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SPECIAL ISSUE: MOLECULA DETECTION OF TROPHIC INTERACTIONS

Molecular assessment of heterotrophy and prey digestion in zooxanthellate cnidarians

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Abstract

Zooxanthellate cnidarians are trophically complex, relying on both autotrophy and heterotrophy. Although several aspects of heterotrophy have been studied in these organisms, information linking prey capture with digestion is still missing. We used prey-specific PCR-based tools to assess feeding and prey digestion of two zooxanthellate cnidarians – the tropical sea anemone *Aiptasia* sp. and the scleractinian coral *Oculina arbuscula*. Prey DNA disappeared rapidly for the initial 1–3 days, whereas complete digestion of prey DNA required up to 10 days in *O. arbuscula* and 5 or 6 days in *Aiptasia* sp. depending on prey species. These digestion times are considerably longer than previously reported from microscopy-based examination of zooxanthellate cnidarians and prey DNA breakdown in other marine invertebrates, but similar to prey DNA breakdown reported from terrestrial invertebrates such as heteroptera and spiders. Deprivation of external prey induced increased digestion rates during the first days after feeding in *O. arbuscula*, but after 6 days of digestion, there were no differences in the remaining prey levels in fed and unfed corals. This study indicates that prey digestion by symbiotic corals may be slower than previously reported and varies with the type of prey, the cnidarian species and its feeding history. These observations have important implications for bioenergetic and trophodynamic studies on zooxanthellate cnidarians.

Keywords: *Aiptasia*, digestion, dla-qPCR, predator–prey interaction, qPCR, scleractinian corals

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Introduction

The association between cnidarians and photosynthetic endosymbiotic dinoflagellates (genus *Symbiodinium*; commonly termed zooxanthellae) is among the most investigated ecological associations (e.g. see reviews by Venn *et al.* 2008; Davy *et al.* 2012). This relationship provides the cnidarian with translocated photosynthates from the zooxanthellae, and the host provides a sheltered light-rich environment with inorganic nutrients (Porter 1976; Falkowski *et al.* 1984). Besides this autotrophy through endosymbionts, zooxanthellate cnidarians are also able to feed heterotrophically, particularly by preying on zooplankton. This form of heterotrophy may account for a significant portion of the nutrition of zooxanthellate cnidarians, particularly when photosynthetic products are unavailable, such as when light is limiting or during bleaching events (Anthony & Fabricius 2000; Grottoli *et al.* 2006; Palardy *et al.* 2008).

Heterotrophy in zooxanthellate cnidarians has been thoroughly investigated, at least in terms of prey capture and ingestion (see Houlbreque & Ferrier-Pagès 2009 for a review). However, lack of quantitative data on prey digestion processes presently limits the understanding of trophic ecology of these organisms. It is also important to understand the digestion dynamics in the deprivation of external food because heterotrophic starvation affects the bioenergetics and bleaching susceptibility of zooxanthellate cnidarians (Titlyanov *et al.* 2000; Borell *et al.* 2008).

Investigations on prey ingestion and digestion in zooxanthellate cnidarians have been challenged by inherent limitations in available methods. Feeding studies on these organisms have largely been based on clearance rates during incubations in experimental chambers and

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visual observations of polyp dissections (e.g. Sebens 1981; Sebens & Johnson 1991; Houlbreque *et al.* 2004; Palardy *et al.* 2005; Grottoli *et al.* 2006). The first method, clearance rate, is an indirect estimate of prey capture as it builds on the assumption that all prey that disappears during incubation have been ingested. The second method, visual identification of prey content, is prone to error as it relies on the visual recognition of partially digested prey, likely causing a substantial underestimation of digestion times, especially for small prey types (see Nejstgaard *et al.* 2008 for thorough discussion on limitations in bottle incubation and microscopical gut analyses in feeding studies).

During the last decade, there has been a dramatic increase in the development and use of molecular methods to study trophic interactions, both in marine and in terrestrial environments (Symondson 2002; Sheppard & Harwood 2005; King *et al.* 2008). PCR-based methods have enabled direct assessment of feeding dynamics through the analysis of prey DNA sequences and provide qualitative and quantitative assessments of prey capture and digestion (e.g. Deagle *et al.* 2006; Troedsson *et al.* 2009; Durbin *et al.* 2012; Roura *et al.* 2012). While these molecular tools have been successfully used to study marine invertebrate trophic interactions (Troedsson *et al.* 2007; Simonelli *et al.* 2009; O'Rorke *et al.* 2012a; Roura *et al.* 2012), to our knowledge they have only been applied once to zooxanthellate cnidarians to investigate the presence/absence of prey ingested by corals (Leal *et al.* this issue). The use of PCR-based methods to investigate heterotrophy in these organisms may provide a more accurate and reliable estimate of prey ingestion and digestion, which may have implications for bioenergetic estimates of zooxanthellate cnidarians. Ultimately, using a molecular approach to study the nutrition of these organisms, researchers may achieve a better understanding of the cnidarian–dinoflagellate symbiosis.

