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Measuring copepod naupliar abundance in a subtropical bay using quantitative PCR

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Abstract Copepod nauplii are important in plankton food web dynamics, but limited information is available about their ecology due to methodological challenges. Reported here is a new molecular method that was developed, optimized, and tested in laboratory and field samples that uses quantitative PCR (qPCR) to identify and estimate the abundance of nauplii of the planktonic copepod, *Parvocalanus crassirostris*. The overall approach included collection of bulk zooplankton samples in the field, size fractionation to create artificial cohorts of relatively few developmental stages, obtaining DNA copy number for each size fraction by qPCR amplification of a target gene region, and estimation of the number of animals in each fraction through application of known DNA copy number across developmental stage. Method validation studies found that our qPCR-based approach has comparable accuracy to microscope-based counts of early developmental stages. Naupliar abundance estimates obtained using the two methods on cultured populations were similar; the regression of qPCR estimates on microscope-based counts resulted in a nearly 1:1 ratio (slope = 1.09). The qPCR-based method is superior to traditional identification and quantification methods for nauplii due to its higher taxonomic resolution, sensitive detection over a range of DNA quantities, and relatively high throughput sample processing.

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Introduction

Copepod nauplii are important members of the marine planktonic community and are often the most abundant component of the microzooplankton (Calbet et al. 2001; Lučić et al. 2003; Turner 2004; Safi et al. 2007). Microzooplankton (20–200 µm) are capable of grazing 65–75 % of primary productivity (Calbet and Landry 2004), but the specific contribution of copepod nauplii relative to protists is unknown. As grazers, nauplii are capable of feeding on a diverse assemblage of prey. They preferentially consume nanoflagellates over the smaller picoplankton, which are grazed in the absence of other prey (Turner and Tester 1992; Roff et al. 1995; Böttjer et al. 2010). The ability of nauplii to feed at the base of the food web is significant, as it may be an avenue by which carbon can be transferred from the microbial loop to higher trophic levels. In subtropical ecosystems, where many adult copepods are <1 mm in prosome length, the importance of crustacean microzooplankton is even greater since all naupliar and early copepodite stages fall into the microzooplankton category. Furthermore, the younger size classes are typically more abundant than the adult stages (e.g., Hoover et al. 2006; Böttjer et al. 2010) and may be capable of exerting considerable grazing pressure on pico- to nanoplankton-sized prey throughout the year. As prey, nauplii are equally important: invertebrate and vertebrate predators, in particular fish larvae, depend on them as food (Sullivan and Meise 1996; Eiane et al. 2002; Sampey et al. 2007).

The realization that microzooplankton, including copepod nauplii, are critically important in pelagic ecosystems

has led to changes in sampling and enumeration methods (e.g., Hopcroft et al. 1998; Gallienne and Robins 2001; Turner 2004). Nevertheless, the role of copepod nauplii in pelagic food webs continues to be poorly understood, in part due to difficulties in identifying and enumerating early life-history stages of copepods in mixed field populations using conventional microscopic methods. Behavioral studies on adults, copepodites, and nauplii underscore the importance of species identification for meaningful population studies. Species-specific behavioral patterns in swimming, feeding, mating, and escape behaviors in copepods, including closely related species, are well documented (Paffenhöfer and Lewis 1990; Tiselius and Jonsson 1990; Burdick et al. 2007; Waggett and Buskey 2007), and many of these behavioral differences are already present in the early nauplii (Paffenhöfer and Lewis 1989; Paffenhöfer et al. 1996; Titelman 2001; Titelman and Kiørboe 2003; Borg et al. 2012; Bruno et al. 2012; Bradley et al. 2013). These differences may contribute to the selective predation that has been seen in ichthyoplankton for copepodites and nauplii of certain copepod genera (Sampey et al. 2007; Paradis et al. 2012). Thus, the role nauplii play in the trophodynamics of the system may differ by species, making identification a priority.

Morphological identification of nauplii can be challenging and potentially inaccurate since there are relatively few morphological differences within taxonomic orders, and there are relatively few taxonomies for the earlier developmental stages of copepods (*but see* Björnberg 1966, 1967, 2001; Lawson and Grice 1973; Takahashi and Uchiyama 2007). As a result, nauplii of all species are often lumped into a single category for enumeration (Paul et al. 1991; Uye et al. 1996; Eiane et al. 2002; Lučić et al. 2003; McKinnon and Duggan 2003; Zervoudaki et al. 2007), or they are assumed to come from a single dominant species (e.g., Landry 1978; Castellani et al. 2008). New methods are required to identify nauplii to species in diverse field populations, in order to fully understand the role of copepod nauplii in pelagic food webs. Ideally, such methods also would enable more rapid data acquisition than microscopic identification of nauplii in preserved plankton samples.

Molecular methods permit accurate species identification (e.g., Kiesling et al. 2002; Bucklin et al. 2003; Lindeque et al. 2004; Savin et al. 2004; Holmborn et al. 2011) and could serve as the basis for new methods for identification and enumeration of nauplii. Here, we focus on quantitative real-time PCR (qPCR), targeting the mitochondrial cytochrome oxidase c subunit I (mtCOI) gene. This gene was selected because intraspecific genetic divergence is typically low in calanoid copepods (<4 % within species), while differentiation between species often ranges from ~9 to >25 % (Bucklin et al. 1999, 2003, 2010b). This characteristic underlies the utility of the mtCOI gene for DNA barcoding studies of zooplanktonic organisms (Bucklin et al. 1999, 2010a, b; Costa et al. 2007; Ortman et al. 2010) and enables qPCR amplification of a single species' DNA in a mixed plankton sample. QPCR-based methods are sensitive to low DNA quantities and highly specific to the gene of interest, but have seen limited application in marine zooplankton research. For metazoan plankton, qPCR has been previously used to detect the presence of rare and/or economically important meroplanktonic larvae in environmental samples (Vadopalas et al. 2006; Dias et al. 2008; Pan et al. 2008; Wight et al. 2009), to detect invasive species (Darling and Blum 2007; Bott et al. 2010), and in the analysis of gut contents for specific target prey (Troedsson et al. 2007, 2008; Durbin et al. 2008, 2012; Nejstgaard et al. 2008; Tobe et al. 2010; Cleary et al. 2012). Few studies have applied qPCR to estimate the abundance of metazoan plankton in field samples. In one such study, Mackie and Geller (2010) found high sensitivity of qPCR-based assays for detection of a single target species in plankton community DNA, with the capability of detecting a single nauplius. Results reported in Durbin et al. (2008) also suggest that a qPCR-based approach has promise, in that they found a linear relationship between body carbon and mtCOI copy number across developmental stage in copepods (N2-N6, *Acartia tonsa*), and high sensitivity of qPCR down to very low DNA copy number (5 mtCOI copies).

The qPCR method developed here targets the planktonic copepod *Parvocalanus crassirostris*, in the subtropical embayment, Kane‘ohe Bay, Hawai‘i. This species is common in subtropical coastal ecosystems worldwide (Lawson and Grice 1973; Hopcroft et al. 1998; McKinnon and Duggan 2001; Razouls et al. 2005–2012), and in some coastal bays, it is the most abundant taxon, contributing the largest fraction to total copepod biomass (e.g., Kingston Harbor, Jamaica, Hopcroft et al. 1998). This copepod species is one of four dominant copepods (calanoid and cyclopoid) at our field site in the southern region of Kane‘ohe Bay (Calbet et al. 2000; Scheinberg 2004; Jungbluth and Lenz 2013).

The overarching goal of this work was to develop and validate the accuracy of a novel qPCR-based approach to enumerating copepod nauplii in mixed plankton samples. We had five primary objectives: (1) to develop species-specific primers that amplify the target species in qPCR, and test the species specificity of amplification, (2) to optimize a sample treatment protocol for maximum reliable recovery and quantification of DNA from environmental samples, (3) to quantify mtCOI DNA copy number for each life stage of the target species, (4) to test whether food concentration had an effect on mtCOI copy number in nauplii, and (5) to validate this new method through comparisons of qPCR-based estimates of naupliar abundance to direct microscope counts of nauplii from both culture and field samples. Here,

we show that our new qPCR-based method has comparable accuracy to microscope counts of copepod nauplii and can be used to estimate the abundance of *P. crassirostris* early-stage nauplii in the field.

