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Richard O’Rorke
A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy in Marine Science,
The University of Auckland,
2013
Abstract

Discovering the natural diet of spiny lobster larvae (phyllosomas) could give insight into how an aquaculture feed could improve their survival. It could also lead to improving the sustainable management of fisheries stocks by enabling us to understand how prey dynamics affect the subsequent recruitment of lobster post-larvae into fisheries.

However, the natural diet of phyllosomas has been difficult to determine because of the low densities, remote offshore habitat, and flat transparent morphology of the larvae that makes them difficult to dissect and because their gut contents contain no morphological clue of their prey. A DNA approach overcomes these problems, but creates alternative problems: phyllosomas are generalist predators, making it difficult to detect prey without co-detecting lobster DNA, guts contain additional ‘noise’ such as fungi, and net sampling increases risk of exogenous contamination being detected by PCR. The present thesis addressed these problems and successfully developed a novel approach to extract uncontaminated gut contents using inexpensive high-grade syringes, and then applying a DNA-based protocol for extracting and determining the diet of phyllosomas. This protocol targeted small hyper-variable regions of the 18S rDNA gene and a lobster-specific peptide nucleic acid clamp (PNA-clamp) suppressed predator PCR amplification. To overcome the potential ‘noise’ that comes from amplifying gut contents, this study generates an excess of reads by high throughput 454 pyrosequencing.

This prey-enriched, amplicon pyrosequencing protocol was used to determine the prey of phyllosomas of two species of spiny lobster: *Jasus edwardsii* and *Panulirus cygnus*. A DNA approach had not previously been applied to these species. It was found that both species have a taxonomically diverse diet, but that some diet items such as hydrozoans
and bony fishes are especially significant. Additionally, colonial radiolarians are
significant diet items for *P. Cygnus*, as are ctenophores for *J. edwardsii*. Other significant,
although less abundant, diet items for both species are salps, arrow worms and krill.
Overall, these results indicate that while phyllosomas are generalist zooplankton
predators they also have preferences for some prey taxa. Significantly, the prey-enriched
pyrosequencing approach that was developed in this thesis revealed that there are no
significant dietary differences between *P. cygnus* phyllosomas in the East Indian Ocean
across distinct water masses. This is despite the fact that phyllosomas sampled from
distinct water masses are in varying nutritional condition, and dietary composition had
previously been hypothesised as contributing to phyllosoma condition.
Acknowledgments

Thank you to my supervisors Shane Lavery and Andrew Jeffs and the co-authors of the papers published from this work: Miao Wang, Scott Nodder, Seinen Chow, Haruko Takeyama, Quinn Fitzgibbon, Peter Tsai, Lynnath Beckley, Peter Thompson and Anya M. Waite. Thank you also to Tony Hickey for laying the foundation for much of the present work.

Thank you to the captains, vessel crew and scientific crew of R. V. Tangaroa for voyages Tan909 (Biophys18), Tan1006 (Biophys19), Tan1103 (Biophys20) and Tan1107 (Biophys21) and to the captain, crew and scientific team of R. V. Southern Surveyor voyage 2010-05 and 2011-04.

Thank you to support from WA Fisheries, particularly Nick Caputi who proof read Chapter 6.

Cultured samples were generously donated by Stephen Battaglene at the Fisheries Aquaculture & Coasts, Institute for Marine & Antarctic Studies, University of Tasmania, Australia and Hirokazu Matsuda and associates at the Fisheries Research Division, Mie Prefectural Science and Technology Promotion Center, Hamajima, Shima Mie, Japan.

University of Auckland Staff: Liam Williams, Kathy (Katya) Ruggiero, Vivian Ward, Murray Birch, Brady Doak, Kristine Boxen, Vibhar Thakur, Angela Little, Bhakti Patel, Letty Joseph, Nigel Birch, Craig Millar, Paul Donaldson.

SBS students, particularly Emma Carroll and Ramon Gallego for their considerable conceptual input and Simon Connell who also worked on the diet of phyllosomas.
And finally, a big thanks to Talia and other family for support.

Funding for this project came from various sources including the Foundation for Research, Science and Technology in New Zealand (FRST, now the Ministry of Business, Innovation & Employment) and the Coasts & Oceans Outcome-Based Investment series program (CO1X0501)
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Chapter 5 has been submitted to a journal for review:
R O’Rourke, S Lavery, M Wang, SD Nodder, AG Jeffs (2013) Determining the diet of larvae of the red rock lobster (Jasus edwardsii) using high-throughput DNA sequencing techniques. In Review

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1 General Introduction

1.1 Introduction

There are critical questions about the natural diet of lobster larvae that need to be addressed to enhance the management of wild lobster fisheries and lobster aquaculture. The difficulties of attempting to do this in such creatures means that a new approach needs to be taken in order to adequately address such questions about their diet. In this thesis, a prey-enriched, next generation DNA sequencing approach is developed that better addresses these issues. This approach is used to address the diet richness and composition of two species of southern hemisphere spiny lobster, Jasus edwardsii and Panulirus cygnus.

This thesis is based on a series of Chapters of published and unpublished manuscripts that individually address the methodological background (Ch 2,3), and the application of new DNA methods to investigating aspects of the wild diet of J. edwardsii (Ch 5) and P. cygnus (Ch’s 6 & 7). This general introduction addresses the broad background and overall rationale for the study, and presents a roadmap for the Chapters that follow. Many of the broad issues outlined in this general introduction are dealt with in more detail in the introductions to each of the subsequent Chapters, and this is indicated where applicable.

1.2 Spiny Lobster Larval Diet

The understanding of trophic interactions between organisms is fundamental to ecosystems ecology because an organism’s connections within a foodweb structure can have deep implications. Characterising the foodweb provides a fundamental schematic for how an environment’s budget of biologically available nutrients and energy can be
transformed into a functioning ecosystem and facilitates the prediction of its carrying capacity. Determining the prey items of a predator can also enable the construction of accurate predictive models of how predator abundance fluctuates. Perhaps the best known example of this is the harmonic oscillations of the Lotka-Volterra equation (Berryman, 1992; Leigh, 1968; Lotka, 1925) that has been used to model such predator-prey interactions as the Canadian lynx and snowshoe hare (Finerty, 1979; Gilpin, 1973; Weinstein, 1977). Being able to predict the fluctuations in predator dynamics, from prey dynamics, or more fundamental physico-chemical and atmospheric processes that determine prey field, can have implications for the conservation and management of that animal. This is particularly true in the case of spiny lobster larvae. These animals have an extended larval duration of between 3 to 24 months (Phillips, Booth, Cobb, Jeffs, & McWilliam, 2006a) and most of this time is spent beyond the continental shelf break, often hundreds of kilometres offshore where they are in extremely low densities (Phillips et al., 2006a), which makes them particularly difficult to study. Determining their prey could improve methods to predict larval abundance and enable successful stock management. The long larval duration of these animals, along with the transparent and flattened morphology of these larvae and their distinctive swimming method might also be explained by understanding their prey and how it is captured.

Discovering the prey of spiny lobster larvae has also been an objective for the commercial aquaculture of these animals. Attempts have been made to culture these animals for over a century (Kittaka, 2000), but have until relatively recently failed, due to high mortalities. A key moment in their aquaculture was the discovery that the gonad tissue of bivalve mussels was, in addition to Artemia, a sufficient food source to raise some species of larvae through to metamorphosis into post-larvae (Kittaka, 1988 in, Kittaka, 2000). However, this was still with a considerable mortality rate (Kittaka,
Therefore, it is clearly the case that further advancements need to be made into understanding the nutritional requirements of phyllosomas and considerable insights can be gained through learning their natural diet.

Spiny lobster larvae have not been observed feeding in the wild (Jeffs, 2007), and a research voyage premised on direct observation of wild phyllosomas would be a difficult undertaking, given their remote environment and small, thin, transparent morphology that makes them difficult to detect. However, larvae of slipper lobsters (family Scyllaridae), which are a close relative of spiny lobsters, have some species that occur near shore, and have been observed in the wild to cling to, and possibly feed on, various pelagic cnidarians (Ates et al., 2007; Herrnkind et al., 1976; Thomas, 1963). By analogy spiny lobster larvae might feed on similar prey. Slipper lobster larvae have also been observed to consume the entirety of cnidarians of various species in captivity (Wakabayashi et al., 2012). Captive and cultured spiny lobster larvae have been fed a variety of prey items including cnidarians (Kittaka, 1997a; Mitchell, et al., 2005; Moe, 1991), fish larvae (Inoue, 1978; Lebour, 1925; Macmillan et al., 1997), chaetognaths, (Mitchell, 1971; Saunders et al., 2012), a variety of crustacea (Saunders et al., 2012) and polychaetes (Batham, 1967). While these feeding trials are informative as to what larvae will feed on in culture, it is difficult to determine if they reflect wild feeding conditions. Prey concentrations are typically more dense in these trials than in the wild and there must be a sensory overload for animals from oligotrophic environments that use chemosensory or mechanosensory cues. The integument of phyllosomas are studded with such receptors (Cox & Bruce, 2003; Lee & Meyers, 1996; Nishida et al., 1990) and it does seem they could respond to chemical feeding cues (Cox & Bruce, 2003; Nelson et al., 2002). Many predators are also difficult to keep in captivity, which is certainly the case with phyllosomas, and predators can exhibit different feeding choice depending on
the type of enclosure they are kept in (Toonen & Fu-Shiang, 1993). This seems to be true for *J. edwardsii*, of which stage-1 phyllosomas have been observed to reject *Artemia* nauplii (Batham, 1967) or to consume them (Tong et al., 1997), depending on the feeding conditions, which differed between these studies, especially prey density which is a crucial factor in predation success (Tong et al., 1997). Approaches to determine trophic interactions, such as examining gut contents or faeces for hard remnants of prey, also appear next to impossible with phyllosomas because their gut contents appear clear because they select transparent prey and/or macerate their prey beyond recognition. An alternative approach to determine the natural diet of phyllosomas is to make inferences from observation of co-occurrence of zooplankton abundances with phyllosomas, because this could quite possibly reveal prey preferences (Cox, 2004).

Some compelling evidence for the kind of diet that phyllosomas eat has been inferred by interpreting their physiological and digestive capacities. Phyllosomas mouthparts are a complex arrangement of grinding plates, incisors and sharp maxillipeds that are well suited to predation (Cox & Johnston, 2003a; Johnston & Ritar, 2001; Mikami et al., 1994). The pereiopods (limbs) of phyllosomas are long and generally end in sharpened dactyli (the number depends of the species and instar of the larvae, Baisre, 1994) and observation of captive phyllosomas has shown that this limb morphology is good for piercing, capturing and manipulating prey items (Cox & Johnston, 2003a; Kittaka & Abrunhosa, 1997; Wolfe & Felgenhauer, 1991). The digestive enzyme profile of multiple stages of phyllosomas is comprehensive, with *J. edwardsii* expressing a range of proteases, chitinases, lipases, and amylases (Johnston et al., 2004b).
Alternative methods can be used to enable the natural prey items to be inferred, for example signature lipid (Jeffs et al., 2004; Limbourn et al., 2009; Phillips et al., 2006b; Wu et al., 2012) and stable isotope analyses (Waite et al., 2007a). These approaches can help to determine the trophic connections of prey organisms. However, these approaches provide limited taxonomic resolution of prey and they are subject to confounding factors because signature molecules can also be dependent on seasonal, environmental and geographical factors (Pompanon et al., 2012). Phyllosomas can also readily modify sterols, which undermines some of the utility of using these as lipid biomarkers (Jeffs et al., 2004).

Another approach, which is that taken in this thesis, is a DNA approach to diet analysis, which involves the extraction of prey DNA from a predator’s gut contents or faeces, and the subsequent PCR amplification of a DNA locus to uniquely identify that prey by DNA sequencing methods. Such DNA approaches have progressively become a common method for tracking interactions between predator and prey or discovering trophic links (Andrew et al., 2013; Carreon-Martinez & Heath, 2010), which reflects the rapid decrease in the cost of molecular analyses and the increasing fluency of ecologists with these techniques. A DNA approach has been highly successful at indicating the diet of Japanese species of spiny lobster larvae (Chow et al., 2010; Suzuki et al., 2006; 2007; 2008). Details of how DNA diet studies are approached and their efficiency with phyllosomas are dealt with in more detail in subsequent Chapters (particularly Chapter 2).

DNA approaches are becoming increasingly popular because they can identity prey precisely, so long as the prey’s sequence is available to be matched on a public database or can be specifically sought out as a reference for this purpose. Additionally, DNA
techniques do not require direct observation of a predator because prey DNA can be directly extracted from the predators gut or faecal contents. However, while a DNA approach solves the problems associated with conventional trophic studies, it introduces some new problems, particularly for generalist predators. The problems of most significance, that must be solved for the research in this thesis to be successful are: i) when the interest is in identifying a broad group of taxa, PCR is likely to amplify more predator than prey, ii) the method is highly sensitive to exogenous contamination, and iii) when a broad taxonomic approach is required, PCR will inevitably amplify non-prey items such as PCR artefacts and non-prey organisms such as commensals. The research presented in this thesis develops a method to deal with these problems so that it can determine the diet of two species of spiny lobster larvae, which are generalist predators whose diet has proved difficult to determine.

The research presented in this thesis therefore aims to solve these issues with applying a DNA diet approach to phyllosomas. In doing so, it can then address the specific issue of discovering the richness and composition of prey items in the midgut of phyllosomas. Once these are determined, then it would be possible to compare spatial and temporal changes in diet that might in turn affect lobster health. It is also of value to determine if there are dietary changes with developmental stage with wild phyllosomas, because it has been experimentally determined that dietary changes increase the survival of phyllosomas in culture (Inoue, 1978). It has also been found that cultured larval survivorship increases dramatically when they are fed multiple prey items (Kittaka, 1997a). Once a successful method is developed for determining gut contents of phyllosomas it should be possible to determine if wild phyllosomas also have a requirement for a diverse range of prey.
1.3 Life history of spiny lobster

Spiny lobsters are relatively long lived organisms, and female adult *Panulirus* reach maturity within 2 to 6 years of settlement and *Jasus* between 3 to 7 years (Booth, 2007) with speed of maturity being correlated to temperature, (Phillips & Melville-Smith, 2007). Their eggs are relatively small with considerably less nutritional reserves than in clawed lobsters (Pollock, 1997) and these eggs are carried under the abdomen of the female for 100-150 days in *Jasus* species (Booth, 2007) and 19-68 days for *P. cygnus* (Chittleborough, 1976) with temperature being highly correlated with incubation time. In some species of *Jasus* there is a very brief naupliosa phase after hatching (Batham, 1967) that lasts a period of hours (Phillips et al., 2006a; Williamson & Rice, 1996), whereas the *Panulirus* genus progress directly to the larval or phyllosoma phase. In general, in spiny lobster there is little investment in yolk and the larvae are almost immediately dependent on capturing and consuming food items (Abrunhosa & Kittaka, 1997). As mentioned previously, the Palinurids have a long larval period that extends from 3 to 24 months in the wild depending on the species (Phillips et al., 2006a). After hatching, the larvae are between 1.5 to 2 mm in length and by the final larval moult they reach up to 30 mm total body length (Kittaka, 2000).

At the completion of the larval phase the animals undergo metamorphosis into a post-larval (puerulus) phase. There is good evidence that the puerulus is non-feeding (secondary lecithotrophy), because they have not been observed to feed in captivity, do not appear to have a fully developed gut system (McWilliam & Phillips, 1997; Nishida et al., 1995), and as pueruli are sampled nearer to shore their lipid energy reserves become increasingly depleted (Phillips et al., 2006b). Furthermore, pueruli have been captured offshore with far less lipid reserves than is estimated to be required to make it onshore (Jeffs, Chiswell, & Booth, 2001a; Phillips et al., 2006b) and the energetics of
swimming to shore clearly push available energy reserves to the limit (Fitzgibbon et al., 2013; Wilkin & Jeffs, 2011). Altogether, the larval and post-larval life history of spiny lobsters explains much of the difficulty that has faced both aquaculture and stock management of this species; the details of which are discussed in the introductions to Chapter 5 for *J. edwardsii* and Chapters 6 and 7 for *P. cygnus*. The lengthy larval phase of spiny lobsters has impeded culture efforts and poor feeding during this phase results in a reduced capacity of post-larva to recruit into fisheries.

1.4 **Determining the diet of a generalist predator; a prey enriched amplicon pyrosequencing approach**

The research presented in this thesis sequentially deals with the methodological issues that will potentially impact on the effective use of a DNA approach to ascertain the diet of lobster larvae: i) when the interest is in identifying a broad group of taxa, PCR is likely to amplify more predator than prey, ii) the method is highly sensitive to exogenous contamination, and iii) when a broad taxonomic approach is required, PCR will inevitably amplify non-prey items such as PCR artefacts and non-prey organisms such as commensals. The research presented in Chapters 5 and 6 applies these techniques to samples taken from two populations of larvae to ascertain what is in their guts, and then (Chapter 7) uses these techniques to ascertain if there are differences in dietary composition due to the spatial location of the larvae or the among the developmental stages of the larvae.

Chapter 2 is a literature review of methods that have been used to enrich low copy DNA, such as prey, and suppress the amplification of high copy DNA, such as the predators’ DNA. There are few of such ecological studies, and these have mostly been applied to predators that do not predate on as diverse array of Phyla as phyllosomas do.
The review therefore looks to other areas of biology for guidance, in particular clinical research, where the enrichment of rare variants of loci from mixed DNA assemblages has been crucial for the early detection of mutant cancer alleles. Using the insights from this literature review, the present thesis developed an enriched amplicon sequencing protocol that used an “elongation arrest” approach where a predator specific clamp is used to prevent predator amplification (refer Fig. 2.1). This approach was considered inefficient, but has recently had some success in clinical literature (Miyazawa et al., 2008). For the present study it was desired that the 18S rDNA gene be targeted because it has excellent database coverage of eukaryotes and has DNA regions that are conserved across almost all eukaryotes, which makes it an excellent loci for research on the prey taxa of phyllosomas (Suzuki et al., 2006; 2008). However, it does not work well with the other PCR enrichment techniques that have been used in previous ecological applications, for instance restriction (Suzuki et al., 2006; 2008) and primer exclusion (Deagle et al., 2009). Identifying this different approach was therefore of value, because it meant that the 18S loci could be used to detect prey.

Chapter 3 addresses the problem of exogenous DNA contamination. PCR is a very sensitive technique and it has recently been observed that techniques used to collect samples for diet studies, such as the net tows used to collect phyllosomas, are highly susceptible to the external surfaces of animals being contaminated by DNA of animals with which they have no trophic connection (Greenstone et al., 2012; King et al., 2012). When these exogenous contaminants are amplified it creates a false positive.

Several solutions to the problem of exogenous contamination have been proposed (Greenstone et al., 2012; King et al., 2008). With small animals the consensus view has been to wash the animal before it is homogenised for DNA extraction (for instance,
Durbin et al., 2008; Meyer & Hoy, 2008; Simonelli et al., 2009). These washes can be carried out with high purity water, sea-water, ethanol (EtOH) or bleach. Unfortunately, the rarity of phyllosomas makes the use of bleach a high risk strategy. It is however questionable whether washing with only water or ethanol is sufficient to remove DNA from the surface of a predator such as a phyllosoma. **Chapter 3** assays the efficacy of washing the exterior of animals as an approach to remove exogenous DNA contaminants. This method is found inadequate and it is proposed that an approach be used that is akin to dissecting out the gut - a technique that has been used in diet studies of animals that are large or have gut systems that are clearly distinct of the integument. Because the guts of phyllosomas are not clearly distinct from the integument and cannot easily be dissected out, a novel syringing technique was developed to remove the midgut content to reduce the risk of DNA contamination from exogenous sources.

**Chapter 4** outlines the final methodological approach determined from the studies presented in the previous Chapters, plus further experiments. These methods were applied to specific biological questions in **Chapters 5, 6 and 7**. A significant methodological aspect of these papers was the use of a high throughput massively parallel sequencing platform (454 pyrosequencing) to overcome the problem that in a DNA diet study of a generalist predator that many unwanted contaminants and artefacts will be sequenced.

Massively parallel sequencing platforms have the potential to enhance the ability of PCR based studies to represent the dietary component of gut or faecal contents. The principal advantage of these technologies is their high throughput, which provides deep sequencing coverage at a drastically reduced cost. Before these technologies, the methods available to ecologists were cloning followed by dideoxy (Sanger) sequencing,
which required a considerable amount of time and expense to generate even a few reads. Alternatively, to characterise a community a genetic ‘fingerprinting’ approach could be used, for example restriction fragment length polymorphism (RFLP). The problem with this approach is that it does not offer much scope for determining the identity of organisms, instead it is a tool to ascertain community richness.

There are multiple next generation technologies (Metzker, 2010) although prior to 2012 the 454 GS pyrosequencer was preferentially used in PCR-based ecological studies because it was readily commercially available and capable of sequencing DNA >100bp and was therefore an excellent choice for sequencing PCR amplicons (Glenn, 2011). A long read length means that short nucleotide identifiers can be incorporated into the sequenced DNA (often called ‘tags’ or ‘MIDs’) so that multiple samples can be sequenced in the same sequencing run and then separated out in a post-sequencing bioinformatic routine (Meyer et al., 2008). Consequently, the 454 GS has been applied to several diet studies (Deagle et al., 2010; Valentini et al., 2009a). However, there have been great advancements in performance, and while the 454 GS is now capable of sequencing reads >650bp, other rival technologies are now able to guarantee high yields of reads >150bp, which makes these technologies applicable to sequencing tagged amplicons (http://www.molecularecologist.com/next-gen-fieldguide-2012/). More recently, there have been two developments in the next generation technologies that will enable molecular ecology laboratories to access this technology, even if constrained by modest budgets. Small, low-volume platforms have been developed such as the Roche GS Junior (http://www.gsjunior.com) and Illumina’s MiSeq Personal Sequencing System (http://www.illumina.com/systems/miseq.ilmn), and, third generation sequencing technologies such as the Ion Torrent (www.iontorrent.com; Rothberg et al., 2011) have entered the marketplace. These third generation technologies are
characterised by non-optical detection systems that greatly reduce reagent costs. These developments will enable molecular ecology laboratories with modest budgets to obtain a depth of coverage required to perform crucial meta-analysis of the efficiency of PCR and assess the contribution of contaminants to datasets. The massive numbers of sequence reads produced by these sequencers reduce the potential for negative impact from non-target amplicons that are caused by artefacts, ‘leaky’ enrichment methods, gut symbionts and DNA contaminants being incidentally co-amplified with genuine diet items.

The primary advantage of generating multiple reads for a DNA diet study is that it enables the researcher to gauge which of a multiple set of reads are authentic diet items and which are methodological artefacts. There are multiple sources of such artefacts. False positives can occur in PCR enrichments through the use of low gDNA concentration, high PCR cycle numbers, polymerase error rate and also the size of the recognition sequence used by the enrichment method (Jacobs et al., 1999). It has been estimated that Taq will incorporate an erroneous base for every $9 \times 10^3$ bases it polymerises (Tindall & Kunkel, 1988). A DNA polymerase with 3’ to 5’ exonuclease activity can substantially reduce the replication error rate compared to Taq polymerase (Gilje et al., 2008), though these can still generate errors. The environment of the gut causes elevated DNA degradation, for example nucleotide deamination occurs in acidic situations, and can cause bases to be miscalled as entirely different ones, most commonly, cystine is deaminated to uracil and therefore sequenced as a thymine (Shapiro & Klein, 1966). Massively parallel sequencing of numerous amplicons from a single specimen has been used to ascertain the extent of variation due to deamination in ancient DNA studies (Hofreiter et al., 2001). Another problem is contamination. The environment is filled with liberated DNA, which can be co-sampled with specimens. It
is also likely that universal primers will amplify gut symbionts and parasites, although
the discovery of such organisms might also be informative about the ecology of the
predator (Chow et al., 2010; Pompanon et al., 2012). A false positive may also arise
through secondary predation, where the food of prey has been mistaken for prey species
(Sheppard et al., 2005). A strategy is therefore to use the relative quantities of different
amplicons generated on a high-throughput platform to sort out potential prey from
contaminants – assuming that the amount of prey DNA in the gut exceeds
contaminating DNA.

After sequencing on a high-throughput technology these artefacts and contaminants can
then be dealt with either by removing reads that occur below a certain threshold (Rayé
et al., 2011; Valentini et al., 2009a) or by applying various algorithms designed to
detect and remove reads that are likely to be artefacts (Bik et al., 2012; Schloss et al.,
2011). For example, Chimera-Perseus (Quince et al., 2011) and preclustering (Huse et
al., 2007) apply algorithms to high-throughput data by searching through low abundant
sequences to see if they could be derived from degraded or chimeric sequences that are
more abundant in the dataset. Massively parallel sequencing therefore offers a
procedure for coping with heterogenous sequences that is not possible with lower
throughput approaches.

Chapter 5 applies the protocol developed in previous Chapters to ascertain the diet of
mid to late stage Jasus edwardsii phyllosomas from the Wairarapa Eddy. This species
of spiny lobster comprises the major commercial and recreational lobster fishery for
New Zealand and southern regions of Australia from southern Western Australia to
northern New South Wales. They are believed to have the longest larval duration of any
arthropod, which can extend to 24 months (Phillips et al., 2006a). This larval phase is a
feeding phase that is spent up to 600 km offshore in oceanic conditions (Booth & Stewart, 1992) and the energy accumulated during this phase is used to fuel the non-feeding postlarvae from metamorphosis through its migration onshore to settle (Fitzgibbon et al., 2013; McWilliam & Phillips, 1997; Wilkin & Jeffs, 2011) over a distance of 200 km on average (Jeffs et al., 2001a). There is massive regional and interannual variation in the magnitude of numbers of postlarva that settle on shore, the basis for which is unexplained (Booth, 1994) and is quite likely to be associated with feeding success during the larval phase. A hypothesis that is reinforced by the discovery that an estimated 16.5% of captured pueruli lacked sufficient energy reserves to successfully complete migration onshore (Jeffs et al., 2001a). Therefore, discovering their prey will enable future testing of the hypothesis that prey field dynamics are driving recruitment success. Also, larviculture of J. edwardsii has been hindered by high mortality and because their artificial diet has been considered a key problem larviculture success discovering their wild diet could also have implications for larvicultue (Cox & Johnston, 2003a; Kittaka, 1997a).

**Chapters 6 and 7** applies the DNA diet protocol to determine the diet of late stage Western rock lobsters (*Panulirus cygnus*) phyllosomas, the adults of which occur down the Western Australian coastline and comprise the most valuable single species fishery in Australia. Like J. edwardsii, the onshore recruitment of *P. cygnus* is highly variable between years. This interannual variation was thought to be contingent on ENSO events, with La Niña years being correlated with higher sea levels at Perth and higher recruitment (Caputi et al., 2001; Pearce & Phillips, 1988). Therefore, the fishery has been sustainably managed for decades by using the abundance of post-larval arrivals to manage future fishery catch quotas. However, since 2007 this correlation has failed to eventuate and recruitment has remained low in La Niña years (ABARES, 2011; Brown,
Discovering the diet of larvae therefore has become an urgent priority, because changes in the prey field of phyllosomas may be limiting the energy available to post-larvae to migrate back onshore.

For **Chapter 6**, a 2010 population of phyllosomas was sampled from a single cyclonic eddy in the Leeuwin Current off Western Australia and had their midgut contents examined to identify what they were feeding on. It is highly likely that phyllosomas in cyclonic eddies consume very different prey items to those in anticyclonic eddies because the counter rotating systems have food-webs driven by very different physico-chemical processes (Feng et al., 2011; Waite et al., 2007a; Waite, Pesant, Griffin, Thompson, & Holl, 2007b). Therefore, in **Chapter 7**, the analyses of the *P. cygnus* diet was extended to compare both cyclonic and anticyclonic eddies to assess the scope of the diet of phyllosomas from within counter-rotating ocean eddies. Discovering if the diets differ between the two eddysystems would answer crucial questions about the survivorship of larvae, because it was found that in 2011 the phyllosomas in cyclonic eddies were in far superior nutritional condition to their counterparts in anticyclonic eddy systems (Wang et al., 2013a; 2013b).

### 1.5 Conclusion

Altogether this thesis involves the development of a new approach in DNA techniques to determine the diet of a highly generalistic predator whose diet has proved historically difficult to characterise. Spiny lobsters are a commercially and ecologically significant group of species globally, and improving our knowledge of their lengthy larval phase, such as their food web position, will ultimately help to improve our understanding of their population dynamics. Therefore, the present study develops a prey-enriched, high-throughput amplicon sequencing approach on DNA obtained from the midgut of larvae.
to discover their most significant prey items, and addresses the significance of variation in prey composition across species and water masses.
2 PCR enrichment techniques to identify the diet of predators

2.1 Introduction
A long-standing and critical goal of ecology is to understand the trophic links between organisms, that is, what eats what? When applied to this goal, the polymerase chain reaction can be of tremendous value. It enables the ecologist to amplify DNA remnants of prey from the regurgitate, dissected gut or faeces of a predator. The amplified DNA is sequenced, and the sequence is then matched to a database to identify its source. The amount of usable prey DNA from these sources is expected to be low because of degradation by digestive processes, but because PCR is a sensitive method, capable of amplifying trace quantities of DNA, it is an ideal technique for diet studies.

