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First evidence of maternal transmission of algal endosymbionts at an oocyte stage in a triploblastic host, with observations on reproduction in *Waminoa brickneri* (Acoelomorpha)

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Abstract. Examination of sexual reproduction in a symbiotic acoelomorph worm, *Waminoa brickneri* from Eilat (Red Sea), presents the first definitive evidence for maternal transmission of dinoflagellate algal symbionts in a triploblastic organism. Sexually mature worms were removed from the stony coral *Plesiastrea laxa* and raised in the laboratory. Eggs were detected 18 d after the collection of the worms and hatched 4 d later. Histological sections performed on sexually mature worms showed an ovary with oocytes containing two distinct types of algal endosymbionts within their ooplasm. Transmission electron microscopy corroborated the presence of algal symbionts within the developing embryos. Our findings regarding maternal transmission of symbionts shed new light on the diversity of modes of algal symbiont acquisition known in triploblastic organisms.

Additional key words: Acoelomorpha, *Waminoa brickneri,* symbiosis

Symbiotic associations are prevalent in terrestrial as well as marine environments and are of great ecological importance. Symbioses of invertebrates with photosynthetic algae encompass a wide array of hosts in several phyla, including Porifera, Cnidaria, Platyhelminthes, and Mollusca (Douglas 1995) as well as members of the recently designated phylum Acoelomorpha (Baguñá & Riutort 2004). Research on symbiotic systems is complex and involves aspects of diversity, morphology, physiology, recognition, and specificity of both partners. A key factor in specificity research is the mode of symbiont acquisition by the host.

The initiation of symbiosis in sexually produced offspring is known to occur either by acquisition of symbionts from the environment (horizontal transmission) or by direct transmission of symbionts from parent to its offspring (vertical transmission, maternal transmission) (Douglas 1995). The latter takes place by way of incorporation of symbionts into the oocyte (Muller-Parker & D'Elia 1997). Vertical transmission of algae from parent to offspring is unusual among marine symbiotic systems and has been described among members of the phylum Porifera and in a few members of the diploblastic phylum Cnidaria (Benayahu et al. 1992; Douglas 1994; Hirose et al. 2000, 2001).

Acoel worms have historically been placed in the triploblastic phylum Platyhelminthes. However, recent molecular and morphological evidence suggests that this taxon falls outside of the Platyhelminthes in the new phylum Acoelomorpha, the most basal bilaterian-triploblastic taxon (Baguñá & Riutort 2004).

Acoelomorphs are hermaphroditic and typically engage in mutual cross fertilization (Apelt 1969). The oocytes of acoelomorphs are produced in homocellular gonads that give rise to entolecithal eggs (Rieger et al. 1991; Tyler 1999). During oogenesis, oocytes are surrounded by accessory cells that contribute to the development of the egg envelope (Falleni & Gremigni 1990; Raikova et al. 1995). Spermatozoa of acoelomorphs are typically elongate and biflagellate. The ultrastructural features of spermatozoa in acoelomorphs have proved to be a rich source of phylogenetic characters that are concordant with studies of molecular systematics (Hooge

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et al. 2002; Petrov et al. 2004). Egg laying in the Acoelomorpha is carried out either through the mouth or through a tear in the epidermis. The eggs undergo direct development, and are often deposited singly, but are sometimes protected by brooding or encapsulation (Apelt 1969; Henley 1974).

Horizontal transmission of algal symbionts in the Acoelomorpha is well documented (Douglas 1992). The sexual reproduction, egg production, and the uptake of algal symbionts by the juveniles of *Symsagittifera roscoffensis* Graf 1891 were extensively studied and became a model for specificity studies in this group (Douglas & Gooday 1982; Douglas 1992). All of the symbiotic flatworms studied to date produce aposymbiotic eggs (McCoy & Balzer 2002) apart from the acoel *Amphiscolops carvalhoi* Marcus 1952, which was described as having zooxanthellae in internally brooded young (Marcus 1954), implying possible maternal transmission. Without information on the presence of algal symbionts in its oocytes, however, evidence of vertical transmission is lacking. In this article, we present the first definitive evidence for maternal transmission of dinoflagellate algal symbionts in a triploblastic organism, a coral-dwelling acoel from the Red Sea.

