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Determining the diet of larvae of the red rock lobster (*Jasus edwardsii*) using high-throughput DNA sequencing techniques

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Abstract The diet of *Jasus edwardsii* during its ~2-year larval (phyllosoma) phase is largely unknown. High mortalities experienced during larviculture might be reduced if their diet were nutritionally modelled on the natural diet. Here, prey species were identified from phyllosoma midgut glands using 454 pyrosequencing of 18S rDNA. We found that gelatinous zooplankton, particularly Siphonophora and Ctenophora, occurred frequently in the midgut glands of phyllosomas, resolving previous conjecture that these animals are in the diet of *J. edwardsii* phyllosomas. A high occurrence of sequencing reads from unicellular microbes may also reflect a reliance on scavenging detritus.

Introduction

Spiny lobster species (family Palinuridae) are distributed widely throughout temperate and tropical waters of the world and make up a significant fishery of over 60,000 t per year with a total value of well over US$700M (Jeffs 2010; Fitzgibbon et al. 2013). For example, the red rock lobster *Jasus edwardsii* was New Zealand's most valuable fisheries species exported in 2011, landing 2,683 t valued at more than US$180M (http://www.seafoodindustry.co.nz/factfile). Despite this value, little is known about the ecology of the larval phase of spiny lobsters, which is characterised by an extended period of development that takes place in offshore oceanic waters (Phillips 2005). In particular, the natural diet of spiny lobster larvae, known as phyllosoma, has been difficult to determine due to a range of factors: phyllosomas occur in low densities in offshore waters, both phyllosomas and much of their potential pelagic prey are translucent and lack hard parts, and phyllosomas are very difficult to culture through their multitude of developmental stages that are punctuated by many moulting events (Cox and Johnston 2003a; Phillips et al. 2006a; Jeffs 2007).

Researchers have inferred the phyllosoma diet from the morphology of their digestive tract (Cox and Johnston 2004; Phillips 2005), feeding limbs (Cox and Johnston 2003a, b; Jeffs 2007; Phillips et al. 2006a) and digestive enzyme profiles (Booth and Phillips 1994; Johnston et al. 2004a), as well as observing the feeding behaviour of phyllosomas that have been cultured (Jeffs and Hooker 2000; Kittaka 1997a; Mitchell 1971) or captured from the wild (Saunders et al. 2012). These various research approaches indicate that phyllosoma is opportunistic and generalist predators of gelatinous zooplankton (Jeffs 2007). Signature lipid analysis has been used to refine this broadly defined category of prey items, but for both *J. edwardsii* (Jeffs et al. 2004) and *Panulirus cygnus* (Phillips et al. 2006b) this technique offered insufficient resolution of prey, possibly due to the diverse range of prey items consumed by the larvae effectively blurring any signature lipid profile from a dominant prey species. A DNA-based diet approach has recently shown promise for the study of phyllosoma diets of *P. japonicus* (Chow et al. 2010; Suzuki et al. 2006,

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2007), *P. longipes bispinosus* (Chow et al. 2010) and *P. cygnus* (O'Rorke et al. 2012a). These three species belong to the same 'subclade' of the genus *Panulirus*, which are characteristic of tropical or subtropical oceans. There are no comparable studies of members of any of the other eleven genera of Palinuridae family, such as the heavily fished *Jasus* species, which are only found in the cool temperate waters of the southern hemisphere.

As with other species of spiny lobster, the larviculture of *J. edwardsii* has been hindered by profoundly high mortality (Kittaka 1997b). Three major research efforts have managed to raise very small numbers of juveniles of *J. edwardsii* from eggs in laboratory culture firstly in Japan, then New Zealand and Australia (Kittaka et al. 1988, 2005; Ritar et al. 2006; Illingworth et al. 1997). However, survival of these phyllosomas through to metamorphosis to the post-larval (puerulus) stage was very low with only 3.0, 0.6 (Kittaka et al. 2005) and 0.6 % (Ritar et al. 2002) of the initial hatch reaching metamorphosis and those that reached metamorphosis died shortly after. Improving the success of larviculture depends not only on managing water quality and disease, but also upon understanding and better meeting the nutritional requirements of the larvae (Kittaka 1997b). There is a belief that modelling an artificial diet on the natural diet will lead to improved diet formulation leading to more successful larviculture (Jeffs and Hooker 2000; Jeffs 2007; Wang et al. 2013).

The reptant adults and juveniles of *J. edwardsii* are found in the coastal waters around all the islands of New Zealand and the southern parts of Australia (Chiswell and Booth 2008). After hatching in shallow coastal waters, the phyllosomas migrate into open ocean waters, where they have been found up to 600 km off the east coast of New Zealand (Booth and Stewart 1992) and across the Tasman Sea, which stretches over 2,000 km between Australia and the New Zealand (Booth and Ovenden 2000). The larval phase of wild *J. edwardsii* is extremely long and extends to as much as 24 months (Booth and Phillips 1994; Lesser 1978), which is believed to be the longest larval duration recorded for any arthropod. At the end of this larval phase, the phyllosoma metamorphoses into a lecithotrophic puerulus that uses energy reserves accumulated during the preceding lengthy phyllosoma phase to fuel active swimming back to the coast, where they settle and moult to become reptant juveniles (Jeffs et al. 1999, 2001a, 2005; Jeffs and Holland 2000; Phleger et al. 2001). This return to coastal waters is a migration distance of around 200 km on average (Jeffs et al. 2001b). Physiological, biochemical and biomechanical measures of the cost of migratory swimming all indicate that most pueruli have limited energy budget for successful migration from offshore to the coast (Jeffs et al. 2001b; Wilkin and Jeffs 2011; Fitzgibbon et al. 2013). For example, based on measurements of chemically stored energy, it has been estimated that up to 16.5 % of captured pueruli lacked sufficient reserves to successfully complete migration onshore (Jeffs et al. 2001b). Measurements of the respiratory rates of pueruli of the sympatric spiny lobster, *Sagmariasus verreauxi*, indicate that as many as 50 % might have insufficient energy reserves to successfully recruit (Fitzgibbon et al. 2013). There is enormous interannual variation in the number of pueruli arriving on the coast, varying by as much as 70 times between years, which in turn greatly affects the subsequent recruitment to wild fisheries (Booth 1994). This interannual variability in recruitment is due to natural fluctuations in the planktonic food resources available to phyllosomas, which in turn affects their nutritional condition and the subsequent survival of the lecithotrophic pueruli (Fitzgibbon et al. 2013).