Consequently, the technique is emerging as a popular method with ecologists to establish trophic links among organisms (Carreon-Martinez & Heath, 2010). However, when PCR is applied to a heterogeneous sample that contains multiple DNA templates, then it is typically the case that the dominant ortholog will be preferentially amplified. This is problematic for diet studies where the target prey DNA may be overwhelmed by the DNA of the predator.

The problem of predator DNA swamping the PCR can be avoided using species- or group-specific primers that are designed to exclusively target a set of predetermined prey DNA sequences (King et al., 2008). This approach is successful and highly sensitive and predominates in the literature. It most frequently enables us to refine what we already understand about the diet of a predator. However, if the goal is to characterise the limits of a predator’s diet, then by anticipating prey items to design prey-specific primers we run the risk of presupposing the results we are testing. This
review aims to discuss the PCR tools that enable ecologists to discover new trophic interactions or reveal the full extent of the diet of generalist predators. There are many studies that have addressed these broader questions, either by using a carefully selected set of group-specific primers (Corse et al., 2010; Jarman et al., 2004) or by screening the amplicons from universal primers with methods such as PCR denaturing gradient gel electrophoresis (PCR-DGGE, Martin et al., 2006; Tollit et al., 2009). These approaches are very successful and their use has been reviewed (Beja-Pereira et al., 2009; Gariepy et al., 2007; King et al., 2008; Sheppard & Harwood, 2005; Symondson, 2002). However, there is another strategy employed by a handful of DNA diet studies that has so far eluded review. This strategy is to use PCR enrichment techniques such as combined amplification and restriction digestion (Blankenship & Levin, 2007; Blankenship & Yayanos, 2005; Dunshea, 2009), DNA blockers (Deagle et al., 2009; 2010; Vestheim & Jarman, 2008) and peptide nucleic acid (PNA) clamps (Chow et al., 2010). Enrichment techniques constitute a very small part of published DNA diet studies, but they are a very promising means to uncovering unknown trophic links.

While ecology has neglected PCR enrichment techniques, cancer research has been at the forefront of research into these methods. This is because genetic mutations can be used as markers to screen for genetic diseases such as cancer, but many mutant cancer alleles typically occur at low frequency in tissues that can be routinely (i.e. non-surgically) biopsied. Enrichment techniques have therefore been perceived as a way to dramatically improve the early detection of cancers and enable clinicians to monitor the efficacy of cancer treatments. This is evidenced by the rich literature documenting highly sensitive assays where activating mutations are detected in peripheral fluids such as blood or sputum (e.g., Lietman et al., 2005; Miyazawa et al., 2008). The clinical literature is vast and there are many good reviews of enrichment techniques (Gocke et al., 2000; Milbury et al., 2009; Parsons & Heflich, 1997), although these tend to focus
on emerging technologies, rather than the optimisation of established techniques. No published review of enrichment techniques surveys the existing PCR enrichment literature to synthesise those results and infer which enrichment methods work best in different experimental frameworks for dietary studies. What motivates this current review is the sheer number of clinical studies that incrementally modify techniques to improve the yield and sensitivities of reactions. These studies are a resource that can be used to rapidly develop PCR enrichment studies in the field of ecology.

The PCR enrichment techniques discussed in this article apply to a broad range of biological problems, including other fields of ecology. For instance, enrichment with competitive primers has been applied to study microbial ecology (Wintzingerode et al., 2000) and parasite detection (Troedsson et al., 2008). The use of this competitive primer technique (one-tube enrichment) is discussed below, and its application to ecology in general is the subject of an excellent review (Vestheim et al., 2011). However, diet studies are distinct from many other fields of ecology because the DNA target is degraded and fragmented by digestive processes (Deagle et al., 2006; Simonelli et al., 2009). This is not a trivial point; for instance, to detect a parasite on a host, a researcher is afforded the advantage of targeting long sequences of DNA that span ideal priming and enrichment sites. Conversely, to detect the host DNA digested inside the parasite, the researcher is constrained to dealing with short DNA fragments where the coincidence of priming, enrichment and taxonomically informative sites can become unlikely. Ancient DNA studies, if they wish to use enrichment techniques, must contend with this problem too (e.g., Gigli et al., 2009). The purpose of this article is to supplement the vast and excellent literature on DNA techniques for diet studies with a review of methods to enrich the DNA of prey in the gut, which greatly ease the task of identification. It is very likely that many of the findings reported here would also be useful to fields other than diet studies.
In the following, a review is presented of PCR-based methods that enable prey discovery in any predator, particularly predators that are generalist or whose trophic roles are uncharacterised. The mainstream approaches to generalist prey discovery are outlined, including the use of multiple group-specific primers in parallel single reactions or multiplexed reactions. PCR involving universal primers and the techniques that have been used to selectively remove predator amplicons to enrich prey amplicons are then discussed. These methods include electrophoretic methods that screen for unwanted amplicons (e.g. DGGE), restriction digestion, PNA clamps and DNA blockers. The clinical research literature is also surveyed to see how they report enhanced enrichment for these techniques. Finally laser capture microdissection (LCM) is discussed as a technique that may mitigate the need for highly sensitive enrichment techniques because it enables the physical dissection of minute biological samples that contain target nucleic acids. LCM has recently been used to study diet in larval fish (Maloy et al., 2010). Altogether, this review is intended to supplement the methodological programmes for DNA diet studies that are prescribed in existing reviews (Beja-Pereira et al., 2009; King et al., 2008; Sheppard & Harwood, 2005; Symondson, 2002) by directly addressing methods suited to the discovery of prey species of generalist and uncharacterised predators.

2.2 Group-specific primers
Species-specific and group-specific primers amplify prey to the exclusion of the predator by targeting prey DNA regions where the prey and predator sequences have diverged. Group-specific primers are usually efficient and sensitive, which is not
surprising, because designing primers to anneal to one sequence and not another is the essence of PCR. The publication that initiated modern PCR with the thermostable DNA polymerase of Thermus aquaticus (Taq) reported PCR sensitivities between $1 \times 10^5$ and $1 \times 10^6$ when amplifying mixed template (Saiki et al., 1988), and such sensitivities are sufficient for diet studies.

Using PCR to characterise the diet of herbivores has been facilitated by the plastid genome of plants, because as Jarman et al. (2004) comment, designing primers to amplify a plastid gene is basically designing group-specific primers to target an entire kingdom. Short fragments of the rbcL gene (~157 bp: Hösß et al. 1992) and the P6 loop of the trnL (UAA) intron (10–143 bp, Taberlet et al., 2007) are sensitive enough to generate amplicons from not only faeces but also ancient coprolites that are as much as 28 500 years old (Hofreiter et al., 2000; Poinar et al., 1998). The P6 loop has been used in conjunction with a massively parallel sequencing approach to yield 97 737 amplicons (Valentini et al., 2009a). Despite being a short fragment (20–85 bp long), the amplicons had sufficient phenetic information to resolve 50% of amplicons to species, 45% to genus and the remainder to family. This use of the P6 loop with massively parallel sequencing has continued with similar success (Kowalczyk et al., 2011; Soininen et al., 2009), indicating that DNA diet studies of terrestrial herbivores have transitioned from pioneering to fully developed methodologies. It is more problematic when a predator is more closely related to the prey they consume.

With a region of DNA that is hyper-variable across taxa, it is relatively straightforward to find a DNA sequence that uniquely identifies a predator. It is more difficult to find a
unique predator sequence in a region that is highly conserved across taxa (i.e. an autapomorphy). However, if a predator and several of their prey belong to the same taxonomic group, then the only way to exclusively amplify all prey with a single primer set and not amplify the predator would be to locate an autapomorphy. The design of group-specific primers and PCR enrichment methods looks for unique predator sequences in hyper-variable regions, whereas a single primer approach, that sets out to discover autapomorphies, is potentially a significant bioinformatic task. Also, the analysis would also need to be performed anew for every predator studied. In one study, such a pseudo-universal primer set has been used to amplify amphipods, krill, bony fishes and squid, while excluding the predator, which was penguin (Deagle et al., 2007). Their primer set did not amplify penguins because of a 3’ T that was not present in birds but was present across the targeted prey taxa (Deagle et al., 2007). Although the primer set did not amplify all taxa with equal efficiency, it did establish this approach as a plausible option. Another option is to design multiple group-specific primers to amplify taxonomic groups excluding the predator (Jarman et al., 2004). These primers can be used in parallel single reactions (Deagle & Tollit, 2007; Jarman et al., 2006; Marshall et al., 2010) or in multiplex reactions (e.g., Greenstone et al., 2005; Harper et al., 2005). By multiplexing, it is possible to have comprehensive taxon coverage, and as many as 34 primer pairs have been used to represent 53 prey families (Corse et al., 2010).

2.3 Universal primer approaches

2.3.1 Fingerprinting
Universal primers have an advantage over group-specific primers for studying uncharacterised and generalist predators because the target does not need to be known a priori for them to be effective. The problem with using universal primers is that predator
amplicons will normally dominate, so that for every prey amplicon there will be potentially thousands of predator amplicons generated. Some diet studies have solved this by following PCR or cloning with an amplicon fingerprinting step that assorts target and non-target sequences by denaturing or temperature gradient gel electrophoresis (DGGE, TGGE) and restriction fragment length polymorphisms (King et al., 2008; RFLP, Symondson, 2002). A range of gene targets, mostly 18S, have been screened by a finger printing method (refer Table 2.1). In addition to RFLP, DGGE and TGGE, a novel method of screening has been used to ascertain the animal portion of an ancient human diet using universal primers to amplify DNA extracted from human coprolites, followed by cloning into a vector for sequencing (Poinar et al., 2001). A second PCR with a human-specific primer informed the researcher whether clones contained host amplicons so they were not sequenced as potential prey.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Predator</th>
<th>Universality</th>
<th>Approach</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>Bivalve</td>
<td>Eukaryote</td>
<td>RFLP</td>
<td>(Duplessis et al., 2004)</td>
</tr>
<tr>
<td>18S</td>
<td>Lobster larvae</td>
<td>Eukaryote</td>
<td>RFLP</td>
<td>(Suzuki et al., 2006)</td>
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<tr>
<td>18S</td>
<td>Lobster larvae</td>
<td>Eukaryote</td>
<td>RFLP</td>
<td>(Suzuki et al., 2008)</td>
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<tr>
<td>18S</td>
<td>Krill</td>
<td>Eukaryote</td>
<td>DGGE</td>
<td>(Martin et al., 2006)</td>
</tr>
<tr>
<td>18S</td>
<td>Eel larvae</td>
<td>Eukaryote</td>
<td>DGGE</td>
<td>(Riemann et al., 2010)</td>
</tr>
<tr>
<td>18S</td>
<td>Bivalves</td>
<td>Eukaryote</td>
<td>DGGE</td>
<td>(Maloy et al., 2009)</td>
</tr>
<tr>
<td>16S</td>
<td>Sea lion</td>
<td>Cephalopoda and teleostii</td>
<td>DGGE</td>
<td>(Deagle et al., 2005b)</td>
</tr>
<tr>
<td>16S</td>
<td>Giant squid</td>
<td>Metazoan (Cephalopoda, teleostii and crustacea)</td>
<td>DGGE</td>
<td>(Deagle et al., 2005a)</td>
</tr>
<tr>
<td>16S</td>
<td>Sea lion</td>
<td>Cephalopoda, teleostii and crustacea</td>
<td>DGGE</td>
<td>(Tollit et al., 2009)2</td>
</tr>
<tr>
<td>COI3</td>
<td>Beetle</td>
<td>Invertebrate (Insect)</td>
<td>TGGE</td>
<td>(Harper et al., 2006)</td>
</tr>
</tbody>
</table>

1 Duplessis et al. (*Duplessis:2004uf) also use 16S universal primers to amplify bacterial DNA.
2 These studies used a nested PCR approach, first amplifying with a universal primer set and then a group specific one.
3 Harper et al. (2006) also use TGGE with 12S primers to earthworms.

Screening amplicons after PCR with RFLP, DGGE or TGGE enables universal primers to be used in diet studies, but it is a labour-intensive approach; therefore, it ultimately narrows the sample size examined. Screening methods also carry the risk that target
amplicons might share the same genotypic profile as the predator, for example, the same RFLP band size. This means that target amplicons might be excluded erroneously from the diet study. These shortcomings have led to recent trials of PCR enrichment methods in diet studies.

2.3.2 Restriction as an enrichment strategy

The method of using restriction enzymes discussed in this section differs from the use described in the previous section. It involves physically removing the non-target amplicons from the workflow rather than visualising amplicons so that the non-target can be avoided. There are a few DNA diet studies that cite restriction as a prey enrichment method to be used with universal primers (Blankenship & Levin, 2007; Blankenship & Yayanos, 2005; Dunshea, 2009; Dunshea et al., 2008). Restriction enzymes can be used to cut non-target amplicons near their centre, reducing them into two short fragments that are removable by electrophoresis or another size selection method (Blankenship & Levin, 2007; Blankenship & Yayanos, 2005). In contrast, genomic DNA can be digested prior to PCR, thereby removing the non-target template (Dunshea, 2009). Additional post-PCR restriction can further reduce the number of non-target amplicons (Dunshea, 2009). While the success of these approaches has been attributed to the restricted fragments being removed by electrophoresis or incapacitated so they do not contribute to PCR, it is important to note that a TA cloning system was used in all studies. This is presumably significant because the restricted predator fragments cannot be incorporated into the clone vector because the restriction enzymes that were used do not provide an A overhang and cannot be utilised in TA cloning (Blankenship & Levin, 2007; Blankenship & Yayanos, 2005; Dunshea, 2009).
Despite its successful application to diet studies, there are potential problems with restriction. Commercially available restriction enzymes have varying efficiencies (Parsons & Heflich, 1997), so there is the danger that a critical amount of predator DNA will not be cut. This problem could be overcome by careful enzyme selection, and to this end, less efficient or poorly targeted enzymes should be avoided. Another possible solution comes from clinical studies that have observed that using thermostable restriction enzymes can improve efficiency (Sandy et al., 1992). The rationale is that thermostable enzymes survive the melting step of the PCR and therefore continue to remove predator template throughout the cycles of a PCR.

More troubling for the use of restriction enzymes in PCR enrichment is that enzyme recognition sequences are generally short (<6 bp) and therefore occur frequently throughout genomes. This means there is the danger that the nuclease restriction site might occur in polymorphic regions of the targeted prey as well as their unwanted orthologs and that the restriction enzyme might accidentally remove target sequences. To minimise the chances of removing a target, a ‘rare cutter’ can be used, which is an enzyme with a recognition sequence of 8 bp or more (Dunshea, 2009). However, there are relatively few commercially available rare cutters, so it is a matter of luck if a rare cutter is available that can be used to discriminate sequences of interest. An alternative is to use at least two of the shorter, more readily available enzymes in separate reactions and combine the results (Blankenship & Yayanos, 2005). However, performing digests in parallel creates additional work and increases the risk of contamination with extraneous DNA. Alternatively, artificial restriction cutters could be designed such as zinc finger nucleases (Porteus & Carroll, 2005) or, more economically, artificial restriction cutters (ARCUT, Yamamoto et al., 2007). Restriction-based enrichment differs from the ‘one-tube’ methods (discussed below) because enrichment and amplification are independent reactions. The advantage of this independence is that both
PCR and enrichment can be independently optimised to maximise the efficiency of both (Dunshea, 2009).

### 2.3.3 ‘One-tube’ enrichment

‘One-tube’ enrichment involves a non-priming oligonucleotide being added to the PCR to specifically prevent the amplification of unwanted DNA sequences. This non-priming oligonucleotide is homologous to the predator so that when it binds it prevents polymerisation of the predator DNA but allows polymerisation of target DNA. Blocking primers are DNA oligonucleotides with the 3’ end modified so that they will not prime amplification. They have been used successfully on both invertebrate and vertebrate predators such as krill (Vestheim & Jarman, 2008), fur seals (Deagle et al., 2009) and penguins (Deagle et al., 2010). Another alternative has been to use a PNA clamp (Ørum et al. 1993). PNA does not prime polymerisation, which in combination with its high affinity for DNA makes it an ideal candidate for specific PCR suppression. In diet studies, PNA has been used to ascertain the diet of lobster larvae (Chow et al., 2010).

Blocking primers are DNA oligonucleotides with the 3’ end modified so that they will not prime amplification. These oligonucleotides can be made from conventional nucleic acids but have a 3’ modification to prevent polymerisation. Such modification has included a dideoxy (ddNTP) terminal base as is used in Sanger sequencing (Parsons, McKinzie, & Heflich, 2005). More commonly used terminals have been phosphorylation (Hu et al., 2009; Ren et al., 2009; Senescau et al., 2005; Thiede et al., 2006) or the ligation of a C3 linker (Laughlin et al., 2008; Vestheim & Jarman, 2008). These DNA blockers very often incorporate a few locked nucleic acid (LNA) nucleotides to increase sensitivity and increase the melting temperature (Tm) of the
blocker above the primer Tm. The LNA molecule imparts these properties because it is an RNA, but with the sugar cross-linked between the 2’ oxygen and the 4’ carbon, which gives the oligonucleotide an ‘open’ conformation that favours hybridisation.

The majority of clinical studies using ‘one-tube’ enrichments use a clamp constructed of peptide nucleic acid (PNA). PNA is an RNA analogue, but with the phosphodiester backbone replaced by an N-(2-amino-ethyl)-glycine backbone (Egholm, Buchardt, Nielsen, & Berg, 1992). Because PNA is neutrally charged, the PNA–DNA base pairs have high thermostability. PNA clamps are ideal for suppressing PCR because they do not prime polymerisation, which in combination with its high affinity for DNA makes it an ideal candidate for specific PCR suppression (Ørum et al., 1993). Mismatched base pair-ing between PNA and DNA is thermodynamically disfavoured (Igloi, 1998), much more so than DNA duplex mis-matches. This makes PNA clamping highly specific, which is generally an advantage for ‘one-tube’ enrichment, although if there is high intra-specific variation in the enriched gene it might be advisable to use DNA blockers to allow some blocker mismatches so that the entirety of the predator amplicons can be suppressed.
A. Primer Exclusion

Stylised representation of two different approaches to 'one-tube' enrichments. In primer exclusion (A) the clamp/blocker (red) does not anneal to the prey DNA so the prey is amplified. The clamp/blocker does bind to the predator, which prevents primer binding and stops the polymerisation of predator amplicons. In elongation arrest (B) prey DNA is amplified. The primer binds to the predator DNA template but because the clamp/blocker binds downstream polymerisation is stopped.

B. Elongation Arrest

Figure 2-1 'One Tube' Enrichments.
Stylised representation of two different approaches to 'one-tube' enrichments. In primer exclusion (A) the clamp/blocker (red) does not anneal to the prey DNA so the prey is amplified. The clamp/blocker does bind to the predator, which prevents primer binding and stops the polymerisation of predator amplicons. In elongation arrest (B) prey DNA is amplified. The primer binds to the predator DNA template but because the clamp/blocker binds downstream polymerisation is stopped.

There are two common approaches to designing PNA clamping or blocking primer reactions: ‘primer exclusion’ and ‘elongation arrest’ (Vestheim & Jarman, 2008; refer Fig. 2.1, Ørum et al., 1993). In primer exclusion, the clamp/blocker prevents the annealing of either the forward or reverse primer of the unwanted (wild-type/predator) strand because it anneals to a region that overlaps with the 3’ end of the primer-binding site. The result is that polymerisation cannot be initiated in the direction of the excluded primer. In elongation arrest, the clamp/blocker anneals to any region between the two primer sites on the unwanted DNA strand. Therefore, the function of the clamp is to impede the polymerisation of the non-target DNA in one direction, preventing the
synthesis of the complementary primer-binding site so that the incomplete strand cannot act as a template in the reverse direction.

2.3.4 Primer exclusion

All published diet studies that have used ‘one-tube’ enrichments successfully have designed the blocker/clamp to partially overlap with a primer-binding site, an approach called primer exclusion (Chow et al., 2010; Deagle et al., 2009; 2010; Vestheim & Jarman, 2008). Other blocking strategies were trialled in one diet study (Vestheim & Jarman, 2008), but these were unsuccessful. Primer exclusion was first used in a ‘one-tube’ reaction to enrich codon-12 mutations in K-ras (Thiede et al., 1996). This was achieved with a PNA clamp and yielded sensitivities of $1 \times 2 \cdot 10^2$. Later, the same PCR primers and PNA clamp achieved a sensitivity of detection $> 1 \times 1 \cdot 10^3$ by being used in conjunction with a restriction digest (Behn et al., 2000). The restriction step was possibly an unnecessary complication, as sensitivities of $1 \times 2 \cdot 10^3$ were later achieved using a PNA clamp alone (Taback et al., 2004). It was also found that while conventional Taq polymerase achieved sensitivities of $1 \times 2 \cdot 10^3$, that this could be improved to $1 \times 2 \cdot 10^4$ by instead using a polymerase with 3’ $\rightarrow$ 5’ exonuclease activity (Gilje et al., 2008). The lengthening of the PNA with two N-terminal C residues was probably another factor in this improved sensitivity, because it would have increased the Tm of the clamp (Gilje et al., 2008). With PNA clamps, it has been found that it is useful to have two annealing steps, one at a higher temperature that the clamp binds at and another step at a lower for primers to bind to the target sequence (Ørum et al., 1993).
There are several limitations in applying primer exclusion enrichments to diet studies. They arise from the constraint that the blocker or clamp can sit in only one of the two locations that overlap with the primer-binding sites. This means there is a fairly good chance that these two small regions will be unsuitable for the design of blocking/clamping oligonucleotides. For example, these regions might contain nucleotide sequences that complement the primers, are self-complementary or have extremely low or high GC content. With PNA, there is also a restriction on the number of purine repeats (Thiede et al., 1996), which make it an even more difficult molecule to accommodate. For example, a highly self-complementary PNA clamp with purine repeats had to be used when isolating prey DNA from oceanic larvae of spiny lobster (Chow et al., 2010), which potentially limited its success. Several strategies have been employed to minimise the difficulties associated with the design of primer exclusion assays, and these are discussed in the later section.

Another problem for using primer exclusion in PCR diet studies is that it requires that the sequence immediately adjacent to the primer-binding site be sufficiently variable to uniquely define the predator. This is a problem because massive alignments of diverse taxa indicate that there are few regions where highly conserved domains are immediately adjacent to regions with high entropy, rather there is gradual erosion from the conserved to the variable. This is most apparent in ribosomal gene alignments, which are the genes used most routinely in diet studies (Ali et al., 1999). Therefore, a conserved primer set designed to amplify all metazoans or all eukaryotes could end up located far from variable regions suited to exclusion blocking/clamping.

Despite the difficulty of applying primer exclusion to the PCR of a gene fragment, the technique has been used for diet studies. Adapting it can be achieved by conceding
complete universality, and this might not be a problem for some biological questions. For example, the diet of fur seals is known to consist largely of chordates and cephalopods, so to discover whether the prey of seals were benthic or pelagic animals, a study restricted their primer set to target mostly chordates and cephalopods (Deagle et al., 2009). Given that the seals are also chordates, the study used a blocking primer, but by limiting their target prey to two phyla, it was possible to find a conserved region adjacent to a variable region that could be exploited for primer exclusion. The same study investigated the possibility of other metazoans being included in the prey, but the investigation was constrained to using a short portion of the highly conserved 18S rRNA gene, which offers no possibility for primer exclusion. Instead of moving primer-binding sites nearer to variable regions that suit blocking, the blocker can be moved nearer the universal primer-binding site. The advantage of this approach is that the universality of the primers is not compromised, but the negative consequence is that the blocker can non-specifically inhibit the PCR of species other than the predator. For example, a blocker trialled for a krill diet study was found to match some species of decapod, but this consequence was not important to the study because those species of decapod were not listed as of interest to the study, which otherwise targeted a diverse range of taxa (Vestheim & Jarman, 2008). An alternative to targeting fewer taxa is to target intergenic regions instead of intragenic regions. In this way, a study can place universal primers on conserved genes that are immediately adjacent to a hyper-variable region that is suitable for clamp/blocker annealing. For example, a diet study has targeted the intergenic region between the 18S rDNA and the 5.8S rDNA (ITS1) for PNA clamping (Chow et al., 2010). The disadvantage with intergenic regions is that they can have massive variation in length and nucleotide composition, which can affect both PCR efficiency and the possibility of species identification (Chow et al., 2010; Chow et al., 2009). Another solution is to make a DNA blocker long enough to stretch
from the conserved to the variable region. A dual priming oligonucleotide (DPO, Chun et al., 2007) with a 3’ C3 blocker was used to successfully enrich prey DNA extracted from Antarctic krill stomachs (Vestheim & Jarman, 2008). The DPO is a long primer punctuated by an inosine bridge that forces the primer to have low affinity to mismatched DNA orthologues (Chun et al., 2007). There are therefore ways in which primer exclusion can be made to work for diet studies, but generally it does not appear to be the optimal strategy for designing a universal prey detection system for generalist or uncharacterised predators. For this, elongation arrest might be a better strategy.

### 2.3.5 Elongation arrest

For elongation arrest, the clamp/blocker anneals between the two PCR primers and prevents amplification by impeding polymerisation (see Fig. 2.1). The method was not initially favoured as a PCR clamping technique because it did not perform as well as primer exclusion in both single nucleotide polymorphism studies (Ørum et al., 1993) and in ecological studies (Wintzingerode et al., 1997). However, recently, elongation arrest has been further developed, has been reported to have very good sensitivity and has become a favoured method in clinical diagnostics (Miyazawa et al., 2008). Elongation arrest of PCR failed to work with DNA blockers in the context of diet studies (Vestheim & Jarman, 2008), but it has been used with PNA clamps to detect parasites of crustaceans (Troedsson et al., 2008).

Although it has been used frequently over the past 3–4 years, elongation arrest was at one point considered an unreliable method for suppressing non-target amplicons. There is even an early study that designed a system to enhance the synthesis of a strand by designing PNA clamps to bind down its length (Demers et al., 1995); that is, it used the
methodology of elongation arrest to achieve the opposite goal. This study emphasises
that when using elongation arrest it is important to optimise the antecedent conditions;
otherwise, the enrichment not only will fail to suppress PCR of unwanted amplicons but
will fail spectacularly.

Through trial and error, it was found that there are three important PCR parameters for
optimising elongation arrest with PNA clamping. The first of these is the relationship
between the clamp/blocker Tm and primer Tm. For example, with K-ras enrichment,
the main difference between the early poor attempts at exclusion and contemporary
successful ones is that the latter use a longer PNA clamp that has a higher Tm. To
enable PNA clamping of EGFR, it was found that dropping the PCR extension
temperature from 72 to 60 °C made elongation arrest of the wild type possible (Luo et
al., 2006). At 72 and 65 °C, the wild type entirely dominated the amplicons, which
indicates the importance of Tm in affecting arrest. The only published diet study to use
DNA blockers for arrest found it unsuccessful (Vestheim & Jarman, 2008). Substituting
LNA monomers into a blocker, which increases the blocker Tm, does enable
enrichment (Laughlin et al., 2008). The blocker used in this study had the 5’ end
modified on the assumption that this would pre- vent the 5 → 3’ proofreading from
digesting away the blocker (Laughlin et al., 2008). However, it was later found that an
LNA blocker with no 5’ modification can affect elongation arrest with sensitivities of
$1.3 \times 10^3$, so the success is almost certainly due to the benefits of LNA increasing Tm
(Ren et al., 2009). The utility of increasing Tm of PNA–DNA binding can be enhanced
by adding positively charged molecules to the N or C termini of the PNA
oligonucleotide (e.g. lysine, Ørum et al., 1993) or by introducing cationic PNA
monomers into the oligonucleotide (Abibi et al., 2004; Dragulescu-Andrasi et al., 2006;
Rapireddy et al., 2007). The other parameter that governs the sensitivity of elongation arrest PCR enrichments is the run-off distance from the PNA to the complementary reverse primer-binding site (Chiou et al., 2007; Luo et al., 2006). A minimum of 50 nt have been suggested as the minimum run-off length for successful elongation arrest (Chiou et al., 2007), and another found that extending run-off from 28 nt to 97 nt dramatically increased enrichment sensitivity (Luo et al., 2006). Fast ramping between PCR cycles has also been found to improve the sensitivity of enrichments (Chiou et al., 2007; Luo et al., 2006).

