Molecular-based diet analysis of the early post-larvae of Japanese sardine *Sardinops melanostictus* and Pacific round herring *Etrumeus teres*

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ABSTRACT: Successful feeding on preferred prey could be important for the survival of fish larvae. However, high taxonomic resolution of prey types from damaged gut contents is difficult to achieve through morphological classification, especially for fragile organisms or immature stages. In this study, <10 mm early post-larvae of Japanese sardine *Sardinops melanostictus* and Pacific round herring *Etrumeus teres* were collected from Tosa Bay (Japan) during their main spawning periods. Diet and plankton communities present in the environment were investigated using molecular operational taxonomic units (MOTUs) clustered using eukaryotic metagenetic data from the 18S V9 region. There were no clear differences in the diets of the co-existing fish species. Fragile protists and gelatinous metazoans occasionally represented large proportions of gut content; however, copepods comprised the majority of the prey items. Among the copepods, the Calanidae MOTU derived from the large copepod *Calanus sinicus* was most consumed, followed by the Paracalanidae MOTU identified as small *Paracalanus parvus* s.l. The *C. sinicus* MOTU largely dominated intermediate (0.5–1.0 mm) and large (1.0–2.0 mm) environmental mesoplankton communities, whereas the *P. parvus* s.l. MOTU was the most dominant in small-sized (0.1–0.5 mm) communities. Early post-larvae fish were considered to prefer eggs or nauplii of *C. sinicus*, which was the second-dominant MOTU in small-sized communities. These results suggested that both food availability and developmental stages of copepods determined prey preference.

KEY WORDS: *Sardinops melanostictus* · *Etrumeus teres* · Larval fish · Diet · Metagenetics

INTRODUCTION

The early post-larval stage is when fish larvae start to replace yolk absorption with exogenous feeding. Feeding success during this period is essential to the survival of fish larvae (Hjort 1914), and growth and nutrient condition are the major factors that allow fish larvae to avoid predation (Anderson 1988, Bailey & Houde 1989, Takasuka et al. 2003). As both quality and quantity of prey significantly affect the growth of

fish larvae (e.g. Beaugrand et al. 2003, Dower et al. 2009, Murphy et al. 2012, Robert et al. 2014a), investigating prey preference is important to understand recruitment dynamics of fish. However, only a limited number of studies have acquired sufficient taxonomic resolution of preferred prey (reviewed by Robert et al. 2014b), as gut contents tend to be damaged through feeding and digestion, preventing high taxonomic resolution of prey in small fish larvae. Soft-bodied zooplankton and protozoa are especially

fragile and easily digested and therefore hard to quantify visually, although they may be important food sources for fish larvae (Montagnes et al. 2010, Llopiz 2013). Many fish larvae feed on small, cryptic, planktonic organisms that are highly diverse (e.g. de Vargas et al. 2015), which further hinders morphological classification and prevents high taxonomic resolution of the dietary analysis of fish larvae.

Japanese sardines *Sardinops melanostictus* are commercially important fish in the northwestern Pacific, and their population dynamics show major long-term fluctuations (Kawasaki 1983). The main spawning season of *S. melanostictus* is February and March in the subtropical Pacific Ocean off Japan (Watanabe et al. 1996), and their major spawning ground is Tosa Bay. Tosa Bay is an open-type bay off the southern coast of Japan, and the hydrography is strongly affected by the Kuroshio Current (Kuroda et al. 2008). During the spawning season of *S. melanostictus*, another commercially important clupeoid species, the Pacific round herring *Etrumeus teres*, also dominates the larval community (Djumanto et al. 2004). A previous morphological study on gut contents showed that the larvae of both species mainly select small copepods, although the compositions varied depending on food availability in the environment (Nakata 1988, 1995, Yasue et al. 2011). However, details of the preferred prey of these 2 species remain unclear, due to the difficulty involved in classifying the immature stage of copepods. A staining method has been used to detect naked protozoan zooplankton in the gut contents of *E. teres* larvae collected from Tosa Bay (Fukami et al. 1999). The importance of these taxa as food items might be underestimated for sardine larvae (Nakata 1988), and important prey for *S. melanostictus* and *E. teres* may therefore not be sufficiently documented, especially for the early post-larval period.

Molecular approaches can achieve high taxonomic resolutions, even from damaged samples or those lacking morphological characteristics. For instance, the metagenetic method — which amplifies a specific genetic region from bulk DNA samples and recovers taxonomic compositions by bioinformatic analysis of mass sequence data (sequence reads) produced by a high-throughput sequencer — is a potentially powerful tool for diet studies (Pompanon et al. 2012). The V9 hyper-variable region in the nuclear gene coding 18S ribosomal RNA (18S rRNA) is a common genetic maker for metagenetic analysis of Eukaryota (e.g. Amaral-Zettler et al. 2009, de Vargas et al. 2015). Registered sequences in public databases are relatively large for the 18S rRNA gene across eukaryotic taxa (e.g. SILVA; Quast et al. 2013), and a universal PCR primer pair is available to amplify the short 18S V9 region (approximately 130 bp) across eukaryotes (Amaral-Zettler et al. 2009). Due to high taxonomic coverage and resolution, metagenetic analysis of the 18S V9 region is frequently used in diet studies to detect preferred prey items, including organisms that cannot be detected by visual observation (O'Rorke et al. 2012, 2014, 2015, Jarman et al. 2013, Albaina et al. 2016).

Here, we identified the major prey of early post-larvae of *S. melanostictus* and *E. teres* during their main spawning season in Tosa Bay, Japan, using metagenetic analysis of 18S V9 to detect dominant eukaryotic taxa present in gut contents. Results were compared with prey availability in metagenetic data from environmental plankton communities. To our knowledge, this is the first study using metagenetic analysis to determine the diet of *S. melanostictus* and *E. teres* early post-larvae; our results contribute towards revealing the details of preferred prey types of both species.

MATERIALS AND METHODS

Sampling

All samples were collected from 4 stations (L3250, N3320, P3320, and Q3310) in Tosa Bay during the SY-15-02 cruise aboard the FRV 'Soyo-Maru' (Fig. 1). All sampling was performed during the day between 15 and 20 February 2015 (Table 1). Early post-larvae were collected at 0 to 50 m depth with an oblique tow using an ORI net (Omori 1965) with a 1.6 m mouth diameter and 335 µm mesh size. A Norpac net (Motoda et al. 1957) with 100 µm mesh size was deployed using vertical tows to collect organisms comprised mainly of mesoplankton in the same depth range (0 to 50 m). Samples collected using ORI and Norpac nets were preserved in 99% ethanol. Ethanol was replaced within 24 h of initial preservation, and samples were stored at −20°C. At Stn L3250, a formalin-preserved sample was also collected for morphological analysis using the Norpac net. We also collected water samples to collect microplankton at the surface (0 m), 10 m, and 30 m using a plastic bucket or Niskin bottles attached to the temperature and depth (CTD) system (SBE-911 plus; Sea-Bird Electronics). A sample of 1 l of seawater was immediately filtered through 1 µm pore-sized polycarbonate filters (Nuclepore membrane; GE Healthcare). All polycarbonate filters were preserved at −20°C.
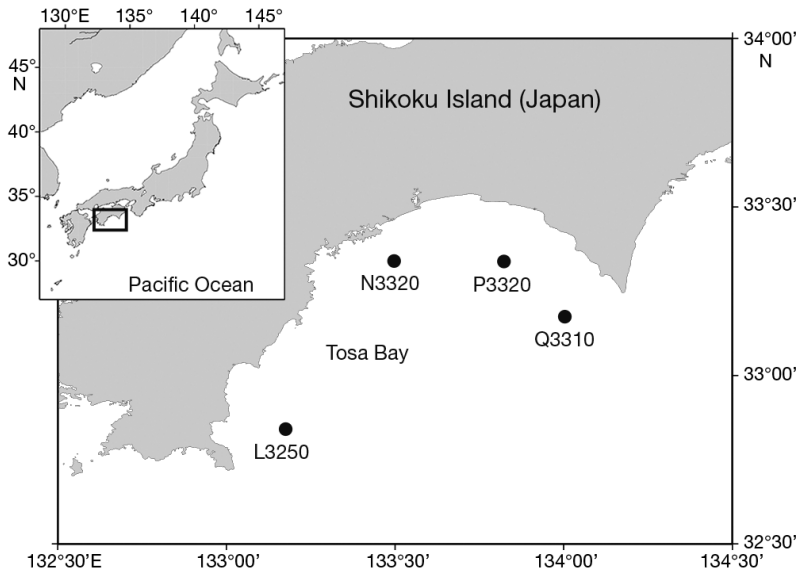
Fig. 1. Sampling locations in this study. Samples were collected from 4 stations during the SY-15-02 cruise in Tosa Bay (Japan). Detailed information on sampling events is listed in Table 1

Identification of fish early post-larvae

Sixteen early post-larvae of clupeoid (<10 mm) were separated from bulk samples at each station to analyze gut contents. In total, 64 early post-larvae were identified to species level by Sanger sequencing of the mitochondrial 16S ribosomal RNA gene (mt16S). Each larval specimen was dissected under a microscope, and the whole gut was separated for metagenetic analysis. Genomic DNA was extracted from the other body parts of early post-larvae using the DNeasy Blood & Tissue Kit (QIAGEN) and preserved in 100 μl of buffer AE. A fragment of mt16S sequence (approximately 610 bp) was amplified by PCR using the primer pair 16Sar and 16Sbr (Palumbi 1996; Table 2). PCR amplification was conducted with TaKaRa Z-*Taq* polymerase (Takara) in 15 μl reaction mixtures containing 6.3 μl distilled water, 1.5 μl 10× buffer, 1.2 μl dNTPs (2.5 mM), 1.5 μl of each primer (5 μM), 0.07 μl Z-*Taq*, and 3 μl of template DNA. PCR cycling included denaturation at 94°C for 2 min, followed by 35 cycles of 5 s denaturation at 94°C, 5 s annealing at 50°C, and 10 s extension at 72°C, with a final extension at 72°C for 2 min. PCR products were purified with ExoSap-IT (GE Healthcare), sequenced directly using dye-labeled terminators, and analyzed on a 3130 DNA Sequencer (Applied Biosystems). Sequencing reactions were performed according to manufacturer protocols using the same primer pair (16Sar and 16Sbr). Consensus mt16S sequences were manually confirmed and edited by comparing aligned sequences for both forward and reverse strands using Geneious R7 (www.geneious.com; Kearse et al. 2012). Each sequence was BLAST searched against the NCBI database to identify fish species.

