JPR Advance Access published October 14, 2013

Journal of
Plankton Research

plankt.oxfordjournals.org

J. Plankton Res. (2013) 0(0): 1–5. doi:10.1093/plankt/fbt104

SHORT COMMUNICATION

A DNA-based method for investigating feeding by copepod nauplii

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Received July 26, 2013; accepted September 13, 2013

Corresponding editor: Roger Harris

We developed an approach to recover prey DNA from gut contents of nauplii of a predatory copepod by blocking the detection of predator with ligase and a blocking oligonucleotide and amplifying prey DNA with universal primers. Adding 25 U of ligase improved predator clamping, while 100 and 200 U reduced efficiency. Field-collected nauplii consumed a broad range of prey. The method paves the way to investigations of the diets of small predators at all life stages.

KEYWORDS: *Tortanus dextrilobatus*; gut contents; diet analysis; LNA clamp; blocking oligonucleotide

Copepod nauplii are arguably more abundant than any other multicellular animal in the sea (Fulton, 1984; Humes, 1994), yet relative to adults, little is known about their feeding. Although nauplii are a key part of the copepod life cycle and the plankton, current conceptual models of aquatic foodwebs may inaccurately assume nauplii feed similarly to adults. The upper size limit of prey is apparently smaller for nauplii than for conspecific adults (Vogt *et al.*, 2013) because nauplii have a greatly reduced feeding apparatus and are much smaller.

Studying feeding by nauplii in the wild is challenging with available methods, each of which imposes certain biases. Gut fluorescence techniques detect only pigmented prey and have limited taxonomic resolution. Microscopic

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inspection of gut contents is best for detecting prey with hard parts and would be difficult with nauplii because they are small. DNA-based techniques, which identify prey from trace amounts of DNA in the gut or remains, may be the best means to characterize feeding by nauplii in nature. These techniques have been applied to adult copepods (Nejstgaard *et al.*, 2003), and here we adapted the approach to nauplii.

Because little is known about naupliar feeding *in situ* and how it may vary seasonally and annually, we chose a method that required minimal prior knowledge about prey. Briefly, the approach used universal primers to amplify prey DNA and a predator-specific blocking oligonucleotide (oligo) to inhibit amplification of predator DNA, which would otherwise overwhelm the signal of prey during PCR (Vestheim and Jarman, 2008; Vestheim *et al.*, 2011). Similar methods have been successfully applied to other small predators including krill (Vestheim and Jarman, 2008; Cleary *et al.*, 2012), lobster larvae (O'Rorke *et al.*, 2012) and bivalve larvae (Maloy *et al.*, 2013), although none as small as copepod nauplii.

Here we applied the approach to nauplii of the copepod *Tortanus dextrilobatus*, the most abundant predatory copepod in the San Francisco Estuary, which preys on other copepods as an adult (Hooff and Bollens, 2004). A predatory copepod was of particular interest because it would be expected to undergo significant changes in diet between the larval and adult stages, effectively occupying two niches in its lifetime.

Nauplii were collected in seven sampling events from four sites in the San Francisco Estuary, USA, at salinities ranging from 10.6 to 19.3. Plankton tows were brief (3–5 min) to minimize the time copepods spent in the net cod end. Samples were preserved in 95% ethanol immediately after the tow and stored at −20°C at the laboratory.

Late-stage *T. dextrilobatus* nauplii (NIV-NVI) were isolated from field samples and rinsed by transfer through three successive dishes of 95% ethanol. Later samples were also rinsed over a sieve with vigorous squirts of ethanol to reduce the potential for carry over of extraneous debris.

Initial tests with 1, 2, 5, 10, 20 and 100 nauplii hatched in the laboratory and fed *Tetraselmis suecica* or *Prorocentrum minimum* indicated that the groups of 20 and 100 nauplii yielded sufficient prey DNA (data not shown). From field samples, nauplii from the same plankton tow were pooled into groups of 100 for DNA extraction.