### 2.3.6 Blocking oligos and clamps

Purchasing 100 nmol of a high-pressure liquid chromatography (HPLC)-purified 25 nucleotide (25 mer) C3 blocker will cost a little over $100 US with one C3 blocker or $145 US if there is a blocker at either end (American price, November 2010). If a shorter blocker is required (to avoid PCR artefacts or to target a short sequence similar to that targeted by PNA clamps), then they can be synthesised with strategically substituted locked nucleic acid (LNA) bases. Each additional LNA base adds approximately $40 US to the cost. An HPLC-purified PNA 14 mer will cost $520US, and the addition of lysine increases the cost to $550. DNA blockers can be synthesised and shipped within a few working days (in my experience orders occasionally take more than 14 days to dispatch, but this is infrequent). PNA will generally take 3 weeks to ship. Because there is only one company that holds the international patent for synthesising LNA, it means that demand can substantially increase wait times. However, on enquiry, the customer is supplied with a realistic estimate of synthesis time, which is reassuring when planning experiments involving LNA. These costs and time frames are for oligonucleotides purchased in late 2010 and early 2011, and global competition and market values will likely see these changes.
Before being purchased, blockers and clamps should be tested in silico to ensure that they have optimal characteristics for hybridisation and that they do not form secondary structures or heteroduplex with primers. Conventional primer design software can be used to design DNA blockers (Vestheim et al., 2011 offer advice for this), but modified nucleotides have additional stringencies; useful websites for testing PNA and LNA oligonucleotides are [http://www6.appliedbiosystems.com/support/pnadesigner.cfm](http://www6.appliedbiosystems.com/support/pnadesigner.cfm) and [http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/default.aspx](http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/default.aspx). The companies that synthesise the latter oligonucleotides are also very helpful.

### 2.3.7 Dissection

Universal primers have been used without PCR enrichment methods to study the contents of shark regurgitate (Barnett et al., 2010; Dunn et al., 2010) and pieces of insects in red bat faeces (Clare et al., 2009). These predators are megafauna whose stomachs contain large pieces of prey and not just a homogenous digestive chyme, and therefore, enrichment techniques are unnecessary because DNA for PCR can be simply extracted from an isolated piece of prey tissue. This approach seems confined to large predators, because at some point the pieces of prey tissue become too small to be isolated with certainty. However, a recent publication has used LCM to excise prey DNA from folds in the gut of cod larvae (Maloy et al., 2010). LCM was designed to specifically remove cells (or one cell) from the bulk phase of very small tumours, therefore avoiding the problems associated with the co-dissection of wild-type cells (Emmert-Buck et al., 1996). A brief account of LCM is that it excises tissue from thin histology sections by firing a low-power laser at the target region. The excited tissue is captured onto a thin piece of film that is placed over the tissue (Emmert-Buck et al., 1996).
1996). The technique is highly accurate and relatively cheap and has been applied to numerous biological problems (e.g. Bonner 1997; Simone 1998). With cod larvae, the application of LCM to isolate cells of prey tissue from the gut produced excellent results using only universal 18S primers on gDNA (Maloy et al., 2010). The percentage of amplicons derived from cod and not prey ranged from 18% for the shortest amplicon (264 bp), to 48% and 52% for the larger amplicons (378 bp, 626 bp). It is possible to purchase purpose-built LCM platforms from commercial vendors (Applied Biosystems, Zeiss). After this initial outlay, the ongoing operating costs are the replacement of the laser burner (most core facilities charge around $30 US/h), common histological consumables and the adhesive caps used to capture DNA ($6 US). An Internet browser search will also reveal that there are several commercial LCM facilities that will perform LCM for upwards of $20 US per sample. The recent introduction of this technology to molecular diet studies has great potential for the field.

### 2.3.8 Technological limitations and solutions

The public DNA databases are still largely incomplete for many metazoans, even for popular ‘barcoding’ genes (e.g., Bucklin et al., 2010). The incompleteness of databases is problematic for the study of uncharacterised predators because an impoverished database limits the ability to identify unknown prey. More importantly for experimental design, the database is required to test the universality of the primers. Enrichment PCR is even more affected by poor databases because a representative database is required to ensure the specificity of the enrichment. However, as the price of sequencing drops and through concerted sequencing efforts, this problem will gradually dissipate.
Perhaps the greatest problem for studying generalist and uncharacterised predators is finding a suitable ‘barcoding’ gene. Most genes are either too conserved or too variable across the metazoa. Having to work in concert with an enrichment method adds to the complication. Mitochondrial genes, such as cytochrome oxidase I, cytochrome b (Cyt B) and the 16S rDNA, have tended to be favoured in PCR-based diet analyses because they are abundant in cells and relatively variable, permitting a high level of species discrimination (King et al., 2008). However, this variability is problematic for designing universal PCR primers and also results in difficulties in taxonomic identification for taxa poorly represented in the databases. Of studies that are truly universal, the majority use a small portion of the 18S rDNA gene. The 18S gene is reasonably conserved across eukaryotes, and most eukaryotic phyla are represented by 18S in public databases. This makes it possible to identify 18S gene fragments to some level of taxonomic resolution. The disadvantage of 18S, which is the flip side of its being so conserved, is that it generally does not resolve to the level of species. However, when dealing with generalist and uncharacterised predators, the discovery of prey items at any level of taxonomic resolution is a valuable insight Further resolution can be obtained by other subsequent PCRs. Fragment length is also very important, especially in ‘one-tube’ enrichments where there is a tension between the requirements that a fragment that is sufficiently long to perform enrichments (Luo et al., 2006) and provide taxonomic information, and the requirements that fragments be short enough to be detectable after the digestive processes of the predator (Deagle et al., 2009). The problem of finding an appropriate barcoding gene can also be addressed by concerted sequencing efforts and intensive bioinformatic analyses.
Pseudogenes are non-functional copies of genes integrated into the genome. They mutate rapidly and create confusing signals if they are inadvertently sequenced. It is a problem for any diet study if prey pseudogenes are present (Dunshea et al., 2008), but it is a particular problem for PCR enrichment if there are predator pseudogenes because the high mutation rate of pseudogenes means that the enrichment site might not be present and therefore predator pseudogenes might feature in the sample (Chow et al., 2010; e.g., Dunshea et al., 2008). The best way around this is careful gene selection. Another method to avoid pseudogenes is to sequence reverse transcribed RNA (e.g., Machida et al., 2009), but RNA is a molecule with a short half-life and its validity for diet studies needs to be assessed.

The increased sensitivity that enrichment enables increases the possibility of false positives owing to contamination. This contamination need not be an artefact of the laboratory or sampling. The environment is filled with liberated DNA, which can be co-sampled with specimens. A false positive may also arise through secondary predation, where the food of prey has been mistaken for prey species. It might be valuable to assess RNA as an alternative target to DNA because RNA is less stable so it is less likely to be present in the gut of the prey or as free RNA in the environment. Another strategy is to use the relative quantities of different amplicons/clones as an approach to sort out potential prey from contaminants — assuming that the amount of prey DNA in the gut exceeds contaminating DNA. However, although it is tempting to treat the distribution of amplicons as quantitative, this is potentially misleading (Acinas et al., 2005). The best way to minimise any misleading signal from exogenous DNA is to pair the enriched PCR with other quantitative molecular techniques. It is also likely that universal primers will amplify gut symbionts and parasites. This can create confusion,
and it is also best dealt with by supplementing the DNA technique with a non-molecular technique (e.g. stable isotope analyses). False positives can occur in PCR enrichments through the use of low gDNA concentration, high PCR cycle numbers, polymerase error rate and the size of the recognition sequence used by the enrichment method (Jacobs et al., 1999). Therefore, although the sensitivities of $1 \times 10^5$ achieved by the enrichment methods are remarkable, these results may be inflated. It has been estimated that Taq will incorporate an erroneous base for every $9 \times 10^5$ bases it polymerises (Tindall & Kunkel, 1988) and Taq error during enrichment has been estimated as being one base in every $1.76 \times 10^5$ (Ohishi et al., 2004). With enrichments, there is a danger that Taq polymerase will induce errors in the predator amplicons, which will then be unrecognised by the enrichment method, and therefore, the unwanted DNA will proliferate. A PCR negative control containing only predator DNA can determine Taq error. Such negative controls suggest that Taq error can lead to false positives even when the target is outnumbered by only $1 \times 1 \cdot 10^2$ (Luo et al., 2006). A DNA polymerase with 3’ → 5’ exonuclease activity can substantially reduce the replication error rate compared to Taq polymerase (Gilje et al., 2008). This is serendipitous, because many diet studies already use proofreading enzymes because they are more sensitive to the nucleotide damage induced by digestion (Deagle et al., 2005a; Jarman et al., 2002) and because choice of enzyme affects the efficiency of PCR from low copy template (Beja-Pereira et al., 2009). To prevent the accumulation of errors in ‘one-tube’ enrichments, clamps could be made to complement both strands of the PCR template. This has already been practised, though for different purposes than to evade Taq error (Gigli et al., 2009; Hancock et al., 2002). However, the bidirectional use of a clamp v blocker seems necessary only when a high degree of sensitivity is required.
One solution to PCR-induced error is to eliminate PCR. Hybridisation capture systems consist of numerous immobilised probes that are highly specific single-stranded oligonucleotides. These capture systems can be used in concert with next-generation sequencing technology to eliminate PCR from a sequencing workflow (see, Bau et al., 2008; Lee et al., 2009). This results in tens or hundreds of thousands of sequences of not only the probed DNA region but also the regions of the captured DNA strand that flank the probed region. For an ecology study, it is possible that an array of probes could target various metazoan groups and could be designed with the software used to design group-specific primers. The regions of DNA that flank the probed region would then provide sufficient genetic information to identify the isolated DNA. Capture arrays have been used to enrich ancient DNA (Burbano et al., 2010), and this is a good indicator that the method will be applied to DNA diet studies.

Hybridisation capture systems can be commercially synthesised so are simple to implement, but despite the fact that their cost is dropping, they are still an expensive option. If PCR is not eliminated, then it might be beneficial to reduce PCR cycle number through technologies that increase the relative quantity of target (e.g. LCM). Also, enrichment techniques such as clamping, blocking and restriction give good results so long as a sufficient ‘predator-only’ negative control is used.

### 2.3.9 PCR enrichment in the context of diet studies

There are numerous methods that have been applied to studies of trophic interactions. Observing the behaviour of predators in situ and in laboratory scale, feeding trials are fairly fundamental approaches, but not always practical. One approach to situate a predator in a broad trophic context is to match the chemical composition of the predator
(e.g. ratio of stable isotopes or lipids) to the chemical composition of various prey and environmental sources. However, there are three methods that are unique because they can identify dietary items with greater taxonomic precision; these are DNA techniques, serological techniques and hard-part analysis. Each of these methods has particular advantages and limitations. The main advantage of using universal PCR primers for DNA amplification is that they can identify a very wide range of prey but also give highly specific information about the prey species. Of course, this advantage lacks power without an effective enrichment strategy to prevent the predator homolog swamping the amplicon assemblage. Alternatively, a species/group-specific PCR approach can be used, but at the risk of overlooking unanticipated diet items. It is generally true that serological techniques have now been superseded by PCR techniques because they take considerably longer to optimise than PCR techniques (Carreon-Martinez & Heath, 2010; Symondson et al., 2002). However, the high specificity of serological techniques can also make them highly informative, especially when used in conjunction with a prey-enriched universal PCR approach. For example, a PCR approach might establish that bony fish are a part of a marine predator’s diet. However, DNA cannot determine whether this predator consumes the adult fish, its larvae or eggs. Monoclonal antibodies can be raised against antigens present at a particular life stage, or tissue type, and can therefore be particularly informative as to the life stage of the prey (see, Symondson, 2002). Hard-part analysis has been used to identify prey remnants for many years and has been successfully applied to samples as small as copepod faeces (e.g., Turner, 1985). One drawback with hard-part analyses is that it can over-represent dietary components owing to differences in gut residence times and digestibility for hard parts (Bowen, 2000; Tollit et al., 2006; Tollit et al., 2003). Hard-part analysis is also time-consuming and a highly specialised skill. However, hard-part analysis can be used to help validate that a PCR approach is not missing any prey
species or to calibrate quantitative DNA methods. For example, a study on the diet of the arrow squid, a generalist predator, found that using DNA techniques and hard-part analysis in combination was more effective than using either method alone (Braley et al., 2010). They detected nineteen unique prey taxa, but only six of these were identified by both methods. Ten taxa were only detected using DNA techniques, and the other three were only detected by a morphological approach (Braley et al., 2010).

Integrating the data derived from prey-enriched PCR methods with other diet-study methods can enable highly supported hypotheses for trophic interactions involving generalist and uncharacterised predators. What techniques can be applied to any study is naturally constrained by the nature of the predator being studied. For example, the habitat of an organism will determine whether observing feeding behaviour is feasible or the digestive structures of an organism (e.g. if it has a specialized gut) will determine how easily samples can be dissected out for molecular analysis. Determining which techniques are suited to studying different clades of predators can involve synthesising a massive number of studies, but reviews of the applicability of techniques to specific predators are available, for example, for seabirds (Barrett et al., 2007) and lobster larvae (Jeffs, 2007).

2.4 Applying enrichment to diet studies: recommendations and conclusions

Of the enrichment methods discussed, the reagents of for restriction digests, DNA blocking and PNA clamping are readily available, at reasonable cost, and do not require any special development to be incorporated into even a rudimentary molecular ecology laboratory. While LCM is not standard laboratory apparatus, its utility means it is
typically a part of the core facilities of larger research institutes. These techniques have been established in the clinical research for at least 15 years. Because of this long period of clinical application, there is a wealth of informative literature on how to optimise these techniques.

Next-generation sequencing technologies have the potential to enhance PCR-based studies’ ability to represent the dietary component of gut/faecal contents. These technologies have enabled deep sequencing coverage at a drastically reduced cost. Diet studies have already benefitted from the application of second-generation sequencing technologies (Deagle et al., 2009; e.g., Valentini et al., 2009b). More recently, there have been two developments in the next-generation technologies that will enable molecular ecology laboratories to access this technology, even if constrained by modest budgets. Small, low-volume platforms have been developed such as the Roche GS Junior (http://www.gsjunior.com) and Illumina’s MiSeq Personal Sequencing System (http://www.illumina.com/systems/miseq.ilmn), and third-generation sequencing technologies such as the Ion Torrent (http://www.iontorrent.com, Rothberg et al., 2011) have come onto the marketplace. These third-generation technologies are characterised by non-optical detection systems that greatly reduce reagent costs. These developments will enable molecular ecology laboratories with modest budgets to obtain a depth of coverage that will enable them to perform crucial meta-analysis of the efficiency of PCR enrichment and assess the contribution of contaminants to data sets. The massive numbers of sequences produced by these sequencers reduce the negative impact of non-target amplicons that are caused by ‘leaky’ enrichment methods, resulting in gut symbionts and DNA contaminants being incidentally co-amplified with genuine diet items.
It could be anticipated that the very recent application of enrichment technologies to study trophic ecology will enable the rapid resolution of questions about what constitutes the diet of any predator. Enrichment techniques will therefore enable molecular studies of organisms that prey on closely related organisms to be conducted with an efficiency and sensitivity similar to that attained by molecular studies of trophic between unrelated organisms, such as herbivory. The study of herbivory still has some advantages over the study of animals that predate upon animals. For example, a study can simultaneously determine the diet of several herbivores using the same universal primer set (e.g., Valentini et al., 2009b), but a study of several predators would require developing several corresponding enrichment techniques, which is potentially expensive. Future research needs to be performed on creating enrichment techniques that are readily transferable between predators. These could come from new technologies, for example capture arrays, but could also come from reinvestigating enrichment methods that have been proposed for clinical screening but have not been adopted. For example ‘headloop’ PCR is a primer mediated technique for enriching target amplicons by inducing hairpins in non-target amplicons (Rand et al., 2005). This technique could be a cheap and quick way to enrich amplicons and therefore would be suited to designing enrichments for various predators. This is not an endorsement of ‘headloop’ PCR, as more research needs to be performed on the technique, but it is an example of the value of investigating other proposed enrichment techniques for ecological applications.

The least tractable biological questions are ones about the relative proportions of prey that constitute the diet of predators. This is a general problem faced by molecular
ecology, largely because PCR does not necessarily preserve the composition of environmental samples (e.g., Acinas et al., 2005), and future research is required to demonstrate whether quantitative data from environmental samples are reliable. Sequencing directly from hybridisation capture arrays is another potential solution to this problem, so long as the array is not saturated. This is because it removes the potential biases introduced through PCR. However, if the enrichment techniques discussed in the present review are only able to establish the presence/absence of potential prey in the diets of uncharacterised predators, it is a significant step forward in understanding the ecology of predation.
3 Extracting DNA from whole organism homogenates and the risk of false positives in PCR based diet studies; a case study using spiny lobster larvae.

3.1 Introduction

The application of the polymerase chain reaction (PCR) to DNA extracted from predator faeces and gut contents has become an established technique to determine dietary composition (Beja-Pereira et al., 2009). However, because PCR is a very sensitive technique, the detection of prey using a PCR diet approach is subject to the possibility that amplified molecules are exogenous contaminants and not valid dietary items (King et al., 2008). DNA contamination that originates in the laboratory can usually be detected using the appropriate negative controls. However, when dealing with predators sampled from the wild, the exterior of the predator is a source of potential contamination and this contaminating DNA might resemble legitimate prey leading to false positives (Greenstone et al., 2012; King et al., 2012). With larger predators it is possible to dissect the gut out and therefore minimise exogenous contamination. This is more difficult to do with small predators that are in continuous contact with their environment, such as insects and marine organisms. The risk of false positives is compounded when many studies of diet in insects and marine larvae have relied on DNA analyses of homogenates of the entire predator because of difficulty accessing or isolating the gut content (Durbin et al., 2008; Meyer & Hoy, 2008; Simonelli et al., 2009).
The problem of exogenous DNA contamination in terrestrial predators has been addressed by treating the outside of the predator with bleach. Fly maggots have been bathed in 20% chlorine bleach solution (Linville & Wells, 2002). Sap feeding arthropods can be soaked in bleach, which has been sufficient to remove exogenous fungal DNA and enable the detection of endosymbionts (Meyer & Hoy, 2008). However, aquatic animals, even those with carapaces, are more permeable than terrestrial animals (Haond et al., 2001; Rodriguez Souza et al., 1999), so the application of soluble bleach presents a much greater risk of sample degradation. In the present study spiny lobster larvae are used to evaluate the potential for exogenous contamination in small aquatic predators, and develop a novel method to overcome this problem.

The larvae of spiny lobsters hatch from eggs of around 1.5-2 mm diameter and subsequently grow to greater than 30 mm over a period of up to 24 months, during which time they are offshore in the pelagic marine environment (e.g., Chittleborough & Thomas, 1969; Dennis et al., 2001; Inoue, 1978; Lesser, 1978; Lewis, 1951 and reviewed in Phillips et al., 2006a). The larvae are known predators of zooplankton but little is understood about the specific composition of their prey because of difficulties in observing feeding in their oceanic habitat (Jeffs, 2007). Considerable attention has focused on using molecular methods to investigate the diet of these and other marine larvae, due to intense commercial interest in developing new methods for the closed-cycle aquaculture of these valuable species (Chow et al., 2010; Jeffs, 2010; O’Rorke et al., 2012b; Suzuki et al., 2006; 2007; 2008). Spiny lobster larvae are known as phyllosomas (i.e., “leaf-like” body), due to their unusually flattened body morphology (Phillips et al., 2006a). As a consequence, it is particularly difficult to dissect away the gut in spiny lobster larvae because their digestive system consists of a series of fine
diverticulae dispersed throughout a very flat disk-shaped cephalothorax with little mesoderm between the gut and permeable exoskeleton (Rodriguez Souza et al., 1999). A common solution to this problem is to forego dissection and homogenise the whole predator (Chen et al., 2000; Fournier et al., 2008; Juen & Traugott, 2005) or a segment of the tissue that encloses the gut (Chow et al., 2010). The advantage of this approach is that it captures the entire gut contents, however, there are also several limitations. For example, it results in significant dilution of the targeted prey DNA with predator (larval) DNA (O'Rorke et al., 2012a), and there is a greater potential for co-extraction of PCR inhibitors. Perhaps more importantly, DNA on the predator’s exterior will be co-extracted and co-amplified with DNA from their gut, and as such an experimental control must be devised to identify this form of contamination.

It has been identified that exogenous contamination is particularly problematic for aquatic predators, because sampling methods such as netting will inevitably bring a wide range of organisms into contact with one another (King et al., 2008). Studies that have homogenised their samples have adopted several approaches to deal with the potential for exogenous contamination. One approach is to trust that DNA contained in the gut exceeds exogenous DNA sources and not use any kind of cleaning, an approach that has been used in studies of both aquatic (Durbin et al., 2008; Simonelli et al., 2009) and terrestrial predators (Fournier et al., 2008; Hoogendoorn & Heimpel, 2001). Other planktonic studies have used highly purified water or sea water to wash their samples before homogenisation, but have not controlled for the efficacy of washing (Schielke et al., 2007; Troedsson et al., 2009). Studies that have controlled for washing efficacy have done so by inspecting the predator by microscope for signs of prey (Oberholster et al., 2006) or using a final wash of water as a PCR template (Suzuki et al., 2006). In the present study a subsample of the final wash was tested to establish if it can act as a
reliable negative control for exogenous DNA contamination. The feasibility of an alternative approach of minimising exogenous DNA contamination by using syringe extraction of gut contents and small swabs to obtain DNA from the larval interior was also determined.

3.2 Material and Methods

3.2.1 Samples
Sagmariasus verreauxi larvae were of developmental instars 15 - 17 (Kittaka, 1997a) and were cultured at the Institute for Marine and Antarctic Studies, Marine Research Laboratories (IMAS-MRL), Hobart, Australia (for details of culture method see, Fitzgibbon & Battaglene, 2012a; 2012b). Isolated individual larvae were starved for 24 h before being offered 5 mm pieces of freshly shucked gonad tissue of Mytilus galloprovincialis. Larvae were allowed to feed for 30-60 min, during which time all larvae were visually confirmed as feeding. Larvae were then sampled and total body length, developmental instar and food contents in the midgut gland were assessed using a profile projector. Food content within the gut was visible through the transparent exoskeleton (Vestheim & Kaartvedt, 2009) and individual larva were assigned a mid-gut score from 0 - 3 based on of the overall estimated quantity of food material in the midgut. A score of 0 is no visible prey and 3 is a high level of visible prey. Larvae were immediately euthanised by being placed in chilled 70% EtOH, in which they were stored at 4 °C and later at -20 °C.

Eight larvae were used in the experiment (Table 3.1). Before dissection, each larva was washed in a total of 2 L of MilliQ filtered (>16 µΩ, Millipore, Billerica, MA) and autoclaved H₂O, which was applied by a squirting action using a 60 ml syringe. The final 2 ml of wash water was retained as a negative extraction control. Each individual
larva was placed on a new sterile, disposable plastic surface and the exterior rubbed with a sterile flocked swab (Copan, Murrieta, CA) to obtain surface DNA contaminants. The contents of the hepatopancreas were then removed by syringing (see below). A piece of hepatopancreas (~3 mm²) was also excised by sterile scalpel, homogenised by mashing with a disposable scalpel blade and transferred to a sterile eppendorf tube. Finally, a sterile flocked-miniswab was used to remove DNA from the interior of the hepatopancreas.

Table 3-1 Larvae used in this study. Length refers to the distance from the anterior margin of the cephalic shield (cephalothorax) from between the eyestalks to the posterior tip of the pleon.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Length (mm)</th>
<th>Instar</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>26.9</td>
<td>16</td>
</tr>
<tr>
<td>B</td>
<td>27.0</td>
<td>16</td>
</tr>
<tr>
<td>C</td>
<td>26.5</td>
<td>15</td>
</tr>
<tr>
<td>D</td>
<td>26.8</td>
<td>15</td>
</tr>
<tr>
<td>E</td>
<td>26.3</td>
<td>15</td>
</tr>
<tr>
<td>F</td>
<td>25.6</td>
<td>15</td>
</tr>
<tr>
<td>G</td>
<td>29.2</td>
<td>16</td>
</tr>
<tr>
<td>H</td>
<td>37.0</td>
<td>17</td>
</tr>
</tbody>
</table>

To prevent contamination, new sterile materials were used with each larva, and each larva was dissected on a new, sterile and disposable plastic surface. DNA was extracted from each sample using the Chargeswitch™ Forensic DNA extraction kit (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. Dissection and DNA extraction were performed in a UV sterilised laminar flow hood to minimise contamination (Blankenship & Yayanos, 2005).

Each larva was mounted in solidified 2% agar (GIBCO BRL) so that the right lateral side of the cephalic region was exposed. Gut content were syringed out of the hepatopancreas using individual, sterile, disposable 31 gauge hypodermic needles (Ultra-fine II, Becton Dickinson, Australia). These were first flushed with Chargeswitch™ DNA extraction buffer (Invitrogen, Carlsbad, CA), mounted on a
micromanipulator and then carefully inserted into the larva’s hepatopancreas, trying to minimise contact with its exterior. A difficulty with using disposable syringes is that the rubber bung cannot be manually drawn out in a smooth action, therefore a small “turn screw” linear actuator was used to incrementally and smoothly move the syringe plunger (Fig. 3.1).

Figure 3-1 Components used in syringing out gut contents. A 31 gauge hypodermic needle and syringe were mounted on a “turn screw” linear actuator that controlled the syringe plunger. These were mounted on a micromanipulator, which was used to gently direct the needle into a larva, which was embedded in 2% agar. Inset photo shows the syringing out of the thin carapace of *S. verreauxi*. 
3.2.2 PCR

The template concentration and quality of DNA from larval guts is low and of poor quality, so to improve sensitivity a semi-nested PCR approach was used. The Amplicon software package (Jarman, 2004) was used to design PCR primers to amplify the prey *M. galloprovincialis*, but not the predator (larva), *S. verreauxi*. PCR primers were designed to amplify a short sequence of DNA spanning the V8 and V9 regions of the 18S rRNA gene (refer Table 3.2 for primer sequences). Reactions contained 1× reaction buffer, 1.5 mM MgCl₂ (Invitrogen) 0.4 mg ml⁻¹ BSA (GIBCO), 0.1 mM dNTPs (Roche), 0.05 µM of forward and reverse primers (IDT), and 1 unit of Platinum Taq (Carlsbad, CA). First round PCRs contained 25 ng genomic DNA and followed a touchdown approach which started with 94 °C for 1 min, followed by 32 cycles of 94 °C for 20 s, 59 °C for 15 s, and 72 °C for 15 s. PCR products were diluted 1:50 and 3 µl used as template in a subsequent reaction with internal primers using the following cycling parameters: 94 °C for 1 min, followed by 32 cycles of 94 °C for 20 s, 61 °C for 15 s, and 72 °C for 15 s, followed by a final extension step of 72 °C for 30 s. Each PCR was carried out in 30 µL volumes using a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA, USA).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 MYT_18S_v8_F2</td>
<td>CCG TTG AAC CTC CTT CGT GC</td>
</tr>
<tr>
<td>2 MYT_18S_v9_R1</td>
<td>GCG TCT TTT CGG CAC ACC G</td>
</tr>
<tr>
<td>3 MYT_18S_v9_Rm</td>
<td>ACG GTT GTT GCC AAC CAT TTC C</td>
</tr>
</tbody>
</table>

Given that predator DNA is not degraded and vastly dominates prey DNA, it was important to confirm that PCR products were of prey origin and not chimeras or artefacts originating from the large number of PCR cycles. Therefore, to determine amplicon provenance the amplicons were subjected to cycle sequencing with BigDye™ Dye Terminator Chemistry (Applied Biosystems) using the manufacturer’s
specifications and then run on an ABI3130XL capillary sequencer (Applied Biosystems). Sequences were aligned to homologous S. verreauxi and M. galloprovincialis sequences.

### 3.3 Results

For all eight samples the final rinse water did not contain M. galloprovincialis DNA (Table 3.3). However, for the swabbed exterior controls, four of the eight samples presented strong amplification for M. galloprovincialis DNA. Syringed gut samples were all positive for M. galloprovincialis DNA, while five of the eight samples derived from homogenised hepatopancreas had positive amplification. The use of a sterile flocked miniswab to remove DNA from the interior of the hepatopancreas was abandoned when it was found to be not reproducible across samples due to the break up of the dissected larvae when put under the pressure of wiping with a miniswab.

Table 3-3 Prey detected in phyllosomas guts.

Midgut score is a qualitative value that refers to the volume of food that can be visually detected in the fed larvae, where 0 = none, 3 indicates a full gut and 1 and 2 are a subjectively estimated intermediate score. The next four columns (Wash, Swab, Syringe and Homogenate) are presence (1) absence (-) scores for PCR success. “Wash” and “swab” are controls for exogenous contamination where “wash” refers to using the final rinse water as a PCR template and “swab” refers to DNA obtained by swabbing the larval exterior. The next two columns refer to prey detected in gut content obtained by syringe or making a homogenate of a part of the larva and its gut.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Midgut Score</th>
<th>Negative Control</th>
<th>Prey Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wash</td>
<td>Swab</td>
</tr>
<tr>
<td>a</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>b</td>
<td>2</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>c</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>d</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>e</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>f</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>g</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>h</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>NA</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

The quantity of prey that could be visually identified in the eight samples varied considerably (Table 3.3), from no visible prey contents (score = 0 out of 3) to there being a reasonably large visible mass of food after feeding (score = 2 out of 3).
However, none of the samples could be described as being completely full of food mass (score = 3 out of 3). There was no correlation between the quantity of prey visible in the midgut and PCR success of homogenised samples.

