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Incubation duration effects on copepod naupliar grazing estimates

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ABSTRACT

Copepod naupliar grazing estimates often appear to represent an insignificant fraction of prey community mortality, despite high naupliar abundances and weight-specific ingestion rates. To address this seeming paradox, the impact of incubation time on grazing by nauplii of the subtropical copepod *Parvocalanus crassirostris* on natural prey assemblages was evaluated. Measurements of prey removal during feeding experiments were taken every 6-h over a 24-h period during two experiments (E1, E2), where the initial 2–35 μm natural prey biomass differed by 3-fold, i.e., 66 μg C L^{-1} (E1) vs. 198 μg C L^{-1} (E2). Results showed that total prey ingestion rate estimates decreased over the course of incubation by up to 75% after 24-h, with highest ingestion rates obtained during the initial 6-h. In no predator controls, total prey biomass also decreased significantly during the 24-h incubation particularly in the smallest prey size groups despite the absence of nauplii, and positive non-significant trends were observed in prey > 10 μm in the experiment with higher initial prey abundances. Predator density effects in this experiment provided additional evidence for trophic cascades. Results of this work suggest that in communities with rapidly changing natural prey assemblages and predators with short development times, short incubations minimize bottle effects and reduce the risk of measuring grazing rates on prey communities that no longer resemble the *in situ* populations.

1. Introduction

Year-round copepod reproduction in subtropical ecosystems (e.g., Webber and Roff, 1995) can lead to large fluctuations in copepod naupliar abundance that are linked to local environmental variation (Hopcroft and Roff, 1998; McKinnon and Duggan, 2003; Hoover et al., 2006). During peaks of abundance, nauplii can exert significant grazing pressure on their prey, for example *A. tonsa* nauplii accounted for up to 50% of the zooplankton grazing in Chesapeake Bay (White and Roman, 1992). Daily carbon rations of nauplii can be much greater than those of adult conspecifics, and many species are capable of consuming well over 100% of their body carbon per day (Paffenhöfer, 1971; White and Roman, 1992; Saiz and Calbet, 2007; Böttjer et al., 2010). In the subtropics, nauplii often do not contribute significantly to plankton biomass (e.g. Roff et al., 1995; Hopcroft et al., 1998). Nevertheless, due to high growth rates (e.g., Kiørboe and Sabatini, 1995; McKinnon and Duggan, 2003) and rapid development times (Hart, 1990; Kiørboe and Sabatini, 1995; Peterson, 2001), nauplii may be an important pathway transferring microbial production to higher trophic levels, given their ability to feed on pico- and nano-sized prey (Roff et al., 1995).

A standard method used to measure zooplankton ingestion rates is using a bottle incubation experiment to study prey removal relative to controls with no predators (Gauld, 1951; Frost, 1972; Paffenhöfer, 1988). Other methods to study zooplankton grazing, which do not require incubations, include gut fluorescence methods (e.g., Mackas and Bohrer, 1976; Kleppel and Pieper, 1984; Vogt et al., 2013), microscopic examination of gut contents or fecal pellets (e.g., Harding, 1974; Turner, 1986; Kleppel et al., 1988), radioisotope tracers (Chipman, 1959; Roman and Gauzens, 1997), and, most recently, molecular methods (Nejstgaard et al., 2008; Craig et al., 2014); and while each method has strengths, most of them do not account for the full spectrum of potential prey. While there are drawbacks to bottle incubations to estimate grazing rates, this method is one of few that measures feeding rates on both pigmented (phytoplankton) and non-pigmented (heterotrophic protist) prey directly, which are important to measure in subtropical environments where both prey types are common (e.g. Takahashi and Bienfang, 1983). This type of experiment requires optimization of incubation conditions to avoid artificial

Abbreviations: Chl *a*, chlorophyll *a*; CV, coefficient of variation (%); DW, dry weight; E1, experiment 1; E2, experiment 2; E, excretion rate (μg N $animal^{-1}$ h^{-1}); ESD, equivalent spherical diameter (μm); *F*, clearance rate (mL $grazer^{-1}$ h^{-1}); *I*, ingestion rate (ng C $grazer^{-1}$ h^{-1}); P_{high}, high predator treatment (100 nauplii L^{-1}); P_{low}, low predator treatment (50 nauplii L^{-1}); t_0, initial timepoint; TL, total length

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estimates of grazing due to 'bottle effects', or conditions that occur in the bottle that may differ from the natural environment. Potential bottle effects include changes in predator grazing or prey growth over time due to differing nutrient, light, or turbulence regimes, crowding of grazers, interactions with container walls, and trophic cascades or food web effects (see Båmstedt et al., 2000). Roman and Rublee (1980) recommended shorter incubations to reduce the impact of bottle effects that can be inconsistent over time based on several grazing indicators such as ATP, chlorophyll *a*, and particle concentration.

Negative relationships between ingestion rates and the duration of the incubation have been reported previously (Mullin, 1963; Roman and Rublee, 1980; Tackx and Polk, 1986). Multiple factors contribute to the reduction in ingestion estimates over long incubations and these include diel rhythms in grazing rates (Kiørboe et al., 1985), a decline in food quality over time (Roman and Rublee, 1980), and trophic interactions in control bottles (Calbet and Landry, 1999; Nejstgaard et al., 2001). This can be a particular concern in studies with natural microzooplankton as prey items, which can directly compete with nauplii for prey, requiring extra consideration of potential trophic interactions in bottle incubations (Nejstgaard et al., 2001).

Thus, grazing rate measurements for nauplii based on 24-h incubations are highly variable. In some temperate regions, naupliar grazing removed a large portion of specific prey items (e.g. 56% ciliates in Chesapeake Bay, [Merrell and Stoecker, 1998]; 54% nanoplankton off the Chilean coast, [Böttjer et al., 2010]) while other grazing estimates from a range of latitudes suggest that nauplii were insignificant grazers on prey (Castellani et al., 2008; Verity et al., 1996; Almeda et al., 2011). While the impact of naupliar grazers may vary by habitat and season, there may be confounding results from the methods used to measure grazing impact.

The primary goal of this work was to optimize conditions and duration of incubation for measurements of naupliar grazing. Naupliar grazing experiments were performed on the natural prey community in order to (1) evaluate the magnitude of changes in the enclosed prey community over the course of 24-h bottle incubation experiments (controls), (2) determine how the length of the incubation affects the resulting estimates of ingestion rates and grazing impacts by copepod nauplii, and (3) test how different predator concentrations affect ingestion rate estimates and the potential for trophic cascades during the experiment. Results of this work suggest that shorter incubation times minimize bottle artifacts, such as declining ingestion rate estimates over time, and give a more clear view of trophic interactions within bottles when compared with longer incubations.

