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Diet and trophic interactions of a circumglobally significant gelatinous marine zooplankter, *Dolioletta gegenbauri* (Uljanin, 1884).

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ABSTRACT

Gelatinous zooplankton play a crucial role in marine planktonic food webs. However, primarily due to methodological challenges, the *in situ* diet of zooplankton remains poorly investigated and little is known about their trophic interactions including feeding behavior, prey selection, and *in situ* feeding rates. This is particularly true for gelatinous zooplankton including the marine pelagic tunicate, *Dolioletta gegenbauri*. In this study, we applied an 18S rRNA amplicon metabarcoding approach to identify the diet of captive-fed and wild-caught *D. gegenbauri* on the mid-continental shelf of the South Atlantic Bight (SAB), USA. Sequencing-based approaches were complimented with targeted quantitative real time PCR (Polymerase Chain Reaction) analyses. Captive-fed *D. gegenbauri* gut content was dominated by pico-, nano- and microplankton including picodinoflagellates (picozoa) and diatoms. These results suggested that diatoms were concentrated by *D. gegenbauri* relative to their concentration in the water column. Analysis of wild-caught doliolids by quantitative real time PCR utilizing a group-specific diatom primer set confirmed that diatoms were concentrated by *D. gegenbauri*, particularly by the gonozooid life stage associated with actively developing blooms. Sequences derived from larger metazoans were frequently observed in wild-caught animals but not in captive-fed animals suggesting experimental bias' associated with captive feeding. These studies revealed that the diet of *D. gegenbauri* is considerably more diverse than previously described, that parasites are common in wild populations, and that prey quality, quantity and parasites are likely all important factors in regulating doliolid population dynamics in continental shelf environments.

INTRODUCTION

Gelatinous zooplankton play a crucial role in marine plankton food webs and, although a subject of considerable debate (Sanz-Martín 2016), it has been speculated that they may become increasingly important in the future warmer and more acidic ocean (Richardson et al. 2009, Brotz et al. 2012, Purcell 2012, Condon et al. 2013, Winder et al. 2017). Although a significant component of most marine systems, the trophic role of gelatinous zooplankton remains poorly investigated due to methodological challenges and persistent misconceptions of their importance (Henschke et al. 2016, Lamb et al 2017). This is especially true for smaller gelatinous organisms, including the pelagic tunicates (salps, larvaceans and doliolids), with respect to their trophic interactions (Jaspers et al. 2015).

It is well known that continental shelf regions of subtropical oceans experience intermittent occurrences of pelagic tunicate blooms (Paffenhöfer et al. 1995). These blooms are understood to be initiated by eddy-related shelf-break upwellings that deliver cool nutrient-rich water onto the adjacent shelf and result in increased pelagic productivity (Yoder et al. 1983, Pelegrí et al. 2006, Castelao 2014). On the broad shelf associated with the South Atlantic Bight (SAB) in the subtropical region of the western North Atlantic, doliolid blooms, most commonly the species *Dolioletta gegenbauri* (Doliolida Dolididae), occur predictably in association with shelf upwelling conditions (Deibel and Paffenhöfer 2009). Doliolids are most abundantly located in the particle and nutrient-rich intruding bottom water and in the thermocline containing higher concentrations of phytoplankton and associated plankton communities (Paffenhöfer and Lee 1987). Typically, once on the continental shelf, doliolid blooms develop within 1 to 2 weeks due to their prolific asexual reproduction (Paffenhöfer and Köster 2011) and therefore contribute significantly to shelf production, pelagic ecology, and pelagic-benthic coupling (Deibel 1998, Nakamura 1998, Martin et al. 2017). Blooms exceeding 1,000 doliolid zooids m^{-3} are frequently reported from most of the world's subtropical continental shelves (Paffenhöfer et al. 1995, Deibel 1998, Nakamura 1998).

Based on laboratory derived estimates of doliolid clearing rates (Deibel 1998) it can be inferred that on the SAB shelf doliolids have the potential to remove a significant fraction of daily phytoplankton production, at times having the capacity to clear nearly 100% of the water

column (Paffenhöfer et al. 1995). These filtration rates are considered conservative as recent *in situ* observations of pelagic tunicate filtration rates are considerably higher than laboratory-derived estimates (Kakani et al. 2017). In addition to high clearance rates and efficient particle capture (Tebeau and Madin 1994), doliolids produce copious amounts of relatively buoyant fecal pellets containing high quantities and quality of organic matter (Paffenhöfer and Köster 2005). The fate and significance of these pellets are poorly understood, but they can be reingested by doliolids and other animals, colonized and remineralized by bacteria; or, reach the seafloor, stimulating benthic bacterial production. Thus, doliolid blooms through their ability to collect, aggregate and release particles have the potential of re-structuring the pelagic food web including shunting a considerable fraction of pelagic water column productivity to the microbial food web (e.g. Pomeroy and Deibel, 1980).

Doliolids exhibit a complex multi-zooid life cycle (Figure 1) and thus, it is likely their diet changes throughout their development. However, due to constraints associated with culture-based approaches, it remains a challenge to identify and quantify doliolid feeding *in situ* without introducing experimental bias associated with cultivation. This challenge, although particularly acute for small delicate gelatinous zooplankton species such as *D. gegenbauri*, has been well recognized in general for zooplankton that prey on microscopic organisms at the base of the grazing food web (Bathmann et al. 2001, Troedsson et al. 2007, Nejstgaard et al. 2008).

In view of these challenges, new methodologies using prey-specific DNA as biomarkers for the study of trophic interactions have yielded promising results. For example, DNA-based gut content analysis approaches have been successfully applied in qualitative and quantitative dietary studies of a wide-range of terrestrial and marine invertebrates and vertebrates (Blankenship and Yayanos 2005, Durbin et al. 2010, Clare 2014, Nielsen et al. 2017). This is especially true for revealing trophic behaviors of cryptic species including insects, deep sea animals and species that forage widely in remote environments and are therefore difficult to observe. Recently PCR-based assays for detection of prey content in the gut of a variety of marine mesozooplankton species including the larvacean *Oikopleura dioica* (Troedsson et al. 2007) and the doliolid *D. gegenbauri* (Frischer et al. 2014) have been developed. In the case of both these pelagic tunicate species, prey DNA digestion appears to be minimal and therefore prey ingestion can be quantitatively estimated using quantitative PCR approaches (Frischer et al. 2014). The availability of such methods provide a culture independent means to assess the diet of these

delicate and cryptic marine animals. To our knowledge all previous investigations that have attempted to directly determine the diet of *D. gegenbauri* have relied cultivated animals in laboratory settings and therefore likely incorporate biases' associated with culture conditions.

In order to improve the understanding of the trophic role of doliolids in continental shelf food webs, in this study we applied an 18S rRNA amplicon metabarcoding approach to identify the diet of captive-fed and wild-caught *D. gegenbauri* across seasons and bloom conditions on the mid-continental shelf of the SAB. The sequencing-based approach was complimented with targeted quantitative real time PCR analyses, providing novel insights into the diet of doliolids.

MATERIALS AND METHODS

Dolioletta gegenbauri Collection

D. gegenbauri zooids were collected frequently throughout the summers of 2011 – 2012 and approximately monthly from 2015 – 2016 from the South Atlantic Bight mid-continental shelf in waters ranging from 20 – 40 m in depth. Animals 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 L non-filtering cod end previously described by Paffenhöfer and Gibson (1999). Following the procedures described in Gibson and Paffenhöfer (2000), *D. gegenbauri* were maintained in culture through their entire life cycle and made available for Molecular Gut Content Analysis (MGCA) enabled feeding studies. 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 ether, Alfa Aesar, Pelham, NH), rinsed three times and placed into ATL buffer with proteinase K (DNeasy Blood & Tissue DNA extraction kit (Qiagen Inc USA, Valencia, CA USA)). Samples were stored at 4°C until DNA was extracted shipboard or in the laboratory within 24 –48 hours after their initial capture. During extended research cruises samples were processed at sea and on shorter cruises (1-2 days), samples were processed in the laboratory.

Quantitative Zooplankton Collection & Enumeration of Doliolids

Zooplankton samples for quantitative analysis of doliolids were collected through the whole water column from a drifting ship by slowly (15 M min^{-1}) raising and lowering a 202 µm

mesh cone net, 0.5 M opening and 2.5 m long (ratio 1:5) equipped with a filtering cod end. A calibrated flowmeter (General Oceanics, Inc. Miami, Florida, 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 immediately fixed in 60% ethanol to a final volume of 1L. Samples were returned to the laboratory for counting and identification. Sample processing was generally completed within 12 months of collection.

Doliolids were identified and counted by microscopy as described by Godeaux (1998). If doliolids were visually abundant, the sample was diluted before counting using a Folsom plankton sample splitter. Doliolids were identified and counted in duplicate 5 mL subsamples (10 ml total) such that 1% of the original 1L sample was examined. Subsamples were transferred to a Bogorov zooplankton counting chamber with a 5 mL Hensen-Stempel pipette and counted under an Olympus SZH10 binocular microscope. Total abundance of doliolids was calculated by multiplying the counted zooids by the final dilution factor and reported as individuals per cubic meter.

