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ORIGINAL PAPER

Parental diets determine the embryonic fatty acid profile of the tropical nudibranch *Aeolidiella stephanieae*: the effect of eating bleached anemones

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Abstract *Aeolidiella stephanieae* is a stenophagous tropical nudibranch that feeds exclusively on glass anemones of the genus *Aiptasia*. These sea anemones usually harbour endosymbiotic photosynthetic dinoflagellates that contribute to the nutrition of their host by providing photosynthetates, such as fatty acids (FA). The present work determined the effect of parental diets on the FA profile of *A. stephanieae* embryos by feeding breeding pairs of this nudibranch with either symbiotic or aposymbiotic *A. pallida*. Contrasting FA profiles, namely in the levels of palmitic acid (16:0) and docosahexaenoic acid (DHA, 22:6n-3), were recorded for both parental diets and egg masses produced by nudibranchs eating either symbiotic or aposymbiotic *A. pallida*. Noteworthy effects of parental dietary FAs on egg masses were also observed, particularly for DHA, which is mainly synthetized by the endosymbionts of *A. pallida*. Additionally, the present study also highlights how bleaching events may promote cascading effects on the nutrition of marine species with a stenophagous diet, such as *A. stephanieae*.

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Introduction

Fatty acids (FA) are among the most relevant chemical compounds in invertebrate embryos, as they play a key role in cell membrane structure and can also be used as a reserve of metabolic energy to fuel embryogenesis (Lee et al. 2006; Arts et al. 2009). Several FAs commonly referred to as essential fatty acids (EFAs) cannot be synthesized in animal tissues and must be provided by diets (Dalsgaard et al. 2003). As an example, linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) are EFAs that form the starting point for the elongation and desaturation of other FAs, such as the highly unsaturated fatty acids (HUFAs), eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6). Most marine invertebrates are unable to elongate and desaturate EFAs to obtain these HUFAs in suitable levels to fulfil species' requirements, which highlight the role of diets as a source of important FAs (Dalsgaard et al. 2003; Arts et al. 2009). Although larval, juvenile and adult stages of marine invertebrates can obtain nutrition from external food sources, embryos' nutrition is primarily regulated by their broodstock. Nudibranch molluscs are no exception to this rule, and FA profiles of early developing embryos are also assumed to be primarily regulated by broodstock nutrition (Mike et al. 2004;

Martínez-Pita et al. 2005). Although FAs have been widely used as biochemical markers for trophic interactions in aquatic ecosystems and to evaluate diet patterns in aquatic animals (Dalsgaard et al. 2003), to our knowledge, only two studies were ever published on nudibranchs FAs (Martínez-Pita et al. 2005; Zhukova 2007). Moreover, only the study by Martínez-Pita et al. (2005) addressed the effect of parental diets on the FAs composition of egg masses.

The present research tested how broodstock diets displaying contrasting FA profiles affect the FA composition of egg masses of the tropical nudibranch *Aeolidiella stephanieae* Valdés 2005. This species has been and continues to be erroneously designated as *Berghia verrucicornis* in the marine aquarium trade and sometimes in scientific literature (Valdés 2005). *A. stephanieae* inhabits the shallow waters of the Florida Keys (Valdés 2005) and feeds exclusively on glass anemones of the genus *Aiptasia* (Carroll and Kempf 1990). Tropical sea anemones of the genus *Aiptasia* harbour endosymbiotic photosynthetic dinoflagellates, commonly known as zooxanthellae, which significantly contribute EFA to their cnidarian host (Muscatine et al. 1984). With the increasing occurrence of severe bleaching events (Burke et al. 2004; Hoegh-Guldberg et al. 2007), and given the dramatic shifts in FA composition displayed by cnidarian hosts when they lose their endosymbiotic dinoflagellates (Bachok et al. 2006), it is possible that marine organisms exclusively preying on symbiotic cnidarians can also be negatively affected by bleaching (Cole et al. 2009). However, no study has ever focused on whether and how the ingestion of bleached cnidarians influences the incorporation of FAs on embryonic yolk reserves of marine organisms that solely rely on cnidarian prey for survival. In the present research, the nudibranch *A. stephanieae* was fed zooxanthellate or azooxanthellate sea anemones *A. pallida* to test the hypothesis—FA profile of egg masses of *A. stephanieae* reflects the FA composition of either the symbiotic or aposymbiotic anemones ingested by the parental organisms.

