Doctoral Dissertation

Behavioral study on larval escapes and taxis in dicyemids

By

Naoki Hisayama

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Naoki Hisayama

Abstract

Dicyemids (Phylum: Dicyemida) are endosymbionts, exclusively found in the renal sacs of benthic cephalopod molluscs. The body structure is quite simple, usually comprising 10 to 50 cells and lacking specialized organs such as genital openings, muscles, digestive and nervous systems. The vermiform body is organized into a two-layer arrangement with a single cylindrical central axial cell surrounded by one layer of ciliated peripheral cells. Dicyemids are considered to be secondarily simplified animals due to their parasitic lifestyle, however, the mechanisms behind their simple body organization are still unknown. Also, they are characterized by two distinct types of embryo: the vermiform embryo, which arises from asexual reproduction, and the infusoriform embryo, which develops from fertilized eggs, both developing within the axial cell of the adult.

The escape means of each embryo from the parent body was studied in *Dicyema japonicum* and *Dicyema misakiense*, living in the renal sac of *Octopus sinensis*. There were no differences in escape means between species or types of embryo, presumably because of morphological constraints that result in adults (nematogen or rhombogen) sharing a similar body plan. Embryos escaped through the gap between neighboring peripheral cells of the adult, rupturing the membranes of the axial cell and the embryo enveloping. After the embryo escaped, the path was sealed by the enveloping membrane remained. Vermiform embryos were able to escape from any region of the axial cell, though more embryos escaped were frequently observed from anterior regions than from posterior regions. Infusoriform embryos escaped from both anterior and posterior regions within the axial cell, with more embryos escaped from the posterior regions. The varied escape regions for the two embryo types are presumably due to the adult body organization lacking a genital opening, therefore each different embryo type bears its proper escape site.

The three types of taxis: chemotaxis, thigmotaxis, and rheotaxis were studied in *D. japonicum* and *D. misakiense*. Vermiform individuals did not exhibit chemotaxis towards the major host components: urine, tissue fluids, or gill extracts. However, they exhibited positive thigmotaxis and positive rheotaxis to slow water flow, probably aiding in their attachment to the renal appendages and remaining in the renal sac. Infusoriform larvae exhibited negative chemotaxis to host blood and negative thigmotaxis, with no evidence of rheotaxis. Negative thigmotaxis may function to facilitate the release of infusoriform embryos from the renal appendages. Negative chemotaxis to the host blood suggests that the infusoriform larvae do not enter the renal sac via the vascular system, therefore the process by which infusoriform larvae enter the cephalopod host is yet to be elucidated.

Introduction

In cephalopods, the circulatory system is well developed and highly efficient. A relatively small blood volume, 6% of the body volume in *Octopus dofleini*, recirculates within an almost closed vessel system in a short circulation time for 30-40 seconds in *Octopus vulgaris* (Martin et al., 1958; O'Dor and Wells, 1984; Budelmann et al., 1997). Because protein is a primary component of the cephalopod diet, significant amounts of ammonia (NH_4^+) are generated as waste. This waste is expelled in solution through various routes. Excretion from the blood system occurs within a well-differentiated renal system surrounding the venous return to the systemic heart (Martin and Harrison, 1966). Filtered nitrogenous waste is produced through ultrafiltration of blood in the pericardial cavity of the branchial hearts, each connected by a narrow canal to the branchial heart appendages. This canal delivers the excretory product to a bladder-like renal sac and reabsorbs glucose plus amino acids (Harrison and Martin, 1965; Martin, 1965). Several outgrowths of the lateral vena cava, renal appendages, project into the renal sac, and are soft and contractile (Wells, 1978), inflating and deflating continuously with the branchial heart beats. This action helps pump the secreted waste into the sacs, which is then released into the mantle cavity through a renal pore. The primary extra-renal organs involved in ammonia excretion are the gills, where waste is directly excreted from the epithelium into the seawater (Guerra, 2019). In short, the excretion is managed by the renal complex (renal and pancreatic appendages) and the branchial heart complex (branchial heart and pericardial appendage). The fluid-filled renal organs, known as renal sacs, provide unique habitats for parasites (Hochberg, 1982). Contrary to most marine invertebrates that expel waste directly into the environment, cephalopod renal sacs are temporarily store urine within the body. The urine is rich in amino acids such as kynurenic acid, hypoxanthine and tryptophan (Lapan, 1975a).

Dicyemids are endosymbionts, exclusively found in the renal sac of benthic cephalopods. Most exhibit high host specificity (Furuya and Tsuneki, 2003). The first observation record is in a brief note, saying "small infusorial organisms shaped like eels in an octopus" (Cavolini, 1787), and the first generic description was by Kölliker in 1849 (Hochberg, 1983; Catalano, 2012). Until now, 149 dicyemid species have been described from cephalopod hosts across various geographic regions (Furuya, 2018; Furuya and Moritaki, 2022; Catalano, 2024). Speciation in dicyemids likely results from a combination of host switching and speciation within the host (Nakajima et al., 2024).

The body organization of dicyemids is quite simple, consisting of 10 to 47 cells, with neither body cavities nor differentiated organs such as genital openings, muscles, digestive and nervous systems (Kölliker, 1849; Beneden, 1882; Furuya, 2018). The vermiform body is organized into a two-layer arrangement with a single cylindrical central axial cell surrounded by one layer of ciliated peripheral cells. The distinct anterior region, termed a "calotte," is inserted into the renal tubules, crypts, or surfaces of the host renal appendages (Furuya et al., 1997, 2003b, 2004b; Furuya and Tsuneki, 2005; Furuya, 2006), likely adhering to the renal epithelium through the cilia on the

calotte peripheral cells (Ridley, 1968). The calotte is differentiated from other regions by its stiffer, shorter, and thicker cilia (Nouvel, 1947; McConnaughey, 1951; Hochberg, 1990; Furuya et al., 2007).

Dicyemid species with various types of calotte configurations can live together inside the renal sacs of a single host individual. *Dicyema japonicum* and *Dicyema misakiense* occupy different parts of the renal appendages and are commonly found together (Furuya et al., 2001). Dicyemids with a conical calotte, such as *D. misakiense*, insert the anterior body region into crypts or folds within the renal appendages. On the other hand, dicyemids with a disc-shaped calotte, such as *D. japonicum*, attach to the broad, flat, or gently rounded inner surfaces of the renal appendages.

The dicyemids have been morphologically debated as either primitive animals and a sister taxon to all metazoans (Beneden, 1876; Hyman, 1940, 1956; Lapan and Morowitz, 1975) or as secondarily reduced animals whose simple body organization results from parasitic specialization (Nouvel, 1947; Stunkard, 1954, 1972; Ginetsinskaya, 1988). DNA analysis supports the latter view, showing that dicyemids are not truly primitive animals warranting the designation “mesozoa”: using 18S rDNA nucleotide sequences (Katayama et al., 1995); *Hox* gene sequences (Kobayashi et al., 1999); *Pax6* and *Zic* (Aruga et al., 2007); innexin (gap junction component protein) amino acid sequences (Suzuki et al., 2010); transcriptomic data and predicted gene models (Lu et al., 2017); genome sequences (Lu et al., 2019b); and the other transcriptomes with genome data (Drabkova et al., 2022), but rather suggests they belong to the Lophotrochozoa by those authors (Kobayashi et al., 1999; Suzuki et al., 2010; Lu et al., 2017, 2019b; Drábková et al., 2022). However, the origin of their simple body plan remains to be investigated.

The life cycle includes two distinct phases of body organization, as illustrated in Figure 1 (modified from Furuya and Tsuneki, 2003): (1) the vermiform stage, in which the dicyemid exists as a vermiform embryo formed asexually from an agamete (axoblast), and as a final form, the nematogen or rhombogen; and (2) the infusoriform embryo, which develops from a fertilized egg produced around a hermaphroditic gonad termed an infusorigen. Development processes, including the formation of infusorigens, gametogenesis around each infusorigen, and the development of both types of embryo, occur within the axial cell cytoplasm (Furuya et al., 1992a, 1993, 1994, 1996, 2001; Furuya and Tsuneki, 2007). After breaking out of the parent body, the infusoriform larvae escape from the renal environment with the passage of the urine (Hochberg, 1983, 1990). That is, the vermiform stages are restricted to the renal sac of cephalopods, whereas the infusoriform larvae escape from the host into the sea and may enter a new host. However, the process by which the infusoriform larva transitions into the vermiform stage within a new host remains to be revealed. Therefore, this study focuses on the detailed observation of embryos escaping from the parent body, which has been noted concisely, and the investigation of taxis aiming to elucidate infecting pathway to the renal organs in a cephalopod host.

Dicyemids possess a distinct membrane that covers all reproductive cells, originating from the membrane of the axial cell during the development of the vermiform embryo (McConnaughey, 1951; Furuya et al., 1994, 2001). This membrane, which lacks any particular name, is referred to here as the ‘enveloping membrane’. The formation of the enveloping membrane is illustrated in Figure 2 (modified from Furuya et al., 1994). In the final stages of vermiform embryo development, the prospective axial cell undergoes unequal division. The larger anterior cell ceases further division and becomes the axial cell, whereas the smaller posterior cell is incorporated into the axial cell and develops into an agamete. Consequently, both the vermiform embryo and the agamete-derived infusorigen are enclosed by the enveloping membrane. Moreover, the enveloping membrane surrounds a zygote resulting from the fertilization of an egg by a sperm within the infusorigen. The infusoriform embryo developing from such a zygote is likewise surrounded by the enveloping membrane (Furuya et al., 1994). Both embryo types develop within the axial cell of an adult individual and eventually escape. The embryo escape behaviors briefly mentioned (McConnaughey, 1951), however, the mechanism remains unclear due to the absence of genital opening. In trematodes and nematodes lacking a genital opening, larvae or juveniles are released either by penetrating the parent body wall or through another natural opening of the parent body (Lie and Umathevy, 1965; Ivanchenko et al., 1999; Ehlers, 2001).

In invertebrates, body size generally correlates with fecundity (Blueweiss et al., 1978; Peters, 1983; Poulin, 1995), and this is true for dicyemids. Larger dicyemids have more hermaphroditic gonads, called infusorigens, leading to higher fecundity (Furuya et al., 2003a). However, in some species of dicyemids, smaller adult individuals contain infusoriform embryos; this phenomenon is called progenesis, and progenetic rhombogens have fewer infusorigens, producing only 10–20% of the larvae compared to normal rhombogens. Despite their reduced size, their oocytes, spermatocytes, and larvae remain the same size as those in normal rhombogens (Furuya, 2024).

Endoparasites typically exhibit two main stages in their life cycle (Smyth, 1994): a stage where they proliferate within the host body, and a free-living larval stage for dispersal. Dicyemids follow this pattern, with the vermiform individuals representing the growth stage within the host, and the infusoriform larvae representing the dispersal stage. The vermiform individuals function to increase their population within the renal sac, in order that they can produce maximum individuals of infusoriform larvae. To avoid being expelled with the host's urine, these vermiform individuals adhere to the renal sacs by either embedding their anterior part into indentations or attaching to the surface of renal appendages. The cilia on their surface create water currents to bring fresh urine to the area around the calotte, and also enable the vermiform individuals to swim in the urine if they become detached from the renal appendage (Hochberg, 1990). Conversely, infusoriform larvae do not stay within the renal sac but must have the ability to exit it to find a new cephalopod host. These

larvae possess specialized cells called apical cells that contain a refractile body. This refractile body holds highly hydrated magnesium inositol triphosphate, which is considered to be used in detecting cephalopods on the seafloor (Lapan, 1975b).

