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SHORT COMMUNICATIONS

Evidence against mutualism in an aeolid nudibranch associated with Symbiodiniaceae dinoflagellates

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Abstract

The symbiotic association with Symbiodiniaceae dinoflagellates has been more investigated for reef-building corals than for other metazoan taxa. Nudibranchs are relevant hosts as they present a wide variety of relationships with Symbiodiniaceae that range from predation to mutualistic association. The aeolid *Berghia stephanieae* is perhaps the best model for ecological studies in the mollusk-dinoflagellate association due to its hardiness, short life cycle and simple aquaculture protocols. However, it remains untested if *B. stephanieae* and Symbiodiniaceae actually engage in mutualism. Therefore, this study experimentally investigated the following aspects pertaining to the relationship between the two organisms: (i) Symbiodiniaceae retention time in the host tissue, (ii) effect of Symbodiniaceae presence in the prey item on host growth, and (iii) host capability to obtain free-living Symbiodiniaceae. Three experiments were performed: (i) monitoring of Symbiodiniaceae concentration in the cerata of starved *B. stephanieae*, (ii) offer of different-sized prey with and without symbionts and measuring *B. stephanieae* growth, and (iii) offer of free-living Symbiodiniaceae to *B. stephanieae*. Results show that the retention time (3–5 days) is much shorter than for many symbiont-associated nudibranchs. *Berghia stephanieae* growth is influenced by prey size, and apparently not affected by symbiont presence. Finally, this species is unable to obtain free-living Symbiodiniaceae. These results indicate that *B. stephanieae* does not meet criteria for a mutualistic relationship with Symbiodiniaceae, such as long-term retention and metabolite or favor exchange. This relationship may be in an evolutionary transitional stage, unlike the fully functional mutualism found in other organisms such as reef-building corals.

Keywords *Berghia stephanieae* · Symbiosis · *Aiptasia* · Zooxanthellae · Opisthobranchia · Sea slug

1 Introduction

Photosynthetic dinoflagellates of the family Symbiodiniaceae are known to establish mutualistic symbiosis with multiple marine invertebrate taxa (Stat et al. 2006; Venn et al. 2008). The association is based on mutual benefit for both host and symbiont. An established mutualism meets specific criteria, including long-term symbiont retention and metabolite/favor exchange (Davy et al. 2012; Mies et al. 2017a; Melo Clavijo et al. 2018). In most cases, symbionts are provided with essential compounds for the photosynthetic process, such as CO_2, phosphorus and nitrogen (Allemand et al. 1998; Leggat et al. 2003), and in return the host is supplied with photosynthates such as glucose, amino acids, fatty acids and glycerol (Muscatine 1990; Grant et al. 1997; Papina et al. 2003; Burriesci et al. 2012). In reef-building corals, this exchange takes place intracellularly, as corals retain symbionts in vacuoles in gastrodermal cells (Weis et al. 2008). Mollusks, such as giant clams and nudibranch gastropods, house their symbionts in diverticulae that extend from the digestive tract (Norton et al. 1992; Burghardt and Gosliner 2006; Burghardt et al. 2008). While giant clams must acquire symbionts by filter-feeding during larval development and keep them in extracellular spaces (Fitt et al. 1986; Mies et al. 2012), nudibranchs usually acquire them by sequestration from their prey and keep them inside digestive gland cells (Rudman 1981; Kempf 1984; Wägele and Johnsen 2001; Burghardt et al. 2008).

Remarkable functional diversity exists in the relationship between gastropods and photosynthetic eukaryotes, ranging from predation (Kempf 1984) and functional kleptoplasty

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(as observed in sacoglossan species – see Trench 1980; Christa et al. 2015) to fully established mutualism with metabolite exchange (Hoegh-Guldberg and Hinde 1986; Hoegh-Guldberg et al. 1986). Within this range, the association between nudibranchs and Symbiodiniaceae may be divided into several degrees, mainly depending on symbiont retention time and its fate in the host digestive tract (Kempf 1991). Therefore, the mere presence of symbionts in the digestive glands does not account for a mutualistic relationship between nudibranchs and Symbiodiniaceae (Wägele and Johnsen 2001; Burghardt et al. 2005).