2. Materials and methods

Naupliar grazing rates were measured on field-collected prey assemblages in bottle incubation experiments in the laboratory. Nauplii used in these experiments were derived from laboratory culture populations of *Parvocalanus crassirostris*, originally established from animals collected in Kane'ohe Bay. This species is a dominant component of tropical, neritic plankton communities (Hopcroft et al., 1998; McKinnon and Klumpp, 1998; Hoover et al., 2006; Jungbluth and Lenz, 2013) and has relatively short development times (e.g. McKinnon et al., 2003); it is capable of completing naupliar development within 3 days and reaching the adult stage (C6) in approximately 8 days (at 23–25 °C). Use of these monospecific cultures enabled us to produce naupliar cohorts of a specific stage for use in grazing incubations. At 18-h prior to the start of each experiment, adults were isolated and fed *Tisochrysis lutea* (formerly *Isochrysis galbana* Tahitian strain [Bendif et al., 2013]) at a concentration of 10^5–10^6 cells mL^{-1} After 6-h, adults were removed, and eggs and nauplii were allowed to develop for 12-h in order to produce a cohort of mid-stage nauplii (N3-N4) with a narrow age-range at the beginning of each experiment. Sets of approximately 50 nauplii were isolated into small volumes (< 10 mL) of 0.2 μm filtered seawater 1–2 h prior to the start of each grazing experiment.

Seawater for the prey assemblage was collected from the central basin of the southern semi-enclosed region of Kane'ohe Bay, Oahu, Hawai'i (21°25′56″N, 157°46′47″W) on two dates: 10 March 2015 (Experiment: E1) and 22 April 2015 (Experiment: E2). Seawater was collected from ~2 m depth using a 5 L General Oceanics Niskin bottle deployed by hand line, and gently transferred using acid-washed silicon tubing directly from the Niskin bottle into 20 L covered (dark) polycarbonate carboys. The seawater was transported to the laboratory within 2-h of collection. The collected water was gently pre-screened (35 μm Nitex mesh), which was intended to remove all *in situ* nauplii and other large grazers, so that the only metazoan grazers in the bottles were the added nauplii. The < 35 μm incubation water was added to pre-washed (10% HCL rinse, followed by 3 rinses with experimental seawater) 1 L polycarbonate bottles (total volume: 1120 mL).

Nutrients were not amended in control or treatment bottles due to the expected low rates of excretion by these small biomass nauplii over the incubation duration as compared with baseline levels in Kane'ohe Bay, and also in order to minimize development of artificially high nutrients given prevailing oligotrophic conditions in the study area. Excretion rates of copepods are a function of biomass (Vidal and Whitledge, 1982; Mauchline, 1998), with excretion by nauplii roughly an order of magnitude lower than conspecific adults. At a nauplius grazer concentration of 50 nauplii in a 1 L volume, excretion rates result in values 2 to 3 orders of magnitude below the average nitrogen concentrations of 0.2–1.0 μM in Kane'ohe Bay (Drupp et al., 2011). Therefore, excretion rates in bottle incubations were expected to have negligible impacts on prey growth rates in experimental bottles, and nutrient amendment would have only altered the prey community further away from *in situ* conditions.

The isolated N3-N4 nauplii were transferred into triplicate < 35 μm incubation water bottles (grazing treatments) and placed on a bottle roller (4–6 rpm) to maintain prey in suspension for the duration of the incubation period. Parallel triplicate control treatments (incubation water without added nauplii) were also placed on the bottle roller. Grazing rates were measured using two densities of naupliar grazers: high (N = 92–97 nauplii L^{-1}) and moderate (N = 45–50 nauplii L^{-1}) densities. All incubations were run for a total of 24-h in the dark, with subsamples taken every six hours to examine changes in ingestion rates over time. Experiments were run at 21 °C, which is at the low end of the range of annual temperature fluctuations for this region of Kane'ohe Bay (20–29 °C in prior 5 years [Franklin et al., 2015]).

During the course of the incubation, triplicate 2-mL volumes of each subsample were measured with a Coulter Counter (Beckman-Coulter Multisizer III) with a 100 μm orifice tube, yielding a spectrum of particle sizes from 2 to 35 μm ESD, as well as quantitative abundance data. In a diverse environment with a variety of autotrophic and heterotrophic pico- to microplankton, standard cell quantification methods (e.g. epifluorescence microscopy, inverted microscopy) do not reliably preserve some components of the community (Omori and Ikeda, 1984; Sherr and Sherr, 1993), requiring a patchwork of methods to quantify the full potential suite of prey items. In the absence of large cells or of abiotic particles that may result in unreliable quantification (e.g. Harbison and McAlister, 1980), the Coulter Counter is an appropriate and more reliable means of describing how the abundance of different sized cells change over the duration of grazing incubations (Paffenhöfer, 1984), with results comparable to methods based on gut fluorescence and egg production (Kiørboe et al., 1985). Water subsamples for Coulter Counter measurements were taken directly from experimental bottles upon addition of nauplii at the start of each experiment (time 0) and at each six-hour time point, being careful to retain nauplii as experimental grazers by recovery of animals on a 35 μm cap filter and washing them back into bottles during subsampling with a small volume of filtered seawater.

Data on prey size (ESD) and abundance from the Coulter Counter were further processed using R (Core Team, 2013). Prey ESD was converted to biovolume (BV, μm^3), then to carbon (C, pg C $cell^{-1}$)

using the relationship $C = 0.216 \times BV^{0.939}$ (Menden-Deuer and Lessard, 2000). Averages (triplicate Coulter Counter measurements) were binned into 5 functionally relevant prey size groupings (2–5, 5–10, 10–15, 15–20, and 20–35 μm), chosen to ensure comparable data to a prior study of adult copepod grazing in Kane'ohe Bay (Calbet et al., 2000). The binned data for initial and final time points for each control and treatment bottle were used to calculate carbon ingestion (I, ng C grazer^{-1} h^{-1}) and clearance (F, mL grazer^{-1} h^{-1}) rates on each prey size group using the equations of Frost (1972), and are reported here only where F or $I > 0$.

