COPEPOD NAUPLII AND THEIR ROLES IN PLANKTONIC MARINE FOOD WEBS

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DEDICATION

For Marcella Heinecke.

For all the love and support she provided to our family throughout my life, for inspiring in me a love of nature through her beautiful gardens, for always being there when I needed her, for thinking of her every time I hear polka music.

Thank you, Grandma, you will always be in my heart.

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And now, a recipe for the cocktail that helped me get through my final dissertation days:

Dark 'N Stormy

1 bottle Crabbie's Ginger Beer

1 shot dark rum (preferably The Kraken)

Mix well and enjoy, preferably while on a British Ship traversing the Atlantic Ocean

ABSTRACT

Copepod nauplii are numerically dominant metazoan plankton in many marine ecosystems and play important ecosystem roles both as grazers and as prey, but have historically been understudied due to small body size and challenges in identification of taxa. Little is known about naupliar grazing and the effects of environmental forcing on early life history stages of copepods in subtropical ecosystems. In this study, I measured the grazing rates and trophic impacts of two calanoid copepod nauplii on natural prey assemblages and investigated the population dynamics of four common species of copepod nauplii in a subtropical embayment, Kaneʻohe Bay, Hawaiʻi, under several ecosystem states. I found that grazing experiments can be biased by methods; extending the duration of grazing incubations from 6 h to 24 h reduced grazing rate estimates by up to 75%. *Bestiolina similis* nauplii were more selective against 2-5 µm prey than the closely related species, *Parvocalanus crassirostris*, and *P. crassirostris* nauplii were capable of significantly impacting prey populations, removing up to 12.9% of chlorophyll from the water column when this species was abundant. Species-level studies of nauplii in mixed field samples required the development of a quantitative PCR (qPCR)-based technique to distinguish species and estimate naupliar biomass across five orders of magnitude. Application of the qPCR method to study naupliar populations during a non-storm period and after two ecosystem perturbation events (i.e. storms), revealed temporal differences in recruitment rates and biomass loss between the four dominant species. After the early-season storm event, naupliar biomass was observed to increase by up to an order of magnitude within days of the storm, biomass was lost by up to 99% in the earliest developmental stages in the days following the storm, and the community structure rapidly returned to levels similar to my observations for a non-storm summer period. My results demonstrate that copepod nauplii can be selective grazers and have important trophic impacts in subtropical marine plankton communities. I also show that ecosystem perturbations alter species-specific copepod recruitment rates and biomass loss across development, promoting ephemeral shifts in species dominance within the naupliar community.

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LIST OF ABBREVIATIONS

Aut	Autotroph
b_f	Biomass at final time point, in calculation of P
b_i	Biomass at initial time point, in calculation of P
BL	Percent biomass lost between sequential artificial cohorts over expected development time
BSA	Bovine Serum Albumin
BV	Biovolume (μm^3)
C.V.	Coefficient of variation (%)
C#	Copepodite developmental stage number
CC	Coulter Counter
Chl *a*,	Chlorophyll *a*
Cq	Cycle quantification threshold
DW	Dry weight
E	Excretion rate ($\mu g\ N\ animal^{-1}\ h^{-1}$)
E#	Experiment number
EFD	Eggs $female^{-1}\ day^{-1}$
EPI	Epifluorescence microscopy
ESD	Equivalent spherical diameter
EtOH	Ethyl alcohol
F	Statistical F-value (ANOVA, not italic)
F	Clearance rate (mL nauplius h^{-1})
FCM	Flow cytometry
Het	Heterotroph
I	Ingestion rate (ng C nauplius h^{-1})
LV	Large volume (250 mL, 8.0 µm) epifluorescence sample
mtCOI	Mitochondrial cytochrome oxidase subunit I
N#	Naupliar developmental stage number
NTC	No template control
p	Statistical p-value
P	Proportional change between sequential artificial cohorts over time
PAR	Photosynthetically active radiation
PCA	Principal component analysis
PEUK	Photosynthetic eukaryotes
P_{high}	High predator treatment
P_{low}	Low predator treatment
qPCR	Quantiative polymerase chain reaction

LIST OF ABBREVIATIONS - continued

t	Transition time (d) between sequential artificial cohorts
TL	Total length
x_{early}	Expected fold-change in biomass between the early cohort to the middle cohort
x_{mid}	Expected fold-change in biomass between the middle cohort to late cohort
SV	Small volume (50 mL, 0.8 µm) epifluorescence sample

Chapter 1:
Introduction and Rationale

1.1 Background

Including nauplii and copepodites, copepods can constitute up to 99% of the zooplankton community abundance (Calbet et al. 2001; Duggan et al. 2008). Copepod nauplii alone contribute 30-70% of copepod abundance (Hopcroft et al. 1998a; Lučić et al. 2003; Turner 2004) and are often the most abundant component of the microzooplankton in ecosystems worldwide, from the Southern Ocean (Safi et al. 2007), through the temperate Adriatic Sea (Lučić et al. 2003), in Boston Harbor (Turner 2004), across the Atlantic Ocean (López and Anadón 2008), and in subtropical Kane'ohe Bay, Hawai'i (Calbet et al. 2001). However, due to their small size they often contribute little to total biomass (e.g. <10% Hopcroft et al. 1998a; Miyashita et al. 2009).

Nauplii are prey for a variety of other zooplankton and can be a preferred food resource for fish larvae (Checkley 1982; Sampey et al. 2007). Species-specific morphology, feeding, swimming and escape behaviors (Borg et al. 2012; Bradley et al. 2013; Bruno et al. 2012; Gauld 1959; Titelman and Kiørboe 2003) suggest that nauplii of different species may have distinct roles as contributors to carbon transfer in marine food webs. Despite their potential importance, due to their small size and morphological similarities (Björnberg 1986), there is little species-specific information available on their abundance or roles in trophic transfer in marine food webs.

The trophic role of nauplii and other small-bodied copepods may be of greater importance in oligotrophic ecosystems, because they serve as links between classical and microbial food webs (e.g.. Roff et al. 1995, Wickham 1995, Calbet et al. 2000). In the oligotrophic subtropics, the relative size of primary producers is smaller (Agawin et al. 2000; Chisholm 1992) and microbes dominate the water column (Gasol et al. 1997). Due to rapid naupliar growth rates and year round high abundance in these ecosystems, the grazing impact of nauplii is potentially high, but is often reported to be an insignificant portion of community grazing.

Microzooplankton grazers, including nauplii, remove 49-77% of primary production in marine pelagic ecosystems worldwide (Calbet and Landry 2004; Schmoker et al. 2013). Nauplii

have an unknown contribution to total microzooplankton grazing, but are capable of consuming prey spanning a range of sizes (Vogt et al. 2013) and nutritional qualities (Meyer et al. 2002). Nauplii may be important grazers to study at the species level, as indicated by the differential feeding behaviors that are exhibited by ambush-feeding and current-feeding nauplii (Bruno et al. 2012). While informative, many prior studies investigated naupliar feeding on monocultured prey items, and did not take into account selective grazing that may occur in the field where communities are characterized by diverse taxa with broad size and motility ranges.

Our knowledge of the role of nauplii in ecosystems is lacking in part because nauplii are undersampled by conventional mesozooplankton nets. Most studies of copepod populations use a 200 µm or larger mesh net (Sameoto et al. 2000). Previous studies on the copepod naupliar contribution to abundance or biomass from oceanic systems primarily used vertical or oblique tows using >53 µm mesh nets (e.g. López and Anadón, 2008; McKinnon and Duggan, 2003; Roman *et al*., 1995) however, a 64 µm mesh net misses 85% of the nauplii of small-bodied subtropical species (Hopcroft et al. 1998a). Studies reporting on nauplii typically report total naupliar abundance (e.g. López and Anadón, 2008), a single species abundance or depth distribution in a low diversity temperate ecosystem (e.g. Takahashi and Uchiyama, 2008; Zamora-Terol *et al.*, 2013), or naupliar abundance at the order level (McKinnon and Duggan 2003). Before we can understand the trophic ecology of nauplii, we need methods that allow us to measure the *in situ* contribution of species to abundance or biomass of the naupliar assemblage. In order to address this, during my Master's Thesis I described the genetic diversity of common copepod species in Kaneʻohe Bay (Jungbluth and Lenz 2013), and used DNA barcodes to develop a quantitative PCR (qPCR) based method to quantify naupliar stages of *P. crassirostris* in mixed field samples (Jungbluth et al. 2013). Results from my prior work showed promise for the development of the qPCR method to quantify additional dominant and ecologically important species in the study area.

The research described here takes place within the southern semi-enclosed region of, Kaneʻohe Bay, located on the windward side of Oʻahu, Hawaiʻi. In subtropical coastal regions such as Kaneʻohe Bay, storm events are a natural perturbation that can result in rapid and significant changes in the physical and chemical properties of the water column (De Carlo et al. 2007), as well as in the plankton community structure over daily time scales (Cox et al. 2006;

Hoover et al. 2006; McKinnon et al. 2003; Ringuet and Mackenzie 2005). These rapid changes may play a key role in supporting local coral reef productivity as well as support for surrounding oceanic waters, since productivity in the bay feeds the 'wall of mouths' living in coral reef habitats (Hamner et al. 2007; Hamner et al. 1988). Southern Kane'ohe Bay is characterized by four dominant copepod species present throughout the year (Scheinberg 2004). The species, *Parvocalanus crassirostris, Bestiolina similis, Oithona simplex,* and *O. attenuata* (Hoover et al. 2006; Jungbluth and Lenz 2013; Scheinberg 2004), are common constituents of (sub)tropical, neritic plankton communities across the Indo-Pacific (Hoover et al. 2006; Hopcroft et al. 1998a; Jungbluth and Lenz 2013; McKinnon and Klumpp 1998). The southern part of Kane'ohe Bay is oligotrophic in the summer (mean chlorophyll (Chl) *a* at 0.5 µg L^{-1}), and mesotrophic to eutrophic in the winter (mean 1.9 µg Chl a L^{-1}), due to storm events introducing nutrients to the bay from the surrounding watershed (De Carlo et al. 2007; Drupp et al. 2011). The waters are dominated by picophytoplankton year-round, with episodic, short-lived diatom blooms after nutrient input events (Calbet et al. 2000; Cox et al. 2006). The average residence time of water in the southern bay (1-2 months, Lowe *et al.*, 2009) is much longer than the growth rates of both autotrophic and heterotrophic microbial populations (>1 d^{-1}; (Hopcroft et al. 1998b; Landry et al. 1984; Laws et al. 1984; Newbury and Bartholomew 1976). Therefore, plankton dynamics observed over daily time scales are likely determined by *in situ* rather than advective processes under most conditions in the bay. During storm perturbations, previous work showed that wind speed and direction are the biggest factors contributing to retention or advection of nutrient-rich freshwater runoff. The upper 2-3 meters of the water column are most affected by wind forcing (Bathen 1968). Given the bathymetry of the southern basin (12-16 meters) it is expected that most of the water column below the surface waters are retained at rates close to the 1-2 month average residence time previously reported. Kane'ohe Bay also has close proximity to highly productive patch reefs that serve as nurseries for a range of marine species. These factors make the bay an interesting and tractable model system to study the impact of anthropogenic and natural perturbations on coastal copepod naupliar communities.

1.2 Research objective and structure

The overall objective of my doctoral research was to provide comprehensive understanding of the ecological significance of copepod nauplii as members of planktonic marine communities.

The dissertation has six chapters. **Chapter 1** is the introductory chapter. In **Chapter 2,** I tested methods for measurement of subtropical naupliar grazing on the natural prey assemblage. A significant decline in ingestion rate estimates was found in response to longer incubation durations, suggesting that shorter incubations result in more biologically relevant ingestion rates in studies of subtropical naupliar grazing. Several previous studies of grazing by nauplii on *in situ* prey communities reported nauplii to be largely insignificant grazers on prey communities, many of which used 24-h experiment incubations on temperate species. Chapter 2 is *in review* in the Journal of Experimental Marine Biology and Ecology, with co-authors K. E. Selph, P. H. Lenz, and E. Goetze. The manuscript was submitted as: Jungbluth, M. J., Selph, K. E., Lenz, P. H., and E. Goetze. *Incubation duration effects on copepod naupliar grazing estimates.* Journal of Experimental Marine Biology and Ecology, August 2016.

In **Chapter 3** experiments were performed to measure naupliar (N3-N4) grazing rates and trophic impacts of two dominant calanoid copepod species, *Bestiolina similis* and *Parvocalanus crassirostris*, on natural prey communities. Bottle incubation experiments were paired with *in situ* dilution experiments and quantitative estimates of naupliar abundance. The two species were able to graze the same range of prey types and sizes. However, *B. similis* nauplii exhibited stronger selection for larger prey in experiments where large prey were more available, and selection against the smallest prey quantified (2-5 µm) throughout the experiments, in comparison to *P. crassirostris*, which selected prey more in proportion to its availability in the environment. Application of genetic methods described in Jungbluth et al. (2013) and Chapter 4 to estimate the abundance of each species *in situ* revealed that due to the higher abundance of *P. crassirostris* nauplii, their grazing impact was potentially high, capable of removing up to 12.9% of the daily chlorophyll *a*, in addition to other prey types and size groups. Chapter 3 is in review in the journal Marine Ecology Progress Series, with K. E. Selph, P. H. Lenz, and E. Goetze as co-authors. The manuscript was submitted as: Jungbluth, M.J., Selph, K.E., Lenz, P.H., and E.

Goetze. *Are copepod nauplii important grazers in marine planktonic food webs*? Marine Ecology Progress Series, September 2016.

In **Chapter 4,** I describe optimization and testing of a quantitative PCR-based method to quantify and identify all four dominant copepod nauplii in Kane‘ohe Bay, with important conversions of mtCOI copy number to animal biomass. Results of this work show that the primers developed accurately amplified DNA of each of the four species, and that quantification of DNA copy number gave an accurate estimate of copepod naupliar biomass of culture populations of *P. crassirostris*. Field validation suggests a range for calanoid and cyclopoid nauplii within 2.5 fold when comparing qPCR-based estimates with count-based biomass of quantitatively split field samples. Future and ongoing work will develop species-specific conversion factors, which may increase the correspondence between these two measures.

This chapter will be submitted for publication with Katherine Hanson and Michelle Jungbluth as co-first authors; we split the work evenly. All coauthors and committee members have agreed that inclusion of this work as a chapter of my dissertation is appropriate. My portion of the work included development and initial testing of the primer pairs for two of the four target species (*Parvocalanus crassirostris* and *Bestiolina similis*), identifying initial potential candidate primer pairs for the two *Oithona* species, measuring the biomass of *P. crassirostris* stages, comparison of culture *P. crassirostris* biomass to copy number, and performing the field validation experiments necessary for comparing paired samples to show the utility of the method. Development and testing of the methods for quantification of copy number and biomass in each target species directly facilitates work done in Chapter 5, and is therefore important to include in the thesis. This work will be submitted for publication with the following authorship: Hanson, K.M., Jungbluth, M.J., Lenz, P.H., Robinson, E., and E. Goetze as coauthors.

In Chapter 5 I investigate copepod early life history responses to event-scale perturbations in Kane‘ohe Bay, using the qPCR method to quantify naupliar biomass for each species. I examine changes in early naupliar production rates, quantify the disappearance of biomass between naupliar developmental periods as an index of mortality, and evaluate differences between populations during a non-storm period and two different types of storms; a first-flush (late

October) and a late-season (early June) storm. The difference in the magnitude of species response to the two storms suggests that all storms do not result in equal forcing of copepod communities despite relatively high levels of total Chl *a* during the period after the two events, that species are not equal in their contribution to the naupliar pool, and that the contribution of species to the naupliar community changes over time, particularly during ephemeral post storm periods. I plan to submit this manuscript for publication with authorship as follows: M. Jungbluth, P. Lenz, K. Hanson, K. Selph and E. Goetze.

Finally **Chapter 6** is a synthesis of the conclusions and significance of the dissertation, as well as future directions of research on copepod nauplii in marine food webs.

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Chapter 2:
Incubation duration effects on copepod naupliar grazing estimates

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2.1. Abstract

Copepod naupliar grazing estimates often appear to represent an insignificant fraction of prey community mortality, despite high naupliar abundance and weight-specific ingestion rates. To address this seeming inconsistency, 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 of incubation. In no predator controls, prey biomass also decreased markedly during the 24-h incubation in many prey groups despite the absence of nauplii, while prey group biomass increased in prey >10 µm in the experiment with higher initial prey abundances. Predator density effects in this experiment provided additional evidence for trophic cascades. Our results 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 little resemble the *in situ* populations.

Keywords: bottle incubation experiments, copepod grazing, nauplii, experimental design, subtropical marine plankton

2.2. 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). However, 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 by which microbial production could be transferred to higher trophic levels, due to a capacity to feed on pico- and nano-sized particles (i.e., 0.2-20 µm equivalent spherical diameter: ESD), which represent the size spectra of the majority of phytoplankton (Roff et al. 1995).

A common and informative method used to measure zooplankton ingestion rates is using a bottle incubation experiment to study prey removal relative to controls with no predator (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, many of these methods do not account for the full spectrum of potential prey. While there are drawbacks to the use of bottle incubation studies 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, a critical factor in subtropical environments where both prey types are common (e.g. Takahashi and Bienfang 1983). However, this type of experiment requires optimization of incubation conditions to avoid artificial 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 turbulence, nutrient, or light regimes, crowding of grazers, interactions with the container walls, and trophic cascades or food web effects (see Båmstedt et al. 2000). Roman and Rublee (1980) found inconsistent bottle effects over time in ingestion estimates based on grazing indicators such as ATP, chlorophyll *a*, and particle concentration. These studies recommended shorter incubations in order to reduce the impact of many types of bottle effects (Roman and Rublee 1980; Båmstedt et al. 2000).

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). Some reduction in ingestion estimates over longer incubations may occur due to diel changes in grazing rates (Kiørboe et al. 1985), declining food quality over time (Roman and Rublee 1980) or from trophic interactions in bottles artificially making it appear as though ingestion estimates decline over time (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).

Grazing rate measurements for nauplii based on 24-h incubations suggest that some nauplii may be able to impact prey items significantly, while others do not. In more 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 Chilean coast, [Böttjer et al. 2010]) while other studies found nauplii to be 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.

Our goal was to identify the optimal conditions and duration of incubation for measurement 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-hour bottle incubation experiments (controls), (2) determine how different incubation durations affect 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. Our results suggest that shorter incubation times minimize bottle artifacts, such as declining ingestion rate estimates over time, and gives a more clear view of trophic interactions within bottles when compared with longer incubations.

2.3. 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 (sub)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. 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-hours 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 hours 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 our added nauplii. The <35 µm-filtered 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 Kaneʻohe Bay, and also in order to minimize development of artificially high nutrients given the prevailing oligotrophic conditions of our 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 (<1 µm) in Kaneʻohe Bay (Drupp et al. 2011). Therefore, excretion rates in our 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 N3-N4 nauplii that had been isolated previously 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 Kaneʻohe 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 – 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 sampling methods to study 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 can be 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 sub-sampling with a small volume of filtered seawater.

Data on prey size (ESD) and abundance from the Coulter Counter were further processed using R (R Core Team, 2016). 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 $nauplius^{-1}$ $hour^{-1}$) and clearance (*F*, mL $nauplius^{-1}$ $hour^{-1}$) rates on each prey size group using the equations of Frost (1972), and report here only where *F* or $I > 0$.

The effects of incubation time on *I*, prey biomass, prey abundance, and differences in prey growth rates between control and treatment bottles were evaluated separately. Linear regressions were used to evaluate whether there was a relationship between control bottle prey abundance and incubation time, and to evaluate the relationship between carbon *I* and incubation time. Two-Way ANOVAs followed by post-hoc Tukey tests were performed to compare interactions of carbon *I* by prey size group and time, and comparing carbon *I* for low and high predator abundance bottles and time. The Coefficient of Variation (CV, %) was calculated for cell abundance estimates followed by a two-way ANOVA and post-hoc Tukey test to evaluate for the effects of prey size group and incubation time on the variation in cell abundance. Some studies of zooplankton grazing (e.g., Atienza et al. 2006; Calbet et al. 2009; Almeda et al. 2011) tested for significant differences in prey growth rates between control and treatment bottles, and then considered only significantly different conditions in further calculations of *I*. In contrast,

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.

2.4. Results

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. 2.1) 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 2.1).

The prey community within the control bottles changed during the 24-h incubation period. Biomass in each prey size group changed linearly as a function of incubation time (0, 6, 12, 18, 24-h) in both experiments, even though there were differences in the initial prey community between E1 and E2 (Fig. 2.1, Table 2.1). During E1, total prey carbon in control bottles decreased by 13% after 6-h, and 34% after 24-h of incubation. Total carbon declined similarly in E2, decreasing 5% after 6-h and 16% after 24-h. In E1, 74% of the initial total prey carbon was 2-10 µm in size and all prey size groups decreased in abundance over time (Fig. 2.1). In E2, 84% of the total prey carbon was 2-10 µm in size (Table 2.1); the contribution of this group declined to 79% of the total after 24-h, due to an increase in the abundance of prey larger than 10 µm over the duration of the experiment (Fig. 2.1). In E1, the available biomass in every prey size group decreased between 6 and 24-h (slopes of -0.01 to -0.66), and in the three smaller size fractions incubation time explained 50 – 95% of the variation in biomass (Table 2.1). 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, where incubation time explained 21 – 65% of the increase in biomass, while in the two smallest size groups time explained 55% and 98% of the reduction in biomass (Table 2.1).

Ingestion and Clearance Rate Estimates Over Time – Estimates of the total ingestion (*I;* ng C $grazer^{-1}$ $hour^{-1}$) rates decreased (negative slope) over the 24-h incubation period (Fig. 2.2, Table 2.2). The highest total *I* rates were observed in low predator treatments (P_{low}) for both

experiments (Fig. 2.2a, b). In E1, the maximum total *I* was 21.5 ± 6.8 ng C grazer^{-1} hour^{-1} (mean ± standard error) at 6-h (Fig. 2.2a), and in E2 it was 45.8 ± 14.2 ng C grazer^{-1} hour^{-1} at 12-h (Fig. 2.2b) which was 7% higher than the E2 6-h estimate. After 24-h of incubation *I* decreased by 75% in E1 and decreased by 39% (from the 6-h estimate) in E2. In high predator treatments (P_{high}), maximum *I* for E1 at 6-h was 10.5 ± 4.6 ng C grazer^{-1} hour^{-1} (Fig. 2.2a), while E2 maximum *I* was 17.6 ± 4.3 ng C grazer^{-1} hour^{-1} at 12-h (Fig. 2.2b) which was 28% higher than the 6-h estimate. The *I* estimates for P_{high} decreased over 24-h of incubation by 48% in E1 and 18% from the E2 6-h time point.

Predator treatment affected the total *I* estimates over time in E2 but not in E1, while incubation time significantly affected total *I* in E1 but not in E2. A two-way ANOVA of *I* by predator treatment (P_{low} and P_{high}) and time (6, 12, 18, and 24-h) for experiment E1 shows a significant effect of time on *I* estimates ($F_{3,16} = 7.15$, $P < 0.01$), but the differences in predator treatment were not significant ($F_{1,16} = 2.98$, $P = 0.10$). Post-hoc comparisons (Tukey test) show that for E1, within P_{low} the 6-h *I* was significantly different from 12, 18, and 24-h ($P < 0.05$), but there were no significant differences over time in P_{high} ($p > 0.05$). Comparison of the difference between P_{low} and P_{high} *I* estimates within each incubation duration found significant differences for 6-h estimates but not comparisons within 12, 18, and 24-h (Tukey test; $P < 0.05$). These results are an indication that differences in predator abundance have significant effects at the 6-h time point but not during longer incubations for E1. A two-way ANOVA of *I* by predator treatment and time for E2 total *I* did not yield the same results; predator type had a significant effect on *I* estimates ($F_{1,16} = 26.95$, $P < 0.001$), however time did not ($F_{3,16} = 2.27$, $P = 0.12$). Post-hoc comparisons show that within 6, 12, and 18-h (each separately) P_{low} and P_{high} *I* estimates were significantly different, but not within 24-h results (Tukey test; $P < 0.05$).

Total clearance (*F;* mL grazer^{-1} hour^{-1}) rate estimates also decreased (negative slope) over the 24-h incubation period (Fig. 2.2c, 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} hour^{-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} hour^{-1} and 1.32 ± 0.17 mL grazer^{-1} hour^{-1} respectively (Fig. 2.2c,d). This declined 33% in E1 and 78% in E2 after 24-h.

The prey size groups that drove the differences in total *I* between P_{low} and P_{high} for E1 and

E2 can be seen when the *I* data as a function of incubation time are examined across different prey size groups. For experiment E1, the patterns of declining *I* over time were similar between the predator abundance (i.e., P_{low} and P_{high}) treatments (Fig. 2.3, Table 2.2). However, in experiment E2 there was more variability in the estimate of *I* between P_{low} and P_{high} treatments. In P_{low} treatments the maximum mean *I* estimate for E1 was found after 6-h at 12.6 ± 4.8 ng C grazer^{-1} hour^{-1} on 20-35 µm prey and decreased by 87% after 24-h. The maximum *I* for E1 P_{high} was also on the 20-35 µm prey group after 6-h at 4.2 ± 3.4 ng C grazer^{-1} hour^{-1} and decreased 44% after 24-h. During experiment E2, in the P_{low} treatment, the greatest *I* was observed on 2-5 µm prey at 17.8 ± 3.1 ng C grazer^{-1} hour^{-1} that decreased by 54% after 24-h. The maximum *I* in experiment E2 in the P_{high} treatment was on 5-10 µm prey at 6.2 ng C grazer^{-1} hour^{-1} at 18-h, and decreased by 14% by the 24-h time point.

Linear regressions of the individual size fractions show a negative or near-zero relationship between *I* and incubation time (Fig. 2.3, Table 2.2). There was a negative correlation (negative slope, $r^2 > 0.5$) between *I* and increasing time of incubation in experiment E1 for two of the five size fractions in P_{low} and three of five size fractions in P_{high}, and in experiment E2 for four of the five size fractions for P_{low} and two of the size fractions in P_{high} (Fig. 2.3, Table 2.2). In E1 this included the 2-5 µm and 20-35 µm prey size groups in P_{low} and P_{high}, as well as the 10-15 µm and 15-20 µm size groups in $P_{high.}$ In E2 this included 15-20 µm, and 20-35 µm prey size groups for both P_{low} and $P_{high,}$ and the 2-5 µm prey in P_{low}. Interestingly, the *I* of 5-10 µm prey increased over time for both P_{low} and P_{high} in E2 but was close to unchanging over time in E1 (Table 2.2). Two-way ANOVAs of *I* by size group (2-5, 5-10, 10-15, 15-20, and 20-35 µm) and time (6, 12, 18, and 24-h) were performed for each experiment and predator treatment separately. For E1 P_{low}, there were significant effects of both prey group ($F_{4,34} = 3.50$, $P = 0.02$) and time ($F_{3,34} = 3.86$, $P = 0.02$) on *I* estimates, however there were no significant effects of prey group or time on *I* in E1 P_{high} (group: $F_{4,33} = 1.11$, $P = 0.37$; time: $F_{3,33} = 2.61$, $P = 0.07$). Post-hoc tests on E1 P_{low} results show that differences between 6 and 18-h were significant (Tukey test; $P = 0.04$) and that the 15-20, and 20-35 µm size fractions were significantly different (Tukey test; $P = 0.02$). For E2 P_{low} and P_{high} the prey group had a significant effect on *I* estimates (P_{low}: $F_{4.34} = 7.50$, $P < 0.001$; P_{high}: $F_{4.26} = 3.90$, $P = 0.01$) but time did not (P_{low}: $F_{3,34} = 2.76$, $P = 0.06$; P_{high}: $F_{3,26} = 1.82$, $P = 0.17$). Post-hoc tests for E2 P_{low} indicate that the ingestion estimates for the 2-5

and 5-10 µm size fractions were each different from all other size fractions (Tukey test; $P < 0.05$) but for E2 P_{high} the 2-5 and 15-20 µm size fractions were the only significantly different estimates (Tukey test; $P < 0.05$).

A two-way ANOVA of *I* by predator treatment (P_{low} and P_{high}) and time for experiment E1 ignoring differences in prey size group shows there was a significant effect of time on *I* estimates but not of predator treatment (time: $F_{3,16} = 7.14$, $P < 0.01$; predator: $F_{1,16} = 2.98$, $P = 0.10$). The same comparison for E2 found a significant effect of predator treatment on *I* estimates and no significant effect of time on *I* estimates (time: $F_{3,16} = 2.27$, $P = 0.12$; predator: $F_{1,16} = 26.95$, $P < 0.001$).

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. This statistic was unaffected by the length of incubation for P_{low} with variable results for P_{high}. For both E1 and E2 P_{low}, the 2-5 µm size group control and treatment growth rates were significantly different at each time point ($t = 6, 12, 18, 24$; *t-test*, $P < 0.05$), with the single exception of the 18-h control and treatment growth in E2, and no significance was found for the other size fractions. For P_{high}, the growth rates of E1 15-20 µm prey were significantly different at 18-h with no other significance for size fractions or time points in E1, and in E2 the 2-5 µm prey 12-h and 24-h control and treatment growth rates were significant, but no other size fractions or time-points (*t-test*, $P < 0.05$). The differences between P_{low} and P_{high} suggest that the higher abundance of predators altered prey abundances in E1 and E2 differently than the low predator abundance treatments, possibly reflecting the effects of copepod nauplii on trophic dynamics occurring in the bottles over time.

In general, the CV (%) was low for the smallest prey size groups, and increased with increasing prey size as a result of the decrease in the number of prey counted per replicate for larger prey. The range of the 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 relationship between the CV and prey size groups, but not with incubation time (two-way ANOVA, $P < 0.05$), indicating confidence in prey abundance

estimates does not differ between 6 and 24-h incubations and higher confidence in estimates of small prey than in estimates of larger prey.

2.5. 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, 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 significantly affected *I* only at the higher prey concentration (E2, ~3-fold higher). These observations suggest that there is not a straightforward way to correct for the effect of incubation time on ingestion estimates. In control bottles, biomass decreased over incubation time in most prey size groups in both experiments, indicating that any *I* estimates based on longer incubations became increasingly artificial. Differences between the two experiments suggest that *I* estimates could be affected by initial prey fields. Due to the combination of the rapid changes in the prey community, decreasing *I* over time, and variable trophic interactions, shorter incubation times are preferable for subtropical-tropical species, particularly for studies on grazing by 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 (Ambler and Miller 1987; Haney 1988; Neill 1992), however diel migration patterns in 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. There was no evidence in the current study of a diel feeding cycle, instead there was

a steady 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 less than 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, it is important to design experiments considering both the rate of variation in the prey community as well as biologically relevant rates of the predator.

Prey biomass and abundance decreased over the duration of the experiments. For example, after 24-h a 34% decrease in total prey biomass in E1 and a 16% decrease in E2 was observed, with a decline in total carbon of only 13% and 5%, respectively, 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 the 24-h incubations. For example, in E1 the individual prey group biomass decreased by 12 - 47% after 24-h, but only decreased by 1 - 24% after 6-h. In E2 this difference was even greater. After 24-h the prey biomass increased by up to 20% in some prey groups and decreased by up to 30% in others, while after 6-h, prey biomass increased by up to 21% in some prey groups and decreased by up to 7% in others (Fig. 2.1). 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.

Additional 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. In E2 controls, the biomass of organisms larger than 10 µm increased over time (Fig. 2.1c, d, e), which would increase the rate of removal of the smallest prey if most of the cells >10 µm were heterotrophic. This expectation is consistent with the

negative trend observed in the <10 µm prey groups. If significant protistan microzooplankton grazing occurred in the control bottles, the rate of decrease in E2 small prey (2-10 µm) biomass would be greater than the rate of decrease in the E1 small prey over the 24-h incubation due to the higher relative abundance of large cells, and the increase in large cells over time. In fact, the rate of decrease in the biomass of the 2-5 µm prey size group for E2 was 1.9 times greater than in E1 (slope for E2 –1.23 vs. E1 -0.66, Table 2.1), and the 5-10 µm prey size group decreased in E2 at a rate 2.5 times greater than in E1 (slope for E2 -0.27 vs. E1 -0.11, Table 2.1). 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. 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). A general correction factor (0.4 day^{-1}, see Saiz and Calbet 2011; Nejstgaard et al. 2001) was applied to the data to assess the effect of correcting for grazing by competing protistan grazers, and resulted in variable effects on grazing rate estimates (i.e. some increases, some decreases).

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). Some previous studies found the Coulter Counter to be unreliable for quantification of large, irregularly sized cells, resulting 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, rarely dominated by large-celled phytoplankton (Cox et al. 2006; Hoover et al. 2006). Ephemeral blooms of larger phytoplankton, such as the diatom *Chaetoceros* sp., may occur in response to rainfall-induced nutrient addition, often lasting 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 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 and use of the

coulter counter was justified for evaluation of the change in cells over the duration of grazing incubations.

Ingestion rates 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). However, in experiments studying nauplii grazers, which have low nitrogen excretion rates relative to in situ concentrations and tend to conserve nutrients more than adults, it is unlikely that nutrient amendments are necessary to obviate this concern in a nitrogen-limited system. 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 0.2-1.0 μM in Kaneʻohe Bay (Drupp et al. 2011).

The prey communities in the control bottles changed little during the initial 6-h, suggesting that the effect of trophic cascades on naupliar grazing was minimized with the shorter incubation time. Compared with 24-h *I* estimates, 6-h estimates of naupliar grazing impacts on the total prey community lead to 2-3-fold higher removal rates, and thus support inferences of a more significant role of copepod nauplii in marine ecosystems. 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 are more appropriate for warmer-water species, these estimates would increase subtropical-tropical grazing impacts to up to 15% of primary production if the differences between 6-h and 24-h were as high as measured in 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 less than 24-h, shorter incubation times are recommended to obtain the most accurate estimate of natural grazing rates. In our study, the use of longer incubation times resulted in significantly lower *I* estimates, divergence of the prey community from the initial assemblage, and differing trophic dynamics that were elucidated by testing multiple predator densities with two prey communities. Future studies on grazing in natural prey communities with widely variable prey densities may benefit from testing multiple predator densities, as was done here, and which allowed us to see trophic dynamics that would otherwise have been invisible. 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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Tables and Figures

Table 2.1: Control bottle mean prey; initial (time = 0) biomass (µg C L^{-1}) and abundance (cells mL^{-1}) (± standard error), and the slope, y-intercept, and correlation coefficient (r^2) of the linear regression of prey biomass (µg C L^{-1}) over time in experiments E1 and E2.

		E1							E2						
		Biomass (µg C L^{-1})			Abundance (cells mL^{-1})			Regression			Biomass (µg C L^{-1})			Abundance (cells mL^{-1})			Regression		
								Slope	Intercept	r^2							Slope	Intercept	r^2
	Total	66.1	±	4.4	11428	±	144	-0.92	63.78	0.93	197.6	±	4.4	34491	±	119	-1.30	195.87	0.97
Prey groups (µm)	**2-5**	32.9	±	0.9	10673	±	100	-0.66	31.77	0.95	95.8	±	0.2	31190	±	64	-1.23	95.58	0.98
	5-10	16.0	±	0.4	675	±	30	-0.11	15.95	0.78	71.0	±	1.2	3127	±	45	-0.27	68.80	0.55
	10-15	8.0	±	1.7	66	±	12	-0.07	7.52	0.50	18.0	±	0.9	150	±	6	0.10	17.78	0.65
	15-20	3.4	±	0.0	10	±	0	< -0.01	3.03	0.06	6.3	±	1.4	18	±	4	0.04	6.37	0.60
	20-35	5.6	±	1.5	5	±	1	-0.08	5.53	0.46	6.5	±	0.8	6	±	0	0.06	7.46	0.21

Table 2.2: Experiment E1 and E2 mean (± standard error) ingestion rates (I, ng C nauplius^{-1} hour^{-1}) for 6-h and 24-h incubations, and the slope, y-intercept, and correlation coefficient (r^2) 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.

		E1									E2								
		6-h I (ng C nauplius^{-1} hour^{-1})			24-h I (ng C nauplius^{-1} hour^{-1})			Regression			6-h I (ng C nauplius^{-1} hour^{-1})			24-h I (ng C nauplius^{-1} hour^{-1})			Regression		
								Slope	Intercept	r^2							Slope	Intercept	r^2
Low Predator																			
	Total	21.5	±	6.84	5.3	±	0.68	-0.85	23.07	0.75	42.6	±	7.54	26.2	±	3.42	-1.00	52.41	0.79
Prey groups (µm)	**2-5**	5.5	±	0.6	1.2	±	0.1	-0.23	6.37	0.92	17.8	±	3.1	8.2	±	1.5	-0.57	20.36	0.87
	5-10	2.1	±	0.9	1.4	±	0.2	-0.03	1.92	0.20	7.7	±	1.0	9.0	±	1.7	0.04	11.79	< 0.01
	10-15	0.1	±	*		-		-	-	-		-		3.2	±	*	-0.10	5.58	0.99
	15-20	1.3	±	0.5	1.1	±	0.3	-0.02	1.43	0.18	6.2	±	3.4	2.7	±	0.2	-0.22	8.38	0.76
	20-35	12.6	±	4.8	1.6	±	*	-0.56	12.93	0.64	11.0	±	*	3.2	±	*	-0.39	11.27	0.66
High Predator																			
	Total	10.5	±	4.69	5.5	±	0.80	-0.29	11.47	0.82	13.7	±	3.23	11.2	±	1.79	-0.22	16.86	0.35
Prey groups (µm)	**2-5**	4.1	±	1.3	1.2	±	0.4	-0.16	4.64	0.87	5.1	±	0.3	4.8	±	0.8	-0.05	5.58	0.18
	5-10	2.2	±	*	1.1	±	0.3	-0.04	2.10	0.44		-		5.4	±	0.7	0.11	3.20	0.37
	10-15		-		0.5	±	0.1	-	-	-		-			-		-	-	-
	15-20		-		0.3	±	0.1	-0.05	1.49	0.98	3.6	±	2.7	0.5	±	0.3	-0.18	4.55	0.95
	20-35	4.2	±	3.4	2.4	±	*	-0.13	4.68	0.57	5.0	±	0.2	0.6	±	0.0	-0.24	5.99	0.94

- not enough observations with $I > 0$.

* only one replicate where $I > 0$.

Figure 2.1. Change in mean carbon (μg C L^{-1}) in replicate control bottles for each size group (a-e) over time. Dark circles and solid lines are data and regression for E1, light circles and dotted lines show results for E2. Error is ± SE. Regression results are reported in Table 1.

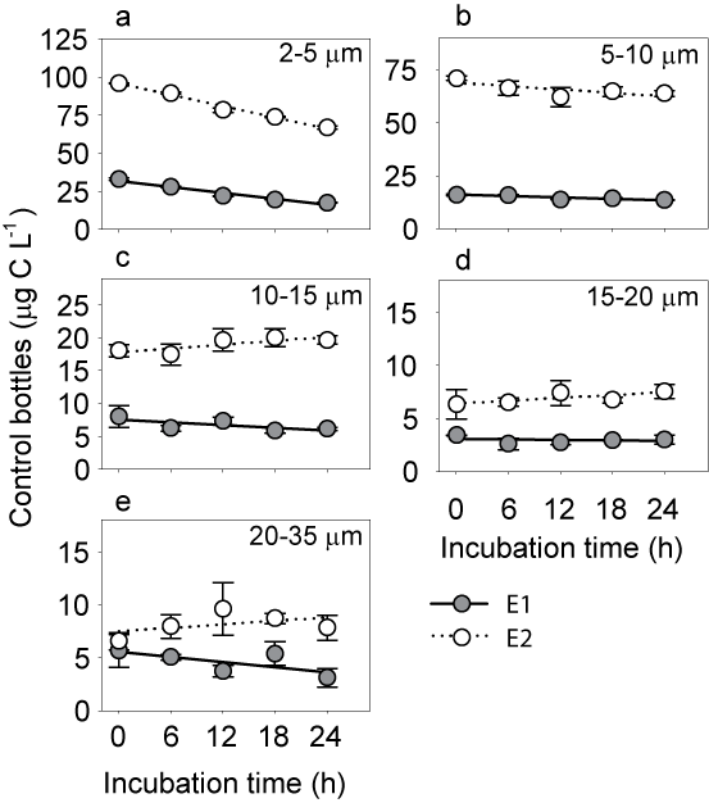


Figure 2.2. Change in estimates of total carbon ingestion (a,b) (ng C grazer^{-1} hour^{-1}), and total clearance (c,d) (mL grazer^{-1} hour^{-1}) over incubation time. Triangles are low predator abundance bottles, circles are high predator abundance bottles. Dark symbols are experiment E1, light symbols are E2. In the legend, dotted regression line is for low abundance predator treatments (~50 L^{-1} = Low), solid regression line is for high abundance predator treatments (~100 L^{-1} = High). Error is ± SE.

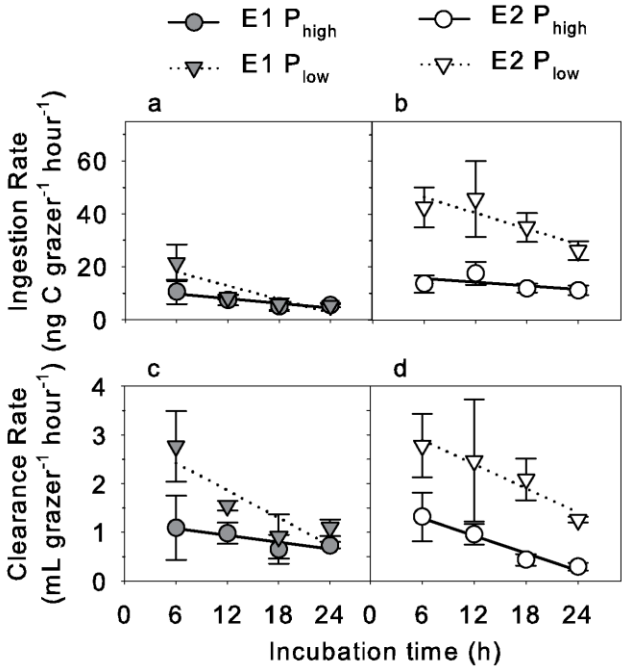
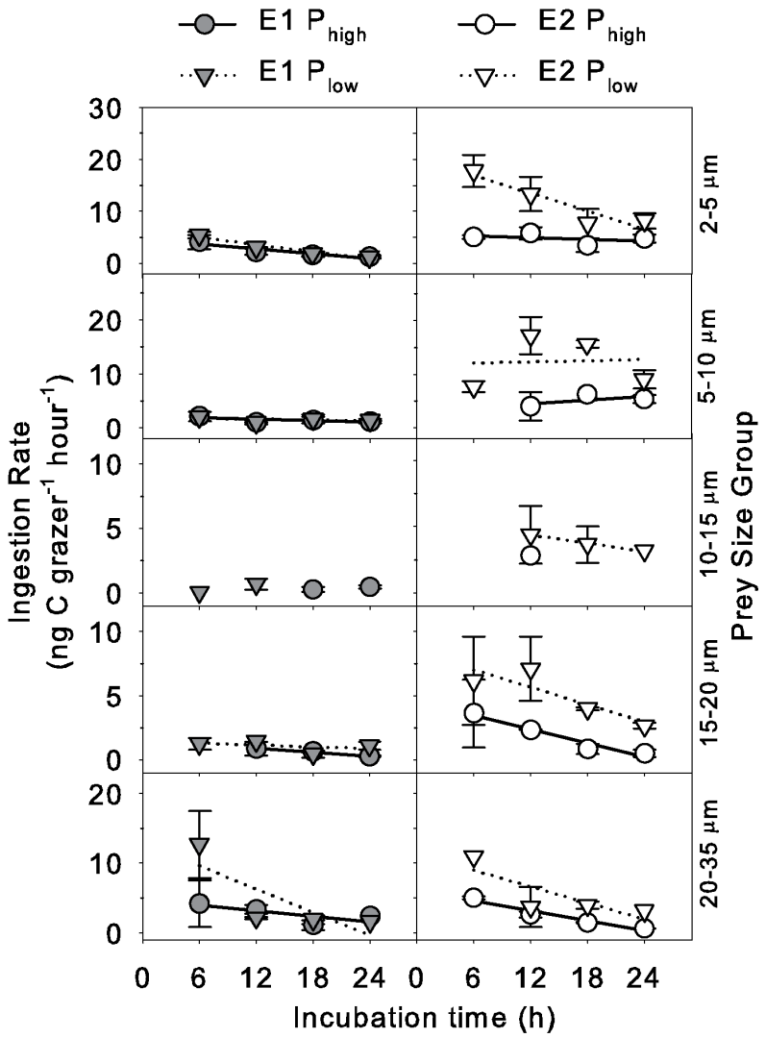


Figure 2.3. Change in estimates of carbon ingestion (ng C grazer^{-1} hour^{-1}) in replicate grazer bottles for each prey size group (right y-axis) over time, for experiments E1 (left panels, dark symbols) and E2 (right panels, light symbols). Lines are linear regressions of the data. In the legend, dotted regression line is for low abundance predator treatments (~50 L^{-1} = Low), solid regression line is for high abundance predator treatments (~100 L^{-1} = High). Error is ± SE.



Chapter 3:
Are copepod nauplii important grazers in marine food webs?

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3.1 Abstract

The ingestion rates of *Parvocalanus crassirostris* and *Bestiolina similis* mid-stage (N3-N4) nauplii feeding on a natural prey assemblage from a subtropical embayment were measured to evaluate differences in prey preferences and estimate the trophic impact of grazing by each species. During the 2-week period of our experiments, the *in situ* 2-35 µm cell biomass increased over time and ranged from 45 – 187 µg C L^{-1}. The two copepod species studied overlapped in the range of prey removed, however *P. crassirostris* nauplii grazed less selectively than nauplii of *B. similis*, which selected against the 2-5 µm prey size group and increased selection over time for 20-35 µm prey. Total ingestion rates were comparable between the two species, and the majority of the grazing impact was due to *P. crassirostris*, which had a 2 to 11-fold higher *in situ* naupliar abundance. Grazing impacts by the two species combined ranged from 1.0 to 8.7% of the total 2-35 µm prey biomass. Prey removal on individual prey size groups by *P. crassirostris* ranged from 0.9 to 23.7%, with maximum impacts on prey >10 µm. Total daily chlorophyll *a* removal was up to 12.9% by *P. crassirostris*, and 5.7% by *B. similis*. Our results suggest that naupliar ingestion rates and prey selectivity can vary on timescales of 2-3 days and differ between two closely related species, and also that nauplii can have a significant grazing impact on prey populations, particularly through positive selection for prey size classes that are less abundant.

3.2 Introduction

Microzooplankton are the dominant phytoplankton consumers in the ocean and can remove up to 75% of primary production in marine ecosystems (Calbet and Landry 2004). Because of the year-round reproduction of subtropical copepods (Webber and Roff 1995), their high growth rates (e.g., Kiørboe & Sabatini 1995; McKinnon & Duggan 2003), rapid development times (Hart 1990; Kiørboe and Sabatini 1995; Peterson 2001), and rapid response to increases in their prey communities (Hoover et al. 2006; Hopcroft and Roff 1998; McKinnon and Duggan 2003), copepod nauplii can be an episodically significant component of the microzooplankton. For example, in response to the first rainfall event after a long dry summer in Hawai‘i an waters, Hoover et al. (2006) found that copepod nauplii increased to roughly 6-fold greater abundance than their pre-storm population within 10 days of the storm. During peaks of abundance, nauplii can exert significant grazing pressure on pico- and nano-sized prey (i.e., 0.2-20 µm equivalent spherical diameter: ESD)(Böttjer et al. 2010; Lonsdale et al. 1996; White and Roman 1992), in part because their specific ingestion rates can be greater than adult conspecifics (Almeda et al. 2011; Böttjer et al. 2010; Paffenhöfer 1971; Saiz and Calbet 2007; White and Roman 1992). Due to episodically high abundance and high specific ingestion rates, nauplii may be an important pathway by which microbial production is transferred to higher trophic levels.

Diverse species of nauplii are capable of consuming prey of a wide range of types and sizes (Saiz et al. 2014; Uye and Kasahara 1983), including phytoplankton (Meyer et al. 2002; Vogt et al. 2013), microzooplankton (Merrell and Stoecker 1998), bacteria (Roff et al. 1995; Turner and Tester 1992) and detritus (Heinle et al. 1977), with diet breadth often overlapping adult conspecifics. For example, *Oithona davisae* nauplii and adults are capable of consuming a similar range of prey types; even though the highest *O. davisae* nauplii clearance rates occurs on the smaller *Oxyrrhis marina* (18 µm ESD, 1.6 mL ind^{-1} d^{-1}), the adults have highest clearance rates on the larger ciliate *Strombidium sulcatum* (30 µm ESD, 11.7 mL ind^{-1} d^{-1}) (Saiz et al. 2014). Additional studies have shown that conspecific nauplii and adults ingest a similar range of different food types (Finlay and Roff 2004; Merrell and Stoecker 1998; Vogt et al. 2013). However, the preferred prey in a mixed assemblage of food has not been well studied for many species, and may differ between nauplii and adults.