To test the hypothesis that the natural diet of phyllosomas fluctuates, the planktonic diet of phyllosoma of *J. edwardsii* must first be reliably determined. Therefore, in the present study, a DNA-based approach was adopted to determine the diet of *J. edwardsii* phyllosoma. DNA methods for reliably determining diets of small invertebrates are improving with advancements in molecular methods (O'Rorke et al. 2012b; Pompanon et al. 2012; Symondson 2002; Vestheim and Jarman 2008), including their evolving application for marine larvae, such as spiny lobster phyllosoma (Chow et al. 2010; O'Rorke et al. 2012a; Suzuki et al. 2006, 2007). However, these methods have not been applied to *J. edwardsii*, nor any member of this important genus.

In the present study, phyllosomas were obtained on several sampling occasions from the Wairarapa Eddy, a seasonally oligotrophic, semi-permanent and mesoscale anticyclonic eddy in subtropical waters off the eastern coast of New Zealand (Murphy et al. 2001) that accumulates and retains a large number of *J. edwardsii* phyllosoma (Chiswell and Booth 1999; Chiswell and Roemmich 1998). Discovering the natural prey of phyllosomas has the potential to improve the ongoing larviculture initiatives for this and other spiny lobster species and to improve our understanding of their ecology.

Methods

Sampling

Samples were opportunistically collected from the research vessel *Tangaroa* operated by the National Institute of Water and Atmospheric Research Ltd on four separate voyages off the eastern coast of New Zealand (Table 1). Surface waters were sampled at night with a surface net (1.6 m^2 opening, 1 mm mesh and cod-end with 355 μm mesh) and tows were made at less than 3 km h^{-1} by slowly lowering

Table 1 Phyllosoma of *J. edwardsii* used in this study and their developmental stage (as per Lesser 1978), length and source location

Sample ID	Date	Stage	Size (mm)	Latitude (°S)	Longitude (°E)	Chla (±SEM) (mg ml^{-3})
Tan01	30/10/2009	9	21.5	41°10.80	178°33.53	0.61 (±0.21)
Tan02	8/05/2010	10	36.0	41°13.09	178°30.29	0.27 (±0.03)
Tan04	8/05/2010	9	23.5	41°13.09	178°30.29	0.27 (±0.03)
Tan05	8/05/2010	6	10.5	41°13.09	178°30.29	0.27 (±0.03)
Tan07	21/02/2011	7	15.0	41°10.50	178°29.00	0.10 (±0.01)
Tan09	21/02/2011	7	12.5	41°10.50	178°29.00	0.10 (±0.01)
Tan10	21/02/2011	7	14.2	41°10.50	178°29.00	0.10 (±0.01)
Tan11	21/02/2011	7	13.0	41°10.50	178°29.00	0.10 (±0.01)
Tan12	21/02/2011	7	12.8	41°10.50	178°29.00	0.10 (±0.01)
Tan13	21/02/2011	8	21.0	41°10.50	178°29.00	0.10 (±0.01)
Tan14	21/02/2011	8	15.0	41°10.50	178°29.00	0.10 (±0.01)
Tan16	10/05/2011	8	14.5	41°11.41	178°30.27	0.12 (±0.02)
Tan17	10/05/2011	7	13.0	41°11.41	178°30.27	0.12 (±0.02)
Tan18	10/05/2011	9	23.5	41°11.41	178°30.27	0.12 (±0.02)
Tan19	10/05/2011	8	15.0	41°11.41	178°30.27	0.12 (±0.02)
Tan20	10/05/2011	8	21.5	41°11.41	178°30.27	0.12 (±0.02)
Tan21	10/05/2011	9	24.5	41°11.41	178°30.27	0.12 (±0.02)
Tan22	10/05/2011	8	20.0	41°11.41	178°30.27	0.12 (±0.02)
Tan23	10/05/2011	9	25.0	41°11.41	178°30.27	0.12 (±0.02)

Date refers to when the sample was collected, and length refers to the distance from the top of the cephalic shield to the bottom of the abdomen. The concentration of Chl*a* averaged over the sampling day is given (±SEM). Chl*a* concentration was derived from MODIS Ocean colour satellite data—NASA

the net down to 50 m depth and then raising it back up to the surface waters over a time interval of between 10 and 20 min. On recovery of the net, the contents of the cod-end were emptied into shallow plastic trays for sorting. Phyllosomas were euthanised in pre-chilled (−20° C) 70 % EtOH, rinsed down with Milli-Q filtered water (Millipore) water, then preserved and stored in fresh pre-chilled 70 % EtOH on board the vessel at −20° C for later analysis in the laboratory. Phyllosomas were staged under a dissecting microscope according to the developmental key of Lesser (1978).