While there is a wide array of forms of association between nudibranchs and Symbiodiniaceae, there is little information on this relationship for the aeolid *Berghia stephanieae* (often confused with *B. verrucicornis* and formerly known as *Aeolidiella stephanieae*; now placed in the superfamily Aeolidioidea – see Valdés 2005; Leal et al. 2012a; Carmona et al. 2013). *Berghia stephanieae* is a commercially relevant species for the marine aquarium trade (Olivotto et al. 2011). Unlike other nudibranchs, this species is very easy to culture because larval development is lecithotrophic and short (less than 48 h) and juvenile and adult stages are hardy (Carroll and Kempf 1990; Leal et al. 2012a; Dionísio et al. 2017; Mies et al. 2017b). Furthermore, it feeds exclusively on the anemone *Exaiptasia pallida* (from where symbionts are obtained), also simple to culture under laboratory conditions (Leal et al. 2012b). However, very little is known about the interaction between *B. stephanieae* and Symbiodiniaceae and it still remains largely untested whether they form a fully established mutualistic relationship.

This study experimentally addresses three aspects pertaining to the relationship between Symbiodiniaceae and *B. stephanieae*: (i) the symbiont retention time in the host; (ii) the effects of the presence of symbionts in the prey on the growth of *B. stephanieae*; and (iii) the capability of *B. stephanieae* to acquire free-living symbionts. Aeolids are relevant model organisms for investigations into the evolution of mutualistic symbiosis (Kempf 1991). These questions may provide insights relevant not only for the evolutionary history of the mollusk-dinoflagellate association, but also for the mutualistic interactions of other organisms (Herre et al. 1999; Foster and Wenseleers 2006; Hillesland and Stahl 2010).

2 Materials and methods

Berghia stephanieae broodstock individuals (1.6 ± 0.1 cm in length, $n = 4$) (Fig. 1a) were kept in a bare and round 60-L tank connected to a 320-L recirculating system. Seawater density was kept at 1024 kg m^{-3} and temperature at 27 °C. Nitrogen (NH_3/NH_4^+, NO_2^- and NO_3^- combined) and phosphorus (PO_4^{-3}) compounds were kept below 0.1 and 0.03 mg L^{-1}, respectively, using an Aviv Reef 600 protein skimmer. The photoperiod was 10 L:14D, using two OSRAM 40 W tubular lamps that generated 80 μmol photons m^{-2} s^{-1} with a spectrum of 5000 K. As *B. stephanieae* is a stenophagous species that feeds exclusively on *Exaiptasia* anemones (Carroll and Kempf 1990; Leal et al. 2012a), five *E. pallida* individuals (0.5−0.8 cm pedal disk diameter and 1−2.5 cm column height) harboring *Symbiodinium microadriaticum* (ITS2 type A1 - previously identified using the methods in LaJeunesse and Trench 2000) were offered twice a week to the broodstock. The anemones were cultured separately in a 250-L recirculated system under the same physical-chemical conditions. Broodstock individuals spawned approximately five egg masses after a week, which were removed and kept in an additional 60-L tank in the recirculating system. After 10 days of incubation under aeration, juveniles were visible and then approximately 25 *Exaiptasia* anemones of variable sizes were offered every week. When individuals reached the size necessary for the experiments described below, they were collected and separated.

The first experiment was conducted to determine the retention time (loss of 90% of the initial quantity) of symbionts in *B. stephanieae*, and if that differs between juveniles and adults. A preliminary experiment tested whether there is a difference in the number of symbionts in the cerata from different parts of the body (anterior, middle and posterior) for a *B. stephanieae* individual (Fig. 1a-b). Three cerata were removed from each part of the nudibranch, then smeared on a Nageotte counting chamber to determine the number of symbiont cells per ceratum. For the main experiment, six individuals (three juveniles of 0.8–1.0 cm and three adults of 1.3–1.6 cm) were kept together for 24 h and offered 30 *E. pallida* anemones of variable sizes (Fig. 1c) to ensure they were satiated. Afterwards, the six nudibranchs were individually isolated in 1-L Erlenmeyer flasks and starved for 10 days to guarantee that no new symbionts would be acquired. Therefore, three replicates were produced for each age class, i.e. juvenile or adult. One ceratum was removed daily from a random area (see section 3) of each of the six nudibranchs and the number of symbiont cells counted. Symbiont data for adult nudibranchs on day 2 was lost because this specific sample experienced contamination and therefore was not considered.

