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Diet composition and feeding habits of larval Pacific bluefin tuna *Thunnus orientalis* in the Sea of Japan: integrated morphological and metagenetic analysis

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ABSTRACT: Diet compositions of larval Pacific bluefin tuna (PBT) *Thunnus orientalis* caught in the Sea of Japan during the summers of 2011–2015 were investigated using both microscopic (n = 149) and metagenetic (n = 120) methods to determine the species' prey and feeding habits. The microscopic analysis revealed that prey abundance in gut contents ranged from 0–10 ind. larva^{-1} (mean ± SD: 0.89 ± 1.5 ind. larva^{-1}, including larvae with empty guts), which was low, similar to levels for larval bluefin tunas in other seas except the Mediterranean. The cladoceran family Podonidae (*Podon* spp. and *Evadne* spp.) represented the most abundant prey (0.73 ± 1.4 ind. larva^{-1}) observed microscopically. Metagenetic analysis of the 18S V9 region detected a taxonomically wide range of prey. In addition to Podonidae, morphologically unidentified taxa were detected as major prey, including Copepoda (*Paracalanus parvus* s.l. and *Labidocera* sp.), Appendicularia (*Oikopleura* spp.), and Doliolida (*Doliolum* sp.). Compared with plankton communities in the sea, the proportions of *Oikopleura* spp. and Podonidae in the guts of the larvae were high, indicating that PBT larvae fed on them selectively. Although copepods were abundant in the sea, PBT larvae only consumed a small number of them. The ability of copepods to escape predation is higher than that of *Oikopleura* spp. and Podonidae, and the guts of the PBT larvae generally contained a low number of copepods considering their abundance and size in the region. This study successfully characterized the prey of PBT larvae in the Sea of Japan, and showed that they were unable to exploit the abundance of copepods, a fact which may lead to high larval mortality.

KEY WORDS: Pacific bluefin tuna · Larval feeding habits · Larval trophic ecology · Diet composition · Zooplankton · 18S rDNA · Metagenomics · Trophic selectivity

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INTRODUCTION

Pacific bluefin tuna (PBT) *Thunnus orientalis* is among the most commercially valuable fisheries resources in the world, along with other bluefin tuna (BT) species, namely Atlantic (ABT) *T. thynnus* and southern (SBT) *T. maccoyii* (Collette et al. 2011). After PBT spawn, larvae and juveniles develop in the western subtropical North Pacific and southern Sea of Japan during May–July and July–August, respectively (Tanaka et al. 2007, Ohshimo et al. 2017). Larval growth is a controlling factor in the recruitment of PBT (Tanaka et al. 2006, Watai et al. 2017), and culture experiments have indicated that appropriate diets are an important factor for PBT larval growth (Tanaka et al. 2014). Other culture experiments have shown that PBT larvae consume more prey than other fish larvae (Miyashita 2002); guts of larvae always contained prey (rotifers) during the daytime in food-rich conditions.

Small crustacean zooplankton, i.e. copepods and cladocerans, are the main food of larval BTs (Llopiz & Hobday 2015). Previous studies based on microscopic observations revealed that small copepods (Corycaeidae, Clausocalanidae, and Paracalanidae) and their nauplii are the main prey of PBT larvae in the western subtropical North Pacific (Uotani et al. 1990), as well as of SBT larvae in northwestern Australia (Uotani et al. 1981). ABT larvae preyed mainly on copepods and cladocerans in the Gulf of Mexico (Llopiz et al. 2015, Tilley et al. 2016) and on copepod nauplii and cladocerans in the Mediterranean (Catalán et al. 2011). In the Gulf of Mexico including the Florida Straits, ABT larvae feed on appendicularians as well as crustacean zooplankton (Llopiz et al. 2010, 2015, Muhling et al. 2017). Stable isotope analyses have indicated that larval ABT are zooplanktivorous in the Gulf of Mexico and the Mediterranean (Laiz-Carrión et al. 2015); however, their dietary habits in the western Gulf of Mexico were unclear based on isotopic discrimination of nitrogen. The primary diet depends on larval size. Piscivory, which is important for rapid growth and survival (Tanaka et al. 2014), starts at >6 mm body length in the case of ABT in the Gulf of Mexico (Llopiz & Hobday 2015, Llopiz et al. 2015).

Morphological identification of prey species has traditionally been used to determine the feeding habits of fishes; however, it is of limited use for the identification of damaged prey samples and gelatinous plankton (Sousa et al. 2016). To overcome this problem, a metagenetic (metabarcoding) technique has recently been applied in dietary analyses of fishes in lieu of microscopic observations (Riemann et al. 2010, Martin et al. 2015, Albaina et al. 2016, Oyafuso et al. 2016, Sousa et al. 2016, Hirai et al. 2017, Sakaguchi et al. 2017). Metagenetic analysis revealed a greater diversity of taxa in the guts of juvenile chum salmon than microscopic observation (Sakaguchi et al. 2017); thus, the metagenetic technique is a powerful tool to analyze feeding habits. However, this technique also has possible biases, including the degree of digestion (which can affect DNA preservation), numbers of registered sequences available in public databases, copy numbers of target genes, and PCR biases (Pompanon et al. 2012); therefore, a combination of microscopic observations and the metagenetic technique are recommended to assess feeding habits (Sakaguchi et al. 2017). In addition, microscopic observations can be used to investigate prey numbers in the gut, and this technique is also necessary for comparisons with the results of previous studies.

The Sea of Japan is one of 2 PBT spawning areas (Okochi et al. 2016), and a quarter of the age-0 PBT caught in Japan may have hatched in the Sea of Japan (Itoh 2009). The zooplankton population dynamics in the Sea of Japan are unique (Iguchi 2004), as are the physical and chemical water properties (Uda 1934, Kodama et al. 2015): the Sea of Japan has a lower water temperature (Okiyama 1974, Ohshimo et al. 2017) and higher biological productivity during summer than the other major PBT spawning ground in the subtropical western North Pacific (Kodama et al. 2015).

Here, we examined the diet composition of PBT larvae in the Sea of Japan using integrated morphological and metagenetic techniques, and evaluated their feeding habits and selectivity.

MATERIALS AND METHODS

Larval PBT and zooplankton were collected during cruises conducted between 2011 and 2015. All collected larval PBT were dissected, and organisms found in their guts were identified under a microscope. Organisms in the guts of larval PBT sampled in 2013–2015 were collected on a filter and identified using metagenetic analysis.

Field observations

On-board observations were conducted in July and/or August from 2011–2015 on the RV 'Shoyo-maru' of the Fisheries Agency (2012 and 2013) and on the RV 'Shunyo-maru' of the Japan Fisheries Re-

search and Education Agency (2011, 2014, 2015) at 273 stations in the southern Sea of Japan (Fig. 1). Larval PBT were collected with a surface horizontal tow of a ring net (340 µm mesh, 2 m mouth diameter) for 10 min towing at a speed of ~1.5 knots alongside the ship. Zooplankton were collected with a vertical haul of a single long Norpac net (Nytal XX25, 64 µm mesh, 0.45 m mouth diameter; Rigo) from 50 m to the surface to avoid underestimating the abundance of nauplius larvae of copepods. The sea surface temperature (SST) was measured using a calibrated mercury thermometer. Zooplankton samples were fixed with neutral formalin immediately after sampling, and individuals were morphologically identified to the lowest possible taxonomic level at selected stations (n = 13) where PBT larvae were also collected (Fig. 1). In addition, seawater was pumped from the bottom of the ship (~5 m depth) at 10 l min^{-1}, and was partly (~2 l min^{-1}) filtered using 100 µm mesh for approximately 60 min at the other 11 stations during the 2015 cruise, and filtered organisms were analyzed using the metagenetic method described below (eDNA, Fig. 1).