Materials and methods

Figure 1 illustrates an overview of our new qPCR-based method to quantify copepod naupliar abundance in mixed field samples with identification to the species level. First, samples from culture were used to determine the number of mitochondrial cytochrome oxidase subunit I (mtCOI) DNA copies in each developmental stage of our target species, and to finalize a size-fractionation protocol using different Nitex mesh sieves (Fig. 1, Part A). These data then were used to determine the expected mtCOI copy number for the 'average' individual in each size fraction (Fig. 1, 'x'), which is required to calculate the number of nauplii in a mixed field sample. Mixed plankton samples from the field (Fig. 1, Part B) were size fractionated, with DNA extracted from each fraction, and the number of mtCOI copies measured using qPCR. The number of nauplii in each size fraction was then estimated by combining these two types of data (field, laboratory).

Field sample collection

Plankton samples were collected from station S3 in Kane'ohe Bay, on the windward side of Oahu Hawaii (21°25′56″N, 157°46′47″W). Vertical net tows from 10 m to the surface were taken with a 0.5-m diameter, 64-µm

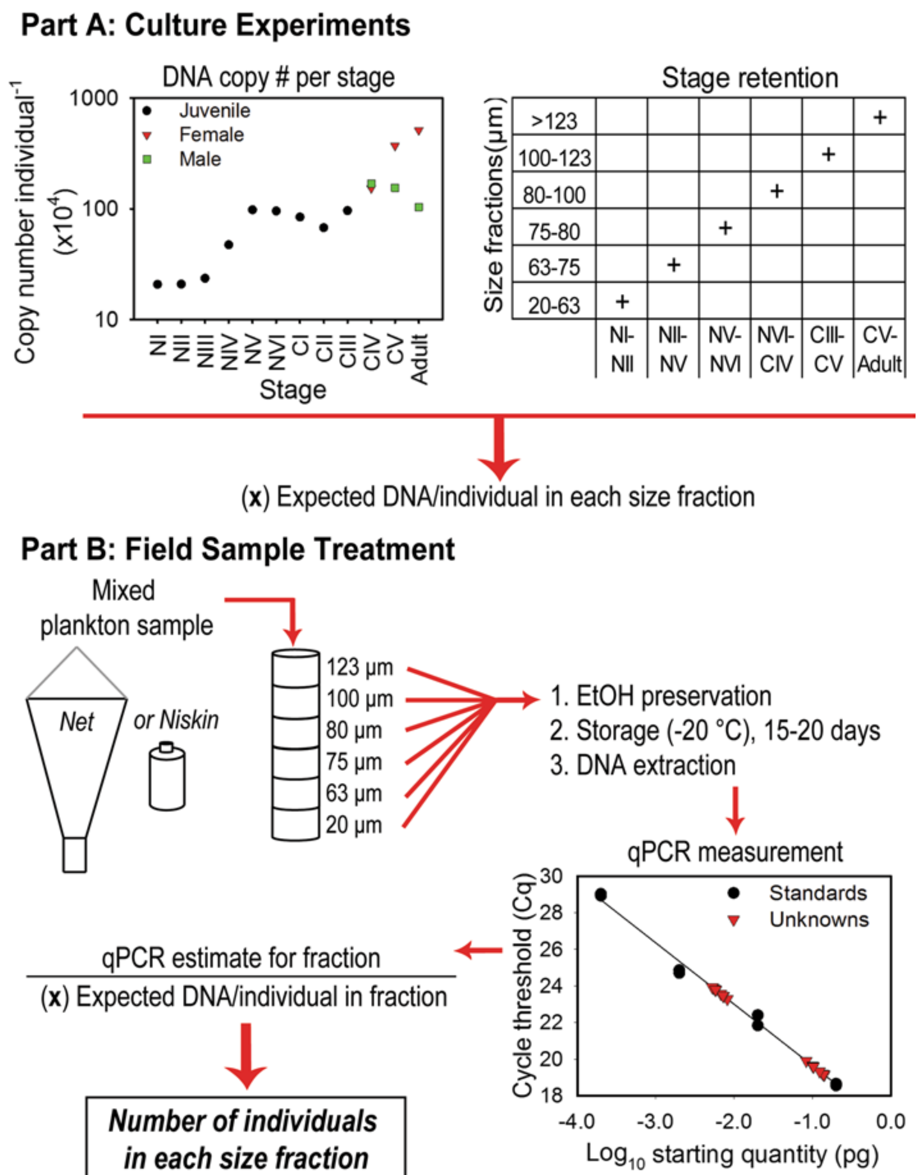

Fig. 1 Overview of our novel qPCR-based approach to identification and enumeration of nauplii. Estimating naupliar numbers in the field requires integration of measurements on field samples (Part B) with data from culture-based experiments (Part A). Part A (*top panel*): Culture experiments. The expected DNA individual^{-1} in a particular size fraction (x) is determined from the DNA copy number per stage and the size fractionation of stages across the 6 mesh sizes. Part B (*bottom panel*): Field sample treatment. To estimate the number of nauplii, the field sample is size fractionated (6 mesh sizes), preserved (EtOH) and stored for 15–20 days, DNA is extracted from each size fraction, and the DNA copy number in each size fraction is quantified using qPCR. This DNA copy number is divided by the expected DNA individual^{-1} for that size fraction (x) to estimate the number of individuals that were present in that size fraction in the field sample

mesh plankton net fitted with a General Oceanics 2030R6 flowmeter. Samples were concentrated and immediately preserved in 95 % non-denatured ethyl alcohol (EtOH), put on ice in the field, and then stored at −20 °C. The alcohol was changed once within 24 h of collection, to maintain the integrity of sample DNA (Bucklin 2000).

Copepod cultures

Parvocalanus crassirostris was maintained continuously in the laboratory on a diet of *Isochrysis galbana* (McKinnon et al. 2003; VanderLugt and Lenz 2008). The *P. crassirostris* culture was initially isolated from Kane'ohe Bay in 2008. Animals from this culture were used in experiments to determine size fractionation efficiency of mixed-stage samples, evaluate DNA extraction methods, determine the extent of DNA degradation during sample storage, quantify the mtCOI copy number at each developmental stage, and for method validation by direct comparison of qPCR-based estimates of naupliar abundance to microscope counts.

Species-specific primer design

mtCOI sequence data were obtained from the eight common copepod species present in Kane'ohe Bay as described in Jungbluth and Lenz (2013). For qPCR species-specific primer development, the DNA sequences of the most common haplotype for each of the eight Kane'ohe Bay copepod species as well as the potentially cryptic *P. crassirostris* lineage described in Jungbluth and Lenz (2013) were aligned to the primary lineage of *P. crassirostris* using ClustalW (Geneious v5.5.7). Sequence accession numbers for each species used for primer design are as follows: *P. crassirostris* (main lineage KC594153, cryptic lineage KC594157), *Bestiolina similis* (KC594124), *Labidocera* sp. (KC594137), *Acartia* sp. (KC594115), *Undinula vulgaris* (KC594160), *Oithona simplex* (KC594150), *Oithona attenuata* (KC594139), and *Oithona oculata* (KC594141). Regions of this gene with the highest differentiation between species were identified for primer design, and primers 15 to 24 bp in length that had maximum mismatch to non-target species (including the cryptic *P. crassirostris*) at their 3′ end were selected to amplify a 50–200 base pair (bp) amplicon in qPCR. Primers were initially analyzed in silico to test for potential amplification of non-target species DNA (using Amplify v3.1.4) and to evaluate the quality of the oligomer pair (using OligoAnalyzer v3.1). Candidate primers were then tested with conventional PCR to optimize the annealing temperature for maximal amplification of the target species and minimal non-target amplification. For conventional PCR tests, DNA was extracted from individual adult females using the lysis buffer method described in Lee and Frost (2002). PCR amplifications were performed in 25 µl reaction volumes using 2.5 µl 10× PCR Buffer minus Mg^{2+}, 1.5 mM $MgCl_2$, 0.3 µM of each primer, 0.2 mM of each dNTP, Invitrogen Taq polymerase (recombinant) at 0.05 units μl^{-1}, and 3 µl of template DNA. Reaction conditions included denaturing at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing temperature (various temperatures tested for optimal amplification) for 30 s, extension at 72 °C for 1 min, then the final extension step at 72 °C for 4 min. The final *P. crassirostris*-specific primers, PCOI424 (5′-GCG GGA GTA AGA TCA ATT CTA GGC-3′) and PCOI588 (5′-AGT AAT GGC CCC TGC TAA TAC GG-3′), produced a single 165-bp band for *P. crassirostris*, with no bands visible in the no-template control (NTC). The optimal annealing temperature was 65 °C for this primer pair.

