Methodology for datasets from project “New molecular methods for studying copepod nauplii in the field” (EAGER: Copepod nauplii) https://www.bco-dmo.org/project/473049

Source: Michelle J. Jungbluth, Karen E. Selph, Petra H. Lenz, Erica Goetze, Species-specific grazing and significant trophic impacts by two species of copepod nauplii, *Parvocalanus crassirostris* and *Bestiolina similis*. Mar Ecol Prog Ser 572:57-76 (2017); DOI: https://doi.org/10.3354/meps12139

**MATERIALS AND METHODS**

**Grazing experiments**

A series of 5 bottle incubation experiments (hereafter referred to as E1 to E5; see Table 1) were conducted over a 10 d period (27 May to 5 June 2013) to measure grazing by copepod nauplii on the natural prey assemblage collected from Stn S3, located in the southern semi-enclosed basin of Kane‘ohe Bay, Oahu, Hawaii (21° 25' 56" N, 157° 46' 47"W; Jungbluth & Lenz 2013). The copepods were N3 and N4 stage nauplii of *Parvocalanus crassirostris* and *Bestiolina similis*. Concurrent experiments were run to measure microzooplankton community grazing, and to quantify in situ predator and prey abundances. The results from these experiments were used to correct for multiple trophic interactions within the bottle incubations, as these interactions can mask the effect of metazoan grazing (Nejstgaard et al. 2001). Salinity and temperature in the field were measured using a YSI 6600V2 sonde prior to collecting water for bottle incubations. Daily rainfall estimates were obtained from a rain gauge located at Luluku (www.prh.noaa.gov), and weather station data from the Hawaii Institute of Marine Biology (HIMB) (www.himb.hawaii.edu/weatherstation/) were used for estimates of the wind magnitude, wind direction, and solar irradiance.

Copepod nauplii used in the incubations were obtained from laboratory culture populations of *P. crassirostris* and *B. similis* established from animals previously collected in Kane‘ohe Bay (P. Lenz lab). Both species are capable of completing naupliar development in less than 3 d and reaching the adult stage (C6) in approximately 7 to 8 d (McKinnon et al. 2003, VanderLugt et al. 2009). Use of these monospecific cultures enabled us to produce high abundance naupliar cohorts of a specific age for grazing incubations. To produce these cohorts, adults of each species were isolated and fed 1 × 106 cells ml-1 *Tisochrysis lutea* (formerly *Isochrysis galbana* Tahitian strain; Bendif et al. 2013) 18 h prior to the start of each experiment to increase naupliar production. The adults were removed 6 h later, resulting in a cohort of nauplii (N3 and N4; note that N3 are the first feeding stage of these nauplii) raised at the experimental temperature of 21°C by the beginning of each experiment. Sets of ~50 nauplii were isolated into small volumes (<10 ml) of 0.2 µm filtered seawater and held for 1 to 3 h prior to the start of each grazing experiment. This procedure resulted in minimal exposure of the N3 and N4 nauplii to prey prior to the start of the grazing experiments. Seawater for the prey assemblage was collected from 2 m depth using a 5 l General Oceanics Niskin bottle deployed by hand line, with the contents gently added (silicone tubing) to two 20 l polycarbonate carboys.

Separate experiments indicated that longer incubation times decreased ingestion estimates within the grazing treatments, likely due to the fast development rates of our nauplii (< 24 h inter-molt period) and the diverse and rapidly changing prey community in this relatively warm (> 20°C) system (Jungbluth et al. 2017). Thus, 6 h incubations were chosen to give the most representative view of naupliar grazing

rates on natural prey, with conditions closest to those in situ, and in order to minimize nutrient remineralization and other food web interaction effects that can be significant during longer incubations (Roman & Rublee 1980).