In this study, we investigated heterotrophy and prey digestion of zooxanthellate cnidarians fed different prey under laboratory settings using three PCR-based approaches. Standard endpoint PCR was used to develop a qualitative assessment of heterotrophic feeding (Leal *et al.* this issue). Prey DNA breakdown was assessed by the development and use of differential amplification length quantitative PCR (dla-qPCR) (Deagle *et al.* 2006; Troedsson *et al.* 2009). Quantitative PCR (qPCR) was used to estimate digestion time (Durbin *et al.* 2012). As experimental organisms we used the brine shrimp *Artemia* sp. nauplii and the rotifer *Brachionus plicatilis* as prey for the symbiotic tropical sea anemone *Aiptasia* sp. and the scleractinian symbiotic coral *Oculina arbuscula*. *Aiptasia* sp. is acknowledged as a model organism for the study of the cnidarian–dinoflagellate symbiosis (Weis *et al.* 2008), and *O. arbuscula* is a facultative symbiotic species that is able to survive when deprived of its photosynthetic endosymbionts and thus represents a coral capable of a high degree of nutritional plasticity (Miller 1995). These prey species (*Artemia* sp. and *B. plicatilis*) were chosen as they are robust prey model organisms for coral feeding studies (e.g. Titlyanov *et al.* 2001; Houlbreque & Ferrier-Pagès 2009; van Os *et al.* 2012; Leal *et al.* 2013a).

In this study, we address the following hypotheses: (i) prey DNA breakdown of zooxanthellate cnidarians is identical for different prey species and (ii) different zooxanthellate cnidarian species have similar digestion rates regardless of prey species and starvation conditions.

Methods

Zooplankton cultures

Artemia sp. nauplii were hatched from cysts (San Francisco Bay strain; Brine Shrimp Direct suppliers, Ogden, UT, USA) by immersion in aerated water (26 °C, 25 ppt salinity). Nauplii were captured after 20–24 h incubation and rinsed with filtered seawater (Whatman GF/F filter, nominal pore size 0.7 μm). Live cultures of *B. plicatilis* ('L' type; Reef Nutrition, Campbell, CA, USA) were maintained in aerated tanks (Hoff & Snell 2008) and fed a mixture of microalgae (RGcomplete APBreed; Reed Mariculture, Campbell, CA, USA). *Brachionus plicatilis* were captured using a sieve (100-μm mesh) and rinsed with filtered seawater (GF/F). Species identification was confirmed by amplification of nearly the complete 18S rRNA gene using the primers Univ18S-15F and Univ18S-1765R (Gruebl *et al.* 2002) and sequencing approximately 900 bp from the 5′-end of the amplified fragments (Table S1, Supporting information). The recovered 18S rRNA gene sequences of *Artemia* sp. (876 bp) and *B. plicatilis* (893 bp) were identical to the sequences reported by Weekers *et al.* (2002) (GenBank no. AJ238061) and by Giribet *et al.* (2004) (GenBank no. AY218118), respectively.

Zooxanthellate cnidarians collection and husbandry

Oculina arbuscula was collected at Gray's Reef National Marine Sanctuary (Georgia, USA, 30.3939°N 80.8885°W, 20 m depth, May 2012) under the manager's permit and fragmented into replicate coral nubbins (Highsmith 1982). Corals were kept unfed in a recirculating tank maintained at 24 °C and 35 ppt salinity. The aquarium system was composed of a 200-L tank connected to a 100-L filter tank equipped with a protein skimmer and a biological filter (Sheridan *et al.* 2013). Partial water changes (10% of total system volume) with fresh filtered seawater were performed weekly. The coral tank was

illuminated from above (two 21-W T5 bulbs: actinic blue and natural daylight; Coralife, Franklin, WI, USA) in a 12-h light/12-h dark cycle.

Aiptasia sp. were supplied by a local marine ornamentals wholesaler and stocked in a recirculating tank (40 L) with an external trickle filter. The tank was illuminated from above (6-W T5 bulb natural daylight; Coralife) in a 12-h light/12-h dark cycle. Water changes were performed as previously described. Apart from light, all other culture conditions followed the protocol to produce monoclonal *Aiptasia* (Leal *et al.* 2012). The anemones were starved 1 week prior to conducting the experiments.

Oculina arbuscula was visually identified to the species level, and its identity was supported by sequencing a fragment of its 18S rRNA gene as described above (GenBank no. JX983594). The resulting sequence was submitted to GenBank (Accession no. JX983594). It was not possible to identify *Aiptasia* specimens used in this study to the species level based on the morphology or the 849-bp 18S rRNA gene sequence. Sequencing results indicated a high degree of similarity (>98%) to *Aiptasia mutabilis* (GenBank no. FJ489438), *Aiptasia insignis* (GenBank no. AY046885) and *Aiptasia pulchella* (GenBank no. EU190846, AY297437). Therefore, we used clones stemming from one individual in all experiments and refer to them as *Aiptasia* sp.