Methods

Husbandry and preliminary propagation of *Aiptasia pallida*

Monoclonal *A. pallida* originating from a single specimen were held in two 250 l tanks (1 m × 0.5 m × 0.5 m) filled with 5 μm filtered and UV-irradiated natural seawater. One tank was placed outdoors under natural sunlight and photoperiod to maintain symbiotic sea anemones, while the second one was kept in total darkness for 6 months to produce aposymbiotic sea anemones. The aposymbiotic status was confirmed through visual and dissecting microscope observations. Although residual zooxanthellae may still have survived in the cnidarian host tissues, *A. pallida* reared in total darkness were considered azooxanthellate (please read the Discussion section for a more detailed analysis on this issue). Ceramic tiles (20 mm × 20 mm) were placed in the tanks to increase available surface area for the growth of cultured anemones and to allow large numbers of *A. pallida* to be quickly collected without inducing any mechanical damage. Water temperature was kept at 26 ± 1 °C (mean ± SD) by using 300 W electrical heaters equipped with a thermostat, and salinity was kept stable at 35 ± 1 by daily adding freshwater to the tanks to compensate for any losses due to evaporation. Water circulation was provided by airlifts placed at each corner of the tanks. Ammonia and nitrite were kept under detectable levels; nitrate was recorded between 10 and 20 mg l^{-1} and pH at 8.0 ± 0.1. All these parameters were determined with colorimetric tests. Every 3 days, newly hatched *Artemia* nauplii (5 nauplii ml^{-1}) were supplied to both tanks in order to feed *A. pallida* and increase anemone production (Leal et al. 2012). The bottom of the tanks was syphoned every week together with a 25 % partial water change.

Husbandry of *Aeolidiella stephanieae*

Six breeding pairs of *A. stephanieae* were imported from a wholesaler trading marine ornamental species for the aquarium industry. Each pair was kept in a 5-l plastic container with constant aeration, inside a 26 °C water bath and illuminated from above with fluorescent white light (12 h light:12 h dark photoperiod). Daily water changes (20 %) were made using 5 μm filtered and UV-irradiated natural seawater, heated to 26 °C and with salinity at 35 ± 1 and pH 8 ± 0.5. Ammonia and nitrite were maintained under detectable levels while nitrates were maintained below 10 mg l^{-1}. Nudibranchs were fed *A. pallida* according to the experimental design detailed in the following section.

Experimental design and sampling

The experiment started 3 weeks after the acclimatization of nudibranchs to laboratory conditions. In the first day of experiment, 10 anemones were randomly collected from each of the 2 anemone propagation tanks, washed with distilled water and stored in liquid nitrogen for later biochemical analysis. *A. stephanieae* breeding pairs, which had not been fed for 3 days, were separated into two groups: 3 pairs (tanks 1–3) were fed with the *A. pallida* reared under natural photoperiod (symbiotic *A. pallida*) and the remaining 3 pairs (tanks 4–6) were fed with *A. pallida* reared in total darkness (aposymbiotic *A. pallida*). At least

two intact anemones were always available as food for breeding pairs, and new anemones were added to the containers as needed, in order to assure that breeding pairs could always feed ad libitum.