Waminoa brickneri Ogunlana, Hooge, Tekle, Benayahu, Barneah, & Tyler 2005, a newly discovered species from the reefs of Eilat (Red Sea), is epizoic on living corals (Ogunlana et al. 2005). Similar worms belonging to the genus *Waminoa* were detected there on 14 species of stony and soft corals at a depth range of 2–50 m (Barneah et al., 2007). The worms possess two distinct types of dinoflagellate algal symbionts within their cells: small symbionts 5–10 μm in diameter, which were identified as belonging to the genus *Symbiodinium,* and larger *Amphidinium*-like symbionts 12–20 μm in diameter (Barneah et al., 2007). The initial hypothesis that the worms receive their algal symbionts from their coral hosts was examined using denaturing gradient gel electrophoresis profiles of the ITS2 region of *Symbiodinium* derived from coral hosts and resident worms (Barneah et al., 2007). However, it was determined that corals and worms possess different phylotypes of *Symbiodinium,* thus suggesting a different source for their symbionts.

In this study, we followed the course of sexual reproduction and mode of algal acquisition in the acoel *W. brickneri.*

Methods

Collection and maintenance of animals

A part of a colony of the stony coral *Plesiastrea laxa* Klunzinger 1879 with individuals of *Waminoa brickneri* was collected from the Inter University Institute in Eilat (March 31, 2004) and transferred to an aerated aquarium at Tel Aviv University. The worms were removed from their coral host; 20 worms were preserved in 2.5% glutaraldehyde in seawater on the day of collection. The remaining worms were kept in 250-mL Pyrex bowls containing 45 μm filtered seawater. Half of the water volume was changed every second day. The bowls were maintained in an incubator (Yihdern, LE-509), at a temperature corresponding to the ambient seawater temperature at the time of collection. The light regime was 12 h light (30 μmol quanta $m^{-2} s^{-1}$): 12 h dark. No food was added. The documentation of events started upon the collection of the coral with the worms, which apparently was 18 d before egg laying, and continued until hatching of the juveniles, which occurred 4 d after laying. The worms were observed under a dissecting microscope twice during the day (morning and evening). Observations on the live worms were carried out daily with the aid of a dissecting microscope.

Histology

The worms preserved in 2.5% glutaraldehyde in seawater were placed into 2% liquid agarose gel. After the gel solidified, it was dehydrated in a graded series of ethanol and butanol, and embedded in paraffin. Serial sections 8 μm thick were cut using MIR microtome (Thermo Fisher Scientific, Waltham,