Water Sample (Prey Field) Collection

To compare the composition of available prey present in the water column to ingested prey, similar molecular-based methods were utilized to assess the plankton community composition. Near bottom water (500 mL) was collected contemporaneously (within 1 hour) of the doliolids and was pre-filtered through a 63 µm sieve and collected onto a 0.8 µm (47 mm) Supor filter (PALL Life Sciences, East Hills, NY). The filter was placed in a sterile 2 mL cryovial and stored at -80°C until DNA was extracted. Estimation of the composition of the available prey for the captive-fed feeding studies was based on the analysis of these samples.

Shipboard Feeding Studies

Experimental feeding studies were conducted aboard the R/V Savannah during 5 summer research cruises in the South Atlantic Bight (2011 – 2012). To initiate feeding, laboratory-reared 6-8 *D. gegenbauri* gonozooids (1 – 7 mm in length) were transferred to 1.9 L glass jars that contained freshly collected near bottom seawater and acclimatized for 2 hours on a rotating plankton wheel (ca. 0.3 rpm) to keep them in constant suspension. Following acclimation,

doliolids were transferred to fresh near-bottom seawater in 1.9L jars and allowed to feed for 2 hours. Assuming average gut residence times of 20-30 min and clearance rates of 0.5 – 1L day^{-1} (Gibson and Paffenhöfer 2000), during the 2 hour feeding period the doliolids would have been expected to have cleared 250 – 700 mL (13 – 35%) of the feeding vessel volume during the experimental feeding period. After the feeding period, the doliolids were immediately removed from the feeding chamber and anesthetized by placing them into 0.2 µm filtered seawater containing 0.4% MS-222 (3-aminobenzoic acid ethyl ester, Alfa Aesar, Pelham, NH). After the animals had been anesthetized they were individually rinsed 3 times in 0.2 µm filtered seawater containing MS-222 and transferred to individual 2 mL tubes containing extraction buffer ATL from the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). Samples were stored at 4°C until DNA was extracted, usually within 24 – 48 hours after initial collection.

Genomic DNA purification.

Genomic DNA from whole animals was extracted using the Qiagen DNeasy Blood & Tissue kit as described by the manufacturer's instructions for total DNA from animal tissues (Qiagen, Valencia, CA). Total community DNA from filtered water samples was also extracted from each filter using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) to make them as consistent as possible with DNA extracted from *D. gegenbauri*. Following extraction, purified DNA was quantified using a NanoDrop™ 3300 fluorospectrometer (Thermo Scientific, Wilmington, DE) after staining with PicoGreen (Invitrogen, Carlsbad, CA) or using a Qubit® 2.0 fluorospectrometer with the dsDNA HS assay reagents (ThermoFisher Scientific). Samples collected during the 2011 - 2012 expedition were quantified using the NanoDrop™ fluorospectrometer. Samples collected during the 2015-2016 expeditions were quantified using the Qbit fluorospectrometer®. Yields ranged from 0.04 – 2 ng DNA $gonozooid^{-1}$ and 0.16 – 0.68 ng DNA per 500 ml water. The suitability of the purified DNA for downstream PCR and sequencing was assessed by determining whether an 18S rRNA gene fragment could be produced utilizing a general (universal) targeted primer set as previously described (Frischer et al. 2014). Each certified DNA sample was archived and stored at -20°C until further analysis. A total of 193, 41 and 12 PCR amendable samples from captive-fed doliolids, wild-caught doliolids and water samples, respectively, were collected over the course of this study.

Development of a Doliolid blocking PNA PCR assay

To detect and identify potential doliolid prey and parasites, the presence of eukaryotic associates was detected using generic eukaryotic 18S rRNA-targeted primers followed by high-throughput next-generation sequencing of resultant amplicon mixtures. An approximately 630 bp amplicon spanning the variable V4 and V5 regions of the 18S rRNA gene was amplified using the universal eukaryotic primers Univ-18S-557F and Univ-18S-1180R (Hadziavdic et al., 2014). To inhibit amplification of the doliolid (*Dolioletta gegenbauri)* 18S gene, amplification reactions were performed in the presence of a doliolid-specific peptide nucleic acid (PNA) oligonucleotide blocker essentially as previously described except that a PNA blocker specific for *D. gegenbauri* was used (Troedsson et al. 2008). The doliolid PNA–blocking oligonucleotide Dg677F PNA (5' Lysine-GGC CAA TGC AGC CTG TG) was designed, developed, and validated in this study essentially as previously described by Troedsson et al. (2008). The blocking efficiency of doliolid PNA was empirically determined to prevent the amplification of 99.99% of all doliolid 18S rRNA gene copies in a PNA-PCR reaction (Supplementary Figure 1). The PNA was synthesized by PNA Bio, Inc (Thousand Oaks, CA). Blocking PCR (PNA-PCR) reactions were conducted in 40 µl reactions and facilitated using 4 ng of template DNA, 130 nM (final concentration) of each primer Univ 18S-557F, Univ 18S-1180R, 1X (final concentration) Taq PCR Master Mix (Qiagen, Valencia, CA) and 0.8 µM of Dg18S-677F PNA blocking primer. Amplification was accomplished using a GeneAmp 9700 Thermocycler (Applied Biosystems, Foster City, CA) and included a 5 min initial denaturation at 94ºC followed by 30 amplification cycles [94ºC (30 s), 60ºC (30 s), 72ºC (1 min)] then a 10 min final extension step at 72ºC. The PNA blocking oligonucleotide was incorporated into the standard PCR cycle by including a PNA annealing step (63°C, 30 s) following denaturation at 94°C.

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). Briefly, barcoded libraries from pooled samples prepared from doliolids and water samples collected from each cruise were prepared from randomly sheared (~ 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). Next, quality-controlled 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) 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, sequences identified as doliolid and human were also removed from each dataset.

Development of Diatom-Specific Quantitative Polymerase Chain Reaction (qPCR) assay.

To determine the quantitative importance of diatoms in the diet of *D. gegenbauri*, a general diatom-specific real time quantitative PCR (qPCR) assay was developed. 18S rRNA sequences were obtained from GenBank and aligned using the ClustalW utility implemented in Bioedit (Hall 1999). Sequence regions that were conserved within the diatom prey group yet distinct from non-diatom groups were identified and screened for suitability as PCR primer targets following best practice PCR primer design criteria (Taylor et al. 2015). Potential primers were screened *in silico* using Primer3Plus (Untergasser et al. 2012) to predict their efficacy. *In silico* specificity was further confirmed using the SILVA TestPrime and TestProbe utilities (Quast et al. 2013).

Empirically optimized annealing temperatures were identified utilizing the Bio-Rad SsoFast™ EvaGreen® Supermix. qPCR reactions were conducted in 20 µl reaction volumes containing a final concentration of 0.3 µmol (of each primer) and template concentrations ranging from $5x10^{-4}$ – 1.2 ng μl^{-1} target genomic DNA. qPCR reaction conditions included an initial enzyme activation step at 95°C for 30 seconds followed by 40 cycles of denaturation (95°C, 5 sec) and annealing/extension (62°C, 5 sec). After cycling, product melt-temperatures were evaluated from 62 –95°C at 0.5°C increments for 5 seconds each. The abundance of diatom

18S rRNA genes was quantified relative to standard curves prepared with quantified plasmid DNA containing an insert of the target 18S rRNA gene from the diatom *T. weissfloggi*. In addition, the quantity of diatom genes in each seawater sample was estimated volumetrically based on a standard curve prepared from a series of filters containing water collected at the time the animals were collected from 1, 5, 10, 50 and 100 mls of water and calibrated to the plasmid standard. This allowed the creation of standard curves based on the diatoms present in the seawater that were presumably being consumed by the *D. gegenbauri* zooids. All qPCR reactions utilized a Bio-Rad CFX96 Real-Time PCR System. All wild-caught animal and water samples were assayed, at a minimum, in triplicate.

Statistical analysis.

To investigate the significance of water quality parameters, prey and doliolid abundance, correlation analyses were used to screen for significant relationships followed by more stringent linear and non-linear regression approaches. Because the abundance of doliolids between sampling dates was not normally distributed (Shapiro-Wilk test, $W=0.209$, $p<0.001$) the Spearman Rank Order correlation procedure was used. Linear and non-linear regression analyses were used to assess the performance of the diatom qPCR assay. Correlation and regression analyses were facilitated in SigmaPlot (Windows version 13.0). Comparison of 18S rRNA gene sequence libraries were facilitated using the R Community Ecology Package VEGAN (version 2.5-1) in the R Software environment (3.2.2) (R Core Team 2013). Community similarity (Morista-Horn, Jaccard and Bray-Curtis) analyses and assembly of heat maps were also facilitated in R using the VEGAN and ggplot2 packages, respectively. The frequency of NGS sequence reads expressed as a relative fraction of the total number of sequence reads recovered were interpreted semi-quantitatively and not subjected to parametric statistical analyses.

