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Selective feeding and linkages to the microbial food web by the doliolid *Dolioletta gegenbauri*

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Abstract

Gelatinous zooplankton play a crucial role in pelagic marine food webs, however, due to methodological challenges and persistent misconceptions of their importance, the trophic role of gelatinous zooplankton remains poorly investigated. This is particularly true for small gelatinous zooplankton including the marine pelagic tunicate, *Dolioletta gegenbauri*. *D. gegenbauri* and other doliolid species occur persistently on wide subtropical shelves where they often produce massive blooms in association with shelf upwelling conditions. As efficient filter feeders and prodigious producers of relatively low-density organic-rich aggregates, doliolids are understood to contribute significantly to shelf production, pelagic ecology, and pelagic–benthic coupling. Utilizing molecular gut content analysis and stable isotope analysis approaches, the trophic interactions of doliolids were explored during bloom and non-bloom conditions on the South Atlantic Bight continental shelf in the Western North Atlantic. Based on molecular gut content analysis, relative ingestion selectivity varied with *D. gegenbauri* life stage. At all life stages, doliolids ingested a wide range of prey types and sizes, but exhibited selectivity for larger prey types including diatoms, ciliates, and metazoans. Experimental growth studies confirmed that metazoan prey were ingested, but indicated that they were not digested and assimilated. Stable isotopic composition ($\delta^{13}C$ and $\delta^{15}N$) of wild-caught doliolids, during bloom and non-bloom conditions, were most consistent with a detrital-supplemented diet. These observations suggest that the feeding ecology of *D. gegenbauri* is more complex than previously reported, and have strong and unusual linkages to the microbial food web.

Gelatinous zooplankton are ubiquitous in marine systems and central players in marine plankton food webs (Martin et al. 2017; Madin and Harbison 2019). Compared to non-gelatinous zooplankton species, the ecological significance of gelatinous zooplankton has received less attention. Specifically, the role of small mucus-net feeding pelagic tunicates is poorly understood. These organisms have long been intriguing components of marine pelagic systems due to their ability to form large blooms capable of impacting the structure of pelagic food webs by depleting prey and consuming eggs and larvae of competing species (Deibel 1998; Haskell et al. 1999; Lucas et al. 2014). Although common in most marine systems, the processes that lead to bloom formation and termination of gelatinous species remain poorly understood despite over a century of study (Deibel and Lowen 2012; Purcell 2012; Pitt et al. 2018).

Three main types of gelatinous zooplankton occur in almost all marine waters: (1) cnidarians, including hydrozoan and scyphozoan medusae, and siphonophores; (2) ctenophores, and (3) mucus feeding pelagic tunicates including appendicularia, pyrosomes, salps, and doliolids. Cnidarians and ctenophores, with some exceptions, are predators of other planktonic organisms, notably copepods and fish (Hyman 1940). For most jelly taxa, their rapid proliferation relies on a complex life cycle involving benthic stages (polyps) and asexual reproduction (Fautin 2002). In contrast, the trophic and ecological role of the pelagic tunicates remains less well understood (Henschke et al. 2016; Lamb et al. 2017), particularly with respect to their trophic interactions (Jaspers et al. 2015; Walters et al. 2018). Pelagic tunicates do not have

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a benthic phase like most jellies, but they do exhibit complex reproductive cycles with obligatory sexual and asexual phases that can lead to blooms.

Blooms of pelagic tunicates are common features of subtropical continental shelves and oceans (Bone 1998). Fundamentally, as is the case for other zooplankton groups, pelagic tunicate blooms result from the delivery of nutrients into the euphotic layer by a variety of physical mechanisms that result in increased phytoplankton production (Boero et al. 2008). In the case of the South Atlantic Bight continental shelf, blooms of pelagic tunicates result from the upwelling of nutrient-rich deep waters onto the shallow shelf and subsequent high primary productivity (Deibel and Paffenhöfer 2009).

The ecological significance of pelagic tunicate blooms, however, can be highly variable and dependent on the location, duration, and blooming species. Because doliolids are efficient filter feeders (Deibel 1998; Takahashi et al. 2015; Ishak et al. 2020) and produce low-density fecal pellets with slow sinking rates (Deibel 1990; Patonai et al. 2011), they have the potential to significantly influence shelf carbon cycling, pelagic ecology, and pelagic–benthic coupling (Deibel 1985; Ishak et al. 2020). Although the exact mechanisms contributing to doliolid bloom formation remain unclear, the presence of fine-scale oceanographic boundaries, including vertical pycnoclines and horizontal fronts, appears to be an important factor (Takahashi et al. 2015; Martin et al. 2017; Greer et al. 2020). Little is known about the predators of doliolids, although predation by some larval fish, cnidarians, ctenophores, pteropods, and sappihrinid copepods have been reported (Harbison 1998; Takahashi et al. 2013). In the South Atlantic Bight, our observations suggest that hydromedusae in the genus *Liriope* are common predators of *Dolioletta gegenbauri* (unpublished observations).

Mucus-feeding pelagic tunicates are generally considered to be passive grazers, feeding indiscriminately on small particles including bacteria and phytoplankton (Crocker et al. 1991; Vargas and Madin 2004). Chi et al. (2021), in a comprehensive investigation of the trophic position of gelatinous organisms in the Eastern Tropical Atlantic, reported that salps and pyrosomes exhibit isotopic values matching those of surface seston supporting their trophic classification as primary consumers. As with other pelagic tunicates, *D. gegenbauri* is an efficient filter feeder that can clear large volumes of water in both low- and high-food concentration environments (Gibson and Paffenhöfer 2000; Lucas and Dawson 2014). Feeding currents are generated by ciliated gill structures that produce water flow through the pharyngeal cavity. Because this flow is independent of swimming, in contrast to salps, doliolids are able to feed while stationary (Alldredge and Madin 1982; Bone et al. 1997; Madin and Deibel 1998). Based on anatomical considerations, laboratory-based experimental studies, and inferred from field observations, doliolids are capable of ingesting particles over a wide size range from <1 μm to >1 mm (Crocker et al. 1991; Tebeau and Madin 1994; Katechakis et al. 2002). In the natural water column, however, larger particles are present in mixtures with nano and micro-sized plankton; thus, it is difficult to extrapolate the relationship between feeding and particle size from simple laboratory studies alone (Troedsson et al. 2007).

Recent investigations, however, have suggested that both doliolids and salps may be capable of selective feeding and rather than being primary consumers in a classical pelagic food web, they are better represented as members of the microbial food web (Conley et al. 2018; Walters et al. 2018; Pakhomov et al. 2019). For example, based on a meta-analysis of stable isotope data, Pakhomov et al. (2019) suggested that pelagic tunicates prefer small heterotrophic prey. Walters et al. (2018), utilizing emerging cultivation-independent Molecular Gut Content Analysis (MGCA) tools, reported selective feeding by the doliolid, *D. gegenbauri*, on relatively rare larger prey including diatoms, heterotrophic protists, and metazoans. These observations suggest that in addition to both autotrophic and heterotrophic protists, metazoans may contribute nutritionally to the growth and reproduction of *D. gegenbauri*, adding further complexity into pelagic food webs. Alternatively, these signals may represent the ingestion of metazoans, but not their assimilation, or may be derived from the consumption of detrital material containing eDNA (environmental DNA) from these organisms. Walters et al. (2019) reported that *D. gegenbauri* can be maintained in cultivation successfully on algal diets, but culture success is enhanced when detrital material is available. These observations suggest that consumption of detritus is beneficial to the growth of this species. Doliolid ontogeny likely also contributes to its trophic diversity. Doliolids undergo a complex life cycle that alternates between sexual and asexual stages and includes both solitary and colonial phases (Braconnot 1971). The life history of *D. gegenbauri* is provided in (Fig. S1) and highlights the diversity of zooid types. In this study, we attempted to ascertain the diet of each of these *D. gegenbauri* life stages.

The objective of this study was to advance the understanding of the trophic role of doliolids in continental shelf food webs. We explored the quantitative ingestion of several representative prey types by different wild-caught *D. gegenbauri* zooids across seasons and bloom conditions on the mid-continental shelf of the South Atlantic Bight in the Western North Atlantic to explore the hypothesis that doliolids are capable of selective feeding. In addition, we investigated the stable isotopic composition ($\delta^{13}C$ and $\delta^{15}N$) of wild-caught and laboratory-raised *D. gegenbauri* to explore the hypothesis that a significant fraction of *D. gegenbauri* nutrition is derived from the consumption of detrital material and that the trophic role of *D. gegenbauri* is more complex than has been previously understood.

Materials and methods

D. gegenbauri collection

D. gegenbauri zooids were collected and quantified approximately monthly from the South Atlantic Bight mid-continental

shelf from August 2015 to December 2017. To establish cultures, assess diet, and measure isotopic composition, live *D. gegenbauri* zooids were collected from 31°N to 29°N aboard the R/V *Savannah* using a 202 μm mesh cone net (2.5 m length) with a 0.5-m opening and equipped with a 4 liter non-filtering cod-end as previously described (Walters et al. 2019). Following the procedures described in Walters et al. (2019), *D. gegenbauri* was maintained in culture through its entire life cycle for multiple generations and made available for molecular gut content analysis, stable isotope analysis, and for experimental feeding and growth studies.

For molecular gut content analysis, wild *D. gegenbauri* zooids were captured as described above and immediately anesthetized in 0.2 μm filtered seawater containing 0.4% MS-222 (3-aminobenzoic acid ethyl ester, Alfa Aesar, Pelham, New Hampshire), rinsed three times in filtered seawater, and placed into ATL buffer with proteinase K (DNeasy Blood & Tissue DNA extraction kit, Qiagen Inc, Valencia, California). Samples were stored at 4°C until DNA was extracted within 72 h after their initial capture. During extended research cruises, samples were processed at sea. On shorter cruises (1–2 d), samples were processed in the laboratory. Zooids used for stable isotope analysis were starved for 4 h in 0.2 μm filtered seawater in a large glass beaker, allowing them to evacuate their guts completely before processing. After the starvation period the length of each zooid was measured, then transferred to 8×10 mm pre-cleaned tin capsules (EA Consumables Inc., Pennsauken, New Jersey) with a minimum of excess seawater, and dried at 60°C for 24–48 h. Samples were stored at – 20°C until analysis.

