to this notion are observed. For example, the southern dumpling squid, *Euprymna tasmanica* Pfeffer, frequently associates with the sea bottom, burying itself in the sand during the day to hide from predators (Norman and Reid, 2000). Such a strategy would allow for ample opportunity to be infected by the infusoriform embryo; however, in the study by Catalano et al. (2014), adding to the weight of evidence but while not conclusive, no dicyemids were recorded from this host species for 6 individuals collected and analyzed. In contrast, Finn et al. (2005) recorded the presence of dicyemids in 14 out of 18 *E. tasmanica* individuals from the same region, however, the 6 individuals collected in the study by Catalano et al. (2014) were small with a mantle length half of what is typically reached for this species. Other authors have also recorded absence of dicyemids from small host individuals (for example, Furuya et al., 1992b; Furuya and Tsuneki, 2005; Castellanos-Martínez et al., 2011). Furuya and colleagues (2004) correlated this to the complexity of the renal appendage, stating large host individuals have a more developed and complicated external surface compared to smaller host individuals, therefore they are able to provide more attachment sites and surface area for vermiform stages.

Interestingly within a single host individual, different stages (either exclusively the asexual stage or exclusively the sexual stage) have been recorded in each renal appendage, such as the adult nematogen (asexual stage) in the left renal appendage and the adult rhombogen (sexual stage) in the right renal appendage (Figure 10) (Finn et al., 2005; Catalano et al., 2014). This suggests that dicyemids infect the renal appendages independently of one another, at different times, and do not or cannot move from 1 renal appendage to the other. This also elucidates that the cue which mediates the transition from the asexual to the sexual stage is parasite driven rather than host-mediated, or else both renal appendages would be equally affected and contain the same stage at any one time (Lapan and Morowitz, 1975; Finn et al., 2005; Catalano et al., 2014).

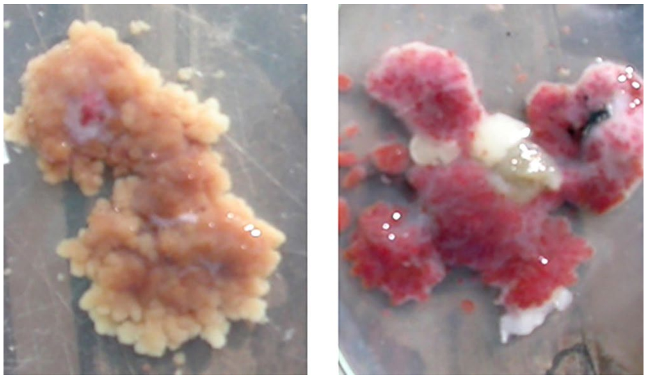


Figure 10. The left (brown) and right (red) paired renal appendages of cuttlefish, which can harbor different stages of dicyemid mesozoans in each renal appendage. Source: S. Catalano. License: CC BY-NC-SA 4.0.

True Parasite or Commensal Organism?

The true nature of the dicyemid mesozoan as a parasitic, commensal, or mutualistic organism remains unresolved with arguments for and against each option presented in the literature. Some authors support the parasitic way of life, stating that the delicate brush borders of the host renal appendage surface is damaged by the dicyemid attaching and maintaining its foothold, while others support the dicyemid as a commensal organism, stating they have little effect, either positive or negative, on the host (Finn et al., 2005; Furuya and Tsuneki, 2005). The third notion is that these organisms are mutualistic, with the beating cilia on their bodies facilitating host excretion of ammonia and urine, while also allowing the dicyemid to receive nutrients, taken up through the peripheral cells by endocytosis (Lapan, 1975; Hochberg, 1990; Furuya et al., 2004).

Molecular Analyses: Mitochondrial Mini-circle Molecules

Few studies have focused on the molecular genetics of the dicyemid mesozoans. For those that have reported molecular analyses, the mitochondrial (mt) cytochrome *c* oxidase complex unit genes (*COI*, *COII*, and *COIII*) have typically been sequenced, although some studies have reported sequences for nuclear genes from dicyemid species (Ohama et al., 1984; Katayama et al., 1995; Pawlowski et al., 1996). Quite unusually, the mt genome architecture of the dicyemids departs from the typical ~ 16 kb circular metazoan genome. In addition to a putative circular genome (Boore, 1999), the mt *COI*, *COII*, and *COIII* genes have been found to be located on separate mini-circle molecules, each with their own non-coding region (NCR) (Watanabe et al., 1999; Awata et al., 2005, Catalano et al., 2015). While the gene coding region defines genome metabolic functionality by specifying proteins, the NCR can define the architecture and regulation of the genome, often harboring the replication origin of the mini-circle and the promoters for transcription (Le et al., 2002; Burger et al., 2012). Although no specific origin of replication was found in the *COI* mini-circle molecules of 10 dicyemid species sequenced in the study by Catalano et al. (2015), palindrome sequences with the potential to form stem loop structures were identified in 5 species, suggesting that these palindrome regions may be involved in initiating mini-circle replication. Nonetheless it is quite bizarre to have single mt genes on single mini-circle molecules, as the typical mt genome, where all the genes are linked together on a chromosome will ensure the complete genetic information is transmitted when the mitochondrion replicates. This is otherwise challenging with a fragmented mt genome structure as observed in the dicyemid mesozoans. Further molecular studies are needed for the dicyemid mesozoans, particularly to confirm the validity of classifications based on traditional morphological traits, with sequences of DNA containing thousands of characters with orders of magnitude more than morphological analyses (Poore and O'Hara, 2007).