In a thirty-day period, the presence of embryos was checked daily in the morning. Freshly laid egg masses with uncleaved fertilized eggs were carefully detached from the walls of the tanks with a small spatula, washed with distilled water and stored in liquid nitrogen for later biochemical analysis. A total of thirty samples, 10 per breeding pair (each with approximately the same wet weight), were collected from each treatment. As biomass of the collected eggs was relatively low, embryos sampled in each treatment were pooled into 4 composite samples per treatment for posterior biochemical analysis ($n = 4$ replicates per treatment). Each composite sample, of either cultured *A. pallida* or *A. stephanieae* eggs, consisted of a pool of several individual samples in order to have identical portions for FA analysis.

Fatty acid analysis

Fatty acid extraction and preparation of methyl esters were carried out according to Lepage and Roy (1986) [modified by Cohen et al. (1988)]. Freeze-dried samples (100 mg) were transmethylated with 5 ml of methanol/acetyl chloride (95:5 v/v). The mixture was sealed in a light-protected Teflon-lined vial under nitrogen atmosphere and heated at 80 °C for 1 h. The vial contents were then cooled, diluted with 1 ml water and extracted with 2 ml of n-heptane. The heptane layer was dried over Na_2SO_4, evaporated to dryness under nitrogen atmosphere and redissolved in heptane, which contained the methyl esters. The methyl esters were then analysed by gas–liquid chromatography, on a VARIAN (Palo Alto, USA) 3800 gas–liquid chromatograph (USA), equipped with a flame ionization detector. Separation was carried out on a 0.32 mm × 30 m fused silica capillary column (film 0.32 μm) Supelcowax 10 (SUPELCO, Bellafonte PA, USA) with helium as carrier gas at a flow rate of 1.3 ml min^{-1}. The column temperature was programmed at an initial temperature of 200 °C for 10 min, then increased at 4 °C min^{-1}–240 °C and held there for 16 min. Injector and detector temperatures were 250 and 280 °C, respectively, and split ratio was 1:100. Peak identification was carried out using known standards (Nu-Chek-Prep, Elysian, USA). Peak areas were determined using Varian software, and the FAs analysed as percentage data.

Statistical analysis

The percentage of FAs and FA ratios obtained for the egg masses of *A. stephanieae* fed with symbiotic and aposymbiotic *A. pallida*, as well as for parental nudibranchs' food sources (symbiotic and aposymbiotic *A. pallida*) were compared using Student's *t* test. When the assumptions of homogeneity of variances and homoscedasticity for the parametric analysis were not met, the nonparametric Mann–Whitney U test was performed. Analyses were performed using R (R Development Core Team 2011). Samples were also analysed using principal coordinates ordination (PCO). The PCO was used to describe overall relationship among the different *A. pallida* and egg masses of *A. stephanieae* based on their fatty acid profile. Different PCOs were performed for the FA percentages using only FAs accounting for more than 1 % of the total FA pool and FA ratios. The raw data matrix of FA per treatment was first log $(x + 0.1)$ transformed, as this procedure places more emphasis on compositional differences among samples rather than on quantitative differences. After this transformation, a similarity/difference matrix was constructed using the Euclidean distance. The obtained plots represented the distribution of all treatments according to their FA profile, together with the eigenvectors with a multiple correlation higher than 0.2 (Clarke and Gorley 2006). The displayed eigenvectors correspond to the obtained eigenvalues, which reflect the amount of variance explained by the PCO. The elements of the eigenvectors are coefficients that reflect the contribution of their respective variables to the variance of the PCO. The larger the absolute value of the coefficient, the greater is the importance of its variable to the PCO. Similarity percentages (SIMPER) were also explored to examine the similarity within the FAs of (1) symbiotic and aposymbiotic *A. pallida* and (2) egg masses of *A. stephanieae* fed with symbiotic or aposymbiotic *A. pallida*. All multivariate analyses were performed using PRIMER v6 with PERMANOVA add-on (Primer-E, Ltd., Plymouth, UK).