The differences in symbiont retention between juvenile and adult *B. stephanieae* were compared using a repeated measures two-way analysis of variance (ANOVA) to account for the dependence of the repeated measures; time (days) and age class (juvenile and adult) were fixed factors and each experimental unit (nudibranch) was considered random and nested within each age class. A one-way ANOVA was used to test differences in the symbiont load in cerata from different parts of *B. stephanieae*.

To assess whether the presence of symbionts in *E. pallida* has an effect on the growth of adult *B. stephanieae*, a population of aposymbiotic *E. pallida* (containing few to no symbionts – Fig. 1d) was produced in the laboratory by being kept in

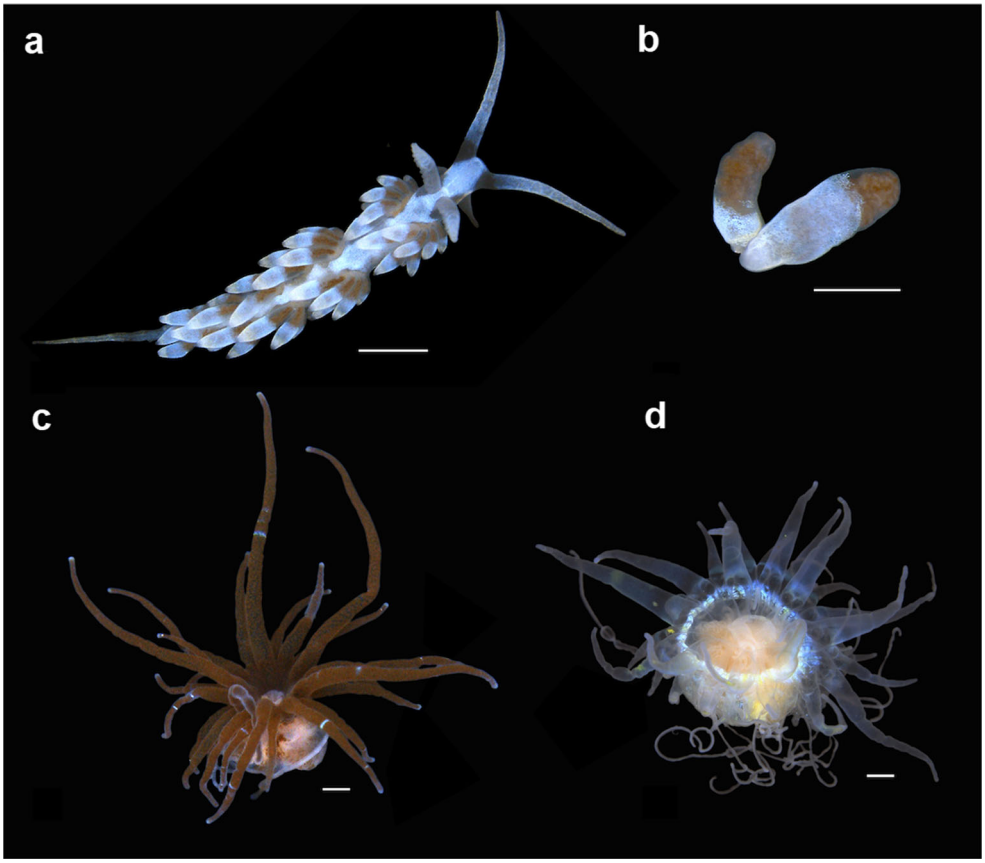

Fig. 1 Organisms used in the experiments. Scale represents 0.1 cm **a** *Berghia stephanieae* nudibranch fully extended **b** *Berghia stephanieae* cerata containing *Symbiodinium microadriaticum* **c** Symbiotic *Exaiptasia pallida* with brownish coloration due to the presence of symbionts **d** Bleached (aposymbiotic) *E. pallida*