qPCR protocols

qPCR was carried out on a Bio Rad iCycler IQ. Preliminary tests determined that the optimal primer concentration was 0.4 µM. The 165-bp region amplified by the *P. crassirostris*-specific primers (+4 bp on each end for more optimal binding) was used as a DNA standard. This standard was resuspended to a concentration of 100 µM, diluted eightfold to 10^{-6} pmol μl^{-1}, and aliquoted to minimize freeze-thawing. A single aliquot was used for each single plate and further diluted for the four-point standard curve. Each plate was run with the same four-point standard dilution series of the synthesized primer amplicon region and with a NTC, all in triplicate. Use of this standard allows direct quantitation of mtCOI gene copies in samples tested in qPCR. Reactions were carried out in 50 µl volumes, with each primer at 0.4 µM concentration, with 25 µl iQ SYBR Green Supermix (Bio Rad), 0.05–1.0 ng total DNA $well^{-1}$ (unless otherwise specified), and the remaining volume of nuclease-free water. Reaction conditions were 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, with melt curve analysis at the end of the experiment to assess purity of the amplicon. Baseline cycles were determined automatically by the iCycler software (v3.1). For all qPCR data analysis, the threshold level was adjusted manually to 150 RFU to allow comparisons of the sample threshold (Cq) across plates. If the standard deviation (SD) of the Cq for triplicate measurements of the same sample was greater than 0.5, the extreme value was considered an outlier and was excluded from analysis (as recommended by Bio-Rad). If the SD of the Cq for remaining duplicate wells after removal of outlying samples was still greater than 0.5, the entire sample was excluded from analysis (<2 % of samples). All remaining data were used in the analyses. The mean DNA copy number per individual for qPCR technical

replicates was used for statistical analysis. Statistical analyses are reported as (mean ± SD) unless otherwise noted.

DNA extraction

Consistent extraction of DNA from environmental samples is critical to the success of the overall approach, and a number of experiments were conducted to maximize the recovery of DNA. Experiments were conducted using cultured *P. crassirostris* and the species-specific primer pair to compare sample extraction methods, homogenization techniques, and solutions used during DNA elution. No significant difference in DNA quantity was found between samples homogenized using pestle-grinding vs bead-beating, so we do not report these experiments in detail here (see Jungbluth 2012 for a full description).

Experiments were conducted to compare two DNA extraction procedures: a lysis buffer method (Lee and Frost 2002), which has been found to be particularly useful on small amounts of starting tissue (Goetze unpub.), and the QiaAmp DNA mini kit (Qiagen). Both DNA extraction methods tested here are commonly used on metazoan plankton (e.g., Goetze 2005, Simonelli et al. 2009; Bucklin et al. 2010b; Durbin et al. 2012). In this experiment, three sets of 10 adult females were preserved in 95 % EtOH, stored at −20 °C for 15 days, placed into 500 µl of lysis buffer, and then ground by bead-beating. In the bead-beating protocol, 0.6 g of 1.0 mm and 0.2 g of 0.1 mm sterile silica–zirconium beads (BioSpec Inc.) were added to each tube (2.0 ml, screwcap), and all samples were subject to three cycles of bead-beating (BioSpec Minibeadbeater-16) for 40 s followed by 1 min on ice, then iced 5 min and centrifuged for 5 min at 14,000 rpm. The 400 µl supernatant was transferred to a new tube (microcentrifuge), incubated at 65 °C for 1 h, followed by 95 °C for 15 min, and stored at −80 °C until used in qPCR. For Qiagen extractions, three additional sets of 10 adult females were preserved in 95 % EtOH, stored for 15 days, placed into 500 µl of Buffer ATL (Qiagen) and ground by bead-beating (as above). DNA was then extracted following the QiaAmp DNA mini kit tissue extraction protocol, with a 16–20-h lysis step, all volumes scaled up for increased starting volume, with addition of RNAse A (Qiagen), and final elution in 400 µl autoclaved deionized (DI) water. All samples were run on a single qPCR plate.

We also tested four solutions for final elution of DNA. The QiaAmp kit protocol recommends DNA elution in AE Buffer. However, this buffer contains EDTA, which is known to interfere with SYBR Green chemistry (Demeke and Jenkins 2010). Therefore, a number of alternative elution solutions were tested to find a solution appropriate for use in qPCR. Sets of 10 adult females preserved in EtOH and stored for 15 days were extracted using bead-beating and the QiaAmp extraction kit, followed by elution of three biological replicates in 400 µl of each of the following four elution solutions: nuclease-free water (Ambion, pH 6.4), nuclease-free water (pH 7.2, pH adjusted with Molecular Grade NaOH), autoclaved deionized (DI) water (pH 7.0), and AE Buffer (Qiagen). All samples were measured on one qPCR plate.

Finally, given the intentions of the field program in which this qPCR method would be applied, samples needed to be preserved rapidly in the field and stored for up to 14 days prior to DNA extraction. The most commonly used sample fixative for molecular work on metazoans in field surveys is preservation and storage in 95–100 % EtOH (e.g., Bucklin 2000; Nagy 2010). Although EtOH preservation is known to be among the most reliable for recovery of high-quality DNA for storage times >1 year (e.g., Quicke et al. 1999; Mandrioli et al. 2006), there also have been reports that the success of this method varies depending on water content in the sample (Fukatsu 1999) and that DNA degradation over longer-term storage can occur (e.g., Reiss et al. 1995; Holzmann and Pawlowski 1996). Therefore, experiments were conducted to assess recovery of DNA over different storage times using samples from laboratory cultures. Triplicate sets of 10 adult females were preserved in 95 % EtOH and stored at -20 °C for 1 day, 22 days, and 41 days. After the specified storage times, each set was extracted using bead-beating and the QiaAmp tissue kit, with elution in 400 µl autoclaved DI water and final storage of DNA extracts at −80 °C.

Finalized sample treatment protocol

Based on results of the experiments described above (additional detail in Jungbluth 2012), all samples for the remaining experiments were extracted using the following procedure. Copepod samples were preserved in 95 % EtOH and stored at −20 °C for 15–20 days prior to DNA extraction. When testing mixed plankton samples or sets of juvenile copepods, the samples were centrifuged, transferred into a 2.0-ml screwcap tube, and centrifuged again for removal of excess EtOH. Samples were further dried by vacuum centrifugation and resuspended in 500 µl Buffer ATL. When testing sets of adults, the adults were placed directly into 500 µl Buffer ATL. After resuspension in Buffer ATL, the samples were bead beat as follows: 0.6 g of 1.0 mm and 0.2 g of 0.1 mm sterile silica–zirconium beads were added to each tube and all samples were homogenized with three cycles of bead-beating for 40 s and icing for 1 min. Samples were then iced for 5 min, centrifuged for 5 min at 14,000 rpm, and 400 µl of supernatant was transferred into a 1.7-ml sterile microcentrifuge tube. The remaining steps were followed as recommended by the QiaAmp DNA mini kit tissue extraction protocol, scaling up for increased

starting volume, with the following modifications: samples were incubated at 56 °C overnight for 16–20 h, the recommended RNAse A step was incorporated, and samples were eluted in 400 µl autoclaved deionized water. DNA extracts were then stored at −80 °C.

Testing species-specific amplification in qPCR

Species specificity of amplification during qPCR was tested across all 8 common copepod species in Kane'ohe Bay. Triplicate sets of 1–10 individuals of each species were extracted with the finalized sample treatment protocol (described above). The DNA concentration of these extracts was measured in a Qubit 2.0 Fluorometer (Invitrogen) using the high-sensitivity (HS) assay. Template DNA concentration was normalized and a total of 0.8 ng total DNA well^{-1} was used across all samples. Samples were run on a single qPCR plate.

mtCOI copy number across development stage

mtCOI copy number was measured across all developmental stages of *P. crassirostris*, in order to use these numbers to estimate the abundance of nauplii in environmental samples (as summarized in Fig. 1). Three biological replicates of 10 individuals of each life stage were obtained from laboratory cultures, preserved for 18 days, DNA extracted, and measured with qPCR. Developmental stages of *P. crassirostris* were identified according to Lawson and Grice (1973). The following equation was used to calculate the number of mtCOI DNA copies in an individual of each life stage from the pg of DNA measured in qPCR determined from the standard curve;

$$\text{DNA copy number} = \frac{\left[(\text{pg DNA}) \times \left(6.022 \times 10^{23}\right)\right]}{\left[165 \times \left(1 \times 10^{12}\right) \times 650\right]}$$

where 165 is the number of base pairs in the amplicon including primers and 650 is the average molar mass per base pair.