Quantitative zooplankton collection and enumeration of doliolids

Zooplankton samples utilized for quantitative analysis of doliolids were collected as previously described by Walters et al. (2018) from the whole water column by slowly lowering and raising a 5 m long, 202 μm mesh cone net with a 1 m opening (ratio 1 : 5) equipped with a filtering cod-end through the entire water column at $\sim$ 15 m min^{-1} from a drifting ship. A calibrated flowmeter (General Oceanics, Inc. Miami, FL, Model 2030RC) was centered in the net opening to estimate the volume of water filtered. After net retrieval, the plankton concentrate was rinsed with surface seawater through a 202 μm mesh sieve and transferred to wide-mouth jars where they were fixed in 60% ethanol to a final volume of 1 liter. Samples were returned to the laboratory for counting and identification.

Doliolids were identified and counted by microscopy as described by Godeaux et al. (1998). If doliolids were visually abundant, the sample was split in parts suitable for examination using a Folsom plankton sample splitter (Griffiths et al. 1984). Samples were diluted to a total known volume, and doliolids were identified and counted in duplicate aliquots, according to sample concentration (Gifford and Caron 2000). Generally, 1–2% of the total sample was counted. Aliquots were transferred to a Bogorov zooplankton counting chamber with a Hensen-Stempel pipette and counted under an Olympus SZH10 binocular microscope. The abundance of doliolids was calculated by multiplying the aliquot's averaged counts of zooids by the final dilution factor (total sample volume/aliquot volume) divided by the net filtered water volume (average counts × dilution factor/filtered volume) and reported as individuals per cubic meter. Sample processing was generally completed within 12 months of collection.

Water sample (prey field) collection

To compare the types and isotopic composition of available prey present in the water column to ingested prey, similar molecular-based and stable isotope methods were utilized to assess the plankton community and particulate organic matter (POM) composition. Near-bottom water was collected in 10 liter Niskin bottles attached to the CTD rosette contemporaneously with water quality parameters, within 1 h, of *D. gegenbauri* collections. For metabarcoding analysis of the prey field, 500 mL was pre-filtered through a 63 μm sieve and collected onto a 47 mm 0.8 μm Supor filter (PALL Life Sciences, East Hills, New York). For qPCR assessment of prey abundance per water volume, replicate water samples of 1, 5, 20, and 100 mL were filtered onto 25 mm 0.2 μm Supor filters. All filters were placed into sterile 2 mL cryovials and stored at – 80°C until DNA was extracted. To compare the isotopic composition of the prey field, triplicate total and < 10 μm size-fractionated POM samples were collected onto combusted 25 mm Whatman glass fiber filters (GFF) (Cytiva, Marlborough, Massachusetts). The < 10 μm fraction was prepared by filtering whole water through a 10 μm Nitex sieve and collecting the filtrate onto a GFF. Water was filtered until the filter was saturated and clogged (100–625 mL). Filters were completely dried at 60°C and stored at – 20°C until analysis. Triplicate total and > 8 μm size-fractionated chlorophyll a (Chl *a*) was collected by filtering 200 mL of water though 25 mm Whatman nucleopore hydrophilic polycarbonate 0.2 and 8 μm (Cytiva, Marlborough, Massachusetts) filters, respectively. Filters were stored at – 20°C in the dark for no more than 48 h. Chl *a* concentration was determined by fluorescence using a 10 AU fluorometer (Turner Designs, San Jose, California) after soaking in 90% acetone at – 20°C overnight (Parsons et al. 1984). In addition to laboratory analyzed water samples, CTD casts allowed the collection of water column profiles of major oceanographic water quality parameters at each sampling station. Parameters included temperature, salinity, oxygen, light, chlorophyll fluorescence, and Chromophoric Dissolved Organic Matter (CDOM) (Frischer and Gibson 2019).

DNA extraction, purification, and PCR amenability

Total genomic DNA extraction and purification from *D. gegenbauri* zooids and water samples were completed within

72 h of collection using the Qiagen DNeasy Blood and Tissue Kit (Valencia, California) as previously described (Walters et al. 2018). Following genomic DNA (gDNA) extraction, purified DNA was quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, California) and a Qubit dsDNA High Sensitivity Assay Kit (Invitrogen, Carlsbad, California). To confirm that the purified DNA was of sufficient quality for downstream PCR, qPCR, and sequencing analysis, every gDNA sample was analyzed by end-point PCR using primers Univ 18S-557F and Univ 18S-1180R (Hadziavdic et al. 2014) and Taq PCR Master Mix (Qiagen). Once samples were identified as containing amplifiable DNA, they were archived at – 20°C until further analysis.

Gut content assessment by metabarcoding next generation sequencing

Sequencing of barcoded amplicon libraries was accomplished using Ion Torrent procedures on a Personal Genome Machine (PGM) as previously described by (Frischer et al. 2017). Barcoded libraries from pooled samples prepared from doliolids and water samples collected from each cruise were prepared from randomly sheared (ca. 400 bp) preparations of the 630-bp 18S rRNA amplicon and were sequenced on a 316v2 chip with 400 bp chemistry. Standard protocols for library preparation (Ion Xpress™ Plus Fragment Library Kit MAN00077044 Revision A.0), library templating (Ion PGM™ Template OT2 400 Kit PGM, template preparation MAN0007218 Revision 3.0) and sequencing (Ion PGM™ Sequencing 400 kit, MAN0007242 Revision 2.0) were followed. Raw sequence reads were filtered using the Ion Torrent Suite software (ver. 4.2.1) to trim adaptor sequences, and to remove polyclonal sequences. Ribosomal sequences were exported into the Mothur pipeline to remove low-quality sequences (Schloss et al. 2009). Following these quality-control procedures, sequences were uploaded to the SILVAngs pipeline (version 1.2) (Quast et al. 2013), where libraries were aligned, de-replicated, and taxonomically classified. Taxonomic classification was facilitated using a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (Quast et al. 2013) release 119; http://www.arb-silva.de; date last accessed: 20 August 2020) using blastn (Altschul et al. 1990) (version 2.2.28+; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences from identified prey that contained less than 10 sequencing reads, and sequences identified as doliolid and human were also removed from each dataset.

Development of prey group-specific quantitative polymerase chain reaction (qPCR) assays

To determine the quantitative importance of different prey items in the doliolid diet, five real-time qPCR assays were developed targeting 18S rRNA genes from representative prey groups including Copepoda, Bacillariophyta (diatoms), Haptophyta, Cryptophyta, and Picozoa. Primer design, optimization, and validation followed previously described strategies (Frischer et al. 2014). To facilitate primer design, approximately 100 18S rDNA representative sequences from GenBank for each target prey group were downloaded from http://www.ncbi.nlm.nih.gov. The sequences were aligned using the ClustalW utility implemented in Bioedit (Hall 1999). Consensus sequences within the representative alignments of each target prey group were created and targeted as potential primers. Once potential target regions were identified, optimal primers for detection of the five potential prey groups were designed using the Primer3Plus open source primer design tool (Untergasser et al. 2007). Potential prey group-specific primers were paired with existing or modified universal-targeted 18S rRNA primers (Hadziavdic et al. 2014) that would be expected to amplify fragments < 200 bp. Amplicons < 200 bp were previously demonstrated to be useful for the quantitative determination of prey ingestion by *D. gegenbauri* (Frischer et al. 2014). Primer pair specificity was further evaluated in silico using the SILVA TestPrime and TestProbe utilities (Quast et al. 2013). Following evaluation, the prey group-specific primer sets were synthesized by Integrated DNA Technologies (Coralville, Iowa) and empirically optimized for qPCR using a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, Hercules, California). Primers were evaluated for their specificity, sensitivity, and efficiency in qPCR reactions as previously described in (Frischer et al. 2014), by targeting cloned 18S rDNA inserts prepared from *D. gegenbauri*, *Thalassiosira weissflogii*, *Rhizosolenia alata*, *Rhodomonas* sp., *Isochrysis galbana*, *Emiliania huxleyi*, *Eucalanus pileatus*, and from an unidentified species of copepod, crytophyte and picozoa (Table S3). qPCR reactions were conducted in 20 μL reaction volumes containing a final concentration of 1X SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, California), 0.3 μmol of each primer, and template concentrations of plasmid DNA (pDNA) containing a cloned copy of the target 18S rRNA gene ranging from 10^1 to 10^8 target gene copies per reaction.

Gut content assessment by qPCR

Prey DNA concentrations associated with each wild-collected *D. gegenbauri* zooid and paired water samples were estimated by real-time qPCR using each of the five prey-group-specific primer sets designed in this study (Table S1a). qPCR reactions were conducted in 20 μL reactions essentially as described above, except that template concentrations ranged from 5×10^{-4} to 1.2 ng μL^{-1} target genomic DNA per reaction. Empirically optimized amplification annealing temperatures for each of the assays are reported in Table S1b. qPCR reaction conditions included an initial enzyme activation step (95°C, 30 s) followed by 40 amplification cycles of denaturation (95°C, 5 s, annealing/extension, 5 s). After cycling, product melt-temperatures were evaluated from 60.5–63°C to 95°C at 0.5°C increments for 5 s each. All qPCR assays were repeated in at least triplicate.

