

Comparison of morphological and DNA metabarcoding analyses of diets in exploited marine fishes

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ABSTRACT: Ecosystem-based management (EBM) is a framework for managing marine resources. EBM strategies can be evaluated with ecosystem models that represent functional components of ecosystems, including anthropogenic factors. Foodwebs are at the core of ecosystem models, but because dietary data can be difficult to obtain, they are often coarsely characterised. High-throughput DNA sequencing (HTS) of diets is a rapid way to parameterise foodwebs at enhanced taxonomic resolution, and potentially, to optimise the functioning of ecosystem models. We evaluated the relative merits of microscopic and HTS analyses of the diets of 8 fish species harvested in Australia's most intensive fishery, viz. the southeast trawl fishery. We compare the taxonomic resolution and phylogenetic breadth of diets yielded by these methods and include a comparison of 3 DNA barcoding markers (mtDNA COX1 Minibar, mtDNA 16S Chord-cephA, nDNA 18S Bilateria). Using paired individual gut samples (n = 151), we demonstrate that HTS typically identified similar taxon richness but at significantly higher taxonomic resolution than microscopy. However, DNA barcode selection significantly affected both the resolution and phylogenetic breadth of estimated diets. Both COX1 Minibar and 16S Chord-cephA barcodes provided higher taxonomic resolution than morphological analysis, but the resolution varied between taxonomic groups primarily due to availabilities of reference data. However, neither barcode recovered the full dietary spectrum revealed by the 18S Bilateria barcode. HTS also revealed the presence of dietary items not previously recorded for target species, and diverse parasite assemblages. We conclude that HTS has the potential to improve structure and function of ecosystem models and to facilitate best-practice EBM.

KEY WORDS: Foodweb · Ecosystem-based management · DNA sequencing · Fisheries management · Ecosystem modelling

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INTRODUCTION

Increasingly, fisheries managers are adopting the principles of ecosystem-based management (EBM). This provides a holistic approach to managing marine resources that contrasts with the more conventional focal-species approach to management (Pikitch

et al. 2004). As a result, tools to monitor the complex and diverse interactions between fisheries and the environment are more in demand (Levin et al. 2009). Ecosystem modelling is among the most important emerging tools for understanding ecosystem dynamics and highlighting major knowledge gaps, and for evaluating EBM strategies (Fulton et al. 2011). Eco-

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system models can represent all of the significant components of an adaptive management cycle: human users, biophysical dynamics and management activities (Fulton et al. 2011). In addition, ecosystem models can be coupled to economic and climate models to consider broader management issues and interrelationships (Fulton 2010).

For ecosystem models to act as informative heuristic tools, they must be underpinned by a sound understanding of ecosystem processes, which in turn relies on well-characterised foodwebs (Kearney et al. 2012). Foodwebs are networks of predator–prey dietary relationships amongst ecosystem components, and in marine systems, they can be challenging to reconstruct because of their complexity and diversity (Ainsworth et al. 2010). If foodwebs are represented too simplistically, either by over-aggregating or omitting groups, model behaviour can be altered to such an extent that it no longer faithfully reproduces system dynamics (Fulton et al. 2003). Conventionally, foodwebs are generated from dietary studies, typically through microscopic analysis of gut contents, which provide a near real-time snapshot of feeding ecology (Hyslop 1980), or from isotopic and fatty acid signatures in tissue samples, which provide longer-term and aggregated information on trophic position (Dalsgaard et al. 2003, Revill et al. 2009, Hardy et al. 2010). Microscopic analysis has 4 key limitations. The first is that it can be time consuming, which can constrain the sample sizes that can be reasonably processed (Hyslop 1980, Williams et al. 2012). Second, it requires specialist taxonomic expertise, often at a regional level, across a diverse range of taxa. Third, even where taxonomic expertise is available, mastication and digestion processes may render stomach contents unidentifiable. A fourth, and related, limitation is the bias introduced by differential digestion of organisms. For example, cartilaginous and soft-bodied organisms digest more completely and rapidly than arthropods and bony fishes, and therefore are likely to be under-represented in diet assessments (Hyslop 1980, Gales & Cheal 1992, Alonso et al. 2014).

DNA-based characterisation of diets and other trophic interactions is an emerging discipline (Pompanon et al. 2012, Symondson & Harwood 2014). Its utility relies on key conceptual and technological advances. Conceptually, DNA-based dietary analysis applies the principles of DNA barcoding, where diagnostic DNA sequences are obtained from an unknown specimen and compared to a reference collection of DNA sequences obtained from identified (ideally vouchered) organisms (Cronin et al. 1991, Hebert et al. 2003, Hollingsworth et al. 2009). The

most significant technological advance has been the development of high-throughput sequencing (HTS) platforms that generate large numbers (typically thousands to millions) of diagnostic DNA barcode sequences from heterogeneous samples, including environmental samples—this approach has been termed metabarcoding (Yu et al. 2012). The key benefits of DNA sequence analysis for the characterisation of dietary samples include: less direct reliance on taxonomic expertise, a capability of detecting organisms lacking diagnostic morphological features (because of absence or digestion) and speed of analysis and suitability for automated processing. Several comparative investigations have demonstrated that HTS delivers superior diversity and taxonomic resolution to microscopic analysis, and is more likely to detect rare species (Clare et al. 2014, Emrich et al. 2014, Krüger et al. 2014), but metabarcoding applications to study fish diets have been limited.

Metabarcoding using HTS has its own intrinsic limitations and biases, and needs to be carefully implemented. An important difference between morphological and DNA-based analyses is that the latter requires DNA barcode selection, which *a priori* necessitates a taxonomic focus for the analysis (Deagle et al. 2014). This will determine both the phylogenetic breadth of the dietary items retrievable and the taxonomic depth (resolution), and there is usually a trade-off between these. Therefore, it is important to select markers with regard for the downstream use of the data (Deagle et al. 2009). Another consideration when dealing with dietary samples is that the DNA is likely to be degraded, and short DNA barcode amplicons should be targeted (Deagle et al. 2006). Short amplicons, however, typically contain fewer informative sites than longer sequences, thereby limiting the ability for the barcode to resolve at either family, genus or species level. A further restriction on DNA-based assignments is the limited availability of reference sequences (Symondson & Harwood 2014). Just as morphology cannot provide identities for species not included in keys, strict DNA barcoding, which relies on making matches to a reference collection, cannot make assignments where reference collections are incomplete or where the underpinning taxonomic framework is unstable. Nevertheless, because DNA sequences contain phylogenetic signals, there is a strong case for the use of molecular operational taxonomic units (MOTUs) as working hypotheses where reference sequences are not available (Burgar et al. 2014, Clare et al. 2014), although there are many views on how best to identify MOTUs (Wilson et al. 2011).

