Copepod feeding and digestion rates using prey DNA and qPCR

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Copepod feeding and digestion rates were measured using quantitative polymerase chain reaction (qPCR) to amplify the prey Thalassiosira weissflogii and Heterocapsa triquetra from the guts of Acartia tonsa. Using species-specific primers, prey 18S rDNA could be detected routinely and quantified in the guts and fecal pellets of A. tonsa. Recovery of gut contents DNA using two fixation methods was compared. Prey 18S copy numbers were >10-fold higher in copepods fixed in 95% ethanol (3260 ± 822 copies copepod⁻¹) compared with anesthetized and frozen copepods (210 ± 19 copies copepod⁻¹). Experiments using 95% ethanol fixation showed rapid prey DNA digestion rates during the initial 2 min after ingestion (0.7 min⁻¹) after which they slowed ≏10-fold. Chlorophyll pigment disappearance rates were slower (≏0.015 min⁻¹). Rates of gut filling measured by DNA and gut pigments differed, reaching 95% of the asymptote, Imax, in 3 and 58 min, respectively, likely reflecting differences in rates at which biomarkers were digested. Gut fullness measured by DNA increased with prey concentration, reaching Imax at 9760 copies copepod⁻¹ and a critical concentration (Icrit) at 1530 cells mL⁻¹. These results demonstrate that qPCR analysis of prey DNA in copepod guts can be used to provide a quantitative index of feeding rates.

KEYWORDS: Acartia tonsa; Thalassiosira weissflogii; Heterocapsa triquetra

INTRODUCTION

The predatory role of mesozooplankton in marine food webs can only be understood through the knowledge of their in situ feeding behavior. Gaining this information has been a major challenge in biological oceanography. Approaches used include bottle incubation with natural plankton, extrapolation of laboratory-derived functional curves to field samples and stomach content analyses of copepods collected in the field (reviewed in Bamstedt et al., 2000). However, the first two methods do not provide a direct in situ measurement of consumption and are subject to various artifacts arising from lengthy bottle incubations and the difficulty of ensuring that a representative prey field is collected. The third approach requires information on stomach contents in the field at intervals over 24 h and a measure of how rapidly food is being digested in the stomachs, i.e. food disappearance rates (Durbin and Campbell, 2007). This latter approach has been applied to copepods using chlorophyll pigments as tracers of gut contents (Mackas and Bohrer, 1976). Although this approach has been used extensively, it is limited to herbivory. HPLC has been used to discriminate among phytoplankton taxa using pigments or other biomarkers (e.g. Kleppel and Pieper, 1984; Kleppel et al., 1988; Head and Harris, 1996; Juhl et al., 1996), while stable isotopes, particularly 15N, may be used to provide information on the trophic level at which the predator is feeding. An approach to estimate the in situ carnivory of individual prey species has been to use immunological analysis of gut contents (Ohman, 1992). More recently, molecular techniques have been
developed using polymerase chain reaction (PCR) to identify prey DNA in the guts of both terrestrial and marine predators (e.g. Jarman et al., 2002; Symondson, 2002; Blankenship and Yayanos, 2005; Pons, 2006; Read et al., 2006). This approach has been used both in the laboratory and in the field together with species-specific primers to detect phytoplankton prey DNA in the guts and fecal pellets of copepods (Nejstgaard et al., 2003; Vestheim et al., 2005; Haley et al., 2010). Although these studies demonstrated the feasibility of using endpoint PCR and species-specific primers to detect prey, they were not quantitative.

To quantify prey-specific DNA in zooplankton guts, quantitative PCR (qPCR) has been used (e.g. Passmore et al., 2006; Troedsson et al., 2007; Durbin et al., 2008; Nejstgaard et al., 2008; Barošky et al., 2010). This approach has the advantage of providing information on the amount of prey DNA present within zooplankton guts. However, both Nejstgaard et al. (Nejstgaard et al., 2008) and Durbin et al. (Durbin et al., 2008) noted that the amount of DNA detected with qPCR was lower than expected. One possible explanation is that digestion of DNA is very rapid, permitting prey DNA to be partially digested during handling (Simonelli et al., 2009). These possibilities would result in an underestimation of stomach contents that in turn would affect the estimates of feeding rates.

Below, we describe experiments in which we use quantitative measures of prey DNA in the guts of copepods to determine the rate of gut filling, the effect of food concentration on stomach contents and the short-term digestion rate of prey DNA. Our results demonstrate that qPCR analysis of prey DNA in copepod guts can be used to provide a quantitative index of zooplankton feeding rates.