Metagenetic analysis of gut contents and environmental samples

Total genomic DNA was extracted using the DNeasy Blood & Tissue Kit. The whole gut was used to analyze the diet of each fish larva to avoid loss of gut contents during dissection. Mesoplankton samples collected by the Norpac net were categorized into 3 size fractions using 0.1, 0.5, 1.0, and 2.0 mm Nitex meshes: small (0.1–0.5 mm), intermediate (0.5–1.0 mm), and large (1.0–2.0 mm). Large fractions at Stns P3320 and Q3310 were not analyzed due to insufficient numbers of zooplankton in this size fraction. DNA samples of fish guts were purified using PowerClean DNA Clean-Up Kit (MO BIO). DNA concentration of each sample was measured with a Qubit 3.0 Fluorometer (Life Technologies).

Table 1. Sampling information collected at each station, including date, location, and sea surface temperature (SST). The number of individuals analyzed (left) and total length (right) are shown for *Sardinops melanostictus* and *Etrumeus teres* at each sampling site. Numbers in parentheses indicate the average total length

Station	Date	Latitude	Longitude	SST (°C)	No. of individuals / total length, mm	
					S. melanostictus	*E. teres*
L3250	15 February 2015	32° 49.8′ N	133° 10.2′ E	16.4	7 / 4.3–9.1 (5.9)	9 / 4.4–7.0 (5.5)
N3320	17 February 2015	33° 20.1′ N	133° 29.9′ E	16.0	5 / 5.0–6.7 (6.2)	11 / 4.3–5.2 (4.7)
P3320	19 February 2015	33° 20.1′ N	133° 49.7′ E	16.1	12 / 5.5–8.6 (6.6)	4 / 5.7–9.5 (7.5)
Q3310	20 February 2015	33° 10.1′ N	133° 59.6′ E	16.4	16 / 4.0–7.2 (4.9)	0

Table 2. PCR primers used in this study. The third round of PCR primers contained an 8 bp index (represented by XXXXXXXX) to discriminate samples

Gene	Primer name	Sequence
mt16S	16Sar (Palumbi 1996)	5-CGC CTG TTT ATC AAA AAC AT-3
	16Sbr (Palumbi 1996)	5-CCG GTC TGA ACT CAG ATC ACG T-3
18S	1389F (Amaral-Zettler et al. 2009)	5-TTG TAC ACA CCG CCC-3
	1510R (Amaral-Zettler et al. 2009)	5-CCT TCY GCA GGT TCA CCT AC-3
	Second primer for 1389F	5-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TTG TAC ACA CCG CCC-3
	Second primer for 1510R	5-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TCC TTC YGC AGG TTC ACC TAC-3
28S	LSU Cop-D2F (Hirai et al. 2015)	5-AGA CCG ATA GCA AAC AAG TAC-3
	LSU Cop-D2R (Hirai et al. 2015)	5-GTC CGT GTT TCA AGA CGG-3
	Second primer for LSU Cop-D2F	5-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT AGA CCG ATA GCA AAC AAG TAC-3
	Second primer for LSU Cop-D2R	5-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TGT CCG TGT TTC AAG ACG G-3
18S & 28S	Third forward primer	5-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACX XXX XXX XAC ACT CTT TCC CTA CAC GAC GC-3
	Third reverse primer	5-CAA GCA GAA GAC GGC ATA CGA GAT XXX XXX XXG TGA CTG GAG TTC AGA CGT GTG-3

Eukaryotic universal primers 1389F and 1510R (Amaral-Zettler et al. 2009; Table 2) were used to amplify the 18S rRNA V9 region. Each PCR sample using the KOD Plus version 2 (Toyobo) was prepared as a 25 µl reaction volume that contained 13 µl distilled water, 2.5 µl 10× buffer, 2.5 µl dNTPs (2 mM), 1.5 µl MgSO$_4$ (25 mM), 1.5 µl of each primer (5 µM), 0.5 µl KOD Plus polymerase, and 2 µl template DNA (1 ng µl^{-1}). When the concentrations of template DNA were low (<1 ng µl^{-1}), we used 5 µl of template DNA and 10 µl distilled water. PCR cycling included initial denaturation at 94°C for 2 min, followed by 30 cycles of 10 s denaturation at 98°C, 30 s annealing at 56°C, and 1 min extension at 68°C, with a final extension step at 68°C for 7 min. PCR products of the target region were confirmed by electrophoresis on a 2.0% Tris-borate-EDTA (TBE) agarose gel (Takara).

Second and third PCRs were conducted to attach adaptors for sequencing on MiSeq and dual-index target sequences to discriminate samples (primers listed in Table 2). PCR products of environmental plankton communities were diluted (1/20) before the second PCR. First PCR products were not diluted as the target region of electrophoresis for samples of fish was not as clearly visible as those of environmental plankton communities. All second PCR products were diluted (1/20) and used for the third PCR. PCR cycles were set at 8 cycles with annealing temperature at 50°C for second PCR and 59°C for the third PCR. Final PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN), and the concentration of purified PCR products was measured with a Qubit 3.0 Fluorometer. The final PCR products were transferred to FASMAC Co., where a single sequencing run was performed using MiSeq Reagent Kit v2 on an Illumina MiSeq to obtain 2 × 250 bp paired-end sequence reads (accession number in the NCBI/EBI/DDBJ, sequence read archive: DRA004678).

28S D2 (approximately 400 bp) metagenetic analysis for the copepod community (Hirai et al. 2015) was also applied to mesoplankton samples. Because 28S analysis was performed to confirm the validity of the 18S V9 analysis, metagenetic data of 28S were only obtained at Stn L3250. As well as 18S metagenetics, the final PCR products of 28S were prepared using primer pairs listed in Table 2, with an annealing temperature of 58°C for the first PCR. A sequencing run was performed using MiSeq Reagent Kit v3 on an Illumina MiSeq to obtain 2 × 300 bp paired-end sequence reads of 28S (same accession number as 18S analysis). Note: this sequencing run contained ap-

proximately 200 amplicon samples, and not all sequence reads in a single MiSeq run were obtained in this study.

Bioinformatic analysis

Raw 18S paired-end reads were initially quality-filtered using Trimmomatic (Bolger et al. 2014) based on the following settings: CROP:135 MINLEN:50 LEADING:20 TRAILING:20 SLIDINGWINDOW:30: 30. We merged paired-end reads and performed a bioinformatic analysis in MOTHUR (Schloss et al. 2009). Both primer sites were eliminated, with criteria of 1 mismatch for 1389F and 3 mismatches for 1510R. Merged sequence reads were further quality-filtered and discarded if they did not meet the following criteria: (1) containing no ambiguous bases (Ns); (2) containing 100−200 bp; and (3) containing ≤6 homopolymers. All quality-filtered sequence reads were aligned against SILVA 119 databases (Quast et al. 2013) in MOTHUR. The aligned sequences were also filtered using single linkage preclustering (Huse et al. 2010). Possible chimeras were removed by both methods, with and without a reference dataset in UCHIME (Edgar et al. 2011). We used the V9_PR2 reference database provided by the Tara Oceans project (de Vargas et al. 2015; available at http://taraoceans.sb-roscoff.fr/EukDiv/). Using the same V9_PR2 reference database, we classified sequence reads into taxonomic groups using a naïve Bayesian classifier (Wang et al. 2007) with a threshold greater than 70%. As this study focused on eukaryotic organisms, only sequences classified as 'Eukaryota' were selected. The taxonomic group 'Craniata', including vertebrates, was removed from gut contents data to avoid sequence reads from the host or other contamination. The final quality-filtered sequences were clustered into molecular operational taxonomic units (MOTUs). We used the 99% similarity threshold for high taxonomic resolution based on the average neighbor algorithm. As this study focused on dominant taxonomic groups, rare MOTUs with less than 0.01% of total sequence reads were eliminated to avoid erroneous inflation of MOTUs.

Raw 28S sequence reads were also quality-filtered and merged according to the method for 18S metagenetics, except for CROP:300 and MINLEN:100 in Trimmomatic. Other bioinformatic analyses for the 28S metagenetic analysis of copepods followed those in previous studies (Hirai et al. 2015, Hirai & Tsuda 2015). As in the 18S analysis, a 99% similarity threshold was used for MOTUs clustering.