3.4 Discussion

Syringing out the gut contents of larvae outperformed the approach of homogenising a segment of the larva and its gut to obtain DNA template for PCR. The poor result for homogenisation has several explanations that are not necessarily exclusive. First, the syringing of the sample prior to homogenising might reduce the amount of detectable prey in the homogenisation, because it is apparent when syringing that the suction draws material from throughout the hepatopancreas. Second, the homogenate approach results in the co-extraction of a massive amount of predator DNA that might vastly exceed the prey DNA so that the latter is undetectable. However, these two explanations are inconsistent with one unexpected result, which was that PCR performed on homogenates was unsuccessful with larvae with very visible gut content (Table 3.3), but was successful on samples with low or no visible gut content. This result is counter-intuitive but may be due to the localisation of food in the midgut gland resulting in the sampling of a gut segment absent of prey. Observations of spiny lobster larvae digestive processes have demonstrated that food passed into the midgut gland can be highly localised, particularly during periods of active food digestion (see Fig. 5 in Smith et al., 2009). This could possibly be compensated for by targeting parts of the gut that appear to contain food for analyses. However, the gut contents of larvae are extremely difficult to see when alive and transparent, and it is virtually impossible to detect gut contents once larvae are fixed and opaque. Therefore, instead of homogenising a gut-segment, it is recommended that the entire part of the predator that contains the gut be homogenised. Of course, increasing the size of the homogenate increases the absolute
amount of the integument that is included in the DNA extraction, which increases the chances of exogenous contamination entering the extraction.

A significant result of this study was the performance of the negative controls for exogenous contamination. Specifically, the extensive washing of larvae with 2 L of water did not remove all exogenous DNA from the larvae as subsequent swabs taken of the exterior of the larvae revealed the presence of prey DNA in half of the larvae sampled. This was despite the absence of prey DNA in the final wash of the exterior of all eight larvae. Therefore, PCR of the final wash is not as good a negative control, compared to taking swabs of the exterior of the predator.

If a whole predator homogenate method is to be used for DNA extraction from the gut it would seem most appropriate to use a swab to detect residual exogenous DNA contaminants. However, if the exterior of a predator is positive for DNA, it does not follow that it cannot be excluded as potential prey. For example, lobster larvae manipulate their food and hold it against their bodies when they feed, so it would be expected that prey DNA is likely to be present on the exoskeleton surface. Furthermore, much of the external body surface and especially the mouthparts and appendages of lobster larvae are covered in erect sensory setae, many of which are thought to be used for sensing, piercing and manipulating prey and other items that it may encounter and review as potential food (Cox & Bruce, 2003; Nishida & Kittaka, 1992). That the outside of the lobster larvae is frequently contaminated with food is indicated by the fouling of these surfaces with food particles and other material, which often creates problems in the culture of these organisms (Kittaka, 1997a; Nelson et al., 2002; Smith et al., 2007). Therefore, any “prey” signal detected from the exterior of the predator cannot simply be subtracted from the prey signal in the gut. If washing the predator is
the only feasible method, for instance if the predator is too small to access gut content by syringe, then a better approach is to wash and then swab the exterior of the predator, perform a PCR on DNA extracted from the swab, but only homogenise the predator once it is known that the exterior swab is clear of exogenous contaminants. If the swab is not clear, then washing can be repeated before homogenisation. However, this approach of performing PCR on an external swab as a check prior to homogenisation will only be useful when a study is using group-specific primers. If a study is trying to identify prey items by using universal primers, then the swab will inevitably contain DNA of the predator and, most likely, microscopic eukaryotes. In that case dissection is the only valid option, or if there are sufficient samples to experiment with, then it might be advantageous to trial bleaching the predators’ exteriors.

While previous studies have successfully removed exogenous contaminants from terrestrial invertebrates through bleaching with chlorine solution (Linville & Wells, 2002; Meyer & Hoy, 2008), the concentration of bleach that will degrade target template is dependent on the species of predator (Greenstone et al., 2012). This approach can be considered to be high risk with marine plankton because of the higher permeability of these animals. Lobster larvae, for example, lack gills until very late in their development and their integument is the site for gas and ion exchange (Haond et al., 2001) as well as the absorption of lipid, carbohydrate and amino acids (Rodriguez Souza et al., 1999; 2010). However, whether the gut of metabolically inactive larvae are permeable to bleach is something that needs to be explored experimentally, especially given that these processes most likely occur by active transport (Rodriguez Souza et al., 2010). The success of bleach with some species of terrestrial predators (Greenstone et al., 2012) suggests that before embarking on a DNA-based diet study on any aquatic species it would be useful to experimentally assess their susceptibility to bleach at
varying concentrations. The only difficulty with trialling bleach is that it is potentially destructive and therefore experimentation might drastically reduce the sample size of a study. Lobster larvae samples are rare and difficult to obtain, which limits experimentation. This is because wild larvae, like many aquatic plankton, are in low densities and ocean-going sampling voyages are expensive undertakings, (Jeffs et al., 2001a) and aspects of their culture continue to be optimised, such as their dietary requirements and disease susceptibility (Kittaka, 1997a; Matsuda et al., 2006). Therefore, a low risk strategy is advantageous with a small number of valuable samples. The use of DNase could overcome the permeability issue, but DNase has been shown to be insufficient at digesting exogenous contaminants from terrestrial predators (Linville & Wells, 2002) and there is no compelling reason that it would be successful on aquatic predators. Washing the exterior of the predator with sterile water (either saline or purified) is a lower risk and therefore a preferable approach with aquatic predators, but based on the results of this study it is not a sufficient approach to remove all exogenous DNA.

The most satisfactory method to obtain gut DNA from lobster larvae and minimise exogenous contamination is dissection following washing to remove loosely adhering exogenous contaminants. Combining the washing and dissection methods has been found to be effective with terrestrial predators (Remén et al., 2010). In some DNA-based diet studies of plankton, dissection has been used to access gut contents (Albaina et al., 2010; Bonnet et al., 2010; Pliru et al., 2012; Vestheim & Jarman, 2008), although the use of forceps and scalpel works best on predators with a gut system that can easily be cut away from other adjoining tissues. The method of using a fine gauge hypodermic needle mounted on a micromanipulator to syringe the gut contents out of lobster larvae is effective as it resulted in the recovery of prey DNA from all larvae examined. This
method of recovering gut contents is both inexpensive and suited to use with DNA
techniques because the fine gauge hypodermic needles are inexpensive, disposable and
sterile. The needle still makes this contact, but because contact with the exterior of the
predator is minimal, the chances of DNA contamination from the exterior of the
predator are greatly reduced compared to homogenising the entire predator. The syringe
method has an additional benefit over the homogenisation method because it introduces
significantly less predator tissue into the DNA extraction, which increases the
proportion of prey DNA introduced into the PCR reaction and therefore increases the
likelihood that PCR will be successful.

The syringe method works best on larvae larger than 8 mm, and has the potential to be
used on other thin, semi-transparent invertebrate and fish larvae. However, for smaller
larvae an alternative method needs to be used. Although homogenisation of the larval
gut is a possible approach, this method was not entirely reliable in recovering prey
DNA, however, this was confounded by the prior syringing of the gut contents, which
may have removed much of the prey DNA. While dissection may be a viable approach
for recovering gut contents in small predators, it is time consuming, difficult to perform
with precision, and may still result in contamination from contact with external
surfaces. Laser capture micro-dissection (LCM) has recently been used to study fish
larval diet (Maloy et al., 2010) and to discover the gut microbiota of echinoderms
(Thornhill et al., 2008). The syringe method for recovering gut contents is less costly
and faster than LCM, which requires the histological sectioning of predators.
4 General Methodology for Prey-enriched Pyrosequencing

4.1 Overview
The following chapter outlines the methods developed, in the light of the preceding chapters, to collect samples, extract DNA from the midgut gland and then sequence that DNA. The methods described are common to those used in chapters 5, 6 and 7 and are therefore collected together here.

4.2 Sampling
Western Australian samples were collected from the RV Southern Surveyor (CSIRO, Australia) on 7, 13 and 14 July 2010 and 27, 28, 29 and 30 August 2011 (refer Tables 6.1 and 7.1 and Fig. 7.1 for geographic co-ordinates). Phyllosomas were sampled from surface waters by net tows at night for around 10 minutes at less than 3.7 km hour\(^{-1}\) using a surface net (1 m\(^2\) opening, 1 mm mesh and cod-end with 355 µm mesh). New Zealand samples were opportunistically collected from the research vessel Tangaroa (NIWA) on four separate voyages off the eastern coast of New Zealand (Table 5.1). 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 hr\(^{-1}\) by slowly lowering 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 minutes. 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. Phyllosomas were immediately removed from this pre-chilled EtOH, measured and Western Australian \textit{P. cygnus} phyllosomas were staged under a dissecting microscope in accordance with the developmental key of Braine (1979) and New Zealand \textit{J. edwardsii} phyllosomas were staged using the
developmental key of Lesser (1978). They were then rinsed down with Milli-Q filtered water (Millipore), then preserved and stored in fresh pre-chilled 70% EtOH on board the vessel at -20° C for later analysis in the laboratory.

Species identity of the 2010 Western Australian phyllosomas was confirmed by sequencing the mitochondrial cytochrome-oxidase I gene (COI). For this, approximately 1 mm of the fifth pereiopod was removed and DNA extracted using the prepGEM™ extraction kit (Zygem, Hamilton, New Zealand) following manufacturer’s instructions, PCR but in 20 ml reagent volume. PCR used the LCO-1490 and HCO-2198 primers and PCR protocol of Folmer et al. (1994) except 20 μL reactions were used.

4.3 DNA extraction
In the laboratory, phyllosomas were rinsed with 600 ml of sterile MQ water using wash bottles to remove any loosely adhering surface contaminants (O’Rorke et al., 2013). Phyllosomas were then mounted in solidified 2% agar gel so that their cephalic region was exposed. The midgut contents of phyllosomas was syringed out using individual, sterile, disposable 31 gauge hypodermic needles (Ultra-fine II, Becton Dickinson, Australia) (O’Rorke et al., 2013). These were first flushed with Chargeswitch™ DNA extraction buffer (Invitrogen, Carlsbad, CA), mounted on a micromanipulator and then carefully inserted into the hepatopancreas of the phyllosomas and care was taken to minimise contact with the animal’s exterior. DNA extraction was performed with the Chargeswitch Forensic™ DNA extraction kit 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 midgut tissue was added. These were run in duplicate. All plasticware used was sterile.
and nuclease free. Dissection and DNA extraction were performed in a UV sterilised laminar flow hood following the recommendation of Blankenship and Yayanos (Blankenship & Yayanos, 2005). PCR reactions were set up in a separate UV sterilised PCR hood. DNA was quantified spectrophotometrically using a Nanodrop ND-1000 (Thermo Scientific).

4.4 Design of prey enriched PCR
Phyllosomas potentially prey on a range of zooplankton from phylogenetically divergent phyla, which restricts potential loci to those gene regions with highly conserved priming sites. The DNA from the digesta removed from the hepatopancreas was also likely to be degraded into short fragments. Therefore, the hyper-variable v7 and v9 regions of the 18S rRNA were targeted (Table 4.1). These are flanked by highly conserved priming sites and diverse assemblages of meiofauna and microscopic eukaryotes have been amplified for v7 (Chariton et al., 2010; Gast et al., 2004) and v9 (Pawlowski et al., 2011; Stoeck et al., 2009). Details on two primers used in this study have been published previously (Hardy et al., 2010; Medlin et al., 1988). The other two primers target a similar region to that used in previously published studies, but have been moved slightly to enable the priming of more metazoan phyla, in particular Cnidaria, Ctenophora and Chaetognatha: 18S_v7_con is a version of Uni1304F primer of Larsen et al. (2005) and 18S_v9_con targets a region slightly 59 (upstream) of the popular NSF1624 primer (Van der Auwera et al., 1994). Because these universal primers would otherwise amplify lobster DNA, they were used in conjunction with a PNA-clamp to suppress the amplification of lobster DNA.

Prey enrichment has been performed in other DNA diet studies using DNA blocking primers with 39 termini modified to prevent polymerisation (Vestheim & Jarman,
2008). However, the 18S rRNA of some potential prey, specifically the arthropods, differs little from that of lobsters and if a modified DNA primer were used then there might be non-specific binding. Therefore, PNA-clamps were used because their low mismatch tolerance would reduce non-specific PCR enrichment (Ørum et al., 1993), thereby building on the use of PNA to ascertain the diet of larval lobsters by Chow et al. (2010). In contrast to them, this study did not use the PNA to competetively exclude the binding of PNA primers to predator template, but rather to bind downstream of the primers and arrest polymerisation. The benefit of using this arrest approach is that the enrichment is not constrained to targeting regions of DNA where priming sites were immediately adjacent to hyper-variable clamping sites, but could independently target regions that were ideal primer and PNA clamping sites, which allowed us to design a very reliable PCR (O’Rorke et al., 2012a). Therefore, the PNA in this study can be used to prevent and enrich the prey of any phyllosomas from the Palinuridae or Scyllaridae.

<table>
<thead>
<tr>
<th>Name</th>
<th>Target</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13F_EukB</td>
<td>18S v9</td>
<td>TGT AAA ACG ACG AGT TGA TCC TGC AGG TTC ACC TAC</td>
<td>(Medlin et al., 1988)</td>
</tr>
<tr>
<td>M13R_18s_v9_Con</td>
<td>18S v9</td>
<td>CAG GAA ACA GCT ATG ACC CCT TTG TAC ACA CCG CCC</td>
<td>This study</td>
</tr>
<tr>
<td>M13R 18S_v7_Con</td>
<td>18S v7</td>
<td>CAG GAA ACA GCT ATG ACG CCG TTC TTA GGT GGA</td>
<td>This study</td>
</tr>
<tr>
<td>M13F.All18SR</td>
<td>18S v7</td>
<td>TGT AAA ACG ACG GCC AGT CAT CTA AGG GCA TCA CAG ACC</td>
<td>(Hardy et al., 2010)</td>
</tr>
<tr>
<td>Lobster_PNA_18S_v7_17mer</td>
<td>18S v7</td>
<td>TTG CGA ACG GAC ACC AC-Lys</td>
<td>This study</td>
</tr>
<tr>
<td>Lobster_PNA_18S_v9_18mer</td>
<td>18S v9</td>
<td>CGC TCT TGG ATG TTC TAC-Lys</td>
<td>This study</td>
</tr>
</tbody>
</table>

| 4.5 PCR and sequencing preparation |

PCR amplification was undertaken in two rounds following the “universal tailed amplicons sequencing” method outlined in the GS Junior System Guidelines for Amplicon Sequencing (Roche, 2010). In the first round of PCR the hyper-variable v7 and v9 regions of the 18S rRNA gene were targeted in separate reactions (refer Table 4.1 for primer sequences). PCR products were diluted 1:50 and 2.5 ml used as template
in a subsequent reaction to add adapter A and B sequences, the 454 GS-FLX Titanium sequencing key and multiplex identifier tags (MIDs, Table 4.2). Each PCR was carried out in 25 ml volumes using a GeneAmp 9700 thermocycler (Applied Biosystems Foster City, CA, USA). Reactions contained 16 reaction buffer, 2 mM of MgSO$_4$ (Invitrogen) 0.46 BSA (NEB), 0.1 mM dNTPs (Roche), 0.1 mM of forward and reverse primers (IDT), 1 mM of PNA-clamp (Panagene) and 1 unit of Hi-fidelity Platinum Taq (Invitrogen). First round PCRs contained 25 ng genomic DNA or were negative (no template) controls for contamination during DNA extraction or contamination. The first round of the v7 region reaction was carried out at 94 °C for 2 min, followed by 28 cycles of 94 °C for 20 s, 56 °C for 20 s, and 68 °C for 12 s followed by a final extension step of 68 °C for 30 s. The first round of the v9 region reaction differed, following a protocol of 94 °C for 2 min, followed by 30 cycles of 94 °C for 20 s, 59 °C for 20 s, and 68 °C for 12 s followed by a final extension step of 68 °C for 30 s. Second round PCR reactions used the following protocol: 94 °C for 2 min, 6 cycles of 94 °C for 20 s, 58 °C for 20 s, and 68 °C for 12 s, followed by 14 cycles of shuttle PCR of 94 °C for 20 s, then 68 °C for 30 s and a final extension step of 68 °C for 30 s. PCR conditions for the New Zealand phyllosomas, *J. edwardsii*, were similar to that above, but it was found that the number of PCR cycles in the second round shuttle PCR needed to be increased from fourteen cycles to twenty-eight 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 separately cleaned using Ampure XP$^{\text{TM}}$ beads (Agencourt) following the manufacturer’s instructions. Ampure XP$^{\text{TM}}$ beads were calibrated according to Roche 454 GS Junior Methods 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 diluted to 1×10⁹ molecules ml⁻¹ ready for sequencing. Sequencing for Chapter 6 was carried out following manufacturers instructions of the Roche 454 GS Junior (Roche, 2011). Sequencing reactions for Chapters 5 and 7 were carried out by Macrogen (Seoul, South Korea) on 1/8th of microtitre plates on a 454 GS FLX platform.

Table 4-2 Tagged fusion primers. Used in second round PCR. MIDs are in bolded text and are numbered following Roche guidelines (Roche, 2010)

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>454_M13F_MID1</td>
<td>CGT ATC GCC TCC CTC GCG CCA TCA G <strong>AC</strong> GAG TGC GT TGT AAA ACG ACG GCC AGT</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>CGT ATC GCC TCC CTC GCG CCA TCA G AT CAG ACA CG TGT AAA ACG ACG GCC AGT</td>
</tr>
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<td>CGT ATC GCC TCC CTC GCG CCA TCA G AT ATC GCG AG TGT AAA ACG ACG GCC AGT</td>
</tr>
<tr>
<td>454_M13F_MID7</td>
<td>CGT ATC GCC TCC CTC GCG CCA TCA G CG TGT CTC TA TGT AAA ACG ACG GCC AGT</td>
</tr>
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</tr>
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</tr>
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<td>454_M13R_MID13</td>
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<td>454_M13R_MID14</td>
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<td>CTA TGC GCC TTG CCA GCC CGC TCA G <strong>CG</strong> TGT CTC TA CAG GAA ACA GCT ATG AC</td>
</tr>
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5 Determining the diet of larvae of the red rock lobster (*Jasus edwardsii*) using high-throughput DNA sequencing techniques

5.1 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 (Fitzgibbon et al., 2013; Jeffs, 2010). 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](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 phyllosomas, have 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 & Johnston, 2003a; Jeffs, 2007; Phillips et al., 2006a).

Researchers have inferred the phyllosoma diet from the morphology of their digestive tract (Cox & Johnston, 2004; Phillips, 2005), feeding limbs (Cox & Johnston, 2003b; 2003a; Jeffs, 2007; Phillips et al., 2006a) and digestive enzyme profiles (Booth & Phillips, 1994; Johnston, Ritar, & Thomas, 2004a), as well as observing the feeding
behaviour of phyllosomas that have been cultured (Jeffs & Hooker, 2000; Kittaka, 1997b; Mitchell, 1971) or captured from the wild (Saunders et al., 2012). These various research approaches tend to indicate that phyllosomas are 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; 2007), *P. longipes bispinosus* (Chow et al., 2010) and *P. cygnus* (O’Rorke et al., 2012b). These three species belong to the same “sub-clade” of the genus *Panulirus*, which are characteristic of tropical or sub-tropical 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, 1997a). 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 (Illingworth et al., 1997; Kittaka et al., 1988; Kittaka et al., 2006). 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, 1997a). There is a belief that discovering the wild diet will lead to
improved diet formulation leading to more successful larviculture (Jeffs, 2007; Jeffs &
Hooker, 2000).

The reptant adults and juveniles of *Jasus edwardsii* are found in the coastal waters
around all the islands of New Zealand and the southern parts of Australia (Chiswell &
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 & Stewart, 1992) and across the Tasman Sea which stretches over
2,000 km between Australia and the New Zealand (Booth & Ovenden, 2000). The larval
phase of wild *J. edwardsii* is extremely long and extends to as much as 24 months
(Booth & 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 phyllosomas
metamorphose 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 & Holland, 2000;
Jeffs et al., 2005; Jeffs et al., 2001b; Jeffs et al., 1999). This return to coastal waters is a
migration distance of around 200 km on average (Jeffs et al., 2001a). Physiological,
biochemical and biomechanical measures of the cost of migratory swimming all
indicate that most pueruli have a tight margin for successful migration from offshore to
the coast (Fitzgibbon et al., 2013; Jeffs et al., 2001b; Wilkin & Jeffs, 2011). For
example, it has been found that up to 16.5 % of captured pueruli lacked sufficient
energy reserves to successfully complete migration onshore (Jeffs & Holland, 2000).
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). It appears likely that 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 begin to be able to test this hypothesis the planktonic diet of phyllosomas 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* phyllosomas. DNA methods for reliably determining diets of small invertebrates are improving with advancements in molecular methods (O’Rorke et al., 2012a; Pompanon et al., 2012; Symondson, 2002; Vestheim & Jarman, 2008), including their evolving application for marine larvae, such as spiny lobster phyllosomas (Chow et al., 2010; O’Rorke et al., 2012b; 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* phyllosomas (Chiswell & Booth, 1999; Chiswell & Roemmich, 1998). By reliably determining the diet of these phyllosomas, it should ultimately be possible to see how their prey is affected by variability in oceanographic processes that in turn could greatly influence the fitness of phyllosomas. Discovering the wild prey of these larvae also has the
potential to help to improve the ongoing larviculture initiatives for this and other spiny lobster species.

5.2 Methods
Sampling, DNA extraction, PCR and sequencing methods are described in Chapter 4.

5.2.1 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 and qfiles 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 samples were uniqued (de-replicated). Uniqued reads were aligned with MAFFT (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 preclustering 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 mid-gut, phyllosomas with less than 300 reads, were eliminated to prevent reads from poorly performing sequencing reactions from disproportionately influencing the results and then the number of reads per phyllosoma were standardised and converted to a percentage.
5.3 Results

5.3.1 Samples

A total of 23 phyllosomas were captured on the four separate sampling periods (Table 5.1). 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 5.1) (Lesser, 1978).

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Date</th>
<th>Stage</th>
<th>Size (mm)</th>
<th>Latitude [°S]</th>
<th>Longitude [°E]</th>
</tr>
</thead>
<tbody>
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<td>9</td>
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<tr>
<td>Tan02</td>
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<td>10</td>
<td>36.0</td>
<td>41° 13.09</td>
<td>178° 30.29</td>
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<td>8/05/10</td>
<td>9</td>
<td>23.5</td>
<td>41° 13.09</td>
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<td>Tan05</td>
<td>8/05/10</td>
<td>6</td>
<td>10.5</td>
<td>41° 13.09</td>
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</tr>
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<td>25.0</td>
<td>41° 11.41</td>
<td>178° 30.27</td>
</tr>
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</table>
5.3.2 PCR efficiency

DNA extracted from the mid-gut 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 100bp indicating primer dimers. This is consistent with there being little template for the PCR reaction besides the host DNA.

5.3.3 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 Palinurid template and 26,732 remained after removal of contaminants. Contaminants were from mammal DNA (human, 628 reads), plant material (417) and insects (96). It was typically the case that Palinurid sequences occurred in the dataset because PCR errors had removed the PNA sequence from these sequences 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.
Most samples produced relatively small proportions of zooplankton DNA, 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 DNA dominated mid-gut content of phyllosomas, comprising the majority of reads in twelve samples. Parasitic Chromalveolata were detected in 10 samples and an arachnid parasite 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.

5.3.1 Taxonomic assignment of reads

Of the total remaining 26,732 reads (i.e., not subsampled or normalised), fungi contributed 69.1% of the reads and were either of the phylum Ascomycota or Basidiomycota (Fig. 5.1). Potential parasites (arachnids and alveolates) contributed 16.4% of the sequencing reads, and zooplankton, which are potential phyllosoma prey, made up the remaining 14.5% of reads and there were 45 OTUs defined by a 98% clustering threshold. After reads were normalised, and phyllosoma samples with <300 reads were discarded, the relative contribution of zooplankton was 27.1%, parasitic Chromalveolata contributed 13.7% and fungi 59.2% of reads. 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. 5.1). 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. 5.1).
Table 5.2 Zooplankton OTUs detected in mid-gut of *J. edwardsii* phyllosomas.
The taxonomy of prey items detected in the mid-gut of phyllosomas and the frequency with which these taxa 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.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>No. of larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiolaria</td>
<td>Acantharea</td>
<td>Arthracanthida (95)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Polycystinea</td>
<td>Spumellaria</td>
<td>2</td>
</tr>
<tr>
<td>Arthropoda</td>
<td>Malacostraca</td>
<td>Euphausiacea</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Maxillopoda</td>
<td>Calanoida</td>
<td>1</td>
</tr>
<tr>
<td>Chordata</td>
<td>Actinopterygi</td>
<td>Pleuronectiformes</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetraodontiformes</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Thaliacea</td>
<td>Salpida</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Salpida (98)</td>
<td>1</td>
</tr>
<tr>
<td>Cnidaria</td>
<td>Hydrozoa</td>
<td>Siphonophora</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Hydrozoa</td>
<td>Trachylina</td>
<td>1</td>
</tr>
<tr>
<td>Ctenophora</td>
<td>Tentaculata</td>
<td>Beroida</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lobata</td>
<td>7</td>
</tr>
<tr>
<td>Echinodermata</td>
<td>Ophiuroidea</td>
<td>Ophiurida</td>
<td>1</td>
</tr>
<tr>
<td>Mollusca</td>
<td>Gastropoda</td>
<td>Thecosomata</td>
<td>1</td>
</tr>
</tbody>
</table>

Based on rank-abundance of reads, the most most frequently occurring zooplankton OTUs detected in the mid-gut 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. 5.2). Siphonophora occurred in 8 of the 19 phyllosomas and Lobata in 7 phyllosomas (Table 5.2). 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...
(Arthropoda) DNA occurred in three, but the OTUs of salps and copepods were different for each phyllosoma (Table 5.2).

Table 5-3 Fungal OTUs.
Fungal OTUs detected in the mid-gut of phyllosomas of J. edwardsii. The taxonomy of fungi detected in the mid-gut of phyllosomas and the frequency with which these taxa occurred among the 19 sampled phyllosomas (i.e., count data). Only members of the saprophytic sub-kingdom Dikarya were detected, which is characteristic of marine fungal assemblages. 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 had bootstrap support between 75% and 100% are indicated in parentheses. The unclassified taxa received support of less than 75%.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>No. of larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascomycota</td>
<td>Dothideomycetes</td>
<td>Pleosporales</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Eurotiomycetes</td>
<td>Eurotiales</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Eurotiales (82)</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Lecanoromycetes</td>
<td>Umbilicariales (68)</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Leotiomycetes</td>
<td>Helotiales</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Pezizomycetes</td>
<td>Pezizales</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Saccharomycetes</td>
<td>Saccharomycetales</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Basidiomycota</td>
<td>Agaricomycetes</td>
<td>Polyporales</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trechisporales</td>
<td>1</td>
</tr>
<tr>
<td>Exobasiomycetes</td>
<td>Malasseziales</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Microbotryomycetes</td>
<td>Sporidiobolales</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Tremellomycetes</td>
<td>Cystofilobasidiales</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Filobasidiales (86)</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Tremellales</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Across the standardised J. edwardsii phyllosomas, a total of 13.7% of reads were assigned to parasitic orders of the kingdom Chromalveolata: Eugregarinida, Apostomatida and Albuginaceae (Table 5.4). DNA from the Apostomatida contributed the second most abundant number of reads (12.7%; Fig. 5.2), and occurred in three phyllosomas (Table 5.4). Eugregarinida occurred in two phyllosomas and Albuginaceae occurred in one (Table 5.4). Arachnid DNA from the order Sarcoptiformes occurred in only one phyllosoma (Tan13), but it was the only organism detected in the mid-gut of that specimen.
Table 5-4 Parasitic organism OTUs. OTUs of parasitic organisms detected in mid-gut of phyllosomas of *J. edwardsii*. Parasitic organisms detected in the mid-gut of phyllosomas and the frequency with which these taxa occurred among the 19 sampled phyllosomas (i.e., count data). Three phyla of the unicellular eukaryotic kingdom Chromalveolata were detected in several phyllosomas and a parasitic metazoan, an arachnid of the sub-class Acari, was detected in one phyllosoma. 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 had bootstrap support between 75% and 100% are indicated in parentheses.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>No. of larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apicomplexa</td>
<td>Gregarinia</td>
<td>Eugregarinida</td>
<td>2</td>
</tr>
<tr>
<td>Ciliophora</td>
<td>Oligohymenophorea</td>
<td>Apostomatida</td>
<td>3</td>
</tr>
<tr>
<td>Stramenopiles</td>
<td>Oomycetes</td>
<td>Albuginaceae</td>
<td>1</td>
</tr>
<tr>
<td>Arthropoda</td>
<td>Arachnida</td>
<td>Sarcopiformes</td>
<td>1</td>
</tr>
</tbody>
</table>

5.4 Discussion

The present study used the hyper-variable v9 region of the 18S loci to detect the gut contents of phyllosomas of *J. edwardsii*. Other DNA diet studies have used a range of other hyper-variable 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, amplicon lengths can be quite variable causing differential PCR amplification of products from different organisms and affect the reproducibility and universality of the results (Acinas et al., 2005; Suzuki & 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 mid-
gut 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., 2012b). 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 phyllosomas.