Dietary studies have generally relied on 1 or more of 3 DNA barcode genes: mitochondrial cytochrome oxidase subunit 1 (COX1), mitochondrial 16S and the nuclear 18S gene. COX1 is the DNA barcoding gene designated for use in animals by the Barcode of Life Consortium, and it is well represented in publically available databases. Conventional COX1 DNA barcoding uses a large fragment, ca. 650 bp, and is poorly suited for dealing with degraded material. Shorter COX1 fragments (mini-barcodes) can be employed to assess such material, although finding universal primer binding sites within the protein coding region is not possible. The mtDNA 16S gene is widely used in microbial analysis of environmental samples (Bowman et al. 2012), but is less well represented in databases for metazoans than COX1. Primer sets amplifying short sequences and targeting specific groups are available (e.g. Deagle et al. 2009). The nuclear 18S gene evolves more slowly than the mtDNA genes, and as a consequence, primer sites can be more conservative, making it less likely to introduce primer-binding bias and more applicable to phylogenetically broad targets. As a trade-off, however, the taxonomic resolution may be reduced (Deagle et al. 2009). Each DNA barcode marker has strengths and weaknesses, and ultimately the combination of multiple markers is likely to be the optimal way to profile complex (multi-species) substrates (O'Rourke et al. 2012).

Here, we conducted a comparative investigation of the breadth and depth of taxonomic resolution of dietary samples collected from 8 fish species harvested in the Commonwealth Trawl Sector (CTS) of the Southern and Eastern Scalefish and Shark Fishery (SESSF), which receives the greatest fishing effort of all Australian fisheries (Georgeson et al. 2014). Until recently, research on the SESSF focused on single-species stock assessments (Williams & Bax 2001). However, over the past decade, several ecosystem models have been developed for southern Australia (Fulton et al. 2007, 2012, Forrest 2008, Savina et al. 2013). These models have relied heavily on dietary data collected in the 1990s via visual inspection of stomach contents and isotopic analyses (Bulman et al. 2000, 2001).

Our research was conducted with a view to establishing a regime for time-series monitoring of trophic structure across the study region. Since the initial implementation of ecosystem models for the SESSF, awareness has increased that

commercial fishing is impacting its viability (Savina et al. 2013). In this study, we applied 3 different DNA barcode markers to dietary samples collected from the SESSF and examined the phylogenetic breadth and taxonomic depth of the identities recovered. We also compared these analyses to results based on conventional morphological analysis. The ultimate aim of this research is to evaluate the strength and weaknesses of these different diet proxies for ecosystem models in order to develop an optimal model structure.

MATERIALS AND METHODS

Sample collection

We revisited an area in eastern Bass Strait to re-sample a subset of the species targeted in earlier surveys of dietary diversity of the SESSF (Bulman et al. 2001, Davenport & Bax 2002) (Fig. 1). Stomachs were collected from 8 dominant and targeted fish species: common jack mackerel *Trachurus declivis*, gemfish *Rexea solandri*, tiger flathead *Platycephalus richardsoni*, reef ocean perch *Helicolenus percoides*, jackass morwong *Nemadactylus macropterus*, pink ling *Genypterus blacodes*, blue warehou *Seriolella brama* and john dory *Zeus faber*. In addition, intestinal tract samples were collected from jackass morwong. Stomach and intestine samples were collected during observer trips on 2 commercial fishing vessels (FV 'Western Alliance' and FV 'Coovara') in the area of the original 1994–1996 survey. The first sampling period was late winter/spring 2010 (August to October), and the second was late autumn 2011 (May).

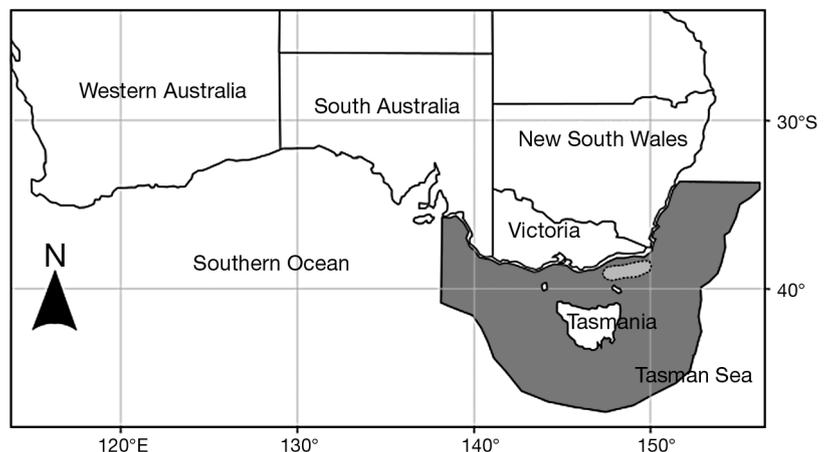


Fig. 1. Region where samples were collected (light grey) within the Commonwealth Trawl Sector of the Southern and Eastern Scalefish and Shark Fishery (SESSF; dark grey) of Australia

During the first sampling trip, fish were sampled from the catches on board the vessel. Stomachs were removed and frozen. During the second trip, whole fish were retained from the catch by the crew, frozen and transported back to the laboratory where stomachs were dissected and contents preserved in 70% ethanol until processed further. We also retained the intestinal tracts of jackass morwong because their stomachs were invariably empty but fragments were always found in the intestines. Sample sizes are given in Table 1.

Morphological analysis

Where prey items were still in the stomach, the stomach contents were emptied, blotted dry on paper towel, identified to the lowest possible taxon, weighed to 0.001 g (wet weight) and enumerated (when possible). Preserved stomach contents dissected from whole fish in the lab were processed similarly. Identification of fish prey digested beyond visual recognition was attempted from otoliths, if present, using an identification guide (Furlani et al. 2007), photographic records and otolith collections. Intestinal contents were also examined microscopically, but weighing and counting contents was not feasible due to very fine fragmentation of the remaining hard parts. All contents were then preserved (or re-preserved) in 70–80% ethanol and stored at room temperature for genetic analyses. Effort was made to minimise opportunities for sample contamination during dissections by processing primarily species by species and using comprehensively washed containers and instruments.

Sample sorting, DNA analysis and extraction

In total, 151 samples were available for DNA sequence analysis (Table 1). They were preserved in 70–80% ethanol for a period of 6 to 12 mo before DNA extraction. Samples were sorted visually into those comprising single dietary items ($n = 61$), and those considered likely to contain more than 1 organism (mixed samples; $n = 90$). Approximately 2 mm³ of tissue was taken from single items, and DNA was extracted with a Qiagen DNeasy Blood and Tissue kit according to the manufacturer's recommendations. DNA was eluted in 200 µl AE buffer. Mixed samples were transferred into disposable Ultra Turrax homogenisation tubes (model DT-20 or BMT-20, IKA) and homogenised at full speed for 60 s. Homogenate (200 µl) was collected with a wide-bore 1000 µl tip and pipetted into a 1.5 ml tube, which was centrifuged at 14 000 × g (60 s). The supernatant was discarded, and the pellet was partially dried in an Eppendorf vacuum concentrator for 3 min at 37°C. DNA was extracted from the pellet according to the standard Qiagen DNeasy kit protocol for animal tissues, but with the addition of 40 µl of Proteinase K. DNA was eluted into 200 µl AE buffer. All DNA extractions took place in a laboratory dedicated to DNA extractions and isolated from workspaces where PCR products were processed. PCR experiments were conducted after the completion of all DNA extractions. Benches and utensils were routinely cleaned with bleach. All PCRs included no-template controls to check for sample cross-contamination.