Feeding experiments

Experiments for each cnidarian species fed *Artemia* sp. or *B. plicatilis* were performed in Plexiglass chambers (Vogel & LaBarbera 1978) with a water volume of 1200 mL (30 × 4 × 12 cm) and water flow (0.1 m/s) provided by an internal pump (TOM Aquarium & Pet Products, Inc., Shawnee KS, USA). Each replicate consisted of a separate feeding chamber with a single coral fragment (approximately 25 polyps) or small anemone (approximately 3–6 mm). *Aiptasia* sp. were fed either *Artemia* or *B. plicatilis* at a concentration of 2000/L that were added once all polyps were expanded. *Aiptasia* sp. was fed for 15 min and *O. arbuscula* for 30 min. Feeding times were different between species because prey capture rates were higher in *Aiptasia* sp. than *O. arbuscula*, and overfeeding the first could lead to prey regurgitation and potentially bias feeding results. After feeding, organisms were sampled and thoroughly rinsed three times in GF/F-filtered seawater.

For the qualitative assessment of heterotrophy, *Aiptasia* sp. and *O. arbuscula* were rinsed and sampled immediately after feeding. For prey digestion assessment, *Aiptasia* sp. and *O. arbuscula* were rinsed and either sampled immediately after feeding (day 0) or transferred to a prey-free chamber and kept in continuous heterotrophic starvation until being sampled after 24 h (day 1), 48 h (day 2), 72 h (day 3), 96 h (day 4), 144 h (day 6), 192 h (day 8) and 240 h (day 10). Unfed *O. arbuscula* and *Aiptasia* sp. were also sampled as negative controls.

To investigate whether continuous starvation after feeding affected the relatively long prey digestion times observed for *O. arbuscula* (see Results section), a subset of experiments was conducted where corals were fed either *B. plicatilis* or *Artemia* sp. and transferred to a prey-free chamber after feeding (day 0). After the first day, the prey type was switched so that corals remained fed but that postexperimental feeding did not interfere with the specific detection of the prey that were provided on day 0. Samples were collected after 2, 3 and 6 days of incubation.

For all experiments involving *O. arbuscula*, individual polyps were sampled (Kemp *et al.* 2008). For all experiments involving *Aiptasia* sp., whole individuals were used for total genomic DNA (gDNA) extraction. For each digestion time point of each experiment with *O. arbuscula,* nine samples were taken: three polyps sampled per coral nubbin and a total of three nubbins from different colonies (n = 3 x 3). For *Aipasia* sp. experiments, a total of three single organisms were sampled.

Genomic DNA extraction and purification

gDNA from zooplankton cultures, corals and anemones were extracted using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA). DNA quantity was estimated using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen) on a NanoDrop ND-3300 fluorospectrometer (NanoDrop products, Wilmington, DE). Each time a feeding experiment was performed, the gDNA of a known number of the zooplankton prey used in the experiment was also extracted for later use as standards in qPCR assessments. This information also allowed the estimation of gDNA content per individual prey, which was used to estimate ingested prey numbers based on prey DNA content at ingestion (day 0).

Assessment of prey DNA breakdown

Prey DNA breakdown was assessed through the dla-qPCR assay (Deagle *et al.* 2006; Troedsson *et al.* 2009). This assay utilizes multiple primer sets that amplify different sized fragments in a single specific area of the prey 18S rRNA gene. Prey DNA breakdown is defined as a decline in amplifiable target DNA strand length over time.

Primers

Primer pairs targeting *Artemia* sp. 18S rRNA were designed using previously referenced nucleotide

sequences (Weekers *et al.* 2002) (GenBank no. AJ238061), apart from the forward (Af18S-1298F) and the reverse (Af18S-1387R) primers (Mackie & Geller 2010). All *B. plicatilis* primer pairs were designed using an alignment of nucleotide sequences (GenBank no. AY218118, no. U29235, no. U49911). Using a single forward primer, sets of reverse primers were designed to amplify fragments of increasing size from 50 to 500 bp. Primer design was facilitated using Primer 3 software (Rozen & Skaletsky 2000). All oligonucleotides were synthesized by Integrated DNA Technologies (www.idtdna.com).

Ten candidate reverse primers targeting *Artemia* 18S rRNA gene were tested with Af18S-1298F, and ten candidate reverse primers targeting *B. plicatilis* 18S rRNA gene were tested with a forward primer (Bp18S-202F). Six reverse primers were ultimately selected for each prey species (Table S2, Supporting information) based on (i) similar annealing temperature with forward primer, (ii) specificity and (iii) reaction efficiency. Primer specificity was confirmed *in silico* in the NCBI database by BLAST searching (http://ncbi.nlm.nih.gov/BLAST/) and empirically in PCR assays against gDNA purified from each predator and prey species. However, these primer sets were not specifically validated for use beyond this study and therefore should be used cautiously in any future studies. Amplification efficiency for each primer set was calculated using the slope of the log standard curve (Heid *et al.* 1996) over a target gDNA template concentration range of 0.02–100 ng/mL. Minimum detection sensitivity was 4 and 3 pg of gDNA from *Artemia* sp. and *B. plicatillis*, respectively. PCRs were performed in 20 μL reaction volumes using the prey-specific primers. All qPCRs were performed using a Bio-Rad CFX96™ C1000™ real-time thermal cycler (Bio-Rad Laboratories, Hercules CA, USA) in 96-well plates with each reaction well containing 10 μL of SsoFast™ EvaGreen® Supermix, 400 nM of primers and template gDNA ranging between 200 and 600 ng/mL. Amplification conditions included an initial denaturation step (2 min, 98 °C) followed by 40 amplification cycles (5 s, 98 °C; 5-s annealing/extension temperature; Table S2, Supporting information). All reactions were run in triplicate, and PCR-grade water was used as template for negative controls. PCR products were visualized by gel electrophoresis on a 1% agarose gel buffered in 1× TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0).