**METHODS**

**Culturing methods**

*Acartia tonsa* adult females were used as the predator in all experiments. A culture was established by isolating adult females collected from the pier at the University of Rhode Island, Graduate School of Oceanography in Narragansett Bay, RI (41°29.5′N, 71°25.1′W). Eggs from these females were collected for initiation of the culture. Copepods were maintained in 20-L polycarbonate containers and fed *Rhodomonas* sp. (CCMP768). When larger numbers of copepods were required for experiments, groups of eggs were collected over 1 or 2 days and a cohort of was reared in 120-L containers. Prey used in the feeding and digestion rate experiments were the diatom *Thalassiosira weissflogii* (CCMP1336) and the dinoflagellate *Heterocapsa triqueta* (CCMP448). These cultures were grown in f/2 media (Guillard, 1975), with (*T. weissflogii*) or without Si (*Rhodomonas* sp., *H. triqueta*). Both *A. tonsa* and phytoplankton prey were raised at 18°C. All experiments were carried out at 20°C.

Phytoplankton cultures were harvested in a mid-exponential phase of growth for determination of cell carbon, nitrogen, chlorophyll and 18S rDNA gene sequence and copy number per cell. Cells of each species were preserved in Lugol’s iodine for later enumeration. Samples (15 mL) for cell carbon and nitrogen were filtered onto 13 mm GF/A pre-combusted glass fiber filters, dried at 60°C and analyzed with a NA1500 CHN Analyzer (Carlo Erba). Samples (3 mL) for chlorophyll a (Chl a) were filtered onto 25 mm diam GF/F glass filters. These were placed immediately in 13 mm diameter glass tubes with 6-mL 90% acetone, incubated at –20°C for 24 h, read on a fluorometer (Turner Designs Model 10) before and after acidification and Chl a and pheopigments calculated (Parsons et al., 1984). Four to five replicate filters were prepared for each different analysis.

**Phytoplankton genomic DNA extraction**

To obtain *T. weissflogii* genomic DNA for 18S rDNA sequencing and determination of cell 18S copy numbers, 2 mL of culture (107 000 cells) were filtered onto 13 mm diam, 3 μm pore size filters (Nucleopore), frozen at –80°C and processed using the DNeasy Plant Kit (Qiagen). Initial attempts to obtain genomic DNA from *H. triqueta* using this method gave low and variable copy numbers cell⁻¹. Three methods to obtain genomic DNA from *H. triqueta* were subsequently compared. Four 2-mL aliquots of culture (22 500 cells) were centrifuged at 4000g for 5 min, the supernatant aspirated and the pellet frozen at –80°C. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) after vortexing (3200 rpm and 1 min) with lysis buffer (DNeasy buffer ATL with proteinase-K) and three to five 425–600 μm acid washed glass beads (Sigma). This method was compared with extraction of DNA from pelleted cells using the DNeasy Plant Kit (Qiagen) with and without glass beads. DNA from these extractions was quantified using species-specific primers and qPCR as described below. Genomic DNA from the former method was used for 18S rDNA sequencing.

**Copepod fixation and DNA extraction**

We compared the efficacy of two copepod fixation methods in recovering ingested prey DNA; 95% ethanol...
and freezing. *Acartia tonsa* were fed *T. weissflogii* at a concentration of 10 000 cell mL$^{-1}$ for 2 h. A portion of these copepods was fixed immediately by collecting them on a sieve and immediately placing this in a beaker containing 95% ethanol. These were rinsed several times with clean ethanol to remove any phytoplankton that may have been present and then transferred to 1.5-mL microcentrifuge tubes with ethanol for later DNA extraction. Prior to DNA extraction, these copepods were sorted in four groups of eight into clean microcentrifuge tubes. The other group was anesthetized in MS-222 (0.5 g L$^{-1}$ SW; Durbin and Durbin, 1978), rinsed in filtered seawater and sorted quickly in four groups of eight into microcentrifuge tubes and frozen at $-80^\circ$C. Copepods processed using each method were ground in the microcentrifuge tubes with a disposable sterile pestle (Kimble Chase Kontes) and genomic DNA purified using the DNeasy Blood & Tissue Kit (Qiagen). The eluted volume was then concentrated by a factor of $\approx 10$ (Thermo Savant DNA110 SpeedVac) to increase our ability to quantify low concentrations of prey DNA.