RESULTS

Doliolid Collection and Near Bottom Water Conditions

Feeding studies and molecular Next Generation Sequencing-based (NGS) gut content analyses were conducted during 5 summertime (June – August) cruises to the mid-continental shelf in the SAB from 2011 and 2012. A total of 193 *D. gegenbauri* gonozooids ranging from 1

– 7 mm in length were analyzed. During the May, August and September 2011 cruises, an additional 41 wild-caught *D. gegenbauri* gonozooids were collected and their gut content analyzed. During these cruises, near-bottom shelf water temperatures ranged from 17.5 – 27.7°C and salinity ranged from 36.1 – 36.5 PSU. Estimates of total chlorophyll *a* are not available from the 2011 cruises but ranged from 2.9 – 3.3 µg L^{-1} in 2012 (Table S1). The abundance of doliolids was not determined during these cruises.

To explore the quantitative importance of diatom prey in the doliolid diet, wild-caught *D. gegenbauri* captured during an additional set of 15 cruises conducted on 20 May, 2015 and approximately monthly from August 2015 – December 2016 were also examined. Over this period a total of 153 *D. gegenbauri* zooids including 108 gonozooids, 8 phorozooids, 35 nurses and 2 oozooids were captured and the abundance of diatoms in the gut of each zooid was estimated by qPCR. Near-bottom water conditions and the abundance of *D. gegenbauri* during this period are provided in Table S1. Over the course of these studies near bottom temperatures ranged from 13.3 – 27.9°C, salinity ranged from 34.2 – 36.5 PSU, total chlorophyll *a* ranged from 0.55 – 3.7 µg L^{-1} and the fraction of total chlorophyll *a* > 8 µm ranged from 2.8 – 70.2% but was typically closer to 30% (29.7±17.9%).

Seasonal Abundance of Doliolids

The abundance of doliolids was estimated during 14 cruises from August 2015 – December 2016 (Figure 2 & Table S1). Quantitative estimates of doliolids were not completed during the May 2015 expedition. Doliolids were observed in nearly every tow but their abundance was highly variable (0.3 – 16,795 m^{-3}). Blooms, defined here as periods when greater than 25 zooids m^{-3} and multiple zooid life stages are present, were common; blooms were observed on 7 out of the 14 cruises. Moderate blooms (25–100 zooids m^{-3}) were observed on 3 of the 14 cruises. Super blooms (100 –1000 zooids m^{-3}) were observed 3 times and a Mega bloom (> 1000 zooids m^{-3}) was observed once. In comparison to the operational definition of a Thaliacea bloom suggested by Martin et al. (2017) based on the fractional contribution of Thaliaceans to total mesozooplankton (> 300 µm) biomass, Super bloom status roughly corresponds to a moderate – intense bloom and a Mega bloom corresponds to the high end of an intense bloom as defined in this study. The abundance of doliolids was significantly correlated with total ($r = 0.838$, $p < 0.001$) and the > 8 µm ($r = 0.901$, $p < 0.001$) chlorophyll a fractions in

near bottom waters (Figure 3). However, these correlations were largely driven by conditions during the Mega bloom on 11 August, 2016. Excluding this period when chlorophyll *a* concentrations in near-bottom water were < 3 µg L^{-1} and the abundance of doliolids was < 16,0000 m^{-3}, there was not a significant correlation between the abundance of doliolids and total chlorophyll a concentration in bottom or surface waters ($p > 0.49$) or any of the other parameters measured (including date, location, depth, surface and bottom temperature, salinity, particulate organic nitrogen and carbon). There was, however, a significant relationship between the abundance of presumed actively growing *D. gegenbauri* gonozooids (< 8 mm) and the fraction of total chlorophyll a in the < 8 µm size class (Figure 3). A similar relationship was not observed for other *D. gegenbauri* zooid life stages.

Doliolid Diet Diversity and Comparison to Available Prey

Ion Torrent sequencing of doliolid gut content and water column amplicon libraries from the 5 collections generated a total of 1,000,746 and 957,562 sequence reads, respectively, prior to applying a QA/QC pipeline (Supplemental Table S2). Following the removal of low-quality sequences, sequences that did not have at least 10 replicates in the dataset and sequences identified as being derived from doliolids or humans, the dataset included 207,116 gut content sequences derived from feeding experiments, 58,827 derived from wild-caught animals and 284,597 water column derived sequences. Curated sequences generated in this studies are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.99p2308 (Walters et al. 2018). Average read lengths for gut content and water column libraries were 328 (227 – 400) bp and 328 (230 – 399) bp, respectively. Sequences classified using the SILVAngs rRNA sequence classification service (Quast et al. 2013) resulted in a total of 40,353 OTUs. These OTUs could be collapsed into 417 unique phylogenetic taxa that could further be classified into 17 functional prey groups containing 353 taxa and 4 major parasite groups containing 64 taxa.

The overall distribution of functional prey and parasite groups from captive-fed and wild-caught *D. gegenbauri* and from the water column samples are provided in Figure 4 A-C. Picodinoflagellates (picozoa) dominated the sequence libraries from both the captive-fed doliolids (28.7%) and the associated water samples (38%). Diatoms were the next most abundant sequences recovered from the captive-fed doliolid libraries (24.7%) but were poorly represented (2.4%) in the water column libraries. Sequences derived from metazoans, especially

hydrozoans, were also commonly retrieved and accounted for 21.5% of all sequences recovered from the captive-fed *D. gegenbauri*. Sequences classified as hydrozoans accounted for 71.1% of the metazoan sequences but sequences derived from copepods, mollusks, chaetognaths, appendicularians and fish were also recovered. Other micro-eukaryotic prey included a variety of microalgae including representatives of Charophyta, Chlorophyta, Cryptophyta, Haptophyta, unclassified flagellates and larger ciliates and radiolarians. Sequences classified as Charophyta were most likely derived from aeolian deposited tree pollen. In addition to the 200,327 prey sequences recovered from the captive-fed animals, 6,789 sequences were classified as probable parasites. Of these, fungal sequences were most common (85.4%). However, representatives of the Discoba-Euglenozoa (12.8%), Amoebozoa (1.3%), and Apicomplexa (0.5%) groups were also detected.

The distribution of classified prey groups identified in the libraries generated from the wild-caught animals exhibited a remarkably different composition than those derived from the captive-fed animals. Whereas picodinoflagellates and diatoms dominated the libraries from the captive-fed animals, these 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 metazoan groups that included chaetognaths (21.8%), mollusks (15.5%), copepods (7.6%) and cnidarians (2.1%).

Potential parasite groups identified based on recovered sequences included fungi, euglenozoid, amoebozoid and apicomplexan groups. Parasite groups recovered from captive-fed animals largely reflected the sequence representation observed in the water column samples with fungal and euglenozoids being most prevalent in both samples types (Figure 3). However, Apicomplexa sequences dominated (72%) the potential parasite libraries derived from the wild-caught animals.

There was not a consistent relationship between the composition of the available prey community (water) and what was recovered from doliolids (guts) (Figure 5). Excluding metazoans and probable parasites that are unlikely to be a component of the doliolid diet, each sequence library could be clustered into groups that were dominated either by pico-

dinoflagellates, diatoms or microzooplankton (radiolarians and ciliates). In no cases were prey communities clustered together in paired water and gut samples (Figure 5).

Examination of the ratio of sequences recovered from paired gut and the water samples suggest that, while the majority prey groups were observed at ratios not significantly different from 1.0 ($p < 0.05$) as would be expected assuming non-selective filter feeding. There were, however, several notable exceptions. Diatoms, ciliates and two groups of metazoans (hydrozoa and chaetognatha) were each over-represented in gut content samples relative to their abundance in the water indicating that these groups were selectively concentrated (Figure 6).

Contribution of Diatoms to the Doliolid Diet

To determine the quantitative importance of diatoms in the diet of *D. gegenbauri*, a general diatom-specific real time quantitative PCR (qPCR) assay was developed and utilized to determine the abundance of diatoms ingested by wild-caught *D. gegenbauri* zooids relative to their availability in the water column. A general 18S rDNA diatom-targeted primer set was designed for this purpose. The primer set consists of two diatom-specific primers including a forward primer 18SF-Diatom-487 (5'- GGTCTGGCAATTGGAATGAGAAC) and a reverse primer 18SR-Diatom-615 (5'- CTGCCA GAAATCCAACTACGAG). This diatom-specific primer pair amplifies a 128 bp fragment of the hypervariable V3 region of the 18S rRNA gene. *In silico* testing utilizing the Silva TestPrime utility (Quast et al. 2013) indicated that this primer set matches with full identity (0 mismatches) with 10.8% of all diatom species included in the Silva reference database v132. In practice, however, 18S rDNA targeted primers are generally effective in amplifying targets containing up to 3 mismatches depending on assay conditions (Frischer et al. 2017). The primer set developed here would be expected to amplify 17.9%, 34.3% and 61.1% of all diatom species in the Silva reference database (r132) allowing for 1, 2 and 3 mismatches, respectively. The primer set appears particularly well matched to amplify diatoms in the Mediophycea including the common marine genera *Skeletonema*, *Stephanodiscus* and *Thalassiosira*. Minimal cross-hybridization with other organisms is expected based on *in silico* analyses. Empirical specificity testing supported these results (Figure 7A). Empirical testing of sensitivity utilizing a cloned fragment of the 18S rRNA gene from two diatom species in a real-time qPCR format, *Thalassiosira weissflogii* and *Rhizosolenia alata*, indicated that this assay could be used to quantify as few as 10 gene copies and was linear ($r^2 = 0.99$) up to 10^7

409 copies in a qPCR assay format for *T. weissflogii*. As anticipated based on *in silico* evaluation, the
410 primer set was significantly less sensitive for *R. alata* producing linear (r^2=0.95) amplification
411 signal from 10^3 – 10^7 18S rDNA target copies (Figure 7B).