The grazing impacts of copepod nauplii in subtropical ecosystems may be higher than those observed in temperate waters. In the subtropics, copepod nauplii may have an equal or greater daily carbon ration than observed in other ocean regions due to the effect of warmer temperatures 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 (Hannides 2007; Hopcroft et al. 1998; McKinnon and Duggan 2003), high daily carbon rations could result in grazing impacts in these ecosystems that are greater than those observed in temperate ecosystems. However, in subtropical ecosystems, the higher diversity, coexistence of closely related species, and year-round production of nauplii leave no distinct copepod cohorts to be followed and studied throughout development. Furthermore, morphological identification of nauplii to species is typically difficult or impossible due to their nearly identical appearance, and therefore knowledge of the ecology of subtropical copepod nauplii at the species level on natural prey is very scarce.

Despite the potential importance of nauplii as grazers in subtropical ecosystems, there are no prior studies of the impacts of subtropical nauplii grazing on natural prey assemblages. The current study focused on grazing by *P. crassirostris* and *B. similis* nauplii in Kaneʻohe Bay, a subtropical embayment on the eastern shore of Oahu, Hawaiʻi . Naupliar grazing on the natural prey assemblage was measured in five experiments over a two-week period in May-June 2013. Concurrent measurements of *in situ* naupliar abundance and their potential prey in field populations were made, as well as parallel seawater dilution experiments to estimate microzooplankton community grazing rates (Landry and Hassett 1982). Data from these latter 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). The objectives of the study were to 1) quantify ingestion by two species of common subtropical copepod nauplii, 2) evaluate species' differences in prey selectivity and how these preferences change with variable prey availability in the field, and 3) estimate the species-specific impact of naupliar grazing on the standing stock of prey to determine whether nauplii are important grazers in subtropical ecosystems. Our results suggest that selectivity on prey differs between species

and this leads to potentially different impacts on their prey depending on species-specific naupliar abundance and prey availability.

3.3 Materials and Methods

Grazing Experiments – A series of five bottle incubation experiments (referred to as E1-E5; Table 3.1) were conducted over a 10 day period (27 May - 5 June 2013) to measure grazing by copepod nauplii on the natural prey assemblage collected from station S3, located in the southern semi-enclosed basin of Kane‘ohe Bay, Oahu, Hawai‘i (21°25′56″N, 157°46′47″W; Jungbluth & Lenz 2013). The copepods chosen for these experiments were N3-N4 stage nauplii of *Parvocalanus crassirostris* and *Bestiolina similis*. In addition to the grazing experiments, concurrent experiments were run to measure microzooplankton community grazing, and to quantify *in situ* predator and prey abundances. Salinity and temperature in the field were measured using a YSI 6600V2 sonde. Daily rainfall estimates were obtained from rain gauge data located at Luluku (www.prh.noaa.gov/), and weather station data from the Hawai‘i Institute of Marine Biology (HIMB) (www.hawaii.edu/himb/weatherstation.html) was 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 have relatively short development times and are capable of completing naupliar development in less than 3 days and reaching the adult stage (C6) in less than 7 days (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 the grazing incubations. To produce these cohorts, adults of each species were isolated and fed 1×10^6 cells mL^{-1} *Tisochrysis lutea* (formerly *Isochrysis galbana* Tahitian strain; (Bendif et al. 2013) 18 h prior to the start of each experiment, with the adults removed 6 h later, resulting in a cohort of nauplii with a narrow developmental stage range (N3-N6) by the beginning of each experiment. Sets of ~50 nauplii were isolated into small volumes (<10 mL) of 0.2 µm filtered seawater 1-3 h prior to the start of each grazing experiment. 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 to two-20 L polycarbonate carboys directly from the sample bottle using silicone tubing.

Pilot experiments indicated that longer incubation times in our system decreased ingestion estimates within the grazing treatments, likely due to the fast development rates of our nauplii (<24 h inter-moult period) and the diverse and rapidly changing prey community in this relatively warm (>20°C) system. 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 (Jungbluth et al. *in review;* Roman and 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; this pre-screening was done to remove *in situ* nauplii and other comparably-sized competing microzooplankton predators. The nauplii were transferred into the 1120 mL grazing bottles at two densities (42-51 (moderate) and 81-95 (high) nauplii, Table 3.1) and placed on a bottle roller (Wheaton) at 5 rpm in the dark for 6 h. These two nauplius densities were tested to ensure we saw removal of prey cells relative to controls over our incubation period. Moderate density bottles resulted in positive ingestion of many prey items and results were comparable to those from higher density bottles, therefore results reported here focus on bottles with ~50 nauplii L^{-1}. This density of nauplii is high relative to individual species densities in Kaneʻohe Bay; however, it is within the range of total nauplius concentrations reported in previous studies in the bay (7-68 total nauplii L^{-1}; Hoover et al. 2006) and within the range we measured following storm run-off events in the bay (*pers. obs.*).

Treatment bottles were run in triplicate, with two to three control bottles for each experiment (two for E1-E2, three for E3-E5). Experiments were incubated at 21°C, which is within the range of the annual temperature fluctuation expected for this area of Kaneʻohe Bay (20-29 °C in prior 5 years; 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, the short incubation times (6 hr), and since nauplii are known to have low

expected nitrogen remineralization (~10-fold lower than adults) due to their small biomass compared to adults (Mauchline 1998; Vidal and Whitledge 1982). At a naupliar grazer concentration of 50 nauplii in a 1L volume, excretion rates result in values 2 to 3 orders of magnitude below the average nitrogen concentrations of 0.2-1.0 µM in Kaneʻohe Bay (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, as well as samples for specific prey types, including chlorophyll *a* (Chl *a*) and the abundance and biomass of pico-, nano- and micro-plankton. Nauplii from each experimental bottle 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. These nauplii counts were used for calculations of clearance and ingestion rates.

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 Coulter Counter (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 Coulter Counter with a 100 µm orifice tube, yielding a spectrum of particle sizes from 2 – 35 µm equivalent spherical diameter (ESD), as well as quantitative abundance data. These raw data were further processed in R (statistical software) to streamline re-binning of prey size groups, for downstream calculations of clearance and ingestion rates, and for statistical analyses.

Prey ESD was converted to biovolume (BV, μm^3) assuming spherical cells, then to carbon (C, pg C $cell^{-1}$), using the relationship $C = 0.216 \times BV^{0.939}$, which applies well to taxonomically diverse protists (Menden-Deuer and 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 Kaneʻohe Bay and also due to use in prior studies of adult copepod grazing in Kaneʻohe Bay

(Calbet et al. 2000). The binned, averaged data for initial and final time points for each control and treatment bottle was 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 Analysis)*.

Photosynthetic eukaryotes (Flow Cytometry) – Flow Cytometry (FCM) samples (1.5 mL) for photosynthetic eukaryote (PEUK) abundance were taken from the 20 mL subsamples (described above), preserved in 0.4% paraformaldehyde (final concentration), then flash-frozen in liquid nitrogen and transferred to a -80 °C freezer until processing. Preserved, frozen FCM samples were thawed in batches, then stained with the DNA dye Hoechst 34442 (1 µg ml^{-1}, final concentration) (Campbell and Vaulot 1993; Monger and Landry 1993), and analyzed using a Beckman-Coulter Altra flow cytometer for phytoplankton population abundances using fluorescence signals from DNA, phycoerythrin and Chl *a*. Resulting data were grouped into relevant populations using FlowJo (Treestar, Inc.). From parallel microscopy samples, we found that most of the phytoplankton in these samples were 2-3 µm ESD, with an average carbon biomass of 1.55 pg C cell^{-1} (biovolume converted to biomass using the Menden-Deuer & Lessard (2000) volume:carbon relationship). This average biomass was used to convert the FCM PEUK abundance data to biomass, assuming spherical cells.

Nano- and microplankton abundance and biomass (Microscopy) – Initial and final samples for nano- and microplankton abundance by epifluorescence microscopy (EPI) were preserved using a final concentration of 0.4% paraformaldehyde, and kept in the dark and cold (4°C) until filtered within 24-48 h. Within 1-2 h prior to filtration, EPI samples were stained with 0.5 nM proflavin, then incubated in the dark at 4°C. Subsequently, the samples (25 or 50 mL) were filtered onto 0.8 µm black polycarbonate filters (Midland Scientific Inc.), until ~10 mL was left in the filter tower, then 4 drops of 50 µg L^{-1} 4',6-diamidino-2-phenylindole (DAPI) was added and incubated for 2 minutes. The sample was then filtered to dryness, and the filter mounted between a slide and coverslip with immersion oil above and below. Slides were frozen at -80°C until digitally imaged.

Digital images of the slides were taken using a color camera (Olympus U-LH100HGAPO) attached to an epifluorescence microscope (Olympus Model BX51 TRF, 400X 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 counted. In two cases, slide and image quality was poor, and as few as 60 cells were counted to estimate abundance (i.e., May 31 *B. similis*: 60 cells counted; May 31 *P. crassirostris*: 95 cells counted). To quantify 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, assuming their shape was an oblate spheroid, and these biovolumes were converted to biomass according to Menden-Deuer and Lessard (2000). Note that diatoms >10 µm were also quantified, but were never abundant during our experiments (Table 3.2).

Samples for measurement of ciliate biomass and abundance by inverted microscopy (INVERTED) were preserved with a 1/20 dilution of acid Lugol's solution (Throndsen 1978), and kept in the dark at room temperature until later analysis by the Utermöhl technique (Sherr and Sherr 1993). Aliquots of 28 mL from a randomly selected control and experimental treatment were settled and their entire contents examined with a Zeiss inverted microscope (400X 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 for each cell, and to carbon biomass according to Menden-Deuer & Lessard (2000).

Chlorophyll a determinations – For Chl *a*, triplicate 305 mL samples were collected and filtered onto GF/F filters (Whatman), flash-frozen in liquid nitrogen, and then transferred to a -80 °C

freezer for storage until measurement. Chl *a* (and phaeopigment) was measured using a Turner Designs (model 10AU) fluorometer, using the standard acidification technique (Strickland and Parsons 1972; Yentsch and Menzel 1963).

Data analyses – Hourly clearance rates on each prey size or type were calculated from cell abundance according to Frost (1972), and converted to biomass ingestion by multiplying cell ingestion by the biomass estimate per cell for that prey group. Negative clearance rates of prey were discarded. According to (Båmstedt et al. 2000; Gifford 1993) the change in prey concentration within grazing experiment treatment bottles must be 20-40% so that the variation in count replicates (C.V. often up to 20%) is less than the difference between initial and final counts. With this in mind, the percent reduction of prey abundance within the experimental bottles between initial and final time points is reported in Supplementary Tables 1 and 2.

Specific ingestion rates were calculated using the carbon biomass of mid-stage nauplii measured directly for a cohort of *P. crassirostris* nauplii produced in the lab and using particulate C analysis (Exeter Analytical CE 440) (Gordon 1969; Sharp 1974). These measurements agreed well with naupliar biomass based on equations using nauplius body length (White and Roman 1992). Given this agreement, and the dimensional similarity of *P. crassirostris* and *B. similis* mid-stage nauplii (McKinnon et al. 2003), the measured value of 0.057 ± 0.019 µg C nauplius^{-1} (mean ± SE) was applied to calculate weight-specific ingestion rates for both species on the different prey types.

Corrections for microzooplankton grazing – Naupliar grazing rates on total phytoplankton (as total Chl *a*) and PEUKS were corrected according to the general method as described in (Nejstgaard et al. 2001), to account for trophic interactions in the bottles. This method uses the change in biomass of potentially competing micrograzers (e.g., ciliates, dinoflagellates, etc.) over the course of the experiment, as well as the community microzooplankton grazing impact on specific prey populations, to correct the estimates of naupliar grazing. Results of the concurrent community microzooplankton grazing experiments (Selph, unpub) were used for total Chl *a* and PEUKs, to derive these correction factors (Supplementary Table 3.3). The Chl *a* and PEUK mortality rates were determined using parallel seawater dilution experiments run with 2 m depth

Kane‘ohe Bay seawater on each experimental date following methods described in Selph et al. (2005), except 1 L incubation bottles were used. Corrections for changes in non-nauplii microzooplankton during the incubation were made for ciliates (INVERTED) and other heterotrophs (>2 µm heterotrophs, EPI), by summing these two groups. Results of this correction are reported as ‘Corrected Rates’ in comparison to ‘Uncorrected Rates’ and the corrected rates are further used in calculations of specific ingestion rates and resulting grazing impacts on these prey types.

Selectivity on Prey – To assess whether the nauplii were selecting for or against specific prey items according to the availability of prey in the environment, the percent of prey in the environment was compared to the percent of prey in the diet, for each species. To do this, biomass ingestion rates were compared to the initial prey biomass separately for two prey groups: prey size groups (CC), and prey types in different autotroph and heterotroph prey size groups (EPI). The percent contribution to the total initial prey biomass was calculated for each non-overlapping prey type (or size group) and compared to the percent contribution of that taxa (or size group) in the nauplius diet (% Diet). This measure was used to indicate a selection for (above 1:1 line) or against (below 1:1 line) a prey type compared to the availability of that prey item. In some cases, there were cell types that were abundant in the environment, but nauplii had negative grazing rates on these potential prey items. These prey were included in this analysis since the prey type was abundant in the environment, and the percent in the nauplius diet was considered to be zero.

In addition to the above comparison, an electivity coefficient was calculated to assess for selective feeding on prey in the different prey quantification groups using prey clearance rates, according to the electivity index (E^*) in Vanderploeg and Scavia (1979):

$$E^* = \frac{W_i - \frac{1}{n}}{W_i + \frac{1}{n}}$$

where n is the number of categories of prey and W_i is defined by:

$$W_i = \frac{F_i}{\sum F_i}$$

with F_i as the clearance rate (mL nauplius^{-1} h^{-1}) on prey type *i* and $\sum F_i$ is the sum of clearance rates on all non-overlapping potential prey types in the experiment. This index was chosen because it gives an accurate measure of electivity in environments where different prey types are rare or of variable abundance (Lechowicz 1982). Electivity was calculated for the two prey quantification groups (size, type) described above. Electivity > 0.5 suggests stronger positive selection for a prey item, and electivity <–0.5 suggests stronger selection against a prey item. Negative clearance rates were omitted from this analysis.

Estimates of in situ naupliar abundance – Naupliar abundances *in situ* were estimated using a qPCR-based method (Jungbluth et al. 2013) allowing application of individual species grazing rates to *in situ* abundances to estimate the total potential grazing impact of each species. The samples for estimating naupliar abundance *in situ* 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) mounted in the mouth of the net. The contents of each net were split quantitatively; one half was size-fractionated through a series of five Nitex sieves (63, 75, 80, 100, and 123 µm) and preserved in 95% non-denatured ethyl alcohol (EtOH), and the second half of the bulk plankton was preserved immediately in 95% EtOH. 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 12-24 h of collection to ensure high-quality DNA for analysis (Bucklin 2000). Along with the net tows, duplicate Niskin bottle samples (5 L) were collected at 2 m depth and filtered through 20 and 63 µm mesh sieves, in order to sample the earliest naupliar stages in the 20-63 µm size range.

The three smallest plankton size fractions from the net collection (63, 75 and 80 µm) and the smallest Niskin bottle fraction (20 µm) were analyzed with qPCR to enumerate *Parvocalanus crassirostris* and *Bestiolina similis* nauplius abundances (Jungbluth et al. 2013). In brief, DNA was extracted from four plankton size fractions (20, 63, 75 and 80 µm, described above) 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 described in Jungbluth et al., (2013). On each qPCR plate, 4-5 standards spanning 4-5 orders of magnitude in DNA copy number were run along with the two 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 uL^{-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 92-102%, and melt-curves indicated amplification of only the target species. The qPCR estimate of each species' mitochondrial COI DNA copy number was converted to an estimate of nauplius abundance using methods described in Jungbluth et al. (2013).

Statistical analysis – For each prey type that had replicate measurements, two-sided t-tests of growth rates were run to test for significant differences of growth in control and treatment bottles for each species. This was done separately for prey size spectra, Chl *a*, and PEUK prey groups (Supplementary Table 3.1 and 2, underlined values). Two-sided t-tests were also performed to test whether the differences were significant between uncorrected and corrected ingestion rate estimates of Chl *a* and PEUKS. To test the null hypothesis that there was no selective grazing behavior by either species, a one-way ANOVA was run on the electivity data across experiments for each species.

3.4. Results

Environmental conditions – The water temperature (26.4-26.6 °C) and salinity (34.1-34.7 PSU) were stable at the sampling depth across all experimental dates. Further, in the three months leading up to our experiments, the sampling area received an average of 0.24 cm of daily rainfall (10.31 cm max., 16 days before E1), which is within the expected trends for the sampling area with respect to recent history (0.47-0.85 cm average daily rainfall, 4.06-19.69 cm max, Mar-May 2010-2012). However, between E1 and E2 (28 May), the Luluku sampling station reported 20.37 cm of rainfall. A decrease in wind speed (6.9 kts) and solar irradiation (229 Cal cm^{-2}) on 29 May 2013 also occurred relative to the other sampling dates (i.e., other dates range between 11.8-15.3 kts, 314-500 Cal cm^{-2}; HIMB weather station).

In situ Community – The potential prey community was assessed using four independent methods: Coulter Counter (CC), epifluorescence microscopy (EPI), inverted microscopy

(INVERTED), and chlorophyll *a* (Chl *a*). As mentioned previously, CC measurements were deemed appropriate to analyze the feeding dynamics in this system, given that the predominant prey cells were spherical, as opposed to elongate or other shapes that one might find in a diatom-dominated community, and spherical cells are ideal for accurate estimates of biovolume.

CC measurements indicated a ~3-fold increase in the total biomass of the prey community in Kane'ohe Bay from E1 (57 µg C L^{-1}) to E5 (187 µg C L^{-1}), with little change in the relative abundance of most prey size groups (Fig. 3.1a, Table 3.1). The size groups contributing the most to the biomass were 2-10 µm, which made up 65-78% of the initial prey biomass and 99% of the abundance. Microscopy measurements of the autotrophic and heterotrophic community, despite being lower over all, also showed an ~2-fold increase in biomass during this period (E2 ~18 µg C L^{-1} to E5 of ~34 µg C L^{-1}), again mostly due to the 2-10 µm size fractions.

When the total biomass of the prey community is divided into size classes, the 5-10 µm autotrophs contributed 24-52% to the available prey biomass (Fig. 3.1b); whereas the 5-10 µm heterotrophs contributed 32-59%. The other prey size classes contributed <13% to the total prey biomass. Peak autotroph biomass and abundance occurred at the start of E4 (20.1 µg C L^{-1}), which was almost three-fold higher than on E2 (7.1 µg C L^{-1}), while peak heterotroph biomass and abundance occurred at the start of E5 (21.8 µg C L^{-1}), roughly two-fold higher than on E2 (10.7 µg C L^{-1}). Prey type measurements indicate 5-10 µm autotrophs and heterotrophs made up a majority of the prey types quantified on the EPI slides, making up 24-59% of the total prey type biomass (Fig. 3.1b), suggesting that prey types 2-5 µm in size were not well represented on the EPI slides.

Chl *a* was low (0.26-0.33 µg Chl L^{-1}; Table 3.1) in the first three experiments (E1 - E3), and increased ~2-fold by E4 and E5 (0.85 and 0.79 µg Chl *a* L^{-1}, respectively). Although heavy rainfall can sometimes result in a diatom bloom in the southern region of Kane'ohe Bay (Cox et al. 2006; Hoover et al. 2006), there was no evidence of a large-celled phytoplankton bloom within our 10 day sampling period, as indicated by the minor increase in the initial community of >10 µm autotroph and diatoms; rather, the close correspondence of the increase in Chl *a* with the increase in 2-10 µm autotrophs suggest that the nanophytoplankton responded most to the rainfall-induced perturbation to the bay (Table 3.1). This was also supported by the trends in

abundance of FCM-derived PEUKS (includes eukaryotic autotrophs ~<5 µm), which generally followed the same trend as the 2-5 µm autotrophs found with microscopy. PEUK biomass (derived using a single average cell:C conversion factor) increased by over 3-fold from E1 to E5, peaking at the start of E5 (33.9 µg C L^{-1}).

Ciliates and diatoms did not contribute significantly to the prey community biomass or abundance (Table 3.1). However, ciliate biomass increased by four-fold from E1 to the peak during E4 (1.0 µg C L^{-1}), while their abundance increased by less than two-fold, peaking at 2.5 cells mL^{-1}, because there was a shift in the community from smaller to larger ciliates.

Total *in situ* naupliar abundance (count-based) over E1-E5 ranged from 77 (±8) to 207 (±4) nauplii L^{-1} including all calanoid and cyclopoid nauplii >63 µm. Application of our qPCR based method to estimate the *in situ* abundance of each of the target species shows that *P. crassirostris* was 2 to 11-fold more abundant than *B. similis* over this time period, and *P. crassirostris* increased in abundance *in situ* ten-fold (0.8 to 8.9 nauplii L^{-1}), while *B. similis* increased by two-fold (0.4 to 0.8 nauplii L^{-1}) from E1 to E5 (Fig. 3.2a). Over this time period, *P. crassirostris* nauplii contributed 0.6 to 7.1% of the total nauplius population, while *B. similis* contributed <1% to the total nauplius abundance.

Naupliar grazing rates – Application of the Nejstgaard correction for microzooplankton grazing resulted in variable and largely non-significant effects on the mean estimates of Chl *a* and PEUK ingestion rates by nauplii (Fig. 3.3a-d). The correction was applied to both positive and negative ingestion rates, given the possibility of decreasing or increasing ingestion rates. The difference between uncorrected and corrected ingestion rates estimated by Chl *a* was non-significant for all experiments (*t-test*; $p > 0.05$), whereas there was a significant difference between *B. similis* ingestion rates only on PEUKs in E2 (*t-test*; $p = 0.01$), where the correction reduced the ingestion estimate by 33% (Fig. 3.3d). The variability observed in the effect of correction is a result of a number of contributing factors: 1) increasing heterotroph availability in the initial prey community from E1 to E5 (2-35 µm EPI heterotrophs plus total ciliates; Table 3.1), differing ingestion rates by each copepod species on the heterotrophs (Supplementary Tables 4 and 5), and 3) differing mortality (m) rates based on Chl *a* and PEUKS by the total microzooplankton community measured in the concurrently run seawater dilution grazing experiments (m, in

Supplementary Table 3.3). Corrected ingestion rates were used in all subsequent analyses on these prey types, and those that were negative were omitted from further analysis.

Corrected nauplii ingestion rates on Chl *a* increased over time from E2-E5; *P. crassirostris* ingestion increased from 0.12 to 0.46 ng Chl *a* nauplius^{-1} h^{-1} (Fig. 3.3a), while *B. similis* Chl *a* ingestion rate increased from 0.06 to 0.46 ng Chl *a* nauplius^{-1} h^{-1} (Fig. 3.3b).

There was no significant trend in PEUK ingestion over time by *P. crassirostris* (3.06 - 5.57 ng C nauplius^{-1} h^{-1}; Fig. 3.3c; regression slope = 0, $p = 0.99$), but *B. similis* ingestion rates showed a significant negative trend over time (Fig. 3.3d; regression slope = -0.022, $p = 0.04$, $r^2 = 0.36$). These trends give further evidence for changes in prey selection over time as prey type availability changed in the environment.

Clearance rates were generally similar between the two species. Mean clearance rates on prey size groups for *P. crassirostris* nauplii ranged from 0.04-2.83 mL nauplius^{-1} h^{-1} (Fig. 3.4a) while *B. similis* ranged from 0.11-2.71 mL nauplius^{-1} h^{-1} (Fig. 3.4b), with the highest clearance rates on the largest 20-35 µm prey in both species. Plots of clearance rate as a function of cell abundance in each size fraction resembled the Type 3 functional response (Fig. 3.4a, b), where clearance rate generally decreases as prey abundance increases within each size group, although prey abundance doesn't approach zero, so it is unclear whether a threshold feeding response exists. Clearance rates on prey types generally supported the trends seen in the CC data, however there were instances of high clearance rates on 5-10 µm heterotrophs by *P. crassirostris*, and on 5-10 µm autotrophs by *B. similis* (Fig. 3.4c, d). By prey types, the maximum average clearance rate measured was slightly higher, up to 8.32 mL nauplius^{-1} h^{-1} for *P. crassirostris* (Fig. 3.4c) and up to 5.71 mL nauplius^{-1} h^{-1} for *B. similis* (Fig. 3.4d).

Both species showed positive ingestion of prey of a range of sizes and types in the five experiments. Total ingestion rates from CC data across the experiments ranged from 14.4 to 87.8 ng C nauplius^{-1} h^{-1} for *P. crassirostris* and 25.4 to 73.8 ng C nauplius^{-1} h^{-1} for *B. similis* (Table 3.2). The two species generally had similar ingestion rates on prey size groups but the size groups they removed at the highest rates differed across experiments (Table 3.2; Fig. 3.5a,b). *Parvocalanus crassirostris* ingestion rates were highest on 10-15 µm prey during E2 (23.9 ± 5.7 ng C nauplius^{-1} h^{-1}) whereas *B. similis* ingestion rates were highest on 5-10 µm prey during E5 (26.5 ± 7.3 ng C nauplius^{-1} h^{-1}). Total ingestion rates on prey types (EPI) were comparable to the

CC data and ranged from 4.4 to 145.4 ng C nauplius^{-1} h^{-1} in *P. crassirostris* and 12.2 to 63.9 ng C nauplius^{-1} h^{-1} for *B. similis* (Supplementary Table 3.5). Ingestion rate estimates on individual prey types were within the range of total ingestion observed on prey size groups, but ingestion estimates on >10 µm autotrophs and heterotrophs were much lower than that for prey size groups >10 µm (Fig. 3.5c, d; Supplementary Table 3.5). This discrepancy could be due to having lower confidence in prey type data from microscopy relative to CC data, since a single slide for each control and treatment was analyzed and therefore we do not have a measure of the variability across treatment bottle replicates. Ciliates were not a significant contributor to the potential prey community, and ingestion rates on ciliates ranged from 0.2-0.4 ng C nauplius^{-1} h^{-1} by *P. crassirostris* and from 0.04-0.8 ng C nauplius^{-1} h^{-1} by *B. similis* (Supplementary Table 3.4).

Diet composition and prey preference – A comparison of the proportion of prey ingested to the proportion available in the environment suggests selective feeding behavior by both species. Non-selective grazing by either species would be indicated by the percentage of a prey group in the diet being roughly equal the proportion of that prey available in the environment (placement along the 1:1 line; Fig. 3.6a, b). While the smallest prey size group (2-5 µm) dominated the biomass of the initial community across all experiments, the proportion of this prey group ingested was always much lower than would be expected given indiscriminate grazing. Prey in the 5-10 µm category tended to be along the 1:1 trend line, whereas, with a few exceptions, larger prey (>10 µm) were on or above the line suggesting preferential selection. The overarching trend was for greater selectivity for larger cells relative to their availability in the environment for both species.

Electivity results also suggest that *B. similis* consistently selected against the 2-5 µm prey across all dates (Fig. 3.7b), while *P. crassirostris* was less selective for or against prey in the 2-35 µm size range (Fig. 3.7a). Both species had electivities < 0 on prey <10 µm in size, while electivity for prey >10 µm was more variable; *B. similis* specifically had more strong negative electivity against 10-15 and 15-20 µm prey during E5, while *P. crassirostris* had more neutral electivity on prey >10 µm in size on all dates. Selection for or against certain prey by *B. similis* may depend more on what other prey are available, as shown by the shift from more positive electivity on 15-20 µm prey in early experiments (E1 - E2) to more positive electivity on 20-35

µm prey in later experiments (E4 - E5). Linear regressions of electivity over time indicate a negative relationship for the 15-20 µm prey size group for *B. similis* (slope = -0.12, r^2 = 0.94), and a positive relationship for the 20-35 µm prey group (slope = 0.07, r^2 = 0.79). Similarly, a negative relationship for *P. crassirostris* electivity over time for the 10-15 µm prey group also was observed (Fig. 3.7a; slope = -0.05, r^2 =0.89), however all but two electivity scores fell well within the -0.5 to 0.5 range for this nauplius species, indicating more neutral grazing behavior of *P. crassirostris*, despite the trend. We tested the hypothesis that electivity was not different across the experiments in each prey size group and species using ANOVA and found that there was no significant difference in electivity over the experiments for any prey size group by *P. crassirostris* (ANOVA; $p \geq 0.06$), despite the negative relationship described above for the 10-15 µm prey. However, *B. similis* did show a significant difference in electivity in the 15-20 (ANOVA; $F_{4,9} = 8.94$, $p = 0.003$) and 20-35 µm prey groups across experiments (ANOVA; $F_{3,6}=13.03$, $p = 0.005$). A *post-hoc Tukey Test* found that electivity for the 15-20 µm prey size group was significantly different in E5 vs. E2 and E1 ($p \leq 0.007$) while electivity of 20-35 µm prey was significantly different in E4 and E5 relative to E2 and E3 ($p \leq 0.04$).

Results for electivity on prey types (EPI) generally agreed with the results of electivity by prey size (Fig. 3.7c, d). Similar to electivity on prey size groups, prey type electivity suggested some selection against 2-5 µm prey by *B. similis*, but the level of selection appeared less strong than was observed in the prey size data (Fig. 3.7d). Similar to prey size data, there was an increase in selection for >10 µm autotrophs by *B. similis* over time (slope = 0.11, r^2 = 0.87). Both species decreased selection of the 2-5 µm heterotrophic prey over time (*P. crassirostris* slope = -0.08, r^2 = 0.78; *B. similis* slope = -0.08, r^2 = 0.82), with the greatest negative electivity during E5 (5 June). Electivity results also suggested that *P. crassirostris* was less selective for or against prey types than *B. similis*, consistent with results from prey size groups. Generally, electivity for prey types decreased with increasing initial prey type abundance for cells <10 µm in size, and electivity increased with increasing cell abundance for those >10 µm in size.

Potential grazing impact – Application of ingestion rates to the *in situ* nauplius abundance for each species resulted in a combined trophic impact for both species ranging from 1.0 - 8.7% of the total 2-35 µm prey community across the 10-day experimental period, with the maximum

total impact during E3 when initial prey was low relative to E2, E4 and E5 (Table 3.2). The trophic impact of *P. crassirostris* on total prey was 0.6 – 7.1%, while the impact of *B. similis* was much lower, 0.4 - 1.6%. Similarly, the impact of *P. crassirostris* on prey size groups was higher than *B. similis* except in E5 on 5-10 μm prey (equal impact), in E1 on 15-20 μm prey, and when ingestion rates of *P. crassirostris* were negative (Table 3.2, Fig. 3.8). The greater impact by *P. crassirostris* was primarily due to higher and increasing abundance of this species in the water column over the 10-day period (Fig. 3.2a). The trophic impact on larger cells was generally higher than that on smaller cells (Fig. 3.8), particularly by *P. crassirostris* whose maximum trophic impact was 23.7% of the standing stock of 20-35 μm cells in E5 and whose minimum impact on that size range was 10.9% in E2. This was due mainly to the lower abundance of larger cells in the water column (Table 3.1), while still maintaining a substantial ingestion rate similar to the smaller size fractions. In contrast, the greatest grazing impact by *B. similis* was much lower, removing 5.7% of the 10-15 μm prey size group on May 31st.

The daily trophic impact on prey type standing stocks and on Chl *a* was potentially high (Supplementary Tables 4 and 5). The maximum impact on Chl *a* standing stock by *P. crassirostris* was 12.9% during E5, in part due to the high abundance of this nauplius species. Despite a similar ingestion rate, *B. similis* was capable of removing only 1.1% total Chl *a* on the same date (Supplementary Table 3.4, Fig. 3.8). As expected given their abundance in the water column, trophic impacts on PEUK stocks were relatively low, up to 3.5% by *P. crassirostris* (E5), while the impact by *B. similis* was <1% in all experiments (Supplementary Table 3.4). The combined trophic impact by both species on 2-35 μm prey type (EPI) standing stocks from E2-E5 was 1.7 to 53.5% daily. The impact of *P. crassirostris* on the total daily prey type biomass was up to 50.6% on E4, while the maximum grazing impact by *B. similis* was 2.9% on E5. There was no clear trend associating prey size or autotroph vs. heterotroph with higher or lower ingestion rates or grazing impacts, likely due to lower confidence in the EPI estimates of prey abundance and grazing rates.

3.5. Discussion

Over a series of five grazing experiments spanning ten days where the *in situ* prey community biomass increased by ~3-fold, our results suggest that when copepod nauplii are

offered a diverse natural prey assemblage, ingestion rates and prey selectivity can vary on time-scales of 2-3 days and differs between two closely related species. *Parvocalanus crassirostris* and *B. similis* were shown to be capable of grazing on the same range of prey types and sizes, yet the two species did not always select for the same prey. When offered the same prey, *P. crassirostris* fed more generally, and ingested many prey close to their availability in the environment, while *B. similis* selected more positively for larger prey and strongly selected against 2-5 µm cells. The two species also had very different impacts on prey populations, largely due to the differences in their *in situ* naupliar abundances. *P. crassirostris* was more abundant over this time period, by E5 reaching ten-fold higher abundances than *B. similis*, and as a result *P. crassirostris* was capable of removing up to 12.9% of Chl *a* standing stock and up to 8.7% of the total 2-35 µm prey size groups. The impact on individual prey size groups was greater for the less abundant larger prey items, and generally higher during later experiments when *P. crassirostris* was more abundant. Together, these results suggest differing impacts of these two species on *in situ* prey populations. Finally, our results suggest significant trophic impacts of nauplii as grazers in subtropical ecosystems, and future work should include these micro-metazoans in studies of food web dynamics in marine systems.

Natural prey field: Methodological considerations – A suite of methods were applied to quantify the potential prey available in the 2-35 µm size range given the known diversity of potential naupliar prey items in Kane‘ohe Bay (Calbet et al. 2000; Cox et al. 2006). The Coulter Counter was chosen to quantify the full potential suite of prey by size groups, while the EPI slides were intended to identify autotrophic and heterotrophic cells, and the other measurements were intended to quantify predation on other specific prey items (e.g., ciliates, PEUKS, total phytoplankton as Chl *a*). There are limitations to each method of quantification, which is why multiple methods were used; the Coulter Counter can bias results if large or irregularly shaped cells are a major component of the prey community (Harbison and McAlister 1980), epifluorescence preservation techniques do not quantify soft-bodied organisms well (Sherr & Sherr, 1993), the quality of staining and amount of Chl *a* per cell can affect confidence in identification of autotrophic and heterotrophic cells, inverted microscopy samples do not distinguish autotrophy or heterotrophy, Chl *a* does not give indications of the taxa involved and

does not measure ingestion of heterotrophs, and FCM PEUK measurements give cell counts, but no indication of biomass per cell or cell sizes. In combination, these factors make direct comparisons across the different prey quantification methods difficult.

The initial abundance and biomass of prey types in the 2-35 µm prey size range from EPI microscopy were lower than that found with the CC data (Table 3.1, Fig. 3.1a). This was likely due to the loss of small, dim (chlorophyll) cells upon preservation for microscopy, and inability to accurately distinguish larger prokaryotes from smaller non-pigmented eukaryotes via microscopy. Since the samples were taken from 2 m depth, very near the surface in a brightly lit water column, little chlorophyll per cell is expected. Corroborating this expectation are the FCM data: PEUK abundances were very high (10^4 cells mL^{-1}), yet few (~<10^3 mL^{-1}) were observed on the slides. Also, little abiotic debris was observed in any of the samples; they were taken at the near surface and the station location is not near any sedimentary sources at the margins of the bay. For these reasons, the CC data is considered to more accurately reflect the total cell counts in the samples, as it is based on biovolume-carbon conversions of individual particles, and thus, in the absence of significant detritus and a dominance of spherical cells, would better reflect the concentration and biomass of available prey.

Correction of copepod ingestion rate estimates for microzooplankton grazing that is co-occurring during bottle incubation experiments is commonly accomplished by applying an average microzooplankton grazing rate from the literature (Calbet et al. 2009; Huo et al. 2008; Vargas and González 2004) or by modeling trophic relationships (Klaas et al., 2008; reviewed in Saiz and Calbet, 2011). In our study, correction for microzooplankton grazing was calculated from concurrent seawater dilution measurements for grazing, based on Chl *a* and PEUKs (flow cytometry, <2 – ~10 µm), and this correction did not significantly increase or decrease estimates of ingestion on these prey in most experiments. Rather, the correction had variable effects, increasing or decreasing the ingestion estimates depending on the experiment, which can be attributed to daily changes in the prey community and to different rates of ingestion on or grazing by the competing microzooplankton. Therefore, it would be inappropriate in our system to apply an average microzooplankton correction factor derived from literature values until more is known about microzooplankton grazing rates in this environment and how the rates vary over time.

Naupliar ingestion rates – Very few studies of naupliar grazing on natural prey assemblages have been conducted, and in particular little is known about grazing on diverse prey assemblages, such as those found in the subtropics. Previous grazing rate measurements have shown that specific ingestion rates vary widely among species, and that some species impact prey communities significantly, while others do not (Table 3.3). Published specific ingestion rates by calanoid copepod nauplii from temperate regions, with cooler water temperatures, slower development times, and less diverse prey range from <0.1 – 2.8 µg C µg-nauplius C^{-1} d^{-1} (Table 3.3), most often quantified as grazing on phytoplankton from a mixed assemblage. Our maximum specific ingestion rates on total prey were often higher: on the prey size groups (Coulter Counter estimates) they were 6.1 - 37.2 µg C µg-nauplius C^{-1} d^{-1} and 1.9 - 61.6 µg C µg-nauplius C^{-1} d^{-1} on prey types. However, assuming a 20% gross growth efficiency, our rates are only 1.3 to 8-fold higher than expected metabolic requirements for growth and development based on body carbon, development time, and growth efficiency in this warm-water ecosystem. For instance, *P. crassirostris* N3-N4 nauplii have a measured mean carbon content of 57 ng C $nauplius^{-1}$, which corresponds well with calculations from nauplius total length based on diverse nauplii (Berggreen et al. 1988; White and Roman 1992). By length, an N3 nauplius at 50 ng body C requires 155 ng C to grow into an N4 nauplius of approximately 81 ng C, assuming a 20% gross growth efficiency (Straile 1997) and using body length differences between stages from (Lawson and Grice 1973) to calculate stage-to-stage biomass. The stage duration for an N3 to molt to N4 is approximately 14-h for this species (Mckinnon and Duggan, 2003); so a N3 nauplius would need to consume 11.1 ng C h^{-1} for 14 h to add 31 ng of carbon to their body weight and molt to the next stage. Our measured rates of total ingestion for *P. crassirostris* (14.4 - 87.8 ng C $nauplius^{-1}$ h^{-1}) and *B. similis* (25.4 - 73.8 ng C $nauplius^{-1}$ h^{-1}; Table 3.2) only marginally exceeded these requirements for growth and development, despite being high compared to published ingestion rates for nauplii of other species in temperate ecosystems. These calculations suggest that our measured ingestions rates are realistic for our species and ecosystem and also that nauplii were not food-limited during this study.

There are a number of factors that also may contribute to our comparatively high ingestion rates, many of which are difficult to quantify and not well understood for nauplii. The

use of shorter incubation times for our grazing experiments likely resulted in more accurate and higher grazing rate estimates than would have been measured with longer incubations. In a prior study of the impact of incubation time on naupliar ingestion rate estimates, we found that rates measured over 24-h were as much as 75% lower than rates measured over a 6-h incubation (Jungbluth et al. *in review*). The significant decline in ingestion rates was attributed to increasingly artificial conditions in bottle incubations due to bottle effects, trophic interactions changing the prey community, and non-feeding during molting, which occurred at least once during the course of the 24-h incubation. Had the current study used the more common 24-h incubation period, our ingestion rates would have been more similar to prior reports for nauplii; 1.5-9.3 µg C µg-nauplius C^{-1} d^{-1} for *P. crassirostris* and 2.7-7.8 µg C µg-nauplius C^{-1} d^{-1} for *B. similis* (prey size groups). Other factors that may contribute to the higher ingestion rates observed here include sloppy feeding by the nauplii, which may reduce actual ingestion rates by up to 30% (Strom et al. 1997), the potential for feeding at high rates until satiation and then cessation of feeding (e.g., in adults: Ishii, 1990; Mackas and Bohrer, 1976), or unaccounted for trophic interactions in the grazing bottles (trophic cascades). Sloppy feeding may be particularly common for nauplii due to their rudimentary feeding appendages.

Selectivity in naupliar grazing – Selective grazing is well known in adult copepods, but is not well understood in nauplii. Adult copepods exhibit highly selective feeding behavior, and are known to select prey based on a range of characteristics, including prey size (Ambler 1986; Hansen et al. 1994; Marshall 1973; Mullin 1963; Poulet 1978), prey motility(Kiørboe et al. 1999; Svensen and Kiørboe 2000), and coarse metrics of prey quality (Schultz and Kiørboe 2009; Teegarden 1999), as well as more subtle differences in the nutritional value of prey (e.g., Cowles et al., 1988; Koski et al., 1998). In a few cases, adults of predatory species also were found to avoid consumption of conspecific nauplii (e.g., Lonsdale et al., 1979; Mullin and Brooks, 1967). Prior studies of nauplii observed selection for specific prey in a few cases (Berggreen et al. 1988; Fernández 1979; Swadling and Marcus 1994), while other studies found no evidence of selective feeding (Allan et al. 1977; Isari et al. 2013). Selective feeding behavior by nauplii would be an advantageous trait, because it would allow them to maximize ingestion of nutrients required for growth, which may be critical for survival in these fast-growing subtropical species.

Our prey electivity results suggest that both copepod species showed an increase in selective feeding on large prey as all prey size groups became more abundant in the field, and that *B. similis* was more strongly selective both for and against particular prey than was *P. crassirostris*. The comparison of the proportion of prey in the diet to the prey available in the environment (Fig. 3.3) also supports the inference of selection for larger prey in both species. The changes in electivity observed across experiments in these two species are likely due to switching predation preferences with changing prey availability in the field. The few prior behavioral studies on feeding in nauplii suggest two different modes of feeding, namely feeding current and ambush feeders, with nauplii exhibiting behaviors broadly similar to adult conspecifics (Bruno et al. 2012; Henriksen et al. 2007; Titelman and Kiørboe 2003). Given the feeding mode of adult *B. similis* and *P. crassirostris*, these nauplii likely feed using a weak feeding current and would first detect prey when the prey touches the setae on the feeding appendages (Bruno et al., 2012). Such close proximity to prey would enable detection of chemical cues and facilitate selection for or against prey items by the nauplius. Over the course of our study, as larger prey items became more abundant in the water column, both species showed increasing selection for larger prey items. This preference could be a function of both higher encounter rates with larger cells as they become more abundant, and selection against the abundant small prey that may have lower nutritional value or incur higher handling costs.

Comparison of our results to prior studies on adult conspecifics suggest that our nauplii are able to feed on a similar range of prey 2-35 µm in size and have similar selective tendencies as adults under the conditions studied here. Calbet et al. (2000) found that ingestion on 2-5 µm and >5 µm size groups of autotrophic and heterotrophic prey by adults of *P. crassirostris* and *B. similis* (previously reported as *Acrocalanus inermis*) in Kane‘ohe Bay also depended on the prey community, and electivity on prey was stronger for *B. similis* on >5 µm autotrophs, and against 2-5 µm heterotrophs. Electivity by *P. crassirostris* adults indicated no strong selection against most prey groups, with one event of positive selection for >5 µm autotrophs when overall prey were more abundant. These results suggest that the selective feeding behavior exhibited in the naupliar stages continues through to adulthood in these two species, although whether the specific prey types targeted are the same across development is unknown. Comparisons of nauplii and adult copepod feeding in other species have found similar selective feeding behavior

(Finlay and Roff 2004; Merrell and Stoecker 1998), and similar abilities to feed on a range of prey sizes across developmental stages (e.g., Saiz et al., 2014; Vogt et al., 2013).

Trophic and ecosystem impact of nauplii as grazers – Previous studies report both high and low trophic impacts by nauplii in a range of environments, and the general ecosystem importance of nauplii as grazers is unclear. For example, *Oithona* spp. nauplii removed up to 54% of nanoplankton and 21% of picoplankton off the coast of Chile (Böttjer et al. 2010), and *Eurytemora affinis* nauplii removed up to 56% of ciliates in Chesapeake Bay (Merrell and Stoecker 1998). A study on micrometazoan community grazing in which the community was dominated by copepod nauplii also found trophic impacts of >40% on total primary productivity in a Long Island bay (Lonsdale et al. 1996). However, a number of other studies of this type report insignificant trophic impacts for nauplii (e.g., Almeda et al., 2011; Verity et al., 1996). Low impacts reported for specific naupliar species include *Oithona similis* and *Calanus finmarchicus,* where the copepod populations, including nauplii, had insignificant impacts on prey standing stocks in the Irminger Sea (Castellani et al. 2008), and *Calanus* spp. nauplii which removed up to only 1.3% of Chl *a* in Disko Bay (Turner et al. 2001).

We found that the trophic impacts of our subtropical nauplii are potentially significant, depending on *in situ* prey densities and naupliar abundance. At the maximum *P. crassirostris* abundance of 8.9 L^{-1} observed during the study, this species removed a significant fraction of the standing stock of some prey: up to 12.4% of Chl *a*, and up to 24% of 20-35 µm prey during E5. *B. similis,* however, was a less significant grazer, largely due to its lower abundance in the south bay, reaching a maximum abundance of 0.8 nauplii L^{-1} and removing up to 1.1% of Chl *a* (E5) and 5.7% of the 10-15 µm prey size group (E3). However, the calanoid copepod species studied here made up <1% to 7% of the total nauplii in Kane‘ohe Bay, with the rest of the naupliar community being dominated by *Oithona* spp. (Jungbluth, unpub). Thus, if the ingestion rates and impacts of the cyclopoids are even a small portion of those measured in the current study, the total nauplius population is a substantial contributor to grazing and may exert top-down control on prey populations within Kane‘ohe Bay.

3.6. Conclusions

Although copepod nauplii are numerical dominants of the zooplankton community in many planktonic systems, relatively little is known about their feeding ecology and potential trophic impacts. Nauplii may play particularly important food web roles in subtropical and tropical ecosystems, due to year-round high abundance and rapid rates of growth and development in warm waters. Results reported here show that when copepod nauplii are offered a diverse natural prey assemblage, ingestion rates and prey selectivity can vary on timescales of 2-3 days and differ between two closely related species, implying complex selective feeding behavior. The trophic impacts estimated for the two calanoid nauplii also were significant, and grazing by the whole naupliar community may exert top-down control on *in situ* prey populations. Future work should include greater consideration of nauplii as potentially important members of the grazer assemblage, and include measurements of abundance and feeding of these micro-metazoans.

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Tables and Figures:

Table 3.1: Initial pico- to microplankton community abundance (Abund; cells mL^{-1}), and biomass (Biom; µg C L^{-1}) by type and size groups (µm) for each grazing experiment. Measurements made by: [a] Fluorometry (µg Chl *a* L^{-1}), [b] Coulter Counter, [c] Inverted Microscopy, [d] Epifluorescence Microscopy. (-) indicates data not available.

			E1 5/27/2013		**E2 5/29/2013**		**E3 5/31/2013**		**E4 6/3/2013**		**E5 6/5/2013**	
			Biom.	**Abund.**	**Biom.**	**Abund.**	**Biom.**	**Abund.**	**Biom.**	**Abund.**	**Biom.**	**Abund.**
Chlorophyll *a* [a]			0.33	-	0.35	-	0.26	-	0.85	-	0.79	-
Total Particles [b]	2-5	µm	24.8	9.4×10^3	33.3	11.1×10^3	21.8	7.1×10^3	52.8	17.0×10^3	86.6	27.7×10^3
	5-10	µm	12.2	513	17.6	803	8.6	389	33.5	1487	59.3	2629
	10-15	µm	10.6	79	8.9	69	6.5	51	13.0	103	21.9	178
	15-20	µm	4.9	14	3.3	9	3.6	10	4.6	13	8.4	24
	>20	µm	4.6	6	5.4	5	4.1	5	6.3	6	10.8	10
	Total		***57.1***	***10.0×10^3***	***68.5***	***12.0×10^3***	***44.6***	***7.6×10^3***	***110.2***	***18.6×10^3***	***187.0***	***30.6×10^3***
Ciliates [c]	<10	µm	0.02	0.32	0.02	0.36	0.01	0.11	0.02	0.43	0.00	0.11
	10-20	µm	0.14	0.82	0.22	1.21	0.11	0.57	0.14	0.96	0.14	0.61
	20-30	µm	0.10	0.21	0.16	0.39	0.02	0.07	0.25	0.50	0.26	0.43
	>30	µm	0.00	0.00	0.00	0.00	0.19	0.04	0.62	0.57	0.36	0.46
	Total		***0.26***	***1.35***	***0.40***	***1.96***	***0.33***	***0.79***	***1.03***	***2.46***	***0.76***	***1.61***
Diatoms [d]	10-35	µm	-	-	0.20	6	0.0	1	0.20	5	0.10	8
Autotrophs [d]	2-5	µm		-	1.53	403	1.52	402	4.16	835	2.64	662
	5-10	µm		-	5.23	314	13.81	586	15.39	725	8.08	499
	>10	µm		-	0.36	11	0.15	3	0.54	15	0.65	22
	Total				***7.12***	***728***	***15.48***	***991***	***20.09***	***1575***	***11.37***	***1183***
Heterotrophs [d]	2-5	µm		-	0.91	206	0.80	175	1.69	480	2.00	530
	5-10	µm		-	9.66	659	10.00	644	10.41	583	19.63	807
	>10	µm		-	0.17	6	0.07	2	0.17	2	0.16	6
	Total				***10.74***	***871***	***10.87***	***821***	***12.27***	***1065***	***21.79***	***1343***

Table 3.2. Ingestion rates (*I*, ng C nauplius^{-1} h^{-1}) and grazing impacts (as % of initial biomass removed by *in situ* population day^{-1}) on the standing stock of prey size groups (µm) by *Parvocalanus crassirostris* and *Bestiolina similis* in each experiment. Total ingestion rate for each experiment (total *I*, ng C nauplius^{-1} h^{-1}) and the mean ingestion rate by each species on a given prey size (± standard error) are also reported. – indicates negative ingestion rate. Prey quantified by Coulter Counter.