Larval treatments

The guts of PBT larvae are generally full during the daytime (Uotani et al. 1990), but we conducted gut content observations regardless of the time of day. The collected PBT and tuna-like larvae were sorted on board soon after collection in ~0°C seawater, and morphologically identified PBT larvae were fixed in 99% ethanol at room temperature (15–25°C) for several months until genetic identification and dissection. After the genetic identification of larval species (Chow et al. 2003, Suzuki et al. 2014), the notochord or standard length (summarized as body length, BL) of each PBT larva was measured using computer software (Image-J; https://imagej.nih.gov/ij/), after taking a photograph under a dissecting microscope. After larval dissection, organisms in the guts were identified to the lowest taxonomic level, and the numbers of identified organisms were counted. The longest and shortest diameters (BL and width, re-

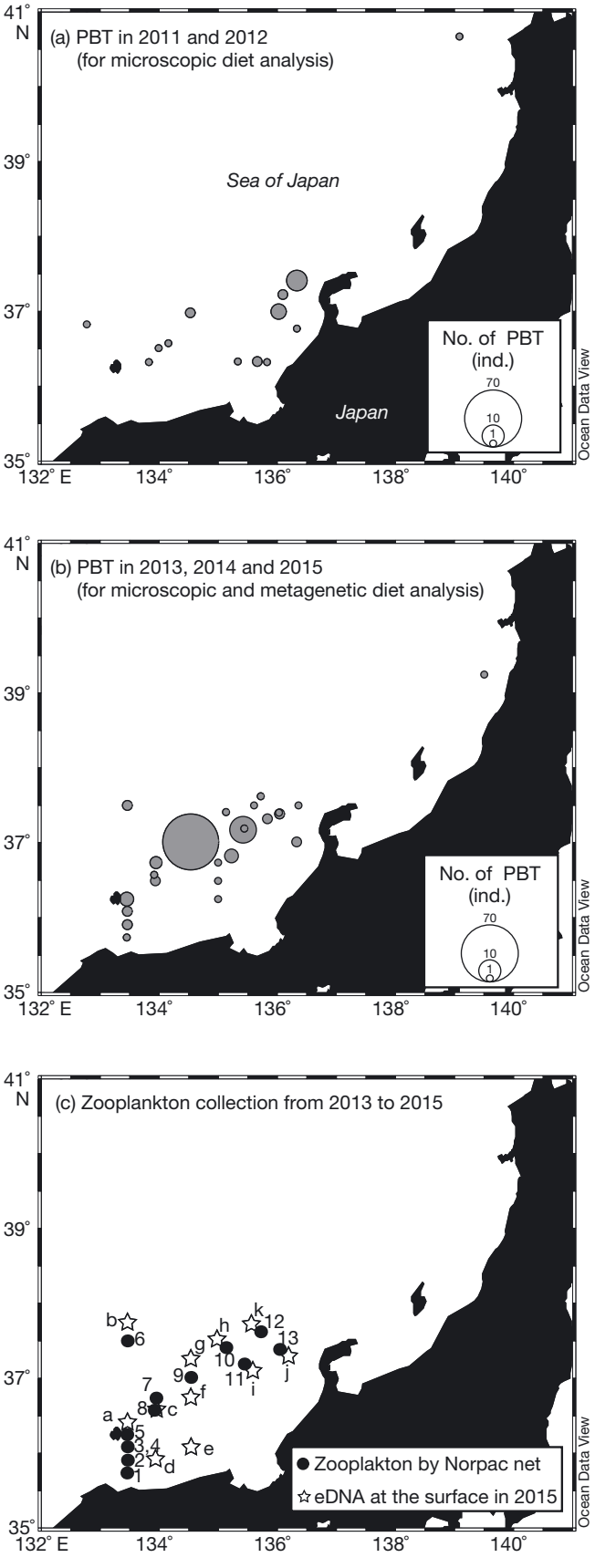

Fig. 1. Study site in the Sea of Japan. The numbers of Pacific bluefin tuna *Thunnus orientalis* (PBT) larvae collected (a) in 2011 and 2012, and (b) in 2013, 2014, and 2015 are indicated by the size of the circles. (c) Stations where zooplankton were collected. Stars with letters a–k denote stations where plankton were collected for the environmental metagenetic analysis. Black dots numbered 1–13 denote stations where plankton were collected with a 0.06 mm-mesh Norpac net from a depth of 0–50 m for microscopic analysis

spectively) of the identified organisms collected during the daytime were measured in 2014 and 2015 as effectively as possible. All gut contents and larvae in ethanol on individual dissecting plates were returned to a bottle until DNA extraction at room temperature, except in 2015, when they were stored in ethanol at –20°C.

Metagenetic analysis of gut contents

After removing the body and large tissues of a PBT larva, the gut contents in ethanol solution were filtered through a 25 mm diameter polytetrafluoroethylene (PTFE) membrane filter with a 0.2 µm pore size (Omnipore, Merck) using gentle suction (<100 hPa). Controls were prepared using the gut content samples: (1) PTFE filter through which only ethanol (without any organisms) was passed, (2) the eye of a larval PBT, (3) Podonidae, which were collected using the Norpac net and preserved in ethanol, and (4) gut contents of a cultured PBT larva that was fed the rotifer *Brachionus plicatilis*. The filter sample was used to determine whether there was contamination from non-target organisms during the experiment; and the other 3 sample types provided information on the success or failure of the PCR amplification. The podonid samples were prepared in 2 ways: one contained 2 individuals of *Podon* sp., and the other contained 2 individuals of *Podon* sp. and *Evadne* sp., respectively. These podonid samples helped interpret the results after a bioinformatics analysis. DNA extraction and purification were carried out using the DNeasy Blood & Tissue Kit (Qiagen) and the PowerClean DNA Clean-Up Kit (MO BIO Laboratories). The concentrated purified DNA was measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific).

Amplicons of the 18S V9 region were prepared using a 3-step PCR for high-throughput sequencing on the Illumina MiSeq. First, each 25 µl subsample was prepared using KOD -Plus- Ver.2 (TOYOBO Life Science). The subsamples comprised 13 µl of distilled water, 2.5 µl of 10× buffer, 2.5 µl of 2 mM dNTPs, 1.5 µl of 25 mM $MgSO_4$, 0.5 µl of KOD Plus polymerase, 1.5 µl of 5 µM forward primer, 1.5 µl of 5 µM reverse primer, and 2 µl of purified DNA solution (1 ng μl^{-1}). When the concentration of purified DNA solution was <1 ng μl^{-1}, the subsamples were prepared in the same way, excluding 10 µl distilled water and 5 µl purified DNA solution that was not diluted. The primer pair 1389F (5′-TTG TAC ACA CCG CCC-3′) and 1510R (5′-CCT TCY GCA GGT TCA CCT AC-3′) was used to amplify the 18S rDNA V9 region (Amaral-Zettler et al. 2009). After the first PCR (94°C for 2 min, followed by 22 cycles of 98°C for 10 s, 56°C for 30 s, and 68°C for 60 s; and ending with 68°C for 7 min) using a thermal cycler (Eppendorf, Mastercycler epgradientS), the amplifications of the target region (approximately 130 bp) were confirmed using electrophoresis on a 1.5% agarose gel with a SYBR Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific). Since some samples had been preserved for >3 yr at room temperature, the target region was not well amplified by PCR in the samples collected in 2011 (n = 4) and 2012 (n = 1), and thus, we removed these samples from the metagenetic analysis. The sample of 2012 was larval *Thunnus tonggol*, which was collected and preserved with PBT larvae; it is not listed in Table S1 in the Supplement at www.int-res.com/articles/suppl/m583p211_supp.xls. The pilot PCR experiment for 2011 and 2012 samples was conducted by using the samples for which the prey were observed by microscopic analysis, and sometimes the DNA concentration was detectable by the Qubit Fluorometer after purification; thus we considered that the DNA in 2011 and 2012 was fragmented.

Second, PCR amplification was performed to attach the Illumina MiSeq tag to the edges of the target sequence. By using combined primer pair MiSeq tag and 18S V9 primer (see details in Hirai et al. 2017), products were amplified with the following thermal cycle: 94°C for 2 min followed by 8 cycles of 98°C for 10 s, 50°C for 30 s, 68°C for 60 s; and ending with 68°C for 7 min. The template, i.e. the product of the first PCR, was diluted 10 times when amplification was confirmed using electrophoresis, and not diluted when amplification was not confirmed.

Third, the PCR used the primer pair attached to the sequence tag for discriminating samples, and was conducted with the following thermal cycle: 94°C for 2 min followed by 8 cycles consisting of 98°C for 10 s, 59°C for 30 s, 68°C for 60 s; and ending with 68°C for 7 min. The quality of the final PCR products was analyzed using electrophoresis, and the DNA concentration was measured using the Qubit fluorometer. After purification of the PCR-amplified samples using a QIAquick PCR Purification Kit (Qiagen), the amplified samples were sequenced and distributed in 2 runs on Illumina MiSeq (Paired-End reads; 2 × 250 bp) (accession numbers in the DNA Data Bank of Japan [DDBJ] Sequence Read Archive: DRA005917 and DRA005918).