412 The concentration of diatoms in 143 wild-caught *D. gegenbauri* zooids and paired water
413 samples (available prey field) from the 10 cruises in 2015-2016 where sufficient numbers of *D.*
414 *gegenbauri* zooids were collected for diet studies was determined by qPCR. Doliolid life stages
415 included gonozooids (97) ranging in size from less than 1 mm – 10.5 mm, oozoids (2), nurses
416 (35) ranging from 1 – 20 mm and phorozooids (9) ranging in size from 4 – 12 mm. Each sample
417 was analyzed at least in triplicate. Diatom concentrations in gut samples ranged from 65 –
418 32,000 copies per animal and from 22 – 294 copies L^{-1} in the water samples. The ratio of diatom
419 18S rDNA gene copies recovered from the gut and water samples normalized on a volume-to-
420 volume basis was calculated for each doliolid zooid life stage (Figure 8). Diatom gene copies
421 were highly enriched in all zooids. Enrichment factors ranged from ~10^5-fold in large (> 8 mm)
422 gonozooids to ~10^7-fold in small (< 8 mm) gonozooids. Actively growing gonozooids (< 8 mm)
423 that typically dominate doliolid blooms in the SAB (Paffenhöfer 2013, Paffenhöfer and Köster
424 2011) exhibited significantly greater diatom concentration factors ($p < 0.001$) compared to other
425 *D. gegenbauri* zooids. In contrast, large (> 8 mm) gonozooids exhibited significantly less
426 diatom enrichment ($p = 0.003$) compared to other zooid stages suggesting that diatoms contribute
427 less to the nutrition of larger gamete producing mature gonozooids.

428

429 DISCUSSION

430 The use of PCR-based assays for qualitative detection of prey consumed by predatory
431 species has become nearly routine in the study of trophic ecology (Pompanon et al. 2012).
432 Increasingly, these methods are also being used to quantify prey consumption although
433 quantification can be considerably more challenging (Nejstgaard et al. 2008, Frischer et al.
434 2014). Results from NGS studies are particularly prone to systematic bias associated with library
435 preparation, sequencing and bioinformatics procedures and thus it is recommended that NGS
436 data be interpreted qualitatively unless appropriate controls or complimentary orthogonal
437 methods are also applied (Hardwick et al. 2017, Bista et al. 2018). In this study NGS data was

interpreted semi-quantitatively and, in the case of diatoms, the results confirmed independently by real time qPCR. Despite a healthy dose of caution, however, the usefulness MGCA tools for investigation of trophic interactions is clear. This is especially true where predator-prey interactions are complex and cryptic. These methods are therefore especially useful for marine zooplankton species that exhibit complex life histories, prey on a high diversity of microbial species and are difficult to investigate in laboratory settings (Frischer et al. 2014). One example of such a species is the pelagic tunicate *Dolioletta gegenbauri*, a bloom forming species found circumglobally on productive sub-tropical continental shelves.

Abundance and Seasonal Distribution of D. gegenbauri in the SAB

Consistent with previous reports (Deibel and Paffenhöfer 2009), over the course of this study *D. gegenbauri* was a persistent component of the mid-shelf SAB zooplankton community. During the 14 sampling expeditions where doliolid abundance was determined, doliolids were encountered on every expedition and at 23 of the 27 (85%) stations samples. There was not a significant correlation between the abundance of doliolids and season (Figure 2, $p = 0.816$). Significant blooms, however, were more likely to be observed during the mid-summer and early-winter periods in association with shelf upwelling as previously reported (Deibel and Paffenhöfer 2009). Of the 4 blooms with zooid abundance that exceeded 100 zooids m^{-3}, 3 occurred in the summer and 1 during the winter. Also consistent with previous reports, the abundance of doliolids varied greatly from being nearly absent to dominating the zooplankton biomass. For example, during the August 2016 expedition the abundance of *D. gegenbauri* zooids, largely gonozooids, was estimated to be greater than 16,000 m^{-3} and accounted for ~ 80% (relative abundance) of the total zooplankton community (López-Figueroa 2017). To our knowledge this is the largest bloom of *D. gegenbauri* that has ever been documented.

The Diet of D. gegenbauri

The availability of appropriate prey, in addition to the gross quantity of potential prey, is likely an important factor controlling the initiation and termination of doliolid blooms. For example, with the exception of the Mega bloom that was observed in August 2016, there was not a significant correlation between doliolid abundance and total chlorophyll (Figure 3, $p = 0.49$, $r^2 = 0.26$) supporting the hypothesis that the gross standing stock of phytoplankton alone is not a sufficient factor to explain the occurrence or termination of bloom events. Quantitatively

468 determining what doliolids actually consume is therefore likely to lead to a better understanding
469 of the processes involved in the formation and termination of doliolid blooms.

470 Utilizing a blocking Peptide Nucleic Acid (PNA) assay (Troedsson et al. 2008, Von
471 Wintzingerode et al. 2000) specific to *D. gegenbauri* in conjunction with a general (universal)
472 18S rDNA-targeted PCR primer set, it was possible to directly determine the diversity of prey
473 ingested by *D. gegenbauri* in both laboratory and field studies. Because prey DNA is poorly
474 digested by *D. gegenbauri* (Frischer et al. 2014), it was also possible to quantify diatoms
475 utilizing a real time quantitative PCR approach with a diatom group-specific primer set.
476 Comparison of Next Generation Sequencing 18S rDNA amplicon libraries from continental shelf
477 water (available prey field) with wild-caught animals and cultured animals exposed to shelf
478 water yielded new insights into the diet of *D. gegenbauri*. As expected, the diversity of ingested
479 prey was high reflecting the diversity of marine eukaryotic microbial communities typical of
480 continental shelf surface waters. Surprisingly, *D. gegenbauri* appeared to have selectively fed on
481 larger-sized prey groups including diatoms, ciliates and, several groups of metazoans relative to
482 their concentration in the water column (Figure 6). As a filter feeding animal capable of
483 capturing a wide range of particle sizes (Tebeau and Madin 1994), *D. gegenbauri* and other
484 doliolid species are understood to be passive rather than selective feeders (Katechakis et al.
485 2004). Therefore, it is not obvious how selective feeding was accomplished.

486 To our knowledge selective feeding behavior by *D. gegenbauri*, either *in situ* or under
487 experimental conditions, has not been previously investigated. Selective feeding, however, has
488 been reported in other pelagic tunicate species including salps (Metfies et al. 2014) and
489 larvaceans (Conley et al. 2017). Using similar molecular gut content analysis approaches
490 Metfies et al. (2014) demonstrated that two different salp species, *Salpa thompsoni* and *Ihlea*
491 *racovitzai*, had different diets despite being sympatric in the Southern Ocean. It was speculated
492 that these dietary differences may be due these species occupying different locations (depths) in
493 the water column. Conley et al. (2018) reported that the larvacean *Oikopleura dioica*, is able to
494 selectively capture and retain prey based not only on size but their shape. Selective feeding has
495 also been reported in many other gelatinous predators including scyphozoan jellyfish and
496 ctenophores that have long been considered to be passive feeders (e.g. Marques et al. 2015,

Álvarez-Tello et al. 2016, Zeman et al. 2016). In general, most gelatinous predators are now understood to exhibit some degree of feeding selectivity (Purcell 1997).

In doliolids, one possible mechanism of feeding selectivity may involve the ability of doliolids to take advantage of micro-heterogeneity and micro-layers in the distribution of micro-plankton. Unlike salps, doliolds are able to feed while motionless and therefore may be able to take advantage of prey-field heterogeneity created by a variety of physical and biological processes (Mouritsen and Richardson 2003, Durham et al. 2013). Feeding in patches of concentrated prey would explain how doliolids might be capable of concentrating prey relative to concentrations estimated from bulk water samples. A second mechanism that may lead to feeding selectivity is the ability of doliolids to stop and even reverse their feeding currents (Deibel and Paffenhöfer 1988). Although the ability of doliolids to select or reject specific prey has not been rigorously investigated, it has been observed that doliolds are capable of rejecting some types of particles including plastic microfibers (personal observations – https://youtu.be/cgVMDUZO7kg). The ability of doliolids to adjust their feeding currents may facilitate the ability to avoid or reject undesirable prey.

