Gut health in European lobster - the gut microbiome as a health marker in offshore sea culture

Submitted by Corey Carrington Holt, to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, September 2019.

I certify that all material in this thesis which is not my own work has been identified and that no material has been previously been submitted and approved for the award of a degree by this or any other University.

Abstract:

The European lobster (*Homarus gammarus*) represents one of the United Kingdom's most valuable fisheries. High market prices and a relatively limited fishery suggests that the species may be a good candidate for aquaculture and/or further stock enhancement. Disease is one of the biggest threats to the development and progression of sustainable aquaculture. However very little is known in regard to diseases of the European lobster. Therefore, better characterisation of potential pathogens is vital to prevent collapse of any future culture initiative. The community of microorganisms within the gut, referred to as the gut microbiome, is involved in key metabolic processes affecting host health and fitness and may serve as a key resource in reducing susceptibility to pathogenesis. Here we utilise a series of histological and molecular techniques to better characterise lobster disease and the lobster gut microbiome and assess the use of sea-based container culture (SBCC) as a means to culture the species and overcome the bottlenecks associated with traditional land-based culture (LBC).

In assessing mortality of land-based larvae and egg clutches entering the hatchery, we first detected the abalone parasite *Haliphthoros milfordensis*. Histological and molecular characterisation of this parasitic oomycete confirm past suspicions that *H. milfordensis* and *Halocrusticida noduliformans* are one and the same, highlighting the lack of available information surrounding animal oomycete pathogens. To facilitate monitoring of the parasite and growth of the literature, we developed and tested general oomycete primers, targeting the 18S ribosomal small sub-unit (SSU) and revealed a wealth of oomycete diversity in freshwater, marine water and soil samples.

Monitoring health of deployed juveniles in SBCC, we observed histological signs of viral infection. Genome characterisation and phylogenetic reconstruction revealed the virus to be a new member of the *Nudivirdae.* Thus, we discovered the first described clawed-lobster virus: *Homarus gammarus* nudivirus (HgNV). The double-stranded DNA (dsDNA) virus, containing 107 063 bp contains the conserved repertoire of genes associated with the nudiviruses yet does not form occlusion bodies, characteristic of its neighbouring lineages. We compared viral prevalence in SBCC to LBC and questioned the transmission strategy of this novel virus in the absence of occlusion.

Utilising high-throughput amplicon sequencing, we characterised the bacterial gut microbiome of the European lobster using the V4 region of the ribosomal SSU. We compared communities assembled in SBCC with those found LBC and assessed culture and time-dependent determinants of community structure and assembly. Typical of other aquatic invertebrates, the gut of juvenile European lobster mainly harboured *Vibrio* spp. but became more diverse as the host ages. Assessing phylogenetic structuring of the community, SBCC encouraged more deterministic assembly of the gut microbiome and reduced stochasticity observed in LBC. Furthermore, the gut microbiome of individuals reared in SBCC was significantly more diverse that those reared in LBC. A more diverse gut is thought to be beneficial to host health. In support of this theory, a reduction in bacterial gut diversity was associated with the detection of HgNV in the digestive tract, which was less prevalent in SBCC populations compared to the hatchery. Together, this information suggests that SBCC may support the culture of a healthy population through the assembly of a more beneficial gut community with the potential to provide resistance against infection.

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I am incredibly grateful to all those who have generously spared their time over the course of this project. This truly has been a collaborative effort. I would like to start by thanking my supervisors: Dr. David Bass, Prof. Mark van der Giezen and Dr. Carly Daniels. All of whom have been unwavering in their support throughout this project. David, you have given me so much throughout this process and I have really felt like I have learnt from one of the best. Mark, thank you for giving me so many wonderful opportunities and always encouraging me to look forward. Carly, thank you for those early mornings on the docks and for always making me feel like a part of the National Lobster Hatchery team.

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Finally, I would like to thank my family. This work is a reflection of the love and encouragement you have never failed to provide. This achievement is as much yours as it is mine.

Honourable mentions: coffee, dry-roasted peanuts, toast.

Contents

List of figures and tables

throughout a year - clone libraries (Meziti *et al*. 2010). Ornate spiny lobster, *Panuliris ornatus*, profiles obtained from each region of the digestive tract - 454 sequencing (Ooi *et al*. 2017). Pacific white shrimp, *Litopenaeus vannamei*, at increasing life stages - 454 sequencing (Huang 2014 *et al*. 2014) and different culture environments – Ion Torrent sequencing (Corejo-Granados *et al*. 2017). Black tiger shrimp, *Penaeus monodon*, at increasing life stages – 454 sequencing (Rungrassamee *et al*. 2013) and different culture environments – 454 sequencing (Rungrassamee *et al*. 2014). Eastern oyster, *Crassostrea virginica*, isolated from Hackberry Bay and Lake Caillou (Louisiana, USA) and analysed with Mothur - 454 sequencing (King *et al*. 2012). Stomach and gut from Chinese mitten crab, Eriocheir *sinensis*, farmed in China, DGGE and Illumina sequencing (Chen *et al*. 2015). An average mammalian gut profile from 60 species living in zoos and the wild, clone libraries (Ley *et al*. 2009). Mollicutes were assigned to the Tenericutes phylum.

Chapter 2: *Halioticida noduliformans* infection in eggs of lobster (*Homarus gammarus*) reveals its generalist parasitic strategy in marine invertebrates

Figure 2.1 Gross pathology of infected eggs of *Homarus gammarus*. Pale, discoloured eggs observed in brood clutch of berried hen. Black eggs indicate healthy, uninfected eggs.

Figure 2.2 Histological sectioning of infected tissues. Light microscopy images of 3 µm tissue sections. A) Hyphae protruding from the surface of the egg. Scale bar = 50 µm. B) Silver staining of the hyphal cell walls within egg tissue. Scale bar = 50 µm. C) Low magnification image of infected gill tissue showing loss of structure and replacement with inflammatory cells and

melanisation. Scale bar = 500 µm. D) Melanised lesion showing multinucleate nature of the hyphae ramifying through gill tissue. Scale bar = 500 µm. E) Silver staining of hyphal cell walls within the melanised lesion of the gill. Scalebar = 50 µm. F) In-situ hybridisation labelling of *H.noduliformans* using universal-oomycete SSU probes. Scale bar = 50 µm

Figure 2.3 SSU gene phylogeny of the Oomycete class. Bayesian phylogeny indicating the range of oomycete diversity detected using Oomycete-specific SSU primers. Shapes accompanying tip labels indicate number of environmental samples grouped with each OTU. Circle = freshwater sample (blue), triangle = soil sample (yellow) and square = marine water sample (green). Red tip labels indicate sequences derived from lobster tissue. Grey highlights cultured, positive control. Nodes labelled with black circles indicate Bayesian/Maximum likelihood (%) support of over 0.95/95. With the exception to nodes surrounding the *Haliphthoros*/*Halocrusticida* clade, only support greater than 0.8/75 is annotated.

Figure 2.4 SSU gene phylogeny of the lineages surrounding NJM 0034. Bayesian phylogeny of NJM 0034 and its close relatives. Accompanying (green) squares indicate number of environmental samples grouped with that OTU. Red tip labels indicate sequences derived from lobster tissue. Nodes labelled with hollow circle indicate Bayesian/Maximum likelihood (%) support of over 0.95/95. Nodes showing support greater than 0.8/75 are annotated.

Chapter 3: The first clawed lobster virus *Homarus gammarus* nudivirus (HgNV n. sp.) expands the diversity of the *Nudiviridae*

Table 3.1 Comparative genomic data of sequenced nudiviruses. † Cell line. *Direct submission to GenBank - number of ORFs, gene density and GC

content estimated from database entry. Accession numbers provided where journal reference of genome annotation is not available.

Figure 3.1 *Homarus gammarus* nudivirus (HgNV) infection within the hepatopancreas. A) Section through the hepatopancreas, haemal sinus (HS) surrounds the tubules, cross section of the tubules shows a clear lumen (*). Infected nuclei within the epithelial cells of the tubules are enlarged, with emarginated chromatin and possess an eosinophilic inclusion body (white arrows). Infected cells (black arrow) may be sloughed into the lumen of the tubules. H&E Stain. Scale bar = 50 µm. B) Infections can be seen within multiple epithelial cells, infected nuclei appearing larger than uninfected nuclei. Margination of the chromatin can form septa leading to the appearance of discrete intranuclear compartmentalisation (arrow). H&E Stain. Scale bar = 20 µm. C) Nucleus from a HgNV infected cell containing rod-shaped virions. Virions accumulate at the periphery of the nuclear membrane (arrow), TEM. Scale bar = 500 nm. D) Longitudinal (white arrow) and transverse sections (black arrow) of HgNV virions within the nucleus. Virions possess an electron dense nucleocapsid surrounded by a trilaminar membrane (envelope). The rod shaped nucleocapsid appears to bend within the envelope forming a “u” or “v” shape in some cases (line arrows). TEM. Scale bar = 500 nm

Figure 3.2 Prevalence of intranuclear inclusions in sea-based and hatchery lobsters over 104 weeks. Prevalence of intranuclear inclusions in sea-based and hatchery lobsters over 104 weeks. Proportion of surviving lobsters displaying histopathological signs of viral infection. Green triangles: hatchery-based animals. Blue circles: sea-based animals. Sample size indicated at each point.

Table 3.2 Direct repeat predictions within the HgNV genome. dr = direct repeat. Tandem repeat alignment score of > 100.

Figure 3.3. HgNV circular genome plot. Visual representation of HgNV layout scaled to the complete 107 063 bp contiguous sequence. Outermost track shows GC content (%) across complete sequence. Dark blue track displays gene predictions localised to the forward strand, whereas light blue displays those on the reverse. The innermost track depicts direct repeat regions. Links highlight genes involved in similar functions; yellow - DNA replication and repair, red - nucleotide metabolism, green – RNA transcription, pale blue – per os infectivity, pink – packaging and assembly, and grey – apoptosis inhibition.

Table 3.3 Supported open reading frame annotations of the HgNV genome. Colours as in Figure 3.3. BLAST annotations with an E-value equal or greater than 1 are not shown. Annotations with an E value >x10^{10} are highlighted in bold. * Pfam annotations with an E-value less than 1. SP = signal peptide, TM = transmembrane domain.

Table 3.4 HgNV homologs to conserved nudivirus sequences. Colours as in Figure 3.3. *fused to a single gene. **multiple copy number. Shaded cells of second column indicate ‘core nudivirus genes’ shared with the Baculoviridae. + Reported present.

Figure 3.4 Single and multigene phylogenies of known nudiviruses. Maximum Likelihood analyses of nudivirus phylogeny, including whispovirus and baculovirus outgroups. Node labels indicate bootstrap support (%). A – Single gene phylogeny of DNA polymerase. B – Single gene phylogeny of DNA helicase. C – Multigene phylogeny of late expression factors *(lef-4, lef-5, lef-8, lef-9 and p47).* D – Multigene phylogeny of *per os* infectivity genes *(pif-0, pif-1, pif-2, pif-3,*

pif-4, pif-5 and pif-6). DiNV – *Drosophila innubila nudivirus,* KNV – Kallithea *virus (D. melanogaster),* ENV – Esparto *virus (D. melanogaster),* TNV – Tomelloso *virus (D. melanogaster),* OrNV – *Oryctes rhinoceros* nudivirus, NleNV – *Nilaparvata lugens* endogenous nudivirus, GbNV – *Gryllus bimaculatus* nudivirus, HzNV-1 – *Heliothis zea* nudivirus 1, HzNV-2 – *Heliocoverpa (syn. Heliothis) zea* nudivirus 2, HgNV – *Homarus gammarus* nudivirus, PmNV – *Penaeus monodon* nudivirus, ToNV - *Tipula oleracea* nudivirus, NeleNPV – *Neodipirion lecontei* nucleopolyhedrovirus, NeseNPV – *Neodipirion sertifer* nucleopolyhedrovirus, AcMNPV – *Autographa californica* multiple nucleopolyhedrovirus, AgseGV – *Agrotis segetum* granulovirus, and WSSV – white spot syndrome virus. Coloured clade groupings refer to proposed genera: yellow – *Alphanudiviruses,* pink – *Betanudiviruses,* green – *Gammanudiviruses,* blue – *Deltanudiviruses,* red – *Baculoviruses,* cream – *Whispovirus.*

Chapter 4: Penaeid health and the microbiome

Table 4.1 List of gut microbiome papers describing the shrimp gut microbiome using high throughput sequencing. Grey indicate eukaryotic studies.

Figure 4.1 Overview of the penaeid shrimp gut microbiome in relation to disease, life stage and culture environment. A) Visual mapping of the tripartite digestive tract. B) Major bacterial phyla associated with gut changes in Pacific white shrimp (*Litopenaeus vannamei*) during pathogenesis; including diseased larvae from China (Zheng *et al*. 2017), Acute Hepatopancreatic Necrosis Disease (AHPND) infected postlarvae (PL) form Vietnam (Chen *et al*. 2017), White Spot Syndrome Virus (WSSV) infected shrimp from China (Wang *et al*. 2019), WSSV

juveniles in clearwater and Biofloc systems (Pilotto *et al*. 2018), adults showing symptoms of White Faeces Syndrome (WFS) (Hou *et al*. 2018) and growth retarded adults from China (Xiong *et al*. 2017a). C) Bacterial gut profiles of black tiger shrimp (*Penaeus monodon*) at increasing life stages (Rungrassamee *et al*. 2013) and different culture environments (Rungrassamee *et al*. 2014). C) Bacterial gut profiles of Pacific white shrimp (Litopenaeus vannamei) at increasing life stages (Huang 2014 *et al*. 2014) and different culture environments (Corejo-Granados *et al*. 2017). Data plotting from original publications. If relative abundances were not stated in manuscript, corresponding bars in original figures were measured as a percentage of the axis scale.

Chapter 5: Spatial and temporal axes impact bacterial gut ecology and assembly of juvenile European lobster (Homarus gammarus): exploration of the gut in a novel sea-based culturing system.

Figure 5.1 Average bacterial profiles of all animals sampled over 52 weeks.

Bacterial genera representing more than 2 % of entire 16S community. Genera coloured according to key. Time increases towards to extremities of the x axis from the pre-deployment control (0) at the centre. Green = land-based culture (LBC). Blue = sea-based container culture (SBCC).

Table 5.1 Exact sequence variant count of genera representing more than 2 % relative abundance.

Figure 5.2 Three-dimensional non-metric multidimensional scaling (NMDS) of all gut samples. Unweighted non-metric multidimensional scaling (NMDS) using the Bray-Curtis measure of dissimilarity over three axes. Stress = 0.130. A:

Coloured according to sample group. B: Coloured according to culture environment.

Figure 5.3 Alpha diversity measures of all sample groups. A: Shannon's measure of species diversity across all sample groups. B: Chao1 estimate of species richness across all sample groups. Green = land-based culture (LBC). Blue = sea-based container culture (SBCC). Environmental comparison 'LBC' (including day 0) and 'SBCC' represent combined data of all corresponding groups. Boxes labelled with groups that are significantly different. ** p <= 0.01. *** p <= 0.001.

Figure 5.4 Standard effect size of mean-nearest taxon index (ses.MNTD) indicating phylogenetic clustering of sequence variants. Standard deviation of mean-nearest taxon index (MNTD) from random model. Ses.MNTD values > 2 indicate phylogenetic overdispersion of taxa, 2 < & > -2 indicate stochastic distribution across phylogeny, < -2 indicate phylogenetic clustering. Green = land-based culture (LBC). Blue = sea-based container culture (SBCC). Environmental comparison 'LBC' (including day 0) and 'SBCC' represent combined data of all corresponding groups. Boxes labelled with groups that are significantly different. *** p <= 0.001.

Figure 5.5 Changes to gut microbiota in the presence of Homarus gammarus nudivirus (HgNV). A: Shannon's measure of species diversity across healthy and infected individuals sampled at 104 weeks. B: Chao1 estimate of species richness. C: Bacterial genera representing more than 2 % of entire 16S community. Yellow = HgNV-negative samples. Pink = HgNV-positive samples. Genera coloured according to key.

Supplementary Figure 5.1 Sequencing coverage across all samples. Estimations of community saturation across all individuals. A: Good's coverage estimates. B: Rarefaction of increasing sequencing effort. Plots coloured according to sample group.

Supplementary Figure 5.2 Shepard plot indicating fit of NMDS. Shepard plot indicating fit of observed dissimilarity to ordination distance.

Supplementary Table 5.1 Percentage abundances from average bacterial profiles of all animals sampled over 52 weeks.

Definitions

0034	*Haliphthoros* sp. NJM 0034
AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
AGD	amoebic gill disease
AHPND	Acute hepatopancreatic necrosis disease
AMPs	antimicrobial peptides
ASV	amplicon sequence variant
BBSRC	Biotechnology and Biological Sciences Research Council
BCBP	beta-glucan binding protein
BCIP	5-bromo-4-chloro-3-indolyphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	Bovine serum albumin
BV	budded virus
C	cytosine
C3	three-carbon
CCS	circular consensus sequencing
Cefas	Centre for Environment, Fisheries and Aquaculture Science
cox 2	cytocrome c oxidase subunit II
cox I	cytocrome c oxidase subunit I
CTAB	Cetrimonium bromide
DGGE	denaturing-gradient gel electrophoresis
DiNV	*Drosophila innubila* nudivirus
DNA	deoxyribonucleaic acid
dNTPs	Deoxynucleotide
drs	direct repeats
dsDNA	double stranded DNA
eDNA	environmental DNA
EDTA	Ethylenediaminetetraacetic acid
EMS	Early Mortality Syndrome
ESD	epizootic shell disease
ESV	exact sequence variant

F cells	fibrilar cells
FAD	flavin adenine dinucleotide
FAO	Food and Agriculture Organisation
FW	Freshwater
G	guanine
GbNV	*Gryllus bimaculatus* nudivirus
GTR	generalised time-reversible
H&E	haematoxylin and eosin
HgNV	*Homarus gammarus* nudivirus
HP	hepatopancreas
hrs	homologous regions
HS	haemai sinus
HTS	high-throughput sequencing
HzNV-1	*Heliothis zea* nudivirus-1
HzNV-2	*Helicoverpa zea* nudivirus-2
IDA	industrial denatured alcohol
IFCA	Inshore Fisheries and Conservation Authority
IGV	Integrative Genomics Viewer
ITS	internal transcribed spacer
LAB	lactic-acid bacteria
LBC	land-based culture
LDA	Linear Discriminate Analysis
lef	late expression factor
LEFSe	Linear Discriminate Analysis Effect Size
LG2	Lobster Grower 2
LPS	lipopolysaccharides
LSU	large subunit
MAFFT	multiple sequence alignment program
MAMPs	microbial associated molecular patterns
MBV	*Monodon baculovirus*
ML	Maximum Likelihood
MMS	Mysis Mold Syndrome
MNTD	mean nearest taxon distance

MOS	mannan oligosaccharide
NBT	nitroblue tetrazolium
NCBI	National Center for Biotechnology Information
NleNV	*Nilaparvata lugens* endogenous nudivirus
NMDS	non-metric multidimensional scaling
nr	non-redundant
NSTI	nearest-sequenced taxon index
nt	nucleotide
ODV	occlusion-derived virus
ORFs	open reading frames
OrNV	*Oryctes rhinoceros* nudivirus
OTU	operational taxonomic unit
Pav1	*Panuliris argus* virus 1
PCR	polymerase chaine reaction
PDC	pre-deployment control
PEG	polethylene glycol
PERMANOVA	Permutational multivariate analysis of variance
pif	per os infectivity
Pir	*Photorhabdus* insect-related
PmNV	*Penaeus monodon* nudivirus
proPO	prophenoloxidase
PRRs	pattern recognition receptorss
qPCR	quantitative PCR
R cells	reserve cells
RNA	Ribonucleic acid
SAF	Centre for Sustainable Aquaculture Futures
SBCC	sea-based container culture
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	Structural Equation Modelling
ses.MNTD	standard effect size of Mean-nearest taxon index
SSC	saline-sodium citrate
SSU	small subunit

TEM	transmission electron microscopy
tk	thymidine kinase
ToNV	*Tipula oleracea* nudivirus
tRNA	Transfer ribonucleic acid
UK	United Kingdom
WFS	White faeces syndrome
WPD	weeks post deployment
WSSV	white spot syndrome virus
ZMWs	zero-mode waveguides

Chapter 1

Introduction

1.1 Invertebrate aquaculture

With the increased reliance on aquaculture to meet growing food demands (FAO, 2016), means by which we can maximise production are becoming more important. The continual and rapid increase in aquaculture production now means that half of all fish produced for human consumption globally are derived from an aquaculture-based setting (World Bank, 2013) (Figure 1.1A). The expanding investment and innovation in aquaculture production have meant growth in the aquaculture industry, in terms of production, has far surpassed that of the capture fishing industry (World Bank, 2013) (Figure 1.1A). Currently, 90 % of all fish stocks are either overfished or fished to the maximum sustainable yield of the population (FAO, 2016) (Figure 1.1B). Assuming a linear increase of 2 million tonnes per year, aquaculture production is required to more than double if it is to meet the expected nutritional demands of the human population in 2050 (Waite *et al.*, 2014).

The use of fish as feed for cultured stocks, however, may limit the sustainability of future aquaculture production (De Silva and Turchini, 2009). Therefore, shifts towards the consumption and production of species occupying the basal levels of the food chain may be required to maintain consumption trends of fish proteins (Waite *et al.*, 2014). Although the vast majority of Crustacean aquaculture is based on shrimp, which is not farmed in a self-sustaining way, Mollusc and Crustacea species can be self-sustaining in terms of their dietary requirements and are a promising resource to fulfil expanding protein demands. Crustacea production is also of high economic value. Despite Crustacea production representing just under 9.9 % (7.9 million tonnes) of total world aquaculture production of food fish in 2016, it generated just under 24.7 % ($57.1 billion) of the estimated first-sale value of the total yield (FAO, 2016). This means, on

average, Crustacea output is worth over 2.68 times more per gram of body weight compared to finfish. As such, high demand and economic potential have meant invertebrate aquaculture has increased in popularity, with shrimp farming, in particular, becoming a major source of income for Asian and Latin American countries (Wurmann, Madrid and Brugger, 2004).

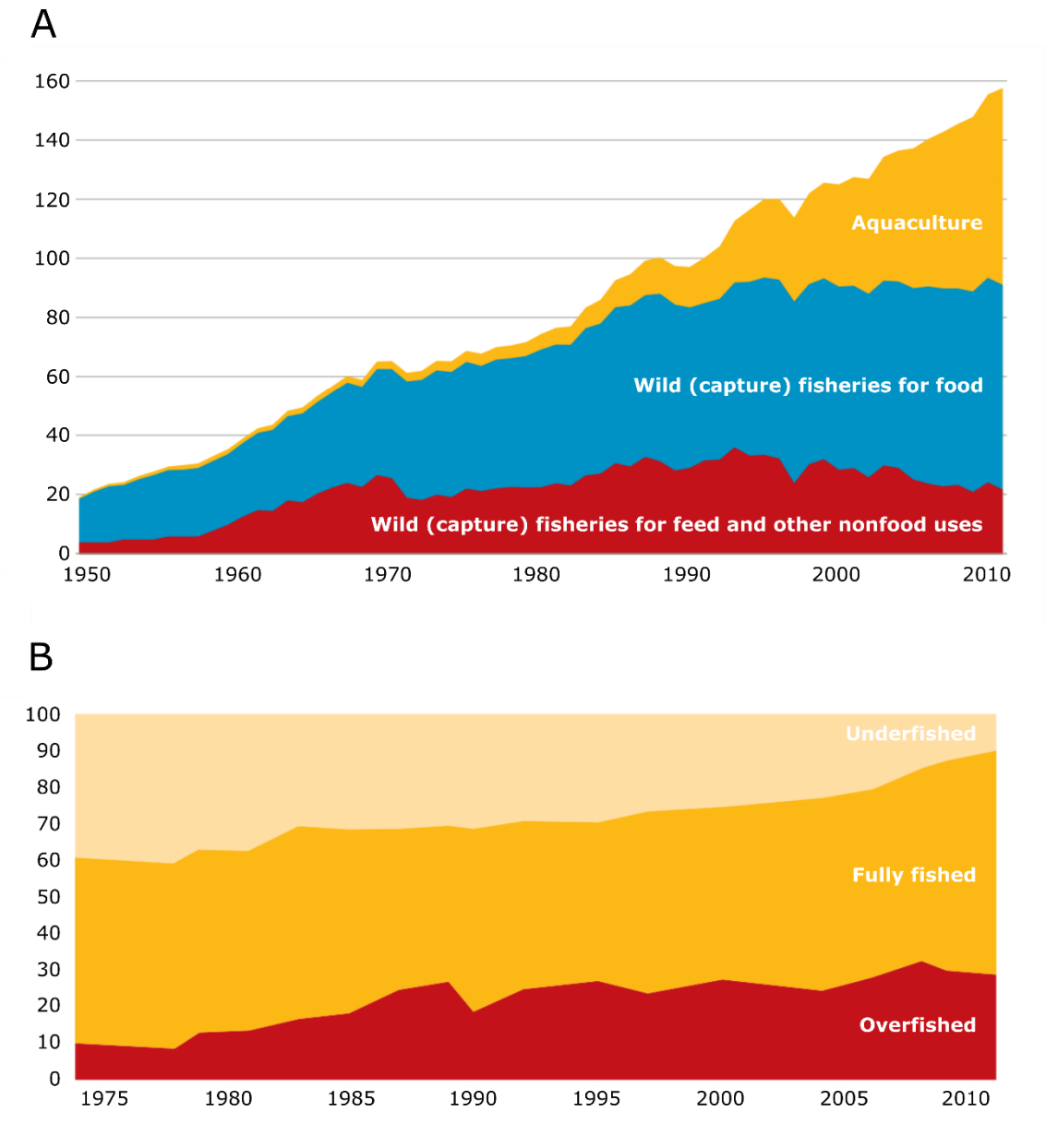

Figure 1.1 The current landscape of aquaculture and the capture fishing industry. A) Global fish production (million tonnes). B) Assessment of marine fish stocks over the past 40 years. Figures taken from Waite, R. *et al*. (2014).

1.1.1 Lobster Grower 2 and the offshore rearing of juvenile lobsters

The European lobster (*Homarus gammarus*) fishery is one of the most valuable in the United Kingdom (UK). The number of reported landings of the European lobster has shown an overall increase since the early 1980s. In 2017, over 80 % of European lobster landings were from the UK (4403 t; Figure 1.2), equating to a value of over £48 million at the first point of sale. However, in recent stock assessments of lobster fisheries in Scotland, all fisheries within the area were noted to be currently fished above, or close to, the maximum sustainable yield of the population (Mesquita *et al.*, 2017). Therefore, hatchery-reared and/or maricultured lobster stocks (reared at sea) are becoming increasingly important (World Bank, 2013).

Between 1983 and 2013, over 1.4 million cultured juvenile European lobsters were released across several countries including: the UK, France, Norway, Ireland, Germany and Italy (Ellis *et al.*, 2015), and the on-growing of juvenile lobsters to adult stages is undergoing continual development (Bannister and Addison, 1998; Drengstig and Bergheim, 2013; Daniels *et al.*, 2015; Halswell, Daniels and Johanning, 2016, 2018). However, early developmental stages of lobsters undergo significant losses, which could serve as a bottleneck to large-scale production, including aquaculture. It is estimated that only 0.005 % of hatchling larvae survive to the benthic phase in the wild (Allen, 1895). This figure increases to 5-10 % when measuring larval survival in a hatchery setting (The National Lobster Hatchery, pers. comm). Although individual variations in fitness and unsuccessful ecdysis likely account for a large proportion of loss in both environments, disease also likely contributes to this initial mortality and predation is a key driver of mortality in wild larval stocks. Lobsters are also cannibalistic in nature and damage caused by cannibalism could increase susceptibility to

disease. Young lobsters have a lesser developed immune system and thinner integument and may therefore succumb to disease much more rapidly compared to older individuals (Vadstein, 1997; Butler, Behringer and Shields, 2008). In this context, infectious disease may be expected to contribute to early-stage die-offs, as observed in other taxa.

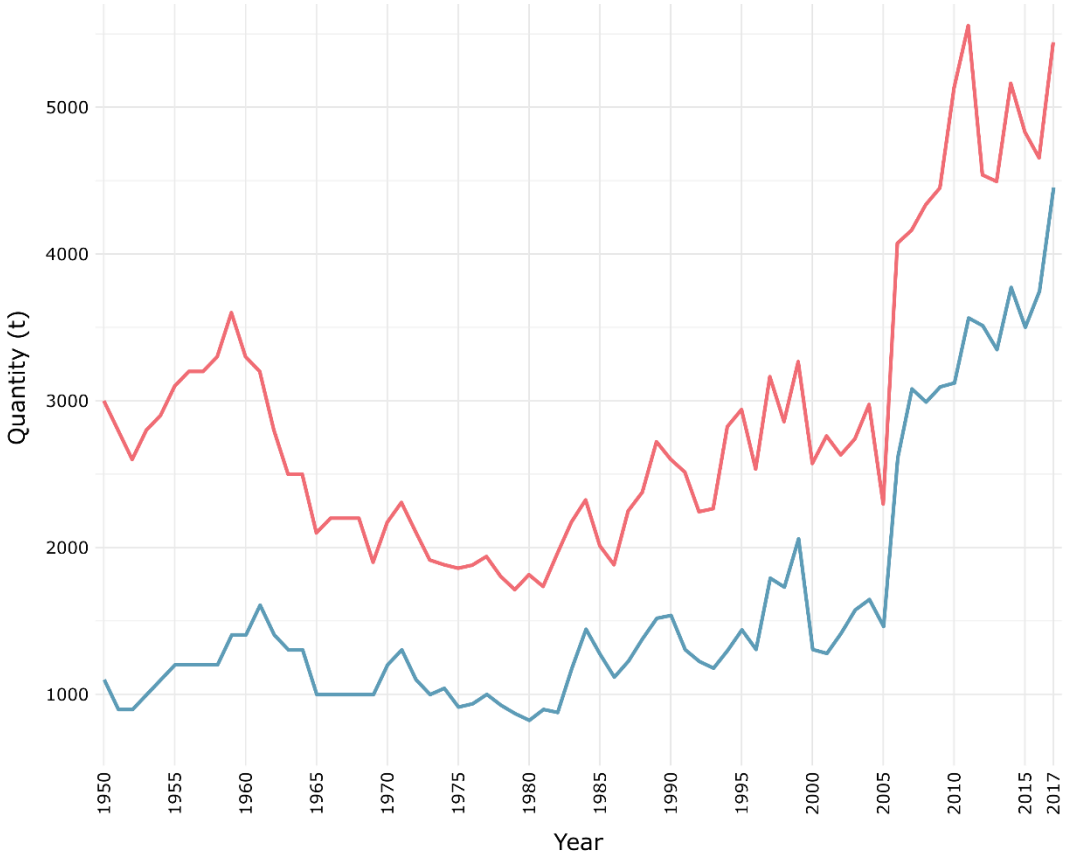

Figure 1.2 European lobster landings in the United Kingdom and Europe. Quantity of European lobsters landing in the United Kingdom* (blue) and Europe** (red) from 1950 – 2017. Data acquired from FAO. *Includes Chanel Islands and the Isle of Man. ** Includes the United Kingdom.

'Lobster Grower' is a collaborative research project partnering the National Lobster Hatchery with the University of Exeter, the Centre for Environment,

Fisheries and Aquaculture Sciences (Cefas), Westcountry Mussels of Fowey and Falmouth University (www.lobstergrower.co.uk). The collaboration aims to test the efficacy and efficiency of the large-scale rearing of juvenile lobsters at sea. The most recent stage of the project, 'Lobster Grower 2' (LG2), utilised novel sea-based container cultures (SBCCs) designed specifically for lobster rearing as part of an earlier project and compared their performance and output with those designed for oyster culture. This mariculture system allows for the on-growing of hatchery-reared animals before release into the wild and can be utilised to support the preservation of local populations. Furthermore, SBCCs could support a growing commercial aquaculture sector and offer a sustainable model for lobster production as they require no feed input after deployment and simply rely on settler species that naturally enter the containers. Early stage lobsters can also utilise filter feeding to obtain a source of nutrition (Lavalli and Barshaw, 1989), therefore non-settling phytoplankton surrounding SBCCs *in situ* will also benefit cultivation. Several pilot studies have already demonstrated the potential of SBCC mariculture models (Beal and Protopopescu, 2012; Daniels *et al.*, 2015). However, commercial-scale lobster mariculture is yet to be tested.

Lobster Grower 2 involved the deployment of 14,507 hatchery-reared lobsters between July 2016 and April 2017 in containers anchored off the coast of Cornwall (St. Austell Bay 50° 18.956 N, 4°44.063 W) (Figure 1.3A) in order to monitor growth and survival throughout production. The majority of these animals (10,987) were deployed during the summer of 2016. The lobster grower site was made up of 6 header lines, each supporting at least 50 'dropper' lines. The remaining lines were used to culture mussels. Containers sampled for the work presented in this thesis were held on lines 4, B and 9 (Figure 1.3B). Novel SBCCs were comprised of 20 compartments (Figure 1.3C) and were hung in pairs

totalling four separate containers per dropper (Fig 1.3E). Oyster containers were made up of 32 compartments (Figure 1.3D) and were hung two to a dropper (Fig 1.3E). To quantify the success of sea-based rearing, the LG2 project monitored growth and survival at regular intervals, tracked throughout the lobsters' development. The project also undertook a histology-lead disease assessment to assess cultivation impacts on lobster health. It has previously been shown that intensive aquaculture can increase the incidence of disease within a population through the positive selection of more virulent strains (Sundberg *et al.*, 2016). Therefore, it is important to tightly monitor the health of the cultivar in any intensive, or even semi-intensive (in the case of LG2) scenario.