The abundance (gene copies) of each targeted prey type was quantified in all animals and in associated water samples such that the relative ratio of each prey group could be determined. To assess selective feeding, the abundance of prey genes was compared to the abundance of each prey type in the surrounding water column on a per-volume basis. Although doliolids are barrel-shaped, doliolid volume was calculated assuming them to be cylinder shaped with an opening diameter of half the length because only zooid length was measured. Ratios of prey gene abundance in animals vs. the water >1 were interpreted as indicative of positive selection while ratios <1 were interpreted as negative selection. Ratios not significantly different than 1 indicate passive feeding. All statistical analyses were performed in SigmaPlot (v13, Systat Software). Descriptive statistics and normality tests were run on all data sets before using a combination of One-Way ANOVA testing, and appropriate post hoc tests dependent on the data set, predominately Kruskal Wallis, Tukey, and Holm Sidak. The abundance of prey gene copies in water samples were estimated based on linear regression of the volumetric samples.

Experimental growth studies

To explore the hypothesis that metazoan prey contributes to the nutrition and growth of doliolids, three 9–10 d experimental growth studies were conducted using *D. gegenbauri* gonozooids reared under optimal cultivation conditions as previously described (Walters et al. 2019) (Table 1). In these studies, the brine shrimp *Artemia* sp. was utilized as a model metazoan prey for doliolids. Prior to conducting growth experiments, 20 mg of *Artemia* sp. eggs (Carolina Biological, Burlington, North Carolina) were partially heat-inactivated by incubating them at 60°C for 24 h. The goal of using partially inactivated eggs was to provide both eggs and nauplii, as prey, throughout the doliolid growth experiment period. Following the heat treatment, eggs were transferred to 200 mL filtered seawater and incubated for ~ 24 h at 20°C to rehydrate them. Seawater was filtered through Whatman GFF filters. After the hydration period, the eggs were filtered through a 300-μm sieve to remove any egg clumps. The eggs were counted under a dissecting microscope using a Bogorov counting chamber and target concentrations were calculated for each experiment.

Prior to the start of each feeding experiment, 30–40 gonozooids were transferred, using a wide-bored glass pipette, from laboratory stock cultures into two 1.9-liter jars containing 0.2 μm filtered seawater. The jars were mounted on a rotating plankton wheel at 0.3 rpm and held ca. 5–16 h with no food. This allowed for gut evacuation prior to the start of feeding with defined algal cultures and *Artemia* sp. eggs. Following the starvation period, five individual animals were immediately processed for stable isotope analysis as described below. Five, ca. 3 mm gonozooids of a similar size were placed into experimental jars containing GFF-filtered seawater enriched with algal culture as described in Table 1. A one-time addition of heat-treated, inactivated *Artemia* sp. eggs was added to the jars to achieve the desired concentrations (Table 1). Jars were then mounted on the plankton wheel and cultured under standard conditions (Walters et al. 2019). At the beginning of each experiment, and every 2 d throughout the experiment, length was estimated visually using a graduated glass pipette to monitor zooid growth. Algal concentrations were monitored daily prior to feeding using a Coulter Counter, and were adjusted to maintain the specified algal

Table 1. Experimental design of *D. gegenbauri* growth studies with *Artemia* sp. eggs and nauplii.

Experiment no	Duration of experiment (d)	Culture jar size (L)	Algal diet*	No. Gonozooids per culture jar	Algal Conc. (μg C L^{-1})	Initial *Artemia* sp. conc. (eggs L^{-1})
1	9	3.8	Ig, Rh, & Tw	5	60	0
1	9	3.8	Ig, Rh, & Tw	5	60	50
1	9	3.8	Ig, Rh, & Tw	5	60	100
1	9	3.8	Ig, Rh, & Tw	5	60	150
2	10	1.9	Rh & Tw	5	10	0
2	10	1.9	Rh & Tw	5	40	0
2	10	1.9	Rh & Tw	5	80	0
2	10	1.9	Rh & Tw	5	10	150
2	10	1.9	Rh & Tw	5	40	150
2	10	1.9	Rh & Tw	5	80	150
3	10	1.9	Rh & Tw	5	40	0
3	10	1.9	Rh & Tw	5	40	25
3	10	1.9	Rh & Tw	5	40	50
3	10	1.9	Rh & Tw	5	40	100
3	10	1.9	Rh & Tw	5	40	150

*Ig, *I. galbana*; Rh, *Rhodomonas* sp.; Tw, *T. weissflogii*. Mixed algal diets were prepared at approximately equal concentrations based on estimated carbon content.

prey concentrations (Table 1). At the conclusion of each experiment, the size of each remaining gonozooid was estimated visually and by microscopy, and then processed for stable isotope analysis. The isotopic composition of the culture water (500 mL) at the conclusion of the experiment containing POM, 5 mL of algal cultures, and 200 μg of heat-inactivated *Artemia* sp. eggs were also determined (Table 1). Algal culture and POM samples were collected onto combusted pre-weighed GFF filters. Dry *Artemia* sp. eggs were place in pre-weighed tin sample cups. Whenever possible, the interaction between doliolids, *Artemia* sp., and doliolid fecal pellets were photographed under a dissecting scope (Fig. 5).

Stable isotope analyses

Total carbon and nitrogen (μg per sample), together with $\delta^{13}C$ (vs. vPDB) and $\delta^{15}N$ (vs. air N_2) of starved *D. gegenbauri* zooids, size fractioned POM samples, and prey from growth experiments were measured using a ThermoFisher Scientific Flash EA coupled to a ThermoFisher Delta V plus isotope ratio mass spectrometer (Fry et al. 1992). Individual *D. gegenbauri* zooids were placed into pre-cleaned tin cups with a minimal amount of water and dried at 60°C. Samples on 25 mm GFF filters (POM and prey) were first dried at 60°C, then excess filter material was removed. Filters were divided in two equal pieces to run in duplicate, and wrapped inside tin cups prior to analysis. Isotopic and elemental composition calibrations were performed using commercially available powdered chitin (MilliporeSigma, St. Louis, Missouri) calibrated against isotopic and composition standards (USGS 40 Glutamic Acid and Elemental Microanalysis B2151 and B2155) before distribution. Chitin standards were run at the start of each analysis across the range of C (μg) anticipated to be present in each sample, and every 10 samples as a drift check. Samples were not acidified or lipid extracted prior to analysis following best practice recommendations for stable isotopic analysis of gelatinous animals (MacKenzie et al. 2017). Carbon and nitrogen curves were calibrated by standard curves generated by the chitin standards. The amount of C and N was calculated as the total area below the peaks in the curve as measured from the output of the instrument. Isotopic values were corrected for blank values and size-fractionation effects. Such corrections were especially important given the very small size of many of the animal samples.

Results

Seasonal abundance of doliolids

The abundance of doliolids, quantification of target prey groups by qPCR, and stable isotope analysis of doliolids and POM were determined during 24 cruises to the South Atlantic Bight middle continental shelf at 31°N from August 2015 to December 2017. Doliolids were observed on every cruise; however, their abundance was highly variable, both temporally and spatially (Fig. 1).

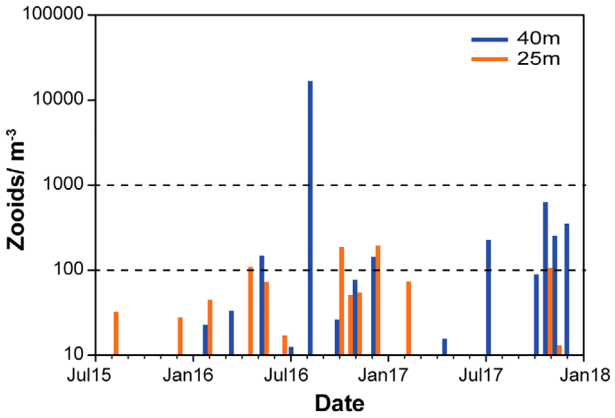

Fig 1. Abundance of *D. gegenbauri* zooids on the mid-continental shelf of the South Atlantic Bight (31°N 80°W) at the 25 and 40 m isobaths during 24 cruises from August 2015 to December 2017. Super (> 100 zooids m^{-3} and Mega (> 1000 zooids m^{-3}) reference lines are indicated (dashed lines).

These abundances varied from < 1 to > 8000 zooids m^{-3} over the study period. Blooms were typically patchy, but generally did not occupy the entire middle shelf region. Doliolid zooids were present in at least one station on every cruise indicating that *D. gegenbauri* is a continuous resident of the mid-continental shelf. Furthermore, doliolid abundances frequently reached Super- and Mega-bloom concentrations, > 100, and > 1000 zooids m^{-3} , respectively, (Walters et al. 2018), and were more common during the fall transition (August), and early winter (November and December). Of the eight Super and Mega blooms observed, seven of them occurred between late summer and early winter. As previously described, the abundance of doliolids was significantly correlated with total ($r = 0.838$, $p < 0.001$) and > 8 μm (r = 0.901, $p < 0.001$) Chl *a* fractions in near-bottom waters (Walters et al. 2018). However, these correlations were largely driven by water column conditions during the single Mega bloom observed on 11 August 2016 when the Chl *a* concentration was extraordinarily high (> 3 μg L^{-1}). Excluding this bloom period, there was not a significant correlation between the abundance of doliolids and Chl *a* or any other water quality parameters in bottom or surface waters (Walters et al. 2018). The absence of a correlation between salinity and the abundance of doliolids indicates that blooms of *D. gegenbauri* are unlikely to be driven by nutrients delivered directly by precipitation events or terrestrial derived materials. A complete understanding of the drivers of doliolid blooms on the South Atlantic Bight shelf is lacking though it is clear that not all phytoplankton blooms result in blooms of doliolids (Walters et al. 2018). Data associated with these cruises are archived at the Biological & Chemical Oceanography Data Management Office (Frischer and Gibson 2019).