Taxonomic compositions of metagenetic data

For 18S metagenetics, proportions of all eukaryotic reads in the gut contents were calculated for each individual and for environmental planktonic communities to examine whether protozoans represent major food sources for early post-larvae of *Sardinops melanostictus* and *Etrumeus teres*. Proportions of sequence reads were investigated within taxonomic groups of Metazoa to evaluate the importance of non-copepod metazoans, including gelatinous zooplankton. Dominant MOTUs were also investigated within Copepoda. The representative sequence of each copepod MOTU was obtained based on the most abundant sequence reads. Then, each sequence was taxonomically assigned to Family level based on the BLAST search against the NCBI database, as not all 18S sequences of copepods were included in the V9_PR2 reference database. These analyses were also performed for environmental samples of microplankton and mesoplankton, with prey availability at each station being compared against the average taxonomic compositions of gut contents at each station. As the main prey size of larvae in this study was less than 0.5 mm in length (Nakata 1988), the averages of water samples (0, 10, and 30 m) were used to determine prey availability for Eukaryota and small mesoplankton (0.1−0.5 mm) for Metazoa and Copepoda.

To investigate the effects of species and sampling stations on diets, differences in gut contents among all individuals were investigated using proportions of sequence reads of all MOTUs. The degree of similarity among gut contents was evaluated using Bray-Curtis similarity, compared among all individuals by multidimensional scaling (MDS). The homogeneity of dispersion was tested using permutational analysis of multivariate dispersions (PERMDISP) and the differences among groups (species or station) were tested using permutational analysis of variance (PERMANOVA). PERMANOVAs were conducted using Type III sums of squares and the unrestricted model. All permutation-based tests were conducted using 999 permutations. Analyses of similarity, MDS, PERMDISP, and PERMANOVA were run in PRIMER version 7 (Clarke & Gorley 2015) with the PERM-ANOVA+ add-on (Anderson et al. 2008).

Food selection index and trophic niche breadth

The food selection index and trophic niche breadth were calculated following Roura et al. (2012, 2016).

The food selection index was calculated to compare selectivity of major copepod prey using the linear index of food selection (*L*) (Strauss 1979), which was calculated as:

$$L = r_i - p_i \tag{1}$$

where r_i is the proportion of copepod MOTUs in the gut and p_i is the proportion in the environment. The values ranged from −1 to +1, with prey preference indicated by a positive value and prey avoidance or inaccessibility by a negative value. Trophic niche breadth was calculated using Czekanowski's Index (CI) (Feinsinger et al. 1981):

$$CI = 1 - 0.5 \sum_i |p_i - q_i| \tag{2}$$

where p_i is the proportion of prey in the individual gut contents and q_i is the proportion in the environmental communities. This index was calculated for each of the 2 fish species at each sampling site using environmental data of Eukaryota, Metazoa, and Copepoda. The value ranged from [min q_i] (specialization to the rarest resources) to +1 (utilization of resources in proportion to their availability). Statistical analysis of the CI and *L* was performed using Student's *t*-test.

Comparisons between 18S and 28S analyses

Only copepod sequences at Stn L3250 were used to compare between 18S and 28S metagenetics, as the 28S metagenetic method (Hirai et al. 2015) was optimized for copepods. MOTUs with less than 0.1% of total sequence reads were eliminated to focus on dominant taxa and avoid inflating MOTUs. The 'best-hit' species were identified using 28S MOTUs based on the BLAST search against the NCBI database, which was shown as the most useful method to detect dominant species in the Kuroshio region off Japan (Hirai et al. 2015). Sequence proportions of MOTUs were compared between 18S and 28S metagenetic analyses for each size fraction of environmental copepods (0.1–0.5 mm, 0.5–1.0 mm, and 1.0–2.0 mm) at Stn L3250.

Developmental stages of *Calanus sinicus*

Metagenetic analyses provided no information on the developmental stages of prey; therefore, we investigated developmental stages of the large dominant copepod *Calanus sinicus* at Stn L3250 to sup-

port metagenetic analyses. The formalin-preserved sample was size-fractioned, and small (0.1–0.5 mm) and middle (0.5–1.0 mm) size fractions were split into 1/32 and 1/4 respectively. *C. sinicus* in each size fraction were classified as egg, nauplii (NI–NVI), early copepodite (CI–CIV), fifth copepodite (CV), or adult (CVI Female/Male) stages. Middle-size fractions of *Calanus*-shaped specimens were classified as nauplii and egg of *C. sinicus* (Koga 1984). The abundance of each developmental stage was counted for each size fraction, and compositions of developmental stages were investigated.

RESULTS

Diet analysis of *Sardinops melanostictus* and *Etrumeus teres*

Mt16S sequences were obtained from all 64 early post-larvae, which were classified as *Sardinops melanostictus* or *Etrumeus teres*. The proportions of the 2 species varied with respect to sampling stations. Of 16 individuals sampled at each station, the number of *S. melanostictus* was 7 (Stn L3250), 5 (Stn N3320), 12 (Stn P3320), and 16 (Stn Q3310) (Table 1). The total length of early post-larvae (mm) at each station was 4.3 to 9.1 (Stn L3250), 4.3 to 6.7 (Stn N3320), 5.5 to 9.5 (Stn P3320), and 4.0 to 7.2 (Stn Q3310). The average total length (mm) of *S. melanostictus* was 5.9 (Stn L3250), 6.2 (Stn N3320), 6.6 (Stn P3320), and 4.9 (Stn Q3310). Average total length (mm) of *E. teres* was 5.5 (Stn L3250), 4.7 (Stn N3320), and 7.5 (Stn P3320).

High proportions of Craniata sequences, which are mainly teleosts, were observed in the gut content metagenetic analysis. The percentages of Craniata ranged from 23.7 to 98.0% (average 78.5%) in all 64 early post-larvae (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m564p099_supp.pdf). Final sequence reads for data analysis were 1092 to 60 264 for fish guts, 15 642 to 42 658 for microplankton, and 44 699 to 61 573 for mesoplankton. These sequence reads were clustered into 430 MOTUs, with >0.01% total sequence reads at the 99% similarity threshold.

18S metagenetic analysis of Eukaryota

Various taxonomic groups of Eukaryota were detected in the metagenetic analysis of gut contents. However, most sequence reads were Metazoa in Opisthokonta, with averages of 64.4 to 77.5% (*S.*

melanostictus) and 66.8 to 75.8% (*E. teres*) at a single site (Fig. 2). Several individuals contained high percentages of Radiolaria (≤82.0%), however, on average, the percentage of Radiolaria was low, with 5.2% (*S. melanostictus*) and 4.8% (*E. teres*) in the gut contents across all stations. Other major taxonomic groups detected in the gut contents included fungi (average 11.9% in *S. melanostictus* and 11.2% in *E. teres*), Streptophyta (average 3.7% in *S. melanostictus* and 6.2% in *E. teres*), and Dinophyta (average 3.9% in *S. melanostictus* and 2.7% in *E. teres*). Metazoa comprised most sequence reads in mesoplankton communities collected by plankton nets, with an average of 89.2% in the 0.1–0.5 mm class, 95.0% in the 0.5–1.0 mm class, and 99.1% in the 1.0–2.0 mm class. Microplankton communities also had high percentages of sequence reads of Metazoa (49.3%); however, the percentages of other taxonomic groups were also high: Dinophyta (22.1%), Chlorophyta (11.0%), and Bacillariophyta (6.9%). Comparison of the average gut contents and prey availability of microplankton communities showed high predation on Metazoa, which had high availability in the environment (Fig. 3a). Radiolaria, fungi, and Streptophyta were detected in the gut contents, but were minimal in the microplankton communities, with averages of 0.4, 0.2, and 0.4% respectively.

18S metagenetic analysis of Metazoa

As Metazoa were the most prevalent taxonomic group within the Eukaryota, taxonomy within Metazoa was further investigated. Copepoda was the primary prey item for both *S. melanostictus* and *E. teres* (Fig. 4). The small mesoplankton communities (0.1 to 0.5 mm) mainly comprised Copepoda (86.3 to 93.5%), while all other taxonomic groups were minor with respect to both the gut contents and prey availability of small mesoplankton communities (Fig. 3b). The average percentage of Copepoda in the gut contents was 86.9 to 94.9% (*S. melanostictus*) and 84.0 to 94.2% (*E. teres*) at a single station. High percentages of other metazoan groups were occasionally ob-
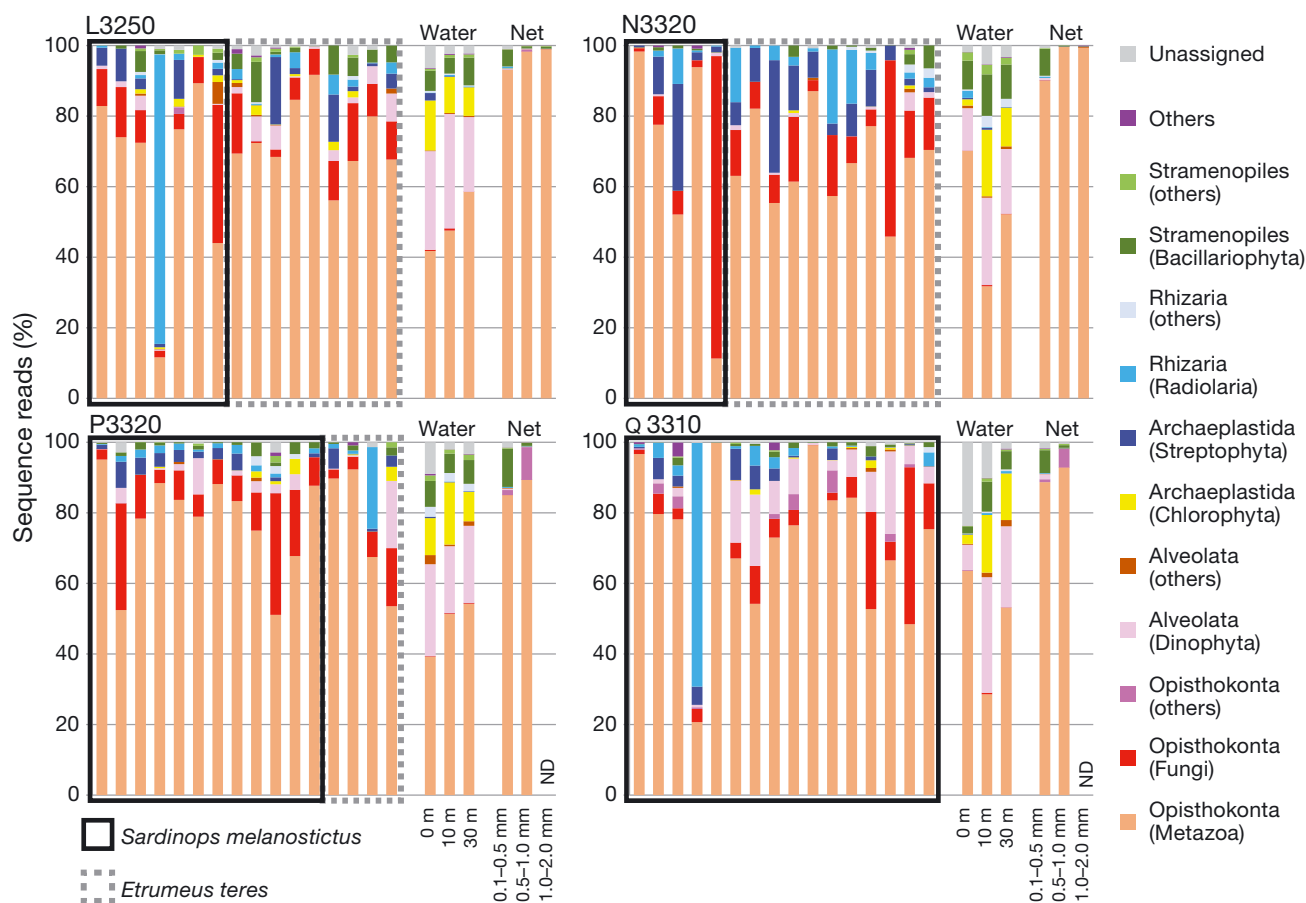