Alternatively, apparent feeding selectivity inferred from the comparison of water and doliolid samples may be an artifact of Molecular Gut Content Analysis (MGCA) prey DNA-based detection methodologies. Compared to water samples, prey DNA recovered post-ingestion may have been impacted by host mediated digestion or amplification interference associated with co-purified doliolid substances. PCR amplification bias is well known to occur, especially when general “universal” primers are utilized due to differences in primer targeting specificity, efficiency and amplification interference by complex amplification substrates (Polz and Cavanaugh 1998, Brooks et al. 2015, Elbrecht and Leese 2015). Variation in prey-species gene target copy abundance and differential DNA digestion may also contribute to bias associated with the detection of prey types (Nejstgaard et al. 2008, Troedsson et al. 2009). However, at least with respect to the enrichment of diatoms, selectivity was also supported by real time qPCR studies that utilized a diatom specific primer set and therefore would be expected to be less influenced by these types of biases. Quantitative estimates of diatom gene copy numbers normalized by volume in wild-caught doliolids and in the water column from where they were captured indicated that diatom genes were concentrated up to 10 million times in doliolids

relative to the concentration estimated present in feeding water (Figure 8). Although all zooid life stages appeared to concentrate diatoms, smaller (< 8 mm) actively growing gonozooids exhibited the highest concentration factors. Additionally, the abundance of actively growing gonozooids was positively correlated with the > 8 µm fraction of total chlorophyll a suggesting that this fraction of the phytoplankton community is associated with the growth of *D. gegenbauri* (Figure 3, $p < 0.05$). The > 8 µm chlorophyll a size fraction is typically composed of diatoms in SAB waters (Verity et al. 1993). Actively growing gonozooids are associated with bloom conditions and therefore this observation is consistent with the hypothesis that diatom production is a likely factor associated with upwelling stimulated doliolid blooms (Paffenhöfer and Köster 2005).

The Diet and Parasites of Wild-Caught Versus Cultured D. gegenbauri

Significant differences were observed between the distribution of prey types detected in captive-fed and wild-caught *D. gegenbauri* gonozooids (Figure 4). Whereas the diet of captive-fed animals was dominated by nano- and micro-plankton, collections of recovered sequences from the wild-caught animals was dominated by larger prey. One possible explanation for these differences is that the experimental procedures involved with captive feeding were responsible. These procedures included 1) raising *D. gegenbauri* in captivity fed on a diet of nano- and micro-phytoplankton and 2) exposing animals to natural prey fields in small (1.9 L) containers for a relatively short period of time (4 hours). Both these factors are likely to have favored the ingestion of smaller over larger prey types. It seems particularly likely that the small volume of the feeding containers and short feeding period resulted in biases that favored more abundant micro-plankton over relatively rare larger prey types. Alternatively, it is possible that the detection of larger prey, including metazoans, in the wild-caught animals, is an artifact of high gene copy numbers and preservation of their DNA signatures once captured by a doliolid. Despite the absolute differences observed in the quantities of prey consumed based on the captive feeding and wild-caught approaches, both approaches indicated that diatoms were selectively enriched from the water column. This conclusion was supported and strengthened based on quantitative qPCR-based estimates of diatom abundance in wild-caught animals.

The distribution of parasite classified sequences was also different between the captive-fed and wild-caught animals. Fungal sequences were the most common parasite sequences

recovered from the cultivated and captive-fed animals and reflected the relative abundance of fungal sequences in the water column. In contrast, sequences classified as Apicomplexa were the most common parasite sequences recovered from wild-caught animals and were enriched relative to their abundance in the water column amplicon libraries. The Apicomplexa phylum is closely related to the Alveolata taxonomic group including the dinoflagellates and ciliates (Yoon et al. 2008). All known Apicomplexa species are obligate intracellular parasites (Morrison 2009). Little is known about the parasites of *D. gegenbauri* but a broad range of protist parasites have been reported in other pelagic tunicates including pyrosomes, larvaceans and salps (Harbison 1998). Lombard et al. (2010) reported the common presence of *Strombidium* sp. ciliates associated with the larvacean *Oikopleura dioica*. The observation that Apicomplexa sequences were enriched in *D. gegenbauri* relative to their concentration in the water column suggests that *D. gegenbauri* is parasitized by this group of parasites.

Because all previous studies that have explored the diet of doliolids have been based on cultivation-dependent laboratory studies or indirect observations of plankton communities in the field, an important motivation of these studies was to compare MGCA determined results from comparable cultivation-dependent and –independent experiments. Based on these studies it is not possible to determine if one method, experimental feeding versus wild collection, better reflects the actual *in situ* diet of these animals, but it is prudent to consider that regardless of the method utilized, methodological artifacts related to experimental design can be introduced. In general, minimizing handling artifacts is likely to result in more accurate observations. With respect to parasites it does appear that examining wild-caught animals are more likely to reflect natural parasitic interactions rather than artifacts associated with culture conditions.

Conclusions

Gelatinous zooplankton comprise a ubiquitous component of all marine systems yet much remains to be learned about their importance, role and ecology. This is well recognized to be due, in part, to the difficulties in sampling and studying this group of delicate and difficult to culture animals. Emerging new MGCA approaches are becoming a useful tool for identifying and quantifying trophic interactions, especially in complex and often cryptic environments typified by marine systems. In this study the ingestion of prey and the presence of potential parasites was examined in the doliolid *D. gegenbauri* utilizing MGCA tools. Perhaps most

interestingly, these studies revealed that *D. gegenbauri* is potentially capable of selective feeding and that bloom dynamics may depend on the composition of prey fields, specifically the availability of suitable diatom species. These observations suggest that by feeding opportunistically doliolids are able to sustain themselves under suboptimal conditions by feeding generally while still able to selectively enrich their diet on nutritious prey items when available. Novel parasitic interactions were also observed suggesting that parasitic interactions may also be an important factor influencing doliolid bloom dynamics.

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FIGURE LEGENDS

Figure 1. Life history of *D. gegenbauri*. Figure re-drawn based on Braconnot (1971), Deibel (1998), Deibel and Lowen (2012), Paffenhöfer and Köster (2011) and Paffenhöfer and Gibson (1999).

Figure 2. Abundance of *D. gegenbauri* (all zooids) on the mid-continental South Atlantic Bight continental shelf during 14 cruises from August 2015 – December 2016. Doliolids were observed on every occasion, half of the time (7 of 14) at bloom levels. Blooms were recognized as periods when zooid abundance exceeded 25 m^{-3} and multiple life history stages were present. Moderate bloom (25-99 m^{-3}), Super bloom (100 – 999 m^{-3}), Mega bloom (> 1000 m^{-3}).

Figure 3. Relationship between *D. gegenbauri* zooid abundance on the SAB mid-shelf, total and > 8 µm chlorophyll fraction. The abundance of doliolids was not correlated with total chlorophyll a (★). The relative proportion of the > 8 µm chlorophyll fraction

was significantly correlated ($p < 0.05$) with the abundance of actively growing gonozooids (< 8 mm) typical of a developing doliolid bloom. The > 8 µm chlorophyll a fraction is largely composed of diatoms.

Figure 4. Relative proportion (%) of classified 18S rDNA sequences recovered from (A) captive-fed *D. gegenbauri* gonozooids, (B) wild-caught *D. gegenbauri* gonozooids and (C) water samples. All samples were pooled from 2011 and 2012 summertime cruises on the mid-continental shelf of the South Atlantic Bight.

Figure 5. Composition of prey species in captive-fed and wild-caught *D. gegenbauri* gonozooids and paired water samples. Metazoans and parasite sequences are not included. The relative abundance (%) of sequences in each sample is indicated by shading in the heatmap. Samples are grouped by similarity.

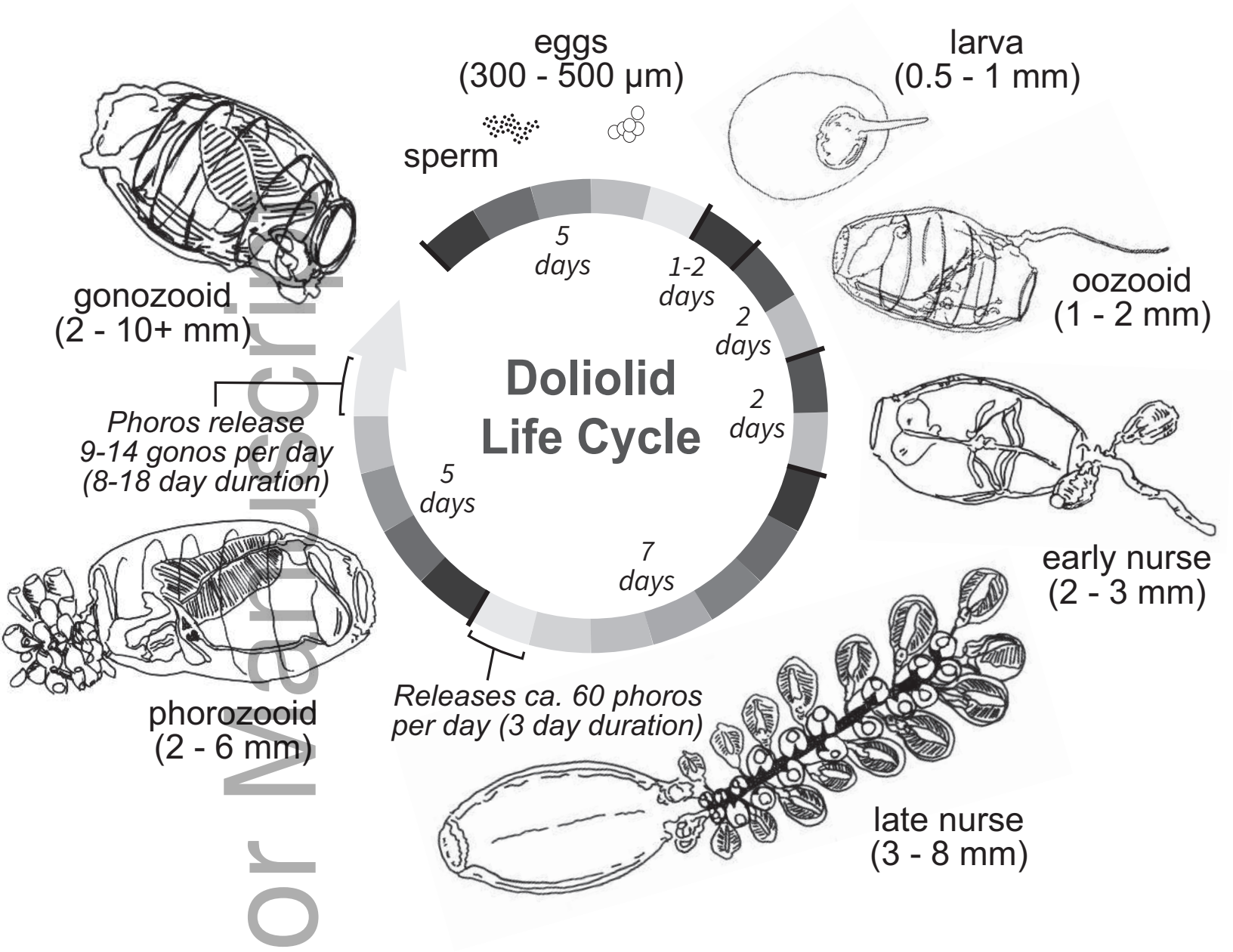
Figure 6. Relative proportion (by volume) of prey and parasites gene copies in the gut vs. water column of paired captive-fed *D. gegenbauri* gonozooids and water samples. Diatoms, ciliates, copepods and chaetognaths were each concentrated in doliolids relative to available prey concentrations. Reference lines (- - -) indicate 1 standard deviation from the mean of the gut to water ratio for each prey and parasite type.