Linear regressions were used to evaluate whether there was a relationship between control bottle prey biomass and incubation duration, and between measured ingestion rates (I) and incubation time. An analysis of covariance (ANCOVA) was used to test for significant ($p < 0.05$) effects of incubation time, predator treatment (P_{low}, P_{high}), and experiment (E1, E2) on carbon ingestion rates (I), and for interactions between variables, accounting for random error due to differences between replicate bottles. The ANCOVAs were performed for each prey size group or total prey using the aov function in the package stat with time, predator treatment, and experiment as potentially interacting factors, and incorporating bottle replicate error as a random effect. The coefficient of variation (CV, %) was calculated for cell abundance estimates and followed by a two-way ANOVA and post-hoc Tukey test to evaluate for the effects of prey size group and incubation time on variation in cell abundance. In many studies of zooplankton grazing (e.g., Atienza et al., 2006; Calbet et al., 2009; Almeda et al., 2011), significant differences in prey growth rates between control and treatment bottles were tested, and then only significantly different conditions were considered in further interpretations of I. Here, significant differences (t-test, $p < 0.05$) between treatment and control prey growth rates were used to evaluate whether the significance of this test was affected by the duration of incubation. All statistical analyses were conducted using the program R (stats package) (R core team, 2016).

3. Results

3.1. Prey community dynamics

The relative prey size distribution for both experiments was typical for the study region, with a dominance of small cells (Calbet et al., 2000; Cox et al., 2006) and a roughly logarithmic decrease in cell biomass (Fig. 1) and abundance (data not shown) with increasing cell size. Total initial carbon during E1 in the 2–35 μm size range was 66 μg C L^{-1} (1.14 × 10^4 cells mL^{-1}), while E2 prey total carbon was 3-fold higher, at 198 μg C L^{-1} (3.45 × 10^4 cells mL^{-1}) (Table 1).

The prey community within the control bottles changed significantly over the course of the 24-h incubation period (Table 2). Biomass in each prey size group changed as a function of incubation time (0, 6, 12, 18, 24-h) in both experiments (Fig. 1). There were significant declines in total prey biomass and in the 2–5 μm prey size group over the incubation duration (linear regression, $p < 0.05$; Table 2). In E1, the available biomass in every prey size group decreased between 6 and 24-h (slopes of −0.01 to −0.66), however some of these trends were non-significant (Table 2). In contrast, during E2 prey carbon in the three largest size groups (10–15, 15–20, and 20–35 μm) increased moderately from 6 to 24-h of incubation, but these changes were not significant (Fig. 1; Table 2).

In both experiments, the highest prey carbon biomass was in the 2–10 μm size range, with 74% in E1 and 84% in E2 (Table 1). During E1, total prey biomass in control bottles decreased by 34% after 24-h of incubation (Table 2). Total biomass declined similarly in E2, decreasing by 16% after 24-h. A one-way ANOVA assessing for the change in biomass in each size fraction over time found the 2–5 and 5–10 μm size groups of E1, and the 2–5 μm size group of E2 to be significant ($p < 0.001$), but the rest of the size fractions were not ($p > 0.29$).

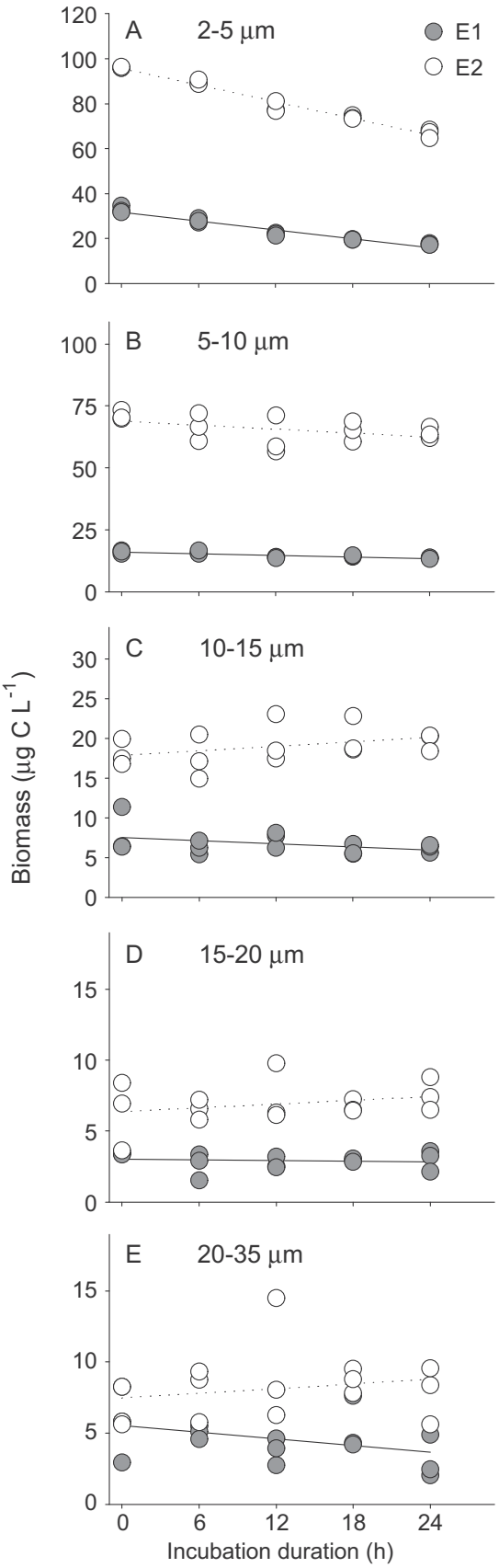

Fig. 1. Mean biomass (μg C L^{-1}) in replicate control bottles for each prey size group (A–E) over the duration of incubation (h). Dark circles and solid lines are data and regression for E1, light circles and dotted lines show results for E2. Regression results are reported in Table 1.

Table 1
Control bottle mean prey; initial (time = 0) biomass (μg C L^{-1}) and abundance (cells mL^{-1}) (± standard error) in experiments E1 and E2, in the different prey size groups.

Prey size (μm)		Biomass (μg C L^{-1})	Abundance (cells mL^{-1})
E1	Total	66.1 ± 4.4	11,428 ± 144
	2–5	32.9 ± 0.9	10,673 ± 100
	5–10	16.0 ± 0.4	675 ± 30
	10–15	8.0 ± 1.7	66 ± 12
	15–20	3.4 ± 0.03	10 ± 0.3
	20–35	5.6 ± 1.5	5 ± 1
	Total	197.6 ± 4.4	34,491 ± 119
E2	2–5	95.8 ± 0.2	31,190 ± 64
	5–10	71.0 ± 1.2	3,127 ± 45
	10–15	18.0 ± 0.9	150 ± 6
	15–20	6.3 ± 1.4	18 ± 4
	20–35	6.5 ± 0.8	6 ± 0.4

A post hoc Dunnett's test was used to test for significant changes in biomass from time t_0, and found that for both E1 and E2, 2–5 μm prey biomass at 6, 12, 18 and 24 h was significantly different from t_0 ($p < 0.001$), while 5–10 μm prey in E1 were significantly different from t_0 after 12, 18 and 24 h incubation durations ($p < 0.006$; Table 2).