PCR/qPCR amplification of zooplankton prey

All endpoint PCRs were performed using the reaction details and the same equipment as previously described. Only one primer set per prey species was used for PCR (*B. plicatilis*: Bp18S-202F and Bp18S-626R; *Artemia*: Af18S-1298F and Af18S-1547R), whereas all the species-specific primer sets were used for qPCR assays (Table S1, Supporting information). The appropriate amount of template DNA in all assays was achieved using 1 μL of either undiluted, 5-fold or 10-fold dilution of gDNA extract. For each qPCR, a dilution series of extracted gDNA from each zooplankton culture was run as a quantitative standard. All reactions were run in triplicate, and PCR-grade water was used as template for negative control. PCR products were visualized by gel electrophoresis as described above.

The number of prey ingested by each *Aiptasia* sp. and *O. arbuscula* polyp on day 0 was estimated using gDNA quantification from culture extracts and qPCR results based on the smallest fragments amplified for each prey.

Statistical analysis

The significance of differences in the prey DNA content obtained using each primer set (i.e. different fragment sizes) was tested for each experimental day using a one-way ANOVA. One-way ANOVA was also used to assess the significance of differences in prey DNA content between digestion times. Tukey HSD test was used when ANOVA results revealed significant differences ($P < 0.05$). Student's *t*-test was used to compare prey DNA contents for the same digestion time of *O. arbuscula* fed only once or continuously. Assumptions of homogeneity of variances and homoscedasticity were tested before all statistical analyses. When the assumptions were not met, square root transformations were performed. All statistical analyses and plots were facilitated using R (R Development Core Team 2012).

Results

Qualitative detection of prey in the predators

Initially, PCR was used to detect the presence of prey 18S rRNA in DNA extracted from the zooxanthellate cnidarian fed the respective prey species: *Artemia* sp. and *B. plicatilis*. These results, although not quantitative, indicated that both *Aiptasia* sp. and *O. arbuscula* ingested both prey species.

Assessment of the quantitative breakdown of prey DNA by dla-qPCR

Immediately after ingestion (day 0), there was no indication of prey breakdown, that is, there was no statistically significant difference in amplified DNA concentration between the dla-qPCR amplicons for any of the prey and predators (Figs 1a, d and 2a, d, one-way ANOVA, $P = 0.87$, 0.91, 0 57 and 0.84, respectively). This indicates

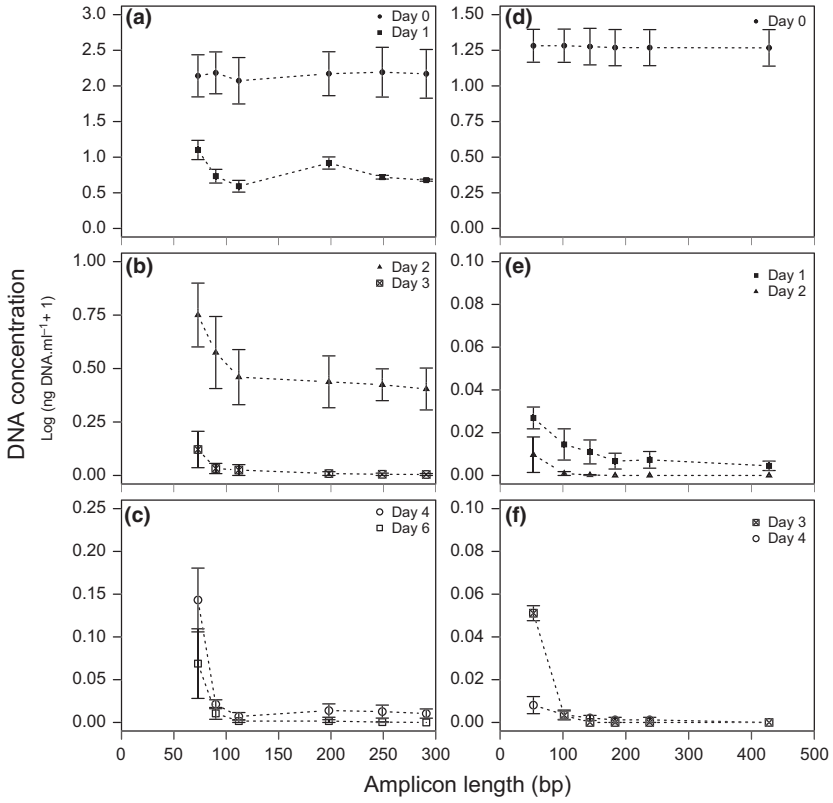

Fig. 1 Prey DNA breakdown based on the amplification of different sizes of DNA fragments of *Artemia* sp. (a, b, c) and *Brachionus plicatilis* (d, e, f) ingested by *Aiptasia* sp. 0, 1, 2, 3, 4 and 6 days after feeding (error bars indicate standard error). Data available in Leal *et al*. (2013b).