		2-5			**5-10**			**10-15**			**15-20**			**20-35**			**Total**
		I		**% day^{-1}**	***I***		**% day^{-1}**	***I***		**% day^{-1}**	***I***		**% day^{-1}**	***I***		**% day^{-1}**	***I* (± SE)**
Parvocalanus crassirostris	E1	-		-	7.7	(±1.2)	1.2%	5.6	-	1.1%	4.4	(±2.1)	1.8%	-		-	**17.7 (±3.3)**
	E2	11.5	(±1.0)	1.1%	17.1	(±1.0)	3.0%	23.9	(±5.7)	8.2%	16.4	(±5.0)	15.5%	18.9	(±0.8)	10.9 %	**87.8 (±13.5)**
	E3	-		-	8.3	(±1.5)	7.0%	17.0	(±1.0)	19.1%	8.3	(±4.4)	16.9%	9.7	(±1.0)	17.5 %	**43.3 (±7.9)**
	E4	16.3	(±2.8)	3.5%	14.9	(±3.8)	5.0%	8.2	-	7.1%	-		-	12.0	(±0.3)	21.4 %	**51.4 (±6.8)**
	E5	-		-	2.4	-	0.9%	-		-	-		-	12.0	(±0.8)	23.7 %	**14.4 (±0.8)**
Mean		***13.9***			***10.1***			***13.7***			***9.7***			***13.2***			
Bestiolina similis	E1	1.9	-	0.1%	4.0	(±0.8)	0.3%	6.7	(±4.0)	0.6%	12.8	(±4.2)	2.4%	-		-	**25.4 (±8.9)**
	E2	9.3	(±3.0)	0.1%	17.8	(±1.2)	0.4%	21.6	(±3.4)	1.0%	15.6	(±5.3)	2.0%	9.5	(±1.4)	0.7%	**73.8 (±14.4)**
	E3	-		-	7.0	(±0.5)	1.5%	19.9	(±2.1)	5.7%	4.9	(±2.8)	2.5%	7.4	-	3.4%	**39.2 (±5.4)**
	E4	6.5	(±1.2)	0.2%	10.1	(±4.7)	0.4%	4.7	(±2.2)	0.5%	2.5	(±1.2)	0.8%	11.1	(±1.8)	2.6%	**35.0 (±11.1)**
	E5	10.7	(±2.1)	0.2%	26.5	(±7.3)	0.9%	2.1	-	0.2%	0.8	(±0.5)	0.2%	20.3	(±8.7)	3.6%	**60.4 (±18.6)**
Mean		***7.1***			***13.1***			***11***			***7.3***			***12.1***			

Table 3.3. Published naupliar specific ingestion (Sp. ingestion) rates on natural (natl.) prey assemblages, along with the results from this study. Total prey biomass (µg C L^{-1}) reported for all prey types quantified. * indicates only phytoplankton ingestion quantified. # indicates Coulter Counter estimates.

Nauplius species	**Prey measured**	**Total prey biomass (µg C L^{-1})**	**Sp. ingestion (d^{-1})**	**Temp (°C)**	**Incubation time (h)**	**Source**
Calanoids						
Parvocalanus crassirostris	natl. prey 2-35 µm#	45-187	6.1-37.2	21	6	this study
Bestiolina similis	natl. prey 2-35 µm#	45-187	10.8-31.2	21	6	this study
Parvocalanus crassirostris	natl. prey 2-35 µm	44-67	1.9-61.6	21	6	this study
Bestiolina similis	natl. prey 2-35 µm	44-67	5.2-27.1	21	6	this study
Calanus finmarchicus	natl. phytoplankton	28-212	0.3	1-12	24	Irigoien *et al.*, 2003
Calanus spp.	natl. prey*	5-20	0.0087-0.012	5	23-47	Turner *et al.*, 2001
Acartia hudsonica	natl. prey*	300-420	0.8	4-7	0.25-1	White and Roman 1992
Acartia tonsa	natl. prey*	300-420	2.8	15-26	0.25-1	White and Roman 1992
Mixed nauplii	natl. prey*	15-68	0.1-0.3			Uitto 1996
Mixed nauplii (50-200 µm)	natl. prey	37-950	0.3-1.2	12-24	24	Almeda *et al.*, 2011
Cyclopoids (*Oithona* sp.)						
O. nana *O. similis*	natl. prey <125 µm natl. prey <100 µm	51-306	0.4-11.1	11-12	24	Bottjer *et al.*, 2010
O. nana *O. similis*	natl. prey <2 µm	14-31	0.5-6.4	11-12	24	Bottjer *et al.*, 2010

Figure 3.1. Biomass (µg C L^{-1}) of the initial prey community on each experimental date from two different methodologies. (a) Prey size groups by Coulter Counter (µm; bars), with epifluorescent microscopy data shown of the combined autotrophic and heterotrophic biomass (open circles). (b) Percent contribution of prey types by epifluorescence microscopy (µm, open circles). Aut = autotroph, Het = heterotroph. Experimental Days: E1 = May 27, E2 = May 29, E3 = May 31, E4 = June 3, and E5 = June 5.

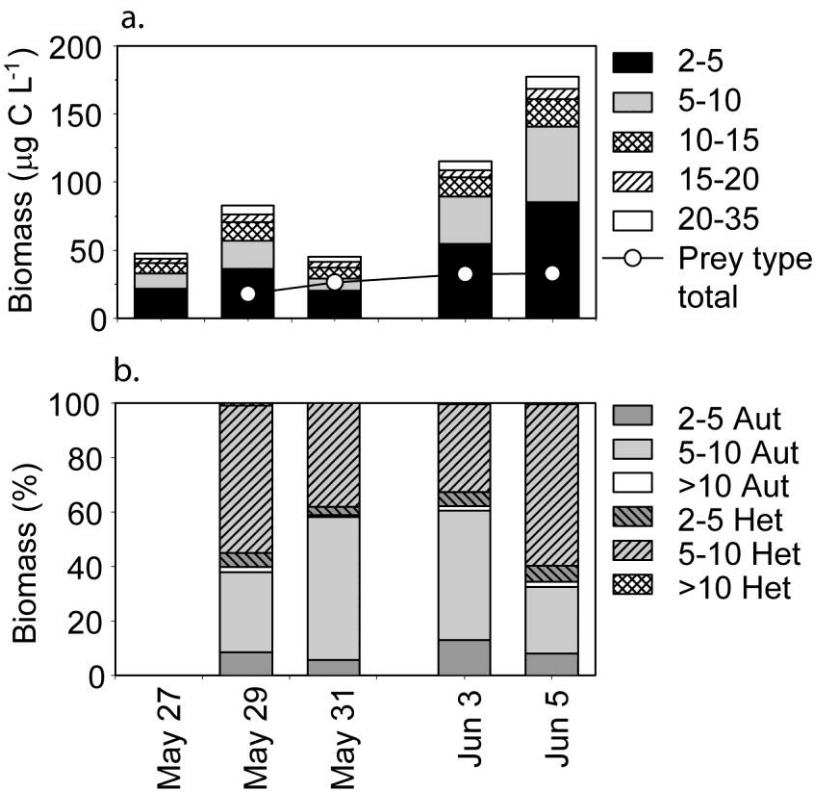


Figure 3.2. a) *In situ* abundances for *Parvocalanus crassirostris* and *Bestiolina similis* nauplii (L^{-1}) in field populations in Kane'ohe Bay on the experimental days, estimated from quantitative PCR, and b) ambient Chl *a* ($\mu g\ L^{-1}$). Error bars are ± standard error. Experimental Days: E1 = May 27, E2 = May 29, E3 = May 31, E4 = Jun 3, E5 = June 5.

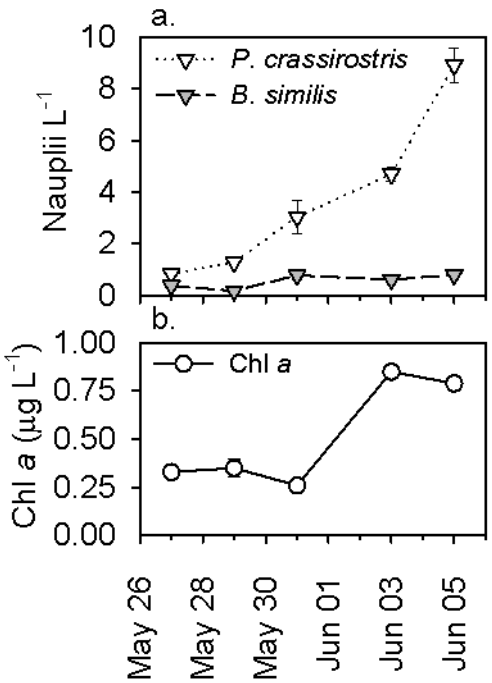


Figure 3.3. Average ingestion rates of nauplii in each experiment on prey measured as chlorophyll a (Chl a) or on photosynthetic eukaryote carbon (PEUKS) as measured by flow cytometry. Data shown are uncorrected (open) and corrected (hatched) following the method of Nejstgaard *et al.* (2001). *Parvocalanus crassirostris* (left) and *Bestiolina similus* (right) data on (a, b) Ingestion as chlorophyll *a* (top, ng Chl-*a* nauplius $^{-1}$ h^{-1}), and, (c, d) Ingestion as carbon biomass of PEUKS (bottom; ng C nauplius^{-1} h^{-1}). * No correction was available for May 27,, so data not shown. Error is ± standard error. Experimental days: E1 = 27 May, E2 = 29 May, E3 = 31 May, E4 = 3 June, E5 = 5 June.

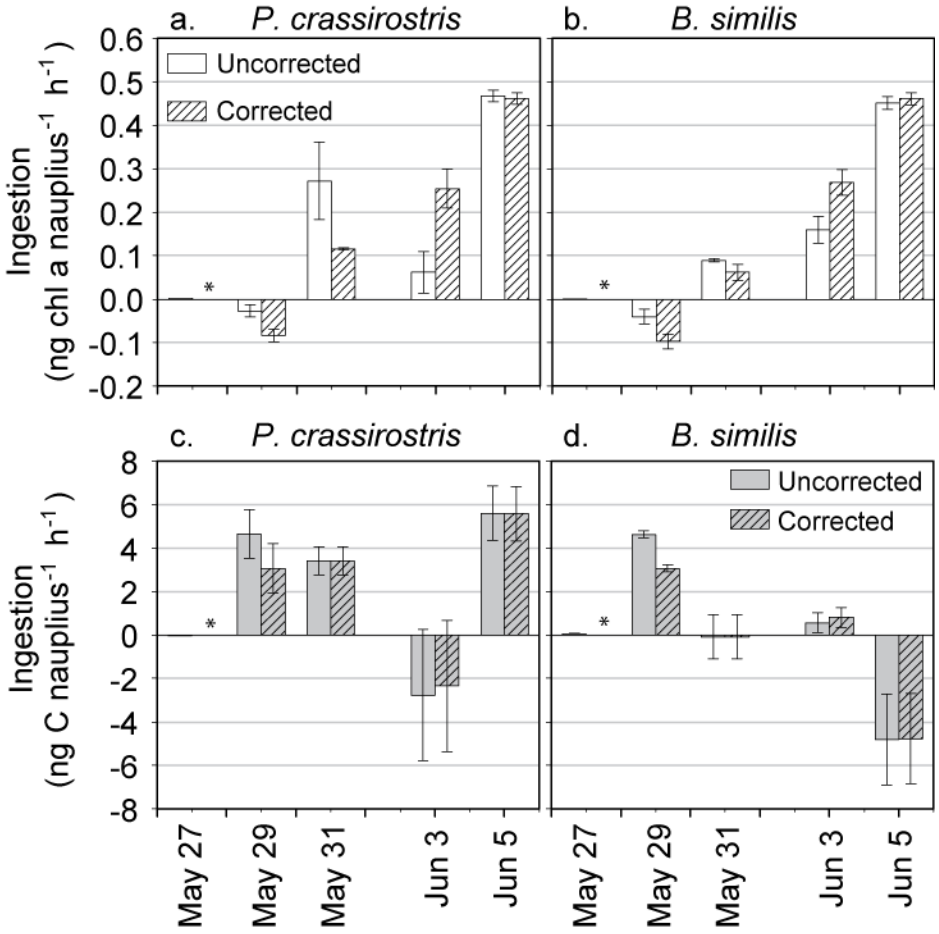


Figure 3.4. Average clearance rates (mL nauplius $^{-1}$ h^{-1}) for *Parvocalanus crassirostris* (left) and *Bestiolina similus* (right) on (a, b) prey size groups (µm) and, (c, d) on prey types (Aut=autotrophs, Het=heterotrophs) as a function of initial prey abundance (cells mL^{-1}). Error is ± standard error.

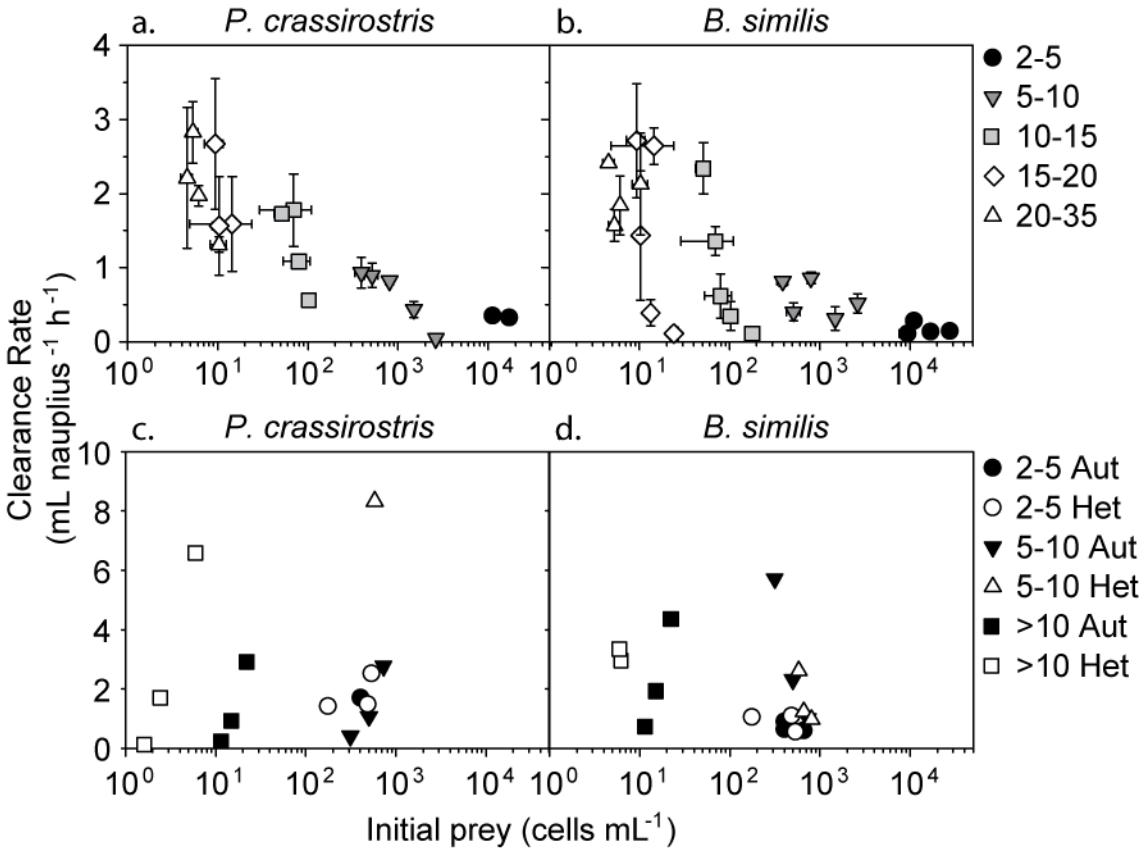


Figure 3.5. Ingestion rates (ng C nauplius^{-1} h^{-1}) for *Parvocalanus crassirostris* (left) and *Bestiolina similis* (right) on each experimental date. (a, b) Ingestion on each prey size group (µm) and, (c, d) on prey types (Aut=autotroph, Het=heterotroph). Error is ± standard error. * indicates ingestion rates not available for prey types on May 27. Experimental Days: E1 = 27 May, E2 = 29 May, E3 = 31 May, E4 = 3 June, E5 = 5 June.

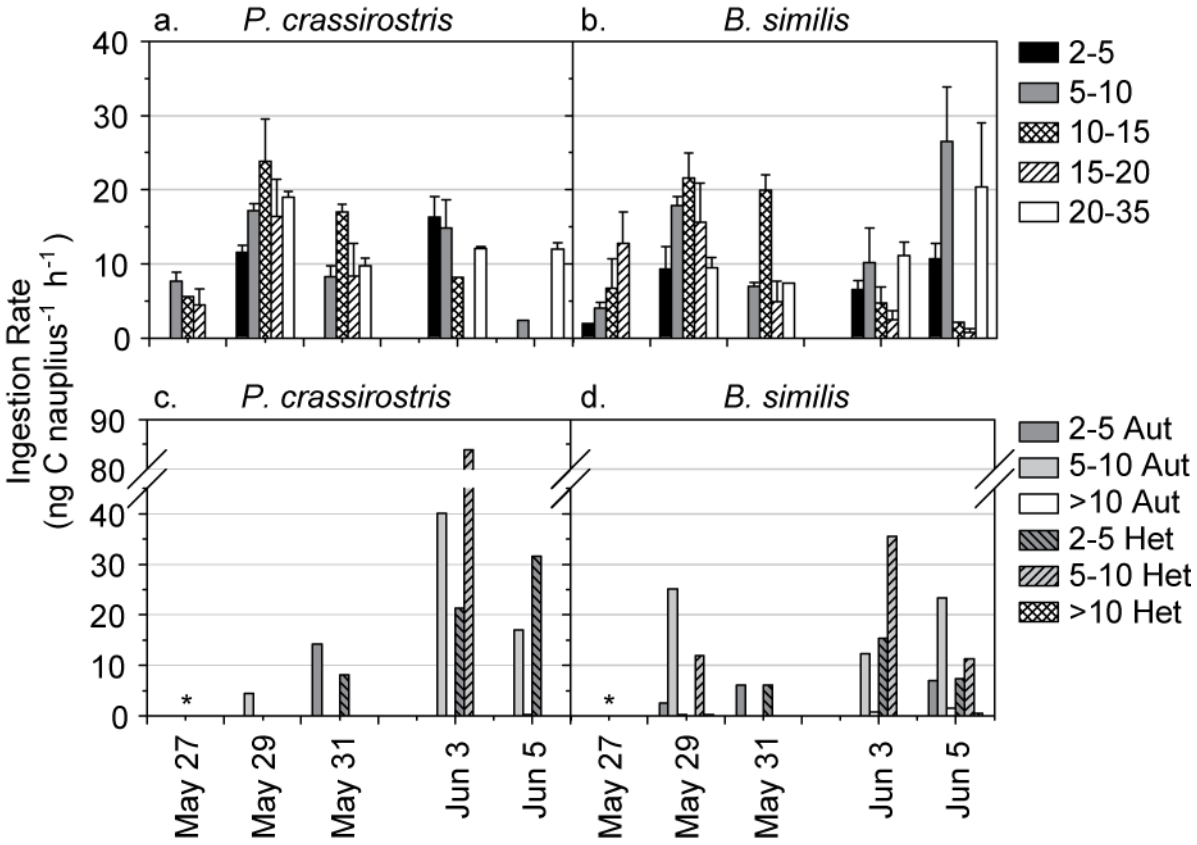


Figure 3.6. Percentage of the biomass of different prey in naupliar diets (Prey in Diet (%)) compared to the initial prey biomass available in the environment (Prey Biomass Environmental Availability (%)), by size groups (µm). a) *Parvocalanus crassirostris*, and b) *Bestiolina similis* Error is ± standard error. Compared to their environmental availability, points lying above 1:1 line indicate selection for the prey item, and those below 1:1 line indicate selection against a prey item.

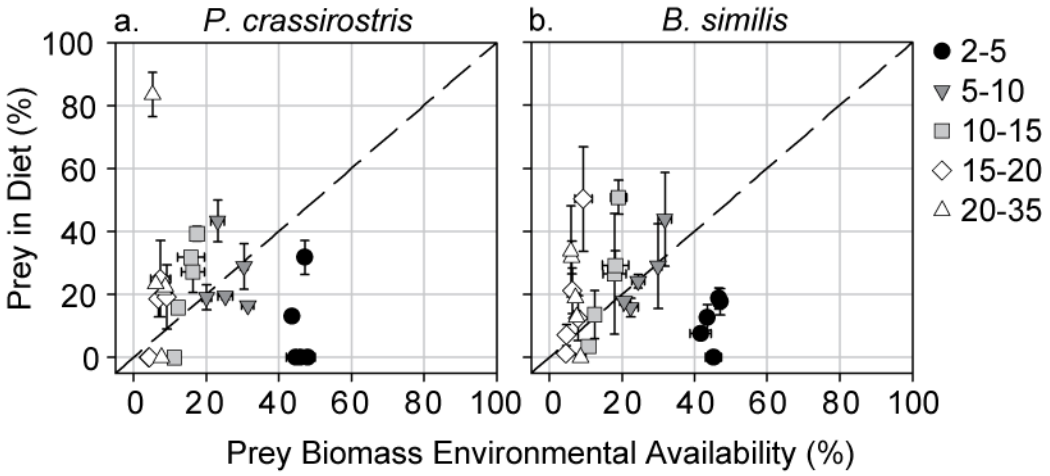


Figure 3.7. Electivity index in grazing by each nauplii species over the experimental days. *Parvocalanus crassirostris* (left) and *Bestiolina similis* (right) electivity on (a, b) prey size groups (µm) and (c, d) on prey types. Shaded grey region indicates the area of low selectivity, between strong positive (E >0.5) or strong negative (E<0.5) electivity of a prey group (Vanderploeg & Scavia, 1979). Error is ± standard error. Lines are linear regressions where r^2 > 0.75 (indicated in legend by line type). Experimental Days: E1 = 27 May, E2 = 29 May, E3 = 31 May, E4 = 3 June, E5 = 5 June.

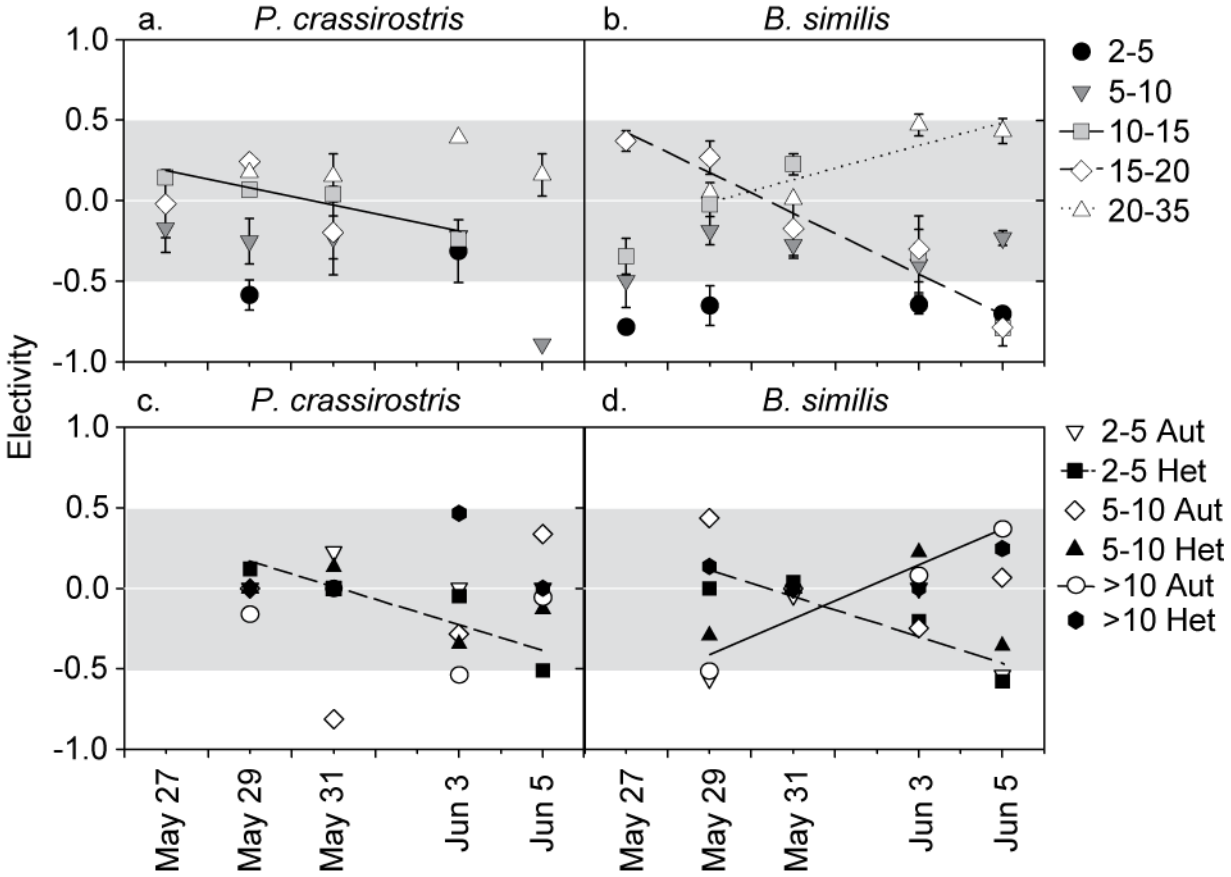
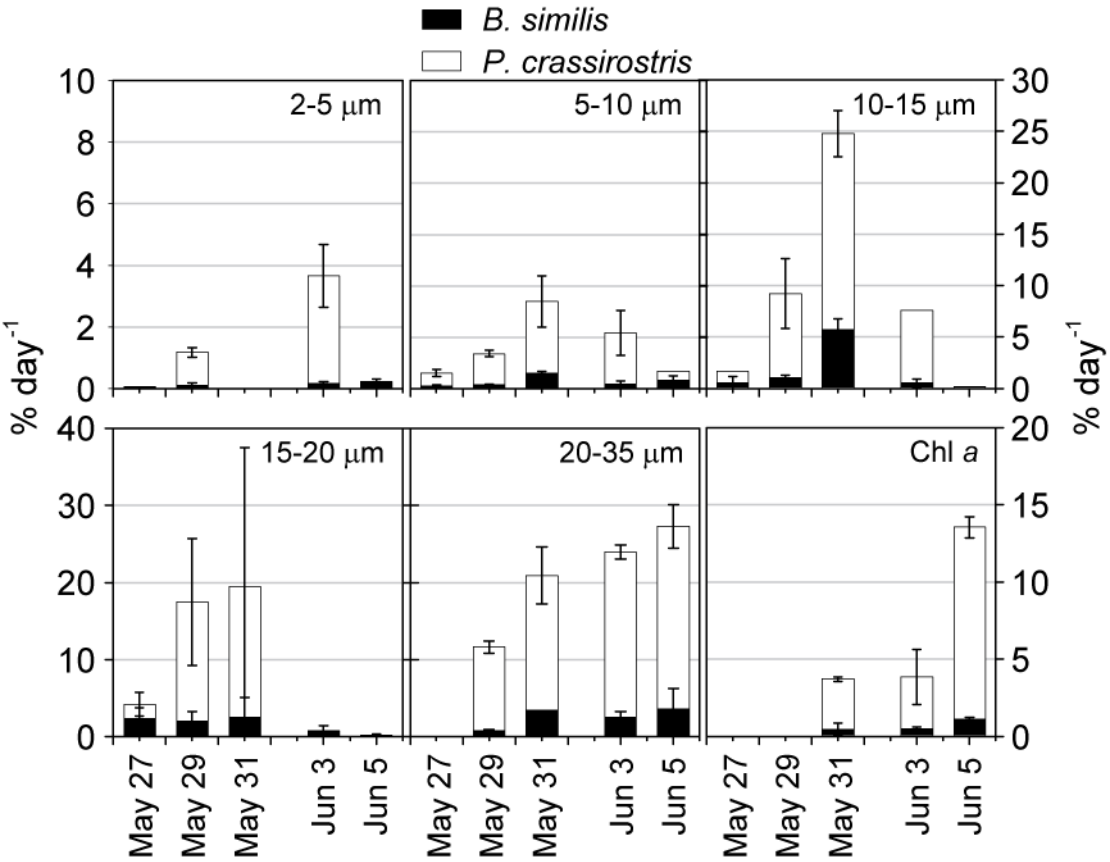


Figure 3.8. Daily estimates of the prey standing stock removed (% d^{-1}) by *Parvocalanus crassirostris* (white) and *Bestiolina similis* (black) nauplii calculated from *in situ* abundance of nauplii and mean ingestion rates measured on each prey size group (data shown in Table 3.2) and Chl *a* (data shown in Supplementary Table 3.4). Note the differing y-scale in size fractions of the top row (2-10 µm figures) vs. the bottom row (10-35 µm figures). Experimental Days: E1 = 27 May, E2 = 29 May, E3 = 31 May, E4 = 3 June, E5 = 5 June.



Supplementary Table 3.1. Mean percent reduction of each prey size group (range in µm) from the final time point relative to the initial concentrations of prey (Coulter Counter particle counts). Negative values indicate growth relative to the initial community. **Bold** values indicate where there was a 20% decrease or increase in prey type or size, relative to initials. Underlined values indicate where the difference between control and treatment prey growth rate was significant (*t-test*, $p<0.05$). Note that from parallel microscopy samples, little debris was seen in the samples, so the particle counts are assumed to be derived mostly from living autotrophic and heterotrophic cells.

	Particle Counts (autotrophs + heterotrophs)				
	2-5	**5-10**	**10-15**	**15-20**	**20-35**
E1 (27 May)					
P. crassirostris	15.1	**40.5**	-2.7	-3.1	-11.9
B. similis	**20.0**	**33.5**	5.4	**22.2**	-3.6
E2 (29 May)					
P. crassirostris	15.0	**25.0**	**21.8**	15.1	13.2
B. similis	13.7	19.3	15.3	19.3	12.8
E3 (31 May)					
P. crassirostris	17.9	**30.1**	-16.4	**-20.1**	**-103.0**
B. similis	15.8	**32.6**	7.8	-10.7	**-39.0**
E4 (3 June)					
P. crassirostris	14.7	**22.7**	-8.5	**-33.7**	-1.2
B. similis	12.1	**22.7**	4.9	3.0	**23.8**
E5 (5 June)					
P. crassirostris	12.2	13.3	-9.8	1.1	-12.7
B. similis	12.4	19.5	-4.6	11.8	**28.7**

Supplementary Table 3.2. Mean percent reduction of each prey type (size range in µm) from the final time point relative to the initial concentrations of prey based on cell abundance or reduction in chlorophyll (Chl) *a*. Autotroph abundances are from epifluorescence microscopy only, whereas photosynthetic eukaryote (PEUK) data are from flow cytometry and heterotroph abundance is from epifluorescence and inverted microscopy. Negative values indicate prey growth relative to the initial community. **Bold** values indicate where there was a ≥20% decrease or increase in prey type or size, relative to initials. Underlined values indicate where the difference between control and treatment prey growth rate was significant (*t-test*, $p<0.05$). – indicates no data.

			Autotrophs			**Heterotrophs**		
	Chl a[#]	**PEUK**	2-5	5-10	>10	2-5	5-10	>10
E1 (27 May)								
P. crassirostris	**39.5**	9.1	-	-	-	-	-	-
B. similis	**34.5**	17.7	-	-	-	-	-	-
E2 (29 May)								
P. crassirostris	**-20.8**	8.4	-13.0	**47.8**	-3.5	**-31.2**	**52.5**	-13.0
B. similis	**-22.0**	8.8	**50.5**	**87.9**	9.8	**-157.7**	**79.8**	**67.5**
E3 (31 May)								
P. crassirostris	**36.5**	2.9	9.6	**88.5**	**-67.4**	**-157.4**	**82.2**	**-95.0**
B. similis	**20.5**	-4.3	-1.5	**90.4**	**-165.1**	**-157.4**	**82.2**	**-210.0**
E4 (3 June)								
P. crassirostris	**20.4**	-5.5	**22.2**	**83.2**	13.4	**-50.2**	**87.7**	**-163.3**
B. similis	**22.6**	-2.0	**-22.1**	**73.8**	**29.9**	**-67.9**	**49.2**	**-320.0**
E5 (5 June)								
P. crassirostris	4.7	10.3	**-61.1**	**54.5**	**55.6**	8.8	**59.5**	**74.3**
B. similis	4.5	2.1	-12.0	**67.4**	**70.0**	**-51.4**	**84.9**	**41.9**

Supplementary Table 3.3. Phytoplankton mortality rates (m) and the coefficient of determination (r^2) for the linear regressions of net growth rate (i.e., net growth rate = gross growth rate – mortality rate) as a function of dilution factor for each experiment (Expt). Data were obtained from parallel 24 h seawater dilution experiments to obtain microzooplankton community mortality rates, from differences in initial and final samples of Chl *a* (total phytoplankton community) and photosynthetic eukaryotes (PEUK, measured by flow cytometry).

	Chl *a*		**PEUK**	
Expt	**m**	**r^2**	**m**	**r^2**
E2 (29 May)	0.59	0.84	0.35	0.79
E3 (31 May)	0.18	0.16	0.22	0.25
E4 (3 June)	0.64	0.75	0.04	0.01
E5 (5 June)	0.39	0.88	0.04	0.07

Supplementary Table 3.4. *Parvocalanus crassirostris* and *Bestiolina similis* percent grazing impacts (% of initial biomass removed by *in situ* population day^{-1}, % d^{-1}) and ingestion rates (*I*) on chlorophyll *a* (Chl *a I*; ng Chl nauplius^{-1} h^{-1}), photosynthetic eukaryotes (PEUKS *I*: ng C nauplius^{-1} h^{-1}), and ciliates (2-35 µm ciliates' *I*; ng C nauplius^{-1} h^{-1}) in each experiment (E1-E5). *I* for Chl *a* and PEUKS is the corrected rate. Also shown are the mean (E1-E5) ingestion rates for each species on a given prey type. – indicates negative ingestion rate. n.d. indicates no data available. Error is ± standard error. Experimental Days: E1 = 27 May, E2 = 29 May, E3 = 31 May, E4 = 3 June, E5 = 5 June.

		Chl *a*			**PEUKS**			**Ciliates**		
		I		**% d^{-1}**	***I***		**% d^{-1}**	***I***		**% d^{-1}**
Parvocalanus crassirostris	E1	0.10	(±0.01)	0.6	-		-	n.d.		n.d.
	E2	-		-	4.54	(±1.07)	0.7	0.16		1.3
	E3	0.30	(±0.04)	8.5	3.32	(±0.60)	2.1	0.30		10.2
	E4	0.06	(±0.05)	0.8	-		-	0.42		4.6
	E5	0.48	(±0.02)	12.9	5.71	(±1.30)	3.7	0.17		4.7
Mean		***0.24***	***(±0.05)***		***4.52***	***(±0.40)***		***0.26***	***(±0.06)***	
Bestiolina similis	E1	0.02	(±0.04)	0	2.64	(±0.67)	0.3			
	E2	-		-	4.63	(±0.21)	0.1	0.04		0
	E3	0.10	(±0.01)	0.7	-		-	-		-
	E4	0.17	(±0.03)	0.3	0.62	(±0.47)	0.03	0.08		0.1
	E5	0.45	(±0.01)	1.1	-		-	0.80		2.0
Mean		***0.18***	***(±0.05)***		***2.63***	***(±0.67)***		***0.23***	***(±0.19)***	

Supplementary Table 3.5. Ingestion rates (*I*, ng C nauplius^{-1} h^{-1}) and percent grazing impacts (as % of initial biomass removed by *in situ* population day^{-1}) on the standing stocks of prey types (EPI) by *Parvocalanus crassirostris* and *Bestiolina similis* in each experiment (E2-E5). Total ingestion rate for each experiment (Total *I*, ng C nauplius^{-1} h^{-1}) and the mean ingestion rate by each species on a given prey type (± standard error) are also reported. – indicates negative ingestion rate. Prey quantified by epifluorescence microscopy. Experimental Days: E1 = 27 May, E2 = 29 May, E3 = 31 May, E4 = 3 June, E5 = 5 June.

		2-5 Aut		**5-10 Aut**		**>10 Aut**		**2-5 Het**		**5-10 Het**		**>10 Het**		**Total**
		I	**% d^{-1}**	***I***	**% d^{-1}**	***I***	**% d^{-1}**	***I***	**% d^{-1}**	***I***	**% d^{-1}**	***I***	**% d^{-1}**	***I***
Parvocalanus crassirostris	E2	-	-	4.40	2.6	0.02	0.1	-	-	-	-	-	-	**4.41**
	E3	14.15	67.9	-	-	-	-	8.09	38.8	-	-	0.001	0.1	**22.24**
	E4	-	-	40.11	29.4	0.08	1.7	21.32	57.8	83.85	61.4	0.03	0.7	**145.39**
	E5	-	-	17.01	45.0	0.23	7.5	31.54	255.4	-	-	0.09	3.1	**48.87**
	Mean	***14.15***	**-**	***20.50***	**(±*6.04*)**	***0.11***	**(±*0.04*)**	***20.32***	**(±*3.92*)**	***83.85***	**-**	***0.04***	**(±*0.02*)**	
Bestiolina similis	E2	2.54	0.7	25.07	2.0	0.24	0.3	-	-	11.93	1.0	0.29	0.3	**40.07**
	E3	6.10	7.5	-	-	-	-	6.07	7.5	-	-	-	-	**12.18**
	E4	-	-	12.26	1.2	0.81	2.2	15.29	5.4	35.56	3.4	-	-	**63.93**
	E5	7.03	5.1	23.42	5.5	1.54	4.5	7.36	5.3	11.28	2.7	0.46	1.3	**51.09**
	Mean	***5.23***	**(±*0.79*)**	***20.25***	**(±*2.32*)**	***0.86***	**(±*0.22*)**	***9.58***	**(±*1.66*)**	***19.59***	**(±*4.61*)**	***0.38***	**(±*0.06*)**	

Chapter 4:
A qPCR-based approach to estimating species-specific biomass of copepod nauplii in mixed field samples

To be submitted as: Hanson[†], K.M., Jungbluth[†], M.J., Lenz, P.H., Robinson, E., and E. Goetze. *A qPCR-based approach to estimating species-specific biomass of copepod nauplii in mixed field samples*.

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4.1 Abstract

The juvenile stages (nauplii) of planktonic copepods play important roles as both grazers and prey in marine food webs. To date, species-level data on the dynamics of nauplii in marine ecosystems have been largely unattainable due to the difficulty of visually identifying nauplii based on morphology. Here, we present a quantitative PCR (qPCR)-based approach to quantifying naupliar biomass and the relative contribution of species in mixed-species field samples. Expanding on a qPCR assay designed for the calanoid copepod *Parvocalanus crassirostris*, we developed qPCR assays for three additional copepod species that together dominate the zooplankton assemblage in Kane‘ohe Bay, Oahu, Hawai’i (USA). The qPCR assays target fragments of the mitochondrial cytochrome *c* oxidase subunit I gene. The assays are highly specific, amplifying a single product in the presence of target DNA at levels as low as 2 pg total DNA, and in mixed-species samples where the target species represented <1 % of the total DNA. Tests on cultured *P. crassirostris* mtCOI copy number compared to measurements of carbon across developmental stage found a strong linear relationship across a five order of magnitude range in animal biomass. Application of the relationship from *P. crassirostris* to paired field samples to estimate the DNA copy number-based biomass compared to count-based biomass resulted in an average of 2.5-fold higher estimate of calanoid and cyclopoid biomass using the qPCR approach than when estimating biomass based on counts and body size alone, which is less than the error associated with other standard means of estimating biomass. The ratio of cyclopoid DNA copies to calanoid copies was 5:1, which matched the count-based biomass ratios. Overall, our results show that the qPCR assay developed here gives accurate estimates of relative contributions of species to the total community and can be used to obtain reliable estimates of species-specific biomass from mixed community samples.

4.2 Introduction

Nauplii, the earliest developmental stages of copepod crustaceans, comprise an important component of the microzooplankton assemblage in a range of marine ecosystems. Nauplii are often numerically dominant within the zooplankton, outnumbering adult copepods (Calbet et al. 2001; Chisolm and Roff 1990; Turner 2004; Vogt et al. 2013), and can have a total biomass comparable to the biomass of protozoan grazers (Paffenhofer 1998). Due to their abundance and high weight-specific grazing rates, the cumulative grazing impact of nauplii can exceed that of larger metazoan zooplankton (White and Roman 1992). Nauplii also feed on heterotrophic protozoans and have been shown to remove upwards of 30 % day^{-1} of standing heterotroph biomass (Paffenhofer 1998). Nauplii are prey for a wide range of larger zooplankton including adult copepods (Durbin et al. 2008), and they are a primary food source for the larval stages of many fish species (Jackson and Lenz 2016; Paradis et al. 2012; Peterson and Ausubel 1984; Sampey et al. 2007a). Nauplii may be particularly important components of the grazer assemblage in subtropical ecosystems, due to year-round high abundance (e.g., Scheinberg 2004).

In this study, we developed a real-time qPCR approach to quantify the species-specific biomass of nauplii in mixed-species field samples using amplification of a fragment of the mitochondrial cytochrome *c* oxidase subunit I (mtCOI) gene. We developed this method in order to facilitate field and experimental studies of the zooplankton assemblage in Kane‘ohe Bay, the largest embayment on the island of Oahu, Hawai’i, USA (45 km^2, 157°48’W, 21°27’N). Kane‘ohe Bay receives episodic freshwater input from storm-associated runoff; these storm flows elevate nutrient levels in the bay and drive phytoplankton and zooplankton blooms (Cox et al. 2006; De Carlo et al. 2007; Drupp et al. 2011; Ringuet and Mackenzie 2005). Within Kane’ohe Bay, copepod nauplii numerically dominate the metazoan zooplankton assemblage (Scheinberg 2004) and naupliar developmental stages show a large numerical response to these storm-associated inputs. For example, following a 2003 storm event naupliar abundance increased from 10 to 60 ind. l^{-1} over a seven day period (Hoover et al. 2006). The Kane‘ohe Bay food web includes both metazoan and protozoan microzooplankton grazers (< 200 µm), with the metazoan micro-grazers primarily comprised of copepod nauplii (Scheinberg 2004). Assessment

of the relative importance of metazoan and protozoan grazers in this and other marine systems requires studies of copepod nauplii in natural field settings. A growing number of studies highlight interspecific variation in swimming and feeding behaviors among copepod nauplii, aspects that likely influence their roles as both grazers and prey (Bradley et al. 2013; Bruno et al. 2012; Titelman and Kiorboe 2003). Methods that allow taxonomic discrimination among species are thus crucial to appraising the influence of copepod nauplii on broader food-web dynamics within pelagic ecosystems.

To date, species-level data on the ecology and dynamics of nauplii in marine ecosystems have been largely unattainable due to the lack of morphological variation among species. The species diversity of the copepod assemblage in Kane‘ohe Bay is relatively low; four species dominate the assemblage in the southern sector of the bay (Jungbluth and Lenz 2013), with an additional four species common in the central and North regions of the Bay (*Undinula vulgaris*, *Labidocera sp.*, *Acartia fossae*, *Oithona oculata*). The four focal South Bay species include two calanoid copepods (*Bestiolina similis*, *Parvocalanus crassirostris)* and two cyclopoid species *(Oithona attenuata*, *O. simplex)* that are common to subtropical and tropical coastal ecosystems in the Indo-Pacific and in some cases Atlantic Oceans (e.g., Kimmerer and McKinnon 1985, Nishida 1985, McKinnon and Klumpp 1998, Kiesling et al. 2002). During the naupliar stages, these species cannot be visually discriminated on the basis of morphology, and determining the population dynamics and changing biomass of these species across early developmental stages is not currently possible.

Molecular methods enable study of morphologically cryptic taxa at the species level, and have been previously used to examine plankton predator-prey dynamics in a range of marine ecosystems (Durbin et al. 2012; Durbin et al. 2008; Nejstgaard et al. 2008; Troedsson et al. 2009). In a prior study, we developed a quantitative PCR method that applies stage-specific calibrations to estimate the abundance of *Parvocalanus crassirostris* in field samples (Jungbluth et al. 2013; Jungbluth and Lenz 2013). However, development of stage-specific calibration factors is unrealistic for difficult-to culture species. In order to support studies with species resolution for the full assemblage in Kane’ohe Bay, we extend the qPCR method and test its effectiveness in estimation of animal biomass rather than abundance. Our approach relies on

targeted amplification of the mitochondrial cytochrome *c* oxidase subunit I (mtCOI) gene, which is routinely used to discriminate species in DNA barcoding applications (e.g., Blanco-Bercial et al. 2014; Nigro et al. 2016), due to the high rates of molecular evolution and resulting high taxonomic resolution obtainable using this locus. Prior work by Durbin et al. (2008) found a linear relationship between mtCOI copy number and body carbon for the copepod *Acartia tonsa*, suggesting mtCOI copy number may be directly translatable to species-specific estimates of biomass.

The goal of the qPCR method developed here is to provide species-specific quantitative estimates of naupliar populations in the field. The objectives of the study were to: (1) develop and optimize mtCOI- based qPCR assays for the copepod species that dominate the Kane'ohe Bay zooplankton assemblage, (2) test the performance of the developed assays in mixed-species samples, (3) establish empirical relationships between mtCOI copy number and biomass, and (4) conduct a field validation of the full plankton sampling and qPCR method.

4.3 Materials and Methods

Quantitative PCR Assay Development – Real-time qPCR assays were developed for identification and estimation of biomass of *Bestiolina similis, Oithona simplex,* and *O. attenuata*. Mitochondrial cytochrome *c* oxidase subunit I (mtCOI) sequence data was used to design primers specific to each target species and tested against the remaining common non-target species in Kane'ohe Bay (*Oithona oculata, Acartia fossae*, *Labidocera* sp. and *Undinula vulgaris*; Genbank accession KC594111 - KC594166)(Jungbluth and Lenz 2013). Primers and qPCR assay conditions for *Parvocalanus crassirostris* were reported in Jungbluth et al. (2013); further optimization and comparisons of assay performance across all four species is described here. The dominant mtCOI haplotype from each target species was aligned to the seven respective non-target species using ClustalW (in Geneious Pro v. 5.5.7), and regions with the highest divergence among species were tested for primer design. Candidate primers were evaluated *in-silico* for their potential to amplify non-target species. For each species, synthesized oligonucleotides (Integrated DNA Technologies, standard desalting purification) that included

the predicted PCR product and 4-6 additional base pairs flanking each end were used as qPCR standards to quantify the number of mtCOI copies in unknown samples.

Candidate primer pairs were tested using single-species DNA extracts generated for each of the four focal species using animals sorted from mixed zooplankton collected in Kane'ohe Bay and from laboratory monocultures of *Bestiolina similis* and *Parvocalanus crassirostris*. Zooplankton samples were bulk-preserved in 95 % non-denatured ethyl alcohol (EtOH) and stored at -20°C prior to sorting and extraction. Biological replicates of 10-50 animals were extracted for each of the four focal species. Samples were homogenized using a bead-beading protocol following Jungbluth et al. (2013) and DNA was extracted using the QIAamp DNeasy Mini Kit (QIAGEN). Samples were incubated at 56°C for 16 – 20 h, treated with 9 µL of RNAse A (100 mg mL^{-1}, QIAGEN) and eluted in Milli-Q water. Total DNA yield for each sample was quantified using Qubit™ dsDNA high-sensitivity assay kits (Invitrogen™/Life Technologies).

Experiments for qPCR assay development were run at 20 µL reaction volumes. Milli-Q water was used to re-suspend synthesized oligonucleotides and dilute DNA extracts. Experiments were performed on a LightCycler® 96 (Roche) using the intercalating fluorescent dye SYBR® Green I and KAPA SYBR® FAST qPCR Kit for LightCycler® 480 (KAPA Biosystems cat. no. KK4611). To prevent PCR inhibition, bovine serum albumin (BSA) was added to each well at a concentration of 0.1 µg/µL. The thermocycling program included pre-incubation of 95°C for 180 s, 45 cycles of 95°C for 10 s, the primer-specific annealing temperature for 60 s, and 72°C for 1 s, followed by melt curve analysis of 95°C for 5 s, 65°C increasing 2.2°C s^{-1} for 60 s, 97°C for 1 s, and cooling 37°C for 30 s. Gradient tests were used to optimize the primer concentrations and annealing temperature used in each assay. Triplicate technical replicates were run for all samples.

