

Studying Cyphonautes Larvae of *Hislopia prolixa* (Bryozoa; Ctenostomata) in Temperate Fresh Water

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ABSTRACT

Cyphonautes larvae are confirmed for the first time among the temperate freshwater zooplankton. They are part of the life cycle of the ctenostome bryozoan, *Hislopia prolixa* Hirose and Mawatari, 2011, known so far from China, Japan, and the Republic of Korea. Until 2008 cyphonautes were thought to be exclusively marine, occurring in three families of ancient ctenostome and cheilostome bryozoans. Since then they have been recognized in several tropical freshwater bryozoan species of the Genus *Hislopia*. Their presence in temperate latitudes now enlarges the area where these unusual larvae can be studied in their natural habitat. Embryological development in fresh water can be easily observed from egg to larva within a span of 12–24 h. Laboratory culture of the larvae has so far been hampered by their small size, hydrophobic body surface, unknown nutritional requirements, and high mortality rate.

Keywords: Bryozoa, Ctenostomata, cyphonautes, embryology, evolution

INTRODUCTION

This paper confirms for the first time the presence of bryozoan cyphonautes larvae among temperate freshwater zooplankton. The larvae are produced by the ctenostome bryozoan, *Hislopia prolixa* Hirose and Mawatari 2011, a species first described from Japan and now reported also from the Republic of Korea (Jung et al., 2017) and at least eight provinces in northern China (Wang, 2016). Previously cyphonautes larvae in fresh water were documented only from *Hislopia malayensis* Annandale, 1916 in Thailand (Wood, 2008). They have also been seen in the Amazon basin (Brandorff, 2005 cited by Wood and Okamura, 2017), probably a product of *Hislopia corderoi* Mané-Garzón, 1960. Otherwise the distinctive cyphonautes is known only from a few widely-distributed marine bryozoans that are generally considered phylogenetically ancient (Nielsen and Worsaae, 2010).

Hislopia Carter, 1858 is one of the few genera of ctenostome bryozoans that occur in fresh water. It is considered mostly a tropical group, with species dominating bryozoan communities over a wide geographic range. Eight species are now recognized (Hirose and Mawatari, 2011), although

the morphological criteria distinguishing several Asian species may not be fully reliable. In addition to *Hislopia prolixa* in northeastern Asia there are *Hislopia* species throughout the south and southeastern regions of the continent (Wood et al., 2006; Hirose and Mawatari, 2011). *Hislopia corderoi* is the most abundant bryozoan species in the Amazon near Santarém (Wood and Okamura, 2017), and its range may extend through much of tropical South America (Mañé-Garzón, 1960; Bonetto and Cordivola, 1963). An endemic hislopiid, *Hislopia placoides* (Korotnev, 1901) occurs in and around Lake Baikal (Kozhov, 2013). It is likely that all of these are disseminated largely through cyphonautes larvae.

Among freshwater bryozoans four other genera are derived from marine ctenostomes, but none has retained a cyphonautes larva. *Paludicella* has a primitive, nonfeeding larva (Braem, 1896); *Victorella*, *Pottsiella* and *Timwoodienella* brood their young (Braem, 1951; Smith et al., 2003; Wood and Okamura, 2017). The reproductive biology of *Arachnoidea* is not yet known.

Several features distinguish *H. prolixa* from others in the genus. One is the dual morphology of its zooids (Hirose and Mawatari, 2011). In addition to the typical flat, sessile zo-

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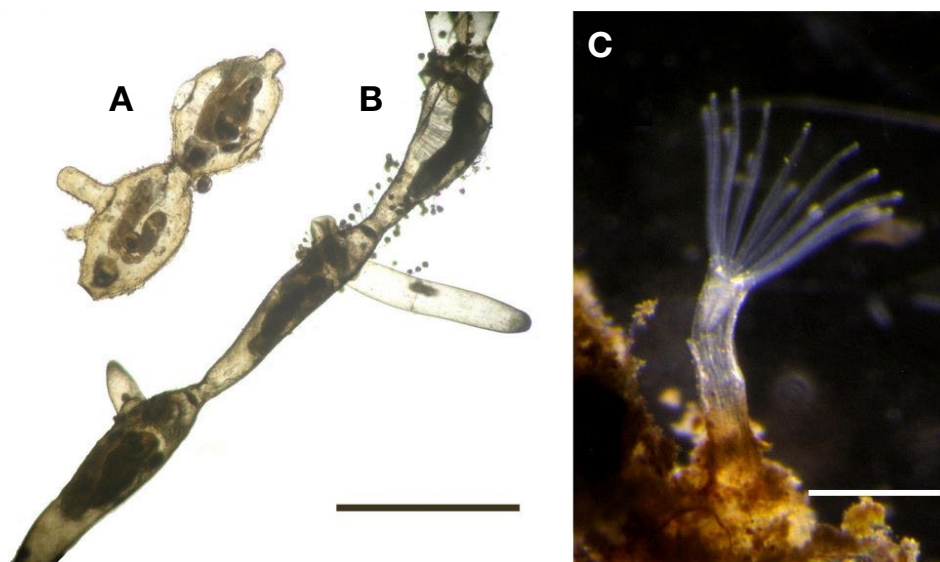