Table 1. Descriptive statistics for the DNA sequence dataset used in the DNA barcoding analysis. These data represent high-quality (post-filtered) sequences

Host species	Number of individuals			Number of sequences			Average sequences ind. ⁻¹		
	COX1 Minibar	16S Chord- ceph A	18S Bilateria	COX1 Minibar	16S Chord- ceph A	18S Bilateria	COX1 Minibar	16S Chord- ceph A	18S Bilateria
<i>Genypterus blacodes</i>	6	8	8	2705	5233	1820	465	654	228
<i>Helicolenus percoides</i>	19	15	20	2973	9931	5393	160	662	270
<i>Nemadactylus macropterus</i>	20	15	27	2584	6796	5686	137	453	211
<i>Platycephalus richardsoni</i>	7	13	12	1341	12139	5237	206	934	436
<i>Rexea solandri</i>	9	16	19	2014	17860	9347	232	1116	492
<i>Serirolella brama</i>	6	4	5	684	2524	621	119	631	124
<i>Trachurus declivis</i>	6	8	8	962	5089	1831	161	636	229
<i>Zeus faber</i>	5	4	4	1029	3106	1017	215	777	254
Total	78	83	103	14292	62678	30952			
Average	10	10	13	1787	7835	3869	212	733	280

Single-item samples

PCR amplification and sequencing. Single items were only screened for COX1, using the standard 'barcode' fragment (Hebert et al. 2003). Attempts were made to amplify the ca. 650 bp DNA sequence with primers HCO2198 and LCO1490 (Folmer et al. 1994; see Table S1 in the Supplement, available at www.int-res.com/articles/suppl/m540p167_supp.pdf). Twenty-five μl reactions consisted of the following: 1 \times PCR Buffer (Bioline), 0.25 mM MgCl_2 , 10 mM dNTPs, 0.4 mg ml^{-1} bovine serum albumin, 1.25 U *Taq* (MangoTaq, Bioline), primers and 2.5 μl DNA extract. Cycling conditions were as follows: 94°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 20 s and 72°C for 30 s; and 72°C for 1 min. Reactions were conducted on an Eppendorf EPS thermocycler. Fragments were visualised on 2.5% Tris-borate EDTA (TBE) agarose gels pre-stained with 0.25 \times Gel-Red (Biotium) run at 90V for 30 min.

Where samples failed to amplify the 650 bp PCR product, an amplification was attempted again, and in addition, amplification of a 130 bp Minibar fragment (Meusnier et al. 2008) was attempted with modified primers Minibar-Mod-F (5'-3') and Minibar-Mod-R (5'-3') (Table S1). Failed amplifications for the 130 bp fragment were also repeated. PCR cycling conditions were identical to those used for the 650 bp fragment, except that an annealing temperature of 45°C was used. PCR products were prepared for sequencing with BigDye Chemistry (Applied Biosystems), and sequenced in forward and reverse on an ABI 3730 DNA sequencer. Sequences were checked by eye and edited with GENEIOUS R7 software (Biomatters; Kearsse et al. 2012).

Sequence analysis. Edited sequences were queried against 2 reference sequence databases with the MegaBLAST algorithm (Altschul et al. 1990). The first query was against the National Center for Biotechnology Information (NCBI) GenBank nucleotide database (accessed June 2012; www.ncbi.nlm.nih.gov) (Benson et al. 2010). The second was against a custom COX1 database made in-house from Barcode of Life Data Systems (BOLD) sequences (www.boldsystems.org, Ratnasingham & Hebert 2007). This comprised publically available Australian echinoderm and fish sequences (BOLD project 'EAR' and BOLD container project 'AUSA', respectively) together with Australian decapod and additional fish sequences (BOLD projects 'DAR' and 'FOAB-FOAN' plus 'FOAMP', respectively). Species in this reference database are associated with lodged voucher specimens.

Mixed-item samples

PCR amplification and sequencing. An HTS approach was used to recover DNA barcode sequences from mixed samples. DNA from mixed samples was amplified with 3 primer pairs targeting 2 mtDNA regions and 1 nDNA region. Fragments of the mtDNA COX1 and 16s genes were amplified with the modified Minibar primer set (COX1 Minibar; as above) and primers chordata/cephalopoda A (16S Chord-cephA; Deagle et al. 2009), respectively. The latter amplified a ca. 260 bp fragment of the mtDNA 16s rRNA gene. A ca. 285 bp fragment of the nDNA 18s gene was amplified with the primers BilSSU1100_F and BilSSU1300_R, which target bilaterian organisms (18S Bilateria; Deagle et al. 2009). Concentrations of starting DNA template were equalised according to estimates made with a qPCR protocol employing a standard curve and EvaGreen intercalating dye (Biotium). Multiple forward ($n = 12$) and reverse ($n = 8$) Fusion primers (incorporating 454 adapters) were manufactured for each primer set, each containing a unique 10 bp multiplex identifier (MID) sequence. These were used in combination to provide 96 uniquely tagged amplicons that could be separated bioinformatically. Fusion-tagged amplicons were generated using an appropriate amount of input copies (determined by qPCR) in triplicate, purified by Agencourt AMPure XP bead PCR purification protocol (Beckman Coulter Genomics) and pooled in approximately equimolar concentrations. An appropriate bead:template ratio was determined by qPCR followed by sequencing according to the manufacturer's guidelines on a 454 GS Junior (Roche). COX1 Minibar primers employed the Lib-L Fusion primers, while the 16S Chord-cephA and 18S Bilateria primers employed the Lib-A Fusion primers. A single run was conducted for each marker.

Bioinformatic filtering. Sequences retaining primer and MID sequences were obtained in standard flowgram format (SFF). GENEIOUS R7 software (Kearsse et al. 2012) was used to filter sequences according to expected size and to annotate incorporated primer sequences with 100% match to expectations. Sequences containing imperfect primer matches were discarded. Sequences were imported into the Galaxy workflow platform and filtered by Phred score with the Filter FastQ function. Sequences containing Phred scores below 20 were discarded. Sequences were demultiplexed based on combined forward and reverse MID sequences using the Separate Sequences by Barcode function in Geneious software. Sequences with imperfect MID barcode sequences were discarded.

Taxonomic assignment. Sequences were analysed both at the level of individual stomach samples, and pooled to species. A GALAXY workflow was used to interrogate the NCBI BLASTn database with DNA sequence datasets. Settings were as follows: Type of BLAST = megablast; no filtering of low-complexity regions, maximum hits = 100; expectation cutoff = 0.01; output format = BLAST XML. BLAST results were imported into MEtaGenome ANalyzer (MEGAN) software (Huson et al. 2007), and taxonomic identities were assigned based on the lowest common ancestor (LCA) algorithm with the following settings: minimum number of reads = 5, minimum bit score equivalent to 90% identity, top percent to be considered 10%, minimum complexity not considered. Taxonomic assignments made with the LCA were evaluated against expert knowledge of species distributions and against the Codes for Australian Aquatic Biota (CAAB) database (www.marine.csiro.au/caab/index.html), which lists species names and catch locations for Australian fishes.