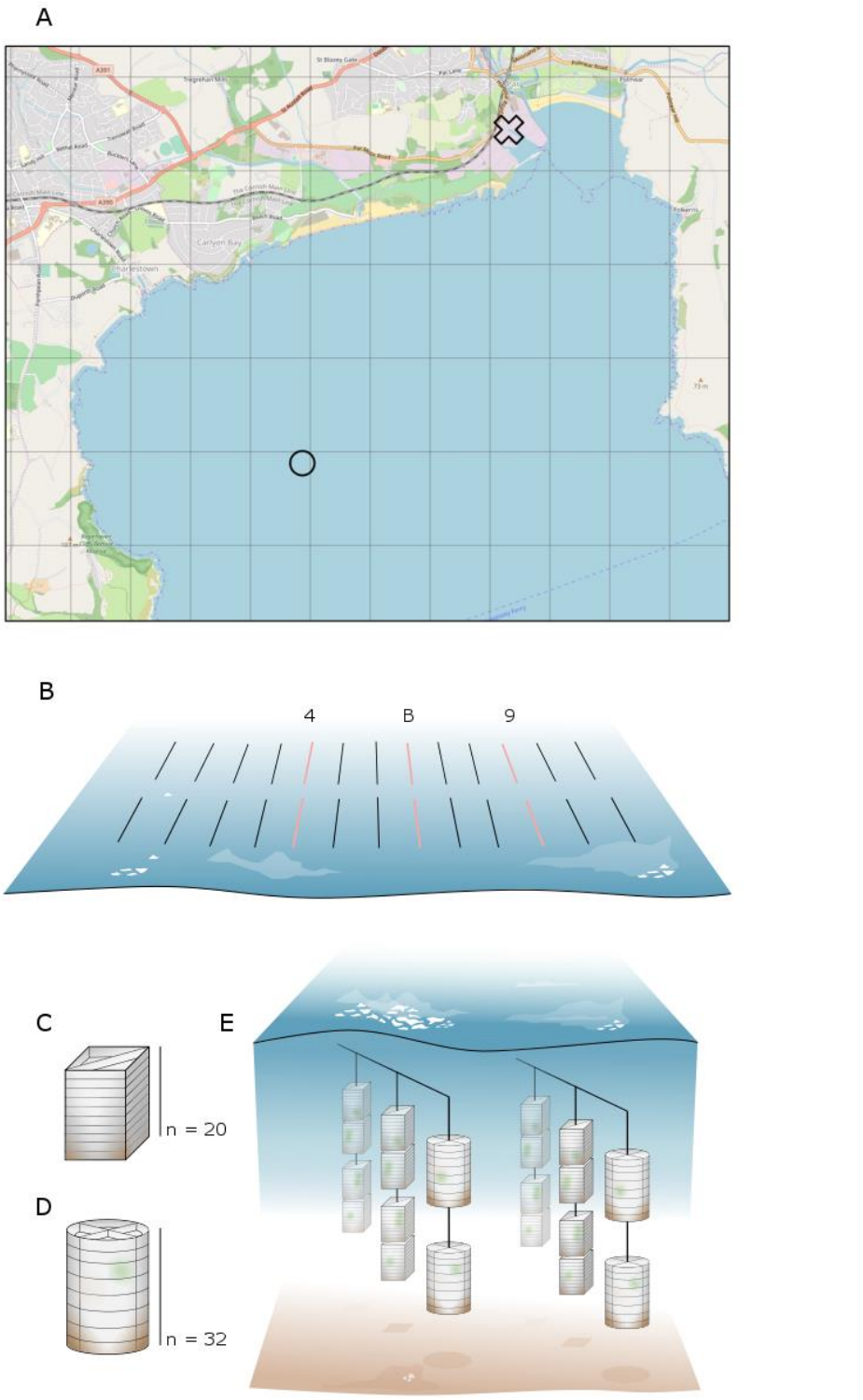

Figure 1.3 LG2 container layout. A) Map of St. Austel Bay (50° 18.956 N, 4°44.063 W) showing location of container site (circle) and docks where animals were processed (X). B) LG2 site plan. Pink lines indicate those used for sampling. C) Novel SBCCs hung in groups of four, totalling 80 animals per dropper. D) Oyster containers hung in groups of two, totalling 64 animals per dropper. E) Containers hung in groups of three.

1.2 Lobster health and disease

Growth in the aquaculture industry is predominantly threatened by disease (Meyer, 1991) and disease in shrimp aquaculture has been reported to be responsible for the loss of $3 billion globally (Farzanfar, 2006). Likely owing to high market prices impacting the obtainment of statistically reflective sample sizes in health screens, very little is known about European lobster disease and the breadth of pathogens to which they are susceptible.

Lobsters, like all other invertebrates, lack a typical adaptive immunity (Arala-Chaves and Sequeira, 2000). They rely on the innate immune system and largely the process of melanisation to fight infection. Melanisation, through a prophenoloxidase (proPO) cascade, is the process by which an invading pathogen is encapsulated and prevented from further affecting surrounding tissues. Microbial associated molecular patterns (MAMPs) such as lipopolysaccharides (LPS) bind to beta-glucan binding protein (BCBP) in the haemolymph and trigger the degranulation of the semi-granulocytes and the release of peroxinectin. Peroxinectin subsequently results in the degranulation of granulocytes and the release of proPO. The prophenoloxidase-activating enzyme catalyses proPO to produce phenoloxidase which then reacts with phenols in the haemolymph to produce quinones. These quinones polymerise to form melanin, which encapsulates the foreign body and prevents the spreading of infection (Cerenius and Söderhäll, 2004).

The exterior integument also serves as an important barrier to pathogen invasion. It is breakage to the integument which facilitates the transmission of *Aerococcus viridans*, a Gram-positive bacterium, into the haemolymph and results in Gaffkaemia – a fatal disease responsible for large losses in lobster holding facilities (Snieszko and Taylor, 1947; Cawthorn, 2011). Gaffkaemia is associated

with septicaemia of the haemolymph and a reduction in the number of circulating haemocytes resulting in lethargy, often characterised by a drooping abdomen and pink colouration of its ventral side (Snieszko and Taylor, 1947; Davies and Wootton, 2018). Gaffkaemia has significant impacts on lobster fisheries in America and Northern Europe (Behringer, Butler IV and Stentiford, 2012). However, it's impact on European lobsters is limited and the lack of variation between isolates suggests its prevalence in the UK resulted from the importation of American lobster (Stebbing *et al.*, 2012).

Shell disease, whereby cuticular damage results in melanisation and erosion of the carapace, may also drive mortality. Shedding of the exoskeleton can result in the full recovery of the animal however, older animals, who moult less frequently, can experience infection of the underlaying tissues (Vogan, Powell and Rowley, 2008). Similarly, epizootic shell disease (ESD) also results in erosion of the carapace however it is much more severe in its gross pathology and coverage (Davies and Wootton, 2018). The syndromic condition is caused by the microbial degradation of carapace proteins, however there is no singular aetiological agent known to be associated with ESD (Whitten *et al.*, 2014). Although ESD has not been detected in wild European lobster, infected American lobster have been observed in European waters (Davies and Wootton, 2018). Shell diseases are of great concern to the global lobster fishery as infected individuals become unsightly and unmarketable resulting in considerable economic loss. Furthermore, increasing sea temperature as a result of global warming is thought to increase the occurrence and severity of lobster diseases such as ESD (Groner *et al.*, 2018). Increasing sea temperatures are noted to be the cause of a suspected *Neoparamoeba pemaquidensis*-associated mortality event affecting American lobster in 1999 (Pearce and Balcom, 2005). *Neoparamoeba*

pemaquidensis, an amoebozoan protist has also been identified on shell-diseased lobsters (Tlusty *et al.*, 2005). *Neoparamoeba pemaquidensis* was originally associated with amoebic gill disease (AGD), a significant cause of loss in farmed finfish (Munday, Zilberg and Findlay, 2001). However, it has since been demonstrated that *Neoparamoeba perurans* was the predominant aetiological agent of AGD across several host species and countries (Young *et al.*, 2008). Protozoan parasites are considered important drivers of disease in crustacea however few have been identified in lobsters. In *H. americanus, Anophryoides haemophila* a ciliate responsible for 'bumper car disease' associated with holding facilities with cold water temperatures can destroy haemocytes and result in large die-offs (Cawthorn, 1997).

Despite their increasingly apparent ubiquity across the marine environment, there have been no naturally occurring viruses found in any clawed-lobster species. *Panuliris argus* virus 1 (Pav1), the first and only virus found in any lobster species, infects the mesodermal cells of the spiny lobster, *Panuliris argus* (Shields and Behringer, 2004), but has not been observed in European or American lobster populations. Similarly, white spot syndrome virus (WSSV), which causes huge production losses in cultured shrimp and has since infected a number of Crustacea, has not been found in lobster. However, experimental challenges demonstrate both American and European lobster are susceptible to infection (Bateman *et al.*, 2012; Clark *et al.*, 2013).

1.3 The gut microbiome and its host

Mutualistic relationships have facilitated the evolution and expansion of numerous species throughout history. Importantly, endosymbiotic organisms

within the gut can be responsible for the pre-digestion of particular foods and sometimes play a pivotal role in utilising an otherwise inaccessible source of energy; such is the role of cellulose-degrading metamonads in the guts of termites (Brune, 2014) and nitrogen-fixing Rhizobiales in ants at the basal end of the trophic scale (Russell *et al.*, 2009). The importance of these host-microbe interactions has facilitated the colonisation and evolutionary maintenance of a diverse community of microorganisms inhabiting the gut of a vast array of species across the animal kingdom.

This community, also known as the *gut microbiota* or *gut microbiome* (in reference to the collection of genomes within the community), may further support the nutritional uptake of the host through the increase of digestive enzyme activity in the gut (Sang, Fotedar and Filer, 2011; Zokaeifar *et al.*, 2012; Dai *et al.*, 2017) and the extension of microvilli and subsequent increase in surface area for nutrient absorption (Daniels *et al.*, 2010). Thus, the application of gut supplements is thought to be a promising tool for increasing production yield in farming.

Changes to the gut microbiome have been associated with several diseases in aquatic invertebrates and other organisms. These are mainly reported in Penaeid shrimp species and these effects are reviewed in Chapter 4. Therefore, manipulating the gut is thought to be one way in which pathogenesis can be reduced or prevented. The presence of a symbiotic microbiota can itself provide a sort of immunity; known as colonisation resistance (Lawley and Walker, 2013). Colonisation resistance essentially limits the proliferation of pathogenic organisms as attachment sites in the gut are ultimately finite and reducing the abundance of certain bacterial classes within the microbiota, disrupting its normal equilibrium, can allow previously symbiotic species to become pathogenic

(Blumberg and Powrie, 2016). Colonisation resistance can also be supported through the microbiota's production of antimicrobial peptides and its stimulation of the host immune system (Lawley and Walker, 2013).

1.4 Gut analysis in the age of high-throughput sequencing

1.4.1 A (brief) history of characterising the gut

It is the development of DNA sequencing approaches that has expedited extensive characterisation of the gut microbiome in recent years (Figure 1.4). Since the development of high-throughput sequencing (HTS) and the fall in sequencing costs, associations between differential compositions of the human gut microbiota and changes to the health of the host have attracted considerable attention.

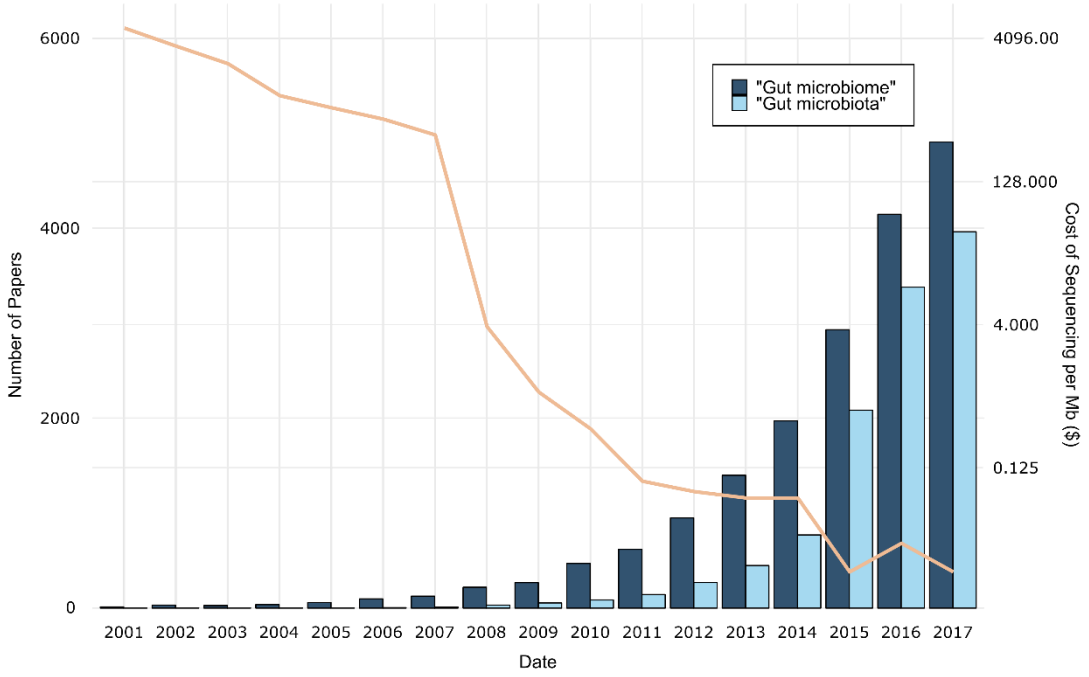

Figure 1.4 Increasing trend in gut research Papers published with the terms “gut microbiome” (dark blue) and gut microbiota (light blue) since 2001. The cost in sequencing per Mb is indicated by an orange line.

The development of HTS approaches has resulted in a progression toward culture-independent techniques in the characterisation of the gut community. It is now recognised that culture-dependent techniques used to identify individuals in diverse communities are inherently biased. The choice of culture media will ultimately define which species grow, as many require specific substrates for their successful proliferation. The difficulty in replicating the precise environmental conditions required for growth means the vast majority of bacteria are deemed unculturable *in vitro*.

Before the development of HTS, denaturing-gradient gel electrophoresis (DGGE) became the go-to method for culture-independent ‘fingerprinting’ of the gut microbiota. The technique involves the amplification of conserved DNA fragments which differ in sequence but not size. These fragments are separated using a polyacrylamide gel which contains a linear gradient of DNA denaturants (urea and formamide). As partially melted amplicons migrate at different rates, the resulting gel profile can be compared to the migration patterns of known strains to identify the taxonomic composition of the query community. However, this technique is time consuming and expensive. And the requirement of reference strains means it too suffers from bias. In that, communities can only be characterised if they have been identified previously.

Before the advent of HTS methods, full-length marker genes were often amplified, cloned into plasmids and transformed into competent bacterial cells. Plasmid DNA was then isolated from positive transformants and sequenced using a

Sanger sequencer. This method was often combined with DGGE to identify taxa within a community however, the cost of this process meant the depth to which these communities were sequenced was limited and less abundant organisms were often missed.

1.4.2 Amplicon vs metagenomic sequencing

Amplicon and shotgun metagenomic sequencing are the two predominant ways in which we now characterise the microbes within the gut using next-generation sequencing. Targeted marker gene, or amplicon, sequencing is a deep-sequencing approach which relies on PCR amplification of a conserved gene and results in the generation of comparative taxonomic profiles constructed around sequence variations within that gene. Several marker genes have been utilised in the generation of these communities however the ribosomal small-subunit (SSU) or 16S gene (in the case of prokaryotes) is by far the most common and is now supported by an extensive reference database. Other genes include the ribosomal large-subunit (LSU) and the *cytochrome c oxidase* I (coxI) gene, as well as the internal transcribed spacer regions (ITS1/2). The SSU gene serves as a good marker gene as it contains hypervariable sequence intermittently spaced between conserved regions of the gene which show homogeneity in the majority of organisms within the target domain. Therefore, primers can be designed to bind to the conserved region and subsequently amplify the succeeding hypervariable sequence. However, there have been no marker genes identified which span multiple domains and a separate amplification is required to compare both prokaryote and eukaryote diversity. Furthermore, each hypervariable region of the SSU is not uniformly informative across all clades of

bacteria, i.e. different regions have different capacities to resolve specific taxonomies.

Shotgun metagenomic sequencing is an untargeted approach which typically does not rely on the amplification of a conserved gene. It instead involves the fragmentation and subsequent sequencing of all genomic DNA, resulting in the generation of sequences from prokaryotes, eukaryotes and even viruses, including those obtained from functional genes. As all genomic DNA is being randomly sequenced, as opposed to singular amplified fragments, the depth to which these communities are sequenced (per run) is considerably less. Furthermore, as shotgun metagenomics is ideally PCR-free, it requires a large amount of template DNA which is sometimes unobtainable when working with small organisms.

For this thesis I predominantly focused on amplicon sequencing to characterise the gut community. Amplicon sequencing has the benefit of requiring very low amounts of DNA (1 ng μL^{-1}) and can be employed in the analysis of individual larva. It does not, however, provide any (reliable) functional annotation of the target community as only the target gene is sequenced.

1.4.2.1 Amplification bias and multiplexing samples

PCR can introduce bias during the amplification of the template DNA. In an ideal world, all target DNA is amplified uniformly. However, this is not the case. The proportion of guanine (G) and cytosine (C) nucleotides within a sequence determines its relative stability. A high GC content can hinder amplification therefore species-specific amplicons that are characteristically low in GC content will be preferentially amplified. Furthermore, amplicons of the same target region can differ in size and smaller amplicons will be amplified more efficiently. These

biases can be reduced by performing amplifications in triplicate before pooling and minimising the number of PCR cycles. Pooling replicates helps minimise stochastic variation in early cycles (Polz and Cavanaugh, 1998).

A custom PCR approach can reduce the number of PCR rounds required. Typically, a one-step PCR approach first amplifies the region of interest and then, in a second PCR, adds sequencing adaptors and unique indexes that can later be used to discern amplicons between samples. Alternatively, a one-step PCR approach can mitigate cross-contamination between PCR reactions and help reduce bias (Kozich *et al.*, 2013). This approach relies on custom primers which include a priming site that targets the region of interest as well as an extension which incorporates the sequencing adaptor that binds to the flow cell (Figure 1.5). These are separated by an indexing sequence, to identify sample-specific sequences; a 10 nucleotide (nt) pad sequence, which boosts the melting temperature of the oligo; and a 2 nt linker which is anticomplementary to known sequences and prevents non-specific binding. This eliminates the need for expensive Illumina indexes, as all primers, which include index and adaptors, are designed by the user.

When specific genes are targeted for sequencing, the sequencing coverage of those genes is far greater than an attempt to sequence the entire genome. Therefore, in tagging DNA from each sample with unique combinations of DNA indexes, amplicon sequencing can be performed on hundreds of samples on a single sequencing run. This is referred to as ‘multiplexing’. Considering a Miseq run can produce ~ 25 M paired-end reads per run and using a conservative estimate of 10,000 (paired) reads per sample to characterise a community, one run could theoretically contain up to 937 samples (allowing ¼ of the run for spiking). In reality, however, the number of reads produced by a low diversity

(amplicon) run and sequencing coverage across samples within a run are much more variable and it would be unwise to commit to such a high number of samples on an individual run.

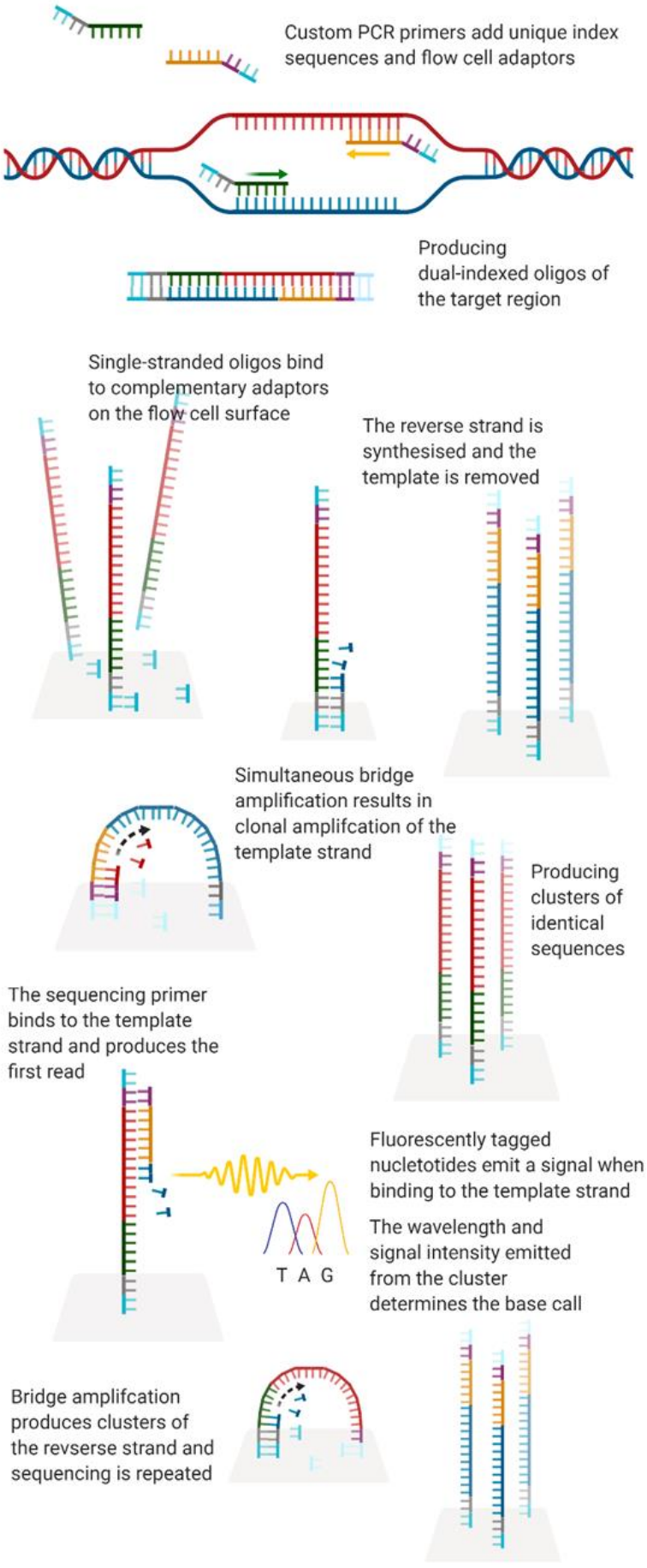
Custom PCR primers add unique index sequences and flow cell adaptors
Producing dual-indexed oligos of the target region
Single-stranded oligos bind to complementary adaptors on the flow cell surface
The reverse strand is synthesised and the template is removed
Simultaneous bridge amplification results in clonal amplifcation of the template strand
Producing clusters of identical sequences
The sequencing primer binds to the template strand and produces the first read
Fluorescently tagged nucletotides emit a signal when binding to the template strand
The wavelength and signal intensity emitted from the cluster determines the base call
T A G
Bridge amplifcation produces clusters of the revserse strand and sequencing is repeated

Figure 1.5 Amplicon-based sequencing on Illumina systems. Workflow describing a dual indexing approach using custom oligos sequenced on the Illumina platform.

1.4.3 OTUs versus ASVs

The traditional way in which amplicon sequences are analysed involves the clustering of similar sequences into operational taxonomic units (OTUs). Popular OTU-based analysis pipelines include 'Mothur' (Schloss *et al.*, 2009) and 'Qiime' (Caporaso *et al.*, 2010). After quality filtering, the remaining sequences are grouped based on a percentage similarity, which is typically 97 %. In doing this, minor amplification and sequencing errors, which would otherwise constitute the classification of multiple erroneous variants, are absorbed. However, clustering overlooks the high-quality of modern sequencing platforms (Levy and Myers, 2016). And it is now recognised that even minor differences in nucleotide sequence can account for large ecological and phylogenetic distances (Eren *et al.*, 2014). Therefore, there is an increasing worry that OTU clustering based methods also absorb meaningful biological information in terms of species and strain-level variation within the community. Finally, as OTU clustering is a function of percentage similarity of the sequences within the community, OTUs are ultimately dependent on the dataset from which they are derived. Therefore, OTUs from separate analyses are technically incomparable.

This fine-scale information is, however, retained by programs utilising an amplicon sequence variant (ASVs), or exact sequence variant (ESVs) approach (the terminology has not yet stabilised, but refers to the same thing), wherein sequences are only grouped if they are 100 % identical. 'Denoising' programs, like DADA2, rely on stringent error correction models to ensure artefactual

sequences are removed during the initial quality control (Callahan *et al.*, 2016). DADA2 builds an error model around the frequency of base transitions, initially using the most abundant sequence which it assumes is correctly sequenced. The error model is then applied to each dereplicated read during sample inference. This process relies on abundance p-values for each unique sequence and tests whether the abundance of that sequence, with its associated rate of error, can be explained by sequencing error or genuine variation. For example, a low p-value would suggest that there are more reads of that sequence that can be explained by the errors introduced in its amplification and sequencing (Callahan *et al.*, 2016).

It should be noted that there is a definitive lack of eukaryotic analysis in studies investigating the gut microbiome. The dominance of host-derived sequences in 18S (the SSU of eukaryotic organisms) and metagenomic libraries often mean that very little information is retrieved from the gut in terms of the diversity of eukaryotic organisms that it supports. However, advancements in the use of blocking primers which prevent complementary sequences from extending along the host template (Vestheim and Jarman, 2008) and more specific 'universal' primers which exclude Metazoa are currently under development (Hartikainen *et al.*, 2016). Nevertheless, both HTS amplicon and shotgun metagenomic sequencing is now routinely applied to the characterisation of disease and has facilitated the exploration of novel pathogens and complex microbial communities

1.5 Invertebrate gut microbiome

There is currently a dearth of studies describing the gut microbiome of aquatic invertebrates compared to humans and other vertebrates. Most invertebrate

study is limited to model organisms, bees, and commercially important species such as shrimp. There are currently no HTS studies characterising either *Homarus* lobster species.

Both culture-dependent techniques and HTS has revealed that Proteobacteria are widespread in aquatic invertebrate gut microbiotas and are often a dominant component of this community (Figure 1.6). In contrast, the microbial analysis of pooled faecal samples from over 60 mammalian species showed that Firmicutes dominated the average mammalian gut microbiota, making up 65.7% of the overall classified sequences. Bacteroidetes were the second most abundant, corresponding to 16.3% of sequences, finally followed by Proteobacteria (predominantly gamma) at 8.8% and other, less abundant phyla (Figure 1.6) (Ley *et al.*, 2008). Compared to humans and other mammals, Bacteroidetes are relatively less abundant within the aquatic invertebrates. This could well be a consequence of the absence of lactation in invertebrates. In addition to providing nutrition, a range of antibodies and direct transmission of certain microbes, lactation can act as a selection pressure within the gut (German *et al.*, 2008). Some Bacteroides (in addition to the Actinobacteria, *Bifidobacterium infantis*) can utilize the oligosaccharides, contained within milk, as a carbon source and can therefore exploit a niche unavailable to many other taxa. This is, perhaps, why Bacteroides have established a strong foothold as a dominant species in the human microbiome (Marcobal *et al.*, 2011). In the absence of this pressure within the aquatic invertebrates, alternative bacteria phyla make up the major constituent of the gut.

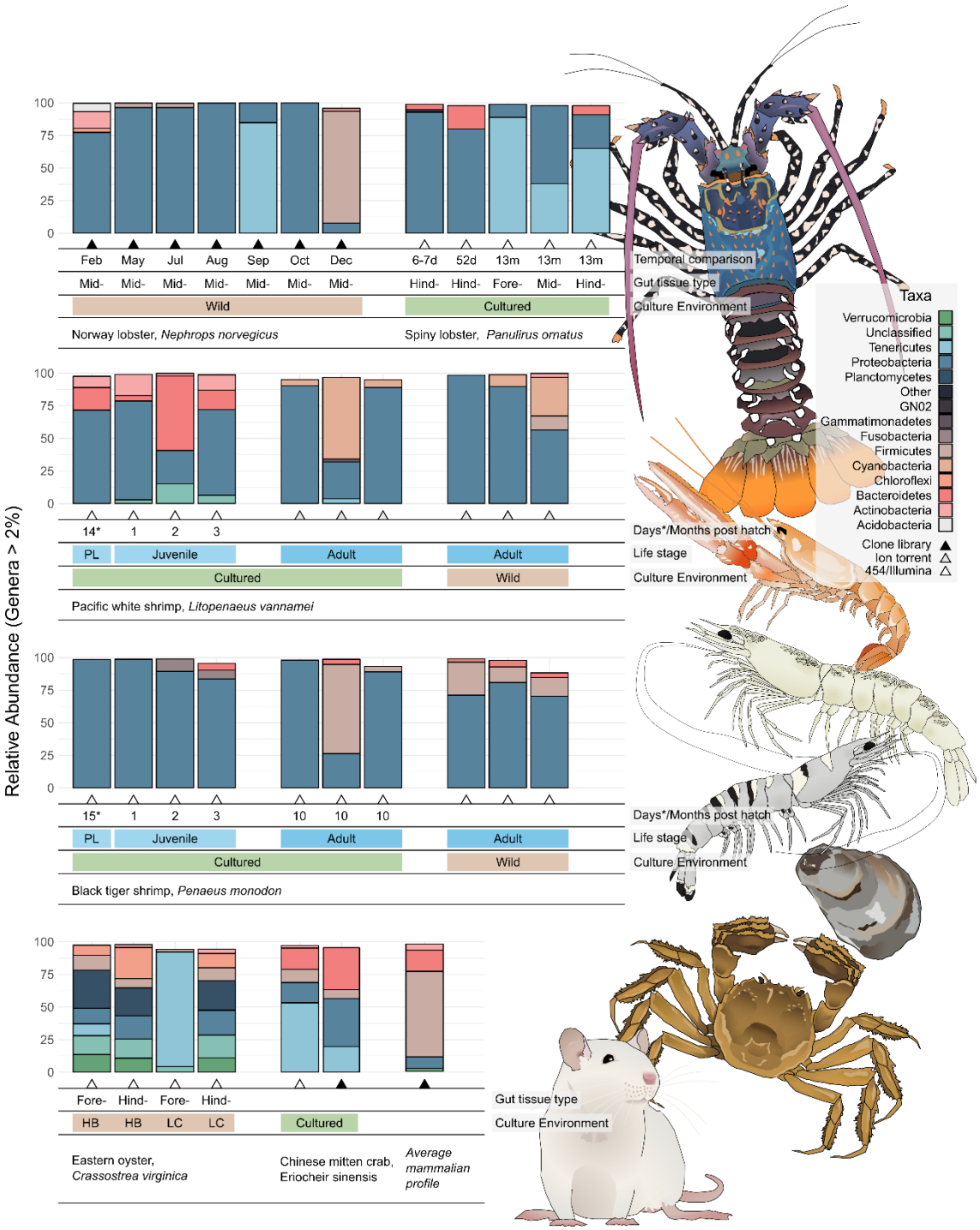

Figure 1.6 Comparing the gut microbiome across multiple species. Representative gut profiles of several species displaying bacterial phylum with relative abundances above 2 %. Norway lobster, *Nephrops norvegicus*, sampled throughout a year - clone libraries (Meziti *et al*. 2010). Ornate spiny lobster, *Panuliris ornatus*, profiles obtained from each region of the digestive tract - 454 sequencing (Ooi *et al*. 2017). Pacific white shrimp, *Litopenaeus vannamei*, at increasing life stages - 454 sequencing (Huang 2014 *et al*. 2014) and different culture environments – Ion Torrent sequencing (Corejo-Granados *et al*. 2017). Black tiger shrimp,

Penaeus monodon, at increasing life stages – 454 sequencing (Rungrassamee *et al*. 2013) and different culture environments – 454 sequencing (Rungrassamee *et al*. 2014). Eastern oyster, *Crassostrea virginica*, isolated from Hackberry Bay and Lake Caillou (Louisiana, USA) and analysed with Mothur - 454 sequencing (King *et al*. 2012). Stomach and gut from Chinese mitten crab, Eriocheir *sinensis*, farmed in China, DGGE and Illumina sequencing (Chen *et al*. 2015). An average mammalian gut profile from 60 species living in zoos and the wild, clone libraries (Ley *et al*. 2009). Mollicutes were assigned to the Tenericutes phylum.

In a study investigating ornate spiny lobster phyllosoma (*Palinurid ornatus*) in the Great Barrier Reef, Alphaproteobacteria were the dominant class, primarily dominated by *Sulfitobacter* and *Roseobacter* species in whole body homogenates using DGGE (Payne *et al.*, 2008). *Roseobacter* spp. can be particularly beneficial to the host and have demonstrated inhibitory action toward pathogenic bacteria (Ruiz-Ponte *et al.*, 1999; Planas *et al.*, 2006). They comprise a group of bacteria that are commonly found in the marine environment and contain species that are involved in carbon and sulphur recycling (Wagner-Döbler and Biebl, 2006). Some phylotypes can cause pathogenesis and have been identified as the causative agents of juvenile oyster disease (Boettcher, Barber and Singer, 2000) as well as black band disease in coral (Cooney *et al.*, 2002). Tracking the gut across host development, using an HTS approach, Gammaproteobacteria were shown to be the most dominant class in 6-7 and 52-day old individuals, with Mollicutes increasing abundance after 13 months (Figure 1.6) (Ooi *et al*. 2017). In Norway lobster (*Nephrops norvegicus*), a bacterium related to the chitinolytic Gammaproteobacteria, *Photobacterium leiognathi* was a dominant sequence from animals in the post-moult stage when comparing mussel-fed, pellet-fed and starved animals (Meziti, Mente and Kormas, 2012). This is noted as a possible explanation for the increase in chitinase activity in the

gut during this period, which facilitates the release of the moult (Lustigman *et al.*, 1996; Vega-Villasante *et al.*, 1999; Gulmann, 2004). *Arcobacter* associated phylotypes were very common in mussel-fed lobsters. *Arcobacter* is, again, a genus belonging to Proteobacteria and contains species of denitrifying bacteria (Heylen *et al.*, 2006). The authors also found that most of the taxa identified were not related to those in the surrounding water and largely belonged to Gammaproteobacteria, Epsilonproteobacteria and Tenericutes.

Although differing from midgut to hindgut, the gut bacterial make-up of the Chinese mitten crab (*Eriocheir sinensis*) was also dominated by Proteobacteria (38.3%), this was closely followed by Bacteroidetes (33.6%), Tenericutes (20.3%) and Firmicutes (7.0%) when composition was analysed based on clone library frequencies (Figure 1,6) (Chen *et al.*, 2015). The Chinese mitten crabs were also shown to harbour (Mollicute) sequences affiliated with *Candidatus* Hepatoplasma, which is thought play a role in the survival of terrestrial isopods under nutritional stress (Fraune and Zimmer, 2008). Analysis of the same dataset using an HTS approach showed a slightly different profile, with Tenericutes representing the most abundant phylum (Figure 1.6) (Chen *et al.* 2015). A second study investigating *E. sinensis* noted that the most dominate OTUs in the gut were those that corresponded to Mycoplasmataceae, the (Tenericute) family of bacteria that contains *Candidatus* Hepatoplsma. These OTUs were barely detected in the water and gills and the crab showed reduced bacterial diversity in the gut when compared to their surrounding water (Zhang *et al.*, 2016).

Gammaproteobacteria were also the most common bacteria found in the gut of giant tiger shrimp (*Penaeus monodon*) which, again, harboured bacterial microbiotas distinct from that of their rearing waters (Chaiyapechara *et al.*, 2012). *Penaeus monodon* were shown to exhibit a diverse range of gut microbes,

however, most of which belonged to the Proteobacteria. This was also true of domestic shrimp of the same species, who mainly harboured Gammaproteobacteria, but also Firmicutes, including the genus *Lactobacillus* which is known for its use as a probiotic (Figure 1.6) (Rungrassamee *et al.*, 2014). A dominance of Gammaproteobacteria during the first three months of culture was also described in Pacific white shrimp (*Litopenaeus vannamei*), with the exception to 2-month old juveniles (Figure 1.6) (Huang, *et al*. 2014). Proteobacteria and Cyanobacteria where most abundant in samples when comparing wild and cultured *L. vannamei* in Mexico (Figure 1.6) (Cornejo-Granados *et al.* 2017) and in a small study of Chinese shrimp (*Fenneropenaeus chinensis*), Proteobacteria were again the most dominant type of bacteria (Liu *et al.*, 2011).

Analysis of the stomach and gut microbiomes of the Eastern Oyster (*Crassostrea virginica*) from two sites in Louisiana, revealed bacterial communities which differ from those of other aquatic invertebrates, showing a reduction in the abundance of Proteobacteria (King *et al*. 2012). Eastern oyster guts appear more diverse compared to Crustacea (Figure 1.6) and this variation could be heavily influenced by associated differences in feeding strategies and digestive physiology.