Fig. 1. *Hislopia prolixa*. A, Typical sessile zooids; B, Elongate zooids on adventitious branch, C, Zooid with extended lophophore. Scale bars: B=1 mm, C=0.2 mm.

oids *H. prolixa* also produce erect, adventitious branches of elongate zooids capable of reaching beyond the colony to access adjacent substrata (Fig. 1A, B). *Hislopia prolixa* is also the only bryozoan known to maintain spherical concretions in the gizzard that may function to help break up food particles (Wood, unpublished).

In this paper we focus on a feature now believed to be shared by all members of the Genus *Hislopia*: the appearance of cyphonautes larvae in the life cycle. The existence alone of these larvae in fresh water is unique and noteworthy. Cyphonautes are independent, heterotrophic life stages developing from microlecithal eggs. The primitive level of their development corresponds to that of marine trochophore larvae, which represent a large number of marine invertebrate species. Cyphonautes feed and grow in the plankton community until there is sufficient accumulated energy for them to transform into the sessile adult form. In tropical fresh waters this can take as few as five days (Wood, 2008), although in marine bryozoans it is thought to be considerably longer, depending on the species (Marcus, 1926; Dudley, 1973).

For more than 125 years the cyphonautes larva has been well known in the marine zooplankton community. It was first described by Prouho (1890, 1892), then in greater detail by Kupelwieser (1905). Although the larva is confined to only 13 marine species, those species are common and widely distributed. They range from the soft-bodied ctenostome, *Alcyonidium albidum* Alder, 1857 to the calcified cheilostome, *Membranipora membranacea* (Linnaeus, 1767).

Morphologically the freshwater cyphonautes is very similar to its marine counterparts (Nielsen and Worsaae, 2010)

except for being much smaller when it transforms to adult form (Wood, 2008). One remarkable feature is the apparent retention of the larval stomach in the adult, which would place hislopiid bryozoans in direct lineage with marine species long extinct (Zimmer and Woollacott, 1977; Wood, 2008). How the freshwater cyphonautes invaded freshwater habitats and survived in apparent evolutionary stasis are interesting and important questions. In this paper, however, we simply wish to draw attention to the presence of these unique larvae in fresh water and describe some of the challenges in studying them.

MATERIALS AND METHODS

The work described here was conducted during late July, 2017 at Woosuk University in Jincheon, Chungcheongbuk-do, Republic of Korea. Small rocks and pieces of plastic sheeting colonized by *H. prolixa* were collected from the Geum River near the ancient Nongdari stone bridge, 3 km E of Jincheon, 36.8275°N, 127.4940°E. The substrata were brought to the laboratory where they were placed in 5-L containers of fresh river water, and inverted over petri dishes, resting on the dish rims so that oocytes released from the colonies would collect in the dishes.

The timing of oocyte release was measured from about 20 cm² of dense *Hislopia* colonies resting above a petri dish in 1 L of water. Eggs were allowed to accumulate in the dish for 1 h. The substrata were then moved to a new container with freshly aerated water and a new collection dish. The process