Parasitic animals utilize taxes to aid in migrating towards and positioning themselves at specific sites within the host (Yasuraoka, 1953, 1954; Haas, 2003). In dicyemids, taxes may facilitate their migration to a new cephalopod host and their movement from seawater through the cephalopod body to the renal sac. Additionally, taxes likely help retain dicyemids within the renal sac, their specialized habitat. Candidate stimuli from the host, which could drive taxes, were investigated to better understand the behavior of vermiform individuals and infusoriform larvae in their respective environments: the cephalopod renal organ and seawater. The involvement of three types of taxes (chemotaxis, thigmotaxis and rheotaxis) were confirmed.

This thesis details the escape behavior and the sites of both vermiform and infusoriform embryos from their respective adult nematogens and rhombogens, as well as the taxes involved in their life history: settled life in renal organs and a free-living life of dispersing into the sea.

Chapter 1: Escape processes in embryos of dicyemids

Materials and methods

Animals

The dicyemid species used in the study were *Dicyema japonicum* and *Dicyema misakiense*, described from the renal sac of the East Asian common octopus *Octopus sinensis* (Gleadall, 2016). Specimens of *O. sinensis* were sourced from a fisherman who collected them in Osaka Bay (Akashi, Hyogo, Japan).

To prevent cross-contamination, dissecting equipment was cleaned and sterilized with absolute ethanol for each renal organ and each host. Each cephalopod was positioned ventral side up in a tray, and the mantle cavity was opened to reveal the paired renal sacs. Dicyemids were isolated from the host octopus kidneys using a pipette. The collected dicyemids were maintained in artificial seawater (Marine Art SF-1; Tomita Pharmaceutical Co., Ltd., Tokushima, Japan) containing the antibiotics streptomycin and penicillin (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Small sections of the renal organ with attached dicyemids were removed by cutting, smeared onto glass slides, fixed immediately in Bouin's solution for 24 hours, and then stored in 70% ethanol. Most preparations were later stained with Ehrlich's hematoxylin and counterstained with eosin. The stained smears were mounted in Entellan (Merck, Darmstadt, Germany). Dicyemids were observed by a light microscope (Olympus BH-2, Tokyo, Japan) at magnifications up to 200x, and measurements were taken with an ocular micrometer. Relative positions of 149 agametes in 20 nematogens and 60 infusorigens in 60 rhombogens were calculated by dividing each central location of the agamete or infusorigen by the axial cell length of the parents. Relative frequency and kernel density curves were constructed using R version 4.1.3 (R Core Team, 2022).

Observation of escaping embryos

Dicyemids typically attach to the surface of renal appendages by their calotte or insert their calotte into a fold of a renal appendage (Furuya et al., 1996; 2004a). When released into artificial seawater, dicyemids swim toward a place to be anchored, necessitating a substitute for the missing renal appendage. The best substitute material identified so far is "nata de coco," a gel produced by *Acetobacter xylinum* through the fermentation of coconut water (Iguchi et al., 2000). Nata de coco was prepared by slicing it into cuboids approximately 10 mm x 10 mm x 5 mm, soaking in distilled water, autoclaving (120°C, 20 min), placing in artificial seawater, autoclaving again, and storing at 4°C with the antibiotics streptomycin and penicillin. Dicyemids were kept in the sterile artificial seawater with a piece of nata de coco in a 4-well dish (Nunc, Roskilde, Denmark). Each well of the dish contained over 100 adult individuals.

Escaping embryos were observed using a high-resolution chilled charge-coupled device color camera VB-7010 (Keyence, Osaka, Japan) and Axiocam 208 color (Zeiss, Oberkochen,

Germany) with inverted microscopes IMT-2 and CKX53 (Olympus) using a 40x objective lens. Measurements were taken with the aid of an ocular micrometer. Escapes were observed in 100 nematogens and 40 rhombogens of *D. japonicum* to determine whether larvae escape from a specific site on the parent body. Relative frequency and kernel density curves were constructed similarly, as described above.

Results

Escaping behavior of vermiform embryos

Vermiform embryos escape from the parent nematogen through one of two methods: either (1) passing through a gap between the membranes of two adjacent peripheral cells or (2) penetrating a peripheral cell. Most escape by the first method (Table I).

Fully developed vermiform embryos are surrounded by an enveloping membrane, until they escape from the parent nematogen. Prior to escaping, vermiform embryos undergo rotations, folds, and turns facilitated by their cilia beating against the axial cell (Fig. 3a_1, a_2). The embryo is positioned near the boundary between membranes of adjacent peripheral cells (Fig. 3a, b) and proceeds to slip into the gap between them (Fig. 3c_1, c_2), ultimately escape from the parent nematogen (Fig. 3d, e). As it departs, the embryo leaves the enveloping membrane behind, alongside some large vacuoles, in the axial cell gap (Fig. 3e_1, e_2). The enveloping membrane within the axial cell contracts and appears to seal the gap between the membranes of the peripheral cells (Fig. 3f_1, f_2). This closure of the escape pathway prevents any harm to the parent nematogen body. Embryos typically take about 10 minutes from the initial signs of escape until complete departure from the parent nematogen.

Signs of the embryo behavior just prior to escape are consistent, whether embryos escape through the gap between peripheral cells or by penetrating a peripheral cell. The vermiform embryo proceeds backward, ruptures the axial cell membrane, and remains positioned within the gap between the axial and peripheral cell membranes (Fig. 3g_1, g_2). Subsequently, the embryo rotates, proceeds backward, and penetrates the peripheral cell to escape from the parent (Fig. 3h, i). Some embryos rotate further and squeeze forward to escape (29.4%) (Table I). One vermiform embryo escaped along with the enveloping membrane (2.9%). In that instance, the enveloping membrane was torn on contact with another individual (not shown). No instances of a vermiform embryo escaping backward were observed. After the escape, the enveloping membrane remains in the gap between peripheral cells, adhering to adjacent peripheral cell membranes (Fig. 3i_1, i_2). Subsequently, the gap between adjacent peripheral cells closes due to the expansion of each cell.

A single embryo lacking the enveloping membrane was observed swimming within the cytoplasm of the axial cell cytoplasm (not shown). This embryo moved randomly within the axial cell cytoplasm and may have been unable to escape.

Escape behavior of infusoriform embryos

Infusoriform embryos escape from their parent rhombogen through one of two methods similar to the escape of vermiform embryos from their parent nematogen. They either (1) pass through a gap between two peripheral cell membranes or (2) penetrate a peripheral cell. Most infusoriform embryos escape by the first method (Table II). After their escape, the gap between the peripheral cells closes through a process akin to that observed when vermiform embryos escape from a parent nematogen.

Fully developed infusoriform embryos surrounded with an enveloping membrane, exhibit imminent escape signaled by the rotational movement of cilia beating within the axial cell (Fig. 4a_1, a_2). Positioned near the boundary between membranes of two adjacent peripheral cells, the embryo proceeds to slip into the gap between them (Fig. 4b_1, b_2), escaping from the parent rhombogen through the gap (Fig. 4c_1, c_2). The enveloping membrane remains in the gap between the peripheral cell membranes, sealing it after the embryo has left (Fig. 4c, d). In some rhombogens, vacuoles are expelled from the axial cell (Fig. 4e_1, e_2). Subsequently, they were incorporated into the axial cell as the hole through which the embryo escaped seals (Fig. 4f_1, f_2). More embryos move forward than backward during the escape (38.7% vs. 32.3%). In two instances, the infusoriform embryos escaped along with the enveloping membrane (6.5%).

Some embryos move backward and slip through the gap between the membranes of peripheral cells (Fig. 4g–i). These embryos ruptured both the axial cell and the enveloping membranes, penetrating a peripheral cell to escape from the parent rhombogen (Fig. 4g_1, g_2). Such escapes leave a hole between the membranes of the peripheral cells, which was subsequently filled by vacuoles dispersed in the axial cell cytoplasm (Fig. 4h_1, h_2). These vacuoles were drawn back into the axial cell once the gap between adjacent peripheral cells had been closed (Fig. 4i_1, i_2). Fewer embryos moved forward compared to those moving backward during escape (6.5% vs. 16.1%).

Site of escape within the parent body

Diagrams illustrating typical escape processes for both vermiform and infusoriform embryos are shown in Figure 5. Because dicyemids lack genital and excretory openings, larvae face the challenge of finding a site from which to escape. Although vermiform and infusoriform embryos were observed to escape from similar sites within the parent nematogen or rhombogen, the frequency distribution of escape events at each site varied between the two types of embryo (Fig. 6).

In nematogens, agametes are distributed throughout the cytoplasm of the axial cell, with a higher concentration in the anterior region (Fig. 6b). Corresponding to this distribution, vermiform embryos escape from various parts of the body, but more embryos escape from anterior than the posterior regions (Fig. 6a, b).

In rhombogens, the infusorigen is located in the anterior region just beyond the center.

Infusoriform embryos escape more frequently from the anterior or posterior regions of the axial cell, rather than from the central region where the infusorigen is situated. However, a greater number of infusoriform embryos escape from the posterior region compared to the anterior site (Fig. 6c, d).

Discussion

Escape routes of embryos from parents without a genital opening

The release of larvae or juveniles has been observed in other invertebrates that lacks genital openings. For instance, in trematodes, the sporocysts of *Schistosoma mansoni* and the rediae of *Echinostoma audyi* escape by penetrating the parent body wall (Ivanchenko et al., 1999 and Lie and Umathevy, 1965, respectively). In entomopathogenic nematodes, the juveniles escape from natural openings after the mother death, a process known as endotokia matricida (Ehlers, 2001). In these cases, the parent organism dies shortly after larval release. In contrast, dicyemid parents repair their bodies and can repeatedly release embryos. Consequently, embryos likely escape through routes that cause minimal damage to the parent, and it seems that a route between cell membranes fulfills such a requirement for both vermiform and infusoriform embryos. Enclosed within a capsule formed by the enveloping membrane, the embryos are not free-swimming in the axial cell cytoplasm, allowing them to slip through the gap between adjacent peripheral cell membranes to escape from the parent. Despite their morphological differences, both types of dicyemid embryo use the same escape mechanism, besides, despite the differences in location and habitat within the renal sac between *D. japonicum* and *D. misakiense*, embryos of both species escape in the same way, likely due to the similar body structure of nematogens and rhombogens.

This study has also showed that the enveloping membrane is involved in repairing the hole left by the escaping embryo, enabling multiple escapes without leaving scars.

Repairing the body with the enveloping membrane

Dicyemids have a unique feature in that all reproductive cell lines are enclosed within an enveloping membrane. This is a unique expansion of the cell membrane, which occurs during the development of vermiform embryos, but remains intact (McConnaughey, 1951; Lapan and Morowitz, 1975; Furuya et al., 1994, 2001). The function of this membrane has not been previously discussed. In this study, it was found that the enveloping membrane significantly aids in repairing the gap through which the embryo has passed. The membrane, left in the gap between the peripheral cell membranes, likely acts as a lid or cover to contain the axial cell cytoplasm. While the enveloping membrane system probably did not evolve specifically to facilitate embryo escape, it serves an essential function in preventing leaks. Without this system, repeated embryo release would be impossible, and the parent would die, as seen in other parasites that lack a specialized opening for offspring release.

After the embryos escape, vacuoles are sometimes observed spilling out from the axial cell. However, these vacuoles are soon reincorporated into the axial cell, leading to the repair of the hole in the axial cell. Several researchers have reported vacuoles arranged in a reticular pattern within the axial cell (Beneden, 1876; Lameere, 1919; Nouvel, 1933; McConnaughey and McConnaughey 1954; Ridley, 1968; Matsubara and Dudley, 1976), though not in connection with embryo escape. These vacuoles typically gather around the embryos, especially those that have left over the enveloping membrane. This observation suggests that the vacuoles help prevent cytoplasm from leaking out of the axial cell. It is likely that the vacuoles function as a substitute for the enveloping membrane in preventing cytoplasm leakage. Therefore, dicyemids possess two structural mechanisms to prevent cytoplasmic leakage after embryo escape.