An important caveat is that there are relatively small number of studies in each case. Therefore, it is difficult to generalise any of these findings. In order to facilitate comparisons between systems (which should be cautionary as discussed earlier in the introduction), microbial profiles are also commonly described at the phylum level. The diversity of organisms within a phylum often makes it very difficult to interpret the possible biological role and effect within the gut environment. Furthermore, a myriad of factors could potentially impact the determination of these communities.

1.6 Selection pressure impacting gut colonisation

1.6.1 Gut morphology

Both environmental and host-related factors may impact the colonisation of bacteria within the gut. The digestive tract of the decapod Crustacea is split into three sections: the foregut, the midgut and the hindgut. The foregut is made up of the oesophagus, containing tegumental glands to lubricate the ingesta; the cardiac stomach, which sometimes contains calcified ossicles for mechanical digestion (the gastric mill apparatus); and the pyloric stomach which filters processed and unprocessed foodstuff and facilitates the movement of liquid digesta into the hepatopancreas (HP). The midgut begins with the junction to the HP, where the filtered food is digested and absorbed, before the intestine extends throughout the cephlathorax and abdomen. The midgut sometimes contains anterior and posterior caeca although their function is largely unknown. Finally, the hindgut connects the midgut to the anus and begins in the posterior end of the abdominal region (Ceccaldi, 1989). However, with the absence of posterior cecae and alternative lengths of the midgut between different animals, it can be difficult to determine the midgut-hindgut boundary, without looking more closely at gut wall composition. For Ceccaldi reports the midgut-hindgut boundary within the last abdominal segment (Ceccaldi, 1989) however, McGaw and Curtis depict the boundary at the junction of the abdomen and cephalothorax (McGaw and Curtis, 2013).

Unlike the midgut, the foregut and hindgut are lined with a chitinous cuticle (McGaw and Curtis, 2013), which could facilitate a compositional shift in the microbiota by allowing the proliferation of chintinolytic bacteria. Many organisms, such as *Vibrio* spp., can utilise chitin as a carbon source (Suginta *et al.*, 2000)

and are often found in the chitin-lined hindgut (Harris, 1993). Therefore, morphological and physiological differences along the gut axis may induce differential pressures on community composition. In cultured juvenile spiny lobster, *Panuliris ornatus,* the hindgut harboured a more diverse microbiome than both the foregut and midgut (Ooi, *et al*. 2017) and although dominant taxa were shared between the mid- and hind-gut of Chinese mitten crab, there was a differential abundance of several bacterial phylotypes when comparing the two regions (Chen *et al.* 2015). The cuticle in the gut, however, is sloughed during ecdysis (Ceccaldi, 1989) and this will likely impact the attachment of bacteria to the gut wall. As moulting occurs less frequently in older animals, this could impact intraspecies variation in later life stages.

1.6.2 The vertical and horizontal transmission of gut endosymbionts: niche versus neutrality

Vertical transmission of endosymbionts through the maternal line occurs across multiple species (Bright and Bulgheresi, 2010). Evolutionary theory predicts that the more important the relationship between host and microbe, the more likely it is to be maintained. If a symbiosis provides an advantage to a host, then this relationship will likely persist across its evolution. In invertebrates, endosymbionts can be inherited via transovarian transmission using specialised vesicles called bacteriocytes, maintaining host-microbe associations through multiple generations (Koga *et al.*, 2012). In a form of maternally-guided horizontal transmission, some flies adopt egg smearing behaviours using faeces, or even a form a milk, which defines the immediate microbial environment prior to hatching and controls initial colonisation of the gut (Attardo *et al.*, 2008; Prado and Zucchi, 2012). Priority effects mean that those microbes which colonise the gut first can establish a strong foothold and subsequently impact additional colonisers

(Sprockett, Fukami and Relman, 2018). Even so, parental mucus feeding in discus fish (*Symphysodon* aequifasciata) results in the transfer of microbes and subsequent taxonomic shifts in the gut microbiota of the offspring even after initial gut colonisation (Sylvain and Derome, 2017). Vertically-transmitted microbes tend to be more important in terms of influencing host fitness. Removing these has a greater effect on reducing host fitness compared to the removal of horizontally-transmitted microbes (Fisher *et al.*, 2017).

It is generally believed that colonisation of aquatic gut endosymbionts is dependent on horizontal transmission from the environment. It has been suggested that an aquatic environment imposes much fewer restrictions on microbial colonisation, compared to a terrestrial one, and the gut of a terrestrial animal provides an 'aquatic' environment for microbial growth which isn't essential for microbes who already inhabit a true aquatic environment (Harris, 1993). However, the microbiota of the invertebrate digestive tract is often noted to be both qualitatively and quantitatively distinct from that of the animals' rearing water (Harris, 1993; Md Zoqratt *et al.*, 2018). The gut and its ingested substrates likely promote the growth of rare lineages within the water column and result in the positive selection of its associated community. First feeding fish fry, for example, harbour a gut community associated with their food (Ingerslev *et al.*, 2014) and coating the eggs of water fleas (*Daphnia magna*), can manipulate the gut microbiota to a desired composition (Sison-mangus, Mushegian and Ebert, 2014).

Biological communities are typically assembled through either stochastic or deterministic processes, or both (Dumbrell *et al.*, 2009; Dana Ofiteru *et al.*, 2010; Langengeder and Szekely, 2011; Stegen *et al.*, 2012). Neutral theory assumes that all species have the same ecological fitness. That is, there are no differences

in death and dispersal rates between members of the community. Therefore, stochastic assembly is governed by ecological drift and random birth-death events (Burns *et al.*, 2016). There is evidence that larval zebrafish tend to rely on these neutral processes to colonise their digestive tracts but as the host ages, deterministic processes become more important (Burns *et al.*, 2016). Deterministic processes result in environmental filtering through host selection and species-species interactions as differential capacities to occupy ecological niches exist within the community (Stegen *et al.*, 2012). It is presumed that as the host develops, and its immune system along with it, selection pressures within the gut are thought to increase. However, there is also evidence to the contrary, whereby environmental filtering has decreased with fish development (Yan *et al.*, 2016). This also appears to be true of shrimp (Zhu *et al.*, 2016; Xiong *et al.*, 2017). However, in shrimp, colonisation becomes more stochastic with the incidence of disease (Zhu *et al.*, 2016). The induction of disease and compromise to host health is thought to impose trade-offs as the host attempts to mitigate pathogenesis. It is likely that the assembly of these communities within the gut are dependent on a combination of both stochastic and deterministic processes.

1.6.3 Spatial/temporal determinants of community structure

If gut colonisation is largely dependent on horizontal transmission of its endosymbionts, then environmental determinants of gut community structure might affect host-microbe interactions and the cultivation of the host.

The gut microbiota contains both autochthonous species, those that are deemed resident and attach to the gut wall, and allochthonous, transient microbes. It is perhaps the presence and characterisation of allochthonous microbes which result in the intraspecies variability often reported in gut microbiome studies.

However, allochthonous microbes can still exert an effect on the host animal (Kim and Lee, 2014) and their relative importance in microbiome-host interaction has not been investigated (in sufficient detail). The transient nature of the allochthonous microbiota may also be responsible for the buffering of environmental conditions. The guts of Norway lobster (*N. norvegicus)* varies with sampling time throughout the year and undergoes a shift from a typical Gammaproteobacteria-dominated profile to that of one mainly comprised of uncultured Mollicutes (Meziti *et al.*, 2010). These temporal changes are thought to be the product of seasonal prey abundance and shifts in feeding strategies. Changes to diet requirements and feeding strategies along with the development of more stringent immune regulation as the host ages likely corresponds to changes in the gut throughout a lifetime.

Diet is also likely to be the key determinant of differential gut microbiomes in environmental comparisons. *Drosophila* reared on a particular diet can show a similar microbiota, regardless of their original environment (Staubach *et al.*, 2013) and different species feeding on the same diet were more similar in terms of their gut microbiota compared to the same species feeding on different diets (Chandler *et al.*, 2011). A change in diet, and ultimately substrate within the gut, will alter the availability of niches and subsequently affect the success of microbes attempting to colonise the gut.

Contrasting diets of wild versus farmed populations may well explain microbiota difference observed in the aquatic invertebrates. In a meta-analysis of shrimp gut microbiotas, culture environment was the strongest biological factor impacting its composition (Cornejo-granados *et al.*, 2018). Wild shrimp have a more diverse gut microbiota compared to those in culture (Rungrassamee *et al*. 2014, Cornejo-Granados *et al*. 2017). Therefore, some culture scenarios have the potential to

detrimentally impact the host via its alterations to the gut microbiome. Furthermore, rapid environmental change can correspond to taxonomic shifts in gut community make-up (Chen *et al.*, 2017).

1.7 Aims

Despite widespread characterisations of beneficial host-microbe interactions, remarkably little is known about the invertebrate gut microbiome. This is particularly true of the European lobster. Furthermore, the range of pathogens to which European lobsters are susceptible is unclear. This information is vital in the assessment of the European lobster's potential as a sustainable aquaculture species. As such, the over-arching aims of the project were; 1) to investigate disease of the European lobster and 2) comprehensively characterise the gut microbiota, contextualising this information in regard to the cultivation of the species in an aquaculture-based setting. By comparing this information with the incidence of disease, we aimed to test whether changes to the gut microbiota reflect that of changes to the health of the host.

To accomplish this, we aimed to:

1) *Employ histological and molecular diagnostics to monitor and characterise pathogens in both land-based and sea-based settings.* Considering the relative lack of information surrounding lobster pathogens we hypothesised that the information gathered from these samples would reveal novel insights into lobster pathology and the pathogens which may affect the cultivation of the species.

2) *Compare the gut microbiota of sea-based and land-based animals to indicate environmental impacts on taxonomic profiles and their subsequent interactions*

with the host. We hypothesised that sea-based culture will impact the composition of the gut community through the availability of a more diverse source of microbial colonisers and a more diverse source of nutrition to sustain these communities within the gut.

3) *Track the composition of the gut microbiota on a temporal scale, exploring correlations with the growing host and its changing needs.* Deterministic processes influencing gut community assembly reportedly become more important as the host ages. If this is true of lobsters, we expected diversity within the gut to be greater in younger individuals, who are governed by more stochastic means of gut colonisation and variation within the gut to subside as the host ages.

4) *Better understand the relationship between the gut microbiome and host health. Comparing differential growth rates in co-habiting animals and incidence of a diseased state.* We anticipated that the ability of orally-transmitted pathogens to initiate infection within the lobster host may be dependent on microbiome-induced resistance within the gut. Following the identification of lobster pathogens within our cultured population, we aimed to compare the gut profiles isolated from the animals with those from otherwise healthy individuals. We also hypothesised that microbes within the gut will have different capacities to support nutritional breakdown within the lobster gut and thus distinct communities within the gut may be responsible for differential growth rates of cohabiting lobsters.

1.8 Contributions to the field

Despite the increase in popularity of studies sequencing the microbial communities of the digestive tract, there are very few studies which aim to characterise this community in aquatic invertebrates. As invertebrate aquaculture

secures its foothold in global aquaculture production, means by which we can maximise aquaculture production are becoming increasingly recognised as important avenues of study. Production loss as a result of disease is one of the main limitations of growth in the sector. However, very little information is known regarding the diseases to which European lobster are susceptible, and even less regarding which of these pathogens naturally infect wild and/or cultured populations.

This body of work provides much needed information to support the growth of *H. gammarus* in both land-based and sea-based systems. Reference histology and diagnostic primers capable of detecting *Halioticida noduliformans* and *Homarus gammarus nudivirus* (HgNV) will allow the characterisation and surveillance of these natural pathogens which may have the capacity to limit production.

We offer the first characterisation of the gut microbiota of *H. gammarus* using an extensive high-throughput sequencing approach. This information not only begins to address the dearth of knowledge surrounding invertebrate gut microbiomes, but offers a strong rationale for sea-based culture as increased diversity within the gut may improve the health of the host by reducing susceptibility towards disease. Characterisation of this community also builds on previous work to improve gut health of the European lobster (Daniels *et al.*, 2010, 2013) and supports the development of novel probiotic supplements by providing a catalogue of microbes known to survive passage to the gut and known to be associated with healthy individuals.

1.9 Thesis roadmap

Chapters two and three describe initial characterisations of novel lobster pathogens isolated from hatchery and or sea-based lobster populations. Chapter two, published in the Journal of Invertebrate Pathology, details the characterisation of an unidentified disease agent causing mass mortalities in the hatchery larvae. These mortalities were coincident with the incoming and development of discoloured eggs in maternal broodstocks. We set out to identify the suspect pathogen by applying a combination of histological and molecular-based diagnostics with the goal of providing reference material for future monitoring. We characterised the pathogen as the parasitic oomycete *Halioticida noduliformans*, previously unreported in any lobster species or indeed any animal in European waters. The analysis helped resolve the confusion regarding *H. noduliformans* and *Haliphthoros milfordensis* and supported the idea that they are one and the same. In the process, we designed novel universal oomycete primers and tested their capacity to detect a diverse range of lineages through a series of environmental screens.

In chapter three, published in Scientific Reports, we track the prevalence of a novel viral infection in LG2 juveniles with the aim to reconstruct its genome and phylogeny. PCR-free shotgun metagenomic sequencing was used in the short-read Illumina sequencing of metagenomes isolated from suspect hepatopancreatic tissue showing histological signs of viral inclusions. We confirm the first ever naturally occurring clawed lobster virus to be a species of Nudivirus, *Homarus gammarus* nudivirus (HgNV) and provide a diagnostic primer set to aid in its future identification.

Chapter four is an invited review for the Journal of Invertebrate Pathology. The issue focuses on penaeid shrimp culture and this review details host-microbe interaction in relation to health and disease of the host. Owing to high economic importance, shrimp guts are the best studied among the aquatic invertebrates and were therefore used as a proxy to investigate the role of the gut microbiome in the maintenance of health of the invertebrate host, with the idea that the same principles likely apply to lobster and similar decapod crustacea.

Chapter five aims to compare gut bacteria profiles between hatchery-reared and sea-based lobsters spanning a one-year period and incorporates the discovery of HgNV to correlate the incidence of disease with differential changes in sequence abundance. The paper is published in The ISME Journal. We sequenced the V4 region of the bacterial SSU and analysed the resulting sequences in respect to exact sequence variants. We found sea-based animals harbour a significantly more species rich and species diverse microbiota which can be indicative of a healthier host. In support of this theory, a reduction in bacterial diversity correlates with the presence of HgNV in the digestive tract.

Chapter six makes up the general discussion, bringing together all aspects of the project in context with the wider literature and limitations affecting the field of invertebrate gut microbiomes. It also addresses important considerations for future study and considers the role of disease and the gut microbiome in aquaculture research and development.

1.10 Author's declaration and contribution to co-authored papers.

Holt, C. C., Bass, D., Daniels, C. L., Stentiford, G. D., & van der Giezen, M. The gut microbiome and the cultivation of aquatic invertebrates. Microbiome. *In preparation.*

CCH: Literature review, production of figures, writing of manuscript. DB: Supervision to CCH, contributed to manuscript. CLD: Supervision to CCH, contributed to manuscript. GDS: contributed to manuscript. MvdG: Supervision to CCH, contributed to manuscript.

(Excerpts within Chapter 1)

Holt, C., Foster, R., Daniels, C. L., van der Giezen, M., Feist, S. W., Stentiford, G. D., & Bass, D. (2018). *Halioticida noduliformans* infection in eggs of lobster (*Homarus gammarus*) reveals its generalist parasitic strategy in marine invertebrates. Journal of invertebrate pathology, 154, 109-116.

CH (CCH): Sample collection, histological processing and analysis, molecular analysis of lobster tissue, phylogenetic analysis, writing of the manuscript. RF: Preparation and sequencing of environmental samples. CLD: Provision of suspect animals, contributed to manuscript. MvdG: Contributed to manuscript. SF: Supervision to CCH during histological analysis. GDS Supervision to CCH during histological analysis, contributed to manuscript. DB: Facilitated analysis of environmental samples, guided phylogenetic analysis, contributed to manuscript.

Holt, C. C., Stone, M., Bass, D., Bateman, K. S., van Aerle, R., Daniels, C. L., van der Giezen, M., Ross, S., Hooper, C., & Stentiford, G. D. (2019). The first clawed lobster virus *Homarus gammarus* nudivirus (HgNV n. sp.) expands the diversity of the *Nudiviridae*. Scientific Reports, 9(1), 10086

CCH: Sample collection, molecular analysis, in-situ hybridisation, bioinformatic analysis, data visualisation, writing of the manuscript. MS: Sample collection, histological processing and analysis, prevalence data. DB: Supervision to CCH, contributed to manuscript. KSB: Histological/TEM imaging, contributed to manuscript. RvA; Bioinformatic analysis. CLD: Sample collection, supervision to CCH, contributed to manuscript. MvdG: Supervision to CCH, contributed to manuscript. SR: Sample collection, TEM processing. CH: Molecular analysis. GDS: Supervision to CCH, histological analysis, contributed to manuscript.

Holt, C.C., Bass, D., Stentiford, G.D., and van der Giezen, M. Understanding the role of the shrimp gut microbiome in health and disease. Journal of Invertebrate Pathology. Under Review

CCH: Literature review, production of figures, writing of manuscript. DB: Supervision to CCH, contributed to manuscript. GDS: contributed to manuscript. MvdG: Supervision to CCH, contributed to manuscript.

Holt, C. C., van der Giezen, M., Daniels., C. L., Stentiford, G. D., & Bass, D. (2019). Spatial and temporal axes impact bacterial gut ecology and assembly of juvenile European lobster (*Homarus gammarus*): ISME. *Under review*

CCH: Sample collection, molecular analysis, sequencing library preparation, bioinformatic analysis, statistical analysis, data visualisation, writing of the manuscript. MvdG: Supervision to CCH, contributed to manuscript. CLD: Facilitated the provision of samples, supervision to CCH, contributed to manuscript. GDS: contributed to manuscript. DB: Supervision to CCH, contributed to manuscript.

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Chapter 2

Halioticida noduliformans infection in eggs of lobster (*Homarus gammarus*) reveals its generalist parasitic strategy in marine invertebrates

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Abstract

A parasite exhibiting Oomycete-like morphology and pathogenesis was isolated from discoloured eggs of the European lobster (*Homarus gammarus)* and later found in gill tissues of adults. Group-specific Oomycete primers were designed to amplify the 18S ribosomal small subunit (SSU), which initially identified the organism as the same as the '*Haliphthoros*' sp. NJM 0034 strain (AB178865.1) previously isolated from abalone (imported from South Australia to Japan). However, in accordance with other published SSU-based phylogenies, the NJM 0034 isolate did not group with other known *Haliphthoros* species in our Maximum Likelihood and Bayesian phylogenies. Instead, the strain formed an orphan lineage, diverging before the separation of the Saprolegniales and Pythiales. Based upon 28S large subunit (LSU) phylogeny, our own isolate and the previously unidentified 0034 strain are both identical to the abalone pathogen *Halioticida noduliformans.* The genus shares morphological similarities with *Haliphthoros* and *Halocrusticida* and forms a clade with these in LSU phylogenies. Here, we confirm the first recorded occurrence of *H. noduliformans* in European lobsters and associate its presence with pathology of the egg mass, likely leading to reduced fecundity.

Keywords: *Halioticida noduliformans*, *Homarus gammarus*, *Haliphthoros*, Oomycete, 18S rRNA gene

2.1 Introduction

The Oomycetes are parasitic or saprotrophic eukaryotes that group within the Stramenopile clade (Phillips *et al*., 2008). They include numerous taxa which infect and cause disease in aquatic invertebrates (Noga, 1990). Several Oomycete genera are known pathogens of lobsters and Crustacea in general. *Lagenidium*, has been identified as a mortality driver in larval American lobster (*Homarus americanus*) (Nilson *et al*., 1976) and other members of the genus have been detected in several commercially significant shrimp and crab species (Armstrong *et al*., 1976, Bian *et al*., 1979, Bland and Amerson, 1973, Lightner and Fontaine, 1973). Species belonging to the genera *Saprolegnia* and *Aphanomyces* are also notable pathogens of freshwater crayfish (Alderman *et al*., 1984, Diéguez-Uribeondo *et al*., 1994); often associated with catastrophic mortalities in natural stocks in Europe (Holdich *et al*., 2009).

The genus *Haliphthoros* comprises three species; *H. milfordensis, H. philippinensis and H. sabahensis.* These typically infect eggs and early life stage marine invertebrates. Infection has been described in spiny rock lobster (*Jasus edwardsii*) (Diggles, 2001), blue crab (*Portunus pelagicus*) (Nakamura and Hatai, 1995a, Nakamura and Hatai, 1995b), mud crab (*Scylla serrata, S. tranquebarica*) (Leano, 2002, Lee *et al*., 2017), American lobster (*Homarus americanus*) (Fisher *et al*., 1975), white shrimp (*Penaeus setiferus*) (Tharp and Bland, 1977), black tiger shrimp larvae (*Penaeus monodon*) (Chukanhom *et al*., 2003), and abalone (*Haliotis* spp.) (Hatai, 1982, Sekimoto *et al*., 2007). Experimental challenges have also demonstrated the susceptibility of pea crab eggs (*Pinnotheres* sp.) (Ganaros, 1957, Vishniac, 1958) the European lobster (*Homarus gammarus*) (Fisher *et al*., 1975), ova of the blue crab (*Callinectes sapidus*) (Tharp and Bland,

1977), adult pink shrimp (*Penaeus duoraram*) (Tharp and Bland, 1977) and, the ova and larvae of brine shrimp (*Artemia salina*) (Tharp and Bland, 1977). Furthermore, *Haliphthoros* has also been isolated from the surfaces of several algae which may give an indication of its lifecycle outside of an invertebrate host infection (Fuller *et al*., 1964). With the exception of *H. sabahensis* in mud crab (Lee *et al*., 2017), all of these descriptions were solely based on the morphological characteristics of cultures isolated from the site of infection. The infection occurring in black tiger shrimp (Chukanhom *et al*., 2003), however, was later sequenced and analysed phylogenetically (Sekimoto *et al*., 2007). It is therefore possible that, based on morphological descriptions alone, several of these infections could have been misdiagnosed as '*Haliphthoros*' and more accurate diagnostics are required (Stentiford *et al*., 2014).

Halocrusticida (*syn. Halodaphnea*) is a closely related genus isolated from marine Crustacea, erected to contain six taxa belonging to the genus *Atkinsiella* (Nakamura and Hatai, 1995a). All six infect invertebrates with *A. hamanaensis, A. okinawaensis* and *A. panulirata* originally isolated from decapods (*Scylla serrata, Portunus pelagicus* and *Panulirus japonica*, respectively) (Bian and Egusa, 1980, Kitancharoen and Hatai, 1995, Nakamura and Hatai, 1995b). *Atkinsiella dubia*, a crab parasite, was the only species not to be reclassified as a member of the *Halocrusticida* (Atkins, 1954, Nakamura and Hatai, 1995a, Sparrow, 1973).

Sekimoto *et al*. (2007) isolated an unidentified Oomycete (NJM 0034) from white nodules in the mantle of an abalone (*Haliotis rubra*) imported to Japan from southern Australia. The pathogen most closely resembled a species of *Haliphthoros* based on characteristic morphological features such as hyphal fragmentation by cytoplasmic restriction. However, zoosporogenesis, which has

traditionally served as the principle method of species identification to discern between *Haliphthoros* and its close relatives, was not observed. Upon discovery of the unidentified NJM 0034 isolate (herein referred to as 0034), Sekimoto *et al*. (2007) analysed three different gene loci; the ribosomal small subunit (SSU), the ribosomal large subunit (LSU) and the cytochrome c oxidase subunit II (cox2). In the SSU and cox2 phylogenies, 0034 branched just prior to the divergence of Peronosporales and Saprolegniales, separately from the other members of *Haliphthoros*. In the LSU phylogeny, 0034 formed a clade with '*Haliphthoros* sp. *NJM 0131*', originally isolated from black tiger prawn (Chukanhom *et al*., 2003, Sekimoto *et al*., 2007). Muraosa *et al*. (2009) later described a second abalone parasite sharing morphological characteristics with *Haliphthoros* and erected a new genus to describe the species as *Halioticida noduliformans*. *H. noduliformans* was later isolated in wild Japanese mantis shrimp (*Oratosquilla oratoria*) from Japan and cultured abalone (*Haliotis midae*) from South Africa (Atami *et al*., 2009, Macey *et al*., 2011) and found to share 100% sequence identity to the previously sequenced 0034 in the LSU gene region (Macey *et al*., 2011).

As part of an ongoing programme considering novel and emerging pathogens of the European lobster (*Homarus gammarus*) in the United Kingdom, we carried out a histopathology and molecular diagnostic survey of lobsters displaying cloudy/discoloured eggs. We designed and applied new Oomycete-specific SSU PCR primers to reveal the presence of 0034 associated with the egg pathology, and generated LSU sequences from the lobster pathogen to determine whether it was the same as that in *Haliotis rubra* in Japan (Sekimoto *et al*., 2007). We also designed and tested 0034-specific SSU primer sets for use as molecular

diagnostic tools. Our SSU analysis confirmed that 0034 cannot belong to the genus *Haliphthoros* and has no directly related SSU sequence types.

2.2 Methods

2.2.1 Sample collection

2.2.1.1 Animal sampling

From July 2015 to October 2016, 323 egg bearing female lobsters were obtained from various fishermen and wholesale facilities around Cornwall and the Isles of Scilly, United Kingdom, originally recruited to take part in a larval rearing program at the National Lobster Hatchery, Padstow (UK). The landing of egg bearing females was carried out under authorisation granted by the Cornwall Inshore Fisheries and Conservation Authority (IFCA). During this period, a total of 21 animals developed abnormal egg colouration (Figure 2.1) (6.5% of the total number of animals that entered the hatchery). Eighteen of the suspect 21 animals were maintained in wholesaler tanks for up to 7 days prior to transport to the hatchery. The remaining three were chilled and immediately transported. Animals that developed pathological signs of infection (n = 21) had spent between 24 and 106 days within the hatchery tank system. In order to understand the nature of the disease, animals were anaesthetised under ice for up to one hour, depending size. Heart, hepatopancreas (HP), gonad, gut, muscle, gill and eggs were removed using sterile dissecting equipment and fixed for DNA extraction, histopathology, and transmission electron microscopy. Six eggs from a subset of animals were cut in half so that histological and molecular analysis could be applied to the same individual egg. From 4th to 9th July 2016, an additional 17 egg bearing lobsters were collected on landing, from wholesalers in the south of

Cornwall and processed in the same manner as above. These animals did not enter any holding tanks and are herein referred to as ‘wild’. Wild lobsters were chilled on landing and sampled that same day.

Figure 2.1 Gross pathology of infected eggs of Homarus gammarus. Pale, discoloured eggs observed in brood clutch of berried hen. Black eggs indicate healthy, uninfected eggs.

2.2.1.2 Environmental sampling

Littoral marine, brackish water and sediment samples were collected from Newton’s Cove and the Fleet Lagoon (Weymouth, SW England) by Hartikainen *et al*. (2014), together with agricultural soil samples (Gosling *et al*., 2014), and

freshwater samples from the River Avon (Bickton) and California Lake (Berkshire) (Hartikainen *et al*., 2016).

2.2.2 Histology

Lobster egg samples were fixed in Davidson's Seawater Fixative for 24–48 h before transferring tissues to 70% industrial denatured alcohol (IDA). Cassettes were processed using a Leica Peloris Rapid Tissue Processor and subsequently embedded in paraffin wax. Sections were cut using a rotary microtome set at 3 µm thickness, adhered to glass slides and stained using a standard haematoxylin and eosin protocol. Slides were screened for any abnormal pathologies using a Nikon Eclipse light microscope and NIS imaging software at the Cefas Laboratory, Weymouth.

Hyphal staining was carried out following a Grocott-Gomori methanamine silver nitrate staining protocol. Slides were de-waxed and rinsed, followed by oxidation in 5% aqueous chromic acid for 1 h. Slides were then washed and rinsed in 1% aqueous sodium bisulphate for 1 min to remove excess chromic acid, washed again and subsequently placed in the incubation solution (5% sodium tetraborate, distilled water, silver solution (5% aqueous silver nitrate, 3% aqueous methenamine)), pre-heated to 50–60 °C and covered in foil, for 10 min. Stain development was checked after 5 min. This was followed by several washes in distilled water, toning in 0.1% gold chloride for 3–4 min and rinsing in distilled water. Sections were then fixed in 2% sodium thiosulphate for 2–5 min and washed under running water before counterstaining with light green dye (light green SF, acetic acid, water) for 20 s and mounting.

2.2.3 DNA extraction

One hundred mg of lobster tissue (or one egg) was transferred to an MPBio FastPrep (Lysing Matrix A) (MP Biomedicals, Santa Ana, CA) tube containing 250 µL of lysis buffer (SDS, EDTA) and homogenised. 100 µg/µL of Proteinase K was added and tubes were incubated overnight at 55 °C. 75 µL of NaCl along with 42 µL of 10% CTAB/0.7 M NaCl was added prior to further incubation at 65 °C for 10 min. DNA was isolated through phase separation with subsequent additions of chloroform and phenol:chloroform:isoamyl alcohol (25:24:1). DNA was then precipitated in 2× volume of cold 100% ethanol at −20 °C for 1 h, centrifuged to form a pellet and washed with 70% EtOH. The pellet was air dried before elution in molecular grade water.

For water samples, up to 100 L of water was serially filtered through 55 µm and 20 µm meshes. Twenty-five L of the filtered water was later serially passed through 3 µm and 0.45 µm filters. The filtrand was dried and DNA extracted using the MoBio PowerSoil DNA extraction kit (MoBio, Qiagen, Carlsbad, CA).

2.2.4 Primer design

Universal Oomycete primers were designed by manually inspecting an alignment of 215 Stramenopile sequences that spanned the 18S rRNA gene: Oom278F (5′-CTATCAGCTTTGGATGGTAGGA-3′) and Oom1024R (5′-CTCATACGGTGCTGACAAGG-3′), producing an amplicon of around 750–800 bp. The 0034-specific primers were also designed using the Stramenopile alignment with added sequence data generated from infected lobster tissue: Hali_312_F2 (5′-TGGTTCGCCCATGAGTGC-3′) and Hali_415_R1 (5′-CACAGTAAACGATGCAAGTCCATTA-3′) giving a product of ∼100 bp.

2.2.5 PCR and sequencing

PCR amplification was performed in 50 µL volumes using 10 µL of Promega 5× Green GoTaq Flexi Buffer, 5 µL of $MgCl_2$, 0.5 µL of each primer (1 µM), 0.5 µL of DNTPs, 0.25 µL of GoTaq DNA Polymerase, 30.75 µL of molecular grade water and 2.5 µL of template DNA. Initial denaturation was carried out at 94 °C for 2 min. This was followed by 30 PCR cycles: denaturation at 94 °C for 1 min, annealing at 64.5 °C (Oomycete) and 67 °C (0034) for 1 min and extension at 72 °C for 1.5 min (Oomycete) and 10 s (0034), followed by a final extension at 72 °C for 5 min before being held at 4 °C.

PCR products from gill and egg tissues were directly sequenced. Amplification of the environmental samples were conducted in 20 µL final volumes with 1 µL of template DNA and was completed at the Natural History Museum, UK. The thermal cycler program was adjusted (95 °C for 5 min, 30 cycles of 95 °C for 1 min, 55 °C for 1 min and extension of 1 min 15 s at 72 °C, with a final extension at 72 °C for 10 min). Amplicons generated from environmental sampling were pooled according to habitat type (soil, marine, freshwater) and cleaned using polyethylene glycol (PEG) ethanol precipitation. Purified amplicons underwent A-tailing to improve cloning efficiency and were subsequently PEG-cleaned once more.

Clone libraries were created using the StrateClone kit (Agilent Technologies, Santa Clara, CA, USA). 32 clones from each habitat type were Sanger sequenced using the M13 forward primer at NHM. LSU gene fragments were amplified using the LSU-0021 (5′-ATTACCCGCTGAACTTAAGC-3′) and LSU-1170R (5′-GCTATCCTGAGGGAAATTTCGG-3′) following the concentrations and conditions described by Macey *et al*. (2011).

Sequences generated by the study are available in GenBank: accession numbers MH040872-MH040907 (Figure 2.3).

2.2.6 Phylogenetic tree construction

Sequenced amplicons were added to the collection of full-length SSU Oomycete sequences with a Labyrinthulomycete outgroup. Distinct OTUs were defined as having at least one nucleotide difference in two variable regions of the gene. Those that did not satisfy this criterion were considered duplicate sequences and grouped together. Closest BLAST hits for each amplicon generated were included before aligning using the multiple sequence alignment program (MAFFT Version 7; (Katoh and Standley, 2013) and the E-INS-I iterative refinement method. The resulting alignment was used to produce a maximum likelihood phylogenetic tree inference using RAxML-HPC BlackBox version 8 (Stamatakis, 2014) on the CIPRES Science Gateway (Miller *et al*., 2010) using a generalised time-reversible (GTR) model with CAT approximation (all parameters estimated from the data). A Bayesian consensus tree was constructed using MrBayes v 3.2.5 (Ronquist *et al*., 2012). Two separate MC^3 runs with randomly generated starting trees were carried out for 5 million generations, each with one cold and three heated chains. The evolutionary model used by this study included a GTR substitution matrix, a four-category auto-correlated gamma correction, and the covarion model. All parameters were estimated from the data. Trees were sampled every 1000 generations. The first 1.25 million generations were discarded as burn-in (trees sampled before the likelihood plots reached stationarity) and a consensus tree was constructed from the remaining sample.

2.2.7 *In-situ* hybridisation (ISH)

One hundred µl of hybridisation probes were generated using 20 µL of Promega 5× Green GoTaq Flexi Buffer, 10 µL of $MgCl_2$, 2 µL of each primer (Oom278F and Oom1024R), 10 µL of DIG-labelled dNTPs, 1 µL of GoTaq DNA Polymerase, 49 µL of molecular grade water and 6 µL of template DNA. Amplification was performed using the previously mentioned thermal cycler settings.

Slides mounted with suspect wax sections were de-waxed as above and air-dried. De-waxed slides were then treated with 100 µg/ml Proteinase K in H_2O for 15 min at 37 °C in an opaque box soaked in 5× saline-sodium citrate (SSC) buffer (trisodium citrate, NaCl, water). The slides were then incubated in 100% IDA for 5 min and subsequently rinsed in 2× washing buffer (20× SSC, Urea, BSA). Gene Frames (Thermo Fisher Scientific) were mounted on to each slide and 300 µL of probe in a 1 in 2 dilution with hybridisation buffer (100% formamide, 50% dextran sulphate, 20× SSC, 10 mg/mL yeast tRNA, 50× Denhardt's solution) was added. Slides were then denatured at 95 °C for 5 min and hybridised overnight at 40 °C. Gene Frames were removed and slides were washed with 2× washing buffer, preheated to 40 °C, for 15 min. Hybridisation was blocked with a one hour incubation using 6% skimmed milk powder in Tris buffer. Slides were then incubated with an Anti-Digoxigenin antibody diluted in Tris buffer (1/300 dilution) for one hour at room temperature. Antibody was removed and slides were washed before staining with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyphosphate (NBT/BCIP) solution. Stained slides were then washed and counter-stained with Nuclear Fast Red before mounting and examination under light microscopy.