### 18S rDNA sequencing

For 18S rDNA sequencing of *A. tonsa*, genomic DNA copepods were starved for 24 h and fixed in 95% ethanol. Individual copepods were then placed in clean microcentrifuge tubes and DNA extracted as described above. An $\approx 1750$-bp segment of the 18S rDNA gene of each prey species and of *A. tonsa* was amplified in reaction mixtures containing 1× Taq-PCR Master Mix (Qiagen), 0.5 μM of primers 18SA and 18SB (Medlin *et al*., 1988) and 3.0 μL (10–15 ng) template of genomic DNA. Amplification was conducted in a Mastercycler Ep gradient S thermal cycler (Eppendorf) with the following thermal regime: 95°C for 30 s (1×); 94°C for 30 s, 60°C for 60 s and 72°C for 2 min (35×); 72°C for 10 min (1×). Amplicons were cleaned using the QIAquick PCR Purification Kit (Qiagen). Sequences were obtained using Big Dye terminators and primers 18SA and 18SB and analyzed on a 3130xl (ABI).

### Design of species-specific qPCR primers and methods

Species-specific qPCR primers for *T. weissflogii* and *H. triquetra* were designed with the aid of PrimerQuest (IDT) and are listed in Table I. These primer sets were tested with genomic DNA from each species by qPCR to confirm specificity and were subsequently used to quantify prey DNA copy numbers in all experiments using procedures described below.

### Table I: Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thalassiosira weissflogii qPCR F</td>
<td>5’-ACC GGT CTC ACA CCC TGT-3′</td>
</tr>
<tr>
<td>Thalassiosira weissflogii qPCR R</td>
<td>5’-TGC CCG ACA ACT GAA TGC CAA ACA-3′</td>
</tr>
<tr>
<td>Heterocapsa triquetra qPCR F</td>
<td>5’-ATG GCC GTC CTT AGT TGG TGG AGT-3′</td>
</tr>
<tr>
<td>Heterocapsa triquetra qPCR R</td>
<td>5’-TAA GAA GTA GCC CAC GGA ACC GAG-3′</td>
</tr>
</tbody>
</table>

Methods used to quantify the 18S copy number in samples were similar to those described in Durbin *et al.* (Durbin *et al*., 2008). Standards for qPCRs were prepared from end-point PCR amplicons for both *T. weissflogii* and *H. triquetra*. The copy number μL$^{-1}$ in both standards was calculated from the amplicon concentration (quantified using a NanoDrop ND-1000) and length. Standard curves were prepared in a 10-fold serial dilution ($5 \times 10^2$, $5 \times 10^3$, $5 \times 10^4$, $5 \times 10^5$, $5 \times 10^6$ and 50 copies per reaction). The reaction volume for qPCR was 25 μL and contained $\approx 50$ ng of template, 1× SYBRGreen Master Mix (Stratagene), 250 nM of each primer (Univ18S-550F and R) and 0.03 μM ROX Reference Dye (Stratagene). For each run of unknowns, we carried out a duplicate standard curve together with a no template control. Amplifications were performed on an Mx3005P (Stratagene) and consisted of 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 1 min at 57°C and 30 s at 72°C. Melting curve analysis was used to verify amplicon purity. Copy numbers were determined from the C$_{\text{t}}$ value (the fractional PCR cycle number where the fluorescence signal crosses a certain threshold) for each prey species, template concentration and the standard curve. Amplification efficiency was calculated from: PCR efficiency = $10^{\frac{1}{C_{\text{t}}}}-1$. In the experiments reported here, all but one (84.4%) were $>96%$.