Environmental data

Surface chlorophyll *a* (Chl*a*) concentrations were obtained from the Moderate Resolution Imaging Spectroradiometer (MODIS) website (http://modis.gsfc.nasa.gov) for the month proceeding each sampling date for the sampling station. The monthly averages of surface Chl*a* (±SEM) were then taken.

DNA extraction from midgut gland

In the laboratory, phyllosomas were rinsed with 600 ml of sterile Milli-Q filtered water (Millipore) using wash bottles to remove any loosely adhering surface contaminants (O'Rorke et al. 2013). As with other marine plankton, care was taken to obtain gut contents from the gut region furthest from the mouth to minimise effects from ingestion of other organisms trapped in the net (Vestheim and Jarman 2008); this was achieved by targeting the midgut gland. Phyllosomas were mounted in solidified 2 % agar gel so that their cephalic region was exposed. In brief, the contents of the midgut gland of phyllosomas were syringed out using individual, sterile, disposable 31 gauge hypodermic needles (Ultra-fine II, Becton–Dickinson, Australia) using the methods outlined in O'Rorke et al. (2013). The syringes were first flushed with Chargeswitch™ DNA extraction buffer (Invitrogen, Carlsbad, CA), mounted on a micromanipulator and the needle was then carefully inserted into the midgut gland of the phyllosoma with care being taken to minimise contact with the animal's exterior. A small quantity of Chargeswitch™ DNA extraction buffer was injected into the midgut gland and then withdrawn with the syringe. The extracted DNA is therefore an integrated sample of the midgut gland, and probably the midgut, of phyllosomas, so a study that wished to target discreet regions of the gut would have to use another technique such as microdissection (Maloy et al. 2010). Subsequent DNA extraction was performed with the Chargeswitch Forensic™ DNA extraction kit following the manufacturer's instructions. Duplicate negative controls for contamination during DNA extraction consisted of tubes of buffer that were treated in a manner identical to extractions except that no material extricated from the midgut gland was added. All plasticware used was sterile and nuclease free. Syringe extraction of midgut gland contents and DNA extraction were performed in a UV sterilised laminar flow hood following the recommendation of Blankenship and Yayanos (2005). PCR reactions were set up in a separate UV sterilised PCR hood.

Universal PCR and 454 GS FLX sequencing

Universal primers were used to target the v9 region of the 18S rRNA as used by O'Rorke et al. (2012a). These primers were used in conjunction with a PNA clamp (peptide nucleic acid clamp) that binds exclusively to spiny lobster DNA to prevent the *J. edwardsii* DNA being amplified and to enrich the amplification of prey DNA (O'Rorke et al. 2012a, b). PCR conditions were similar to that of O'Rorke et al. (2012a) except that the number of PCR cycles in the second round shuttle PCR needed to be increased from 14 cycles to 28 to ensure that a detectable amount of PCR product was generated.

PCR amplicons, which contained 454 GS FLX *Titanium* fusion primers and MID sequences, were cleaned separately using Ampure XP™ beads (Agencourt) following the manufacturer's instructions. Ampure XP™ beads were calibrated according to Roche *454 GS FLX* Technical Manual (Roche 2011), and amplicons over 200 bp were size selected. Amplicons were run on the Agilent Bioanalyzer (Agilent Technologies, Germany GmbH) with DNA 1000™ chips to check the quality and size distribution of amplicons. Amplicons were then diluted, pooled, re-cleaned with Ampure XP™ and triplicate samples were quantified the using Qubit Fluorometer (Invitrogen) and quality control repeated on the Agilent Bioanalyzer. After quality control, the pooled amplicons were sent to Macrogen (Seoul, South Korea) where they underwent further quality control and were sequenced on a 1/8th of microtitre plate on a 454 GS FLX platform using Titanium chemistry (Roche).

Bioinformatics

Assorting reads to their respective samples using multiplex identifiers (MIDs) was performed on 454 GS FLX data using Geneious v5.6 (Kearse et al. 2012). FASTA (FAST—All) and qfiles (quality files) were then exported into the MOTHUR pipeline (Schloss et al. 2009) for clustering and taxonomic assignment. Inside MOTHUR, the trim.seqs command was used to remove sequences with phred scores less than 35, and replicated sequences were reduced to a single representative sequence (de-replicated). These reads were aligned with MAFFT (Multiple Alignment using Fast Fourier Transform: Katoh et al. 2002) and the alignment visualised in Geneious. In MOTHUR, the alignment was treated with a further series of algorithms to detect and minimise sequencing and PCR artefacts including pre-clustering and chimera checking using Chimera Perseus (Quince et al. 2011). Sequences were then clustered (Nearest Neighbour) and the operational taxonomic units (OTUs) defined at 98 % homology, singletons were removed and OTUs were classified against a database of non-redundant 18S sequences downloaded from Genbank (Retrieved October 2012: http://www.ncbi.nlm.nih.gov/genbank/). Environmental sequences were not included in the reference database. After taxonomic assignment of reads, each Eukaryotic OTU was counted for each individual phyllosoma it was discovered in (i.e. presence/absence counts). Also, to ascertain which reads were most abundant in the midgut gland, phyllosomas with less than 300 reads were eliminated to prevent reads from poorly performing sequencing reactions from disproportionately influencing the results. The number of reads per phyllosoma was standardised and converted to a percentage.