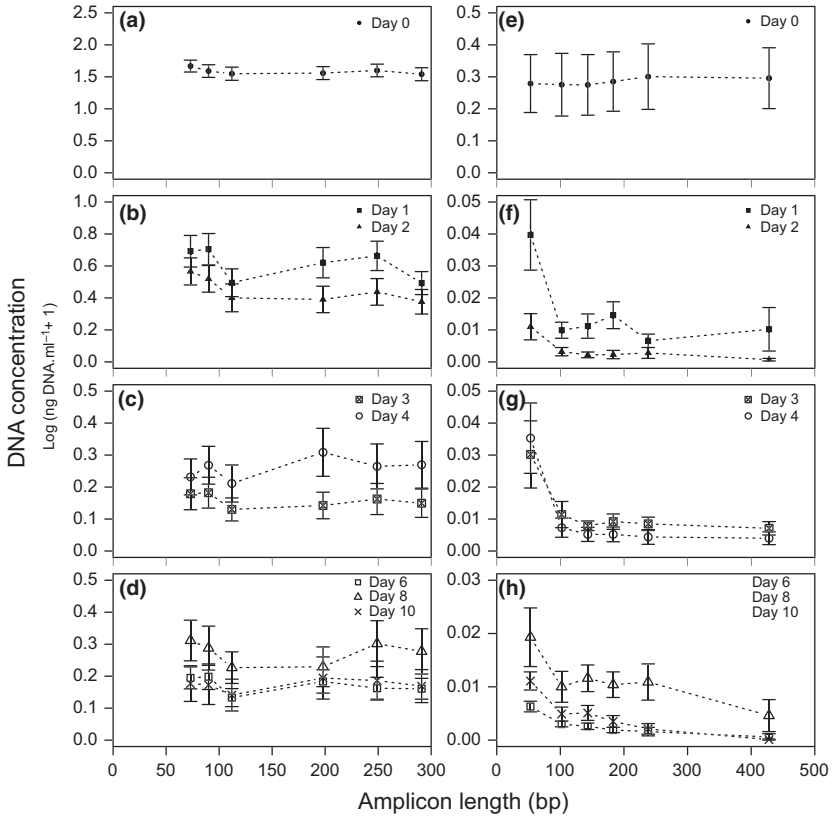

Fig. 2 Prey DNA breakdown based on the amplification of different sized DNA fragments of *Artemia* sp. (a, b, c, d) and *Brachionus plicatilis* (e, f, g, h) ingested by *Oculina arbuscula* 0, 1, 2, 3, 4, 6, 8 and 10 days after feeding (error bars indicate standard error). Data available in Leal *et al*. (2013b).

that in all predator–prey combinations, the full length of the target region of the prey DNA was intact and amplifiable immediately after ingestion. However, the dla-qPCR assessment of *Artemia* sp. DNA ingested by *Aiptasia* sp. 1 day after ingestion (Fig. 1a) demonstrated that DNA breakdown had occurred, that is, the shorter fragments displayed a higher concentration compared with the longer fragments, although the trend was not significant ($P = 0.24$; Fig. 2a). Over the next 6 days, the quantity of prey DNA declined (Fig. 1b, c; Tukey HSD test, $P < 0.05$) and was not detectable by day 8 (data not shown).

Degradation of *B. plicatilis* DNA was also evaluated in *Aiptasia* sp. (Fig. 1d–f). Similar to *Artemia* sp., by day 1 significant *B. plicatilis* DNA breakdown was apparent and DNA degradation continued through day 4 ($P < 0.05$), decreasing consistently over time (Tukey HSD test, $P < 0.05$). *Brachionus plicatilis* DNA was undetectable in the experimental animals after 6 days (data not shown).

Similar to *Aiptasia* sp., prey DNA breakdown was also observed for *O. arbuscula* fed *B. plicatilis* (Fig. 2e–h). Compared with the first observation shortly after ingestion (day 0; Fig. 2e), the breakdown of *B. plicatilis* DNA was significant at day 1 ($P < 0.01$) and breakdown increased with time (Tukey HSD test, $P < 0.05$). In contrast to *Aiptasia* sp., however, even after 10 days, prey DNA was still detectable (Fig. 2h). Furthermore, when the coral ingested *Artemia* sp., no differential DNA breakdown between the shortest and longest fragments could be detected over the observation period of 10 days (Fig. 2a–d; $P \geq 0.17$).