![Figure 5-2](image)

**Figure 5-2** Rank abundance of phylogenetic orders. Rank abundance of phylogenetic orders of prey organisms detected in the mid-gut of *J. edwardsii* phyllosomas. Phylogenetic order of organisms detected using PCR of the v9 region of the 18S rDNA locus. 300 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.

### 5.4.1 Zooplankton

The zooplankton DNA isolated from the mid-gut of the phyllosomas was dominated by Cnidaria from the order Siphonophora, and Ctenophora from the order Lobata, which is demonstrated by both rank abundance of DNA sequences (Fig. 5.2) and by the
frequency with which these taxa occurred in the mid-gut of the phyllosomas (Table 5.2). Euphausiids, colonial radiolaria and bony fish were also 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. 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, 1997a) including J. edwardsii (Kittaka et al., 2005; Macmillan et al., 1997).

Multiple zooplankton OTUs were found in the mid-gut 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 mid-gut of phyllosomas of both P. cygnus (O’Rorke et al., 2012b) 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 & 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 & Bruce, 2003; Jeffs, 2007; Saunders et al., 2012). Siphonophores, which appear to be a large proportion 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.
While the present study corroborates the hypothesis that *J. edwardsii* phyllosomas are generalist predators, it appears that they might preferentially target siphonophores and ctenophores (collectively known as coelenterates). 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 mid-gut 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* were 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 phyllosomas, in an otherwise low density of zooplankton prey that is characteristic of the often oligotrophic oceanic waters where spiny lobster phyllosomas typically dwell. This would help to explain the consistent presence of pelagic coelenterates in the mid-gut of phyllosomas and the corresponding strong vertical migration behaviour commonly observed in the phyllosomas of many species of spiny lobster (Bradford et al., 2005; Minami et al., 2001; Rimmer & Phillips, 1979).
The present study also identified krill (Euphausiacea) in the mid-gut of two stage 7, one stage 8 and one stage 9 phyllosoma. 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 phyllosomas in the Wairarapa Eddy and are associated with the DSL there, while predation of krill by mid- and late-stage phyllosomas has been related to the accumulation of nutritional reserves in phyllosomas (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 & Smetacek, 1994).

While coelenterates have historically been thought of as a nutritionally poor food source because of their high water content, they have more recently been found to be readily digested by predators (Arai et al., 2003). Cnidarians, such as siphonophores, may suit the nutritional requirements of *J. edwardsii* phyllosomas because of their relatively high protein and unsaturated fatty acid content (Kittaka, 1997a). Siphonophores from the Wairarapa Eddy are consistent with this, having high protein and fatty acid content relative to carbohydrate (Jeffs et al., 2004; Wang et al., 2013c). This reflects the composition of successful feeds that have been used in larviculture experiments (Cox & Johnston, 2003a; Goldstein & Nelson, 2011) as well as the major nutrient composition of phyllosomas (Ritar et al., 2003). Additionally, in larval culture conditions cnidarians have proved to be equally attractive prey to phyllosomas as chopped mussel gonad, which is one of the most successful foods for larviculture of phyllosomas of many species of spiny lobster, including *J. edwardsii* (Kittaka, 1997b).
The presence of coelenterate DNA in the mid-gut of phyllosomas confirms the hypothesis that gelatinous zooplankton are suitable food for phyllosomas (Jeffs, 2007; Kittaka et al., 2005) and is consistent with the observation that siphonophores, especially the species *Chelophyes appendiculata*, occur in high densities in the Wairarapa Eddy (Cox, 2004). However, some gelatinous zooplankton that are a major component of most oligotrophic plankton assemblages were only present as a minor component of the range of prey DNA sampled from the mid-gut of phyllosomas, namely; salps (urochordates) (four OTU’s found in four independent phyllosomas, Table 5.2), colonial radiolarians (the most abundant OTU occurred in three phyllosomas, Table 5.2) and arrow worms (Chaetognatha), which were not detected at all. The low occurrence of salps is particularly surprising because salps occur in high concentrations in oligotrophic waters including the Wairarapa Eddy where they have been found to be the most abundant members of that assemblage (Cox, 2004) and they have been hypothesised to be an ideal food for phyllosomas (Heron et al., 1988). Furthermore, salps were identified as a major component of DNA from the mid-gut of phyllosomas of *P. cygnus* along with chaetognaths and colonial radiolarians (O’Rorke et al., 2012b). Therefore, their low representation of salps in the mid-gut content of phyllosomas of *J. edwardsii* is unanticipated and may represent a dietary preference in this species.

### 5.4.2 Fungi

Fungal DNA provided the most abundant reads from the mid-gut of *J. edwardsii* phyllosomas (59.2% of standardised reads), and fungal OTUs were detected in fifteen of the nineteen phyllosomas (Fig. 5.1). Fungal strains sampled from marine and terrestrial habitats do not assort into phylogenetically distinct clades (Richards et al., 2012), which makes it difficult to characterise the fungal reads by sequence homology.
alone. However, marine fungal assemblages taken from marine surface waters have a distinct community composition, where they are almost exclusively populated 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., 2012b), and in *J. edwardsii* in the present study. This, along with the measures taken to minimise contamination, indicates that these sequences originate from the phyllosoma mid-gut and are very unlikely to be post-sampling artefacts.

Fungi digest and assimilate nutrients osmotically and do not phagocytise particles (as do many other pelagic picoplankton), which means they must always be in contact with a food substrate (Richards et al., 2012). Therefore, the fungi detected inside the mid-gut of phyllosomas are parasites, mid-gut endosymbionts, are potentially competing with the phyllosomas for nutrients, or it is evidence for phyllosomas consuming detritus from the water column. It is possible that the fungus detected in the mid-gut are parasitic because, although neither of these fungal phyla have been identified as lobster pathogens (Shields et al., 2006), a strain of 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). It is also highly probable that the high proportion of Ascomycota and Basidiomycota detected in the mid-gut of phyllosomas is because fungi are ingested along with particulate organic matter (POM) often in the form of marine snow and this may also explain the overall low presence of zooplankton DNA reads discovered in the mid-gut. Nutrient recycling, such as through consumption of POM, is a major feature of the Wairarapa Eddy food web (Bradford-Grieve et al., 1999). Also, eel larvae (leptocephalii), which like phyllosomas are characteristic of oligotrophic waters, have been found to consume POM such as faeces and other detritus
in situ (Mochioka & Iwamizu, 1996; Otake et al., 2013). Leptocephali have been found to have the same signature fungal community of Ascomycota and Basidiomycota DNA inside their gut (Riemann et al., 2010; Terahara et al., 2011). Therefore, it is highly likely that POM is an important part of the diet of phyllosomas of some species of spiny lobsters. This might have implications for the culture of phyllosomas, suggesting that artificial feeds might benefit from incorporating compositional characteristics of degraded or fermented food sources. It has been demonstrated that phyllosomas 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 culture of phyllosomas (Rodriguez Souza et al., 1999; 2010). The present study reinforces the potential benefit of testing this approach.

5.4.3 Parasites
DNA from parasitic organisms was detected in the mid-gut of several phyllosomas (Table 5.4). These included Acari mites and, more frequently, DNA from single celled heterotrophs from the kingdom Chromalveolata. Chromalveolata have been observed in the gut of leptocephalii and have been hypothesized to be a component of their POM scavenging diet (Govoni, 2010) and their presence in the mid-gut of phyllosomas may indicate scavenging on POM. However, Chromalveolata were co-detected with other zooplankton in all but one phyllosoma and it is therefore likely that parasitic Chromalveolata are co-ingested with other prey items, 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 parasites from the two classes of Oomycete and Oligohymenophorea have been
identified in cultured phyllosomas (Kitancharoen & Hatai, 1995; Kittaka, 1997a). The Chromalveolata class Gregarinia, which was detected in two phyllosomas, parasitises arthropods including crustaceans where they occur in the mid-gut and digestive tract, (Rueckert et al., 2011; Takahashi et al., 2008a; 2008b). In the Antarctic krill, Euphausia superba, it was revealed that between 90-100% of the krill from separate populations were infected with Gregarinia (Takahashi et al., 2008b). DNA from an Acari (mite) was also detected in one phyllosoma. The Acari are predominantly a marine clade of parasitic mites (Bartsch, 2006) and their presence suggests that J. edwardsii phyllosomas are being parasitised by multicellular as well as unicellular organisms. It would be informative to isolate all these commensal species and assess their negative (and possibly positive) effects on development and survival of phyllosomas. For example, Lesser (1978) estimates 98% mortality for phyllosomas, 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 & Sastry, 1980), but understanding their susceptibility to parasites and monitoring parasite dynamics might provide an alternative explanation.

The migratory behaviour of spiny lobster phyllosomas into oligotrophic offshore waters where they survive at very low densities may be an evolutionary strategy to minimise parasitism. There is growing evidence that some animal migrations might be driven by a parasite avoidance strategy (Altizer et al., 2011) and migration into unproductive waters to avoid parasites would circumvent investment in physically costly innate immunity strategies, for which crustaceans are known to have severe limitations (Vazquez et al., 2009). Analogously, while two species of non-migratory freshwater fish (Galaxias depressiceps and G. gollumoides) have been found to suffer from a high incidence of
parasitism in larvae, but their migratory congener (*G. brevipinnis*) does not (Kelly et al., 2010; Poulin et al., 2012). For *J. edwardsii* this hypothesis has already been used to explain the observation that early benthic juveniles exhibit solitary behaviour during a brief post-settlement period when they are highly susceptible to cross-infection with disease (Butler et al., 2008) and this is in stark contrast to the gregarious behaviour of later juveniles and adult lobsters, which share shelters. The identification of a suite of hitherto unknown parasites in phyllosomas highlights a potentially significant obstacle for aquaculture efforts, which necessarily involves keeping phyllosomas in high densities, which is ideal for parasite transmission. Therefore, characterising the parasites of wild phyllosomas provides invaluable information about what measures need to be taken to avoid and monitor outbreaks of parasites under culture conditions. These parasitic organisms appear to have gone unnoticed in both wild and cultured phyllosomas despite many years of research on spiny lobster phyllosomas.

### 5.4.4 Low DNA recovery and the developmental ecology of *J. edwardsii*

In comparison to a study of *P. cygnus* phyllosomas that used analogous methods (O’Rorke et al., 2012b), where the overall percentage of sequencing reads from zooplankton was 77.0%, the present *J. edwardsii* study had a much lower contribution from zooplankton of 27.1% of standardised reads (and 14.5% of total reads). Here the assemblage was instead dominated by microscopic organisms from the kingdoms Fungi and Chromalveolata. Furthermore, the PCR efficiency was relatively poor and in order to achieve a reasonable yield of PNA-clamp enriched PCR product, the present study needed to double the PCR cycle number over that of the *P. cygnus* study that otherwise used the same methodology (O’Rorke et al., 2012b). This indicated that *J. edwardsii* phyllosoma midguts contained much less amplifiable DNA than in *P. cygnus*. 
One explanation for the observed differences in the abundance of zooplankton DNA in the mid-gut of *P. cygnus* versus *J. edwardsii* phyllosomas may relate to the latter species having a more effective digestive system that is capable of degrading prey with greater rapidity. Certainly *J. edwardsii* phyllosomas have been found to have effective digestive enzymes which could break down DNA quickly (Johnston et al., 2004b). Furthermore, both acidity and temperature increase the rate of nucleic acid decay (Lindahl, 1996). The crustacean mid-gut is typically acidic and these two genera may vary in mid-gut acidity, which might explain DNA degradation rates. Unfortunately, there is no information about the mid-gut acidity of these phyllosomas. It is known that slipper lobster phyllosomas have a mid-gut pH of 5.9 (Johnston & Yellowlees, 1998), which suggests that acidity might be a factor in DNA degradation.

Phyllosomas do not feed before and after moulting (Tong et al., 1997) and the low prey signal in some of the phyllosomas from this study might be explained by their having been captured near moult. However, this not a likely explanation for the discrepancy between *J. edwardsii* and *P. cygnus* because the former species moults at a lower frequency than the other: 17 moults in 12-24 months compared to 15 moults in 9-11 months, respectively (Phillips et al., 2006a). The habitat of *P. cygnus* is warmer than for *J. edwardsii*, so temperature is also excluded as an explanation for the observed differences in mid-gut content as it could be expected that DNA would degrade faster at the warmer temperature, and the poikilothermic digestive metabolism would operate more quickly, not vice versa.

A more likely explanation for both the low percentage of zooplankton reads and the reduced PCR amplification with *J. edwardsii* is an ecological one. Phyllosomas are passive encounter predators and the phyllosomas of *J. edwardsii* may encounter
zooplankton prey with less frequency than *P. cygnus* and therefore are more likely to have empty mid-guts. Hence, the mid-gut content of the phyllosomas are merely a reflection of the natural availability of zooplankton in the pelagic habitat of the phyllosomas. Wild *J. edwardsii* take almost twice as long to reach metamorphosis as cultured phyllosomas (Booth & Phillips, 1994; Phillips et al., 2006a) and while factors such as incubation temperature contribute to this, much of it can be explained by food availability (Smith et al., 2007; Smith et al., 2010; Tong et al., 1997). Developmental responses to diet restriction are not unusual and have been observed in many invertebrates, vertebrates and yeast (Bishop & Guarente, 2007; Guarente & Kenyon, 2000). Furthermore, the metabolic pathways that link calorific restriction to delayed development are conserved throughout the eukaryotes, where restriction prolongs both larval phases and life expectancy (Hafen, 2004; Kabil et al., 2011; Kapahi et al., 2004; Lucanic et al., 2011). 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 relative stages of phyllosomas become asynchronous (Moss et al., 1999; Tong et al., 1997). Within water bodies 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 are relatively synchronised (Phillips et al., 1979), which probably reflects their development in feeding conditions with greater prey densities. For example, coastal waters of the East Indian Ocean experience elevated primary production from autumn, which is closely tracked by increased heterotroph production through to spring (Caputi et al., 2003; Strzelecki et al., 2007; Waite et al., 2007a), so that *P. cygnus* phyllosomas that reside in
anticyclonic, as well as cyclonic, eddies experience a more prolonged respite from the usual oceanic oligotrophic conditions. By contrast, the Wairarapa Eddy experiences only a brief spring diatom bloom, when Chlorophyl $a$ doubles, although it does not exceed 1 mg m$^{-3}$ during which time mesozooplankton densities spike (Bradford-Grieve et al., 1999). However, the Wairarapa Eddy is otherwise characteristic of a predominantly oligotrophic anticyclonic eddy (Bradford et al., 1982; Chiswell, 2011; Murphy et al., 2001) and therefore, relatively minor variations in productivity within this eddy greatly influence zooplankton prey availability for phyllosomas and they may rely heavily on these brief periods of elevated productivity in order 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; 2005; Jeffs & Holland, 2000; Jeffs et al., 2001b). There is evidence that pueruli with poor nutritional condition fail to complete this transition (Fitzgibbon et al., 2013; Jeffs et al., 2001a; Wilkin & Jeffs, 2011) and this may be important contributor to the enormous interannual variability in recruitment of $J. \text{edwardsii}$ pueruli observed in a number of coastal locations around New Zealand (Booth, 1994). The apparently constrained prey availability for phyllosomas of $J. \text{edwardsii}$ as determined by DNA methods, tend to support the hypothesis that the large interannual variability in recruitment of this species is driven by natural fluctuations in the planktonic food resources available to phyllosomas, which in turn greatly influences the survival of the subsequent lecithotrophic pueruli (Fitzgibbon et al., 2013).

5.5 Conclusion
The results of the present study are consistent with the hypothesis that $J. \text{edwardsii}$ phyllosomas are generalist predators of gelatinous zooplankton, but that coelenterates are a significant and perhaps targeted component of their diet. This contrasts with a
recent study of *P. cygnus* where greater quantities of DNA were consistently detected in the mid-gut of phyllosomas that originated from a more diverse range of zooplankton taxa, such as colonial Radiolaria, Thaliacea and Sagittoidea (O’Rorke et al., 2012b). 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 phyllosomas are also consuming decaying matter, such as faecal material and marine snow, or they are endosymbionts of the mid-gut of phyllosomas. 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 larviculture of phyllosomas. The detection of a number of unicellular eukaryote and multicellular parasites in phyllosomas indicates the potential stressors that impact on phyllosomas and 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.
6 Determining the Diet of Larvae of Western rock Lobster (*Panulirus cygnus*) Using High-Throughput DNA Sequencing Techniques

6.1 Introduction

Despite considerable research into the biology of spiny lobsters (Family Palinuridae), the larval phase of their lifecycle remains enigmatic. In particular, the composition of the diet of larvae remains uncharacterised for all species of spiny lobster (Jeffs, 2007), including the western rock lobster (*Panulirus cygnus*), which is the basis of the second largest commercial spiny lobster fishery in the world (ABARES, 2011).

Spiny lobsters have an unusually long planktonic larval phase and *P. cygnus* has an estimated larval duration of 9 to 11 months that is spent in oceanic waters extending from the continental shelf margin to over 1,500 km offshore from Western Australia (Phillips et al., 1979). After this oceanic phase the larvae, which are known as phyllosomas, are thought to be carried shoreward by ocean currents and eventually undergo metamorphosis into nektonic post-larvae, or pueruli, which actively migrate back onshore (Phillips & McWilliam, 2009). For *P. cygnus* pueruli there is good evidence that the magnitude of recruitment to the coast of Western Australia is positively correlated with westerly winds (Caputi et al., 2001), La Niña events, and the associated increase in strength of the Leeuwin Current (Caputi, 2008; Caputi et al., 2001; Pearce & Phillips, 1988). However, the underpinning causal details of how these...
oceanic events impact lobster ecology and recruitment to the coastal benthic stock remain to be fully established.

All evidence indicates that the puerulus is a non-feeding phase that fuels its shoreward migration by metabolising extensive lipid reserves built up during the preceding phyllosoma phase which actively feeds in the pelagic environment (Limbourn et al., 2009; Limbourn & Nichols, 2009; Phillips et al., 2006b). For example, the puerulus of _P. cygnus_ caught closer to shore have markedly lower lipid reserves than those caught further offshore (Phillips et al., 2006b), a phenomenon consistent with observations of the pueruli of other species of spiny lobster (Jeffs, 2001; Phleger et al., 2001). Settled _P. cygnus_ pueruli also display seasonal fluctuations in lipid reserves indicating environmental effects on available energy that may be due to increased metabolic activity from elevated temperature, having to traverse ocean currents and mesoscale oceanic features, or seasonal changes in pelagic prey availability to phyllosomas (Limbourn et al., 2009). Pueruli that have traversed a greater distance to settle on the coast tend to have more depleted lipid reserves than those that have travelled shorter distances, but this pattern is not consistent (Limbourn et al., 2009). The inconsistency could be due to final stage phyllosomas undergoing metamorphosis without sufficient lipid reserves to migrate successfully to shore. This hypothesis is supported by observations of the red rock lobster, _Jasus edwardsii_, for which 16.5% of nektonic pueruli were estimated to have insufficient lipid to reach settlement sites on the coast (Jeffs et al., 2001a). Likewise, estimates based on the biomechanics of swimming by spiny lobster pueruli confirm that the lipid energy reserves are marginal for ensuring settlement success (Wilkin & Jeffs, 2011). Puerulus mortality due to exhaustion of lipid reserves is presumably more of a risk in _P. cygnus_, which has significantly smaller
larvae and post-larvae and therefore a reduced ‘‘capacity to store lipid’’ (Phillips et al., 2006b). Any hypothesis based on the feeding of phyllosomas and their nutritional status at metamorphosis is contentious and, currently, not experimentally testable because it is neither known what the prey of phyllosomas are, nor what ‘triggers’ metamorphosis (McWilliam & Phillips, 2007; Phillips & McWilliam, 2009). The present study examines the efficacy of a molecular approach to identify the prey so that future studies can assess the impact of oceanographic events on prey abundance and health. This would be timely because a recent collapse in puerulus recruitment to the Western Australia coast has dramatically impacted the fishery (Brown, 2009), which has financial as well as ecological implications. Spiny lobsters are Australia’s most valuable fishery and western rock lobsters contributed over 60% of the catch up to the 2003 – 2004 financial year, but this contribution has trended down to 50% for 2009 – 2010 (ABARES, 2011). Following this trend the total export value of rock lobsters has declined from over AUS $600 million for 2003 – 2004 to under AUS $400 million for 2009 – 2011 (ABARES, 2011).

Determining the diet of spiny lobster larvae has been difficult, because the low densities and patchy distribution of these animals in the open ocean makes them difficult to observe and it is perceived to be prohibitively expensive to conduct blue water field studies (Ritz, 1972). Microscopic analysis of the gut contents of phyllosomas is also a difficult way to determine diet because many potential prey lack hard parts and often have transparent body morphology (Jeffs, 2007). Researchers have therefore relied on various methods to infer the diet of phyllosomas of several species. These methods include fatty acid profiling of wild phyllosomas (Jeffs et al., 2004; Phillips et al., 2006b), captive feeding trials (Mitchell, 1971; Saunders et al., 2012), examining limb, gut and mouthpart physiology (Cox & Johnston, 2004; Lemmens & Knott, 1994; Nishida et al., 1990), enzyme profiling (Johnston et al., 2004a), stable isotopes (Waite
et al., 2007a), and the sequencing of DNA from gut contents (Chow et al., 2010; Suzuki et al., 2006; 2007; 2008). These methods, combined with insights from the artificial culture of phyllosomas, suggest that these larvae may be generalist predators that consume gelatinous zooplankton such as Chaetognatha (arrow worms), Cnidaria, fish larvae, Salpa and soft-bodied arthropods. However, many details are still uncertain, including which species or taxonomic groups are targeted in the wild, and how this changes spatially, temporally and with developmental stage. Of the methods used to date, the DNA-based approaches have proved to be the most powerful to study the diet of phyllosomas because they have the capability to assign taxonomy to digesta obtained from the larval gut with a degree of resolution that other methods are not capable of.

In previous DNA studies PCR has been performed with universal primers on DNA extracted from the gut contents and a small random selection of PCR amplicons were cloned and sequenced using traditional techniques. In the first study of its type, one *Panulirus japonicus* phyllosomas from the Atlantic Ocean and two from the Pacific were analysed and returned amplicons from the phylum Cnidaria and the sub-phylum Urochordata (Suzuki et al., 2006). The same study found DNA of Urochordata and Cnidaria in the gut of one of two slipper lobsters (Scyllaridae) that were also analysed (Suzuki et al., 2006). A subsequent study used the same methods on eleven phyllosomas and detected DNA from teleost fish in three larvae (Suzuki et al., 2008). However, these PCR reactions generated an overwhelming quantity of lobster (host) PCR amplicons and numerous clones had to be screened prior to sequencing. The problem of host derived amplicons swamping the prey amplicons was significantly reduced in a subsequent study, that utilised a lobster specific peptide nucleic acid clamp (PNA-clamp) (Chow et al., 2010), a method that selectively blocks predator PCR
amplification and therefore enriches prey signal. Although such PCR enrichment techniques have been common in medical contexts for over two decades, they are only a recent innovation in ecological studies (Deagle et al., 2009; O’Rorke et al., 2012a; Vestheim et al., 2011; Vestheim & Jarman, 2008). Another improvement was to target a shorter genomic region (Chow et al., 2010), an approach that is consistent with the consensus approach to DNA diet studies (Beja-Pereira et al., 2009; King et al., 2008). By using PNA-clamping, ten out of thirty-nine phyllosomas were found to contain DNA from the phyla Cnidaria, Ctenophora, Arthropoda, Vertebrata (Teleostei) and Chaetognatha (Chow et al., 2010).

Despite the success of these DNA-based dietary studies, their authors have indicated several shortcomings that require additional innovations to enable an efficient DNA-based method to study the diet of phyllosomas. In particular, their results contained a high occurrence of PCR sequences that are unlikely to be credible prey sequences (Chow et al., 2010; Suzuki et al., 2006; 2008). Such sequences included host sequence variants (either PCR artefacts or pseudogenes), chimeras and also a high percentage of amplicons derived from microscopic eukaryotes with a questionable role in phyllosoma nutrition (Chow et al., 2010; Suzuki et al., 2008). Also, the small quantities of DNA obtained from the guts of phyllosomas makes DNA analyses highly susceptible to exogenous DNA contamination. These issues can be circumvented through sequencing a greater number of amplicons, which would allow genuine sequences to be identified amongst sequences that are PCR and laboratory artefacts (Pompanon et al., 2012). Therefore, the present study employs a high-throughput DNA sequencing approach to attempt to overcome these shortcomings, and represents a significant advance for this methodology for a wide range of such dietary studies. Using these methods over 30,000
amplicons were sequenced from the gut contents of eighteen phyllosomas of *P. cygnus* (stage VI and VII) taken from a single water mass 150 km offshore of Western Australia in an effort to establish if these methods can more accurately determine the composition of the diet of phyllosomas.

### 6.2 Methods

Sampling, DNA extraction, PCR and sequencing methods are described in Chapter 4.

#### 6.2.1 Bioinformatics

Basic bioinformatics were performed using custom PERL scripts and a brief summary follows. Amplicon reads were assorted to individual samples based on 454 MIDs and then split into the v7 and v9 18S rRNA loci using the PCR primer sequence. To quality control the sequencing output, any reads that did not perfectly match the MID or primer sequences were discarded. Reads were dereplicated, trimmed and then BLASTed (Altschul et al., 1990) against *P. cygnus*. A script was subsequently used to BLAST reads that did not match *P. cygnus* to the NCBI nucleotide database as at August 2011. The ten top-scoring BLAST hits were then returned, or in the case where the top BLAST hits were unannotated environmental sequences, the top one hundred hits were returned. Due to the nature of the BLAST (multiple good hits for a given query sequence), a method was adopted that summarised the taxonomic classification by checking for consistency amongst the top 10 hits. In the cases where the most closely-related sequence could not be easily determined, it was required that 6 out of the 10 hits belonged to the same taxon otherwise the taxonomic identity was labelled as “No consensus”. This was summarised at each of the seven taxonomic levels (Kingdom, Phylum, Class, Order, Family, Genus and Species). In the cases where there was a single best BLAST hit, then this was used as the final taxonomic classification. The
frequency of different organisms was then returned for each of the seven taxonomic
levels. This approach, of clustering sequences based on their best taxonomic hits also
mitigates the problem of grossly overestimating taxon richness that can result from
directly clustering sequences into OTUs (Kunin et al., 2010).

Table 6-1 Samples
Stage, length and source location of phyllosomas larvae used in study as well as duplicate negative (no
template) controls for contamination originating from DNA extraction (ext. neg) and PCR (PCR neg). 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. MIDs refer to Roche’s multiplex identifiers that uniquely identify samples in
the 454 GS reaction (Roche, 2010).

<table>
<thead>
<tr>
<th>ID</th>
<th>MID</th>
<th>MID</th>
<th>Date</th>
<th>Stage</th>
<th>Length (mm)</th>
<th>Latitude</th>
<th>Longitude</th>
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<td>1</td>
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<td>MID9</td>
<td>7-Jul</td>
<td>VII</td>
<td>14.2</td>
<td>30.72</td>
<td>113.52</td>
</tr>
<tr>
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<td>MID10</td>
<td>7-Jul</td>
<td>VII</td>
<td>15.5</td>
<td>30.72</td>
<td>113.52</td>
</tr>
<tr>
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<td>MID11</td>
<td>7-Jul</td>
<td>VII</td>
<td>17</td>
<td>30.72</td>
<td>113.52</td>
</tr>
<tr>
<td>4</td>
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<td>MID13</td>
<td>7-Jul</td>
<td>VII</td>
<td>17.1</td>
<td>30.72</td>
<td>113.52</td>
</tr>
<tr>
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<td>MID14</td>
<td>13-Jul</td>
<td>VI</td>
<td>14.5</td>
<td>30.70</td>
<td>113.83</td>
</tr>
<tr>
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<td>VI</td>
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<tr>
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<tr>
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<td>VI</td>
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<td>30.21</td>
<td>113.09</td>
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<tr>
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<td>MID14</td>
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<td>VI</td>
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<tr>
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<td>MID9</td>
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<td>VI</td>
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<td>30.21</td>
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<tr>
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<td>MID13</td>
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<td>13.5</td>
<td>30.14</td>
<td>113.34</td>
</tr>
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<td>MID10</td>
<td>ext neg</td>
<td>MID7</td>
<td>PCR neg</td>
<td>MID11</td>
<td>PCR neg</td>
</tr>
</tbody>
</table>

To assess if there was sufficient sequencing coverage to capture prey richness a
rarefaction curve was generated for both the v7 and v9 loci. For this, an OTU approach
was determined to be preferential because resolving taxonomy to fine levels is not
reliant on the completeness of the reference database. OTUs were generated at 93%,
95% and 97% similarity thresholds and curves were calculated with the software.
Analytic Rarefaction 2.0 (S. M. Holland, n.d.) that implements the equations of Tipper (1979).