Development of prey group-specific qPCR assays

To explore the importance of different prey in the doliolid diet, five real-time qPCR assays were developed targeting 18S

rRNA genes from representative prey groups. Target prey groups included Copepoda, Bacillariophyta (diatoms), Haptophyta, Cryptophyta, and Picozoa. Primer sequences, amplicon product size, empirically determined optimal annealing temperature, and amplification sensitivity for each of the assays developed in this study are provided in Tables S1a, S1b. The results of in silico estimates of assay specificity and false amplification rates (Mismatch) based on comparison to the Silva reference database SSU r138 (Quast et al. 2013) are also provided in Table S1b. The assays developed in this study amplified 18S rDNA fragments ranging in size from 121 to 229 bp. With the exception of the Cryptophyta-targeted assay, all qPCR assays had a dynamic range from 1 to 10^7 target copies (data not shown). The linear dynamic range of the Cryptophyta-targeted assay was from 10^2 to 10^7 target copies (data not shown). Empirical testing by end-point PCR against a small panel of representative prey types indicated that each assay exhibited the expected taxonomic specificity with the exception of the Haptophyta-targeted assay that also amplified the diatom species *R. alata*. The Haptophyta assay, however, did not amplify the diatom *T. weissflogii* and only weakly amplified the Haptophyte *E. huxleyi* (Table S4). In silico estimation of assay specificity supported the empirical results, but indicated that none of the assays would be expected to amplify all species within the broad taxonomic groups targeted by the primers and that a low level of non-specific amplification could be expected (Table S1b). Specificity ranged from 87.5% for the Cryptophtya assay to 99.9% for the Bacillariophyta primer set. Matches to sequences not belonging to target taxonomic group (Mismatches) are also provided in Table S1b. The mismatch range for primer sets ranged from 0.01% for the Copepoda assay to 6.37% for the Picozoa assay.

Feeding selectivity

Recently, we reported that *D. gegenbauri* appears to be capable of selective feeding (Walters et al. 2018). This conclusion was based on a comparison of prey DNA (barcoded 18S rDNA amplicons) associated with captive-reared animals that had been fed freshly collected seawater containing natural prey in 1.9 liter microcosm experiments. Associated with these experimental feeding studies, the gut contents of a collection of wild-caught animals captured during the summer of 2011 were analyzed for evidence of feeding selectivity by comparing the abundance of prey amplicons, volume normalized, associated with the animals to their concentration in the water column (Fig. 2).

The prey species associated with these wild-caught animals were significantly different from the captive-fed animals that were fed the same waters where the wild-caught animals were collected (Fig. 2a). Interestingly, picozoa and diatoms dominated the libraries of the captive-fed animals, but these prey

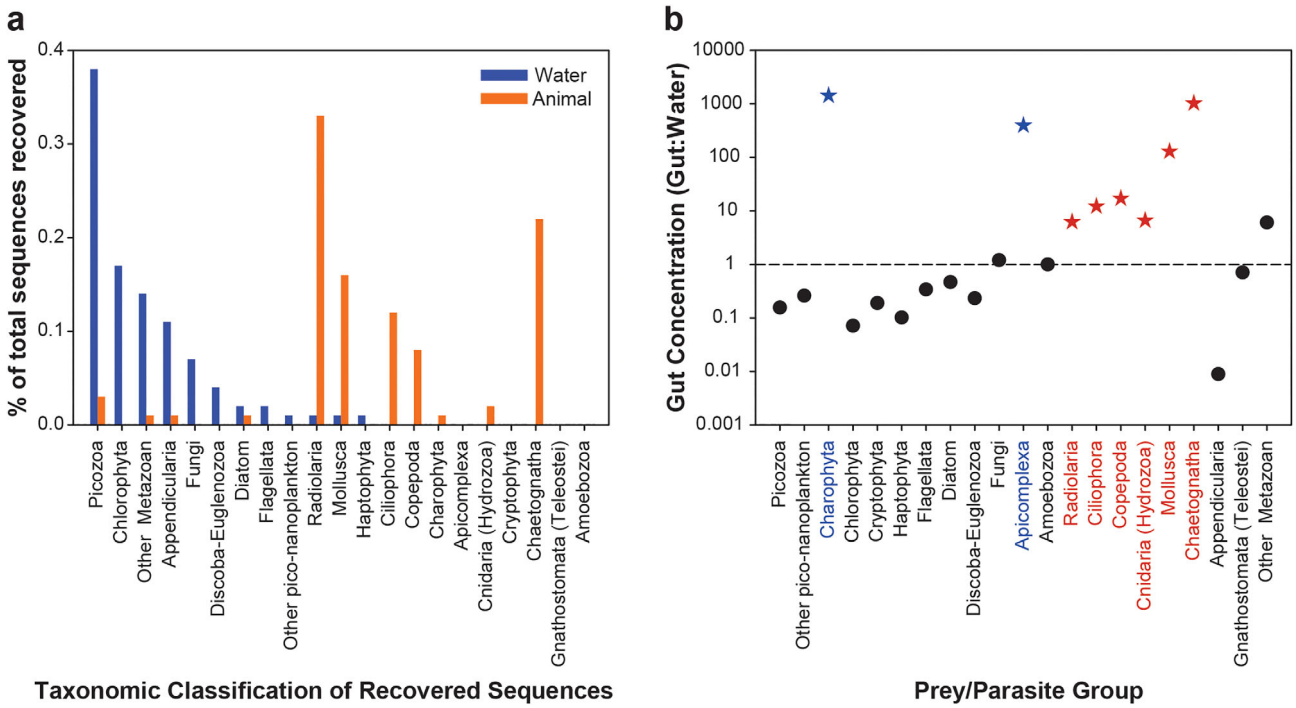

Fig 2. (**a**) Composition as a % of 18S rRNA amplicon sequences recovered from paired water (blue bar) and animal (orange bar) samples. Prey types are based on taxonomic classified 18S rRNA amplicons. (**b**) Feeding selectivity of wild-caught *D. gegenbauri* based on the relative proportion (by volume) of prey and parasite gene copies recovered from metabarcoded 18S rRNA amplification in the gut vs. water column of paired wild-caught *D. gegenbauri* gonozooids and water samples. The dashed line indicates gut : water 1 : 1 reference line. Positive feeding selection (orange star) of several metazoan groups and Ciliophora was detected. Significantly ($p < 0.05$) gut vs. water column ratios were also detected for Charophyta, most likely pollen, and the parasitic Apicomplexa group (blue stars). All other prey types were either neutrally or non-significantly negatively selected (black circles).

groups accounted for only a small fraction (combined 3.3%) of the sequences recovered from the wild-caught animals. The majority of the recovered sequences from the wild-caught doliolids were classified as larger microzooplankton including radiolarians (32.5%), ciliates (11.7%), and metazoans. Metazoan sequences accounted for nearly half (48.6%) of the sequences recovered from the guts of the wild-caught animals, relative to the abundance of metazoan sequences recovered from associated near-bottom water samples. The metazoan sequences were enriched in the gut, suggesting selective feeding on metazoan prey or on detritus containing metazoan environmental DNA (eDNA) (Fig. 2). Relative selectivity estimated as the ratio of sequences retrieved from the animal and the surrounding water indicated positive selectivity for Chaetognatha (2996.1), Radiolaria (29.8), Ciliophora (25.1), Copepoda (19.3), Mollusca (18.0), and Cnidaria (10.0). All other prey types were neutrally selected (Fig. 2).

Based on these initial observations of wild-caught *D. gegenbauri* gonozooids, the abundance of 18S rRNA amplified genes from five targeted prey groups including copepoda as a representative metazoan prey group were quantified by real-time quantitative PCR. The abundance of 18S rDNA amplicons of the target prey groups were estimated in 196 individual *D. gegenbauri* zooids and paired water samples collected approximately monthly over the study period. Zooids (see Fig. 1) included 70 mature gonozooids, 66 immature gonozooids, 4 oozooids, 12 immature nurses, 31 mature nurses, and 13 phorozooids. With the exception of the mature and immature nurses, in which cryptophyta was not detected, all prey groups were detected in each *D. gegenbauri* life stage, confirming that they ingest a wide range of particle sizes (Deibel 1985; Tebeau and Madin 1994).

The relative selectivity towards each prey group by each *D. gegenbauri* life stage is provided in Fig. 3 and Table S2a–f. Relative selectivity, defined as the ratio of gene target copies per volume of animal per volume of the surrounding seawater, ranged from 0.2 for mature gonozooids feeding on picozoa prey to > 3000 by immature gonozooids ingesting diatoms. Relative selectivity values above one indicates positive selection, while values below one suggests negative selectivity.

Metazoan (Copepoda) prey selectivity

With the exception of oozooids, the gut content of all life stages were enriched with copepod DNA relative to the surrounding water. Oozooids were not selective for copepod DNA (relative selectivity = 1.1). Relative selection ratios ranged from 22.9 to 3431.2 for other zooids, with mature gonozooids exhibiting significantly higher relative selectivity ($p < 0.001$) compared with other life stages, with the exception of phorozooids ($p = 0.359$). Phorozooids exhibited the second highest copepod DNA relative selectivity ratio of 925.3 (Fig. 3; Table S2).

Flagellate prey selectivity

Overall, haptophyta and cryptophyta prey groups were positively selected by all *D. gegenbauri* life stages, but were significantly less positively selected for than the larger sized prey groups including Copepoda ($p < 0.001$) and Bacillariophyta ($p = 0.006$) and more positively selected than the smaller-sized Picozoa group ($p < 0.001$). There were no significant differences between relative selectivity ratios between Cryptophyta and Haptophyta ($p = 1.00$), as relative selectivity for haptophyte prey ranged from 1.6 by mature gonozooids to 907.3 by oozooids. The average relative selectivity for all zooid stages ingesting flagellates was 217.6 ± 112 (SE) (Fig. 3; Table S2).