3.2. Ingestion and clearance rate estimates over time

Linear regressions suggest that estimates of the total ingestion (*I*; ng C $grazer^{-1}$ h^{-1}) rate significantly decreased over the 24-h incubation period for P_{low} in both experiments, but was not significant for either P_{high} (Fig. 2A, B; Table 3). The highest total *I* rates were observed in low predator treatments (P_{low}) at 6–12 h of incubation for both experiments and decreased by up to 75% after 24-h of incubation (Fig. 2A, B). In E1, the maximum total *I* was 21.5 ± 6.8 ng C $grazer^{-1}$ h^{-1} (mean ± standard error) at 6-h (Fig. 2A), while in E2 the maximum total *I* was 45.8 ± 14.2 ng C $grazer^{-1}$ h^{-1} at 12-h of incubation, which was 7% higher than the 6-h estimate (Fig. 2B). In high predator treatments (P_{high}), maximum *I* at 6-h was one half to one third of the P_{low} 6-h ingestion rate (Fig. 2A, B). The *I* estimates for P_{high} decreased over 24-h of incubation by 18 to 48% from the 6-h time point.

Total clearance (*F*; mL $grazer^{-1}$ h^{-1}) rate estimates also decreased over the 24-h incubation period (Fig. 2C, D). Total *F* after the first 6-h for P_{low} was at a maximum, and equal in both E1 and E2 (2.8 mL $grazer^{-1}$ h^{-1}). After 24-h of incubation total *F* for P_{low} decreased 61% in E1 and 55% in E2 from the 6-h estimate. Total *F* for P_{high} after 6-h was similar between E1 and E2; 1.09 ± 0.66 mL $grazer^{-1}$ h^{-1} and 1.32 ± 0.17 mL $grazer^{-1}$ h^{-1} respectively (Fig. 2C, D). This declined 33% in E1 and 78% in E2 after 24-h.

The relationships between the ingestion rates of individual size fractions over time were negative or near-zero in many cases (Table 3). There was a significant negative relationship between *I* and increasing time of incubation in experiment E1 for two of the five size fractions in P_{low} and one size fraction in P_{high}, and in experiment E2 for one size fraction for P_{low} and one size fractions in P_{high} ($p < 0.05$; Table 3). In E1 this included the 2–5 μm prey size group in P_{low} and P_{high}, as well as the 20–35 μm group in P_{low}. In E2 the regression of ingestion over time was significant and negative for the 2–5 μm group in P_{low} and the 20–35 μm prey size group for P_{high}.

The two experiments resulted in significant differences in ingestion rates over the duration of incubation in the majority of prey size groups (ANCOVA; Table 4). There was a significant interaction between time and experiment for the 15–20 μm size group, which was driven by the difference in ingestion between the two experiments at the 18-h time point. Incubation time significantly impacted ingestion rate in the 2–5, and 20–35 μm size groups for both experiments, while predator treatment impacted ingestion rates in the 2–5 and 5–10 μm groups (Table 4). Significant interactions occurred between incubation time and predator treatment and between predator treatment and experiment for the 2–5 μm prey size group, and between predator treatment and experiment for the 5–10 μm prey size group. Further analyses of the 2–5 μm group found a significant interaction between incubation time and predator treatment on ingestion rates for E2 (ANCOVA; $F_{1,17} = 6.5$; $p = 0.021$) but not E1, and between predator treatment and experiment only for the 24 h time point (ANCOVA; $F_{1,6} = 6.7$; $p = 0.04$) (Table 4). This result suggests that in E2 where the prey biomass was higher, there was a significant difference in prey removal between the low and high predator treatments after 24 h but not after 6 h.

3.3. Additional statistical tests

The control and treatment bottle prey growth rates of each time point were compared to assess whether statistical significance was more likely with increasing duration of incubation. There was no clear effect of incubation duration on the significance of control and treatment prey growth rates (Table 3). In E2, growth rates were significantly different for the 2–5 μm prey size group at 6, 12 and 24-h in P_{low}, and at 12 and 24-h in P_{high}. In E1, growth rates were significantly different for the 2–5 μm prey at 6 and 12-h in P_{low}, and not at all in P_{high} treatments. The differences between the two experiments E1 and E2 are likely a result of differing initial prey biomass, with E2 total prey biomass almost three

Table 2
Changes in control bottle prey over time; linear regression equation, *p*-value (*p*-val) of the linear regression of prey biomass (μg C L^{-1}) over time in experiments E1 and E2, and percent change in biomass from t_0 in experiments E1 and E2. * denotes where biomass was significantly different from t_0 (Dunnett's test).

Prey size (μm)		Regression	*p*-val	Change from t_0 (%)			
				6-h	12-h	18-h	24-h
E1	Total	$y = -0.92x + 63.78$	< 0.001	−13.3**	−25.6**	−27.8**	−34.4**
	2–5	$y = -0.66x + 31.77$	< 0.001	−14.9**	−33.6**	−40.6**	−47.0**
	5–10	$y = -0.11x + 15.95$	< 0.001	−1.1	−14.0**	−10.5**	−15.7**
	10–15	$y = -0.07x + 7.52$	0.14	−22.5	−8.7	−26.9	−23.4
	15–20	$y = -0.01x + 3.03$	0.67	−24.1	−20.2	−13.8	−12.1
	20–35	$y = -0.08x + 5.53$	0.15	−10.8	−33.8	−4.9	−45.0
E2	Total	$y = -1.30x + 195.87$	< 0.001	−5.2	−10.4	−11.9*	−16.4**
	2–5	$y = -1.23x + 95.58$	< 0.001	−6.7**	−18.3**	−23.1**	−30.4**
	5–10	$y = -0.27x + 68.80$	0.09	−6.5	−12.6	−8.9	−10.1
	10–15	$y = 0.10x + 17.78$	0.16	−3.1	9.0	11.1	9.0
	15–20	$y = 0.04x + 6.37$	0.32	3.1	16.9	6.4	19.4
	20–35	$y = 0.06x + 7.46$	0.45	21.5	46.7	33.1	19.5