6.3 Results

6.3.1 Samples
A total of 18 phyllosomas were recovered from five sampling events (Table 6.1). Phyllosomas were confirmed to be *P. cygnus* using the COI marker (Folmer et al., 1994) and were then determined to be either stage VI or VII (Table 6.1).

6.3.2 PCR amplicon sequences from the hepatopancreas of phyllosomas
A total of 100,693 sequencing reads were returned by the 454 GS Junior. Out of these reads 36,800 passed the stringent quality control requirements that both MIDs and primers had no mismatched bases. The average number of reads for each sample was 2041, and of these, the average number for the v7 locus was 752 (n=18) and 1289 for the v9 (n=18).

6.3.3 Taxonomic identity of DNA from samples
Of the quality controlled reads, 8,628 were a close match to lobster, with 3,837 reads coming from the v7 loci and 4,791 reads coming from v9. Although numerous lobster reads occurred in the dataset, none of these reads contained the PNA-clamp sequence. The negative controls for PCR reactions were empty for v9, but v7 yielded a small number of reads from vascular plants (probably pollen) and mammals (probably humans). The negative controls for DNA extractions yielded more DNA in the v7 and v9 loci, these were also from mammals, vascular plants (v9) and fungi. The negative control for the extraction also contained nine unidentified sequences and seven sequences for Bacillariophyta. As none of these taxa are relevant to this study and are most likely sampling or laboratory contaminants, the negative controls were excluded.
from the study, as were any occurrences of the taxa identified in the negative controls if they occurred in other samples.

Figure 6-1 Source of sequence reads among individual larvae. Relative distribution of combined reads for each sample to ascertain the proportion of potential prey reads against other kinds of reads. Samples 1, 2, 3, 5 and 11 had very little potential prey DNA, whereas samples 4, 7, 8, 10, 12, 13, 15 and 17 contained over 50%. Fungal DNA could originate from laboratory contamination, but along with algae, it is just as likely to originate from the gut and is not relevant to the current study. Contamination was either mammalian (probably human) or plant material and sequencing artefacts were mostly very short reads.

Samples 1, 2, 3, 5 and 11 yielded almost no zooplankton sequences and consequently a proportionately higher percentage of contamination, fungus or Palinuridae (i.e. predator) sequence (Fig. 6.1). This is consistent with there being no metazoan prey tissue in the hepatopancreas of the larvae and the PCR reaction amplifying any available template. These samples were excluded from further analysis. Sample 7 was negative for the v7 loci but yielded ample prey-reads in the v9 region. The sequencing results for sample 7 in the v7 region were similar to those for the v7 negative controls (i.e., contained reads for mammals and fungi); this suggests that there was insufficient template in the initial v7 PCR reaction. Accordingly, sample 7 was only included in subsequent analysis using the v9 region and where the v7 and v9 regions were compared it was excluded.
Once non-prey reads had been determined and removed from samples the average number of prey reads for the v7 locus was 546.7 (n = 12, range: 62–1280) and 1103.1 reads for the v9 locus (n=12, range: 34–2805). The slopes of the saturation curves rapidly approached asymptotes, which indicates that although there are multiple read types, sufficient sequencing reads were generated to capture major prey items and trends towards capturing full taxon richness (Fig. 6.2). Rank abundance of DNA reads showed that five taxa were very highly represented in both loci, that less than ten taxa were highly represented overall, and the remainder were represented in very small numbers (Fig. 6.3). The most abundant reads in both loci, were from the phyla and classes: Radiolaria (Polycistinea), Chordata (Thaliacea), Chordata (Actinopterygii), Cnidaria (Hydrozoa) and Chaetognatha (Sagittoidea), with these five taxa together composing 97.1% of v7 reads and 93.2% of v9 reads. In addition to these top-ranking classes, Malacostraca and Gastropoda contributed about 2% and 0.5% to each locus respectively.

There were minor differences in the results between the v7 and v9 loci with slightly more taxa uncovered using the v9 locus than the v7 locus (Table 6.2), although, these taxa were generally represented by less than 1% of reads. Asciidaeae (Chordata) occurred exclusively in the v9 locus. Discrepancies are most likely due to PCR amplification biases from primer binding, but these biases are small and do not impact on the detection of significant taxa, or even mildly significant taxa, because there is sufficient coverage to ascertain the range of taxa in a sample, which is evidenced by the species accumulation plot (Fig. 6.2). The v9 region is more variable than the v7. Therefore, although the same orders of taxa were identified with these loci when a clustering approach is used the v9 assigns reads into more OTUs at similar similarity
thresholds (Fig. 6.2). Unfortunately, because of gaps in the reference database for v9 it was not possible to resolve any higher order taxonomic assignments with this higher level of information.

![Rarefaction curves representing the number of OTUs detected in pooled samples for the (a) v7 and (b) v9 loci. OTUs are defined at 93%, 95% and 97% respectively and as the percentage threshold increases so does the number of OTUs detected. However, the estimate of OTU richness for each OTU threshold tends toward an asymptote indicating that there is sufficient sequencing coverage to detect most taxa.](image)

**Figure 6-2 Sampling saturation of (a) v7 and (b) v9 loci.**

6.4 Discussion

6.4.1 Pyrosequencing

The present study indicates that high throughput sequencing of short 18S rDNA PCR amplicons is a robust solution to the problem experienced in previous DNA diet studies of phyllosomas where the prevalence of non-target amplicons inhibited detection of prey amplicons (Chow et al., 2010; Suzuki et al., 2006; 2008). High throughput sequencing technologies have had an immense impact on studying bacterial community composition through mass sequencing of the 16S rDNA (e.g., Armougom & Raoult, 2008) and have gradually become significant for studies of complex eukaryotic assemblages (Amaral-Zettler et al., 2009; Behnke et al., 2010; Bråte et al., 2010; Fonseca et al., 2010). Compared to other high throughput sequencing technology the Roche 454 genome sequencer (454 GS-FLX) enables relatively long read length (Metzker, 2010). While other sequencing technologies are increasing their read length, the substantial lengths achieved with the 454 GS-FLX have made this platform an
attractive approach for ‘‘bar-coding’’ based ecological studies including diet studies (Deagle et al., 2009; Soininen et al., 2009; Valentini et al., 2009a). The 454 GS has been re-released in a smaller scale ‘‘Junior’’ platform that is affordable for a moderate-sized laboratory (Roche 2011). Using this affordable technology the present study was able to exclude contaminants and chimeras during bioinformatic analyses without having a dramatic impact on the quantity of sequence reads for analysis. This would not be possible with the number of sequence reads afforded in a traditional cloning effort.

By targeting the 18S ribosomal gene the present study overcame the problems that Chow et al. (2010) identified using the ultra-variable ITS1 region, that is, being unable to find sufficiently homologous sequences and the problem of length variation influencing PCR efficiency. However, a disadvantage of targeting short 18S regions is that the reads generally contain only sufficient information to determine their taxonomy to either the order or family level. This disadvantage was a deliberate trade-off considered in the experimental design, but it was more important for the hypothesis being tested that the PCR primers were capable of amplifying all metazoans, which is amply demonstrated by the wide range of taxa revealed from the digesta of the phyllosomas. Few loci besides the 18S rRNA gene have high coverage in public DNA sequence databases and also have primer-binding sites that are conserved across the Cnidaria, Ctenophora, Chaetognatha, Urochordata and Vertebrata. An unexpected benefit of choosing such conserved loci was the discovery of a predominance of DNA from colonial radiolaria, which was otherwise unanticipated and has rarely, if ever, been considered as a potential prey for phyllosomas (Jeffs, 2007). Another advantage of the conserved nature of the 18S rRNA is that the PNA-clamps work with all lobster species from the families Palinuridae and Scyllaridae. This is particularly attractive because this
protocol and its reagents can be reused to study the structure of the diets of the larvae of other species of lobster, which makes the approach very economical.

There was DNA for multiple taxa in the hepatopancreas of each phyllosoma, with the exception of phyllosoma 7, which returned 339 reads that exclusively matched chaetognatha, indicating Chaetognatha tissue as its sole dietary source. The temptation with community amplicon sequencing is to treat the sequence reads as quantitative or semi-quantitative, and therefore to treat higher frequency operational taxonomic units (OTUs) as having greater biological significance. However, the numbers of each sequence type can be influenced by a multitude of factors beyond prey concentration in the gut. Multi-copy genes such as ribosomal genes vary in copy number across different animals (Prokopowich et al., 2003). Read number may also vary due to PCR amplification bias prior to sequencing (Acinas et al., 2005; Suzuki & Giovannoni, 1996). Starting with a low quantity of genomic DNA template can also cause stochastic sampling errors to distort amplicon composition (Budowle et al., 2009; van Oorschot et al., 2010). To a limited extent amplification bias was anticipated and controlled by targeting two loci on the same gene. If there were little amplification bias it would be predicted that the same taxa could dominate rank abundance for v7 and v9 (Fig. 6.3) and that taxa would be ranked in the same order. The concordance between the results for these two loci indicates that PCR bias had little negative impact on determining prey items, and therefore relative abundance of reads at the level of class and order may reflect some relative differences in the consumption of prey species for each phyllosoma. This inference on the relative abundance of reads also tends to be supported by the overall general concordance of these data with the dietary profile.
among multiple phyllosomas provided by the presence/absence of DNA for the same range of taxa (Table 6.2).

### Table 6-2 Presence/absence of prey items across samples.

The frequency of prey taxa occurring across the twelve phyllosomas that contained traces of prey in the gut. Prey were identified to the hierarchical level of order and brackets denote the family-level of assignment to a sequence where it could be determined. “No consensus” refers to taxa that have too little database coverage to confidently assign a taxonomic order to the sequences.

<table>
<thead>
<tr>
<th>Class</th>
<th>Order</th>
<th>v7 18S rDNA</th>
<th>Frequency (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malacostraca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphipoda</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Decapoda</td>
<td></td>
<td>2 (Pandalidae)</td>
<td>2 (Pandalidae)</td>
</tr>
<tr>
<td>Euphausiacea</td>
<td></td>
<td>8 (Euphausiidae)</td>
<td>8 (Euphausiidae)</td>
</tr>
<tr>
<td>Maxillopoda</td>
<td>Calanoida</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Calanoida</td>
<td></td>
<td>1 (Metridinidae)</td>
</tr>
<tr>
<td>Sagittoidea</td>
<td>Aphragmaphora</td>
<td></td>
<td>8 (Sagittidae)</td>
</tr>
<tr>
<td>Actinopterygii</td>
<td>No Consensus</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Thaliacea</td>
<td>Dololida</td>
<td>2 (Doliolidae)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Pyrosomata</td>
<td>2 (Pyrosomatidae)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Salpida</td>
<td>9 (Salpidae)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>No Consensus</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Hydrozoa</td>
<td>Hydroida</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>No Consensus</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Siphonophora</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Trachylina</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>No Consensus</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Scyphozoa</td>
<td>No consensus</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Tentaculata</td>
<td>No consensus</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Holothuroidea</td>
<td>Apodida</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Cephalopoda</td>
<td>Teuthida</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Gastropoda</td>
<td>Thecosomata</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>No Consensus</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Polycystinea</td>
<td>Spumellaria</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

This study targeted very short reads of around 150 bp (v7) and 180 bp (v9). Although larger DNA fragments are useful for taxonomic assignment, they are in low concentrations in digesta (Deagle et al., 2006) and have proven difficult to amplify in diet studies of phyllosomas (Suzuki et al., 2006). Using very little DNA template and 50 PCR cycles this study generated sufficient amplicons to profile DNA from the hepatopancreas of the larvae, which is an achievement made possible by targeting shorter reads. Results from this study indicate that there are a sufficient number of amplicons generated through PCR, suggesting that fewer PCR cycles could be used in
future work, which would be advantageous because it would reduce the accumulation of PCR artefacts. This would not only reduce the number of base miscalls in prey DNA, but also reduce the number of lobster amplicons that have incorporated a replication error in the PNA-clamp region, which enables them to elude enrichment. It might also reduce the discrepancies between the v7 and v9 loci.

The approach presented in this study not only builds on DNA-based approaches to study the diet of phyllosomas, but it is a promising alternative to other methods. The cost of this method was around US $200 per locus per sample. However, because this value includes the initial optimisation of reactions, the purchase of bulk reagents, and due to the ever-decreasing cost of sequencing, it is anticipated that this cost will drop to less than US $100 per locus per sample. This price is more expensive than stable isotope analysis and comparable to the price of lipid analyses that have much less power for taxonomic resolution (Jeffs et al., 2004; Phillips et al., 2006b). Therefore, the better resolution of an enriched meta-genetic approach makes it a valuable tool for trophic analyses of phyllosomas and other pelagic predators.

6.4.2 Larval diet: taxa from gut DNA

The dataset from this study indicates that gelatinous zooplankton are significant in the trophic ecology of *P. cygnus*. This is entirely consistent with the hypothesised diet items that have been inferred from examining the mouthparts and feeding structures of phyllosomas (Jeffs, 2007). Tunicates, fish larvae, siphonophores and chaetognaths have been indicated as prey in the DNA diet studies of *P. japonicus* (Chow et al., 2010; Suzuki et al., 2006; 2008) as well as in experimental feeding of *P. interruptus* (Mitchell, 1971).
The discovery of DNA from colonial radiolarians in the guts of phyllosomas is a new addition to the groups of organisms associated with the diet of phyllosomas. Colonial radiolarians fit the profile of potential prey for middle to late stage phyllosomas because they are gelatinous zooplankton that cannot easily evade capture (Jeffs, 2007). The zooplankton assemblages in East Indian Ocean eddies have typically been assessed using plankton nets, a sampling technique that tends to destroy delicate zooplankton, and probably greatly under-represents colonial radiolarian density (Dennett et al., 2002). Less destructive sampling techniques such as video plankton recorders (VPR) demonstrate that net tows of identical water bodies under-represent the true concentration of colonial radiolarians by at least an order of magnitude and often by more than several orders (Dennett et al., 2002). The only video plankton recorder study of East Indian Ocean oceanic waters offshore from the coast of Western Australia found that colonial radiolarians were by far the most dominant member of the zooplankton assemblage (Stemmann et al., 2008). This abundance is not surprising because colonial radiolarians are very well suited to the extreme oligotrophic conditions found in this oceanographic region. The colonial radiolarian DNA identified in this study came from the families Sphaerozoidae and Collosphaeridae, which are monophyletic clades that are exclusively colonial and lack skeletons (Amaral-Zettler & Anderson, 1999). The Sphaerozoidae has species whose colonies can reach dimensions of over 2–3 m (Swanberg & Anderson, 1981; Swanberg & Harbison, 1980) making them large targets for passive encounter by drifting phyllosomas in the water column, and they could serve as both a refuge and food source for phyllosomas.
6.4.3 Interpreting multiple reads from hepatopancreas

Recovering amplicons from multiple taxa in each phyllosoma sample was not unprecedented. In their cloning based study Chow et al. (Chow et al., 2010) detected 0–4 taxa for each phyllosoma and one phyllosoma contained 10 different prey types. Of these reads, thirteen were from Ctenophora, six and four were unidentified homologues and the other seven reads were singletons. The presence of multiple reads could have several explanations; some reads are possibly sampling artefacts, phyllosomas may prey on multiple taxa, some taxa may be from secondary predation, and phyllosomas may engage in coprophagy. These possibilities are discussed in detail below.

Sampling artefacts could result from phyllosomas opportunistically feeding on animals in the cod-end of the zooplankton net or subsequently in sorting trays. Opportunistic net feeding has been reported in ecology studies for other predators (Harwood, 2008), however, this seems unlikely as phyllosomas appear to become incapacitated once captured in nets due to the fragility of their feeding appendages. Phyllosomas of slipper lobsters (of the same infra-order as P. cygnus) have been observed in the wild to cling to Cnidaria (Herrnkind et al., 1976; Shojima, 1963; Thomas, 1963). The Cnidaria could be prey, transport or both. This close association between the animals means that there is a risk that exogenous DNA adhering to the predator’s exterior could be detected through PCR and subsequent sequencing. To minimise this risk the exterior of the animals were washed when collected and prior to dissection, also the gut contents were syringed out of the hepatopancreas through a small gauge needle. By taking these measures the overwhelming majority of DNA extracted for this study came from the predator’s interior. The presence of multiple taxa inside each phyllosoma may be explained by a recent history of feeding on several different prey items. Examination of phyllosomas in culture conditions has demonstrated that they can be voracious feeders.
that are capable of rapidly processing prey (Cox & Johnston, 2003a; Johnston et al., 2008; Saunders et al., 2012). The digestive gland structure also includes a series of blind diverticula within which digestion processes may progress relatively slowly in comparison to the rate at which prey may be consumed (Cox, 2004). Therefore, it is conceivable that the gut contents of phyllosomas are a representation of recent prey feeding history in terms of the residual DNA retained throughout the extent of the digestive gland. Of the eighteen phyllosomas examined, five had no prey DNA signal, possibly due to recent absence of prey encounter, or a temporary halt to feeding which is known to occur when phyllosomas enter a moulting event (Tong et al., 1997). Of the remaining thirteen phyllosomas, colonial radiolarian DNA was predominant and was detected in twelve phyllosomas suggesting that it is a commonly consumed prey species. Thaliacea, predominantly Salpa, were the next most abundant class and were present in ten phyllosomas. Actinopterygii, Hydrozoa and Sagittoidea were each detected in nine larvae (Fig. 6.3).

DNA dietary studies can be very sensitive to secondary predation, where the food species that remains present in the digestive tract of the prey is co-amplified with prey DNA (Sheppard & Harwood, 2005). Phyllosomas can rapidly consume their entire prey (Jeffs, 2007), so it is likely that phyllosomas are also consuming the gut content of their prey. Directly targeting the gut of zooplankton is not an unusual trophic strategy in oligotrophic water (Janssen & Harbison, 1981), as the gut contains a conveniently concentrated and partially digested source of plankton. The gut/pseudo-gut contents of Urochordata, Cnidaria and Ctenophora are limited only by mouth size and many of the low abundance amplicons detected, such as Bacillariophyta, could well have been the food species of prey. Likewise, colonial radiolarian are known to consume various
zooplankton upon making contact with them (Angel, 1991). Although Chaetognatha are most often described as preying on copepods, this is most likely because most studies on chaetognatha have taken place in eutrophic waters where they are often an abundant and important predator of grazing copepods (Froneman & Pakhomov, 1998). However, the Chaetognatha have been found to consume various other prey such as Tinnitids, Appendicularia (Baier & Purcell, 1997) and Euphausiacea (Giesecke et al., 2010).

Chaetognatha also digest only around 80% of their prey (Dilling & Alldredge, 1993), which would provide sufficient residual DNA for it to be detected as secondary predation.

Many zooplankton opportunistically consume detritus (marine snow) such as faecal pellets, discarded appendicularian ‘houses’ and zooplankton carcasses (Dilling et al., 1998; Turner, 2002). In captive feeding studies P. cygnus were observed to frequently capture small pieces of suspended detritus to feed on (pers ob). If phyllosomas consumed detritus it would provide a further explanation for the detection of multiple taxa taken from gut samples. If phyllosomas are coprophagic then the most abundant DNA reads from the hepatopancreas are likely to be from the animal that excreted the

Figure 6-3 Rank abundance of potential prey reads after standardisation.
Rank abundances for the (a) v7 and (b) v9 loci. The x-axis shows the prey classes that constituted more than 0.5% of the sequencing reads. Samples were standardised into ratios of prey per predator prior to combining them into rank abundance.

Many zooplankton opportunistically consume detritus (marine snow) such as faecal pellets, discarded appendicularian ‘houses’ and zooplankton carcasses (Dilling et al., 1998; Turner, 2002). In captive feeding studies P. cygnus were observed to frequently capture small pieces of suspended detritus to feed on (pers ob). If phyllosomas consumed detritus it would provide a further explanation for the detection of multiple taxa taken from gut samples. If phyllosomas are coprophagic then the most abundant DNA reads from the hepatopancreas are likely to be from the animal that excreted the
particle and the sequences in lower abundance would be traces of that animal’s prey. However, because DNA is an excellent source of carbon, phosphate and nitrogen it is unlikely to last long in these conditions, so if the present study detected DNA from faecal pellets then they would have been recently excreted. For example, faecal pellets from tunicates are rapidly colonised and assimilated by microbes and other zooplankton that could rapidly digest residual DNA (Pomeroy et al., 1984). If it occurs, then coprophagy is likely to be an opportunistic feeding strategy by phyllosomas and not their primary dietary source because captive feeding studies and the physiology of phyllosomas all demonstrate active predation of zooplankton (Cox & Johnston, 2003a; Saunders et al., 2012). However, it is logical that a passive encounter feeder in oligotrophic waters will opportunistically consume marine snow. So despite the strong evidence that phyllosomas are active predators, marine snow and faecal pellets may potentially act as dietary subsidies. Eel larvae, or leptocephali, which like phyllosomas are a common feature of oligotrophic ocean waters, appear to have specialised in feeding on marine snow, especially the faecal pellets of zooplankton and discarded larvacean houses (Mochioka & Iwamizu, 1996; Terahara et al., 2011).

6.5 Conclusion.
The variety of gelatinous zooplankton that this study detected, and the possibility of a faecal subsidy, support the hypothesis that phyllosomas are generalist, opportunistic feeders of this type of prey. However, while this opportunism might provide a basal level of sustenance, it may be insufficient to deliver excess energy to store as lipids that are needed to fuel the migration of the pueruli to shore. Further work is required to ascertain the optimal food for phyllosomas and whether oceanographic processes impact the density of optimal prey groups and/or the ability of phyllosomas to capture these prey. Attempts to culture phyllosomas have found that feed is a strong determinate of larval viability, and supplementation of feed with mussel gonad has been found to be
essential for phyllosomas to advance through multiple instars (Kittaka 1997). More recently, a study of wild-caught mid-late stage P. cygnus phyllosomas reared under culture conditions and offered live freshly caught chaetognatha, salpa or krill, showed a clear preference for, and an ability to consume significant numbers of chaetognatha (Saunders et al., 2012). It was also shown that phyllosomas experimentally fed on chaetognatha over six days tended to accumulate lipid, which is known to be important for energy storage in phyllosomas for subsequent use during the pueruli migration to the coast (Jeffs et al., 2001a; Phillips et al., 2006b; Saunders et al., 2012). The spatial and temporal variability in the availability of these significant prey items could result in phyllosomas in poor nutritional condition, which in turn may contribute to pueruli having insufficient lipid reserves to recruit back to the shallow coastal environment (Phillips et al., 2006b; Wilkin & Jeffs, 2011).

Without knowing the diet of phyllosomas it is not possible to test the hypothesis that spiny lobster larval recruitment depends on successful feeding of the oceanic phyllosomas to fuel the shoreward migration of non-feeding pueruli. The DNA approach presented in the present study identifies a group of key animals that are clearly important in the trophic ecology of P. cygnus phyllosomas. While this approach cannot unequivocally establish that an animal is directly preyed upon, it is highly informative for providing an indication of the potential nutritional importance of prey taxa. For example, the results indicate colonial radiolarians are a predominant constituent of the prey DNA of phyllosomas. Although it cannot be confirmed that phyllosomas feed directly on colonial radiolarians, or via coprophagy or secondary predation, such as through chaetognatha, it remains the first time that colonial radiolarian have been associated with the diet of phyllosomas. Other taxa for which DNA was commonly
found in phyllosomas, included salpa, fish larvae, siphonophora and chaetognatha suggesting they are also likely to be significant to the trophic ecology of *P. cygnus*. The high-throughput amplicon sequencing approach presented in the present study enables further discovery of how the trophic connections of *P. cygnus* are spatially and temporally structured and how this relates to the nutritional condition of the phyllosomas.
Differences in the diet of larvae of the spiny lobster, *Panulirus cygnus*, from cyclonic versus anticyclonic eddies of the Leeuwin Current.

### 7.1 Introduction

The discovering of the diet of spiny lobster larvae, known as phyllosomas, has proved to be elusive despite extensive investigation (Jeffs, 2007). This is due to the offshore pelagic habitat of these larvae (Phillips et al., 1979), their transparent morphology, and the difficulty of their culture in the laboratory (Cox & Johnston, 2003a; Jeffs, 2007). In the last decade DNA techniques for determining the identity of material found in the gut have been used to ascertain the diet of a wide range of predators (Pompanon et al., 2012; Symondson, 2002) including the phyllosomas of the Japanese spiny lobster, *Panulirus japonicus* (Chow et al., 2010; Suzuki et al., 2006; 2008). Recently, an advanced DNA approach was successfully used to ascertain the diet of a small sample of phyllosomas of the Western Australian rock lobster, *P. cygnus*, in an effort to demonstrate further advancement of these DNA techniques for this species for the first time (O’Rorke et al., 2012b). The development of a technique to ascertain larval diet is timely for *P. cygnus* because a recent unexplained and dramatic decline in the fishery (Brown, 2009) necessitates improving our understanding of the ecology of this lobster species. Particularly because it is the basis of the most valuable commercial fishery in Australia and had an export value of over AUS $600 million for 2003–2004, which dropped to under AUS $400 million for 2009–2011 (ABARES, 2011). The *P. cygnus* fishery has been successfully managed for several decades, helped greatly by understanding the strong relationship between interannual of climatic cycles and the
large fluctuations of post-larval recruitment to coastal reefs and subsequent abundance of adults (Caputi, 2008; Caputi et al., 2001; Caputi et al., 1995; Pearce & Phillips, 1988). In 2007/08 the post-larval recruitment of *P. cygnus* deviated dramatically from these historical environmental correlations, and in the following year recruitment dropped to the lowest in 40 years (Feng et al., 2011). The cause of the dramatic decline in post-larval recruitment to this fishery is uncertain, but is thought to be a result of changing ocean conditions during the larval phase (Saunders et al., 2012).

Understanding how environmental and oceanographic factors affect the success of the larval phase could be the key to the future sustainable management of the stock. In the late Austral Autumn, persistent mesoscale eddies begin to form in the East Indian Ocean due to interaction of the strengthening of the seasonal poleward tropical current down the Western Australian coast (the Leeuwin Current) interacting with topological features (Pattiaratchi, 2006) and other currents with distinct thermohaline properties (Batteen et al., 2007; Rennie et al., 2007). Between latitudes 28°S to 31°S these mesoscale eddies, are formed as counter-rotating dipoles, which for their first 8 months average 100 km in diameter (Fang & Morrow, 2003) and can travel over 10° longitude (1,000 km) oceanward (Fang & Morrow, 2003) with anticyclonic, warm core eddies veering equatorward and cyclonic cold core eddies poleward (Morrow et al., 2004). The highest densities of developing phyllosomas are also found at this latitude, at distances of up to 1,500 km offshore (Phillips et al., 1979) apparently entrapped in these persistent eddies for a significant period of their larval development (Waite et al., 2007a). In 2011 it was found that phyllosomas were almost twice more abundant in the anticyclonic eddies than in cyclonic eddies (Chan, 2012), but that these phyllosomas were in far worse nutritional condition than their cyclonic counterparts (Wang et al., 2013a; 2013b). In particular, stage VIII phyllosomas on average contained 53% more
energy comprised of greater lipid reserves and protein levels than larvae in cyclonic eddies (Wang et al., 2013b).