### Prey DNA and chlorophyll disappearance rates in copepod guts

Experiments were carried out to measure the short-term disappearance rates of prey (*T. weissflogii* and *H. triquetra*) DNA and Chl a pigments in copepod guts. For each experiment, copepods were anesthetized with MS-222 (0.5 g L$^{-1}$ SW) and sorted in groups of 30 into 0.15 mm sieves (40 mm i.d.) sitting in 100-mL glass beakers to which 60 mL of filtered seawater had been added. Beakers (13 to 17) were prepared providing 1 control each (no food) for gut pigments and DNA and 12 and 16 experimentals (8–11 for DNA and 3–5 for gut pigments). After $\approx 24$ h, food was added to each
experimental beaker to provide a final concentration of either 8000 cells mL$^{-1}$ ($T$. weissflogii) or 3000 cells mL$^{-1}$ ($H$. triquetra). Prior to each experiment, the appropriate volume of food was dispensed into 13 mm diam glass tubes to speed the addition of food to beakers containing copepods. This food was added rapidly and after a brief feeding period (2–60 min depending on experiment), one of the sieves containing copepods was transferred to a beaker containing 95% ethanol, while the copepods in another were rinsed with MS-222 into a Petri dish and sorted in three groups of 8 x 25 mm diam GF/A glass fiber filters for initial gut pigment determination. The remaining sieves were immediately transferred to 100-mL beakers containing 60 mL of filtered seawater. A sieve was transferred from the filtered seawater to 95% ethanol at either 30 s ($T$. weissflogii) or 20 s ($H$. triquetra) intervals for 2 min, and then longer intervals for the next 20–60 min. At 5, 10, 20 and 30 min, an additional sieve in filtered seawater was rinsed with MS-222 into a Petri dish and sorted for gut pigments. Copepods without food were also either fixed in 95% ethanol or processed for background gut pigment measurements. Chl a and pheo gut pigments (as Chl a equivalents) were determined using the methods described above for phytoplankton chlorophyll and expressed as ng pigment copepod$^{-1}$ (Durbin et al., 1995). Copepods in 95% ethanol in beakers were rinsed several times with clean ethanol to remove any phytoplankton that may have been present and then transferred to 1.5-mL microcentrifuge tubes with ethanol for later DNA extraction. DNA was extracted (three groups of eight copepods; DNeasy Blood & Tissue Kit, Qiagen), and the 18S gene copy number determined through qPCR using the methods described above. While it was not logistically possible to replicate treatments within an experiment, by dividing copepods into 3 groups of 8 for analysis (pseudo replication), chances of loss of a whole sample during processing were minimized. However, each short-term disappearance rate experiment was repeated two ($H$. triquetra) or three ($T$. weissflogii) times.

**Gut filling rate and food concentration experiments**

An experiment was carried out to determine the rate of gut filling as measured by the 18S copy number and by gut pigments. In this experiment, $T$. weissflogii was used as prey. Groups of 30 copepods were placed in filtered seawater in sieves as described above and left for 24 h. One group was transferred to 95% ethanol to provide an initial no food DNA gut content measure while another was processed for background gut pigment measurements. *Thalassiosira weissflogii* culture was then added to the remaining beakers to provide a final concentration of 10 000 cells mL$^{-1}$. At 2.5, 5, 10 15, 20, 25 and 30 min, the groups of copepods were fixed with 95% ethanol, while other groups were anesthetized and processed for gut pigment determination as described above.

Another experiment was carried out to determine the relationship between food concentration and gut fullness as measured in terms of copy numbers. The experimental design was similar to that described above. After the 24 h food-deprivation period, $T$. weissflogii was added to eight beakers in final concentrations of 0, 200, 400, 800, 1200, 2000, 5000 and 10 000 cells mL$^{-1}$. The copepods were allowed to feed for 5 min and then sieves immediately transferred to 95% ethanol. 18S copy numbers were determined as described above.

**Fecal pellet copy numbers**

Fecal pellets produced by *A. tonsa* females after being fed *T. weissflogii* at 10 000 cells mL$^{-1}$ for 30 min were placed in groups of 16–23 onto 13 mm x 3 μm filters (Nucleopore) and frozen in microcentrifuge tubes. 18S copy numbers were determined as described above.

**Statistical analysis**

The negative exponential curves describing the rate of gut filling and the effect of food concentration on gut fullness were fitted using Proc NLIN (SAS 9.2). Similar procedures were used to fit the exponential decline in gut fullness as measured by gut pigments in all experiments and for DNA in the 60-min feeding experiment. Data for the short-term feeding experiments were analyzed using a segmented model with two exponential curves and a breakpoint ($C$) in SAS (V9.2). For the left line $Y_1 = A_1 \times \exp(-B_1 \times X)$ for $X \leq C$, while for the right line $Y_2 = A_2 \times \exp(-B_2 \times X)$ for $X \geq C$. For the left and right regression to be continuous at point $C$, $Y_1 = Y_2$, allowing $B_2$ to be calculated.

**RESULTS**

**Comparison of fixation and DNA extraction methods**

We compared the number of 18S copies obtained using different fixation and extraction methods. Prey 18S copy numbers were over 10-fold higher in copepods that had been fixed in 95% ethanol (3260 ± 822 18S copies copepod$^{-1}$) compared with those that had been anesthetized, sorted and frozen (210 ± 19 18S
copies copepod\(^{-1}\)). This indicated that prey DNA was lost during sorting prior to, and/or after, freezing. In all subsequent experiments, copepods were fixed in 95\% ethanol.