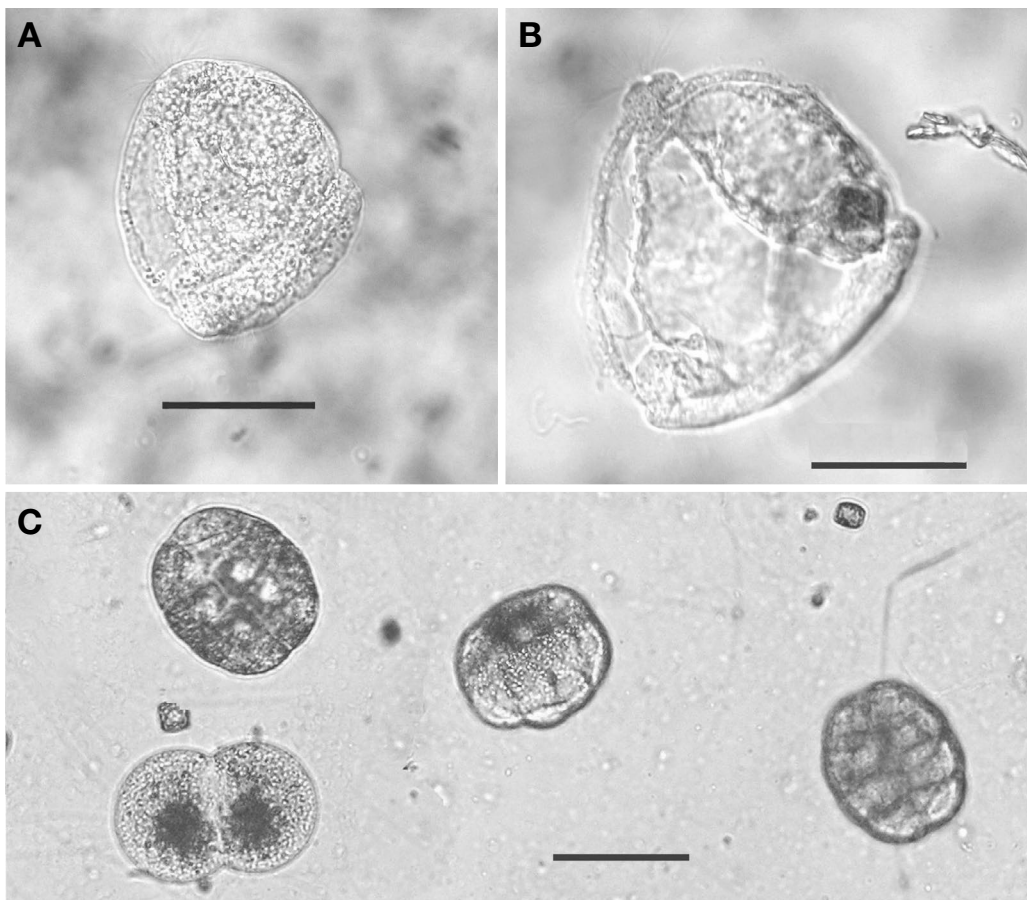


Fig. 2. Stages of development in *Hislopia prolixa*. A, Cyphonautes larva, 2 h after becoming motile; B, Larva 24 h after becoming motile; C, Early stages of cleavage. Scale bars: A-C=0.05 mm.

was repeated every 0.5 h for 24 h, starting at 16:00. Eggs were enumerated immediately after each transfer.

To obtain and maintain larvae eggs and developing embryos, the material collected overnight was separated from fecal pellets and other debris using a Spemann pipet and transferred in groups of 100 to containers with 8 mL river water. Containers included 15 mL polypropylene Falcon tubes slowly oscillated at five cycles per minute; also Falcon tubes held in a stationary horizontal position; and 20 mm diameter covered styrene petri dishes.

For observation and enumeration, the swimming larvae were transferred to small petri dishes where the shallow depth made it easier to find them under a dissecting microscope. In searching for larvae it helped to occasionally swirl the water in the dish because the larvae otherwise tended to gravitate to the sides where they could not be seen.

Other developing embryos were sealed in hanging drop preparations for observation with a compound light microscope. Assembly of the hanging drop began with a small pe-

tri dish containing a small amount of water. A 2.5 cm nylon bushing was placed in the center of the dish, a thin film of casein glue smeared across the upper surface, and a 20 × 30 mm coverlip placed on top along with the hanging drop containing oocytes. A good preparation could be used for more than 12 h.

To limit the growth of filamentous bacteria erythromycin was added to the water daily (E.M. Erythromycin powder, Aquarium Pharmaceuticals, Chalfont, PA, USA) at the twice the dose recommended for fish diseases.

RESULTS

The pigmented frontal wall made it difficult to detect the presence of oocytes inside the zooids of sessile colonies. However, when zooids were dislodged intact from the rock surface ova could be observed through the clear underside. As in *H. malayensis* Annandale, 1916, the oocytes developed

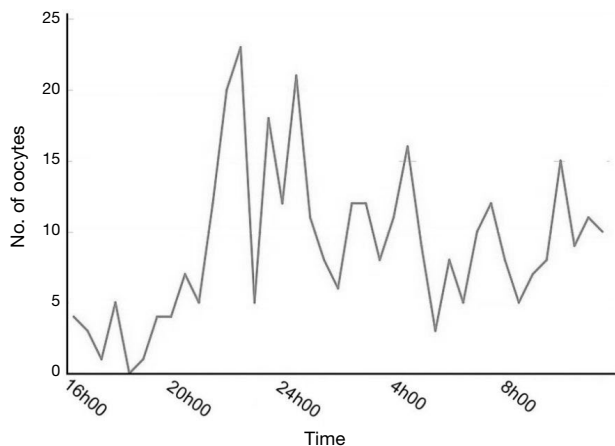


Fig. 3. Release of 350 oocytes counted every 30 min from 16:00 to 10:00 the following day. The releasing colonies measured 90 cm² in surface area.