Estimation of prey digestion time

Prey digestion by *Aiptasia* sp. (Fig. 3) and *O. arbuscula* (Fig. 4) over time was assessed by quantifying the amount of the PCR amplicon using the prey-specific dla-qPCR primer sets that produced the smallest amplified fragments. Significant differences in prey DNA content over the digestion time were observed for both zooxanthellate cnidarians fed either prey ($P < 0.05$). *Aiptasia* sp. fed *Artemia* sp. (Fig. 3a) exhibited significant differences in prey DNA content between day 0 and all other days, between day 1 and days 3, 4 and 6 (Tukey HSD test, $P < 0.01$), and for day 2 compared with days 3, 4 and 6 (Tukey HSD test, $P < 0.1$). *Artemia* sp. DNA was digested more slowly by *O. arbuscula*, but its DNA content in the coral was significantly higher on day 0 than on all other days (Fig. 4, Tukey HSD test, $P < 0.01$). Compared with *Artemia* sp., *B. plicatilis* digestion was faster. *Brachionus plicatilis* DNA content in both *Aiptasia* sp. (Fig. 3b) and *O. arbuscula* (Fig. 4b) was significantly higher on day 0 compared with all other days (Tukey HSD test, $P < 0.01$). No significant difference in

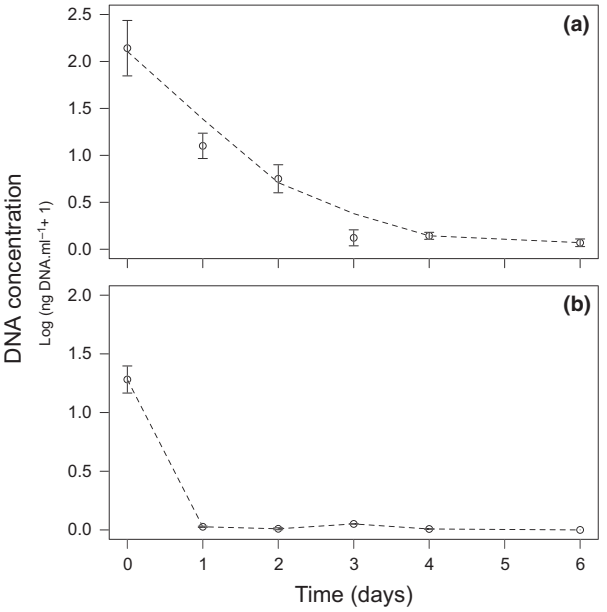

Fig. 3 Digestion time of *Aiptasia* sp. fed either *Artemia* sp. (a) and *Brachionus plicatilis* (b) based on the prey DNA content estimated by qPCR amplifying a 73-bp (*Artemia* sp.) and 53-bp (*B. plicatilis*) fragment of the 18S rRNA. Error bars indicate standard errors.

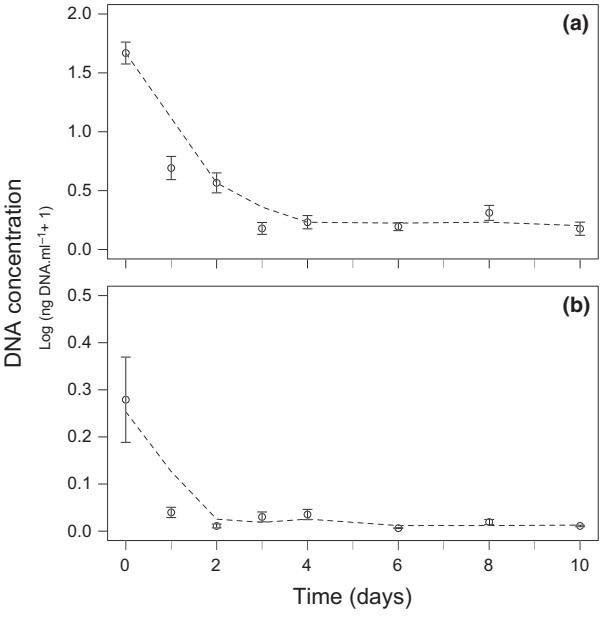

Fig. 4 Digestion time of *Oculina arbuscula* fed either *Artemia* sp. (a) or *Brachionus plicatilis* (b) based on the prey DNA content estimated by qPCR amplifying a 73-bp (*Artemia* sp.) and 53-bp (*B. plicatilis*) fragment of the 18S rRNA. Error bars indicate standard errors.

B. plicatilis DNA content was observed among all other days for both zooxanthellate cnidarians (Tukey HSD test, $P \geq 0.65$).

Prey digestion time may also be associated with the number of prey initially captured and ingested. The estimation of prey numbers ingested by each *Aiptasia* sp. individual during feeding experiments (sampled on day 0) indicated that the number of ingested *Artemia* was higher (ca. 36) than for *B. plicatilis* (ca. 11). *Oculina arbuscula* individual polyps showed similar rates (ca. 21 and 22 for *Artemia* sp. and *B. plicatilis*, respectively).