2.3 Results

2.3.1 Clinical signs

Infected eggs often appeared white, pink or grey relative to uninfected eggs (Figure 2.1). Upon dissection, necrotic lesions were also commonly observed within the gill chambers of infected animals. Copepod parasitism within the gill chamber was observed in all animals.

2.3.2 Histopathology

Egg samples from 8 out of the original 21 animals showed abnormal pathology (38% of animals in total) (Figure 2.2A and B). Eggs showed a reduction or complete lack of egg yolk protein and were instead filled with large, hyphal structures. In some cases, thalli made up the entire egg mass and structures were seen protruding out of the egg membrane, potentially representing zoospore discharge tubes (Figure 2.2A). Hyphae were irregular in shape and multinucleated.

Gill samples from 5 out of the 21 (24%) animals showed similar thallic structures (Figure 2.2C–F). Nine of the 21 gills showed evidence of an immune response characterised by the presence of haemocyte aggregation (not shown) and melanisation (Figure 2.2C and D). Hyphal cell walls were stained with silver (Figure 2.2E). *In situ* hybridisation with general *Oomycete* SSU probes demonstrated the localisation of the gene target in infected tissues (Figure 2.2F).

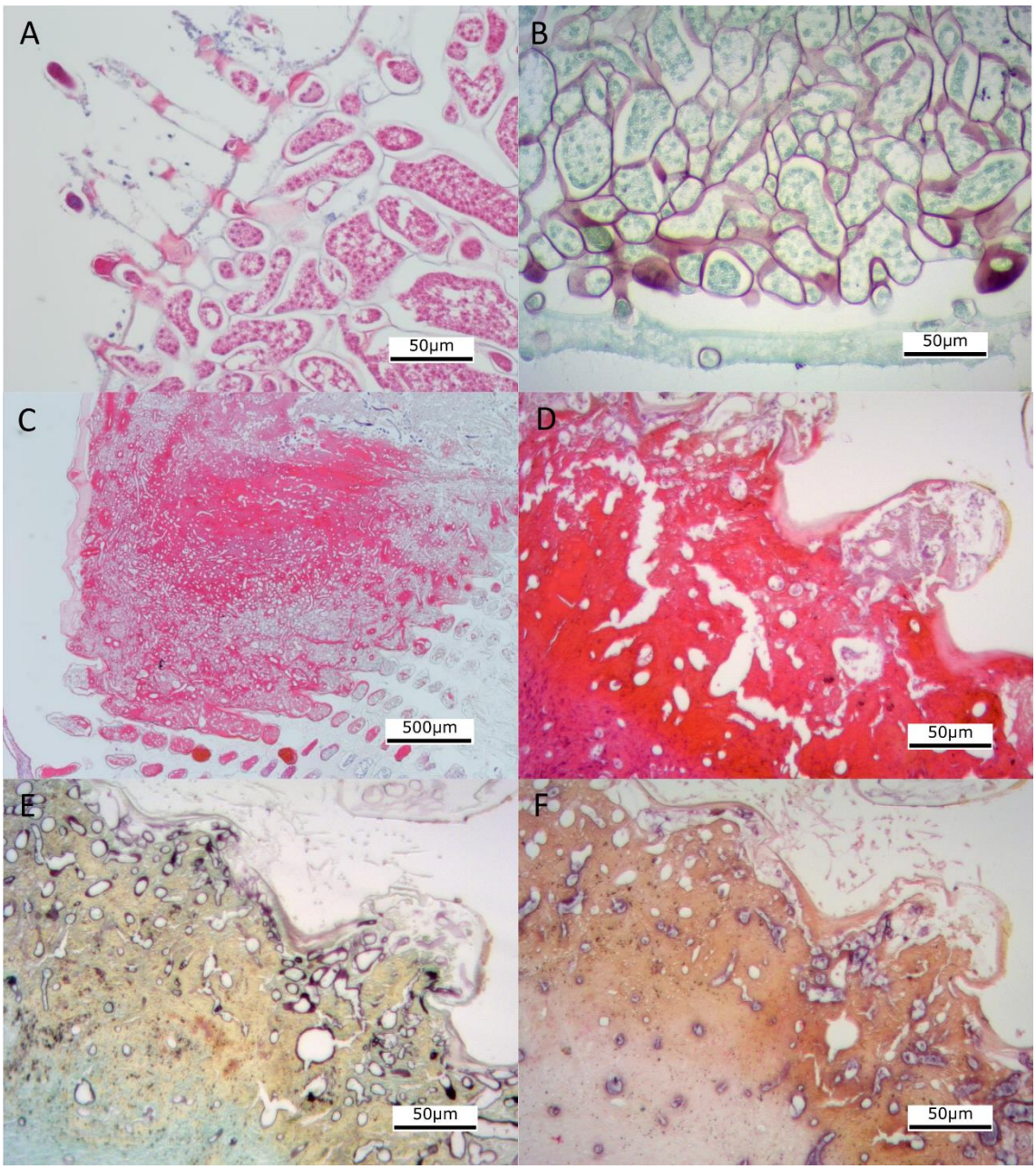

Figure 2.2 Histological sectioning of infected tissues. Light microscopy images of 3µm tissue sections. A) Hyphae protruding from the surface of the egg. Scale bar = 50 µm. B) Silver staining of the hyphal cell walls within egg tissue. Scale bar = 50 µm. C) Low magnification image of infected gill tissue showing loss of structure and replacement with inflammatory cells and melanisation. Scale bar = 500 µm. D) Melanised lesion showing multinucleate nature of the hyphae ramifying through gill tissue. Scale bar = 500 µm. E) Silver staining of hyphal cell walls within the melanised lesion of the gill. Scalebar = 50 µm. F) In-situ hybridisation labelling of H.noduliformans using universal-oomycete SSU probes.Scale bar = 50 µm

2.3.3 Molecular characterisation of the 18S ribosomal SSU in infected eggs

Oomycete-specific PCRs on all but three of the 13 eggs from the initial group produced positive PCR products (~800 bp). Sequences obtained from excised positive bands were 99–100% identical to the *Haliphthoros* sp. NJM 0034 GenBank entry (AB178865.1). Both positive control DNA samples, *Aphanomyces invadans* and *Saprolegnia parasitica*, also amplified. Further individual egg samples (30 eggs from 5 individuals) were each bisected; one half used for histological analysis, the other for molecular analysis. All the eggs containing hyphal structures were PCR-positive using Oomycete-specific primers. In some cases, histology-negative samples produced a positive but weaker PCR product. All amplicons were sequenced and all but one of the histology positive samples produced a sequence identical to the 0034 sequence. The remaining egg, (sample 5.3) along with two histology negative samples produced PCR products which, when sequenced, showed 98–99% sequence identity with *Lagenidium callinectes* (AB284571) (Figure 2.3).

Several of the histology and Oomycete PCR-positive samples were tested using the 0034-specific primer set and produced a positive amplicon of around 100 bp. Additionally, the *Lagenidium*-positive egg, sample 5.3, produced a positive, but very weak PCR product with the 0034-specific primers. Sequence data from the 0034-specific primer set confirmed the additional presence of this lineage.

2.3.4 Environmental sequencing using oomycete-specific primers

To test the specificity of the Oomycete primers we used them to amplify DNA extracted from a range of environmental samples: 16 samples from filtered coastal sea and brackish water, 24 samples from agricultural soil, and 48 samples from filtered freshwater. Eighty of the 88 environmental samples (90.9%)

amplified using the Oomycete primers: 16/16 of the marine water samples, 22/24 of the soil samples and 42/48 of the freshwater samples. These sequences clustered into 71 operational taxonomic units (OTUs), which branched across the full range of Oomycete diversity as shown in Figure 2.3. Thirty seven of these were identical or very similar to GenBank sequences using the same grouping criterion as described in the methods. The other 34 OTUs were novel and are indicated by FW, Soil, and Marine prefixes in Figure 2.3.

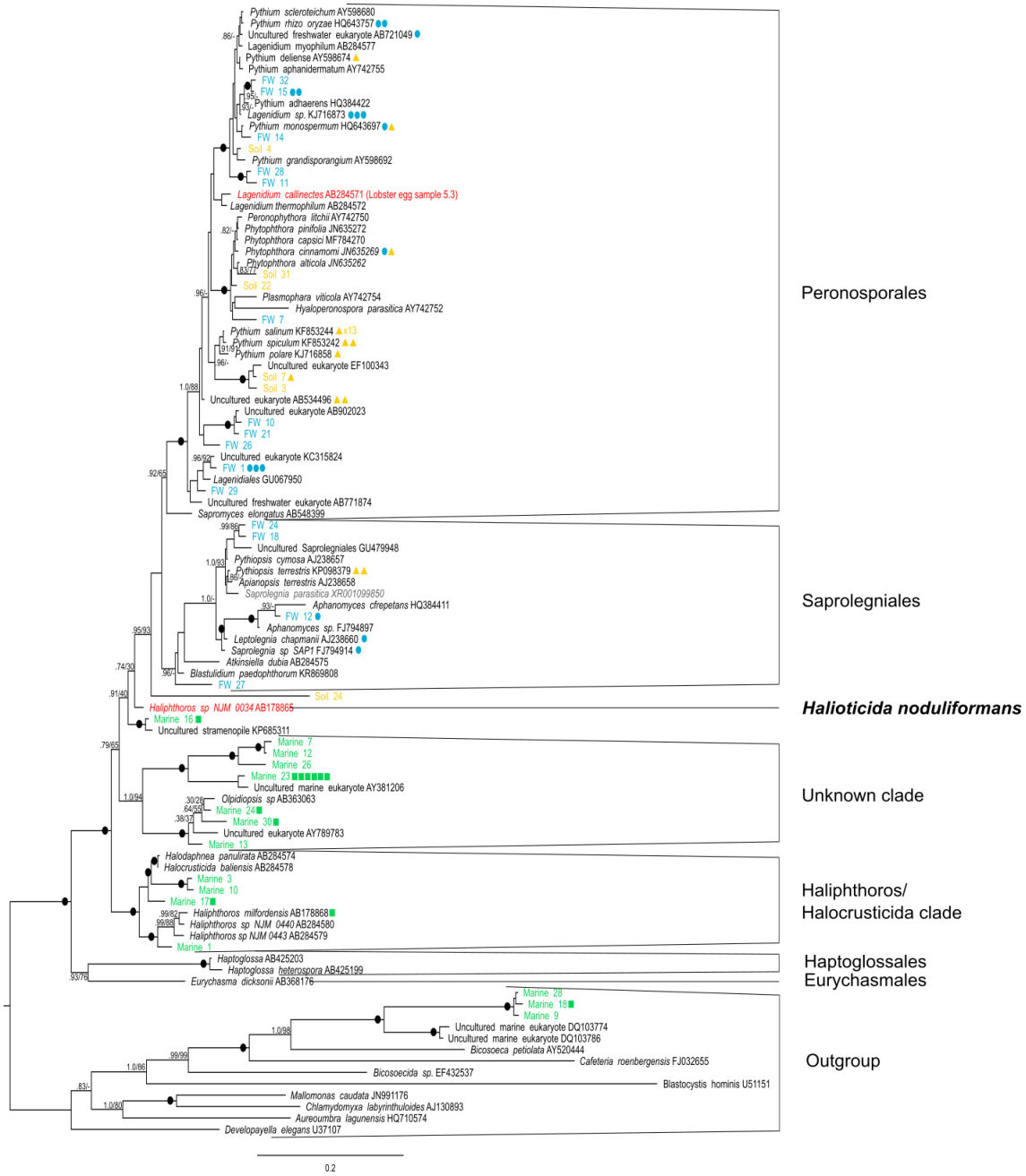

Figure 2.3 SSU gene phylogeny of the Oomycete class Bayesian phylogeny indicating the range of oomycete diversity detected using Oomycete-specific SSU primers. Shapes accompanying tip labels indicate number of environmental samples grouped with each OTU. Circle = freshwater sample (blue), triangle = soil sample (yellow) and square = marine water sample (green). Red tip labels indicate sequences derived from lobster tissue. Grey highlights cultured, positive control. Nodes labelled with black circles indicate Bayesian/Maximum likelihood (%) support of over 0.95/95. With the exception to nodes surrounding the Haliphthoros/Halocrusticida clade, only support greater than 0.8/75 is annotated.

Twenty six of the OTUs generated in this study grouped within the Peronosporales (12 FW, 10 Soil and two in both FW and Soil), 7 in the Saprolegniales (6 FW, one Soil) and one Soil sample (Soil 24) branched prior to the divergence of these two orders (Figure 2.3). All the sequences generated from marine water samples also branched before the radiation of the Peronosporales and Saprolegniales. Three out of the 31 OTUs generated from marine sampling branched outside of the Oomycete radiation, near the Bicosoeca.

In phylogenetic analyses of a comprehensive taxon sampling of early-branching oomycete diversity, including 0034, *Haliphthoros, Halocrusticida, Olpidiopsis* and *Anisolpidium*, lineages cluster to some extent according to known host (Figure 2.4). Four OTUs branch in a clade with the brown algae parasites *Anisolpidium* spp., three OTUs form a clade with the red algae parasites *Olpidiopsis*, and a further four OTUs in a clade with *Haliphthoros, Halocrusticida,*and *Haliphthoros* (parasites of marine invertebrates), including one grouping strongly with (AB178868) *Haliphthoros milfordensis*.

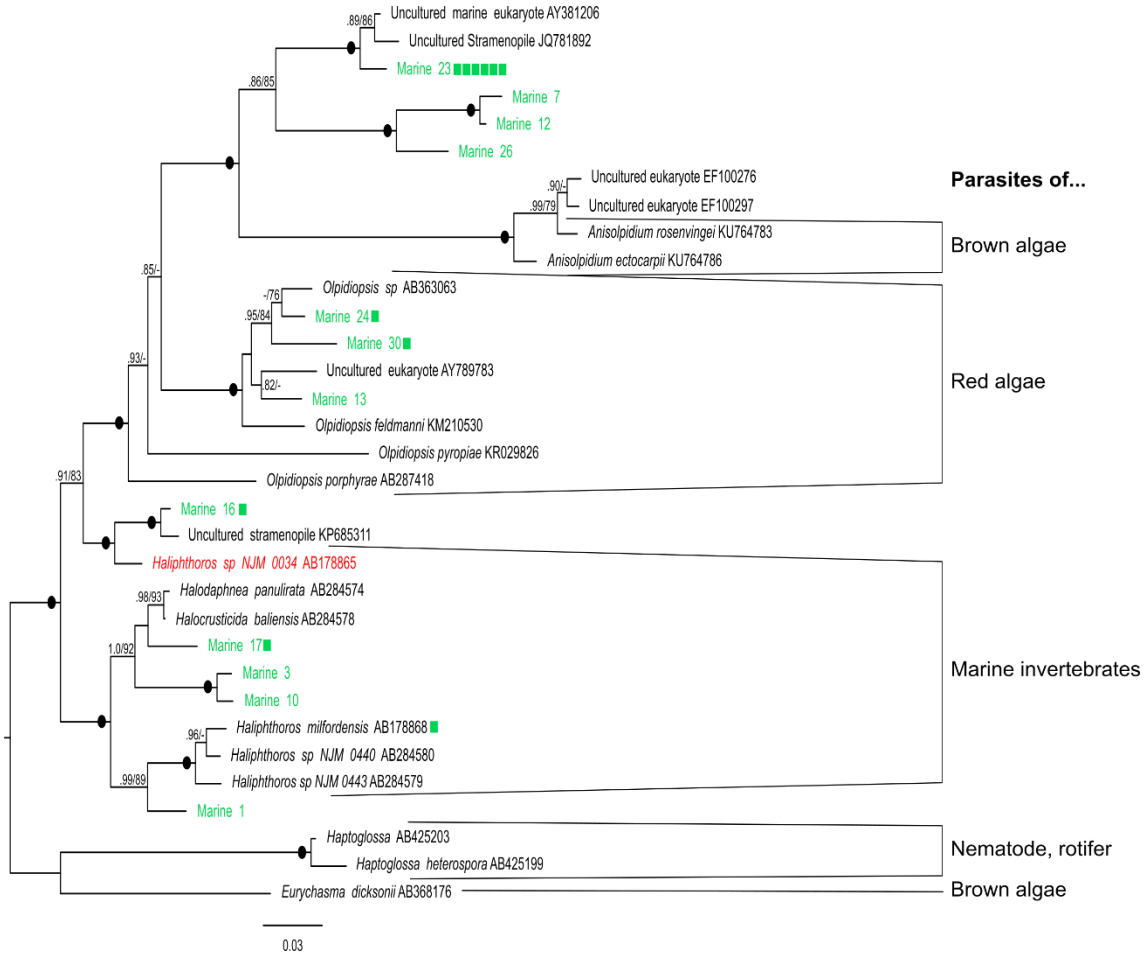

Figure 2.4. SSU gene phylogeny of the lineages surrounding NJM 0034. Bayesian phylogeny of NJM 0034 and its close relatives. Accompanying (green) squares indicate number of environmental samples grouped with that OTU. Red tip labels indicate sequences derived from lobster tissue. Nodes labelled with hollow circle indicate Bayesian/Maximum likelihood (%) support of over 0.95/95. Nodes showing support greater than 0.8/75 are annotated.

2.3.5 Phylogenetic relationships of the *Haliphthoros*-like samples

Twenty two out of the 27 sequences generated from lobster egg samples were 99–100% similar to *Haliphthoros* sp. NJM 0034 (AB178865). In ML and Bayesian phylogenetic analyses of the consensus sequence (Figure 2.3), this lineage branched separately from the three other described *Haliphthoros* sequences (AB178868, AB284580, AB284579) and as a sister to the Saprolegniales but without strong support (Bayesian PP 0.74; ML bootstrap 40%). The remaining

three sequences grouped with AB284571 (*Lagenidium callinectes*) (98–99% identity) isolated from marine crustacea (unpublished). Two further low-quality sequences were not analysed. LSU PCR amplification of three heavily infected eggs produced an amplicon of around 1 kb in length. Sequences generated from the isolation and purification of these products aligned with *Halioticida noduliformans* sequences (GU289906, AB506706, AB285230, AB285227) and 0034 (AB178866) with 99–100% identity. Phylogenetic analysis of the LSU region by Macey *et al*. (2011) showed how this *H.noduliformans* sequence branches alongside *Haliphthoros* and *Halocrusticida* species.

2.3.6 Follow-up health screen of wild lobsters

Histology of 17 wild lobster tissues did not show any abnormalities or Oomycete-related pathology. No amplicons were generated when Oomycete and *H. noduliformans* (*0034*) primers were applied to the eggs and guts. However, 5 out of the 17 gill samples weakly amplified using the Oomycete primers. Three of these tested positive for *H. noduliformans* using the species-specific primer set. There was no evidence of infection by histology in any of these samples other than the copepod parasitism of the gills as observed in the previous group of animals.

2.4 Discussion

2.4.1 Phylogenetic position of *Halioticida noduliformans*

In this study, we confirm the presence of the Oomycete pathogen *Halioticida noduliformans* as an egg parasite of the European lobster (*Homarus gammarus*). By application of improved Oomycete diagnostic primers and, by phylogenetic

analysis of the amplicon derived from these primers applied to infected lobster eggs, we show that the parasite is also the same as the abalone pathogen 0034 (previously described as a *Haliphthoros* sp. NJM 0034) (Sekimoto *et al*., 2007). Isolation and amplification of the SSU region of the parasite from a number of eggs produced amplicons that shared 99–100% identity with 0034 (AB178865). This sequence has only previously been associated with diseased abalone in Japan (Sekimoto *et al*., 2007). Despite similar morphological characteristics, 0034 did not group with *Haliphthoros milfordensis* (*AB178868*) from black tiger prawn (*Penaeus monodon*) or *Haliphthoros* sp. (*AB284580, AB284579*) isolated from marine Crustacea in our SSU phylogenetic analyses and in already published analyses using the same marker gene (Beakes and Sekimoto, 2009, Sekimoto *et al*., 2007). The lobster egg parasite instead branched before the radiation of the more derived Saprolegniales clade. We therefore agree with the suggestion by Sekimoto *et al*. (2007) in that, although 0034 shares morphological similarities to *H. milfordensis* (both in terms of their wet mount observations and our histological sectioning), the isolate is clearly distinct from already described *Haliphthoros* species.

LSU rRNA gene phylogenies provide further insight into the position of the 0034 sequence type. The LSU Maximum-Likelihood phylogeny of Sekimoto *et al*. (2007) showed the original 0034 isolate branching as a sister to the *Haliphthoros milfordensis NJM 0131* strain (AB178869). In Macey *et al*.'s (2011) LSU phylogeny, 0034 is apparently identical to *Halioticida noduliformans* (GU289906); an Oomycete also isolated from nodules in the mantle of abalone and described as a causative agent of abalone tubercle mycosis disease, which causes significant mortalities in South Africa (Macey *et al*., 2011). There is no available corresponding SSU sequence belonging to *H.*

noduliformans to allow the comparison of both gene markers however, it is likely that 0034 is *Halioticida noduliformans*, based on its LSU sequence and phylogeny and that the *Halioticida, Haliphthoros* and *Halocrusticida* genera are mutually related, together comprising parasites of aquatic invertebrates. LSU amplicons from our own isolate were identical to *Halioticida noduliformans* sequences isolated from both shrimp and abalone along with the 0034 isolate.

2.4.2 Pathology of *Halioticida noduliformans* and its relatives

To our knowledge, this is the first report of *Halioticida nofuliformans* in the European lobster or any host species from the United Kingdom and Europe. Very few references exist in terms of the histopathological descriptions of *H. noduliformans* and its closely related Oomycetes, such as *Haliphthoros.* Atami *et al*. (2009) offered the first histological descriptions of *H. noduliformans* in their shrimp host. They described the presence of hyphae in the gill filaments and base of those filaments. In our lobster hosts, we first detected the pathogen in discoloured egg samples. Infiltration of the egg had resulted in a mass of vegetative hyphae and the breakdown of the egg yolk protein within. Infection in adult tissues was similarly confined to the gills where growth was likely halted by the surrounding areas of melanisation; a key defence mechanism of the host. It should be noted however, that gill fouling may have contributed to the presence of necrotic tissue. No other negative health effects were observed, however severe melanisation and subsequent necrotic lesions may well interfere with ecdysis or compromise respiratory function (Diggles, 2001, Fisher, 1977, Fisher *et al*., 1975). Macey *et al*. (2011), who reported the pathogen in abalone, also conducted a histological examination. They describe large numbers of hyphae penetrating the affected areas. However, in contrast to our own observations,

where infected lobsters showed vast areas of melanisation, they note that there was ‘very little inflammation and in most cases no reaction zone’. *Haliphthoros* pathology in juvenile spiny rock lobster (*Jasus edwardsii*) shows a similar histology of the gills with the presence of multinucleate hyphae within the filaments. Hyphae and melanised lesions were also observed within the leg musculature and hepatopancreas (Diggles, 2001).

Low levels of *Halioticida noduliformans* were detected by PCR in the gills from our wild lobster health screen. However, we did not observe any pathological evidence of an infection. It is likely that adult lobsters in the wild are better able to combat the pathogen and infected eggs are prematurely dispersed to make way for the next brood (Leano, 2002). Although we do not understand its effect on the wild population, *H. noduliformans* and other similar pathogenic Oomycetes are likely to pose an increased threat to hatchery and/or aquaculture-based lobsters and other invertebrates in culture situations. Intensive culture systems/sub optimal culture situations can cause physiological stresses which can increase the disease susceptibility of cultured organisms (Robohm *et al*., 2005). With increasing food demands and the continual growth in the world’s aquaculture industry, it is estimated that by 2030, 62% of consumed seafood will come from a farming environment (World Bank, 2013). It is therefore becoming increasingly important to better understand the health risks associated with such a shift and identify simple means in which we can detect and monitor them within these environments. That is particularly true of the Oomycetes and, more specifically the *Halioticida/Haliphthoros/Halocrusticida* clade, as they have demonstrated their ability to dramatically affect commercially important invertebrate species.

2.4.3 Oomycete-specific PCR primers

The cytochrome *c* oxidase subunits (cox) and internal transcribed spacers (ITS) have been suggested as DNA barcodes for the Oomycetes. However, these loci can be problematic for phylogenetic reconstruction, which is an important element of the interpretation of amplicon diversity (metabarcoding) data (Hartikainen *et al*., 2014). Choi *et al*. (2015) report that the ITS regions can contain large insertions exceeding 2500 bp for some species which will introduce biases in PCR amplification. Furthermore, together with an insufficient reference database, cox1 amplification does not identify all known Oomycete lineages (Choi *et al*., 2015). The authors demonstrate how amplification of the sequence region spanning the cox2 gene and the hypervariable cox2-1 spacer amplified all the lineages tested (n = 31) and therefore suggest that the cox2 region is better suited as a gene marker. However, these primers were not tested through means of an environmental survey and were only applied to individual lineages belonging to the Peronosporales.

LSU primers have also been used to analyse the molecular characteristics of the Oomycetes and, based on its ability to separate *Halioticida* sequences within the Haliphthoraceae, the D1/D2 region of the LSU has been suggested as a useful marker to discern between members of this family (Muraosa *et al*., 2009). Although the cox2 and LSU gene regions have proven beneficial in the identification of the Haliphthoraceae and the Oomycetes in general, their reference databases are not as extensive as that of the SSU gene marker. The SSU primers that we present here will facilitate better phylogenetic comparisons to be made as comparative gene sequences are more readily available. Environmental testing of the primer set has indicated their ability to detect a wide phylogenetic range of Oomycetes across all sample types tested (freshwater,

marine water and soil). Thus, we were able to detect *H. milfordensis* for the first time in a UK marine water sample. We have also developed a second primer set that is specific to *Halioticida noduliformans*. Using a combination of these primers, we detected cases of co-infection with *Lagenidium* in several lobster egg samples. It is possible that *H. noduliformans* infection commonly occurs in tandem with other pathogenic Oomycetes as previously reported in mangrove crab; where co-infection with *Lagenidium callinectes, Haliphthoros milfordensis* and *Halocrusticida baliensis* caused mortality rates of nearly 100% in tanked larvae (Hatai *et al*., 2000).

2.5 Conclusions

We provide the first evidence of infection of European lobsters (*Homarus gammarus*) by *Halioticida noduliformans* causing a destructive pathology of the eggs. To our knowledge, this is also the first report of the parasite in any animal collected from European waters. Potentially due to the unavailability of *Halioticida* and *Halocrusticida* SSU sequences, the AB178865 sequence does not resolve the phylogenetic positioning of this parasite in SSU trees. However, LSU analysis confirms its clustering within the Haliphthoraceae clade, which also contains the *Haliphthoros* and *Halocrusticida* genera.

Incidence of *Halioticida noduliformans* in the European lobster not only demonstrates its ability to impact animals outside of its known hosts (abalone and Japanese mantis shrimp) but also, highlights the far-reaching geographical distribution of the pathogen, which has not been previously reported in Europe. This relatively newly discovered Oomycete has proven its ability to impact commercially important species and may pose a threat to future aquaculture

efforts. Based on its similarity and relatedness to the genus *Haliphthoros*, it is possible that *Halioticida noduliformans* can impact a range of invertebrate species (as does *Haliphthoros milfordensis*) and therefore further work is required to highlight the extent of its host range and subsequent effects on the hatchery and aquaculture industry.

The general Oomycete and *H. noduliformans*-specific primer sets we have developed during this study should better facilitate the identification of this and other potentially problematic Oomycetes and allow the exploration of other susceptible host species. They have been subject to environmental testing on a range of different sample types and have demonstrated their ability to identify a diverse spectrum of species that span the entire Oomycete diversity.

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Chapter 3

The first clawed lobster virus *Homarus gammarus* nudivirus (HgNV n. sp.) expands the diversity of the *Nudiviridae*

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Abstract

Viral diseases of crustaceans are increasingly recognised as challenges to shellfish farms and fisheries. Here we describe the first naturally-occurring virus reported in any clawed lobster species. Hypertrophied nuclei with emarginated chromatin, characteristic histopathological lesions of DNA virus infection, were observed within the hepatopancreatic epithelial cells of juvenile European lobsters (*Homarus gammarus*). Transmission electron microscopy revealed infection with a bacilliform virus containing a rod shaped nucleocapsid enveloped in an elliptical membrane. Assembly of PCR-free shotgun metagenomic sequencing produced a circular genome of 107,063 bp containing 97 open reading frames, the majority of which share sequence similarity with a virus infecting the black tiger shrimp: *Penaeus monodon* nudivirus (PmNV). Multiple phylogenetic analyses confirm the new virus to be a novel member of the Nudiviridae: *Homarus gammarus* nudivirus (HgNV). Evidence of occlusion body formation, characteristic of PmNV and its closest relatives, was not observed, questioning the horizontal transmission strategy of HgNV outside of the host. We discuss the potential impacts of HgNV on juvenile lobster growth and mortality and present HgNV-specific primers to serve as a diagnostic tool for monitoring the virus in wild and farmed lobster stocks.

3.1 Introduction

Viral pathogens and the diseases that they impart are a particularly significant source of production loss in the cultivation of crustaceans (Stentiford *et al.*, 2012). Despite the preponderance of known viruses in a wide range of crustacean hosts and their ubiquity in the aquatic environment, with abundance estimates of ~10^8 viruses per ml of productive coastal waters (Suttle, 2005), there have been no reported examples of naturally-occurring viruses infecting any clawed-lobster species (Decapoda; Nephropidae).

The currently unclassified *Panulirus argus* virus 1 (PaV1) is so far the only virus described from lobsters, in this case infecting mesodermal cells of the Caribbean spiny lobster (*Panulirus argus)* in Florida and throughout the Caribbean (Shields and Behringer, 2004). The infection, characterised by a milky colouration of the haemolymph and lethargy of the host, initially infects fixed phagocytes in the hepatopancreas (HP), prior to spreading to cells of the connective tissues. Since its initial discovery in juvenile life stages in the United States, PaV1 has been found in wild and cultured host populations throughout the Caribbean (Shields, 2011). Remarkably, healthy lobsters demonstrate avoidance behaviours towards those infected with the PaV1 virus (Behringer, Butler and Shields, 2006). As with several other invertebrate pathogens, mortality rate is higher in animals with a carapace length of less than 16 mm (Butler, Behringer and Shields, 2008). Until now, there have been no reports of PaV1-like viruses or any other viruses in any species within the clawed lobster genera of the family Nephropidae. In experimental conditions, White spot syndrome virus (WSSV), a double stranded DNA (dsDNA) virus of the family *Nimaviridae*, has been demonstrated to infect (and cause disease in) both American lobster *(Homarus americanus*)

(Clark *et al.*, 2013) and the European lobster (*H. gammarus*) (Bateman *et al.*, 2012) as well as numerous other decapod crustacean taxa (Pradeep and Rai, 2012). However, WSSV has not been detected in wild or cultured Nephropidae.

Viral diseases have led to substantial bottlenecks to shrimp aquaculture production. Monodon baculovirus (MBV), the causative agent of spherical baculovirosis, was the first virus reported in penaeid shrimp (Lightner and Redman, 1981). Phylogenetic analyses and genomic reconstruction has since suggested that MBV be reclassified as *Penaeus monodon* nudivirus (PmNV) and be reassigned to the *Nudiviridae* (Wang and Jehle, 2009; Yang *et al.*, 2014), a family of dsDNA viruses which to that point was exclusively comprised of viruses infecting insects. Although initially named to reflect a lack of occlusion body formation (large protein lattices which protect the bacilliform-shaped virions and facilitate transmission outside of the host), there are now multiple examples within the *Nudiviridae* where occlusion bodies have been observed, or where sequence and structural homologs of the *polyhedrin* gene have been found within the genome (Cheng *et al.*, 2002; Yang *et al.*, 2014; Bézier *et al.*, 2015). Seven fully sequenced virus species have been characterised as nudiviruses: *Penaeus monodon* nudivirus (PmNV) (Yang *et al.*, 2014); *Gryllus bimaculatus* nudivirus (GbNV), infecting the nymph and adult stages of several cricket species (Wang *et al.*, 2007); *Heliothis zea* nudivirus-1 (HzNV-1), a persistent pathogen of insect cell lines (Cheng *et al.*, 2002); *Helicoverpa (*syn. *Heliothis) zea* nudivirus-2 (HzNV-2), the sexually transmitted corn earworm moth virus which can cause sterility in the host (Burand *et al.*, 2012); *Oryctes rhinoceros* nudivirus (OrNV), a biological control agent used to manage palm rhinoceros beetle populations (Wang *et al.*, 2011); *Tipula oleracea* nudivirus (ToNV) a causative agent of nucleopolyhedrosis in crane fly larvae (Bézier *et al.*, 2015); and *Drosophila*

innubila nudivirus (DiNV) (Unckless, 2011; Hill and Unckless, 2018), which causes significant reductions to fecundity and lifespan (Unckless, 2011). Three further viruses isolated from metagenomic sequencing of *Drosophila melanogaster* (Kallithea virus (Webster *et al.*, 2015), Tomelloso virus and Esparto virus) have also been described as nudiviruses (Table 3.1). However, the genomes of these three *Drosophila* viruses have yet to be analysed with respect to their phylogenetic position. There is also evidence of ancestral nudivirus integration into the host genome (*Nilaparvata lugens* endogenous nudivirus (NleNV) (Cheng *et al.*, 2014) and a sister group of the nudiviruses, the bracoviruses, associated with Braconid wasp hosts, where viral genes are also integrated into the host genome (Bézier *et al.*, 2009). Finally, a large DNA virus infecting the hepatopancreas of the European brown shrimp, *Crangon crangon* has also been proposed as a putative member of the *Nudiviridae* albeit based upon limited genomic information (Bateman and Stentiford, 2017; Van Eynde *et al.*, 2018).

As part of a large UK-based lobster rearing study assessing the growth of hatchery-reared European lobsters in novel sea-based container culture (SBCC) systems (Lobster Grower, www.lobstergrower.co.uk), we conducted a histology-led health screening of a large cohort of individuals (n = 1,698), sampled at several time points throughout a multi-year production cycle. We observed a distinctive histopathology of the hepatopancreas of juvenile lobsters in both hatchery and sea container phases of production. Intranuclear inclusions appeared within the hepatopancreatocytes of affected individuals; later confirmed by transmission electron microscopy (TEM) as of viral aetiology. Genome assembly of PCR-free shotgun metagenomic sequences confirmed the presence of a novel member of the *Nudiviridae*; hereby named *Homarus*

gammarus nudivirus, the first virus described infecting any clawed lobster genus. Here, we present the fully annotated genome of HgNV, comprising a single contiguous sequence, together with diagnostic primers and reference histology and ultrastructure to aid in future identification in natural and aquaculture settings. HgNV is now the second confirmed aquatic nudivirus.