Timing of rupture of the enveloping membrane

The enveloping membrane ruptures when the embryo moves into the gap between two peripheral cells. In one instance, a vermiform embryo was observed to shed its enveloping membrane and swim freely within the axial cell cytoplasm. Typically, vermiform embryos within the enveloping membrane cannot move freely in the axial cell cytoplasm, restricting them to the region where they developed. Infusoriform embryos, although slightly mobile within the enveloping membrane, also remain confined to their development region. Despite the activity of their cilia, the propulsive force is not transmitted outside the enveloping membrane, preventing the embryos from swimming freely within the axial cell cytoplasm. An embryo moving randomly within the axial cell cytoplasm may therefore be unable to escape.

When part of an infusoriform or vermiform embryo contacts the peripheral cell membrane, the embryo appears to move toward it. Eventually, the embryo finds and opens a gap between two peripheral cells. Despite the restricted movement of the embryo, how it navigates to this gap remains unclear. One possibility is that constant internal pressure inside the axial cell, caused by the increasing volume from developing larvae, facilitates the escape.

The timing for the enveloping membrane to be ruptured is when the embryo enters the gap between the peripheral cells. However, it is unknown whether hatching enzymes or other factors contribute to the rupture of the enveloping membrane at this location.

Position at which embryos escape

Most invertebrates release eggs and larvae through a reproductive opening, such as a genital pore or oviduct. However, dicyemids lack a specific orifice equivalent to a genital pore. Despite their simple body structure, it is considered that a gradient exists to determine the site of embryo escape. This consideration is based on their elongated cylindrical shape, the presence of an anterior–posterior axis, and the differences in the types of internal reproductive cell (agametes in nematogens or infusorigens

with gametes in rhombogens).

In nematogens, agametes are found throughout the axial cell but are more abundant in the anterior region. As the axial cell grows, an agamete proliferates and disperses through repeated divisions. Generally, the agamete is centrally located within the axial cell and not particularly biased towards the anterior in vermiform embryos (Nouvel, 1947; McConnaughey, 1941; Furuya, 1999, 2006, 2018; Furuya et al., 2007; Catalano, 2013a, 2013b; Furuya and Moritaki, 2022). Therefore, agametes in the anterior part of the axial cell presumably undergo cell divisions more actively than those in the posterior part. As a result, vermiform embryos escape from various sites throughout the body, but more embryos escape from the anterior than from the posterior. Escaping from the anterior region is advantageous for vermiform larvae, because it is closer to the renal appendage tissue. If vermiform larvae remain too long in the surrounding urine, they expend energy for swimming and risk being expelled with the urine. Therefore, it is presumably beneficial for vermiform larvae to be released near the anterior region of the parent nematogen, which is in close contact with the settlement site.

In rhombogens, the infusorigen is located slightly forward from the center where an agamete differentiates into an infusorigen (Furuya et al., 1993, Furuya and Tsuneki, 2007). Infusoriform embryos often escape from both the anterior and posterior regions of the axial cell rather than around the central region where the infusorigen is located. This results in a distribution of fully formed embryos at the anterior and posterior ends of the axial cell. Constant gametogenesis and fertilization occur in the infusorigen, with zygotes subsequently leaving the infusorigen. Embryos farther from the infusorigen are more developed, resulting in fully formed larvae at the anterior and posterior ends of the axial cell (Furuya et al., 1992b). More infusoriform embryos escape from posterior regions compared to the anterior regions, in contrast to vermiform embryos (although direct comparison of the two types is challenging due to their distinct forms). Infusoriform embryos are dispersed into the sea after escaping from the rhombogen. Thus, escape from the posterior body region is preferable over the anterior region, which is closer to renal appendage tissue and where entanglement could occur. Under culture conditions, escaped embryos are often observed entangled in kidney fragments left after dissection, suggesting an advantage to escaping from the posterior regions of the free-living rhombogen in urine.

Vermiform and infusoriform embryos do not escape from the same regions of their respective parent bodies. This suggests a significant implication for adult that lacks both genital and excretory openings. Lacking a genital opening requires the embryos to escape from other areas of the parent body. If dicyemids possessed genital or excretory openings, these would need to be located at two different sites on the body for each type of embryo escape. It would be adaptive not to have a specific genital opening site, enabling embryos with different characteristics to be released using a unified body structure. The absence of a genital opening facilitates the release of embryos from appropriate locations for each type, despite sharing a common body plan.

Future perspectives

This chapter has revealed the mechanisms by which embryos are released and the parent body is subsequently repaired in dicyemids, which do not possess a reproductive opening. The function of the enveloping membrane, previously unclear, has been identified as serving to seal or obstruct the leakage of cytoplasm from the parent axial cell. These features, during the embryo escape, may be an inevitable consequence of the simple body structure of animals without a genital pore.

There are still some remaining issues concerning the process of embryo escape. Fully developed embryos consistently rotate due to ciliary movement within the enveloping membrane, which itself does not rotate alongside the embryo. Therefore, it appears that no propulsive force is transferred to the enveloping membrane. Reticular structures within the axial cell cytoplasm form a cytoskeleton and potentially anchor to the embryos, perhaps thereby providing a defined pathway for escape.

As embryos move outward from the axial cell, they open a gap between the cell membranes, establishing a pathway for escape. It is documented that cell adhesion structures, such as septate junctions, gap junctions, and adherens junctions, exist between peripheral cells and axial cell membranes (Furuya et al., 1997). However, it remains unclear whether these adhesive structures are physically disrupted or enzymatically digested to facilitate embryo passage between the membranes. Additionally, the presence and mechanism of any hatching enzymes involved in rupturing the enveloping membrane during the escape of both vermiform and infusoriform embryos have yet to be investigated.

Chapter 2: Taxes of dicyemids

Materials and methods

Animals

The dicyemid species studied were *Dicyema misakiense* and *Dicyema japonicum*, collected from the renal sac of the East Asian common octopus, *Octopus sinensis*, obtained from commercial catches in Osaka Bay (Akashi, Hyogo, Japan). These two dicyemid species are easily distinguished by the calotte morphology of their vermiform adults (Furuya et al., 1992a). However, their infusoriform larvae cannot be differentiated based on morphological characteristics (Furuya et al., 2004a). In some experiments, a few vermiform individuals of *D. japonicum* were found among predominantly *D. misakiense* specimens, and there is a possibility that some infusoriform larvae of *D. japonicum* were also present among those of *D. misakiense*. However, based on the population of parental *D. japonicum* vermiform individuals, the number of mixed individuals of *D. japonicum* is considered to have been negligible.

The dissection equipment was cleaned and sterilized with absolute ethanol for each renal sac and each host to prevent cross-contamination. Each octopus was placed ventral side up in a tray, and the mantle cavity was opened to reveal the pair of renal sacs. Dicyemids were extracted from each renal organ of the host octopus using a pipette. The collected dicyemids were kept in artificial seawater (Marine Art SF-1; Tomita Pharmaceutical Co., Ltd., Tokushima, Japan) with added antibiotics, streptomycin and penicillin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and observed using an inverted microscope (Olympus CKX53; Tokyo, Japan) equipped with a CMOS camera (Axiocam 208 color; Zeiss, Oberkochen, Germany).

Plates for taxis experiments

Customized acrylic plates for the taxis experiments were crafted by the Center for Scientific Instrument Renovation and Manufacturing Support at Osaka University (see Fig. 7). Each plate features a long groove along its midline designed for microscopic observation. To ensure there was no shape bias in the groove, it was filled with artificial seawater, and a suspension of dicyemids was placed at the center. This confirmed their random movement. For the purpose of defining individual migration and identifying a taxis reaction, the groove was divided into three zones of equal length.

Experiment for phototaxis

If dicyemids exhibit phototaxis, whether positive or negative, it could affect experiments and observations under a light microscope. To address this, the transmitted light intensity through the plate was measured under both light conditions (normal observations) and dark conditions using a blackout curtain with a light analyzer (LA-105; Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan). One end of a taxis plate was irradiated for 10 minutes, after which the number of dicyemids at each end of the plate was counted. Experiments using plates with both light and dark regions are referred to as light and dark conditions (LD), while experiments with plates kept entirely in the dark are referred to as dark conditions (DD). For DD experiments, a dicyemid suspension was added to the groove, and the number of dicyemids in both end zones was counted after 10 minutes. Each experiment was performed 10 times, and the data were analyzed statistically using the Brunner-Munzel test.

Experiment for chemotaxis

In experiments on taxis within host tissue, 60µl of host body fluids (blood: taken from systemic heart, gill extraction: extracted from 2mL of artificial seawater, body surface mucus: taken from arms of host, saliva: posterior salivary gland extracted in 2mL of artificial seawater, or urine: filtered with PVDF filter (Millex-GV 0.22µm; Merck, Cork, Ireland) were placed at one end of a groove filled with artificial seawater, while dicyemids were placed in the central region of the groove. After 10 minutes, the number of dicyemids that had moved into the zones at each end of the groove was counted.

For chemotaxis experiments, individual chemical components were selected based on their reported abundance in octopus urine (Lapan, 1975a; Lapan and Morowitz, 1975). The selected chemicals were guanine (GA; 0.2 mg/ml), hypoxanthine (HXA; 0.3 mg/ml), kynurenic acid (KYNA; 1.2 mg/ml), taurine (Tau; 0.3 mg/ml), and tryptophan (Trp; 0.3 mg/ml), all presented within 1.5% agar gel. During different trials, each chemical was tested at one end of the groove, 15 mm from the center, with artificial seawater in 1.5% agar used as a control at the opposite end. The chemicals were observed to diffuse within the first 10 minutes of the experiment, as preliminarily checked with methylene blue solution. After 10 minutes, the number of dicyemids that had moved to each end was counted. The chemotaxis experiments were conducted 10 times, and the data were analyzed statistically using the Brunner-Munzel test.

Experiment for thigmotaxis

Vermiform individuals of *D. misakiense* insert their calotte into indentations in the renal appendages, whereas those of *D. japonicum* attach to the surface of the renal appendage. When released into artificial seawater, they swim to find a place to anchor themselves. Once isolated from the renal sac, they consistently swim in contact with the petri dish until they find a renal appendage or an equivalent substitute. In experiments, when a renal sac was not available, a substitute material was required for anchorage. Nata de coco in sterile artificial seawater was used as a substitute for a renal appendage (Hisayama and Furuya, 2023).

A suspension of dicyemids was added to the center of a customized acrylic plate (also used for the chemotaxis experiments). Two pieces of nata de coco were placed into the groove, which was then filled with seawater. Since the dicyemids complete their movements within 10 minutes from the start of the experiment, the number of individuals attached to and not attached to the nata de coco was counted after 10 minutes. The experiments were performed 10 times, and the data were analyzed statistically using the Brunner-Munzel test.

Experiment for rheotaxis

To investigate the response of dicyemids to the flow of urine expelled from the renal sac, a modified serological pipette (bent at right angles in two places) was placed on the stage of an inverted microscope (Fig. 8). The setup included a flow of artificial seawater (simulating urine flow) released by an adjustable tap from a reservoir connected to a vertically fixed Pasteur pipette via silicone tubing. Dicyemids were introduced through a pipette inserted into a 3 mm diameter hole at the outflow side of the horizontal part of the modified serological pipette. The microscope stage was leveled using a spirit level (ED-FL; Ebisu, Niigata, Japan).

Artificial seawater was allowed to flow until the horizontal section of the serological pipette was full, and then the flow was stopped. A sample of dicyemids was added through the 3 mm diameter hole, and then artificial seawater was allowed to flow at one of three current speeds: 9.8 μm/s (slow), 30 μm/s (medium), or 752 μm/s (fast). Data on flow speed and dicyemid swimming were estimated by analyzing 30-second video recordings using the Manual Tracking function of Fiji (Schindelin et al., 2012), and a circular plot was constructed using R version 4.3.1 (R Core Team, 2023). The velocity of the water current in the setup was measured using the flow speed of debris unavoidably introduced with the dicyemids.