the dark for 3–4 months. Symbiodiniaceae cells in *E. pallida* tissues were counted and compared to establish the difference between symbiotic and aposymbiotic anemones. Then, anemones were offered to nudibranchs and two factors were tested: anemone size (large: 0.5–0.8 cm pedal disk diameter and 1–2.5 cm column height; and small: 0.2–0.4 cm and 0.5–1.5 cm) and anemone symbiont content (symbiotic or aposymbiotic). Combined treatments were designated LS (large and symbiotic anemones), LA (large and aposymbiotic), SS (small and symbiotic) and SA (small and aposymbiotic). Each of these combinations had three replicates (1-L flasks) containing a pair of adult *B. stephanieae*. The experiment lasted for 20 days, during which an anemone from the assigned feeding regimen was offered daily to each nudibranch pair and daily body length measurements were taken of fully extended *B. stephanieae* individuals.

For the effects of anemone size and symbiont content on the growth of *B. stephanieae* among the experimental combinations (LS, LA, SS and SA), differences in nudibranch size at the end of this experiment (20th day) were tested using two-way crossed ANOVA. Post-hoc Tukey's HSD tests were performed to pinpoint differences between levels for each factor. Because of uneven sample sizes, a non-parametric Wilcoxon rank-test was used to compare differences in the quantity of symbiont cells in symbiotic and aposymbiotic *E. pallida*. All data were tested for normality and log-transformed to meet criteria of homogeneity of variance when necessary. Results, of both experiments, are expressed as mean ± standard error (SE).

To test whether *B. stephanieae* is able to acquire free-living symbionts, two nudibranchs (0.8 and 0.9 cm in length) were starved under regular photoperiod (10 L:14D) until all symbionts were lost. They were inspected daily under a Leica microscope at 100X magnification in a small water volume to maintain the nudibranch still. After 10 days, symbionts were entirely lost and nudibranchs were then placed separately in 1-L flasks filled with a mixed Symbiodiniaceae culture (*Symbiodinium microadriaticum, Breviolum minutum, Cladocopium goreaui* and *Durusdinium glynni* - ITS2 types A1, B1, C1 and D1, respectively) at 10^4 cells mL^{-1}. Every five days for two weeks the nudibranchs were checked for the presence of symbionts in the cerata.

3 Results

3.1 Symbiont retention time

No differences were found in the number of symbiont cells in cerata removed from different areas of the dorsum of *B. stephanieae* (one-way ANOVA: $F = 0.218$, $df = 2$, $P = 0.809$). Only a small percentage (< 10%) of

the observed symbiont cells were in division stages. The number of cells found in the cerata of juvenile and adult *B. stephanieae* decreased after starvation (Fig. 2a). The day after feeding on the anemones, cerata in juvenile individuals contained 69% more symbiont cells than adults. In the following days, symbiont concentration decreased in both juveniles and adults. However, it was noted that juveniles expelled larger quantities of symbionts in their faecal pellets. Therefore, no significant difference was found in symbiont loss for juvenile and adult *B. stephanieae* (Table 1). Also, the retention time was similar for both life stages; juveniles lost 90% of symbiont cells by day 3 and adults by day 5. Nearly 100% of the symbiont cells were lost within 10 days from acquisition (Fig. 2a).

3.2 Effects of symbiont presence in prey on *B. stephanieae* growth

In the preliminary test, differences were found in the symbiont content of symbiotic and aposymbiotic *E. pallida* (Wilcoxon rank test: chi-square = 7.5, df = 1, $P = 0.006$). Bleached (aposymbiotic) *E. pallida* ($N = 5$) contained $0.90 \times 10^3 \pm 0.47 \times 10^3$ symbiont cells, while symbiotic individuals ($N = 6$) contained $205.72 \times 10^3 \pm 115.92 \times 10^3$ cells. For the main experiment, *Berghia stephanieae* grew under all feeding regimens (Fig. 2b). At the end of the experiment, only prey size correlated significantly with *B. stephanieae* length differences between feeding regimens (Table 1; two-way crossed ANOVA $F_{size} = 25.4737$, df = 1, $P = 0.001$). The presence of