Grazing incubations were performed in pre-washed (10% HCl rinse, followed by 3 rinses with ambient 0.2 µm seawater) polycarbonate bottles (total volume: 1120 ml) with 35 µm gently pre-screened bulk seawater offered as prey. It is possible that our nauplii would consume prey > 35 µm given the opportunity, however the small size of the copepod species in our study (~40 µm wide, ~70 µm long; *P. crassirostris* N1 dimensions) necessitated the removal of prey > 35 µm to ensure removal of other nauplii from the field. Our initial expectations were that the optimum prey size for our species would be 2 to 7 µm (Berggreen et al. 1988, Hansen et al. 1994), therefore the prey included here (< 35 µm) should represent a majority consumed naturally by our species.

The experimental nauplii were transferred into the 1120 ml grazing bottles at 2 densities (42-51 [moderate] and 81-95 [high] nauplii; see Table 1) and placed on a bottle roller (Wheaton) at 5 rpm in the dark for 6 h. The 2 nauplius densities were tested to ensure that we could detect removal of prey cells relative to controls over our incubation period, since a predator density that is too low may result in insignificant prey removal relative to control bottles. Removal of cells in treatments relative to control bottles was detected in the moderate density treatments and ingestion rate estimates were comparable to those from higher density bottles. Since results were comparable between moderate and high density treatments, results reported here focus on bottles with ~50 nauplii l-1, also because treatment replication was better with moderate density bottles (n = 3 per experiment) than high density bottles (n = 2). This density of nauplii is well within the range of total nauplius concentrations reported in previous studies in Kane‘ohe Bay (7 to 68 total nauplii l-1; Hoover et al. 2006) and within the range of each species abundance we have previously measured following storm run-off events in the bay (M.J.J. pers. obs.).

Treatment bottles were run in triplicate, with 2 or 3 no-nauplii control bottles for each experiment (2 for E1 to E2, 3 for E3 to E5). Experiments were incubated at 21°C, which is within the range of the annual temperature fluctuation in Kane‘ohe Bay (20 to 29°C during the previous 5 yr; HIMB weather data). No nutrients were added to the bottles, because controls and experimental bottles were considered approximately equally influenced by nitrogen remineralization from grazing processes, due to the presence of other < 35 µm microzooplankton grazers in all bottles and the short incubation times (6 h). Nauplii are known to have low expected nitrogen remineralization (~10- fold lower than adults) due to their small biomass compared to adults (Vidal & Whitledge 1982, Mauchline 1998); at 50 nauplii l-1, excretion rates were estimated to be 2 to 3 orders of magnitude below the in situ average nitrogen concentrations in Kane'ohe Bay (0.2 to 1.0 µM; Drupp et al. 2011). Initial and final time-point measurements included samples to quantify particle size and abundance in the 2-35 µm size range from the Coulter counter (CC), as well as samples for specific prey types, including chlorophyll a (*chl a*) and the abundance and biomass of types of nano- and microplankton. Prey types and CC-quantified potential prey were not expected to be equal; some prey types include cells < 2 µm, while the lower limit of the CC was 2 µm. Nauplii were recovered at the end of the experiments to check their condition (alive/dead; no dead nauplii were found), then preserved in 10% paraformaldehyde, stained with 1% Rose Bengal, and enumerated using microscopy for use in clearance and ingestion rate estimates.

**Prey size spectra and abundance (Coulter counter)**

Due to the ambient prey community being largely spherical cells (few diatoms present, verified microscopically), initial and final time-point CC samples were taken for prey particle spectra by gently pouring 20 ml from each incubation bottle through a 35 µm cap filter into a clean beaker, then gently back-washing the filter into the experimental bottle to return any nauplii using a small volume of 0.2 µm filtered seawater. From this subsample, triplicate 2 ml volumes were measured with a Beckman Coulter Multisizer III CC with a 100 µm orifice tube, yielding a spectrum of particle sizes from 2-35 µm ESD, as well as quantitative abundance data. These raw data were further processed in R (R Core Team 2016) to streamline binning of prey size groups, for calculations of clearance and ingestion rates, and for statistical analyses.