** $p < 0.05$.
* $p < 0.1$.

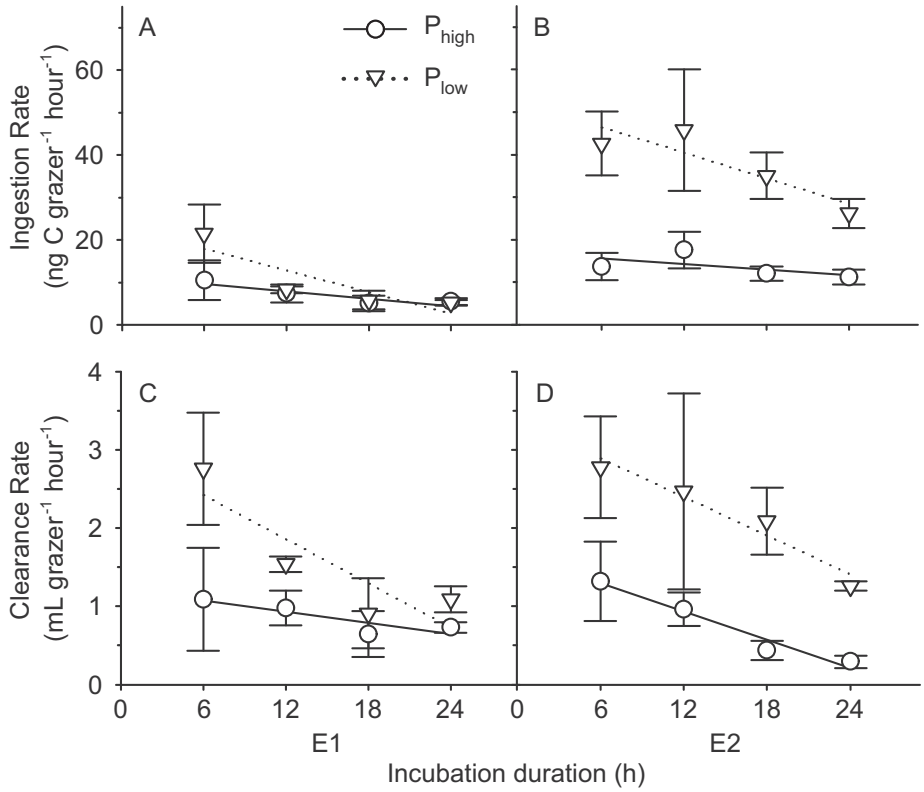

Fig. 2. Mean estimates of total carbon ingestion (A,B) (ng C grazer^{-1} h^{-1}), and total clearance rates (C,D) (mL grazer^{-1} h^{-1}) over incubation duration (h) in experiment E1 (A, C) and E2 (B, D). Triangles are low predator abundance bottles, circles are high predator abundance bottles. In the legend, dotted regression line is for low abundance predator treatments (~50 L^{-1} = P_{low}), solid regression line is for high abundance predator treatments (~100 L^{-1} = P_{high}). Error is ± SE.

times higher than E1, primarily due to higher biomass of prey < 10 μm. The differences between P_{low} and P_{high} within experiments suggests that the higher abundance of predators may have had a different effect on prey abundances than the low predator treatments in the two experiments (E1 and E2), possibly reflecting the effects of copepod nauplii on trophic dynamics occurring in the bottles over time.

The CV (%) was low for the smallest prey size groups and increased with increasing prey size, due to the decrease in the number of prey

Table 3
Experiment E1 and E2 (mean ± standard error) ingestion rates (I, ng C grazer^{-1} h^{-1}) for 6-h, 12-h, 18-h, and 24-h incubation duration, the equation and p-value (p-val) of the linear regression of ingestion rate over time. Total ingestion rates and estimates for prey size groups are shown for both low and high predator abundances. - no observations with $I > 0$. * only one replicate where $I > 0$. p-val = 0.05 in **bold**. [a] indicates values with significantly different control and treatment prey growth rates.

Prey (μm)			6-h Ingestion	12-h Ingestion	18-h Ingestion	24-h Ingestion	Regression	p-val
			(ng C grazer^{-1} h^{-1})	(ng C grazer^{-1} h^{-1})	(ng C grazer^{-1} h^{-1})	(ng C grazer^{-1} h^{-1})		
		Total	21.5 ± 6.8	8.4 ± 0.8	5.9 ± 2.2	5.3 ± 0.7	$y = -0.84x + 21.72$	**0.02**
E1	P_{low}	2–5	[a]5.5 ± 0.6	[a]3.0 ± 0.2	1.9 ± 0.4	1.2 ± 0.1	$y = -0.23x + 6.37$	**0.05**
		5–10	2.1 ± 0.9	1.0 ± 0.04	1.6 ± 0.9	1.4 ± 0.2	$y = -0.03x + 1.91$	0.85
		10–15	*0.1	0.7 ± 0.4	–	–	$y = 0.10x - 0.54$	0.85
		15–20	1.3 ± 0.5	*1.5	0.5 ± 0.3	1.1 ± 0.3	$y = -0.02x + 1.32$	0.88
		20–35	12.6 ± 4.8	2.2 ± 0.2	1.9 ± 0.6	*1.6	$y = -0.69x + 14.80$	< **0.001**
		Total	10.5 ± 4.7	7.4 ± 2.1	5.1 ± 1.8	5.5 ± 0.8	$y = -0.28x + 10.02$	0.41
	P_{high}	2–5	4.1 ± 1.3	2.2 ± 0.6	1.6 ± 0.4	1.2 ± 0.4	$y = -0.16x + 4.64$	**0.05**
		5–10	*2.2	1.0 ± 0.3	1.4 ± 0.6	1.1 ± 0.3	$y = -0.03x + 1.83$	0.76
		10–15	–	–	0.3 ± 0.2	0.5 ± 0.1	$y = 0.04x - 0.37$	0.90
		15–20	–	0.9 ± 0.5	[a]0.6 ± 0.02	[a]0.3 ± 0.1	$y = -0.05x + 1.51$	0.70
		20–35	4.2 ± 3.4	3.3 ± 0.7	1.1 ± 0.7	*2.4	$y = -0.16x + 5.03$	0.11
		Total	42.6 ± 7.5	45.8 ± 14.2	35.0 ± 5.5	26.2 ± 3.4	$y = -0.75x + 41.21$	**0.03**
E2	P_{low}	2–5	[a]17.8 ± 3.1	[a]13.3 ± 3.3	7.7 ± 2.8	[a]8.2 ± 1.5	$y = -0.57x + 20.36$	**0.005**
		5–10	7.7 ± 1.0	[a]17.2 ± 3.5	15.6 ± 0.7	9.0 ± 1.7	$y = -0.07x + 13.64$	0.73
		10–15	–	4.4 ± 2.2	3.7 ± 1.4	*3.2	$y = -0.11x + 5.63$	0.79
		15–20	6.2 ± 3.4	7.1 ± 2.5	4.0 ± 0.1	2.7 ± 0.2	$y = -0.23x + 8.38$	0.33
		20–35	*11.0	3.7 ± 2.8	4.0 ± 0.5	*3.2	$y = -0.35x + 10.14$	0.26
		Total	13.7 ± 3.2	17.6 ± 4.3	12.0 ± 1.7	11.2 ± 1.8	$y = -0.12x + 11.61$	0.73
	P_{high}	2–5	5.1 ± 0.3	[a]5.8 ± 1.2	3.4 ± 1.2	[a]4.8 ± 0.8	$y = -0.06x + 5.66$	0.54
		5–10	–	4.0 ± 2.7	*6.2	5.4 ± 0.7	$y = 0.12x + 2.79$	0.43
		10–15	–	*2.8	–	–	–	–
		15–20	3.6 ± 2.7	2.3 ± 0.02	0.8 ± 0.4	0.5 ± 0.3	$y = -0.18x + 4.55$	0.10
		20–35	5.0 ± 0.2	2.6 ± 0.4	1.5 ± 0.1	0.6 ± 0.001	$y = -0.24x + 5.99$	**0.03**