A plausible hypothesis as to why phyllosomas are in poorer condition in anticyclonic eddies could be because they are feeding on very different prey. This is highly likely because although eddy formation is a mechanism through which highly oligotrophic waters such as the East Indian Ocean can be invigorated, counter-rotating eddy dipoles are invigorated in very different ways: Cyclonic eddies upwell nutrients from the deep, but anticyclonic eddies draw in nutrient-rich coastal water, as they are formed (Letelier et al., 2000; Waite et al., 2007b). Therefore, anticyclonic and cyclonic eddies in the East Indian Ocean form different food webs based on distinctly different phytoplankton communities with quite dissimilar capacities to utilise the differences in the available nutrients (Moore et al., 2007; Strzelecki et al., 2007; Thompson et al., 2007; Waite et al., 2007a; Waite et al., 2007b). In the late Winter and Spring these eddy systems are pronounced features of the East Indian Ocean (Fang & Morrow, 2003; Waite et al., 2007c), at which time the phyllosomas are developing into their later stages (Phillips et al., 1979) and this coincides with peak zooplankton densities in the East Indian Ocean (Feng et al., 2011; Tranter & Kerr, 1969). It is crucial that phyllosomas feed on a high volume of zooplankton prey during their lengthy larval phase because the energy that is accumulated during this phase is used to fuel the energetically demanding migration of the lecithotrophic (non-feeding) post-larvae from beyond the continental shelf into shallow coastal reefs where they settle (Lemmens, 1994; Lemmens & Knott, 1994). It also appears that a significant proportion of P. cygnus post-larvae typically have insufficient or borderline energy reserves to recruit onshore (Limbourn et al., 2009; McWilliam & Phillips, 1997; Phillips et al., 2006b), which has also been observed for
post-larvae of other species of spiny lobster (Fitzgibbon et al., 2013; Jeffs et al., 2001a; 2001b; Wilkin & Jeffs, 2011). The emergence of effective DNA techniques for determining composition of midgut contents of phyllosomas of *P. cygnus* provides an excellent opportunity to better understand if spatial and temporal variation of ocean conditions influence larval diet, which in turn may influence their recruitment success (O’Rorke et al., 2012b).

The initial aim of this study is to use DNA methods to characterise the diet of *P. cygnus* phyllosomas sampled from across cyclonic and anticyclonic eddies, because their diet is likely to be distinct. This study extends previous research of the diet of some *P. cygnus* phyllosomas that were sampled from a single cyclonic eddy in 2010, conducted 13 months prior to the present study (O’Rorke et al., 2012b). This previous study confirmed the effectiveness of the advanced DNA methods for determining prey from larval midgut contents. Therefore the present study builds on the goal of discovering the prey items of phyllosomas of *P. cygnus*, evaluating their diet in different environmental contexts, and also addresses the hypothesis that cyclonic and anticyclonic eddies influence the composition of zooplankton prey consumed by phyllosomas. To achieve this aim, phyllosomas of different mid- to late-developmental stages (VI, VII and VIII) were sampled from two pairs of counter-rotating eddies in the Leeuwin Current in 2011 and their midgut contents were determined using DNA methods (O’Rorke et al., 2012b).

### 7.2 Methods

Sampling, DNA extraction, PCR and sequencing methods are described in Chapter 4.
7.2.1 Samples and Sampling water controls

DNA was extracted from 48 phyllosomas in total. These phyllosomas were selected so that 12 were obtained from each of two cyclonic (CC1, CC2 - Fig. 7.1) and two anticyclonic eddies (WC1, WC2 - Fig. 7.1). From each eddy, four phyllosomas of stages VI, VII and VIII were randomly selected for further analysis, with the exception of WC1, where only three stage VIII phyllosomas were collected so a stage 9 phyllosoma was substituted in its place (Table 7.1).

![Figure 7-1 Map of sampling locations.](http://www.cmar.csiro.au/remotesensing/oceancurrents/SW/; accessed April 2012). Arrows indicate direction and velocity of geostrophic surface water flow and colour indicates the difference in temperature (°C) as measured by satellite.
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 techniques bring into close proximity animals that might not be trophically linked (Greenstone et al., 2012; King et al., 2012). It is conceivable that contaminating DNA could passively enter through the mouths of phyllosomas and into the midgut system when the phyllosomas are in the cod-end or sorting tray. To ascertain if this did occur, DNA was also extracted from sampling water taken from the net cod-end. For this, 50 ml of water was drawn from the sorting tray into a sterile syringe, then a 0.5 µm syringe filter (Millipore) was fitted to its end and 5 ml of water pushed through the filter 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 of late stage phyllosomas (Simon et al., 2012; Smith et al., 2009). A single sample water control (SW-control) was extracted for each eddy (4 in total). Before extraction, the EtOH preserved SW-controls were evaporated down using a Concentrator 5301 Centrifugal Evaporator (Eppendorf).

Table 7-1 Phyllosomas collected from each sample site.

<table>
<thead>
<tr>
<th>Eddy</th>
<th>Stage 6</th>
<th>Stage 7</th>
<th>Stage 8</th>
<th>Stage 9</th>
<th>Date</th>
<th>Lat</th>
<th>Long</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n / n'</td>
<td>Length (±SD) (mm)</td>
<td>n / n'</td>
<td>Length (±SD) (mm)</td>
<td>n / n'</td>
<td>Length (±SD) (mm)</td>
<td>Date</td>
</tr>
<tr>
<td>WC1</td>
<td>4 / 2</td>
<td>16.3 (±0.5)</td>
<td>4 / 4</td>
<td>17.3 (±1.2)</td>
<td>3 / 3</td>
<td>21.3 (±0.6)</td>
<td>0 / 0</td>
</tr>
<tr>
<td>WC2</td>
<td>4 / 1</td>
<td>16.2 (±1.3)</td>
<td>4 / 4</td>
<td>16.9 (±2.1)</td>
<td>4 / 3</td>
<td>19.4 (±0.6)</td>
<td>0 / -</td>
</tr>
<tr>
<td>CC1</td>
<td>4 / 4</td>
<td>16.3 (±0.7)</td>
<td>4 / 3</td>
<td>20.1 (±1.0)</td>
<td>4 / 2</td>
<td>22.0 (±2.2)</td>
<td>0 / -</td>
</tr>
<tr>
<td>CC2</td>
<td>4 / 4</td>
<td>17.3 (±1.7)</td>
<td>4 / 4</td>
<td>18.1 (±1.1)</td>
<td>4 / 1</td>
<td>19.1 (±1.7)</td>
<td>0 / -</td>
</tr>
</tbody>
</table>

7.2.2 Bioinformatics

Assorting amplicons to their respective samples using multiplex identifiers (MIDs) was performed on 454 GS FLX data using Geneious v5.6 (Kearse et al., 2012). FASTA and qfiles 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 samples were uniqued (de-replicated). Uniqued reads were aligned with MAFFT (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 preclustering 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. Fungi were left in for the analysis because they might be food, or they might alternatively indicate the feeding condition of phyllosomas because of the potential that fungi are amplified in the absence of any alternative prey item.

There are several equally valid approaches to grouping and analysing data, which can affect the results of analyses. In addition to the approach used here, where reads are first clustered into OTUs and then assigned a taxonomic identity, the taxonomic identity of each read can be assigned first and then are clustered second based on the similar taxonomic identities. Also, because PCR can distort abundances, it is common to transform all OTUs to presence/absence data for each sample. This is an extreme transformation and loses a good deal of information and instead, where possible, the compositional component of the dataset was retained, which is why the present study uses two linked loci to independently test hypotheses. However, statistical tests on each of these possible data treatments have been performed to confirm that the results are supported under all data treatment scenarios.
### 7.2.3 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 & Warwick, 2005). Analyses were run on the v7 and v9 datasets independently. Non metric multi-dimensional scaling and cluster analysis were then used to evaluate the relatedness of samples to SW-controls to samples 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 rho correlation, and associations with a magnitude exceeding 0.6 were plotted as vectors to indicate which OTUs structure the MDS ordination. Also in Primer 6, permutational multivariate analysis of variance (PERMANOVA, Anderson, 2005; Anderson et al., 2008) was applied to the Bray Curtis matrices of fourth root transformed OTU data to test the validity of the null hypotheses that no factors structured the diet of phyllosomas ($\alpha=0.05$). Three factors were tested in the PERMANOVA: Dipole, Stage and Site. Dipole refers to whether samples came from an anticyclonic or cyclonic eddy, Stage to whether the samples were of stage VI, VII or VIII, and Site refers to the whether phyllosomas were sampled from the same eddy site. As such, Site was nested inside Dipole, and Stage was crossed with both Dipole and Site(dipole) to see if interactions between these factors accounted for variance. Type III (partial) sums of squares were used for comparisons because the Dipole factor only has one degree of freedom and the sampling design was unbalanced because of the exclusion of some samples due to low reads. Jacobs’ modification of Ivlev’s electivity index $D$ (Jacobs, 1974) was calculated for each phyllosoma for each prey type and then averaged ($\pm$SD). The index is given by: $D = (r - p)/(r + p - 2pr)$, where $p$ is the proportion of prey in the environment and, as is used by Kowalczyk et al., (2011), $r$ is the proportion of pyrosequencing reads from in the diet.
7.3 Results

7.3.1 Sampling, DNA extractions and sequencing
Yields of DNA extracted from each phyllosoma were relatively high at an average of 76.84 ng µL⁻¹ ± 47.32 (Table 7.1). Yields of DNA from extraction negative controls and water from cod-end matched water samples were negligible and within the margins of error around zero for accurate measurement (Fig. 7.1). 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*. 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.

7.3.2 Zooplankton assemblages from the midgut of phyllosomas
For each DNA loci, 500 reads were subsampled from each phyllosoma and phyllosomas with <500 reads in either loci were removed from subsequent analyses. This meant that two samples were removed from WC1, four samples were removed from WC2, and three each removed from CC1 and CC2. One of the sample water controls (SW-CC2) also fell below 500, but was included in subsequent analyses because of its importance as a control.

The midgut contents of phyllosomas from each sampled eddy were visualised in one of two ways: by the number of phyllosomas containing a particular kind of prey item, (Table 7.2) or by the relative abundance of reads that assigned to separate prey items (Fig. 7.2). Across all the phyllosomas the most abundant class of prey amplified from their midguts was from the zooplankton class Hydrozoa (v7: 21.9% reads; v9: 23.3%...
reads), predominantly the order Siphonophora (16.7%; 14.7% reads), which was
detected in phyllosomas from all four eddies and detected in the midgut of all but one
phyllosoma (Table 7.2). The Class Actinopterygii (bony fish) was also detected in all 36
phyllosomas across all four eddies for the v7 loci and the v9 loci (Table 7.2). 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. 7.2). Polycystinea (almost entirely from the order Spumellaria)
were also abundant (v7: 13.1%; v9: 17.1%), especially in phyllosomas in WC2, CC1
and CC2 (Fig. 7.2) and although in low abundance was present in most phyllosomas in
WC1 (Table 7.2).

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 WC1, CC1 and CC2 (Fig. 7.2, Table 7.2).
Anthozoa occurred in phyllosomas sampled from anticyclonic eddies and a single
phyllosoma in CC1 and CC2 (but only one loci), but were numerically greater in
phyllosomas sampled from WC1 (Fig. 7.2). There was a general pattern that the most
abundant taxa were detected in phyllosomas across all sampled eddies. The main
exception to this was the scyphozoan Semaeostomeae, which was present in 8 of 9
phyllosomas in CC2, and in a single phyllosomas in CC1, but in neither WC1 nor WC2,
(Table 7.2). Scyphozoa were also highly abundant in phyllosomas from CC2, but no
scyphozoan order contributed any more than a negligible number of amplicons inside
phyllosoma sampled from any of the other three eddies.
7.3.1 Sampling water control

Three of the four SW controls (SW-WC1, SW-CC1, SW-CC2) were dominated by one or two organisms (Fig. 7.2). Polycystinea was a relatively abundant taxon in all cod-end matched samples, but dominated those of SW-WC1 and SW-CC1 (~80% of amplicons). Polycystinea contributed only marginally to the composition of DNA detected in phyllosomas sampled from SW-CC1, but this taxon did contribute around 10% to the midgut contents of phyllosomas in CC1, indicating that this might have been an influencing factor. The v7 and v9 loci in SW-CC2 were quite variable with the v9 being dominated by amplicons from Ascomycota and then Polycystinea, but the v7 loci was equally dominated by Malacostraca and Polycystinea. The abundances of zooplankton
Table 7-2 Prey from midgut.
The taxonomy of prey items detected in the midgut of phyllosomas and the frequency with which these taxa occurred among the sampled phyllosomas (i.e., count data). The four main columns (WC1, WC2, CC1 and CC2) each represent an eddy that was sampled. The colon separates the frequencies for each of the v7 and v9 loci.

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>WC1 (10)</th>
<th>WC2 (8)</th>
<th>CC1 (9)</th>
<th>CC2 (9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromalveolata</td>
<td>Apicomplexa</td>
<td>Gregarinia</td>
<td>Eugregarinida</td>
<td>0:5</td>
<td>1:3</td>
<td>0:2</td>
<td>1:3</td>
</tr>
<tr>
<td></td>
<td>Ciliophora</td>
<td>Oligohymenophorea</td>
<td>Apostomatida</td>
<td>0:0</td>
<td>1:1</td>
<td>0:0</td>
<td>0:0</td>
</tr>
<tr>
<td>Excavata</td>
<td>Euglenozoa</td>
<td>Kinoplastida</td>
<td>unclassified</td>
<td>0:5</td>
<td>0:3</td>
<td>0:8</td>
<td>0:3</td>
</tr>
<tr>
<td>Fungi</td>
<td>Ascomycota</td>
<td>Dothideomyctes</td>
<td>Capnodiales</td>
<td>1:0</td>
<td>2:0</td>
<td>1:0</td>
<td>1:0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pleosporales</td>
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<td>0:0</td>
<td>0:0</td>
<td>1:0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Eurotiales</td>
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<td>6:6</td>
<td>7:6</td>
<td>9:7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>8:9</td>
<td>6:6</td>
<td>7:8</td>
<td>2:2</td>
</tr>
<tr>
<td></td>
<td>Basidiomycota</td>
<td>Exobasidiomyctes</td>
<td>Malasseziales</td>
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<td>0:5</td>
<td>3:0</td>
<td>1:2</td>
</tr>
<tr>
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<td></td>
<td></td>
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<td>1:2</td>
<td>1:0</td>
<td>1:0</td>
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<td>Malacostraca</td>
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<td>0:0</td>
<td>0:0</td>
</tr>
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<td></td>
<td></td>
<td></td>
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<td>0:1</td>
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</tr>
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<td></td>
<td></td>
<td>Euphausiaclae</td>
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<td>7:8</td>
<td>8:9</td>
<td>5:7</td>
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<tr>
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<td>Aphragmaphora</td>
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<td>5:3</td>
<td>8:8</td>
<td>6:8</td>
</tr>
<tr>
<td></td>
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<td>Actinopterygii</td>
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<td>8:8</td>
<td>9:9</td>
<td>9:9</td>
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<tr>
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<td>1:0</td>
<td>2:2</td>
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<tr>
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<td></td>
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<td>1:0</td>
<td>0:0</td>
<td>2:0</td>
<td></td>
</tr>
<tr>
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<td>8:9</td>
<td>9:9</td>
<td></td>
</tr>
<tr>
<td>Cnidaria</td>
<td>Anthozoa</td>
<td>Ceriantharia</td>
<td>10:10</td>
<td>6:5</td>
<td>1:0</td>
<td>0:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hydrozoa</td>
<td>Anthomedusae</td>
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<td>0:0</td>
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<tr>
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<td></td>
<td>Leptomedusae</td>
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<td>1:1</td>
<td>1:1</td>
<td>0:1</td>
<td></td>
</tr>
<tr>
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<td>1:0</td>
<td>6:6</td>
<td>7:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Siphonophora</td>
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<td>10:10</td>
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<td>9:9</td>
<td>8:8</td>
<td></td>
</tr>
<tr>
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<td>1:0</td>
<td>0:0</td>
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</tr>
<tr>
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<td>6:6</td>
<td>5:3</td>
<td></td>
</tr>
<tr>
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<td>Coronatae</td>
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<td>4:7</td>
<td>1:4</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Semaeostomeae</td>
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<td>0:1</td>
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</tr>
<tr>
<td></td>
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<td>Tentaculata</td>
<td>Lobata</td>
<td>8:5</td>
<td>0:0</td>
<td>3:2</td>
<td>1:1</td>
</tr>
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<td>2:1</td>
<td>4:3</td>
<td>1:1</td>
</tr>
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<td>unclassified</td>
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<td>Mollusca</td>
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<td>Hypsogastropoda</td>
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<td>0:0</td>
<td>0:0</td>
<td>0:1</td>
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<td>Thecosomata</td>
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<td>2:3</td>
<td>6:7</td>
<td>6:7</td>
<td></td>
</tr>
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<td>Rhizaria</td>
<td>Cestoidea</td>
<td>Phaeodarea</td>
<td>Phaeosphaerida</td>
<td>1:0</td>
<td>1:0</td>
<td>0:1</td>
<td>0:0</td>
</tr>
<tr>
<td></td>
<td>Radiolaria</td>
<td>Polycystinea</td>
<td>Spumellaria</td>
<td>6:8</td>
<td>8:8</td>
<td>9:9</td>
<td>7:8</td>
</tr>
</tbody>
</table>

taxa in the SW-WC2 control 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 WC2
phyllosomas and their matched cod-end sample (SW-WC2) was that the phyllosomas contained ~15% fungal DNA from the Ascomycota, while the matched cod-end sample contained much less than 1%.

7.3.2 Does stage determine diet?

Diet did not vary between the three consecutive developmental stages of phyllosoma, i.e., VI, VII and VIII (Table 7.4). This statistic is corroborated by inspection of the MDS plot (Fig. 7.3), which shows that the distance between the different stages is not consistent. Neither is the interaction between Stage and Dipole or Stage and Site significant (Table 7.4). However, several phyllosomas were excluded from analyses, which resulted in an unbalanced design that meant some of these crossed factors contained empty cells and were not analysed.

Table 7-3 Prey preference metrics for phyllosomas.

Groups of zooplankton and fungi and their total proportion in the phyllosoma midgut contents. Proportion of zooplankton in water column refers to percentage of total biovolume that each group contributes. Jacobs’ modification of Ivlev’s electivity index gives a value from 1 for preferred prey to -1 for least preferred prey. Electivity indices were calculated for each individual phyllosoma for each prey group and then averaged.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Proportion of Diet % v7 : v9</th>
<th>Proportion of zooplankton Biomass</th>
<th>Electivity Index* v7 : v9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siphonophora</td>
<td>13.5 : 15.4</td>
<td>29.05</td>
<td>-0.30(±0.10) : -0.24(±0.10)</td>
</tr>
<tr>
<td>Polycystinea</td>
<td>13.1 : 17.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinopterygii</td>
<td>24.8 : 10.4</td>
<td>0.05</td>
<td>0.99(±0.00) : 0.99(±0.00)</td>
</tr>
<tr>
<td>Medusae**</td>
<td>12.44 : 10.5</td>
<td>3.29</td>
<td>0.36(±0.11) : 0.45(±0.09)</td>
</tr>
<tr>
<td>Ascomycota</td>
<td>7.1 : 8.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thaliacea</td>
<td>6.3 : 8.1</td>
<td>58.49</td>
<td>-0.90(±0.03) : -0.89(±0.04)</td>
</tr>
<tr>
<td>Anthozoa</td>
<td>5.5 : 7.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euphausiacea</td>
<td>4.3 : 7.5</td>
<td>3.75</td>
<td>-0.21(±0.11) : 0.19(±0.09)</td>
</tr>
<tr>
<td>Sagipitoidea</td>
<td>1.2 : 2.9</td>
<td>0.21</td>
<td>0.22(±0.13) : 0.39(±0.13)</td>
</tr>
<tr>
<td>Appendicularia</td>
<td>1.9 : 1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastropoda</td>
<td>1.2 : 1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basidiomycota</td>
<td>2.8 : 0.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Biovolume prey per cubic meter of water rather than per total biomass.
[Makes the assumption that fish: 0.1 ml, Siphonophore: 20 ml, krill: 0.6 ml, Arrow worm: 0.1 ml, Thaliacea: 50ml, Medusa: 30 ml]

**(Scyphozoa+Antomedusae+Narcomedusae+Leptomedusae+Trachymedusae)
7.3.3 Does eddy dipole determine diet?

Whether phyllosomas are feeding in anticyclonic or cyclonic eddies has no significant effect on their midgut composition, because any significant variation at the dipole level can be entirely accounted for by the variation between sampling sites (Table 7.4).

Furthermore, there is considerable similarity among the four sites with the MDS plot indicating only small distances between the mean centroids for each eddy (Fig. 7.3) and reasonably high residuals indicate that much of the variation is not accounted for. This indicates the consistent contribution of particular OTUs across all eddies. Significantly, there were particular clades that occurred across all sampling sites: siphonophores, fish and colonial radiolaria. The repeated occurrence of these taxa indicates that they are an important component of diet of phyllosomas and it is indicated that there is strong selective feeding on bony fish (electivity index v7: 0.99, v9: 0.99) and, to a lesser extent,

Table 7.4 PERMANOVA
Results of permutational multivariate analysis of variance (PERMANOVA) for v7 (i) and v9 (ii) loci. Factors are dipole, refers to whether samples are located in an anticyclonic (WC1, WC2) or cyclonic eddy (CC1, CC2), site refers to the particular eddy sampled and stage refers to the developmental stage of the phyllosoma sampled.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>Pseudo-F</th>
<th>P (perm)</th>
<th>P (Monte Carlo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. Dilution</td>
<td>1</td>
<td>8533.6</td>
<td>1.2153</td>
<td>0.0001</td>
<td>0.3268</td>
</tr>
<tr>
<td>Site</td>
<td>3</td>
<td>1301.1</td>
<td>0.9326</td>
<td>0.5749</td>
<td>0.5914</td>
</tr>
<tr>
<td>Site(Dipole)**</td>
<td>2</td>
<td>7023.0</td>
<td>4.5230</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Dipole x Stage**</td>
<td>2</td>
<td>1382.1</td>
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<tr>
<td>Total</td>
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<td>Total</td>
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</table>

*Term has one or more empty cells
arrow worms (electivity index v7: 0.22, v9: 0.39), but the relatively high biomass of siphonophores in the water column suggests the high proportion of midgut DNA from siphonophores is due to opportunistic feeding (electivity index v7: -0.30, v9: -0.24; Table 7.4).

Figure 7-3 An nMDS based on taxa occurring in midgut of phyllosomas. Ordination is based on a Bray Curtis resemblance matrix of log transformed data derived from sequencing i) the v7 18S rDNA, and ii) the v9 18S rDNA loci. Stages 6 ( ), 7 ( ), 8 ( ) and a single stage 9 ( ) as well as the matched cod-end sample (×). Each eddy is colour coded (refer key). Samples inside the black line share over 30% resemblance by cluster analysis. 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 vector OTUs are from the taxonomic orders: i) 1 Ceriantharia 2 Calanoida 3 Siphonophora 4 Copelata 5 unclassified Cnidaria 6 Coronatae 7 Trachymedusae 8 Semaeostomeae, and for ii) 1 Ceriantharia 2 Calanoida 3 unclassified Cnidaria 4 Trachymedusae 5 Siphonophora 6 unclassified Tunicate 7 Spumellaria 8 Semaeostomeae 9 Salpida.

7.4 Discussion

7.4.1 Midgut Contents of Phyllosomas

Overall, the prominent prey items of all phyllosomas, in descending order of abundance, were Hydrozoa (21.3% reads), Actinopterygii (bony fish; 17.2% reads) and
Polycystinea (colonial radiolarians; 15.7% reads) (Fig. 7.2). These three taxa were also the most abundant in the midguts of twelve phyllosomas of the same developmental stages taken from a single cyclonic eddy in the same vicinity as WC2 but 13 months earlier (O’Rorke et al., 2012b), which indicates the relative importance of these prey items. A total of 67.6% of Hydrozoa were from the order Siphonophora, a group which has also been observed to be a significant prey taxa for phyllosomas of *J. edwardsii* (O’Rorke et al., 2013b). Siphonophores play a major role in the diurnal formation of the deep scattering layer (DSL, Barham, 1963; 1966) so the benefit of phyllosomas targeting these animals could be that they represent a localised maxima of biomass in the water column. In the present study Polycystinea (colonial radiolaria) dominated the midgut contents of phyllosomas in WC2 and CC2 more than in CC1, and were barely present in phyllosomas in WC1 (Fig. 7.2). They were also the most dominant reads in phyllosomas in the 2010 cyclonic eddy (O’Rorke et al., 2012b). Colonial Radiolarians are highly fragile and therefore difficult to quantify using traditional net tow methods (Dennett et al., 2002; Stemmann et al., 2008), but it was noticed whilst sampling that there appeared to be more associated with eddies and waters with a greater intrusion of subtropical water (*Personal observ.*).

Arrow worms (Sagittoidea) were not as abundant in the midgut contents of phyllosomas in the present study (1.9% reads) as they were in samples taken in the previous year (9.3% reads) (O’Rorke 2012), which may reflect the overall lower abundance of arrow worms in the 2011 plankton assemblages (Chan, 2012) compared to the 2010 assemblage (Sawstrom unpubl. data). However, despite the lower abundance of arrow worm reads in midgut contents, they did occur in many of the midguts of phyllosomas within all the eddies that were sampled in the present study (Table 7.2). The present study also uncovered some taxa from the midgut of phyllosomas that had not previously
been identified as prey items, with Anthozoa being a particular feature of WC1 (22.1% of reads at WC1, <1% elsewhere). These Anthozoa were from the order Ceriantharia (tube anemones), which have an extended pelagic larval stage of up to several months in duration (Molodtsova, 2009) and some are only known as pelagic species (Molodtsova, 2004; Shanks & Walters, 1997). Anthozoan larvae are often associated with marine snow (Shanks & Walters, 1997) and their presence in the midgut of phyllosoma is consistent with phyllosomas scavenging on detritus. Multiple OTUs were detected in all phyllosomas and could indicate the presence of secondary predation, that is, that the method is also detecting the prey of prey (Sheppard & Harwood, 2005). Phyllosomas typically consume the entirety of their prey (Cox & Bruce, 2003; Saunders et al., 2012; Wakabayashi et al., 2012) and prey items such as chaetognaths, siphonophores, polycystinea and krill contain numerous prey items and the partially digested matter could be a rewarding meal (Angel, 1991; Biggs, 1976).

Thaliacea (6.9% reads) were not as abundant in midgut contents as they were in 2010 phyllosoma samples (O’Rorke et al., 2012b) and mostly occurred in the cyclonic eddies, particularly CC2 (Fig. 7.2). A small amount of DNA from Hyperiidea amphipods was however observed in four phyllosomas in WC2 (Table 7.2; although a similar amount of Hyperiidea was in the corresponding SW-control), but such Amphipods are typically associated with Thaliaceans and might be evidence of their presence. Scyphozoa were also a conspicuous prey item in CC2 (26.9%), but were not prominent in the phyllosoma samples from the other three eddies (<1%).

### 7.4.2 Detection of fungi and parasites

Fungi were a common feature of the midgut assemblage of *P. cygnus* phyllosomas (~10% reads), but was significantly less abundant than the levels of 60% of reads
discovered in a comparative DNA diet study of phyllosomas of *Jasus edwardsii* (O’Rorke et al., 2013b), which is an Australasian temperate species with a larval duration more than twice that of *P. cygnus* (Phillips et al., 2006a). The fungi detected in the present study were from the sub-kingdom Dikarya, which is typical of marine fungal assemblage (Richards et al., 2012). Dikarya must always be in contact with a food substrate, which indicates that they are either commensals in the midgut of phyllosomas or are co-ingested with prey and POM. If it is the latter, then it is interesting that *P. cygnus* contains less fungi than *J. edwardsii*, because this suggests that in the East Indian Ocean that POM is allowed less time to be colonised by saprotrophic microbes. This may indicate that detritus might be quickly cycled in this environment, possibly because of a higher density of zooplankton. The midgut contents of phyllosomas that had consumed salps, which are filter feeding detritivores, were not correspondingly enriched in fungus, as would be expected if detritus was allowed time to be colonised (Fig. 7.2). Furthermore, it has been found that POM in both anticyclonic and cyclonic eddies is highly enriched in $\delta^{15}N$, to levels as high as those of heterotrophs, which indicates recycling of nutrients (Waite et al., 2007a).

Gregarinia are common parasites of oceanic crustaceans and were detected in 16 phyllosomas (Table 7.2) distributed across all eddies. Gregarinia have been detected in wild phyllosomas of *J. edwardsii* (O’Rorke et al., 2013b), but were not found in the *P. cygnus* phyllosomas sampled in 2010 (O’Rorke et al., 2012b). There is good evidence that migration to regions of low biomass, as undertaken by phyllosomas of *P. cygnus*, are a means to improve the survival of disease susceptible larvae (Altizer et al., 2011; Kelly et al., 2010; Poulin et al., 2012) because migration removes them from high density areas where disease transmission is more probable (Behringer & Butler, 2009; Butler et al., 2008). Assessing whether these parasites have an impact on phyllosomas
would potentially have high utility for understanding and predicting \( P. \text{cygnus} \)
population dynamics.

### 7.4.3 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 (Acinas et al., 2005; Suzuki & Giovannoni, 1996), although the extent of this distortion varies between PCR primer sets and loci (Deagle & Tollit, 2007; Tollit et al., 2006). The justification behind using two linked loci is that discrepancies between the read compositions of the loci would help detect the extent to which PCR and sequencing artefacts distort the detection of the prey items of phyllosomas, but in general the composition of reads was fairly consistent between the v7 and v9 loci for the most abundant taxa, which is therefore grounds to assume that PCR and sequencing errors did not significantly distort the abundances.