Position in the Tree of Life

The position of the dicyemids in the Tree of Life has long fascinated researchers, with the Belgian biologist Édouard Van Beneden providing the first attempt to classify the dicyemids (Van Beneden, 1876). His belief was that this group occupied an evolutionary intermediate position between the Protozoa (unicellular animals) and the Metazoa (multicellular animals), and hence he created the intermediate name Mesozoa Van Beneden, 1876. Since then, numerous attempts have been made to classify the dicyemids, however, often leading to additional confusion rather than resolution. In particular, the dicyemid mesozoans have been suggested to be members of the Spiralia (based on developmental studies and encoding of a 'spiralian peptide' in the dicyemid *DoxC* gene (Furuya et al., 1992a; Kobayashi et al., 1999), highly simplified bilaterians (based on tool-kit *Pax6* and *Zic* genes; Aruga et al., 2007), ancient multicellular animals (based on 5S rRNA gene; Ohama et al., 1984), relatives of nematodes (based on 18S rRNA sequences; Pawlowski et al., 1996), closely affiliated to annelids (based on 18S and 28S rRNA sequences; Petrov et al., 2010), and a sister group to the clade consisting of annelids and molluscs (amino acid sequences of innexin; Suzuki et al., 2010). Recently, the transcriptome of *Dicyema japonicum* was sequenced with the authors presenting support for the placement of Dicyemida with the Orthonectida in phylum Mesozoa, which then forms a sister group to the clade of Mollusca and Annelida (Lu et al., 2017). However, differences in internal features and stages of their respective life cycles still shed a level of doubt on the dicyemids and orthonectids grouping together within a single phylum. Further transcriptome sequence data from additional dicyemid and orthonectid species, including representatives from all of the described genera, will be required to validate these findings and confirm the definite position in the Tree of Life of the dicyemid mesozoans.

Collection and Staining Methods

As dicyemid mesozoans are minute and comprise only a few cells, they rapidly degrade following host death so collection should be targeted from fresh material. After euthanasia, place the body of the cephalopod ventral side up in a tray and open the mantle cavity with a sterile scalpel blade to expose the paired renal sacs. Using forceps and scissors sterilized in absolute ethanol to avoid cross contamination, remove the left and right renal appendages, and smear small pieces onto glass microscope slides. A smear is made by holding the renal appendage between the forceps in one hand and the glass slide in the other, and then with slight pressure, moving the renal appendage across the glass slide from left to right covering the slide surface from the top to the bottom in straight parallel lines. If the host is infected by dicyemids, typically small white strands will be seen on the glass slide when it is held up to the light. At a minimum, 4 smears should be made per renal appendage (8 smears per host), although the number of smears can be increased for larger host individuals. Smears will then need to be fixed immediately in 70% ethanol to avoid parasite desiccation, with Lock-Mailer$^{\mathrm{TM}}$ jars (Ted Pella, Inc.) proving to be ideal for field sampling and storage before slides are stained and mounted upon returning to the laboratory.

Although a range of staining methods have been used by past authors, a trial performed by Catalano et al. (2014) suggests staining with Ehrlich's acid haematoxylin diluted 20 parts of MilliQ water to 1 part stain for 20 minutes, dehydration in an ethanol series and counterstaining in eosin (70% ethanol for 10 minutes, 90% ethanol for 10 minutes, eosin 1% alcoholic solution diluted 20 parts of MilliQ water to 1 part stain for 2 minutes, and 100% ethanol for 15–20 minutes) as ideal for visualization of distinguishing morphological characters. Stained smears can then be mounted in Canada balsam, dried on a hot plate at 50 °C and examined with a compound microscope at magnification up to × 1,500, with drawings and measurements made with the aid of an ocular micrometer and drawing tube.

To confirm host species identification, it is desirable to collect a section of the host tissue (for example, mantle tissue) in 100% DNA-grade ethanol for molecular analysis. A section of each renal appendage can also be collected and stored in 100% DNA-grade ethanol for molecular analysis of the dicyemid parasite to complement traditional morphological classification. Depositing stained slides in registered museum collections should be mandatory for all new dicyemid species descriptions.

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