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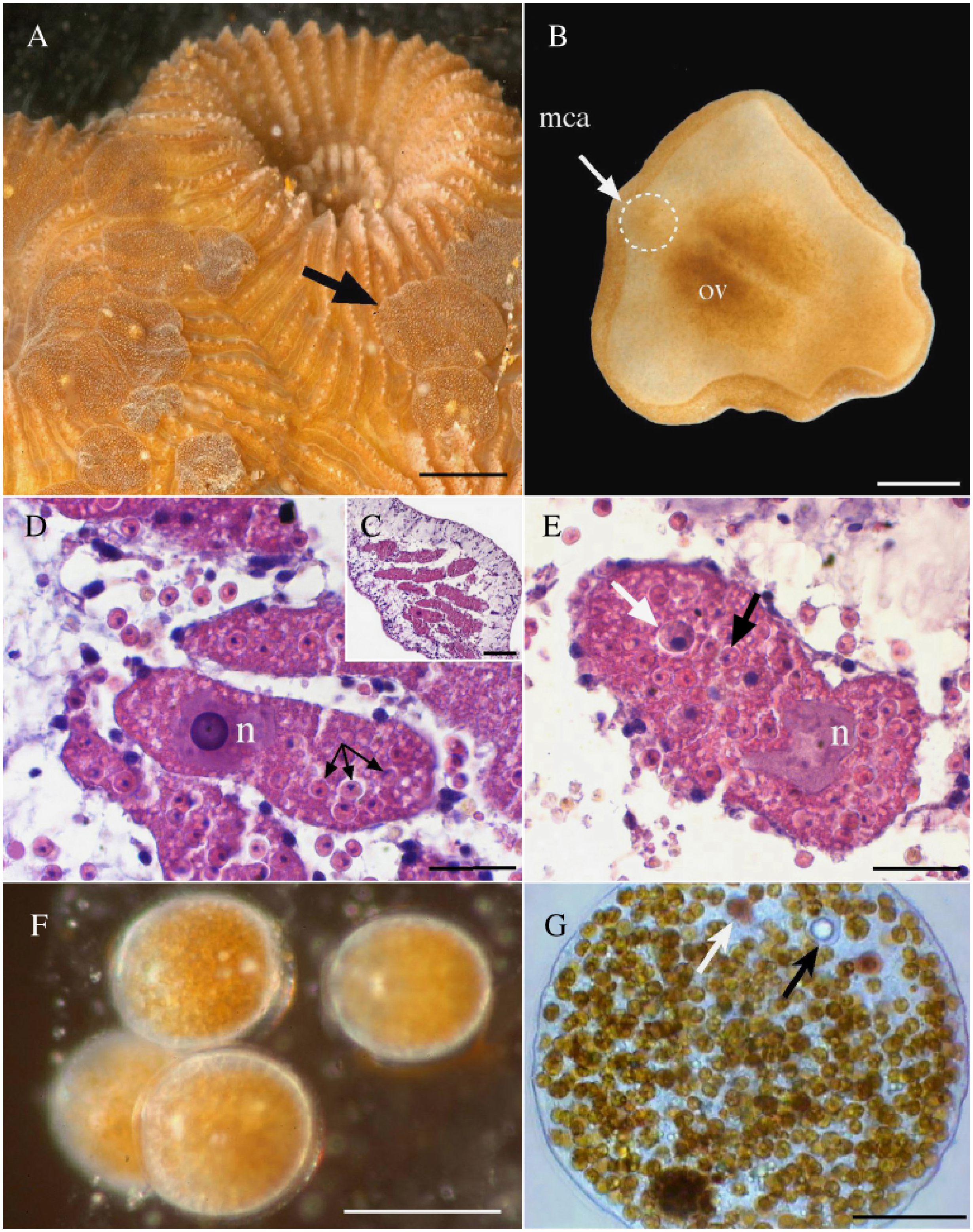
Fig. 1. A. The stony coral *Plesiastrea laxa* with worms of *Waminoa brickneri* (black arrow). Scale bar = 3 mm. **B–F.** Stages of sexual reproduction in *W. brickneri*. B. Ventral view of a sexually mature specimen with a paired ovary (ov) and the male copulatory apparatus (mca) marked with a dashed line and white arrow. Scale bar = 1 mm. C. Histological section of worm containing gonads, 5 d before egg laying, showing elongate oocytes. Scale bar = 110 μm. D. Oocyte containing nucleus (n) with prominent nucleolus and algal symbionts (arrows). Scale bar = 30 μm. E. Oocyte containing two symbiont types: *Symbiodinium* sp. (black arrow) and single larger symbiont (white arrow). Scale bar = 30 μm. F. Gelatinous egg mass. Scale bar = 200 μm. **G.** Worm hatchling containing numerous algal symbionts, statocyst (black arrow), and eyespot (white arrow). Scale bar = 45 μm.

MA). Sections were stained with Delafield hematoxylin–eosin. Specimens were preserved on the day of collection (March 31, 2004), on April 14 and April 19 (a day after the egg masses were detected).

Transmission electron microscopy

Worms preserved in 2.5% glutaraldehyde in seawater were rinsed in buffer phosphate, stained with



1% OsO_4, dehydrated through a graded ethanol series, and embedded in Epon. Sections were cut with a diamond knife, stained with lead citrate, and viewed with a JEOL 1200 EX transmission electron microscope (TEM, Tokyo, Japan).

Results

Figure 1A shows a polyp of the stony coral *Plesiastrea laxa* with worms of *Waminoa brickneri* (black arrow). Sexually mature worms, containing paired ovaries and testes, were observed on the day of collection (Fig. 1B). TEM images of these worms show a male gonad with sperm in close proximity to a female gonad containing oocytes with prominent nuclei and nucleolus (Fig. 2A). Sperm cells appear elongated (Fig. 2A) and biflagellate (Fig. 2B). The axonemes of both flagella have a 9+0 arrangement. Also observed in TEM micrographs of a specimen fixed on the day of collection was an algal symbiont engulfed by cytoplasmic processes originating from an oocyte (Fig. 2C).

Histological sections of specimens fixed 14 d after the initial collection (April 14, 2004) revealed the presence of a few elongated oocytes within the ovary. These oocytes were occasionally stretched dorsoventrally (Fig. 1C), and large nuclei with a distinct nucleolus were detected (Fig. 1D). Algal symbionts were observed within the ooplasm of developing oocytes (Fig. 1D). In most cases, the oocytes contained two distinct types of algal symbionts: an abundant small symbiont and a larger symbiont that rarely occurred (Fig. 1E).