Table 4
Results of ANCOVA analyses of prey ingestion rates over time, testing for the effects of predator treatment (Pred: P_{low} and P_{high}) and experiment (Expt: E1 and E2), as well as for interactions between variables. The degrees of freedom (d.f.), sum of squares (s.s.), mean of squares (m.s.), *F*-statistic (*F*) and *p*-value (*p*-val) are shown for each source of variation individually, as well as for interactions (:) between them. Superscript numbers refer to the significant interactions, and the source of each significant interaction is noted below the table.

Prey group	Source of variation	d.f.	s.s.	m.s.	*F*	*p*-val
2–5 μm	Time	1	0.083	0.083	24.2	1.8E-05***
	Pred	1	0.097	0.097	28.4	5.1E-06***
	Expt	1	0.228	0.228	66.6	8.5E-10***
	Time:Pred[a]	1	0.023	0.023	6.8	0.01*
	Time:Expt	1	0.006	0.006	1.8	0.19
	Pred:Expt[b]	1	0.067	0.067	19.7	7.9E-05***
	Time:Pred:Expt	1	0.015	0.015	4.4	0.04*
	Residuals	37	0.126	0.003		
5–10 μm	Time	1	0.007	0.007	1.0	0.32
	Pred	1	0.076	0.076	10.7	0.003**
	Expt	1	0.285	0.285	40.3	1.2E-06***
	Time:Pred	1	0.001	0.001	0.1	0.72
	Time:Expt	1	0.000	0.000	0.0	0.91
	Pred:Expt[c]	1	0.069	0.069	9.7	0.005**
	Time:Pred:Expt	1	0.002	0.002	0.2	0.63
	Residuals	25	0.177	0.007		
10–15 μm	Time	1	0.004	0.004	1.5	0.28
	Pred	1	0.003	0.003	1.2	0.32
	Expt	1	0.010	0.010	3.7	0.11
	Time:Pred	1	0.001	0.001	0.3	0.61
	Time:Expt	1	0.001	0.001	0.4	0.58
	Pred:Expt	1	0.000	0.000	0.03	0.87
	Residuals	5	0.013	0.003		
15–20 μm	Time	1	0.019	0.019	11.3	2.7E-03**
	Pred	1	0.007	0.007	4.4	0.05*
	Expt	1	0.032	0.032	19.2	2.2E-04***
	Time:Pred	1	0.000	0.000	0.0	0.96
	Time:Expt[d]	1	0.008	0.008	5.0	0.04*
	Pred:Expt	1	0.005	0.005	3.2	0.09
	Time:Pred:Expt	1	0.000	0.000	0.2	0.67
	Residuals	23	0.038	0.002		
20–35 μm	Time	1	0.092	0.092	11.4	0.003**
	Pred	1	0.020	0.020	2.5	0.13
	Expt	1	0.001	0.001	0.1	0.70
	Time:Pred	1	0.024	0.024	3.0	0.10
	Time:Expt	1	0.003	0.003	0.4	0.54
	Pred:Expt	1	0.000	0.000	0.0	0.84
	Time:Pred:Expt	1	0.009	0.009	1.1	0.31
	Residuals	20	0.161	0.008		
Total prey	Time	1	0.310	0.310	8.7	0.01*
	Pred	1	0.947	0.947	26.4	8.5E-06***
	Expt	1	1.077	1.077	30.1	2.9E-06***
	Time:Pred	1	0.111	0.111	3.1	0.09
	Time:Expt	1	0.005	0.005	0.1	0.71
	Pred:Expt[e]	1	0.491	0.491	13.7	6.7E-04**
	Time:Pred:Expt	1	0.000	0.000	0.0	0.92
	Residuals	38	1.360	0.036		

Description of significant interactions:
[a] interaction is driven by how both P_{low} and P_{high} have significantly different *I* during E1 and E2.
[b] interaction is driven by E2 P_{low}, which shows significantly different *I* over time, however E2 P_{high} does not.
[c] interaction is driven by significant differences between E1 and E2 for P_{low}.
[d] interaction is driven by 18 h difference in *I* between experiments.
[e] interaction is driven by the difference in total *I* estimates in P_{low} between experiments, and this change is significant over time in E1, but not in E2 which shows significant differences in total *I* between P_{low} and P_{high}, but E2 does not have a significant change in total *I* over time.
* $p \leq 0.05$.
** $p \leq 0.005$.
*** $p \leq 0.0005$.

counted per replicate for larger prey. The range in CV increased with increasing prey size: 2–5 μm was 0.3–4.3%, 5–10 μm was 0.8–12.1%, 10–15 μm was 1.7–35.7%, 15–20 μm was 4.4–37.5%, and 20–35 μm was 3.8–63%. There was a significant difference in the mean CV across prey size groups, but not over incubation duration (two-way ANOVA, $p < 0.05$), indicating that confidence in prey abundance estimates does not differ between 6 and 24-h incubations and supporting higher confidence in estimates of small prey than in estimates of larger prey.