After being trimmed using Trimmomatic version 0.36 (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 bioinformatics analysis in MOTHUR (Schloss et al. 2009). The following quality

filtering criteria were applied mainly based on Hirai et al. (2017): (1) contained no ambiguous bases; (2) contained <3 mismatches per primer; (3) contained ≤5 homopolymers; and (4) archived 97.5% of the minimum and maximum sequence length. Sequences were aligned in MOTHUR against the SILVA database (Quast et al. 2013). The aligned sequences were filtered using single-linkage pre-clustering (Huse et al. 2007). To reduce the data size, we removed rare sequences (≤1 and ≤100 reads in gut contents and eDNA samples, respectively); rare organisms could not be detected in our analysis. Possible chimeras were checked by UCHIME (Edgar et al. 2011) without a database. We classified sequence reads into taxonomic groups using a naïve Bayesian classifier (Wang et al. 2007) and the *V9_PR2* reference database provided by the Tara Ocean project (de Vargas et al. 2015) with a threshold >80%. In this study, we focused on eukaryotic organisms, thus only sequences classified as 'Eukaryota' were selected, and the taxonomic group 'Craniata' was removed from the gut content data to avoid sequence reads from the host or contamination. In addition, the taxonomic groups 'Fungi' and 'Embryophyceae' were contained in the control samples, and thus, we removed these taxonomic groups as well. The quality-filtered sequences were clustered into molecular operational taxonomic units (MOTUs) at the 99% similarity threshold using the average neighbor algorithm in MOTHUR. The MOTUs were taxonomically assigned to the lowest level possible based on a BLAST search against the NCBI database at the 99% similarity threshold. When similarity was <99%, we used the lowest taxonomic groups classified by the *V9_PR2* reference.

Plankton community and diet composition indices

To evaluate the importance of metagenetically identified organisms in the eDNA samples and PBT larval guts, we used indices showing the proportion of total sequences of target species (MOTUs) to the total number of organisms (%N), proportion of presence/absence of target species (MOTUs) to the number of non-empty guts (%O), and feeding incidents (FI, proportion of non-empty guts to total) (Hyslop 1980). To calculate %N based on the results of the metagenetic analysis, the numbers of sequence reads in MOTUs were used instead of the number of individuals; however, read numbers are largely affected by proportions of host DNA or non-target DNA, such as bacteria. Therefore, %N values based on the metagenetic analysis were calculated using the following 2 steps: (1) the %N of each larva or sample was calculated (individual %N), and (2) they were averaged. In addition, assuming that 18S rDNA concentration is associated with total DNA concentration, and that DNA concentration is associated with body size (de Vargas et al. 2015), we calculated an index (percent mass, %M), using the following equations.

$$\mathrm{cor}N_{n,m} = DNA \times N_{n,m} \div N_m \quad (1)$$

$$\%\mathrm{M}_n = \sum \mathrm{cor}N_{n,m} \div \sum \mathrm{cor}N_m \quad (2)$$

where $\mathrm{cor}N_{n,m}$ is the correlated read number of target organisms, *DNA* is the extracted DNA concentration (ng μl^{-1}), $N_{n,m}$ is the read number of target organisms (n) in 1 sample (m), and N_m represents the total read numbers contained in the host and contamination in 1 sample. When the DNA concentration was undetectable, we used the detection limit values of the fluorometer, 0.1 ng μl^{-1}. The %M of the target organism ($\%\mathrm{M}_n$) was calculated from $\mathrm{cor}N_{n,m}$ and $corN_m$, which are the re-calculated total read numbers (Eq. 2) and averaged absolute dominancy of target organisms in the gut contents. The %M values of eDNA samples were also calculated, as well as those of the diet analysis.

Food selectivity was determined using Chesson's index (Chesson 1983). Chesson's index (α_n) of a target organism (n) was calculated using the following equation.

$$\alpha_n = (r_n / p_n) \Big/ \sum (r_n / p_n) \quad (3)$$

In this equation, r_n and p_n indicate the proportion of target organisms in the larval gut and eDNA, respectively. Proportionally, r_n was calculated using $\%\mathrm{M}_n$ or %N of larval guts (not individual ones), and p_n was calculated using all station values (n = 13). The selectivity analysis was dependent on whether α_n was higher (preferred) or lower (nonpreferred) than the neutral value, which is the inverse number of taxa in the diet. We only used larval samples from 2015 (n = 70) because the eDNA samples were collected only during the 2015 cruise.

RESULTS

PBT larval collection

We collected a total of 149 larvae at 40 stations during 2011–2015, and 120 larvae from 29 stations were analyzed using the metagenetic approach during 2013–2015. PBT larvae ranged from 2.7–7.6 mm BL

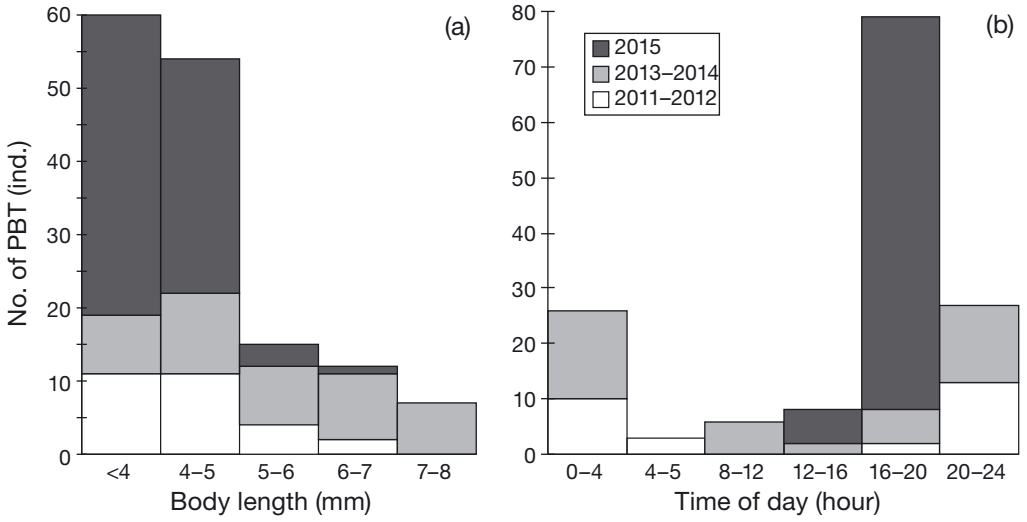

Fig. 2. (a) Numbers of Pacific bluefin tuna *Thunnus orientalis* (PBT) larvae collected and categorized into 5 classes based on body length, and (b) 4-hourly variation in numbers of PBT larvae collected. White, gray, and black bars denote larvae collected during 2011–2012, 2013–2014, and 2015, respectively

(n = 148; 1 larva was missing its head), and larval lengths were classified into size classes of 3.0–<4.0 and 4.0–<5.0 mm (n = 47 and 40, respectively, during 2013–2015, Fig. 2a). Larval PBT samples were sampled both during the day and at night (Fig. 2b). From 05:00 to 08:00 h, no PBT larvae were collected throughout the cruises. From 04:00 to 05:00 h, no PBT larvae were collected during 2013–2015 (Fig. 2b); thus FI in metagenetic analysis was not calculated in this period. At Stn 9 (37° 00′ N, 134° 30′ E) in 2015, we collected 63 larvae with BLs of 2.9–4.8 mm (Fig. 1). Six of 7 large larvae (≥7.0 mm BL) were collected only at Stn 11 in 2013, and only 1 of 76 larvae collected in 2015 had a BL ≥6.0 mm (Fig. 2a). SST ranged from 23.3–28.8°C; only 2 larvae were collected from water with SST >28°C, and the others were collected at SSTs below 27°C. These results are also shown in Table S1 in the Supplement.