Fig. 2. Taxonomic percentages of sequence reads in Eukaryota, represented by the gut contents, microplankton (water), and mesoplankton (net) at each sampling station. 'Others' include Amoebozoa, Excavata, and Hacrobia. ND: no data
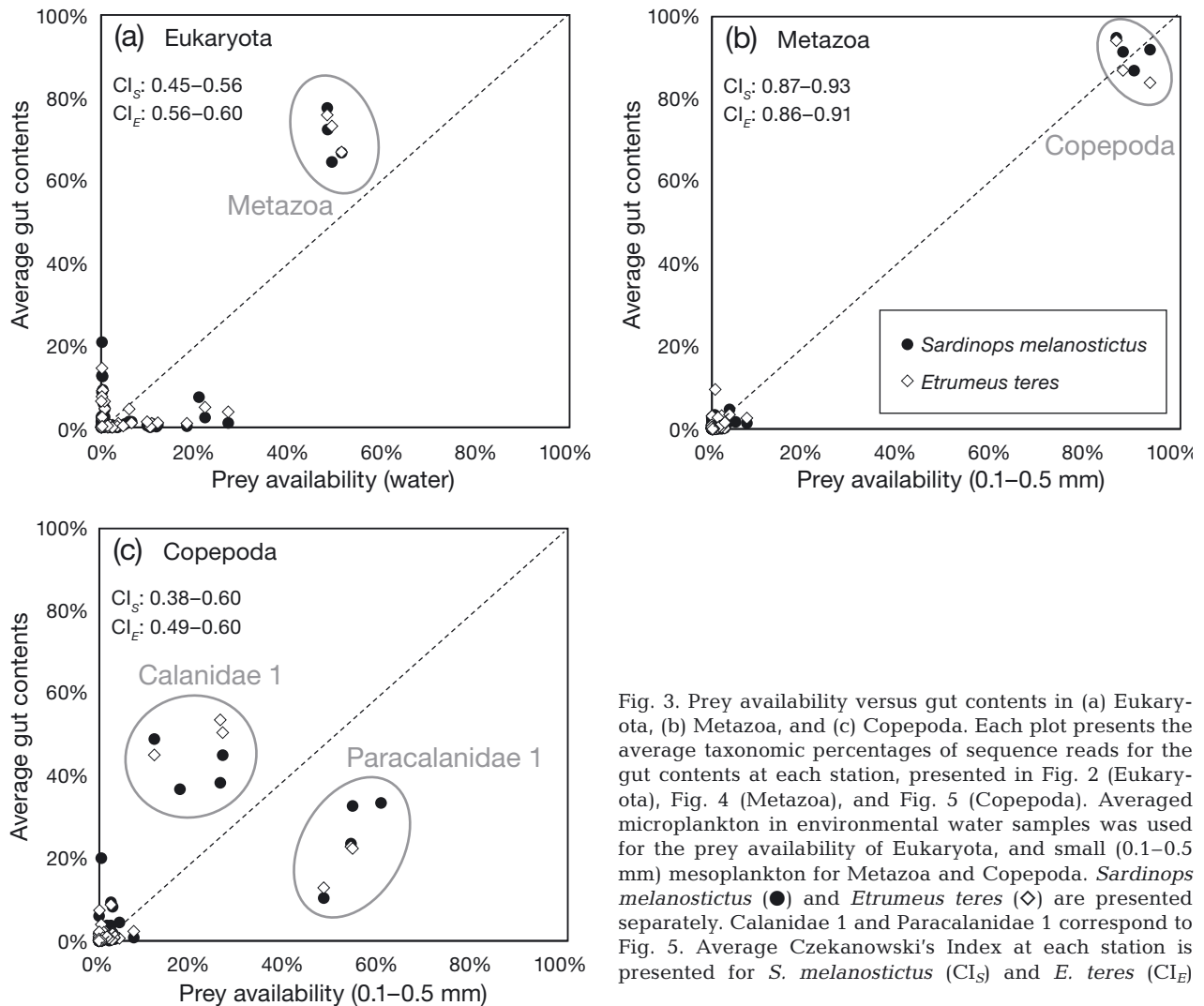
Fig. 3. Prey availability versus gut contents in (a) Eukaryota, (b) Metazoa, and (c) Copepoda. Each plot presents the average taxonomic percentages of sequence reads for the gut contents at each station, presented in Fig. 2 (Eukaryota), Fig. 4 (Metazoa), and Fig. 5 (Copepoda). Averaged microplankton in environmental water samples was used for the prey availability of Eukaryota, and small (0.1–0.5 mm) mesoplankton for Metazoa and Copepoda. *Sardinops melanostictus* (●) and *Etrumeus teres* (◇) are presented separately. Calanidae 1 and Paracalanidae 1 correspond to Fig. 5. Average Czekanowski's Index at each station is presented for *S. melanostictus* (CI_S) and *E. teres* (CI_E)

served, including Ostracoda (≤61.7%), Appendicularia (≤14.0%), Chaetognatha (≤25.3%), and Hydrozoa (≤19.4%). However, these groups represented small percentages in the average gut contents of all early post-larvae; namely, 3.2% (Ostracoda), 1.6% (Appendicularia), 0.7% (Chaetognatha), and 2.9% (Hydrozoa). High percentages of Copepoda were also observed in the intermediate (0.5–1.0 mm) and large (1.0–2.0 mm) mesoplankton communities, although other taxonomic groups, including Hydrozoa (≤29.4%) and Appendicularia (≤14.0%) were occasionally present in these size fractions.