4. Discussion

Despite the potential importance of copepod nauplii to marine food webs and carbon transfer in marine ecosystems, little work has been done to understand the grazing impact of nauplii on natural prey assemblages. Even less has been done to assess experimental protocols such as the impact of incubation duration and other conditions on measurements of naupliar grazing rates, despite numerous studies and reviews on adult copepod grazing (e.g., Saiz and Calbet, 2007, 2011; Hansen et al., 1997). For *Parvocalanus crassirostris* nauplii grazing on natural prey assemblages, extended incubations (24-h) resulted in 39–75% lower total *I* estimates (significant regressions; Table 3), similar to previous observations in grazing experiments with adults (Mullin, 1963; Roman and Rublee, 1980; Tackx and Polk, 1986). In addition, the magnitude of this effect varied with initial prey biomass and grazer density, the latter significantly affected *I* only at the higher prey concentration (E2, ~3-fold higher). These observations suggest that there is no simple way to correct for the effect of incubation time on ingestion estimates. Changes in the control bottles suggest that the prey community became increasingly artificial during longer incubations as smaller prey abundances decreased disproportionately. Differences in ingestion rates between the two experiments indicate that *I* estimates also could be affected by initial prey fields. The combination of the rapid changes in the prey community, decreasing *I* over time, and variable trophic interactions, make shorter incubation times preferable for studies on grazing in subtropical-tropical environments, particularly for grazer species with fast-developing early developmental stages.

The primary justification for 24-h bottle incubations in grazing experiments is to account for possible diel periodicity in grazing rates, which is well known for many adult copepods (e.g., Dagg et al., 1989; Peterson et al., 1990; Saito and Taguchi, 1996; Calbet et al., 1999; Calliari and Antezana, 2001). Patterns of grazing activity in adult copepods are often associated with diel vertical migratory behavior, which is absent in naupliar stages (Ambler and Miller, 1987; Haney, 1988; Neill, 1992). Diel grazing patterns by nauplii are not well understood: Båmstedt et al. (2000) note that copepod nauplii may feed most intensely during daytime, but there is limited evidence to support this conclusion. In the current study, there was no evidence of a diel feeding cycle, instead there was a nearly linear decline in *I* for most treatments and prey size groups (Figs. 2, 3). This observation suggests that diel changes in feeding for these copepod nauplii were less important than other sources of variability in the bottles. In addition, warm-water species such as *P. crassirostris* undergo rapid development, growing from N1 to the adult stage in under 8 days (at 23–25 °C), with some developmental stages lasting < 12-h in the inter-molt period (see McKinnon et al., 2003 for published development times at 27 °C). Given the brevity of each developmental stage for this species, changes in feeding rates are more likely linked to molting, when feeding activity slows or even temporarily stops to allow for shedding of the exoskeleton (e.g., Lipcius and Herrnkind, 1982). Thus, when studying fast-developing predator species and rapidly changing prey communities, the experimental design needs to consider both the rate of variation in the prey community as well as biologically relevant changes in the predator.

Total prey biomass decreased significantly over the duration of the experiments, which was largely driven by a significant decline in 2–10 μm prey in E1 and 2–5 μm prey in E2 (Table 2). For example, after 24-h a 34% decrease in total prey biomass in E1, while the decrease was only 13% after a 6-h incubation. The changes in total carbon capture only part of the pattern; prey size groups changed at different rates in the control bottles and this was particularly evident in

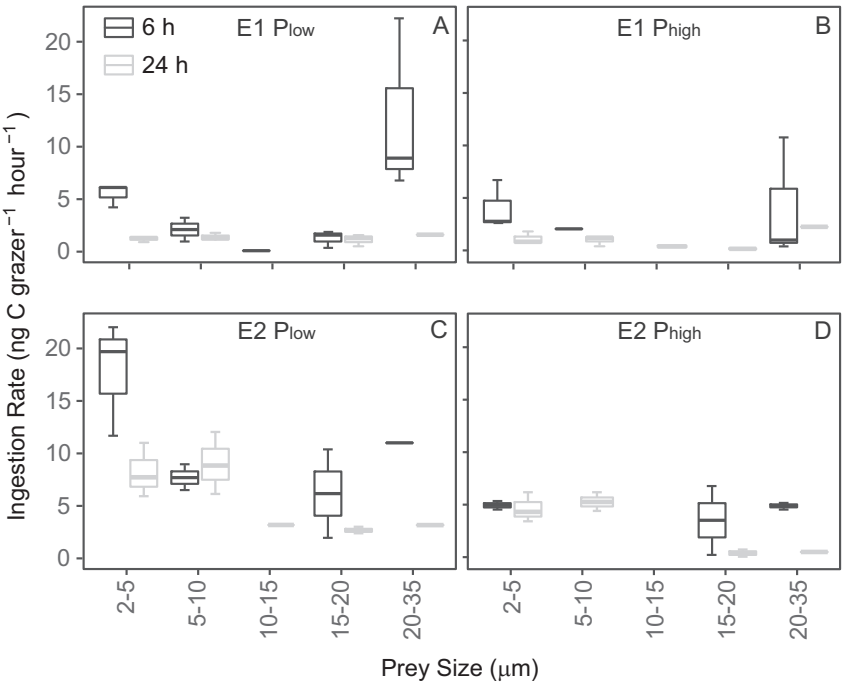

Fig. 3. Ingestion rates (ng C grazer^{-1} h^{-1}) in replicate grazer bottles for each prey size group after 6 (black) and 24 (grey) hours of incubation in low (P_{low} = ~50 L^{-1}) and high (P_{high} = ~100 L^{-1}) predator treatments during experiments E1 and E2. (A) E1 P_{low}, (B) E1 P_{high}, (C) E2 P_{low}, and (D) E2 P_{high} treatments.

the 24-h incubations. For example, considering only significant changes in biomass from t_0, in E1 the 2–5 µm prey biomass decreased by 47% after 24-h, but only decreased by 15% after 6-h. In E2 the 2–5 µm biomass decreased by 7% after 6-h and by 30% after 24-h. Though non-significant, there were positive trends in E2 larger prey biomass, which increased by up to 20% in some prey groups. One interpretation of the changes observed in the controls is that trophic interactions between the other microzooplankton grazers, primarily heterotrophic ciliates and flagellates, were occurring in the bottles, and longer incubation times resulted in greater divergence away from the initial prey community, leading to increasingly artificial estimates of naupliar grazing rates.