Cleaned nauplii were collected on autoclaved 20-μm Nitex and placed with the Nitex into a tube for DNA purification (QIAamp DNA Microkit, Qiagen). Manufacturer's instructions were followed with two modifications: 8 μg of carrier RNA was added per reaction instead of 1 μg (Durbin *et al.*, 2008) and the incubation with 20 μL elution buffer was increased from 5 to 30 min (Eberl *et al.*, 2007).

An approximately 350 nt, variable portion of 18S was amplified using forward primer 14 adapted from Hendriks *et al.* (Hendriks *et al.*, 1991) and reverse primer 18SCom1R modified from Zhang *et al.* (Zhang *et al.*, 2005). A third oligo, the blocking oligo TortStop1, was designed to bind to predator DNA at a site where predator sequence differed from prey sequence in several neighboring positions, based on a sequence alignment of *T. dextrilobatus* with representatives from potential prey groups. The selected site was 52 nt downstream of the forward primer (Fig. 1) and acted by arresting polymerization of the extending strand, i.e. elongation arrest (Vestheim and Jarman, 2008). We modified the elongation arrest by addition of DNA ligase, which permanently binds extending strands of *T. dextrilobatus* DNA to the blocking oligo during PCR (McCoy and Palumbi, 2007), preventing partial amplicons of predator DNA from acting as primers in later cycles of the PCR (Fig. 1). Ligase binds only pieces of DNA that are correctly base paired at the junction (Landegren *et al.*, 1988) and is sensitive to single nucleotide mismatches. The blocking oligo was therefore designed with mismatches to prey sequence at the 5′ end, where ligation would occur, to prevent binding to prey DNA which would inhibit prey amplification in later cycles.

The blocking oligo also contained seven locked nucleic acids (LNAs), RNA molecules modified into a locked conformation that increases the melting temperature of the oligo (Obika *et al.*, 1997; Koshkin *et al.*, 1998), making its binding more discriminatory between predator and prey. Blocking oligos of this type are also called LNA clamps. The blocking oligo was purified by high-performance liquid chromatography, used a C3 spacer as the blocking molecule on the 3′ end of the oligo, and was phosphorylated on the 5′ end for the ligation component of the reaction.

Fig. 1. The blocking oligo, TortStop1, binds downstream of the universal forward primer and is ligated to the extending strand by DNA ligase. TortStop1 contains three sites that make the oligo specific to *T. dextrilobatus* (nucleotides under asterisks), locked nucleic acids (underlined nucleotides), a C3 spacer as the blocking molecule, and a phosphorylated 5′ end for ligation.

PCRs were carried out in 25 μL volumes with 1× Stoffel buffer, 1× 9°N ligase reaction buffer, 4.0 mM $MgCl_2$, 0.8 mM dNTPs, 1 μg BSA, 0.1 mM of the forward and reverse primers (Exiqon), 0.4 mM of the blocking oligo (Exiqon), 0.05 enzyme units (U) AmpliTaq® DNA Polymerase Stoffel Fragment (Applied Biosystems), 25 U 9°N™ DNA Ligase [(NotI-HF restriction enzyme (NEB)] and 1 μL template DNA (100–300 ng). The amplification primers were 5′-GGTCTG TGATGCCCTTAGA-3′ (14_M_3) and 5′-CCTACGGA AACCTTGTTACG-3′ (18SCom1R_M_3). The thermal cycling protocol consisted of a denaturing step of 95°C for 1 min followed by 35 cycles of 95°C for 30 s and 60°C for 3 min (combined annealing and extension step). The method was designed to detect trace amounts of DNA, which made it sensitive to contamination, most of which was avoided following techniques of Champlot *et al.* (Champlot *et al.*, 2010).

Clone libraries of the PCR amplicons were created with the TOPO TA Cloning Kit or the pGEM-T Vector System. To assess the number of colonies needed to capture taxon richness, a rarefaction curve was generated with 41 sequences obtained from the first set of nauplii (data not shown). Based on the results of this analysis, in subsequent samples the plasmid DNA from an average of 40 *E. coli* colonies was examined.