Prey ESD was converted to biovolume (BV; µm3), then to carbon (C; pg C cell-1), using the relationship C = 0.216 × BV0.939, which applies well to taxonomically diverse protists (Menden-Deuer & Lessard 2000). Averages of cell abundance and biomass from the triplicate CC measurements were binned into 5 prey size groupings (2-5, 5-10, 10-15, 15-20, and 20-35 µm), chosen based on their relevance to known prey sizes in Kane'ohe Bay and also due to use in prior studies of adult copepod grazing in Kane'ohe Bay (Calbet et al. 2000). The binned, averaged data for initial and final time points for each control and treatment bottle were used to calculate carbon ingestion (I; ng C nauplius-1 h-1) and clearance rates (F, ml nauplius-1 h-1) on each prey size group using the equations of Frost (1972) (see 'Data analyses' below).

Photosynthetic eukaryotes (flow cytometry) Flow cytometry (FCM) samples (1.5 ml) for photosynthetic eukaryote (PEUK) abundance were preserved in 0.4% paraformaldehyde (final concentration), flash-frozen in liquid nitrogen and transferred to a -80°C freezer until processing. Preserved, frozen FCM samples were thawed in batches, stained with the DNA dye Hoechst 34442 (1 µg ml-1, final concentration) (Campbell & Vaulot 1993, Monger & Landry 1993), and analyzed using a Beckman-Coulter Altra flow cytometer for phytoplankton population abundances using fluorescence signals from DNA, phycoerythrin and *chl a*. Data were grouped into relevant populations using FlowJo (Treestar). PEUK cell abundances were converted to biomass using data from parallel microscopy samples, which showed that the eukaryotic phytoplankton in these samples were dominated by 2-3 µm ESD spherical cells, with an average biomass of 1.55 pg C cell-1 (biomass conversions as in Menden-Deuer & Lessard 2000).

**Nano- and microplankton abundance and biomass (microscopy)**

Initial and final samples for nano- and microplankton abundance by epifluorescence microscopy (EPI) were preserved (0.4% paraformaldehyde, final concentration), and kept in the dark and cold (4°C) until filtered within 24 to 48 h. EPI samples (25 or 50 ml) were stained with 0.5 nM proflavin (1 to 2 h prior to filtration), then filtered onto 0.8 µm black polycarbonate filters (Midland Scientific), stained with 4', 6-diamidino-2-phenylindole (DAPI) for 2 min and mounted on a slide. These slides were frozen at -80°C until digitally imaged within 2 mo of collection.

Digital images of the slides were taken using a color camera (Olympus U-LH100HGAPO) attached to an epifluorescence microscope (Olympus Model BX51 TRF, 400× total magnification), and the software program Microfire(TM) (Optronics). For each slide, 3 sequential digital images were taken of 30 random fields, using 3 different excitation/emission filters; one each to illuminate *chl a*/proflavin (EX450-480;

DM500, EM=515), phycoerythrin (primarily due to Synechococcus), and DNA (EX330-385, DM400, EM> 420) fluorescence. Living cells were distinguished from dead cells and debris by the presence of nuclei, and autotrophic and heterotrophic cells were distinguished by the presence of *chl a*.

Images were analyzed by sizing, counting, and identifying autotrophic and heterotrophic cells 2-10 µm in size until >100 cells were characterized. In 2 cases, slide and image quality was poor, and as few as 60 cells were characterized (i.e. 31 May *B. similis*: 60 cells counted; 31 May P. crassirostris: 95 cells counted). To evaluate the large (>10 µm) cell abundance and biomass, all >10 µm cells on ¼ of one randomly selected control and treatment slide from each experiment were counted, identified as an autotroph or heterotroph, and measured using a calibrated ocular micrometer. Cell dimensions were used to estimate biovolume (oblate spheroid), and converted to biomass (Menden-Deuer & Lessard 2000). Diatoms >10 µm were also quantified, but they were never abundant during our experiments (see Table 2).

Ciliate biomass and abundance was estimated by inverted microscopy on samples preserved with a 1/20 dilution of acid Lugol's solution (Throndsen 1978), and kept in the dark at room temperature until analysis (~1 yr later) by the Utermöhl technique (Sherr & Sherr 1993). Aliquots of 28 ml from randomly selected control and experimental treatments were settled and their entire contents examined (18 to 87 total cells in sample volume, median 55) with a Zeiss inverted microscope (400× magnification), with digital images taken (Moticam camera and software) for subsequent dimensional analyses. The measured length and width of each cell was converted to biovolume based on the appropriate geometric shapes, and converted to carbon biomass (Menden-Deuer & Lessard 2000). Note that larger dinoflagellates were removed by pre-screening, and the acid Lugol's technique does not allow differentiation between autotrophs and heterotrophs; thus we focused on ciliates here.