A subset of qPCR products were selected from mixed-species field sample qPCR plates to confirm amplification of the target species' DNA and a PCR product of the expected length. Four wells for each species, representing one high-concentration standard and three animal size-fractions (20-63, 63-75, and 80-100 µm), were selected to visualize amplicon size on a 2% agarose gel run with a DNA Ladder (Hyperladder II, Bioline). From these products, the standard, 20-63, and 80-100 µm wells from each species were further processed for sequencing to evaluate

amplification of the target species in the largest (80-100 μm) and smallest (20-63 μm) size fractions, and of pure amplicon standard. Each product was purified using shrimp alkaline phosphatase and exonuclease I (30 min at 37 °C followed by 95 °C for 15 min), and sequenced on an Applied Biosystems 3730XL (BigDye terminator chemistry v3.1).

Assay Specificity and Performance – The specificity of each qPCR assay was tested using single-species DNA extracts for each of the four focal species (see above). Biological replicates (at 50 pg μL^{-1} total DNA) of the target and non-target species were amplified on the same plate to evaluate the potential for amplification of non-target products in the absence of target DNA.

To verify assay performance on mixed-species samples of known composition, DNA from the target species (400 pg μL^{-1}) was diluted 10-fold into mixed DNA (50 pg μL^{-1}) that was created by combining equivalent amounts of DNA from each of the four respective non-target species. For comparison, target species DNA was diluted in parallel into Milli-Q water. Melt curves and copy number estimates along the dilution series into non-target DNA were compared with those from the dilutions into water. A dilution series of mixed DNA extracted from a single field sample (zooplankton tow conducted in Kane‘ohe Bay, 63 μm net) was included in each plate to evaluate assay performance on a mixed-species sample that included copepod and non-copepod DNA from a natural assemblage.

To test for the potential amplification of non-target products in mixed-species field samples, we analyzed melt curves from 101 field samples collected from Kane’ohe Bay across a range of environmental conditions that were size-fractionated and DNA extracted according to methods described in Jungbluth et al. (2013). These samples included total DNA extracted from field-collected plankton, and the relative dominance of the four target copepod species varied widely across the samples. Total field sample DNA concentrations run in qPCR ranged from reaction concentrations of 0.01-1.25 ng μL^{-1} with the same total DNA concentration within each plate and the protocol described above for each target species. The proportional contribution of each species DNA to the total DNA in each sample was measured to evaluate the range over which clean melt-curves could be obtained in the field samples with variable *in situ* abundance of each species.

Mitochondrial Copy Number and Conversion to Copepod Biomass – Durbin et al. (2008) reported a linear relationship between mtCOI DNA copy number and biomass for *A. tonsa* nauplii, suggesting that quantitative PCR data may be useful for estimating biomass of targeted species in mixed samples. In order to determine whether DNA copy number could be applied to estimate the biomass of copepods from mixed species communities, experiments with cultures and field samples were performed to estimate biomass from DNA copy number and compare that to body size-based estimates of biomass.

Biomass was measured directly for cohorts of N1-N2, N3-N4, C1-C2, C5, and adult *P. crassirostris* from laboratory cultures to compare the biomass individual^{-1} to previously measured mtCOI DNA copy number individual^{-1} (Jungbluth et al. 2013). Stock laboratory copepod cultures were maintained in 18 L each of aerated, UV sterilized seawater at 24 °C, on a 12:12 light:dark cycle. Copepods were fed 5 mL L^{-1} of mature algal culture of *Tisochrysis lutea* (10^6-10^8 cells mL^{-1}) every two days. To isolate adult copepods for production of naupliar cohorts, the density of adult copepods in each stock culture was measured and the volume of stock culture necessary to sieve out the desired number of adults for cohort production was calculated and gently poured through a 210 μm sieve. Adults (on the sieve) were rinsed into 3-5 L of UV sterilized seawater, fed 20 mL L^{-1} of mature algal culture, and aerated in the same conditions described above. After 4-5 hours, adults were sieved out using a 123 μm mesh and the remaining eggs retained. The egg- cohort culture was maintained under the same conditions described above and checked daily for development to N1-N2, N3-N4, C1-C2, and C5 stage cohorts. Once the target developmental stage was reached, the density of the culture was assessed in 3 replicate subsamples (100 mL for nauplii; 250 mL for copepodites). To harvest naupliar and copepodid stages, the desired volume of each cohort culture was gently sieved through 123 μm to exclude adults, and through 20 (N1-N2 stage) or 63 μm (>N3 stage) to concentrate the culture. The number of individuals isolated for replicate measurements of biomass (carbon content) and the corresponding durations of incubation for each of the developmental stages are reported in Table 4.3. The concentrated copepods were rinsed with GF/C filtered and UV sterilized seawater into a glass crystallization dish and examined under a

dissecting scope to remove debris (feces, molts) with a glass pipette. Adult copepods for biomass estimates were sieved out of the bulk copepod cultures using a 123 μm sieve, and rinsed into a 200 mL glass dish with GF/C filtered seawater, and three sets of 500 adult females and males were isolated into GF/C filtered seawater. Before being filtered and dried, all copepods were rinsed thoroughly with GF/C filtered seawater through a 20 μm sieve to remove any remaining algae or detritus. All filtration equipment was pre-rinsed with Milli-Q water. Each replicate of copepods at a particular developmental stage was filtered onto a pre-combusted, weighed and labeled GF/F-filter, and rinsed briefly with a small volume of Milli-Q water to remove salts. The cohort filters were carefully removed with forceps and placed in labeled foil wrappers, and frozen at -20 °C until CHN analysis (Exeter Analytical CE 440; S-Lab, UH Mānoa).

Measured biomass for each *P. crassirostris* cohort was then compared to biomass estimated from copepod body length. Body length estimates for *P. crassirostris* from McKinnon et al. (2003) were applied to calculate animal dry weight (μg) using the general equation for nauplii (calanoid, cyclopoid and barnacle nauplii) from White and Roman (1992) and applying a body carbon (μg C) to dry weight ratio of 0.32 (Wiebe et al. 1975). Published body lengths for our species from McKinnon et al. (2003) are within the range of body sizes observed in Kane'ohe Bay, Hawai'i (pers. obs., VanderLugt and Lenz 2009).

In order to determine if there is a consistent relationship between mtCOI copy number and animal biomass, we compared the mtCOI copy number individual^{-1} to biomass measured for *P. crassirostris* with that for *A. tonsa* (Durbin et al. 2008). Linear regression was performed to evaluate whether the relationship between mtCOI copy number and biomass is linear, and whether the relationship is significantly different between the two (evaluated as a difference in slopes).

Culture Validation of the Copy-Biomass Relationship – The relationship between *P. crassirostris* DNA copy number individual^{-1} and biomass individual^{-1} was then compared to DNA copy number replicate^{-1} and biomass replicate^{-1} from split culture samples in a prior study (Jungbluth et al. 2013) to evaluate whether the individual-based relationship between DNA copies and biomass scales up to measurements on 10s-1000s of individuals of different stages of the same

target species. Detailed descriptions of culture treatment can be found in Jungbluth et al. (2013). Briefly, mature cultures of *P. crassirostris* were split in half quantitatively to directly compare counts from one half to qPCR estimates of DNA copy number. Because prior work showed that mtCOI copy number varies with animal size (Durbin et al. 2008, Jungbluth et al. 2013), we applied a sieving method to separate developmental stages into size fractions for each quantitative split. Each volume was poured through a stacked tower of sieves to separate developmental stages into the following size-groups; 20-63, 63-75, 75-80, and 80-100 μm. Each size fraction was immediately preserved in 95% EtOH and stored in -20 °C, until either counting or DNA extraction for qPCR analysis of DNA copy number. DNA extraction was performed as described in Jungbluth et al. (2013). For the counted half, quantitative subsamples were taken to count the number of each developmental stage (N1-adult) present in each size fraction. Estimates of count-based biomass for each size fraction were calculated based on applying the count of each developmental stage to the stage length-biomass relationship as described above. DNA copy number in the remaining half was measured, biomass was estimated from copy number, and these results were compared to the count-based estimate of biomass.

Field Validation of the Copy-Biomass Relationship – The relationship between mtCOI DNA copy number and biomass based on *P. crassirostris* cultures was then applied to field samples to compare the total mtCOI copy number-based biomass to count-based biomass for calanoid and cyclopoid size fractions, and estimate the contribution of each species to the total naupliar community.

On 18 September 2011 the following replicate samples were collected from South Kane'ohe Bay: three vertical net tows with a 63 μm net towed from 10 m depth, and three 2 L (Niskin) seawater collections (at 11 m depth) in order to sample the full spectrum of naupliar stages expected in our field site. Each biological replicate was immediately concentrated using 20 μm mesh, resuspended with filtered seawater, and split in half quantitatively. Each half was size-fractionated with a tower of fine mesh sieves to separate early, mid, and late stage nauplii from copepodites and adults as described in Jungbluth et al. (2013). Each net-collected half was then poured gently through a 100, 80, 75, and 63 μm connected sieve tower, then rinsed with

GF/F-filtered seawater to ensure maximum settling to the proper size fraction. Each half of each size fraction was then preserved separately in 95% EtOH. The split seawater samples were each poured through a tower of a 63 and 20 μm mesh, rinsed with GF/F-filtered seawater, and preserved in 95% EtOH. All samples were placed on ice in the field and stored at -20 °C until DNA extraction or counting and sizing. All sample EtOH was replaced within 24-h of collection to prevent spoiling.

Subsamples were taken from size fractions of one half of each net sample and of each seawater sample for counting and sizing. Counts were performed by subsampling each size fraction with a stempel pipet and counting in a ward counting wheel (net samples), with > 350 animals counted per replicate. For the 20-63 μm seawater sample, animal density was much lower and entire samples were counted (72-94 animals counted). During counting, animals were identified as calanoid or cyclopoid nauplii or calanoid or cyclopoid copepodites (no adults were identified in the 20-100 μm size fractions); juveniles of the species present in Kane'ohe Bay cannot be identified to species morphologically. Body length was measured in each size fraction for 20-50 calanoid and cyclopoid nauplii or copepodites (total length (TL) for nauplii, prosome length (PL) for copepodites). Estimates of count-based biomass of each type of calanoid or cyclopoid (nauplii or copepodite) in each size fraction were calculated by applying the length-biomass relationship for nauplii from White and Roman (1992) and conversion from dry weight to biomass from Wiebe et al. (1975) to the calanoid and cyclopoid nauplii and to calanoid copepodites. Cyclopoid copepodite count-based biomass was estimated using the equation for length to dry weight for cyclopoids in Webber and Roff (1995) with the same conversion of dry weight to biomass as noted above.

The other half of each net tow and seawater sample was processed after 17 days of preservation following methods in Jungbluth et al. (2013) to extract total DNA for analysis in qPCR for mtCOI DNA copy number of each species using primers optimized in the current study. DNA was extracted from the 20-63 μm seawater fraction, and the 63-75, 75-80, and 80-100 μm net size fractions for one half of each of the three replicate whole water and net tow samples. One of the replicate 20-63 and 80-100 μm size fractions were each lost during DNA extraction, leaving two biological replicates for the 20-63, and 80-100 μm size fractions, and

three 63-75 and 75-80 μm size fraction biological replicates for qPCR measurement of DNA copy number and direct comparison to preserved paired fractions. Total DNA was measured in each size fraction using the Qubit™ dsDNA broad-range assay kit (Invitrogen™/Life Technologies), and aliquots of each sample were diluted into Milli-Q water to 0.5 ng μL^{-1} total DNA to run in qPCR. Assay conditions were as listed above for each species (Table 4.1), with reaction total DNA concentrations of 0.125 ng μL^{-1}, and standards spanned a range of 1.51×10^{2} to 1.51×10^{7} mtCOI copies μL^{-1}.

Data analysis – Amplification curves, reaction efficiency and melt curves for all qPCR runs were analyzed using the LightCycler® 96 software (Roche, v. 1.1.0.1320). Baseline setting and fluorescence threshold values used to calculate the cycle quantification threshold (Cq) were determined automatically by the software. Any qPCR replicates with Cq standard deviations that exceeded 0.5 were excluded from subsequent analyses. Further analysis was conducted using R statistical software (R Core Team 2016) to evaluate relationships between DNA copy number and sample biomass, and perform linear regression analysis.

4.4 Results

Quantitative PCR Assay Development and Optimization – The primers selected for *Bestiolina similis*, *Oithona attenuata*, and O. *simplex* were 19 – 25 bp in length and targeted regions of the mtCOI gene with amplified products that ranged from 109 – 169 bp in length (Table 4.1). For all species, sequences at both forward and reverse priming sites were identical across known mtCOI haplotypes from Kane'ohe Bay (2 - 7 haplotypes per species)(Jungbluth and Lenz 2013). The priming regions for the target species differed from aligned regions in the respective non-target copepod species by 4 – 15 bases. Annealing temperatures and primer concentrations for each qPCR assay (Table 4.1) were optimized to maximize reaction efficiency and minimize non-target amplification.

The developed assays each amplified a single target product. All assays produced clean melting curves with a single peak across Cq levels from 20 – 30 cycles (Fig. 4.1), corresponding to a range from ca. 3×10^{2} to 3×10^{5} copies of the target mtCOI fragment. Gel electrophoresis

results from a subset of the mixed field samples all showed a single strong qPCR product of the expected size. Sequenced qPCR products resulting from amplification of each target species' DNA from mixed field samples matched the targeted species mtCOI sequence.

Assay Specificity and Performance in Mixed-Species Samples – All five assays produced a single, consistent product when amplifying target species DNA (Figs. 4.1a-d, 4.2a-d). Assay optimization did not fully eliminate non-target amplification in the absence of target DNA, however in these instances the melt curves for non-target products were distinct from the expected target product (Fig. 4.1f, g) and occurred at a much higher Cq than for target species (ca. 30 – 40 cycles for non-targets vs. ca. 20 cycles for target species, all at 50 pg μL^{-1} total DNA). Distinct melt curves for non-target amplifications suggest that non-target amplification that might occur in field samples would produce indicative melting curves and be distinguishable. For the assays targeting *B. similis* and *P. crassirostris*, all non-target amplification in the absence of target DNA occurred above our chosen Cq threshold of 35 cycles. The assays for *O. attenuata* and *O. simplex* amplified non-target DNA in the absence of target DNA at Cq values below 35 cycles (Fig. 4.1b, c).

To verify that the presence of non-target DNA does not influence the qPCR estimates of mtCOI copy number, we compared mtCOI copy number estimates across paired 10-fold dilution series (200 – 2 pg total target DNA; Fig. 4.2). In the first series, target copepod DNA was diluted into Milli-Q water. In the second series, target DNA was diluted into an artificial mixture of DNA from the respective non-target copepod species (400 pg μL^{-1}). In this series, the range of target DNA was 200 - 2 pg and the target species comprised 1% - 0.01% of the total DNA. For all four qPCR assays, the presence of non-target DNA did not affect the mtCOI copy number estimates resulting from amplification of a known amount of target DNA (Fig. 4.2).

We used a 10-fold dilution series of a field-collected plankton sample to examine the efficiency with which the developed qPCR assays amplified target DNA in mixed-species field samples. The same dilution series was amplified with each of the four qPCR assays, and reaction efficiency for each assay was calculated from linear regressions of the resulting Cq values against total DNA concentrations (Fig. 4.3, Table 4.2). The resulting efficiencies fell within the

acceptable range and varied from 91% (*O. attenuata*) to 101% (*O. simplex*) with R^2 >0.99 for each run.

In the 101 size-fractionated field samples, the proportional contribution of target DNA to total copepod DNA (as summed mtCOI copies across the four species) ranged from less than 1% to 5% for *Bestiolina similis*, 1% – 56% for *Oithona attenuata*, 3% - 85% for *O. simplex* and 3% - 89% for *Parvocalanus crassirostris* (Fig. 4.4 e-h). All samples analyzed produced clean melt curves with the single, expected melt peak for each target product (Fig. 4.4 a-d). There was no evidence of amplification of secondary or non-target products, even when the target species was rare compared to the respective non-target copepods in the sample.

Estimating Copepod Biomass from MtCOI Copy Number – Copepod biomass was measured from cohorts of cultured *P. crassirostris*, with 500 to 8300 individuals replicate^{-1} (Table 4.4), and compared to biomass estimated from the mean mtCOI copy number for the same stage-ranges from our prior studies of the same species (Jungbluth et al. 2013) and the relevant length-biomass relationships (Fig. 4.5). The mean biomass for *P. crassirostris* cohorts increased from 0.022 (±0.001) µg C for each N1-N2 nauplius to 0.720 (±0.011) µg C adult^{-1} (Table 4.4, Fig. 4.5). The length-dry weight relationship for nauplii from White and Roman (1992) with a biomass to dry weight ratio of 0.32 (Wiebe et al. 1975) was the best fit to our measurements of nauplius through copepodite cohort biomass, and was used in all future estimates of *P. crassirostris* length-biomass analyses. A calanoid-specific conversion from another subtropical environment (Webber and Roff 1995) was tested for the copepodite calculation of biomass from length, but did not fit well to our measurements of *P. crassirostris* copepodite biomass (Fig. 4.5).

Body carbon (µg C animal^{-1}) was linearly related to copy number (animal^{-1}) for *P. crassirostris* reared in the laboratory (Fig. 4.6). The relative ratio of copy number µg C^{-1} decreased with increasing developmental stage; N1-N2 nauplii highest at 19.93 $\times 10^6$ copies µg C^{-1} and adults the lowest at 8.84$\times 10^6$ copies µg C^{-1} (Table 4.3). In order to test whether the relationship between measured biomass and biomass calculated from body length was significantly different, linear regressions of copy number and biomass were compared using an ANOVA. There was no significant difference in the slopes of the two regressions (ANOVA; $F_{1,7}$

= 2.27, *P*=0.18), and the biomass calculated from body length was used for all further calculations of animal biomass. The relationship of *P. crassirostris* DNA copy number to body carbon also was compared to prior work by Durbin et al. (2008), who reported a linear relationship between copy number and biomass for *Acartia tonsa* nauplii (Fig. 4.6). Copy number to biomass ratios in *P. crassirostris* early nauplii (Table 4.3) were 5-fold higher than *A. tonsa* (Durbin et al. 2008), and the difference in this ratio declined to 3-fold at the mid-stage nauplii through adults. Regression slopes of copy number versus biomass were significantly different between the two species (ANOVA; $F_{1,9} = 15.63$, P=0.003), implying a different relationship between mtCOI copy number and biomass for *A. tonsa* and *P. crassirostris* nauplii.

Culture Validation Copy-Biomass Relationship – Cultures of *P. crassirostris* were used to further evaluate the relationship of mtCOI DNA copy number to count and size-based biomass in paired size-fractionated samples (Fig. 4.7). There is a strong positive relationship between DNA copy number and biomass (slope = 9.2×10^6, $P < 0.001$, $r^2 = 0.93$) over a range in biomass of 0.8 – 161.1 μg C sample^{-1} and from $5.8\times10^6 – 1.7\times10^9$ copies sample^{-1}.

The nauplius mtCOI copy animal^{-1} to biomass animal^{-1} regression relationship (Fig. 4.6) was compared to the culture validation regression of copy sample^{-1} to biomass sample^{-1} (Fig. 4.7) to evaluate the strength of the relationship from individual-level estimates up to bulk samples of *P. crassirostris*. There was no significant difference between regressions of the individual copy versus count-biomass relationship and bulk size fraction copy number versus biomass for *P. crassirostris* (ANOVA; $F_{1,16} = 2.75$, P = 0.12). This is further evidence in support of a strong relationship between mtCOI copy number and biomass, from single individuals to bulk-samples across over five orders of magnitude of animal biomass and DNA copy number. The overall linear regression relating DNA copy number to biomass (copies = $9.19\times10^6 \times$ μg C – 5.09×10^7) was therefore used to compare DNA copy number-based biomass to count-based biomass in field samples.

Field Validation of the Copy-Biomass Relationship in Mixed Samples – Counts of calanoid and cyclopoid nauplii and copepodites in each separate size-fractionated field sample ranged from

0.02 – 48 L^{-1} (Fig. 4.8). Cyclopoid nauplii were the most abundant group, with maximum abundances in the 75-80 µm size fraction and total cyclopoid nauplius abundance of 90 L^{-1} in the 63-100 µm net size range and up to 47 L^{-1} in the Niskin 20-63 µm size fraction (Fig. 4.9). Calanoid nauplii had a maximum total abundance of 17 L^{-1} in the 63-100 µm net size range and 10 L^{-1} in the Niskin 20-63 µm size fraction. Cyclopoid copepodites were the most abundant category in the 80-100 µm size fraction (up to 19 L^{-1}), while calanoid copepodites were not a substantial contributor (up to 3 L^{-1}) to the 20-100 µm size fractions in our study due to presumably being retained in the >100 µm sample not analyzed here (due to our focus on naupliar stages).

In order to estimate the average biomass $animal^{-1}$ of each type (calanoid nauplius, cyclopoid nauplius, calanoid copepodite, cyclopoid copepodite) in the paired samples to be compared to their respective copy number estimates, measurements of animals of each type were taken and converted to the average biomass (±SD) for an animal of each type in each size fraction (Fig. 4.10). Count-based biomass $animal^{-1}$ in split field samples ranged from 0.017 µg C $animal^{-1}$ for the smallest calanoid nauplius to 0.169 µg C $animal^{-1}$ for the largest cyclopoid copepodite (Fig. 4.10), with the distribution of sizes in each split size-fraction as shown in Figure 4.10. There was a broad range of copepod types and sizes in the largest size fraction (80-100 µm); calanoid and cyclopoid copepodites were present in the 75-80, and 80-100 µm size fractions but not in the 20-63 or 63-75 µm size fractions which only contained nauplii of both groups.

In order to make an independent comparison of species-specific copy number to count based biomass in mixed field samples, the count-based biomass estimates (total calanoid biomass, total cyclopoid biomass per sample) then were compared to the paired sample copy number data. Since animals cannot be identified to species in the counted half of samples, the species-specific DNA copy number (m^{-3}) was combined for calanoids (*P. crassirostris, B. similis*) and cyclopoids (*O. simplex, O. attenuata*) for an independent comparison of mtCOI copy number (m^{-3}) to count-based biomass (µg C m^{-3}) of calanoid and cyclopoid nauplii and copepodites derived from the paired split of each sample (Fig. 4.11). The mean count-based biomass $animal^{-1}$ of each type (calanoid or cyclopoid, nauplius or copepodite; Fig. 4.10) by each

size fraction was multiplied by the abundance of each type (number m^{-3}) in each size fraction (Fig. 4.8) to calculate the biomass group^{-1} for each type (calanoid or cyclopoid; Fig. 4.11). There is a positive relationship between DNA copy number (m^{-3}) and biomass (µg C m^{-3}) across calanoid and cyclopoids in the different size fractions (linear regression; slope = 1.5×10^7, $P < 0.001$, $r^2 = 0.82$) but this relationship is significantly different from the relationship determined strictly from *P. crassirostris* (ANOVA; $F_{2, 25} = 21.0$, $P < 0.001$). Copy number and biomass were up to ~3-fold higher in the cyclopoids than in the calanoids (Fig. 4.11).

When field validation experiments are plotted along with the culture validation and individual data, we can see that the relationship between biomass and mtCOI copy number continues into higher total DNA copy numbers and animal biomass (Fig. 4.12). In order to test the accuracy of using DNA copy number to estimate the biomass in field samples, the relationship determined for *P. crassirostris* cultures was applied to calculate copy number-based biomass in the field validation data and compared to count-based biomass of calanoids and cyclopoids in each size fraction (Fig. 4.13A,B). The mean ratio of DNA-based biomass to count-based biomass within size fractions was 2.5 for both calanoids (range 0.9 - 7.3) and cyclopoids (range 1.4 - 4.2).

Figures 4.14 and 4.15 illustrate the percent contribution of calanoids and cyclopoids to the total count-based biomass (Fig. 4.14) and the percent contribution of each species to the total mtCOI copy number (Fig. 4.15). Both methods (copy number per species and count-based biomass) result in a ~5:1 ratio of cyclopoids to calanoids. The percent contribution of each species to the total copy number shows that *O. simplex* is a dominant contributor to the community providing 50% or greater of the total mtCOI copies in each size fraction.

4.5 Discussion

In the present study, a highly sensitive qPCR assay was developed for quantifying mtCOI DNA copy number of four common subtropical copepod species with the aim of applying the method to quantify biomass of larval stages of individual species, which would otherwise be impossible given that they cannot be distinguished morphologically. The qPCR assay performed well for all four species; quantification of mtCOI copies was sensitive down to as few as 300

copies of target DNA, and the presence of non-target DNA did not affect qPCR quantification or sensitivity of the assays. Given the consistent melt curves and copy number estimates in the parallel dilution series, the specificity of each assay is sufficiently high to prevent spurious mtCOI copy number estimates in mixed species field samples even when the target species is rare (<1% of total mtCOI copies in mixed field samples) relative to respective non-target species. When mtCOI copy number was directly compared to count-based estimates of biomass for *P. crassirostris*, we found a linear relationship, similar to that reported by Durbin et al. (2008) for *A. tonsa* nauplii. The linear relationship extended from individual estimates of copy number per unit biomass up to bulk-cultured *P. crassirostris*, suggesting no change in accuracy over more than five orders of magnitude difference in biomass. In paired field samples, we found a ratio of 5:1 of cyclopoids to calanoids in our 20-100 µm size fraction by both copy number estimates and count-based estimates of biomass, illustrating a useful future application; evaluation of changes in the ratio of species over temporal sampling. Application of the *P. crassirostris* copy number-biomass relationship to mixed field samples overestimated calanoid and cyclopoid naupliar biomass by ~2.5-fold as compared to count-based estimates of biomass. A likely source of this discrepancy is application of the *P. crassirostris* relationship to all species. Ongoing work will develop species-specific relationships for *B. similis* and the two *Oithona* species, and we have every hope that this will improve the match between counts and qPCR based estimates of biomass.

Similar to results of Durbin et al. (2008), we found a linear relationship between mtCOI DNA copy number and body carbon for the early developmental stages (including N1-C1) of *P. crassirostris.* Because a linear relationship exists for both *A. tonsa* and *P. crassirostris* (Fig. 4.5) and the presence of non-target DNA does not affect the quantification of target DNA (Figs. 4.2-4.4), these results suggest that it should be possible to estimate the biomass of a target species in a mixed field sample from mtCOI DNA copy number alone. Application of the relationship between mtCOI copy number and biomass for *P. crassirostris* overestimated the count-based biomass estimate 2.5-fold on average, which is fairly accurate in comparison to the range observed for other methods comparisons for estimates of biomass. For example, applying a different length-based biomass equation for subtropical nauplii from Hopcroft et al. (1998a)

results in a 7-fold lower biomass estimate for *P. crassirostris* as compared to our measured biomass. Additionally, there can be over an order of magnitude difference in biomass estimates between settling volume and displacement volume based methods (Postel et al. 2000). Overall, the range of error attributable to counts and conversion of length to biomass was far greater than the error from qPCR measurements of copy number. This suggests that the overestimation of biomass from copy number may be due to either error associated with calculating biomass of the other species from length-biomass equations, or be due to the use of the relationship of copy number to biomass determined for *P. crassirostris*. However, despite the 2.5-fold difference to count-based biomass estimates, our qPCR-based method is still an accurate estimator of animal biomass in comparison to the variation expected with other methods.

Knowledge of the accuracy of the mtCOI copy to biomass conversion for one of our four dominant species (*P. crassirostris*) allows us to develop some hypotheses about the relationships for the remaining three dominant species in our study area. The overestimation of field-sample calanoid biomass of ~2.5-fold suggests that *B. similis* must have a higher ratio of mtCOI DNA copies per unit of biomass than *P. crassirostris*, in order to account for the difference between qPCR-based and count-based estimates. As nauplii, *Bestiolina similis* and *P. crassirostris* begin life at the same lower limit of metazoan size, but *B. similis* grows to a larger body size (females to ~520 μm; *P. crassirostris* females to ~400 μm) over roughly the same development time (McKinnon et al. 2003; *pers. obs.*). Mitochondria are the powerhouse of cellular processes, so it may be beneficial for *B. similis* to pack more mitochondria into each cell to facilitate higher growth rates. Early studies on DNA content in copepod species have shown that the larger of two closely related *Pseudocalanus* species contained seven-fold higher DNA in the nuclei of early embryos, whose relative ratio of DNA to body size declined with development (McLaren et al. 1966). The authors suggested that an increase in DNA content may facilitate development of a larger organism, which would help explain our 2.5-fold higher biomass estimate for the total calanoid nauplii in our study. Figure 4.6 and Table 4.3 support the hypothesis that mitochondrial copy number per unit biomass may be linked to the different physiology of each species; the ratio of copies to biomass in *P. crassirostris* is 3-4-fold higher than for *A. tonsa* (Durbin et al. 2008). *Acartia tonsa* development from egg to adult is roughly twice as long as *P. crassirostris*

but also reaches a ~7-fold higher biomass in a cooler, subtropical to temperate environment (Berggreen et al. 1988), so the relationship of mtCOI copy number to animal biomass is likely a balance within the physiology of each species. However the growth rate-based hypothesis cannot be the reason for higher copy number per unit biomass in the *Oithona* species, because prior work suggests that metabolic rates in cyclopoid copepods are lower than calanoids in similar environments (Hopcroft et al. 1998b; Peterson 2001). Further application of this method to make measurements of copy number in individual cyclopoid nauplii will help resolve the copy number-biomass relationship for our *Oithona* species.

Due to the difficulty in identifying larvae, and to their small size relative to the standard mesh size of nets (standard net is 200 μm), copepod nauplii are an understudied component of marine planktonic communities. Most prior studies reporting naupliar populations *in situ* lump all nauplii into a single category and measure a subset of animals (e.g. Turner 1982, López et al. 2007, Paradis et al. 2012) or calculate their contribution to total copepod populations based on egg production rates relative to quantification of adult abundances (e.g. Zervoudaki et al. 2007, Ohman and Hirche 2001, Eiane et al. 2002). These lumping approaches give an overall view of the total naupliar biomass or abundance, but do not allow any level of interpretation that would account for species-level differences in prey susceptibility (Borg et al. 2012; Bruno et al. 2012). Species-specific prey susceptibility might lead to success or declines in specific predator populations, since nauplii are important prey items for invertebrate and vertebrate predators (Eiane et al. 2002; Jackson and Lenz 2016; Sampey et al. 2007b; Sullivan and Meise 1996). Studies on the diets of larval fishes have found clear selective feeding on certain species of copepod nauplii, for example by fish larvae of the Atlantic mackerel (Paradis et al. 2012; Peterson and Ausubel 1984) and the walleye pollock (Hillgruber et al. 1995). Application of methods like the one described here on species-level copepod naupliar dynamics has the potential to clarify whether the success or decline of a fish population is related to temporal or spatial patterns of abundance of a specific type of larval copepod.

Development and application of qPCR-based methods such as the assay described here have enormous potential to tease apart relationships between animals that are difficult to identify and understand their interactions in marine ecosystems. For example, many morphologically

similar copepod species such as *Calanus finmarchicus, C. helgolandicus,* and *C. glacialis* have shifted their seasonal peak in abundance and distribution in response to warming oceans (Barnard et al. 2004; Chust et al. 2013; Mackas et al. 2012). Genetic techniques have provided a more reliable means of distinguishing these congeners in the field in order to better understand changing abundance and distribution of both adults and early life stages (Hill et al. 2001; Lindeque et al. 2006; Lindeque et al. 1999). *Acartia tonsa* is a classic example of a dominant and ecologically important cryptic species complex that has a broad geographic distribution. With genetic methods, Chen and Hare (2011) identified distinct distributions between genetically different lineages across the eastern coast of the US, some of which overlap spatially but whose life cycles differ temporally. Complexities regarding unknown rates of hatching of resting eggs and survival of the following generations to adulthood continue to limit our understanding of causes for observed variation in different populations over time; application of a qPCR-based method to estimate cryptic naupliar biomass could clarify juvenile mortality schedules that influence observed adult abundances.

The use of DNA as an indicator for biomass is not a new idea for marine species, but using quantification of a species-specific gene as an estimate of biomass is a new application to measure species-specific biomass of animals that cannot be identified by morphology. One of the earliest papers describing a DNA to dry weight relationship in marine species found that total DNA was 40-66% of cellular dry weight and roughly 1.2% of organic carbon content in some phytoplankton species (Holm-Hansen et al. 1968). Dortch et al. (1983) described the bulk plankton (1-333 µm) DNA to be 1.54% of biomass of mixed plankton in Dabob Bay, Washington. Application of our field sample total DNA concentrations (fluorometrically measured) compared to the combined calanoid and cyclopoid count-based biomass sample^{-1} results in total DNA as 2.91% (range 1.21-5.85%) of total copepod biomass across size fractions. Our smaller size fractions actually had a higher DNA:biomass ratio than larger size fractions. Average ratios by size fraction were 5.35% for 20-63 µm, 3.14% for 63-75 µm, 1.82% for 75-80 µm, and 1.72% for 80-100 µm. Application of our mtCOI-based biomass estimate per species and summing across species for each sample to calculate the total DNA to mtCOI-based biomass ratio results in a similar mean ratio of 2.24%.

With our current size-fractionation and DNA-based method we can estimate the biomass of the naupliar size-fraction and the relative contribution of different species to the total by quantifying mtCOI copy number. This suggests that it may be possible to use amplification of the mtCOI gene in a similar manner with high-throughput methods, but this has not yet been shown for eukaryotes. Higher throughput sequencing methods enable investigations of diverse communities at a greater resolution than single animal barcoding or even our described qPCR approach; and provide a means to investigate communities at high resolution over longer time-series without extensive taxonomic expertise required with traditional methods. Community sequencing methods have been used for the past decade to estimate the relative contribution of microbes to communities with a higher resolution than is possible with culture or microscope-based methods (e.g. Lloyd et al. 2013, Staley et al. 2013). Our results suggest this may be possible with eukaryotes as well. Careful application of next-generation sequencing methods to metazoan samples would expand the application of whole community diversity sequencing to also estimate the relative biomass contribution of different species to the community.

We developed species-specific primers for quantitative amplification of four dominant copepod species in a productive subtropical embayment. Tests on cultured *P. crassirostris* show that qPCR estimated copy number is linearly related to animal biomass over at least five orders of magnitude range in biomass from individuals up to bulk cultured material. Application of the copy-biomass relationship from cultured *P. crassirostris* to mixed species field samples estimated count based biomass accurately within a factor of 2.5, suggesting that quantification of copy number alone can provide an accurate estimate of species specific biomass. When traditional methods would only report the total naupliar abundance or biomass, we were able to apply this method to mixed field samples to show that not only did cyclopoid copepods dominate the community by 5:1, but that the smallest species *O. simplex* contributed >50% to the community mtCOI DNA and biomass. Application of this method to study dynamics of naupliar communities over time will allow us to better understand natural rates of variation in the dominance of species, and eventually a better understanding of how changes in naupliar populations may be affecting variation in success of predator populations that rely on specific species to survive.

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Tables and Figures

Table 4.1. Primer sequences, amplicon lengths, and reaction conditions for the qPCR assays developed for four target copepod species. Temp indicates the annealing temperature used in the reaction, and [Primers] indicates optimal primer concentrations. All assays were run as 20 µL volumes. Assay conditions for *Parvocalanus crassirostris* are from Jungbluth et al. (2013).

Species	**Primer sequence (5' – 3')**	**Amplicon Length (bp)**	**Temp (°C)**	**[Primers] (µM)**
Bestiolina similis	Fwd-AGGTGCCTCAGTTGACTTTGCG Rev-TCCAAACACTCGAAGATTACCC	109	65	0.2
Oithona attenuata	Fwd- GTGTCCCACAGAGGAGCTGCAGTG Rev-AATCCCAAGTGCTCGGAGGTTG	123	68	0.2
O. simplex	Fwd-TGACTACGCTATTTTTTCA Rev-AAGAGATAACAAGAGAAGTACTGCC	169	55	0.4
Parvocalanus crassirostris	Fwd-GCGGGAGTAAGATCAATTCTAGGC Rev-AGTAATGGCCCCTGCTAATACGG	165	65	0.4

Table 4.2. Amplification efficiencies resulting from qPCR analysis of a field-collected plankton sample. Statistics shown are for dilutions of the same field sample amplified using qPCR assays for four target copepod species. Slope and R^2 values were derived for each run from linear regressions of Cq values against the logarithm of the total DNA concentration (see Fig. 2). E indicates the efficiency of the reaction calculated as $10^{(-1/\text{slope})}$, and % E is the percent efficiency or (E -1) x 100.

Species	**Slope**	**R^2**	**E**	**% E**
Bestiolina similis	-3.34	0.9993	1.99	99
Oithona attenuata	-3.55	0.9991	1.91	91
Oithona simplex	-3.31	0.9999	2.01	101
Parvocalanus crassirostris	-3.44	0.9999	1.95	95

Table 4.3. Average mitochondrial COI DNA copy number (Copies $\times 10^3$ indiv.$^{-1}$) and measured body carbon (µg C indiv.$^{-1}$) for developmental stages of *Parvocalanus crassirostris.* Individuals replicate^{-1} are the mean numbers of animals used to obtain biomass estimates for each cohort of the specified development time (days) and stage (N=nauplius, C= copepodite). Copies $\times 10^6$ µg C^{-1} is the ratio between copy number and biomass for a given stage. *n* = number of replicates measured for each biomass estimate.

Stage	*n*	Dev. time (d)	Indiv. replicate^{-1}	µg-C indiv.$^{-1}$	Range µg C indiv.$^{-1}$	†Copies $\times 10^3$ indiv.$^{-1}$	Copies $\times 10^6$ µg C^{-1}
N1-N2	2	0.71	8300	0.022	0.021-0.023	439	19.93
N3-N4	3	1.71	1700	0.057	0.031-0.095	747	13.17
C1-C2	3	5.0	1200	0.174	0.162-0.180	1597	9.19
C5	3	7.1	690	0.542	0.526-0.559	5366	9.90
Adult	3	>8	500	0.720	0.713-0.733	6365*	8.84

†*Copy number indiv^{-1} from Jungbluth et al., (2013).*

**Adult copy number is based on the average of replicate measurements of adult males and females*

Figure 4.1. Amplification curves (left) and melt curves (right) resulting from qPCR amplification of total DNA extracted from focal copepod species. Panels represent runs with four separate qPCR assays developed for the copepods *Bestiolina similis* (a,e), *Oithona attenuata* (b,f), *O. simplex* (c,g) and *Parvocalanus crassirostris* (d,h). Red lines indicate data for the target species; data for the four respective non-target copepods are shown in blue. Three technical replicates are shown for each sample (all samples at 50 pg/µL total DNA). Non-target samples with Cq > 35 cycles are excluded.

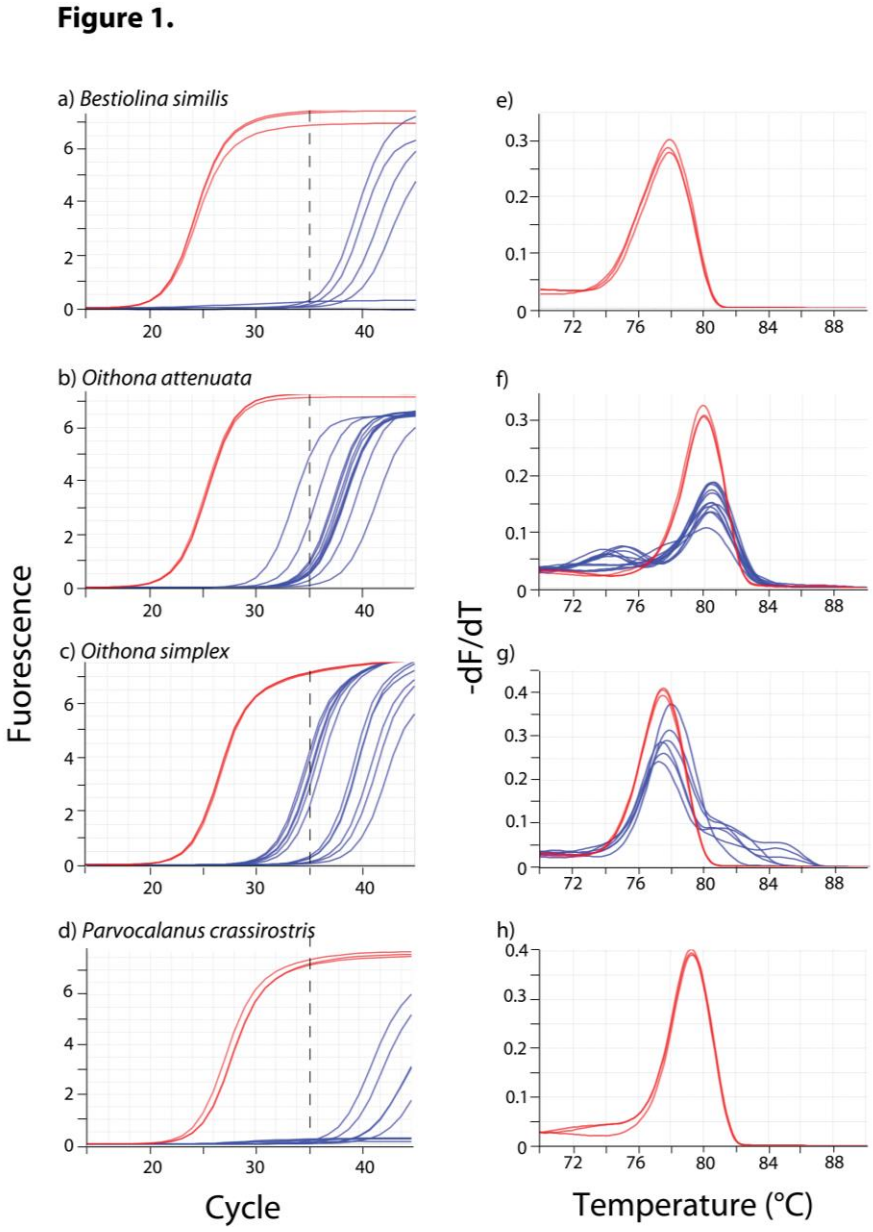


Figure 4.2. qPCR estimates of the number of target mtCOI copies in serial dilutions of target species DNA into water (white) and into mixed DNA from non-target copepod species (black). Bars span 95% confidence intervals on mean copy number estimated from three technical replicates per sample.

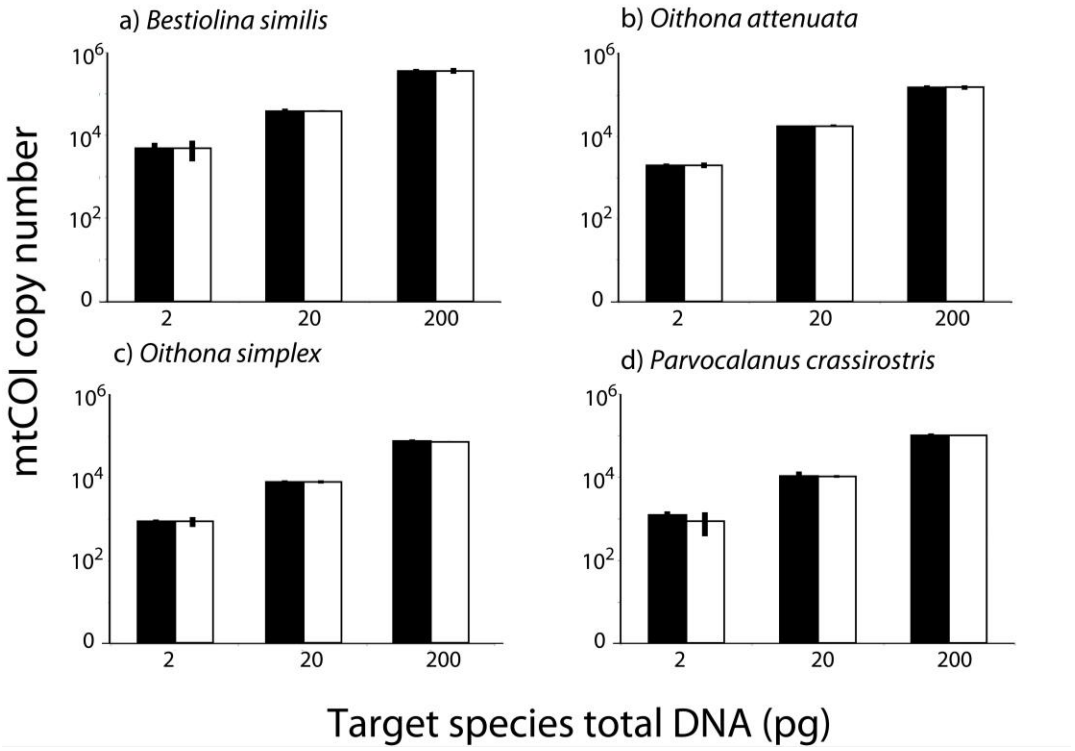


Figure 4.3. Amplification curves resulting from qPCR analysis of a field-collected plankton sample using developed qPCR assays for target copepod species *Bestiolina similis*, *Oithona attenuata*, *O. simplex* and *Parvocalanus crassirostris*. Data are the Cq values corresponding to points along a 10-fold dilution series of the field sample (shown as total DNA per well). Fitted lines are linear regressions of Cq against log(total DNA) (see Table 4.2).

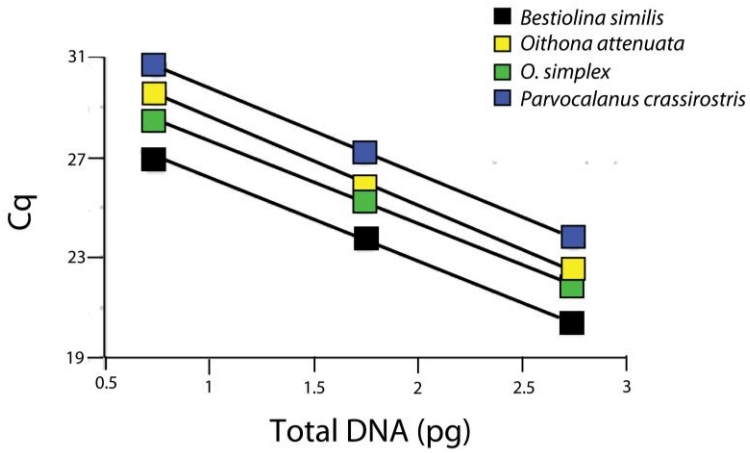


Figure 4.4. (a-d) Melt curves resulting from amplification of total DNA extracted from 101 mixed-species field samples. Each field sample was amplified for all four qPCR assays, developed for the copepods *Bestiolina similis* (a), *Oithona attenuata* (b), *O. simplex* (c) and *Parvocalanus crassirostris* (d). Three technical replicates are shown for each sample; plots are composites of data from multiple plates. (e-h) Histograms of the proportional contribution of mtCOI copies estimated for the target species to total mtCOI copies, summed across the four copepod species, for the 101 field samples.