Gut contents based on microscopic observations

In the microscopic observations, 132 individual prey organisms were observed from the guts of 66 larvae (mean ± SD: 2.0 ± 1.7 ind. larva^{-1}), whereas the guts of 83 larvae were empty; thus, the FI was 44%, and overall mean prey number was 0.9 ± 1.5 ind. larva^{-1}. The 4-hourly calculations of FI and mean prey number showed diel variation, with high values recorded during the daytime, decreasing to 0 just before dawn (Fig. 3). FI from 12:00–16:00 h was 100% (Fig. 3), whereas FI was 4 and 0% during 00:00–04:00 and 04:00–05:00 h, respectively. The mean prey numbers showed the same trend: the highest values (2.0 ± 1.2 ind. larva^{-1}) were observed during 12:00–16:00 h (Fig. 3). The most frequent morphologically identified taxon in larval guts was Cladocera (Podonidae including *Podon* spp. and *Evadne* spp.), with a total of 110 individuals from 55 larvae (the maximum was 10 ind. larva^{-1}). *Paracalanus* spp. (11 individuals from 9 larvae) and *Oithona* spp. (8 individuals from 7 larvae) nauplii were also detected from guts, although their mean abundance was 1 order of magnitude lower than that of Podonidae. Rare taxa included the eggs (observed twice) and larvae (observed once) of

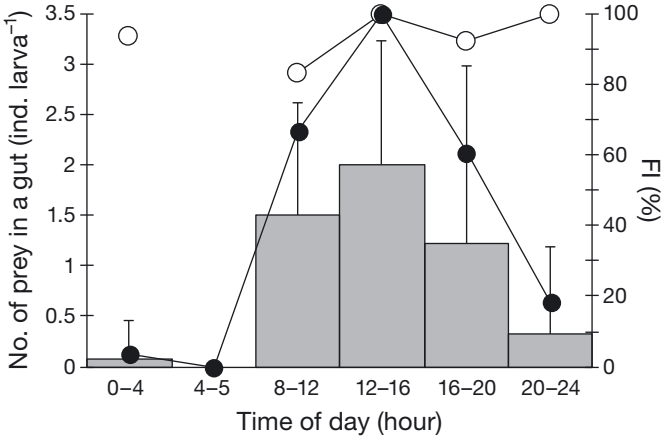

Fig. 3. Four-hourly variation in mean (+ SD) numbers of prey in guts of Pacific bluefin tuna *Thunnus orientalis* larvae (including empty guts), feeding incidents (FI) based on microscopic observations (closed circles), and FI based on metagenetic analysis (open circles). FI based on the metagenetic analysis was not evaluated during 04:00–05:00 h because no larvae were collected during this period in 2013–2015

Cephalopoda, which could not be identified to the genus level, as well as some other organisms, not including amorphous digested prey that could not be identified by morphological observations.

The sizes of identified organisms were mostly 0.2–0.3 mm BL and width in the larval guts collected in 2014 and 2015 (n = 81). From these BLs and widths, we calculated the surface area (size) of organisms based on the assumption that the organisms had an elliptical shape. This showed that 85% (69 of 81) organisms in guts were <0.1 mm^2 and 95% (79 of 81) were <0.2 mm^2 (Fig. 4a). The largest organism in this study was a cephalopod larva of 1.42 mm^2 (2 mm long and 0.9 mm wide), which was consumed by a PBT larva of 6.3 mm BL. Sizes of prey organisms were pooled for every PBT larva, and the pooled sizes were then grouped into size classes. The mean pooled size of the prey organisms in the PBT larvae were <0.3 mm^2, and 50 prey individuals (in 54 larvae) were <0.3 mm^2 (Fig. 4b); organisms that were not intact were assigned the mean organism values.

Metagenetic data for detecting prey organisms

In the metagenetic analysis, 0–75.7% of sequence reads from prey organisms (0–109 501 reads) remained after removal of the host and contaminants. The larval sample with 109 501 reads was an extreme outlier; the read numbers of the other samples were all <1000.

In the negative control samples (filter and larval eye), no sequence reads were detected after removal of the host and contaminants (Fig. 5). The positive controls included target taxa (Podonidae, and rotifer prey), although small proportions of other non-target organisms were also detected (Fig. 5). MOTU numbers of *Podon* sp., *Evadne* sp., and Branchiopoda were higher than the species numbers of each taxon in the Podonidae samples (Fig. 5). Thus, the MOTUs were overestimated, and the MOTUs identified as Branchiopoda, but not *Penilia avirostris*, were regarded as Podonidae in the following analyses.

The bioinformatics analysis indicated a different number of MOTUs; however, at least 155 MOTUs remained at their original taxonomic level based on the TARA-Ocean database and BLAST. A high FI value of 94.1% was observed, and no prey MOTUs were detected from 7 larval guts. The 4-hourly variations in FI values based on the metagenetic analysis were >88%, and no data were collected during 04:00–05:00 h. Regarding %O, copepods identified as *Paracalanus parvus* s.l. were observed most frequently (47.8%O, Fig. 6a), followed by Appendicularia (identified as *Oikopleura* spp. at the genus level, 36.2%O), Branchiopoda (Podonidae, 30.1%O), Chaetognatha (Sagit-

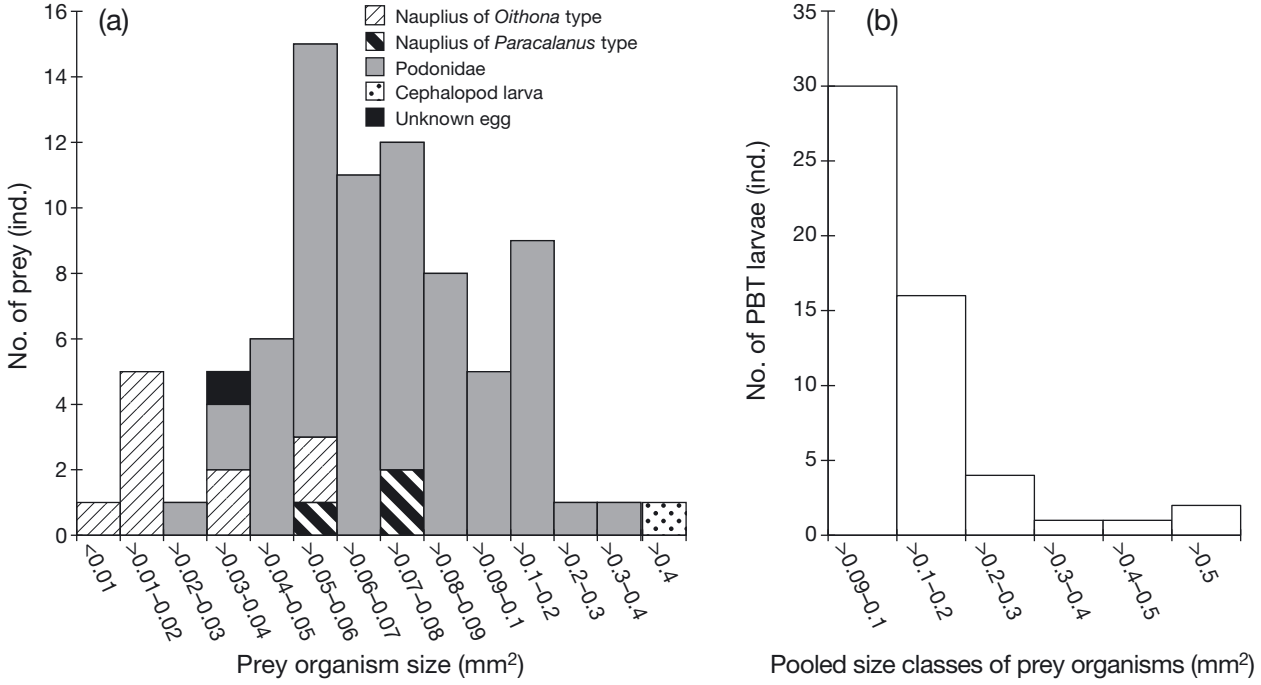

Fig. 4. (a) Number of prey organisms classified by size (surface area, mm^2) in the guts of larval Pacific bluefin tuna *Thunnus orientalis* (PBT) by microscopic observations in 2014 and 2015 collected during the daytime (total n = 81) and (b) number of larvae classified by the pooled sizes of prey organisms (total n = 54)