Results

For both PCOs based on FAs percentages and ratios, the first two axes explained together 93–96 % of the variation in the data sets (Fig. 1). Both ordinations evidenced the differences between both *A. pallida* and *A. stephanieae* egg masses. The horizontal axis of variation in both PCOs separated symbiotic and aposymbiotic groups, while the vertical axis maximized the differences between *A. pallida* and egg masses (Fig. 1). Palmitic acid (PA, 16:0), EPA and DHA had a relevant role on the horizontal and vertical displacement of samples. ALA was closely associated with aposymbiotic *A. pallida*, while DHA and PA were relatively associated with symbiotic *A. pallida* and with egg masses from *A. stephanieae* fed symbiotic anemones, respectively. All ratios presented in Fig. 1b made an important contribution to displayed differences. Aposymbiotic *A. pallida* were associated with a higher n-3/n-6

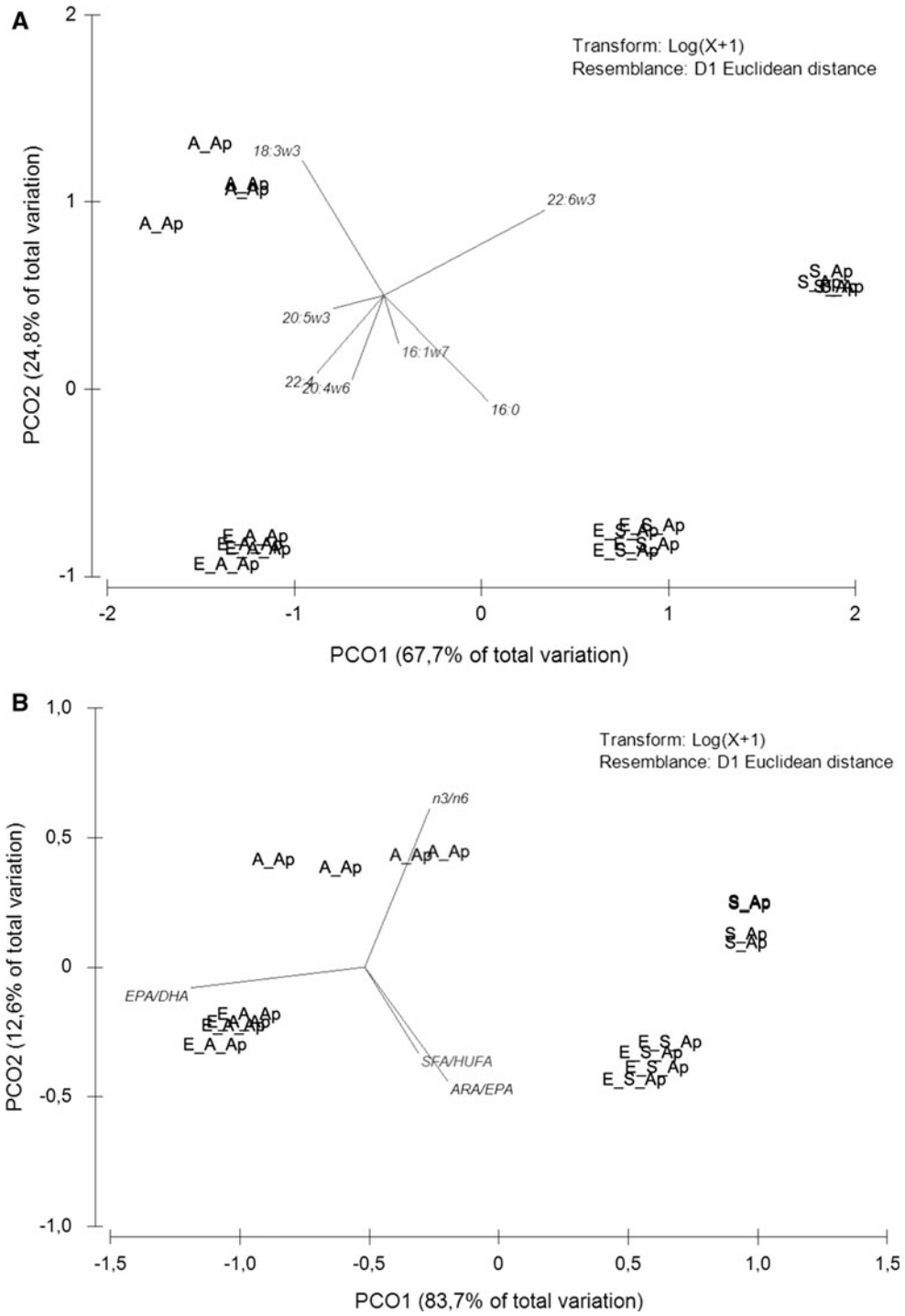

Fig. 1 Principal component ordination based on (**a**) fatty acid profiles and (**b**) fatty acid ratios of symbiotic and aposymbiotic *A. pallida* (S_Ap and A_Ap, respectively), and egg masses of *A. stephanieae* fed with symbiotic and aposymbiotic *A. pallida* (E_S_Ap and E_A_Ap, respectively). Eigen vectors of multiple correlations (>0.2) are also represented

ratio, whereas egg masses of *A. stephanieae* fed aposymbiotic anemones were associated with a higher EPA/DHA ratio.

Fatty acid profile of *Aiptasia pallida*