Bacillariophyta (diatom) prey selectivity

Consistent with earlier qualitative metabarcoding studies (Walters et al. 2018), these quantitative studies confirmed that Bacillariophyta are positively selected by all *D. gegenbauri* zooids ($p < 0.001$). Relative selectivity ranged from 321.7 by immature nurses to 3062.1 by immature gonozooids. Bacillariophyta DNA was detected in all of the immature gonozooids that were collected, but was highly variable among the 66 individuals examined, ranging by eight orders of magnitude from 0.001 to > 134,000 (Fig. 3; Table S2).

Picozoa prey selectivity

Although picozoa were the most abundant species in sampled waters, and the smallest size of the prey group we investigated, the relative selectivity of picozoa was near neutral and indicative of passive feeding. Relative prey selectivity ranged from 0.2 to 8.2 (average = 2.5 ± 2.6). Immature nurses, mature gonozooids, and mature nurses exhibited slightly negative

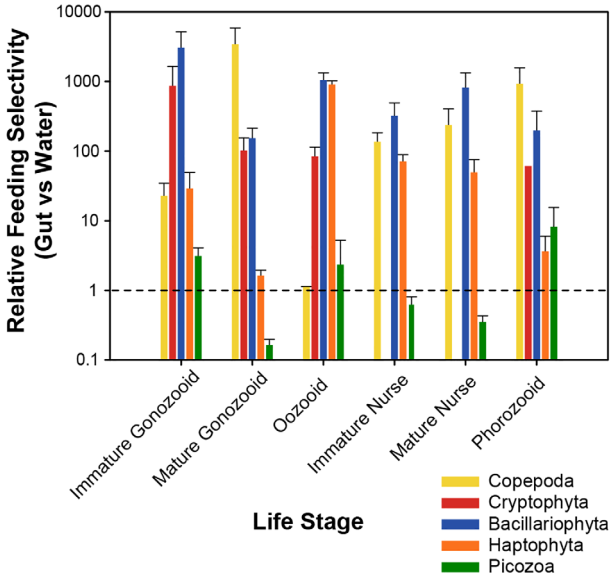

Fig 3. Feeding selectivity by different wild-caught *D. gegenbauri* life stages of target prey groups based on qPCR quantification of prey and parasite gene copies in the gut vs. water column of paired wild-caught *D. gegenbauri* zooids and water samples. The dashed line indicate gut : water 1 : 1 reference line. Error bars represent standard error.

feeding selectivity for picozoa on average, while oozooids, immature gonozooids, and phorozooids exhibited slightly positive selectivity for picozoa prey. Immature gonozooids were significantly more selective of picozoa than larger mature gonozooids ($p < 0.001$) (Fig. 3; Table S2).

Contribution of metazoan prey to the nutrition and growth of *D. gegenbauri* gonozooids

To explore the hypothesis that metazoan prey contributes to the nutrition of *D. gegenbauri*, experimental growth studies were conducted with *D. gegenbauri* gonozooids. Growth and mortality rates of developing *D. gegenbauri* gonozooids were estimated in the presence and absence of *Artemia* sp. eggs and nauplii (Fig. 4) under previously described optimal cultivation conditions (Walters et al. 2019).

Artemia sp., provided as a mixture of heat-inactivated eggs and developing nauplii, was utilized as an experimental model for metazoan (copepod) prey. In the absence of eggs and nauplii, growth rates increased in proportion to the concentration of algal prey (Fig. 5a). Growth rates increased significantly from 0.15 mm d^{-1} when algal concentrations were maintained at 10 μg C L^{-1} to 0.53 mm d^{-1} when algal concentrations were maintained at 60 μg C L^{-1} ($r^2 = 0.88$, $p = 0.04$). Above 60 μg C L^{-1} (80 μg C L^{-1}) growth rates declined to 0.35 mm d^{-1}. These growth rates are consistent with previously reported growth rates of *D. gegenbauri* maintained under similar conditions in culture (Gibson and Paffenhöfer 2000; Walters et al. 2018). In the presence of relatively high concentrations of eggs (initial concentration of 150 eggs L^{-1}) growth rates were significantly decreased ($p = 0.008$) compared to egg-free controls (Fig. 5a). Growth rates were unaffected by the concentration of crustacean eggs and nauplii ($p = 0.146$) although mortality increased in the presence of higher concentrations of eggs and nauplii (Fig. 5b). Due to the small number of experimental estimates, however, the observed increase in mortality at egg/nauplii concentrations above 50 liter^{-1} was not significant ($p = 0.2$).

Visual examination of *D. gegenbauri* zooids and fecal pellets grown in the presence of *Artemia* sp. confirmed that eggs and nauplii were ingested and egested by growing *D. gegenbauri* gonozooids (Fig. 5).

To determine if this material was digested and assimilated, the isotopic fractionation of $\delta^{15}N$ and $\delta^{13}C$ was investigated in each of the growth experiments where *Artemia* sp. eggs and nauplii were provided (Fig. 6).

Average algal prey $\delta^{13}C$ isotopic composition ranged from – 20.2 to – 16.5 and average $\delta^{15}N$ composition ranged from – 6.0 to 0.87. Relative to the algal prey, *Artemia* sp. eggs were depleted with respect to $\delta^{13}C$ (average – 22.5 ± 1.4) and enriched with respect to $\delta^{15}N$ (average 9.8 ± 0.7). The isotopic composition of detrital material (POM) produced during the 9–10 day experimental periods was intermediate. Compared to algal prey, POM fractions were significantly depleted with respect to $\delta^{13}C$ ($p = 0.004$ and enriched with respect to $\delta^{15}N$ ($p = 0.012$). Doliolid gonozooids that had not been exposed to crustacean materials exhibited average $\delta^{13}C$ composition of – 18.8 ± 2.2 and $\delta^{15}N$ composition of 1.3 ± 1.4. Gonozooids grown in the presence of *Artemia* sp. eggs and nauplii had an average $\delta^{13}C$, and $\delta^{15}N$ isotopic composition of – 17.4 ± 0.9 and 1.4 ± 1.9, respectively. There was not a significance difference in either the $\delta^{13}C$ ($p = 0.15$) or $\delta^{15}N$ isotopic composition ($p = 0.97$) between gonozooids exposed to *Artemia* sp. eggs and nauplii and those that were not, supporting the null hypothesis that *Artemia* sp. material does not contribute to the growth of *D. gegenbauri*.

The trophic position of *D. gegenbauri* with respect to life history and bloom status

To investigate the dynamics of *D. gegenbauri* trophic relationships over its complex life history and variable bloom

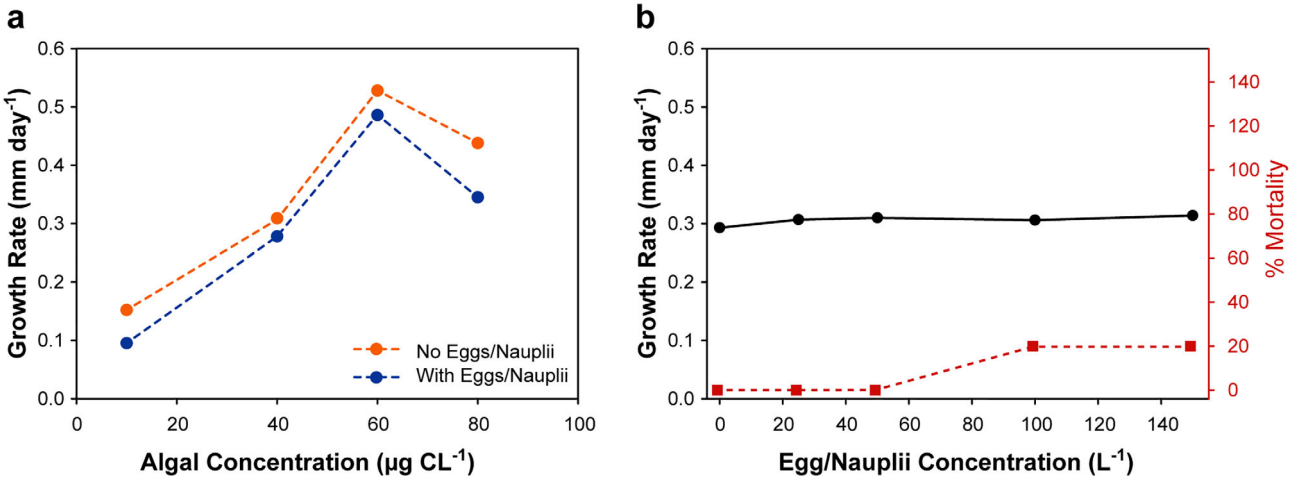

Fig 4. Growth and mortality of *D. gegenbauri* gonozooids in the presence and absence of *Artemia* sp. eggs and nauplii. Growth rate as a function of (**a**) algal concentration and (**b**) egg and nauplii concentration with a fixed algal concentration of 40 μg C L^{-1} .