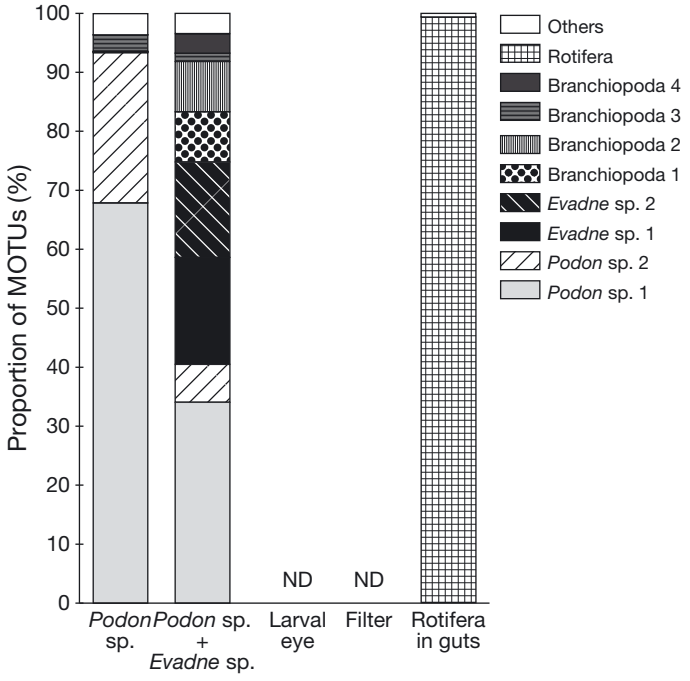

Fig. 5. Proportions of sequence reads in the metagenetic analysis of control samples (2 individuals of *Podon* sp., 2 of *Podon* sp. + 2 of *Evadne* sp., Pacific bluefin tuna *Thunnus orientalis* [PBT] larval eye, the polytetrafluoroethylene [PTFE] filter, and the guts of larvae that had fed exclusively on rotifers in the lab). Proportions of clustered molecular operational taxonomic units (MOTUs) are represented in each sample. ND: no data after quality-filtering processes

toidea, 30.1%O), and a calanoid copepod identified as *Labidocera* sp. (23.9%O). Phytoplanktonic prasinophytes (Prasino-Clade), Dinophyta (Dinophyceae), and diatoms were also detected, with %O values >10. Cyclopoid copepods identified as Oithonidae, calanoids identified as *Clausocalanus* sp., and Poecilostomatoida identified as Oncaeidae also had %O values >10.6.

Regarding %N, copepods were most abundant in sequence reads (22.5%N), followed by Podonidae (10.0%N), *Oikopleura* spp. (8.7%N), and Sagittoidea (5.4%N; Fig. 6a). The %O and N of *P. parvus* s.l., *Labidocera* sp., and Calanidae (*Calanus* sp., *Neocalanus* sp., or *Cosmocalanus* sp.) were high in the calanoid copepods, and their %N was 16.4, 4.6, and 2.9, respectively (Fig. 6). The %O of *Clausocalanus* sp. was >15, but its %N was only 0.3. %N values were >1 in Hydrozoa *Liriope*, Ascidae *Doliolum* sp., Appendicularia *Megalocercus*, and Oithonidae (Fig. 6a). Only 0.3%N was detected in Oncaeidae, whereas their %O was 10.6 (Fig. 6a). The abundance of Insecta was relatively large (3.8%N). Phytoplanktonic prasinophytes, dynophytes, and Bacillariophyceae were also detected (%N >1).

The abundant organisms based on %M were different from those based on %N and %O; *Labidocera* sp. and *Doliolum* sp. were abundant based on %M at 49.0 and 17.5, respectively. Although the %M depended on the outlier data, they still showed high values of 19.2 and 28.1, respectively, after removal of the larval sample with 109 501 reads, which were mostly composed of *Labidocera* sp. These groups were followed by *Oikopleura* spp., Podonidae, *P. parvus* s.l., Sagittoidae, and *Liriope* sp., which also showed different %M values (that were not influenced by high outliers; Fig. 6a). *Labidocera* sp. and *Doliolum* sp. were mainly observed in large larvae (≥6 mm long): *Labidocera* sp. and *Doliolum* sp. comprised 32 and 49%M in large larvae, respectively, whereas they comprised <5.2%M when larval BL was <6 mm.

When the samples were limited to those collected in 2015, trends of %O, N, and M were different from all data combined (Fig. 6b): *Oikopleura* spp. were the most frequently observed from the guts (47%M), and *P. parvus* s.l. had the highest %O and %N (50 and 22%, respectively). *Labidocera* sp. and *Doliolum* sp., which were dominant organisms in the whole samples based on %M (Fig. 6a), decreased to 4%M (*Labidocera* sp.) or were not detected (*Doliolum* sp.) in the samples collected in 2015 (Fig. 6b).

Plankton community structure in the field

In the microscopic observations of the environmental community samples, copepods and their immature stages were dominant in zooplankton (Fig. 7a). In particular, *Microsetella norvegica*, *Paracalanus parvus* s.l., *Oithona similis*, *Oncaea media*, and *Clausocalanus pergens* and their presumed nauplii and copepodites were abundant (Fig. 7a). In the Calanidae, *Calanus* sp. nauplii were the only species identified. Except for copepods and their larvae, abundances of *Penilia avirostris* (Cladocera) and *Oikopleura longicauda* (Appendicularia) were high (~2% of total zooplankton individuals). In Cladocera, Podonidae species, i.e. *Podon schmackeri*, *Evadne spinifera*, and *E. tergestina*, were also present, although their abundance was always <1% of all zooplankton individuals at all stations.

From the metagenetic analysis for eDNA on the 0.1 mm filter during the 2015 cruise, copepods were abundant, similar to the microscopic observations (Fig. 7b): the MOTUs identified Calanidae as the most abundant (27.4%N and 34.0%M), followed by *P. parvus* s.l. (12.1%N and 7.7%M), *Labidocera* sp. (10.3%N and 8.5%M), and Clausocalanidae *Clauso-*