Figure 7. Empirically determined specificity (A) and sensitivity (B) of the diatom-specific primer set 18SF-Diatom-487 (5'- GGTCTGGCAATTGGAATGAGAAC) 18SR-Diatom-615 (5'- CTGCCA GAAATCCAACTACGAG). Specificity was assessed by end-point PCR against cloned full length 18S rDNA fragments from a variety of representative algae and *D. gegenbauri*. Lane 1, *Thalassiosira weissflogii*; Lane 2, *Rhizosolenia alata*; Lane 3, *Isochrysis galbana*; Lane 4, *Emiliania huxleyi*; Lane 5, *Eucalanus pileatus*; Lane 6, unidentified copepod; Lane 7, *Rhodomonas* sp.; Lane 8, *Cryptomonas* sp.; Lane 9, Picodinoflagellate clade I; Lane 10, Picodinoflagellate clade VII; Lane 11, *Dolioletta gegenbauri*; Lane 12, no template (control); MW 100 bp molecular weight ladder. Sensitivity was assessed by qPCR utilizing plasmid standards of cloned full-length 18S rDNA fragments from *T. weissflogii* and *R. alata*.

Figure 8. Ratio of volume normalized gut and water concentration of diatom 18S rDNA gene copies recovered from each life history stage of *D. gegenbauri*. Diatom genes were

enriched in all zooids with the highest concentration factors observed in actively growing gonozooids.

Figure S1. Empirical determination of the PCR blocking efficiency of the 18S rDNA targeted doliolid-specific Peptide Nucleic Acid (PNA) oliogonucleotide Dg 18S-667F (5' Lysine-GGC CAA TGC AGC CTG TG). A plasmid cloned fragment of nearly the complete 18S rRNA gene from *D. gegenbauri* was amplified in the (A) presence and (B) absence of the doliolid-specific blocking PNA Dg18S-677F utilizing universal 18S rDNA targeted primers Univ 18S-557F (5'- CCC GTG TTG AGT CAA ATT AAG C -3') and Univ 18S-1180R (5'- CAG CAG CCG CGG TAA TTC C -3'). This primer set generates a ~630 bp product. Products produced after 30 cycles of PCR amplification were visualized via electrophoresis on a 2% agarose gel. Lane 1, molecular weight ladder (100 bp); lanes 2–8, serial dilution from 10^8 to 10^2 copies of the 18S rRNA gene from *D. gegenbauri*. Approximately four orders of magnitude of blocking were achieved.



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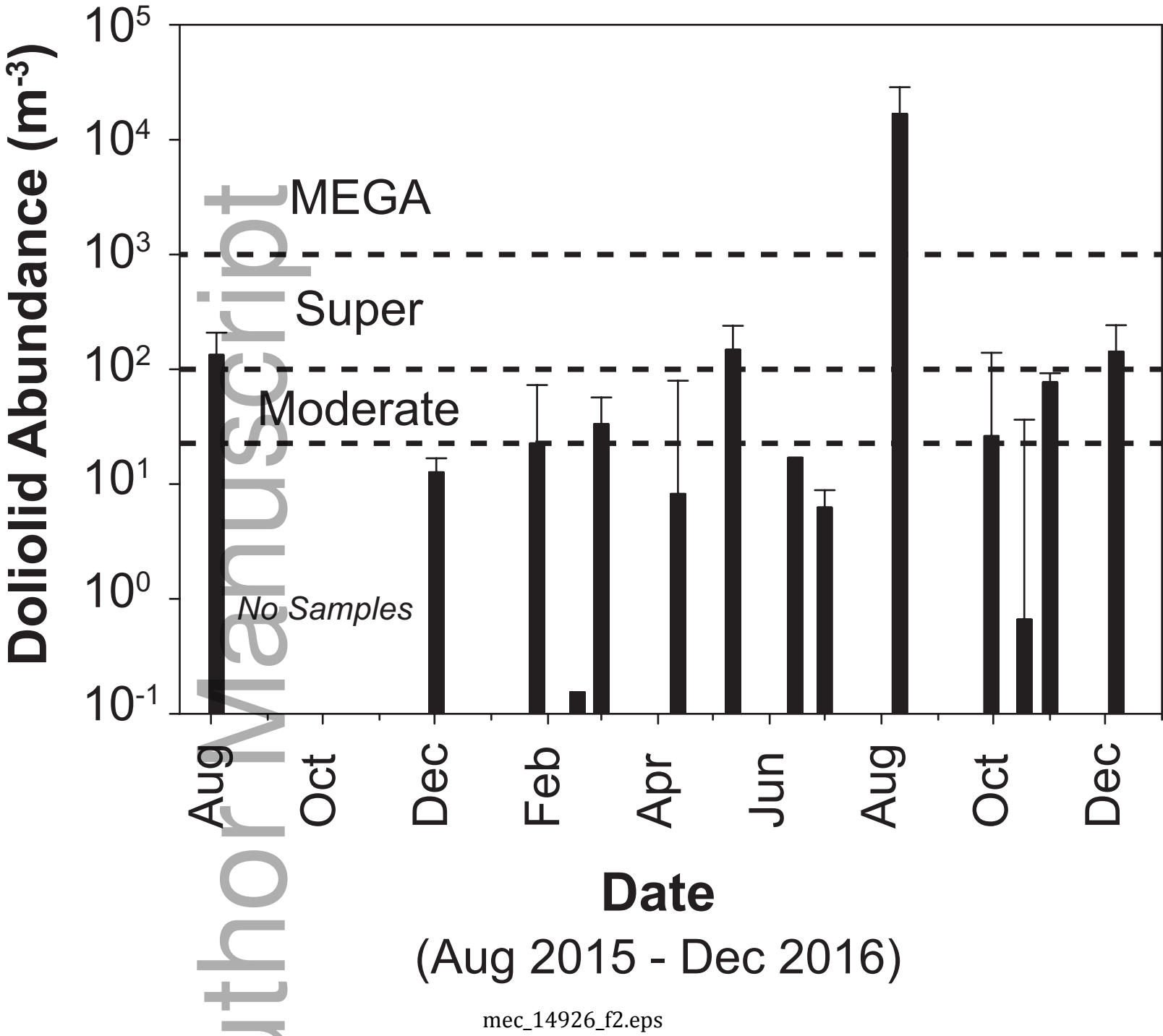