There was a small amount of variation between loci for the classes Malacostraca and Polycystinea, and substantial variation between loci for the teleost fish (Table 7.3 and Fig. 7.2). In WC1, WC2 and CC1 eddies the abundance of teleost reads relative to other reads for the v9 locus was less than half that in the v7 locus (Fig. 7.2). For samples from eddy CC2, the Actinopterygii contributed almost 14% of reads for the v7 loci, but the contribution for the v9 loci was 5.2% (Fig. 7.2), although Actinopterygii were detected in four of the nine samples taken from CC2. The fourth root transformation of data is sufficient to down weight this discrepancy in analyses. For the application of the electivity index (Table 7.4), the absence of reads from a taxa appears to have a more dramatic impact on the result than if there is variation in the proportion of reads. For
this reason there is a substantial discrepancy between the v7 and v9 electivity indices for krill, which are -0.21 and 0.19 respectively (Table 7.4), this is because there were seven phyllosomas in which krill was detected in the v7 but not the v9 loci (Table 7.3), which alters the index severely. In future studies it would be beneficial to use a third loci to resolve the electivity indices for such prey items that “drop out”. Across all phyllosoma 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.

The parasitic class Gregarinia (Kingdom: Chromalveolata) was detected in the v9 but not the v7 loci (Table 7.2). A small number of Echinodermata, Annelida, and Hemichordata occurred in one locus but not the other, but the total abundance of these reads contributed less than 2% to the overall number of reads which makes them likely to be an opportunistic rather than a targeted prey item or else secondary prey. Also, there was a substantial discrepancy between the v7 and v9 regions for the sampling water control SW-CC2 (Fig. 7.2). This is probably because the undetectably small yields of DNA extracted from all SW controls. Small quantities of DNA template can lead to highly random PCR outcomes (Jacobs 1999), so it is surprising that the discrepancy occurred in only one sample.

A comparison of the composition of the SW controls with the composition of the phyllosomas midgut contents vindicates that the results of this study are unlikely to be artefacts of sampling by net. The relative abundances of plankton detected in the SW controls appeared quite distinct from that of the phyllosomas midgut for eddies CC1, CC2 and WC1(Fig. 7.2) and, 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. 7.3). Therefore, the distinctive character of SW-controls at CC1, CC2 and WC1 show that if there was any passive ingestion of DNA by any phyllosoma, that it was not sufficient to bias the results. However, SW-WC2 clustered with some of the phyllosomas from WC2, indicating that passive ingestion of DNA after sampling could have unduly influenced the midgut contents of these individuals.

There was considerable variation in the absolute number of reads that were detected in each phyllosoma. While this might be evidence for variable feeding success between phyllosomas the present study can be taken 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 read abundances are preserved through multiple PCR rounds, emulsion PCR (particularly with MIDs) and pyrosequencing. Some phyllosoma samples were removed from subsequent analyses because they did not reach a critical read threshold of 500. Low yielding PCR’s of mixed assemblages typically give stochastic and unreproducible results and are therefore undesirable for analysis. The most likely reason for these low yielding results is insufficient DNA template, probably due to the midguts of phyllosomas being empty.

Roughly the same number of samples were removed from each sampled site and because six samples were removed from each of the anticyclonic and anticyclonic eddies this cannot be taken as an explanation for the variable nutritional condition of phyllosomas under this factor. There are multiple reasons why the midgut of a phyllosoma might be empty (O’Rorke et al., 2012b), but one likely explanation is that they have not encountered prey. Therefore, a quantitative approach to characterise midgut fullness would be a valuable study for the future because it could be used to
ascertain the extent of food deprivation. The ratio of prey amplicons to predator-derived PCR artefacts might be an approach to achieving this, but requires validation.

### 7.4.4 Ecology

*Does diet differ between late developmental stages?*

There is no evidence that the different late stages (i.e., VI, VII, VIII) of *P. cygnus* phyllosomas feed on different prey. This is because there is no significant differences in midgut contents either within eddies or overall (Table 7.4), despite the plausibility that the developmental stages of the phyllosomas influences their ability to capture and feed on various prey items as has been indicated for other species of spiny lobster (Jeffs et al., 2004; Kittaka, 2000; Saisho, 1966). This finding cannot be extrapolated to earlier stages, given that there are strong indications that the diet of phyllosomas differs between early, middle and later stages. For example, the different levels of development of gut and mouthpart physiology (Cox & Johnston, 2003b; 2004), and because late stage *P. cygnus* occupy different positions in the water-column than the early (Rimmer & Phillips, 1979) and middle stages (Chittleborough & Thomas, 1969), which would expose them to different zooplankton assemblages.

*Does diet vary between anticyclonic and cyclonic eddy dipoles?*

In 2011, the phyllosomas in cyclonic eddies were in distinctly better condition than those in anticyclonic eddies (Wang et al., 2013a; 2013b) with energy levels of stage VIII phyllosomas from cyclonic eddies more than 50% more than phyllosomas from anticyclonic eddies. This energy difference was made up mostly of stored lipid, which is the major fuel for subsequent non-feeding post-larvae, and compromised nutritional condition is likely to affect the likelihood of the animals survival and eventual recruitment into the fishery (Fitzgibbon et al., 2013; Phillips et al., 2006b). The present
study aimed to test whether this could be caused by dietary differences between the two
dipoles. However, the results indicate that eddy dipole has no effect on the diet of
phyllosomas, because variation between eddy dipoles can be entirely accounted for by
the overall variation between sampling sites (Table 7.4).

Fish larvae and arrow worms are highly preferred prey items of *P. cygnus* phyllosomas
(Table 7.4). Fish larvae and chaetognaths are relatively dense in lipid and protein
(Wang et al., 2013c), which are the energy substrates used to fuel the onshore migration
of pueruli after metamorphosis (Fitzgibbon et al., 2013; Limbourn & Nichols, 2009;
Phillips et al., 2006b). The very high electivity index for bony fish is because of the
disproportionately high abundance of reads from Actinopterygii (Fig. 7.2) caused by
neither bony fish adults or larva being particularly abundant in any of these eddy
systems (Chan, 2012) (Table 7.4). This low abundance of fish has been observed in a
previous study of an East Indian Ocean eddy, particularly in anticyclonic systems
(Waite et al., 2007a), and the significant presence of Actinopterygii in the phyllosomas
midgut in the present study, and Actinopterygii along with chaetognaths in the 2010
study (O’Rorke et al., 2012b) is an indication that phyllosomas could respond to certain
prey cues and manoeuvre in the water column to enhance their chance of encountering
this prey (Table 7.4). Bony fish DNA was also one of the few zooplankton prey items
detected in the midgut of some *J. edwardsii* larvae (O’Rorke et al., 2013b), *P. longipes
bispinosus* (Chow et al., 2010) and *P. japonicus* (Chow et al., 2010; Suzuki et al.,
2008). This, in combination with the success of feeding fish larvae to both wild
(Lebour, 1925) and cultured (Inoue, 1978; Kittaka, 1997a; Macmillan et al., 1997)
phyllosomas, indicates that fish are an important component of their diet.

Siphonophores and Thaliacea are not actively targeted by phyllosomas (Table 7.4), but
can occur in quite high abundance in their midguts (Fig. 7.2). The most likely reason why less preferred prey occur in the midgut of some phyllosomas is that phyllosomas are encounter predators (Cox & Bruce, 2003), but they suffer from nutritional limitations if their prey density falls below a critical threshold (Liddy et al., 2004; Ritar et al., 2002). Therefore, in an oligotrophic environment, persistently feeding on a large prey item such as siphonophores or salps, even if it is not an optimal food, is an optimal foraging strategy because it overcomes the problem of being in a low density prey field.

There are two ways in which diet might have caused the poor nutrient condition of phyllosomas in anticyclonic eddies in 2011 (Wang et al., 2013a). The results of the present study rejects the hypothesis that this is because they feed on different prey in anticyclonic eddies. Instead, it is possible that the nutrient condition of the prey that they are consuming differs between the anticyclonic and cyclonic systems. The present study does not address this hypothesis, but by finding that phyllosomas consume particular prey items (siphonophores, colonial radiolarians; Table 7.4) and actively target others (bony fish and arrow worms; Table 7.4) this study has identified the prey groups on which nutrient analysis would be best performed. In 2003, it was found that the siphonophores and colonial radiolaria were enriched in the $\delta^{13}$C stable isotope in an anticyclonic eddy, but not in a cyclonic eddy (Waite et al., 2007a), which indicates that these macrozooplankton were storing lipids. Siphonophores accumulate lipid “pockets” in their feeding cysts in abundant feeding conditions (Lee et al., 2006), potentially these plankton were enriched in lipids in cyclonic eddies in 2011, which were more productive (Chan, 2012). Colonial radiolaria are also known to store excess lipids from their photosynthetic symbionts (Anderson & Botfield, 1983; Anderson et al., 1983), which might also vary with eddy systems.
The physical environment of these eddies is the other remaining explanation for the differences in the phyllosomas condition. The warmer average temperature of water in anticyclonic eddies (~1 °C, Chan, 2012) is likely to have a negative impact on phyllosomas health (Liddy et al., 2004; Liddy et al., 2003; Matsuda & Yamakawa, 1997) and, because phyllosomas control their shoreward horizontal movement by vertical migration into shoreward currents (Butler et al., 2011), the offshore trajectory and much deeper anticyclonic thermocline mean that a phyllosoma must forgo feeding and expend energy to descend to the deeper undercurrents to assist its migration onshore. The latter point is not trivial, because although late stage phyllosomas migrate vertically at a rate of around 20 meters h\(^{-1}\), the mixed surface-layer temperature of anticyclonic eddies is constant to quite some depth (300m in 2003, Thompson et al., 2007) and penetrate from 1500 to 2500m depth (Morrow et al., 2003). By contrast, cyclonic eddies have a shallow thermocline, so phyllosomas can feed and then, if needed, vertically migrate over a short depth to control their onshore trajectory. This means that phyllosomas in cyclonic eddies can favourably regulate their temperature. It also explains why there are significantly more phyllosomas in anticyclonic eddies (Chan, 2012); the unfavourable depth of the thermocline makes them far more entrapped in the eddy than their cyclonic counterparts.

7.5 Conclusion
The diet of *P. cygnus* phyllosomas across two pairs of counter-rotating eddies in the Leeuwin Current is dominated by colonial radiolarians, bony fish, hydrozoans (particularly siphonophores), scyphozoans, salps, arrow worms and krill. With the first three prey items being highly abundant and consistently detected in both the v7 and v9 loci of the 18S rDNA gene, which indicates that the results can be taken with a high degree of confidence. Sampling water controls also indicate that these prey items are
legitimate and not artefacts of net sampling. In addition to prey items, prokaryotic parasites of crustaceans were detected. Parasite avoidance might be a factor that drives phyllosomas to occupy low density, offshore prey fields and it would warrant further study to ascertain if these organisms are pathogenic and, if so, if there are interannual fluctuations in the rate of infection of phyllosomas.

In 2011 more phyllosomas occupied anticyclonic than cyclonic eddies by more than five times (Chan, 2012), but on average the phyllosomas in cyclonic eddies were in superior nutritional condition (Wang et al., 2013a). The present study indicates that in 2011 the diet of phyllosomas in anticyclonic eddies did not differ to those feeding in cyclonic eddies and therefore, was unlikely to be the cause of the marked difference in nutritional condition. Additionally, the high abundance of large and typically colonial gelatinous animals in the midgut of phyllosomas indicates that phyllosomas might use an optimal foraging strategy of targeting large prey items in an otherwise low density prey field. However, the prevalence of bony fish DNA in conjunction with earlier observations of high chaetognath consumption and preference (O’Rorke et al., 2012b; Saunders et al., 2012), indicate that phyllosomas target smaller organisms with a higher nutrient payoff.
8 General Discussion

8.1 Introduction
DNA diet studies involve the sequencing of phylogenetically informative loci from PCR amplified DNA that is extracted from the gut contents or faeces of predators (King et al., 2008; Pompanon et al., 2012; Symondson, 2002). The sequenced DNA can then be identified by being matched to a database of known prey items. The benefit of this approach is that it enables the identification of trophic links for predators that are difficult to observe, or that eat prey items that cannot be identified from undigested morphological remnants. The research presented in this thesis designed and applied a DNA diet approach to determine the diet of spiny lobster larvae (phyllosomas), that are zooplankton predators whose diet has been historically difficult to determine (Cox & Johnston, 2003a; Jeffs, 2007).

Improved understanding of their diet could lead to improvements in artificial feeds for larviculture (Cox & Johnston, 2003a) and provide insights into processes that cause the highly variable settlement and subsequent recruitment of post-larvae into valuable spiny lobster fisheries (Fitzgibbon et al., 2013; O’Rorke et al., 2012b; Saunders et al., 2012). The diet of the phyllosoma phase has been historically difficult to discover because much of their extended larval development occurs in offshore waters where they are found in low densities (Phillips et al., 2006a). Furthermore, the larvae have a flat transparent morphology and there are no hard prey remnants in the digestive tract of phyllosomas from which prey can be morphologically identified (Phillips et al., 2006a). Using a DNA approach solves these problems, but the DNA approach has its own set of difficulties; i) the extremely flattened morphology of phyllosomas makes it difficult to
dissect the midgut out and whole sample homogenates run the risk of exogenous contamination, ii) phyllosomas probably consume a wide range of metazoan prey, but designing a PCR to amplify such a broad taxonomic range of metazoans (protozoa to vertebrates) will inevitably amplify phyllosoma DNA, and iii) DNA diet studies are made difficult in the case of generalist predators because an assay that detects a wide range of prey items is also likely to generate a large quantity of noise, such as commensal organisms and especially PCR artefacts, which are particularly problematic when using degraded prey as a template (Deagle et al., 2006; Pompanon et al., 2012; Suzuki et al., 2008). The research presented in this thesis developed a DNA protocol to overcome these issues and then applied the protocol to improve the understanding of the natural diet of phyllosomas of two species of spiny lobster from the Southern Hemisphere, *Jasus edwardsii* and *Panulirus cygnus*.

### 8.2 Principal Outcomes from the Study

#### 8.2.1 Methodological development

**8.2.1.1 Exogenous contamination (Chapter 3)**

Recent DNA diet studies have identified that the sensitivity of the PCR technique makes it prone to generating false positives due to exogenous DNA contaminating the sample (Greenstone et al., 2012; King et al., 2012). Many previous DNA studies of plankton diets extract DNA from a homogenate of the entire predator and its gut contents and either do not report measures for dealing with contamination, or they make do with washing the animal before extracting DNA (Chen et al., 2000; Fournier et al., 2008; Juen & Traugott, 2005). Research presented in this thesis for spiny lobster phyllosoma found that even in a small number of samples, washing the exterior of the
animal was insufficient for controlling DNA contamination in contrast to a novel syringing approach used to extract the midgut contents of phyllosomas.

8.2.1.2 **PCR prey enrichment (Chapter 2)**

Clamps or blockers have been used previously for enrichment of prey DNA (see Chapter 2 and, O’Rorke et al., 2012a) in diet studies by binding the clamp/blocker to a DNA region that partly overlapped with the binding site of a PCR primer, and therefore ‘excluded’ the primer from binding. One of the difficulties with this approach is that it requires a highly conserved region (for the universal primer) immediately adjacent to a hyper-variable region that will exclusively bind to the blocker. Such an abrupt change from highly conserved DNA region to hyper-variable region is difficult to find in the sequence of a DNA locus, particularly across multiple phyla. Therefore, the present study developed a novel alternative approach. This involved using a very specific, high affinity PNA clamp that would bind downstream of the primer and ‘arrest’ polymerisation (the PNA-clamp was attached to a positively charged lysine to increase the strength of its binding to the negatively charged DNA and thereby minimise self-binding of PNA (Ørum et al., 1993) which can otherwise cause problems for this approach. This alternative novel approach made it possible to use very highly conserved primers capable of amplifying an extremely broad range of prey.

**454 amplicon pyrosequencing (Chapters 5, 6, 7)**

In a previous DNA diet study of phyllosomas, a large number of unexpected organisms were detected in the midgut of phyllosomas (Chow et al., 2010). Traditional sequencing methods, such as cloning and Sanger sequencing, require considerable time and expense to generate a few dozen reads per sample. When the read assemblage is quite diverse and very few reads are used, it is difficult to assess whether many of these reads are random and non-representative artifacts. Use of next generation sequencing
technologies makes it possible to generate a sufficient number of reads in order to detect which items are in high abundance and which are likely to be the result of sampling ‘noise’ (O’Rorke et al., 2012a; Pompanon et al., 2012).

To ascertain the diet of *P. cygnus* phyllosomas, two linked loci were used. This was a second way to detect the potential stochasticity of PCR because the second linked locus can be used to validate the fidelity of the composition of reads given by the first locus. This was particularly the case for the investigation of the midgut contents of *P. cygnus* where there was interest in determining whether diet composition varied in relation to other variables, such as phyllosoma developmental stage and the location of capture.

### 8.2.2 Novel insights into larval diet of spiny lobsters

Determining the taxonomic composition of the natural diet of late stage phyllosomas of spiny lobsters could have important implications. There is considerable unexplained inter-annual variation in the recruitment of lobster post-larvae to fisheries (Booth & Phillips, 1994; Brown, 2009). The post-larval stage in spiny lobsters, known as the puerulus, is lecithotrophic (non-feeding) and depends on energy reserves accumulated during the feeding of the preceding larval phase (Fitzgibbon et al., 2013; McWilliam & Phillips, 1997; Wilkin & Jeffs, 2011). Determining the prey of larvae therefore enables the formulation of testable hypotheses about whether oceanographic events limit prey availability, and may therefore, determine long-term larval nutritional well-being.

Determining the diet will also have implications for advancing the larviculture of spiny lobsters. Attempts to culture spiny lobster larvae over the last century have consistently struggled to raise larvae past early instars and through to pueruli (Kittaka, 1997a; 1997b). This failure has often been attributed to insufficient insight into the dietary
requirements of later instars (Duggan & McKinnon, 2003; Robertson, 1968) and, according to Kittaka (2000), who has been a pioneer and the most senior researcher in the field, diet is considered to be the most important factor in progressing culture of phyllosomas.

**Jasus edwardsii (Chapter 5)**

For this study 19 mid to late stage *J. edwardsii* phyllosomas were sampled between October 2009 and May 2011 on four voyages to the Wairarapa Eddy, which is an anticyclonic oligotrophic eddy that experiences a short spring bloom (Bradford-Grieve et al., 1999). Only one phyllosoma was sampled in spring while the remainder came from late summer and autumn. The midgut contents of phyllosomas were dominated by fungi (59.9%) and parasites of crustacea (arachnids and alveolates; 13.5%) with potential phyllosoma prey making up the remaining 26.7% of sequence reads. Hydrozoans and ctenophores occurred frequently in phyllosoma midguts, resolving previous conjecture that these animals are significant contributor to the diet of *J. edwardsii* phyllosomas (Cox, 2004; Jeffs, 2007; Jeffs et al., 2004). Siphonophores (Hydrozoa), bony fish and Lobata (Ctenophora) were the most abundant, occurring in 7.7%, 6.9% and 6.0% of reads. The coelenterates are most likely an excellent food source for these phyllosomas because their large size means that are more likely to be encountered by phyllosomas despite the low prey density in the Wairarapa Eddy, and once encountered, they would provide a significant food source (Arai et al., 2003). Low prey density might be an issue for phyllosomas, and consequently the predominance of fungal DNA in their midgut is probably because it is amplified in the absence of any suitable zooplankton DNA. This may also reflect a dietary subsidy on marine snow and detritus. The relatively common occurrence of DNA from crustacean parasites in the
midgut of the phyllosomas indicates a further stress that might be affecting phyllosomas.

**Panulirus cygnus 2010 (Chapter 6)**

A total of 18 late stage *Panulirus cygnus* phyllosomas were sampled from a cyclonic eddy in the East Indian Ocean in July. Compared with the *J. edwardsii* midgut content, the number of standardised reads obtained from zooplankton was considerably greater (77.0% as opposed to 26.7%, Fig. 8.1). Fungal DNA, and a small amount of algal DNA made up the remainder of sequences and parasitic eukaryotic microbes were not detected. Of the 18 phyllosomas, 6 had a considerable quantity of DNA derived from artifacts and were excluded from subsequent analyses. Of the remaining 12 phyllosomas, multiple prey OTUs were detected and DNA from colonial radiolarians was the most frequently detected prey (detected in 12, i.e. all, phyllosomas), followed by Thaliacea (10 phyllosomas), bony fish (9) arrow worms (8), krill (8) and hydrozoans from the trachylina (9) and siphonophores (7). The presence of multiple prey types is possibly evidence that phyllosomas feed on multiple prey, or that they are subsidising their diet with detritus, or that it is the result of secondary predation (the prey of prey) because phyllosomas consume the entirety of their prey including the gut contents of prey (Saunders et al., 2012; Wakabayashi et al., 2012). The dominance of the colonial radiolarians among the midgut contents is intriguing, because these organisms have not previously been identified as prey items for phyllosoma. They are also difficult to quantify in the water column using conventional net sampling techniques and therefore their role in zooplankton dynamics in the East Indian Ocean ecosystem has probably been under-appreciated (Dennett et al., 2002; Stemmann et al., 2008).
Figure 8-1 Distribution of reads across studies. Comparison of the number of zooplankton, fungal, parasitic, scyllarid and unidentified DNA reads from i. J. edwardsii (stages 6 - 10) from New Zealand ii. P. cygnus (stages 6 - 7) from Western Australia in 2010, and iii. P. cygnus (stages 6 - 9) from Western Australia in 2011 and, for comparison, the Japanese species iv. P. japonicus (stages 7 - 10) from Japan and v. P. longipes bispinosus (stages 7 - 10) from Japan taken from Chow et al., (2010).

Panulirus cygnus 2011 (Chapter 7)
In 2011, late stage phyllosomas of Panulirus cygnus were sampled from two pairs of cyclonic and anticyclonic eddies to gauge the variance in the diet across water bodies. This study coincided with another that found that phyllosomas from cyclonic eddies were in better nutritional condition than their anticyclonic counterparts, with
significantly higher lipid and protein content (Wang et al., 2013a; 2013b). It is plausible that differences in primary productivity and the resulting zooplankton communities between anticyclonic and cyclonic eddies cause phyllosomas to feed on different prey, which would explain this difference in nutritional condition. However, when the prey taxa found in the mid-gut of 18 phyllosomas from two cyclonic and 18 from two anticyclonic eddies were compared, it was found that all significant variance in the diversity of prey taxa could largely be accounted for by differences between the four sampling sites rather than the eddy dipoles they were situated in. Anticyclonic eddies could be more physiologically demanding for phyllosomas because they have higher average temperatures, which are known to reduce feeding and nutritional condition in cultured phyllosoma of other species of spiny lobster (Greenwood et al., 2007; Strzelecki et al., 2007; Waite et al., 2007a). Alternatively, it is possible that although phyllosomas consume the same prey in different water bodies, the nutritional content of these prey items vary among water bodies. This hypothesis needs to be tested and the present study has identified those significant prey items that should have their nutritional values measured between water bodies in order to test this hypothesis.

8.3 Comparisons among species and studies

The most striking difference between the midgut compositions of J. edwardsii and P. cygnus was the relative ratio of zooplankton to fungus. As mentioned above, the amount of zooplankton DNA detected in the P. cygnus samples amounted to well over 75% of the sequencing reads, but was less than 25% in J. edwardsii (Fig. 8.1). A study of two Japanese species of Panulirus (Chow et al., 2010) found that their midgut contained less than 25% zooplankton. However, it is unclear how to best compare the present studies with that one because it targeted a different locus, the internal transcribed spacer 1 (ITS1). The 18S and ITS1 loci are linked, and so they should be in equimolar
concentrations in the cell, and therefore they should be theoretically comparable. However, the ITS1 is an extremely variable region, which means it can resolve to at least species level and possibly subspecies (Chow et al., 2009). However, with extremely variable (saturated) genes such as ITS1, it is very difficult to assign taxonomy to organisms that have not yet been sequenced, even at higher taxonomic classifications. Therefore, this genetic region allows comprehensive taxonomic identification only when the corresponding gene database is fairly complete. This resulted in the ITS1 study having a considerable number of unassigned reads, because there were insufficiently similar homologues on public databases for these sequences to be matched reliably (Fig. 8.1). Also the variability of ITS1 length means that species with shorter ITS1 gene regions are preferentially amplified, so taxa with long ITS1 regions are less likely to be amplified and identified (Chow et al., 2010). Taxa with long ITS1 include bony fish (Chow et al., 2009), which appear to be prey of phyllosomas. By contrast, although the 18S gene can, on average, resolve only to the taxonomic level of order, the present study has shown very this locus has very good reproducibility.

8.4 Future directions
The greatest constraint on metabarcoding projects such as the research presented in this thesis is the depth of public sequence databases that can be used as a reference for identifying species. Building databases requires the concerted effort of taxonomists and molecular biologists, and this appears to be lacking to some degree for pelagic organisms from the realms of the open ocean. For spiny lobster phyllosoma diet studies the next important step will be characterising the diets of their major prey species in order to begin to establish a foodweb. This will also help to resolve the problem of potential secondary predation being a cause of the multiple prey items being detected in phyllosomas, because if the prey of their prey are known, then it will clarify whether
multiple prey items are from secondary predation or from multiple feeding events. The decreasing cost of sequencing will also enable this problem to be addressed more cost effectively. The 18S assay worked very well for phyllosomas because it can amplify a broad range of taxa and it is less prone to PCR bias that could distort the results. However, it would be informative to supplement this with a more informative locus that resolves prey to the species level, although reliable use of such a marker is highly dependent on the completeness of reference databases.

8.1 Fisheries and Aquaculture Implications

The results of this study have very direct implications for the improvement of aquaculture. This study has determined that phyllosomas are generalist predators, but appear to be optimal foragers that will transfer their predation to a more favoured prey type, such as larval fish. This suggests that future experiments with artificial feeding regimes should aim to find a balance between a basic feed, that generally reflects the nutritional profile of a pelagic system, and whether this needs to be supplemented with a richer ‘preferred’ prey. The identification of key prey items, such as fish larvae and siphonophores in the diets of *P. cygnus* and *J. edwardsii* means that the nutrient profiles of these specific groups can be nutritionally matched in artificial feeds (Wang et al., 2012a).

The results of the present study also extend the information available for effectively regulating the lobster fisheries and predicting future catches. The list of known prey items determined by this study is much reduced from the previous list of possible prey. This smaller subset of prey offers a tractable number of organisms to monitor *in situ* or investigate in laboratory scale experiments. Consequently, variations in the distribution and abundance of specific organisms such as fish larvae, siphonophores and chaetognaths can now be tracked in greater detail over time and space and correlated to
variations in larval recruitment success. Therefore how oceanographic and biological factors that impact on these prey organisms can be used to enhance models to predict larval recruitment into fisheries. In fact, by identifying prey items such as colonial radiolarians, which had not previously been considered as prey items, the present study has not only narrowed the species that should be tracked, but also extended it in new directions.

8.2 Conclusion
The research presented in this thesis has successfully extended DNA diet techniques to work on generalist zooplankton predators by developing a prey-enriched amplicon pyrosequencing protocol to determine the midgut contents of late stage phyllosomas of *J. edwardsii* and *P. cygnus*. Not only was this method able to identify the prey items of the phyllosomas, but a comparison was made of two linked loci, the v7 and v9 regions of the 18S rDNA gene, which showed a greater than expected concordance in the composition of prey items. This suggests that the approach is reasonably trustworthy in its fidelity at reporting the relative quantities of DNA molecules detected in the midgut of phyllosomas. The most important prey items for phyllosomas of *J. edwardsii* appear to be hydrozoans, ctenophores and bony fish. For *P. cygnus* hydrozoans and bony fish are also important, as well as colonial radiolarians. Also of importance are salps, arrow worms and krill. Furthermore, because phyllosomas of individual species of spiny lobster appear to target particular prey, they will continue to consume fairly similar diets across various water bodies, despite variance in the community assemblages of those water bodies. This is significant, because it indicates that variable larval health between water bodies is not due to diet composition. Overall, while similar zooplankton prey were identified in the midgut of *J. edwardsii* and *P. cygnus*, the phyllosoma of *J. edwardsii* contained considerably less zooplankton DNA than the Western Australian
species and considerably more fungal reads, which is most likely due to the different productivity of the respective ocean environments from which they were sampled.

A DNA-based technique is a powerful tool for detecting the prey of small animals, such as marine larvae. The technique does have limitations, such as a susceptibility to contamination. However, these can be controlled for and overcome by careful execution of laboratory techniques. Also, any limitation is far outweighed by the benefits, such as the ability to detect prey across a wide range of taxa and the detection of subtle differences amongst the prey for individual predators. Together, the results of these studies indicate that if the most nutritionally significant prey items of phyllosomas can be determined, it would allow for the monitoring and modelling of how oceanic dynamics impact on these prey that would improve in the understanding of recruitment and assist in managing the rock lobster fishery.
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