Sequences were identified using the BLASTN algorithm on GenBank or by comparison with a sequence library of local copepod species constructed for this project. BLASTed sequences were identified to the taxonomic level at which there was consensus among matches within 10% of the best score.

The method was evaluated in tests with artificial mixtures of DNA from *T. dextrilobatus* and a known prey of adult *T. dextrilobatus*, the copepod *Oithona davisae*. To prevent artifacts that can arise during re-amplification of PCR products, template DNA was generated by inserting PCR amplicons into bacterial plasmids (Vestheim *et al.*, 2011): predator and prey were PCR amplified individually with the universal primers, the PCR products were cloned and the plasmids were linearized with a NotI-HF restriction enzyme (NEB). The linearized plasmids were used in the following tests: (i) to check for unintended blocking of prey DNA, amplification of prey DNA was tested in PCR with and without the blocking oligo and (ii) to test the strength of blocking, amplification of 0.25 pg prey DNA was tested with predator DNA present in increasing amounts, 1 part prey to 1 part predator, 1:10, 1:100, 1:1000, $1:10^4$, $1:10^5$ and $1:10^6$. PCR products were directly sequenced, and ratios that appeared to have both template types (double peaks in chromatogram) were tested again with different amounts of ligase (0, 25, 100, and 200 U) to determine if more or less ligase would improve the blocking of predator. These PCR products were cloned, and the plasmid DNA from 10 *E. coli* colonies was sequenced.

In these tests, the blocking oligo was highly specific to *T. dextrilobatus* and showed no inhibition of prey amplification. In mixtures of predator and prey DNA, complete blocking of predator DNA was possible up to a prey:predator ratio of $1:10^4$. Above this predator concentration, 25 U ligase improved blocking, with predator DNA comprising 10% of recovered sequences at the $1:10^4$ ratio vs. 35% without ligase (Fig. 2). Increasing ligase reduced blocking efficiency, although the reason for this is unclear. Neither predator nor prey DNA was detected at the $1:10^6$ ratio. These results are likely affected by a second variable, among-treatment variation in the concentration of DNA, a factor known to affect the success of PCR. Repeating the experiment with the DNA concentration held constant would better test the efficiency of clamping. However, the results still provide a useful empirical estimate of the amount of prey DNA in wild nauplii and the amount of ligase to use for this system.

From field samples, 302 sequences were obtained from seven samples of 100 nauplii. Predator DNA was detected in all samples except one but represented a minority (2–28%) of sequences in these samples (Fig. 2). The ratio of prey to predator DNA in wild nauplii of this species appears to be $\sim 10^{-4}$–10^{-5}, well within the range of the blocking oligo's ability to block amplification of predator DNA. Sequences of predator DNA were PCR artifacts, consisting of two fragments of *Tortanus* DNA spliced together, in almost every case. The fragments did not overlap with the LNA-clamp binding

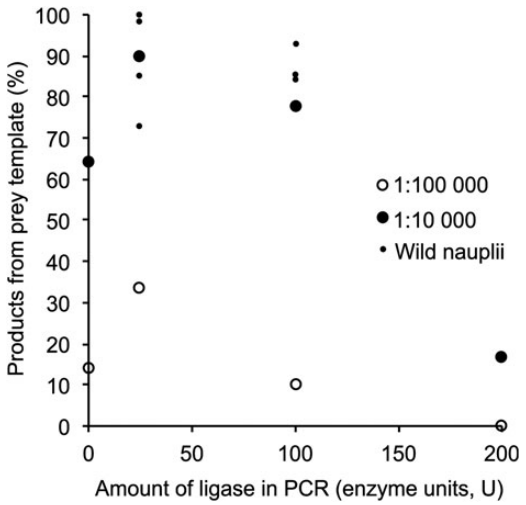

Fig. 2. Performance of the blocking oligo with increasing amounts of ligase and at two ratios of prey to predator DNA concentration. Data are also shown for wild nauplii at 25 and 100 U of ligase.

region and so the recombinant piece was missing the blocking oligo-binding region.

