Journal of
Plankton Research

plankt.oxfordjournals.org

J. Plankton Res. (2015) 37(3): 542–553. First published online April 22, 2015 doi:10.1093/plankt/fbv026

Spinning in different directions: western rock lobster larval condition varies with eddy polarity, but does their diet?

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Received January 22, 2015; accepted March 19, 2015

Corresponding editor: Roger Harris

Larvae of the western rock lobster (*Panulirus cygnus*) that occur in the south-east Indian Ocean offshore of Western Australia have been found to be in poorer nutritional condition in anticyclonic compared with cyclonic mesoscale eddies. The reason for this is unknown, but culture-based experiments have shown that diet composition and water temperature are key determinants of phyllosoma health and survival. Whether differences in prey composition are the cause of poor phyllosoma condition *in situ* was tested in the present study by high-throughput sequencing of 18S rDNA amplified from the gut contents of 48 lobster larvae from two cyclonic and two anticyclonic eddies. We determined that phyllosoma prey composition did not vary significantly between anticyclonic and cyclonic eddies and that any variation was at the level of sample site (permutational multivariate analysis of variance $F_{2,35} = 5.12$, $P < 0.0001$). We therefore reject the hypothesis that prey composition alone during the late larval phase determines larval condition. This indicates that the competing hypotheses are more likely: for example, that eddy water temperature is responsible for reducing the condition of phyllosomas directly, or indirectly by impoverishing the nutritional value of their foodweb.

KEYWORDS: DNA diet study; beta-diversity; oceanic foodweb; amplicon sequencing; larval ecology

INTRODUCTION

The *Panulirus cygnus* fishery is the most valuable commercial fishery in Australia (ABARES, 2011) and has been successfully and sustainably managed for several decades, helped greatly by understanding the strong relationship between climatic cycles and the large inter-annual fluctuations of post-larval settlement to coastal reefs and

subsequent catch of adults (Caputi *et al.*, 1995, 2001; Pearce and Phillips, 1988; Caputi, 2008). Between 2007 and 2011 the post-larval settlement of *P. cygnus* deviated dramatically from these historical environmental correlations, and in the following years recruitment dropped to the lowest in 40 years of careful monitoring (Feng *et al.*, 2011). The cause of the dramatic decline in post-larval recruitment to this fishery is uncertain, although the timing of spawning explains 71% of the variance in post-larval settlement that occurs 9–11 months later (de Lestang *et al.*, 2014). However, the reliability of this predictive correlation would be greatly improved by understanding what occurs over this 9–11-month long larval phase to so dramatically influence settlement.

Understanding how ecological and oceanographic factors affect the success of the lobster larval phase (during which they are referred to as phyllosomas) could be the key to the future sustainable management of this, and other stocks. In the late Austral Autumn, phyllosomas are aggregated in persistent mesoscale eddies (Saunders *et al.*, 2012; Säwström *et al.*, 2014) that begin to form in the East Indian Ocean due to the strengthening of the seasonal poleward tropical current down the Western Australian coast (the Leeuwin Current) which interacts with topological features (Pattiaratchi, 2006) and other currents with distinct thermohaline properties (Feng *et al.*, 2007). Oceanic conditions are likely to affect the accumulation of energy stores in phyllosomas across their nine developmental stages and the subsequent ability for the non-feeding post-larvae to subsequently swim back onshore and settle (Olson and Olson, 1989; Wilkin and Jeffs, 2011; Fitzgibbon *et al.*, 2013).

In 2011, it was found that phyllosomas sampled from the anticyclonic eddies were in a markedly worse nutritional condition than their cyclonic counterparts (Wang *et al.*, 2015). In particular, the lipid reserves were on average 67% less and protein levels 39% less in developmental stage VIII phyllosomas in cyclonic eddies (Wang *et al.*, 2014). This result was surprising because phyllosomas were almost twice as abundant in anticyclonic eddies in 2011 (Chan, 2012) and anticyclonic eddies have consistently been shown to be more productive overall and to typically have richer prey-fields than their cyclonic counterparts (Moore *et al.*, 2007; Strzelecki *et al.*, 2007; Thompson *et al.*, 2007; Waite *et al.*, 2007a, b; Säwström *et al.*, 2014). Not surprisingly, culture-based experiments, in which an excess of food was available to phyllosomas from various species, have shown that the health and survival of phyllosomas is dramatically impacted by the composition of their diet (Kittaka, 1997, 2000), but no such culture-based experiments have been performed on *P. cygnus*.

The analyses of foodweb variance in these animals is impractical with traditional observational approaches, given that phyllosomas are small, transparent and occur at low densities up to over 1500 km or more offshore (Phillips and McWilliam, 2009). However, the advent of high-throughput molecular techniques enables an approach involving detecting changes in phyllosoma feeding ecology without direct observation (Pompanon *et al.*, 2012). Therefore, to assess if dietary composition is the basis of poor condition of phyllosomas in anticyclonic eddies and if dietary diversity differed among phyllosomas from between the eddy dipoles we sampled phyllosomas from the same eddies as those in Wang *et al.* (Wang *et al.*2014) and used next-generation sequencing of a short region of the 18S rDNA gene from their gut contents to identify their prey.

METHOD

Sampling

Samples were collected at night with a surface net (1 m^2 and 1 mm mesh) from the RV Southern Surveyor (CSIRO, Australia) on 27, 28, 29 and 30 August 2011 (Table I and Fig. 1 give geographic co-ordinates) and sampling methods and sites were the same as used by Wang *et al.*, (Wang *et al.* 2014). Phyllosomas were immediately placed in chilled 70% EtOH (<20°C) to minimize deterioration of DNA and then staged using the key of Braine *et al.* (Braine *et al.*1979).