Results

Phototaxis

Under the light vs. dark conditions (LD), the transmitted light intensity was measured at 143 lx at the illuminated end and 0.292 lx at the opposite (dark) end (Fig. 9c, d, e). In the completely dark (DD) condition, the light intensity was 0.807 lx throughout. Neither the vermiform individuals of both species nor the infusoriform larvae exhibited any noticeable phototaxis (Fig. 9c, d, e). Thus, it was concluded that light did not interfere with subsequent microscopic observations and experiments.

Chemotaxis

Vermiform individuals of both species displayed no noticeable chemotaxis towards host organ fluids (such as blood, gill extract, saliva, or body surface mucus) or towards host urine components, including GA, HXA, KYNA, Tau, or Trp (Figs. 10, 12). In contrast, infusoriform larvae exhibited significant movement away from octopus (host) blood ($P < 0.05$, Fig. 11) but did not show any apparent chemotactic response to other host organ fluids, host urine, or its components (Fig. 13).

Thigmotaxis

For both dicyemid species, a significant number of vermiform individuals attached to the nata de coco (Fig. 14), indicating strong positive thigmotaxis ($P < 0.0001$, Fig. 15b, c). In contrast, infusoriform larvae did not attach and exhibited significantly negative thigmotaxis ($P < 0.0001$, Fig. 15d).

Rheotaxis

When the flow was "fast," both vermiform individuals and infusoriform larvae of both species were carried downstream (Fig. 16a, d, g). Some individuals reoriented their anterior part (the calotte for vermiform individuals or the apical cell for infusoriform larvae) against the direction of the water flow. The mean swimming speeds were 593 μm/s for *D. misakiense* vermiform individuals, 666 μm/s for *D. japonicum* vermiform individuals, and 512 μm/s for infusoriform larvae.

At "medium" flow speed, 56.8% of *D. misakiense* vermiform individuals (n = 44), 53.8% of *D. japonicum* vermiform individuals (n = 39), and 34.7% of infusoriform larvae (n = 75) swam upstream. The remaining swam downstream: 43.2%, 46.2%, and 65.3%, respectively (Fig. 16b, e, h). The mean speeds were 210 μm/s for *D. misakiense*, 213 μm/s for *D. japonicum*, and 239 μm/s for

infusoriform larvae.

Under "slow" flow conditions, the majority of vermiform individuals swam upstream: 85.2% vs. 14.8% downstream for *D. misakiense* (n = 27) and 92.3% vs. 7.7% for *D. japonicum* (n = 52), indicating positive rheotaxis (Fig. 16c, f). The infusoriform larvae swam randomly in any direction, with 43.6% moving upstream and 56.4% downstream (n = 55), showing no clear rheotaxis (Fig. 16i). The mean speeds were 255 µm/s for *D. misakiense*, 229 µm/s for *D. japonicum*, and 200 µm/s for infusoriform larvae.

Discussion

Regarding the taxis of small organisms in fluid environments, a great deal of information has been gathered from studies on the unicellular *Paramecium* (Van Houten, 2023). Parasitic organisms employ various types of taxis to navigate to their biological niche, aiding parasite larvae in locating their host and allowing parasites within the host to reach their target organ. However, the specific mechanisms of these processes are yet to be fully understood.

In parasitic nematode species, host-seeking behavior at the larval stage is complex and involves several sensory modalities, such as olfaction, gustation, thermosensation, and humidity sensation, although their behavior remains poorly understood (Gang and Hallem, 2016). In monogeneans, the anterior adhesive organs play a potential role in host-specificity (Whittington et al., 2000). This study investigated how dicyemids living in the unique habitat of a cephalopod renal organ respond to their environment. The responses of the two morphological forms, vermiform and infusoriform, were expected to exhibit taxis according to the requirements of their mode of life.

For vermiform individuals living in the renal sac, adhesion to and interaction with the outer surface of the renal epithelium are crucial. Their strongest response was thigmotaxis, which helps them stay attached within the host renal sac. They showed no response to light, likely because light cues are not useful for orientation inside the host body. Additionally, they did not react to specific metabolites abundant in the urine, as their environment is stable, nutrient-rich, and homogenous, lacking particular substances to avoid or seek for nourishment. The primary requirement for vermiform individuals is to adhere to the renal sac and avoid being expelled with the urine.

The renal appendages rhythmically contract due to a muscle-fiber network, facilitating the

flow of hemolymph and urine (Budelmann et al., 1997). This rhythmic contraction may cause some vermiform individuals to be detached from the renal appendage. Positive rheotaxis against the urine flow helps them re-establish their position within the renal sac, while thigmotaxis enables them to find and adhere to an appropriate site on the renal appendage.

The infusoriform larvae are expelled with urine through the host renal pore and disperse into the sea. Typically, phototaxis and gravitaxis are necessary for locating a benthic host (Latz and Forward, 1977; Yamaguchi et al., 2018). However, infusoriform larvae lack these taxis. Instead, they possess unique apical cells containing a refringent body composed of a highly hydrated magnesium salt of inositol hexaphosphate. This results in a high specific gravity, giving the larvae negative buoyancy, allowing them to navigate the benthic environment effortlessly (Lapan, 1975b). Additionally, the renal pore of cephalopods is located at the base of the renal sac and opens downward, facilitating the larvae escape through the renal pore.

Some infusoriform larvae swam against the current, which seems to be a trait distinct from rheotaxis. When observed in vitro, these larvae swim in various directions. Therefore, among the many larvae, there will always be some swimming against the current. Unlike vermiform individuals, the infusoriform larvae displayed negative thigmotaxis. They have cilia only on the posterior half of their bodies, and their anterior lacks structures for adhesion to the renal appendages, unlike the calotte present in vermiform individuals. These morphological characteristics likely facilitate their departure from the host renal sac and prevent them from attaching to the renal appendages, which they are unable to do due to the absence of specialized attachment structures.

It is unclear how infusoriform larvae reach the host renal sac after swimming freely in the sea, or what stimuli ('markers') they use to approach the host when nearby. In this study, the larvae only exhibited negative chemotaxis to host blood. This aversion suggests that larvae may escape into open seawater from urine containing blood elements or avoid hosts that are injured and near death. Lapan and Morowitz (1975) reported a 10% infection rate in an experiment with infusoriform larvae and their host Sepia. If larvae directly invade the host, they likely do not use the circulatory system for entry. Additionally, the lack of positive chemotaxis to host body surface mucus suggests that larvae may not directly enter a host cephalopod. Instead, another life stage from urn cells (germinal cells) or intermediate hosts may be involved. Urn cells are released from infusoriform larvae (McConnaughey and McConnaughey, 1954), and asymmetric division of urn cells has been observed, though not conclusively (Lapan and Morowitz, 1975). If urn cells develop into another

larval type, studying their taxis may be crucial for understanding the life history of dicyemids.

The two reproductive modes of dicyemids exhibit distinct morphologies and taxis adapted to their roles in the life cycle. Sensory organs in dicyemids have not been identified, even at the electron microscope level. However, Lu et al. (2019a) suggested that infusoriform larvae might possess sensory functions based on gene ontology research and immunohistochemical assays of neurotransmitters and neuropeptides. Infusoriform larvae could potentially detect changes in water velocity, possibly through bristles on the apical cell serving as sensory structures. Additionally, the calotte of vermiform individuals, with its dense short cilia, might function as a sensory organ. Identifying these sensory cells in dicyemids will require further studies, including research on neurotransmitters and gene expression.

Summary

This thesis has revealed the escape behavior and the sites of both vermiform and infusoriform embryos from their respective adult nematogens and rhombogens, which lack genital opening for embryo release, as well as the taxes involved in their life history.

There were no differences in escape methods between species or embryo types, likely due to morphological constraints that result in both nematogen and rhombogen adults having a similar body plan. Embryos escaped through the gaps between neighboring peripheral cells of the adult, rupturing the membranes of both the axial cell and the enveloping membrane of the embryo. After the embryo escaped, the path was sealed by the remaining enveloping membrane. It is a novel finding that the involvement of this membrane and the vesicles within the axial cell help prevent the leakage of the cytoplasm of the axial cell. Because of this system, the adult can expedite the repair of the axial cell membrane and continue to release larvae.

The varied escape regions for the two embryo types are presumably due to the adult body lacking a genital opening, resulting in each embryo type having its specific escape site. Vermiform embryos could escape from any region of the axial cell, although more embryos were frequently observed escaping from the anterior regions than the posterior regions. The vermiform individuals, including adults and vermiform larvae developed, attach their calotte to renal appendage surface. In the host renal sac, the positive rheotaxis to the urine flow caused by the ciliary movement of the individuals already attached to the renal appendages and the positive thigmotaxis to the substances suggest that vermiform larvae escaped from the anterior region of the adult body can adhere to the surface of the renal appendages more quickly than those escaped from the posterior region. In contrast, infusoriform embryos escaped from both anterior and posterior regions within the axial cell, with more embryos escaping from the posterior regions. It is conceivable that the negative thigmotaxis allows them to move away from the surface of the renal appendages and closer to the renal pore, facilitating their release into seawater.

The three types of taxes were investigated: chemotaxis, thigmotaxis, and rheotaxis. In addition, the response to light intensity under normal observation was tested. Vermiform individuals and infusoriform larvae did not show any response to light, likely because light cues are not useful for orienting themselves within the host body. Vermiform individuals did not exhibit chemotaxis toward major host components such as urine, tissue fluids, or extracts of host gills. However, they showed positive thigmotaxis and positive rheotaxis to slow water flow. These two taxis responses seem to be more crucial than others, likely aiding their attachment to the renal appendages and retention in the renal sac, respectively. The infusoriform larvae exhibited negative chemotaxis to host blood and negative thigmotaxis, with no evidence of rheotaxis. Negative thigmotaxis may help release infusoriform embryos from the renal appendages through contact aversion to the surface of the appendage or the surrounding vermiform individuals attached to it. Negative chemotaxis to the

host blood suggests that the infusoriform larvae can sense the urine environment containing hemolymph and avoid entering through the vascular system to access the renal sac, although no sensory organs have been identified in dicyemids, even when examined with an electron microscope. Consequently, the method by which infusoriform larvae enter the cephalopod host remains undetermined. Additionally, the infusoriform larvae did not show the positive chemotaxis to the host body surface mucus, implying that another life stage developed from urn cells (germinal cells), or intermediate hosts may be involved before entering a cephalopod host.

Dicyemids utilize taxis, an ancestral trait also observed even in protozoans, for settlement and dispersal within their habitat. In contrast, regarding larval release, the absence of a specific reproductive pore results in larvae being released through the gaps between peripheral cells across the entire body surface. This derived trait, involving a unique system of an enveloping membrane encasing the larvae and surrounding vacuoles, prevents damage to the parent body and enables continuous larval release. The specialized habitat within the renal sacs of cephalopods has likely exerted selective pressure, leading to the development of this system.