Extraction of *H. triquetra* DNA using the DNeasy Blood & Tissue Kit (Qiagen) with disruption using glass beads yielded 1690 ± 405 18S copies cell\(^{-1}\). Considerably fewer 18S copy numbers cell\(^{-1}\) were recovered using the DNeasy Plant Kit (Qiagen) (430 ± 282 18S copies cell\(^{-1}\) with beads and 303 ± 106 18S copies cell\(^{-1}\) without beads). All subsequent extractions of *H. triquetra* DNA for 18S sequencing and copy number determination were performed using the DNeasy Blood & Tissue Kit (Qiagen) with disruption using glass beads.

**Phytoplankton cell characteristics**

*Heterocapsa triquetra* contained between 4- and 8-fold more carbon, nitrogen and chlorophyll cell\(^{-1}\) than *T. weissflogii* (Table II). Differences in 18S copy numbers cell\(^{-1}\) were much less (1690 and 760 copies cell\(^{-1}\), respectively). The 18S sequence obtained for *H. triquetra* was identical to that already in GenBank (accession numbers AB183670 and AF022198) while those for *T. weissflogii* and *A. tonsa* were submitted (accession numbers GU594638 and FJ422281).

**Gut filling rate and effects of food concentration on gut contents**

Food-deprived adult female *A. tonsa* were offered *T. weissflogii* cells at 10 000 cell mL\(^{-1}\) as prey for differing time periods. Gut contents (as prey 18S copy numbers) increased with feeding time reaching an asymptote (\(I_{\text{max}}\)) of 11 190 18S copies copepod\(^{-1}\), equivalent to 14.7 cells for a copy number cell\(^{-1}\) of 760 (Fig. 1). The rate of increase was very rapid and reached 95\% of the maximum, \(I_{\text{max}}\), in 3 min. In contrast, gut pigments increased more slowly reaching 95\% of the \(I_{\text{max}}\) (0.97 ng Chl \(a\) equiv. copepod\(^{-1}\), equivalent to 324 cells copepod\(^{-1}\)) in 58 min (Fig. 1).

Gut fullness also increased rapidly with concentration of *T. weissflogii* reaching an asymptote (\(I_{\text{max}}\)) of 9580 copies copepod\(^{-1}\), equivalent to 12.6 cells (Fig. 2). The critical concentration (\(I_{\text{crit}}\)) 90\% of this asymptotic value, was reached at a concentration of 1530 cells mL\(^{-1}\).

**Prey DNA and prey chlorophyll disappearance rates in the guts of copepods**

Disappearance of prey DNA in *A. tonsa* that were fed for short periods (2–6 min) showed a rapid decline for ~2 min after removal from food (Figs 3 and 4). After this initial rapid decline, prey DNA declined more slowly and was still present at 30 min when each experiment was terminated. This pattern of decline was best fit by a segmented regression providing a slope for each phase of decline. The initial slopes (\(B_1\)) varied between 0.30 and 1.17 min\(^{-1}\) (Table III) with no significant difference (t-test, \(P = 0.73\)) between prey species.

**Table II: Average and standard deviations of carbon, nitrogen, Chl \(a\) and 18S copies per cell of *T. weissflogii* and *H. triquetra* fed to *A. tonsa* in feeding and digestion rate experiments**

<table>
<thead>
<tr>
<th></th>
<th>Carbon (pg cell(^{-1}))</th>
<th>Nitrogen (pg cell(^{-1}))</th>
<th>Chl (a) (pg cell(^{-1}))</th>
<th>18S copies (cell(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thalassiosira weissflogii</em></td>
<td>77.3 ± 5.4</td>
<td>17.5 ± 0.85</td>
<td>2.99 ± 0.35</td>
<td>760 ± 9.8</td>
</tr>
<tr>
<td><em>Heterocapsa triquetra</em></td>
<td>671 ± 37</td>
<td>134 ± 6.8</td>
<td>11.4 ± 0.54</td>
<td>1690 ± 414</td>
</tr>
</tbody>
</table>