The percentages of saturated FAs (SFA; $t = 3.126$, $P < 0.05$) were significantly higher in symbiotic anemones, which were able to sustain their photosynthetic symbiont population, compared with dark-cultured anemones that bleached in about 2 months (Fig. 2). PA supported most differences in the SFA content, whereas DHA was the FA that mostly contributed to the observed differences in HUFA content. While PA and DHA were the most important FA for symbiotic *A. pallida*, ALA displayed the highest FA levels in aposymbiotic anemones. EPA/DHA ($W = 1.5$, $P < 0.05$)

and ARA/EPA ($t = 26.261$, $P < 0.01$) ratios were considerably higher in anemones cultured in total darkness (Fig. 3). Significant differences were observed for the whole FA profile of symbiotic and aposymbiotic *A. pallida* (ANOSIM, $R = 1$, $P = 0.029$). A high degree of similarity within groups was displayed for both anemone groups (SIMPER: 97 and 95 % similarity, for symbiotic and aposymbiotic *A. pallida*, respectively). DHA (16 %) and ALA (12 %) were the FAs that most contributed to the difference observed between anemones' FA profiles (26 % dissimilarity).

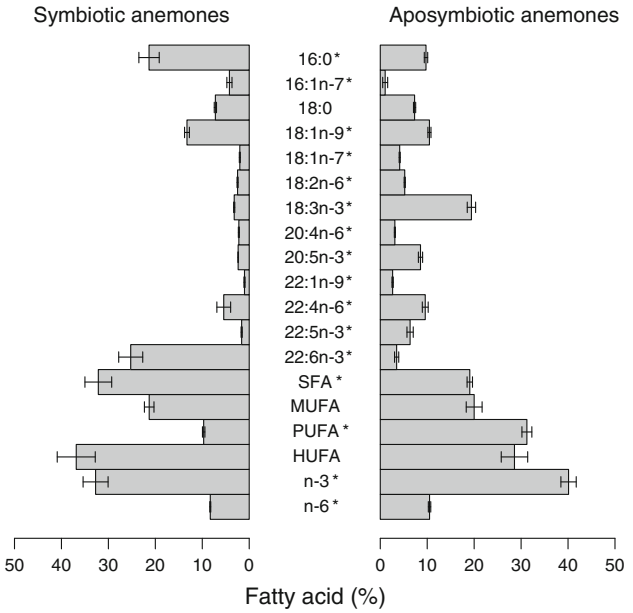

Fig. 2 Fatty acid profile (%) of symbiotic and aposymbiotic *Aiptasia pallida*. Significant differences ($P < 0.05$) are marked *asterisk*. *SFA* sum of saturated fatty acids, *MUFA* sum of monosaturated fatty acids, *PUFA* sum of polyunsaturated fatty acids, *HUFA* sum of highly unsaturated fatty acids, n-3 sum of omega-3 fatty acids, n-6 sum of omega-6 fatty acids