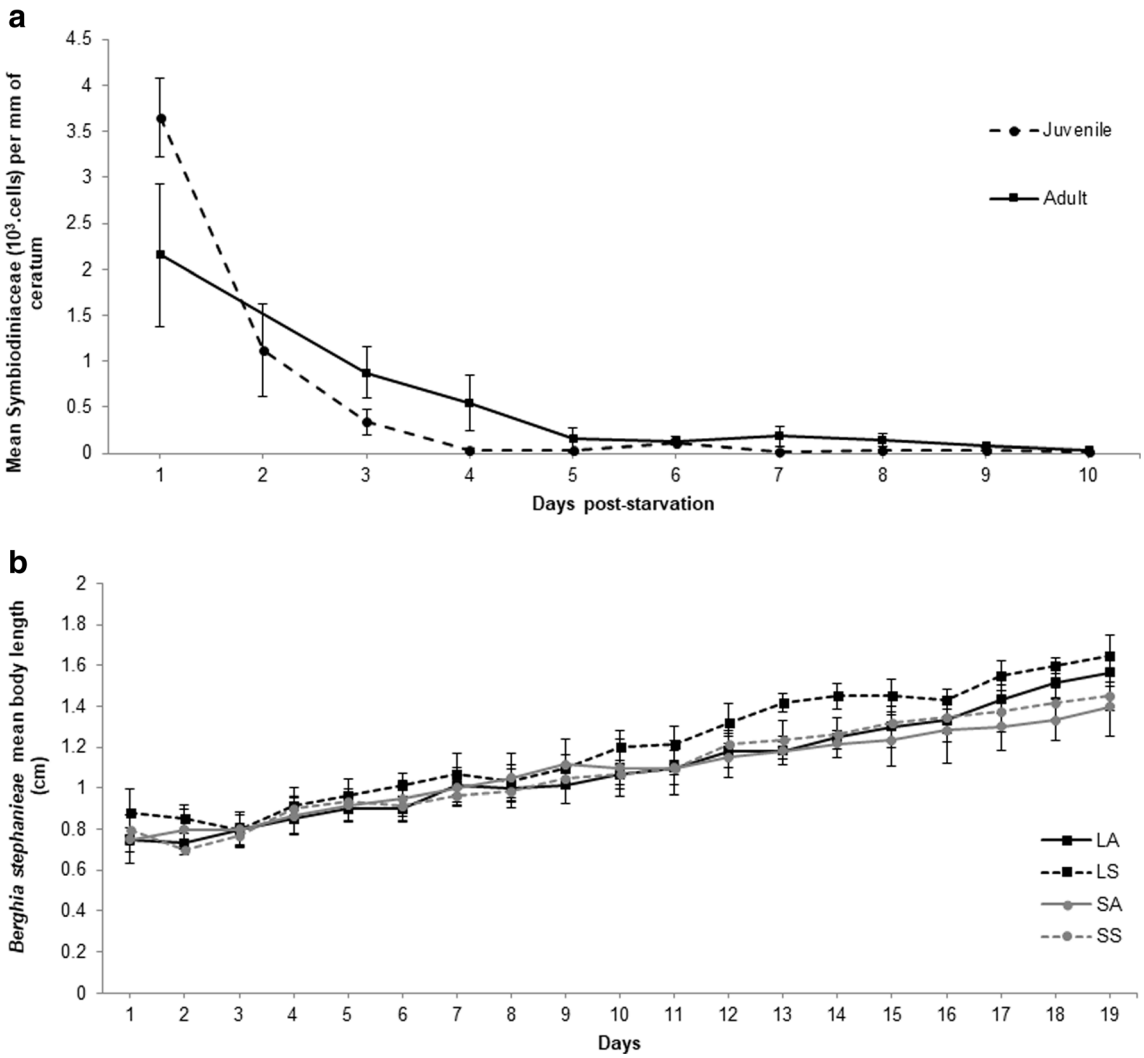

Fig. 2 **a** Retention time of *Symbiodinium microadriaticum* cells in the cerata of juvenile and adult *Berghia stephanieae* during a 10-day starvation period. **b** Mean body length (cm) of fully extended *Berghia stephanieae* individuals fed with large symbiotic *Exaiptasia pallida* anemones (LS), large aposymbiotic (without symbionts) anemones (LA), small symbiotic anemones (SS) and small aposymbiotic anemones (SA). Error bars express standard error