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Figures

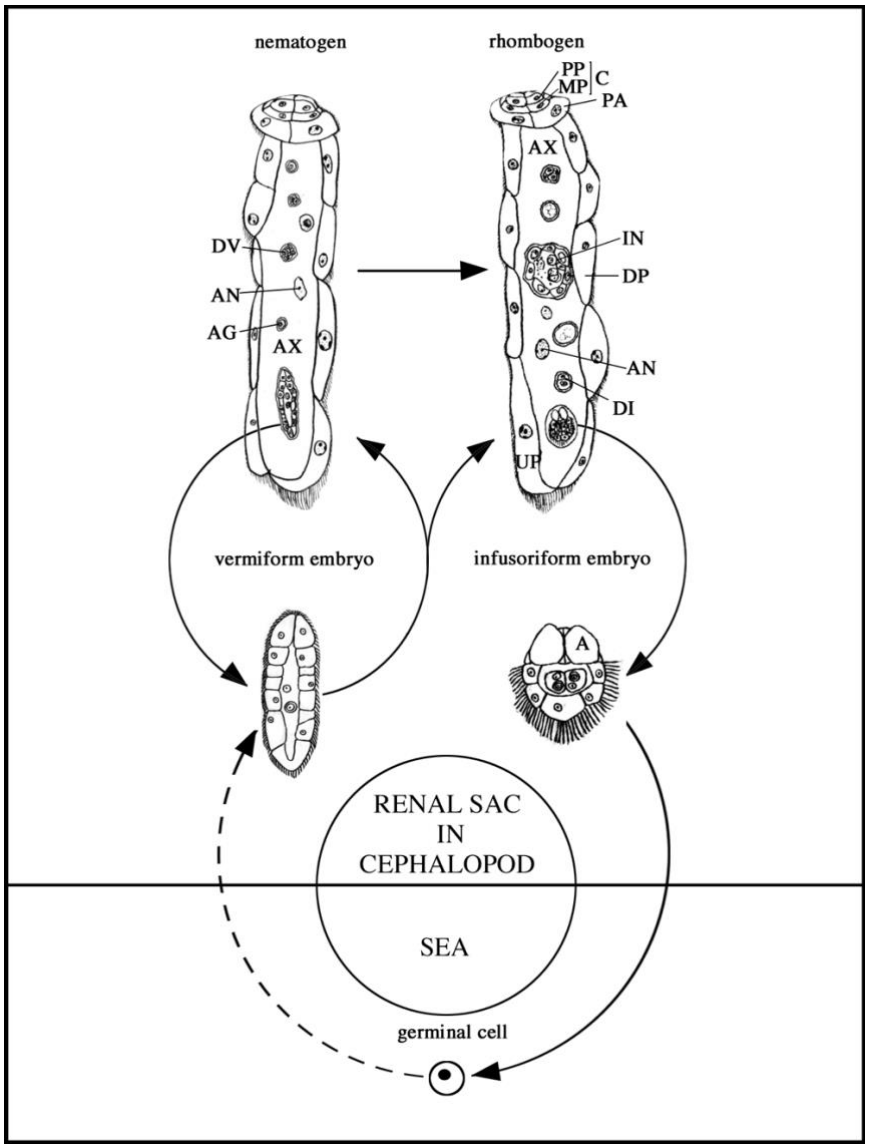

Figure 1. The morphology and life cycle of dicyemids (modified from Furuya and Tsuneki, 2003). The life cycle is characterized by two phases with different body organizations: (1) the vermiform stage, where an agamete (axoblast) asexually develops into a vermiform embryo, eventually forming a nematogen or rhombogen; and (2) the infusoriform embryo, which develops from a fertilized egg produced near a hermaphroditic gonad called an infusorigen. In the vermiform stage, a layer of peripheral cells surrounds a large cylindrical axial cell. A calotte is formed by four to ten anterior peripheral cells (propolars and metapolars). The remaining peripheral cells are called diapolars, with two posterior diapolars specialized as uropolars, often bearing numerous granules. During infusorigen development, gametogenesis occurs around each infusorigen, and the development of the two types of embryo proceeds within the axial cell cytoplasm. Vermiform stages are confined to the renal sac of cephalopods, while infusoriform larvae escape from the host into the sea and may

enter a new host (dashed line). However, it is not known how the infusoriform larva transitions to the vermiform stage in the new host. Abbreviations: apical cell, A; agamete, AG; axial cell nucleus, AN; axial cell, AX; calotte, C; developing infusoriform embryo, DI; diapolar cell, DP; developing vermiform embryo, DV; infusorigen, IN; metapolar cell, MP; parapolar cell, PA; propolar cell, PP; uropolar cell, UP.

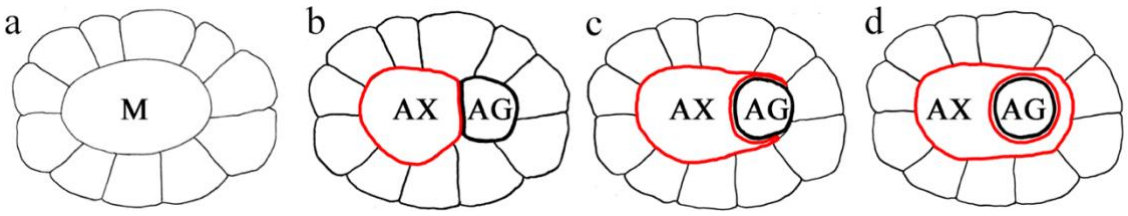

Figure 2. Schematic diagrams illustrating the formation of enveloping membranes during the final stage of development in the vermiform embryo (modified from Furuya et al., 1994; Hisayama and Furuya, 2023). Sagittal section.

(**a**) A prospective axial cell (M) is located in the center of the embryo. (**b**) An axial cell (AX) and an agamete (AG) are produced after the unequal division of the prospective axial cell. (**c**) The agamete is incorporated into the axial cell through the formation of an enveloping membrane surrounding the agamete and integrating it into the axial cell. (**d**) The agamete is fully enclosed by the enveloping membrane derived from the axial cell membrane. Abbreviations: agamete, AG; axial cell, AX; prospective axial cell, M.

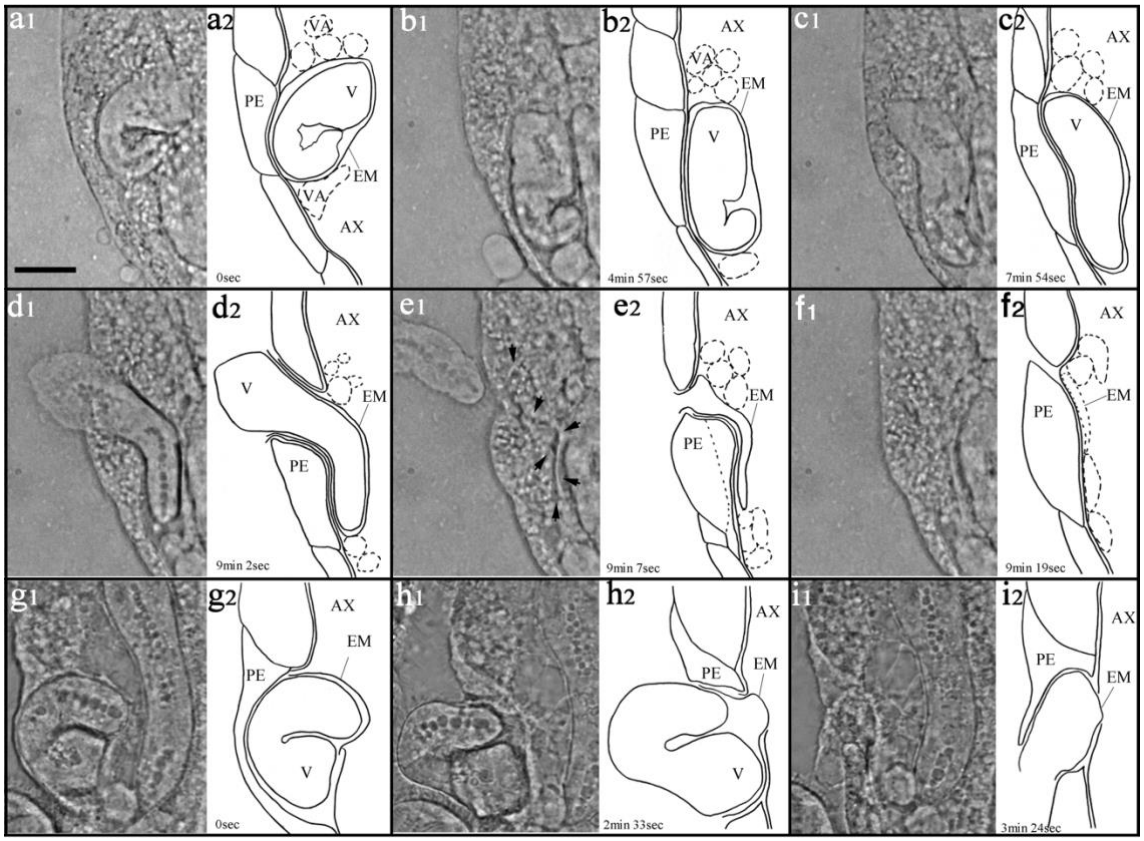

Figure 3. Light micrographs and corresponding diagrams of a vermiform embryo escaping from a nematogen of *Dicyema japonicum* (modified from Hisayama and Furuya, 2023). (**a**–**c**) The vermiform embryo (V), with its enveloping membrane (EM), is located within the axial cell (AX). The letter V, denoting the vermiform embryo, is placed on the calotte. Some vacuoles (VA) are visible within the axial cell. (**d**, **e**) The vermiform embryo escapes through the gap between two peripheral cells (PE). (**d_1**, **d_2**) The vermiform embryo ruptures the enveloping membrane and opens the gap between the two peripheral cells. (**e_1**, **e_2**) After the escape, the enveloping membrane remains in the gap between the peripheral cell and the axial cell, indicated by the arrows. (**f_1**, **f_2**) The gap closes after the escape, and the enveloping membrane retracts back within the axial cell. (**g**–**i**) The vermiform embryo penetrates through a peripheral cell. (**g_1**, **g_2**) The vermiform embryo backs out of the axial cell, and (**h_1**, **h_2**) penetrates through the peripheral cell. (**i_1**, **i_2**) The enveloping membrane remains in the gap between the peripheral cell and the axial cell. Scale bar = 20 μm. Abbreviations: axial cell, AX; enveloping membrane, EM; peripheral cell, PE; vermiform embryo, V; vacuole, VA.

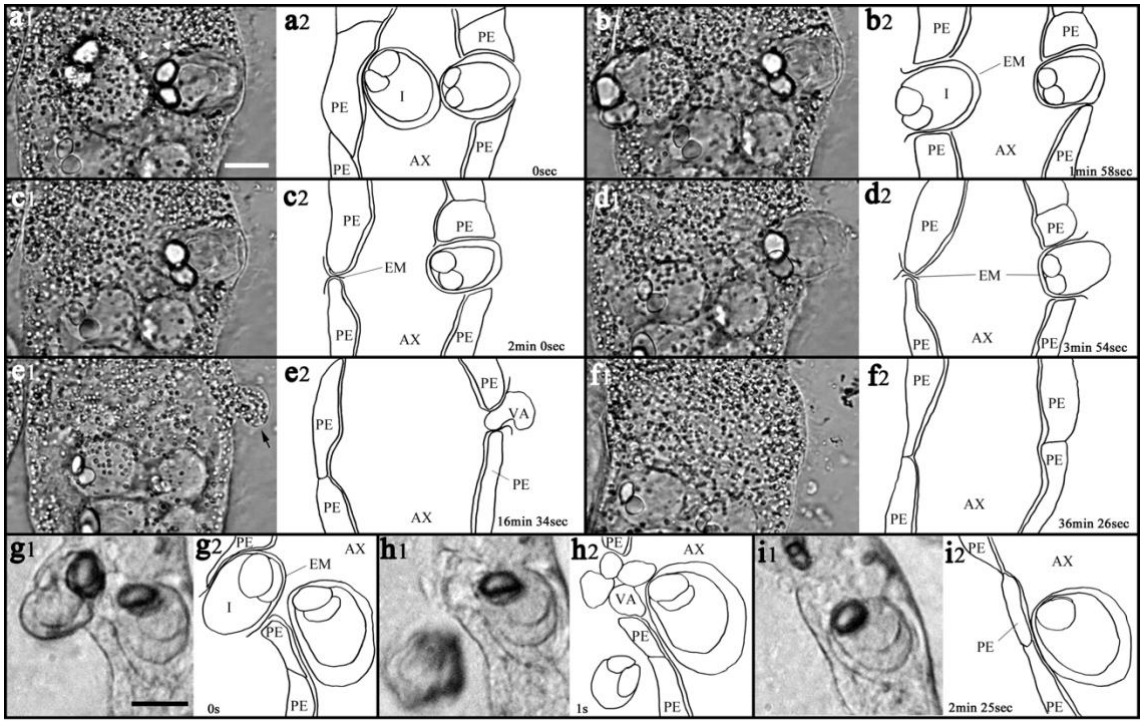

Figure 4. Light micrographs and corresponding diagrams of infusoriform embryos escaping from rhombogens in *Dicyema misakiense* (**a**–**f**) and *Dicyema japonicum* (**g**–**i**) (modified from Hisayama and Furuya, 2023). (**a**$_1$, **a**$_2$) Two infusoriform embryos (I) with the enveloping membrane. (**b**$_1$, **b**$_2$) The infusoriform embryo on the left ruptures the enveloping membrane (EM) and escapes forward through the gap between peripheral cells, while the infusoriform embryo on the right escapes backward through the gap between peripheral cells (PE). (**c**, **d**) After the embryo on the left escapes, the enveloping membrane remains in the gap between the two peripheral cells. (**e**$_1$, **e**$_2$) After the embryo on the right escapes, vacuoles (VA) can be seen protruding from the axial cell (AX). (**f**$_1$, **f**$_2$) The gap between peripheral cells closes. (**g**–**i**) The infusoriform embryo penetrates through the peripheral cell (PE). (**o**, **p**) After the escape, vacuoles (VA) temporarily block the gap between peripheral cells. Note that the vacuoles combine into the axial cell cytoplasm (AX). (**i**$_1$, **i**$_2$) The gap closes. Scale bars = 20 μm. Abbreviations: axial cell, AX; enveloping membrane, EM; infusoriform embryo, I; peripheral cell, PE; vacuole, VA.