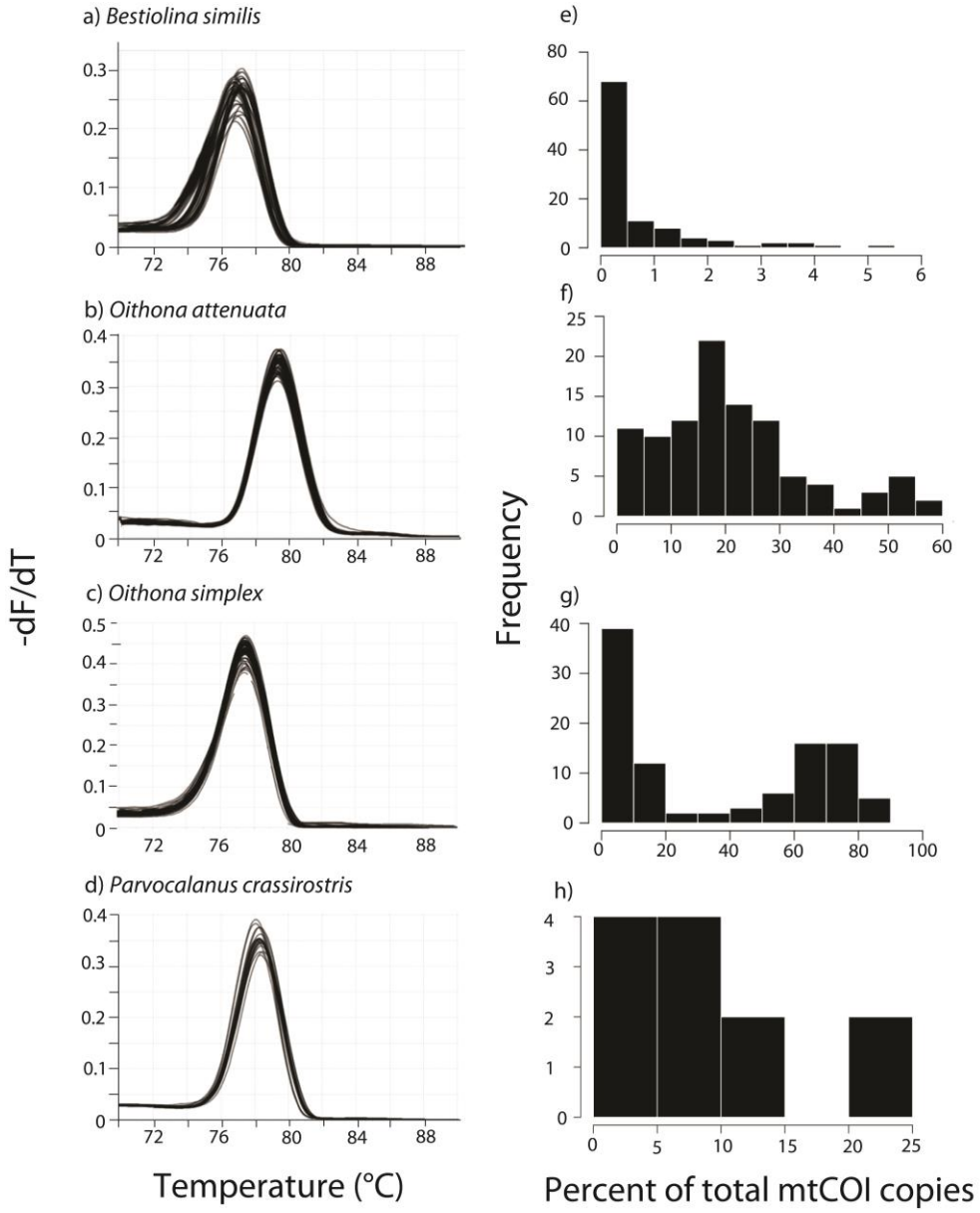
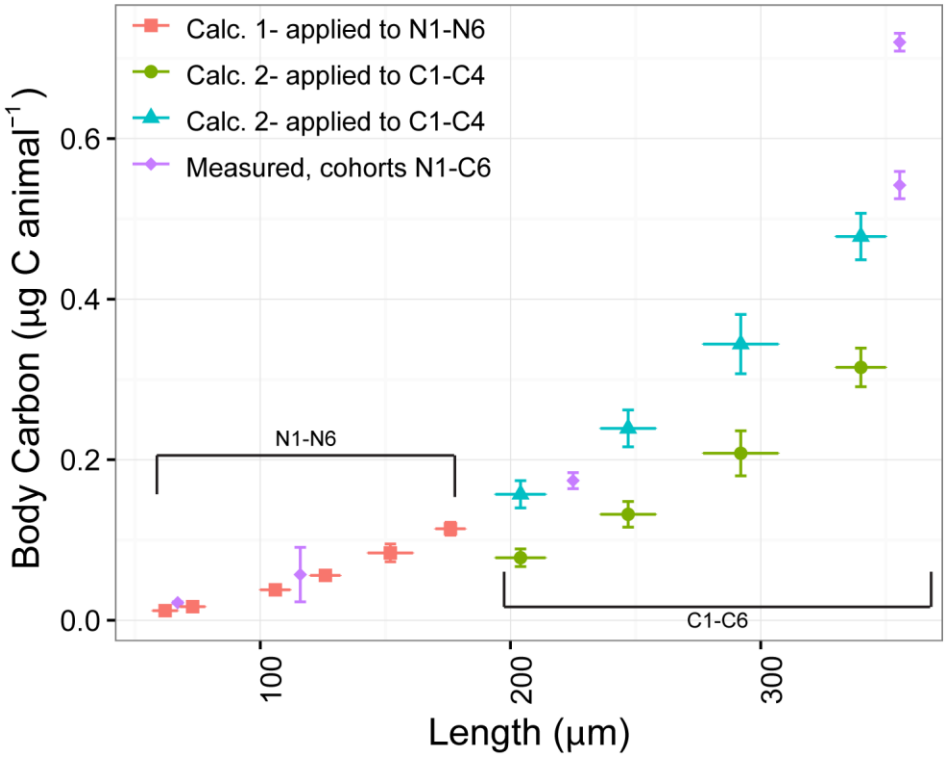


Figure 4.5. Comparison of *P. crassirostris* body length (total length (TL) for nauplii, prosome length (PL) for copepodites; µm) to different estimates of body carbon (µg C animal^{-1}). Red squares apply White and Roman's (1992) equation for nauplii to N1-N6 body sizes. Green circles apply the Webber and Roff (1995) equation for calanoid copepods to copepodite C1-C4 body sizes (PL). Blue triangles apply White and Roman's (1992) calculation for nauplii to copepodite C1-C4 body sizes (PL). Purple diamonds are measured body carbon for cohorts of nauplii and copepodites N1-C6 in this study (Table 4.3). Body length estimates are from McKinnon et al. (2003). Error is ± standard deviation. See below for equations.



Calc. 1 (White and Roman 1992; dry weight (DW) in µg, length (L) in mm):
$DW=15.53\times L^{2.17}$

Calc. 2 (Webber and Roff 1995; dry weight (DW) in µg, length (L) in µm):
$ln(DW)=2.73\times ln(L)-15.93$

Both apply a ratio of body carbon:DW of 0.32 (Wiebe et al. 1975)

Figure 4.6. Comparison of mtCOI copies and measured biomass animal^{-1} for naupliar stages of two species; *Parvocalanus crassirostris* (blue; solid regression line; this study) and *Acartia tonsa* (pink; dashed regression line; Durbin et al. 2008). Linear regression lines, equations, and coefficient of determination (r^2) are shown for each species. Error in both x and y-directions are ± standard deviation, no error was available for *A. tonsa* (see Durbin et al, 2008).

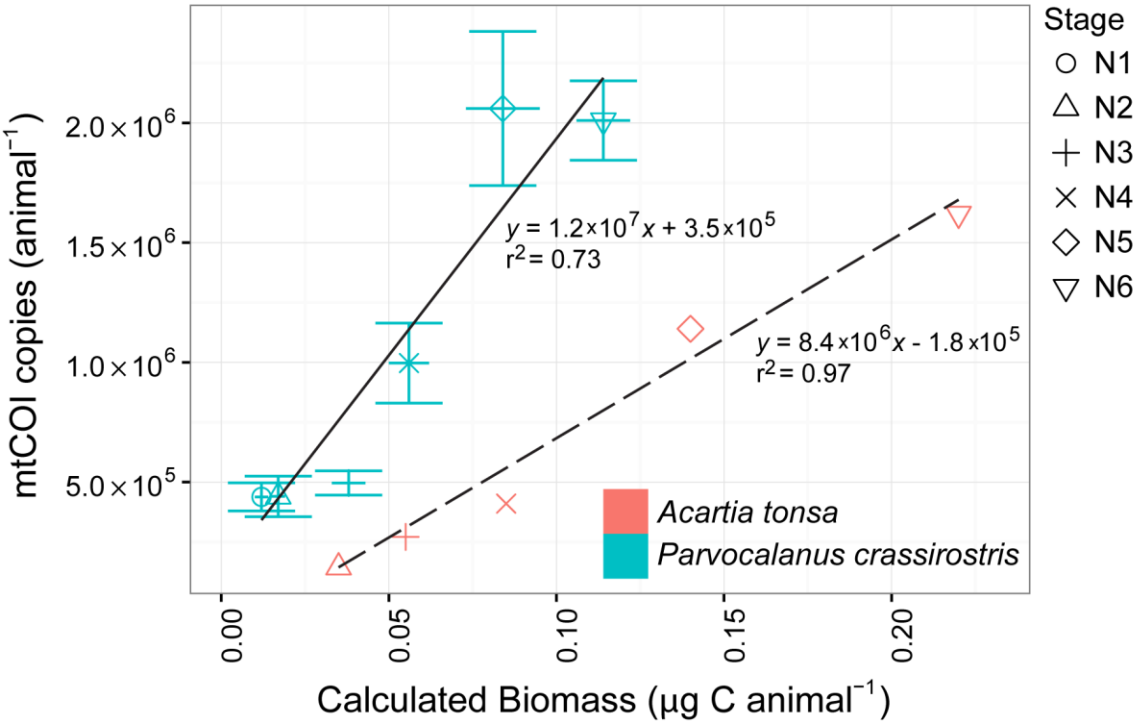


Figure 4.7. Relationship between copy number and biomass sample^{-1} from body length for *P. crassirostris* mixed stage cultures (copy number data from Jungbluth et al., 2013). Dashed line is the linear regression of all size fractions. Error bars are ± standard deviation. N=3 replicate split samples for each size-fraction; 20-63 µm values overlap and have low error.

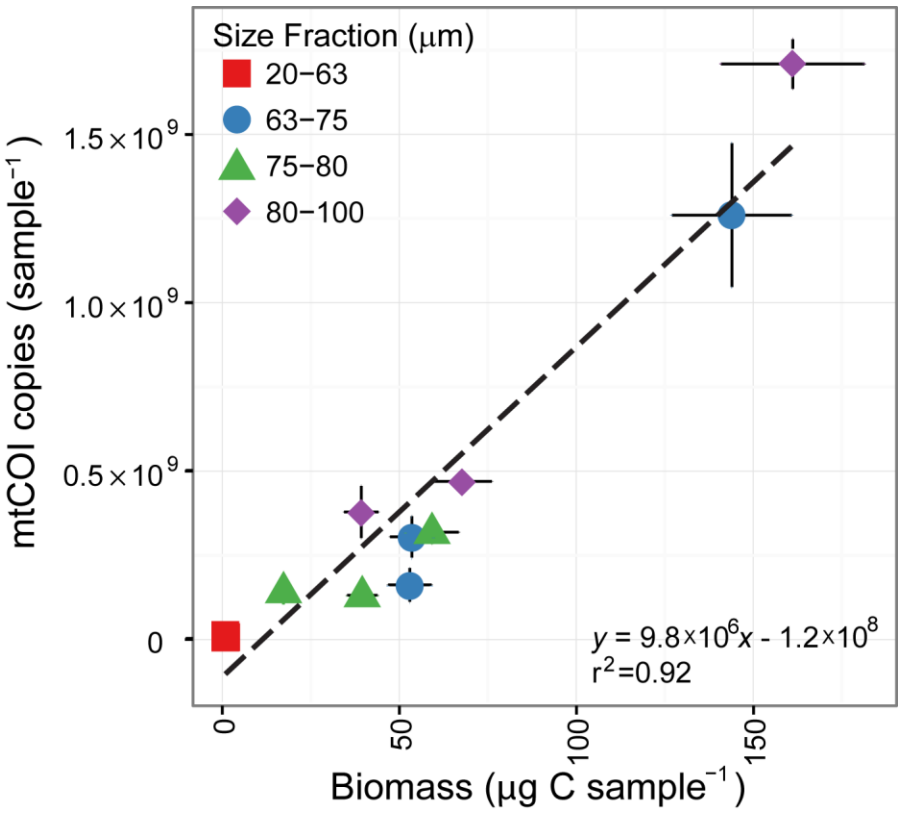


Figure 4.8. Counts of the calanoid (Cal) and cyclopoid (Cyc) nauplii and copepodite abundance (L^{-1}) in each size fraction from field Niskin (A) and net (B-D) samples; A) 20-63 µm, B) 63-75 µm, C) 75-80 µm, and D) 80-100 µm. Calanoid nauplii (green), cyclopoid nauplii (purple), calanoid copepodites (red), and cyclopoid copepodites (blue). Error bars are ± standard deviation.

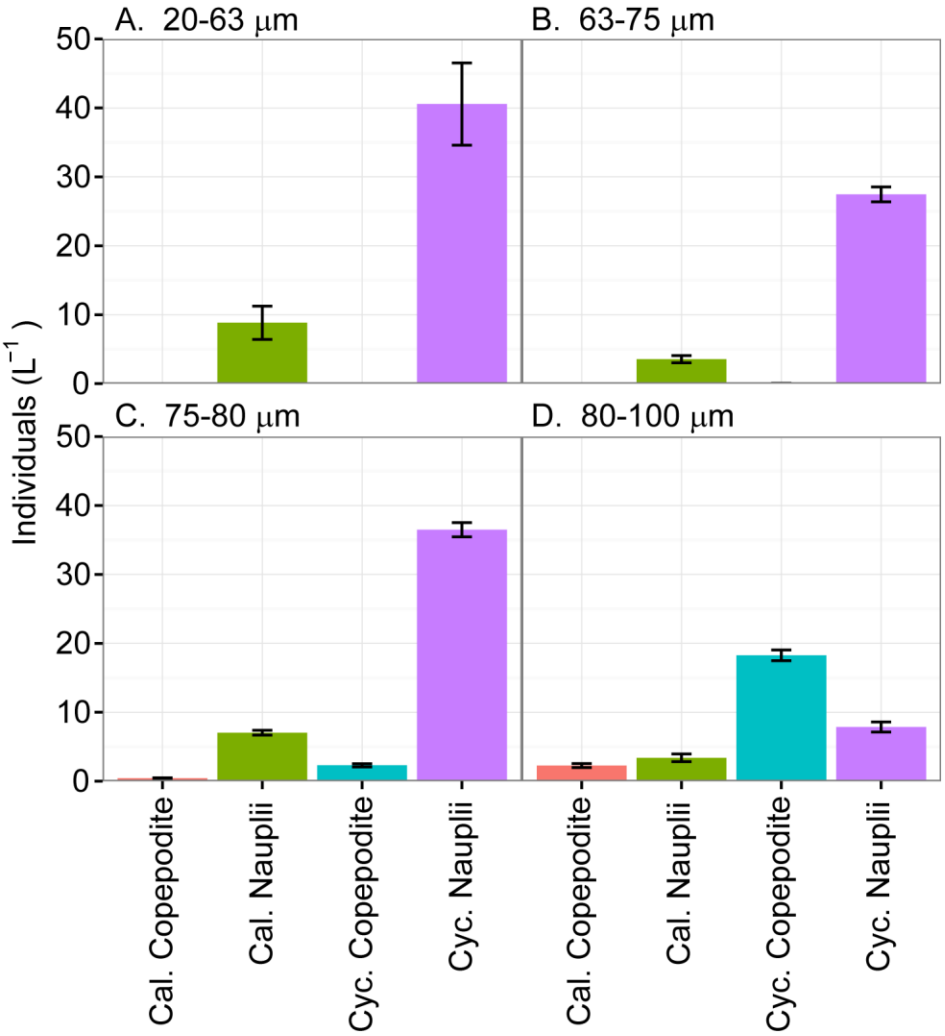


Figure 4.9. The total counted abundance (L^{-1}) of calanoid (Cal) and cyclopoid (Cyc) nauplii and copepodites in net (63 µm mesh) and Niskin replicate collections. Niskin only includes those animals in the 20-63 µm size fraction, net sums across 63-100 µm size fractions. Error bars are ± standard deviation.

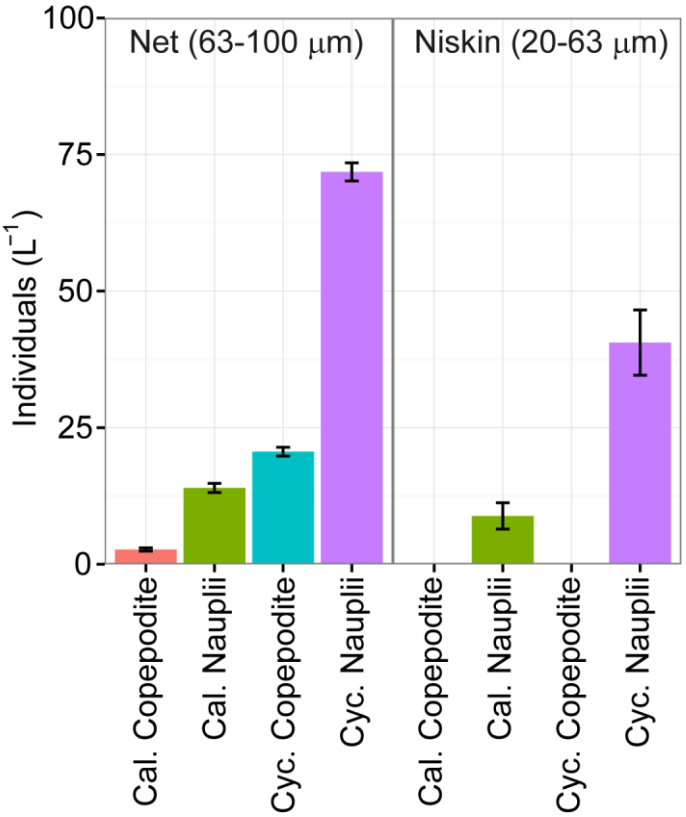


Figure 4.10. Body size (µm) and calculated biomass (µg C animal^{-1}) fractionation of individual calanoid and cyclopoid nauplii (based on total length) and copepodites (prosome length) across size-fractionated field validation samples. Size fractions include the 20-63 µm (red), 63-75 µm (blue), 75-80 µm (green), and 80-100 µm (purple) ranges. Broad taxa identified and measured with microscopy are plotted separately; calanoid nauplius (circle), cyclopoid nauplius (upright triangle), calanoid copepodite (cross), cyclopoid copepodite (inverted triangle). Each point is the body size and calculated biomass of an average individual in a sample replicate. Error bars are ± standard deviation.

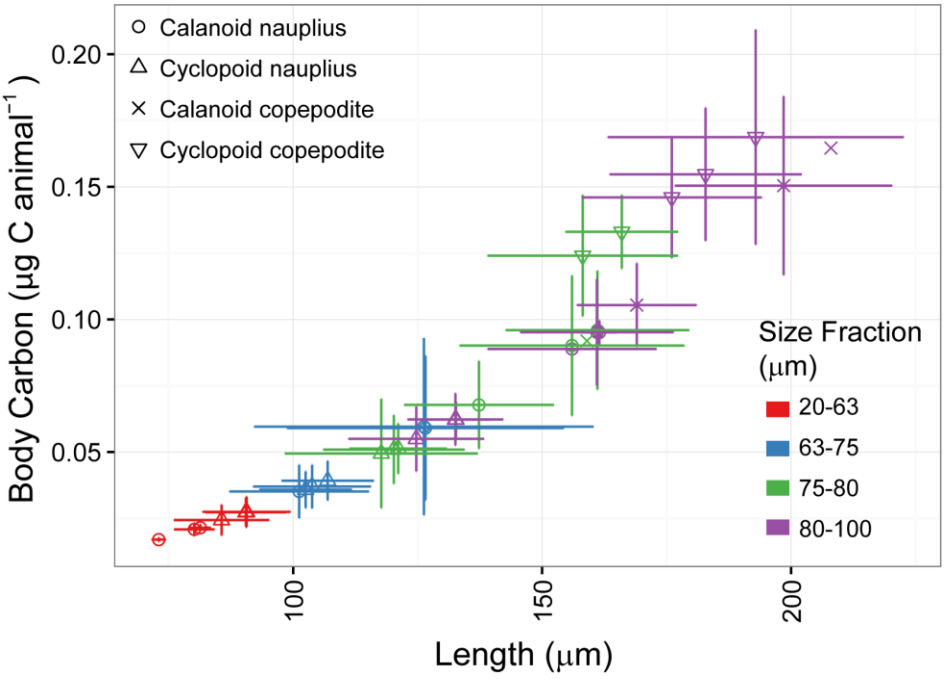


Figure 4.11. DNA copies (m-3) vs. count-based biomass (µg C m-3) in paired, size-fractionated field samples. Size fractions include 20-63 µm (red), 63-75 µm (green), 75-80 µm (blue) and 80-100 µm (purple). Species-specific DNA copy number was combined for calanoids (circles; *Parvocalanus crassirostris, Bestiolina similis*) and cyclopoids (triangles; *Oithona simplex, Oithona attenuata*) for comparison to count-based biomass estimates of calanoid and cyclopoid nauplii and copepodite biomass. Black line is a linear regression of all data points. Error bars in x- and y-direction are ± standard deviation.

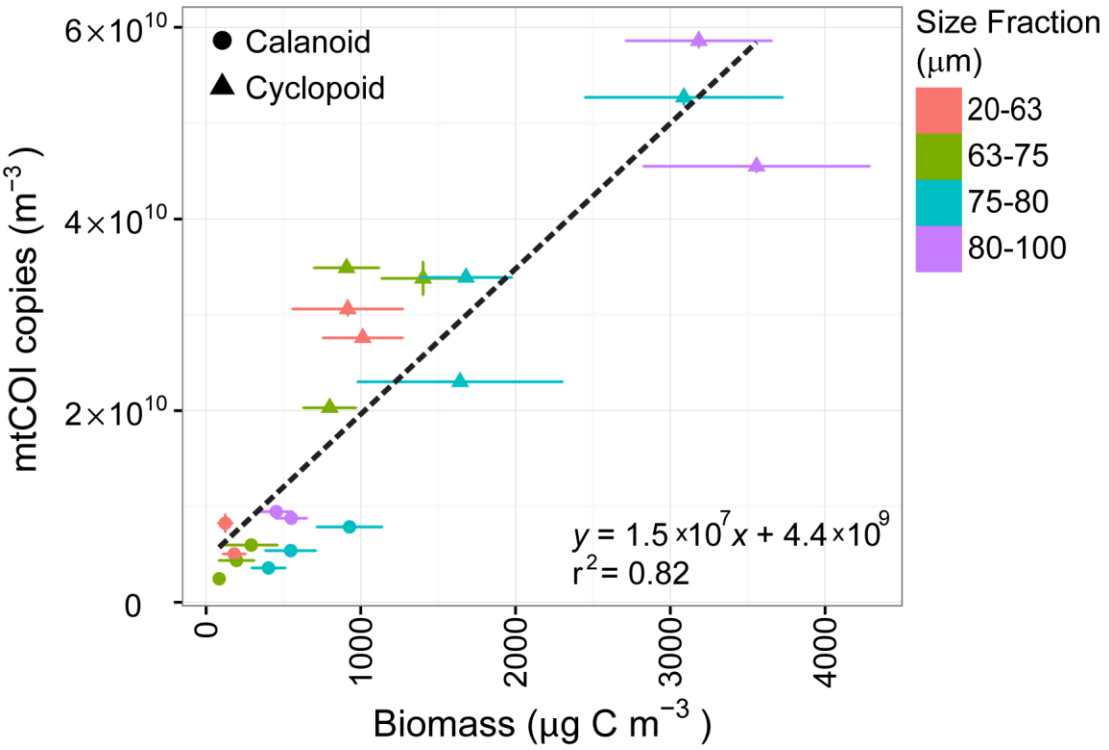


Figure 4.12. Combined copy number biomass comparisons from individual *P. crassirostris* (squares) to monocultured *P. crassirostris* (circles) and mixed species field samples (triangles). Stages and size fractions are plotted separately. In the field validation data, calanoids and cyclopoids were combined to illustrate the overall relationship of mtCOI copies to biomass. Axes are log-transformed to expand the view at the lower biomass and copy number in the individuals and culture validation data. Error is ± standard deviation.

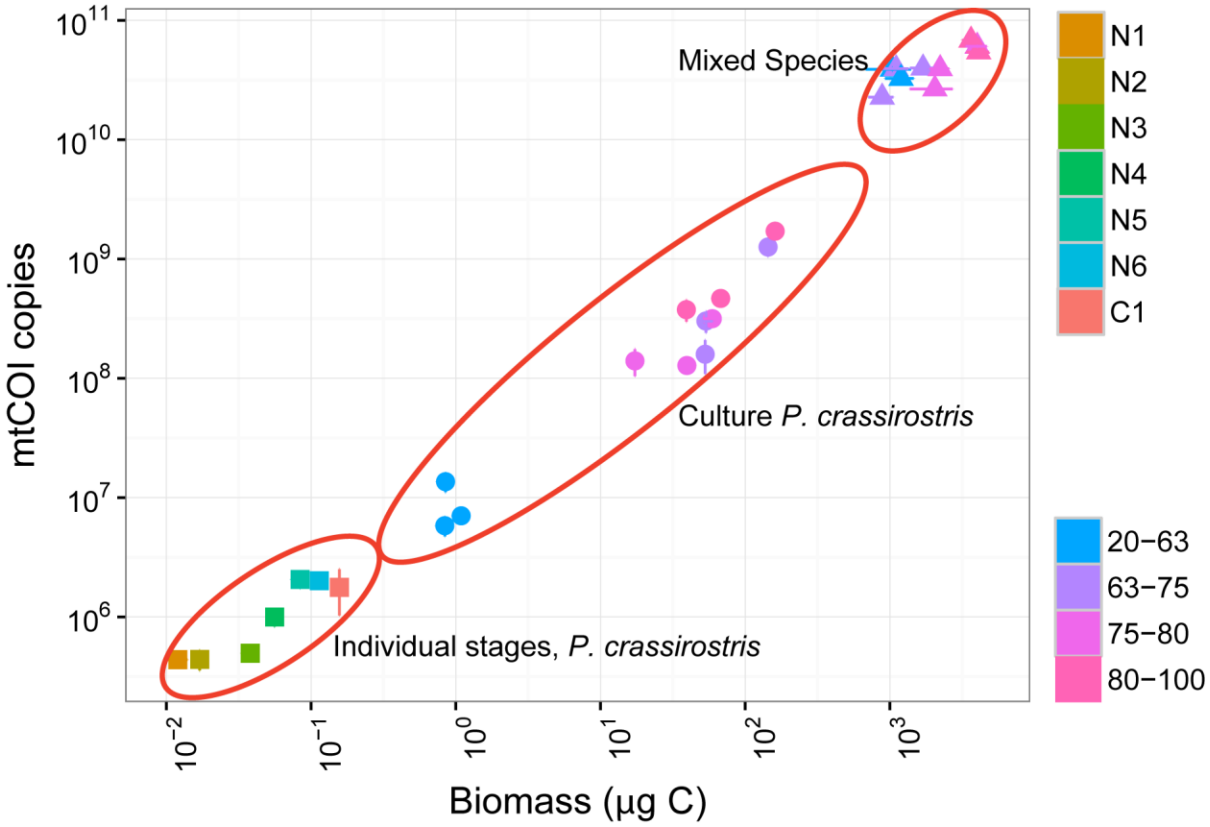


Figure 4.13. qPCR-derived biomass compared to count-based biomass of total (A) calanoids and (B) cyclopoids in each size fraction (μm; 20-63 is red, 63-75 is blue, 75-80 is green, and 80-100 is purple), based on the copy number – biomass conversion estimated from *P. crassirostris* (in Fig. 4.6). Dotted line is 1:1, short-dashed line is 2:1, in (B) compact dashed line is 3:1, and in (A) dash-dotted line is 4:1. Error bars are ± standard deviation.

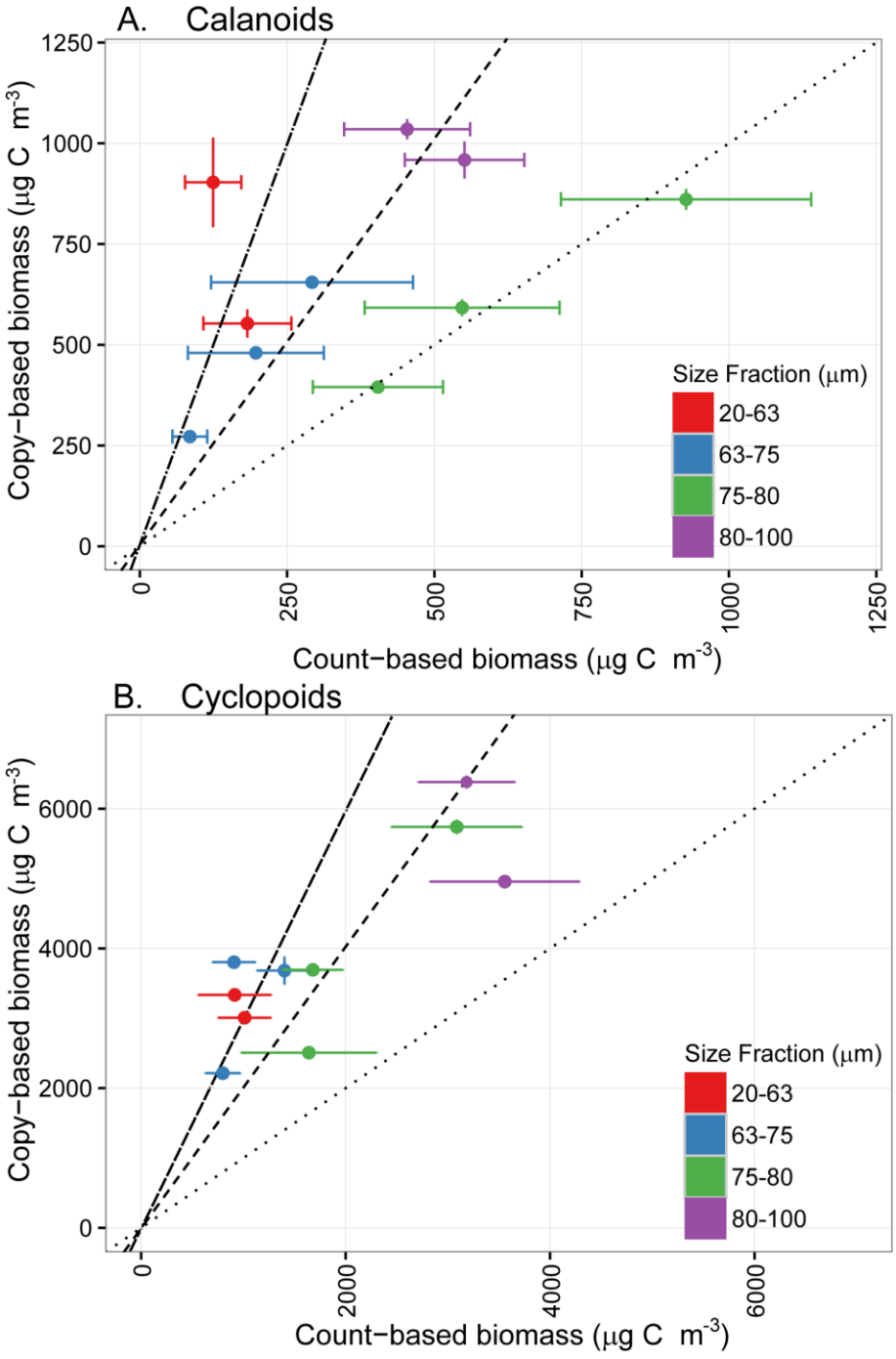


Figure 4.14. Percent contribution of cyclopoids (blue) and calanoids (red) to the total count-based biomass in each field-collected size fraction (µm); (A) 20-63 µm, (B) 63-75 µm, (C) 75-80 µm, (D) 80-100 µm. Error bars are ± standard deviation.

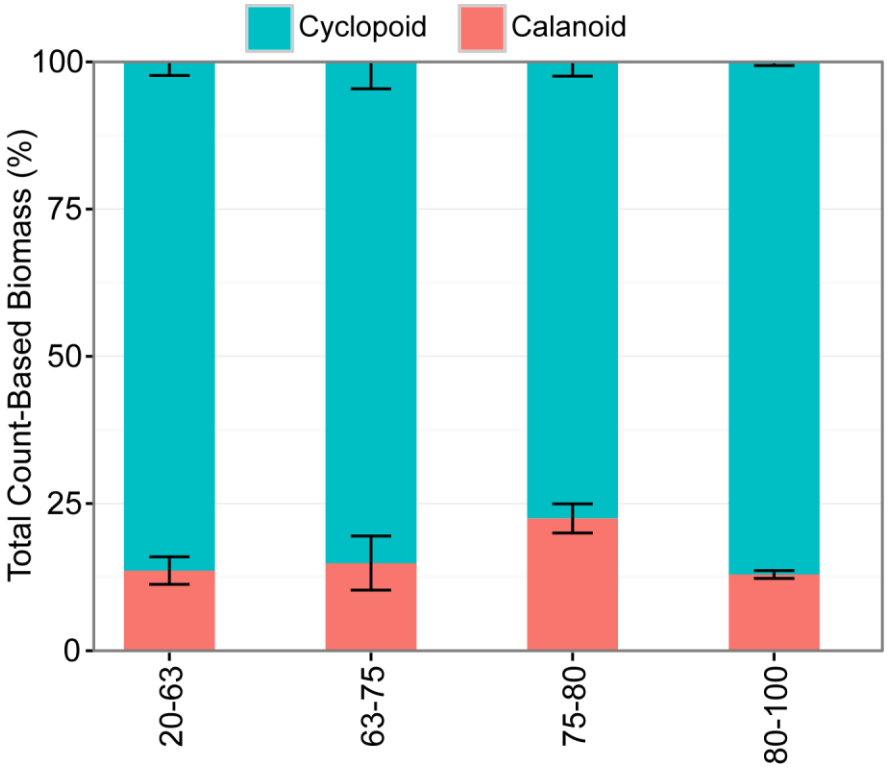
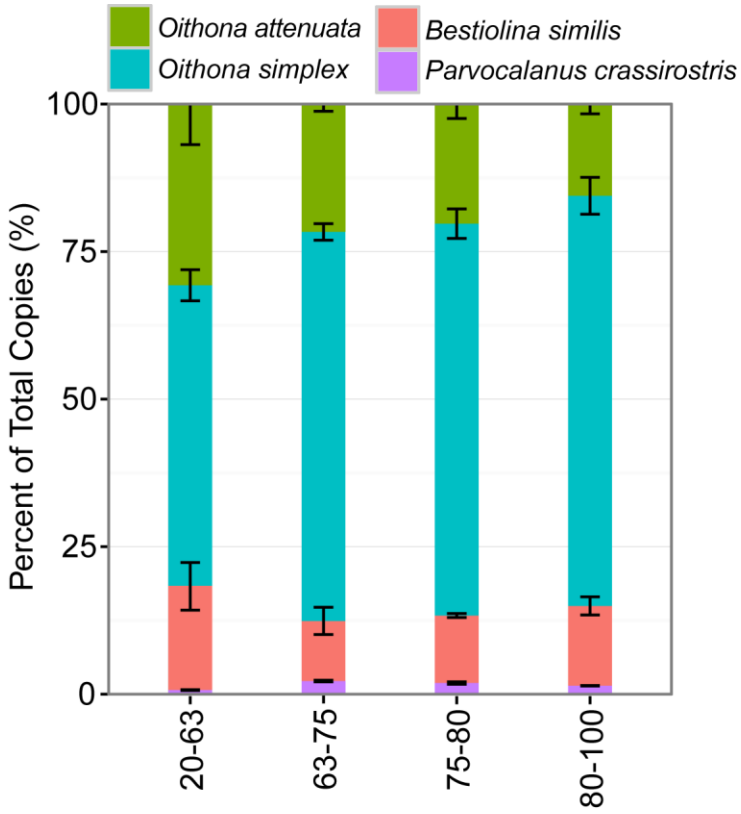


Figure 4.15. Percent contribution of each copepod species to the total mtCOI DNA copy number in each field-collected size fraction (µm); (A) 20-63 µm, (B) 63-75 µm, (C) 75-80 µm, (D) 80-100 µm. Species include *Oithona attenuata* (green), *Oithona simplex* (blue), *Bestiolina similis* (red), and *Parvocalanus crassirostris* (purple). Error bars are ± standard deviation.



Chapter 5:
Copepod early-life history responses to event-scale perturbations in the coastal zone

To be submitted as: M.J. Jungbluth, P.H. Lenz, K. Hansen, K. Selph, E. Goetze. *Copepod early-life history responses to event-scale perturbations in the coastal zone.*

5.1 Abstract

Event-scale perturbations, such as storms, are important drivers of plankton community dynamics in the coastal zone. In subtropical Kane‘ohe Bay, Hawai‘i , storm-associated runoff provides a pulse of nutrients, fueling rapid spikes in phytoplankton production that are often followed by dramatic increases in zooplankton populations within days of a storm event. Within the zooplankton community, a strong numerical response to storm events is often seen in the early life stages of copepods (copepod nauplii). Here we examine species-specific responses of copepods to event-scale storm perturbations by quantifying daily abundance of the nauplii of four copepod species in both a background ecosystem state and following two types of storms (spring and fall). Using a qPCR-based approach to enumerate early-stage copepods, we found that copepod populations were stable in the non-storm ecosystem state for all species except for *Bestiolina similis,* which was under decline and contributed little to the naupliar population over the course of the study. The first-flush storm resulted in the largest increase in early-stage nauplii. Within 2-4 days after the storm, *Oithona simplex* and *Parvocalanus crassirostris* naupliar DNA quantities were up to an order of magnitude greater than pre-storm levels, however, a majority of the early naupliar production was lost. Up to 99% of daily early naupliar biomass and a maximum of 13 mg C m^{-3} d^{-1} of *Oithona simplex* was removed from the naupliar community before reaching the mid stage nauplii, suggesting that there were high rates of early naupliar mortality across all species shortly after the storm. A lagged increase in *O. attenuata* total naupliar abundance indicates distinct population dynamics for this species. Despite a high amount of freshwater runoff and a similar peak in chlorophyll *a*, the spring storm resulted in a moderate response in all species, with a maximum response by *P. crassirostris* nauplii of up to 3-fold. Our results suggest that there were distinct responses of the copepod community to early vs. late season storms, and that the contribution of each species to the community changes over time, particularly during ephemeral post-storm periods.

5.2 Introduction

Plankton populations in oligotophic ocean regions are characterized by greater stability over an annual cycle, with standing stocks of plankton changing by 2 or 3-fold throughout the year (Webber and Roff 1995; Hopcroft et al. 1998; Youngbluth 1976; Coker and Gonzalez 1960), in comparison to temperate regions where plankton biomass varies by orders of magnitude across season (e.g., Kiorboe and Nielsen 1994). Production in oligotrophic ecosystems is supported primarily by nutrient recycling with occasional injections of new nutrients from mesoscale features (e.g., eddies, wind-driven transport, internal waves), which transport nutrients into the photic zone from depth and drive ephemeral increases in community productivity and carbon export (Karl 1999). With persistent nutrient limitation and rapid nutrient recycling, the dynamics of subtropical plankton communities in coastal waters are often driven by temporal variation in rainfall and the supply of allochthonous nutrients to the community primarily from land (Hopcroft and Roff 1990; Chisholm and Roff 1990; Youngbluth 1976). Tropical and subtropical embayments support nearby reef ecosystems and serve as nursery habitat for fishes and larger predators in surrounding oceanic waters (e.g. Clarke 1971; Johannes 1978; Kobayashi 1989); therefore, a better understanding of the effects of ephemeral resource inputs on the plankton community is necessary to understand how these ecosystems may be impacted by the changes in the magnitude and timing of rainfall events that are predicted in the next century (Chu et al. 2010; Timm and Diaz 2009).

Storm runoff can significantly enhance phytoplankton and zooplankton productivity in coastal environments (Flint 1985; Hoover et al. 2006; Ringuet and Mackenzie 2005), but differences in recent rainfall history and in climatic conditions prior to and following a storm influences the magnitude of the numerical response in the plankton community. For example, after an extended duration of drought, "first-flush" storm events transport sediment, detritus, and high levels of land-derived nutrients into coastal waters (De Carlo et al. 2007; Hoover 2002), often fueling in a phytoplankton bloom dominated by large-celled diatoms, which is then followed by an increase in zooplankton abundance (Cox et al. 2006; Hoover et al. 2006; Ringuet and Mackenzie 2005). In contrast, storms that occur following recent rainfall will transport less sediment and nutrients into coastal waters, due to the shorter accumulation period between

storms (e.g. De Carlo et al. 2007); These events more often result in a phytoplankton bloom of smaller, non-diatom species or no bloom at all (Ringuet and Mackenzie 2005). The magnitude and timing of rainfall events are expected to change in the next century (Chu et al. 2010; Pachauri et al. 2014; Timm and Diaz 2009), and will alter marine communities in response to warming and acidifying waters (Hoegh-Guldberg and Bruno, 2010). Considering the seasonality of reproductive events in many marine species (e.g. Johannes 1978; Platt et al. 2003), shifts in the magnitude and timing of rainfall could result in decreased plankton abundances during critical periods of the life history of fish populations that are important for coastal fisheries. Understanding how storms affect different species in the planktonic food web is important to our ability to understand variation in fish stocks over time, and to predict responses of these communities to changing climate.

Copepod nauplii are often a numerically-dominant yet understudied component of zooplankton communities in a range of marine ecosystems around the world (Calbet et al. 2001; Lučić et al. 2003; Safi et al. 2007; Turner 2004). The abundance and small size of nauplii make them a critical food resource, in particular for early stages of fish larvae (Checkley 1982; Jackson and Lenz 2016; Sampey et al. 2007). Studies of fish gut contents have shown that many fish prefer prey of a particular copepod species or within a restricted prey size range (Checkley 1982; Morote et al. 2011; Sassa and Tsukamoto 2012), suggesting that species-specific differences in the contribution of nauplii to marine food webs are likely. This hypothesis is supported by evidence of species-specific morphology, feeding, swimming, and escape behaviors in nauplii (Borg et al. 2012; Bruno et al. 2012; Gauld 1959) that may make them more or less susceptible to predation (Titelman and Kiørboe 2003). However, due to their small size, morphological similarities, and difficulty in identification of nauplii to species (Björnberg 1986), there is little to no species-specific information available on *in situ* naupliar populations or their role in carbon transfer in marine food webs.

Differences in life history characteristics and trophic ecology between the dominant orders of copepods, Calanoida and Cyclopoida, are likely to contribute to differences in the response rates and magnitudes of copepod species to ephemeral environmental forcing in coastal ecosystems. Many calanoid copepods are broadcast spawners, and are capable of producing a

greater number of eggs per female per day than egg-bearing cyclopoid copepods. For example, the calanoid copepod *Bestiolina similis* can produce up to 50 eggs female^{-1} day^{-1} (EFD)(McKinnon et al. 2003), while the cyclopoid copepod *Oithona simplex* can produce up to 16 EFD (McKinnon and Ayukai 1996). Broadcast-spawned eggs and early nauplii typically experience higher mortality rates than those from egg-bearers (Kiørboe and Sabatini 1994; Ohman et al. 2002), due to predation avoidance behavior by the female. In addition, differences in feeding preferences and behaviors between taxa may drive differential response of species to changes in bottom-up forcing, if pico- to microplankton potential prey communities change. Calanoid copepods are often suspension feeding on a range of nano- to microplankton sized cells, including both autotrophs and heterotrophs, and adults would be expected to increase their reproductive rate in response to a post-storm phytoplankton bloom (Calbet et al. 2000; Jungbluth et al. *in review-a;* Jungbluth et al. *in review-b*). In contrast, adult cyclopoids typically prefer motile prey including ciliates and other microplankton, as well as copepod nauplii (Castellani et al. 2005; Lampitt 1978; McKinnon and Klumpp 1998; Nakamura and Turner 1997). Changes between major prey types in the response to environmental perturbation would be expected to drive differences in naupliar production across copepod species and may lead to shifts in species dominance over time.

This study aims to characterize day-to-day changes in recruitment and of biomass loss of co-occurring species of copepod nauplii, towards developing a better understanding of the role of copepod nauplii in marine planktonic ecosystems. Species-specific population dynamic responses to ecosystem perturbations in the coastal zone are expected but are poorly understood, due to a lack of methods to distinguish early naupliar stages in prior studies. In general, the magnitude and duration of the increased resources, the type of ecosystem, and the body mass of the consumer are thought to be important predictors of the magnitude, duration, and timing of consumer responses to increased resources (Yang et al. 2010). In order to characterize the response of four nauplii species to two storm events as compared to a non-storm period, a genetic method was used to distinguish between species and quantify animal biomass. A number of hypotheses regarding the species and stage-specific responses to these event-scale perturbations are outlined below. In order to address these hypotheses, field studies were

conducted over three two-week periods in which diel changes in the copepod naupliar community, the environmental state, and the abundance of picoplankton to microzooplankton food web components were measured in Kaneʻohe Bay, Oahu, Hawaiʻi.

Hypotheses

In order to assess the species and stage-specific naupliar responses to event-scale perturbations, a number of hypotheses were tested regarding copepod population and community dynamics (recruitment, mortality) prior to and following storm runoff into Kaneʻohe Bay. Differences in copepod recruitment rates are expected to be primarily driven by environmental factors affecting adult reproductive output, such as food resources. Since the early nauplii of calanoids are non-feeding, mortality between early and mid-stage nauplii of the calanoids is expected to be due primarily to predation, viral infection, or parasitism and not by food resources. Early nauplii of cyclopoids may feed immediately after hatching (Almeda et al. 2010a; Eaton 1971; Uchima and Hirano 1986), therefore starvation may be an additional source of mortality for early cyclopoid nauplii. Mortality between the mid and late stage nauplii of both calanoids and cyclopoids could be due to food limitation in addition to predation, viral infection, parasitism; however, nauplii are often less food-limited than later juveniles or adults (Kiorboe 1997), so the other sources of death are more likely.

Community dynamics

H_{01}: The copepod community composition is characterized by variable species contributions to the population whose contributions change over time.

H_{1A}: The composition of the copepod community has a stable background state during periods of little meteorological change where species contribute constant proportions to the total population, and storms result in a perturbation away from this stable state.

H_{02}: The age structure of the naupliar population is always the same within each species, reflecting constant rates of mortality and recruitment year-round.

H_{2A}: The age structure of each copepod species is constant during the background community state, and storms result in a perturbation from the stable age-structure due to increased recruitment or mortality.

Recruitment

H_{01}: There are no differences among the four dominant copepod species' recruitment to early nauplii in response to storms or non-storm periods.

H_{1A}: Calanoid copepods have a rapid numerical recruitment response to storms, while cyclopoids show a lag in the timing of their response.

Mortality – General

H_{01}: There is no difference in the mortality schedule across the four species, and no difference in mortality schedules in response to different ecosystem states.

H_{1A}: There are two distinct types of mortality schedule following storms: calanoid and cyclopoid.

H_{2A}: The highest rates of naupliar mortality occur during the early naupliar stages.

Mortality – Early nauplii

H_{01}: The four dominant copepod species show no difference in mortality of early naupliar stages.

H_{1A}: Calanoid early stage nauplii will have higher mortality rates than cyclopoids.

Mortality – Late nauplii

H_0: Four dominant copepod species show no difference in mortality between the mid and late naupliar stages.

H_{1A}: The mortality between mid and late naupliar stages following storms differs across copepod species.

To test these hypotheses, qPCR-based methods developed in Chapter 4 were applied to copepods sampled during different ecosystem states, in order to illuminate differences in the dynamics of the dominant species during both stable periods and two storm perturbations.

5.3 Methods

Field Sampling – Zooplankton, phytoplankton, and environmental data were collected daily from station S3 in southern Kaneʻohe Bay, Oahu Hawaiʻi (Fig. 5.1; 21°25′56″N, 157°46′47″W) during three temporally separated 13-day sampling periods (total number of sample days: 39). Dates sampled were as follows: the non-storm period from June 19-July 2 2011, the fall storm from October 24-November 6, 2011, and the spring storm from May 27-June 8, 2013. Sampling was conducted in the same region of southern Kaneʻohe Bay that was studied in prior work, with physical (Ostrander et al. 2008), chemical (De Carlo et al. 2007; Drupp et al. 2011), and biological (Calbet et al. 2000; Cox et al. 2006; Hoover et al. 2006; Kimmerer 1984; Ringuet and Mackenzie 2005; Smith et al. 1981) parameters examined. This location in Kaneʻohe Bay is 12-16 m in depth.

On each sampling date, duplicate vertical zooplankton samples were collected with a 0.5-m diameter, 64 µm mesh plankton net fitted with a General Oceanics 2030R6 flowmeter towed vertically from 10 m to the surface. Triplicate samples were collected on September 18, 2011. Zooplankton were concentrated in the cod end, then split quantitatively in the field (small boat) using known seawater volumes and a Stempel Pipet (WildCo.) to mix and subsample a known volume (3/4- or 1/2 of the sample, depending upon plankton concentration and likelihood of sieves clogging in subsequent steps), followed by transfers to another container and immediate preservation in 95% non-denatured ethyl alcohol (EtOH). Samples were placed on ice for transport to the lab. The other portion was size-fractionated by pouring through a series of mesh sieves (123, 100, 80, 75, and 63 µm), then rinsed with GF/F-filtered seawater to ensure accurate separation by size, and then each size fraction was individually preserved in 95% EtOH. Additional samples for the earliest naupliar stages were collected with either a 2 or 5 L Niskin bottle at 2 and 11 meters depth, and the entire volume was poured through 63 and 20 µm stacked sieves, size fractionated, and preserved as above. Due to incomplete sampling coverage for the 2

m depth, the 11 m, 20 µm data were used in the final analysis. EtOH in all samples was changed for fresh EtOH within 24 h of collection, and samples were stored at -20°C until processing.

The split, non-size fractionated portion of each zooplankton tow was used to quantify total >63 µm copepods for each of the sampling periods (this fraction included later-stage nauplii, copepodites, and adults). Four to five dates from each of the four sampling periods were selected for counting and one replicate tow for each date was counted in triplicate.

Before sample collection, water column salinity, temperature, and Chl *a* were measured using a YSI 6600 V2 series SONDE (usage courtesy of S. Brown, UH) at the surface and at 2 m increments to 10 m, and at 11 m depth to avoid hitting the bottom with the equipment (~12 m depth). The SONDE was calibrated at the start of each sampling run and every 3-4 days as follows: the Chl *a* response was blanked to deionized water (0 Chl *a* concentration), and salinity was calibrated with a 58.7 mS cm^{-1} KCl salinity solution. Rain data were obtained from online records of a US Geological Survey rain gage at Luluku stream (www.prh.noaa.gov), and wind speed and direction were obtained from the National Weather Service Marine Corps Air Station archives (www.weather.gov). Nearby streamflow rates were obtained from the closest active stream gage at He‘eia Stream, which flows into the central bay but is the closest stream gage maintained by the USGS during the current study and is used to further illustrate the low rates of flow observed during the non-storm period (Fig. 5.1; http://waterdata.usgs.gov).

Phytoplankton – During each sampling period, phytoplankton community composition and abundance were assessed from seawater collected with a 2 L (2011: non-storm, fall storm) or 5 L (2013: spring storm) Niskin bottle from 2 m and 11 m depths, to bracket the range of potential prey for the naupliar population in the water column. Duplicate samples were gently poured into clean containers: 50 mL Falcon tubes, 250 mL and 305 mL (x 3) opaque bottles. These were immediately put on ice in the dark. Upon return to the laboratory (within 2 h of collection), these samples were processed for epifluorescence microscopy, flow cytometry, and fluorometry.