Results

Samples

A total of 23 phyllosomas were captured on the four separate sampling periods (Table 1). Upon capture, samples were sequentially labelled Tan01 through to Tan23. Subsequent to capture it was determined all phyllosomas were *J. edwardsii* except Tan03, Tan06, Tan08 and Tan15, which were from the Scyllaridae family (slipper lobsters) and were then excluded from further analysis. *J. edwardsii* phyllosomas were in mid- to late-stages of development, ranging from larval stages 7 through to 10 (Table 1) (Lesser 1978). Four phyllosomas (Tan04, Tan09, Tan13, Tan14) returned <300 sequencing reads and were therefore excluded from semi-quantitative analyses.

Environmental data

Mean surface Chl*a* concentrations (±SEM) for the month proceeding the sample dates were 0.61 (±0.21), 0.27 (±0.03), 0.10 (±0.01) and 0.12 (±0.02), with the higher value occurring in the austral spring and the other values in summer and autumn (Table 1).

PCR efficiency

DNA extracted from the midgut gland content of phyllosomas generated high yields of PCR amplicons under standard PCR conditions. However, on addition of the PNA clamp the PCR amplicon yield was poor and PCR was characterised by a high number of bands less than 100 bp indicating primer dimers. This is consistent with there being little template for the PCR reaction besides the host DNA.

454GS Titanium sequencing results

The total number of reads returned from the 454 GS FLX run was 113,040. Of these, 68,971 reads were retained after

assigning reads to samples and initial sequence quality control (removing sequences with more than one error in either the MID or fusion primer sequences). A total of 27,873 sequences remained after the removal of sequences with a phred score of less than 35, singletons (of which there were 37) and the removal of incomplete sequences derived from a palinurid template. It was typically the case that palinurid sequences occurred in the dataset because PCR errors altered the DNA sequence in these fragments that matched the PNA, so that the PNA clamp was no longer effective at suppressing them. However, a palinurid sequence that did include the PNA sequence occurred six times in the 68,971 sequences, this was twice in samples Tan01 and Tan 17 and once each in samples Tan12 and Tan14. This indicates a very low rate of failure of the PNA clamp. Average reads per sample was 1,478.6 ± 275.4 SEM.

Taxonomic assignment of reads

After reads were normalised, and four phyllosomas that contained <300 reads were discarded, the relative contribution of Metazoa was 28.4 %, which included Mammalia and Insecta, that respectively contributed 0.4 and 2.1 % of standardised reads, but are not considered mesozooplankton. Chromalveolata contributed 13.3 % of reads, fungi 57.0 %, colonial radiolarians 0.1 % and reads from plants contributed 1.1 %. The extraction negative controls generated strong primer dimers, but were negative for PCR products in the template size range. Negative PCR controls were negative for PCR products. Six phyllosoma samples returned no amplicons derived from zooplankton, i.e., Tan04, Tan05, Tan13, Tan14, Tan16 and Tan21 (Fig. 1). Two samples contained zooplankton exclusively; Tan07 containing DNA derived only from a bony, ray-finned fish (order Tetraodontiformes), and Tan17 containing a mixture of two OTUs from Cnidaria (order Siphonophora) and Ctenophora (order Lobata; Fig. 1).

Based on rank-abundance of reads, the most frequently occurring zooplankton OTUs detected in the midgut gland of all the phyllosomas sampled were Siphonophora (Cnidaria), Tetraodontiformes (ray-finned fish: Chordata) and Lobata (Ctenophora), which were the fifth, sixth and seventh most abundant reads, respectively (Fig. 2). Siphonophora occurred in 8 of the 19 phyllosomas and Lobata in 7 phyllosomas (Table 2). The abundant Tetraodontiformes reads occurred in only two phyllosomas, but were highly represented in one of these. The next most abundant zooplankton was from another ctenophore of the order Beroida, which was detected in two phyllosomas. Salpida (Chordata) DNA occurred in four phyllosomas, and calanoid copepod (Arthopoda) DNA occurred in three, but the OTUs of salps and copepods were different for each phyllosoma (Table 2). Krill were detected in four phyllosomas (Table 2).

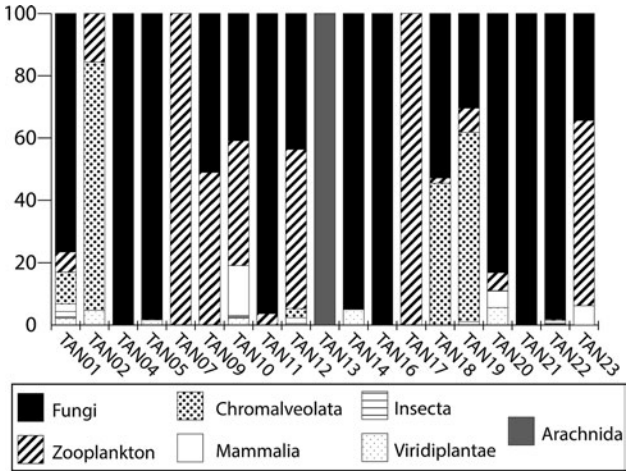

Fig. 1 Origins of sequenced PCR amplicons occurring in each *J. edwardsii* phyllosoma. Most samples produced relatively small proportions of zooplankton, with eight samples containing over 10 % zooplankton reads. Only zooplankton DNA was recovered from two samples, with TAN07 exclusively containing teleost fish DNA, and TAN17 containing both siphonophore and ctenophore reads. Overall, fungal dominated phyllosoma, comprising the majority of reads in twelve samples. Chromalveolata were detected in 6 samples and an arachnid was the only sequence detected in TAN13. Contaminating DNA was predominantly of insect or plant origin, both of which are not uncommon occurrences in other metagenomic marine studies