Gelatinous egg masses were found 18 d after collection (April 18, 2004), attached to the bottom of the bowl, each containing two to nine eggs (Fig. 1F). The eggs were 200 μm in diameter and brown in color due to the presence of algal symbionts. A transparent capsule, 0.8 μm thick, coated each egg. TEM micrographs of the embryo within the egg capsule revealed a ciliated epidermis, numerous nuclei of the parenchymal cells, and algal symbionts within the parenchyma (Fig. 3A). Electron-dense bodies, attached to the inner face of the egg capsule and projecting into the gap between the embryo's epidermis and the capsule, were detected (Fig. 3). Three days after egg laying (April 21, 2004), embryos were observed to be

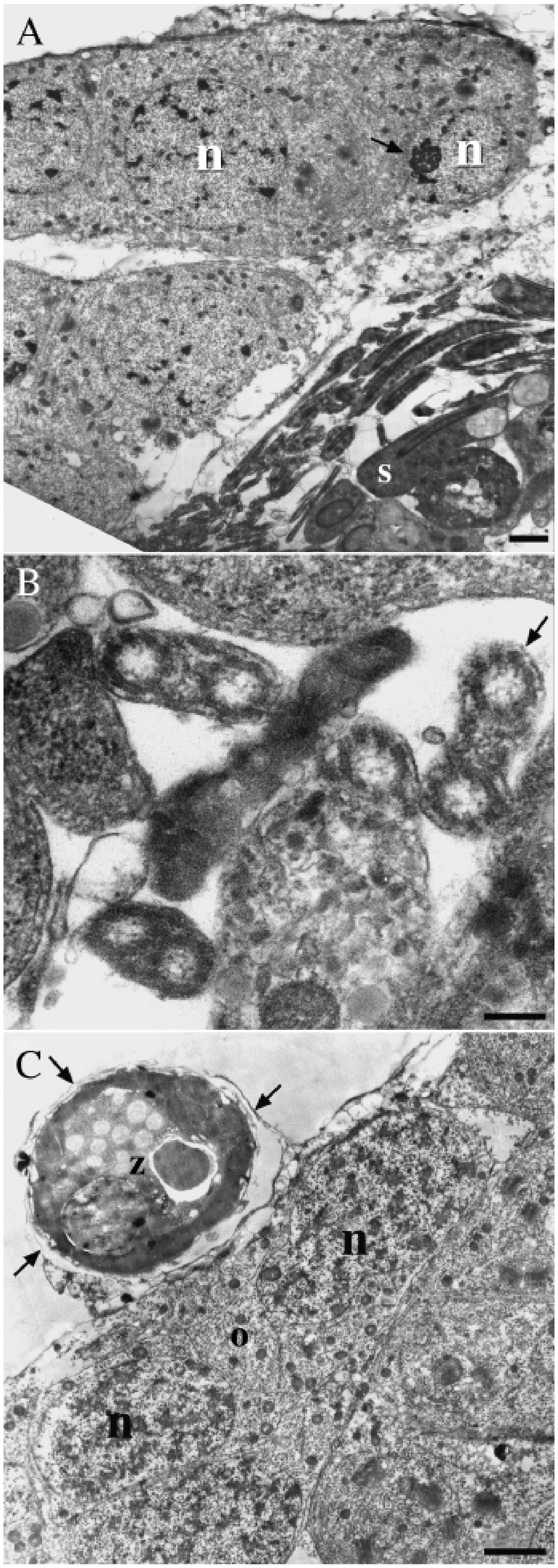


Fig. 2. Transmission electron micrographs of gonads in *Waminoa brickneri,* 18 d before egg laying. **A.** Ovary containing oocytes with prominent nuclei (n) and nucleolus (black arrow) in close proximity to testes containing sperm (s). Scale bar = 2 μm. **B.** Cross section of biflagellate sperm cells characterized by a 9+0 axoneme arrangement (black arrow). Scale bar = 200 nm. **C.** Zooxanthella (z) engulfed by cytoplasmic processes (arrows) originating from adjacent oocyte (o). Scale bar = 2 μm.