18S metagenetic analysis of Copepoda

Copepods were the most prevalent taxonomic group within the Metazoa, therefore detailed taxon-omy within Copepoda was investigated further. In the MOTU-based analysis of Copepoda, 2 MOTUs (Calanidae 1 and Paracalanidae 1) were the major prey items for the early post-larvae of both *S. melanostictus* and *E. teres* (Figs. 3c & 5). BLAST results showed 100% identity for Paracalanidae 1 to *Paracalanus parvus* s.l. However, Calanidae 1 showed 100% identity to several Calanidae species, including *Calanus sinicus* and *Cosmocalanus darwini*, both of which are common in Tosa Bay. Calanidae 1 was the most dominant MOTU in the intermediate (0.5–1.0 mm) and large (1.0–2.0 mm) mesoplankton communities, with 69.4 to 83.4% and 77.2 to 92.9% sequence reads, respectively. Paracalanidae 1 was the most dominant in small (0.1–0.5 mm) mesoplankton communities, with 47.9 to 60.1% sequence reads, followed by Calanidae 1 (11.8 to 26.4% sequence reads). Despite Paracalanidae 1 having higher avail-
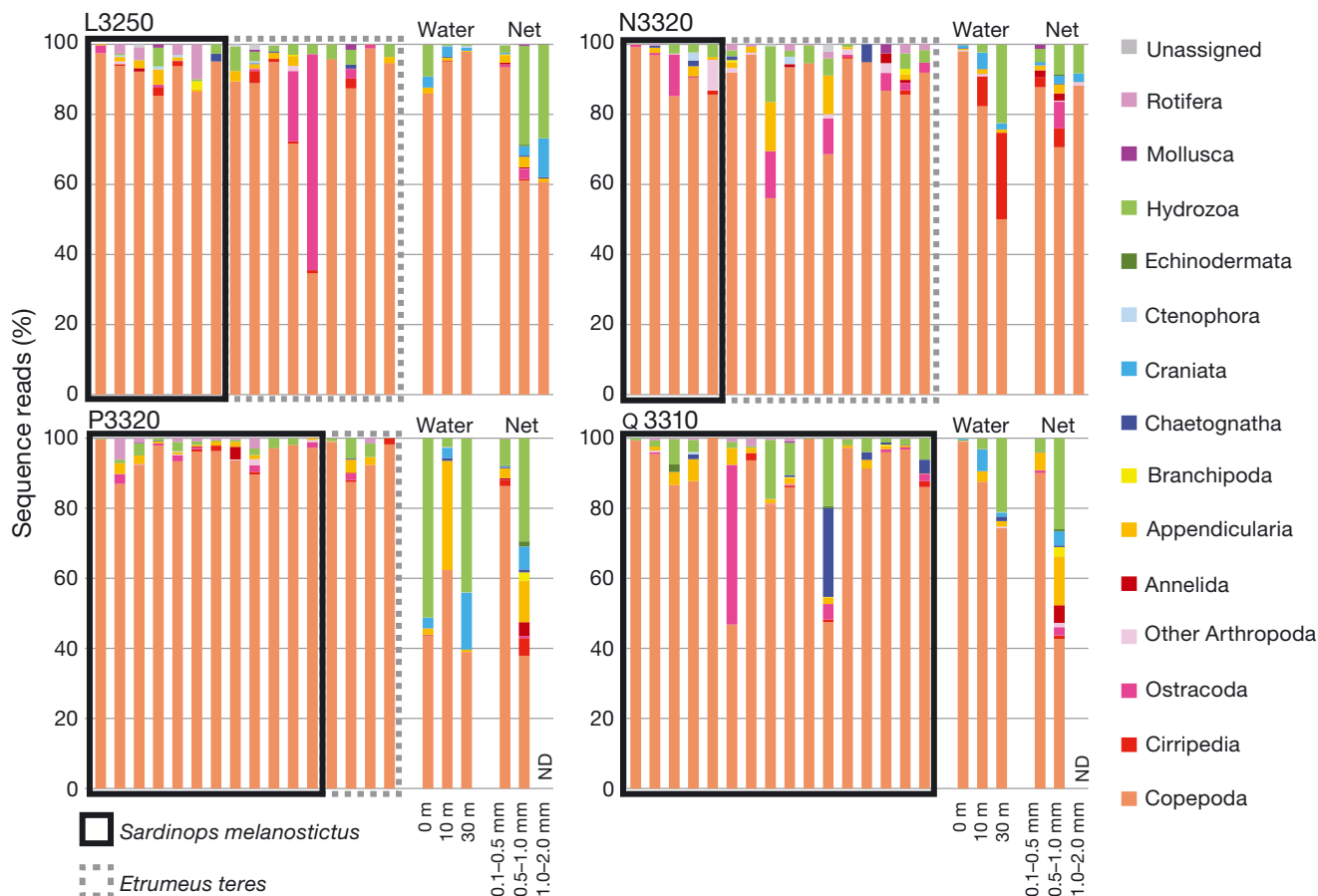
Fig. 4. Taxonomic percentages of sequence reads in Metazoa, represented by the gut contents, microplankton (water), and mesoplankton (net) at each sampling station. ND: no data

ability than Calanidae 1 in small mesoplankton communities, a greater average percentage of Calanidae 1 (36.8 to 48.9% in *S. melanostictus* and 45.1 to 53.6% in *E. teres*) than Paracalanidae 1 (10.3 to 33.4% in *S. melanostictus* and 12.9 to 22.8% in *E. teres*) (Fig. 3c) was detected in the gut contents at each sampling station. The linear indexes of food selection (i.e. *L*) were all positive for Calanidae 1 (0.12 to 0.37 in *S. melanostictus* and 0.24 to 0.33 in *E. teres*) and all negative for Paracalanidae 1 (−0.38 to −0.21 in *S. melanostictus* and −0.35 to −0.31 in *E. teres*). The value of *L* was significantly larger for Calanidae 1 than Paracalanidae 1 at Stns L3250, N3320, and P3320 (p < 0.01). Water samples also typically showed high percentages of Paracalanidae 1 and Calanidae 1 MOTUs, although a high percentage of other copepod MOTUs (e.g. Eucalanidae) was occasionally detected. Fourteen MOTUs with >10% reads in any sample belonged to the Eucalanidae, Oithonidae, Oncaeidae, Clausocalanidae, Paracalanidae, Candaciidae, and Euchaetidae families.

Dietary differences by species and sampling stations

No clear differences were observed between *S. melanostictus* and *E. teres* in the taxonomic compositions Eukaryota, Metazoa, and Copepoda in sequence reads (Figs. 2–5). The MDS analysis based on all MOTSs also showed no clear dietary difference by species or sampling stations (Fig. S2 in the Supplement). PERMDISP showed no significant (p > 0.05) differences in dispersions between *S. melanostictus* and *E. teres* at Stns L3250, N3320, and P3320 (Table S1 in the Supplement). In addition, PERM-ANOVA indicated no significant (p > 0.05) differences in gut contents between each species at each sampling station. The PERMDISP and PERMANOVA analyses also indicated no significant (p > 0.05) dispersions or differences in gut contents among sampling stations in either *S. melanostictus* or *E. teres* (Table S1).

Trophic niche breadth was estimated using CI for *S. melanostictus* and *E. teres* (Fig. 3). Modest values
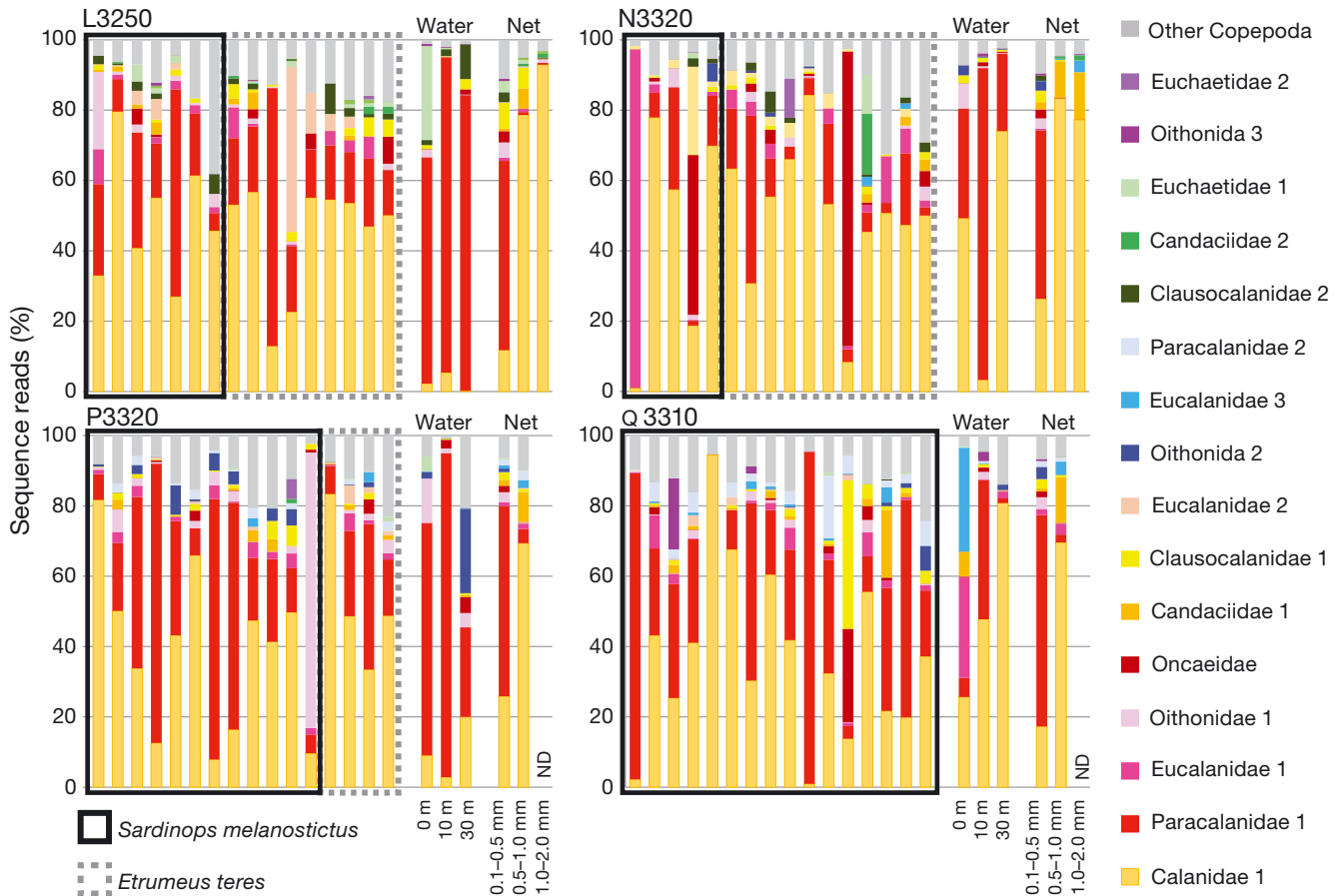
Fig. 5. Taxonomic percentages of sequence reads in Copepoda. Molecular operational taxonomic units (MOTUs) are classified to Family level based on BLAST searches against the NCBI database. Only MOTUs with >10% sequence reads in any sample were selected. All other MOTUs were included as 'other Copepoda.' Percentages of sequence reads in Copepoda are represented by the gut contents, microplankton (water), and mesoplankton (net) in each sampling station. ND: no data

were observed for Eukaryota (0.45 to 0.56 in *S. mela-nostictus* and 0.56 to 0.60 in *E. teres*) and Copepoda (0.39 to 0.61 in *S. melanostictus* and 0.49 to 0.60 in *E. teres*), but higher values were observed for Metazoa (0.87 to 0.93 in *S. melanostictus* and 0.86 to 0.91 in *E. teres*). There were no significant (p > 0.05) differences in CI between *S. melanostictus* and *E. teres* at Stn L3250, N3320, or P3320.