Nauplii are able to graze on a range of prey sizes (Jungbluth et al. in press, Vogt et al., 2013, Almeda et al., 2010), which means that in Kane'ohe Bay they are feeding on small autotrophs as well as on heterotrophs that are grazing on the autotrophs. Thus, the nauplii in this community are both competitors and predators of heterotrophs. Evidence for trophic interactions altering prey size distribution was found by comparing the changes in control bottles over time with relative ingestion rates. In E1 controls, all prey size groups decreased over time, but this trend was only significant for prey smaller than 10 µm and for total prey (Table 2). In E2 controls, there was a non-significant (Table 2) positive trend in the biomass of organisms larger than 10 µm over time (Fig. 1C–E). If these larger cells were consumers, they would decrease the biomass of the smallest (< 10 µm) prey groups, which is what was observed. The same dynamics did not occur in the E1 controls. In fact, both the 2–5 µm and 5–10 µm prey biomass decreased roughly twice as fast in E2 than it did in E1, as indicated by regression slopes (Table 1). These results support the inference that the higher and increasing biomass of large cells in E2 was able to remove smaller prey at a higher rate than those in E1, and suggests there was a potential combined effect of grazing by heterotrophic cells > 10 µm and by the copepod nauplii in the grazing incubations. These dynamics would affect estimates of naupliar *I* in treatment bottles over longer incubations if nauplii were preferentially preying on the larger cells (microzooplankton), which is likely given known prey preferences for some nauplii (Mullin and Brooks, 1967; Meyer et al., 2002). The effects of these trophic interactions were minimized over shorter incubation times (6 h).

Prey were quantified using a Coulter Counter, facilitating rapid enumeration of the abundant small, spherical cells at the study site and avoiding laborious microscopy methods which may also underestimate cells that do not preserve well or which have low levels of autofluorescence (Gifford and Caron, 2000). Flow cytometry, while useful for differentiating phytoplankton from other suspended particles (Cucci et al., 1985; Sieracki et al., 2005), would not provide accurate sizing information for mixed prey populations, and would require preservation and staining of samples to evaluate heterotrophic biomass in the samples. The Coulter Counter was highly effective for these experiments, since the goal was to determine optimum incubation times for ingestion rate estimates on prey of a range of types and sizes. The primary concern with the use of the Coulter Counter occurs when a community is dominated by large, irregularly sized cells (i.e., pennate diatoms), which can result in biased estimates of ingestion rates and selectivity (Harbison and McAlister, 1980). Kane'ohe Bay is an oligotrophic subtropical embayment dominated by pico- to nano-sized plankton and spherical microzooplankton that do not preserve well (Cox et al., 2006; Hoover et al., 2006), making methods that rely on preservation unreliable. Ephemeral blooms of larger phytoplankton such as *Chaetoceros* sp. can occur in response to fall/winter rainfall-induced nutrient addition, but they last only for a few days before returning to a system dominated by small cells (Cox et al., 2006; Hoover et al., 2006; Drupp et al., 2011). In the time leading up to the current experiments, rainfall to the bay was not sufficient to trigger a phytoplankton bloom, with < 3 cm rainfall in 24-h period leading up to each experiment and rainfall < 8 cm cumulatively in the two weeks prior to each sampling event. Therefore, large irregularly shaped prey were unlikely to be a significant component of the < 35 µm prey community during the experiments.

Estimates of ingestion during grazing incubations could be underestimated in the absence of nutrient amendment if remineralization of nutrients by grazers increases the apparent growth rate of the phytoplankton during the incubation in treatments but not in the no-grazer control bottles (Landry and Hassett, 1982; Roman and Rublee, 1980).

Nutrient amendments in grazing experiments with nauplii might introduce artifacts, since nauplii have low nitrogen excretion rates relative to *in situ* concentrations and they tend to conserve nutrients more than adults (Meunier et al., 2016). Excretion rates of copepods are a function of biomass (Vidal and Whitledge, 1982; Mauchline, 1998), which allows a simple calculation of expected excretion rates from animal body size. For a paracalanid nauplius 130 μm in length, dry weight (DW, μg) is related to length (TL, μm) as Log DW = 2.285 × log TL − 5.965 (Mauchline, 1998; Hay et al., 1991), resulting in a DW of 0.0733 μg. With a relationship between excretion rate (E, μg N animal^{-1} h^{-1}) and DW (mg) of Log E = 0.844 × log DW − 0.385 (tropical calanoid copepods; Ikeda, 1974), excretion is estimated as 0.13 ng N animal^{-1} h^{-1}. At a nauplius grazer concentration of 50 nauplii in a 1 L volume, this excretion rate results in 0.7 nM N excreted in 6 h, and 2.5 nM N excreted in 24 h; values 2 to 3 orders of magnitude below the average nitrogen concentrations of 200–1000 nM in Kaneʻohe Bay (Drupp et al., 2011).

The higher ingestion rate estimates obtained from the 6-h incubation experiments suggests that copepod nauplii play a more significant role in marine ecosystems than is currently reported, particularly in tropical-subtropical waters. Compared with 24-h *I* estimates, 6-h estimates of naupliar grazing impacts on the total prey community lead to 2–4-fold higher total prey removal rates (Table 3). In subtropical waters, copepod nauplii could have an equal or greater daily carbon ration than observed in other ocean regions due to the effect that warmer temperatures have on ingestion (White and Roman, 1992) and growth rates (Ota and Landry, 1984), as well as the inverse relationship between carbon rations and body sizes (Mauchline, 1998). In combination with higher year round abundance in the subtropics (Hopcroft et al., 1998; McKinnon and Duggan, 2003; Hannides, 2007), high daily carbon rations could result in grazing impacts in these ecosystems that are higher than those observed in temperate ecosystems. Furthermore, nano- and microplankton communities in the subtropics are typically complex including a diverse community of auto- and heterotrophs (Takahashi and Bienfang, 1983; Barton et al., 2010). Thus, nauplii in warm water environments may also be adapted to exploit a wide range of prey and respond to rapidly changing prey resources with high naupliar grazing rates. Therefore, it is reasonable to conclude that other studies of naupliar grazing would similarly have higher, and potentially significant, grazing impacts if shorter incubations had been used. For example, Calbet et al. (2009) reported that the microzooplankton size fraction dominated by copepod nauplii (63–200 μm) across a transect of the Atlantic Ocean removed < 5% of primary production per day based on 24-h bottle incubation experiments spanning temperate to tropical latitudes. If 6-h incubations had been applied, these estimates would increase subtropical-tropical grazing impacts in the prior study by to up to 15% of primary production based on the results of this study.

In summary, grazing incubations containing a prey assemblage that is closest to the natural prey community give better estimates of trophic interactions occurring in nature. Therefore, especially in diverse and rapidly changing communities and for nauplii that transition between developmental stages in < 24-h, shorter incubation times are recommended to obtain the most accurate estimate of natural grazing rates. In the current study, the use of longer incubation times resulted in significantly lower *I* estimates for total ingestion and some prey size groups, divergence of the prey community from the initial assemblage, and differences in trophic dynamics that were evident by testing multiple predator densities with two prey communities. By more careful consideration of the natural rates of variability relevant to the predator of interest, future studies may discover that copepod nauplii are more important grazers in marine systems than previously thought.

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