To qualitatively evaluate broad changes in the nano- and microplankton community, large volume (LV; 250 mL) and small volume (SV; 50 mL) seawater samples were preserved (0.4% paraformaldehyde, final concentration) for epifluorescence microscopy, and kept in the

dark and cold (4°C) until further processed within 24-48 h. Within 24 h prior to filtration, epifluorescence samples were stained with 0.5 nM proflavin, then incubated in the dark at 4°C. Samples were filtered onto 0.8 µm (SV) or 8.0 µm (LV) black polycarbonate filters (Midland Scientific Inc.), until ~10 mL was left in the filter tower, then 4 drops of 50 µg L^{-1} 4',6-diamidino-2-phenylindole (DAPI) were added and samples incubated for 2 minutes in the tower. Then, the sample was filtered to dryness, and the filter mounted on a glass microscope slide with immersion oil. Slides were stored at -80°C until digitally imaged. Digital images were taken using a color camera (Olympus U-LH100HGAPO) attached to an epifluorescence microscope (Olympus Model BX51 TRF, LV: 200X total magnification; SV: 400X total magnification), and the software program Microfire™ (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*.

Flow cytometry (FCM) samples (1.5 mL) for photosynthetic eukaryote abundance were taken from the 50 mL water subsample (SV), preserved in 0.4% paraformaldehyde (final concentration), flash-frozen in liquid nitrogen, and stored at -80 °C until processing. Preserved, frozen FCM samples were thawed in batches, then stained with the DNA dye Hoechst 34442 (1 µg ml^{-1}, final concentration) (Campbell and Vaulot 1993; Monger and Landry 1993), and analyzed using a Beckman-Coulter Altra flow cytometer using fluorescence signals from DNA, phycoerythrin and Chl *a*. Resulting data were grouped into relevant populations using FlowJo (Treestar, Inc.).

For Chl *a*, the triplicate 305 mL samples were collected at both 2 and 11 m depth during the 2011 storm and the 2013 storms. Within four to six hours of collection, samples were filtered in the laboratory onto 25 mm GF/F filters (Whatman), flash-frozen in liquid nitrogen, and transferred to a -80 °C freezer for storage until measurement. Chl *a* (and phaeopigment) was measured using a Turner Designs (model 10AU) fluorometer, using the standard acidification technique (Strickland and Parsons 1972; Yentsch and Menzel 1963). During the 2011 non-storm

sampling period Chl a was estimated using the YSI-SONDE rather than discrete water samples, however the Chl *a* sensor is unreliable below 2.0 µg L^{-1} (E. De Carlo, *pers. comm.*) therefore it has to be assumed that summertime Chl *a* was within the range previously reported in southern Kaneʻohe Bay unless epifluorescence slides or flow cytometry measurements suggest there was a phytoplankton bloom during this time.

Quantitative PCR Methods – DNA extraction for quantitative PCR (qPCR) analysis of naupliar abundance was performed after 15-21 days for the 20-63, 63-75, 75-80, and 80-100 µm size fractions as described in Jungbluth et al. (2013). Briefly, each EtOH-preserved fractionated sample was concentrated to a 2 mL centrifuge tube and excess ethanol was evaporated by vacuum centrifugation. The samples were homogenized using a bead-beading protocol following Jungbluth et al. (2013) and DNA was extracted using the QIAamp DNeasy Mini Kit (QIAGEN). Samples were incubated at 56°C for 16 – 20 h, treated with 9 µL of RNAse A (100 mg mL^{-1}, QIAGEN) and eluted in Milli-Q water. Total DNA yield for each sample was quantified using Qubit™ dsDNA high-sensitivity assay kits (Invitrogen™/Life Technologies).

DNA extracts from all size fractions for each of the four target species were measured for DNA copy number using qPCR. Assay optimization for each species is described in Jungbluth et al. (2013) and Chapter 4 of this dissertation. qPCR primers and DNA sequence accession numbers corresponding to the four targeted copepod species present in Kaneʻohe Bay are shown in Chapter 4 (Table 4.1). All plates were run with a 3-5 point standard curve of amplicon DNA, spanning up to five orders of magnitude difference in concentration to encompass the entire range of target DNA amplification. Triplicate technical replicates were run for all samples, standards and no-template controls (NTC). Standard curves were run on every plate, which circumvents the need to account for inter-plate variability.

Early experiments (63-100 µm size fractions of *P. crassirostris*) were run on an iCycler IQ (BioRad) in 50 µL well volumes with iQ SYBR Green Supermix (Greenwood Molecular Biology Facility, UH Manoa) with the following reaction conditions: pre-incubation of 95.0 °C for 10 min; 50 cycles of 95.0 °C for 30 s, 65.0 °C for 1 min, and 72.0 °C for 30 s; followed by 95.0 °C for 1 min, then by melt curve analysis of 95°C for 5 s, 65°C increasing 2.2°C s^{-1} for 60 s,

97°C for 1 s, and cooling 37°C for 30 s to assess the purity of the amplicon. After this instrument failed, remaining plates were run on a Roche Light Cycler 96 (LC96) in 20 µL well volumes as recommended by Roche technicians, with detailed species-optimized reaction chemistry, as described in Chapter 4. LC96 thermal cycling followed these reaction conditions: pre-incubation of 95°C for 180 s, 45 cycles of 95°C for 10 s, the primer-specific annealing temperature for 60 s, and 72°C for 1 s, followed by melt curve analysis. All samples to be run on a plate set were diluted to the same total DNA concentration in order for amplification to fall within the standard curve for each species.

All plates with amplification efficiency of 90-110% were deemed acceptable as recommended by MIQE guidelines (Bustin et al. 2009). QPCR data were exported and processed further in Excel and R, and sample cycle quantification threshold (Cq) values were converted to mtCOI DNA copy number as described in Chapter 4 and Jungbluth et al. (2013). In order to reduce the complexity of analysis, copy number data for the 63-75 and 75-80 µm size fractions were combined to include animals 63-80 µm. Size fractions hereafter will be referred to in the following manner: the 20-63 µm as 20 µm, the 63-80 µm as 63 µm, and the 80-100 µm as 80 µm.

Given the close relationship between copy number and biomass described in Chapter 4, application of the mtCOI DNA copy number - biomass relationship to our field sample DNA copy number allows us to use copy number as a proxy for biomass of each species. Here the same relationship was applied to size-fractionated field samples to estimate the biomass of each species and evaluate how biomass changes over time during sampling events. The equation relating DNA copy number to µg C biomass is: $copy\ number = 9.19\times10^{6} \times biomass - 5.09\times10^{7}$.

Early Naupliar Recruitment – To test the population dynamics hypothesis for differences in each species recruitment to the earliest naupliar stages and for changes in recruitment over time, linear regressions (Model I) were performed on the 20 µm copy number data for each species. The slopes were tested to determine whether the slope of the change in copy number over time was significantly different from zero ($p < 0.05$, suggesting growth or decline), and to evaluate the direction of change (positive or negative slope) in copy number as a proxy for recruitment over

time. Copy number data were log-transformed prior to analysis. To test the hypothesis that a species did not change the rate of naupliar recruitment across sampling events, a two-way ANOVA was run separately for each species on log normal copy number over time and sampling event, followed by a Tukey test of the difference between sampling events. To test the hypothesis that the rate of naupliar recruitment by all species was equal within an event, a two-way ANOVA was run on log normal copy number over time and species, followed by a Tukey test of the difference between species.

Naupliar Biomass Across Size Groups – To test the population dynamics hypotheses for evidence of mortality for each species, the change in biomass between sequential size groups was calculated; from the 20 µm group to the 63 µm group, and from the 63 µm group to the 80 µm group. The raw disappearance of biomass from one day to the next was used to illustrate the addition or loss of biomass with respect to the previous size group, and incorporates no growth assumptions between subsequent size groups. This was calculated by subtracting the initial biomass of a species on one day from the final biomass the next day, which results in positive values that represent addition of biomass to the naupliar pool and negative values that represent loss of biomass from the naupliar pool. Linear regressions of the difference in biomass over time were used to illustrate periods with increases in biomass over time (positive slope) or decreases in biomass over time (negative slope) relative to the biomass in the previous developmental size group. Regressions were calculated separately for "early", "mid", and "late" time spans of each sampling period (non-storm 2011, storm 2011, storm 2013) in order to assess for differences in trends during different ranges of time after the storms and to compare slopes to the non-storm period. During the post-storm periods, "early" includes the day of the storm (for the 2013 storm) plus three days, "mid" includes post-storm days four through eight, and "late" includes the remaining days beyond post-storm day nine. For the non-storm 2011 period, "early", "mid" and "late" correspond to the data from the first four sampling days, middle five, and last four sampling days, respectively.

To account for the expected naupliar growth between sequential size fractions with time, changes in the expected biomass as a proxy for mortality were calculated based on the

relationship between DNA copy number and biomass established in Chapter 4. This metric will be called the percent biomass lost (BL). Based on the change in the mean biomass animal^{-1} in each size range as reported in Chapter 4, Fig. 4.10 (20 µm, 63 µm, and 80 µm means of 0.02, 0.07, and 0.11 µg C animal^{-1} respectively), the expected fold-change in biomass (x) from the 20 µm (initial) to the 63 µm (final) size group is x_{early} = 3.5, and from the 63 µm (initial) to the 80 µm (final) size group is x_{mid} = 1.6. The proportional change (P; d^{-1}) from the initial to the final size group on a diel timescale was first calculated for each species by:

$$P = (b_f \div b_i) \times t^{-1}$$

where b_i is the initial observed size group biomass each day, and b_f is the final observed size group biomass, both estimated from qPCR measurements of mtCOI copy number, with a transition time t of either 1 or 2 days. The BL (%) was than calculated for early and mid stage nauplii separately by:

$$BL = (1 - (P \div x)) \times 100$$

where x is the expected fold change in biomass between sequential size groups noted above (either x_{early} or x_{mid}). It is likely that a majority of the 20 µm animals molt to the 63 µm size group within t=1 day for the paracalanids (McKinnon et al. 2003a), but the amount of time from the 63 to 80 µm group and the development rate of the two cyclopoids is less well known. Therefore, the biomass moving between sequential cohorts was calculated using both t=1 and t=2 to bracket the expected range of time that the majority of animals in each size group should move to the next size group.

Statistical Methods – Principal component analysis (PCA) was used to visualize differences in environmental state among the three sampling periods, and to identify environmental measures that were colinear. PCA was performed in R (R Core Team 2016) using the vegan community ecology package for diversity and ordination analysis (Oksanen et al. 2016). Measurements of biological and abiotic properties of the water column were included, and were normalized prior to analysis to minimize the effects of differences in units of measure. Abiotic properties include the means of water column (0-11m) SONDE measurements for salinity and temperature, and the 24-h cumulative value for rainfall. Biotic properties include the mean of the 2 and 11 m depth

FCM measurements for *Synechococcus*, *Prochlorococcus*, and heterotrophic bacteria (Het. Bacteria), the mean Chl *a* measured at 2 and 11 m during storm 2011 and storm 2013, and the Chl *a* depth averages from the *in situ* fluorometer for the non-storm 2011 dates.

Mantel tests were performed to test whether variation in copy number between species across sample dates was linearly correlated with variation in environmental parameters across dates. A log copy number (Bray-curtis) distance matrix based on mtCOI copy number was calculated separately for each size fraction (20, 63, and 80 µm). The combined (Euclidean) distance matrix of scaled environmental data, as well as Euclidean distance matrices of each of the following environmental measurements were each tested against the copy-number distance matrix: temperature, salinity, wind speed, rainfall, Chl *a*, *Prochlorococcus*, *Synechococcus*, photosynthetic eukaryotes, and heterotrophic bacteria. Mantel tests were conducted in R with the vegan package (R Core Team 2016; Oksanen et al. 2016).

5.4 Results

Summer Background Ecosystem State – Environmental conditions during and leading up to the summer non-storm 2011 sampling period were relatively stable and dry. Rainfall at Luluku station ranged from 0-1.6 cm d^{-1} (Fig. 5.2a) and mean streamflow at the He'eia stream gage was 0.06 m^3 s^{-1}, suggesting low, baseline flow during this time. Typical summer northeasterly trade winds prevailed, averaging 8.3 knots and resulting in a well-mixed water column at our sampling station, with surface seawater temperatures from 25.2-25.9°C, salinity averaging 35.4 psu, and mean Chl *a* (SONDE measured) 0.7 µg Chl a L^{-1} (Fig. 5.2a). Flow cytometry measurements indicate *Synechococcus* dominated the phytoplankton community with a mean (± standard deviation) abundance of 1.8 $\times 10^5$ (±0.7) cells mL^{-1}. *Prochlorococcus* abundance was an order of magnitude lower at 0.1 $\times 10^5$ (±0.04) cells mL^{-1}, as were photosynthetic eukaryotes, which were at an abundance of 0.19 $\times 10^5$ (±0.02) cells mL^{-1}. Heterotrophic bacteria were present at 14.0 $\times 10^5$ (±2.4) cells mL^{-1}. Visual inspection of a subset of LV epifluorescence images suggests that microplankton (20-200 µm diameter) occurrence was generally low and invariant. Small (<10 µm length) single pennate diatoms were present in the samples, and some were in chains of up to

10 cells per chain, but the occurrence of these cells in the LV samples was low (chains observed on <5% of images scanned).

Storm 2011 Environment – On 23 October 2011, the Luluku rain gage measured 6.7 cm rain in a 24-h period and the He'eia stream gage recorded a maximum flow of 0.27 m^3 s^{-1} that day, returning to baseline levels of 0.06 m^3 s^{-1} by the next day, illustrating the rapid flushing that occurs after storms in the study area. The conditions after this storm were dynamic. Winds from the day of the storm until 27 Oct were northwesterly at 6.4 knots (mean; Fig. 5.2b), which is a relatively low wind speed compared to the average trade winds (~9 knots) for this area. The water column was stratified, as can be seen in the salinity and Chl *a* values (Fig. 5.2c, d). Salinity over the 2011 storm period ranged from 33.8-35.3 PSU, and the minimum (33.8 PSU) occurred in the surface layer on 24 October due to the freshwater lens remaining from high rainfall and runoff the previous day (Fig. 5.2a). By 25 October, salinity at the surface was closer to average levels at 34.6 PSU. Mean seawater temperatures ranged from 25.1 – 26.0 °C. On 27 October, winds returned to northeasterly for 5 days at an average of 6.1 knots, resulting in a more well-mixed water column. A second small rain event occurred on 1 November coinciding with a single day of northwesterly winds, followed by a return of northeasterly winds for the remainder of the period, and a peak wind speed of 9.3 knots was observed on 2 November (Fig. 5.2b) after which the water column became well-mixed.

A maximum of 3.0 µg Chl *a* L^{-1}, as measured from discrete samples collected at 2 and 11 m, occurred at 11 m on 25 October (sampling day 2 for this event), ~four-fold higher than the non-storm mean, with a secondary peak of 2.3 µg Chl a L^{-1} on 28 October at (Fig. 5.2a). Water column sensor data (SONDE) from 24 October indicated that on sampling day 1, the concentration of Chl *a* at 11 m was 2.8 µg Chl L^{-1} (Fig. 5.2c), close to the observed values on 25 October. Generally, trends in water column SONDE Chl *a* data matched the discrete Chl *a* samples (Fig. 5.2a, right axis and 5.2c); however the sensor data tended to be higher than the discrete sample values. For instance, SONDE-measured Chl *a* also indicated high Chl *a* on 25 October (4.4 µg Chl a L^{-1}), with a second peak on 28 October (4.8 µg Chl a L^{-1}). Discrepancies

between the two methods of Chl a measurement may be due in part to high resolution vertical features measured by the SONDE that were missed in discrete water collections.

LV epifluorescence slide data from after the storm suggest that there were rapid changes in the phytoplankton community immediately following the storm. On 24 October, one day after the start of heavy rainfall, a dense bloom of the diatom *Chaetoceros* occurred (Suppl. Fig. 5.1). These cells were roughly 145 µm in length and formed long (~13 cell) chains. This bloom lasted for only a few days: on 28 October the few large diatoms that remained were clearly dead and degrading (e.g., lack of Chl visible in the frustules), and *in situ* fluorescence indicated lower Chl *a* (2.3 µg Chl *a* L^{-1} at 11 m). On 31 October, 3 days later, the water column was relatively clear of larger cells, with a few remaining *Chaetoceros* skeletons (frustules without chloroplasts or nuclei), and some single spherical centric diatoms.

On 3 November, 10 days after the diatom bloom, there were generally more ciliates and dinoflagellates, including *Ceratium* sp.. Flow cytometry measurements indicate *Synechococcus* increased from 0.96 $\times 10^5$ cells mL^{-1} on 24 October (which is ~3-fold lower abundance than the non-storm period), peaking at a maximum of 4.2 $\times 10^5$ cells mL^{-1} on 3 November. *Prochlorococcus* abundances were low, and did not change significantly over the storm 2011 period, remaining at an average of 0.14 $\times 10^5$ cells mL^{-1}. Photosynthetic eukaryote abundances gradually increased from 0.17 $\times 10^5$ cells mL^{-1} on 24 October, to 0.31 $\times 10^5$ cells mL^{-1} on 6 November. Surprisingly, the abundance of heterotrophic bacteria did not change significantly over the course of the event, despite the rise and fall of the diatom bloom; bacteria occurred at 15 $\times 10^5$ cells mL^{-1} on 24 October, increased to 21 $\times 10^5$ cells mL^{-1} on 28 October, and remained within that range for the remainder of the sampling event.

Storm 2013 Environment – The sampling period in May-June 2013 was not intended to be a storm chase. However, after initiating a sampling series on 27 May 2013, a storm serendipitously occurred on 28 May and the Luluku gage recorded a relatively large amount of rainfall, with an accumulation of 20.4 cm of rain on that day (Fig. 5.2a), and a maximum flow of 9.7 $m^3 s^{-1}$ at He‘eia stream. On 12 May, 15 days prior to this sampling period, the Luluku gage recorded 10.3 cm rain, illustrating how the period leading up to the May 2013 storm was very different from

the 2011 storm sampling described above, which was preceded by three months of low rainfall (average 0.3 cm d^{-1}; < 5 cm on any given day). Water temperatures were 25.7 – 26.7 °C during the 2013 storm sampling period.

Wind over the 2013 storm sampling period was northeasterly; on 27 May wind speed was 7.6 knots, holding steady until 29 May when it dropped to 5.3 knots, then gradually increased to a maximum of 10.2 knots on 7 June (Fig. 5.2b). The decline in the winds on 29-30 May promoted post-storm water column stratification, which is evident in the salinity plot (Fig. 5.2d) during the low wind velocity period. Wind speeds increased in the latter half of the sampling period, and the water column gradually became well mixed by 31 May. Discrete chlorophyll samples show maximum concentration of 2.5 µg Chl a L^{-1} at 11 m on 31 May (Fig. 5.2a), a value supported by the SONDE data (Fig. 5.2c), a sharp decline and gradual increase after 2 June, followed by values of 1-1.5 µg Chl L^{-1} for the last 6 days of the sampling period. The peak in Chl *a* at 11 m may have been promoted by both the onset of mixing of the water column on that date.

Epifluorescence slides indicate, however, that there was no large-celled diatom bloom during the entire period after this storm event, despite the large volume of freshwater run-off into the bay and increase in Chl *a*. On 27 May 2013, there were very few phytoplankton visible on the LV slide (i.e., >8 µm diameter cells), not even the small diatoms that were found in the 2011 non-storm period. On the day of the storm, 28 May, there were still very few organisms on the LV slide images, however some autotrophic dinoflagellate *Prorocentrum* cells were present at low abundance. On 3 June, *Prorocentrum* cells were still observed along with a generally greater occurrence of small heterotrophic flagellates and small dinoflagellates. On 6 June, there was an increase in flocculant and more small (<10 µm) heterotrophic flagellates were present.

Synechococcus gradually increased from 0.5 $\times 10^5$ cells mL^{-1} on the day before the storm, to 2.4 $\times 10^5$ cells mL^{-1}on 9 June, while photosynthetic eukaryotes increased from 0.07 $\times 10^5$ cells mL^{-1} to 0.24 $\times 10^5$ cells mL^{-1} over the same time period, and heterotrophic bacteria doubled from 7.1 $\times 10^5$ cells mL^{-1} on 27 May, to 14.0 $\times 10^5$ cells mL^{-1} on 6 June.

The three sampling periods of this study (1 non-storm and 2 storm-influenced) were well separated by environmental variables in the PCA biplot (Fig. 5.3), indicating greater

environmental variation between than within sampling periods. Seawater salinity and temperature were the strongest drivers of differences between the three periods (non-storm, storm 2013, and storm 2011). Biological variables (heterotrophic bacteria, photosynthetic eukaryotes, *Synechococcus, Prochlorococcus*) and rainfall drove the main differences between the two storm events. The first two components of the PCA explained 60% of the total variance across all samples. These results establish that the oceanographic environment during the two storm events was distinct, and that the non-storm period also differed from both storm periods.

Copepod Abundance – Total net-caught copepod abundance (includes >63 µm nauplii, copepodites, adults) ranged between 48-501 $\times 10^3$ ind. m^{-3} over the course of the study (all sampling periods, Fig. 5.4a). Abundances during the non-storm period ranged from 48-171 $\times 10^3$ ind. m^{-3}. An abundance of 347 $\times 10^3$ ind. m^{-3} was measured on 24 October, the first day after the 2011 storm. The maximum total copepod abundance of 501 $\times 10^3$ ind. m^{-3} was measured on 29 October 2011(6 days after 2011 storm), which was followed by a decline to 164 $\times 10^3$ ind. m^{-3} on 5 November (13 days after the storm). During the 2013 storm sampling period, copepod abundance was 244 $\times 10^3$ ind. m^{-3} on 27 May 2013, the day before the 2013 storm, and was 456 $\times 10^3$ ind. m^{-3} on the day after the 2013 storm, 29 May. By 8 June (11 days after the storm), total copepod abundance had declined to 164 ind. m^{-3}.

Naupliar abundances (count-based, >63 µm net collected, mid-late nauplii only) during the study were up to an order of magnitude greater than adults. During the 2011 non-storm period, naupliar counts were 5- to 10-fold more abundant than adults. Abundances of nauplii ranged from 19- 99 $\times 10^3$ m^{-3}, copepodites ranged from 22-76 $\times 10^3$ m^{-3}, and adults from 3-26 $\times 10^3$ m^{-3}, with maximum nauplius abundance on 20 June (non-storm day 1) and maximum adult abundance on 30 June (Fig. 5.4a). After the 2011 storm, abundances of nauplii and copepodites on the first sampling day were 2-fold higher than the average over the non-storm period, but adult abundances were not elevated relative to the non-storm levels. Total naupliar abundance reached a peak on 26 October of 299 $\times 10^3$ (± 3) nauplii m^{-3} and declined thereafter to 157 $\times 10^3$ (± 16) nauplii m^{-3} on 3 November. Copepodite stages were at maximum abundance on 29 October at 141 $\times 10^3$ (± 51) copepodites m^{-3}. The adult abundances indicated a muted increase in

the adult community relative to non-storm conditions. The initial adult abundance was similar to the non-storm period at 30 $\times 10^3$ ($\pm$13) adults m^{-3} and similar to copepodites, adults peaked on 29 October at 65 $\times 10^3$ ($\pm$ 6) adults m^{-3}. Total copepod abundances after the 2013 storm suggest a slight and brief response compared to the 2011 storm. There was a 2-fold increase in naupliar abundance from 109 $\times 10^3$ ($\pm$ 6) nauplii m^{-3} the day before the storm to 207 $\times 10^3$ ($\pm$ 4) nauplii m^{-3} on 29 May, and a decline afterwards to 78-126 $\times 10^3$ nauplii m^{-3} between 3 and 8 June 2013. Copepodite and adult abundances during the 2013 storm period were similar to the 2011 storm (40-184 $\times 10^3$ copepodites m^{-3} and 13-65 $\times 10^3$ adults m^{-3}), and decreased by 69-78% by the end of the sampling period.

Counts of the adults to the species level were measured on a subset of samples. Adult abundances of each species were lower during the 2011 non-storm period than during either storm event (Suppl. Fig. 5.2). During the 2011 non-storm period, *O. simplex* was dominant at an abundance of 4.3 - 6.8 $\times 10^3$ m^{-3}, *O. attenuata* and *P. crassirostris* exhibited abundances of 1.0 $\times 10^3$ m^{-3} and 0.6-1.1 $\times 10^3$ m^{-3} respectively, and *B. similis* was least abundant at 0.3-0.6 $\times 10^3$ m^{-3}. Overall adult species abundances during the 2011 storm were ~8-fold higher than non-storm levels for all species on the first sampling date, 24 October; 36.3 *O. simplex* $\times 10^3$ m^{-3}, 7.1 *O. attenuata* $\times 10^3$ m^{-3}, 9.3 *P. crassirostris* $\times 10^3$ m^{-3}, and 3.9 *B. similis* $\times 10^3$ m^{-3}. Eight days later on 1 November the adult abundances were similar. By 5 November *O. simplex* abundance had increased to 43.9 $\times 10^3$ m^{-3}, *O. attenuata* had changed little at 8.4 $\times 10^3$ m^{-3}, *P. crassirostris* increased by ~4 fold to 15.5 $\times 10^3$ m^{-3}, and *B similis* was relatively unchanged at 3.3 $\times 10^3$ m^{-3}. In contrast, during the 2013 period, on 27 May *O. simplex* adults were abundant but slightly lower overall at 25.6 $\times 10^3$ m^{-3} and declined by 50% by the end of the sampling period, while the three other species were roughly equal at 4.7-5.8 $\times 10^3$ m^{-3}. By 8 June, *O. attenuata* declined by ~75%, *B. similis* declined by 50%, and *P. crassirostris* remained at 5.6 $\times 10^3$ m^{-3}.

QPCR Assay Results – Bulk extracted DNA concentrations of each naupliar size fraction ranged from 0.24 to 158 ng DNA μL^{-1}. Sample total DNA concentrations of 0.01 to 1.25 ng μL^{-1} were run on each plate. All qPCR plates used in the analysis had an amplification efficiency of 90-103%, with an r^2 of 0.99 - 1.0 (Suppl. Table 5.1) and, with the exception noted below, all

samples amplified within the range of the standard curve. DNA copy number detection in the qPCR reaction was efficient down to 7 copies μL^{-1} for the *B. similis* assay, and up to 2.4×10^{7} copies μL^{-1} for *P. crassirostris*. In the 20 μm whole water size fraction, the total DNA concentration was lower (0.24-3.15 ng μL^{-1}) than that of the net samples, resulting in low target species copy abundance for *B. similis* and cycle amplification values higher than the lowest concentration standard. Based on the conclusions of Chapter 4, the melt-curves of the low-concentration *B. similis* samples indicated even the low level of amplification consisted of only the target species (i.e., no aberrant shoulders in the melt curve). Additionally, the Chapter 4 conclusion that the amplification of target species was reliable even when the target DNA made up 0.01% of the total suggested that copy number quantification should still be accurate. These data were therefore included in my analyses.

DNA Copy Number – Total mtCOI DNA copy number in the 20-100 μm combined size range (Fig. 5.4b) shows similar overall patterns to the total naupliar abundance across the three sampling periods (Fig. 5.4a). Average non-storm total copy number ($4.8 \pm 0.7 \times10^{10}$ m^{-3}; mean ± SD) was 3-fold lower than the total copy number on the first day after the 2011 storm event ($15.0 \pm 0.3 \times10^{10}$ m^{-3}) and 6-fold lower than the maximum total copy number observed during this study ($32.0 \pm 0.4 \times10^{10}$ m^{-3}) on 31 October 2011. The total copy number at the start of the 2013 storm (27 May 2013) was similar to non-storm levels ($3.2 \pm 0.04 \times10^{10}$ m^{-3}) increased 3-fold by 5 June ($9.2 \pm 0.08 \times10^{10}$ m^{-3}) and decreased rapidly thereafter.

Temporal changes in the contribution of each species to the total DNA copy number (20-100 μm) across all three periods suggest distinct early life history responses for each species (Fig. 5.4c). *Oithona simplex* dominated the 20-100 μm DNA over the course of the study and contributed from 41-81% to the total mtDNA across all dates (Fig. 5.5). During the June 2011 non-storm period the contributions of the four species to the naupliar community were relatively stable; *O. simplex* contributed 55-72%, *P. crassirostris* contributed 14-24%, *O. attenuata* contributed 10-23%, and *B. similis* contributed 0.03-3% to the total 20-100 μm copy number. It is not surprising that *B. similis* nauplii were the least abundant, since adult abundances were less than half that of the other species. After the 2011 storm, *O. simplex* made up 81% of the total

DNA, declining to 42% of the total by 28 October when *P. crassirostris* briefly became dominant at 49% of total DNA copies (Fig. 5.5). After 28 October (5 days post-storm) the contribution of *P. crassirostris* to the total declined for the remaining period, reaching 24% of the total copy number on 5 November. *Oithona attenuata* increased from an average of 11% from 24 October through 1 November, up to 35% at the end of the sampling period on 5 November. A very different dynamic of the relative contribution of species occurred after the 2013 storm; *O. simplex* remained dominant during the entire period, contributing an average of 56% to the total. The contribution of *P. crassirostris* slowly increased over the course of this event, unlike its response to the 2011 storm, and contributed 8% to the naupliar community on 27 May, up to 33% on 8 June.

DNA copy numbers in the different size fractions reflect changes in the early stage nauplii (20 µm), mid-late stage nauplii (63 µm), and late nauplii and early copepodite stages (80 µm). During the 2011 non-storm period, *O. attenuata*, *O. simplex*, and *P. crassirostris* exhibited little overall change in DNA copy number in all naupliar size fractions (Fig. 5.6). *Bestiolina similis* exhibited a reduction in copy number in all size fractions; up to three orders of magnitude decrease in the 20 µm size group, and a 30-fold decrease in the 80 µm size group.

The most dramatic changes in DNA copy number over time were observed after the first-flush 2011 storm (Fig. 5.6). *O. attenuata*, *O. simplex*, and *P. crassirostris* showed a clear response to the storm event; with DNA copy number in the 20 µm size fraction 10-100-fold greater than both larger size fractions for the first 1-3 days, followed by an increase in the copy number in the 63 and 80 µm size fractions for the next ~8 days. *B. similis* responded similarly but to a lesser extent with only a 10-fold difference in copy number between the 20 µm and larger size fractions. After 31 October, both *O. simplex* and *P. crassirostris* copy numbers declined, but less so for the *P. crassirostris* 20 µm size fraction, while *O. attenuata* and *B. similis* copy numbers in all size fractions varied more widely. The ephemeral shift in dominance to the early nauplii immediately following the 2011 storm, and rapid changes over time are further illustrated in Fig. 5.7, a plot of the percent contribution to the total of the different naupliar size fractions of each species.

Despite the large volume of freshwater input after the 2013 storm event (rainfall=20 cm d^{-1}) (a late spring/early summer storm), the response of most species evident in the size fraction DNA copy numbers is very different from the 2011 storm event (first-flush storm). On the day of the 2013 storm, 28 May, the 20 µm copy number was at the same level or lower than the larger size fractions and, the day after the storm event (29 May), DNA copy number of the 20 µm size fraction in all species increased by 3-fold (*B. similis*, *O. simplex*), 6-fold (*O. attenuata*), and 2-fold (*P. crassirostris*), suggesting that there may have been a slight increase in early naupliar production. However the 2013 storm response was far lower than the 1-2 orders of magnitude response of most species to the 2011 storm event. After 6 June there was a clear decline in the DNA copies of the 63 and 80 µm size groups for all species, while the 20 µm copy numbers remained relatively constant.

Recruitment Variability – Analysis of changes in 20 µm size group (early nauplii) suggest that there were differences across species in the recruitment response to storms, as well as in the recruitment occurring during the 2011 non-storm period. Because the 20 µm size fraction contains early stage nauplii (N1-N2), and the animals in the 20 µm size fraction are expected to develop in one day to the next size group (see size-fractionation in Jungbluth et al. 2013, Chapter 4 for size ranges of calanoid and cyclopoid nauplii), changes in the 20 µm size group from day to day give an indication of changes in naupliar production by each species. Log transformed 20 µm copy number regressions suggest there were species-specific differences in early naupliar production rates during the three events (Fig. 5.8). Many of the linear regressions were significant ($p < 0.05$; Table 5.1) suggesting increasing or decreasing recruitment over time during each event, however, the r^2 values suggest that time was a good predictor of copy number in only some cases. During the non-storm 2011 period, there was a 3-order of magnitude decrease in *B. similis* copy number ($p < 0.001$, $r^2 = 0.72$). All other species had approximately stable recruitment rates during the 2011 non-storm period. Following the 2011 storm, all species had high 20 µm copy number on day 1, with decreasing recruitment over time. *Oithona simplex* naupliar production declined significantly after the 2011 storm ($p<0.001$, $r^2 = 0.62$), *O. attenuata* and *P. crassirostris* had significant negative slopes ($p=0.04$ and $p<0.001$, respectively) but a poor

correlation between 20 µm copy number and time (r^2 = 0.11, r^2=0.24), and *B. similis* did not have a significant slope nor a strong correlation with time (p=0.21, r^2=0.05). Naupliar recruitment of all species generally increased after the 2013 storm, all with significant p-values ($p < 0.001$). *Parvocalanus crassirostris* and *O. simplex* both had stronger correlations between 20 µm copy number and time during the 2013 storm event (r^2 = 0.49 and 0.47, respectively) than *B. similis or O. attenuata* (r^2 = 0.30 and r^2 = 0.14, respectively).

Linear regressions of the log-transformed 20 µm copy numbers (early naupliar recruitment) for each species suggest there were differences in the recruitment rate of all species across the three sampling events (Fig. 5.8). Two-way ANOVA for each species comparing the copy number slope over sampling events suggests that the change in recruitment rates over time (slope) differed across sampling events for *P. crassirostris, O. simplex,* and *B. similis* (Table 5.2a; $p < 0.001$), but marginally non-significant changes occurred over time across the three events for *O. attenuata* ($p = 0.087$). *Post-hoc* Tukey tests of the slope of copy number over time across events suggest that *P. crassirostris* and *O. simplex* had significantly different recruitment rates across all three events ($p < 0.01$), *B. similis* recruitment rates differed between the 2011 non-storm and each storm ($p < 0.001$) but not between the 2011 storm and the 2013 storm ($p = 0.741$), while *O. attenuata* recruitment rates differed marginally between the 2011 storm and the non-storm 2011 period ($p = 0.068$) but not between the 2013 storm and non-storm period or the 2013 storm and 2011 storm period ($p \geq 0.448$). Two-way ANOVA analyses testing for the difference in recruitment rate between species suggest that recruitment rates differed across species in all events (Table 5.2b; $p \leq 0.02$). Post-hoc Tukey tests also indicate that the change in recruitment of each species was significantly different within each event ($p < 0.05$), except for that of both *P. crassirostris* and *O. attenuata* during the 2011 non-storm and 2011 storm ($p > 0.73$).

Mantel tests suggest that there was a significant relationship between sample-to-sample variation in log normal transformed 20 µm copy number (early naupliar recruitment) and some environmental variables (Table 5.3). These include wind speed, Chl *a*, *Synechococcus,* photosynthetic eukaryotes, and heterotrophic bacteria. Generally, mtCOI copy number in the 20 µm size fraction was more highly correlated with both abiotic and biotic environmental variables

and had lower p-values than was observed for the larger (63 and 80 µm) size fractions. For all size fractions, the copy number correlated significantly with the variation in the environment ($p \leq 0.041$). Individual environmental factors whose variation did not correlate with copy number included water temperature, salinity, rainfall, and *Prochlorococcus* abundance. While 20 µm copy number varied at a highly significant level with chlorophyll (p=0.002), the variation in this size fraction was less strongly correlated to variation in the cyanobacteria *Synechococcus* (p=0.043) and photosynthetic eukaryotes (p=0.032). The variation in *Synechococcus* abundance was more highly correlated to the 63 µm size group (p=0.003) than to any other naupliar size groups. The degree of linear correlation (r^2) across all environmental variables tested was <0.12, indicating that there was not a simple linear relationship between the relative changes in copy number across dates and any single environmental variable, such as chlorophyll and DNA copy number.

Naupliar Biomass – Changes observed in DNA copy number and conversions of that copy number to biomass are consistent with the trends in the total count-based abundance of the naupliar community over the different sampling periods. The biomass of 20-100 µm nauplii estimated by qPCR measurement of the mtCOI copies in the four dominant species was 4.2 – 6.4 mg C m^{-3} during the non-storm period, 11.9-34.9 mg C m^{-3} during the 2011 storm period, and 3.5-10.1 mg C m^{-3} during the 2013 storm period (summed across 20-100 µm size fractions of all species).

The biomass estimated for the different size groups of each species is shown in the series of Figures 5.9-5.12. Generally, biomass in the early naupliar size fraction for all species was up to an order of magnitude higher than non-storm levels immediately following the 2011 storm (except for *B. similis*) and declined by over 50% by the last sampling date to levels close to non-storm and 2013 storm levels. The biomass in mid and late stage size groups reached a peak for most species between 28 October and 1 November declining by the last sampling date (except in *O. attenuata*) to a level higher than the first 2011 storm sampling date. During the 2013 storm, the early naupliar biomass the day after the storm (29 May) was higher than 2011 non-storm

levels, but the increase in biomass after the 2013 storm was barely observable in comparison to the 2011 storm.

Specific observations on the changes in biomass across size groups over time are detailed here. The biomass of *B. similis* was 1-3 orders of magnitude lower than the other species, with a clear decline in the biomass of all size fractions during the 2011 non-storm period (Fig. 5.9). The 2011 storm biomass of *O. attenuata* shows a transfer of biomass from the 20 µm fraction (~2 mg C m^{-3}) into the mid-late nauplii, which gradually increased in the week following the storm (to maximum of 3.7 mg C m^{-3} on 5 November; Fig. 5.10). Over the 2011 storm event, *O. simplex* early naupliar biomass was the highest across all species on 24 October (13.1 mg C m^{-3}; Fig. 5.11), ~60-fold higher than mid-late nauplii, but early naupliar biomass declined over time by an order of magnitude by 5 November (1.3 mg C m^{-3}). During the summer, *P. crassirostris* average 20 µm biomass was 0.3 mg C m^{-3}, which was ~3 fold higher on the first day of the 2011 storm (1.0 mg C m^{-3}; Fig. 5.12). *P. crassirostris* mid-late nauplius biomass was 0.2 mg C m^{-3} on the first day of the 2011 storm, increased by ~60-fold within one week to 12.5 mg C m^{-3} then declined after 31 October to 2.5 mg C m^{-3} on 5 November.

Naupliar Biomass Loss – The range and median biomass gained or lost over t = 1 day from the 20 to the 63 µm size group, and the 63 to the 80 µm size group for each species and sampling event are shown in Table 5.4. A majority of the median biomass points were negative, suggesting that there was often more biomass lost between the 20 µm and 63 µm size fraction than gained. Since this number is the raw biomass change from one size group to the next and does not incorporate any assumptions of growth of animals from one size to the next, this indicates that only a minor fraction of the 20 µm biomass grew to the 63 µm size group on most dates for most species.

An assessment of mortality was done by calculating the raw difference in biomass between subsequent size groups over time for t = 1 day (Fig. 5.13-5.14). Linear regressions of the difference in biomass between cohorts during early, middle and late periods of sampling illustrate the relative increase in biomass moving to the next size group or the decrease in biomass moving to the next group over time. Generally, results from this analysis suggest that

the relative changes in biomass were less extreme over the summer non-storm period and the 2013 storm period than during the 2011 storm. The greatest gain or loss of biomass occurred after the 2011 storm for all species except *B. similis*, which had overall lower variation. The greatest change in biomass over time in *O. simplex* and *O. attenuata* were experienced during the mid and late periods of the 2011 storm, while for *P. crassirostris* all three 2011 storm time-periods were highly variable. The greatest overall disappearance of biomass (indicated by the greatest negative value in Fig. 5.13-5.14) occurred between the 20 and 63 µm group in *O. simplex* after the first day after the 2011 storm (estimated loss of 13.0 mg C m^{-3}), where 99% of the 20 µm biomass did not make it to the 63 µm size fraction within the expected development time of one day (Suppl. Fig. 5.3). A notable period of biomass addition across all species occurred on 25-30 Oct of the 2011 storm in the 80 µm size group, where up to 6.0 µg C m^{-3} d^{-1} were added to *P. crassirostris* relative to the previous size fraction (at t=1) and up to 5.3 µg C m^{-3} d^{-1} were added to *O. simplex*.

The difference in biomass between 20 µm early nauplii and 63 µm mid-stage nauplii over the three events suggests that loss of biomass between these stages was relatively constant during the non-storm period and 2013 storm period for most species, while the 2011 storm resulted in rapid changes in biomass between size groups (Fig. 5.13). *B. similis* was different from the others, with almost no variation in response to the 2011 storm, and greater variation after the 2013 storm, however the overall magnitude of change in biomass of this species was roughly an order of magnitude lower than the other species. In the other three species, the maximum negative difference in biomass was observed the first two days after the 2011 storm. During days 4 through 8 after the 2011 storm (the "middle" period), all species had positive slopes in the difference in biomass that show an increase in the biomass developing into the 63 µm size group over time. During the final 5 days sampled after the 2011 storm, the slope suggests *P. crassirostris* decreased the relative amount of biomass developing into the 63 µm group, while in *O. simplex* the relative amount of biomass developing into 63 µm continued to increase. *O. attenuata* was more variable in the remaining five days than the other species.

The average percent biomass loss (including both t=1 and t=2) of early nauplii transitioning to the mid-stage nauplii during the non-storm period was 81% of *B. similis* early

nauplii, 80% of *O. attenuata* early nauplii, 61% of early *O. simplex* nauplii, and 73% of *P. crassirostris* (Suppl. Fig. 5.3). There was a high rate of loss between the 20 µm early nauplii and the 63 µm mid-stage nauplii after the 2011 storm in all species. Up to 99% of early nauplii biomass did not make it to the mid stage size group for the first 3-4 days, suggesting that despite the increased rate of naupliar production by adults, only a small portion (<1%) of the early naupliar population grew to the mid and late stage nauplii in those first few days following the storm. The rate of species loss after the 2011 storm decreased over time, in *P. crassirostris* to as low as 7%, suggesting increased survival between early and mid naupliar stages, possibly due to lower predation rates since this species is non-feeding during the first two naupliar stages. After the 2013 storm, BL were more similar to the 2011 non-storm period for most species, with average BL of 60-81% across species. There was a higher BL in *O. simplex* and *O. attenuata* in the last 2 sample dates of the 2013 storm period. Generally, increasing the assumed transition time decreased the estimate of the percent biomass lost.

The difference in biomass between 63 µm mid-stage nauplii and 80 µm late-stage nauplii over the three events suggests the magnitude of variation in biomass was smaller than during the transition from early to mid-stage nauplii for most species (Fig. 5.14). Variation in biomass during the 2013 storm was similar to the 2011 non-storm period except for *B. similis,* whose difference in biomass was again about an order of magnitude lower than the other species. During the "middle" time period after the 2011 storm event (sampling days 4-8) *O. attenuata, O. simplex,* and *P. crassirostris* all showed a negative slope suggesting there was an increase in the loss of biomass between the mid to late-stages over time, which continued into the last sampling dates for *O. simplex*. The most negative difference in biomass between the mid to late stage nauplii occurred within the last five sampling days of the 2011 storm for all species.

The average BL (%) (including both t=1 and t=2) between the 63 and 80 µm size group during the non-storm period was 60% of *B. similis*, 81% of *O. attenuata*, 69% of early *O. simplex,* and 60% of *P. crassirostris* (Suppl. Fig. 5.4). Storm 2011 mid stage BL estimates were lower than non-storm rates. Average BL during the five days after the storm were 28% for *B. similis*, -270% for *O. attenuata* (i.e., a 2.7 fold increase in naupliar biomass relative to that expected with 100% developing to the next stage), -367% for *O. simplex*, and -340% for *P.*

crassirostris. The timing of the return of BL rates to levels observed during the non-storm period differed between species. In *O. attenuata*, BL was positive after 29 October, suggesting a more rapid return to levels similar to that seen during the non-storm period, while the BL of other species increased after 31 October (*B. similis* and *P. crassirostris*) and 1 November (*O. simplex*). Changes in BL over time during the 2013 storm period were more variable than during the non-storm period, and fewer negative BL were observed than during the 2011 storm. Notably, *O. attenuata* had more consistent patterns of BL from the 63 to the 80 µm group over the entire 2013 storm period, while the other species had lower loss and some days with negative BL. Periods of negative BL suggest that either the expected biomass increase of 1.6 fold between mid and late nauplii (as observed in Fig. 4.10, Chapter 4) is not accurate for estimating the biomass added during development to late-stage nauplii, or that there was a buildup of biomass in the late-naupliar early copepodite transition. A buildup could occur if food resources were limiting developmental rates. Nauplii in the laboratory have been observed to die after a few days of starvation.

5.5 Discussion

Event-scale perturbations, such as storm events, are important drivers of variation in plankton community dynamics in the coastal zone, and can provide critical injections of new nutrients that drive ephemeral increases in community productivity and carbon transfer. Within the community-level response to an ephemeral increase in productivity, it is unclear whether calanoid and cyclopoid nauplii would experience a similar magnitude and timing of changes in biomass that would reflect increased recruitment or mortality. By applying a qPCR-based method, species and stage-specific responses to different event-scale perturbations were quantified, and brief shifts in dominance of species in the naupliar community were observed. We hypothesized that, during periods of instability due to meteorological perturbation events (high rainfall), changes in the nauplius community would be observed, characterized by variation over time in loss of biomass from system, an increase in naupliar naupliar biomass over time, and increasing copepod population biomass or abundance. In general, there was no clear delineation between calanoids and cyclopoids in their response to the two different storm events;

instead, the differences in the magnitude and timing of responses were species-specific. Very few studies have made species-specific naupliar measurements from field samples due to challenges in identification and sampling of early stages in high diversity communities (but see Hopcroft et al. (1998a)). Using the qPCR-based method, many of the primary observations that would be made using traditional methods were detected, in addition to species-level properties that would otherwise be unattainable using conventional techniques in a diverse ecosystem. The difference in the magnitude of the naupliar response to the two storms suggests that storms are distinct in the bottom-up forcing of copepod communities. During the 2011 storm period, the relative contribution of species to the naupliar pool changed over time, due to different rates of survival of species after the storm, either from differential feeding success or species-specific mortality. High rates of biomass loss from the population were observed after the 2011 storm as compared to other events studied here, up to 13 mg C m^{-3} d^{-1} of *O simplex* early nauplii, suggesting nauplii may contribute substantially to trophic transfer, particularly during the ephemeral bloom of resources after first-flush storm events.

Early Nauplii Recruitment – Calanoid and cyclopoid copepods were expected to exhibit distinct patterns of recruitment in response to the different storm events, due to differences in life history and feeding ecology differences between these groups. We observed broad similarity in the response of the four species to each event, but different response magnitudes to the different storms. The number of early naupliar DNA copies for all species was higher during the 2011 storm than during the non-storm period or the 2013 storm period, except for *B. similis* (Fig. 5.8). In all four species, copy number in the early nauplii (20 µm size group) was higher than non-storm copy number, and ratios of early nauplii to later stages were significantly higher within 24 h of elevated storm runoff, suggesting there was a rapid bottom-up forced response to the storm perturbation (Fig 5.6-5.8). The immediate increase in early nauplii is not surprising for the two calanoids since adult females are capable of increasing egg production within 8-12 hours in the presence of elevated food resources and will start hatching into early nauplii 4-6 hours after egg release (Kline and Laidley 2015; VanderLugt and Lenz 2008). These dynamics are less well-known for the cyclopoids, but our data suggest that a similarly rapid egg production and hatching

response is possible for the two cyclopoid species described here. Despite broad similarities across species, there were species-level differences in the duration of elevated naupliar copy number (number of days elevated post-storm) and in the rate of change in copy number (slope over time) over the two storm sampling periods (Fig 5.8, Table 5.1).

Cyclopoid copepods appeared to have a longer period of elevated naupliar recruitment following the first-flush event than did the calanoids, as suggested by the age structure shown in Fig 5.7, which indicates a higher proportion of the cyclopoid naupliar population was in the early-stage nauplii during this period (Fig 5.7). Rates of egg production and recruitment to nauplii could have been extended for the cyclopoids if the adult *Oithona* fed on higher quality prey for the first few days following the storm (Tang and Taal 2005). In general, *Oithona* spp. are described as feeding primarily on motile prey, including protists and copepod nauplii (Lampitt 1978; Landry and Fagerness 1988; Nakamura and Turner 1997; Paffenhöfer 1993). Given the pulse of elevated nauplii of both cyclopoids and calanoids found in our study, if both *Oithona* spp. adults are capable of feeding on nauplii there would have been no shortage of food resources to support naupliar production following the 2011 storm period. Alternatively, the signal suggesting extended dominance of early nauplii in the Oithonids could have been due to longer development times in the cyclopoids than in the calanoids. The development times of the two cyclopoid species have not been previously described, but given a Q_{10} of 1.6 for a related species, *O. davisae* (Almeda et al. 2010b), and development rates for similar species (*O. nana, O. davisae*) described in Sabatini and Kiorboe (1994), the development time of *O. simplex* and *O. attenuata* in Kane‘ohe Bay would be 13-16 days at ambient temperatures of ~24 °C in the Bay at the time of study, which is 5-8 days longer than the calanoids and suggests we may follow only a single generation of the cyclopoids over the two week sampling period.