Comparison between 18S and 28S metagenetic analyses of copepods

A total of 5864 sequence reads from 28S regions was obtained for each size fraction of copepods at Stn L3250. Major MOTUs in the 28S metagenetic analysis showed high identity (99 to 100%) to particular copepod species based on BLAST results (Fig. 6a). Metagenetic analysis of 28S showed high dominance (39.2% sequence reads) of a MOTU with

100% identity to *P. parvus* s.l. (family Paracalanidae) in the 0.1–0.5 mm copepod community. A MOTU with 100% identity to *C. sinicus* (family Calanidae) was dominant in the 0.5–1.0 mm (55.1%) and 1.0–2.0 mm (79.9%) communities. This MOTU was discriminated from the other Calanidae MOTU that had 100% identity to *C. darwini* and much smaller sequence proportions. Therefore, the dominance of Calanidae 1 and Paracalanidae 1 in the 18S metagenetic analysis (Fig. 6b) almost corresponded with the *C. sinicus* MOTU and *P. parvus* s.l. MOTU in the 28S analysis. Other major MOTUs in 18S (e.g. Candaciidae, Clausocalanidae, and Oithonidae) were also represented in the 28S analysis.

Developmental stages of *C. sinicus*

Morphological analysis showed compositions of developmental stages of *C. sinicus* in each size frac-

(a) 28S L3250

Legend:
- Other Copepoda
- *Cosmocalanus darwini* 100% (Calanidae)
- *Euchaeta indica* 100% (Euchaetidae)
- *Subeucalanus crassus* 100% (Eucalanidae)
- *Oithona similis* 99% (Oithonidae)
- *Oncaea* sp. 100% (Oncaeidae)
- *Paracalanus aculeatus* 100% (Paracalanidae)
- *Candacia bipinnata* 100% (Candaciidae)
- *Oithona atlantica* 99% (Oithonidae)
- *Clausocalanus lividus* 100% *C. arcuicornis*
- *Paracalanus parvus* 100% (Paracalanidae)
- *Calanus sinicus* 100% (Calanidae)

(b) 18S L3250

Legend:
- Other Copepoda
- Euchaetidae 1
- Eucalanidae
- Oithonidae 2
- Oncaeidae 1
- Paracalanidae 2
- Candaciidae 1
- Oithonidae 1
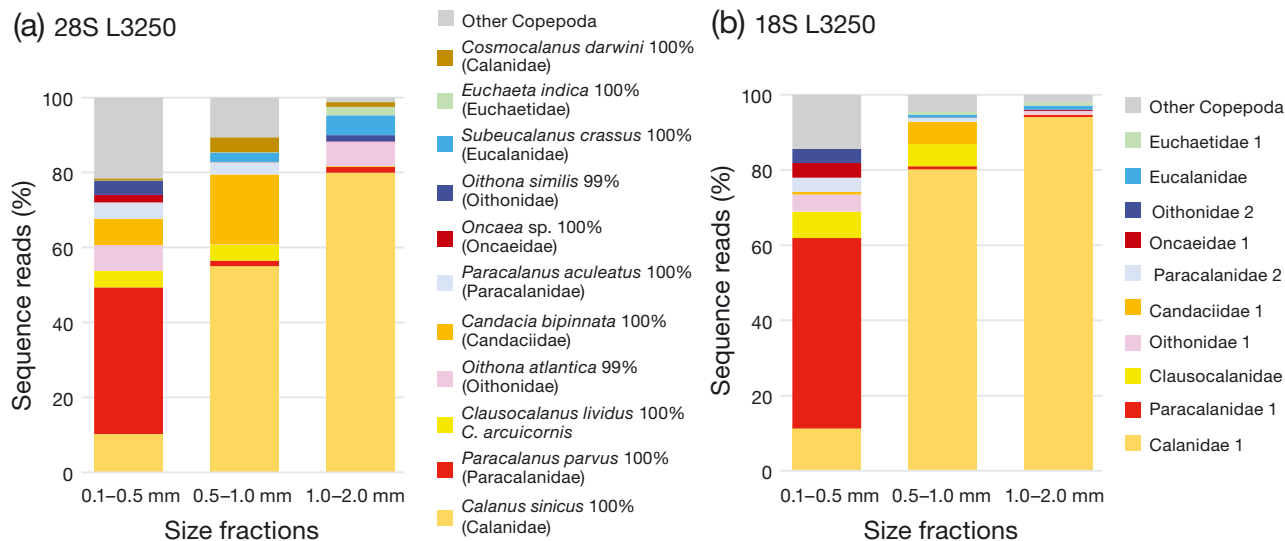- Clausocalanidae
- Paracalanidae 1
- Calanidae 1

Fig. 6. (a) 28S and (b) 18S sequence percentages of the copepod community at Stn L3250. Taxonomy of molecular operational taxonomic units (MOTUs) is based on BLAST searches against the NCBI database. The best BLAST-hit species and identities are presented for the MOTUs of 28S (a). MOTUs of 18S (b) correspond to data presented in Fig. 5

tion (Fig. 7). Adult stages were only observed in the large size fraction (1.0–2.0 mm) and occupied 19.0% (female) and 11.2% (male) of total abundance in this fraction. Individuals of CI–CIV and CV were found both in the 1.0–2.0 mm and 0.5–1.0 mm size fractions, but not in the small (0.1–0.5 mm) size fraction. In the 0.5–1.0 mm fraction, 83.3% was CI–CIV. No NI–NVI were observed in communities >0.5 mm. Only eggs (15.6%) and NI–NVI (84.4%) occupied this small size fraction.

Legend:
- CVI Female
- CVI Male
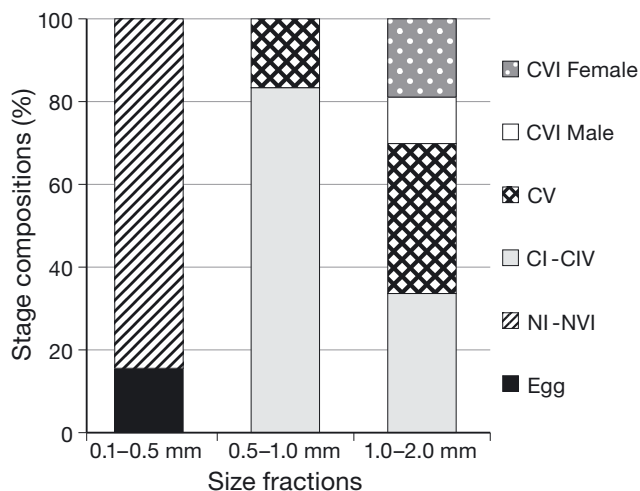- CV
- CI -CIV
- NI -NVI
- Egg

Fig. 7. Compositions of developmental stages of *Calanus sinicus* at Stn L3250. *Calanus sinicus* were classified into eggs, nauplii (N1–NVI), early copepodite (CI–CIV), fifth copepodite (CV), or adult (CVI Female/Male) stages. Abundances of each stage were counted, and compositions of developmental stages are shown for each size fraction

DISCUSSION

Validity of the metagenetic method for diet studies

Our study validated the metagenetic approach to molecular-based diet analysis, which provided a good snapshot of gut contents in early post-larvae of *Sardinops melanostictus* and *Etrumeus teres*. As 18S V9 includes all eukaryotic organisms, both taxonomic coverage and resolution of preferred prey were superior to previous morphological and stable isotope analyses (e.g. Nakata 1988, 1995, Yasue et al. 2011, 2014). However, species-level analysis is more difficult with the slow-evolving 18S rRNA gene compared with the mitochondrial gene (Tang et al. 2012, Zhan et al. 2014, Mohrbeck et al. 2015, Wu et al. 2015). Although previous metagenetic studies of the 18S region normally used a 97% similarity threshold for MOTU clustering (e.g. O'Rorke et al. 2012, Pearman et al. 2014), we used a more strict cutoff value of 99% to obtain higher taxonomic resolution of MOTUs. Comparison with the more variable 28S marker showed that dominant species were successfully reflected in the 18S metagenetic analysis of Copepoda in the study area. Quantitative aspects of the 18S V9 metagenetic analysis have been previously evaluated in environmental eukaryotic communities (de Vargas et al. 2015) and the dietary analysis of fish (Albaina et al. 2016). Despite possible methodological biases (e.g. copy number of the rRNA gene), metagenetic data tend to be correlated with biomass, providing a useful semi-quantitative method (Hirai et al. 2015, Albaina

et al. 2016). This study also showed concordance between 18S and 28S metagenetic analyses of dominant copepods, validating the 18S V9 metagenetic method as a means to investigate the quantity of preferred prey type in the diets of early post-larvae of *S. melanostictus* and *E. teres*.

In this study, whole guts were used for DNA extraction and no blocking primer to avoid amplification of predator sequences was applied. Despite small percentages of prey sequence reads (average 21.5%), deep-sequencing depth by Illumina MiSeq revealed the dominant taxonomic groups. Variations in percentages of prey sequence reads among individuals might derive from differences in gut fullness and phase of digestion. Although an empty gut is frequently observed in the morphological-based diet analysis of clupeoid larvae (Morote et al. 2010), prey information was obtained from all early post-larvae examined in this study. This phenomenon might have been due to metagenetic methods that can recover taxonomic information from degraded or digested material, such as feces (Deagle et al. 2009, Murray et al. 2011).