Name	Initial Host	Genome Size (bp)	Number of ORFs	Gene Density (per kb)	GC Content (%)	Reference
HgNV	European lobster (*Homarus gammarus*)	107 063	97	1.10	35.3	This study
GbNV	Field cricket (*Gryllus bimaculatus*)	96 944	98	0.99	28.0	Wang *et al*. 2007
HzNV-1	Corn earworm (*Heliothis zea*) †	228 089	155	1.47	41.8	Cheng *et al*. 2002
HzNV-2	Corn earworm (*Heliocoverpa zea syn. Heliothis*)	231 621	113	2.05	41.9	Burand *et al*. 2012
OrNV	Rhinoceros beetle *(Oryctes rhinoceros)*	127 615	139	0.92	42.0	Wang *et al*. 2011
PmNV	Black tiger shrimp (*Penaeus monodon*)	119 638	115	1.04	34.5	Yang *et al*. 2014
ToNV	Crane fly (*Tipula oleracea*)	145 704	131	1.11	25.5	Bézier *et al*. 2015
DiNV	Drosophilid fly *(Drosophila innubila)*	155 555	107	1.45	30.0	Hill & Unckless, 2018
***KNV**	Common fruit fly (*Drosophila melanogaster*)	152 388	95	1.6	38.9	KX130344
***TNV**	Common fruit fly (*Drosophila melanogaster*)	122 307	93	1.32	39.6	KY457233
***ENV**	Common fruit fly (*Drosophila melanogaster*)	183 261	87	2.11	29.5	KY608910

Table 3.1 Comparative genomic data of sequenced nudiviruses. † Cell line. *Direct submission to GenBank - number of ORFs, gene density and GC content estimated from database entry. Accession numbers provided where journal reference of genome annotation is not available.

3.2 Methods

3.2.1 Experimental design and sample collection

Over the period of July 2016 to April 2017, 14,507 hatchery-reared juvenile lobsters were deployed in SBCCs anchored off the coast of Cornwall (St. Austell Bay 50° 18.956 N, 4°44.063 W). The majority of those deployments (10,987 animals), including those used in the current study, occurred in the summer of 2016. Routine sampling (3, 6, 28, 39, 52 and 104 weeks post deployment) was carried out to monitor the incidence of disease in SBCC populations. In total, 1,698 animals were sampled over the 2-year period. Another set of lobsters were retained within the National Lobster Hatchery, Padstow UK, and sampled at the same time points, over this period. Carapace length and survival were measured at each time point. Upon sampling, larger animals (39–104 weeks post deployment) were anaesthetised on ice for several minutes prior to bisection through the dorsal line. One half was fixed in Davidson's Seawater fixative for histological processing, the other fixed in molecular grade ethanol for sequence analysis. A small piece of hepatopancreas was removed and fixed for transmission election microscopy. Smaller animals (0–28 weeks post deployment) were fixed whole and underwent separate analyses.

Six juvenile lobsters displaying pronounced histopathology associated with viral infection were selected from hatchery and SBCC settings, allowing for comparative molecular and transmission election microscopy analyses. Five of the six animals had spent one to two years growing in controlled hatchery raceways. The remaining individual had spent 52 weeks in SBCC in the open sea.

3.2.2 Histopathology

Bisected lobsters were placed into histological cassettes and fixed in Davidson's Seawater Fixative for 24–48 h before transfer to 70% industrial denatured alcohol (IDA). Cassettes were processed using a Leica Peloris Rapid Tissue Processor and subsequently embedded in paraffin wax. Histological sections were cut using a rotary microtome set at 3 µm thickness, adhered to glass slides and stained using a standard haematoxylin and eosin (H&E) protocol. Slides were examined using a Nikon Eclipse light microscope and NIS imaging software at the International Centre of Excellence for Aquatic Animal Health at the Cefas Laboratory, Weymouth, UK.

3.2.3 Transmission electron microscopy

Hepatopancreas samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and later rinsed in 0.1 M sodium cacodylate buffer prior to processing. Post-fixation was carried out in 1% osmium tetroxide/0.1 M sodium cacodylate buffer for 1 h. Tissues were washed in three changes of 0.01 M sodium cacodylate buffer and were subsequently dehydrated through a graded acetone series before embedding in Agar 100 epoxy resin (Agar Scientific, Agar 100 pre-mix kit medium). Embedded tissues were polymerised overnight at 60 °C. Semi-thin (1–2 µm) sections were cut and stained with Toluidine blue for viewing with a light microscope to identify suitable target areas. Ultra-thin sections (70–90 µM) of targeted areas were mounted on uncoated copper grids and stained with 2% aqueous uranyl acetate and Reynold's lead citrate (Reynolds, 1963). Grids were examined using a JEOL JEM1400 transmission electron microscope

and digital images captured using an AMT XR80 camera and AMT V602 software.

3.2.4 DNA extraction and sequencing

DNA for genomic reconstruction was extracted using the CTAB/phenol:chloroform extraction protocol as described in Chapter 2 (Holt *et al.*, 2018). DNA for HgNV screens of HP tissue was extracted using the EZ1 Advanced XL and DNA Tissue Kit (Qiagen). Extracted DNA was cleaned with polyethylene-glycol (PEG) precipitation and submitted to the sequencing service at the University of Exeter, UK for shotgun library preparation using the TruSeq DNA PCR-Free Library Prep Kit. Pooled libraries underwent high-throughput sequencing using an Illumina Miseq (2 × 300 bp).

3.2.5 Sequence analysis

The raw Illumina paired-end sequence reads generated were quality-checked using FastQC v0.11.4 (Simon, 2010) and subsequently trimmed to remove adapter sequences and low-quality bases using Trimmomatic v0.36 (Bolger, Lohse and Usadel, 2014). Sequence reads were error-corrected and digitally normalised using bbnorm (part of BBMap v38.22) (Bushnell, 2016) and reads of each sample were assembled individually with Unicycler v0.4.7 (using default parameters and–no_correct) (Wick *et al.*, 2017). Quality-trimmed paired reads from individual samples were also assembled *de novo* using the A5-miseq assembly pipeline (Coil, Jospin and Darling, 2015). Contigs representing putative HgNV were aligned using progressiveMauve (build date Jun 26 2018) (Darling, Mau and Perna, 2010) in order to obtain a consensus sequence. In order to identify viral contigs, Prokka (Seemann, 2014) was used to identify protein-coding regions spanning the assembled contigs and these were subsequently annotated

using the BLASTp algorithm of Diamond v0.7.9 (Buchfink, Xie and Huson, 2014) and the full NCBI non-redundant (nr) protein database (20170515).

Sequences representing dsDNA viruses were identified by visualising the Diamond output in MEGAN6 Community Edition v6.5.5 (Huson *et al.*, 2016) and corresponding contig sequences were extracted. Paired reads from all samples were subsequently mapped to the candidate genome contigs using BWA-MEM 0.7.12-r1039 (Li and Durbin, 2009) and visualised with the Integrative Genomics Viewer (IGV) v2.3.68 (Robinson *et al.*, 2011) Assembly quality and accuracy were assessed with QualiMap v2.0 (Okonechnikov, Conesa and García-Alcalde, 2015) and REAPR (version 1.0.18) (Hunt *et al.*, 2013). Predicted open reading frames (ORFs) were identified using three different tools, including Prokka, FGenesV0 (softberry.com) and GeneMarkS (Besemer, Lomsadze and Borodovsky, 2001)(amino acid size of 50, circular genome). ORFs that were supported by two or more programs were analysed further. In cases where multiple ORFs were predicted to overlap, the largest sequence was chosen. Supported ORFs were annotated using NCBI BLASTp and the full NCBI nr protein sequence database (20180803).

Tandem repeats within the final assembled genome were identified using the tandem repeats finder using default parameters (Benson, 1999). Repetitive regions with an alignment score of 100 or more were further analysed for palindromic sequences using the MEME program and a minimal size of 20 bp (Bailey *et al.*, 2009). Promoter sequences were located within 300 nucleotides upstream of ORF start codon predictions using the Geneious software package v.11.1.4 (Kearse *et al.*, 2012). Early promoters contain TATA[AT][AT][AT] sequences. TATA boxes may also associate with CA[TG]T (E1) or CGTGC (E2) 20–40 nucleotides downstream. The baculovirus late promotor (L) corresponding

to [ATG]TAAG and the HzNV-1-specific late promoter (HL) were also queried using the sequence TTATAGTAT.

A circular map of the HgNV genome was plotted using shinyCircos (Yu, Ouyang and Yao, 2018). The assembled HgNV genome and corresponding ORF predictions are deposited in GenBank under the genome accession number MK439999.

3.2.6 Molecular confirmation of genome assembly

To resolve ambiguous regions of the genome assembly, primers were designed that span areas of lower coverage and INDEL queries. PCR amplification was performed in 50 µL volumes using 10 µL of Promega 5X Green GoTaq Flexi Buffer, 5 µL of $MgCl_2$, 0.5 µL of each primer (Final concentration; 1 µM), 0.5 µL of DNTPs, 0.25 µL of GoTaq DNA Polymerase, 30.75 µL of molecular grade water and 2.5 µL of template DNA. Initial denaturation was carried out at 94 °C for 2 min. This was followed by 30 PCR cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Sequenced amplicons were aligned to the candidate genome using the multiple sequence alignment program (MAFFT Version 7)(Katoh and Standley, 2013) and assembly was assessed across query regions.

Diagnostic primers were constructed from the alignment of *DNA polymerase* gene sequences. HgNV_DNAPol_F1: 5′ACTTGAAGCTGTGCGTGACT 3′ and HgNV_DNAPol_R1: 5′ TGTATGTCTTGCGGCCCATT 3′ produce an amplicon of 383 bp and only anneal to HgNV when queried with Primer-BLAST and the nr database. PCR conditions were as above. Amplicons were cleaned with the GeneJET PCR Purification Kit (Thermo, US) and sequenced via the Eurofins TubeSeq service. HgNV_DNAPol

primers were tested on 150 SBCC and 12 hatchery lobsters (sampled at 52 weeks post-deployment). Shrimp tissues infected with WSSV and a putative nudivirus were tested as negative controls and did not amplify.

3.2.7 Phylogenetic tree construction

Homologous target genes were aligned using the multiple sequence alignment program MAFFT Version 7 (Katoh and Standley, 2013); and the E-INS-I iterative refinement method. Multigene alignments were constructed by concatenating gene sequences prior to alignment. A maximum likelihood phylogenetic tree inference was constructed using RAxML-HPC BlackBox version 8 (Stamatakis, 2014) on the CIPRES Science Gateway (Miller, Pfeiffer and Schwartz, 2010) using a generalised time-reversible (GTR) model with CAT approximation (all parameters estimated from the data).

3.2.8 *In-situ* hybridisation

An extended HgNV-specific *DNA polymerase* probe which spanned and the HgNV_DNAPol amplicon sequence was designed to optimise the hybridisation protocol. HgNV_DNAPol_ISH_1838f: 5′ AGATTGAGCAGAGTGTAGCCC 3′ and HgNV_DNAPol_ISH_2799R 5′ ACCTTCCGATGATAGTTCTTCC 3′ produce an amplicon of 961 bp. *In-situ* hybridisation of the extended HgNV probe was carried out following the protocol described by (Bojko *et al.*, 2018) using a 2X washing buffer (20X SSC, 0.2% BSA, 6 M Urea). However, NBT/NCIP incubation was limited to 15 minutes and slides were instead counterstained with 0.1% Fast Green solution.

3.3 Results

3.3.1 Histological sectioning reveals virus-associated pathology

Lobsters did not appear to display any clinical signs of infection with HgNV. Histopathology of the virus infection was apparently limited to the tubule epithelial cells of the hepatopancreas (HP), observed in fibrilar (F) and reserve (R) cells. Infected cells contained hypertrophic nuclei occupied by a single, large eosinophilic inclusion. This inclusion displaced the host chromatin resulting in the latter's emargination against the nuclear envelope (Figure 3.1A,B). In some cases, this margination of the chromatin causes the formation of septa leading to the appearance of intranuclear compartmentalisation. Viral infection occurred either within the nuclei of isolated cells, within the closely opposing cells of a single tubule, within numerous cells of several closely opposed tubules, or generally throughout the tubules of the hepatopancreas. Often, epithelial cells containing virus-infected nuclei detached from the basement membrane of the tubule and were sloughed to the tubule lumen, presumably for excretion via the gut.

3.3.2 Infection prevalence in hatchery and sea-based juvenile lobsters

Intranuclear inclusions were observed in 12.72 % of all samples processed for histology (145/1140) across the two-year sampling period. In sea-based lobsters the prevalence of intranuclear inclusions was highest at 39 weeks post deployment (17 %) (Figure 3.2). At this time point, the percentage of individuals displaying histological signs of viral aetiology was the same in both the hatchery and sea-based populations. However, whereas intranuclear inclusions were not evident in sea-based lobsters at 104 weeks (0 %), prevalence had peaked in hatchery reared lobsters (53 %) at this time (Figure 3.2). Prevalence was

generally observed to be higher in hatchery-based individuals compared to those retained in SBCC systems.

Three of the 150 (52 week) sea-based lobsters tested were PCR positive for HgNV, all of which were histologically positive. An additional four sea-based samples, displaying histopathological signs of intranuclear inclusions, did not amplify with HgNV-specific primers. Of the 12 hatchery-based animals tested, five were PCR positive for HgNV, three of which were histologically positive. An additional hatchery-based sample showed histological signs of nuclear infection but did not test positive with PCR. *In-situ* hybridisation of the HgNV-specific amplicon probe confirms detection and demonstrates localisation of the target HgNV DNA polymerase gene in infected tissues (Figure 3.1C,D).

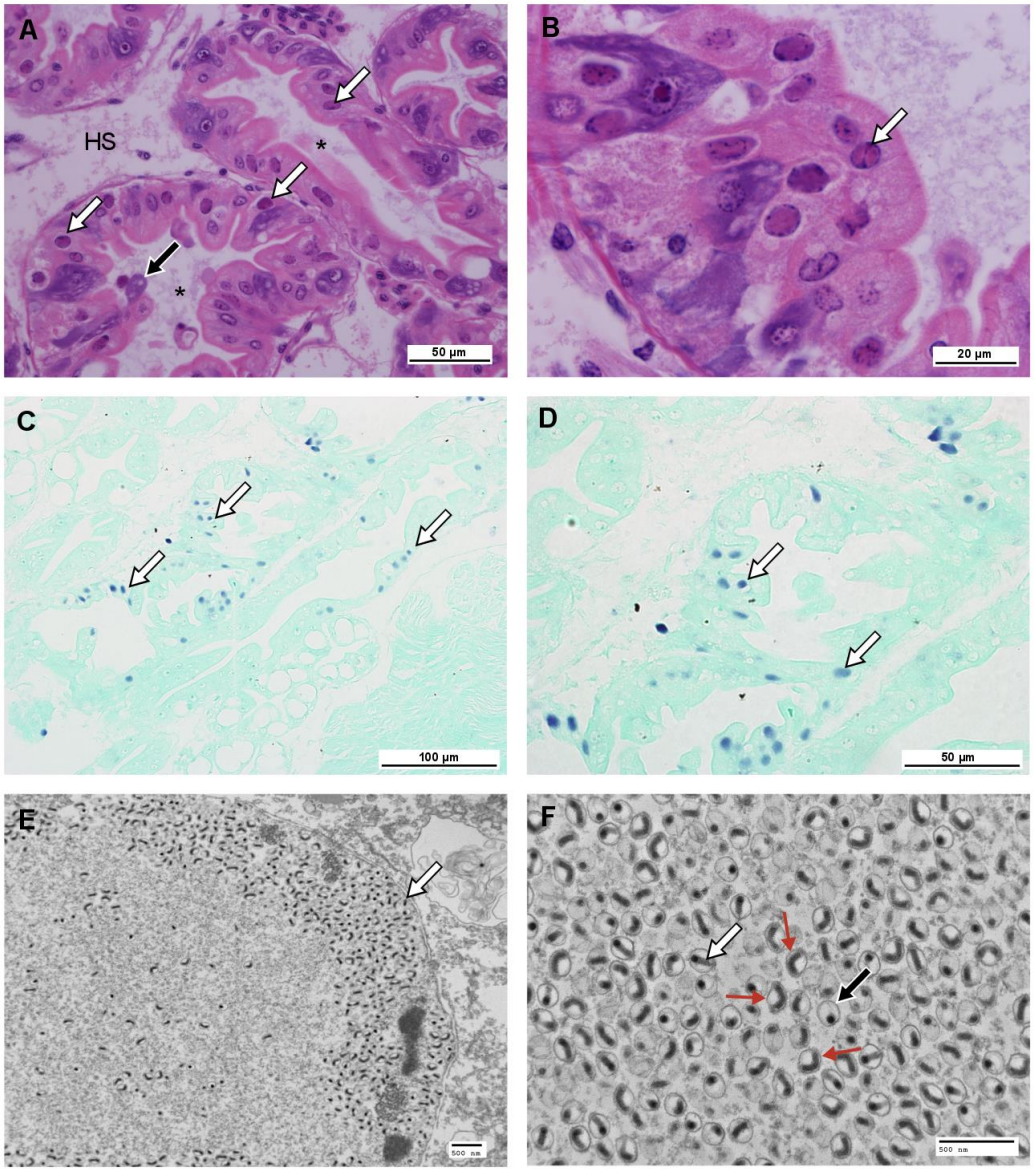


Figure 3.1 Homarus gammarus nudivirus (HgNV) infection within the hepatopancreas. *Homarus gammarus* nudivirus (HgNV) infection within the hepatopancreas. A) Section through the hepatopancreas, haemal sinus (HS) surrounds the tubules, cross section of the tubules shows a clear lumen (*). Infected nuclei within the epithelial cells of the tubules are enlarged, with emarginated chromatin and possess an eosinophilic inclusion body (white arrows). Infected cells (black arrow) may be sloughed into the lumen of the tubules. H&E Stain. Scale bar = 50 µm. B) Infections can be seen within multiple epithelial cells, infected nuclei appearing larger than uninfected nuclei. Margination of the chromatin can form septa leading to the appearance of discrete intranuclear compartmentalisation (arrow). H&E Stain. Scale bar = 20 µm. C,D) HgNV-

specific *DNA polymerase* probe hybridised to infected nuclei (arrows) within epithelial cells of the hepatopancreas. *In-situ* hybridisation. Scale bar = 100 µm, 50 µm respectively. E) Nucleus from a HgNV infected cell containing rod-shaped virions. Virions accumulate at the periphery of the nuclear membrane (arrow), TEM. Scale bar = 500 nm. F) Longitudinal (white arrow) and transverse sections (black arrow) of HgNV virions within the nucleus. Virions possess an electron dense nucleocapsid surrounded by a trilaminar membrane (envelope). The rod shaped nucleocapsid appears to bend within the envelope forming a “u” or “v” shape in some cases (line arrows). TEM. Scale bar = 500 nm

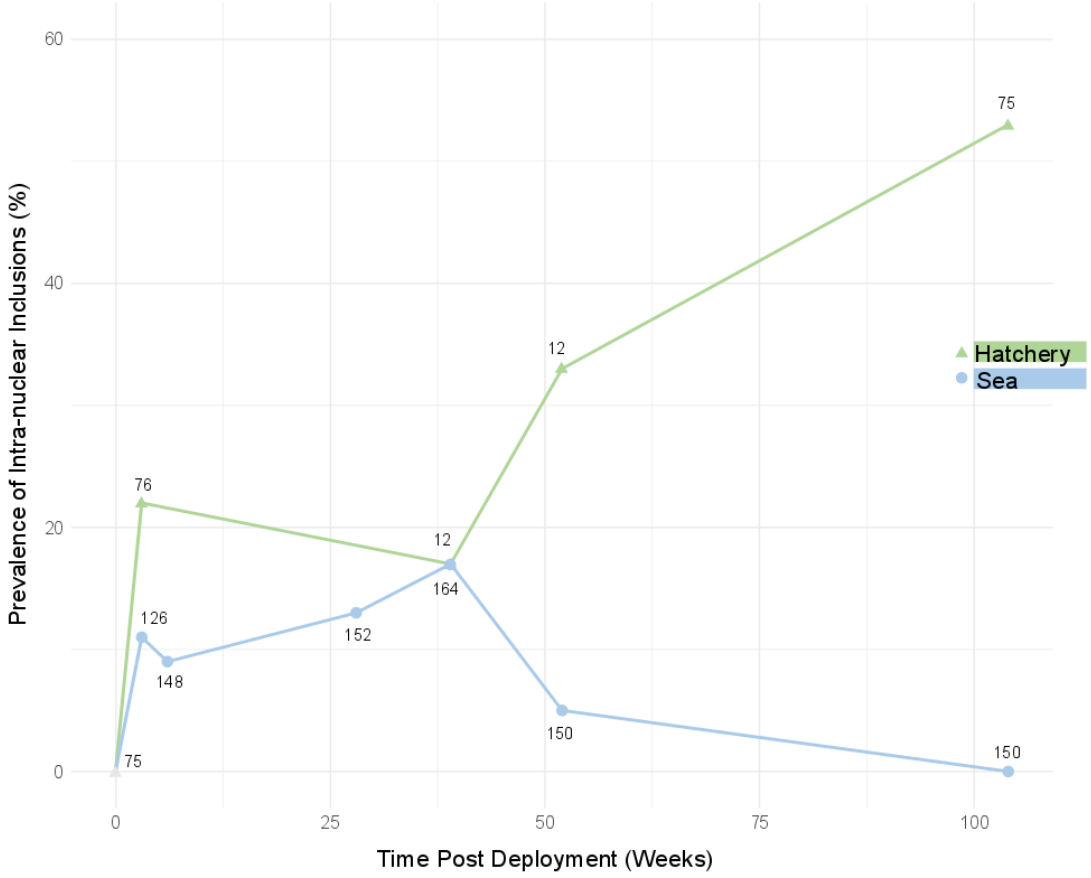


Figure 3.2 Prevalence of intranuclear inclusions in sea-based and hatchery lobsters over 104 weeks. Prevalence of intranuclear inclusions in sea-based and hatchery lobsters over 104 weeks. Proportion of surviving lobsters displaying histopathological signs of viral infection. Green triangles: hatchery-based animals. Blue circles: sea-based animals. Sample size indicated at each point.

3.3.3 Transmission electron microscopy (TEM) confirms the presence of viral infection

Transmission electron microscopy revealed the presence of masses of enveloped virions accumulated at the nuclear membrane and surrounding the virogenic stroma (Figure 3.1E,F). Virions exhibited an electron-dense nucleocapsid showing a bacilliform morphology and were contained within an elliptical membrane. In some cases, the rod-shaped nucleocapsids appeared “u” or “v” shaped within the envelope (Figure 3.1F). The mean length of the enveloped virions was 180.43 ± 16.9 nm, with a mean diameter of 136.07 ± 11.28 nm (n = 20). The mean length of the nucleocapsids was 154 ± 20 nm, with a mean diameter of 36 ± 4 nm, mean envelope width was 5.2 ± 0.2 nm (n = 20).

3.3.4 Complete genome assembly of candidate virus

The alignment of multiple independent assemblies produced a full genome consensus sequence of 107,063 bp (Accession: MK439999). Reassembling the concatenated reads from all samples, after mapping to the candidate consensus sequence, increased coverage to an average of 400.50× (SD: 65.16). The assembled contig of 107,063 bp is concordant with the size of other known nudivirus genomes, as is the estimated GC content of 35.34% (Table 3.1). REAPR detected no errors or breaks in the assembled genome. PCR confirmation and sequencing of reduced coverage areas revealed the presence of repeating units, which sometimes varied in copy number between independent samples. Sanger reads sequenced from three separate samples confirmed correct assembled sequence.

3.3.5 Tandem repeats associated with viral replication

The HgNV genome does not contain any A/T-rich, palindromic, homologous regions (hrs) that are known to support the origin of replication in baculoviruses and play important roles in viral transcription (Guarino and Summers, 1986; Pearson *et al.*, 1992). However, seven direct repeats (drs), ranging from 58.8 to 188 bp were detected (Table 3.2), two of which fall within protein coding regions. *Eco*RI centres or significant palindromic regions, both typical of hrs, were not detected within these repeating regions. However, dr1-dr4 are clustered within 3.3% of the entire genome; a region of 3,531 bp (Figure 3.3). A cluster of drs also appear within the PmNV genome (Yang *et al.*, 2014).

ID	Position		Consensus Repeat Size (bp)	Copy Number	dr Size (bp)	Percent Identity (%)	Consensus pattern
	Start	End					
dr1	26450	26573	27	4.6	124.2	100	GGAAGCTACACTGGTATTAGATGTAGC
dr2	26856	27043	20	9.4	188	100	GAGCTGAGTTAGTACTGCTG
dr3	27351	27453	36	2.9	104.4	97	CTTATCATGAGAGATTGCCCGGCCACCTGCAGTGGT
dr4	29923	29981	21	2.8	58.8	100	TGTTGATTTTGGATTGTATTG
dr5	59936	60081	32	4.6	147.2	100	TATGACTGATTCTCTGATATATGTACTGTGAT
dr6	71612	71718	51	2.1	107.1	96	CATCGACATCGGAACGATCACCAGAGATTCCACACATACCAACACCCCCAC
dr7	79269	79344	36	2.1	75.6	97	CCACCACCAATGTCCGAAGCCACACTCACTCCACCA

Table 3.2 Direct repeat predictions within the HgNV genome. dr = direct repeat. Tandem repeat alignment score of > 100.

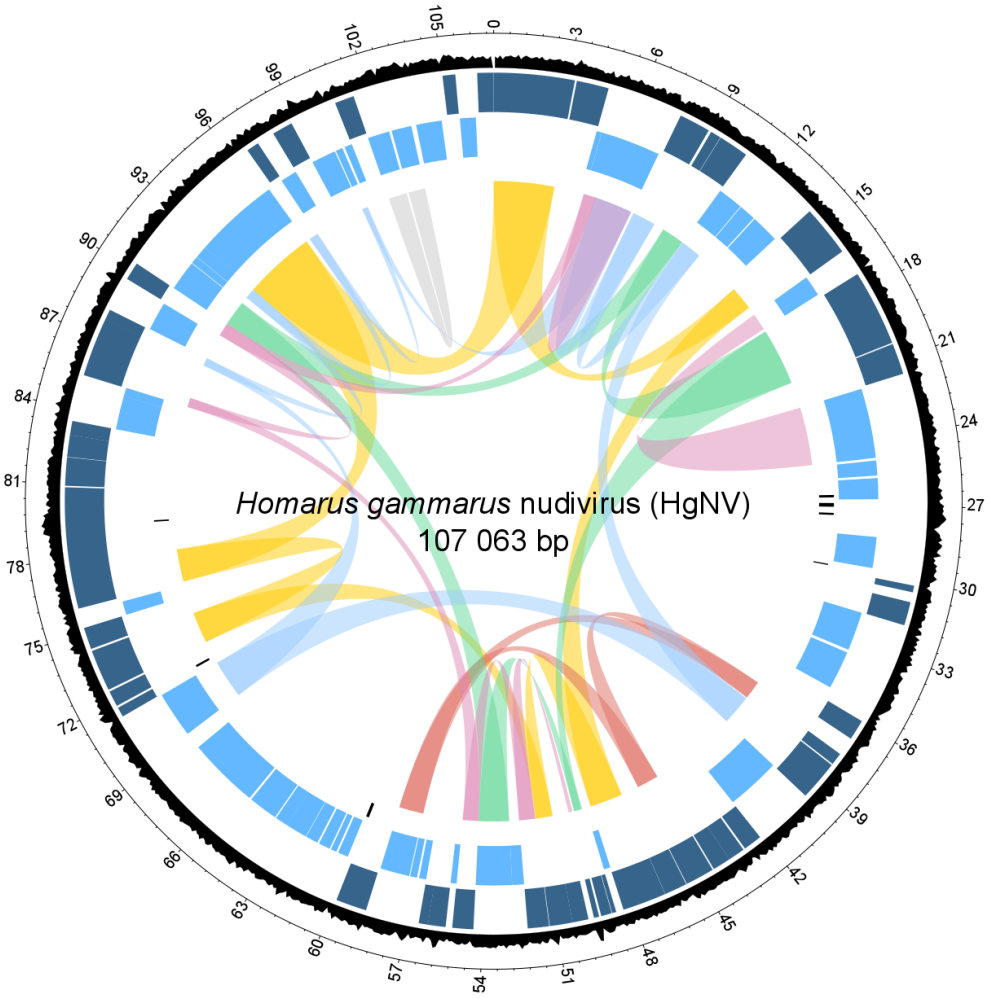


Figure 3.3 HgNV circular genome plot. Visual representation of HgNV layout scaled to the complete 107 063 bp contiguous sequence. Outermost track shows GC content (%) across complete sequence. Dark blue track displays gene predictions localised to the forward strand, whereas light blue displays those on the reverse. The innermost track depicts direct repeat regions. Links highlight genes involved in similar functions; yellow - DNA replication and repair, red - nucleotide metabolism, green – RNA transcription, pale blue – per os infectivity, pink – packaging and assembly, and grey – apoptosis inhibition.

3.3.6 Open reading frame (ORF) prediction and genome annotation

Prokka predicted 101 protein coding regions in the HgNV genome. FGenesV0 and GeneMarkerS predicted 103 and 89 ORFs, respectively. Ninety-seven ORFs were supported by two or more programs and were distributed evenly across both strands (Figure 3.3, Table 3.3); 49 on the plus strand and 48 on the minus. The gene density of the HgNV genome was estimated to be 1.10 per kb and 69% of ORFs aligned most closely with predicted genes from the PmNV genome. The exact number of genes conserved across all the nudiviruses is somewhat unclear. However, re-analyses of all sequenced nudivirus genomes revealed a set of 21 core genes conserved between baculoviruses and nudiviruses (Bézier *et al.*, 2015). The core genes were typically grouped into one of five functional groups: DNA processing, RNA transcription, *per os* infectivity, package and assembly and conserved genes of unknown function. The HgNV genome contained 7 genes involved in DNA processing; *DNA polymerase*, *helicase*, two copies of *helicase2*, *integrase*, *fen-1* and *ligase*. Gene predictions similar to three of the four thymidine kinase (*tk*) genes involved in nucleotide metabolism were also found. All five core baculovirus/nudivirus genes involved in RNA transcription were found; *p47*, *lef-4 (late expression factor)*, *lef-5*, *lef-8* and *lef-9*. As were 8 genes involved in *per os* infectivity: *pif-0*(*p74*), *pif-1*, *pif-2*, *pif-3*, *pif-4* (*19 k/odv-e28*), *pif-5* (*odv-e56*) and *pif-6* (*ac68*) and *pif-8 (vp91/p95)*. The *11K*-like gene was also found (Beperet *et al.*, 2015). HgNV contained less than half of the genes encoding packaging, assembly and release processes conserved amongst the *Baculoviridae*. These include a *38 k* gene, *p6.9*, two copies of *vlf-1*, *vp39*, *p33* (*ac92*) and *ac-81*. Similar to PmNV, HgNV also possessed 2 copies of the *Iap* genes involved in apoptosis inhibition.

Furthermore, HgNV encoded sequences similar to PmNVorf99 and PmNVorf62, reported to be common in nudiviruses (Table 3.4). Other genes typically common to baculoviruses, including *methyltransferase* and two neighbouring copies of *odv-e66*, were also found in the HgNV genome.

ORF	Strand	Position		Promoter motif	Best BLAST hit		Key HMM/ InterPro feature/ GO term
		Start	End		Description	%	
01	+	1	3249	TATA, E1, L	DNA polymerase	48	DNA_pol_B
02	+	3325	4623	TATA, L	methyltransferase	36	FtsJ, TM
03	-	4624	4854	TATA	PmNV_007	57	TM, Chondroitin AC/alginate lyase
04	-	4857	5504	L	Ac92-like protein	39	Evr1_Alr
05	-	5488	7548	TATA	Vp91	38	CMB_14*, TM
06	+	7689	8948	TATA, L	ODV-E56	52	Baculo_E56*, TM, Chondroitin AC/alginate lyase
07	+	9062	9487	E1, L	PmNV_012	34	
08	+	9500	10717	L	P47	39	DNA-directed 5'-3' RNA polymerase activity
09	-	10735	11868	-	Pif-2	58	PIF2, TM
10	-	11900	12652	TATA, L	HZV_115-like protein	29	HAD-like superfamily;P -loop containing nucleoside triphosphate hydrolase
11	-	12706	13965	TATA	PmNV_018	24	
12	+	14103	14459	TATA, L	PmNV_019	30	
13	+	14432	15709	TATA, L	PmNV_020	40	DNA repair
14	+	15675	16163	TATA, L	PmNV_021	40	
15	-	16171	17094	TATA	Vp39/31 k	33	
16	+	17244	20309	E1	LEF-8	49	DNA-directed 5'-3' RNA polymerase
17	+	20362	21618	TATA, L	P51	41	
18	-	21707	24835	TATA, L	PmNV_025	34	Protein AC81, baculovirus

	19	-	24952	25623		PREDICTED: E3 ubiquitin-protein ligase TRIM39-like	34	zf-RING_UBOX, metal ion binding, acid-amino acid ligase activity
	20	-	25742	26152				FSA_C, SP, TM
	21	-	28358	29749	TATA			
	22	+	30149	30418	E1, L			
	23	+	30822	31772	E1, L, HzNV-1	serine/threonine protein kinase	30	Pkinase
	24	-	31787	33529	TATA, L	ODV-E66	33	Chondroitin AC/alginate lyase, SP, TM
	25	-	33662	35383	TATA, E1, L	ODV-E66	38	Chondroitin AC/alginate lyase, TM
	26	+	35948	36772	L	dihydroxy-acid dehydratase	29	EF-hand domain pair
	27	+	37022	38011	-	guanosine monophosphate kinase	41	Phosphorylation, kinase activity
	28	+	38069	39673	TATA	PIF-1	47	PIF, TM
	29	-	39670	40035	-	PmNV_040	30	Chondroitin AC/alginate lyase
	30	-	40016	42100	TATA, L	PmNV_042	30	ERV/ALR sulfhydryl oxidase domain superfamily
	31	+	42114	42866	L	hypothetical protein	40	MqsR_toxin
	32	+	42943	43623	TATA	PmNV_044	40	
	33	+	43620	44348	TATA, L	PmV-like protein	26	
	34	+	44405	45574	TATA, L	p-loop NTPase	41	P-loop containing nucleoside triphosphate hydrolase
	35	+	45601	46521	TATA, L	PmNV_047	46	
	36	+	46527	48293	TATA	PmNV_048	36	DNA_ligase_A_M
	37	-	48281	48577	TATA, L	hypothetical protein	33	
	38	+	48594	48761	TATA	PmNV_051	31	TM
	39	+	48781	49212	TATA	hypothetical protein	53	Baculo_LEF5_C
	40	+	49299	49508	L			Ribonuclease H-like superfamily
	41	+	49733	50383	TATA, L	PmNV_054	33	
	42	+	50380	51297	TATA	integrase	50	Phage_integrase
	43	+	51319	52179	E1, L	VLF-1	41	Phage_integrase
	44	-	52185	52547	E1, L	surface-associated interspersed protein (SURFIN)	24	

	45	-	52492	52713	L			
	46	-	52713	54332	TATA	LEF-9	54	RNA_pol_Rpb1_2
	47	+	54340	55185	-	38K protein	42	NIF
	48	-	55182	55448	TATA			
	49	+	55450	56160	TATA, L	PmNV_061	41	YopH_N*
	50	+	56153	56533	TATA, L	PmNV_062	32	TM
	51	-	56543	56863	TATA, L	PmNV_063	34	
	52	-	56956	57270	TATA, L			TM
	53	-	57311	58627	-	p-loop NTPase	34	
	54	+	58684	59928	-	PmNV_066	38	Chondroitin AC/alginate lyase
	55	-	60168	60629	TATA			membrane
	56	-	60717	60980	TATA			Per os infectivity factor
	57	-	61046	61567	E1	PmNV_067	32	
	58	-	61649	62212	TATA, L	PmNV_068	28	
	59	-	62233	63204	TATA, L	PmNV_069	45	
	60	-	63197	63781	L	PmNV_070	30	
	61	-	63840	65159	TATA, L	PmNV_071	26	
	62	-	65256	68456	TATA	Conserved hypothetical protein*	41	Ribonuclease H-like superfamily, ubiquitin-protein transferase activity
	63	-	69215	71284	TATA, L, HzNV-1	P74	55	Baculo_p74_N, Baculo_p74, TM
	64	+	71332	71727	TATA, E1			Zinc finger, RING-type, TM
	65	+	71814	72452	TATA			
	66	+	72535	74214	L	helicase 2	48	PIF1, Viral_helicase 1*, P-loop containing nucleoside triphosphate hydrolase
	67	+	74299	75180	TATA, L	PmNV_077	24	Zinc finger, RING-type
	68	-	75187	75795	L	PmNV_078	38	Ribonuclease H superfamily
	69	+	75980	77722	-	helicase 2	41	Viral helicase1*, S-adenosyl-L-methionine-dependent methyltransferase
	70	+	77724	80855	L			P-loop containing nucleoside

							triphosphate hydrolase
	71	+	80914	82050	E1, L	PREDICTED: uncharacterized protein LOC108666550	36	S-adenosyl-L-methionine-dependent methyltransferase
	72	+	82064	82945	L	PmNV_082	39	
	73	+	82939	83511	E1, L			
	74	-	83508	85004	TATA, L	PmNV_084	27	
	75	-	84998	85399	-	PmNV_085	34	
	76	+	85386	85892	TATA, L	Ac81-like protein	60	Ac81, TM
	77	+	85867	87768	TATA, E1, L	PmNV_087	34	
	78	+	87755	88186	-	Ac68-like protein	46	TM
	79	-	88212	89504	TATA, L	PmNV_089	32	
	80	+	89589	90314	L	VLF-1	42	DNA binding, DNA integration, DNA recombination
	81	-	90317	91672	TATA, E1	LEF-4	38	regulation of transcription
	82	-	91700	92032	E1, L	PmNV_092	55	
	83	-	92043	92606	TATA, E1, L	PIF-3	50	PIF3, TM
	84	-	92599	96429	TATA, L	helicase	39	helicase activity
	85	+	96641	97144	TATA	ODV-E28	53	ThrE*
	86	-	97106	97846	TATA, L	PmNV_097	33	
	87	+	97864	98697	TATA	esterase	54	Alpha/Beta hydrolase fold, TM
	88	-	98701	99804	TATA, E1, L	PmNV_099	40	ERV/ALR sulfhydryl oxidase domain superfamily
	89	-	99855	100166	TATA	11K virion structural protein	55	TM
	90	-	100274	100618	E1			
	91	+	100608	101390	-	PmNV_102	34	
	92	-	101387	102367	TATA	death-associated inhibitor of apoptosis 1	30	BIR, zf-C3HC4_3
	93	-	102481	103380	TATA, L	PREDICTED: baculoviral IAP repeat-containing protein 7-like	31	Zf-C3HC*, BIR
	94	-	103599	104747	TATA, L			SP, TM
	95	+	105021	105533	TATA, E1, L			RNA polymerase, beta subunit, conserved site
	96	-	105557	106270	TATA, L	PmNV_107	51	
	97	+	106404	107060	TATA, E1, L	PmNV_108	39	Membrane

Table 3.3 Supported open reading frame annotations of the HgNV genome. Colours as in Figure 3.3. BLAST annotations with an E-value equal or greater than 1 are not shown. Annotations with an E value >x10^{10} are highlighted in bold. * Pfam annotations with an E-value less than 1. SP = signal peptide, TM = transmembrane domain.