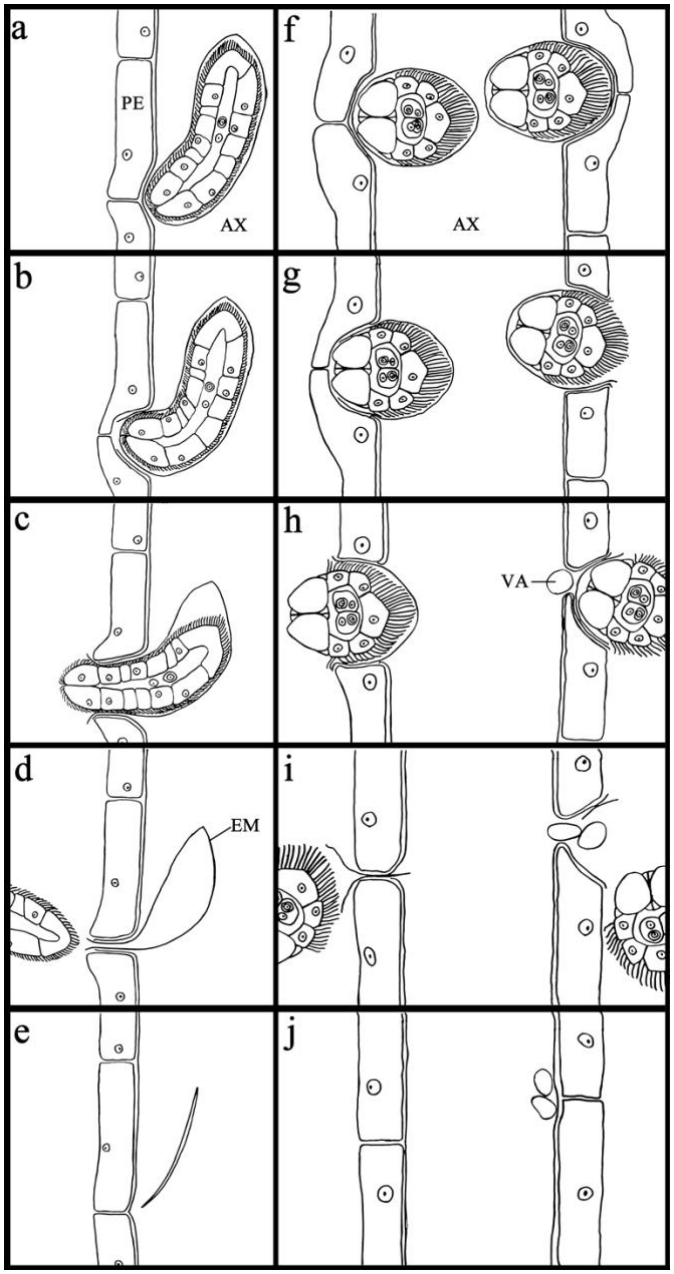

Figure 5. Diagrammatic summary of the events during escape. (**a**–**e**) Escape of a vermiform embryo from a nematogen (modified from Hisayama and Furuya, 2023). (**a**) The vermiform embryo within the axial cell (AX) is surrounded by the enveloping membrane and situated close to the gap between two peripheral cells (PE). (**b**) The axial cell membrane ruptures, and the vermiform embryo moves through the gap between the peripheral cells. (**c**) The vermiform embryo ruptures the enveloping membrane and escapes. (**d**) After escape, the enveloping membrane remains in the gap between the peripheral cells. (**e**) The enveloping membrane shrinks and is resorbed within the axial cell cytoplasm. (**f**–**j**) Escape of an infusoriform embryo from a rhombogen. Embryos can escape either forward (left) or backward (right): one of each is shown here. (**f**) Infusoriform embryos surrounded

by the enveloping membrane are close to the gap between peripheral cells. (**g**) Infusoriform embryos move through the gap between peripheral cells, rupturing the axial cell membrane. The infusoriform embryo on the right ruptures the enveloping membrane. (**h**) During escape, a vacuole (VA) blocks the gap between the peripheral cells of the escaping infusoriform embryo on the right. (**i**) After escape, the remains of the enveloping membrane are in the gap between the peripheral cells on the left, while on the right, vacuoles associate with the enveloping membrane to block the gap. (**j**) The enveloping membrane and vacuoles are resorbed within the axial cell cytoplasm. Abbreviations: axial cell, AX; enveloping membrane, EM; peripheral cell, PE; vacuole, VA.

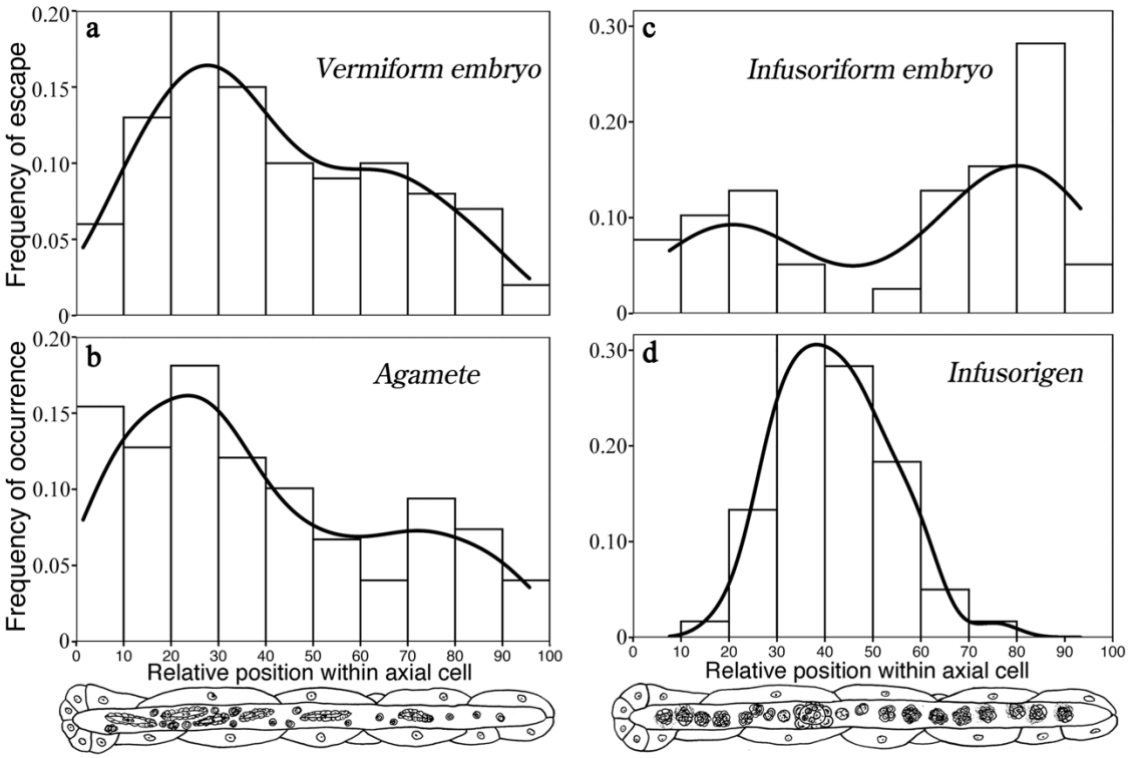

Figure 6. Frequency distribution of the sites where vermiform and infusoriform embryos escape from agametes and infusorigens, respectively, displayed as histograms and kernel density curves (modified from Hisayama and Furuya, 2023). (**a**) Frequency of vermiform embryo escapes in different regions of the nematogen body. (**b**) Position of agametes within the nematogen body. (**c**) Frequency of infusoriform embryo escapes in different regions of the rhombogen body. (**d**) Position of infusorigens within the rhombogen body. Schematic diagrams of the nematogen (bottom left) and rhombogen (bottom right) bodies indicate the positions of agametes, infusorigens, and embryos.

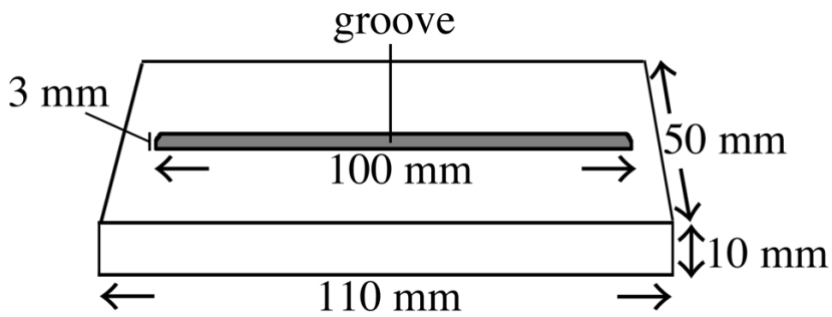

Figure 7. Schematic diagram of the custom acrylic plate designed for taxis investigation, featuring a groove to hold samples.

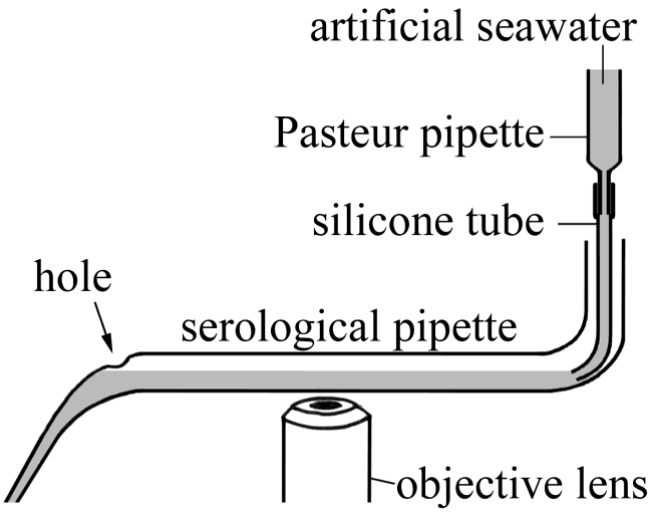

Figure 8. Schematic diagram of the rheotaxis apparatus. A Pasteur pipette is fixed in place with a clamp (not shown). Below the pipette is an adjustable tap on a silicone tube (not shown) to regulate the flow and speed of artificial seawater. After filling the serological glass pipette with a static load of artificial seawater, samples of dicyemids are introduced through the hole.