### *Chl a* determinations

For *chl a*, triplicate 305 ml samples were filtered onto GF/Fs (Whatman), flash-frozen (LN2), and kept at -80°C freezer until measurements were made 4 mo later. *Chl a* (and phaeopigment) was measured using a Turner Designs (model 10AU) fluorometer, using the standard extraction and acidification technique (Yentsch & Menzel 1963, Strickland & Parsons 1972).

### Data analyses

Hourly clearance rates on each prey size or type were calculated from cell abundance (Frost 1972), and converted to biomass ingestion by multiplying cell ingestion by the biomass estimate per cell for that prey group. Prey type ingestion rates were calculated on ciliates, PEUKs (FCM), and 2-5, 5-10, and >10 µm size classes of autotrophs and heterotrophs (EPI). Negative clearance rates of prey were omitted from further analysis. According to Gifford (1993) and Båmstedt et al. (2000), the change in prey concentration within grazing experiment treatment bottles must be 20 to 40% so that the variation in count replicates (CV often up to 20%) is less than the difference between initial and final counts. Thus, the percent reduction of prey abundance within the experimental bottles between initial and final time points is reported in Tables S1 & S2 in the Supplement at www.int-res.com/articles/suppl/m572p057_supp.pdf).

### Estimates of in situ naupliar abundance

Naupliar abundances of the 2 target species in situ were estimated using a quantitative polymerase chain reaction (qPCR)-based method (Jungbluth et al. 2013), as well as microscopic counts of calanoid and cyclopoid nauplii. The qPCR-based method allows application of individual species grazing rates to in situ abundances to estimate the total potential grazing impact of each species. Samples were collected by duplicate vertical microplankton net tows (0.5 m diameter ring net, 63 µm mesh) from near bottom (10 m depth) to the surface with a low speed flow meter (General Oceanics). The contents of each net were split quantitatively. One half was size-fractionated through a series of 5 Nitex sieves (63, 75, 80, 100, and 123 µm) to separate size groups of nauplii from later developmental stages, and each was preserved in 95% non-denatured ethyl alcohol (EtOH). The second half of the sample was preserved immediately in 95% EtOH for counts of total calanoid and total cyclopoid nauplii, which were used for comparison to the qPCR-based results of the abundance of each calanoid species. All samples were stored on ice in the field until being transferred to a -20°C freezer in the laboratory. EtOH in the sample bottles was replaced with fresh EtOH within 12 to 24 h of collection to ensure high-quality DNA for analysis (Bucklin 2000).

The 3 smallest plankton size fractions from the net collection were analyzed with qPCR to enumerate *P. crassirostris* and *B. similis* nauplius abundances (Jungbluth et al. 2013). In brief, DNA was extracted from 3 plankton size fractions (63, 75, and 80 µm) using a modified QIAamp Mini Kit procedure (Qiagen). The total number of DNA copies in each sample was then measured using species-specific DNA primers and qPCR protocols (Jungbluth et al. 2013). On each qPCR plate, 4 to 5 standards spanning 4 to 5 orders of magnitude in DNA copy number were run along with the 2 biological replicates of a size fraction for each sampling date along with a no template control (NTC), all in triplicate. A range of 0.04 to 1 ng µl-1 of total DNA per sample was measured on each plate ensuring that the range of standards encompassed the amplification range of samples, with equal total DNA concentrations run in each well on individual plates. In all cases, amplification efficiencies ranged from 92 to 102%, and melt-curves indicated amplification of only the target species. The qPCR estimate of each species' mitochondrial cytochrome oxidase c subunit I (COI) DNA copy number was converted to an estimate of nauplius abundance using methods described in Jungbluth et al. (2013).