Additional analyses were conducted on the COX1 Minibar and 18S Bilateria datasets. For COX1 Minibar, comparisons were made to available BOLD datasets. Nodes and tips in the MEGAN tree were extracted, and a BLAST search was conducted against a customised database as defined earlier. Where the BOLD database provided a higher BLAST identity than the GenBank database, that identification was substituted. For 18S Bilateria, taxonomic resolution was low when queried against the NCBI database and processed with MEGAN, reflecting in part the limited availability of reference sequences. In order to provide an indication of the taxonomic spectrum of prey in the diets of the host fishes and the number of MOTUs within the dataset, nodes and tips were extracted from the MEGAN tree and subjected to the Uclust MOTU picking algorithm grouping sequences with similarity ≥ 0.97 . This analysis was conducted with QIIME scripts with default parameters (Caporaso et al. 2010) within a Galaxy workflow. The same MOTU analysis was conducted on nodes identified in the COX1 LCA analysis at class or higher taxonomic level. Only MOTUs with 5 or more reads were accepted. MOTUs common between host fish species were identified.

Comparison of taxonomic resolution

We compared the taxonomic resolution derived from morphological assessment with that from each of the DNA barcode markers. Taxonomic resolution

was ranked as follows: species = 1, genus = 2, family = 3, infra-order = 4, order = 5, infra-class = 6, class = 7, phylum = 8. For the morphological analysis, we added the category 'none' = 9, where no identification could be reasonably made. Infra-class and infra-order represent taxonomic rankings between family and order, and between class and order, respectively. Taxonomic resolution of the DNA markers was based on the rank determined by the LCA in the MEGAN software, and based on expert opinion for the morphological analysis. We used paired *t*-tests to directly compare the resolution for individual stomach samples for morphology and DNA barcodes. Where multiple items were present in stomach samples, the mean taxonomic rank was used in comparisons.

DNA sequencing effort and dietary richness

We determined the degree to which each DNA barcode characterised the accessible dietary richness of the target fishes. Based on the complete dataset for each DNA barcode, we used rarefaction incorporating 100 re-samples of individual stomach samples to establish the rate of increase in dietary richness (S_{est}) with increasing number of samples analysed (all species pooled). Rarefaction curves were extrapolated to 500 samples. In addition, we determined how adequately the sequencing effort recovered the dietary richness in each fish species for each DNA barcode. We pooled sequences for each species and conducted rarefaction analysis with 100 random re-samples of DNA sequences. Rarefaction analysis was conducted with the program EstimateS (Colwell 2013).

RESULTS

Single-item samples

Forty-eight of the 61 isolated individual items yielded DNA sequences (24 samples with COX1 Minibar fragments; 22 with full Folmer COX1 barcode fragments; 2 with both fragments). Twenty-two sequences (45.8%) were derived from the host species (Table S2 in the Supplement at www.int-res.com/articles/suppl/m540p167_supp.pdf). Sequences could be assigned with $\geq 98\%$ identity in 89.6% of cases (Fig. S1 in the Supplement). The median percentage match to reference sequences on GenBank was 100% (mean 95.4%). The lowest match was

74.8%. The custom BOLD database yielded similar results, with a median similarity of 100% (mean 96.4%). The Folmer and Minibar fragments yielded similar levels of identity to reference databases (Fig. S1; paired t -test $p > 0.05$).

Mixed-item samples

DNA analysis

The final filtered sequence dataset consisted of 107 922 DNA sequences derived from the 3 primer sets and the 8 host fish species (Table 1 and Tables S3–S5 in the Supplement). These sequences have been deposited in the CSIRO Data Access Portal at <https://data.csiro.au/dap/landingpage?pid=15592>. On average, host species made up 60.9% and 63.7% of the COX1 Minibar and 16S Chord-cephA sequences, respectively. Host sequences could not be resolved with sufficient taxonomic resolution for the 18S Bilateria primer set. 18S Bilateria sequences from parasites were present in 6 of 8 fish species, and formed a significant fraction ($>38\%$) of 18S Bilateria sequences obtained from both *Genypterus blacodes* and *Nemadactylus macropterus* (Table S3). Parasite sequences were not observed in the COX1 Minibar and 16S Chord-cephA datasets.

Comparison of taxonomic resolution

We found substantial differences in the mean taxonomic rank assigned to stomach samples between the different methods of identification (Fig. 2). The 18S Bilateria DNA barcode yielded the lowest resolution with a median rank between class and infra-class. The highest resolution was provided by the COX1 Minibar DNA barcode, which provided a median rank of species, although it exhibited significantly more variance than the 16S Chord-cephA DNA barcode marker, which yielded a median rank approximating genus. Omitting samples for which no identification could be made, the median taxonomic rank for morphological identification was between order and infra-order, but where non-assignments were included, the median taxonomic resolution for morphological assessment was between infra-class and order.

Direct comparisons of taxonomic rank in individual stomach samples between morphological and the 3 DNA-based analyses showed that both COX1 Minibar and 16S Chord-cephA provided significantly

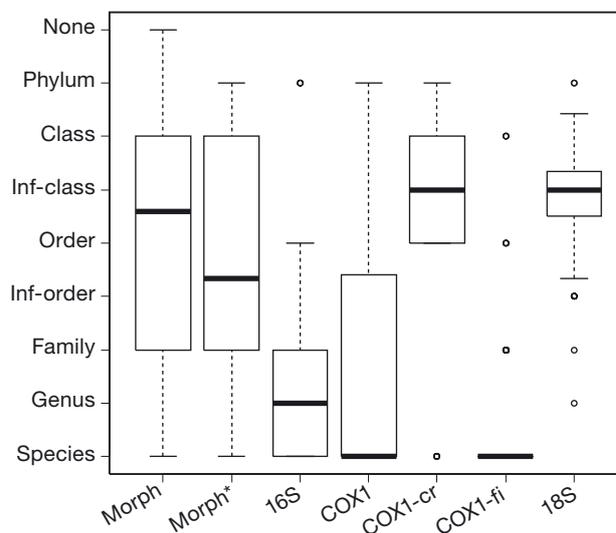


Fig. 2. Comparison of the mean taxonomic rank assigned to items within individual stomachs. Morph: morphological analysis; Morph*: morphological analysis disregarding non-assigned items; 16S: assigned by 16S Chord-cephA barcode; COX1: assigned by COX1 Minibar barcode (all items); COX1-cr: assigned by COX1 Minibar barcode (crustacean items); COX1-fi: assigned by COX1 Minibar barcode (fish items); 18S: assigned by 18S Bilateria barcode. Midline = median, box limits = inter-quartile range, whiskers = 95% confidence intervals, circles = outliers

higher resolution when 'none' assignments for morphology were excluded (COX1 Minibar, $t_{76} = 6.35$, $p < 0.01$; 16S Chord-cephA, $t_{46} = 5.041$, $p < 0.01$). Morphological analysis provided significantly higher resolution than the 18S Bilateria DNA barcode when 'none' samples were excluded (18S Bilateria, $t_{52} = 2.76$, $p = 0.01$), but not if they were included ($t_{61} = 1.14$, $p = 0.26$).

Analysis of pooled COX1 Minibar data also revealed a significantly larger fraction of assignments to species level than was evident in the morphological assessment (Fig. 2). Division of the COX1 Minibar data into bony fishes and crustaceans revealed that fishes were typically assigned to a higher taxonomic rank than crustaceans (Fig. 2; median rank = species and infra-class respectively; $t_{115} = 11.52$, $p < 0.01$).