**Fig. 1.** Gut filling rates for adult female *A. tonsa*. The copepods were fed *T. weissflogii* at 10 000 cell mL\(^{-1}\) for different times before fixing. (A) Gut contents as 18S copies copepod\(^{-1}\) and (B) gut contents as gut pigments (ng Chl \(a\) equiv. copepod\(^{-1}\)). The equations for a negative exponential fit to the data are shown.
During the second phase the slopes \(B_2\) varied between 0.016 and 0.18 min\(^{-1}\), again with no significant difference (\(t\)-test, \(P = 0.89\)) between prey species (Table III). In two experiments, one each with \(T\). weissflogii and \(H\). triquetra, the copepods were fed for 60 min. Compared with the short-term feeding experiments, prey DNA disappearance rates were more variable with a lower initial slope and a longer period (\(\sim 5\) min) during which prey DNA remained relatively high before declining to low levels (Fig. 5). A single exponential curve provided the best fit to the data.

Gut pigment disappearance rates were measured over longer time intervals between samples because of the time required to sort them after anesthesia. However, there was no indication of a rapid decline between 0 and 5 min in contrast with the rapid decline in prey DNA (Figs 3–5). While rates of decline in the short-term feeding experiments were approximately twice as fast when \(T\). weissflogii was the prey compared with \(H\). triquetra (0.025 and 0.012 min\(^{-1}\) respectively; Table IV), these differences were not significant (\(t\)-test, \(P = 0.07\)).

Measurement of phytoplankton DNA in fecal pellets of copepods

We were routinely able to quantitatively measure the amount of phytoplankton prey DNA (as 18S gene copy numbers) in fecal pellets of adult female \(A\). tonsa. Fecal pellets collected from copepods that had been fed \(T\). weissflogii at 8000 cells mL\(^{-1}\) contained an average of 625 \pm 160 18S copies pellet\(^{-1}\).

**DISCUSSION**

**Digestion rates of prey DNA by copepods**

Our study confirms previous suggestions that prey DNA is digested very rapidly (Durbin et al., 2008; Nejstgaard et al., 2008). In experiments where copepods were deprived of food then fed for a brief period, we observed an initial, very rapid, disappearance during the first few minutes after ingestion. This was followed by a slower phase in which DNA disappears at \(\sim 1/10\) of the initial rapid rate. Because of this clear break in the data, we fit a segmented model rather than a single exponential curve as has been more traditionally used for evacuation rate data. We suggest that these two phases reflect two quite different processes controlling the decline in DNA in the guts of copepods. First, there is a very rapid enzyme-mediated decline in DNA after maceration of the food and exposure to digestive enzymes in the gut. Second, there is a slower decline in DNA resulting from possible lower rates of enzymatic degradation after incorporation of food into fecal pellets inside the gut. However, the measurable quantities of DNA in the feces indicate that the DNA was not totally broken down in the gut and that this slower phase also represents fecal pellet release.

The very rapid initial decline in DNA is a result of the very efficient system copepods have for macerating food and exposing it to digestive enzymes as they ingest it with mandibles that have a spined (toothed) distal edge with abrasive tips of silica (Miller et al., 1980). After maceration, food passes through the copepod alimentary canal where cellular contents are exposed to non-specific nucleases, or DNases, that cleave the DNA strands (Linn and Roberts, 1982; Linn et al., 1993). Similar to most other enzyme-catalyzed reactions, these nucleases act very rapidly, cleaving nucleic acids internally (endonucleases), chewing in from the ends (exonucleases) or attacking in both of these modes. Reaction rate constants for reactions catalyzed by these DNases were between 0.03 and 0.05 s\(^{-1}\) (Light et al., 1974). By comparison, the DNA disappearance rates that we
observed were a little slower (0.85 min$^{-1}$ or 0.014 s$^{-1}$ for *T. weissflogii* and 0.011 s$^{-1}$ for *H. triquetra*) but still have the same order for an enzyme-catalyzed reaction. The slower disappearance rates we observed may have resulted from incomplete mixing of reactants.

The copepod alimentary canal has a mid-gut that is divided into three regions; Region 1 extending from the esophagus to approximately the posterior end of the cephalosome, Region 2 behind this to the first or the second metasomal segment and Region 3 from there to
the hindgut in the posterior part of the uresome (Mauchline, 1998). DNA digestion takes place in Regions 1 and 2. Extracellular digestion probably takes place in Region 1 through secretion of digestive enzymes by F-cells lining the gut in this region (Arnaud et al., 1980), while intracellular digestion appears to occur in Region 2 through absorption into B-cells lining the gut where material is digested (Nott et al., 1985). These B-cells subsequently burst releasing contents into the gut. There appears to be cyclical changes in the presence of B-cells in the gut wall between feeding and non-feeding periods (Nott et al., 1985), and we suggest that this may affect feeding activity of individual copepods.