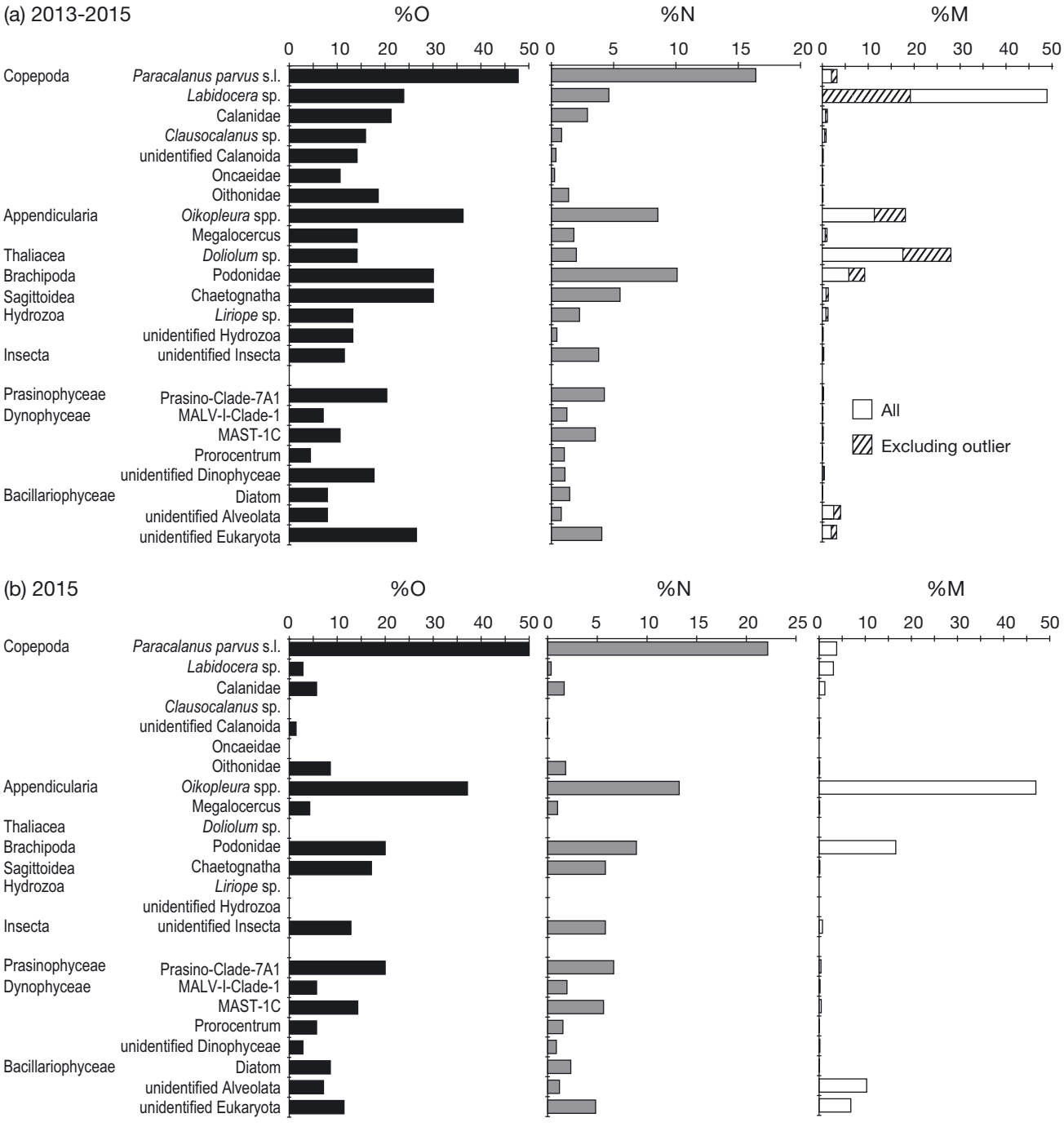

Fig. 6. Proportion of presence/absence of target species (molecular operational taxonomic units, MOTUs) to the number of non-empty guts (%O, black bars, left panel), proportion of total sequences of target species (MOTUs) to total numbers of organisms (%N, gray bars, center panel), and % mass (%M, white bars, right panel) of organisms detected by metagenetic analysis from (a) guts of all Pacific bluefin tuna *Thunnus orientalis* larvae and (b) guts collected in 2015. The hatched bars in panel (a) indicate the %M calculated after the exclusion of the larval guts whose read numbers were extremely high

calanus sp. (7.8 %N and 4.0 %M; Fig. 7b). Cyclopoida (Oithonidae; 3.4 %N and 2.9 %M) and Poecilostomatoida (Oncaeidae; 2.8 %N and 1.7 %M) were also detected. Harpacticoida (unidentified to the family level; 0.75 %N and 0.44 %M) and Poecilostomatoida (Corycaeidae; 0.37 %N and 0.33 %M) were rare (summarized as 'others' in Fig. 7b). Apart from copepods, unidentified Hydrozoa were abundant, as were

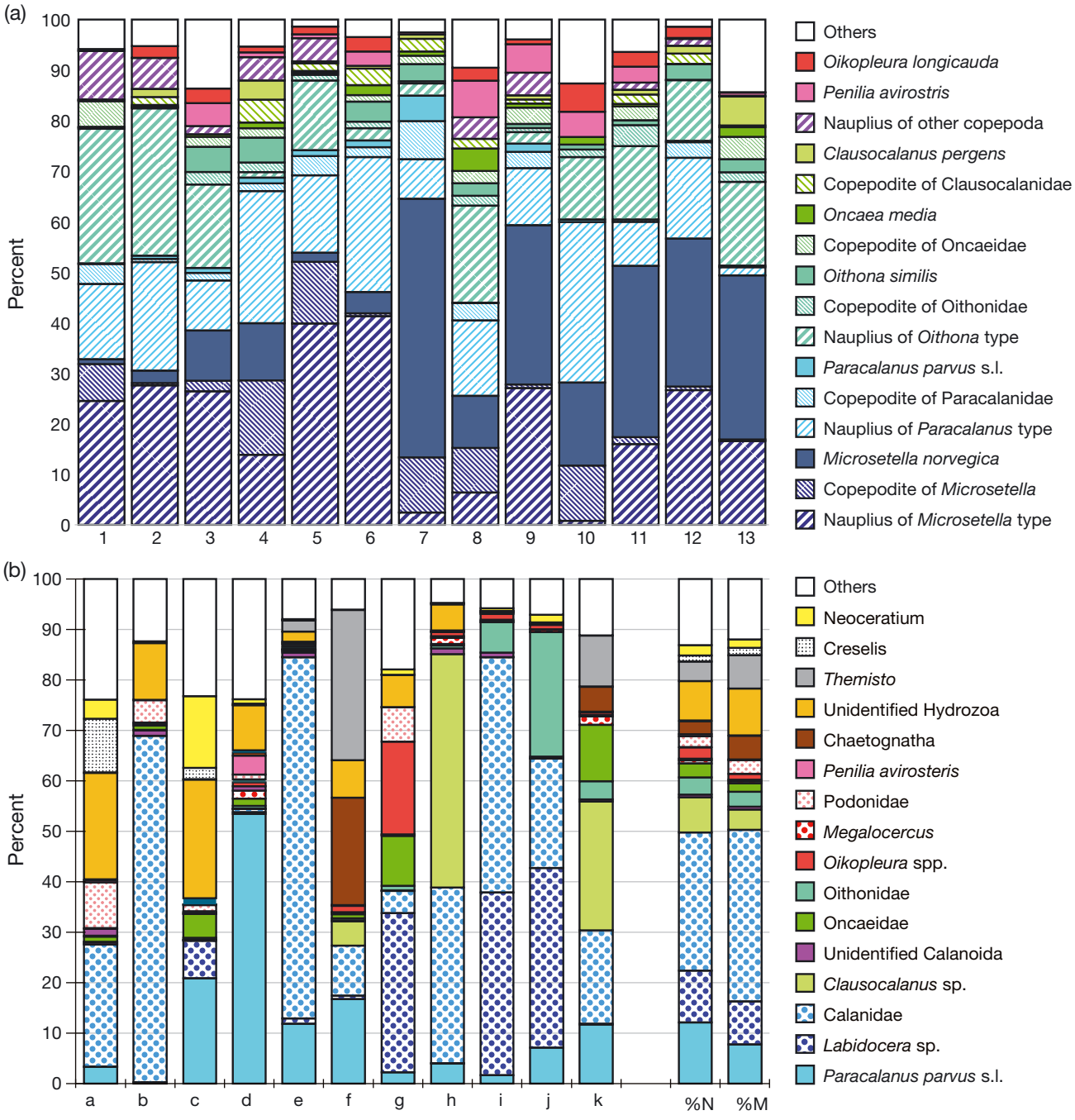

Fig. 7. Individual proportions of total sequences of target species (molecular operational taxonomic units, MOTUs) to total numbers of organisms (%N) of the environmental plankton community structure estimated by (a) microscopic observations of samples collected using a 0.06 mm mesh plankton net at 0–50 m depth (based on number of individuals), and (b) metagenetic analysis of samples collected using a 0.1 mm mesh in the surface water (based on sequence reads of MOTUs). In the metagenetic analysis, the %N and % mass (%M) of all environmental samples were also calculated. The numbers (1–13) and letters (a–k) denote the sampling stations shown in Fig. 1

Liriope sp. (7.8%N and 9.2%M), *Themisto* (3.8%N and 6.6%M), Chaetognatha (2.5%N and 4.6%M), *Oikopleura* spp. (2.2%N and 1.1%M), and Podonidae (2.1%N and 2.7%M). The cladoceran *Penilia avirostris* showed only 0.4%N and 0.15%M (Fig. 7b).

Comparison of organisms between environments and diets

First, we simply compared the results of %M and %N in guts and eDNA (Fig. 8). The relationships

indicated that *Oikopleura* spp. and Podonidae were more dominant in the guts than in the environment based on %M and %N (Fig. 8). For %N, *P. parvus* s.l. and Chaetognatha were higher in guts than in the environment (Fig. 8b).

Nine zooplankton groups which were major prey or species in the water column (*P. parvus* s.l., *Labidocera* sp., Calanoidae, Oithonidae, *Oikopleura* spp., *Megalocercus* spp., Branchiopoda, and Chaetognatha) were used for the calculation of Chesson's selectivity index in the metagenetic analysis. Since the phytoplankton (e.g. Prasinophyceae) observed in the guts could be <0.1 mm, they were removed from this analysis. The neutral value (i.e. the inverse number of taxa in the diet) was 0.125, and a 1-sample *t*-test with a Bonferroni correction was applied to determine whether α was > or <0.125.

The results indicated, based on the %M, that the α values of *P. parvus* s.l., Calanidae, Oithonidae, and Sagittoidea were significantly less than 0.125 ($p < 0.003$) and the other groups were not significantly different from 0.125. The highest and second highest mean α were observed in *Oikopleura* spp. and Podonidae (Fig. 8c). Based on %N, 4 taxa of copepods were significantly less than 0.125 ($p < 0.003$). The α values of *Megalocercus* and Chaetognatha were highest and second highest, respectively, and were not significantly different from 0.125.