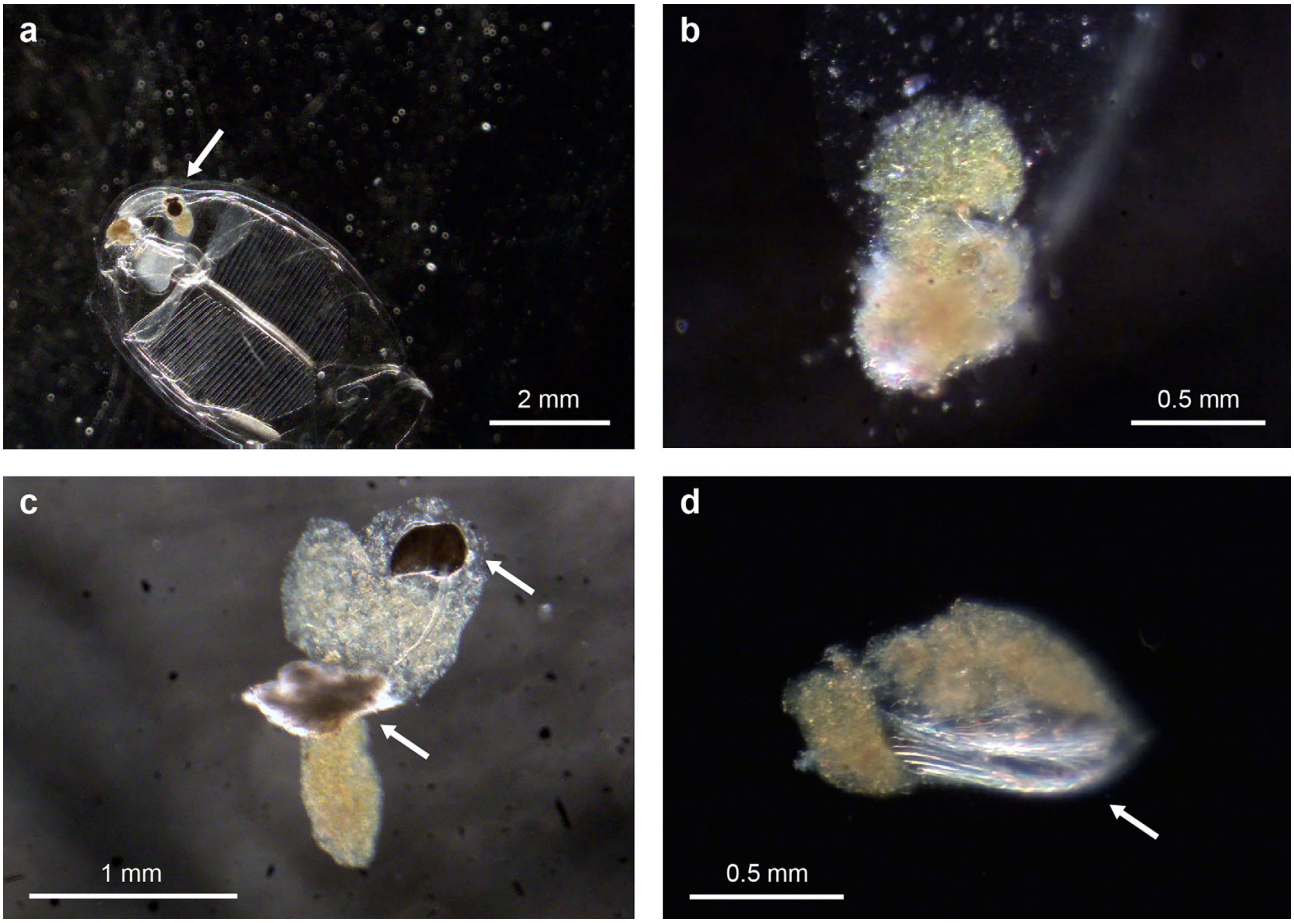

Fig 5. Ingestion and egestion of *Artemia* sp. eggs and nauplii by *D. gegenbauri* gonozooids. (**a**) Captured *Artemia* sp. egg incorporated into a fecal pellet and about to be expelled. (**b**) Fecal pellet produced when *Artemia* sp. eggs and naupli were not present. *D. gegenbauri* was feeding on a mixture of *Rhodomonas* sp., *Isochryis galbana*, and *T. weissflogii*. (**c**) Two viable *Artemia* sp. hatching eggs encased in a *D. gegenbauri* fecal pellet that had been released. (**d**) *Artemia* sp. nauplii encased in a released *D. gegenbauri* fecal pellet. The viability of the nauplii was not determined. Eggs and nauplii are indicated by white arrows. Size scale bars are shown.

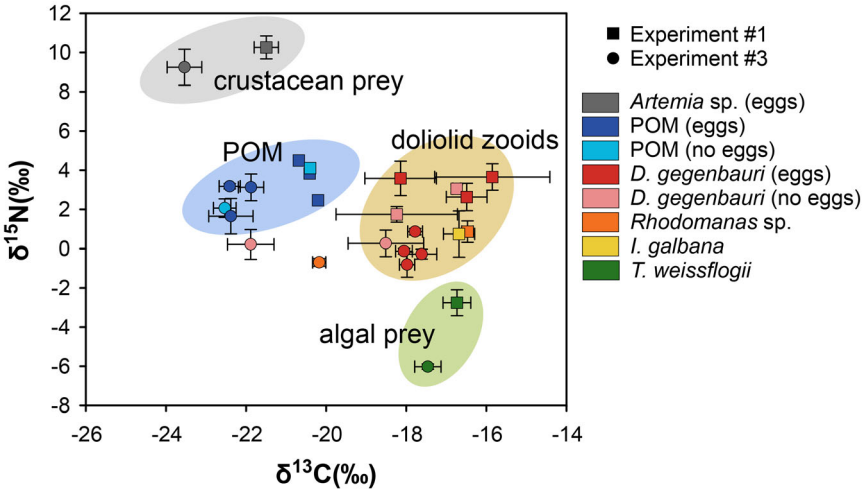

Fig 6. Biplot of stable isotope signatures ($\delta^{13}C$ and $\delta^{15}N$) of cultured *D. gegenbauri*, *Artemia* sp., algal prey, and POM (particulate organic matter) at the end of the 9–10-day experimental periods (Table 2). Bars indicate standard errors. The $\delta^{13}C$ values on the *x*-axis reflect potential carbon sources, while the $\delta^{15}N$ values on the *y*-axis reflect trophic position. Ellipses highlight isotopic composition ranges of doliolid zooids, POM, and algal and crustacean prey.

conditions, the isotopic composition of C and N were determined in starved wild-caught *D. gegenbauri* zooids and size fractioned Particulate Organic Matter (POM). Samples were derived from a representative subset of 10 of the cruises including 5 when doliolid blooms were absent and 5 during blooms of different magnitudes. A total of 60 POM samples and 51 starved zooids comprised of 33 gonozooids, 14 nurses, and 4 phorozooids were analyzed (Table S2). Live oozooids were so rare in the sample collection that none were available for isotopic analysis.

The carbon and nitrogen isotopic profiles of size-fractionated POM and each life stage of wild-caught *D. gegenbauri* are presented in Fig. 7. There was considerable variability in the isotopic composition of both doliolid zooids and POM over the course of the study. *D. gegenbauri* zooids, however, were significantly enriched with $\delta^{13}C$ relative to the POC fractions ($p < 0.001$) indicative of a diet consisting of a mixture of fresh algae and POM. Zooid $\delta^{15}N$ composition was not significantly different from PON fractions ($p = 0.074$) and therefore does not support the hypothesis that metazoan prey contribute significantly to the diet of *D. gegenbauri*.

The isotopic composition of wild-caught *D. gegenbauri* zooids is provided in Fig. 7. The isotopic composition of *D. gegenbauri* zooids was examined relative to the life stage to address the hypothesis that the diet of *D. gegenbauri* varies over its life cycle. Statistically sufficient sample size to address this hypothesis was only available for gonozooids. Reasonable numbers of nurses (14), however, were available and so were also examined. There were no significant differences between different zooid types in $\delta^{15}N$ composition (Fig. 7b, $p = 0.09$) indicating that all zooid types examined occupied the same trophic position regardless of bloom status. The % $\delta^{15}N$ ranged from 2.5 to 7.9 with an average of 4.8 ± 1.2 (Fig. 7).

Comparison of $\delta^{13}C$ composition suggested significant differences with respect to doliolid bloom status. Significant

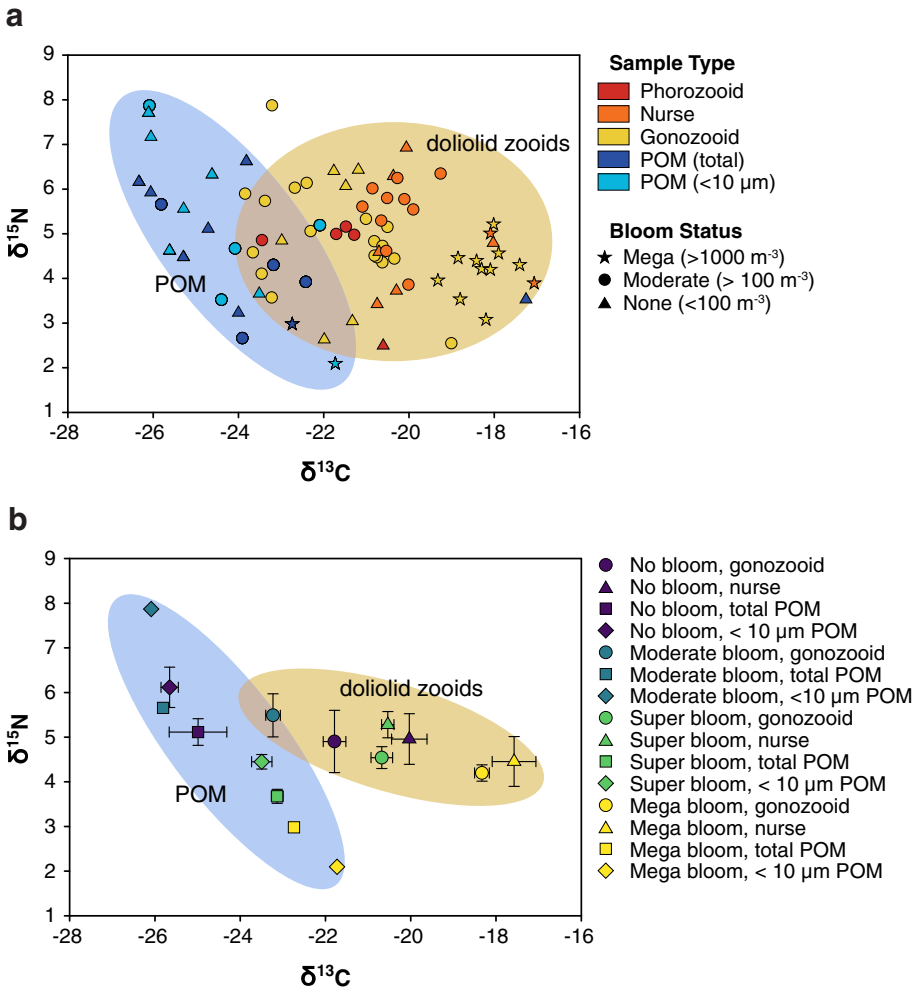

Fig 7. Biplots of stable isotope composition ($\delta^{13}C$ and $\delta^{15}N$) of starved wild-caught *D. gegenbauri* zooids, and sized fractionated POM (particulate organic matter). (**a**) All zooids and (**b**) average isotopic composition of gonozooids, nurses, and size-fractionated POM. Error bars reflect the standard error. *D. gegenbauri* zooids were collected on the South Atlantic Bight mid-continental shelf from August 2015 to December 2017. Ellipses highlight isotopic composition ranges of doliolid zooids and POM.