Food availability, via bottom-up forcing, was a strong driver of the observed variation in early-stage nauplii across events (Table 5.3), which is consistent with previous work in Kane‘ohe Bay and other systems (Hoover et al. 2006; McKinnon et al. 2003b). Variation in early naupliar copy number was significantly correlated with indicators of food availability, such as Chl *a, Synechococcus*, heterotrophic bacteria, and wind speed. Wind speed is not directly related to

food but is an indicator of water column mixing; relaxation of winds after storms promotes stratification and can prolong the duration of phytoplankton blooming in the surface waters.

Differences in adult prey availability and feeding preferences may drive the observed differences in recruitment across species and sampling events. The paracalanids are suspension feeders, and previous work suggests *P. crassirostris* (adult length 400 µm) may be better equipped to handle small cells (~4 µm *Isochrysis* sp.) than the larger copepod *B. similis* (adult length 520 µm; McKinnon et al. 2003). The adults of copepod species in Kane'ohe Bay have been previously described as opportunistic feeders. Adult *O. simplex, P. crassirostris,* and *O. nana* (likely *O. attenuata*; Jungbluth and Lenz (2013)) fed at high rates on cells in the 2-5 µm size range, and adult *B. similis* (formerly *Acrocalanus inermis*) had lower feeding rates than the other species (Calbet et al. 2000). Prior to the diversion of sewage inputs in 1978 when productivity was higher and the bay was more prone to large celled phytoplankton blooms, the southern sector of Kane'ohe Bay was dominated by *B. similis* (described as likely *Acrocalanus* sp.), *O. simplex*, and *O. nana* (Hirota and Szyper 1976; Newbury and Bartholomew 1976). After sewage diversion, an additional copepod species was reported (*P. crassirostris*), and there has been a shift to dominance of the cyclopoids and *P. crassirostris* (Calbet et al. 2000; Scheinberg 2004). Success of the smaller species after sewage diversion suggests that *O. simplex, O. attenuata,* and *P. crassirostris* may be better adapted to feeding and surviving in lower nutrient, smaller prey conditions than *B. similis*, or that it is being out-competed in the South Bay. Australian *B. similis* thrives in highly turbid waters around mangroves where detritus may make up a significant component of their diet (McKinnon and Klumpp 1998). A diet of large cells and detritus may help explain the decline of *B. similis* during the summer non-storm 2011 period, when the waters were relatively clear of large-celled phytoplankton or detritus. Alternatively, predation may play a role in controlling the population of *B. similis* (e.g., (Kimmerer 1984)). *B. similis* is the largest species that is commonly found in the South Bay, and may be under heavier predation by visual predators (e.g., fish larvae, chaetognaths) during the summer when runoff is low and water clarity is higher.

Naupliar Biomass Loss – Copepod population dynamics are more sensitive to mortality than to changes in egg production (Kiørboe 1998); therefore, it was important to assess for indicators of changes in mortality over time, using stage-specific naupliar biomass estimates. Calanoid and cyclopoid nauplii were expected to have different mortality schedules due to life history differences between them. The calanoid nauplii were expected to have higher mortality rates due to differences in swimming, and feeding (mid-late stages) behaviors. For example, there is an extended flow field created around suspension-feeders (many calanoids), whereas raptorial feeders (many cyclopoids) create a much smaller fluid disturbance of shorter duration, due to their intermittent jump-swimming behavior (Borg et al. 2012; Kiørboe et al. 2010). Non-feeding early calanoid nauplii, however, have been found to spend a majority of their time stationary (>75%; Bradley et al. 2013), which may allow the nauplii to hide from mechanosensory-based predators more effectively and decrease mortality rates. In addition, and consistent with the well-accepted r/K selection strategy in ecological theory (MacArthur and Wilson 1967), there is evidence that broadcast-spawned nauplii also may have higher mortality than those of egg-bearing species (Kiørboe and Sabatini 1994; Ohman et al. 2002). Finally, the stage at which the nauplii begin to feed may occur in different stages in different species, suggesting that predation and food limitation would influence mortality rates for calanoids and cyclopoids during different periods of development. In many calanoids including the two species in this study, feeding starts in the third naupliar stage, while in *Oithona* spp. feeding may start as early as the first naupliar stage (Almeda et al. 2010a).

We found that calanoids and cyclopoids had distinct mortality schedules in the non-storm ecosystem state. Overall, *O. simplex* nauplii experienced the lowest mortality during the non-storm period (~60%; Suppl. Fig. 5.3-5.4), which could significantly contribute to the dominance of this species in the community over the longer term. In other *Oithona* species, a combination of lower metabolic expenditures and behaviors contributing to greater predator avoidance were hypothesized to be the primary factors resulting in low mortality and thus ubiquity of the genus (Eiane and Ohman 2004; Paffenhöfer 1993). The lower BL in *O. simplex* nauplii during the non-storm period supports this prior work.

The cyclopoid copepods may have experienced greater loss of biomass during the storm events than the calanoids, particularly after the 2011 storm event, which did not follow our expectations. During the 2011 storm, cyclopoids showed high BL between the early and mid naupliar stages over the first 4 days with up to 99% of expected early naupliar biomass not developing to later stages, while calanoid BL declined within the first few days (Fig 5.13). The high percentage BL in the cyclopoids during the 2011 storm may have been due to high rates of predation (e.g. due to higher visibility or conspicuous swimming behavior) or if the early cyclopoid nauplii are feeding stages, perhaps there were not sufficient food resources to develop to later stages, resulting in higher mortality than the non-feeding calanoid early nauplii. Diatoms are not suitable food for many *Oithona* species which prefer motile prey (Paffenhöfer 1993; Uchima 1988), and the diatom bloom during the early 2011 storm period may have left insufficient food for the *Oithona* nauplii. Lower relative loss of biomass in the calanoids during the 2011 storm period could also be due to higher quality maternal resources providing higher survival of the early non-feeding stages. This pattern does not appear in the 2013 storm, where the BL in the last 2-3 days was similar to the non-storm BL.

Ecosystem Dynamics –Temperate and polar marine planktonic ecosystems show strong seasonality and are characterized by seasonal phytoplankton bloom events triggered by a more extended availability of increased resources that are not present in oligotrophic, subtropical ecosystems. Tropical and subtropical coastal populations are known to be more stable over annual timescales and have less of a seasonal signal in the plankton community (Webber and Roff 1995). The broader dynamics of the plankton community in Kaneʻohe Bay are similar to those in diverse regions of the world. For example, the zooplankton community of Guayanilla Bay, Puerto Rico has a similar pattern of species diversity where species richness decreases from offshore into the main bay basin, and variation in zooplankton density (two to eight-fold) is most associated with wind and rainfall patterns, in part due to the short generation times of the dominant species (Youngbluth 1976). Variation in plankton community structure in subtropical coastal waters are most often related to variable rainfall patterns influencing coastal nutrient inputs, supplying allochthonous resources to the neritic plankton community that are rapidly

utilized (days in duration) and assimilated by trophic interactions in the plankton (Grahame 1976; Hopcroft and Roff 1990; McKinnon et al. 2003b; Youngbluth 1976).

A comparison of our results from the non-storm period suggest that *O. simplex, O. attenuata,* and *P. crassirostris* populations were most stable during the summer non-storm period and the two storm events resulted in higher variation but with no consistent pattern of change when comparing the two storms. During the non-storm period, a more stable copepod population is illustrated by little change over time in the percent contribution of naupliar size groups to each species' nauplius population (Fig. 5.7), in relatively shallow slopes of the change in early naupliar copy number over time (Fig. 5.8; Table 5.1), and smaller differences in biomass between subsequent size groups between early, mid, and late time ranges (Fig 5.13-5.14). However, the *B. similis* naupliar population declined over the same time, suggesting that this particular species was in a state of decline, as mentioned previously. During the 2013 storm, the higher contribution of early nauplii in *P. crassirostris* (over 50%) to the nauplius population (Fig. 5.7) suggests that species was in a state of growth for first ~5 days following the 2013 storm, but the overall magnitude of response was much lower than the 2011 storm. During the late 2013 period, there may have been an increase in mortality of mid-stage nauplii in all species that is evident in the reduction of mid-late nauplius biomass in all species (Figs. 5.9-5.12), a negative difference in biomass between mid to late nauplii of all species (Fig. 5.14), and an increased contribution of early nauplii to the total (Fig. 5.7). The increased late nauplius mortality during the late 2013 period was more likely to be due to predation, parasites, or viral infection rather than starvation, since water column Chl *a* was ~1.0 µg L^{-1} and because nauplii are less prone to food limitation than later stages (Kiørboe 1997). During the 2011 storm period, copepod populations were highly variable during the early and middle stages of the post-storm period. However, by the end of the 2011 sampling period, the percent contributions of stages returned to levels similar to non-storm levels (Fig 5.7) and the difference in biomass between subsequent cohorts returned to non-storm levels (Figs. 5.13-5.14), suggesting that the copepod community may have been recovering from the perturbation by the end of the 13-day sampling period.

Understanding Kane'ohe Bay –The southern, semi-enclosed region of Kane'ohe Bay serves as a useful model study system to understand the response of subtropical coastal plankton communities to ephemeral ecosystem perturbations due to its relatively long water residence times (1-2 mo.; Lowe et al., 2009) and low zooplankton diversity compared with the central and northerly Bay waters and the offshore North Pacific Subtropical Gyre (Hoover et al. 2006; Jungbluth and Lenz 2013; McGowan and Walker 1979). The copepod species in Kane'ohe Bay are commonly found coexisting in other warm coastal waters around the world, including Australia (McKinnon et al. 2003b; McKinnon and Thorrold 1993), Jamaica (Hopcroft et al. 1998b), the South China Sea (Jianquiang et al. 2006), the northern Gulf of Mexico (Turner 1986), and Florida Bay (Kiesling et al. 2002). The qPCR method applied in our study may be directly applicable to these other communities to provide a comparison of naupliar dynamics in different ecosystems. In addition, Kane'ohe Bay has been relatively well-studied, which provides useful background on chemical, physical, and biological dynamics to compare with the current study.

In the southern basin of Kane'ohe Bay, wind speed and direction are the biggest factors contributing to the retention or advection of nutrient-rich storm runoff. Winds primarily push the upper 2-3 meters of the water column, while the bathymetry of the southern basin permits retention of waters below the wind-forced surface waters (12-16 meters deep; Bathen 1968). These features suggest that during storm runoff conditions or aberrant southerly winds, the bulk volume of the plankton population within the southern region of the bay would be retained rather than advected out of the basin, and time-course studies likely reflect *in situ* changes in the south bay plankton population. Low winds during and after storm events result in a stratified water column, maintaining recently-introduced high surface concentrations of nutrients, and can permit persistence of the phytoplankton bloom in the southern region of Kane'ohe Bay. However if trade winds occur during or shortly following the storm, the nutrient rich, buoyant surface plume may be rapidly mixed into the water column lowering nutrient concentrations in surface waters and reducing the magnitude of the phytoplankton response, or if the wind direction is offshore, the surface plume could be advected out of the bay (Drupp et al. 2011; Ostrander et al. 2008; Smith et al. 1981). In addition to wind effects, the amount of coastal nutrient loading that occurs

due to storms is also a result of the length of time prior to the storm event where little rainfall has occurred. Extended dry periods allow for accumulation of eroded minerals, anthropogenic nutrients, and decayed organic matter that are flushed into the bay during storms (Cox et al. 2006). Submarine groundwater reservoirs are a particularly important source of silica and can provide a substantial amount of biologically useful silica to coastal waters after storm events, especially after the first heavy rainfall of the year that can drive the accumulated silica in the groundwater out into the bay (De Carlo et al., 2007; Drupp et al., 2011).

The three sampling periods reported here were very different, but are comparable to prior studies of nutrient and plankton dynamics in Kaneʻohe Bay. During the non-storm period of the current study, the waters in the study region were well-mixed as a result of the summertime northeasterly trade winds (Fig. 5.2b) and as a result nutrient concentrations were expected to be relatively low and stable (e.g., from previous work ~ 0.2 µM NO_3^-, 0.1 µM PO_4^{3-} and 5 µM $Si(OH)_4$), at levels reported in previous studies of summer nutrient dynamics in Kaneʻohe Bay (e.g., Drupp et al., 2011). Both of the 2011 and 2013 storms described here resulted in increases in Chl *a* within days of the storm, similar to what was described in previous studies (Cox et al., 2006), but the phytoplankton community and timing of changes in Chl *a* were very different between the two events likely as a result of differing nutrient inputs between first-flush events and later season storms.

The 2011 storm occurred after over two months of relatively low precipitation that allowed for accumulation of material on land and in groundwater to then be flushed into the coastal waters after the storm (i.e., the first-flush storm event). Hoover et al., (2006) also followed a first-flush storm in the same study area, and immediately observed an order of magnitude increase in dissolved nitrate, silicate, and soluble reactive phosphorous, which then declined rapidly in the days following the storm. The nutrient dynamics after the 2011 storm described here are expected to be similar to the previous study. The relatively low wind speeds observed in the ~8 days following the 2011 storm provided an opportunity for retention and slow mixing of the nutrient-rich runoff in the bay, facilitating the formation of a dense bloom of phytoplankton in the days following the storm. The bloom was dominated by the silica-rich chain-forming diatom, *Chaetoceros,* in the days immediately following the 2011 storm event,

which likely provided excess food resources for the adult copepod population, supporting an increase in egg production and subsequent increase in copepod nauplii. The density of the diatom bloom likely provided substantial food for both the calanoid and cyclopoid copepods, despite the preference of cyclopoids for motile prey. The occurrence of a diatom bloom may have been linked to silica-rich groundwater being flushed into the bay in addition to the surface runoff that primarily provides nitrate and phosphate (Cox et al., 2006; De Carlo et al., 2007; Drupp et al., 2011; Ringuet and Mackenzie 2005). With regard to the suitability of these chain-forming diatoms for the feeding naupliar stages, our results of the percent biomass lost between mid and late nauplii during the first four days of the 2011 storm suggest that diatoms may be a food source for the three dominant nauplii (Suppl. Fig. 5.4), however the elevated mortality of nonfeeding stages suggests there may be a negative effect of the diatom bloom on early naupliar survival, perhaps due to entanglement, or low viability of offspring (e.g., Koski et al. 2008) .

As opposed to the large diatom bloom after the 2011 storm, the peak in chlorophyll on 31 May 2013, 3 days after the storm, was primarily from small autotrophic cells, given the lack of large autotrophs observed on the epifluorescence slides. The lack of large diatom cells in this case may have been due to less groundwater-derived silica (De Carlo et al. 2007; Hoover and Mackenzie 2009), given previous flushing from recent rainfall. In addition, the peak in Chl *a* could be from an increase in picophytoplankton fluorescence per cell, previously reported to occur within 12-24 h after nutrient inputs, which could contribute to higher chlorophyll readings after storms (Cox et al., 2006). Given the lack of large phytoplankton after the 2013 storm, it seems that the adult copepod community was affected to a much lesser magnitude by the late season storm, however there was an overall positive recruitment response for all species (Table 5.1), though the correlations were weak. In the mid-late stage nauplii, higher availability of smaller cells may have enhanced survival of mid-late nauplii; the percent loss of biomass decreased (and became negative for some species, implying growth) for all four species as compared to non-storm levels (Suppl. Fig. 5.4).

Conclusions – Using a novel molecular approach, we were able to observe species-specific variation in naupliar recruitment rates in response to ecosystem perturbations, and we saw brief

shifts in the structure of the copepod naupliar population. High biomass disappearance rates between the early to mid-stage nauplii and mid to later nauplii also suggest that they played a significant role in the trophic transfer of biomass to higher trophic levels in the days immediately following the 2011 storm event, with some species (*O. simplex*) contributing disproportionately to naupliar recruitment and mortality. While the current study focused on subtropical plankton, the genetic methods applied here could be applied to address challenging questions in other study areas that are dominated by sibling species complexes. In the future, further studies can be done looking at the potential causes of increased mortality and survival in these highly variable coastal communities through more specific quantification of the potential predator and prey community. Our results suggest that there were distinct responses of the copepod community to early vs. late season storms, and that nauplii may be particularly important members of the plankton community during ephemeral post-storm periods where naupliar biomass can increase substantially.

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Tables and Figures.

Table 5.1. Trends in recruitment over time. Results of linear regression of log-transformed 20 µm copy number over time portraying the change in recruitment to the early naupliar 'cohort' each day. Positive values indicate an increase in recruitment, whereas negative values indicate a decrease. Regression p value (p val) indicates whether the slope is significantly different from zero ($p < 0.05$), with slope and r^2 as reported.

Species	Event	Slope	SE slope	p val	r^2
Bestiolina similis	Non storm 2011	-0.638	(±0.048)	<0.001	0.72
	Storm 2011	-0.110	(±0.087)	0.212	0.05
	Storm 2013	0.119	(±0.021)	<0.001	0.30
Oithona attenuata	Non storm 2011	0.011	(±0.012)	0.351	0.01
	Storm 2011	-0.053	(±0.025)	0.041	0.11
	Storm 2013	0.064	(±0.019)	<0.001	0.14
Oithona simplex	Non storm 2011	-0.038	(±0.014)	0.009	0.09
	Storm 2011	-0.222	(±0.029)	<0.001	0.62
	Storm 2013	0.136	(±0.017)	<0.001	0.47
Parvocalanus crassirostris	Non storm 2011	0.034	(±0.016)	0.035	0.06
	Storm 2011	-0.051	(±0.015)	<0.001	0.24
	Storm 2013	0.135	(±0.016)	<0.001	0.49

Table 5.2. Testing for differences in recruitment across events, and across species. a) Two-way ANOVA testing for differences in log transformed 20 µm copy number of sampling events and over time (date; slope). b) Two-way ANOVA testing for differences in 20 µm copy number of species and time (date; slope).

a)

Species	Source	df	SS	MS	F	p-val
B. similis						
	Date	1	129.6	129.6	65.9	<0.001
	Event	2	105.9	52.9	26.9	<0.001
	Date:Event	2	131.6	65.8	33.4	<0.001
	Residuals	93	183.0	2.0		
O. attenuata						
	Date	1	0.6	0.6	3.5	0.063
	Event	2	22.6	11.3	67.6	<0.001
	Date:Event	2	0.8	0.4	2.5	0.087
	Residuals	102	17.0	0.2		
O. simplex						
	Date	1	1.7	1.7	5.9	0.017
	Event	2	41.4	20.7	71.6	<0.001
	Date:Event	2	20.1	10.0	34.7	<0.001
	Residuals	102	29.5	0.3		
P. crassirostris						
	Date	1	0.0	0.0	0.2	0.643
	Event	2	49.1	24.6	296.1	<0.001
	Date:Event	2	4.4	2.2	26.6	<0.001
	Residuals	101	8.4	0.1		

Table 5.2 (continued). b) Two-way ANOVA testing for differences in 20 µm copy number of species and time (date; slope).

b)

Species	Source	df	SS	MS	F	p-val
Non Storm 2011						
	Date	1	25.7	25.7	48.5	<0.001
	Species	3	618.3	206.1	388.8	<0.001
	Date:Species	3	156.9	52.3	98.7	<0.001
	Residuals	144	76.3	0.5		
Storm 2011						
	Date	1	31.3	31.3	30.5	<0.001
	Species	3	1291.4	430.5	418.8	<0.001
	Date:Species	3	10.6	3.5	3.4	0.02
	Residuals	143	147.0	1.0		
Storm 2013						
	Date	1	3.0	3.0	15.9	<0.001
	Species	3	267.6	89.2	471.0	<0.001
	Date:Species	3	2.0	0.7	3.6	0.02
	Residuals	135	25.6	0.2		

Table 5.3. Mantel tests on the linear correlation of copy number variation (distance matrix between species across time) with variation in environmental parameters (distance matrix across time). Analyses were separated by naupliar size fractions (20-63, 63-80, and 80-100 µm). The *Combined Environment* tested copy number variation against the combined distance matrix of all variables listed in under Environmental Variables.

	20-63 µm		63-80 µm		80-100 µm	
Environmental Variables	**r^2**	**p-value**	**r^2**	**p-value**	**r^2**	**p-value**
Combined Environment	0.05	0.007**	0.03	0.041*	0.03	0.016*
Temperature	0.00	0.579	0.00	0.553	0.00	0.358
Salinity	0.00	0.497	0.02	0.996	0.00	0.662
Wind Speed	0.04	0.006**	0.03	0.025*	0.04	0.003**
Rainfall	0.00	0.502	0.00	0.602	0.01	0.677
Chlorophyll	0.08	0.002**	0.05	0.02*	0.01	0.071*
Prochlorococcus	0.00	0.496	0.00	0.466	0.00	0.617
Synechococcus	0.02	0.043*	0.06	0.003**	0.02	0.024*
Photosynthetic Eukaryotes	0.03	0.032*	0.07	0.011*	0.06	0.003**
Heterotrophic Bacteria	0.10	0.001***	0.07	0.001***	0.12	0.001** *

*Significance values: *** (p ≤0.001); ** (p≤ 0.01); * (p≤ 0.1)*

Table 5.4. Biomass (*b* µg C m^{-3} d^{-1}; range and median) gained (positive) or lost (negative) between sequential size groups assuming a transition time of *t* = 1 day from the 20-63 µm (S1) size fraction into the 63-80 µm (S2) size fraction, and from 63-80 µm (S2) to the 80-100 µm (S3) size fraction. Negative changes in biomass imply disappearance **(Bold)**.

		S1 to S2		S2 to S3	
Species	Event	Range *b*	Median *b*	Range *b*	Median *b*
Bestiolina similis	Non Storm 2011	-36 – 6	**-6**	-14 – 6	**-2**
	Storm 2011	-11 – 7	5	-6 – 14	2
	Storm 2013	-142 – 46	**-4**	-45 – 95	**-3**
Oithona attenuata	Non Storm 2011	-270 – 215	**-65**	-383 – -52	**-203**
	Storm 2011	-1,829 – 2,244	**-325**	-2,140 – 1,121	**-83**
	Storm 2013	-605 – 34	**-339**	-411 – 42	**-182**
Oithona simplex	Non Storm 2011	-22 – 1,497	484	-1561 – 171	**-493**
	Storm 2011	-12,987 – 2,614	**-2,865**	-1,453 – 5,288	1,465
	Storm 2013	-1,705 – 1,143	**-40**	-1,473 – 985	**-330**
Parvocalanus crassirostris	Non Storm 2011	-263 – 306	46	-284 – 180	**-66**
	Storm 2011	-1,992 – 3,750	270	-1,143 – 6,022	943
	Storm 2013	-592 – 536	**-96**	-613 – 471	**-81**

Figure 5.1. Map of Kane‘ohe Bay showing the sampling station S3 in the southern semi-enclosed region. Primary stream inputs are as labeled in the South and central Bay: Kane‘ohe Stream (South), Kea‘ahala Stream (South) and He‘eia Stream (central). Image: Google Maps.

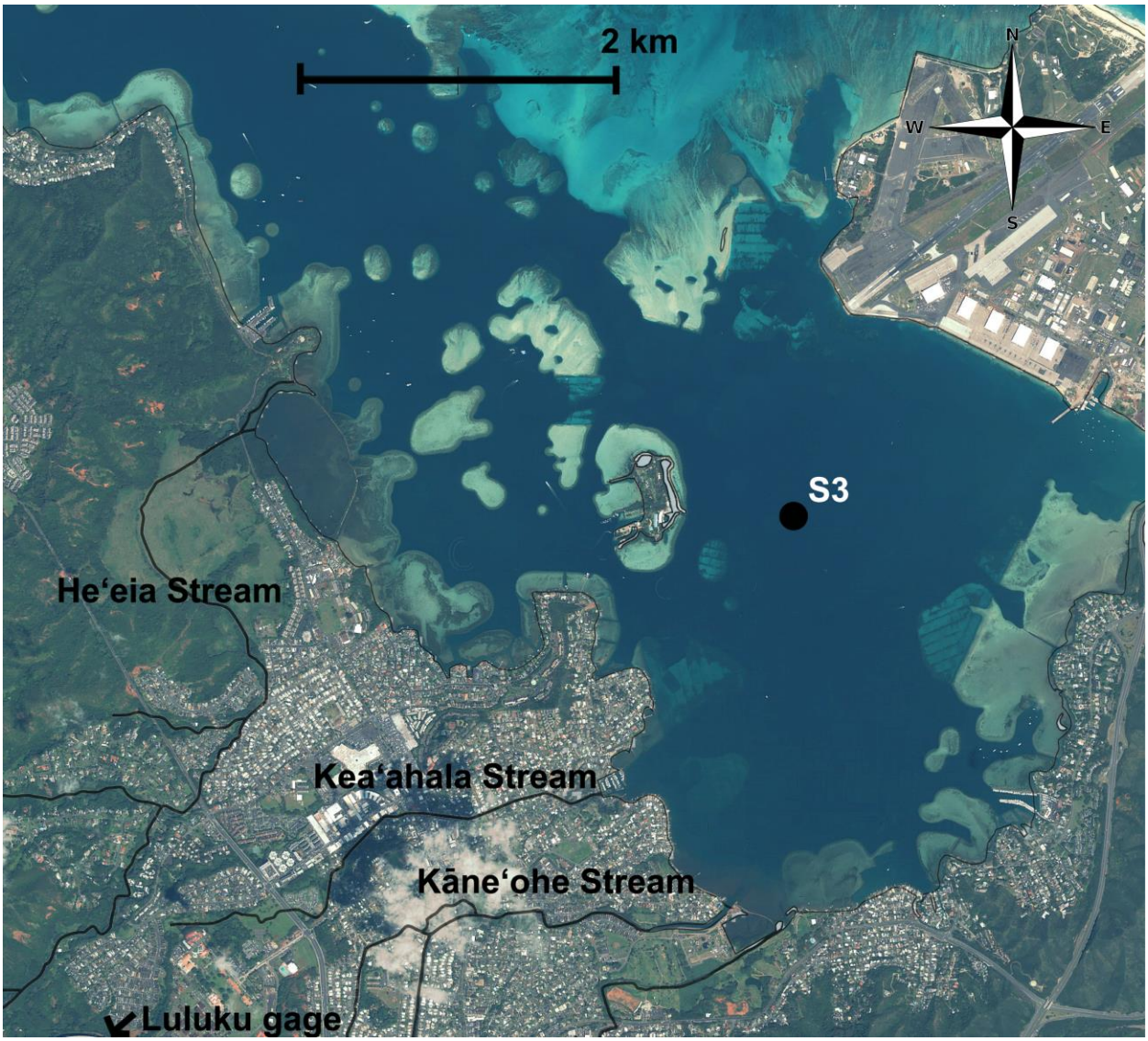


Figure 5.2. Environmental conditions during each sampling event: Non-storm 2011, storm 2011, and storm 2013. Row a) Rainfall at Luluku gage (left axis, grey bars, cm day^{-1}) and chlorophyll *a* (right axis, Chl *a* µg L^{-1}). Chl *a* discretely sampled at 2 m (black circles) and 11 m (white circles) in Storm 2011 and Storm 2013 events. Row b) Wind speed (left axis, open triangles, knots), and wind direction (right axis arrows, arrow length corresponds to magnitude of wind speed, North is up). Vertical dashed lines indicate the first day of sampling. Row c) chlorophyll *a* (µg Chl *a* L^{-1}), from an *in situ* fluorometer, and Row d) salinity (PSU), both plotted over 11 m depth.

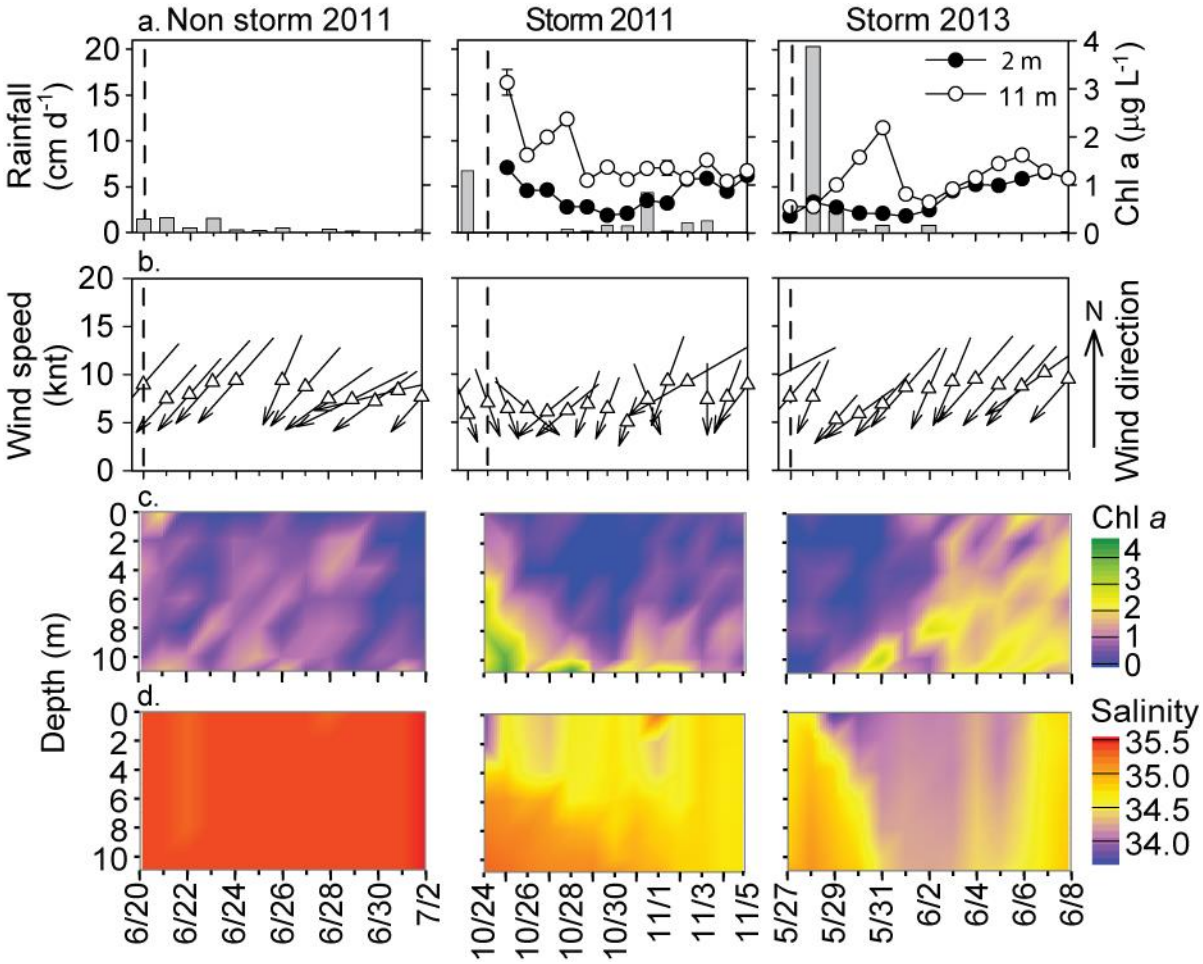


Figure 5.3. PCA biplot of environmental data for each sampling date. Colored points denote sampling event types; Nonstorm 2011 (red), storm 2011 (blue), and storm 2013 (green). Grey arrows indicate the direction of increase for environmental parameters, while arrow length indicates the degree of correlation with the ordination axis. Only the first two principal component axes are shown (PC1, PC2). PC1 and PC2 explain 60% of the variance in differences of environmental samples.

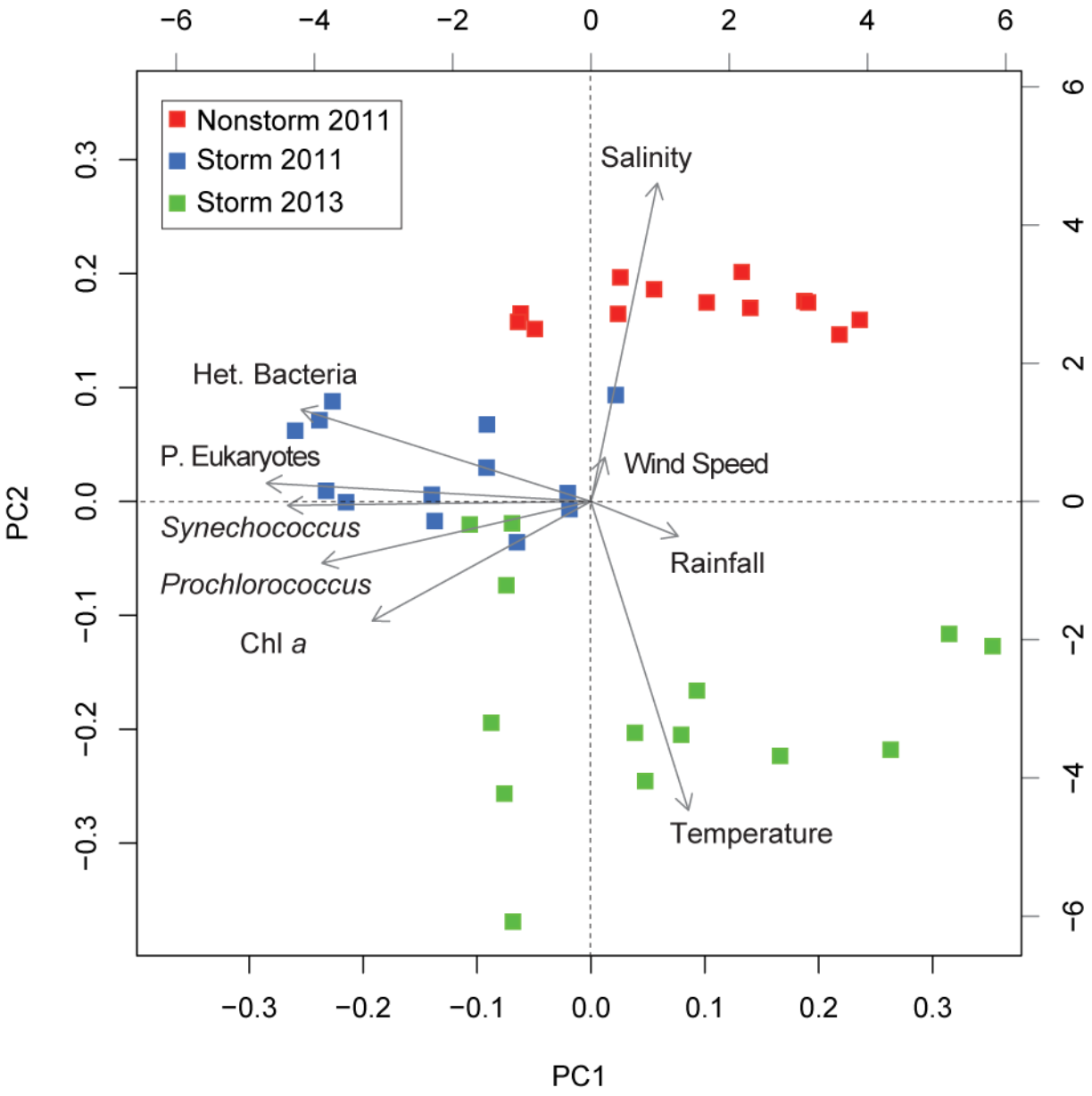


Figure 5.4. a) Abundance ($\times 10^3$ m^{-3}) of nauplii, copepodites, and adult copepods based on 63 µm net microscope counts (does not account for 20-63 µm nauplii) across the sampling events, left to right: non-storm 2011, storm 2011, and storm 2013. Calanoids and cyclopoids are combined. b) Total DNA copies during each event over time in the 20-100 µm size fractions and c) total copies of each species in the combined 20-100 µm size range. Error bars are ± standard deviation.

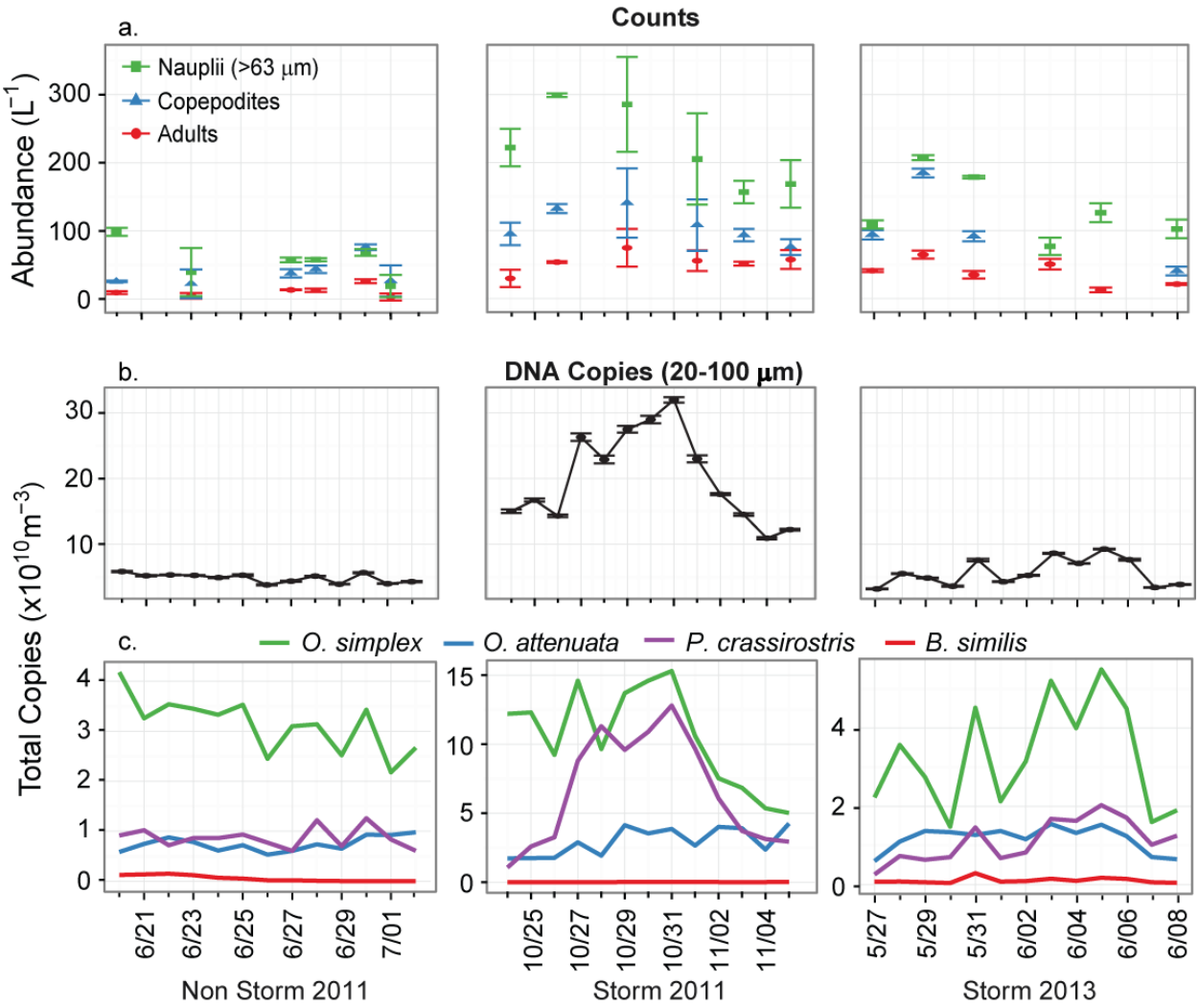


Figure 5.5. Percent contribution of each species to the total 20-100 µm copy number.

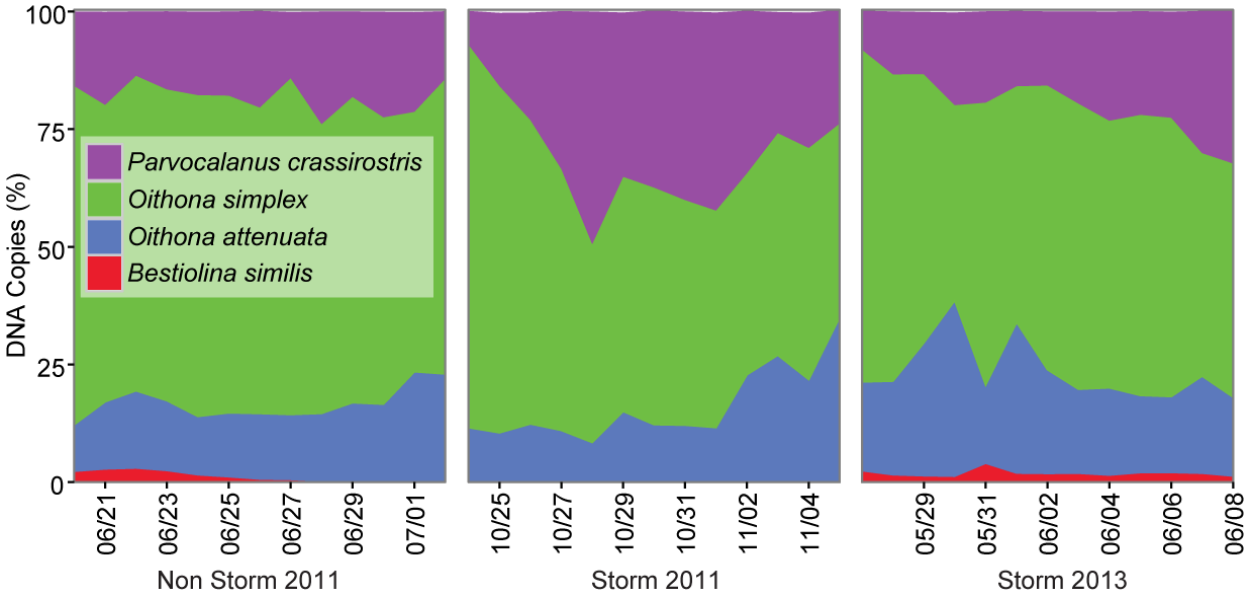


Figure 5.6. DNA copy number in each fraction (20-63 pink, 63-80 blue, 80-100 µm green) for the 4 target species (rows) measured daily across each event (columns): *Parvocalanus crassirostris*, *Oithona simplex*, *Oithona attenuata*, and *Bestiolina similis*. Error is ± SD. Note the y-axis is condensed to a log-scale.

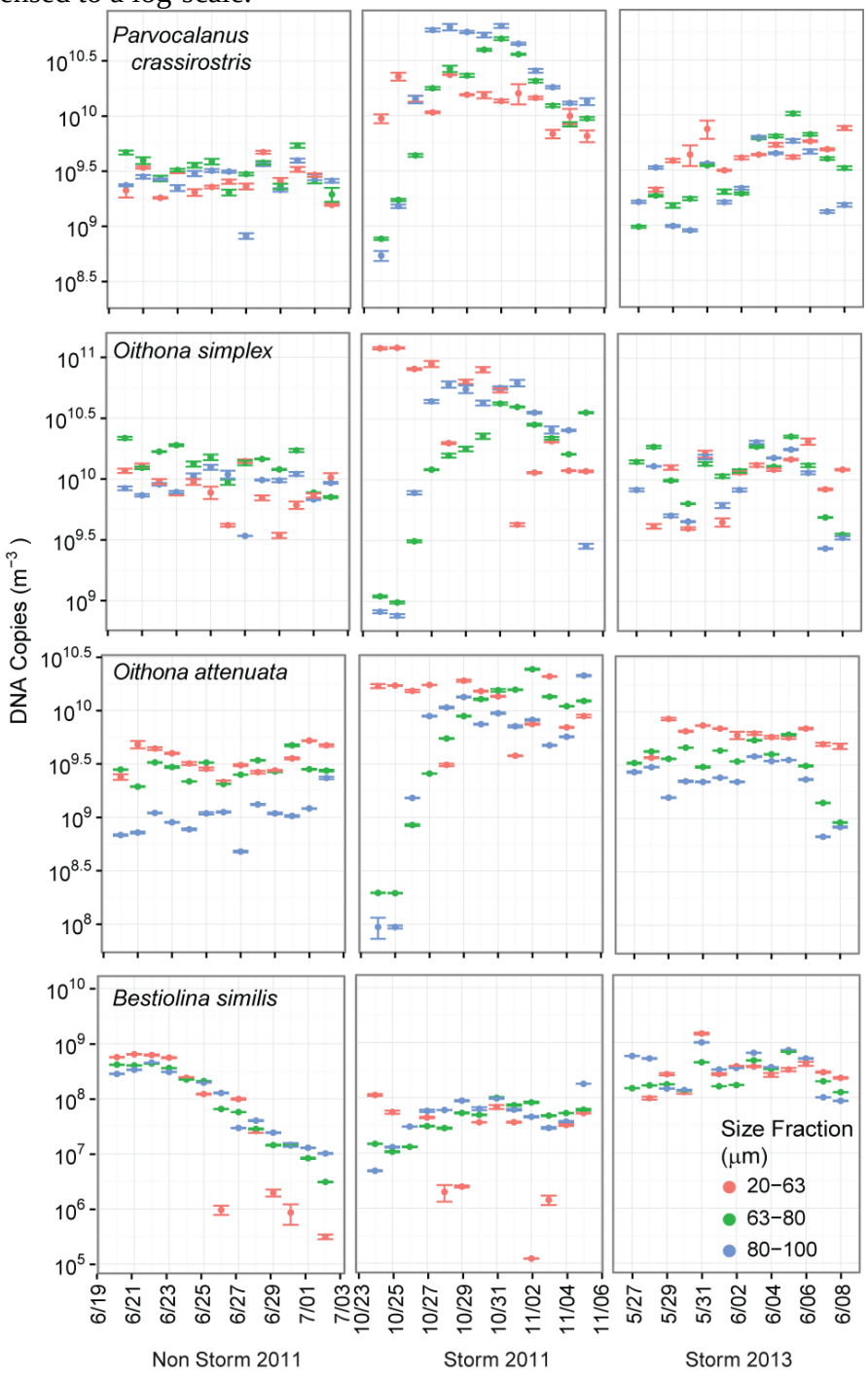


Figure 5.7. Percent contribution of naupliar size groups to the total 20-100 µm mtCOI copy number in each species. Light colors are for the smallest size fraction (20-63 µm), medium hues are the middle size fraction (63-80 µm) and dark colors are the largest size fraction (80-100 µm).

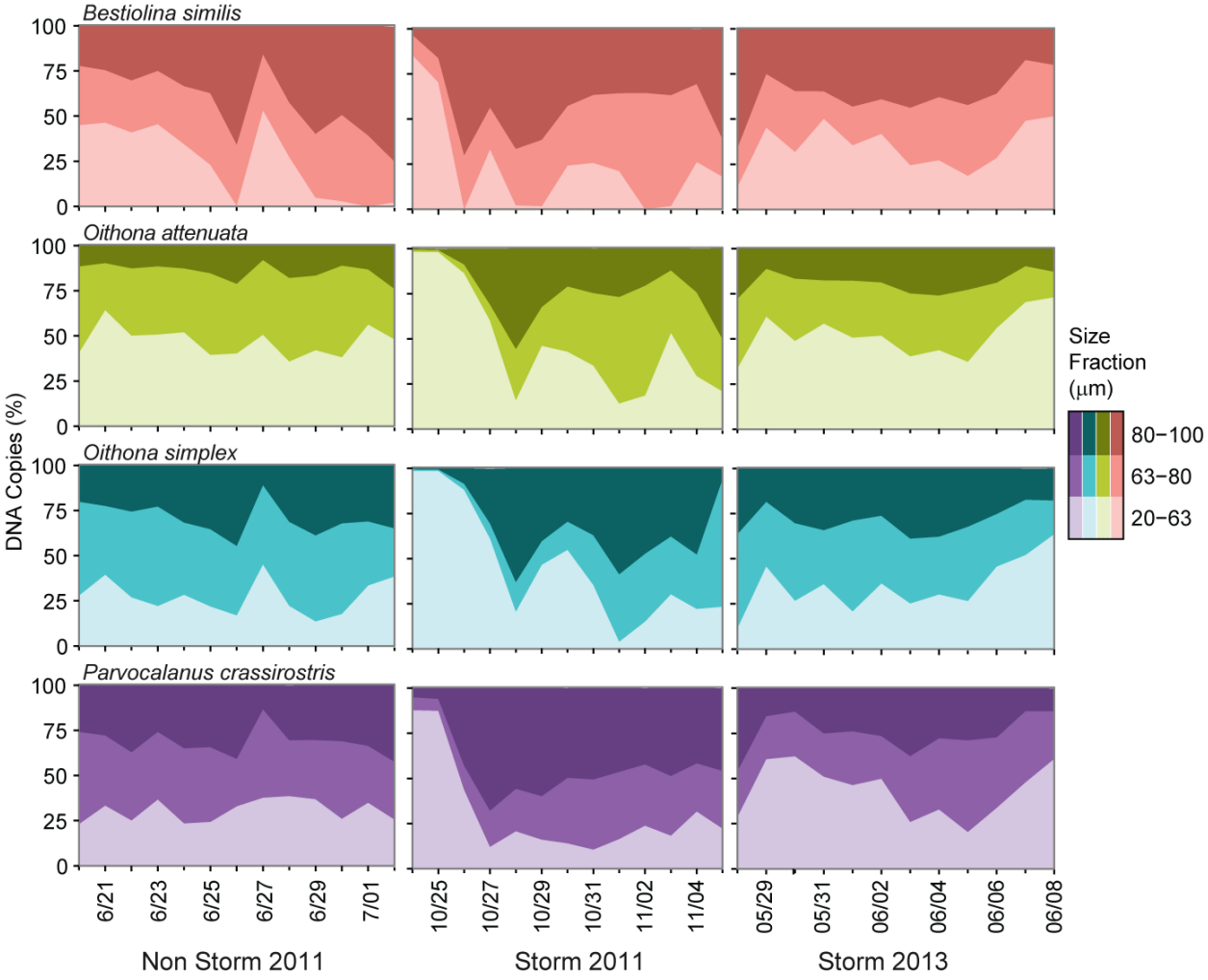


Figure 5.8. Trends in naupliar copy number over time during each event, shown by linear regression of log transformed DNA copy number in the 20-63 µm size fraction. Events are shown in separate columns, and species in rows. Summary of relationships shown in Table 5.1.