DISCUSSION

Prey organisms detected by morphological and metagenetic analyses

The integrated methods of microscopic observation and metagenetic analysis provided detailed information on the prey organisms of PBT larvae in this study (Fig. 6). The microscopic observation detected Podonidae as the major prey group; in addition, *Paracalanus parvus* s.l., *Labidocera* sp., *Oikopleura* spp., and *Doliolum* sp. were also dominant in the gut contents in the metagenetic analysis. Fish larvae were not detected using morphological methods in this study,

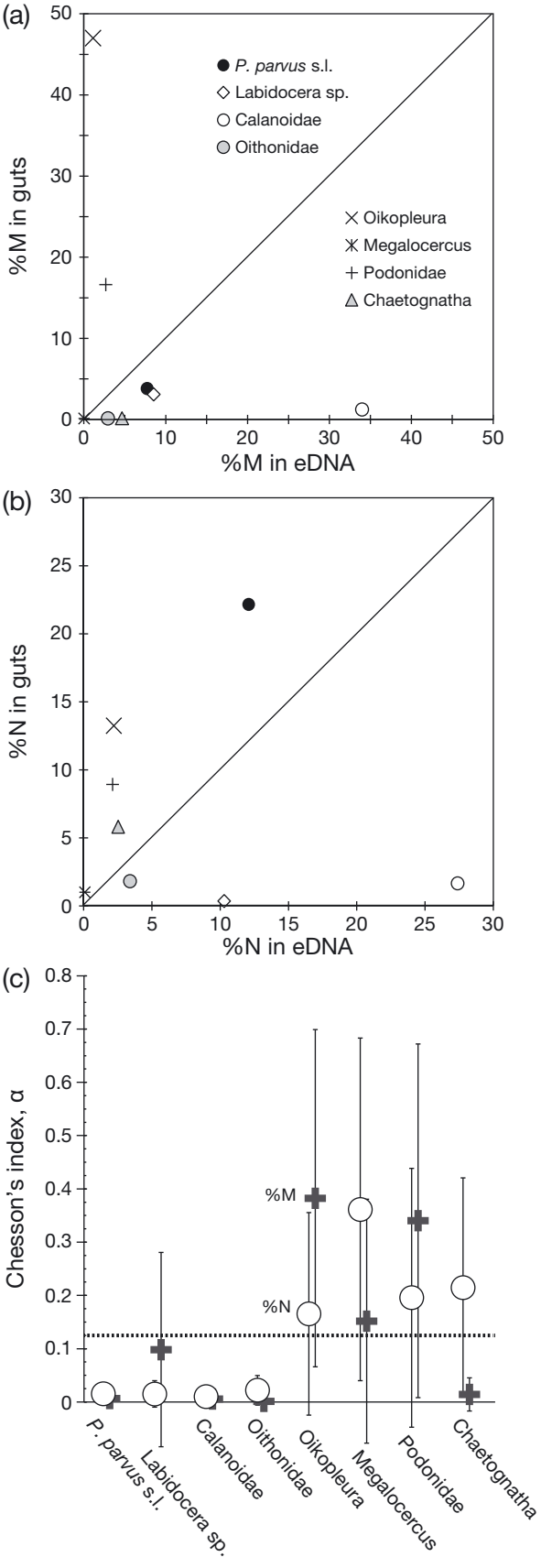

Fig. 8. Comparison of (a) % mass (%M) and (b) the proportion of total sequences of target species (molecular operational taxonomic units, MOTUs) to total numbers of organisms (%N) of the major prey of larval Pacific bluefin tuna *Thunnus orientalis* in 2015 between gut contents and eDNA samples. Major prey are shown in panel (a). (c) Mean ± SD Chesson's index (α) of major prey based on %M (crosses) and %N (open circles). Horizontal line shows the neutral value (0.125), i.e. the inverse number of prey taxa

and size classes of PBT or ABT larvae in this study (<6 mm) were zooplanktivorous (Uotani et al. 1990, Llopiz et al. 2010, Catalán et al. 2011, Llopiz & Hobday 2015, Tilley et al. 2016, Muhling et al. 2017). Therefore, there would be no biases in removing fish sequences from our metagenetic analysis. We could not determine whether small phytoplankton (e.g. Prasinophyceae) were directly utilized by PBT larvae; it is possible that the larvae could prey on phytoplankton, because in a culture experiment larval PBT were able to obtain energy from the microbial loop (mainly dinoflagellates) (Nakagawa et al. 2007), as were other fish larvae in natural environments (de Figueiredo et al. 2007).

FI values were higher in the metagenetic than in the morphological analyses; the FI based on metagenetic analysis was higher than that based on microscopic observation, and prey could usually be identified by the metagenetic analysis even from larvae collected at midnight. This indicated that metagenetic analysis can be used to detect trace prey organisms, and is thus powerful for diet analysis. In particular, appendicularians were only detected by the metagenetic approach. Microscopic observations in previous studies detected appendicularians in the guts of larval Scombridae (Uotani et al. 1981, Llopiz et al. 2010, Llopiz et al. 2015), thus we assumed in our samples that they would be detectable if they were present and in good condition. However, appendicularians are usually divided into head and tail in the larval guts (Uotani et al. 1981), and have no hard exoskeleton structure, thus they may be digested faster than crustacean zooplankton and may be more likely overlooked. It might be possible to detect appendicularian houses but not their bodies by metagenetic analysis, but our results support the ability of this technique to detect soft zooplankton such as appendicularians and hydrozoa, which is one of the merits of the metagenetic approach (Sousa et al. 2016).

However, there was a mismatch in the %O of Podonidae between the 2 approaches (Fig. 6). Podonidae were only detected metagenetically in 17 of 48 larvae in which Podonidae were observed morphologically, whereas in 16 larvae, Podonidae were only detected by the metagenetic analysis. Metagenetic analyses are not free of biases in detecting all eukaryotic taxa (Pompanon et al. 2012); however, experiments of control samples verified the detection of Podonidae by metagenetic analysis (Fig. 5). In contrast, a cephalopod larva identified by microscopic observation was classified as an unidentified taxon, and was removed during the bioinformatics procedure in the metagenetic analysis, owing to limited available data of the 18S V9 region. DNA is degraded to half in several hours, even in water (Maruyama et al. 2014), and the mismatch between morphology and metagenomics has been reported by Sakaguchi et al. (2017). Therefore, as conducted in this study, integrated methods of metagenetic and morphological analyses were an effective approach for understanding PBT diets.

Comparison of major PBT prey among other areas

The results of our metagenetic diet analysis showed the following: a low contribution of copepods and high contribution of Podonidae and *Oikopleura* spp. (Fig. 6), although copepods were dominant in the environment. Compared with the results of diet analysis in the western Pacific (Uotani et al. 1990), the predominance of copepods in gut contents was low in the Sea of Japan (Fig. 6). In addition, predominant copepod species in larval guts were different: *P. parvus* s.l. and *Labidocera* sp. in the Sea of Japan (Fig. 6), and Corycaeidae in the western Pacific (Uotani et al. 1990). Carbon biomass of Corycaeidae was sometimes >50% of zooplankton in the environment in the western Pacific (Uotani et al. 1990, Nakata et al. 2001), but Corycaeidae were rare in eDNA samples in our study (0.37%N and 0.33%M). The low abundance in the Sea of Japan could result in the low contribution of Corycaeidae as prey in the Sea of Japan, while oceanic conditions have changed in the past 3 decades both in the subtropical western North Pacific and the Sea of Japan (Aoyama et al. 2008, Kodama et al. 2016).

The predominance of Podonidae and *Oikopleura* spp. based on %N and %O of the metagenetic analysis in larval guts was the other significant result in our study (Fig. 6). In particular, Podonidae were the most frequently observed on the basis of the microscopic observations (see Fig. 4). Podonidae are prey of ABT larvae in the Gulf of Mexico (Tilley et al. 2016) and the Mediterranean (Catalán et al. 2011), and *Oikopleura* spp. are dominant in the Florida Strait (Llopiz et al. 2010). Therefore, major prey organisms detected in this study corresponded with previous studies of BT larvae.