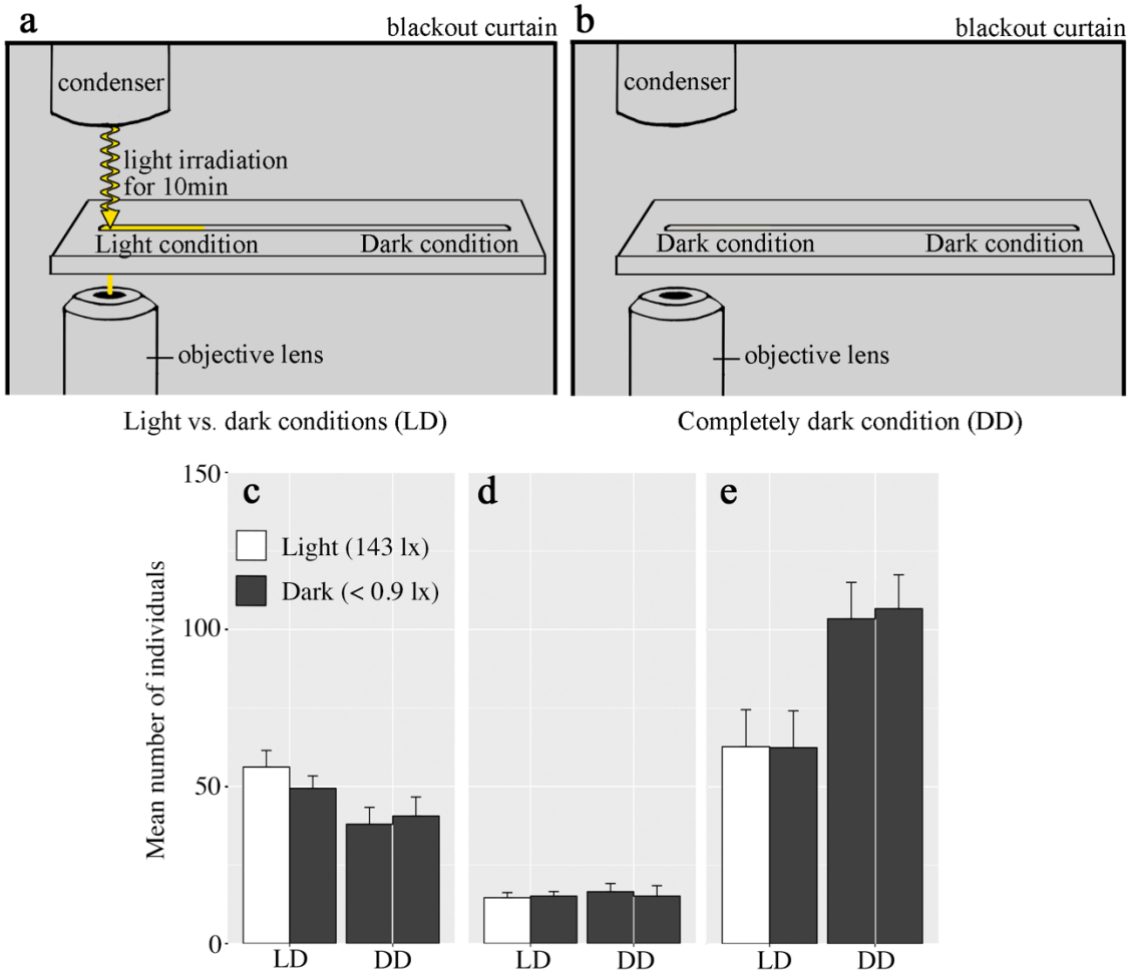

Figure 9. Schematic diagrams and the mean number of individuals (with SE) at each end of the groove in phototaxis experiments. (**a**) Light vs. dark conditions. (**b**) Completely dark condition. (**c**) *Dicyema misakiense* vermiform individuals. No significant differences were observed among all tests (Brunner-Munzel test. n = 10. LD, $P = 0.319$; DD, $P = 0.699$). (**d**) *Dicyema japonicum* vermiform individuals. No significant differences were observed among all tests (Brunner-Munzel test. n = 10. LD, $P = 0.703$; DD, $P = 0.744$). (**e**) infusoriform larvae of *D. misakiense*. No significant differences were observed among all tests (Brunner-Munzel test. n = 10. LD, $P = 1.00$; DD, $P = 0.944$).

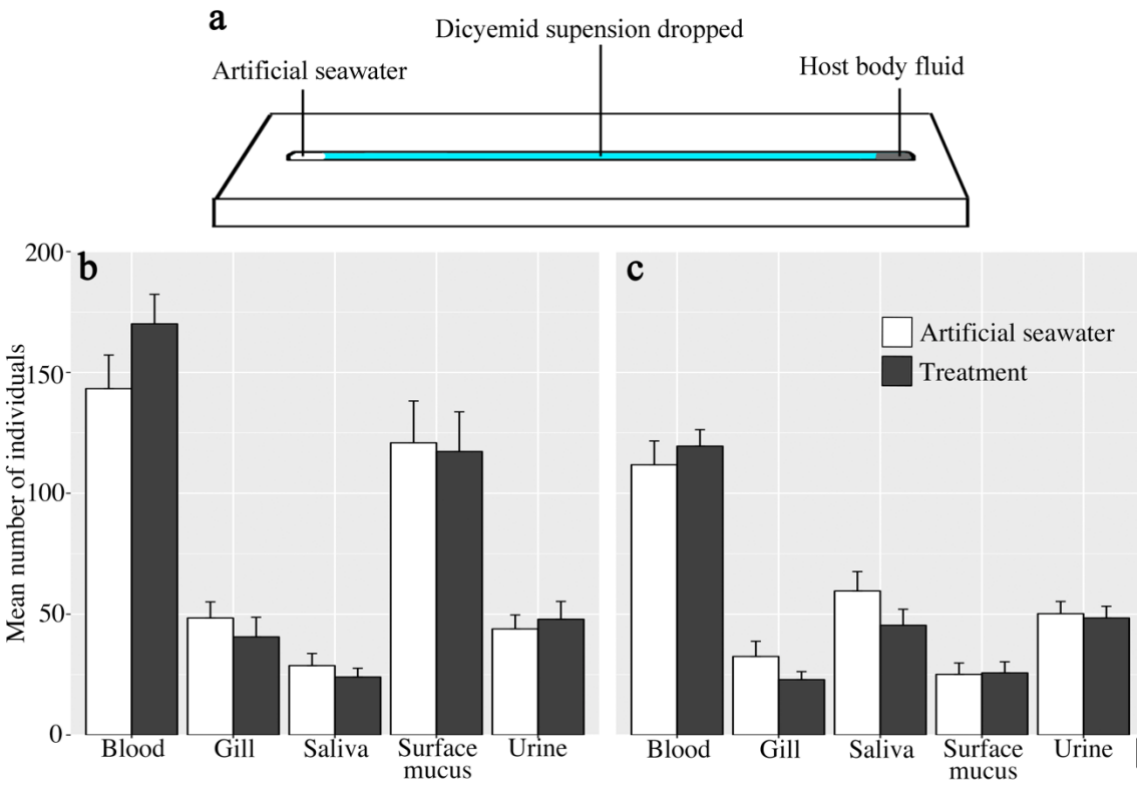

Figure 10. Schematic diagram and the mean number (with SE) of vermiform individuals moving in response to different body fluid stimuli in chemotaxis experiments. (**a**) Schematic diagram of experiment. (**b**) *Dicyema misakiense*. No significant differences were observed among all tests (Brunner-Munzel test. n = 10. Blood, $P = 0.137$; gill, $P = 0.403$; saliva, $P = 0.600$, surface mucus, $P = 0.944$, and urine, $P = 0.887$). (**c**) *Dicyema japonicum*. No significant differences were observed among all tests (Brunner-Munzel test. n = 10. Blood, $P = 0.594$; gill, $P = 0.327$; saliva, $P = 0.215$, surface mucus, $P = 0.858$, and urine, $P = 0.801$).

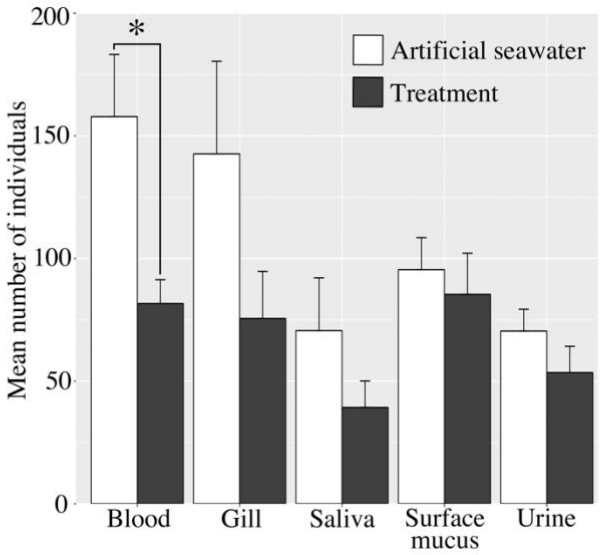

Figure 11. The mean number (with SE) of *Dicyema misakiense* infusoriform larvae responding in chemotaxis experiments with different body fluids. A significant difference was observed in the blood of the host octopus (Brunner-Munzel test. n = 10. Blood, $P = 0.023$); however, no significant differences were observed among the others tested (Brunner-Munzel test. n = 10. Gill, $P = 0.277$; saliva, $P = 0.659$, surface mucus, $P = 0.511$, and urine, $P = 0.208$). *: $P < 0.05$.

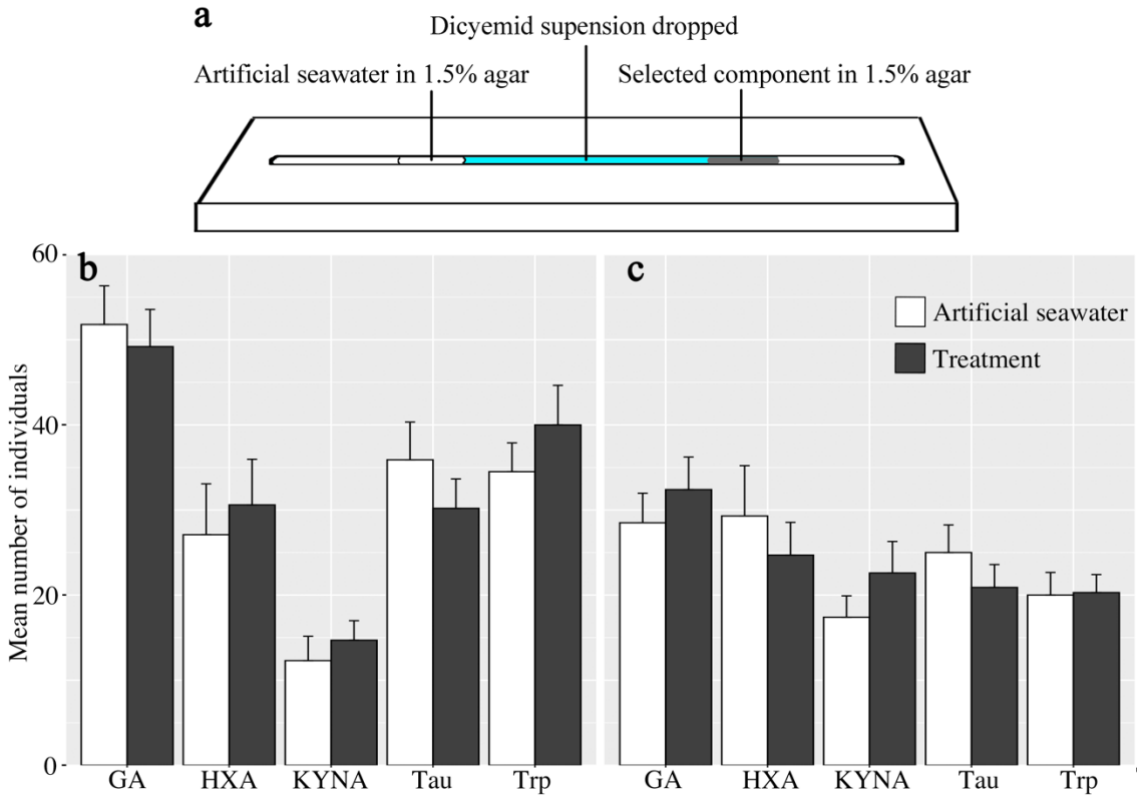

Figure 12. Schematic diagram and the mean number (with SE) of vermiform individuals in chemotaxis experiments with urine compounds. (**a**) Schematic diagram of experiment. (**b**) *Dicyema misakiense*. No significant differences were observed among all tests (Brunner-Munzel test. n = 10. GA, $P = 0.615$; HXA, $P = 0.451$; KYNA, $P = 0.316$, Tau, $P = 0.403$, Trp, $P = 0.271$). (**c**) *Dicyema japonicum*. No significant differences were observed among all tests (Brunner-Munzel test. n = 10. GA, $P = 0.664$; HXA, $P = 0.757$; KYNA, $P = 0.357$, Tau, $P = 0.318$, Trp, $P = 0.678$). Abbreviations: guanine, GA; hypoxanthine, HXA; kynurenic acid, KYNA; taurine, Tau; tryptophan, Trp.