Comparison of dietary diversity revealed by different methods

We observed a significant difference in the mean number of items identified per individual stomach between the different methods of analysis (Fig. 3; Single-factor ANOVA $F = 25.41$, $p < 0.01$). A Tukey-Kramer HSD test indicated that this was largely due

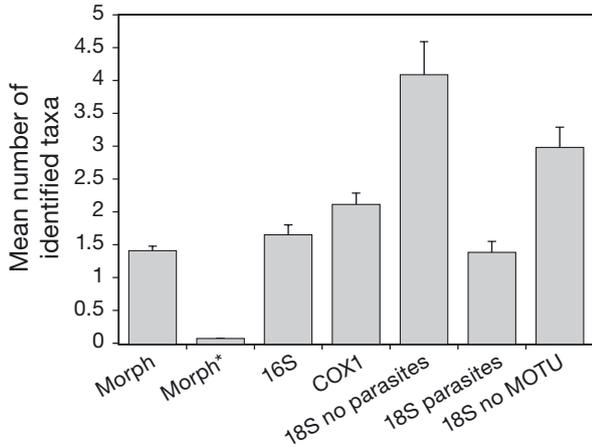


Fig. 3. Mean number of taxa (+SE) identified from individual stomachs of fishes in the SESSF by morphological and DNA barcode analysis. '18S no MOTU' indicates the number of identified items without considering molecular operational taxonomic units (MOTUs)

to the high richness present in the 18S Bilateria dataset, which was significantly greater than any of the remaining methods ($p < 0.01$), whereas none of the remaining comparisons were significantly different.

There was a weak correlation between the dietary richness revealed in individual stomachs by morphology and COX1 Minibar analysis ($R = 0.24$, $p < 0.05$), but correlations were not statistically significant between dietary richness revealed by morphology and 16S Chord-cephA or 18S Bilateria barcodes. The dietary richness revealed by each of the DNA barcodes was significantly correlated (COX1 Minibar vs. 16S Chord-cephA $R = 0.44$, COX1 Minibar vs. 18S Bilateria $R = 0.59$, 16S Chord-cephA vs. 18S Bilateria $R = 0.30$, $p < 0.05$).

Phylogenetic breadth of DNA barcode identifications

The 3 DNA barcode markers differed in the phylogenetic breadth of the revealed dietary items (Fig. 4). Only the COX1 Minibar marker identified multiple kingdoms (Animalia and Plantae). COX1 Minibar and 18S Bilateria identified 5 of the major animal phyla; 16S Chord-cephA identified 4. Within the vertebrate lineage, 18S Bilateria did not resolve the

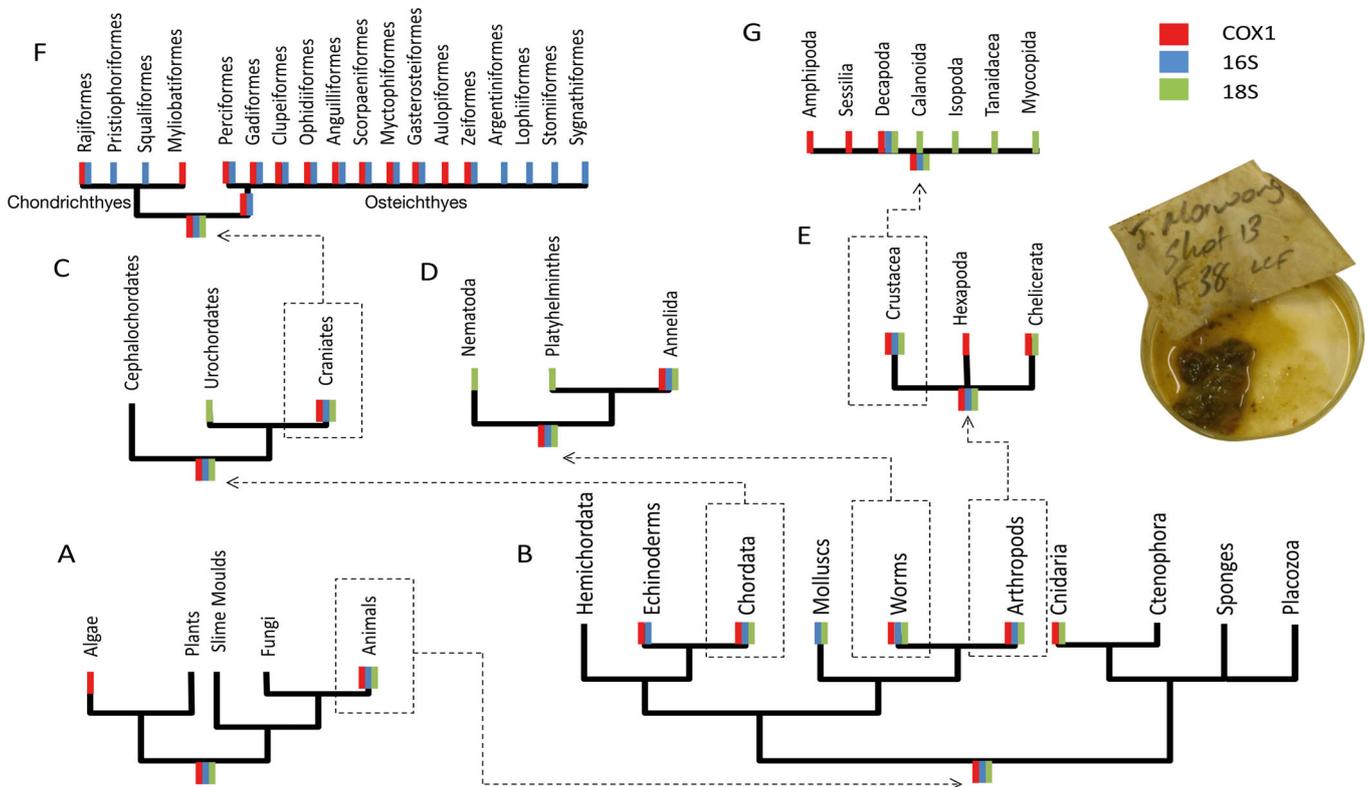


Fig. 4. Phylogenetic breadth of dietary items identified with 3 different DNA barcodes (COX1 Minibar, 16S Chord-cephA, 18S Bilateria). (A) Kingdoms; (B) Phyla; (C) Chordata; (D) Worms; (E) Arthropods; (F) Fishes (order); (G) Crustacea (order). Photo shows typical gut contents recovered from *Nemadactylus macropterus*, illustrating the highly degraded nature of the material and difficulty of visual identification