Selective feeding

Comparisons between guts and eDNA showed that *Oikopleura* spp. were selectively and preferentially preyed upon in the Sea of Japan in 2015 (Fig. 8), fol-

lowed by Podonidae. However, the most abundant taxa in the environment during this study were copepods (Fig. 7), which were not the significantly preferred taxa for PBT larvae in the Sea of Japan (Fig. 8); thus preferential feeding on Podonidae and *Oikopleura* spp. could not be explained only by environmental abundance. Two reasons for the selection of Podonidae and *Oikopleura* spp. were considered: larvae were either passively or actively selecting.

When prey selection is active, Podonidae and *Oikopleura* spp. should be more effective food sources of nutrients than copepods. The ratios of carbon or nitrogen to dry weight were compared among *Podonidae*, *Oikopleura*, *P. parvus* s.l., and Calanidae. These were calculated using equations between dry, carbon, and nitrogen weights and BL (for *Podon leuckarti* and *Calanus sinicus*), or by percentages of these contents (for *P. parvus* s.l. and *O. dioica*) (Uye 1982), with the assumption of a 200–300 µm BL for Podonidae and Calanidae based on the morphological observation of gut contents. The results indicated that carbon and nitrogen of Podonidae (35–39% and 5.8–7.6% of dry weight, respectively) were lower than those of Calanidae (42–43% and 15–16%, respectively), *P. parvus* s.l. (42.9–52.4% and 9.9–14.0%, respectively), and *Oikopleura* spp. (46.3% and 12.7%, respectively). However, when the coefficient of digestibility or essential nutrition was ignored, Podonidae and *Oikopleura* spp. were not more nutritious food than *P. parvus* s.l. or Calanidae for PBT larvae, limiting the possibility of active feeding.

As regards passive selection, swimming ability (escape behavior) of prey is an important factor. The swimming ability of Podonidae is unknown, but the swimming speed of the freshwater cladoceran *Daphnia pulex* (size: 2.1 mm) was slower than that of the smaller copepod *Temora longicornis* (1.1 mm; Seuront et al. 2004). Thus it is possible that Podonidae swim more slowly than copepods. Although fast escape of the appendicularians *Oikopleura* spp. from their houses has been observed (Alldredge 1976, Bochdansky & Deibel 1999), they rarely abandon their houses and are frequently preyed upon by carnivorous zooplanktons (Alldredge 1976). This suggests that PBT larvae feed on Podonidae and *Oikopleura* spp. more easily than on copepods of the same size, and thus passive selection could be more important than active selection.

Passive selection may account for *P. parvus* s.l. being the most preferred taxon of all copepods. *P. parvus* s.l. is small (Uye 1982), and escape velocity has a positive relationship with size of copepods (Kiørboe et al. 2009). In addition, nauplii of *Paracalanus* sp. were frequently observed in our study. The detection of *Labidocera* sp. and *Doliolum* sp. in large larvae could also be explained by their high swimming ability or simply their large body size (e.g. several mm in *Doliolum* sp.).

Feeding conditions in the Sea of Japan

PBT larvae fed selectively on Podonidae and *Oikopleura* spp.; however, their guts were not considered to be full in the Sea of Japan. On the basis of culture experiments (Sawada et al. 2000), larvae (BL: 4–5 mm) with full guts contained 10–15 rotifers larva^{-1}, and the median lorica length of rotifers was 160–200 µm. Therefore, based on a lorica length of 180 µm, the average sizes of prey organisms were 0.25–0.38 mm^2. When a size of 0.3 mm^2 was considered the threshold for a full gut, the guts of >94.4% of the larvae that we collected in 2014 and 2015 during the daytime were not full (Fig. 4b). In addition, ~5 individuals of Podonidae were necessary for a larva to have a full stomach (<0.3 mm^2), (Fig. 4a); this estimated value was the same level as the number of prey in ABT larvae collected in the Mediterranean (3–11 ind. larva^{-1}) (Catalán et al. 2011, Muhling et al. 2017). This was also supported by the low FI in our microscopic observations (44%).

In this study, considering only non-empty guts, gut contents comprised on average 2.0 ± 1.7 ind. larva^{-1}, and during the daytime from 12:00 to 16:00 h this value was 2.0 ± 1.2 ind. larva^{-1}. This is the same level as the larval BTs collected in the western Pacific (1946 organisms from 1939 larvae; Uotani et al. 1990), in western Australia (93 organisms from 85 larvae, and 319 organisms from 231 larvae; Uotani et al. 1981), and in the Gulf of Mexico (1.1 ± 1.3 ind. larva^{-1}; Tilley et al. 2016), but was lower than that reported from the Mediterranean (3.0–11.1 ind. larva^{-1}, Catalán et al. 2011). Llopiz & Hobday (2015) compared the oceanographic conditions of the BT nursery grounds in the Gulf of Mexico, Florida Straits, Mediterranean, western part of Australia, and western North Pacific (except the Sea of Japan), and found that the Mediterranean had the lowest SST and highest chlorophyll *a* (chl *a*) concentration of these nursery grounds. Temperature is generally related to the swimming activity of larvae through physiology and water viscosity (von Herbing 2002), and chl *a* concentration is usually used as an index of productivity. SST at the stations in the Sea of Japan where we caught PBT larvae ranged from 23.3–28.8°C, and the sea surface chl *a* concentration was >0.2 µg l^{-1} during the 2013

cruise (Kodama et al. 2015). These oceanographic features were more similar to the Mediterranean than to the other 4 nursery grounds, whereas the gut contents in the Sea of Japan were more similar to the other 4 grounds than to the Mediterranean. Thus is appears that oceanographic features did not influence the gut contents.

In the metagenetic approach, prey DNA per host was lower in PBT larvae (median of 0.45%) than in the larvae of other pelagic fishes, namely Japanese sardine and round herring (Hirai et al. 2017). Based on morphological identification, Japanese sardine and anchovy feed on copepods, including Paracalanidae and Oithonidae, in the Sea of Japan (Hirakawa & Goto 1996, Hirakawa et al. 1997). This suggests that the PBT larvae do not effectively feed on copepods, which are abundant in the water. In fact, the swimming velocity of zooplanktivorous larval sardines relativized to BL (~3 BL s^{-1}) (Silva et al. 2014) is faster than that of larval PBT (~2 BL s^{-1}) (Sabate et al. 2010) in culture conditions. Therefore, although the amount of slow swimmers was limited in the environment (Fig. 7), the small PBT larvae were limited to this prey. This could explain why PBT larvae in incubation experiments fed at a higher rate (Miyashita 2002): the PBT larvae should feed on prey when and where they can in the natural environment for survival. In addition, the growth of most larval PBT is slow compared to those which successfully survive to become juveniles, and generally, only the rapidly growing larvae survive (Tanaka et al. 2006, Watai et al. 2017). Food densities positively influence the growth of PBT larva in the western North Pacific, as does temperature (Satoh et al. 2013). Therefore, the poor feeding capability of PBT larvae in the Sea of Japan might lead to a high mortality during the larval stage.

CONCLUSIONS

We first investigated the diets of PBT larvae using metagenetic analysis and microscopic observation, revealing that PBT larvae in this study were zooplanktivorous. A wide range of taxa was detected in the gut contents, and the main prey species for PBT larvae in the Sea of Japan were *P. parvus* s.l., Podonidae, *Oikopleura* spp., *Labidocera* sp., and *Doliolum* sp. *Labidocera* spp. and *Doliolum* sp. were important prey organisms for large larvae. Among the major prey, Podonidae and *Oikopleura* spp. were especially preferred prey species for PBT larvae. The escape behaviors of Podonidae and *Oikopleura* spp. are likely not sufficient to avoid predation by PBT larvae. Most of the PBT larvae guts were not full, suggesting that PBT larvae were not capable of catching fast-swimming but nutrient-rich prey, which were abundant in the surface layer of the Sea of Japan. This study showed specific features of the diets of PBT larvae in the Sea of Japan, which is highly productive and has a lower SST than the other BT spawning grounds except the Mediterranean, where productivity and temperature are similar to those in the Sea of Japan. Further dietary analysis covering wider ranges of larval sizes, oceanographic variability driving prey field composition and abundance in the Sea of Japan, and inter-area comparisons of zooplankton community structure, diets, and growth of larval PBT will help to understand key factors influencing the feeding capability of larvae, and the survival and recruitment of PBT in future studies.

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