Maternal effects of food concentration on copy number in nauplii

Body size and dry weight are known to vary across environmental conditions in copepods (e.g., Durbin and Durbin 1978; Campbell et al. 2001; Vestheim et al. 2005), and there also may be environmentally dependent shifts in the number of mitochondria within cells. If this occurs, the number of mitochondrial gene copies (mtCOI) individual^{-1} will vary as a function of environmental condition. In order to determine whether food concentration affects the mtCOI copy number in nauplii (the target developmental period), an experiment was performed to test whether mtCOI copy number in non-feeding (NI) and late stage feeding nauplii (NVI) differs under high and low food conditions. Two equally dense (250 adults l^{-1}) *P. crassirostris* cultures were isolated from laboratory culture. For 7 days, each culture was fed every other day with either 2.5×10^5 or 2.5×10^6 cells ml^{-1} of *I. galbana*. On day 7, each was gently sieved (100 µm) to retain late-stage copepodites and adults only, which were placed back into fresh UV-sterilized seawater and again kept on the respective low and high food diets. Two days later, each culture was harvested onto a 20-µm sieve, preserved in 95 % EtOH, and stored in −20 °C until DNA extraction. Three sets of 10 individual NI and three sets of 4 individual NVI were picked from each preserved culture, extracted after 15 days of storage, and measured with qPCR at 0.4 ng total DNA per well.

Sample size-fractionation

In order to use this qPCR-based approach on mixed field samples, it was necessary to separate developmental stages so that a single large-bodied, late-stage animal would not dominate the signal of mtCOI copy number in samples containing primarily early-stage nauplii. Based on measurements of body size at each developmental stage for the target species (from McKinnon et al. 2003), it was determined that size fractionation should be sufficient to isolate early nauplii from mid- and late-stage nauplii. Preliminary experimentation with an array of mesh sizes determined that the following set of Nitex meshes, mounted in PVC, resulted in the most effective stage separation of cultured *P. crassirostris* (in µm); 123, 100, 80, 75, 63, and 20. To estimate the distribution of stages expected on each sieve, live mixed-stage culture was gently poured through sieves stacked from coarse to fine, and a 300–500 ml volume of GFF-filtered seawater was used to rinse the remaining animals to their proper size level. Each size fraction was preserved immediately in 95 % EtOH, and stage retention on each Nitex sieve was established by replicate counting of subsamples. The EtOH-preserved sample was thoroughly mixed and subsampled with a Stempel pipet into a Ward counting wheel. The final proportions of each stage in each sieve were determined by counting three size-fractionated samples from culture, with three replicate counts of each size fraction. Results of this experiment were used to establish a final sieving protocol for field samples that maximally separates *P. crassirostris* stages into ~2 primary developmental stages per Nitex mesh size.

The expected distribution of stages in each sieve was calculated from the count data for each size fraction. This was done in order to account for potentially large variations in the stage distribution across samples (field and culture) and assumes that in a particular size fraction, the

most abundant stage is likely to be the median stage captured by that size fraction. To calculate the expected distribution of stages in each sieve, the proportion of a single stage in a size fraction out of the total individuals of that stage in the sample was divided by the sum of the proportions of all stages retained in the size fraction. The expected distribution of stages across each sieve, along with the mean copy number individual^{-1} of each stage, was used to calculate the weighted mean (or expected) copy number individual^{-1} for that sieve range by multiplying the expected proportion of each stage by the copy number for that stage, then summing across all stages captured in the size fraction. The standard error associated with the weighted mean copy number individual^{-1} was calculated by taking into account both the error in the proportion of stages in each sieve, as well as the error in copy number per individual. The weighted mean copy number was then used in experiments with mixed-stage samples to estimate the number of individuals in a sieve by dividing the qPCR-measured total mtCOI copy number by the weighted mean copy number for that sieve.

Assessment of PCR inhibition in field samples

Compounds that inhibit the PCR reaction are common in mixed environmental samples (Wilson 1997: Demeke and Jenkins 2010). This experiment tested whether addition of a protein, bovine serum albumin (BSA), which is known to counteract some forms of inhibition (Kreader 1996) would increase the quantity of DNA measured in field samples. The effect of BSA on qPCR inhibition was tested by comparing measured DNA copy number in replicate measurements of environmental samples with and without addition of BSA. Triplicate plankton tows were collected from Station S3, each of which was size fractionated prior to EtOH preservation, and DNA was extracted from each of four size fractions. The four Nitex size fractions (20–63, 63–75, 75–80, and 80–100 μm) that contain nauplii of the target species were measured in qPCR for total DNA copy number both without BSA and when BSA was added to each qPCR well at 0.1 μg μl^{-1}. For the largest size fraction (80–100 μm), a higher concentration of 1.0 μg μl^{-1} also was tested to determine whether a higher concentration would be more effective at removing inhibition for these samples.

Method validation: using samples from culture

In this experiment, we tested the accuracy of the qPCR method by comparing estimates of naupliar abundance obtained from the new qPCR-based method to direct microscope counts for replicate size-fractionated samples taken from culture. Three samples were taken from the copepod monoculture, concentrated onto a 20-μm sieve and resuspended to 200 ml with GFF-filtered seawater, and then split in half quantitatively with a Stempel pipet. Each half was then individually size fractionated and all individual size fractions were preserved with 95 % EtOH. One size-fractionated half of each culture sample was preserved for 15 days at −20 °C, and DNA was extracted for qPCR, while the other half was subsampled for direct count comparisons. Counts were performed by subsampling 2–4 ml of well-mixed sample into a counting wheel using a Stempel pipet and enumerating the number of individuals in each subsample. For qPCR estimation of total copy number, 1 ng total DNA was added per well and the total number of mtCOI DNA copies was determined in each fractionated sample. The number of animals in qPCR-measured samples was estimated by dividing the total size fraction mtCOI copy number by the weighted mean copy number individual^{-1} for that size fraction (as described above, and in Fig. 1).

Method validation: using field samples

Testing the qPCR method with field samples is more complicated than using laboratory monocultures, because the species identity of field-collected nauplii cannot be determined morphologically (between *B. similis* and *P. crassirostris*, Kane‘ohe Bay). Environmental samples were split and size fractionated, and prepared either for microscope counts or for qPCR measurements. For microscope counts, the total number of calanoids (including both *Bestiolina* and *Parvocalanus*) was determined for each size fraction. The proportion of early copepodites of *P. crassirostris* present in the sample (from the 100–123 μm size fraction) was obtained using a multiplex PCR method (described below). This proportion was then used to estimate the number of *P. crassirostris* within each size fraction and provided a ‘count’-based estimate of abundance that was compared to the qPCR method to assess accuracy of the new molecular approach.

On September 18, 2011, triplicate 10-m vertical plankton tows were conducted at Station S3 with a 64-μm mesh net. Each tow was concentrated onto a 63-μm mesh sieve, resuspended to 200 mL in GFF-filtered seawater, and split quantitatively using a Stempel pipet. Each half was size fractionated with a 123, 100, 80, 75, and 63-μm sieve tower, and all samples were preserved in 95 % EtOH and placed on ice. One half of each triplicate tow was used to count the number of calanoid nauplii, calanoid copepodites, cyclopoid nauplii, and cyclopoid copepodites in each fraction by subsampling with a Stempel pipet and enumerating under a microscope using a counting wheel (as above). From a 100–123 μm net collected size fraction, 96 individual calanoid copepodites (CI–CII) were

randomly selected by taking a well-mixed subsample and picking every calanoid that could be identified as a CI or CII. The DNA from each of these was extracted using the lysis buffer protocol, and identified as either *P. crassirostris* or *B. similis* using multiplex PCR (see below). The ratio of *P. crassirostris* to *B. similis* CI–CII was determined by scoring specimens to species on the agarose gel. This ratio was multiplied by the total calanoid copepods counted in the 80–100 μm size fraction to determine the expected number of *P. crassirostris* in this size fraction of each sample. The 80–100 μm size fraction was analyzed and described here since it is the most appropriate for comparison to the ratios derived from multiplex PCR of copepodites. The remaining half of each triplicate net sample was concentrated and extracted after 17 days of preservation with the optimized qPCR sample treatment protocol and stored at −80 °C. For the 80 μm net size fraction, extract DNA concentration was measured with a fluorometer, 1.0 ng of DNA was added per qPCR well, and triplicate wells of each sample were measured in qPCR. Total DNA measured per sample (pg) was converted to total mtCOI copy number, and this was divided by the weighted mean copy number expected for that size fraction to get the qPCR-estimated number of individuals in each sample.