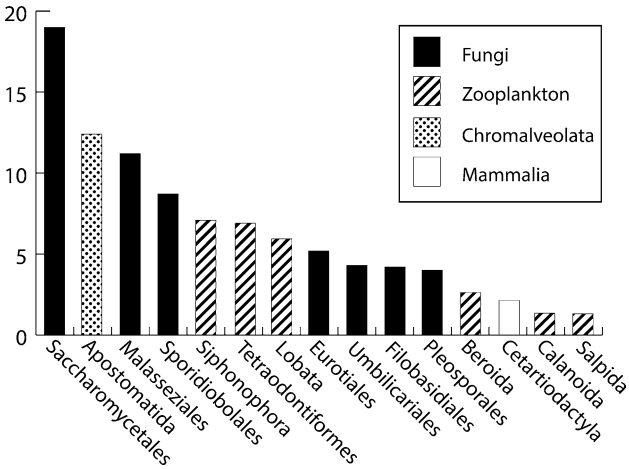

Fig. 2 Rank abundance of phylogenetic orders of prey organisms detected in the midgut gland of *J. edwardsii* phyllosomas. Phylogenetic order of organisms detected using PCR of the v9 region of the 18S rDNA locus. Three hundred reads were randomly subsampled from each phyllosoma, while those with less than 300 reads were excluded from analysis, so that reads from fifteen phyllosomas are represented. The *y* axis shows percent of total reads that each taxonomic order contributes

A total of 13.3 % of reads assigned to orders of the kingdom Chromalveolata Eugregarinida, Apostomatida and Albuginaceae. DNA from the Apostomatida contributed the second most abundant number of reads (12.7 %; Fig. 2)

Table 2 OTUs detected in midgut gland of *J. edwardsii* phyllosoma

Kingdom	Phylum	Class	Order	Count
Metazoa	Arthropoda	Malacostraca	Euphausiacea	4
		Maxillopoda	Calanoida	1
			Calanoida	1
			Calanoida	1
		Arachnida	Acariformes	1
		Insecta	Neuroptera	1
			Coleoptera	1
			Thysanura	1
			Isoptera	1
	Chordata	Actinopterygii	Perciformes	1
			Tetraodontiformes	2
		Thaliacea	Salpida (98)	1
			Salpida	1
			Salpida	1
			Salpida	1
		Mammalia	Cetartiodactyla	6
		Mammalia	Primates	1
	Cnidaria	Hydrozoa	Siphonophora	8
			Trachymedusae	1
	Ctenophora	Tentaculata	Beroida	2
			Lobata	7
	Echinodermata	Ophiuroidea	Ophiurida	1
	Mollusca	Gastropoda	Euthyneura	1
Rhizaria	Radiolaria	Acantharea	Arthracanthida (95)	1
		Polycystinea	Spumellaria	2
Fungi	Ascomycota	Dothideomycetes	Pleosporales	1
				1
				1
				1
		Eurotiomycetes	Eurotiales	6
			Eurotiales (82)	4
		Lecanoromycetes	Umbilicariales (68)	1
		Leotiomycetes	Helotiales	1
		Pezizomycetes	Pezizales	1
		Saccharomycetes	Saccharomycetales	10
			Saccharomycetales	1
	Basidiomycota	Agaricomycetes	Polyporales	1
			Trechisporales	1
		Exobasidiomycetes	Malasseziales	8
		Microbotryomycetes	Sporidiobolales	3
		Tremellomycetes	Cystofilobasidiales	1
			Filobasidiales (86)	2
			Tremellales	1
Chromalveloata	Apicomplexa	Gregarinia	Eugregarinida	2
	Ciliophora	Oligohymenophorea	Apostomatida	3
	Stramenopiles	Oomycetes	Albuginaceae	1
Viridiplantae	Streptophyta	Liliopsida	Poales	1
		Magnoliopsida	Fabales	3
		Magnoliopsida	Malvales	4
		Magnoliopsida	Solanales	2
		Liliopsida	Asparagales	1
		Polytrichopsida	Polytrichales	1

The taxonomy detected in the midgut gland of phyllosomas and the taxa that occurred among the 19 sampled phyllosomas (i.e. count data). Each OTU clustered at ≥98 % similarity, and more than one OTU was detected within some orders. Bootstrapping was performed to indicate the probable support for assignment of each taxonomic rank. Almost all OTU's received 100 % support at the taxonomic rank of order, but those that had bootstrap results of less than 100 % are indicated in parentheses

and occurred in three phyllosomas (Table). Eugregarinida occurred in two phyllosomas and Albuginaceae occurred in one (Table 2).