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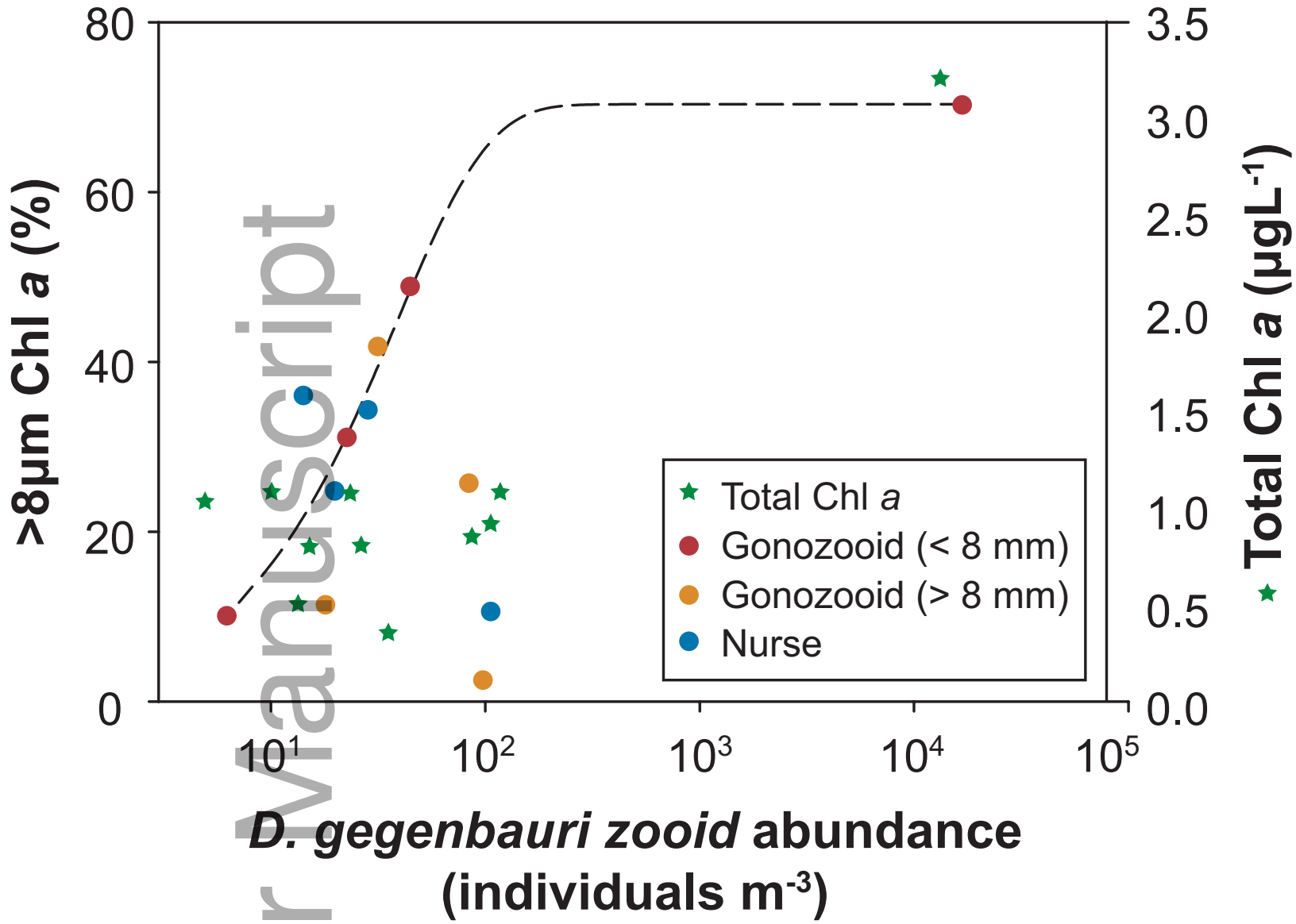

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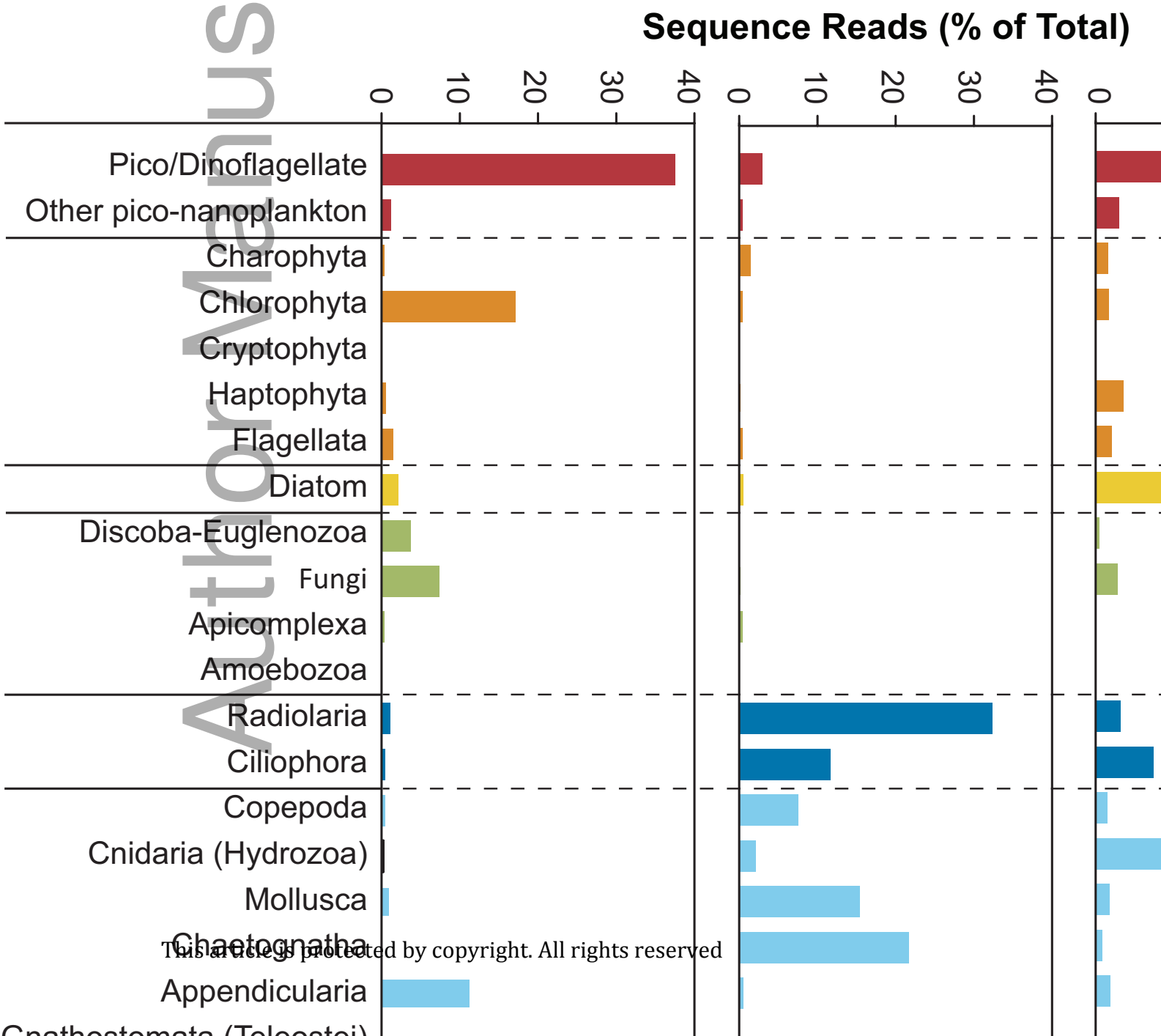

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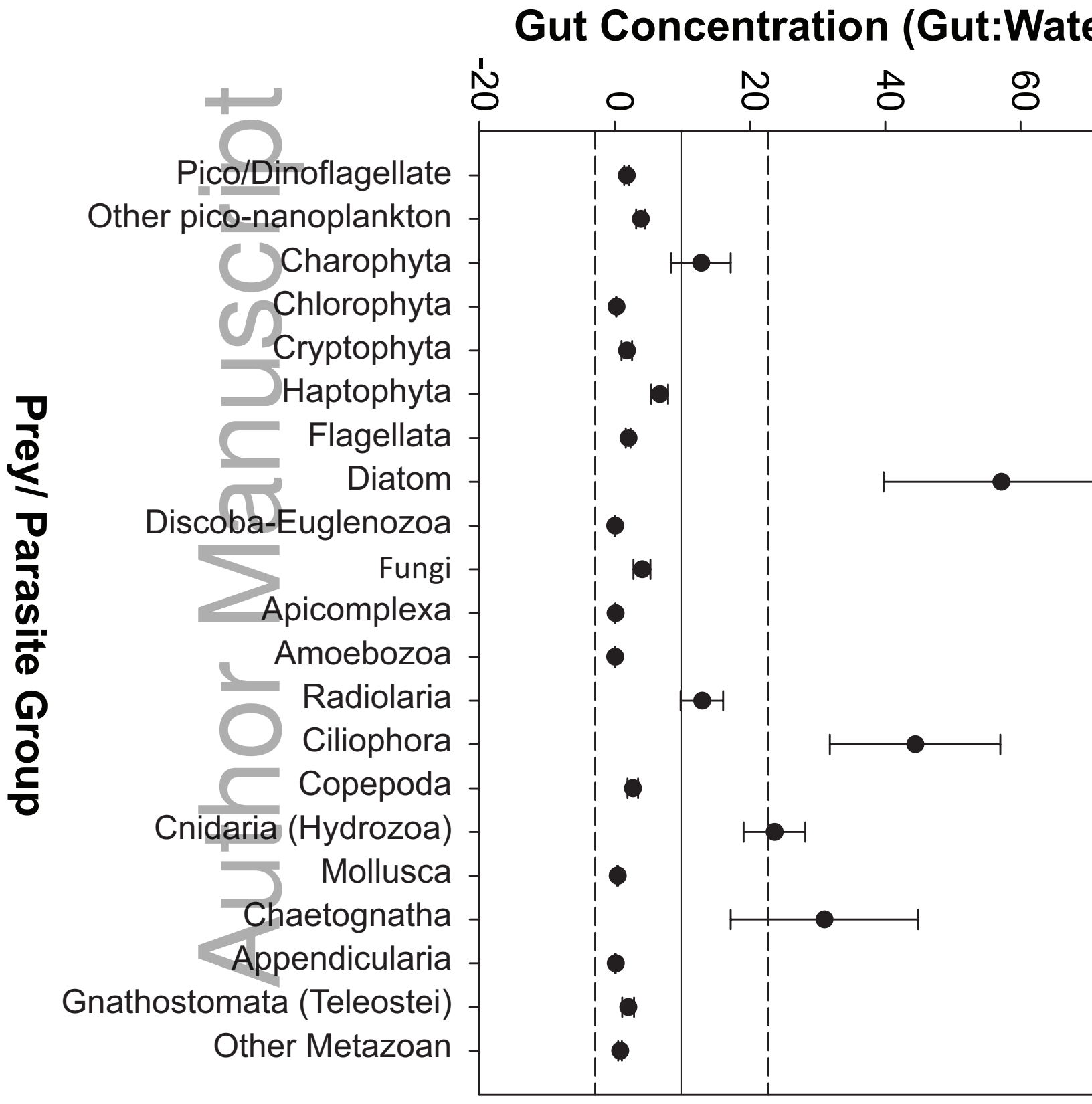