Sampling water controls

There has recently been scrutiny of the susceptibility of DNA diet studies to false-positive errors if nets and traps are used to capture predators (King *et al.*, 2008) because these sampling techniqes bring into close proximity animals that might not be trophically linked (Greenstone *et al.*, 2012; King *et al.*, 2012). DNA was thus also extracted from sampling water (SW) taken from the cod-end of the zooplankton net to control for the remote possibility that the phyllosomas' midguts might be contaminated by free DNA in the cod-end. For this, 5 mL of mixed SW water from the cod-end was passed through a 0.5 μm syringe filter (Millipore) into 10 mL of pre-chilled EtOH and stored at −20°C. A 0.5 μM filter was used because this is the exclusion size of the filter press at the entry to the digestive tract of late developmental stage phyllosomas (Smith *et al.*, 2009; Simon *et al.*, 2012).

DNA extraction from midgut and matched SW cod-end samples

In the laboratory, DNA was extracted from 48 phyllosomas in total. They were selected so that 12 were obtained

Table I: Developmental stage and size of phyllosomas of P. cygnus *collected (*n*) from four mesoscale eddies off Western Australia in August 2011 and successfully used for midgut content analyses (*n′*) in this study*

	Stage VI		Stage VII		Stage VIII		Stage IX				
Eddy	*n*/*n*′	Length (±SD) [mm]	*n*/*n*′	Length (±SD) [mm]	*n*/*n*′	Length (±SD) [mm]	*n*/*n*′	Length (±SD) [mm]	Date	Lat [S]	Long [E]
AE1	4/2	16.3 (±0.5)	4/4	17.3 (±1.2)	3/3	21.3 (±0.6)	1/1	25	28/8/11	31 14.485	111 38.049
AE2	4/1	16.2 (±1.3)	4/4	16.9 (±2.1)	4/3	19.4 (±0.6)	0	–	27/8/11	30 32.200	113 11.378
CE1	4/4	16.3 (±0.7)	4/3	20.1 (±1.0)	4/2	22.0 (±2.2)	0	–	31/8/11	29 57.534	112 10.515
CE2	4/4	17.3 (±1.7)	4/4	19.1 (±1.1)	4/1	19.1 (±1.7)	0	–	30/8/11	31 16.953	114 17.684

Column rows consist of the paired anticyclonic/cyclonic eddies, which are designated as AE1/CE1 and AE2/ CE2. Length is the distance from the baseplate between the phyllosoma eyes to the bottom of the pleon.

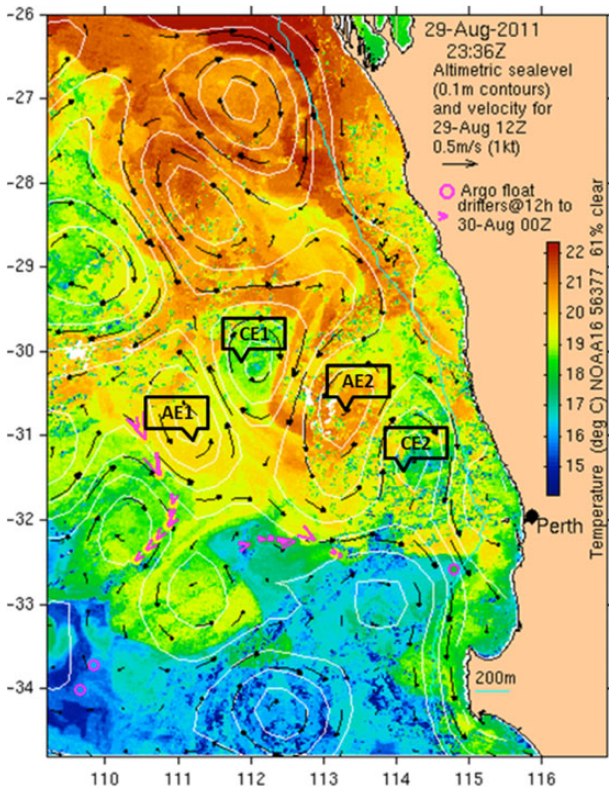

Fig. 1. Map of sampling locations from CSIRO Marine and Atmospheric Research (http://www.cmar.csiro.au/remotesensing/oceancurrents/SW/; accessed April 2012) from 29 August 2011. Arrows indicate direction and velocity of geostrophic surface water flow and colour indicates the difference in sea-surface temperature (°C) as measured by satellite. The same eddy dipoles were sampled as in Wang *et al.* (Wang *et al.*, 2014). Anticyclonic eddies are labeled as AE1, AE2 and cyclonic eddies as CE1 and CE2.

from each of two cyclonic (CE1, CE2—Fig. 1) and two anticyclonic eddies (AE1, AE2—Fig. 1). From each eddy, four phyllosomas of developmental stages VI, VII and VIII were randomly selected for further analysis, with the exception of AE1, where only three phyllosomas of developmental stage VIII were collected, so a stage IX phyllosoma was included (Table I). A single sample cod-end water control (SW-control) was extracted for each eddy (four in total). Before extraction, the EtOH preserved SW-controls were concentrated using a Concentrator 5301 Centrifugal Evaporator (Eppendorf).

Phyllosomas were rinsed with 600 mL of sterile Milli-Q filtered water (Millipore) (O'Rorke *et al.*, 2013a), then their gut contents 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.* (O'Rorke *et al.*, 2013a). DNA extraction was performed with the Chargeswitch Forensic™ DNA extraction kit (Invitrogen) following the manufacturer's instructions. Negative controls for contamination during DNA extraction consisted of tubes of buffer that were treated in a manner identical to extractions except that no gut tissue was added. These were run in duplicate. PCR reactions were set up in a separate UV sterilized PCR hood. DNA was quantified spectrophotometrically using a Nanodrop ND-1000 (Thermo Scientific).