A multiplex species-specific PCR protocol was designed to discriminate between individuals of *P. crassirostris* and *B. similis,* the two dominant calanoid copepods at station S3. DNA sequences (>1,000 bp) obtained using primers Primers L1384 and H2612 (Machida et al. 2004) (42 °C annealing temperature) were aligned, and a common forward primer (PCBS [5′—CTG GTA TAA TTG GAA CAG G—3′]) was designed that is identical to *B. similis* and has a 1-bp difference from *P. crassirostris*. Species-specific reverse primers were designed (PCRP [5′—ACT CCC GCA AGG TGT AAA G—3′], BSRP [5′—GAG CTC ATA CTA CAA ATC CTA AC—3′]) such that if an unknown specimen is *P. crassirostris,* a 388-bp region of DNA is amplified, and if the specimen is *B. similis*, a 792-bp region is amplified. Multiplex PCR amplification conditions were as described above (in *Species-Specific Primer Design*), with a 54 °C annealing temperature. The distinct PCR product length allows differentiation between these two morphologically similar copepods by simple visualization of these PCR products on an agarose gel. This multiplex PCR protocol was used to identify unknown copepodites to species in the method validation experiments described above. Copepodites of these two species can be distinguished morphologically from other calanoid species in the bay (e.g., *U. vulgaris*, *Acartia* sp.), but not easily from each other, so this approach was tested only to discriminate between these two species.

Results

qPCR run statistics

Validity of qPCR data is typically assessed by amplification efficiency, which is based on the slope of the standard curve and the quality of within-plate technical replicates. Amplification efficiencies in qPCR experiments ranged between 91 and 108 %, within the acceptable range (Bustin et al. 2009). A single experiment, comparing different concentrations of BSA, had an amplification efficiency of 86 %. However, these results were used only qualitatively for comparisons across samples of different treatments that were run on the same plate and thus are acceptable for this purpose. Quantification of DNA from unknown samples is based on the standard curve, which is a linear plot of the Cq against the $\log_{10}$ starting quantity (log SQ) of DNA, based on known quantities of DNA in a dilution series of the qPCR standard. All standard curves had regression coefficients (r^2) greater than 0.99. In all cases, the NTCs either did not amplify, or the Cq was at least six cycles higher than the lowest concentration of the standard curve, which would not interfere with quantitative estimates of mtCOI DNA copy number. In all cases, melt curve analysis showed one strong peak, indicating a single PCR product. Within-plate measurements of sample replicates had an average coefficient of variation (C.V.) for Cq values of 1.1 % (range 0.12–3.6 %); the across-plate sample replicate average C.V. was 16 % (range 1.3–33 %). The level of variability observed across plates is expected given the sensitivity of the qPCR reaction and within the range reported by other studies for qPCR inter-assay variability (Karlen et al. 2007; Saikaly et al. 2007).

qPCR tests of species specificity in amplification

Although primer specificity initially was tested with conventional PCR, the species-specific primers also were tested in qPCR to determine the level of amplification of non-target DNA. The mean Cq for the target species (*P. crassirostris*) was 19.5, which is 15 cycles earlier than the mean non-target Cq of 34.8 (Fig. 2; all samples with equal amounts of template DNA). An increase in 3 amplification cycles is equal to approximately an order of magnitude less DNA amplification; therefore, all non-target samples amplified at a rate equivalent to 1/10,000 of one adult and at 1/1,000 of a single nauplius of the target species. These results demonstrate that non-specific amplification using the primer set PCOI424 and PCOI588 was sufficiently low that this would not interfere with quantitative estimates of mtCOI copy number of the target species *P. crassirostris*.

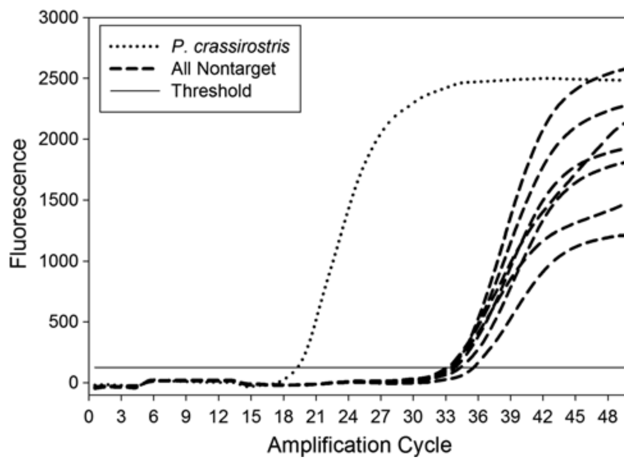

Fig. 2 qPCR primer specificity to the target species, *P. crassirostris*. Non-targets include all planktonic copepod species reported to occur in Kane'ohe Bay (see 'Materials and methods'); 0.08 ng of template DNA was used for each sample. The point where each curve crosses the threshold line is the Cq value for that sample. Non-target species amplified at approximately 0.01 % of one adult and 0.1 % of one nauplius of the target species (mean Cq for target species was 19.5, while non-targets ranged from 34.1 to 35.4). Fluorescence is expressed as a relative measure of fluorescence units

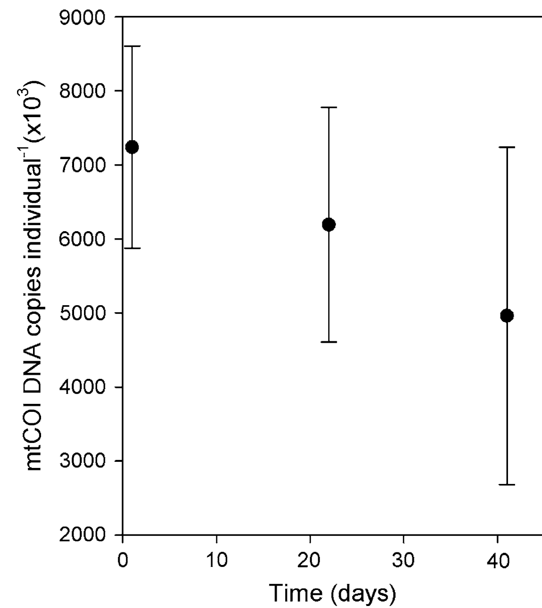

Fig. 3 Effect of the duration of sample storage on DNA copy number individual^{-1}. Samples were preserved for 1 day, 22 days, and 41 days in 95 % EtOH and stored at −20 °C prior to DNA extraction. Error bars represent the standard deviation (SD) of triplicate samples measured ($N = 10$ adult females in each sample)

DNA extraction

The QiaAmp extraction protocol resulted in a significantly higher mtCOI copies individual^{-1} measured in qPCR in comparison with the lysis buffer extraction method, (mean ± SD of $4.3 \times 10^6 \pm 1.1 \times 10^6$ and $2.0 \times 10^6 \pm 0.7 \times 10^6$, respectively; *t* test, $P = 0.023$). However, different elution solutions showed no significant difference in mean copies individual^{-1} (one-way ANOVA $F_{(3,\ 8)} = 0.62$, $P > 0.621$). Autoclaved deionized water resulted in the highest mean measurement of mtCOI copy number individual^{-1} (4.3×10^6) and had the lowest overall C.V. (25 %, Table 1). Autoclaved dH_2O was chosen for use in all subsequent experiments.