($p < 0.001$) differences in % $\delta^{13}C$ were also observed between zooids types captured during the Mega bloom event and in the absence of blooms. During the August 2016 Mega doliolid bloom, the greatest % $\delta^{13}C$ enrichment was observed. Enrichment of $\delta^{13}C$ in zooids and POC fractions varied significantly with doliolid bloom status (Fig. 7b, $p < 0.001$). PO $\delta^{13}C$ was highest during the Mega bloom, intermediate during Super and no bloom periods, and lowest in zooids collected from moderate bloom periods. With respect to life stage, nurses were more enriched with respect to $\delta^{13}C$ than were gonozooids in the absence of doliolid blooms ($p = 0.005$), but nurses were not significantly different during doliolid bloom events suggesting possible feeding selection only when prey availability is low. Insufficient numbers of phorozooids were available for robust analysis ($n = 4$), but based on the limited dataset consisting of three phorozooids and three gonozooids collected from the Super bloom encountered in December 2016 (DolDiet_015, 181.5 individuals m^{-3}), there was no evidence for differential isotopic composition between gonozooids and phorozooids. Neither PO $\delta^{13}C$ ($p = 0.850$) or PO $\delta^{15}N$ ($p = 0.826$) differed between these gonozooids and phorozooids.

In contrast to the *D. gegenbauri* zooids, PO $\delta^{15}N$ and PO $\delta^{13}C$ compositions both varied significantly with respect to doliolid bloom status ($p < 0.001$) reflecting both the source of the carbon and trophic processing processes (Fig. 7b). During the Mega bloom event coincident with a significant phytoplankton bloom, $\delta^{13}C$ was most enriched in the < 10 μm fraction (avg = −21.73) and $\delta^{15}N$ was least enriched (avg = 2.10). In contrast, during periods when doliolid blooms were not present, PO $\delta^{13}C$ composition was the least enriched (avg = −25.66) while PO $\delta^{15}N$ composition was the most enriched in the < 10 μm fraction of POM associated with moderate doliolid blooms (avg = 7.87) and in the absence of a doliolid blooms (avg = 6.12). These observations are consistent with the hypothesis that on the South Atlantic Bight shelf, *D. gegenbauri* blooms are associated with nutrient upwelling induced phytoplankton blooms.

Discussion

D. gegenbauri is a permanent component of the SOUTH ATLANTIC BIGHT zooplankton community

Doliolids and other pelagic tunicate species are common members of continental shelf zooplankton communities found in warm and temperate waters worldwide, but blooms also occur in the Southern Ocean and in oceanic regions where upwelling occurs (Lucas et al. 2014). Although it is well documented that doliolids, including the species *D. nationalis* and *D. gegenbauri*, are common members of South Atlantic Bight zooplankton communities (Paffenhöfer and Lee 1987; Paffenhöfer et al. 1995), it is not known whether these species are always present. During this study that included 24 expeditions to the South Atlantic Bight mid-continental shelf over a two-year period, *D. gegenbauri* was present on all occasions supporting the hypothesis that *D. gegenbauri*, although its abundance and distribution was highly variable, is a permanent resident of the South Atlantic Bight zooplankton community. It is not clear, however, whether *D. gegenbauri* populations are self-sustaining or reliant on continuous recruitment from more southerly locations including the Florida Straits and the Gulf of Mexico where *D. gegenbauri* is also common and abundant (Atkinson et al. 1978; Esnal and Simone 1982; Greer et al. 2020). To discriminate between these possibilities, population genetic studies would likely be required.

Regardless of the origin of South Atlantic Bight doliolid populations, as Deibel and Paffenhöfer (2009) proposed, doliolid blooms are most likely dependent on the upwelling of cool nutrient-replete waters from aphotic depths below the mixed layer onto the shallow continental shelf that stimulates primary production and result in secondary zooplankton production. Consistent with the Deibel and Paffenhöfer (2009) proposal, we observed that the abundance and distribution of *D. gegenbauri* populations were highly variable and patchy, but blooms were most frequent during the summer to fall transition and in the winter when upwelling and deep-water intrusion events are most common (Atkinson et al. 1978). The poor correlation between *D. gegenbauri* abundance and phytoplankton standing stocks, estimated as Chl *a* concentrations (Walters et al. 2018), however, suggests that *D. gegenbauri* blooms are also dependent on poorly understood and complex trophic interactions and contribute to the uncertainty in the prediction of doliolid bloom dynamics.

The diverse diet and selective feeding of *D. gegenbauri*

Confirming recent reports of feeding selectivity (Walters et al. 2018), in this study we observed that *D. gegenbauri* zooids also exhibit selective feeding behavior in situ. Both semi-quantitative analysis of prey DNA sequences retrieved from wild-caught *D. gegenbauri* gonozooids (Fig. 2), and quantification of representative target prey groups by real-time PCR in different life stage zooids (Fig. 3) support the hypothesis that *D. gegenbauri* exhibits selective feeding behavior. Relative to the abundance of target prey genes in bulk water samples collected contemporaneously with animals, the abundance of target sequences associated with metazoans and microplankton were most enriched in the doliolid samples, suggesting that these prey types had been preferentially ingested. Nanoplankton were slightly enriched relative to water column concentrations and picoplankton (picozoa) were present at concentrations consistent with their abundance in the surrounding water, suggesting that although these smallest prey types were efficiently captured by the *D. gegenbauri*, they were not preferentially selected. The mechanisms that Thaliaceans use to selectively capture rarer large prey particles remain poorly understood (Conley et al. 2018), but it is likely that the ability of doliolids to feed while stationary allows them to take advantage of micro-scale prey patches. The physical

distribution of picozoans has not, to our knowledge, been well studied, but it is well established that larger plankton and aggregated materials can be highly patchy due to a wide variety of physical and biological mechanisms (Durham and Stocker 2012). For example, Greer et al. (2020) recently reported that in the northern Gulf of Mexico, a massive doliolid bloom was associated with diatom and marine snow enriched thin layers that formed due to a surface convergence and vertical shear. The availability of prey aggregations and physical structures likely contributes significantly to the dynamics of *D. gegenbauri* distributions.

All life stages of *D. gegenbauri* exhibited feeding selectivity (Fig. 3), but the variability between individuals was high, likely reflecting both environmental and behavioral differences over the period that zooids were collected. Overall, diatoms and metazoans (Copepoda) were the most highly selected prey groups. Due to the high level of variability in feeding selectivity observed, with the exception of gonozooids and nurses that were the most abundantly collected zooid type, differences in relative selectivity were rarely statistically significant. With respect to gonozooids, the developing immature gonozooids were most selective for diatoms while mature gonozooids were most selective for Copepoda (Fig. 3; Table S2). A possible explanation for these observations may be related to the successional patterns of blooms of phytoplankton and resulting zooplankton production. Diatoms often dominate phytoplankton blooms on the South Atlantic Bight shelf (Verity et al. 1993) and are readily ingested by maturing gonozooids that dominate when doliolids reach bloom abundances (Deibel 1985). As a doliolid bloom matures and the phytoplankton bloom senesces, mature gonozooids increase in abundance. As fresh phytoplankton prey resources are diminishing, these zooids can take advantage of the availability of accumulating phytodetritus and zooplankton production to enrich their diet. Additionally, the ability to locate and capture prey likely varies between zooid life stages. For example, the mature nurse is the largest and most mobile life stage and may be better able to hunt for and take advantage of prey patches than the less mobile gonozooids and phorozooids, especially when prey availability is low. The versatile ability to capture and utilize different prey sources including detritus, and the ability of *D. gegenbauri* to take advantage of heterogeneous distributions of prey particles by locating prey patches and feeding while stationary, may contribute to the observed selectivity and efficiency of *D. gegenbauri*. To our knowledge the ability of doliolids to sense prey from a distance has not been investigated, but is it plausible that if a prey-rich patch is encountered, an individual would reduce its swimming while feeding as long as sufficient prey was being captured. Doliolids also have the ability to reverse their feeding current and reject prey particles (Deibel and Paffenhöfer 1988), suggesting that active prey selection may also be possible.

Although in these studies the hypothesis that *D. gegenbauri* is a selective feeder with a preference for larger prey types including protists and metazoans was supported, it remains difficult to extrapolate the absolute contribution of these prey types to the diet of *D. gegenbauri*. The ability to make quantitative diet estimates based on prey DNA-based approaches including qPCR and high throughput sequencing metabarcoding approaches is widely debated because it is not clear that the number of target gene copies present in a DNA extract is always proportional to biomass consumed. This is especially true when multi-cellular eukaryotic prey are the target and group-—rather than species-specific PCR primers are utilized (Nielsen et al. 2018; Deagle et al. 2019).

Does the consumption of metazoan prey contribute to the nutrition of *D. gegenbauri*?