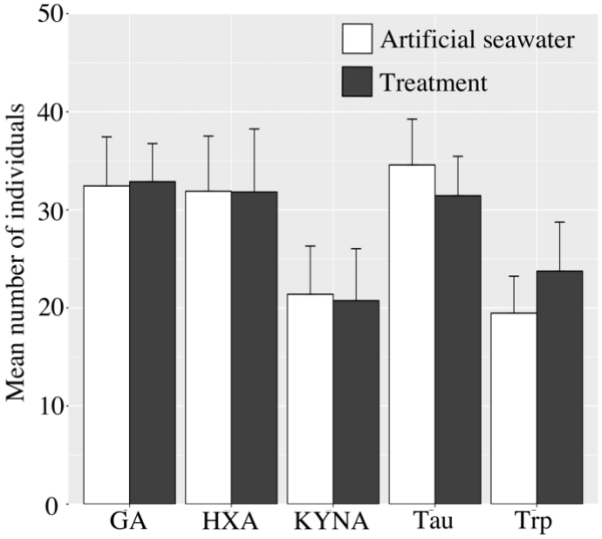

Figure 13. The mean number (with SE) of individuals in chemotaxis experiments against urine compounds for *Dicyema misakiense* infusoriform larvae. No significant differences were observed among all tests (Brunner-Munzel test. n = 10. GA, $P = 0.889$; HXA, $P = 0.832$; KYNA, $P = 0.915$, Tau, $P = 0.615$, Trp, $P = 0.453$). Abbreviations: guanine, GA; hypoxanthine, HXA; kynurenic acid, KYNA; taurine, Tau; tryptophan, Trp.

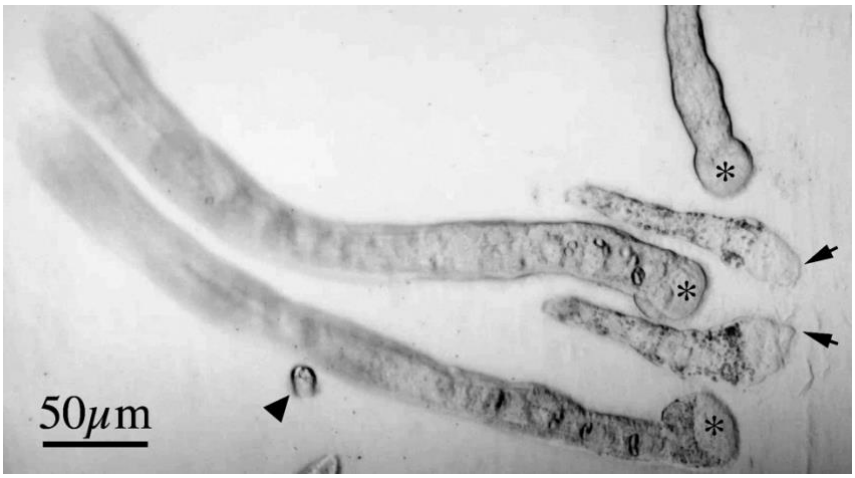

Figure 14. Dicyemids associated with nata de coco, positioned to the right. Vermiform individuals of *Dicyema japonicum* (marked with asterisks) are attached to the surface, while vermiform individuals of *Dicyema misakiense* (indicated with arrows) are embedded within it. The arrowhead points to an unattached infusoriform larva.

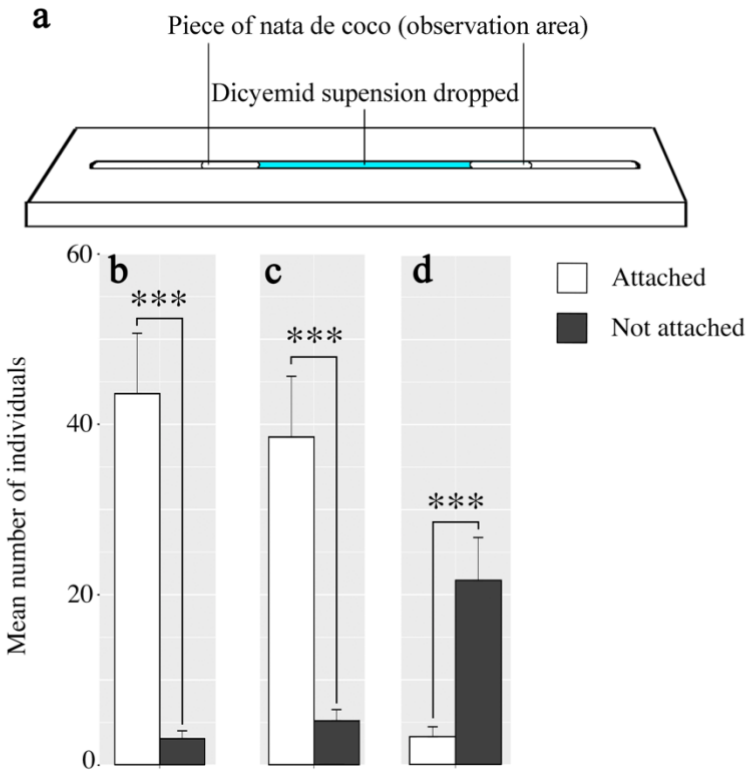

Figure 15. Schematic diagram and the results of thigmotaxis experiments. (**a**) Schematic diagram of experiment. (**b**) *Dicyema misakiense* vermiform individuals. (**c**) *Dicyema japonicum* vermiform individuals. (**d**) Infusoriform larvae of *D. misakiense*. Significant differences were observed in all the experiments (Brunner-Munzel test. n = 10. ***: $P < 0.0001$).

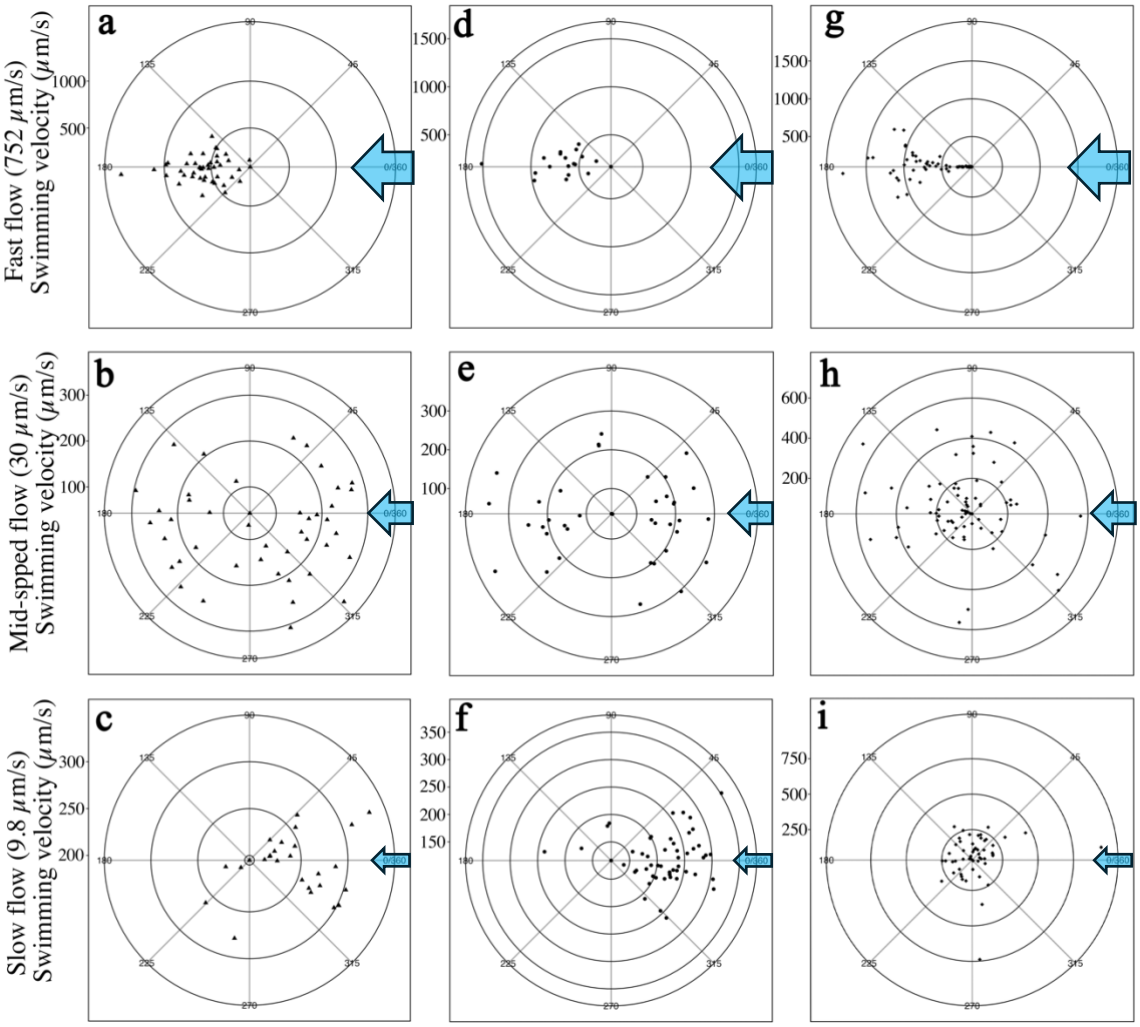

Figure 16. Circular plot distributions for swimming direction and mean velocity at three flow speeds in artificial seawater (flowing from the origin, indicated by the arrows). (**a**–**c**) *Dicyema misakiense* vermiform individuals. (**d**–**f**) *Dicyema japonicum* vermiform individuals. (**g**–**i**) Infusoriform larva of *D. misakiense*.

Tables

Table I. Methods of vermiform embryo escape and site of escape from parent nematogen (n = 34; modified from Hisayama and Furuya, 2023).

Escape method/site	Anterior region	Posterior region
Pass through a gap between peripheral cells	19 (55.9%)	9 (26.5%)
Penetrate a peripheral cell	4 (11.8%)	1 (2.9%)

Table II. Methods of infusoriform embryo escape and site of escape from parent rhombogen (n = 31; modified from Hisayama and Furuya, 2023).

Escape method/site	Anterior region	Posterior region
Pass through a gap between peripheral cells	12 (38.7%)	10 (32.3%)
Penetrate a peripheral cell	2 (6.5%)	5 (16.1%)