In experiments to test for DNA degradation during storage, an overall decrease in mean mtCOI copies individual^{-1} was observed with increasing storage times (Fig. 3). However, these differences were non-significant, given high variation across replicate samples preserved for the same length of time (SD range 1.4×10^6 to 2.3×10^6; one-way ANOVA, $F_{(2,6)} = 1.35$, $P = 0.328$). The decline in the mean measured mtCOI copy number over time with preservation in 95 % EtOH and storage at −20 °C was 14 % after 22 days, and 31 % after 41 days. Given the observed decrease in mean copy number,

Table 1 Comparison of four elution solutions: autoclaved deionized water, nuclease-free water (pH 6.4), nuclease-free water (pH 7.2), and buffer AE. Results are mean and standard deviation (SD) of each biological replicate, the overall mean copy number individual^{-1}, as well as the overall coefficient of variation (C.V.) for each treatment

Elution solution	Mean mtCOI DNA copies individual^{-1} ($\times 10^6$)	SD ($\times 10^6$)	Overall mean copies individual^{-1} ($\times 10^6$)	Overall C.V. (%)
Autoclaved deionized water	4.9	1.2	4.3	25
	5.0	0.38		
	3.4	0.59		
Nuclease-free water (pH 6.4)	4.5	0.11	2.9	47
	1.4	0.47		
	3.3	0.20		
Nuclease-free water (pH 7.2)	3.1	0.46	4.1	28
	4.9	1.5		
	4.1	0.46		
Buffer AE	5.0	1.5	3.2	61
	1.0	0.14		
	3.7	0.50		

samples to be compared in all experiments reported here were consistently extracted after a standard storage time of 15–20 days.

mtCOI copy number across developmental stage

The mean (±SD) number of mtCOI gene copies for *P. crassirostris* was found to increase from 0.21 × 10^6 (±0.03 × 10^6) copies individual^{-1} at the first nauplius stage (NI) to 5.1 × 10^6 (±1.9 × 10^6) copies individual^{-1} in the adult female (Fig. 4; Table 2). Mean copy number changed significantly over naupliar development (ANOVA, $F_{(5, 12)} = 107.30$, $P < 0.001$). A post hoc Tukey test showed that naupliar stages NI, NII, and NIII (0.21 × 10^6, 0.21 × 10^6, and 0.24 × 10^6 copies individual^{-1}, respectively) were significantly different from NIV ($P = 0.005$) (0.47 × 10^6 copies individual^{-1} NIV) and that NIV was significantly different from NV and NVI ($P < 0.001$) (0.98 × 10^6 and 0.95 × 10^6 copies individual^{-1} NV and NVI, respectively). However, NI, NII, and NIII were not significantly different from each other ($P > 0.992$), nor were NV and NVI different from each other ($P = 0.997$). No significant differences in copy number were found between late nauplii and early copepodites (NV through CIV; Tukey Test, $P > 0.050$), or between any sequential life stages above the CI (e.g., between CIV and CV, or CV and adult female). Interestingly, after the CIV stage, the copy number for males and females diverged, and there was a significant difference between the CIV female and adult female ($P = 0.007$) as well as a significant difference between adult females and adult males (5.1 × 10^6 and 1.0 × 10^6 copies individual^{-1}, respectively; Tukey Test, $P = 0.021$).

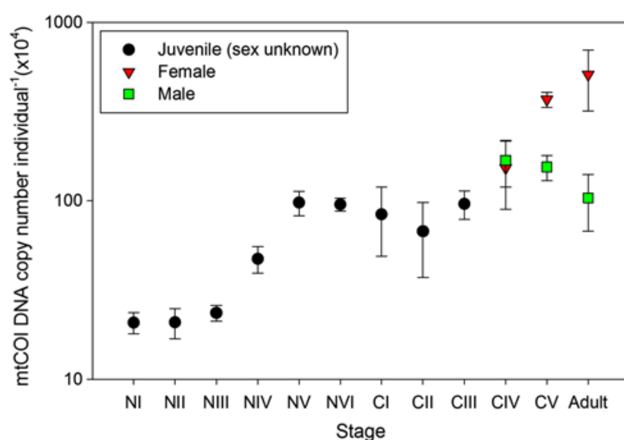

Fig. 4 Mean mtCOI DNA copy number individual^{-1} for each developmental stage in *P. crassirostris*. NI through CV correspond to all naupliar and copepodite stages. *Black circle* juvenile of unknown sex, *red diamond* female, *green square* male. *Error* is standard deviation (SD) of triplicate samples

Maternal effects of food concentration on mtCOI copy number in nauplii

For the earliest stage nauplii (NI, non-feeding), food concentration had no significant effect on mtCOI copy number (*t* test, $P > 0.173$). Mean (±SD) mtCOI copy numbers individual^{-1} for the early nauplius stage NI were 0.50 × 10^6 (±0.06 × 10^6) and 0.40 × 10^6 (±0.06 × 10^6) for high and low food experiments, respectively. In the late nauplius stage NVI, mean (±SD) copy numbers individual^{-1} for each treatment were 1.6 × 10^6 (±0.99 × 10^6) and 0.56 × 10^6 (±0.39 × 10^6) for high and low food experiments, respectively. For the NVI stage, the difference in means for the two treatments was non-significant (*t* test, $P > 0.162$); however, the trend in the means suggests that food concentration may affect mtCOI copy number for feeding stages. The lack of significance may be due to low statistical power (power = 0.19); therefore, we consider it conservative to conclude that there might be an effect of food condition on mtCOI copy number.

Sample size-fractionation

Size fractionation was necessary to separate life stages of the target species in order to accurately estimate the number of individuals in a mixed field sample. Of a range of 8 mesh sizes tested for their ability to separate life stages of the target species, sieve sizes of 20, 63, 75, 80, 100, and 123 µm provided maximum separation of naupliar stages of *P. crassirostris* using samples from culture. Using this combination of sieve sizes, we found that the 20–63 µm size fraction captured primarily NI and NII, the 63–75 um size fraction captured NI, NII, NIII, NIV, and NV, with the majority of animals in stages NII–NV, the 75–80 µm size fraction captured NV and NVI, and the final sieve size, 80–100 µm, captured NVI nauplii (Table 3A). qPCR experiments focused on size fractions 20, 63, 75, and 80 µm, since these retained the naupliar stages of the target species. The 100 and 123 µm size fractions primarily contained mid-to-late copepodites and adults that could be identified morphologically, or differentiated to species (*Parvocalanus*, *Bestiolina*) using multiplex PCR.

The expected contribution of a stage to the total animals within a sieve (Table 3B) was calculated based on the retention values reported in Table 3A. For example, from Table 3A, the expected contribution of NIV to the total animals in the 63–75 µm size fraction is 0.981/[0.316 + 0.865 + 0.950 + 0.981 + 0.628 + 0.023] = 0.261 ± 0.005 (±2 × standard error), which is the expected proportion of the total animals within the 63–75 µm size fraction that are NIV. The values shown in Table 3B and the mean DNA copy number individual^{-1} of each stage were used to estimate a weighted mean mtCOI DNA copy

Table 2 The mean mtCOI DNA copy number individual^{-1} measured for life stages of *P. crassirostris* (this study) compared to copy number reported for *A. tonsa* by Durbin et al. (2008), as well as the range in copy number for both studies (applying the standard deviation to the mean)

Stage	Sex	*Parvocalanus crassirostris* mtCOI copy # ($\times 10^6$)	Range ($\times 10^6$)	*Acartia tonsa* mtCOI copy # ($\times 10^6$)	Range ($\times 10^6$)
NI	–	0.21	0.18–0.24	0.20	0.20–0.25
NII	–	0.21	0.17–0.25	0.14	0.12–0.17
NIII	–	0.24	0.21–0.26	0.27	0.20-0.31
NIV	–	0.47	0.39–0.55	0.41	0.27–0.66
NV	–	0.98	0.82–1.1	1.1	0.62–1.9
NVI	–	0.95	0.88–1.03	1.6	1.6–1.7
CI	–	0.84	0.49–1.2	–	–
CII	–	0.68	0.37–0.98	–	–
CIII	–	0.96	0.79–1.1	–	–
CIV	Female	1.5	0.90–2.2	–	–
	Male	1.7	1.2–2.2	–	–
CV	Female	3.7	3.3–4.1	–	–
	Male	1.5	1.3–1.8	–	–
Adult	Female	5.1	3.2–7.0	17	6.9–30
	Male	1.0	0.68–1.4	–	–

number individual^{-1} (±SE) of the target species for each sieve range; with the 20–63 μm fraction at 0.21 × 10^6 (±0.10 × 10^6) mtCOI copies individual^{-1}, the 63–75 μm fraction at 0.42 × 10^6 (±0.08 × 10^6) copies individual^{-1}, the 75–80 μm fraction at 0.91 × 10^6 (±0.22 × 10^6) copies individual^{-1}, and the 80–100 μm fraction at 0.98 × 10^6 (±0.27 × 10^6) mtCOI copies individual^{-1}. The weighted mean, or expected copy number individual^{-1} was used to estimate the number of nauplii, as demonstrated by Fig. 1, by dividing the qPCR-estimated total mtCOI DNA in that size fraction by the expected copies individual^{-1}. Use of these values will underestimate the number of individuals in a particular sieve if there are an unusually high number of small individuals (earliest stages retained) or a low number of intermediate-sized individuals relative to a stable age distribution for the stages in that sieve size.