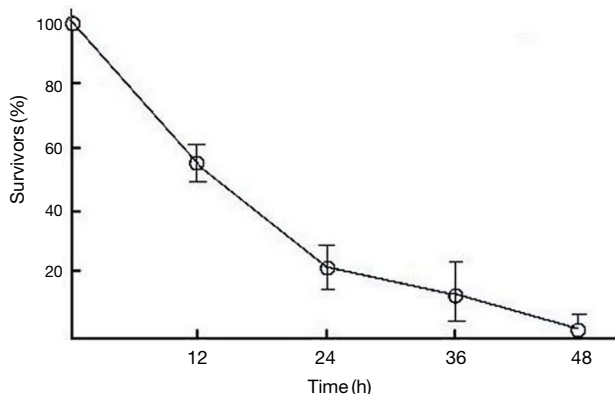


Fig. 4. Survivorship of 900 larvae developing successfully from *Hislopia* eggs and maintained in the laboratory. Error bars indicate the range of 95% confidence among nine replicates of 100 larvae.

along the lateral walls and then detached to move freely within the coelom. In some cases, mature oocytes appeared ready for release from zooids in which the polypides were degenerating. Only those zooids flattened along the substratum were found to have oocytes. The elongate, adventitious zooids appeared not to be sexually active.

Embryonic development was observed in the laboratory at a temperature of 23°C, which was cooler than their natural habitat (26°C). Within 20 min of release a fertilization membrane was raised. The first cleavage began about 30 min later and the second cleavage 45 min after that. Subsequent divisions occurred at diminishing time intervals (Fig. 2C).

During a single observation period in the laboratory oocytes were released from the bryozoan colonies throughout the day and night, although 47% were released at night between

23:00 and 7:00 (Fig. 3). Mortality was high. On average 12% of the oocytes failed to develop at all, and only 52% resulted in swimming larvae. Most of the unsuccessful embryos could be detected in the early stages of cleavage by their abnormal morphological asymmetry. At 23°C it took about 16 h for an oocyte to develop into a swimming larva (Fig. 2A). It was then another 8–12 h before internal organs appeared fully developed and green food was observed in the larval gut (Fig. 2B).

The cyphonautes were not strong swimmers. They generally wobbled through the water in an upright position, leading with the anterior wall and occasionally leaning sideways. In the laboratory they spent much time along the sides or bottom of the petri dish.

Despite persistent efforts we were unsuccessful in maintaining larvae in the laboratory. Nine groups of 100 larvae were maintained under various conditions of temperature and water movement, but the differences in survivorship were not statistically significant. Most of the surviving larvae stopped feeding after the second day, then weakened and died (Fig. 4). Many became entrapped in strands of filamentous bacteria. Those larvae in oscillating Falcon tubes remained more active than those held in static conditions but their longevity was no better. Aeration of the water was not an option, since the larvae are hydrophobic and are easily trapped at the water surface.

DISCUSSION

The cyphonautes larvae described here are biologically unique. In the transition from egg to adult most animals pass through transitional stages of varying complexity (Wray, 2000). Vertebrates are among the exceptions, although even they exhibit such transient structures as a notochord and placenta. At the other extreme are those invertebrates with autotrophic larvae that are anatomically simple, with hardly any true tissues and with very few adult features (Nielsen, 1987; Davidson et al., 1995). Cyphonautes larvae fit this description, as do the trochophore larvae of marine annelids and molluscs and the some of the planktotrophic larvae of echinoderms. Virtually all of the larvae in this category are marine with the singular exception of the cyphonautes from a few hislopiid bryozoans.

In freshwater habitats all other invertebrate species have either modified or eliminated the earliest embryonic larvae. For example, the freshwater veliger larvae of certain molluscs by-pass the earlier trochophore stage of their marine relatives and hatch with the already recognizable body plan of the adult. Planula larvae of certain freshwater hydroids are essentially passive, nonfeeding disseminules. The nauplius lar-

vae of copepods and eubranchipods are basically swimming mouthparts that gradually add posterior segments to achieve adult form. Clearly, “larva” is a generic term that covers a wide variety of ontogenies and evolutionary pathways. However, in fresh water nothing compares to hislopiid bryozoans ejecting microlecithal ova into the water to develop into distinct, self-feeding stages that bear no resemblance to the adult form.