Importance of protozoa and gelatinous plankton as potential prey

As expected, various eukaryotic groups, including fragile protozoa and gelatinous zooplankton, were detected as potential prey from the gut contents of early post-larvae of *S. melanostictus* and *E. teres*. High percentages of protozoa were occasionally detected in the gut contents (Radiolaria and Dinophyta, including MOTUs identified as unarmored dinoflagellates), and the niche breadth of Eukaryota was modest both for *S. melanostictus* and *E. teres*. Thus, these taxa cannot be excluded as prey, although their overall contribution to diet was low compared to copepods. This is consistent with previous studies using morphological classifications (Nakata 1995) or staining methods (Fukami et al. 1999) that reported low abundances of protozoa in the gut contents of *S. melanostictus* and *E. teres* early post-larvae. Despite low availability in the environments, we detected some eukaryotic groups such as fungi in the gut contents. Previous metagenetic analyses of gut contents in marine zooplankton also detected possible parasitic organisms, secondary predations, or even contamination during experiments (O'Rorke et al. 2012, Pompanon et al. 2012, Cleary et al. 2016). Thus, both gut contents and the environmental plankton community should be investigated in metagenetic diet

analysis of fish larvae. Abundance of prey and environmental plankton communities by morphological analysis would also help to support the accuracy and utility of metagenetic analysis.

The soft-bodied chaetognaths, appendicularians, and hydrozoans were not previously considered as potential prey of *S. melanostictus* and *E. teres* early post-larvae (e.g. Yasue et al. 2011). Although these taxa were detected as potential prey in this study, the overall contributions of these taxa, as well as protists, were lower than copepods. Although digestion rates might differ for different prey items (Deagle & Tollit 2007), metagenetic methods presented a robust snapshot of gut contents; therefore, this study highlighted the importance of copepods in the main diet of these 2 fish species. This was also supported by a high value of CI in the Metazoa analyses, suggesting preference on copepods of high abundance in the environment. At a study site in central Chile, early-stage sardine *Sardinops sagax* larvae preferred mollusk larvae in their diets (Llanos-Rivera et al. 2004), while in the northwest Mediterranean, *Sardina pilchardus* selected tintinnids (Morote et al. 2010). Therefore, the importance of protozoa and non-copepod zooplankton might vary both spatially and temporally, depending on predator species, location, and season.

Important species of copepods

This study demonstrated that copepods are a major source of prey for early post-larvae stages of *S. melanostictus* and *E. teres*, which is consistent with previous morphological-based analyses (e.g. Nakata 1988, 1995, Yasue et al. 2011). Two 18S MOTUs with 100% similarity to *Calanus sinicus* (Calanidae 1) and *Paracalanus parvus* s.l. (Paracalanidae 1) were especially dominant in both the gut contents and mesoplankton communities. The 28S region showed higher variabilities in sequences than the 18S region in copepods (Blanco-Bercial et al. 2011), and concordance of BLAST results between 18S and 28S showed that *C. sinicus* and *P. parvus* s.l. were major food sources for fish larvae. These 2 species are key in the coastal areas associated with the Kuroshio Current off Japan, and are important prey for planktivorous fish in this region (Nakata & Hidaka 2003). Other MOTUs were also intermittently abundant in gut contents, including both small copepods (families Clausocalanidae, Oithonidae, Oncaeidae, and Paracalanidae) and large copepods (Eucalanidae and Euchaetidae). These copepods comprised a small percentage of the mesoplankton communities compared with *C. sinicus*

and *P. parvus* s.l. The diet composition of early post-larvae was mainly dependent on the composition of copepods in the immediate environment, although availability was not a significant factor, as suggested by the modest niche breadth in Copepoda analysis.

Copepod eggs and nauplii represented major food items for the early stages of clupeoid larvae in regions associated with the Kuroshio Current (Nakata 1988). However, taxonomic resolution in previous studies was limited, and not fully resolved to the species level. Species-level taxonomic resolutions are presented in this study, and showed that *C. sinicus* were the most prevalent in gut contents. The *C. sinicus* MOTU was dominant in intermediate (0.5–1.0 mm) and large (1.0–2.0 mm) copepod communities, which were size-fractioned using Nitex meshes, while *P. parvus* s.l. were dominant in small (0.1–0.5 mm) copepod communities. These differences may be linked to differences in the body sizes of adult females of the 2 species (which were 2.1 to 3.3 mm in *C. sinicus* and 0.7 to 1.0 mm in *P. parvus* s.l.) around the waters off Japan (Chihara & Murano 1997). According to Nakata (1988) and Morote et al. (2010), the small size fraction (<0.5 mm) was the main prey size for clupeoid larvae. In this study, developmental stages of *C. sinicus* in this size fraction were comprised of eggs and nauplii stages, and copepodite stages of *C. sinicus* were observed only in the >0.5 mm size fraction. Copepod nauplius is a suitable prey for the early larval period that has weak swimming ability (Nakata 1995). The nauplii and eggs of the dominant *C. sinicus* might be more preferred than the copepodids of the most dominant small copepod *P. parvus* s.l., which fall into a similar size range. The preference for *C. sinicus* was also supported by the food selection index, with a positive value for *C. sinicus*, and a negative value for *P. parvus* s.l. The egg production rate of *C. sinicus* peaks in February and March in the Kuroshio region off Japan (Shimode pers. comm), and might be important for the survival of early post-larvae of both *S. melanostictus* and *E. teres*. Thus, metagenetic analysis suggested that the eggs and nauplii of dominant large copepods are important during the spawning period of *S. melanostictus* and *E. teres* in Tosa Bay, Japan.

Differences in prey preferences between *S. melanostictus* and *E. teres*

This study indicated no clear differences in preferred prey of Eukaryota, Metazoa, and Copepoda between *S. melanostictus* and *E. teres*. PERMANOVA

also indicated no significant differences in gut contents, and niche breadth was not significantly different between the 2 species where they co-existed. These results support previous studies of late larval stages (Yasue et al. 2011, 2014) that reported no significant difference in the larval diet between these 2 species. The compositions of *S. melanostictus* and *E. teres* differed among sampling stations, indicating spatial separation in distributions, as highlighted by Oozeki et al. (2007). However, these 2 fish species should be competitive when they co-occur at a single station during the early larval period. In addition, no significant differences in gut contents of each species were observed among sampling stations. This study collected samples during a single cruise in the Tosa Bay, and oceanographic conditions were not largely different among sites. Further sampling efforts are necessary to investigate spatial and temporal differences in diets of both *S. melanostictus* and *E. teres* larvae.

CONCLUSIONS

High-taxonomic resolution of the diet of early post-larvae is particularly difficult to achieve. Here, we used metagenetic methods to successfully detect food sources of early post-larvae of *Sardinops melanostictus* and *Etrumeus teres*, which had not been shown in morphological analyses. The semi-quantitative data of the metagenetic method provided a significant picture of the importance of quantity (food availability) and quality (taxonomic groups and developmental stages of prey) for early post-larvae of *S. melanostictus* and *E. teres*. Various potential food sources included fragile protists and gelatinous zooplankton. However, the eggs and immature stages of dominant large copepods were suggested as important food items. Large *Calanus sinicus* was a major prey source during the spawning period of *S. melanostictus* and *E. teres* in Tosa Bay, followed by small *Paracalanus parvus* s.l., both of which are dominant copepods in these marine waters. This study only provided a snapshot of preferred prey items during a single spawning season. Thus, we could not discuss how changes in plankton communities affect the spatial and temporal population dynamics of *S. melanostictus* and *E. teres*. Although the mechanisms controlling fish recruitment are complex, optimal foraging conditions are important to enhance survival rates during the larval and fish recruitment stages. Future molecular-based studies should be conducted at various spatial and temporal scales to understand how prey availability and preferred prey items

change over time and space. If possible, morphological data should be used to support molecular-based analyses. Higher sampling coverage is becoming more accessible as costs of high-throughput sequencing become lower. These types of future studies would provide novel insights into the population dynamics of commercially important fish.

LITERATURE CITED

Albaina A, Aguirre M, Abad D, Santos M, Estonba A (2016) 18S rRNA V9 metabarcoding for diet characterization: a critical evaluation with two sympatric zooplanktivorous fish species. Ecol Evol 6:1809–1824

Amaral-Zettler LA, McCliment EA, Ducklow HW, Huse SM (2009) A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. PLOS ONE 4:e6372

Anderson JT (1988) A review of size dependent survival during pre-recruit stages of fishes in relation to recruitment. J Northwest Atl Fish Sci 8:55–66

Anderson MJ, Gorley RN, Clarke KR (2008) PERMANOVA+ for PRIMER: guide to software and statistical methods. PRIMER-E, Plymouth

Bailey KM, Houde ED (1989) Predation on eggs and larvae of marine fishes and the recruitment problem. Adv Mar Biol 25:1–83

Beaugrand G, Brander KM, Lindley JA, Souissi S, Reid PC (2003) Plankton effect on cod recruitment in the North Sea. Nature 426:661–664

Blanco-Bercial L, Bradford-Grieve J, Bucklin A (2011) Molecular phylogeny of the Calanoida (Crustacea: Copepoda). Mol Phylogenet Evol 59:103–113

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120

Chihara M, Murano M (1997) An illustrated guide to marine plankton in Japan. Tokai University Press, Tokyo

Clarke KR, Gorley RN (2015) PRIMER v7: user manual/tutorial. PRIMER-E, Plymouth

Cleary AC, Durbin EG, Rynearson TA, Bailey J (2016) Feeding by *Pseudocalanus* copepods in the Bering Sea: trophic linkages and a potential mechanism of niche partitioning. Deep-Sea Res II 134:181–189

de Vargas C, Audic S, Henry N, Decelle J and others (2015) Eukaryotic plankton diversity in the sunlit ocean. Science 348:1261605