Table 1 Results for the statistical analyses for experiments addressing (i) Repeated Measures Two-way ANOVA for symbiont retention time in juvenile and adult *Berghia stephanieae*. Symbiodiniaceae content data was log-transformed. (ii) Two-way crossed ANOVA for the effect of size and symbiont content in *Exaiptasia pallida* tissue on *B. stephanieae* growth on the last day of the experiment (19th day)

Source	df	F ratio	*p*
Time	9	13.2080	<0.0001
Age class	1	0.3722	0.5459
Time x Age class	9	1.6178	0.1472
Error	40		
Size	1	25.4737	0.0010
Symbiont content	1	3.3684	0.1038
Size x Symbiont content	1	0.2105	0.6586
Error	8		

symbionts in the prey was not correlated significantly with nudibranch growth (Table 1; $F_{Symbiodiniaceae} = 3.3684$, df = 1, $P = 0.1038$). A single nudibranch died in one of the replicates in the treatment with large aposymbiotic anemones (for no apparent cause) on day 15.

3.3 Acquisition of free-living symbionts

After two weeks of exposure to a mixed culture of free-living symbionts, *B. stephanieae* individuals did not present any Symbiodiniaceae cells inside their cerata. Apart from the suspended cells, a biofilm of deposited symbiont cells was formed on the flask walls. While this biofilm was also available for potential grazing, no evidence of such was noticed.

4 Discussion

Mutualism requires both parties to benefit and this work experimentally approached the relationship from the host nudibranch perspective. The questions are ecologically-driven, as opposed to the investigations into molecular interactions between these organisms in Mies et al. (2017b). The results obtained from our three experiments, especially those related to retention time, suggest no mutualistic relationship between Symbiodiniaceae and *B. stephanieae*. Most symbiont cells were lost in three to five days after *B. stephanieae* starvation. By the tenth day, 99% had been lost and without differences according to host life stage (Fig. 2a). This is similar to the six-day symbiont retention time reported for *B. verrucicornis* (Kempf 1991). When compared to other nudibranchs, it seems that symbiont retention time is considerably shorter within the *Berghia* genus. For example, adult *Pteraeolidia ianthina*, a confirmed mutualist (Hoegh-Guldberg and Hinde 1986), is known to preserve *Symbiodinium* in their cerata for at least 70 days (Burghardt et al. 2005), *Phyllodesmium colemani* for 74 days and *P. longicirrum* for 116 days (Burghardt et al. 2008). After retention, symbionts are discarded mostly intact in faecal pellets, differently from *B. major*, that actively digests symbiont cells (Kempf 1984).

The presence of symbiont cells obtained from the prey did not influence the growth of *B. stephanieae*. In a truly mutualistic association, it would be expected that consuming prey containing symbionts would have contributed to host growth. Therefore, higher growth rates in nudibranchs fed with symbiotic anemones would serve as an indirect measure of symbiont metabolite translocation to host. However, no differences were found between nudibranchs fed anemones with and without symbionts and heterotrophic feeding seems more important for growth. Metabolite translocation from symbiont to host cannot be entirely ruled out, but, if active, it would play a much smaller role than in the well-established mutualism described for corals and giant clams. In these cases, symbiont metabolite translocation is critical for host development and more important than heterotrophy (Muscatine 1990; Klumpp et al. 1992). Evidently, *B. stephanieae* feeds on *E. pallida* tissue as they approximately doubled their size in all four feeding regimens, regardless of anemone size or symbiont presence (Fig. 2b). This lack of symbiont influence on growth differs from the reports of established mutualism and positive physiological influence of Symbiodiniaceae on other nudibranchs such as *Melibe engeli* (Burghardt and Wägele 2014) and *Pteraeolidia ianthina* (Hoegh-Guldberg and Hinde 1986).