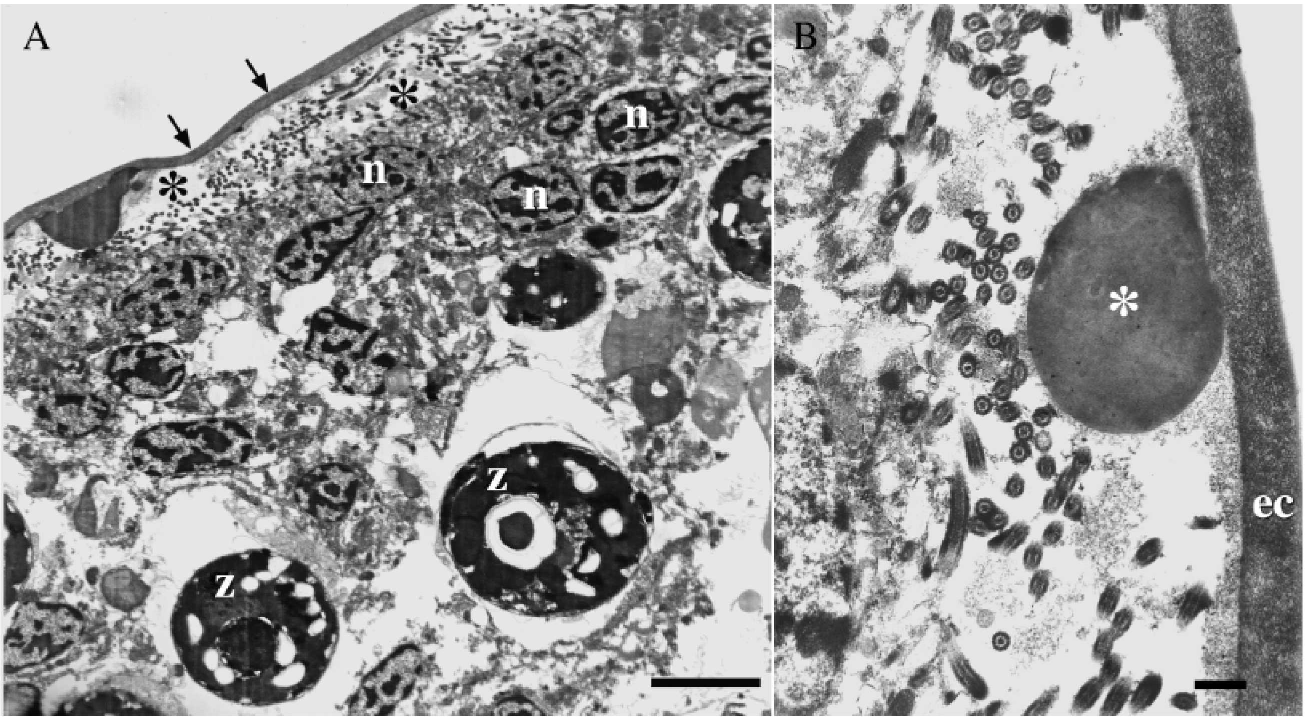


Fig. 3. Transmission electron micrographs of embryo within an egg capsule in *Waminoa brickneri*. **A.** Egg capsule (black arrows), ciliated (asterisks) portion of an embryo containing algal symbionts (z), and nuclei (n) of parenchyme cells. Scale bar = 5 μm. **B.** Inner face of the egg capsule (ec), sections through epidermal cilia in the gap between the embryo and the egg capsule and electron-dense body (asterisk). Scale bar = 500 nm.

moving within the egg capsule. One day later, with the use of a dissecting microscope, we observed a worm hatching from one of the eggs. The actively swimming hatchling was 190 μm in diameter and contained a statocyst, two eyespots, and abundant algal symbionts (Fig. 1G).

Discussion

Our study provides the first definitive evidence of maternal transmission of algal symbionts at the oocyte stage in any triploblastic organism studied so far. To date, all host flatworms studied are known to produce aposymbiotic eggs within a mucilaginous egg case (McCoy & Balzer 2002). Potential symbionts, and other algae, have been found on the egg cases of several host species (McCoy & Balzer 2002), but never inside them. Furthermore, this study represents the first documentation of egg release in the acoelomorph genus *Waminoa*.