Deagle BE, Tollit DJ (2007) Quantitative analysis of prey DNA in pinniped faeces: Potential to estimate diet composition? Conserv Genet 8:743–747

Deagle BE, Kirkwood R, Jarman SN (2009) Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. Mol Ecol 18:2022–2038

Djumanto, Kinoshita I, Bito C, Nunobe J (2004) Seasonal abundance of three clupeoid larvae and juveniles occurring in the shirasu fishery ground in central Tosa Bay, Japan. Mer (Paris) 42:95–106

Dower JF, Pepin P, Kim GC (2009) Covariation in feeding success, size-at-age and growth in larval radiated shanny (*Ulvaria subbifurcata*): insights based on individuals. J Plankton Res 31:235–247

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200

Feinsinger P, Spears EE, Poole RW (1981) A simple measure of niche breadth. Ecology 62:27–32

Fukami K, Watanabe A, Fujita S, Yamaoka K, Nishijima T (1999) Predation on naked protozoan microzooplankton by fish larvae. Mar Ecol Prog Ser 185:285–291

Hirai J, Tsuda A (2015) Metagenetic community analysis of epipelagic planktonic copepods in the tropical and subtropical Pacific. Mar Ecol Prog Ser 534:65–78

Hirai J, Kuriyama M, Ichikawa T, Hidaka K, Tsuda A (2015) A metagenetic approach for revealing community structure of marine planktonic copepods. Mol Ecol Resour 15:68–80

Hjort J (1914) Fluctuations in the great fisheries of northern Europe viewed in the light of biological research. Rapp P-V Reùn Cons Int Explor Mer 20:1–228

Huse SM, Welch DM, Morrison HG, Sogin ML (2010) Ironing out the wrinkles in the rare biosphere through improved OTU clustering. Environ Microbiol 12:1889–1898

Jarman SN, McInnes JC, Faux C, Polanowski AM and others (2013) Adélie penguin population diet monitoring by analysis of food DNA in scats. PLOS ONE 8:e82227

Kawasaki T (1983) Why do some pelagic fishes have wide fluctuations in their numbers? Biological basis of fluctuation from the viewpoint of evolutionary ecology. In: Sharp GD, Csirke J (eds) Proceedings of the expert consultation to examine changes in abundance and species composition of neritic fish resources. FAO Fish Rep 291:1065–1080

Kearse M, Moir R, Wilson A, Stones-Havas S and others (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649

Koga F (1984) Morphology, ecology, classification and specialization of copepods nauplius. Bull Nansei Reg Fish Res Lab 16:95–229

Kuroda H, Shimizu M, Hirota Y, Ambe D, Akiyama H (2008) Surface current and vertical thermal structure on the continental slope in Tosa Bay. J Oceanogr 64:81–91

Llanos-Rivera A, Herrera G, Bernal P (2004) Food size selectivity and diet overlap in larvae of Clupeiform species from central Chile. Cah Biol Mar 45:1–8

Llopiz JK (2013) Latitudinal and taxonomic patterns in the feeding ecologies of fish larvae: a literature synthesis. J Mar Syst 109–110:69–77

Mohrbeck I, Raupach MJ, Martínez Arbizu P, Knebels-

berger T, Laakmann S (2015) High-throughput sequencing—The key to rapid biodiversity assessment of marine Metazoa? PLOS ONE 10:e0140342

Montagnes DJS, Dower JF, Figueiredo GM (2010) The protozooplankton–ichthyoplankton trophic link: an overlooked aspect of aquatic food webs. J Eukaryot Microbiol 57:223−228

Morote E, Olivar MP, Villate F, Uriarte I (2010) A comparison of anchovy (*Engraulis encrasicolus*) and sardine (*Sardina pilchardus*) larvae feeding in the Northwest Mediterranean: influence of prey availability and ontogeny. ICES J Mar Sci 67:897−908

Motoda S, Anraku M, Minoda T (1957) Experiments on the performance of plankton samplings with net. Bull Fac Fish Hokkaido Univ 8:1−22

Murphy HM, Jenkins GP, Hamer PA, Swearer SE (2012) Interannual variation in larval survival of snapper (*Chrysophrys auratus*, Sparidae) is linked to diet breadth and prey availability. Can J Fish Aquat Sci 69:1340−1351

Murray DC, Bunce M, Cannell BL, Oliver R and others (2011) DNA-based faecal dietary analysis: a comparison of qPCR and high throughput sequencing approaches. PLOS ONE 6:e25776

Nakata K (1988) Alimentary tract contents and feeding conditions of ocean-caught post larval Japanese sardine, *Sardinops melanostictus*. Bull Tokai Reg Fish Res Lab 126:11−24

Nakata K (1995) Feeding conditions of Japanese sardine larvae in and near the Kuroshio examined from their gut contents. Bull Natl Res Inst Fish Sci 7:265−275

Nakata K, Hidaka K (2003) Decadal-scale variability in the Kuroshio marine ecosystem in winter. Fish Oceanogr 12: 234−244

O'Rorke R, Lavery S, Chow S, Takeyama H and others (2012) Determining the diet of larvae of western rock lobster (*Panulirus cygnus*) using high-throughput DNA sequencing techniques. PLOS ONE 7:e42757

O'Rorke R, Lavery SD, Wang M, Nodder SD, Jeffs AG (2014) Determining the diet of larvae of the red rock lobster (*Jasus edwardsii*) using high-throughput DNA sequencing techniques. Mar Biol 161:551−563

O'Rorke R, Jeffs AG, Wang M, Waite AM, Beckley LE, Lavery SD (2015) Spinning in different directions: Western rock lobster larval condition varies with eddy polarity, but does their diet? J Plankton Res 37:542−553

Omori M (1965) A 160-cm opening-closing plankton net. J Oceanogr Soc Jpn 21:212−220

Oozeki Y, Takasuka A, Kubota H, Barange M (2007) Characterizing spawning habitats of Japanese sardine (*Sardinops melanostictus*), Japanese anchovy (*Engraulis japonicus*), and Pacific round herring (*Etrumeus teres*) in the Northwestern Pacific. CCOFI Rep 48:191−203

Palumbi SR (1996) Nucleic acids II: the polymerase chain reaction. In: Hillis DM, Moritz C, Mable BK (eds) Molecular systematics. Sinauer Associates, Sunderland, MA, p 205−247

Pearman JK, El-Sherbiny M, Lanzén A, Al-Aidaroos AM, Irigoien X (2014) Zooplankton diversity across three Red Sea reefs using pyrosequencing. Front Mar Sci 1:27

Pompanon F, Deagle BE, Symondson WOC, Brown DS, Jarman SN, Taberlet P (2012) Who is eating what: diet assessment using next generation sequencing. Mol Ecol

21:1931−1950

Quast C, Pruesse E, Yilmaz P, Gerken J and others (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590−D596

Robert D, Pepin P, Dower JF, Fortier L (2014a) Individual growth history of larval Atlantic mackerel is reflected in daily condition indices. ICES J Mar Sci 71:1001−1009

Robert D, Murphy HM, Jenkins GP, Fortier L (2014b) Poor taxonomical knowledge of larval fish prey preference is impeding our ability to assess the existence of a 'critical period' driving year-class strength. ICES J Mar Sci 71: 2042−2052

Roura Á, González ÁF, Redd K, Guerra Á (2012) Molecular prey identification in wild *Octopus vulgaris* paralarvae. Mar Biol 159:1335−1345

Roura Á, Álvarez-Salgado XA, González AF, Gregori M, Rosón G, Otero J, Guerra A (2016) Life strategies of cephalopod paralarvae in a coastal upwelling system (NW Iberian Peninsula): insights from zooplankton community and spatio-temporal analyses. Fish Oceanogr 25: 241−258

Schloss PD, Westcott SL, Ryabin T, Hall JP and others (2009) Introducing MOTHUR: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537−7541

Strauss RE (1979) Reliability estimates for Ivlev's electivity index, the forage ratio and a proposed linear index of food selection. Trans Am Fish Soc 108:344−352

Takasuka A, Aoki I, Mitani I (2003) Evidence of growth-selective predation on larval Japanese anchovy *Engraulis japonicus* in Sagami Bay. Mar Ecol Prog Ser 252: 223−238

Tang CQ, Leasi F, Obertegger U, Kieneke A, Barraclough TG, Fontaneto D (2012) The widely used small subunit 18S rDNA molecule greatly underestimates true diversity in biodiversity surveys of the meiofauna. Proc Natl Acad Sci USA 109:16208−16212

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261−5267

Watanabe Y, Zenitani H, Kimura R (1996) Offshore expansion of spawning of the Japanese sardine, *Sardinops melanostictus*, and its implication for egg and larval survival. Can J Fish Aquat Sci 53:55−61

Wu S, Xiong J, Yu Y (2015) Taxonomic resolutions based on 18S rRNA genes: a case study of subclass Copepoda. PLOS ONE 10:e0131498

Yasue N, Takasuka A, Shirakihara K (2011) Interspecific comparisons of growth and diet among late larvae of three co-occurring clupeoid species in the Kii Channel, Japan. Mar Biol 158:1709−1720

Yasue N, Doiuchi R, Takasuka A (2014) Trophodynamic similarities of three sympatric clupeoid species throughout their life histories in the Kii Channel as revealed by stable isotope approach. ICES J Mar Sci 71:44−55

Zhan A, Bailey SA, Heath DD, MacIsaac HJ (2014) Performance comparison of genetic markers for high-throughput sequencing-based biodiversity assessment in complex communities. Mol Ecol Resour 14:1049−1059