Fatty acid profile of *Aeolidiella stephanieae* embryos

Figure 4 shows the FA profiles of embryos of *A. stephanieae* fed either aposymbiotic or symbiotic *A. pallida*. The percentages of SFA ($W = 16$, $P < 0.05$) were significantly higher in embryos produced by nudibranchs fed symbiotic anemones, while the percentages of MUFA ($t = -4.350$, $P < 0.05$), PUFA ($t = -7.904$, $P < 0.01$) and HUFA ($t = -3.640$, $P < 0.05$) were significantly higher in embryos produced by broodstock provided with aposymbiotic *A. pallida*. All SFA, particularly PA, were present in higher percentages in embryos produced by *A. stephanieae* fed symbiotic *A. pallida*, while all other FAs, apart from DHA and 22:1n-9, were higher in embryos from nudibranchs fed aposymbiotic *A. pallida*, particularly 18:1n-9 and 22:4n-6 that displayed relatively higher levels. The most divergent ratios between both groups of embryos were EPA/DHA, which was significantly higher in *A. stephanieae* embryos fed aposymbiotic *A. pallida* ($W = 0$, $P < 0.05$; Fig. 3), and n-3/n-6, which was higher in *A. stephanieae* embryos fed symbiotic *A. pallida* ($t = 3.541$, $P < 0.05$; data not shown). A significant difference was observed when considering the whole FA profile of egg masses from both groups (ANOSIM, $R = 1$, $P = 0.029$). A