Effect of starvation conditions

The patterns of prey DNA breakdown for *O. arbuscula* fed once and then starved (Fig. 2) did not differ from when the corals were fed continuously with an alternative prey (data not shown). In contrast, prey digestion time differed for *O. arbuscula* starved after initial ingestion compared with individuals fed continuously with alternative prey (Fig. 5). Starved corals exhibited lower prey DNA content on day 3 when initially fed *Artemia* sp. ($t = 3.163$, d.f. = 6.895, $P < 0.05$; Fig. 5a) and on day 2 when initially fed *B. plicatilis* ($t = 3.71$, d.f. = 5.158, $P < 0.05$; Fig. 5b). However, no differences were observed between feeding regimes by day 6. Thus, starvation significantly increased the digestion rate of the investigated coral at least during the first 2/3 days after feeding.

Discussion

Development of molecular tools has transformed our ability to study zooxanthellate cnidarians, in particular the puzzling relationship between the cnidarian host and the endosymbiotic zooxanthellae (Davy *et al*. 2012; Meyer & Weis 2012). Molecular-based approaches can also be used to investigate heterotrophy of zooxanthellate cnidarians, particularly PCR-based methods that are already widely used to examine trophic relationships in terrestrial and marine invertebrates (e.g. Hoogendoorn & Heimpel 2001; Nejstgaard *et al*. 2003; Weber & Lundgren 2009; Lundgren & Fergen 2011). In this study, we applied for the first time these PCR-based approaches to investigate heterotrophic feeding and digestion in zooxanthellate cnidarians, demonstrate the utility of these methods and provide new insights into the trophic ecology of these organisms.

Artemia sp. ingested by *O. arbuscula* did not exhibit the differential length prey breakdown pattern observed for the three other predator and prey combinations (Fig 2a–d). The slow overall decrease in *Artemia* sp. DNA in the coral could indicate a reduced digestion and/or assimilation of *Artemia* sp. or, alternatively, a gradual egestion of undigested prey DNA. In contrast to the coral, *Aiptasia* sp. exhibited differential prey DNA breakdown when feeding on *Artemia* sp. (Fig. 1a–c), suggesting that different zooxanthellate cnidarian species may use different mechanisms to digest their prey. Further, differential DNA breakdown was observed for *B. plicatilis* in both predator species. This suggests species-specific prey DNA degradation or digestion, and/or an unequal assimilation of prey DNA as a source of nutrition. Exploration of this hypothesis will require further study.

The dla-qPCR assay also provides information regarding the optimal amplicon size to use in qPCR

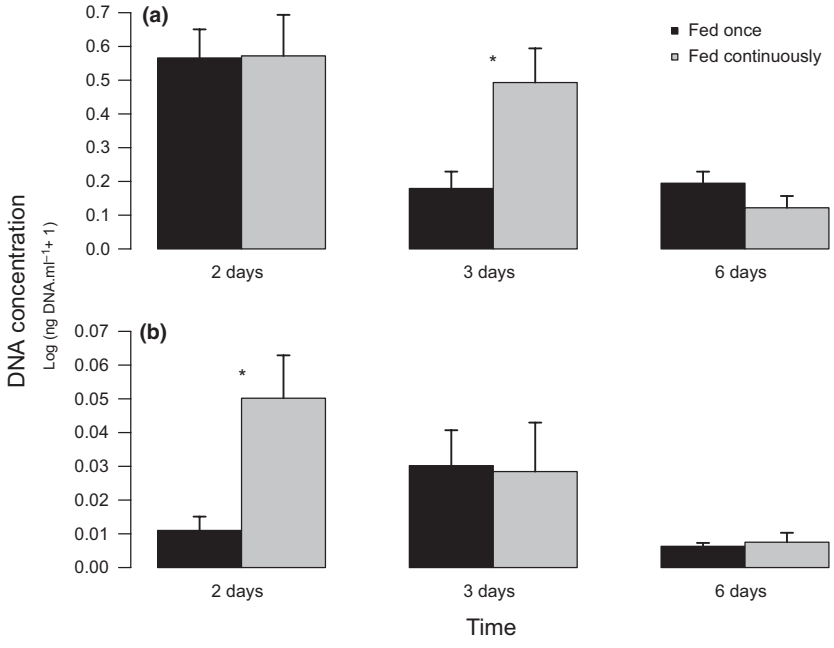

Fig. 5 Prey DNA content of *Oculina arbuscula* fed once and continuously. *Oculina arbuscula* fed once was only fed either *Artemia* sp. (a) or *Brachionus plicatilis* on day 0 (b). *Oculina arbuscula* continuously fed were fed *B. plicatilis* or *Artemia* sp. every day depending whether they were fed *Artemia* or *B. plicatilis* on day 0, respectively. Error bars indicate standard errors, and significant differences ($P < 0.05$) between prey DNA content for the same day are marked (*).