The experiment with free-living symbionts showed that *B. stephanieae* is not capable of acquiring symbionts from outside of prey tissues, either by filtering suspended cells or grazing on deposits. While not a formal criterion for mutualism establishment, this contrasts with the established mutualism described for cnidarians and giant clams, of which the vast majority, if not all of the species, are capable of acquiring free-living symbionts (Stat et al. 2006; Mies et al. 2017a). This is also the case for *Melibe engeli*, a nudibranch species for which both mutualism and free-living symbiont acquisition have been confirmed (Burghardt and Wägele 2014). The acquisition of free-living Symbiodiniaceae zoospores is crucial for maintaining robust mutualistic associations. In being exposed to a diverse symbiont gene pool in the plankton, hosts are able to acquire and select Symbiodiniaceae phylotypes that confer relevant functionality including thermal tolerance, adaptation to high or low irradiance and efficient metabolite translocation (Baker 2003; Stat et al. 2008; Mies et al. 2017a). Because of its inability in acquiring free-living symbionts, *B. stephanieae* is restricted to the low diversity found in their specific prey item.

Our findings do not support a symbiotic interaction and are in accordance with molecular reports in Mies et al. (2017b) that symbiosis-specific genes are not expressed by

S. microadriaticum associated with *B. stephanieae* and therefore the mutualism is not active. Together with our data, these results show that this association fails to meet criteria required for mutualism establishment, which includes metabolite/favor exchange and long-term persistence of the association (Davy et al. 2012; Mies et al. 2017a). In fact, from the symbiont perspective, the relationship seems of little benefit. Symbiodiniaceae cells are removed from the safe and well-lit environment within the anemone by *B. stephanieae*, which has a nocturnal habit, and shortly afterwards are eliminated in the surrounding substrate. This deprives the non-motile symbiont cysts from light and makes them an easy prey for benthic feeders. Therefore, this species does not seem to be "solar-powered" like others among Nudibranchia (Wägele et al. 2010) and contradicts previous non-experimental reports of mutualism (Banger 2011).

Aeolids present diverse forms of association with Symbiodiniaceae, including intraclade variation. *Berghia major* digests the symbionts (Kempf 1984), *B. stephanieae* keeps them for short periods of time, and *Pteraeolidia ianthina* fully engages in mutualism and metabolite exchange (Hoegh-Guldberg and Hinde 1986). Such variety may be strongly related to the branching morphology of the digestive gland, as species with extensive branching have larger surface areas and a more suitable environment for housing symbionts (Rudman 1991; Burghardt and Wägele 2004; Wägele et al. 2010). However, *B. stephanieae* does not possess high branching; cerata are roundish, which may hamper sunlight from reaching the majority of symbiont cells, unlike other species with dorsoventrally flattened cerata, such as *Phyllodesmium longicirrum* (Burghardt et al. 2008). Furthermore, the digestive gland does not possess any special features for retaining symbionts, such as the cistern found in *Melibe engeli* (Bughardt et al. 2008; Burghardt and Wägele 2014). The relationship between Symbiodiniaceae and *B. stephanieae* is most likely in an evolutionary transitional stage prior to a truly mutualistic association, similarly to *B. verrucicornis* (Kempf 1991) and *Aeolidia papillosa* (McFarland and Muller-Parker 1993). This broad range of morphology and association may represent distinct stages in the complex evolution of mollusk-dinoflagellate symbiosis (Wägele and Johnsen 2001; Wägele 2004; Burghardt et al. 2008).

The findings in this work show that a stable-state mutualism is not active between Symbiodiniaceae and *B. stephanieae*. Further investigation is necessary to properly describe the nature of this relationship and the metabolic influence of the presence of symbionts in *B. stephanieae*. Overall, the data presented are relevant for understanding aspects related to the biology, ecology and evolution of the association between Symbiodiniaceae and metazoan hosts, as this appears to be a transitional stage of a mutualistic association.

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Compliance with ethical standards

Conflict of interest On behalf of the authors, the corresponding author states that there is no conflict of interest.

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