Universal PCR and 454 GS FLX sequencing

Universal primers were used to target the v7 and v9 regions of the 18S rRNA as used by O'Rorke et al. (O'Rorke et al., 2012b). These loci can discriminate the taxonomic families or orders of plankton prey, but have limited efficacy at resolving most pelagic taxa to finer taxonomic levels. This is a trade off because the conserved nature of the 18S loci allow across phyla allows us to detect a greater diversity of potential prey that other loci might not (O'Rorke *et al.*, 2012b; Deagle *et al.*, 2014; Jarman *et al.*, 2013), but our analyses will not be sensitive to any differences between factors that occur at a species or sub-species level. These primers were used in conjunction with a PNA-clamp (peptide nucleic acid clamp) that preferentially blocks PCR amplification of spiny lobster (predator) DNA and therefore enriches the PCR amplification of prey (Chow *et al.*, 2010; O'Rorke *et al.*, 2012a). PCR conditions were the same as those of O'Rorke *et al.* (O'Rorke *et al.*, 2012b).

PCR amplicons, which contained 454 GS FLX *Titanium* fusion primers and MID sequences, were separately cleaned using Ampure XP^{TM} beads (Agencourt) following the manufacturer's instructions to select amplicons over 200 bp. Amplicons were run on the Agilent Bioanalyzer (Agilent Technologies, Germany GmbH) with DNA 1000^{TM} chips to check the quality and size distribution of amplicons. The amplicons were then diluted, pooled at equal concentrations, re-cleaned with Ampure XP^{TM} and triplicate samples were quantified using a 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 were sequenced on 1/8th microtitre plates on a 454 GS FLX platform using Titanium chemistry (Roche, 2010). Samples were randomly assigned to plate regions so that each region contained samples from phyllosomas of different developmental stages and from different sampling sites so as to minimize any possible bias due to differential sequencing efficiencies between plate regions. Sequence data were clustered into OTUs at 97% similarity and assigned taxonomy using the MOTHUR pipeline (Schloss *et al.*, 2009) using the same procedure as in O'Rorke *et al.* (O'Rorke *et al.*, 2013b).

Statistical tests

Abundance data of reads per OTU were fourth root transformed to down weight the influence of highly abundant prey items and then transformed into a Bray Curtis dissimilarity matrix using PRIMER-6 (Clarke and Warwick, 2005). Analyses were run on the v7 and v9 datasets independently. Non-metric multi-dimensional scaling and cluster analyses were then used to evaluate the relatedness of samples to SW-controls and assess whether samples clustered into groups based on the prey assemblage. The strength of association of variables (OTUs) to the MDS ordination was explored using Spearman's ρ correlation, and associations with a magnitude exceeding 0.6 were plotted as vectors to indicate which OTUs structure the MDS ordination. Also in in PRIMER 6, permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001; Anderson *et al.*, 2008) was applied to the Bray Curtis matrices of fourth root transformed OTU data to test the validity of the null hypothesis that no factors structured the diet of phyllosomas ($\alpha = 0.05$, type III (partial) sums of squares). The PERMANOVA tested three factors; Dipole, Developmental Stage and Site, with Dipole referring to whether samples came from an anticyclonic or cyclonic eddy, Developmental Stage referring to whether the samples were of developmental stages VI, VII or VIII, and Site referring to whether the phyllosomas were sampled from the same eddy site. As such, Site was nested inside Dipole, and Developmental Stage was crossed with both Dipole and Site (dipole) to see if interactions between these factors accounted for variance. Statistical significance (*P*-value) for the Dipole statistic was generated through random draws (Monte Carlo) because too few permutations could be created when testing this factor.

RESULTS

Sampling, DNA extractions and sequencing

Yields of DNA extracted from each phyllosoma were relatively high, on average 76.84 ng $\mu L^{-1} \pm 47.32$. Yields of DNA from extraction negative controls and SW-controls were negligible and within the margins of error around zero for accurate measurement indicating that the DNA from cod-end water was unlikely to significantly contaminate DNA recovered from the midguts of the larvae. A total of 268 451 reads were returned from the three 1/8th plates. Of these, 132 169 reads passed QC and were not artefacts of degraded *P cygnus* DNA. Demultiplexed reads are available on NCBI SRA under accessions: SAMN03068084, SAMN03068085, SAMN03068086, SAMN03068087. No reads contained the sequence corresponding to the PNA-clamp, which demonstrates the efficiency of this enrichment technique. There was considerable variability in the number of DNA sequence reads obtained per sample, which ranged from 6 to 5622 reads per sample and averaged 2528 reads per sample.

Zooplankton assemblages from phyllosoma midgut

For each DNA locus, 500 reads were randomly subsampled from each phyllosoma and phyllosomas with <500 reads in either locus were removed from subsequent analyses. This meant that two phyllosoma samples were removed from AE1, four samples were removed from AE2, and three removed from each of CE1 and CE2. One of the sample water controls (SW-control for CE2) also fell below 500, but was included in subsequent analyses because of its importance as a control.

The prey contents of phyllosoma midguts from each sampled eddy were visualized in one of two ways: by the number of phyllosomas containing a particular kind of prey item, (Table II) or by the relative abundance of sequence reads that were assigned to separate prey items (Fig. 2). Across all the phyllosomas the most abundant prey taxon amplified from midguts was the zooplankton Class Hydrozoa (v7: 21.9% reads; v9: 23.3% reads, respectively), predominantly the Order Siphonophora (16.7%; 14.7% reads, respectively), which was detected

Table II: The taxonomy of prey items detected in the midgut of phyllosomas and the frequency (i.e. number of phyllosomas containing that taxon) of each taxon in the midgut of the sampled phyllosomas of P. cygnus *sampled from four mesoscale eddies off Western Australia in 2011 based on v7 loci and v9 loci data (separated by colon)*