Although diatoms and metazoan prey are ingested by doliolids, the degree to which they digest these large particles and assimilate them nutritionally is unclear. Paffenhöfer and Köster (2005) reported that small diatoms are poorly assimilated by *D. gegenbauri*. They speculated that a considerable fraction of POM ingested by doliolids is released as fecal pellets, which can then be re-consumed by doliolids and other organisms (Köster and Paffenhöfer 2017). Similar observations have been reported for salps with fecal pellets containing apparently intact diatoms, metazoans, and exhibiting isotopic compositions characteristic of detrital feeding (Décima et al. 2019). Although it has previously been reported that *D. gegenbauri* has the ability to capture larger particles including eggs and larvae of other metazoans making them potential agents of trophic cascades in pelagic food webs (Deibel 1998), to our knowledge, it is not known if this material is digested and assimilated after ingestion.

To address this question, this study investigated the growth and isotopic composition of *D. gegenbauri* in culture-based laboratory studies in which growing *D. gegenbauri* gonozooids were provided diets enriched with crustacean eggs and nauplii. The isotopic composition of wild-captured *D. gegenbauri* zooids was also explored as a means to estimate the trophic position of *D. gegenbauri* in the South Atlantic Bight continental shelf pelagic food web.

Growth rates of developing gonozooids in culture were not enhanced when minimal algal diets of 40 $\mu g\ C\ L^{-1}$ were supplemented with a mixture of *Artemia* sp. eggs and nauplii indicating that this material was not assimilated and did not contribute to growth (Fig. 4a). The effect on reproduction, if any, was not evaluated and may have responded differently than gross growth, as occurs in the larvacean *Oikopleura dioica* (Troedsson et al. 2002; Lobon et al. 2013). When egg concentrations were environmentally unrealistically higher than 100 eggs L^{-1}, increased mortality was observed likely due to mucus-net clogging (Gibson and Paffenhöfer 2000; Fig. 4b). The concentration where increased mortality was observed was higher than *D. gegenbauri* would be expected to encounter in nature, and therefore unlikely to be of ecological significance. Consistent with the results of the growth studies, the isotopic composition of *D. gegenbauri* grown in the presence of *Artemia* sp. eggs and nauplii did not reflect the

assimilation of this material (Fig. 7). Rather, these studies suggest that the animals assimilated carbon and nitrogen largely from the consumption of algal and detrital material.

The isotopic composition of wild-caught *D. gegenbauri* zooids was consistent with experimental studies suggesting that the doliolid diet in nature consists of a mixture of fresh algae and detrital material rather than higher trophic level prey (Fig. 7a). With respect to carbon, the $\delta^{13}C$ values were most enriched in zooids captured during the August 2016 Mega bloom event and significantly less enriched in zooids captured during lesser or non-bloom periods (Fig. 7b). These observations suggest that when fresh algal production is available, it is preferentially consumed. However, during periods when fresh algal material is less abundant, *D. gegenbauri* may rely more heavily on detritivory. In contrast, the $\delta^{15}N$ composition of *D. gegenbauri* zooids was independent of bloom status. While there is evidence that doliolids do not digest all ingested particles equally (see discussion above), the lack of controlled studies on these and other organisms that employ similar feeding strategies highlights the need for quantitative investigations of the importance of detrital material as a source of C and N in the diet of doliolids and other pelagic tunicates. Décima et al. (2019) noted in a study of pyrosomes in the Eastern Tropical Pacific that these filter feeders also isotopically resembled N and C of sinking particles, and not living phytoplankton or zooplankton caught in the same locations. They also observed the same pattern we report here, that N isotopes did not appear to be fractionated, while there was some evidence of enrichment of C isotopes from a putative detrital/particulate source. However, it must be stressed that even fundamental ecological assumptions such as isotopic fractionation between trophic levels may not hold with gelatinous-bodied filter feeders (Pakhomov et al. 2019). Furthermore, poor quality detrital C and N sources may produce minimal isotopic fractionation upon ingestion (Fantle et al. 1999; Boecklen et al. 2011; Shipley and Matich 2020). Additional studies, especially controlled doliolid feeding experiments to determine the isotopic fractionation associated with food sources of different quality, are needed.

Because a sufficient number of individual gonozooids and nurses was collected, comparisons of isotopic composition between these life stages were made. During the Mega bloom event, gonozooids were more $\delta^{13}C$ enriched than nurses were, and while in non-bloom conditions, gonozooids were less $\delta^{13}C$ enriched (Fig. 7b). Additional studies are required, but these differences suggest that feeding behavior and preferences may vary over the life history of *D. gegenbauri* allowing them to maintain a continuous presence, as prey species populations shift on the South Atlantic Bight shelf.

Revisiting the trophic role of doliolids

Historically, *D. gegenbauri* and other thaliacea have been understood to be passive grazers with a diet reflective of the composition of available prey (Crocker et al. 1991; Vargas and Madin 2004; Sutherland et al. 2010). The fate of the particles captured by *D. gegenbauri*, however, has rarely been investigated. For example, although diatoms and larger particles are ingested by doliolids, it is still unclear if these large particles are digested and assimilated. In fact, Paffenhöfer and Köster (2005) reported that some diatoms are poorly assimilated by *D. gegenbauri* and speculated that a considerable fraction of POM ingested by doliolids is released as fecal pellets. More recently, these authors suggest that the consumption of fecal pellets contribute to the doliolid diet, especially when phytoplankton abundance is low (Köster and Paffenhöfer 2017). Carbon assimilation efficiencies of

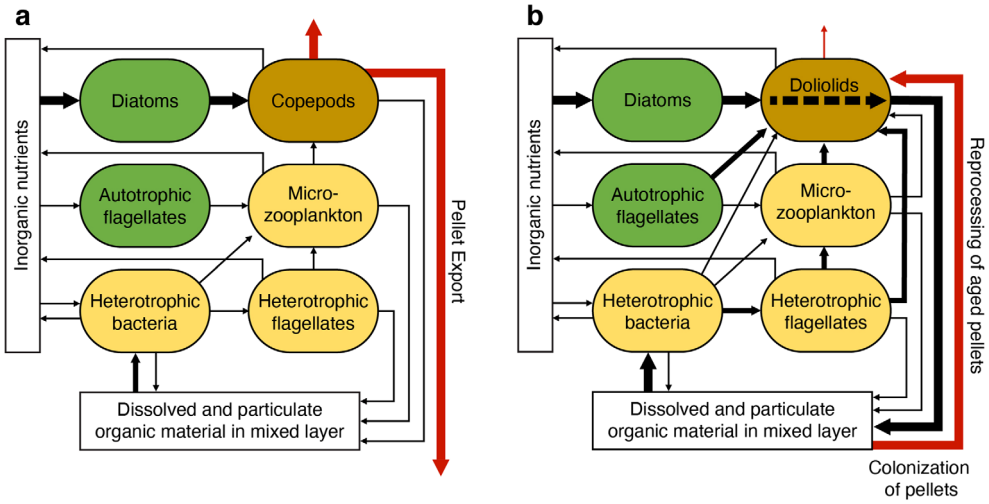

Fig 8. Hypothesized structure of pelagic food web when (**a**) copepods or (**b**) doliolids dominate zooplankton communities. Doliolid dominance is hypothesized to lead to increased complexity of the microbial network, decreased trophic transfer to higher trophic levels, and increased recycling of organic matter in shelf waters. The width of vector arrows indicate the hypothesized relative magnitude of flux. Red vector arrows indicate export flux pathways.

D. gegenbauri are typically much lower than copepod assimilation efficiencies (Paffenhöfer and Köster 2005). Based on the quantification of gut content and isotopic composition, it is apparent that *D. gegenbauri*, rather than being a passively feeding planktivore, is better described as a selective feeder that exhibits variable trophic interactions over its complex life history and is reflective of oceanographic conditions. Furthermore, the isotopic composition of *D. gegenbauri* and its inability to digest a significant fraction of the particles it consumes suggests that detritivory may be the most common trophic mode exhibited on the South Atlantic Bight continental shelf. During periods when suboptimal prey species are present, from a trophic perspective, *D. gegenbauri* and other doliolid species may more accurately be described as facultative detritivores with a unique "trophic twist" in that they contribute significantly to the production of detritus that they re-consume.

Potential ecological significance

Doliolids occur circumglobally on wide subtropical shelves where upwelling, eddies, or other processes generate nutrient input that promotes phytoplankton blooms (Paffenhöfer et al. 1995; Deibel 1998; Nakamura 1998). Because doliolids have high filtering rates and efficiencies and can reach great abundances, they have the potential to remove a significant fraction of shelf water column primary production and can restructure shelf pelagic food webs (Deibel 1985; Paffenhöfer et al. 1995; Takahashi et al. 2015; Ishak et al. 2020). Many investigators have speculated on the significance of blooms of small gelatinous zooplankton species and, the few studies that have been conducted, generally support the idea that doliolids and other thaliaceans can act to intensify microbial loop processes. For example, Katechakis et al. (2002) demonstrated in semi-continuous two-stage chemostats that doliolid grazing can result in trophic cascades and affect the composition and activity of auto- and heterotrophic microbes. Blooms of small gelatinous species may also decouple grazing linkages between the mesozooplankton and higher trophic levels (Sullivan and Kremer 2011) and affect the vertical export of carbon to depth, and biogeochemical cycling (Lebrato et al. 2019; Richardson 2019). If these assumptions are also true for doliolid blooms, consequences could include lower trophic transfer efficiency and yield to higher trophic levels and diminished C sequestration (Fig. 8). The results of this study support the conclusion that doliolids preferentially ingest some food sources over others, and that they do not appear to digest all ingested particles equally. These observations imply a more complex relationship with both the microbial loop and with other organisms than is generally reported for more intensively studied and modeled non-gelatinous zooplankton.

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Conflict of Interest

None declared.

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