Discussion

The present study used the hypervariable v9 region of the 18S loci to detect the gut contents of phyllosoma of *J. edwardsii*. Other DNA diet studies have used a range of other hypervariable loci, including mitochondrial and intergenic regions (reviewed in King et al. 2008). These loci, which are much more variable than the 18S v9, have the advantage that they can typically resolve prey items to the species level. However, the greater variability that confers this higher resolution comes with trade-offs: that the priming sites are not conserved across all animal taxa and that amplicon lengths can be quite variable causing differential PCR amplification of products from different organisms, affecting the reproducibility and universality of the results (Acinas et al. 2005; Suzuki and Giovannoni 1996). Highly variable loci also suffer from the problem that it is impossible to assign even a broad taxonomic classification to an amplicon if no closely related organisms have been sequenced for that locus (Chow et al. 2010). This is often the case for marine organisms from the open ocean, particularly gelatinous ones (Bucklin et al. 2010). Therefore, the present study used the 18S v9 region because the priming sites and fragment lengths are highly conserved which makes the locus highly universal, despite it resolving to the taxonomic level of order or family in most organisms. This universality is highly desirable for determining the source of the midgut gland content of phyllosomas, which are consumers of prey from a diversity of phyla (Jeffs 2007; Phillips et al. 2006a) and also kingdoms (O'Rorke et al. 2012a). Unlike most other commonly used universal PCR primer sets, the 18S v9 primer set has the potential to amplify organisms from across the Eukaryotic domain (Pawlowski et al. 2011; Stoeck et al. 2009), and the present study has been able to detect organisms from unanticipated Eukaryotic kingdoms, such as Chromalveolata and Fungi, and therefore give unexpected clues into the ecology of phyllosoma.

Interpreting the occurrence of multiple OTUs is complicated by the fact that multicopy genes, such as the 18S rRNA, vary in copy number across taxa (O'Rorke et al. 2012b; Pompanon et al. 2012). The problem of using multicopy genes is likely to be exacerbated when making comparisons between Kingdoms, for the microbial eukaryotes are known to have highly heterogeneous quantities of ribosomal genes (Rooney and Ward 2005; Zhu et al. 2005). This means that the relative abundance of sequence reads can at best be considered a 'semi-quantitative' guide to the importance of prey items and that it is much more reliable to reduce reads to presence/absence of reads and observe the OTUs that occur most frequently in samples (Table 2). The ratios of sequence reads cannot be considered a straight-forward indication of organism biomass and semi-quantitative ratios are best used to compare populations (Amend et al. 2010).

The acidity and digestive enzymes of the larval midgut gland (Johnston et al. 2004b; Johnston and Yellowlees 1998) will also degrade DNA (Lindahl 1996), which would potentially affect the quality of genomic DNA and could be the cause of the high number of discarded sequences. Furthermore, if phyllosomas are eating zooplankton infrequently, then sequence reads from their gut system are more likely to be dominated by microbes that are resilient to digestion. Along with low amplification efficiency and a high proportion of microbial sequences, a moderate number of reads were returned for plant, insect and mammals. Plant DNA is not unusual in open ocean studies (Maloy et al. 2013; Riemann et al. 2010; Suzuki et al. 2007) and, if it is not a contaminant, it is possibly evidence of wind-driven processes introducing terrestrial DNA into oceanic systems. The same is possibly true of the insect DNA. The reads from mammals were in one instance primate (probably human), but predominantly Cetartiodactyla (Table 2), which could be due to Cetacean DNA recirculating in the ocean system, but is more likely to be post-sampling contamination.

Zooplankton

Multiple zooplankton OTUs were found in the midgut gland of ten of nineteen phyllosomas, with five out of ten phyllosomas containing more than two zooplankton OTUs and up to eight separate zooplankton OTUs were present in one phyllosoma. This heterogeneity has been observed in DNA from the midgut gland of phyllosomas of both *P. cygnus* (O'Rorke et al. 2012a) and *P. japonicus* (Chow et al. 2010) and is evidence for predation on either multiple prey items, or feeding on particles of detritus and faeces that contain DNA from multiple sources. Secondary predation (detecting the prey of their prey; Sheppard and Harwood 2005) is also a possibility because phyllosomas are voracious predators and have been observed in captivity to often consume their prey entirely, including its gut (Cox and Bruce 2003; Jeffs 2007; Saunders et al. 2012). Siphonophores, which appear to be an important component of the diet of phyllosomas, are capable of consuming hundreds of zooplankton in a minute, and their feeding polyps can contain a multitude of zooplankton prey items (Biggs 1976). Consistent with this observation, eight of the nine phyllosomas that contained DNA from a siphonophore contained DNA from at least one other zooplankter.

Siphonophores, ctenophores, krill, colonial radiolaria and bony fish were detected in more than one phyllosoma. One phyllosoma exclusively contained DNA derived from a ray-finned fish, most likely from predation on a fish larvae or fertilised fish eggs, so this is a reliable indication that fish was the principal prey item of this phyllosoma. Fish larvae were the first prey to be observed being captured and consumed by captive wild phyllosomas (Lebour 1925) and have also been used to feed cultured phyllosomas of some species of spiny lobster through specific developmental stages (Inoue 1978; Kittaka 1997b) including *J. edwardsii* (Kittaka et al. 2005; Macmillan et al. 1997).