Kingdom	Phylum	Class	Order	AE1 (10)[a]	AE2 (8)[a]	CE1 (9)[a]	CE2 (9)[a]
Chromalveolata	Apicomplexa	Gregarinia	Eugregarinida	0:5	1:3	0:2	1:3
	Ciliophora	Oligohymenophorea	Apostomatida	0:0	1:1	0:0	0:0
Excavata	Euglenozoa	Kinetoplastida	Unclassified	0:5	0:3	0:8	0:3
Fungi	Ascomycota	Dothideomycetes	Capnodiales	1:0	2:0	1:0	1:0
			Pleosporales	1:0	0:0	0:0	1:0
		Eurotiomycetes	Eurotiales	8:8	6:6	7:6	9:7
		Saccharomycetes	Saccharomycetales	8:9	6:6	7:8	2:2
			Hypocreales	0:0	0:0	1:0	1:0
	Basidiomycota	Exobasidiomycetes	Malasseziales	2:3	0:5	3:0	1:2
		Tremellomycetes	Filobasidiales	1:0	1:2	1:0	1:0
			Tremellales	0:0	1:0	2:0	0:0
Metazoa	Arthropoda	Malacostraca	Amphipoda	0:1	0:4	0:0	0:0
			Decapoda	0:2	0:1	0:0	0:0
			Euphausiacea	7:10	7:8	8:9	5:7
		Maxillopoda	Calanoida	7:7	1:2	1:1	2:1
	Chaetognatha	Saggitoidea	Aphragmophora	9:10	5:3	8:8	6:8
	Chordata	Actinopterygii	Unclassified	10:10	8:8	9:9	9:9
		Appendicularia	Oikopleura	9:0	1:0	2:2	1:0
			unclassified	2:3	2:1	2:1	0:0
		Thaliacea	Doliolida	4:5	1:0	0:0	2:0
			Salpida	10:10	3:5	8:9	9:9
	Cnidaria	Anthozoa	Ceriantharia	10:10	6:5	1:0	0:1
		Hydrozoa	Anthomedusae	0:0	0:1	0:0	4:4
			Leptomedusae	4:4	1:1	1:1	0:1
			Narcomedusae	8:6	1:0	6:6	7:0
			Siphonophora	10:10	8:8	9:9	8:8
			Stylasterina	0:0	2:0	1:0	0:0
			Trachymedusae	5:10	0:4	6:6	5:3
		Scyphozoa	Coronatae	7:8	0:5	4:7	1:4
			Semaeostomeae	0:0	0:0	0:1	8:8
	Ctenophora	Tentaculata	Lobata	8:5	0:0	3:2	1:1
	Echinodermata	Echinoidea	Unclassified	5:3	2:1	4:3	1:1
		Holothuroidea	Unclassified	2:2	0:0	0:0	0:0
		Ophiuroidea	Unclassified	0:2	0:0	0:0	0:0
	Mollusca	Gastropoda	Hypsogastropoda	0:1	0:0	0:0	0:1
			Thecosomata	1:4	2:3	6:7	6:7
Rhizaria	Cercozoa	Phaeodarea	Phaeosphaerida	1:0	1:0	0:1	0:0
	Radiolaria	Polycystinea	Spumellaria	6:8	8:8	9:9	7:8

The four main columns represent the paired anticyclonic (AE) and cyclonic (CE) eddies sampled in this study.
[a]Total number of successfully analyzed phyllosomas in the sample.

in phyllosomas from all four eddies in all but one phyllosoma (Table II). The Class Actinopterygii (bony fish) was detected in all 36 phyllosomas across all four eddies for the v7 locus and the v9 locus (Table II). For major taxa there was considerable agreement in the relative composition of reads. The major exception was for Actinopterygii, which although being dominant for both loci was less abundant in v9 (10.8% of reads) than v7 (24.8% of reads), perhaps because of variable PCR efficiency (Fig. 2). Polycystinea (almost entirely from the Order Spumellaria) were also abundant (v7: 13.1%; v9: 17.1%), especially in phyllosomas in AE2, CE1 and CE2 (Fig. 2) and although in low abundance, were also present in most phyllosomas in AE1 (Table II).

Malacostraca (predominantly krill, Order Euphausiacea: v7: 4.9%; v9: 8.9%), Scyphozoa (8.1%; 7.1%) and Thaliacea (6.3%; 8.1%) were relatively abundant and present in almost all phyllosomas from AE1, CE1 and CE2 (Fig. 2 and Table II). Anthozoa occurred in almost all phyllosomas sampled from anticyclonic eddies (Table II) and a single phyllosoma in each of CE1 and CE2 (but only one locus), but were numerically greater in phyllosomas sampled from AE1 (Fig. 2). There was a general pattern that the most abundant taxa were detected in phyllosomas across all four of the sampled eddies, i.e. both cyclonic and anticyclonic. The main exception to this was the scyphozoan Order Semaeostomeae, which was present in eight of nine phyllosomas in CE2, and in a single phyllosoma in CE1, but was