Functional group	Gene name	ORF							
		HgNV	OrNV	GbNV	HzNV-1	HzNV-2	PmNV	ToNV	DiNV
DNA processing	*dnapol*	1	1	12	131	18	5	12	60
	helicase	84	34	88	104	38	94	118	11
	helicase2	66	108	46	60	76	76	105	83
	*helicase2**	69	108	46	60	76	79	105	83
	integrase	42	75	57	144	8	55	43	39
	fen-1	13	16	65	68	70	20	1	93
	ligase	36	121	38	141	10	48	121	72
Nucleotide metabolism	*tk1*	53	137	17	51	85	65	22	62
	tk2	27	117	34	111	34	38	44	76
	tk3	34	125	44	115	32	46	14	68
RNA transcription	*lef-4*	81	42	96	98	43	91	25	+
	lef-5	39	52	85	101	40	52	50	24
	lef-8	16	64	49	90	51	23	88	31
	lef-9	46	96	24	*75	*63	58	131	52
	p47	8	20	69	*75	*63	14	115	97
Per os infectivity	*pif-0 (p74)*	63	126	45	11	106	72	45	67
	pif-1	28	60	52	55	82	39	69	35
	pif-2	9	17	66	123	26	15	7	94
	pif-3	83	107	3	88	53	93	13	84
	pif-4 (19 k/odv-e28)	85	33	87	103	39	96	119	10
	pif-5 (odv-e56)	6	115	5	76	62	10	74, 96	78
	pif-6(ac68)	78	72	55	74	64	88	56	+
	pif-8 (vp91/p95)	5	106	2	46	89	9	16	85
	11K-like	89	41	95	124	25	100	28	16
Packaging and Assembly	*38K*	47	87	1	10	108	59	63	46
	p6.9	40	+	73	142	+	+	51	
	vlf-1	43	30	80	121	28	56	65	8
	***vlf-1*	80	30	80	140	11	90	65	8
	vp39 (31K in PmNV)	15	15	64	89	52	22	87	92
	p33 (ac92)	4	113	7	13	104	8	99	80
	Ac-81	76	4	14	33	96	86	123	57
Apoptosis inhibition	*Iap*	93	134	98	138	12	106	103 105	
	*Iap**	92	134	98	138	15	106	105	
Unknown function	*P51*	17			64	73	24	112	
	PmNVorf99-like	88	18	67	122	27	99	6	95
	PmNVorf62-like	50	61	51	+	79	62	19	34

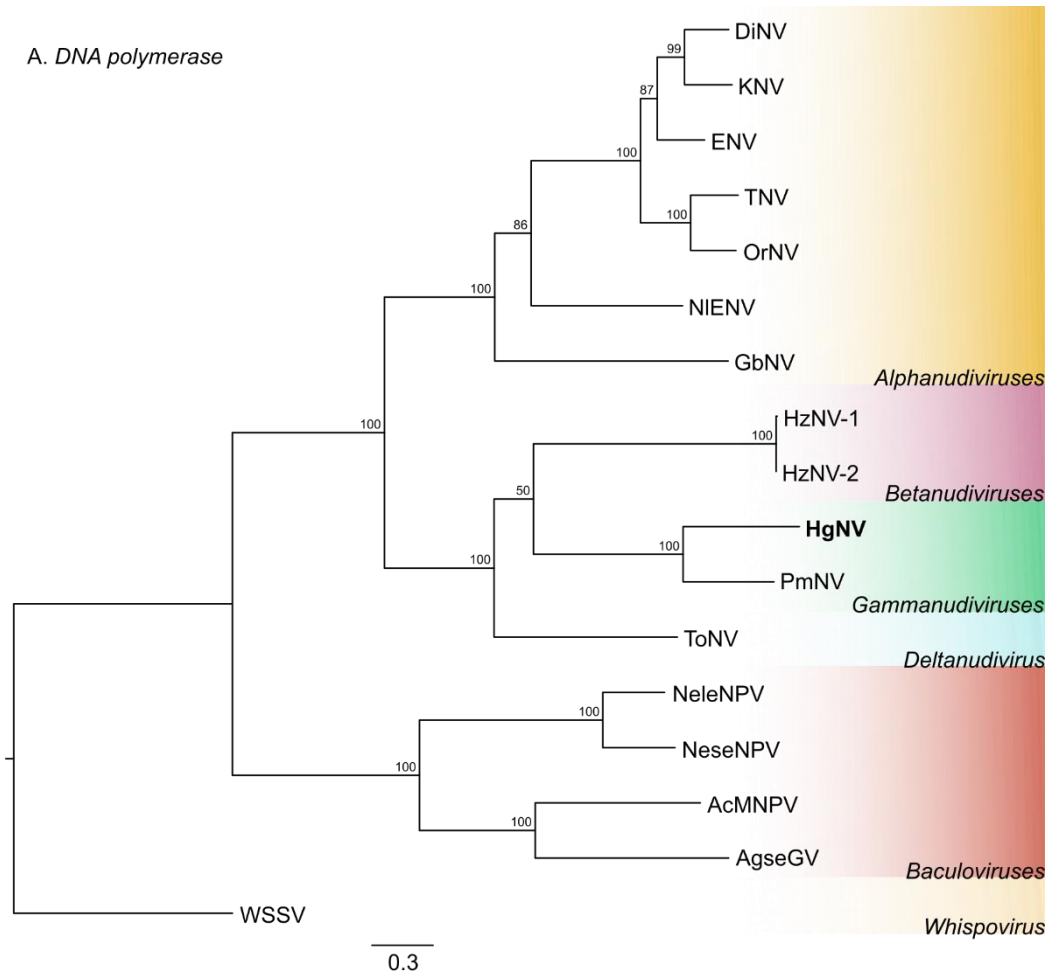
Table 3.4 HgNV homologs to conserved nudivirus sequences. Colours as in Figure 3.3. *fused to a single gene. **multiple copy number. Shaded cells of second column indicate 'core nudivirus genes' shared with the Baculoviridae. + Reported present.

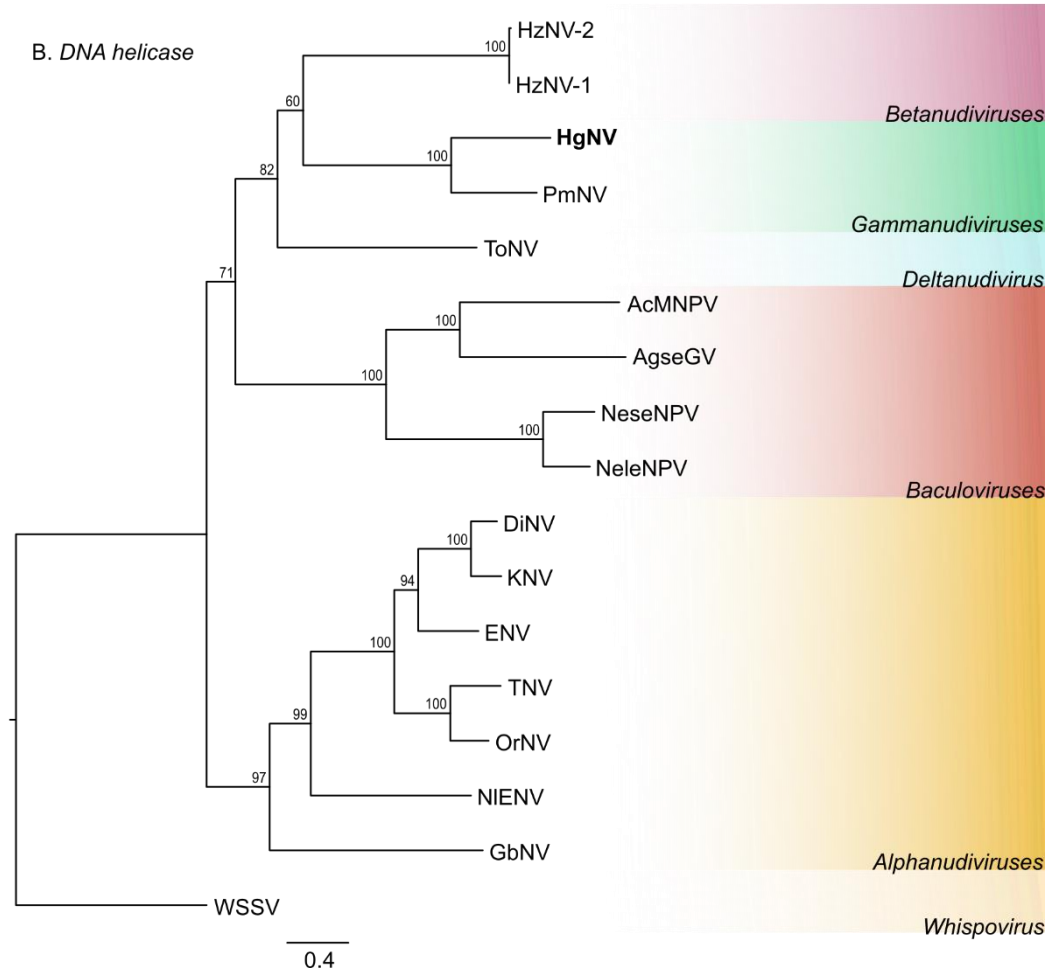
3.3.7 Promotor regions preceding ORF predictions

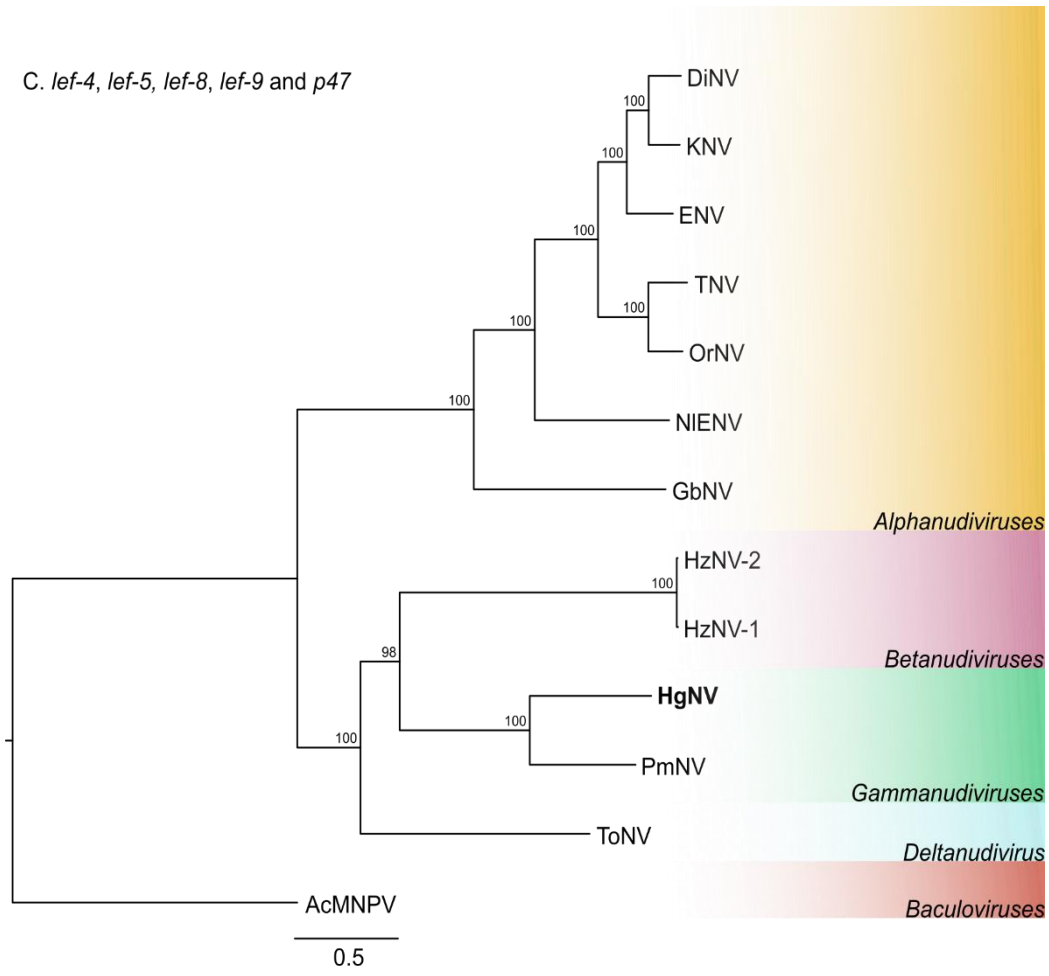
Analysis of the 300 bp region upstream of each ORF start codon revealed the presence of promotor motifs in all but 12 of the predicted coding regions (Table 3.3). Early promotors defined by a TATA box with or without E1 motifs were predicted for 73 ORFs. No E2 motifs were detected. Late (L) promoters were predicted for 59 ORFs with HzNV-1 specific late promoters predicted for two coding regions; HgNV_ORF23, exhibiting a protein kinase structural domain and HgNV_ORF63 coding for the *p74* gene. A combination of early and late promoters were predicted to precede 47 potential coding regions.

3.3.8 Phylogenetic characterisation of HgNV

Single gene phylogenies using the *DNA polymerase* and *helicase* genes showed contrasting positioning of the *Nudivirus* and *Baculovirus* clades, however both grouped HgNV with PmNV, together with ToNV, HzNV-1 and HzNV-2 (Figure 3.4A,B). Multigene analyses of all shared genes involved in transcription (*lef*-4, *lef* -5, *lef* -8, *lef* -9 and p47) and *per os* infectivity (*pif-0*, *pif*-1, *pif*-2, *pif*-3, *pif*-4, *pif*-5, *pif*-6) within the nudiviruses very robustly supported this grouping, with Maximum Likelihood (ML) bootstrap values of 100% and 98% respectively (Figure 3.4C,D).


A. DNA polymerase
DiNV
KNV
ENV
TNV
OrNV
NlENV
GbNV
HzNV-1
HzNV-2
HgNV
PmNV
ToNV
NeleNPV
NeseNPV
AcMNPV
AgseGV
WSSV
Alphanudiviruses
Betanudiviruses
Gammanudiviruses
Deltanudivirus
Baculoviruses
Whispovirus
0.3


B. DNA helicase
HzNV-2
HzNV-1
HgNV
PmNV
ToNV
AcMNPV
AgseGV
NeseNPV
NeleNPV
DiNV
KNV
ENV
TNV
OrNV
NlENV
GbNV
WSSV
Betanudiviruses
Gammanudiviruses
Deltanudivirus
Baculoviruses
Alphanudiviruses
Whispovirus
0.4


C. lef-4, lef-5, lef-8, lef-9 and p47
DiNV
KNV
ENV
TNV
OrNV
NlENV
GbNV
HzNV-2
HzNV-1
HgNV
PmNV
ToNV
AcMNPV
100
100
100
100
100
100
100
100
98
100
100
0.5
Alphanudiviruses
Betanudiviruses
Gammanudiviruses
Deltanudivirus
Baculoviruses

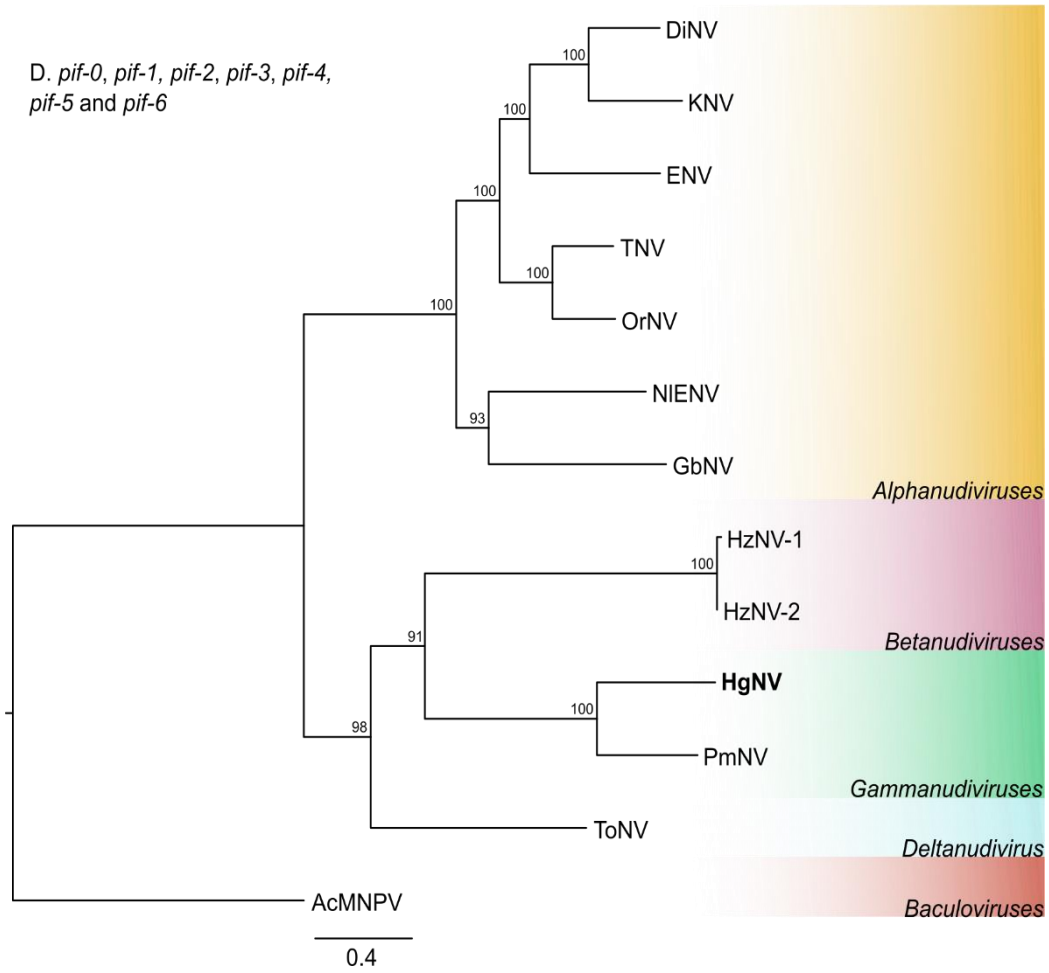

D. pif-0, pif-1, pif-2, pif-3, pif-4,
pif-5 and pif-6
DiNV
KNV
ENV
TNV
OrNV
NlENV
GbNV
HzNV-1
HzNV-2
HgNV
PmNV
ToNV
AcMNPV
100
100
100
100
100
100
93
100
91
100
98
0.4
Alphanudiviruses
Betanudiviruses
Gammanudiviruses
Deltanudivirus
Baculoviruses

Figure 3.4 Single and multigene phylogenies of known nudiviruses. Single and multigene phylogenies of known nudiviruses. Maximum Likelihood analyses of nudivirus phylogeny, including whispovirus and baculovirus outgroups. Node labels indicate bootstrap support (%). A) – Single gene phylogeny of *DNA polymerase*. B) – Single gene phylogeny of *DNA helicase*. C) – Multigene phylogeny of late expression factors (*lef-4*, *lef-5*, *lef-8*, *lef-9* and *p47*). D) – Multigene phylogeny of *per os* infectivity genes (*pif-0*, *pif-1*, *pif-2*, *pif-3*, *pif-4*, *pif-5* and *pif-6*). DiNV – *Drosophila innubila* nudivirus, KNV – Kallithea virus *(D. melanogaster*), ENV – Esparto virus (*D. melanogaster*), TNV – Tomelloso virus (*D. melanogaster*), OrNV – *Oryctes rhinoceros* nudivirus, NleNV – *Nilaparvata lugens* endogenous nudivirus, GbNV – *Gryllus bimaculatus nudivurs*, HzNV-1 – *Heliothis zea* nudivirus 1, HzNV-2 – *Heliocoverpa* (syn. *Heliothis*) *zea* nudivirus 2, HgNV – *Homarus gammarus* nudivirus, PmNV – *Penaeus monodon* nudivirus, ToNV - *Tipula oleracea* nudivirus, NeleNPV – *Neodipirion lecontei* nucleopolyhedrovirus, NeseNPV – *Neodipirion sertifer* nucleopolyhedrovirus, AcMNPV – *Autographa californica* multiple nucleopolyhedrovirus, AgseGV – *Agrotis segetum* granulovirus, and WSSV – white spot syndrome virus. Coloured clade groupings refer to proposed genera: yellow – *Alphanudiviruses*, pink – *Betanudiviruses*, green – *Gammanudiviruses*, blue – *Deltanudiviruses*, red – *Baculoviruses*, cream – *Whispovirus*.

3.4 Discussion

Here we provide the first description of a naturally-occurring virus infection of nephropid lobsters. The virus, *Homarus gammarus* nudivirus (HgNV) is a new species within the family *Nudiviridae*; a group of dsDNA viruses that infect arthropod (mainly insect) hosts. Histopathology and ultrastructure of HgNV is similar to numerous other bacilliform viruses described to infect Crustacea, wherein viral replication within the host nucleus displaces host chromatin and results in aberrant, hypertrophied nuclei, visible in routine histological preparations. In many cases, infected epithelial cells are sloughed off the basement membrane of the tubule into the lumen, for excretion via the faeces. It

is important to consider that intranuclear inclusions may also be indicative of other pathogens as this may explain discrepancies in prevalence when comparing PCR and histology data. Furthermore, digestive tissues are known to contain inhibitors which can impact PCR success (Schrader *et al.*, 2012; El-maklizi *et al.*, 2014). However, *in-situ* hybridisation confirms that HgNV is inducing this pathology in infected cells (Figure 3.1C,D).

Comprehensive genome analysis of infected lobsters revealed that HgNV is most closely related to PmNV, a virus infecting the black tiger shrimp, *Penaeus monodon*. However, despite a high degree of conservation in gene order, the percentage identity of HgNV gene predictions to known annotations was fairly low, averaging just over 38%. Our Maximum Likelihood phylogenies were concordant with previously published trees, which indicate that PmNV may belong to a separate genus within the *Nudiviridae*; the *Gammanudiviruses* (Yang *et al.*, 2014). Our phylogenetic analyses show that HgNV also belonged to this clade and, together with PmNV, could represent a radiation of nudiviruses infecting diverse aquatic crustacean taxa. Based on the long branch lengths of the neighbouring lineages, it is likely that ToNV and both HzNV-1 and HzNV-2 belong to separate genera; provisionally referred to as *Deltanudivirus* and *Betanudivirus* respectively (Figure 3.4). We also show that the newly sequenced *Drosophila* nudiviruses belong to the *Alphanudivirus* clade and present the most substantial nudivirus phylogeny to date. The multigene phylogeny of the late expression factors provides bootstrap support of 100% in all but one node (98%) (Figure 3.4C).

Nudiviruses contain a distinct repertoire of genes involved in DNA processing, compared to the baculoviruses. Two *lef* genes and an *alk-exo* gene are absent in HgNV. *Lef-1* has been shown to be associated with DNA primase activity which

aids in polymerisation, and *lef-2* is thought to stabilize the binding of *lef-1* to the DNA molecule (Mikhailov and Rohrmann, 2002), amplifying replication (Wu *et al.*, 2010). HgNV contains two copies of the *helicase-2* gene which are also found in the PmNV genome (HgNV_ORF66, HgNV_ORF69). Both genes are predicted to contain features characteristic of helicase activity. An *integrase* gene is also common to all sequenced nudiviruses and is represented by HgNV_ORF42, which contains the phase_integrase domain involved in the integration of viral DNA into the host genome. This is noted to facilitate persistent infection of HzNV-1 in its host (Lin *et al.*, 1999).

Of the five core genes involved in RNA transcription, *P47*(HgNV_ORF08) encodes a viral transcription regulator, involved in late stage infections, reported to make up one of the four subunits of RNA polymerase, whereas the four remaining *lef* genes are thought to regulate late and very late gene expression, and are named to reflect their synthesis during infection. In contrast, early gene expression is instead initiated by host-derived RNA polymerase (Acharya and Gopinathan, 2015).

Baculovirus life cycles are typically split between occlusion-derived virus (ODV) and budded virus (BV) stages. *Per os* infectivity genes, conserved within the HgNV genome, are required for the infectivity of ODVs that facilitate the transmission of viral particles from one host to the next, whereas BVs spread virions to neighbouring cells. *Pif-0*,*1* and 2 encode envelope proteins vital for oral infection and are thought to bind virions to the midgut cells (Haas-Stapleton, Washburn and Volkman, 2004). However, *pif-3* does not affect midgut binding. *Pif-3* is instead hypothesised to interact with the viral cytoskeleton and play a role in translocation of the capsid (Slack and Arif, 2006). It is believed that *pif-1*, *2*, 3 and 4 form a multimolecular protein complex that is vital for oral

infectivity, with other *pif* genes associating with the core complex at a lower affinity (Boogaard, van Oers and van Lent, 2018). *Pif-7*¸originally described as the ODV-envelope protein Ac110, also associates with the complex but was not found in the HgNV genome. However, Pif-8, previously described as the structural protein vp91/p95, was detected (HgNV_ORF05) and is predicted to contain chitin-binding peritrophin-A domains. The peritophic membrane surrounds the food bolus and lines the gut of most crustaceans and serves to separate large particulate matter from the epithelial cells and limits the penetration of microbes (Martin, Simcox and Nguyen, 2006). HgNV also encodes a homolog to an 11 K protein noted to enhance oral infection. These 11 K proteins typically contain binding motifs common to mucins, peritrophins and chitinases and could facilitate midgut binding, typically occurring after the alkaline dissolution of the occlusion lattice (Lapointe *et al.*, 2004).

The reduction in genes encoding packaging, assembly and release is likely a reflection of the lack of occlusion bodies in the transmission strategy adopted by the *Nudiviridae*. HgNV_ORF47 encodes a *38K*-like gene which mediates the dephosphorylation of the C terminus of the *p6.9* gene; a gene responsible for the encapsulation of the viral genome. Although not detected through BLAST alignment, likely a result of its highly repetitive sequence, HgNV_ORF40 was identified as the *p6.9* gene after alignment with other annotated sequences. Furthermore, alignment of the hypothesised PmNV coding region of the *p6.9* gene to the HgNV genome corresponds to a region within the HgNV_ORF40 predicted ORF. Similarly to PmNV, HgNV shares two separate sequence homologs to the *vlf-1* gene (HgNV_ORF43, HgNV_ORF80), responsible for very late gene expression and proper formation of the nucleocapsid (Vanarsdall *et al.*, 2006). HgNV also encodes a second major

capsid protein: *p33/ac92* (HgNV_ORF04). HgNV_ORF04 reports an Erv1_Alr feature, belonging to a family of sulfhydryl oxidases. Prior analyses and purification of ac92 suggests it is a flavin adenine dinucleotide (FAD) containing sulfhydryl oxidase (Long, Rohrmann and Merrill, 2009). The major viral capsid protein *vp39* is thought to be a core baculovirus/nudivirus gene and was reportedly mislabelled as a *31K*-like structural protein in PmNV (ORF_022). HgNV_ORF15 shares 33% identity across 99% of the PmNV_022 sequence and 21% identity across 91% of HzNV-1 ORF89 and HzNv-2 ORF52, also annotated in GenBank as 31K-like proteins. However, the similarity of HgNV_ORF15 with the *vp39* genes of other nudiviruses is much lower. Protein domains could not be predicted to aid in its clarification.

The PmNV_099-like coding region is also shared amongst the nudiviruses and HgNV_ORF88 indeed shares 40% sequence identity with PmNV_099, which is described as 'microtubule-associated-like' (Yang *et al.*, 2014; Bézier *et al.*, 2015). This gene could play a role in the rearrangement of the host nucleus during viral replication, whereby host chromatin is translocated to the inner nuclear membrane, a process thought to be dependent on viral interaction with host tubulin (Slack and Arif, 2006). Much like *ac92*, HgNV_ORF88 is also predicted to contain the ERV/ALR sulfhydryl oxidase feature which can play a role in virion assembly by catalysing disulphide bond formation between cysteine residues (Hakim, Mandelbaum and Fass, 2011). In regard to HgNV gene predictions found in the baculoviruses, HgNV_ORF02 shares 35.86% identity to a methyltransferase annotated in the PmNV genome, hypothesised to be involved in viral RNA capping (Koonint, 1993). As is the case with PmNV, HgNV encodes two neighbouring *odv-e66* predictions responsible for the trafficking of viral proteins during infection (Braunagel *et al.*, 2004). Odv-e66 was also reported as

the first chondroitin lyase (Kawaguchi *et al.*, 2013). Chondroitin is an extracellular matrix polysaccharide and its degradation by pathogenic bacteria facilitates access to the target cell (Kawaguchi *et al.*, 2013). Chondroitin AC/alginate lyase Interpro features were also identified in HgNV_ORF24 and HgNV_ORF25.

There are 37 core genes reported to be conserved amongst the baculoviruses (Garavaglia *et al.*, 2012). Assuming the 31K-like gene is in fact a *vp39* homolog, HgNV encodes all 21 core baculovirus genes proposed to be conserved across the nudiviruses (Figure 3.4). Nearly half of HgNV predicted coding regions were preceded by both early and late promoter regions (Table 3.3), suggesting plasticity in the way HgNV can regulate gene expression. However, as stated by Bezier *et al*. gene expression chronology should not be generalised to promoter motifs alone. Transcriptome analysis of the baculovirus AcMNPV failed to associate reliable sequence motifs with gene expression patterns (Chen *et al.*, 2013).

We did not observe evidence of occlusion body formation within our histological or TEM analyses. Similarly, we did not detect sequence homologs of the *poly/gran* gene, which encodes the protein that forms the structural lattice. Ingestion of occlusion bodies allows passage to the digestive tract, where alkalinity of the gut causes the proteinous lattice to dissolve, releasing the virions within and initiating infection (Acharya and Gopinathan, 2015). Much like the baculoviruses, the nudiviruses surrounding HgNV (HzNV-2, ToNV and PmNV) can rely on occlusion bodies to facilitate transmission outside of the host. As it would seem that HgNV does not form occlusion bodies, it begs the question of how viral particles remain viable during horizontal transmission. An alternative infection strategy would be that HgNV persists as a latent virus within the host and its evolution has favoured the maintenance of low virulence, which

subsequently translates to an increase in transmission through longer lasting infections, as infection doesn't incapacitate the host. Viruses infecting cells of the digestive tract sloughed out of the animal may remain viable until the degradation of the excreted cell. The ingestion of faeces may therefore serve as possible route of transmission for HgNV (Lavalli and Barshaw, 2009). Latency within the host is a shared strategy true of several other shrimp viruses and supported by field data relating to the infection of the marine shrimp *Crangon crangon* by a putative nudivirus, where prevalence can reach 100% in wild populations (Stentiford, Bateman and Feist, 2004; Walker and Winton, 2010; Van Eynde *et al.*, 2018). Alternatively, HgNV may persist in reservoirs outside of its currently known host.

Due to the short life-cycle and seasonal development of their host, insect viruses, like the baculoviruses, are unable rely on either latency or reservoir strategies (Slack and Arif, 2006). Therefore, resistant occlusion bodies would ensure viability outside of the host and facilitate transmission to the next. However, compared to penaeid shrimp, lobsters have very long life-cycles (decades). As such, a virus infecting these animals *can* rely on latency and is not required to survive long periods within the environment. In further support of this theory, occlusion-derived viruses infecting the insect midgut rely on occlusion body-associated enhancins, or similar factors, that digest the chitin lining of the midgut and facilitate entry (Slack and Arif, 2006). However, the hepatopancreas of the lobster is not chitinous (Barker and Gibson, 1977). Therefore, HgNV would not depend on OB-associated proteases to gain entry into hepatopancreatic cells. Slack and Arif (2007) hypothesise that baculovirus ancestors were not occluded and instead relied on alkaline proteolytic activation during infection. It is hypothesised that contrasting ecological niches occupied by the insect host life cycle, limit baculoviruses infection to larval stages (Wang *et al.*, 2007). Therefore,

occlusion body-facilitated horizontal transmission is vital for its longevity within the environment. The non-occluded nudiviruses, however, have demonstrated their ability to infect adult life stages. Therefore evolutionary maintenance of occlusion body transmission offers little benefit over vertical transmission or latency within the aging host (Wang *et al.*, 2007).