estimations of prey DNA and, consequently, prey digestion time. Prey DNA may be quickly degraded and affect quantification of ingested prey by leading to an underestimation of DNA levels (Deagle *et al.* 2006; Troedsson *et al.* 2009). In contrast to data available for other marine invertebrates, including copepods and pelagic tunicates that display prey digestion times up to a couple of hours (Durbin *et al.* 2012; ME Frischer, unpub. data), prey digestion in zooxanthellate cnidarians is significantly slower. This suggests that prey DNA can successfully be used to detect ingested prey in both laboratory and field studies, as long prey DNA digestion times are likely to maximize prey detectability. The laboratory assessment of the dla-qPCR profile of a particular predator–prey species combination thus provides important information for the *in situ* quantification of feeding rates using qPCR, as gut content estimates can be corrected based on prey DNA digestion (Troedsson *et al.* 2009). However, this species-specific approach may only be useful in the field when certain prey species are known to occur in the sampling site. Future studies using qPCR to quantify feeding in the field and laboratory should use specific primer sets for the target prey that amplify short sequences to minimize potential underestimation, as has been shown for many other organisms (Zaidi *et al.* 1999; Chen *et al.* 2000; Hoogendoorn & Heimpel 2001; Agustí *et al.* 2003). For purposes other than the assessment of specific prey ingestion and digestion, for example the general identification of diet components, it is important that the information contained in the region between the primers also exhibits sufficient variability to distinguish the potential prey species in the system. The use of this approach in the field would require sequencing techniques with group-specific primers (see reviews by O'Rorke *et al.* 2012b; Pompanon *et al.* 2012).

Digestion time has been largely overlooked as a parameter in trophic studies of zooxanthellate cnidarians. Based on the visual observation, it has been suggested that digestion of zooplankton in zooxanthellate cnidarians can take up to 6 h, although prey may remain in coelenterons for up to 12 h (Lewis 1982; Clayton 1986; Fabricius *et al.* 1995; Sebens *et al.* 1996). In this study, prey DNA content in both *Aiptasia* sp. (Fig. 3) and *O. arbuscula* (Fig. 4) continues to decrease substantially for up to 1 or 3 days when fed *B. plicatilis* or *Artemia* sp., respectively. The discrepancies between early studies (Fabricius *et al.* 1995; Sebens *et al.* 1996) and our work are most likely associated with the use of the highly sensitive and specific molecular approaches. We suggest that digestion times of 1–3 days more likely represent the time of complete digestion compared with the shorter digestion times reported in previous studies. However, the extended periods up to 6 or 10 days (Figs 4 and 5) for which trace amounts of prey DNA was detected may reflect incomplete gastric evacuation of residual prey material rather than active digestion. This slower digestion strategy may also be associated with the presence of symbiotic microorganisms colonizing the cnidarians gut that maximize nutrient assimilation through a slow digestion, as observed in some terrestrial invertebrates (Douglas 2009). Similarly, as the gastric cavity of corals has a high load of endosymbiotic bacteria (Agostini *et al.* 2011), prey digestion in zooxanthellate cnidarians may also be supported by a relatively slow endosymbiotic process similar to what have been observed in true bugs and spiders (Sheppard *et al.* 2005; Douglas 2009). Moreover, high nutrient concentrations were recorded in the coral gastric cavity, particularly phosphorus (Agostini *et al.* 2011). DNA is a phosphorous-rich nutrient source, and it is possible that the long detection times of prey DNA may be associated with a strategy of these zooxanthellate cnidarians to store prey DNA as an energy/nutrient source. Although extracellular DNA is known to be an important nutrient source for bacteria (Finkel & Kolter 2001), further investigation is needed to assess the role of prey DNA in the nutrition of zooxanthellate cnidarians.

A second set of experiments with continuous feeding was performed to investigate whether the long digestion times observed in this study were associated with postfeeding starvation. The results show similar DNA contents on day 6 regardless of the heterotrophic starvation condition of the coral (Fig. 5; approximately 8% and 1%, respectively, of *Artemia* sp. and *B. plicatilis* DNA content recorded at day 0). These results suggest that prey DNA presence in coral polyps after 6 days (Figs 4 and 5) is not associated with a slower digestion of prey caused by the absence of other heterotrophic sources of food. However, differences in prey DNA content on days 2 and 3 between *O. arbuscula* starved after initial feeding and fed continuously (Fig. 5) may suggest a differential prey digestion associated with heterotrophic starvation.

This study provides new insights into the feeding and digestion mechanisms of zooxanthellate cnidarians. It also provides data of relevance for designing molecular approaches to study cnidarian heterotrophy in the laboratory and field. The long digestion times observed for zooxanthellate cnidarians have important implications for the definition of heterotrophic starvation in these organisms. We therefore suggest that future investigations addressing the effect of heterotrophic starvation in the ecophysiology of zooxanthellate cnidarians carefully consider the full time span of the starvation period when designing experiments. On the other hand, the long residence time of prey DNA may facilitate

detection of prey DNA from field-collected specimens. Finally, sensitive and selective PCR-based approaches, such as shown here, open new possibilities to study nutritional plasticity in the cnidarian–algal symbiosis and its response to stress events.

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Data accessibility

DNA sequences: GenBank Accession no. JX983594.

Prey ingestion and digestion data: doi:10.5061/dryad.rp4q6

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Primers used in this study.

Table S2 PCR product length, assay annealing temperatures and PCR performance characteristics used in this study.