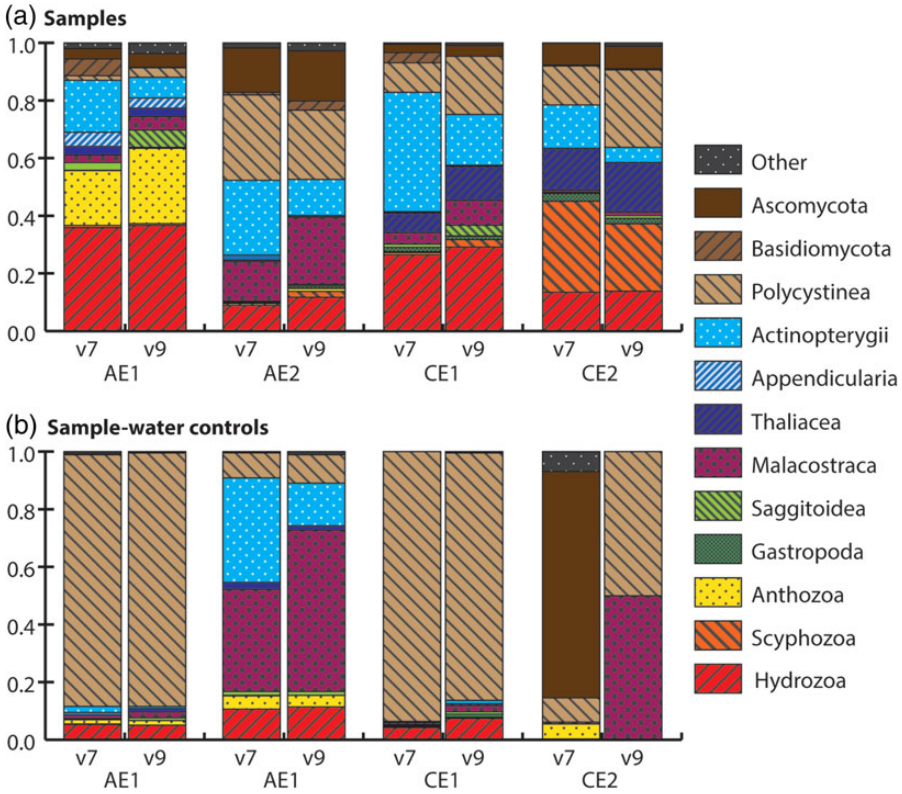

Fig. 2. Relative sequence abundances for two loci of different taxa amplified from the midgut of phyllosoma of *P. cygnus* sampled from four mesoscale eddies off the Western Australian coast in 2011. The v7 locus is on the left and v9 on the right. (**a**) The proportion of prey types in samples. (**b**) The proportion of prey types extracted from the cod-end water of the net that sampled each eddy after it had been passed through a 5 μm filter. The matched cod-end sample is a pseudo-negative control, because if DNA sequencing is detecting soluble DNA that the phyllosomas passively acquired from the net, then the matched cod-end assemblage composition should resemble the midgut assemblage composition.

not present in sampled phyllosomas in either AE1 or AE2, (Table II). Scyphozoa generally were also highly abundant in phyllosomas from CE2, but no scyphozoan Order contributed more than a negligible number of amplicons inside phyllosomas sampled from any of the other three eddies.

SW control

Three of the four SW-controls (i.e. SW-control for AE1, CE1, CE2) were dominated by one or two organisms (Fig. 2). Polycystinea was a relatively abundant taxon in all cod-end matched samples, but dominated those of SW-controls for AE1 and CE1 (~80% of amplicons). Polycystinea contributed only marginally to the composition of DNA detected in phyllosomas sampled from AE1, but this taxon did contribute ~10% to the gut contents of phyllosomas in CE1. The v7 and v9 loci in SW-control for CE2 were quite variable with the v9 being dominated by amplicons from Ascomycota and then Polycystinea, but the v7 locus was equally dominated by Malacostraca and Polycystinea. The abundances of zooplankton taxa in the SW-control for AE2 also resembled the abundances for midgut contents of the phyllosomas from that eddy, with the same top four taxa occurring in both; Hydrozoa, Actinopterygii, Polycystinea and Malacostraca. The main difference between the AE2 phyllosomas and their matched SW-control was that the phyllosomas contained ~15% fungal DNA from the Ascomycota, while the matched cod-end sample contained much <1%.

Does developmental stage determine diet?

Diet did not vary between the mid-to-late developmental stages of phyllosoma (Table III). This statistic is corroborated by inspection of the MDS plot (Fig. 3), which shows that the "distance" between the different larval developmental stages is not consistent in any manner. Interaction terms between the factors Dipole and Developmental Stage, and between Site and Developmental Stage are also not significant (Table III).

Does eddy dipole determine diet?

Whether phyllosomas fed in anticyclonic or cyclonic eddies had no significant effect on their gut composition, because any significant variation at the Dipole

Table III: Results of permutational multivariate analysis of variance (PERMANOVA) for: (i) v7 loci and, (ii) v9 loci from taxa occurring in the midgut of the sampled phyllosomas of P. cygnus *sampled from four mesoscale eddies off Western Australia in 2011.*

Source	df	MS	Pseudo-*F*	*P* (perm)	*P* (Monte Carlo)
i.					
Dipole	1	8864.7	1.24	–	0.3115
Stage	2	1092.4	0.78	0.7333	0.7660
Site(Dipole)	2	7135.5	4.60	0.0001	0.0001
Dipole × Stage	2	1424.4	1.01	0.4797	0.4772
Site(Dipole) × Stage	4	1391	0.90	0.7096	0.6720
Residuals	24	1552.7			
Total	35				
ii.					
Dipole	1	9033.4	1.23	–	0.3192
Stage	2	1234.6	0.87	0.6472	0.6569
Site(Dipole)	2	7360.4	5.12	0.0001	0.0001
Dipole × Stage	2	1197.8	0.84	0.6762	0.7012
Site(Dipole) × Stage	4	1419.5	0.99	0.5101	0.4952
Residuals	24	1438.3			
Total	35				

Factors are: Dipole (i.e. source of samples from an anticyclonic (AE1, AE2) or cyclonic eddy (CE1, CE2), Site (i.e. the particular eddy sampled) and Developmental Stage (i.e. developmental stage of the phyllosoma sampled—VI, VII, VIII).

level was entirely accounted for by the variation between sampling sites (Table III). Furthermore, there is considerable similarity between sites, with the MDS plot indicating only small distances between the mean centroids for each eddy regardless of its dipole (Fig. 3) and the reasonably high residuals indicate that much of the variation of prey content of the midgut of phyllosoma cannot be explained by the experimental factors, Dipole, Developmental Stage or Site. This reflects the consistent contribution of particular OTUs across all eddies and the variability in the prey taxa of individual phyllosoma. Most significantly, there were particular prey clades represented in the midguts of phyllosomas that were pronounced across all sampling sites: siphonophores, fish and colonial radiolarian (Table II).

DISCUSSION

Does diet vary between anticyclonic and cyclonic eddy dipoles?