Assessment of inhibition

Addition of BSA at 0.1 μg μl^{-1} reduced inhibition of amplification during qPCR of all size fractions in mixed environmental samples (Nested ANOVA, $F_{(4,16)} = 6.35$, $P < 0.003$; Fig. 5). A higher concentration of BSA, 1.0 μg μl^{-1}, did not result in higher mtCOI copy number in the 80–100 μm size fraction (Fig. 5). There was some variation across samples. For the smallest size fraction (20–63 μm; Fig. 5A), there was no effect due to the addition of BSA. However, in all other size fractions there was an increase in amplification with the addition of BSA (Fig. 5B–D). In addition, samples taken on different days seemed to be affected differentially. In the absence of BSA, amplification of sample *a* was more inhibited in 3 of 4 size fractions than that of samples *b* and *c*. The 20–63 μm size fraction contained copepod nauplii and detrital material only, which is a potential reason for there being no clear difference after the addition of BSA to this size fraction. The larger size fractions contained a mix of copepods and other planktonic organisms, increasing the diversity of material potentially able to inhibit the PCR reaction. Since addition of BSA either increased DNA measurement or had no effect, we concluded that BSA should be added at a concentration of 0.1 μg μl^{-1} when using qPCR to measure any field samples as a precaution against chemical inhibition.

Method validation: using samples from culture

Abundance estimates from the qPCR method and direct microscope counts were compared for three samples obtained from the copepod monoculture, each of which had four size fractions (Fig. 6). Overall, there was a close relationship between qPCR and count-based estimates over >2 orders of magnitude in naupliar abundance (range 22–3,390; Fig. 6). The linear regression of naupliar abundance estimated from qPCR on microscope counts for all size fractions was highly significant ($r^2 = 0.93$, $F_{(1,10)} = 135.36$, $P < 0.001$), with nearly a 1:1 correspondence between measurement methods (regression slope = 1.09). The overall C.V. of the technical replicates of qPCR measurements ranged from 4.3 to 32 % (mean = 16 %), and the C.V. for technical replicates of counts ranged from 2.9 to 47 % (mean = 16 %), showing almost equal error for both methods. Across all size fractions, qPCR estimated 73–120 % of the number from microscope counts in 8 of 12 size fraction comparisons. In 4 samples, qPCR estimates differed substantially from counts, ranging from 32 to 270 % of the count estimates.

Table 3 Size fractionation of *P. crassirostris* developmental stages using sieves of 5 mesh sizes (20, 63, 75, 80, and 100 μm). Sieves of 100 μm and greater captured primarily copepodite stages and are not shown

Sieve Fraction (μm)	NI	NII	NIII	NIV	NV	NVI
A.						
20–63	0.684 (±0.633)	0.135 (±0.113)	0.048 (±0.031)	0.001 (±0.002)		
63–75	0.316 (±0.633)	0.865 (±0.113)	0.950 (±0.032)	0.981 (±0.018)	0.628 (±0.209)	0.023 (±0.046)
75–80			0.002 (±0.004)	0.016 (±0.018)	0.356 (±0.195)	0.528 (±0.089)
80–100				0.002 (±0.003)	0.016 (±0.017)	0.449 (±0.120)
Total counted	27	172	1835	1781	1522	403

Sieve fraction (μm)	NI	NII	NIII	NIV	NV	NVI	CI	CII	CIII	CIV
B.										
20–63	0.788 (±0.730)	0.155 (±0.131)	0.055 (±0.036)	0.001 (±0.002)						
63–75	0.084 (±0.168)	0.230 (±0.030)	0.252 (±0.009)	0.261 (±0.005)	0.167 (±0.055)	0.006 (±0.012)				
75–80			0.002 (±0.003)	0.012 (±0.014)	0.280 (±0.153)	0.415 (±0.070)	0.249 (±0.095)	0.043 (±0.021)		
80–100				0.000 (±0.001)	0.004 (±0.004)	0.119 (±0.032)	0.182 (±0.032)	0.251 (±0.007)	0.266 (±0)	0.177 (±0.177)

(A) Mean proportion of a particular naupliar stage captured in each sieve ±2 × standard error (SE). (B) Expected distribution of stages within each size fraction ±2 × SE, calculated from the total proportions of a stage from the vertical sieve retention of that stage for *P. crassirostris*

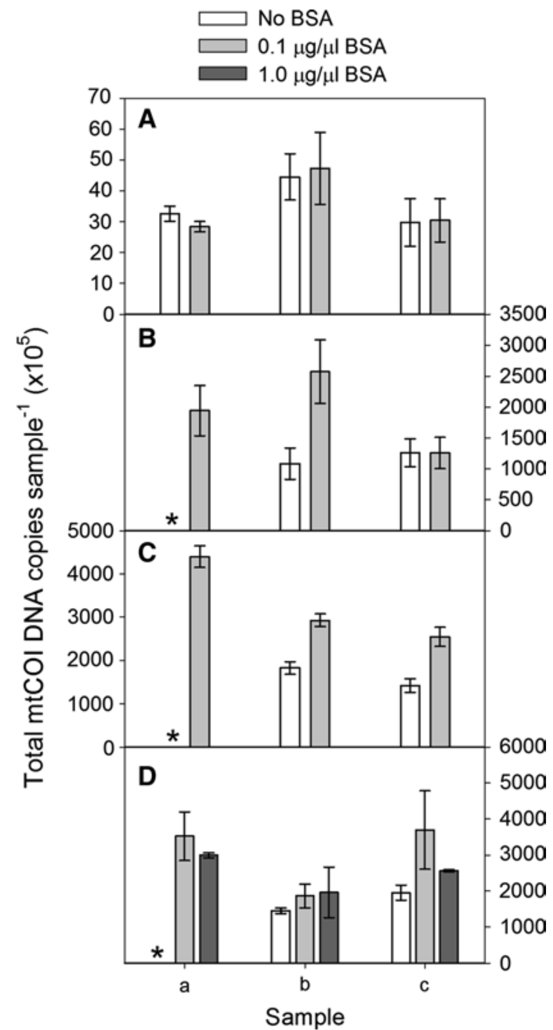

Fig. 5 Testing for qPCR inhibition in field samples. qPCR amplification of size-fractionated field samples. **A** 20–63 μm size fraction, **B** 63–75 μm size fraction, **C** 75–80 μm size fraction, and **D** 80–100 μm size fraction. Bovine serum albumin (BSA) was tested at 0.1 μg μl^{-1} (*light gray*), 1.0 μg μl^{-1} (*dark gray*), and without BSA (*white*). Samples *a*, *b*, and *c* are individual samples collected from the field on separate days. *Error* shown is standard deviation (SD) of mean qPCR measurements for each sample ($n = 3$). *Asterisk* sample was omitted from analyses due to high SD across technical replicates

Method validation: using field samples

Using multiplex PCR, the proportion of juvenile *P. crassirostris* to total calanoids was found to be 19 % based on 84 successful PCR amplifications out of 96 randomly selected individual paracalanid CI and CII copepodites. This ratio was used to calculate the abundance of *Parvocalanus* out of the total calanoids counted in each size fraction, for comparison to the qPCR estimated number of individuals in field samples (Table 4). In the 80–100 μm size fraction, which contained largely the same developmental stages that were used to determine the 0.19 proportion, the abundance

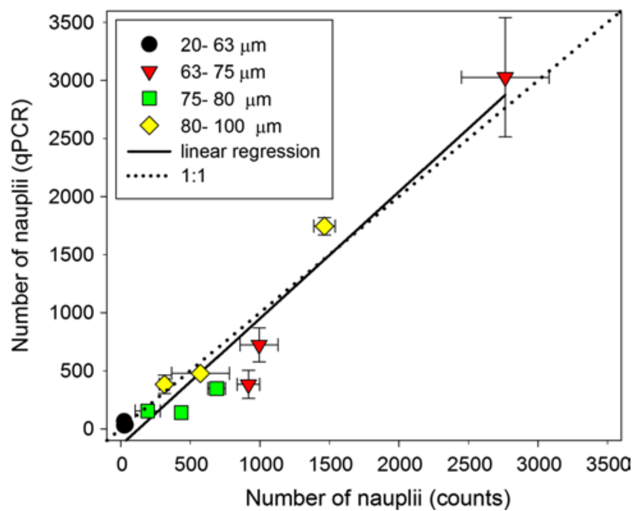

Fig. 6 Method validation. Direct comparison of microscope counts to qPCR-based estimates of the number of *P. crassirostris* nauplii in four size fractions in samples from culture. Samples were quantitatively split at initial collection, and ½ of the sample was processed using each method (qPCR, microscope counts). Dotted diagonal line represents a 1:1 relationship between counts and qPCR estimates. *Black circle*: 20–63 µm size fraction, *red triangle*: 63–75 µm, *green square*: 75–80 µm, *yellow diamond*: 80–100 µm. *Error bars* indicate the standard deviation of the mean ($n = 3$). The linear regression is described by the equation $y = 1.09x–141.22$, $r^2 = 0.93$ ($P < 0.001$)

estimates of the target species by qPCR gave very close comparisons to counts (with the 0.19 ratio applied), with no significant difference between the two methods (ANOVA, $F_{(4,10)} = 0.71$, $P = 0.604$). The microscope counted number of total calanoid nauplii in this size fraction ranged from 5.4×10^3 to 6.3×10^3 nauplii m^{-3}, while the qPCR-based estimate ranged from 0.96×10^3 to 1.2×10^3 nauplii m^{-3}. When the 19 % proportion of *P. crassirostris* was applied to the total calanoid nauplii counts in this size fraction, the qPCR technical replicates estimated 81–110 % of the count-based number of *P. crassirostris*.