The expanding diversity of the *Nudiviridae* suggests that lack of occlusion alone is not a distinguishing characteristic of these viruses; several occlude prior to horizontal transmission whereas others do not. It is therefore likely that other characteristics of the genome underlie the separation of the group from the baculoviruses. Little is known about the nudivirus lifecycle and so this, and the means by which they gain entry into the host cell and cause infection, may also serve as discernible features of the proposed genus.

We did not observe any accompanying clinical signs in HgNV-infected individuals. Evidence suggests a persistent asymptomatic virus may even offer benefit to the individuals within an infected population. Invertebrates lack a typical adaptive immune system, however, host cells infected with latent Hz-1 virus (HzNV-1) are resistant to a more virulent infection of the same virus via homologous interference (Lin *et al.*, 1999). Nevertheless, despite widespread latency within the *Nudiviridae*, many cause delayed development and eventually death (Bézier *et al.*, 2015). Whether HgNV has an effect on growth development or mortality of the European lobster remains to be shown. Furthermore environmental and/or physiological stimuli can result in massive viral amplification which give even low virulent viruses the potential to cause mass mortalities within a population (Walker and Winton, 2010). This may be of particular importance as invertebrate aquaculture grows in popularity. The increased prevalence of HgNV in hatchery vs SBCC lobsters suggests either that

conditions within SBCC are not conducive to high prevalence (e.g. lower transmission potential) or, that lobsters infected with HgNV have higher mortality during early deployment and thus are not present at later stage sampling points. However, in relation to the latter, given that early mortality in SBCC and hatchery populations did not differ (data not shown), HgNV as a driver of mortality in SBCCs appears unlikely. It should be noted that recirculating systems likely serve as drivers for increased prevalence in older hatchery-reared stocks (52–104 weeks post deployment controls) and juvenile lobsters are not typically on-grown in hatchery environments for such extended periods. Further work on the role of HgNV in early life stage growth and mortality is now required.

3.5 Data Availability

Sequences have been deposited in GenBank under the BioProject PRJNA516791.

3.6 Acknowledgements

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Chapter 4

Understanding the role of the shrimp gut microbiome in health and disease

Invited Review:

Holt, C.C., Bass, D., Stentiford, G.D., and van der Giezen, M. Understanding the role of the shrimp gut microbiome in health and disease. *Journal of Invertebrate Pathology. Under Review*

Abstract

With rapid increases in the global shrimp aquaculture sector, a focus on animal health during production becomes ever more important. Animal productivity is intimately linked to health, and the gut microbiome is becoming increasingly recognised as an important driver of cultivation success. The microbes that colonise the gut, commonly referred to as the gut microbiota or the gut microbiome, interact with their host and contribute to a number of key host processes, including digestion and immunity. Gut microbiome manipulation therefore represents an attractive proposition for aquaculture and has been suggested as a possible alternative to the use of broad-spectrum antibiotics in the management of disease, which is a major limitation of growth in this sector. Microbiota supplementation has also demonstrated positive effects on growth and survival of several different commercial species, including shrimp. Development of appropriate gut supplements, however, requires prior knowledge of the host microbiome. Little is known about the gut microbiota of the aquatic invertebrates, but penaeid shrimp are perhaps more studied than most. Here, we review current knowledge of information reported on the shrimp gut microbiota, highlighting the most frequently observed taxa and emphasizing the dominance of Proteobacteria, within this community. We discuss involvement of the microbiome in the regulation of shrimp health and disease and describe how the gut microbiota changes with the introduction of several economically important shrimp pathogens. Finally, we explore evidence of probiotic supplementation and consider its role in the future of penaeid shrimp production.

4.1 Introduction

Gut-inhabiting microbes are recognised as important drivers of several metabolic processes in the host. As such, the characterisation and subsequent manipulation of this microscopic community is an attractive proposition for aquaculture research. Penaeid shrimp aquaculture is an important source of economic gain for many Asian and Latin American countries (Hernández-Rodríguez *et al*., 2001) and shrimp research has subsequently dominated the field of marine-based invertebrate gut microbiomes. However, in comparison with mammals and terrestrial invertebrates, relatively very little is known about the bacteria living in the gut of aquatic invertebrates such as penaeid shrimp.

In this review, we summarise gut microbiome sequence data from currently available penaeid shrimp studies that utilise a high-throughput sequencing (HTS) approach, in order to investigate the diversity of gut-associated bacteria in shrimp grown under a range of conditions across the world. Proteobacteria were the dominant phylum in the majority of studies from Thailand, Mexico, Vietnam, Malaysia, China and Brazil (Table 4.1). Proteobacteria are widespread in aquatic invertebrate gut microbiotas and are often a dominant component of this community in Crustacea (Hakim *et al*., 2015; Huang *et al*., 2016; Meziti *et al*., 2010; Rungrassamee *et al*., 2014, 2013; Zhang *et al*., 2014). They are a diverse phylum of bacteria in terms of their physiologies and morphologies, however most are Gram-negative, and most are facultative or obligate anaerobes (Stackebrandt *et al*., 1988). Gammaproteobacteria, the largest class in the phylum, are often described as the most common bacteria in the gut of giant tiger shrimp (*Penaeus monodon*) (Chaiyapechara *et al*., 2012; Rungrassamee *et al*., 2016, 2014, 2013), Pacific white shrimp (*Litopenaeus vannamei*) (Rungrassamee *et al*., 2016; Tzuc *et al*., 2014; Zheng *et al*., 2017) and Chinese shrimp (*Fenneropenaeus chinensis*)

(Liu *et al*., 2011). Gammaproteobacteria, mainly *Vibrio* and *Photobacterium* spp., have also been reported to account for more than 70 % of sequences isolated from the guts of wild-caught and domesticated *P. monodon* with the remaining classified sequences attributed to other high-level taxa: Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria (Figure 4.1C) (Rungrassamee *et al*., 2014). Many *Vibrio* spp. produce chitinolytic enzymes (Sugita and Ito, 2006) which may explain their dominance in a chitin-rich environment such as the gut, which provides a niche substrate for their utilisation. However, this enzymatic potential can also be responsible for negative effects on the carapace of the animal and other health implications (Jayasree *et al*., 2006). As such, several *Vibrio* spp. have historically caused large losses to the aquaculture industry, with vibriosis often causing mass mortalities (Lavilla-Pitogo *et al*., 1998) and seemingly non-pathogenic *Vibrio* having expressed virulence in compromised hosts (Manilal *et al*., 2010). Despite this, *Vibrio* spp. are often described as the dominant genus within the shrimp gut microbiota (Liu *et al*., 2011; Tzuc *et al*., 2014) and many exist harmoniously with the host.

Reference	Species	Location	Comparison	Hypervariable region/ Primers	Sequencing Platform	Data Accession
CULTURE ENVIRONMENT						
(Rungrassamee *et al*., 2014)	*Penaeus monodon*	Andaman Sea	Wild	V3-4/338F-518R	454	KF329429–KF334451,KF334452–KF344403,KF344404–KF355928
		Surat Thani province, Thailand	Domesticated			KF322280–KF325238,KF325239–KF328420,KF328421–KF329428
(Oetama *et al*., 2016)	*Penaeus monodon*	Bali	Wild	V4/515F-806R	Illumina	SRP059721
		Jakarta Bay	Wild			
		Pejarakan, Singaraja, Bali	Aquaculture farm			
(Cornejo-Granados *et al*., 2017)	*Litopenaeus vannamei*	Nayarit coast, Mexico	Wild	V2-4-8 mix, V3-6-7-9 mix/ Unpublished	Ion Torrent	SRR5585664-84. BioProject: PRJNA387510
		Sonora state, Mexico	Healthy Domesticated			
			APHND +			
(Md Zoqratt *et al*., 2018)	*Litopenaeus vannamei*	Quang Yen, Quang Ninh, Vietnam	Vietnamese farms	V3-V4/S-D-Bact-0341-b-S-17(F)-S-D-Bact-0785-a-A-21	Illumina	SRP126985. BioProject: PRJNA422950
		Sitiawan, Perak, Malaysia	Malaysian farms			
GROWTH STAGE						
(Rungrassamee *et al*., 2013)	*Penaeus monodon*	Surat Thani province, Thailand	Postlarvae 15 days	V3-6/338F-786R	454	JX919344-JX926388
			Juvenile 1 month			JX916289-JX919343
			Juvenile 2 months			JX926389-JX939518
			Juvenile 3 months			JX939519-JX941408
(Huang *et al*., 2014)	*Litopenaeus vannamei*	Xiamen, Fujian Province, China	Postlarvae 14 days	V3-5/338F-907R	454	BioProject: PRJNA248559
			Juvenile 1			
			Juvenile 2			
			Juvenile 3			
		Field pond, Zhangzhou, Fujian Province, China	Pond 1			
			Pond 7			
(Xiong *et al*., 2017b)	*Litopenaeus vannamei*	Ningbo, China	Larvae	V3-V4/341F-806R	Illumina	DRA005256
			Postlarvae			
			Juvenile			
			Preadult			
			Adult			
(Zheng *et al*., 2017)*	*Litopenaeus vannamei*	Hainan, China	Zoea 1	V3-V6/341F-1073R	454	SRP080243
			Zoea 3			
			Mysis 1			
			Mysis 3			

			Postlarva 1			
			Postlarva 6			
(Zeng *et al*., 2017)	*Litopenaeus vannamei*	Maoming, Guangdong, China	1 (15 dph)	V4/515F-806R	Illumina	SRX2946975
			2 (30 dph)			
			3 (45 dph)			
			4 (60 dph)			
			5 (75 dph)			
(Gainza *et al*., 2018)	*Litopenaeus vannamei*	El Oro, Ecuador	Nursery	V2-V3/341F-518R	Ion Torrent	BioProject: PRJNA352369
			Harvest			
(Xiong *et al*., 2019)	*Litopenaeus vannamei*	Xianhshan, Ningbo, China	Larvae	V3-V4/341F-806R	Illumina	DRA007714
			Juvenile			
			Adult			
		Zhannqi, Ningbo, China	Larvae			
			Juvenile			
			Adult			
DIET						
(Zhang *et al*., 2014)	*Litopenaeus vannamei*	Shenzhen, China	Soybean oil (Diet)	V4-5/515F-907R	Illumina	PRJNA253075
			Beef tallow			
			Linseed Oil			
			Fish Oil			
			SBL			
			SBF			
(Qiao *et al*., 2017)	*Litopenaeus vannamei*	Shenzhen, China	Glucose	V4-5/515F-907R	Illumina	PRJNA291010
			Sucrose			
			Corn starch			
(Fan *et al*., 2019)	*Litopenaeus vannamei*	Shan-Wei, China	Fishmeal (Week 1)	V3-V4	Illumina	SRP136220
			Fishmeal (Week 2)			
			Fishmeal (Week 3)			
			Fishmeal (Week 4)			
			Fishmeal (Week 5)			
			Fishmeal (Week 6)			
			Fishmeal (Week 7)			
			Fishmeal (Week 8)			
			Krill meal (Week 1)			
			Krill meal (Week 2)			
			Krill meal (Week 3)			
			Krill meal (Week 4)			
			Krill meal (Week 5)			
			Krill meal (Week 6)			
			Krill meal (Week 7)			
			Krill meal (Week 8)			
HEALTH/DISEASE						
(Xiong *et al*., 2015)	*Litopenaeus vannamei*	Zhanqum Ningbo, China	Black intestine (Healthy)	V4/515F-816R	Illumina	DRA002398
			Red intestine (Sub-healthy)			
			Empty intestine (Diseased)			

(Zheng *et al*., 2016)	*Litopenaeus vannamei*	Hainan, China	Healthy larvae	ALL/B8F-B1510		
			Diseased larvae			
(Rungrassamee *et al*., 2016)	*Penaeus monodon*	Shrimp Biotechnology Business Unit (SBBU), Thailand	0 hours post exposure	V3-4/338F-786R	454	KP944208-KP944681
			6HPE			KP948364-KP948529
			12HPE			KP944682-KP946571
			24HPE			KP946572-KP946691
			48HPE			KP946692-KP948363
			72HPE			KP948530-KP948831
	Litopenaeus vannamei		0HPE			KP948832-KP951735
			6HPE			KP953299-KP953763
			12HPE			KP951736-KP952247
			24HPE			KP952248-KP952978
			48HPE			KP952979-KP953298
			72HPE			KP953764-KP953903
(Xiong *et al*., 2017a)	*Litopenaeus vannamei*	Xiangshan, Ningbo, China	Normal	V3-V4/338F-806R	Illumina	DRA005153
			Retarded			
			Overgrown			
			Water			
(Zheng *et al*., 2017)*	*Litopenaeus vannamei*	Hainan, China	Healthy	V3-V6/341F-1073R	454	SRP080243
			Diseased			
(Dai *et al*., 2017)	*Litopenaeus vannamei*	Xiangshan, Ningbo, China	Normal	V2-V3/18S_F82-Euk_R516	Illumina	DRA005322
			Retarded			
			Overgrown			
(Chen *et al*., 2017)	*Litopenaeus vannamei*	Ben Tre Province, Vietnam	AHPND - HP	V3-V4/S17-A21	Illumina	SRP102384
			AHPND + HP			
(Xiong *et al*., 2018a)	*Litopenaeus vannamei*	Ningbo, China	Healthy larvae	V3-V4/341F-806R	Illumina	DRA005782
			Healthy juveniles			
			Healthy adults			
			Diseased adults			
(Xiong *et al*., 2018b)	*Litopenaeus vannamei*	Zhanqi, Ningo, China	Healthy postlarvae	V4/3NDf-V4_Euk_R2	Illumina	DRA005998
			Healthy juveniles			
			Healthy adults			
			Disease emergence			
			Diseased exacerbation			
(Yao *et al*., 2018)	*Litopenaeus vannamei*	Ningbo, China	Healthy (sampled at 70 days)	V3-V4/338F-806R	Illumina	SRP131736
			Healthy 80 days			
			Healthy 85 days			
			Diseased 70 days			
			Diseased 80 days			
			Diseased 85 days			
(Pilotto *et al*., 2018)	*Litopenaeus vannamei*	Florianópolis, Brazil	Healthy Biofloc	V3-V4/341F-806R	Illumina	
			Healthy Clear seawater			

			WSSV + Biofloc			
			WSSV - Clear seawater			
(Hou *et al*., 2018)	*Litopenaeus vannamei*	Guangzhou, China	WFS +	V4/515F-806R	Illumina	SRR6286523
			Healthy			
(Le Bris *et al*., 2018)	*Penaeus monodon*	Dong Hai district, Bac Lieu province, Vietnam	Asymptomatic gut	V3-V4/338F-806R	Illumina	SAMN06062067
			Symptomatic gut			
(Wang *et al*., 2019)	*Litopenaeus vannamei*	Maoming, Guangdong Province, China	Control	V4/515F-806R	Illumina	SRP145560
			WSSV +			
(Li *et al*., 2019)	*Litopenaeus vannamei*	Guangdong, China	White feces	ITS1/ITS1F-ITS2	Illumina	PRJNA495902
			Black gill			
			Retarded frowth			
			Healthy			
SUPPLEMENTATION						
(Sha *et al*., 2016)	*Litopenaeus vannamei*	Qingdao, China	Basal diet	V1-V2/8F-338R	Illumina	SRP071046
			Lactobacillus pentosus			
			Enterococcus faecium			
			Lactobacillus pentosus supernatant			
(He *et al*., 2017)	*Litopenaeus vannamei*	Xiamen, China	Control		Illumina	
			AviPlus ® 0.6 g/kg			
			AviPlus ® 1.2 g/kg			
(Vargas-Albores *et al*., 2017)	*Litopenaeus vannamei*	Empalme, Sonora, Mexico	Control 30 day	V3-V4/341F-805R	Illumina	https://www.dropbox.com/home/Helgoland%20Marine%20Research
			Control day			
			Eco-AQUAPROTEC 30 day			
			Eco-AQUAPROTEC 60 day			
(Liu *et al*., 2018)	*Litopenaeus vannamei*	Zhanqi, Ningo, China	Control	V4/515F-806R	Illumina	SRR3944126
			Microbial agent treatment			

Table 4.1 List of gut microbiome papers describing the shrimp gut microbiome using high throughput sequencing. Grey indicates eukaryotic studies

With the majority of HTS microbiome studies focussing on the midgut, or in some cases, an unspecified region of the gut, relatively few describe the community of the foregut and hindgut. The penaeid digestive tract, and the digestive tract of all Crustacea, is made up of three sections (Figure 4.1A); the foregut, containing the oesophagus and the two chambered stomach; the midgut; which begins at the junction of the hepatopancreas and traverses the length of the cephalothorax and

the majority of the abdomen; and finally the hindgut, containing the rectum and anus responsible for the excretion of host ingesta. These regions of the gut differ in their cell structure and function (Ceccaldi, 1989). There are few studies describing communities inhabiting the foregut, however Alphaproteobacteria along with Planctomycetales dominated the stomach of healthy Pacific white shrimp (*L. vannamei*) in Vietnam (Chen *et al*., 2017). Microbial profiles are likely influenced by the longitudinal axis of the gut itself as regional morphology and functionality induce differential pressures on selection. These internal pressures are perhaps why wild-caught and domesticated *P. monodon* share similar taxa in the gut (Figure 4.1C) (Rungrassamee *et al*., 2014) and *L. vannamei* guts from different farms are similar, despite differences in the community structure of their respective rearing waters (Md Zoqratt *et al*., 2018). Wild type *L. vannamei* from Mexico were also shown to harbour a more diverse bacterial community compared to healthy cultured animals but unlike *P. monodon*, contained substantial proportions of Cyanobacteria (Figure 4.1D) (Cornejo-granados *et al*., 2017). The availability and diversity of the diet likely impacts spatial comparisons. Mode and location of feeding will determine availability of substrate and the subsequent proliferation of microbial taxa within the gut. Furthermore, studies tracking gut composition over development stages have implicated changes in feeding to be the cause of family-level changes throughout development. Although Gammaproteobacteria dominated the gut throughout the different life-stages of *P. monodon* in Thailand (Figure 4.1C), there were shifts from a Photobacterium-based population to a Vibrio one (Rungrassamee *et al*., 2013). Gammaproteobacteria also dominated the guts of *L. vannamei* at different life-stages in a holding facility in China, with the exception of 2-month old juveniles which mainly harboured Bacteroidetes (Figure 4.1D)(Huang *et al*., 2014).

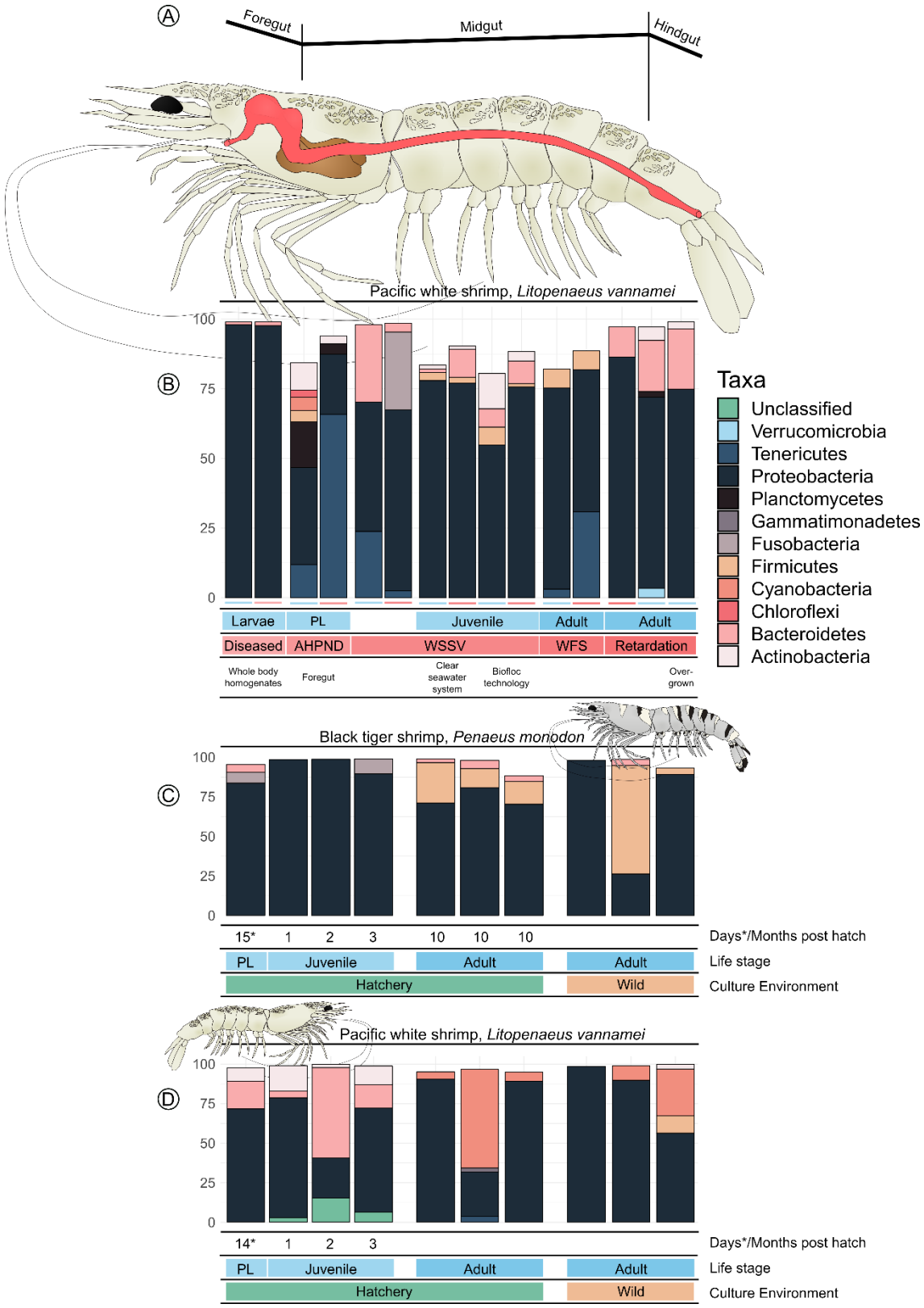
Ⓐ
Foregut
Midgut
Hindgut
Pacific white shrimp, Litopenaeus vannamei
Ⓑ
100
75
50
25
0
Larvae
PL
Juvenile
Adult
Adult
Diseased
AHPND
WSSV
WFS
Retardation
Whole body homogenates
Foregut
Clear seawater system
Biofloc technology
Over-grown
Taxa
Unclassified
Verrucomicrobia
Tenericutes
Proteobacteria
Planctomycetes
Gammatimonadetes
Fusobacteria
Firmicutes
Cyanobacteria
Chloroflexi
Bacteroidetes
Actinobacteria
Black tiger shrimp, Penaeus monodon
Ⓒ
15*
1
2
3
10
10
10
Days*/Months post hatch
PL
Juvenile
Adult
Adult
Life stage
Hatchery
Wild
Culture Environment
Pacific white shrimp, Litopenaeus vannamei
Ⓓ
14*
1
2
3
Days*/Months post hatch
PL
Juvenile
Adult
Adult
Life stage
Hatchery
Wild
Culture Environment

Figure 4.1 Overview of the penaeid shrimp gut microbiome in relation to disease, life stage and culture environment. A) Visual mapping of the tripartite digestive tract. B) Major bacterial phyla associated with gut changes in Pacific white shrimp (Litopenaeus vannamei) during pathogenesis; including diseased larvae from China (Zheng *et al*. 2017), Acute Hepatopancreatic Necrosis Disease (AHPND) infected postlarvae (PL) form Vietnam (Chen *et al*. 2017), White Spot Syndrome Virus (WSSV) infected shrimp from China (Wang *et al*. 2019), WSSV juveniles in clearwater and Biofloc systems (Pilotto *et al*. 2018), adults showing symptoms of White Faeces Syndrome (WFS) (Hou *et al*. 2018) and growth retarded adults from China (Xiong *et al*. 2017a). C) Bacterial gut profiles of black tiger shrimp (Penaeus monodon) at increasing life stages (Rungrassamee *et al*. 2013) and different culture environments (Rungrassamee *et al*. 2014). D) Bacterial gut profiles of Pacific white shrimp (Litopenaeus vannamei) at increasing life stages (Huang 2014 *et al*. 2014) and different culture environments (Corejo-Granados *et al*. 2017). When relative abundances were not stated in manuscript, corresponding bars in original figures were measured as a percentage of the axis scale.

4.2 Patterns and processes relating shrimp health to gut microbiota

One of the biggest threats to shrimp aquaculture is the onset of disease and subsequent mortality in cultured stocks (Seibert and Pinto, 2012; Stentiford *et al*., 2012). Even in cases where the clinical signs of disease are well described, little is known about how the presence of a pathogen may impact or interact with the microbial communities in the gut and subsequently compromise the metabolic processes within the host. On the other hand, it is unclear whether changes to the gut microbiome may predispose the gut to invasion by (a) pathogen(s). This could also facilitate the progression of enteric pathogens that rely on translocation through the gut epithelia to initiate infection in the target tissue.

In humans, changes to the gut microbiota have been implicated in a wide range of health conditions. Characterisation of the interplay between the microbiota and

the host immune system is becoming increasingly well-defined (Sekirov *et al*., 2010). Pattern recognition receptors (PRRs) such as Toll-like receptors on the surface of the gut epithelia are in close proximity to microbial associated molecular patterns (MAMPs) of the microbiota such as lipopolysachharides (Chu and Mazmanian, 2014). Although there are key differences between the vertebrate and invertebrate immune system, the gut microbiota likely has important roles to play in maintaining the health of the shrimp. However, a causal relationship in shrimp has not yet been demonstrated. The presence alone of a symbiotic microbiota could itself provide a kind of immunity. A general theory true of all hosts is that attachment sites within the gut are ultimately finite and colonisation resistance may limit the proliferation of pathogenic organisms through competitive exclusion (Lawley and Walker, 2013). Furthermore, colonisation resistance may be further supported through microbiota-derived antimicrobial compounds, which may limit the establishment and proliferation of transient microbes in the digestive tract (Kobayashi and Ishibashi, 1993). A more species-diverse microbiota in the gut may facilitate resistance to a greater degree of potentially problematic colonisers, as there is a greater number of available species-species antagonisms. Indeed, reducing the abundance of certain bacterial classes within the microbiota can allow previously symbiotic species to become pathogenic, defined as ‘pathobionts’ (Blumberg and Powrie, 2016).

Because of the links between the gut microbiota and the host immune system, it is often suggested that a reduction in bacterial diversity within the gut or the differential abundance of particular taxa may be responsible for the onset of pathogenesis. However, without follow-up studies involving gut supplementation and/or gnotobiotic organisms (germ free animals and/or organisms that harbour a defined microbial community) it is often impossible to discern between cause

and effect. Nevertheless, these correlations should not be dismissed without merit and several studies have described such correlations in shrimp under the affliction of important pathogens which cause massive economic loss (Table 4.1, Figure 2B).

4.3 Changes to the gut microbiome correlate with the incidence of disease

The following section summarises what is known of the microbiome in relation to key diseases which can impact production. Although we have collated these studies in Figure 4.1. It is important to recognise that these samples were analysed independently of each other. Therefore, methodological and/or analytical biases (in the region of the gene sequenced and the bioinformatic approaches used) may in turn bias comparisons between disease studies. Unfortunately, short read data were not accessible for all studies when we attempted a meta-analysis to directly compare the results.

In ‘diseased’ Pacific white shrimp raised in a commercial marine shrimp hatchery in Hainan, China, there were no significant differences in the gut microbiota when compared to healthy individuals up to and including 18 days post-larvae (Figure 4.1B) (Zheng *et al*., 2017). However, Linear Discriminate Analysis (LDA) Effect Size (LEFSe) highlighted several taxa that were indicative of the disease state. Species of the *Nautella* genus (Rhodobacteraceae), which can be pathogenic to algae and brine shrimp (Gardiner *et al*., 2015; Zheng *et al*., 2016) showed the greatest association with diseased individuals and the water in which they were reared. Unlike the shrimp samples themselves, water from healthy and diseased ponds formed distinct clusters when ordinated with non-metric multidimensional scaling (NMDS) therefore environmental DNA (eDNA) assessment of the

microbiome within the rearing environment may be a useful indicator of disease in the cultivar.

For clarification, in a meta-analysis of disease-associated taxa in developing *L. vannamei*, this disease was incorrectly described as Mysis Mold Syndrome (MMS) (Wu *et al*., 2018). The disease state was originally characterised by poor growth, inactivity, lack of appetite, empty digestive tracts and/or low survival rate (Zheng *et al*., 2017) and affected individuals were not known to be suffering from MMS specifically (Yanfen Zheng, Ocean University of China, pers. comm).

4.3.1 White spot syndrome virus (WSSV)

White spot syndrome virus is the biggest threat to shrimp health worldwide (Stentiford *et al*., 2009). The double-stranded DNA (dsDNA) virus infects nuclei of mesodermal- and/or ectodermal-derived tissues and results in lethargy of the infected host and a reduction in food intake (Pradeep and Rai, 2012). Although predominantly infecting shrimp, its severe pathogenesis results in a reduction in growth and ultimately high mortality rates in a wide range of cultured species (Bateman *et al*., 2012; Stentiford *et al*., 2009). The gut microbiota of *L. vannamei,* obtained from a farm in Maoming, China, was recently shown to be significantly altered in association with WSSV infection (Wang *et al*., 2019). Individuals infected with WSSV saw a significant increase in Proteobacteria and Fusobacteria in the gut, including potentially pathogenic bacteria belonging to the *Arcobacter* genus, together with a reduction in Bacteroidetes and Tenericutes (Figure 4.1B). However, no change in bacterial richness and/or diversity of the gut was reported in the animals infected with WSSV (Wang *et al*., 2019). Compositional changes in response to WSSV infection are also impacted by external factors in relation to the culture environment, which might obscure

microbiome changes specifically associated with the disease and/or presence of the virus. When comparing clear seawater and biofloc systems before and after WSSV infection, there were inconsistent changes in phyla abundance and diversity (Pilotto *et al*., 2018). Furthermore, although Protobacteria did increase after WSSV challenge in the biofloc system, a decrease in Bacteroidetes was not observed in either culture condition, indicating a degree of disparity between both studies.

4.3.2 Acute hepatopancreatic necrosis disease (AHPND)

Sometimes referred to as Early Mortality Syndrome (EMS), AHPND has also been responsible for large production losses of cultured shrimp. The disease results in atrophy of the hepatopancreas and ultimately necrosis of the HP tubules and is caused by several species of *Vibrio*, and the acquisition of a plasmid which results in the production of *Photorhabdus* insect-related (Pir) binary toxins (Lee *et al*., 2015; L. Liu *et al*., 2018; Restrepo *et al*., 2018). The incidence of AHPND in *L. vannamei* corresponded to a significant reduction in bacterial diversity of the hepatopancreas compared to that of healthy individuals, with those infected with AHPND showing a reduction in diversity of over 53 % within 7 days. Several *Vibrio* clusters were associated with AHPND positive individuals, along with a high abundance of *Candidatus* Bacilloplasma-like sequences. Analysing interaction networks within the community, it is suggested that commensal *Candidatus* Bacilloplasma spp., which are found in several aquatic invertebrates, interact with the pathogenic *Vibrio* strains and enhance or inhibit infection (Chen *et al*., 2017).

4.3.3 White faeces syndrome (WFS)

White faeces syndrome, characterised by white-golden gut contents and white faecal strings, is a syndromic condition of unknown aetiology. WFS was initially thought to be linked to the presence of the microsporidian *Enterocytozoon hepatopenaei*. However, PCR and *in situ* hybridization has since demonstrated that ponds highly abundant in *E. hepatopenaei* often lack characteristic symptoms of the disease in the corresponding stocks (Tangprasittipap *et al*., 2013). It has since been determined that the gregarine-like vermiform bodies that give WFS its appearance are formed through the transformation, sloughing and aggregation of microvilli within the hepatopancreas (Sriurairatana *et al*., 2014). The cause of this phenomenon is unknown. However, when comparing bacterial gut profiles of WFS infected shrimp and asymptomatic individuals, there was an increase in *Candidatus* Bacilloplasma and *Phascolarctobacterium* and a decrease in *Paracoccus* and *Lactococcus* species, which correlated with a significant reduction in overall diversity of the bacterial community (Hou *et al*., 2018). *Candidatus* Bacilloplasma is commonly found in the shrimp gut. Considering how well adapted this genus is for living in the gut environment (Kostanjšek *et al*., 2007), its increased abundance in diseased individuals is likely a consequence of the reduction in other taxa, and overall diversity of the gut microbiota. White faeces was also associated with changes to the mycobiota, with an in increase in pathogenic *Candida* in symptomatic individuals (Li *et al*., 2019).

4.3.4 Nutritional acquisition and slow growth

The bacterial gut microbiome can also impact the growth of the shrimp through the modification of digestive enzyme activity. After rearing larval Pacific white

shrimp for 70 days in ponds located in Xiangshan, China, body size and weight significantly and positively correlated with amylase, pepsin and lipase activity (Xiong *et al*., 2017a). Structural equation modelling (SEM) described how gut community composition of both bacteria and eukaryotes accounted for significant positive effects on enzymatic activity (Dai *et al*., 2017; Xiong *et al*., 2017a). Bacterial diversity was significantly reduced in retarded shrimp as the relative abundance of Gammaproteobacteria dramatically increased (Figure 4.1B) (Xiong *et al*., 2017a). Retarded shrimp also harboured less phylogenetically clustered gut communities compared to normal individuals, indicating a reduction in host determinism in the assemblage of bacterial gut communities (Xiong *et al*., 2017a).

Despite a commonality of Proteobacteria in the water column, gut microbiotas are repeatedly noted to be distinct from that of their rearing waters (Harris, 1993; Meziti *et al*., 2012; Xiong *et al*., 2015; Zhang *et al*., 2016). This may be explained by the importance of deterministic processes, such as environmental filtering, in the colonisation of the shrimp gut during early life stages (Xiong *et al*., 2018a, 2017b). The onset of disease, however, can cause compositional shifts from the 'normal' microbiota, often referred to as dysbiosis (Xiong *et al*., 2015, 2017b, 2018a; Zhu *et al*., 2016). The emergence of a host disease may also correlate to a reduction in deterministic processes that influence microbiota composition and a more stochastic assembly of gut colonisers (Xiong *et al*., 2017b; Zhu *et al*., 2016). Therefore a dysbiosis may indicate (or precede) the presence of a disease (Zhu *et al*., 2016). Furthermore, considering healthy, sub-healthy and diseased *L. vannamei*, based on characteristic gross pathology of the gut, the severity of disease can correlate to the degree of dysbiosis and the onset of disease can be modelled based on the composition of the gut microbiota (Xiong *et al*., 2017b,

2015). The shift in foregut microbiota associated with AHPND, specifically, was hypothesised to be a result of the stochastic processes influencing gut assembly (e.g. random dispersal) outweighing the deterministic process to shape the host microbiota (Chen *et al*., 2017). Gut profiles of shrimp challenged with vibriosis showed a lower degree of similarity compared to the uninfected, control group (Rungrassamee *et al*., 2016) possibly suggesting a shift to more stochastic determination of the gut flora. We hypothesise that early stochastic outcomes result in intraspecies variation which then predispose individuals to pathogenesis.