In 2011, the phyllosomas in cyclonic eddies were in distinctly better nutritional condition than those in anticyclonic eddies (Wang *et al.*, 2015). Significantly, lipid levels of developmental stage VIII phyllosomas from cyclonic eddies were almost twice those of anticyclonic phyllosomas, and because lipid is the major fuel for the non-feeding post-larvae, this will affect the likelihood of survival of the animals, especially during the subsequent non-feeding puerulus stage (Jeffs *et al.*, 2001; Fitzgibbon *et al.*, 2013). The present study aimed to test whether this could be caused by dietary differences between the two eddy dipoles by taking phyllosoma from the same eddies as Wang *et al.* (Wang *et al.*, 2015) and in the same year and then determining if their diets differed. However, based on the evidence of the present study, eddy dipole has no apparent effect on the diet of phyllosomas, because most variation between eddy dipoles can be entirely accounted for by the variation among the individual eddy sampling sites regardless of their dipole (Table III).

There are two obvious ways in which diet might have caused the poor nutritional condition of phyllosomas in anticyclonic eddies in 2011 (Wang *et al.*, 2015). The results of the present study reject the hypothesis that this is because they feed on different prey in anticyclonic eddies. However, the present study only looked at a limited number of samples, and at a particular time-point in a single year. It is quite possible that there could be dietary differences between phyllosomas in different dipoles when they are in earlier developmental stages and that these are the cause of the dramatic variation in the condition of phyllosomas. It is also possible that greater differences in midgut content might have been detected had phyllosomas been sampled at a different time of day than around midnight. It is also possible that the correlation between larval condition and eddy dipole could be because larvae that are in poor condition accumulate in cyclonic dipoles. Further research needs to be done to determine if any of these antecedent conditions could explain our data. It is also possible that the nutrient condition of the prey that they are consuming differs between the anticyclonic and cyclonic systems. The present study cannot address this hypothesis, but by finding that phyllosomas consume large proportions of particular prey items (bony fish, siphonophores, colonial radiolarians) this study has identified the