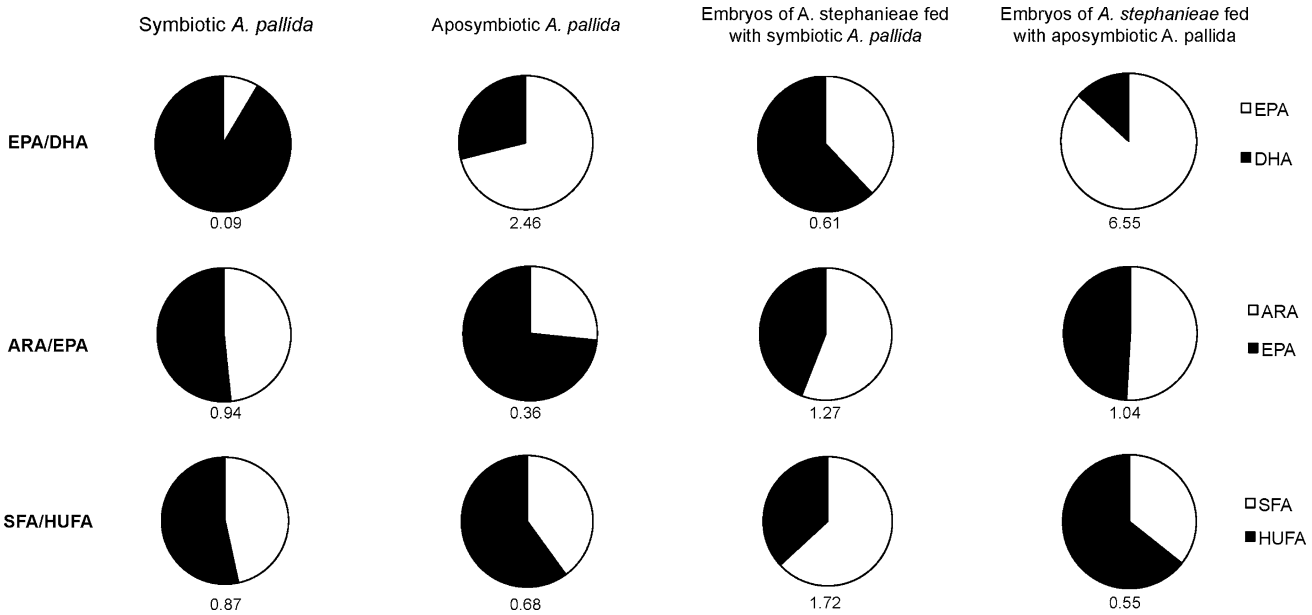

Fig. 3 Fatty acid ratios of symbiotic and aposymbiotic *A. pallida* and of embryos of *A. stephanieae* fed with symbiotic and aposymbiotic anemones. Average ratios are presented *below* each pie ($n = 4$). *EPA* eicosapentaenoic acid (20:5n-3), *DHA* docosahexanoic acid (22:6n-3), *ARA* arachidonic acid (22:4n-6), *SFA* saturated fatty acids, *HUFA* highly unsaturated fatty acids

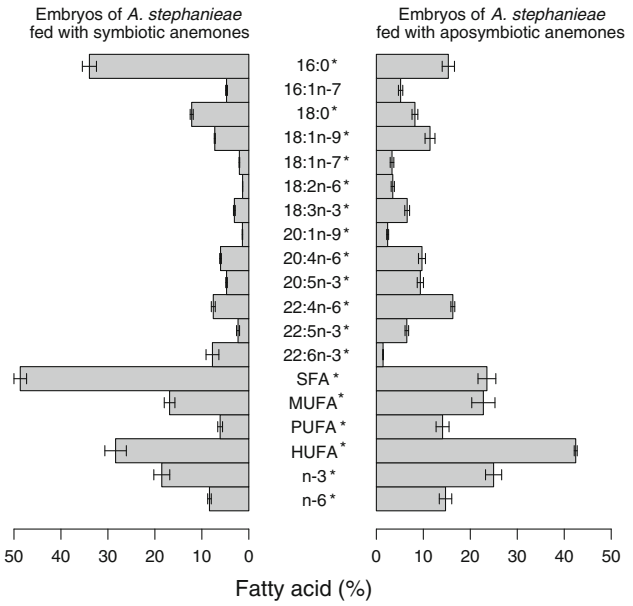

Fig. 4 Fatty acid profile (%) of embryos of *A. stephanieae* fed with symbiotic and aposymbiotic anemones. Significant differences ($P < 0.05$) are marked with *asterisk*. *SFA* sum of saturated fatty acids, *MUFA* sum of monosaturated fatty acids, *PUFA* sum of polyunsaturated fatty acids, *HUFA* sum of highly unsaturated fatty acids, n-3 sum of omega-3 fatty acids, n-6 sum of omega-6 fatty acids

high degree of within-group similarity was displayed for each egg masses' category (SIMPER: 97 and 94 % similarity, for embryos of *A. stephanieae* fed symbiotic and aposymbiotic anemones, respectively). PA (12 %) and DHA (10 %) were the FAs that most contributed to the differences between FA profiles (21 % dissimilarity).

Discussion

The FAs present in marine invertebrates usually originate from biosynthesis, performed by the organisms themselves, or from diet sources (Muscatine et al. 1984; Dalsgaard et al. 2003). Another potential source of FAs is the photosynthetic endosymbionts that live in the tissues of some cnidarians. In the present study, we used symbiotic and aposymbiotic *A. pallida* as a food source. Although symbiotic cnidarians lose their zooxanthellae when kept in total darkness for long periods (i.e. more than 1 month) (Titlyanov et al. 2001), they may not lose all their photosynthetic endosymbionts (Titlyanov and Titlyanova 2002). In the present study, residual zooxanthellae may have not been detected through visual observations in aposymbiotic anemones, but if zooxanthellae were present, they did not conduct photosynthetic activity in total darkness. Therefore, no photosynthetates were available to be translocated to the cnidarian host. In this view, although aposymbiotic *A. pallida* used in the present study may have eventually harboured a residual number of zooxanthellae, there was no FA contribution from its photosynthetates to the cnidarian host.