This is one reason why the freshwater cyphonautes are worthy of further study. Another reason is the strong possibility that hislopiid bryozoans have an extremely long history. The cyphonautes is seen as an ancient relic, most likely the ancestral larval type for 90% of the extant bryozoan species (Nielsen and Worsaae, 2010). Most other bryozoan larvae are lecithotrophic, having lost the ability to feed and deriving their energy instead from an internal supply of yolk. Typically, bryozoan embryos are also brooded before being released into the water (Ostrovsky, 2013). In general, planktivory among larvae is considered a primitive condition, and once lost it is never regained (Strathmann, 1985). Moreover, the apparent retention of the cyphonautes stomach in the adult bryozoan is a feature unique to hislopiids that appears to pre-date all other extant bryozoan species (Wood, 2008).

The detailed study of freshwater cyphonautes is somewhat simplified by its wide availability, not only in tropical habitats but now in temperate climates as well. However, the challenges to detailed study include its small size. It is not by accident that the larvae have remained hidden for so long in the freshwater zooplankton. The maximum dimensions are seldom more than 0.2 mm long and 0.16 mm in height.

When oriented laterally to the observer the broad, triangular larval shell is almost conspicuous under the dissection microscope, but when the larva presents its thin frontal profile it all but disappears from view. Finding and counting live cyphonautes in a container with other zooplankton can be difficult and time consuming.

In temperate latitudes the appearance of larvae is seasonal. In Japan the oocytes were reported in mid-August (Hirose and Mawatari, 2011); in Korea the larvae are also abundant at this time, but by mid-September they are gone. By contrast, tropical hislopiids produce larvae continuously throughout the year (Wood et al., 2010). Because the frontal zooid wall is often opaque it may be difficult to observe the oocytes inside without dissection. However, this is not always the case: colonies collected in the Han River near Seoul in October, 2016 were nearly transparent.

Other challenges to studying the living *Hislopia* cyphonautes include the difficulty in maintaining laboratory populations. In large containers the larvae become lost; in small con-

tainers the contact with surfaces seems harmful. Nutritional requirements are unknown, and even when fresh habitat water is provided the larvae often stop feeding altogether.

Laboratory rearing may not be practical or even necessary. In Thailand various sized larvae of *H. malayensis* were easily obtained with a plankton net from a pond at any time of the day or night. Returning to the laboratory, after phototropic copepods were drawn away with strong light, the cyphonautes could be found and separated with a Spemann pipet.

The appearance of a cyphonautes larva in the life cycle of *H. malayensis* and *H. corderoi* prompted Wood and Okamura (2017) to consider this as a key diagnostic feature of the entire Genus *Hislopia*. This moved *Hislopia natans* Wood et al., 2006, into a new genus named *Timwoodiellina* by d’Hondt (2014), partly because cyphonautes are lacking in that species. This leaves seven described hislopiid species in which the important morphological features have been nicely compared in tabular format by Hirose and Mawatari (2011). However, many of these features are highly variable, such as zooid shape, and the dimensions of internal organs. Those species with overlapping geographic ranges are the very ones that are barely distinguishable by any reliable criteria. In South and Southeast Asia these include *H. malayensis*, *Hislopia cambodgiensis* (Jullien, 1880), *Hislopia lacustris* Carter, 1858, and *Hislopia moniliformis* Annandale, 1911, together forming the “*H. lacustris* group.” *Hislopia corderoi* in South America, *H. placoides* around Lake Baikal, and *H. prolixa* in northeast Asia are distinctive in both morphology and range. Of these it is only in *H. placoides* where the cyphonautes larva has not actually been seen.

At this time *Hislopia prolixa* and its cyphonautes offer many interesting avenues for further study. Among these are:

- The current range and geographic status of the species. *Hislopia prolixa* was recently reported independently in Japan and Korea. It is the only hislopiid capable of surviving the harsh winters of northern Asia, and presumably it could also invade northern Europe and North America. How is the species dispersed, and is the range currently expanding?
- What physiological and morphological adjustments have enabled *Hislopia* species to thrive in a freshwater environment as both embryos, larvae, and adults? Why is the group so successful having retained this ancient life cycle while other freshwater invertebrates have undergone significant modifications?
- If hislopiid bryozoans are truly “living fossils,” as suggested by Wood (2008) and supported indirectly by Todd (2000) and Zimmer and Woollacott (1977), what can be learned about the factors in its persistence? Does the genome, embryology, physiology, etc. offer clarity to our understanding of the evolution of the earliest bryozoans?

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CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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