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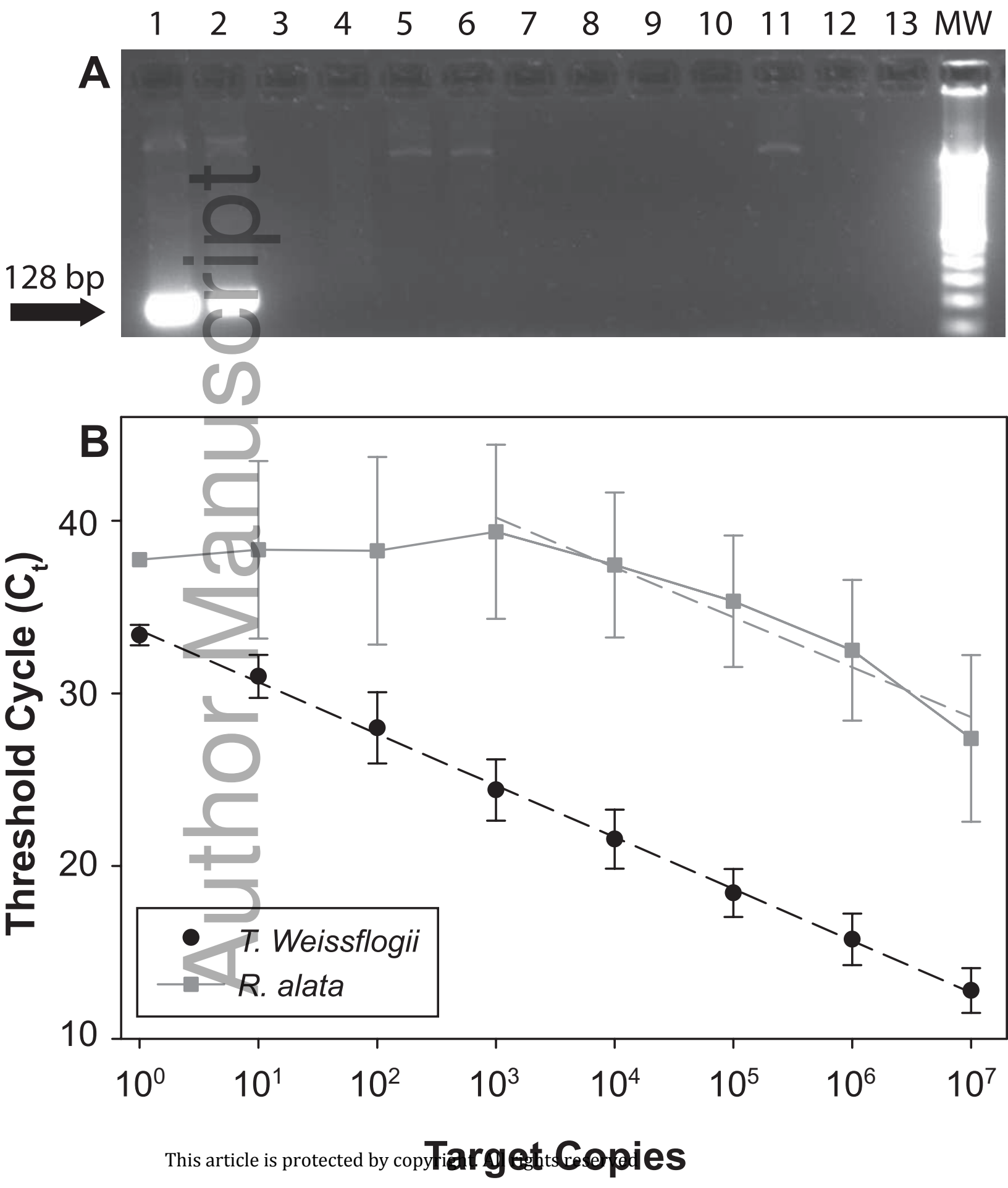

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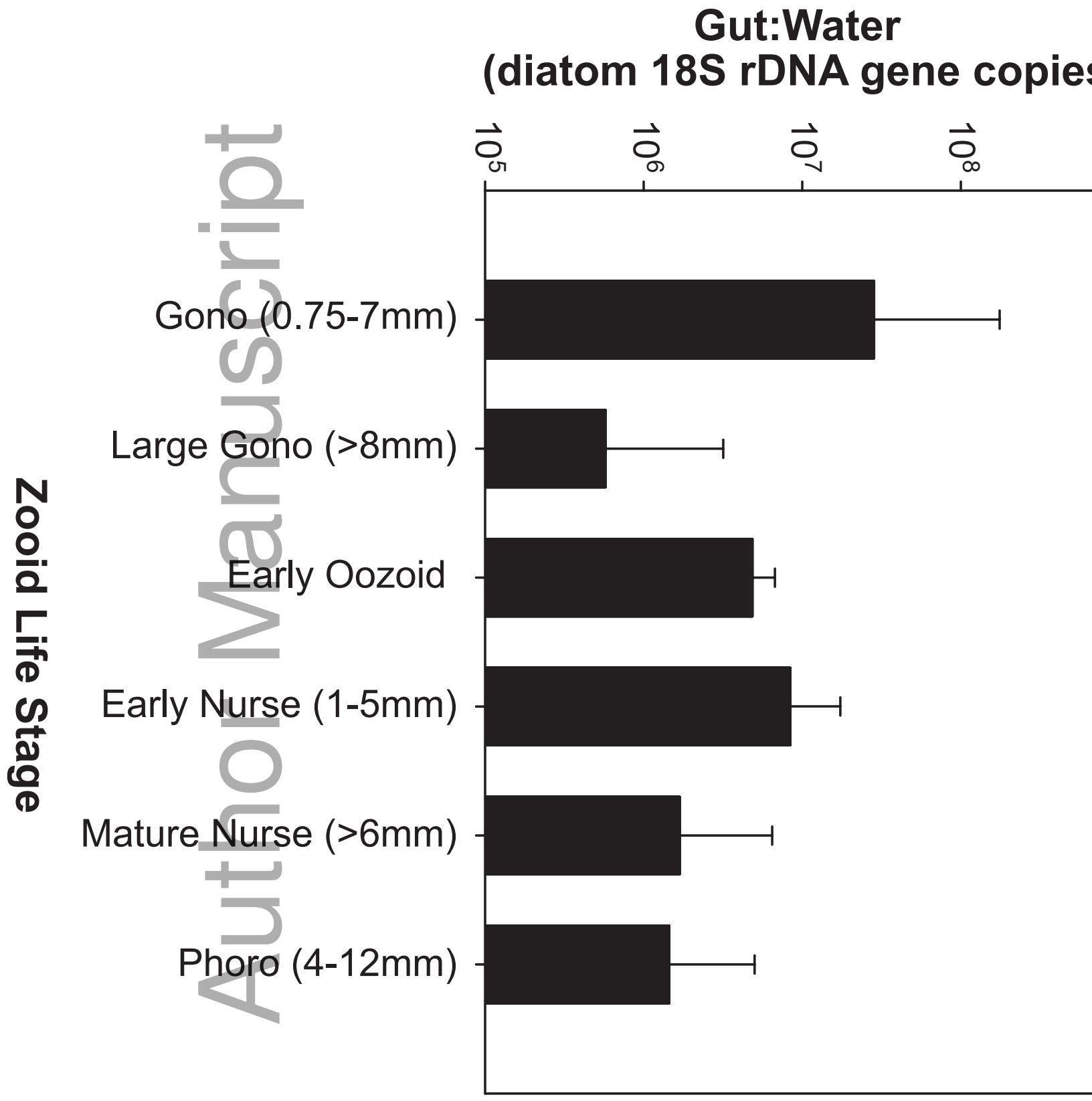

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