Discussion

The new qPCR-based approach described here was developed to identify and quantify nauplii in mixed plankton samples. Conventional techniques, such as microscopy, cannot be used to estimate the abundance of a single species of nauplii in a diverse ecosystem. Our method has a comparable level of technical accuracy to microscope counts (Fig. 6), but provides a higher level of taxonomic resolution than is possible using conventional techniques in our subtropical coastal ecosystem. This new qPCR-based approach provides a quantitative assessment of the number of mtCOI gene copies for a specific copepod species present in the sample. For the population in Kane'ohe Bay, this number can be used to estimate naupliar abundances for that species and thus distinguish early developmental stages of the two dominant calanoid species, *B. similis* and *P. crassirostris*. The method lays the foundation for a broader range of studies investigating species-specific population responses to environmental variation at our study site (e.g., storm events, ecosystem perturbations).

To our knowledge, this is the first study to attempt to use qPCR to quantify the abundance of a common metazoan plankton species in bulk community samples. In contrast to qPCR-based methods for enumeration of single-celled marine organisms (e.g., as in Church et al. 2005; Zhu et al. 2005; Beman et al. 2012), using qPCR to measure the abundance of metazoans presents unique challenges. Two biological sources of variation in DNA copy number individual^{-1} need to be further quantified and accounted for in any DNA-based approach that aims to enumerate individuals in mixed field samples based on DNA copy number (quantified by qPCR).

The number of mtCOI copies individual^{-1} varies as a function of developmental stage (Table 2; Fig. 4, this study; Durbin et al. 2008), due to increasing body size and cell number individual^{-1}. In *P. crassirostris*, mtCOI copy number increased over 3 orders of magnitude across development, with a fourfold change observed among naupliar stages (Table 2). This illustrates the need for size fractionation of bulk plankton prior to DNA extraction (Table 3) in order to obtain estimates of naupliar abundance by stage based on mtCOI copy number. The change in mtCOI copy number individual^{-1} between early-stage nauplii and adults was even greater in *A. tonsa* (Durbin et al. 2008), the only other calanoid species in which mtCOI copy number has been quantified across development. The differences in mtCOI copy number between *P. crassirostris* and *A. tonsa* was greatest in the adults, which is likely related to body size (adult female prosome length: 0.4 mm and 1 mm, respectively).

A second challenge is that mtCOI copy number individual^{-1} may vary with environmental conditions. Preliminary evidence suggests that food quantity may influence mtCOI copy number among individuals of the same developmental stage (feeding stage VI nauplii). This is consistent with a large literature demonstrating that copepod body carbon (or dry weight) is heavily influenced by food quantity, food quality, and seawater temperature (e.g., Durbin and Durbin 1978; Huntley and Lopez 1992; Hirst and Bunker 2003, and references therein). mtCOI copy number is linearly related to body carbon in *A. tonsa* (Durbin et al. 2008). If the variation found in this study in mtCOI copy number among individuals of the same development stage is caused by a higher number of mitochondria within cells under higher food conditions, rather than due to changing cell numbers in each stage as a function of environmental

Table 4 Comparison of qPCR to count-based estimates of *P. crassirostris* abundance in field samples

Size fraction (μm)	Sample replicate	Total counted calanoids (m^{-3})	(A) Count-based abundance (m^{-3})	SD	(B) qPCR-based abundance (m^{-3})	SD	Difference |(A–B)|
80–100	I	5,750	1,090	138	1,230	152	140
	II	6,260	1,190	327	963	149	227
	III	5,400	1,030	313	–	–	–

The count-based estimate (A) is derived from a microscope count of total calanoids ×0.19, where the 0.19 fraction is derived from multiplexed end-point PCR to determine the fraction of calanoids that are *P. crassirostris* in the early copepodite stages (same sample; see 'Materials and methods' and 'Results' text for details). |A–B| is the difference in abundance between the two methods, the absolute value of count-based abundance minus the qPCR estimate. Three subsamples were counted for each sample, and each qPCR sample was run in triplicate. SD: standard deviation; -: no measurements available

condition (McLaren and Marcogliese 1983), then one possible solution would be to use a nuclear instead of a mitochondrial gene as a marker. Further studies will be needed to assess how environmental conditions influence mtDNA and/or nuclear DNA copy number in order to minimize the error associated with estimating the number of individuals from gene copy number.

Despite these challenges, our results suggest that this qPCR-based approach could be used as a higher throughput and higher taxonomic resolution alternative to microscopic counts of nauplii. Both laboratory- and field-based method validation studies found close correspondence between qPCR and count-based estimates of *P. crassirostris* naupliar abundance. We also found similar precision in qPCR and microscope-based measurements (C.V. for technical replicates, 16 % both methods). In the field-based method validation study, the abundance estimates from qPCR also showed close correspondence to microscope counts for the 80–100 μm size fraction (Table 4). In this study, multiplex PCR was used to estimate the proportion of *P. crassirostris* in total calanoid CI–CII copepodites (0.19), since even these stages are difficult to accurately identify using morphological characters. Despite the many potential sources of error in this field study, the difference in abundance as estimated by these two methods was comparable to the standard deviation of biological replicates for each method (Table 4). In sum, these results demonstrate that the qPCR-based approach provides a viable alternative method of quantifying nauplii with identification to the species level in pelagic ecosystems with diverse naupliar populations.

This novel qPCR-based approach has potential for elucidating the roles of copepod nauplii in pelagic food webs. By developing species-specific primers for all species of interest from a given ecosystem, this method will enable assessments of shifts in species dominance at the naupliar level. Population shifts between congeners at short spatial and temporal scales are well documented among adults of *A. tonsa* and *A. hudsonica* in Narragansett Bay, Rhode Island USA (Durbin and Durbin 1981; Sullivan et al. 2007), *Pseudocalanus moultoni* and *P. newmanii* on Georges Bank, Gulf of Maine USA (Bucklin et al. 1998), as well as other congeneric species pairs (e.g., Wesche et al. 2007; Grabbert et al. 2010). Seasonal or spatial shifts in the abundance of copepods and their nauplii can have important ecosystem consequences and, for example, have been suggested to contribute to declines in some commercially important fish stocks (e.g., Atlantic cod, Beaugrand et al. 2003; Möllmann et al. 2008). The qPCR method could be used in these ecosystems to identify and measure the abundances of morphologically indistinguishable nauplii, and obtain greater insight into the population dynamics of these key species.

Within Kane'ohe Bay, copepod nauplii are persistently the most abundant metazoan plankton throughout the year (Scheinberg 2004), but their role in the food web remains uncharacterized. For example, there appear to be strong event-scale shifts in naupliar abundance in response to storm-driven runoff into the bay (Cox et al. 2006; Hoover et al. 2006), but there is no information at the species level on which taxa are responding to changing plankton community structure. This new method will enable direct measurement of species composition in size-fractionated 'cohorts' across all life stages in response to short-term environmental perturbation or seasonal shifts in plankton community structure and function (e.g., Scheinberg 2004; Cox et al. 2006). Late-stage nauplii may be important grazers (e.g., Landry and Hassett 1982; White and Roman 1992; Roff et al. 1995; Böttjer et al. 2010), but little is known regarding trophic differences among species. The qPCR approach could be combined with grazing experiments and analyses of in situ microbial and phytoplankton communities to quantify the contribution of nauplii by species to the transfer of material from the microbial food web to higher trophic levels in Kane'ohe Bay as well as other ecosystems worldwide.

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