The vertical transmission of algal symbionts in *Waminoa brickneri* is the first recorded among triploblastic bilaterians. This mode of symbiont transmission was documented in several species of sponges (Oren et al. 2005), which are multicellular organisms but their cells are not organized in tissues. An absence of records of a free-living stage of sponge-associated cyanobacterial symbionts (Usher et al. 2004a,b) suggests that vertical transmission could be the main (or even the only) mode of acquisition in this phylum (Oren et al. 2005). In cnidarians, the majority of zooxanthellate species acquire their symbionts via horizontal transmission and the minority via vertical transmission (Trench 1987). The timing and process of cellular events accompanying the entry of zooxanthellae into oocytes of several coral species were documented (Benayahu et al. 1992; Hirose et al. 2000, 2001). Our study provides the first evidence for the existence of dual modes of zooxanthellae acquisition in triploblastic animals, which so far were known to acquire photosynthetic symbionts solely from their surroundings (McCoy & Balzer 2002). The exact cellular mechanism and timing of the transmission process of zooxanthellae into oocytes in *W. brickneri* are yet to be investigated.

The observation of two symbiont types within the oocytes of members of *W. brickneri* from Eilat is the first known occurrence of simultaneous vertical transmission of two distinct algal symbiont species. A congeneric of *W. brickneri, Waminoa litus* Winsor 1990, was also reported to have two distinct algal symbionts within the same host's cell (Trench &

Winsor 1987; Winsor 1990), and it is likely that this condition is unique to the genus *Waminoa*. Trench & Winsor (1987) speculated that *Amphidinium* sp. was the natural symbiont, and that *Symbiodinium* was acquired from the corals on which the worms live. However, the authors specifically mentioned that there was no evidence that the worms fed on any of the corals they were found on (Trench & Winsor 1987). Our finding, regarding vertical transmission of both symbionts types in *W. brickneri,* demonstrates that the juvenile worms are equipped with a pool of symbionts from a maternal source upon hatching, without a need to rely on any external sources. Hence, the habitat range of populations of *W. brickneri* does not seem to be constrained by the distribution of free-living populations of suitable symbionts as known from systems with horizontal transmission (Douglas 1992).

Waminoa brickneri is known to associate with 14 species of stony and soft corals in Eilat (Barneah et al. 2007). Sexually mature specimens seem to be rather scarce and the event of sexual reproduction in laboratory-raised specimens during the month of April is regarded as exceptional. Further attempts to re-document the sexual reproduction scenario in the following months were unsuccessful. It is known that the development and maintenance of the female reproductive system in members of the Turbellaria may be affected by exogenous factors, among which seasonal factors and particularly temperature may cause the alteration between sexual and asexual states (Ax 1977). It should be mentioned that asexual reproduction was documented in individuals of *Waminoa* sp. and *W. brickneri* isolated from three species of coral hosts from Eilat (*Acropora hemprichii* EHRENBERG 1834*, Stylophora pistillata* ESPER 1797, and *Plesiastrea laxa*) in 3 consecutive months (O. Barneah, unpubl. data). Therefore, we hypothesize that asexual reproduction is the common mode of reproduction in *W. brickneri* from Eilat, while sexual reproduction might be seasonal.

Sperm in *W. brickneri* appears elongate and biflagellate, characterized by a 9+0 arrangement of the axonemes. Such an arrangement is typical of "large-bodied convolutids," including those containing algal endosymbionts (Hooge et al. 2002; Petrov et al. 2004). Recently, Ogunlana et al. (2005) re-assigned *Waminoa* to the family Convolutidae, based on morphological and molecular features. The 9+0 sperm morphology, as seen in our study, further corroborates this assignment.

The basal position of the Acoelomorpha and the consequent polyphyly of the Platyhelminthes are being tested using both morphological and molecular techniques (Glenner et al. 2004; Ruiz-Trillo et al. 2004). Analyses of mitochondrial amino acid sequences concurrent with morphological characters, and a growing number of molecular studies, argue for a monophyletic Acoelomorpha as a basal bilaterian group, occupying a pivotal position between diploblasts and triploblasts (Ruiz-Trillo et al. 1999). The discovery of maternal transmission of algal symbionts in the acoelomorph *W. brickneri* may act in support of the novel positioning of Acoelomorpha between diploblast and triploblast animals.

Our study is the first to record the sequence of events occurring during the sexual reproduction in the genus *Waminoa*. The discovery of maternal transmission of two distinct types of algal symbionts in *W. brickneri* is the first documentation of this mode in any triploblastic organism studied to date. This finding suggests that members of *W. brickneri,* epizoic on corals, can maintain their algal symbionts from generation to generation without relying on acquisition from external sources, such as their coral host, for supply of symbionts. The symbiosis between *W. brickneri* and its endosymbionts as well as its intimacy with another symbiotic system involving corals and algal symbionts is intriguing and complex. Further study is still required in order to categorize the nature of this symbiosis and to elucidate the possible relationship between the worms and the corals they inhabit.

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