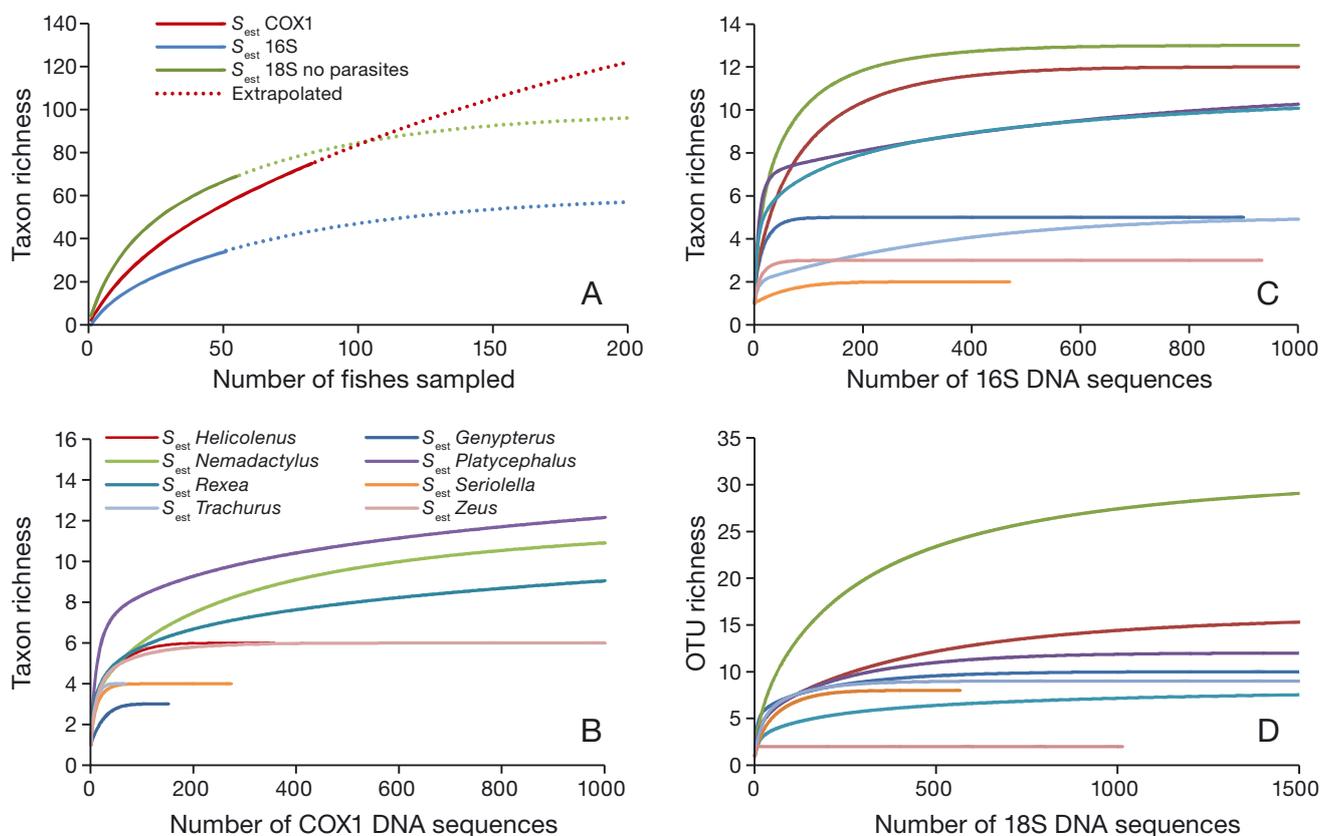


Fig. 5. (A) Accumulation of taxon richness (S_{est}) with increasing number of fish dietary samples analysed (all species combined) for 3 different DNA barcodes (COX1 Minibar, 16S Chord-cephA, 18S Bilateria). Dotted lines indicate extrapolated rarefaction curves to 500 samples. (B) S_{est} accumulation curve for increasing number of COX1 Minibar DNA sequences analysed for each species. (C) S_{est} accumulation curve for increasing number of 16S Chord-cephA DNA sequences analysed for each species. (D) S_{est} accumulation curve for increasing number of 18S Bilateria DNA sequences analysed for each species. Species in panels B–D use the same colour code

major fish lineages (Chondrichthyes and Osteichthyes), whereas COX1 Minibar and 16S Chord-cephA did. COX1 Minibar revealed the presence of 12 fish orders, including 2 chondrichthyans. 16S Chord-cephA revealed the presence of 16 fish orders, including 3 chondrichthyans. Within the arthropod lineage, the 16S Chord-cephA marker only identified the presence of the decapod order, whereas COX1 Minibar revealed the presence of 3 orders (including decapods), and 18S Bilateria yielded 5 orders (including decapods). All markers revealed the presence of the annelid lineage, but only the 18S Bilateria marker revealed the presence of nematodes and platyhelminthes (both parasites).

DNA sequencing effort and dietary diversity

Based on extrapolation of rarefaction curves to 500 samples, the number of stomach samples analysed

was sufficient to capture the majority of the dietary richness yielded by both the 16S Chord-cephA and 18S Bilateria DNA barcodes (56.9 and 69.8%, respectively; Fig. 5A). For the COX1 Minibar data, 44.7% of the predicted dietary richness was captured by the number of samples analysed. The number of COX1 Minibar, 16S Chord-cephA and 18S Bilateria sequences analysed was sufficient to capture the majority of the available dietary richness in all species (Fig. 5B–D).

DISCUSSION

The reliability of predictions made by ecosystem models depends on the validity of the underpinning foodwebs. The 8 predatory fish species examined here consumed a diverse assemblage of organisms, yet the diets attributable to each fish differed significantly in both taxonomic resolution and phylogenetic

breadth according to the method employed to characterise their diets. These results are important considering the potential for model behaviour to be influenced by decisions on how dietary items are aggregated (Fulton 2001). Taken together, our results provide a valuable comparative dataset for evaluating the relative merits of molecular and morphological approaches to fish diets and marine foodwebs.

Taxonomic resolution

DNA sequence analysis revealed the diets of all host fishes at significantly higher taxonomic resolution than analysis of morphological characteristics. Typically, microscopic analysis yielded identities at the rank of order, whereas both 16S Chord-*cephA* and COX1 Minibar DNA barcodes typically yielded identities with rank better than family, and often with assignment to species. Similar results have been documented previously in analyses of seabird diets (Bowser et al. 2013, Alonso et al. 2014), underscoring the utility of DNA metabarcoding approaches for characterising marine foodwebs. The difficulty of identifying items via microscopy is not surprising considering the often highly degraded and fragmentary nature of the material (Fig. 4), which contributed to a significant fraction of cases (ca. 11%) where no items were visually identifiable (see also Dunn et al. 2010). As anticipated, the 18S Bilateria DNA barcode provided low taxonomic resolution in comparison to both morphology and other DNA barcodes (Deagle et al. 2009).

Another characteristic of the taxonomic rank analysis was the different variance in assignment of taxonomic rank for different methods of identification. The 18S Bilateria marker exhibited consistently low resolution, typically between order and class, which accords with an investigation of Australian fur seal diet (Deagle et al. 2009). Morphological analysis exhibited the broadest variation in assignment of identities, with the interquartile range incorporating class to family. While the COX1 Minibar DNA barcode on average exhibited the highest taxonomic resolution, it also had a higher variance than the 16S Chord-*cephA* DNA barcode. An explanation for this is that the 16S Chord-*cephA* DNA barcode largely targets the phylum Chordata (Deagle et al. 2009), which is relatively well represented in available DNA sequence databases (Bucklin et al. 2011). By contrast, the COX1 Minibar DNA barcode targets a broader suite of organisms (Meusnier et al. 2008), including chordates, but also many invertebrate

groups, which are typically poorly represented in other DNA sequence datasets (Bucklin et al. 2011). The large number of MOTUs revealed for the 18S Bilateria barcode (Table S3 in the Supplement at www.int-res.com/articles/suppl/m540p167_supp.pdf) also illustrates the limits of the available 18S Bilateria reference database.

