Project: Feeding and food limitation in copepod nauplii, the neglected life stage

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Sampling and Analytical Methodology:

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 minutes) 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^{o}C at the laboratory.

Late-stage *Tortanus 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 lab and fed *Tetraselmis suecica* or *Prorocentrum minimum* indicated that 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 minutes (Eberl *et al.*, 2007).

A 350 nt, variable portion of 18S was amplified using forward primer 14 adapted from Hendriks *et al.* (1991) and reverse primer 18SCom1R modified from 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 nucleotides downstream of the forward primer and acted by arresting polymerization of the extending strand, i.e. elongation arrest (Vestheim & 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 & Palumbi, 2007), preventing partial amplicons of predator DNA from acting as primers in later cycles of the PCR. 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 (Koshkin *et al.*, 1998; Obika *et al.*, 1997), 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 (HPLC), 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.

PCRs were carried out in 25 µl volumes with 1X Stoffel buffer, 1X 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 (NEB), and 1 µl template DNA (100-300 ng). The amplification primers were 5'-GGTCTGTGATGCCCTTAGA -3' (14_M_3) and 5'-CCTACGGAAACCTTGTTACG-3' (18SCom1R_M_3). The thermal cycling protocol consisted of a denaturing step of 95°C for 1 minute followed by 35 cycles of 95°C for 30 seconds and 60°C for 3 minutes (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.* (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 results of this analysis, in subsequent samples the plasmid DNA from an average of 40 *E. coli* colonies was examined. DNA was sequenced on an ABI 3130 Genetic Analyzer.

Sequences were identified using the BLASTN algorithm on GenBank or by comparison to a sequence library of local copepod species constructed for this project. Blast searches of the GenBank database were conducted on May 16, 2013. 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: 1) to check for unintended blocking of prey DNA, amplification of prey DNA was tested in PCR with and without the blocking oligo, and 2) to test 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 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 $1:10^4$ times excess. 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 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.

References:

Champlot, S., Berthelot, C., Pruvost, M. et al. (2010) An efficient multi-strategy DNA decontamination procedure of PCR reagents for hypersensitive PCR applications. PLoS One, 5, e13042.Durbin, E., Casas, M., Rynearson, T. et al. (2008) Measurement of copepod predation on nauplii using qPCR of the cytochrome oxidase I gene. Mar. Biol., 153, 699–707.

Eberl, R., Cohen, S., Cipriano, F. et al. (2007) Genetic diversity of the pelagic harpacticoid copepod *Macrosetella gracilis* on colonies of the cyanobacterium *Trichodesmium* spp. Aquat. Biol., 1, 33–43.

Hendriks, L., De Baere, R., Van de Peer, Y. et al. (1991) The evolution- ary position of the rhodophyte *Porphyra umbilicalis* and the basidiomycete *Leucosporidium scottii* among other eukaryotes as deduced from complete sequences of small ribosomal subunit RNA. J. Mol. Evol., 32, 167–177.

Koshkin, A., Singh, S., Nielsen, P. et al. (1998) LNA (Locked Nucleic Acids): Synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition. Tetrahedron, 54, 3607–3630.

Landegren, U., Kaiser, R., Sanders, J. et al. (1988) A ligase-mediated gene detection technique. Science, 241, 1077–1080.

McCoy, A. M. and Palumbi, S. R. (2007) Template specific inhibition of PCR. US Patent Application No. 11/486,329. U.S. Patent and Trademark Office, Washington, DC, pp. 1–16.

Obika, S., Nanbu, D., Hari, Y. et al. (1997) Synthesis of 20-O,40-C-methyleneuridine and -cytidine. Novel bicyclic nucleosides having a fixed C3,-endo sugar puckering. Tetrahedron Lett., 38, 8735 – 8738.

Vestheim, H. and Jarman, S. (2008) Blocking primers to enhance PCR amplification of rare sequences in mixed samples—a case study on prey DNA in Antarctic krill stomachs. Front. Zool., 5. http://www .frontiersinzoology.com/about/faq/citation.

Vestheim, H., Deagle, B. E. and Jarman, S. N. (2011) Application of blocking oligonucleotides to improve signal-to-noise ratio in a PCR. Methods Mol. Biol., 687, 265–274. Clifton, NJ.

Zhang, H., Bhattacharya, D. and Lin, S. (2005) Phylogeny of dinoflagellates based on mitochondrial cytochrome b and nuclear small subunit rDNA sequence comparisons. J. Phycol., 41, 411–420.

Data Processing:

The data from tests of the blocking oligo are presented without modification in Figure 2 (Fig2.xlsx).

Taxa detected in nauplii are presented in Table I (TableI.xlsx). Attached are the raw sequence files in FASTA format (Nauplii_gutcontent_sequences.TXT). The sequences will be submitted

to GenBank, along with sequences from adult gut contents and COI barcodes of San Francisco Estuary copepods not currently on GenBank, when we submit the second the manuscript for publication.

The sequences were identified as described above, either by comparison to our sequence library of local copepod species or using a sequence similarity approach. Sequences were Blasted using NCBI's web_blast.pl script (http://ncbi.nlm.nih.gov/blast/docs/web_blast.pl). The Blast output was manipulated with perl scripts we wrote to isolate the accession numbers for the BLAST hits, use the accession numbers to get the full records using NCBI's Batch Entrez (http://www.ncbi.nlm.nih.gov/sites/batchentrez), and get the taxonomic information from the full records. See Nauplii_gutcontent_sequence_ids.TXT for the output of these programs. We then compared the taxonomic information among the top 10% BLAST scores to assign the sequence to the lowest, taxonomic level shared by the top scoring hits.