While the present study corroborates the hypothesis that *J. edwardsii* phyllosomas are generalist predators, it appears they might preferentially target siphonophores and ctenophores. Phyllosomas of the closely related family Scyllaridae, known as slipper lobsters, have been observed adhering to the bells of variously sized cnidarian medusae, and it has been hypothesised that these are a source of food, transportation and, in the case of smaller jellyfish, protection (Ates et al. 2007; Shojima 1963; Thomas 1963). That the phyllosomas feed on cnidarians in situ is indicated by the inclusion of cnidarian coloured pigment in the midgut gland of slipper lobster phyllosomas (Thomas 1963), and voracious feeding on a variety of cnidarian species, including toxic species, has been confirmed in laboratory experiments (Wakabayashi et al. 2012). A previous survey of zooplankton from the Wairarapa Eddy offshore of eastern New Zealand found that the abundance of phyllosomas of mid- and late-stages of *J. edwardsii* was correlated with the biomass of gelatinous zooplankton, a large component of which consisted of siphonophores (Cox 2004; Jeffs et al. 2004). Siphonophores are the zooplankters that principally drive the diurnal formation of the deep scattering layer (DSL; Barham 1963, 1966), and in the Wairarapa Eddy siphonophores and ctenophores also form a key part of this vertically migrating biomass (Cox 2004). The vertical migration behaviour of pelagic gelatinous zooplankton concentrates biomass and provides improved feeding opportunities for predatory phyllosoma, in an otherwise low density of zooplankton prey that is characteristic of the often oligotrophic oceanic waters where spiny lobster phyllosomas typically dwell. This might explain the consistent presence of pelagic cnidarians and ctenophores in the midgut glands of phyllosomas, and the corresponding strong vertical migration behaviour commonly observed in the phyllosoma of many species of spiny lobster (Minami et al. 2001; Rimmer and Phillips 1979; Bradford et al. 2005).

The present study also identified krill (Euphausiacea) in the midgut glands of two stage 7, one stage 8 and one stage 9 phyllosomas. Krill are associated with seasonal dynamics in the Wairarapa Eddy and are more abundant in Spring (Bradford-Grieve et al. 1998). They have also been found in association with mid- and late-stage phyllosoma in the Wairarapa Eddy and are associated with the DSL there (Cox 2004). However, krill sequences were identified in samples that contained DNA derived from other metazoans and fungus, indicating that their presence might be the result of secondary predation or they may be consumed along with faecal material, as coprophagy is thought to be commonplace in pelagic ecosystems (González and Smetacek 1994).

Eukaryotic microbes

DNA from single-celled heterotrophs from the kingdom Chromalveolata were detected in the midgut glands of several phyllosomas (Table 2) and fungal DNA provided the most abundant reads from the midgut glands of *J. edwardsii* phyllosomas (59.2 % of standardised reads), and fungal OTUs were detected in fifteen of the nineteen phyllosomas (Fig. 1). Fungal assemblages taken from marine surface waters have a community composition populated almost exclusively by the phyla Ascomycota and Basidiomycota (Richards et al. 2012); and these were the only fungi that were discovered in phyllosomas of *P. japonicus* (Chow et al. 2010), *P. cygnus* (O'Rorke et al. 2012a) and in *J. edwardsii* in the present study. It is quite possible that these microbes are just spores that are passing through the digestive tract of phyllosomas, but they might also be diet items, commensals or parasites and therefore could play a role in the ecology of *J. edwardsii* phyllosomas.

Particulate organic matter

A high proportion of eukaryotic microbe sequences detected in this study could be ingested along with particulate organic matter (POM). Nutrient recycling, such as through consumption of POM, is a major feature of the Wairarapa Eddy food web (Bradford-Grieve et al. 1999). Eel larvae (leptocephalii), which like phyllosoma is characteristic of oligotrophic waters, have been found to consume POM such as faeces and other detritus in situ (Mochioka and Iwamizu 1996; Otake et al. 1993). They also have the same signature fungal community of Ascomycota and Basidiomycota DNA inside their gut (Riemann et al. 2010; Terahara et al. 2011), and members of the Chromalveolata have also been observed in their gut (Govoni 2010). Therefore, it is highly likely that some species of spiny lobster phyllosomas are suspension feeders that feed on POM, which could be an important source of nutrition for them. POM is an important part of the diet of phyllosoma of some species of spiny lobsters. This might have implications for the culture of phyllosoma, suggesting that artificial feeds might benefit from incorporating compositional characteristics of degraded or fermented food sources. Phyllosoma

can absorb nutrients from POM and dissolved organic matter (DOM) through their digestive tract and integument, which has led to the recommendation that POM be used to augment live and pelletised aquaculture feeds for phyllosoma culture (Rodriguez Souza et al. 1999, 2010).

Parasitism?

It is possible that the fungi and chromalveolates could be commensal organisms living in the midgut gland of phyllosomas, competing for nutrients or parasitising them, although further work is needed to determine whether this occurs. Neither of the fungal phyla detected in this study have been identified as lobster pathogens (Shields et al. 2006), but an Ascomycota has been found to cause disease in clawed lobster (Cawthorn 2011) and another in a species of crab (Hibbits et al. 1981; Sparks 1982). Chromalveolata were detected with zooplankton in all but one phyllosoma, and it is therefore likely that Chromalveolata are co-ingested with other prey items (Lindley 1978) and therefore are either an incidental food source, or infect phyllosomas through the food chain as they do with other crustacean species (Ohtsuka et al. 2004, 2009). Various classes of Chromalveolata are already known to infect adult spiny lobsters (Shields et al. 2006), and Chromalveolata from the two classes of Oomycete and Oligohymenophorea have been identified in cultured phyllosoma (Kitancharoen and Hatai 1995; Kittaka 1997a).