The main FAs present in the egg masses were SFAs (mainly PA; Fig. 4) and HUFAs, as documented for other nudibranchs (Martínez-Pita et al. 2005; Zhukova 2007). While SFA have high caloric content and are primarily used as a source or storage form of energy, HUFAs affect animal physiology. The higher dominance of PA in embryos produced from *A. stephanieae* fed symbiotic anemones, when compared to embryos from adults fed aposymbiotic anemones, might be associated with the similar trend recorded for symbiotic and aposymbiotic *A. pallida*. However, this rationale does not apply to all FAs recorded, namely HUFAs (Figs. 2, 4). It is important to note that this study is addressing the percentage of FAs. Although a given FA, such as ALA, can be notably higher in aposymbiotic anemones, this percentage can be associated with a lower content of other FAs that were present in higher amounts in symbiotic anemones, such as DHA. Nevertheless, although the comparison of percentage data between *A. pallida* and egg masses should be cautious, results reveal a notable effect of dietary FAs on *A. stephanieae* egg masses.

Besides the noteworthy differences on individual FA profiles between symbiotic and aposymbiotic anemones, as well as between embryos produced from nudibranchs provided with different diets (Fig. 1), differences between FAs ratios were also evident (Fig. 3). Although *A. pallida* with contrasting FA profiles were provided to broodstock and different FA content was also expected on *A. stephanieae* egg masses, it would be possible to observe similar ratios between egg masses of both treatments. Similar ratios could mean that the reproductive effort would vary in terms of egg clutches numbers and/or frequency and not in embryonic lipid quality. However, our results suggest that the nutritional composition of produced embryos is directly dependent on the nutritional profile of the broodstock, which is strongly associated with their food sources. The most noteworthy example of this feature is DHA, as differences observed in its relative abundance in *A. pallida* were reflected in *A. stephanieae* egg masses (Figs. 2, 4). These results are in agreement with other studies demonstrating that dietary FAs strongly affect reproductive success of marine invertebrates (Hendriks et al. 2003; Marshall et al. 2010).

Our study focused on nudibranch nutrition and biochemistry, on a species with considerable market value in the marine aquarium trade (Carroll and Kempf 1990; Olivotto et al. 2011). The importance of providing preys with a significant content of EFA to the broodstock in order to produce embryos with similar profiles was evidenced here. Nevertheless, it should also be noted that several key

questions remain to be addressed. First, will hatched larvae also show the same fatty acid profile of their parents and parental diets? Second, what is the hatching success and larval fitness when the broodstock is fed with different diets? Third, do parental organisms compensate a low-quality diet by increasing feeding rates? Fourth, do different diets affect spawning frequency and embryo biomass? Finally, how significantly does embryonic development shift the initial FA composition and will newly hatched specimens display similar FA contents regardless of initial maternal reserves present in the egg yolk?

The present work also highlights the contribution of endosymbiotic zooxanthellae to the FA profile of the cnidarian host, as different percentages of EFA (e.g. ALA, EPA and DHA) were observed between symbiotic and aposymbiotic *A. pallida*. Particularly, the photosynthetic products derived from the zooxanthellae are probably an important source of DHA to the cnidarian host. Several cnidarians, such as corals, depend on the symbiotic relationship with zooxanthellae to survive. The loss of zooxanthellae commonly referred to as bleaching, and the increasing frequency of massive bleaching events is a key issue to coral reef conservation (Kleypas et al. 2010; van Woesik et al. 2011). If bleaching events continue to occur, besides the direct consequences on these invertebrate populations, bleaching may also affect the nutrition of species that prey on these organisms. Those most directly impacted may be highly specialized species that feed exclusively on a single zooxanthellate prey, such as the tropical nudibranch *A. stephanieae*. Bleaching events may consequently promote cascading effects on the reproduction of these specialized predator species and, consequently, affect the resilience of their populations.

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