The taxon-specific effect of available DNA references on taxonomic resolution is also observable within the mixed-sample COX1 Minibar dataset. When considering bony fishes in isolation, a large fraction of the dietary items were attributable to species rank (Fig. 2). By contrast, few crustaceans could be assigned to species, and instead the majority were assigned at ordinal level or class (similar to assignment by the 18S Bilateria marker). This likely reflects the relative completeness of the reference DNA databases for bony fishes and incompleteness of the crustacean dataset (Bucklin et al. 2011). For example, in our custom-made BOLD database, 6809 fish sequences are available for the Australian region, but only 321 decapod crustacean sequences. Considering the ubiquity of crustaceans in the diets of harvested fishes in the SESSF (Bulman et al. 2001), and their known diversity, this potentially represents a significant limitation to our ability to accurately parameterise ecosystem models. It is unclear how different levels of taxonomic resolution for different components of the foodweb might affect models, but it is unlikely to be helpful or optimal (de Young et al. 2004). A related problem with generating reference DNA databases for diverse and relatively unstudied taxa such as crustaceans is that taxonomic uncertainty exists for many taxa (Lefébure et al. 2006). These cases affect the ability of taxonomic assignment algorithms such as the LCA to resolve identities (Alonso et al. 2014). Efforts to develop DNA databases for poorly referenced groups therefore need to be completed in tandem with conventional taxonomic assessments of taxa (Hajibabaei et al. 2007).

The inadequacy of DNA reference databases is a general problem in environmental HTS sequencing (Burgar et al. 2014), but equivalent problems exist for morphological analysis where expertise is missing, digestion removes key diagnostic characters, or vouchers are not available (Alonso et al. 2014). In the case of the SESSF, a concerted effort to boost the representation of crustaceans in reference DNA databases is warranted, especially considering the importance of this group in fish diets generally (Edgar & Shaw 1995). Researchers have pragmatically adopted the MOTU approach where databases are incomplete (Smith et al. 2005, Burgar et al. 2014, Symond-

son & Harwood 2014). However, for parameterising ecosystem models where attribution of functional grouping is required rather than just assessments of species diversity, MOTUs need to be assigned to a taxonomic resolution sufficient to identify a relevant functional grouping. This may be at the family level or below, and further argues for building reference databases for DNA barcodes with high resolving power (Deagle et al. 2014), especially databases suited to metabarcoding degraded prey items. Nevertheless, in the absence of reference sequences, specific MOTUs and MOTU diversity may still be tracked to provide metrics for ecosystem change.

Dietary richness

On average, morphology, 16S Chord-cephA and COX1 Minibar (mixed samples) identified between 1 and 2 items per gut sample. By contrast, 18S Bilateria DNA barcode typically identified twice that number even without the inclusion of gut parasites. This occurred despite fish taxa not being resolvable with the 18S Bilateria marker. A partial explanation for this may be the use of MOTUs based on sequence similarity for the 18S Bilateria dataset, a procedure not used for the other DNA sequence datasets. The 18S Bilateria MOTUs were identified based on clustering DNA sequences by similarity to each other rather than to reference sequences. In this situation, DNA sequencing errors (primarily homopolymer related) may inadvertently appear to be distinct taxa (Jones et al. 2011), despite a concerted effort to identify and remove such artefacts. Nevertheless, even without inclusion of MOTUs, the 18S Bilateria data revealed more dietary items per stomach (Fig. 3).

Phylogenetic breadth

The 3 DNA barcode markers revealed different components of the dietary spectrum in the host fishes. In part this results from the different phylogenetic breadth targeted by each marker, with the 16S Chord-cephA barcode in particular targeting Chordata and the COX1 Minibar and 18S Bilateria amplicons able to capture a broader taxonomic spectrum (Meusnier et al. 2008, Deagle et al. 2009). Within this general pattern, however, some more idiosyncratic patterns emerged. For example, the 16S Chord-cephA barcode identified 4 more fish orders than the COX1 Minibar barcode (see Bowser et al. 2013), indicating that bias may exist in the COX1 Minibar

primer binding sites. Similarly, while both COX1 Minibar and 18S Bilateria identified multiple crustacean orders, 18S Bilateria identified 2 more orders than the COX1 Minibar barcode. The 18S Bilateria marker also revealed that a large fraction of the taxon richness in gut samples originates from parasitic organisms. These were not reflected in the COX1 Minibar or 16S Chord-cephA analysis.

The difference in phylogenetic breadth yielded by the different DNA barcodes underscores the importance of selecting markers, primers and amplicon lengths appropriate for dietary analysis. In our case, the 16S Chord-cephA marker provides the broadest representation of fish groups, and would be therefore suitable to investigate piscivorous diets; however, it would fail to amplify other key components of the diet, in particular, the Crustacea. Based on past surveys, this group represents the most important dietary component by volume in the SESSF (Bulman et al. 2001). The 18S Bilateria barcode, in contrast, identified all of the classes known to feature in the diets of the target SESSF fishes (Bulman et al. 2001). Nevertheless, the resolution of this marker was insufficient to discriminate the major orders of fishes. A 2-phased approach to covering both breadth and depth could be developed. In phase one, a marker with broad taxonomic scope such as 18S Bilateria, or morphological analysis, would provide an overview of dietary breadth. The outcome would inform phase 2, which would employ primers targeting markers with narrower taxonomic focus and better resolution, or explicitly identifying the groups not represented earlier. Morphological analysis can also provide a quantitative assessment based on molecular identities (Alonso et al. 2014). Alternatively, the primers targeting the COX1 Minibar marker yield both phylogenetic breadth approaching that of the 18S Bilateria marker and high taxonomic resolution, suggesting that it is a useful, yet imperfect, dual-use marker. The difficulties of designing truly universal COX1 Minibar markers owing to its lack of conserved regions are well recognised (Deagle et al. 2014), and in our case, it failed to identify key groups known to be present, including molluscs.

Sequencing effort

Most dietary investigations aggregate samples from multiple individuals. By using double-barcoded fusion primers, combined with sequencing of single items, we distinguished 90 individual stomach sam-

ples. This permitted direct comparisons between the different DNA barcodes and between the DNA barcodes and the morphological analysis. Rarefaction analysis enabled us to evaluate the adequacy of the DNA sequencing coverage for each DNA barcode, and sample size of fishes, to reveal the dietary assemblage for the SESSF. Considering the complete dietary assemblage of the combined host species, the 3 DNA barcodes performed differently. The 16S Chord-cephA DNA barcode captured close to all of that marker's 'available' species richness of the diets from a sample of 51 fish, whereas the COX1 Minibar and 18S Bilateria, for which data from 83 and 67 fish, respectively, were available, yielded less complete diet assemblages. This difference likely reflects the 16S Chord-cephA primers targeting a narrower component of the diet (Chordata and Cephalopoda) than the other markers. Again, this highlights the importance of selecting DNA barcode markers appropriate for the question of interest.