Several members of the Chromalveolata class Gregarinia, which was detected in two phyllosoma, parasitise the midgut and digestive tract of Crustacea, (Rueckert et al. 2011; Takahashi et al. 2008) as do some apostome ciliates in rare cases (Gómez-Gutiérrez et al. 2006, 2010; Grimes and Bradbury 2007). In the Antarctic krill, *Euphausia superba*, it was revealed that between 90 and 100 % of the krill from separate populations were infected with Gregarinia (Takahashi et al. 2008), and endoparasitic apostome ciliates have been found in krill in similar frequencies (Capriulo et al. 1991; Capriulo and Small 1986; Gómez-Gutiérrez et al. 2012). It would be informative to isolate all these commensal species and assess their negative (and possibly positive) effects on phyllosoma development and survival. For example, Lesser (1978) estimates 98 % mortality for phyllosoma in the wild, but this could be an underestimate due to the difficulties of estimating natural mortality in an ocean-going larva. The causes of high phyllosoma mortality are largely unknown, although they are likely to include predation by pelagic fish, such as Ray's bream (Phillips and Sastry 1980) and starvation. However, it has recently been shown that mass mortalities of krill, which have traditionally been attributed to predation or starvation, have been shown to be caused by endoparasitic apostome ciliates (Gómez-Gutiérrez et al. 2003). Lobster phyllosoma may not suffer from parasitism to the same extent as krill, as the former occur at much lower densities, but it is worth exploring whether pathogens affect phyllosoma ecology.

Low zooplankton reads

In a comparable study of *P. cygnus* phyllosomas, which used analogous methods to the present one (O'Rorke et al. 2012a), the overall percentage of sequencing reads was dominated by zooplankton (77.0 %). By comparison, the present *J. edwardsii* study shows a much lower contribution from zooplankton of 27.1 % of standardised reads (and 14.5 % of total reads). While copy number of the 18S gene is likely to be highly variable between the microbial and metazoan kingdoms, which makes comparisons difficult, the discrepancy between the two genera of phyllosoma is striking. An explanation for the low percentage of zooplankton reads in *J. edwardsii* compared to *P. cygnus* could be because of physiological differences between the two, but another likely explanation is that *J. edwardsii* may encounter zooplankton prey less frequently than *P. cygnus.*

In support of the hypothesis that *J. edwardsii* phyllosomas are encountering fewer zooplankton is that they take almost twice as long to reach metamorphosis in the wild than when cultured (Booth and Phillips 1994; Phillips et al. 2006a), because although incubation temperature can contribute to delayed maturation, much can be explained by low food availability (Smith et al. 2007, 2010; Tong et al. 1997). Significantly, it has been shown that the development of *J. edwardsii* phyllosomas in batch culture becomes rapid and synchronised if prey density is above a minimum threshold (Ritar et al. 2002), but if the prey density falls below this threshold then larval development becomes slowed and the stages of phyllosomas become asynchronous (Moss et al. 1999; Tong et al. 1997). In the wild, the stages of phyllosomas of *J. edwardsii* are asynchronous despite a relatively narrow hatching period (MacDiarmid 1989), indicating a possible delay of moulting and metamorphosis due to diet restriction (Booth 1994). By contrast, the development of phyllosomas of *P. cygnus* sampled inside any water body is relatively synchronised (Phillips et al. 1979), which probably reflects feeding conditions with greater prey densities due to prolonged periods of elevated primary production (Caputi et al. 2003; Strzelecki et al. 2007; Waite et al. 2007). The Wairarapa Eddy experiences only a brief spring diatom bloom, when Chl*a* doubles, although it does not exceed 1 mg m^{-3}, when mesozooplankton densities spike (Bradford-Grieve et al. 1999). The Wairarapa Eddy is otherwise characteristic of a predominantly oligotrophic anticyclonic eddy (Bradford et al. 1982; Chiswell 2011; Murphy et al. 2001). Eighteen of the nineteen phyllosomas sampled in the present study were

taken in summer and autumn when surface Chl*a* concentrations were 0.27, 0.1 and 0.12 mg ml^{-1} (Table 1), and only a single phyllosoma was sampled in Spring waters containing 0.64 mg ml^{-1} Chl*a* (Table 1). It is difficult to draw general conclusions from such a small sample set, but sampling more phyllosomas all year round might determine whether phyllosomas rely heavily on these brief periods of elevated productivity to provide sufficient prey for accumulating the required energy reserves to fuel the lecithotrophic pueruli to actively swim back to the coast (Jeffs et al. 1999, 2001a).

Conclusion

The results of the present study are consistent with the hypothesis that *J. edwardsii* phyllosomas are generalist predators of gelatinous zooplankton, but that cnidarians and ctenophores are a significant and perhaps targeted component of their diet. This contrasts with a recent study of *P. cygnus* where greater quantities of exogenous DNA were consistently detected in the midgut gland of phyllosoma that originated from a more diverse range of zooplankton taxa, such as colonial Radiolaria, Thaliacea and Sagittoidea (O'Rorke et al. 2012a). Another insight into the ecology of *J. edwardsii* phyllosomas comes from the unanticipated amplification of large numbers of Eukaryotic micro-organisms from the kingdoms Fungi and Chromalveolata. The fungal phyla Ascomycota and Basidiomycota are either an indication that phyllosoma is also consuming decaying matter, such as faecal material and marine snow, or they are endosymbionts of the phyllosoma midgut gland. This possible versatility of feeding on inert POM of low density should be examined further as it may provide a model for the development of artificial feed for advancing phyllosoma larviculture. Determining the ecological roles of the microbial eukaryotes may also be of particular importance for improving larviculture. Overall, the results suggest that *J. edwardsii* phyllosomas survive in an environment that is much more challenging for feeding than that occupied by *P. cygnus*, and that *J. edwardsii* may rely on smaller numbers of prey subsidised by scavenging on particulate organic matter. These depauperate feeding conditions may help to explain the extended larval period and extremely variable interannual recruitment observed in this species.

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