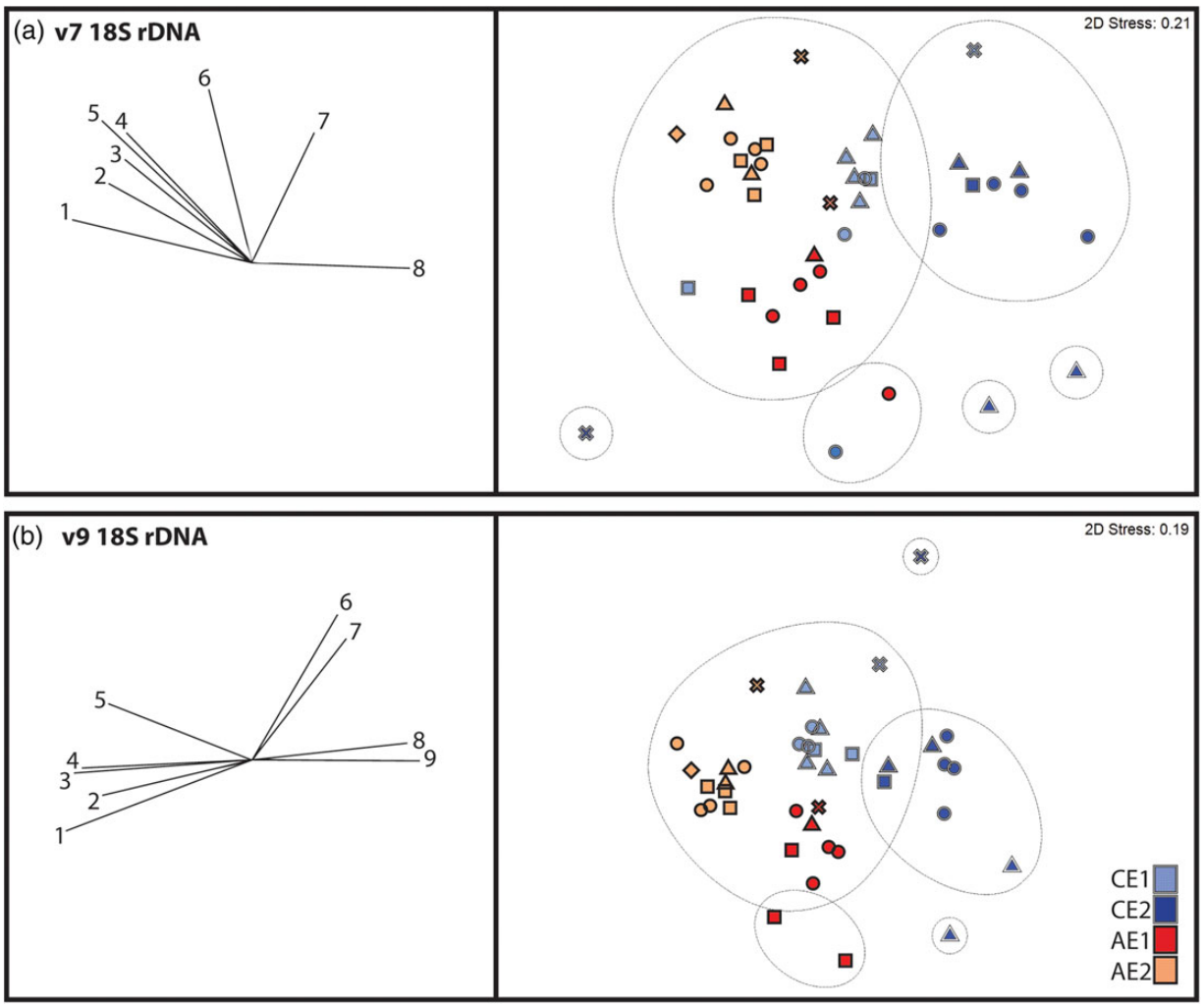

Fig. 3. An nMDS based on taxa occurring in midgut of phyllosomas of *P cygnus* sampled across four mesoscale eddies off the Western Australian coast in 2011. Ordination is based on a Bray Curtis resemblance matrix of log transformed data derived from sequencing (**a**) the v7 18S rDNA, and (**b**) v9 18S rDNA loci. Phyllosoma developmental stages VI (filled triangles), VII (filled circle), VIII (filled squares) and a single IX (filled diamonds) as well as the matched cod-end sample (cross marks). Each eddy is coded by tone and outline (refer key). Samples inside the black line share over 30% resemblance by cluster analysis. Note that the stress values for (a) and (b) are 0.21 and 0.19, respectively. Stress is a goodness-of-fit measure and a plot of 0.2 or less is considered a good fit, therefore these plots are at the upper limit of acceptability. Vector diagram is of Spearman dissimilarity associations of OTU variables that structure the ordination with a magnitude exceeding 0.6. Vector size indicates correlation strength of direction of the correlation. For the vectors the OTUs are from the taxanomic orders: (a) 1:Ceriantharia, 2:Calanoida, 3:Siphonophora, 4:Copelata, 5:unclassified Cnidaria, 6:Coronatae, 7:Trachymedusae, 8:Semaeostomeae, and for (b) 1:Ceriantharia, 2:Calanoida, 3 unclassified:Cnidaria, 4:Trachymedusae, 5:Siphonophora, 6:unclassified Tunicate, 7:Spumellaria, 8:Semaeostomeae, 9:Salpida.

prey groups on which nutrient analysis would be best performed. Results from fatty acid profiles of lipid extracted from phyllosomas co-sampled from the same eddies indicate that there could be some differences in the foundation of the food chain between the eddy dipoles, with a higher level of production from flagellates in cyclonic versus anticyclonic eddies (Wang *et al.*, 2014). However, overall primary productivity is generally thought to be consistently much higher in anticyclonic versus cyclonic eddies (Moore *et al.*, 2007; Strzelecki *et al.*, 2007; Thompson *et al.*, 2007; Waite *et al.*, 2007a).

Differences in the physical environment of eddies with different polarity is another possible explanation for the differences in the condition of phyllosomas. Anticyclonic eddies on average have warmer temperature (~1°C, Chan, 2012), which is likely to have a negative impact on the nutritional condition of phyllosomas (Matsuda and Yamakawa, 1997; Liddy *et al.*, 2003, 2004; Fitzgibbon *et al.*, 2013). Ritz (Ritz, 1972) found that body size of *P cygnus* of developmental stage VI was inversely correlated with temperature, which Wang *et al.* (Wang *et al.*, 2014, 2015) have confirmed to accord with the

nutritional condition of phyllosomas in dipolar eddies. Cyclonic eddies of the East Indian Ocean have a shallow thermocline (<50 m) but anticyclonic eddies have a mixed surface layer that is constant to some depth (300 m in 2003, Thompson *et al.*, 2007) and penetrates as far as 1500–2500 m depth (Morrow *et al.*, 2003). Given that late developmental stage (VI–IX) phyllosomas of *P. cygnus* migrate diurnally to depths >100 m (Rimmer and Phillips, 1979), this means that phyllosomas in cyclonic eddies will experience considerably less physiological stress from temperature during the daylight hours. Therefore, our results suggest it may be important that both changing mean sea-surface temperatures and the seasonal formation of mesoscale oceanic features be built into models that predict *P. cygnus* stock densities.

Midgut contents of phyllosomas

Overall, the prominent prey items of all phyllosomas were Hydrozoa (21.3% reads), Actinopterygii (bony fish; 17.2% reads) and Polycystinea (colonial radiolarians; 15.7% reads) (Fig. 2). These three groups of organisms were also the most abundant in the midguts of the 12 phyllosomas of the same developmental stages taken from a single cold-core cyclonic eddy in the same vicinity as AE2 but 13 months earlier (O'Rorke *et al.*, 2012b), which indicates the relative importance of these prey items. Siphonophores comprised 67.6% of Hydrozoa. Siphonophora have also been reliably identified as important prey of *Jasus edwardsii* phyllosomas (O'Rorke *et al.*, 2013b) and have relatively high nutritional content (Wang *et al.*, 2013).

Chaetognaths were not as abundant in the midgut contents of phyllosomas in the present study (1.9% reads) as they were the previous year (O'Rorke *et al.*, 2012b) which may reflect their overall lower abundance in the 2011 plankton assemblages (Chan, 2012) compared with the 2010 assemblage (Säwström *et al.*, 2014), although this may also represent differences in sampling methods used during the two studies. However, despite the lower abundance of chaetognath sequences in gut contents, they did occur in many of the phyllosomas within all the eddies sampled in the present study (Table II).

While several prey taxa were common features of all sample sites across eddy dipoles, there were some taxa that only occurred in particular sample sites and therefore contributed to the variation at the site level (Fig. 3). Thaliacea (6.9% reads) were not as abundant in midgut contents as they were in samples from a single anticyclonic eddy taken in 2010 (O'Rorke *et al.*, 2012b) and mostly occurred in the cyclonic eddies, particularly CE2 (Fig. 2). Scyphozoa were a conspicuous prey item in the midgut of phyllosomas sampled at CE2 (26.9%), but were not prominent elsewhere (<1%). The present study also uncovered some animals from the gut of phyllosomas that have not previously been identified as prey items, with Anthozoa being a particular feature of AE1 (22.1% of reads at AE1, <1% elsewhere). These Anthozoa were from the order Ceriantharia (tube anemones), which have an extended pelagic larval phase of up to several months in duration (Shanks and Walters, 1997; Molodtsova, 2004).