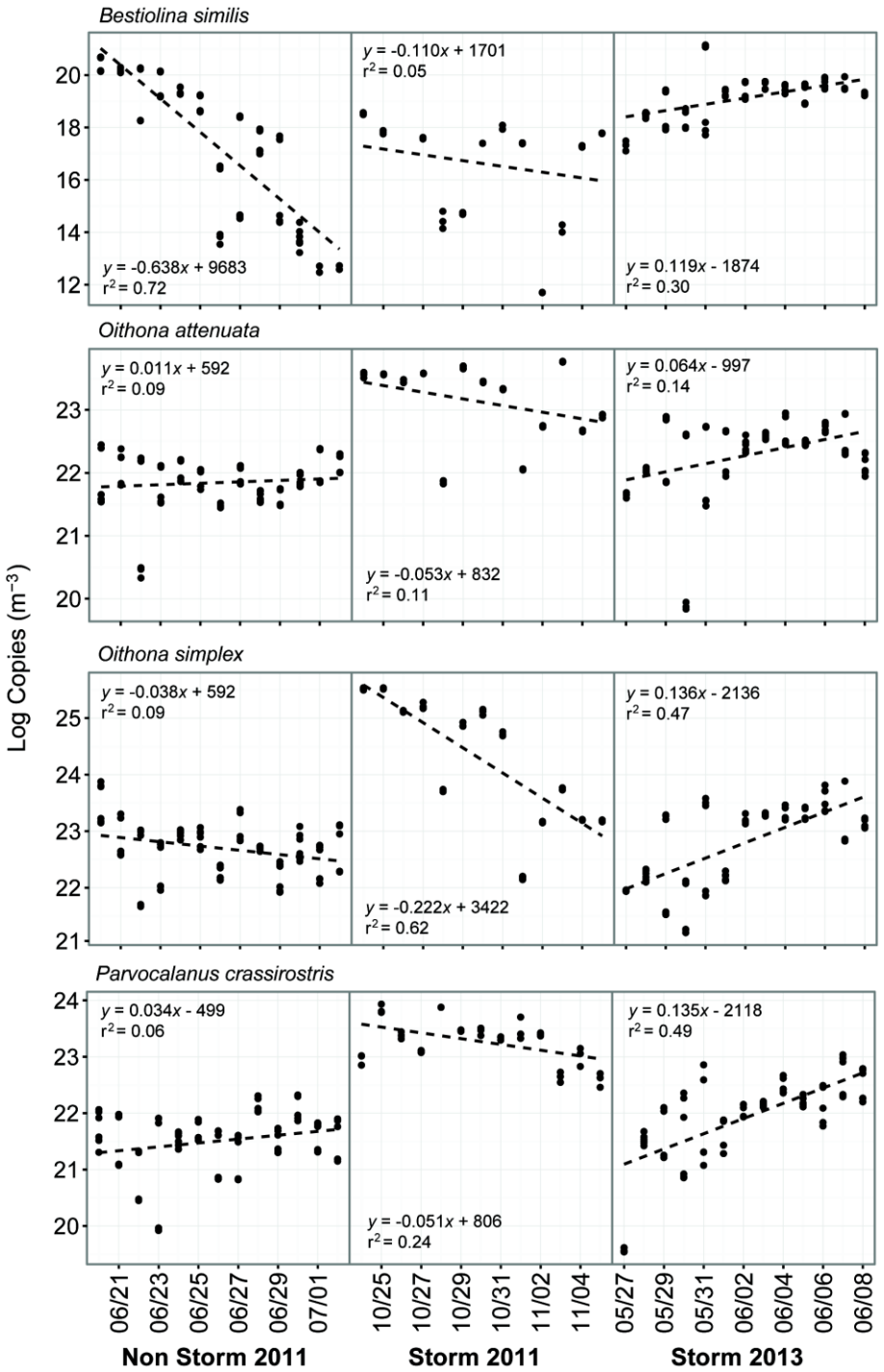


Figure 5.9. Biomass (mg C m^{-3}) of *Bestiolina similis* collapsed to two size fractions (20-63 µm "Early Nauplii", and 63-100 µm "Mid-Late Nauplii") during the events: Non-Storm, 2011 Storm, and 2013 Storm. Error is ± standard deviation. Biomass is estimated from mtCOI copies, measured by qPCR.

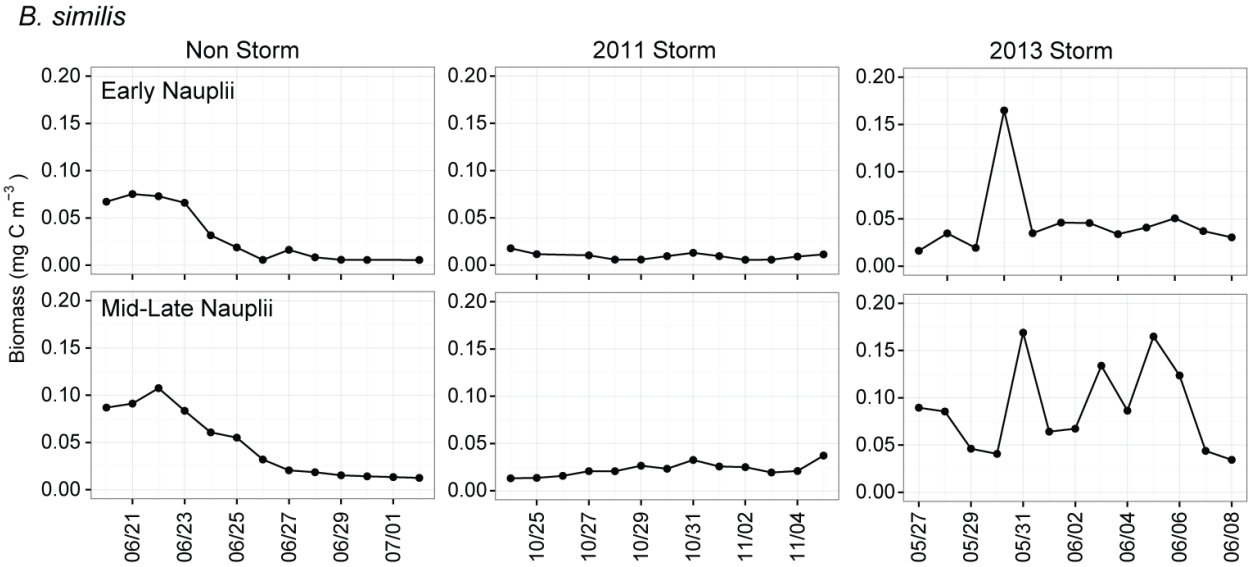


Figure 5.10. Biomass (mg C m^{-3}) of *Oithona attenuata* collapsed to two size fractions (20-63 µm "Early Nauplii", and 63-100 µm "Mid-Late Nauplii") during the events: Non-Storm, 2011 Storm, and 2013 Storm. Error is ± standard deviation. Biomass is estimated from mtCOI copies, measured by qPCR.

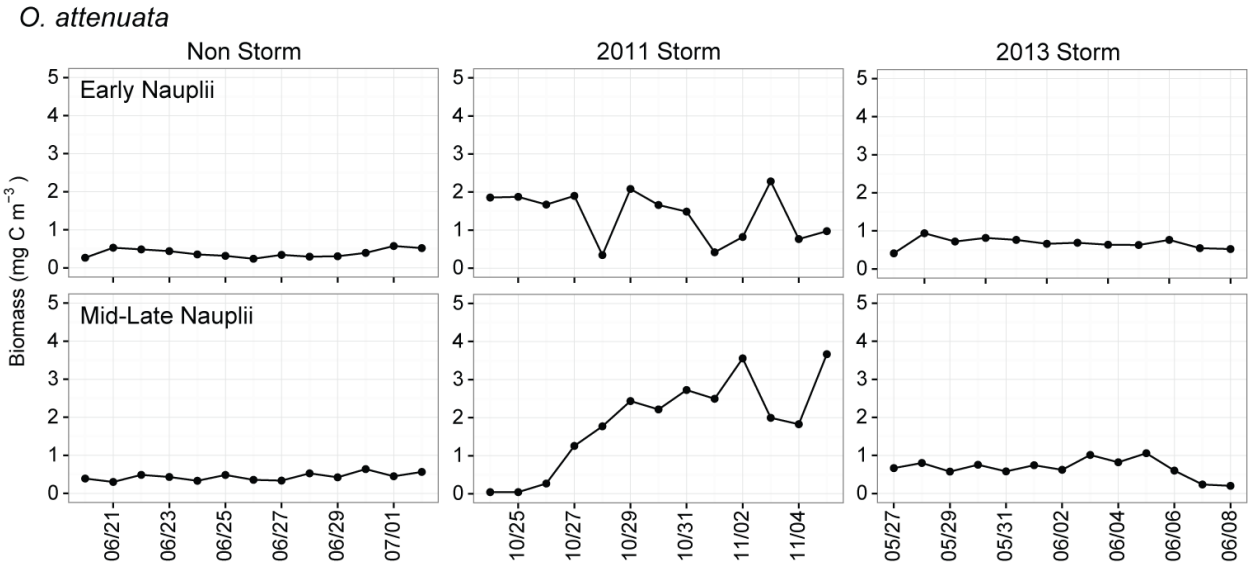


Figure 5.11. Biomass (mg C m^{-3}) of *Oithona simplex* collapsed to two size fractions (20-63 µm "Early Nauplii", and 63-100 µm "Mid-Late Nauplii") during the events: Non-Storm, 2011 Storm, and 2013 Storm. Error is ± standard deviation. Biomass is estimated from mtCOI copies, measured by qPCR.

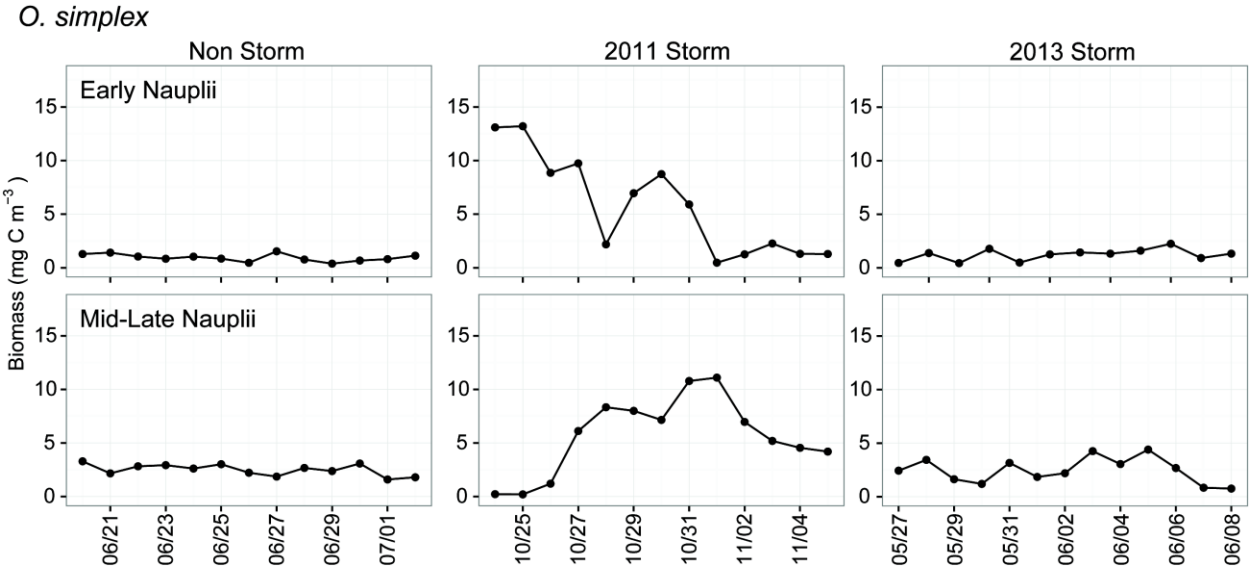


Figure 5.12. Biomass (mg C m^{-3}) of *Parvocalanus crassirostris* collapsed to two size fractions (20-63 µm "Early Nauplii", and 63-100 µm "Mid-Late Nauplii") during the events: Non-Storm, 2011 Storm, and 2013 Storm. Error is ± standard deviation. Biomass is estimated from mtCOI copies, measured by qPCR.

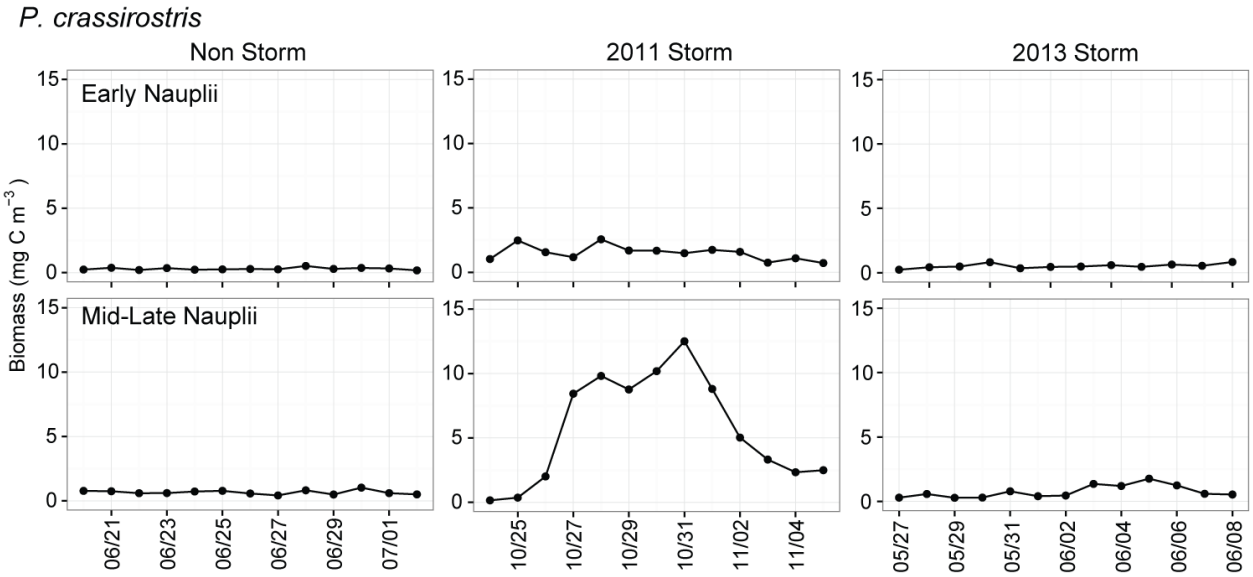


Figure 5.13. Difference in biomass (mg C m^{-3} d^{-1}) between the 20 and 63 µm artificial cohorts. Negative indicates loss of biomass, positive indicates growth between subsequent cohorts over an assumed development time of t = 1. Lines are linear regressions separated by early, middle, and late stages of sampling to illustrate a relative increase in growth (positive slope) or increase in mortality (negative slope) over time.

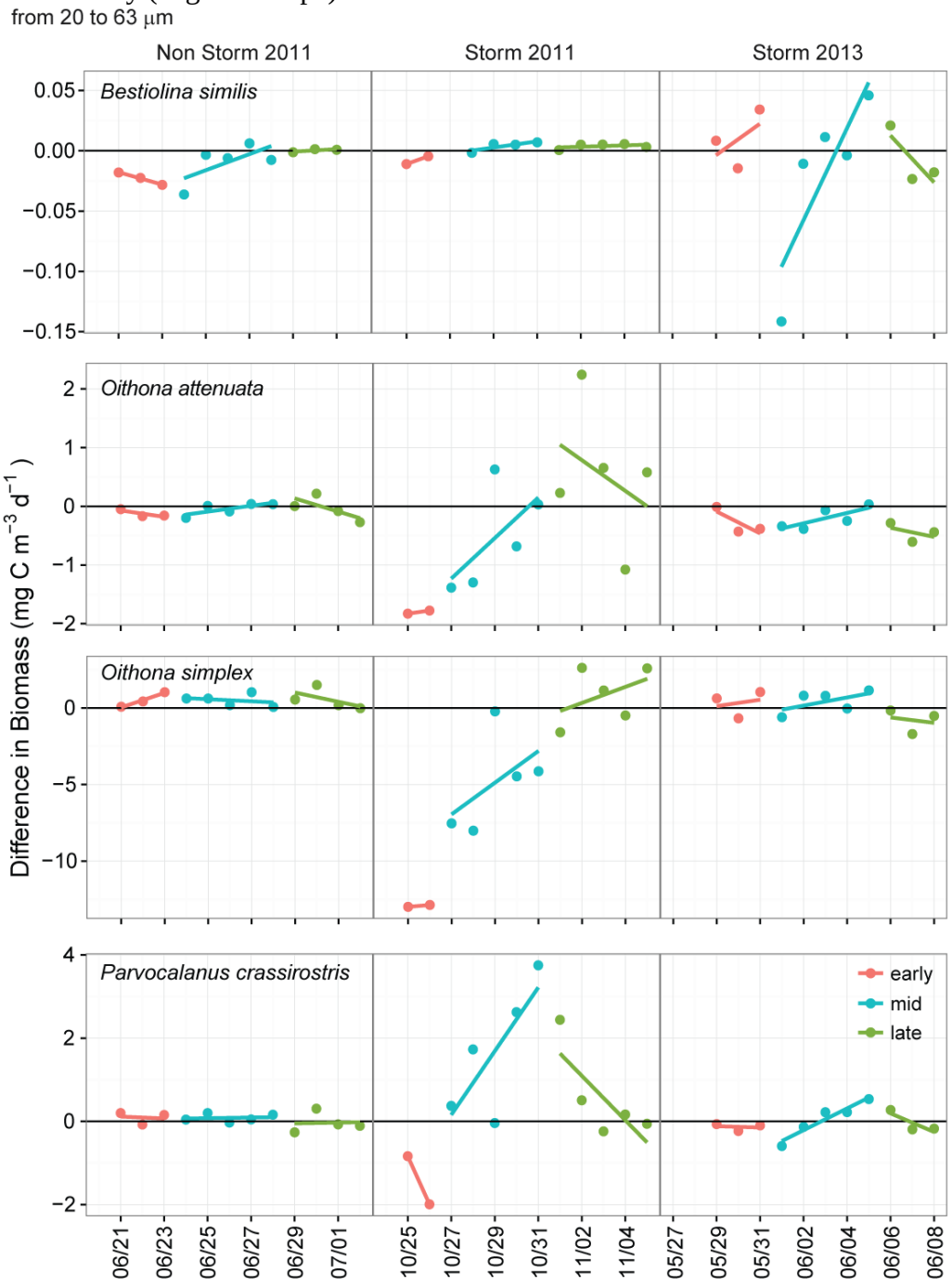
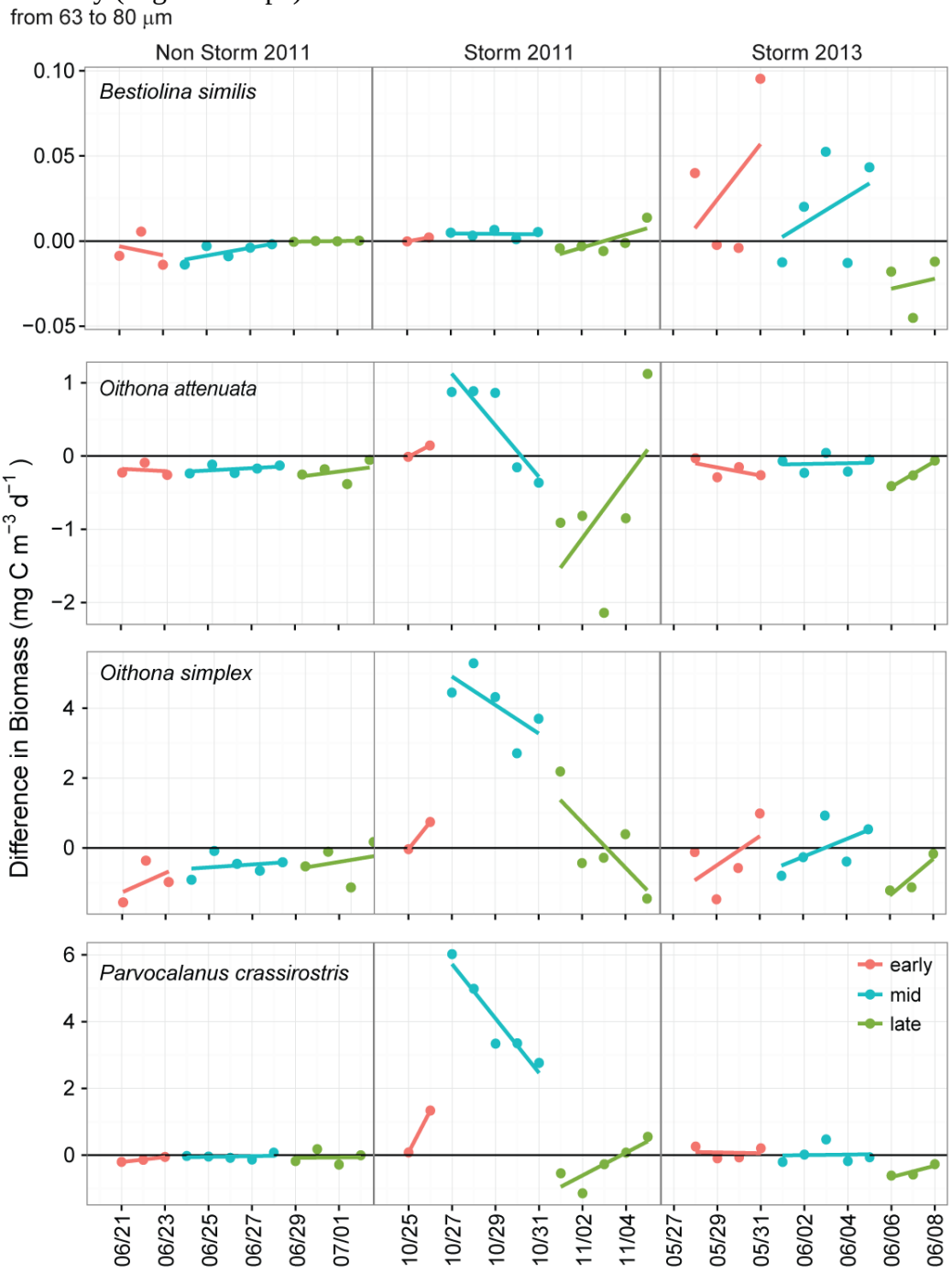


Figure 5.14. Difference in biomass (mg C m^{-3} d^{-1}) between the 63 and 80 µm artificial cohorts. Negative indicates loss of biomass, positive indicates growth between subsequent cohorts over an assumed development time of t = 1. Lines are linear regressions separated by early, mid, and late stages of sampling to illustrate a relative increase in growth (positive slope) or increase in mortality (negative slope) over time.



Supplementary Tables and Figures

Supplementary Table 5.1. QPCR assay reagents (SYBR Green) and results: amplification efficiency (AE%), correlation coefficient (r^2), standard Cq range (cycle threshold) and standard concentration (reaction copies μL^{-1}).

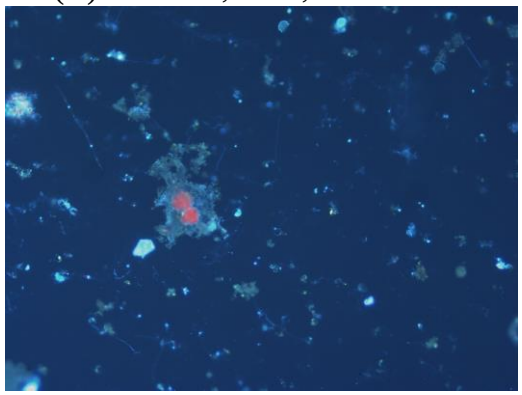
Species	AE (%)	R^2	St. Cq	St. concentration (copies μL^{-1})	SYBR Green	Machine
Bestiolina similis	90-102	0.98-1.0	13.11 – 27.87	$0.7\times10^1 – 1.2\times10^5$	FastStart Essential DNA Green Master	LC96
Oithona attenuata	90-99	0.99-1.0	9.66 – 27.01	$9.5\times10^1 – 5.7\times10^6$	FastStart Essential DNA Green Master	LC96
O. simplex	90-103	0.99-1.0	9.34 – 28.07	$1.5\times10^2 – 1.5\times10^7$	FastStart Essential DNA Green Master	LC96
Parvocalanus crassirostris	90-96	0.99-1.0	14.53 – 26.58	$1.3\times10^3 – 2.4\times10^7$	*iQ SYBR Green Supermix,	*iCycler IQ
P. crassirostris	91-96	1.0	16.42 – 27.16	$1.3\times10^2 – 1.3\times10^5$	FastStart Essential DNA Green Master	LC96

*iCycler IQ was used with 2011 63, 75, and 80 µm samples, machine broke and remaining samples and species including 20 µm 2011 samples were run on LC96

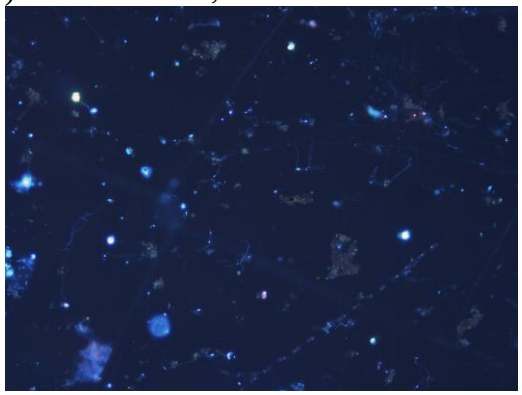
Supplementary Figure 5.1. Assortment of large volume (8.0 µm pore size) UV filter-DAPI and proflavin stained epifluorescence slide images from select dates during the 2011 non storm sampling period (A-B), 2011 storm sampling period (C-F), and 2013 storm period (G-I) next page). Red coloration indicates autotrophy, bright blue indicates recently live DNA. Scale bar ~100 µm applies to all images.

Non Storm 2011

(A) June 20th, 2011, Non-storm

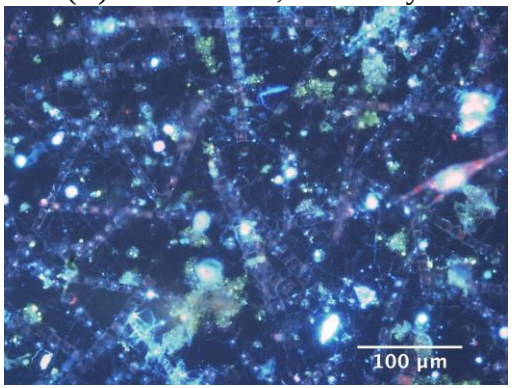


(B) June 28th 2011, Non-storm

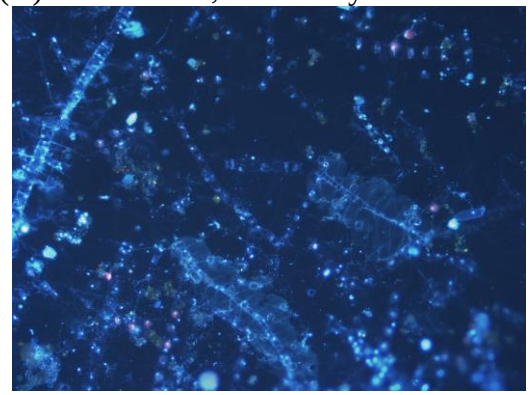


Storm 2011

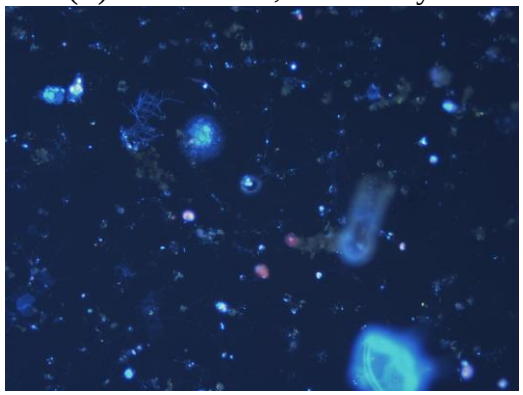
(C) October 24th, 2011 -day 1



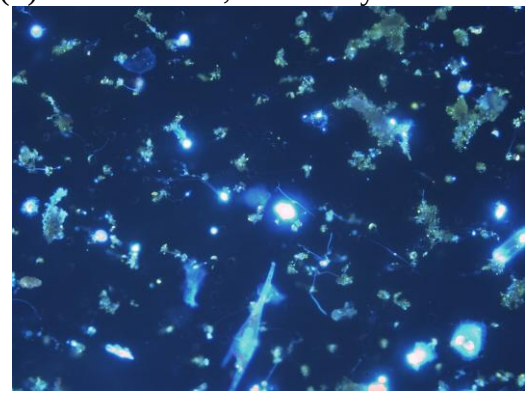
(D) October 28th, 2011 - day 5



(E) October 31st, 2011 – day 8



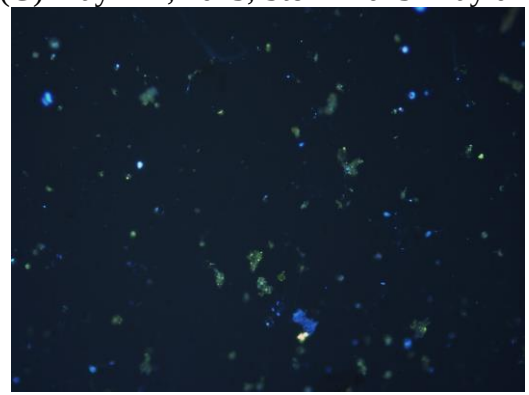
(F) November 3rd, 2011 - day 11



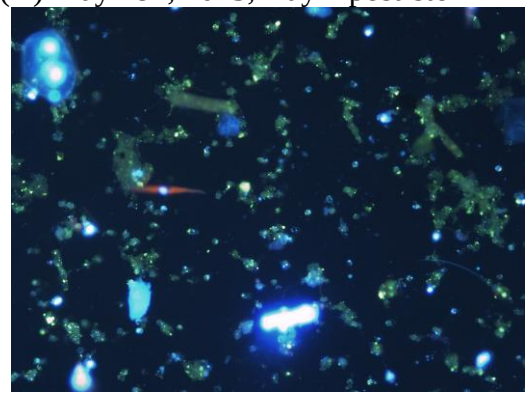
Supplementary Figure 5.1 (continued).

Storm 2013

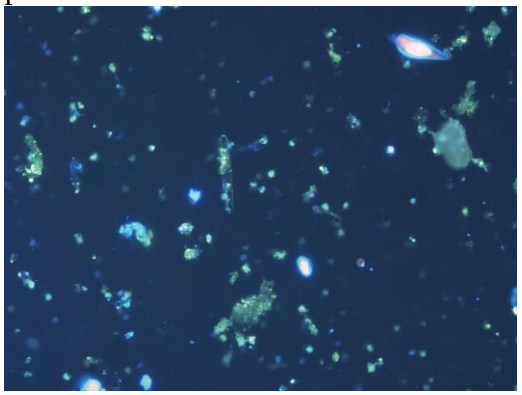
(G) May 27th, 2013, Storm 2013- Day 0



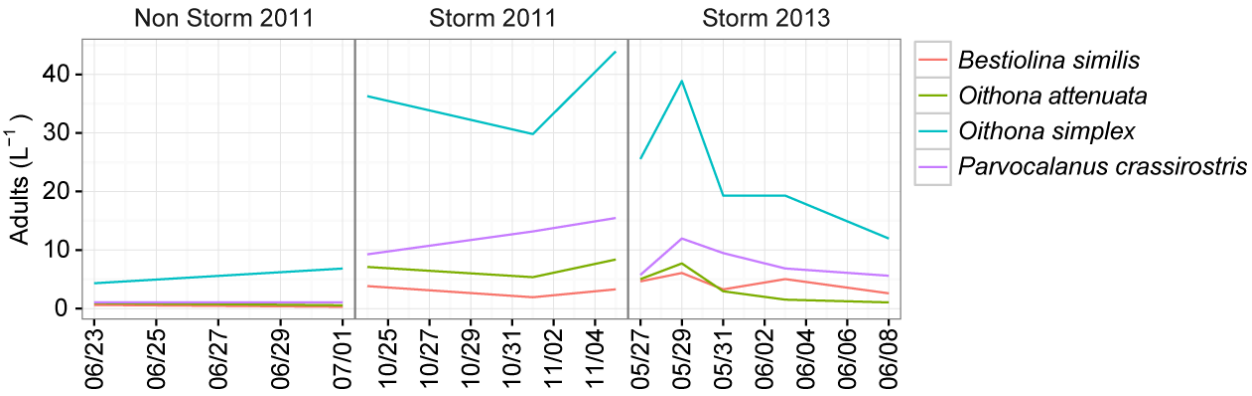
(H) May 28th, 2013, Day 1 post-storm



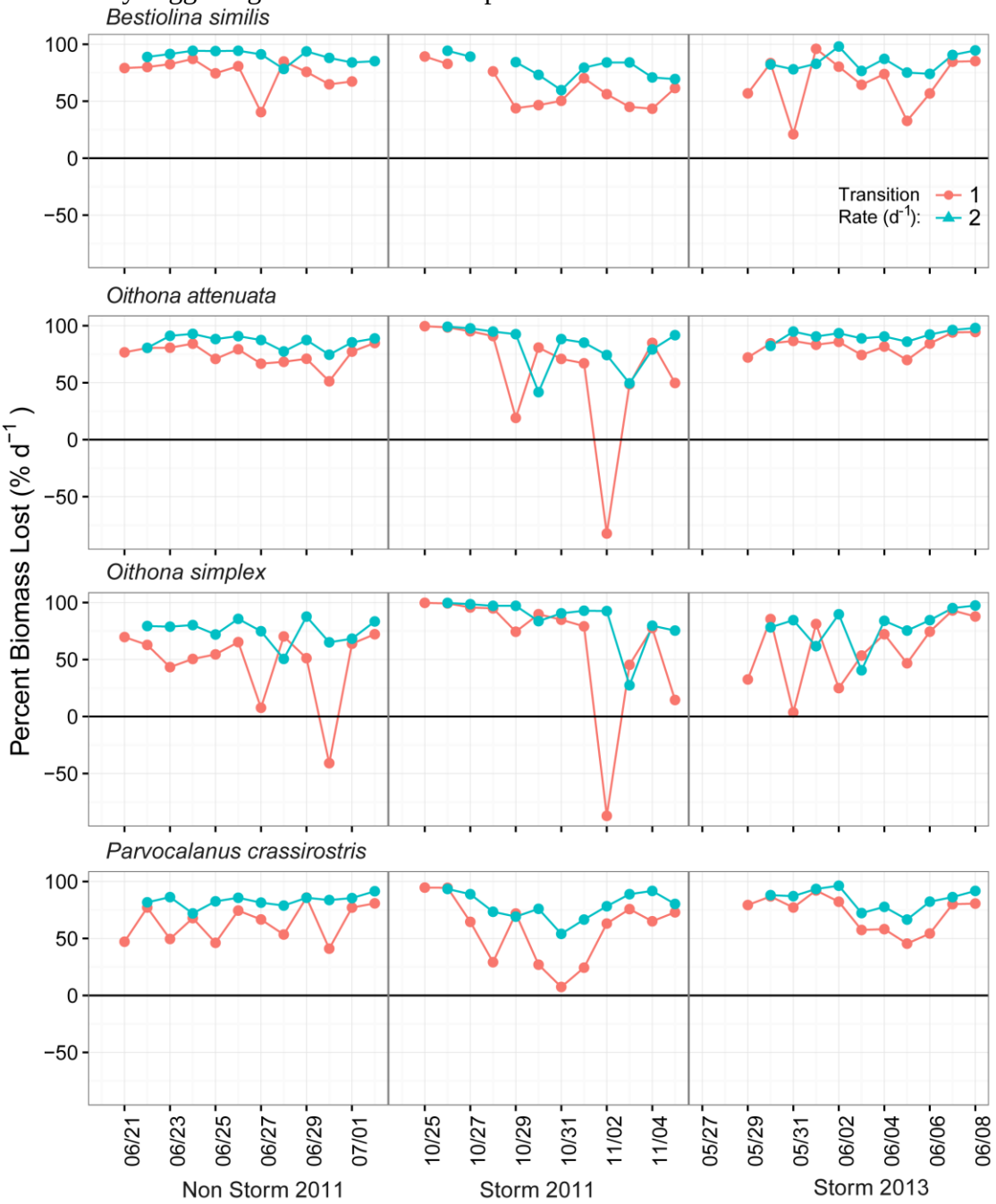
(I) June 6th, 2013, Storm 2013, day 10 post-storm



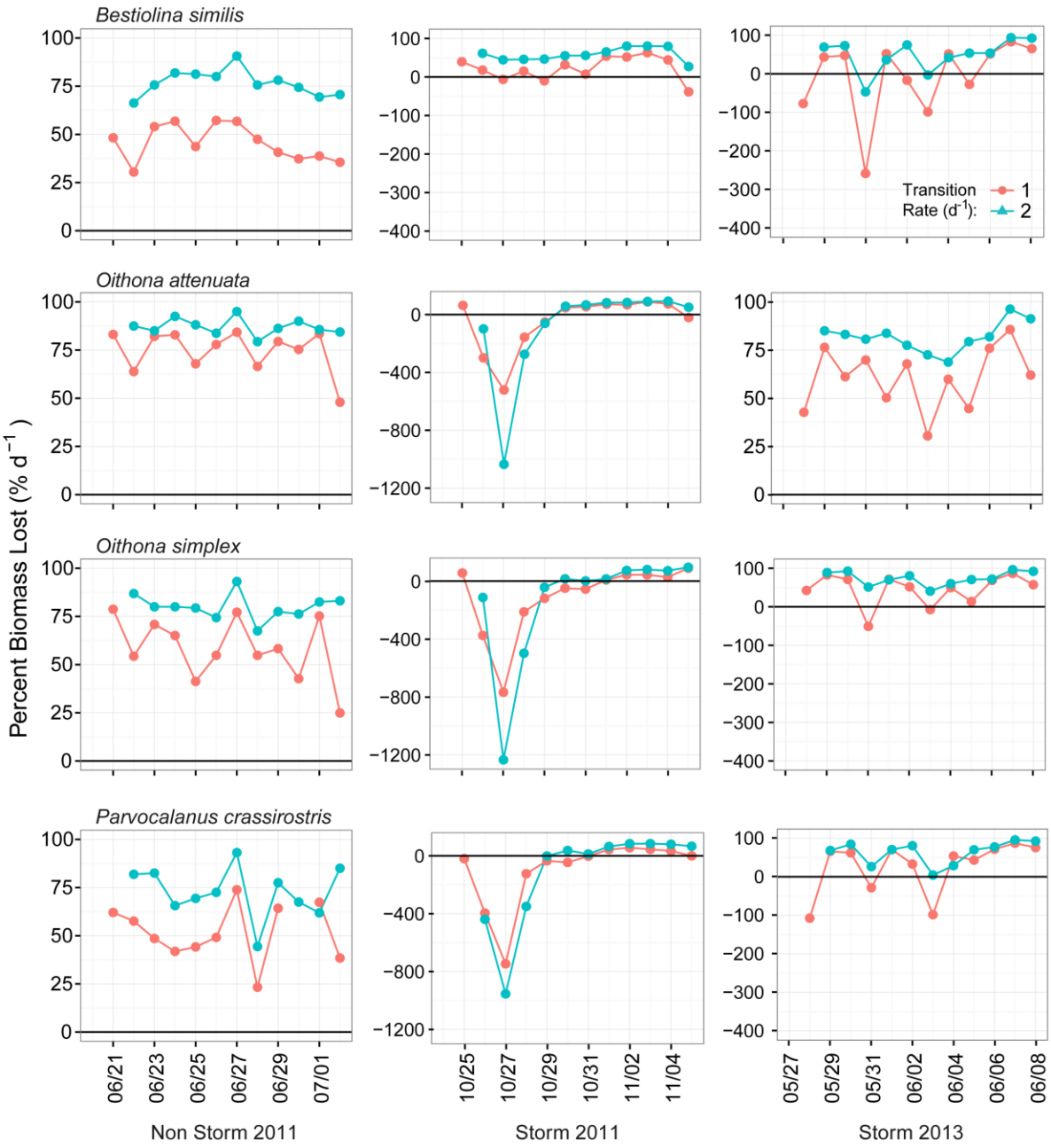
Supplementary Figure 5.2. Adult copepod abundances (number L^{-1}), by species, across all events.



Supplementary Figure 5.3. Percent biomass lost (positive) or gained (negative) of early-stage nauplii (from the 20µm to the 63 µm groups) over two transition times (*t*; 1 or 2 days) for each species (rows) during each event (columns). This assumes an average 20 µm naupliar biomass/animal of 0.02 µg C growing by 3.5 fold to 0.07 µg C in the 63 µm size group. Negative % mortality suggests growth relative to expected 3.5 fold increase in biomass from initial.



Supplementary Figure 5.4. Percent biomass lost (positive) or gained (negative) of the 63 µm mid-stage naupliar population disappearing per day over two transition times (*t*; 1 or 2 days) for each species (rows) during each event (columns). This assumes an average 63 µm naupliar biomass/animal of 0.07 µg C growing by 1.6 fold to 0.11 µg C in the 80 µm size group. Negative % mortality suggests growth relative to expected 3.5 fold increase in biomass from initial.



Chapter 6: Conclusions

6.1 Summary and Conclusions

The principal objectives of this work were to build upon current knowledge of the role of copepod nauplii in subtropical marine ecosystems where small plankton dominate, and to develop a greater understanding of the conditions under which nauplii play important roles in marine food webs. Copepod nauplii are a challenge to study in marine planktonic ecosystems due to their small size and morphological similarities across species, and as a result, have long been under sampled and largely ignored in studies of zooplankton community dynamics. With advances in imaging (Culverhouse et al. 2006; Le Bourg et al. 2015),genetic techniques (Durbin et al. 2008; Jungbluth et al. 2013), and staining technologies (e.g., (Henzler et al. 2010), the quantification and enumeration of small taxa is becoming more feasible in diverse marine ecosystems.

In diverse and rapidly changing plankton communities such as those in the subtropics, copepod nauplii can graze a substantial portion of potential prey, but the results can be biased if methods do not account for rates of natural variability in the environment. Through evaluation of methods for studies of naupliar grazing (Chapter 2), we found significantly lower estimates of ingestion with longer grazing incubations, and evidence for trophic cascades confounding confidence in results with longer incubation times. Previous work on adult copepods with natural prey communities found similar results (Mullin 1963; Roman and Rublee 1980; Tackx and Polk 1986), but our work was the first to investigate these grazing dynamics for grazing rate estimates in rapidly-developing subtropical nauplii and natural prey communities. In addition to trophic cascade effects occurring in the bottle over time, the development times in *P. crassirostris* N3-N4 nauplii are rapid, and they molt to the next stage within <24 h; therefore extended incubation durations result in additional unknowns associated with non-feeding during molting. As a result of this work, it is clear that short incubation times are necessary for more accurate ingestion rate estimates for nauplii of tropical and subtropical species.

Investigation of the grazing rates and trophic impacts of two co-occurring, closely related copepod nauplii show that similar species can have distinct impacts on prey communities (Chapter 3). In their naupliar stages, *P. crassirostris* and *B. similis* are morphologically

indistinguishable and are the same size, yet *B. similis* was more selective against 2-5 µm prey, and selective for larger prey when large prey was more abundant, while *P. crassirostris* was a less selective feeder. Previous studies on naupliar feeding support our evidence for differences in naupliar feeding preferences for different species, however, much of the prior work was conducted on temperate species and with monocultured prey (e.g., Bruno, 2012; Paffenhofer and Lewis 1989; Henriksen et al. 2007). The *in situ* population of *P. crassirostris* nauplii had a greater impact on the *in situ* prey population than the less abundant *B. similis*, and was capable of removing up to 12.9% of the Chl *a* standing stock and up to 8.7% of the total 2-35 µm prey population, illustrating that during periods of higher naupliar abundance nauplii can remove a substantial amount of prey populations. These results provide further support for the inference that species-specific trophic interactions are important to consider in marine food web models if we aim to predict the response of communities to changing conditions and plankton community structure.

Through development (Chapter 4) and application of a qPCR-based method for quantification of species-specific naupliar biomass from mixed plankton communities (Chapter 5), we found differences between species both in a measure of mortality and in estimates of naupliar recruitment in response to different ecosystem perturbations that suggest naupliar populations change on day to day timescales. Comparison of the qPCR-measured DNA copy number to animal biomass revealed a linear relationship, suggesting that qPCR can be used for quantitative estimates of species-specific biomass. We found that applying different equations relating body-size to biomass yields a wider range of biomass estimates than the application of this qPCR-based method, suggesting the method we developed is more accurate than traditional means of estimating animal biomass from body length. This study was the first to show that species-specific DNA copy number has a strong relationship with animal biomass for metazoans, and application of qPCR to quantify species-specific biomass has the potential for broad applications in studies of aquatic plankton communities. Quantitative PCR-based methods are already used to detect harmful algae (Zamor et al. 2012), native species (Wight et al. 2009), and invasive species in planktonic communities (Cary et al. 2014). Future work could extend the use

of qPCR to assess whether the relationship between amplification of a target gene and animal biomass is strong for other taxa.

With the knowledge that copepod nauplii could be important grazers and that different species could impact prey populations differently, we wanted to know how species contribute to the naupliar population *in situ* and how naupliar populations change in response to ecosystem perturbations (Chapter 5). Analysis of naupliar copy number variation over time revealed species-specific differences in early naupliar production rates, suggested differences in the rates of biomass lost between early and late naupliar stages and between species, and differences in the response of the naupliar community to different types of storm events. The proportions of early, mid and late naupliar stages during the non-storm period suggested that copepod naupliar production and mortality were approximately in equilibrium during the summer for three of the four species, (*B. similis* exception), where waters were well-mixed and there was little allochthonous input of nutrients to the coastal zone. However, during the otherwise stable summer period, the population of *B. similis* was in decline. Within the non-storm period, the dominant cyclopoid species, *O. simplex*, had the lowest average loss of biomass of the four species that may reflect that it has lower mortality rates than the other species. Prior work has suggested that lower overall mortality rates in cyclopoids permit their dominance in marine ecosystems (Eiane and Ohman 2004; Paffenhöfer 1993), and our work provides an additional example of a dominant cyclopoid species that may have lower naupliar mortality rates than co-occurring species.

Ecosystem perturbations such as storm events are important drivers of temporal variation in subtropical plankton communities through bottom-up forcing, and we found distinct responses of the copepod naupliar community to two different storm events. The first-flush storm of the rainy season, in particular, resulted in an order of magnitude increase in naupliar biomass relative to non-storm levels, only a fraction of which reached later developmental stages. These first-flush storms are an important source of productivity for coastal marine communities. The storm that followed recent rainfall (May-June 2013) resulted in only a fraction of the copepod numerical response, suggesting that more frequent storms may lead to reduced secondary productivity in the subtropics. However, the increase in chlorophyll suggested enhanced small

phytoplankton abundance. Perhaps more frequent storms provide resources for smaller microplanktonic grazers such as ciliates and dinoflagellates, which are a preferred food resource for many cyclopoid copepods that dominate the ecosystem. Therefore frequent rain events could further promote the dominance of cyclopoid copepods in these ecosystems. With expected changes in the magnitude and intensity of rainfall over this century, a decrease in the frequency of intense rain events could enhance coastal productivity by prolonging dry periods between each storm and may support higher populations of calanoid copepods that graze large phytoplankton blooms that would result from such infrequent flushing events. It would be interesting to investigate whether this trend is evident in the copepod population during wet and dry years, but few studies have performed a long-enough time series to investigate longer-term dynamics.

My dissertation research has shown that, despite being commonly under-studied, copepod nauplii can be important grazers in subtropical marine plankton communities, and that ecosystem perturbations lead to changes in species' mortality and recruitment rates that can promote shifts in species dominance in the naupliar community. Closely-related species with otherwise similar morphology had different selective predation preferences and were able to impact prey populations when naupliar abundances were high. Species-specific population dynamics were observed that provide new information on the biomass of nauplii in a mixed-species assemblage, and on temporal variation in the contribution of species to the naupliar community that suggests that nauplii have an increased role as grazers and prey after ephemeral storm events. While the current study focused on subtropical plankton, the genetic methods applied here have relevance to other study areas that are dominated by difficult to identify species complexes. In particular, the ability to measure DNA copy number to estimate animal biomass is an exciting new direction that could enable the application of community genetic techniques to estimate the biomass contributions of individual species more complex communities.

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