Considering the sequencing effort on a host-species basis, fewer than 1000 sequences were generally required to capture most of the available dietary richness for all of the DNA barcodes for most species. However, the adequacy of sequencing varied between species, and therefore it would be prudent to include redundancy in sequence coverage to accommodate species with greater diversity. In addition, seasonal or geographic variation in diets (e.g. Alonso et al. 2014) may necessitate greater sequence coverage. These figures cannot be readily extended to other cases, as they are based on available sample sizes and temporal windows for the analysed individuals. Nevertheless, they offer rules of thumb for future metabarcoding studies of temperate fish diet.

We observed significant differences in the number of sequences obtained for different dietary items (Tables S3–S5 in the Supplement). There has been interest in whether such data can be viewed as representing diets quantitatively (Murray et al. 2011), although the consensus appears to be that too many biases remain for it to be interpreted in this manner without major caveats (Symondson & Harwood 2014). This consensus is underscored by our comparative analysis with the different barcodes, which in some cases revealed completely non-overlapping components of the diet. It is clear that any semi-quantitative interpretation of HTS data needs to be fully cognisant of primer bias, contamination and input DNA quality, and would benefit from validation with species-specific qPCR assays and/or digital PCR (Murray et al. 2011).

DNA from non-target organisms

A large fraction of the DNA sequences obtained from the dietary samples was derived from the host. This was true for all DNA barcodes, and whether the sequences were obtained via HTS or Sanger sequencing of individual dietary items. The presence of contaminating host sequences is a well-recognised problem in dietary studies (Shehzad et al. 2012, Piñol et al. 2014). One potential solution is the use of blocking primers (Vestheim et al. 2011). However, blocking primers have the potential to introduce biases by screening out components of the diet (Pompanon et al. 2012). This may be particularly true for the case here, where fish prey formed large components of the diets of the host fishes. The pragmatic approach is the incorporation of redundancy in the number of sequences obtained, and the post hoc bioinformatic removal of host sequences. With the rapid increase in the capacity of DNA sequencing technologies, this is becoming increasingly practical. One potential limitation is that cannibalism, which is well documented in fishes (Smith & Reay 1991), would not be identified.

Another consequence of using universal primers is the amplification of gut parasites (Pompanon et al. 2012). In general, owing to the phylogenetic distinctiveness of most gut parasites, this problem will be most prevalent for markers capturing the greatest phylogenetic breadth, such as the 18S Bilateria marker used here (Deagle et al. 2009). Parasites were identified from the gut contents of all host fishes with this marker, but not with the COX1 Minibar and 16S Chord-cephA markers. Whilst parasites are rarely incorporated into marine foodwebs, their ubiquity in the fishes examined here, and their potential to cause significant mortality (Sindermann 1987) and/or to consume significant nutritional resources (Hiscox & Brocksen 1973), indicates that their role in foodwebs deserves further attention.

Another issue that potentially complicates the attribution of diet is secondary consumption, where the diets of prey, parasites of prey or material consumed incidentally during feeding are observed amongst the sequences of a host species (e.g. Dunn et al. 2010, Bowser et al. 2013). In some cases, such as the detection in our data of single-celled Pycnococcaceae algae, it is straightforward to identify these instances based on feeding biology, but this is not always possible. This underlines that DNA barcoding does not represent a replacement for conventional biological knowledge in food web analysis, because the information it yields will always rely on well-informed biological interpretation (e.g. Jarman et al. 2013).

Foodweb linkages in the SESSF

Although the focus here is on the comparison in performance among different methods for dietary analysis, a number of notable dietary items were revealed. For example, multiple chondrichthyans (skates, sawshark) were recorded from multiple host species (*Helicolenus percooides*, *Nemadactylus macropterus*, *Platycephalus richardsoni*) with the COX1 Minibar and 16S Chord-cephA markers. Cartilaginous species such as these are often difficult to identify because they digest readily (Gales & Cheal 1992). In the case of *H. percooides*, the chondrichthyans that were identified are at least as large as the host species (≥ 50 cm), so the predator fishes were either taking bites of flesh from living fish, scavenging carcasses or eating egg cases. *Deania calcea* that was visually identifiable had been eaten by *H. percooides* in a study on the eastern Tasmania slope (Blaber & Bulman 1987) but had not been identified from *H. percooides* from the Eastern Bass Strait shelf either in the 1990s or in the present study (Bulman et al. 2001, C. Bulman unpubl. data). Other soft-bodied organisms that can be difficult to recognise via microscopy but were recorded here include squids (18S Bilateria, 16S chord-ceph A), jellyfish (COX1 Minibar) and salps (18S Bilateria). Cephalopods were frequent sequences in *P. richardsoni* and *Rexea solandri* but comprised only 2% of total diet in morphological analyses (C. Bulman unpubl. data).

Summary

Although there has been a surge in HTS approaches to understanding diets (Pompanon et al. 2012), few investigations have compared HTS with alternative methods on a sample by sample basis. Our paired comparisons therefore enable a more direct evaluation of the merits of the different approaches, and across multiple species. The key conclusion of our analysis is that for the 8 target fish species investigated here, HTS can resolve foodwebs at better taxonomic resolution than conventional morphological analysis. It follows that DNA-based analysis should be better able to provide the resolution necessary to detect ecosystem changes, including those reflecting anthropogenic impacts (e.g. Hardy et al. 2010). Anthropogenic impacts on marine ecosystems are numerous (e.g. fishing, acidification, invasive species and climate change). Our results demonstrate that the incorporation of DNA

metabarcoding into foodweb analysis has the potential to improve the function of ecosystem models and consequently, to facilitate best-practice EBM for marine systems.

Future research should evaluate the impacts of higher-resolution foodwebs on the function of ecosystem models, in addition to the effects of variable resolution among different ecosystem components (e.g. fishes vs. crustaceans). The sequencing effort required to obtain these high-resolution data is easily within the reach of standard dietary studies, and the speed with which results can be obtained suggests that costs associated with HTS analysis should, in many cases, be equivalent to morphological analysis. However, as a broad-spectrum tool that also has the capacity to estimate relative abundances of dietary items, morphology remains an invaluable tool. Indeed, the combination of morphological and DNA approaches is necessary if the value of HTS is to be maximised. One of the key contributions for morphology would be to focus on resolving the underpinning taxonomy of important dietary groups, so that additional reference DNA sequences are made available to more accurately reflect dietary composition. The crustaceans, in particular, are a highly diverse group that forms a significant component of the diets of the SESSF fishes, yet have limited reference sequences, and in many cases poorly resolved taxonomy. Two limitations of this study are first, the relatively low sequencing effort deployed in comparison to more recent DNA sequencing technologies. Although this does not invalidate the comparisons made, it is likely that the DNA-based estimates of species richness were conservative, and would increase with higher sequencing effort. A second unexplored limitation is the potential for sub-sampling from large-volume DNA extractions to lead to stochastic differences in the composition of the DNA sequences retrieved. Again, this would not invalidate comparisons of taxonomic resolution or richness, but likely means that the DNA analyses were conservative. These limitations could be addressed through use of greater sequencing effort, coupled with rarefaction analyses, as well as replicate DNA extractions.

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