Reliability of results

The present study PCR amplified and then sequenced DNA from two linked loci (i.e. v7 and v9) on the 18S rDNA gene. There is strong evidence that the PCR and sequencing process can selectively amplify different loci and distort the composition of mixed templates (Suzuki and Giovannoni, 1996; Acinas *et al.*, 2005), although the extent of this distortion varies between PCR primer sets and loci (Tollit *et al.*, 2006; Deagle and Tollit, 2007). However, the composition of reads was generally consistent between the v7 and v9 loci for the most abundant taxa, which is therefore grounds to assume that PCR and sequencing artefacts did not significantly distort the abundances of prey taxa in phyllosomas. There was a small amount of variation between loci for the Classes Malacostraca and Polycystinea, and substantial variation between loci for the teleost fish (Fig. 2). In samples from AE1, AE2 and CE1 eddies the abundance of teleost reads relative to other reads for the v9 locus was less than half that in the v7 locus (Fig. 2). For samples from eddy CE2, Actinopterygii contributed almost 14% of reads for the v7 locus, but the contribution for the v9 locus was 5.2% (Fig. 2), although Actinopterygii were detected in four of the nine samples taken from CE2. The fourth root transformation of data is sufficient to down weight this discrepancy in analyses. Across all samples more taxa (such as Chromalveolata) were detected in the v9 region than the v7 region and this would have diluted the overall contribution of v9 reads across abundant taxa, but this is also likely to represent PCR efficiency biases for some taxa.

A comparison of the composition of the SW-controls with the composition of the phyllosoma midgut contents indicates that the results of this study are unlikely to be artefacts of sampling by net. The composition of plankton OTUs detected in the SW-controls was distinct from the composition detected in the midguts of phyllosomas from eddies CE1, CE2 and AE1 (Fig. 2). Also, in the nMDS plots, it was not the case that any SW-controls were associated with the mean centroid of the cluster of phyllosomas from the corresponding eddy (Fig. 3). Therefore, the distinctive character of SW-controls at CE1, CE2 and AE1 show that if there was any passive ingestion of DNA by any phyllosoma, that it was not

sufficient to bias the results. However, SW-AE2 clustered with some of the phyllosomas sampled from AE2, indicating that passive ingestion of DNA after sampling could have influenced the gut contents of these individuals.

There was considerable variation in the absolute number of reads detected from each phyllosoma. While this might be evidence for variable feeding success between phyllosomas, the present study can be taken only as a survey of prey richness and, with some provisions, prey composition. However, it cannot be taken as a score of absolute abundance because PCR products were diluted to a standard concentration before they were sequenced. Also, it is a matter of debate whether or not read abundances are preserved through multiple PCR rounds, emulsion PCR (particularly with MIDs) and pyrosequencing. Some phyllosoma samples were removed from analyses because they did not reach a critical read threshold of 500. Low yielding PCR's of mixed assemblages can give stochastic and irreproducible results and are therefore undesirable for analysis (Smith and Peay, 2014). The most likely reason for these low yielding results is insufficient DNA template, possibly due to the midguts of the phyllosomas being empty (O'Rorke *et al.*, 2012b). Roughly the same number of samples was removed from each sampled site and in total six phyllosoma samples were removed from both the cyclonic and anticyclonic eddies. There are a number of reasons why the midguts of phyllosomas might be empty (O'Rorke *et al.*, 2012b), but one likely explanation is that they have not encountered prey. Therefore, a quantitative approach to characterize midgut fullness would be valuable study for the future because it could be used to ascertain the extent of food deprivation. Also, the ratio of prey amplicons to predator-derived PCR artefacts might be an approach to doing this, but requires validation.

CONCLUSION

The diet of phyllosomas of *P. cygnus* in the mid-late developmental stages is highly varied and predominantly consists of colonial radiolaria, bony fish, hydrozoans (particularly siphonophores), scyphozoans, salps, chaetognaths and krill. The first three prey items were highly abundant and consistently detected in both the v7 and v9 loci of the 18S rDNA gene across samples taken from a wide range of locations and the two eddy dipoles, which indicates that the results can be taken with a high degree of confidence. SW controls also indicate that these prey items are legitimate and not artefacts of net sampling. Although the principal prey items were consistently found in all eddies sampled, the additional prey items led to significant variation among all sites.

In 2011, more phyllosomas occupied anticyclonic than cyclonic eddies (Chan, 2012), but the phyllosomas in cyclonic eddies were in superior physical condition (Wang *et al.*, 2014, 2015). The present study indicates that in 2011 the anticyclonic eddies did not offer greatly distinct feeding conditions for phyllosomas in terms of overall prey diversity to those of cyclonic eddies and that dietary composition is unlikely to be the cause of differences in phyllosoma condition between counter-rotating eddy dipoles. The results therefore indicate that the competing hypothesis may have merit for further investigation: that is, that environmental temperature differences underpin the impoverished condition of phyllosomas in anticyclonic eddies. The water temperature of eddies may be responsible for reducing the condition of phyllosomas either directly by reducing growth, or indirectly by impoverishing the nutritional value of their foodweb. Therefore, to improve predictive models of recruitment of lobster stocks into the fishery, we suggest future research and management needs to concentrate on temperature effects on larvae and their prey, rather than only the effects of eddy dipole *per se*. Here, we have identified the principal prey taxa to monitor and investigate further in terms of their role in driving the marked differences in the nutritional condition of phyllosoma between eddy dipoles.

DATA ARCHIVING

Sequences from pyrosequencing are uploaded to NCBI SRA under accession numbers SAMN03068084–SAMN03068087

ACKNOWLEDGEMENTS

Phyllosoma for this study were collected under Western Australian Department of Fisheries Research Permit number 1724-2010-38 and Murdoch University animal ethics permit number R2338/10. We are grateful to Nick Caputi and Fisheries WA for their support and advice. Satellite data were sourced from NOAA and analyzed by the Integrated Marine Observing System, which is supported by the Australian Government through the National Collaborative Research Infrastructure Strategy and the Super Science Initiative.

FUNDING

The field research component of this project was funded by a grant from the Fisheries Research and Development

Council of Australia (http://www.frdc.com.au/) under FRDC Project Number: 2010/047. The RV Southern Surveyor provided by the Australian Marine National Facility under grant SS05–2010 (http://www.csiro.au/Organisation-Structure/National-Facilities/Marine-National-Facility.aspx). This research is a contribution to the World University Worldwide Universities Network project; Ocean Eddies in a Changing Climate: Understanding the Impact of Coastal Climates and Fisheries Production.

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