Prey sequences were identified most often to order, except for local copepods, which were identified to species using our barcode library. The most consistently recovered taxa were other copepods, ciliates and dinoflagellates (Table I). Of the ciliates, tintinnids were common, followed by those in the subclass Oligotrichia and *Myrionecta* sp., the phototrophic ciliate. Diatoms in several groups (Coscinodiscophyceae, Fragilariophycidae, Thalassiosiraceae) were detected in three of seven samples. Less common taxa included platyhelminthes, polychaetes, rotifers, bivalve and gastropod mollusks, barnacles, larvaceans and fish. Potential prey spanned a considerable size range, as found for nauplii of two other copepod species fed on cultured phytoplankton (Vogt *et al.*, 2013). The large size range may reflect feeding on juveniles, eggs or pieces of larger organisms that nauplii encounter, or a shift in consumption from smaller to larger prey by the naupliar stages pooled in this study (NIV-NVI, 225–350 μm in length). It may also indicate that further improvements are needed to minimize the detection of exogenous DNA (O'Rorke *et al.*, 2013). Fungus detected in one sample, representing 6% of the sequences in that sample, could have come from fungi associated with the copepod, laboratory contamination or detritus collected with the sample or ingested by the animals.

Table I: Taxa recovered from field-collected nauplii

Category	Taxon	Level of ID	Count
Cryptophyte	Cryptophyta	Phylum	2
	Teleaulux	Genus	1
Dinoflagellate	Dinophyceae	Class	4
	Peridiniales	Order	1
Diatom	Coscinodiscophyceae	Class	1
	Fragilariophycidae	Subclass	1
	Thalassiosiraceae	Family	2
Ciliate	Choreotrichia	Subclass	1
	Myrionecta	Genus	2
	Oligotrichia	Subclass	3
	Tintinnida	Order	5
Copepod	*Acartia*	Genus	3
	Calanoida	Order	1
	Harpacticoida	Order	2
	Limnoithona tetraspina	Species	2
	Oithona davisae	Species	3
	Pseudodiaptomus marinus	Species	1
Platyhelminthes	Macrostomida	Order	1
	Polycladida	Order	1
Polychaete	Phyllodocida	Order	2
	Polychaeta	Class	1
Mollusk	Euthyneura	Clade	1
	Heterodonta	Subclass	2
Rotifer	Monogononta	Slass	1
Barnacle	Cirripedia	Infraclass	1
Larvacean	Oikopleuridae	Family	1
Fish	Neopterygii	Subclass	1
Fungus	Corticiaceae	Family	1
Vascular plant	Liliopsida	Class	1
Predator	*Tortanus dextrilobatus*	Species	6

Here we report the number of samples that contained each taxon out of seven total plankton samples.

In conclusion, the DNA-based approach we developed detected a wide range of potential prey consumed by nauplii *in situ*. The strength of this technique lies in its ability to survey for many prey taxa with universal primers, allowing the detection of unanticipated prey and saving effort by requiring only one PCR per sample. Because this technique can also be used on adults and juveniles of the same species without modifications, it is ideal for carnivorous species that may undergo the most striking ontogenetic shifts in diet, changes that would most expand our understanding of a species' niche. The method may be readily applied to other copepod species because the blocking oligo binds to a region that differentiated between copepods, a good region for species-specific blockers. Finally, the addition of ligase improved blocking, which could be useful when it is difficult to detect prey, e.g. in small predators containing small amounts of prey DNA, and when it is important to block as much predator as possible, e.g. if clone libraries rather than next-generation sequencing are used. Overall, these tools have the potential to contribute to understanding the significant and largely neglected role of nauplii in aquatic food webs.

ACKNOWLEDGEMENTS

We gratefully acknowledge A. McCoy, T. Ignoffo, A. Slaughter, L. Sullivan, J. Donald, E. Carpenter, E. Lau, F. Cipriano and four anonymous reviewers.

FUNDING

This work was supported by the National Science Foundation [0929075 to W.K. and C.S.C., 0435033 to C.S.C.] and SFSU support to the RTC Molecular facility. This is contribution number 31 for the RTC Molecular facility.

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