### Table III: Exponential disappearance rates of 18S prey DNA for adult female *A. tonsa* fed *T. weissflogii* and *H. triquetra*

<table>
<thead>
<tr>
<th>Feeding time (min)</th>
<th><em>A. tonsa</em></th>
<th><em>H. triquetra</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>T. weissflogii</em></td>
<td><em>H. triquetra</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4.973 (4093–5952)</td>
<td>1.17 (0.67–1.67)</td>
</tr>
<tr>
<td>4</td>
<td>11.765 (6068–17.462)</td>
<td>1.01 (0.24–2.26)</td>
</tr>
<tr>
<td>6</td>
<td>12.800 (10.060–15.540)</td>
<td>0.38 (0.099–0.14)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.853</td>
<td>0.11</td>
</tr>
<tr>
<td>60</td>
<td>15.520</td>
<td></td>
</tr>
<tr>
<td><em>Heterocapsa triquetra</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>17.820 (14.683–20.950)</td>
<td>1.055 (0.66–1.45)</td>
</tr>
<tr>
<td>4</td>
<td>11.600 (8253–14.950)</td>
<td>0.300 (0.028–0.57)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.88</td>
<td>0.11</td>
</tr>
<tr>
<td>60</td>
<td>4.470</td>
<td></td>
</tr>
</tbody>
</table>

For the experiments where the copepods were fed for a short-time (2–6 min) segmented regressions were fit to the data. A1 is the y-intercept and B1 the slope for the initial exponential decline, c the time at which the breakpoint occurs and B2 the slope of the second period of exponential decline. Approximate 95% are given. For the experiments where the copepods were fed for 60 min a segmented regression did not provide a good confidence intervals fit to the data and a single exponential curve was fit for the whole period of measurement (0–30 min).

### Table IV: Exponential disappearance rates of chlorophyll pigments in adult female

<table>
<thead>
<tr>
<th>Feeding time (min)</th>
<th>Obs. time (min)</th>
<th>Exp. slope (min$^{-1}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food: <em>T. weissflogii</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0–30</td>
<td>0.019</td>
<td>0.62</td>
</tr>
<tr>
<td>4</td>
<td>0–30</td>
<td>0.030</td>
<td>0.44</td>
</tr>
<tr>
<td>6</td>
<td>0–10</td>
<td>0.026</td>
<td>0.15</td>
</tr>
<tr>
<td>Mean</td>
<td>0–30</td>
<td>0.025 ± 0.001</td>
<td>0.28</td>
</tr>
<tr>
<td>60</td>
<td>0–30</td>
<td>0.033</td>
<td></td>
</tr>
<tr>
<td>Food: <em>H. triquetra</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0–30</td>
<td>0.011</td>
<td>0.98</td>
</tr>
<tr>
<td>5</td>
<td>0–30</td>
<td>0.013</td>
<td>0.54</td>
</tr>
<tr>
<td>Mean</td>
<td>0–30</td>
<td>0.012 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0–30</td>
<td>0.010</td>
<td>0.99</td>
</tr>
</tbody>
</table>

*Acartia tonsa* fed *T. weissflogii* and *H. triquetra*.
We hypothesize that rapid digestion terminates when digested material is incorporated into fecal pellets in Region 3. The DNA disappearance rate then decreases to about one-tenth of the initial rate, representing both a low rate of continued DNA digestion and fecal pellet release. In copepods, fecal pellets are lined with a peritoneal membrane that binds fecal material together (Gauld, 1957; Brunet et al., 1994). During this process, undigested DNA may become protected from enzyme activity. However, even with potential enzyme activity during the second phase, DNA remains in fecal pellets after release by copepods (Nejstgaard et al., 2003; Vestheim et al., 2005; this study). In our experiments, fecal pellet release began between 5 and 10 min after feeding terminated consistent with this explanation.

In contrast to the rapid decline in DNA, the disappearance rate of chlorophyll pigments in copepod guts during the same time frame was slower by a factor of 3–8 and was best described by a single exponential curve. We suggest that these differences reflect the role of two different processes controlling these rates; a decrease in chlorophyll pigments following feeding solely as a result of defecation (Durbin and Campbell, 2007), whereas most of the DNA is degraded enzymatically via DNases followed by the release of the remaining amount through defecation. Interestingly, there have been suggestions that chlorophyll may also be very rapidly degraded to colorless compounds (pigment destruction; Head and Harris, 1996). If so, there should be an initial rapid disappearance similar to that which we observed for DNA. Unfortunately, however, the methods we used for gut pigment determination where copepods were anesthetized, sorted into tubes and acetone added, were not rapid enough to have detected any such possible rapid disappearance. Notably, the rates of decline in gut pigments we observed were consistently lower than the rate predicted (0.061 min$^{-1}$) for the same temperature (20°C) from an equation presented by Mauchline (Mauchline, 1998). This may have been due to the relatively small amount of food ingested during the short feeding time used in the experiments (2–6 min). Gut evacuation of copepod rates appear to be negatively affected by the amount of food in the guts (Dagg and Walter, 1967; Pasternak, 1994; Durbin et al., 1999; Irigoien, 1998).

For copepods fed for 60 min on both prey species, there was a suggestion of a slower DNA digestion rate and a slightly longer period during which prey DNA remained elevated. This may be due to the utilization of B-cells as they burst (Nott et al., 1985) with the cyclical changes in the gut wall between feeding and non-feeding periods affecting the digestion capabilities of the copepods. All copepods are presumed to be in the same stage prior to feeding with unutilized B-cells. The initial rapid digestion may reflect the synchronized utilization of all of these cells. During longer feeding periods the copepods may lose this synchrony and be in differing stages of this cycle. This could reduce the availability of digestive enzymes resulting in lower digestion rates.

### Quantification of prey DNA in guts and fecal pellets of copepods

This study demonstrates that prey DNA can be detected routinely and quantified in the guts and fecal pellets of copepods using species-specific primers and qPCR. The relationships we observed between gut fullness as measured by DNA, and prey concentration and time, were similar to what we would have expected using traditional bottle incubation techniques and cell counts.

The concentration of *T. weissflogii* at which gut contents reached 90% of the asymptotic value ($C_{crit}$) was 1530 cells mL$^{-1}$ when gut contents were measured as DNA; similar to the value for $C_{crit}$ of 940 cells mL$^{-1}$ when adult female *A. tonsa* were fed the same prey (*T. weissflogii*) at 20°C and ingestion determined by cell counts (Thompson et al., 1994; Fig. 2).

The rate of gut filling as measured by DNA was very rapid, reaching 95% of the maximum gut content ($S_{max}$) at 3 min. This contrasts with a time of 58 min for prey chlorophyll pigments in the gut to reach 95% of $S_{max}$. While this appears to be a large difference, it is what we would expect based on a consideration of digestion rate on the rate of gut filling. With copepods previously deprived of food the rate of gut filling is controlled by the rate of disappearance of the food tracer through either digestion or evacuation. The effect of tracer disappearance rate on gut filling time is illustrated in Fig. 6 where the rate of gut filling was calculated from:

$$S_t = S_0 e^{-Rt} + \frac{F}{R} (1 - e^{-Rt})$$

where $S_t$ is the gut content at time $t$, $S_0$ the initial amount of food, $R$ the exponential stomach content disappearance rate and $F$ the rate of food consumption (Elliott and Persson, 1978). For this example, we used two exponential disappearance rates, 0.5 min$^{-1}$ a rate similar to our results for DNA digestion and 0.061 min$^{-1}$ for the disappearance rate of prey pigments in copepods at 20°C (Mauchline, 1998), and expressed the gut contents as a percent of maximum gut fullness.
rate of gut filling as shown above, different rates of feeding have no effect on the rate of gut filling for the same exponential disappearance rate $R$. For example, using a value of $R = 0.5 \text{ min}^{-1}$, and feeding rates of 100, 50 and 10 DNA copies s$^{-1}$ (Fig. 6), the same proportional stage of gut filling was reached at the same time for each feeding rate.

**CONCLUSIONS**

We have shown that the digestion rates of prey DNA in copepods can be measured directly. The very rapid rates we saw in experiments where copepods were deprived of food and then fed for only a few minutes suggests that DNA degradation is an enzyme-mediated reaction. However, it should be noted that our understanding of factors such as temperature, short-term feeding periodicity and prey type controlling these rates is still very limited. These rapid DNA digestion rates have implications for using prey DNA to estimate in situ feeding by zooplankton. First, tow lengths should be kept to a minimum and zooplankton fixed in 95% EtOH as quickly as possible. Second, the prey DNA present in the guts represents recent feeding, perhaps only over the previous 20–30 min at the temperatures examined here.

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