4.4 Improving shrimp production with gut supplementation

In light of these compositional changes, it is perhaps unsurprising that manipulating the gut microbiota has been shown to produce a number of positive effects on the shrimp host. The addition of live, beneficial microorganisms (probiotics) have been explored in a range of farmed animals for decades and is now becoming commonplace in shrimp aquaculture. Probiotic supplementation can increase competition in the gut, potentially supporting colonisation resistance against pathogenic microbes (Farzanfar, 2006). Furthermore, supplemental bacteria can directly affect and antagonise pathogens. *Streptomyces* spp., for example, have demonstrated a protective effect in *Artemia* and *P. monodon* when challenged with pathogenic *Vibrio* strains, and an increase in survival was reported for both crustaceans when inoculated with *Streptomyces* (Augustine *et al*., 2016; Das *et al*., 2010). Isolation of lactic acid bacteria from wild shrimp guts led to experiments showing that application of *Lactobacillus plantarum* MRO3.12 can also cause a reduction of *V. harveyi*. Shrimp supplemented with *L. plantarum* in their diet showed a significant increase in growth and survival rates, along with

an increased abundance of haemocytes and a reduction of *V. harveyi* in the haemolymph (Kongnum and Hongpattarakere, 2012). Infection with *V. harveyi* was also shown to alter the intestinal bacterial profiles of both *P.monodon* and *L. vannamei*. Interestingly, the altered profiles of infected *L. vannamei* reverted back to that of a healthy animal after 72 hours post infection. This was not observed with the infected *P.monodon*. The ability to regain intestinal normality was noted as a possible explanation for the greater survival rate of *L. vannamei* infected with *V. harveyi* (Rungrassamee *et al*., 2016).

There is now a range of probiotic complexes that are marketed to the farming industry, however blind application of general combinations may not be beneficial to the host. Firstly, probiotics must be able to survive passage through the gut. Common probiotic mixtures used in shrimp aquaculture often contain bacterial species that are not indigenous to the marine environment and subsequently have limited proliferation potential (Vargas-Albores *et al*., 2017). Identifying candidate probiotics from shrimp guts themselves, much as in the case of *Lactobacillus plantarum* MRO3.12 above, reduces the uncertainty surrounding survivability in the host environment. Despite probiotics being an attractive alternative to the use of broad-spectrum antibiotics, their use should be tightly monitored. For example, antibiotic resistant genes have been identified in probiotic supplements (Wong *et al*., 2015), including those often applied to shrimp culture (Uddin *et al*., 2015). However, the latter study did not identify any genetic elements associated with horizontal gene transfer.

Prebiotic supplementation (inert sources of bacterial nutrition) may also offer benefit to the microbiome and can encourage the proliferation of beneficial microbes within the gut. In an eight-week feeding trial using juvenile *L. vannamei*, mannan oligosaccharide (MOS), one of the most common prebiotics, significantly

improved weight gain and growth rate. The prebiotic also significantly increased the length of the microvilli in the intestine which could account for increased surface area for nutrient absorption, subsequently improving growth (Zhang *et al*., 2012). Although MOS did not significantly improve survival, its addition did significantly increase the activity of phenoloxidase and superoxide dismutase – both important pathways in the invertebrate immune system. Inulin, a prebiotic oligosaccharide isolated from grain, fruits and vegetables has also demonstrated positive effects on the gut microbiota. An inulin-enriched diet can significantly increase the abundance of lactic-acid bacteria (LAB), which are recognised as beneficial to host health, correlating to a significant increase in survival (Hoseinifar *et al*., 2015).

Co-application of both pre- and probiotics, termed synbiotics, can stimulate an immune response in *L. vannamei* infected with WSSV, subsequently increasing survival (Li *et al*., 2009), and could offer a potential alternative to the traditional yet ineffective use of antibiotics to treat viral infection. Twenty-seven per cent (15/56) of shrimp farmers interviewed in Thailand incorrectly used antibiotics as antiviral preventions and treatments (Holmström *et al*., 2003) therefore gut supplementation may serve as a more effective means to manage (particularly viral) disease in aquaculture production and prevent unnecessary antibiotic pressures on the environment.

As well as being ineffective in the treatment of several of the above-mentioned diseases, antibiotics can have a direct impact on the gut microbiome which may further detriment the host. Antibiotic application can decrease colonisation resistance within the gut, alter its microbial composition and facilitate the emergence of disease (Jernberg *et al*., 2010). The addition of ciprofloxacin and sulphonamide, which are commonly used to treat bacterial diseases in

aquaculture, caused a short-term reduction in bacterial richness and diversity of the gut along with a significant increase in antibiotic resistant genes in healthy *L. vannamei* (Zeng *et al*., 2019). Antibiotic resistant genes have been detected in aquaculture facilities throughout the world and can persist in bacterial reservoirs even after the initial pressure for their selection (Tamminen *et al*., 2011). Phylogenetic analysis suggests resistance genes are transferred from intestinal bacteria to those in the culture environment (Zeng *et al*., 2019) and horizontal gene transfer can spread resistance between microbes in the environment, including those that are serious human pathogens (Tomova *et al*., 2015).

4.5 Conclusions

The gut microbiomes of penaeid shrimp are becoming increasingly well characterised in comparison to other aquatic invertebrates. There are, however, still substantial gaps in the literature across all the penaeid species, and from the range of farming systems utilised in their culture. We currently lack enough data to make generalisations about the gut microbiome of different shrimp species in regard to growth conditions and health status. We propose that a concerted global effort to increase our understanding of microbial complexity in these systems is now needed. Inferences made from small datasets may not be representative of a true change or general patterns in terms of differential compositions in relation to disease and provide little to go on for the development of positive interventions. The contexts in which different microbiome states arise (shrimp species, culture conditions, treatments, pond ecology, etc.) are very varied and their own influences on shrimp microbiomes are largely unknown. What is ‘normal’ in a wide range of situations needs to be known before abnormal

conditions, for example associated with or predisposing to disease, can be reliably identified. Furthermore, the ability to access sequencing reads and experimental information needs to improve in order to undertake meta-analyses and generalise across studies. This information is vital as demand for aquatic-based protein increases and shrimp aquaculture becomes more intensive. Better characterisation of the microbiota across the entire length of the gut and across growth and development cycles will likely facilitate the improvement of shrimp probiotics to aid in improving growth and reducing the susceptibility towards disease, which will ultimately maximise the sustainable production of these key species.

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Chapter 5

Spatial and temporal axes impact ecology of the gut microbiome in juvenile European lobster *(Homarus gammarus)*

Under review:

Holt, C. C., van der Giezen, M., Daniels., C. L., Stentiford, G. D., & Bass, D. (2019). Spatial and temporal axes impact bacterial gut ecology and assembly of juvenile European lobster (Homarus gammarus): *The ISME Journal*, 1-13

Abstract

Microbial communities within the gut can markedly impact host health and fitness. To what extent environmental influences affect the differential distribution of these microbial populations may therefore significantly impact the successful farming of the host. Using a sea-based container culture (SBCC) system for the on-growing of European lobster (*Homarus gammarus*), we tracked the bacterial gut microbiota over a one-year period. We compared these communities with lobsters of the same cohort, retained in a land-based culture (LBC) system to assess the effects of the culture environment on gut bacterial assemblage and describe the phylogenetic structure of the microbiota to compare deterministic and stochastic assembly across both environments. Bacterial gut communities from SBCCs were generally more phylogenetically clustered, and therefore deterministically assembled, compared to those reared in land-based systems. Lobsters in SBCCs displayed significantly more species-rich and species-diverse gut microbiota compared to those retained in LBC. A reduction in the bacterial diversity of the gut was also associated with higher infection prevalence of the enteric viral pathogen *Homarus gammarus* nudivirus (HgNV). SBCCs may therefore benefit the overall health of the host by promoting the assembly of a more diverse gut bacterial community and reducing the susceptibility to disease.

5.1 Introduction

The gut microbiome is a community of microorganisms that demonstrates complex interactions with both the host organism and within itself. Changes in microbiome structure can correlate with digestive enzyme activity and the subsequent pre-digestion of host ingesta. Consequently, the gut microbiota can aid in nutritional breakdown and contribute to the performance of the host (Harris, 1993; Zokaeifar *et al.*, 2012; Xiong *et al.*, 2017). A diverse microbiome can provide resistance against the proliferation of potentially pathogenic microbes, contributing to host immunity and improving survival (Daniels *et al.*, 2010; Lawley and Walker, 2013). How the gut is colonised and maintained is somewhat unclear. However, considering its association with host processes, environmental determinants of gut community composition may subsequently impact growth and survival of the host (Payne *et al.*, 2008; Chaiyapechara *et al.*, 2012; Rungrassamee *et al.*, 2014; Chen *et al.*, 2015).

The advent of high-throughput sequencing technologies and development of novel analytical approaches to profiling microbial communities has led to a rapid increase in studies of microbiomes and their impact upon their host organism (Metzker, 2010; Kozich *et al.*, 2013; Callahan *et al.*, 2016). Most gut microbiome studies focus on humans or other vertebrates (Petersen and Osvatic, 2018). There are relatively very few studies of invertebrate gut microbiomes as most invertebrate studies are limited to bees and other terrestrial insects (Petersen and Osvatic, 2018), or economically important aquatic species such as penaeid shrimp. Anatomical and functional differences in the invertebrate digestive tract, compared to vertebrates, likely impose different influences on microbiome composition (Karasov and Douglas, 2013). Furthermore, contrasting immune systems, *i.e.* the lack of adaptive immunity in invertebrates, may also impact

bacterial colonisation of the gut along with host tolerance and retention of its commensals (Stagaman *et al*., 2017; Ley *et al*., 2008). Therefore, generalisations about vertebrate gut microbiomes may not reliably be extended to invertebrates. Invertebrates, however, are becoming increasingly important in modern-day aquaculture, comprising a multi-billion dollar global industry (Stentiford *et al.*, 2012, 2017). Furthermore, poor gut health is an increasing issue for development of syndromic conditions which significantly reduce aquaculture production (Stentiford *et al.*, 2017; Stentiford, Bass and Williams, 2019).

With high market prices as a result of a relatively limited fishery, the European lobster (*Homarus gammarus*) has significant potential as a high value aquaculture species in Europe (Drengstig and Bergheim, 2013). To aid stock enhancement and restocking of populations targeted by fisheries, hatchery-rearing of larval and early juvenile stages and their release to the fishery has been utilised as an approach to support European populations (Ellis *et al.*, 2014). Given the relatively high cost of juvenile lobster production in land-based culture (LBC), the on-growing of juvenile life stages in so-called sea-based container culture (SBCC) systems has produced promising results in terms of growth and survival in recent years (Daniels *et al.*, 2015). Sea-based container cultures are proposed to offer a reproducible and sustainable model for open sea rearing of lobsters given that once deployed, lobsters require relatively little management and, importantly, rely on naturally settled feed organisms in their diet (Daniels *et al.*, 2015).

Earlier studies on the gut microbiota of *H. gammarus* using Denaturing Gradient Gel Electrophoresis (DGGE) described a *Vibrio*-dominated community (Daniels *et al.*, 2010). However, there have been no attempts to utilise high-throughput sequencing approaches to comprehensively characterise the gut microbiota of

this economically important decapod. Analysis of faecal samples obtained from *H. gammarus* revealed significant changes in microbial composition between 6 and 12 months of age (Kristensen, 2015). However, these communities were not analysed with respect to their taxonomic composition. Vibrionaceae and Pseudoalteromonadaceae were also the dominant inhabitants of the majority of spiny lobster (*Panulirus ornatus*) guts across different developmental life stages with Mollicute sequences also accounting for a large proportion of the hindgut community sampled at the age of 13 months (Ooi *et al.*, 2017). Temporal shifts in the dominance of Gammaproteobacteria and Mollicutes were also evident in gut libraries isolated from Norway lobster (*Nephrops norvegicus*) (Meziti *et al.*, 2010). However, to date, no comparisons in relation to culture environment have been made for any lobster species.

Here, we characterise the gut microbiota of juvenile European lobster over a one-year period, comparing a cohort retained in an LBC system with another originating in the land-based system but subsequently retained in SBCCs moored off the coastline of Cornwall, UK. We analyse the bacterial composition of the gut by comparing exact sequence variants (ESVs; (Callahan *et al.*, 2016)) derived from the bacterial V4 region of the ribosomal small-subunit (SSU) generated from individual animals, and use diversity indexes to compare the gut microbiomes of those individuals and the groups to which they belong. By assessing phylogenetic-based mean nearest taxon distance (MNTD), we test the role of deterministic assembly of the gut by analysing the phylogenetic relationships of its bacterial inhabitants. Finally, we compare the gut microbiome of healthy individuals with those displaying as histology-positive for the recently described *Homarus gammarus* nudivirus (HgNV), the first characterised clawed-lobster virus (Holt *et al.*, 2019). HgNV translocates through the gut to establish infection

within cells of the associated hepatopancreas of its host and therefore may be influenced by the presence and composition of the gut microbiota.

5.2 Methods

5.2.1 Sample collection

Experimental design was that of described in Holt *et al*. 2019, limited to a one-year period (Holt *et al.*, 2019). Over the period of July 2016 to April 2017, 14 507 hatchery-reared juvenile lobsters were deployed in SBCCs anchored off the coast of Cornwall (St. Austell Bay 50° 18.956 N, 4°44.063 W). The majority of those deployments (10 987 animals), including those used in the current study, occurred in the summer of 2016. Routine sampling (3, 6, 28, 39, 52, 104 weeks post deployment (WPD)) was carried out to monitor the incidence of disease in SBCC populations. In total, 1 698 animals were sampled over the 2-year period. A second set of lobsters (n = 400) from the same cohort were retained within the National Lobster Hatchery, Padstow UK, and sampled at the same time points, over this period. Carapace length and survival were measured at each time point. Upon sampling, larger animals (39 - 104 WPD) were anaesthetised on ice prior to bisection through the dorsal line and the removal of the intestinal tract using sterile instruments. One half was fixed in Davidson's Seawater fixative for histological processing, the other fixed in molecular grade ethanol for sequence analysis. Smaller animals (0 – 28 WPD) were fixed whole and underwent separate analyses. The gut was later aseptically removed using a dissecting microscope.

Twenty-four animals, representing a range of carapace lengths and two container types, were sequenced from each of the five sea-based time points up to and including 52 WPD. Owing to space constraints within the hatchery, 12 individuals

from 3, 39 and 52 WPD time points were chosen from the LBC group and sequenced. Nine individuals that had spent 104 weeks in LBC and suspected to be unwell were also sequenced.

5.2.2 DNA extraction

DNA from individual guts was extracted using a CTAB/phenol:chloroform extraction method as described in Chapter 2 (Holt *et al.*, 2018). Precipitated DNA was eluted in molecular grade water and quantified fluorometrically. DNA quality was assessed by measuring absorbance at 260 nm using a NanoDrop spectrophotometer (Thermo Scientific).

5.2.3 Amplicon library preparation

Gut DNA was diluted to 1 ng/µL and transferred to two 96-well plates. Amplicon libraries were generated using the one-step custom PCR protocol and indexing primers described by Kozich *et al*. (Kozich *et al.*, 2013) and the 515fB (5' GTGYCAGCMGCCGCGGTAA 3') and 806rB (5' GGACTACNVGGGTWTCTAAT 3') V4 primers. All samples were amplified in triplicate in order to minimise PCR bias. Reactions were composed of 12.5 µL NEBNext PCR mix (New England BioLabs), 1.25 µL of both forward and reverse primers (10 µM), 7.5 µL of molecular grade water and 2.50 µL of template DNA (1 ng/µL). Initial denaturation was carried out at 98 °C for 30 s, followed by 30 cycles of 10 s denaturation at 98 °C, 30 s of annealing at 55 °C and 30 s of extension at 72 °C. Final extension was carried out at 72 °C for 2 minutes. Triplicate PCR reactions were then pooled prior to purification.

Amplicon libraries were purified with an Agenourt AMPure XP bead-based clean-up, in order to remove primer dimers and free primers. Cleaned DNA was resuspended in resuspension buffer (Illumina). Amplicon length was assessed

using the D1000 ScreenTape system (Agilent). Expected fragment size was around 400 bp. Libraries were quantified using the Promega Glomax kit. To account for the low yield of some libraries, two separate library pools were made, diluted to 2 µM and mixed in accordance with the ratio of samples between them. The concentration of the final pool was determined using qPCR. One hundred and ninety-six libraries, including two controls, were sequenced using 250 bp reads (v2 chemistry) and the Illumina Miseq.

5.2.4 Bioinformatics analysis

All reads were processed with the DADA2 analysis package in R (Callahan *et al.*, 2016). Paired end reads were trimmed according to visualized quality scores and DADA2's standard filtering parameters: maxN=0, truncQ=2, rm.phix=TRUE, and maxEE=2. DADA2's parametric error model was fitted using the first 100 million bases. Sequences were dereplicated and sequence variants inferred using the associated error model and pseudo-pooling. Filtered reads were merged and used to construct the amplicon sequence variant table. Denoised full length sequences were subsequently trimmed and chimeras removed. Taxonomy was assigned using the Silva database (v.132). Accuracy of the run was determined using a mock community of known samples, sequenced alongside a negative control. The negative control library contained no measurable DNA and produced less than 2 % of sequences compared to the average read count.

All reads were BLASTed against the full nr database using the blastx function of DIAMOND v0.7.9 (Buchfink, Xie and Huson, 2014). Classified reads were then visualised in MEGAN6 Community Edition v6.5.5 (Huson *et al.*, 2016) and non-bacterial sequences were removed. NA taxonomic assignments were labelled with the lowest characterised taxonomic rank. Alpha diversity matrices were

analysed within the phyloseq package (McMurdie and Holmes, 2013). ESVs were pruned prior to non-metric multidimensional scaling; ESVs that were not present in at least one sample were removed, as were samples that contained less than 1000 reads. Seed set at 2209.

Phyloseq and ggplot2 packages were used to visualise taxonomic profiles and diversity measures. The rgl package was used to visualise three-dimensional ordinations (Adler, Nenadić and Zucchini, 2003).

5.2.5 Statistical Analysis

Statistical analyses were conducted in the R statistical environment (Team and R Development Core Team, 2016). A series of linear models with interaction terms were used to correlate variation in the dataset. When comparing culture location, 'Day 0' samples were included within the 'LBC' grouping. Permutational multivariate analysis of variance (PERMANOVA) was analysed using the adonis function of the vegan package. The same package was used to compute the multivariate homogeneity of group dispersions (Betadisper), both analyses were performed using 999 permutations (Oksanen *et al.*, 2016).

5.2.6 Phylogenetic analyses

Exact sequence variants from all individuals were aligned using the DECIPHER package in R (Wright, 2015). A maximum likelihood phylogenetic tree inference was constructed with a generalised time-reversible (GTR) model with gamma rate variation using the phangorn package (Schliep, 2011) and subsequently used to calculate phylogenetic structuring.

5.2.7 Phylogenetic community structure

Mean-nearest taxon index (MNTD) computes the mean of the phylogenetic distance between an ESV in a given community and its closest relative within that sample. The standard effect size of phylogenetic community structure (ses.MNTD) compares the divergence away from a random, null model of distribution, measured in standard deviations, which can then be used to assess assemblage of the community as a reflection of their phylogenetic relationship. For individual samples, a ses.MNTD value of > -2 and < 2 indicates that coexisting taxa are no more related than expected by chance. Values > 2 indicates phylogenetic overdispersion and taxa are more distantly related than the null model. Values < -2 indicates phylogenetic clustering and taxa are more closely related than expected by chance. A mean ses.MNTD value representing multiple communities that is significantly greater or less than 0 is said to represent phylogenetic overdispersion and clustering respectively. ses.MNTD values are equivalent to the negative of the nearest-taxon index (NTI) and were computed using the picante package with null model = 'taxa.names', abundance.weighted=FALSE and 999 random permutations.

5.2.8 Molecular confirmation of viral infection

The HgNV_DNAPol_F1: 5'ACTTGAAGCTGTGCGTGACT 3' and HgNV_DNAPol_R1: 5' TGTATGTCTTGCGGCCCATT 3' diagnostic primer set was used to confirm viral infection in HP and gut tissues of 104 LBC animals. PCR reactions and thermal cycler settings were as described in Chapter 3 (Holt *et al.*, 2019).

5.3 Results

5.3.1 Temporal and spatial changes affect bacterial profiles of the lobster gut

A total of 7 928 959 bacterial SSU V4 region reads from 183 samples remained after filtering. On average, each sample was represented by 43 328 ± 1529 reads. Sequencing depth ranged from 2698 – 148 629 reads across all samples. Good's coverage index exceeded 0.99 in all filtered samples, indicating less than 1 % of reads in each sample only appear once in that sample (Supplementary Figure 5.1A) and rarefaction curves indicated near-saturation of community coverage (Supplementary Figure 5.1B). It should be noted, however, in a bid to remove artefactual sequences, singletons were only retained if they were present in multiple samples.

The average profile of a 0-week pre-deployment control (PDC) individual was composed of 96 ESVs comprising four bacterial genera with over 2 % relative abundance. Vibrio spp. dominated this community (58 ESVs), followed by Photobacteria (33 ESVs), Kiloniella (1 ESV) and Candidatus Hepatoplasma (4 ESVs) (Figure 5.1, Table 5.1, Supplementary Table 1). The latter was not detected in average profiles at 3, 39 and 52 WPD in the LBC system but made up substantial proportions of all SBCC group profiles post-deployment. Eighteen ESVs belonging to this assignment were shared across all SBCC profiles. Sequence variants aligned with Candidatus Hepatoplasma in the Silva database with relatively low identity, ranging from 78.7 % to 90.1 %. Vibrio spp. continued to comprise significant proportions of the guts sampled at all subsequent time-points, but with a general decline in relative abundance over time. With the exception of the 0 PDC group, the number of ESVs attributed to

Vibrio relatives at each timepoint, however, was relatively constant (Table 5.1). The Aliivibrio genus was first detected three weeks after deployment, and then constituted an average of 22 ± 4 % of the community make-up for the rest of the sampled period (Supplementary Table 1). Spongiimonas was also present in all sea-based group profiles, in addition to 3- and 52-week LBC groups. Conversely, Photobacterium lineages were detected in all hatchery group profiles, in addition to 3- and 6-week SBCC groups, which included considerably more sequence variants compared to LBC groups (Table 1). A single *Carboxylicivirga* sequence and four shared *Arcobacter* ESVs made up substantial proportions of the 39- and 52-week LBC groups.

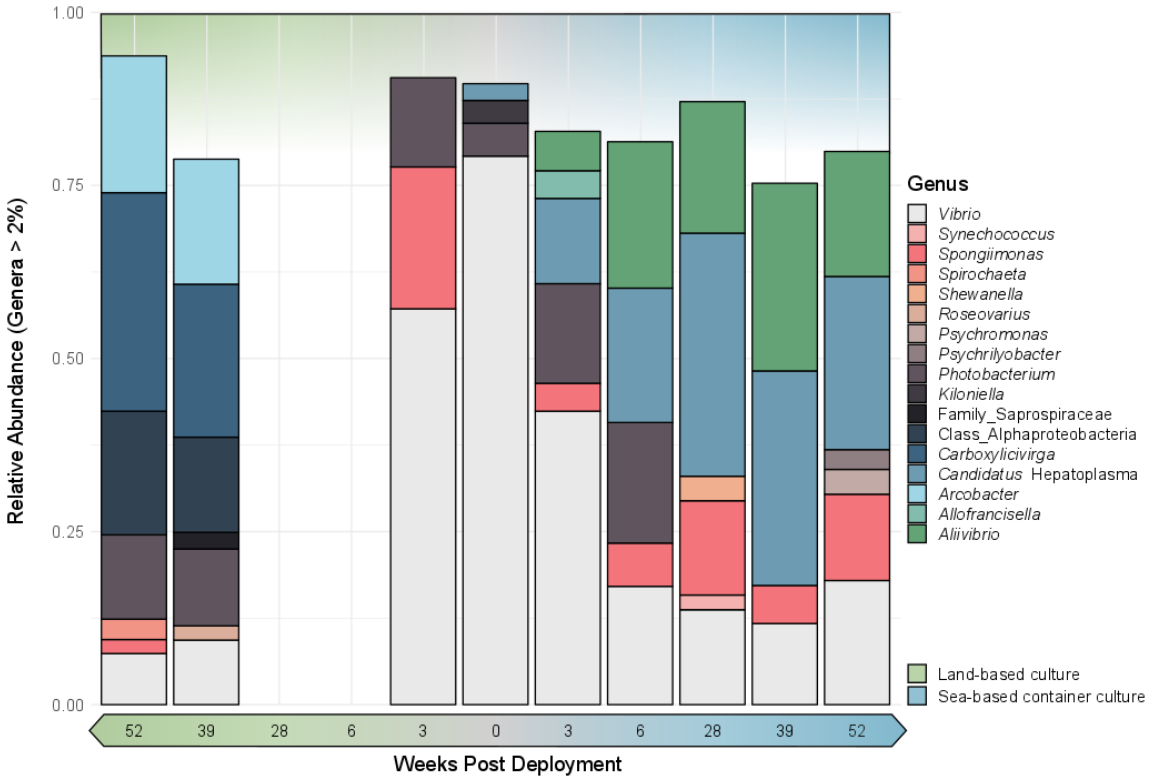

Figure 5.1 Average bacterial profiles of all animals sampled over 52 weeks. Bacterial genera representing more than 2 % of entire 16S community. Genera coloured according to key. Time

increases towards to extremities of the x axis from the pre-deployment control (0) at the centre. Green = land-based culture (LBC). Blue = sea-based container culture (SBCC).

Several genera were limited to one or more time point and only *Vibrio* spp. were isolated from all sample groups, regardless of culture environment (Figure 5.1). Two ESVs were not assigned a taxonomy by the analysis pipeline. Manual classification of these sequences later resolved their identity. The most abundant unclassified ESV from the 39 and 52 LBC groups corresponded to a genus of Sphingomonadaceae (Class_Alphaproteobactera). The remaining ESV making up the 39LBC group were assigned as an uncultured Saprospiraceae.

Genus	Number of ESVs										
	52LBC	**39LBC**	**28LPC**	**6LBC**	**3LBC**	**0PDC**	**3SBCC**	**6SBCC**	**28SBCC**	**39SBCC**	**52SBCC**
Vibrio	17	22	-	-	15	58	33	32	32	23	30
Synechococcus	-	-	-	-	-	-	-	-	6	-	-
Spongiimonas	1	-	-	-	1	-	2	2	4	3	3
Spirochaeta	2	-	-	-	-	-	-	-	-	-	-
Shewanella	-	-	-	-	-	-	-	-	15	-	-
Roseovarius	-	7	-	-	-	-	-	-	-	-	-
Psychromonas	-	-	-	-	-	-	-	-	-	-	14
Psychrilyobacter	-	-	-	-	-	-	-	-	-	-	2
Photobacterium	2	2	-	-	3	33	10	11	-	-	-
Kiloniella	-	-	-	-	-	1	-	-	-	-	-
Family_ Saprospiraceae	-	44	-	-	-	-	-	-	-	-	-
Class_ Alphaproteobacteria	9	20	-	-	-	-	-	-	-	-	-
Carboxylicivirga	1	1	-	-	-	-	-	-	-	-	-
***Candidatus* Hepatoplasma**	-	-	-	-	-	4	6	7	10	8	9
Arcobacter	3	2	-	-	-	-	-	-	-	-	-
Allofrancisella	-	-	-	-	-	-	8	-	-	-	-
Aliivibrio	-	-	-	-	-	-	8	7	13	6	9

Table 5.1 Exact sequence variant count of genera representing more than 2 % relative abundance.

Non-metric multidimensional scaling shows that all samples clustered according to group, defined by age and culture environment (Figure 5.2A; Stress: 0.130). A corresponding stressplot indicates the non-metric fit (R^2) of the ordination distance to the observed dissimilarity was 0.983 (Supplementary Figure 5.2). Centroid analysis of groups within the ordination demonstrates observed clustering was statistically significant (PERMANOVA, $p < 0.001$). Pairwise analysis showed that all groups were significantly different from each other (PEMRANOVA, $p <= 0.002$), with the exception to the 39H – 52H comparison (PERMANOVA, $p = 0.223$). Dispersion of samples within clusters, i.e. variation within each group, was also significant (Betadispersion, $p < 0.001$). The same ordination grouped by culture environment alone shows that LBC and SBCC clusters were significantly distinct ($p < 0.001$; Figure 5.2B). However, variation between samples within environments is not significant ($p = 0.921$), suggesting centroid analysis of clusters representing culture environment is not confounded by differential rates of dispersion, *i.e.* the sample variation within each location is comparable.

The averages of both species richness (Chao1) and species diversity (Shannon's Diversity) of the gut were significantly higher in lobsters from SBCC systems compared to LBC (Figure 5.3. Chao1; p-value < 0.001. Shannon's; p-value = 0.004). The progression of time in LBC did not correlate to any significant changes in bacterial richness or diversity, according to the linear model. However, in SBCC, there was significant reduction species richness with time after deployment (p-value < 0.001). Bacterial diversity remained relatively constant in

SBCC. It should be noted that, according to the linear model, culture environment does not explain all of the variability found in the data.

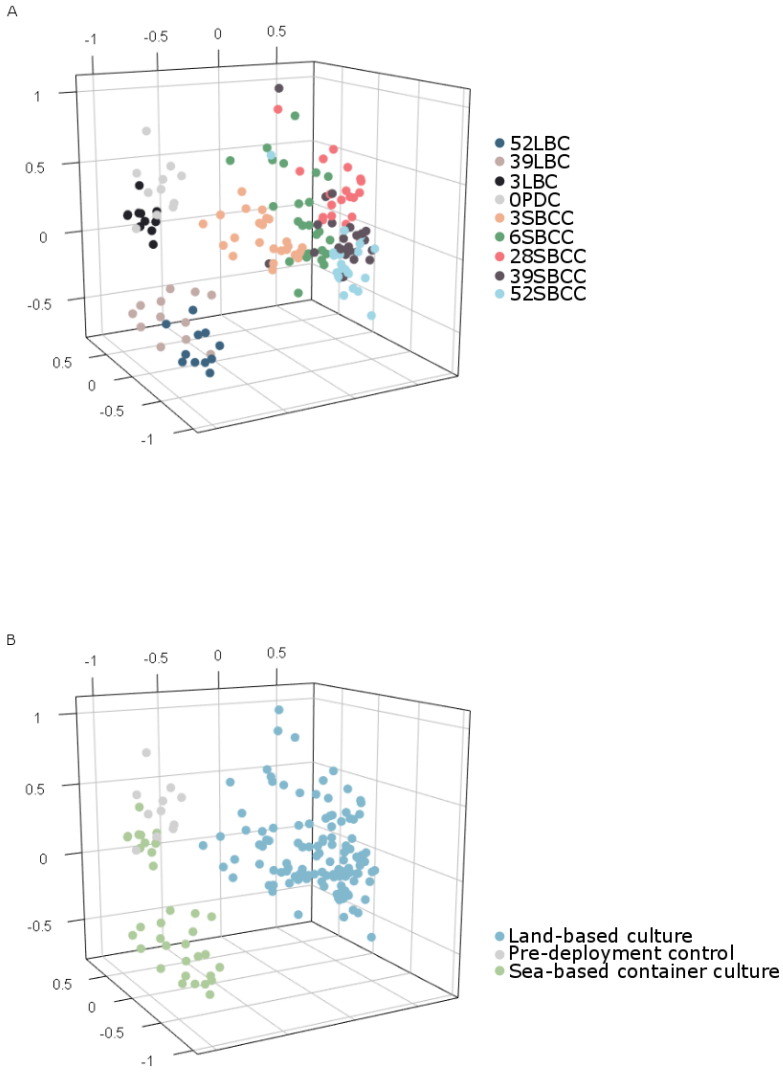

Figure 5.2 Three-dimensional non-metric multidimensional scaling (NMDS) of all gut samples. Unweighted non-metric multidimensional scaling (NMDS) using the Bray-Curtis measure of dissimilarity over three axes. Stress = 0.130. A: Coloured according to sample group. B: Coloured according to culture environment.

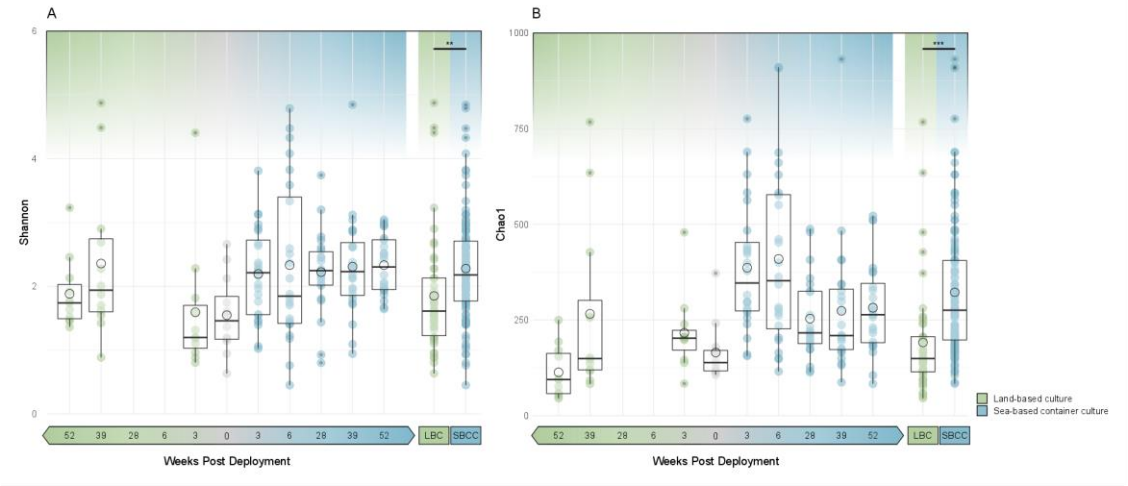

Figure 5.3 Alpha diversity measures of all sample groups. A: Shannon's measure of species diversity across all sample groups. B: Chao1 estimate of species richness across all sample groups. Green = land-based culture (LBC). Blue = sea-based container culture (SBCC). Environmental comparison 'LBC' (including day 0) and 'SBCC' represent combined data of all corresponding groups. Boxes labelled with groups that are significantly different. ** p <= 0.01. *** p <= 0.001.

5.3.2 Deterministic processes impact gut assembly in SBCC

The ses.MNTD representing the 0 WPD control group was -2.091 ± 0.268, indicating that bacterial taxa within these animals are on the border between random distribution and phylogenetic clustering (Figure 5.4). The average ses.MNTD value for remaining LBC groups remain within the limits of implicit stochasticity (-2 > x < 2) and become more indicative of random assemblage (*i.e.*, the null model) as time increases from 3 WPD to 52 WPD (3LBC = -1.432 ± 0.244, 39LBC = -0.991 ± 0.528, 52LBC = -0.721 ± 0.450) (Figure 5). Average ses.MNTD values for all SBCC groups are less than -2 implying a greater degree of phylogenetic clustering of bacteria and deterministic assembly. The degree of phylogenetic clustering, however, does not correlate with an increase in the age of the sample group (3SBCC = -3.403 ± 0.209, 6SBCC = -3.076 ± 0.331, 28SBCC

= -3.047 ± 0.241, 39SBCC = -2.061 ± 0.344, 52SBCC = -3.608 ± 0.272) (Figure. 5.4). Overall, bacterial colonisers of SBCC lobster guts are significantly more phylogenetically clustered compared to those in LBC (p value = < 0.001).

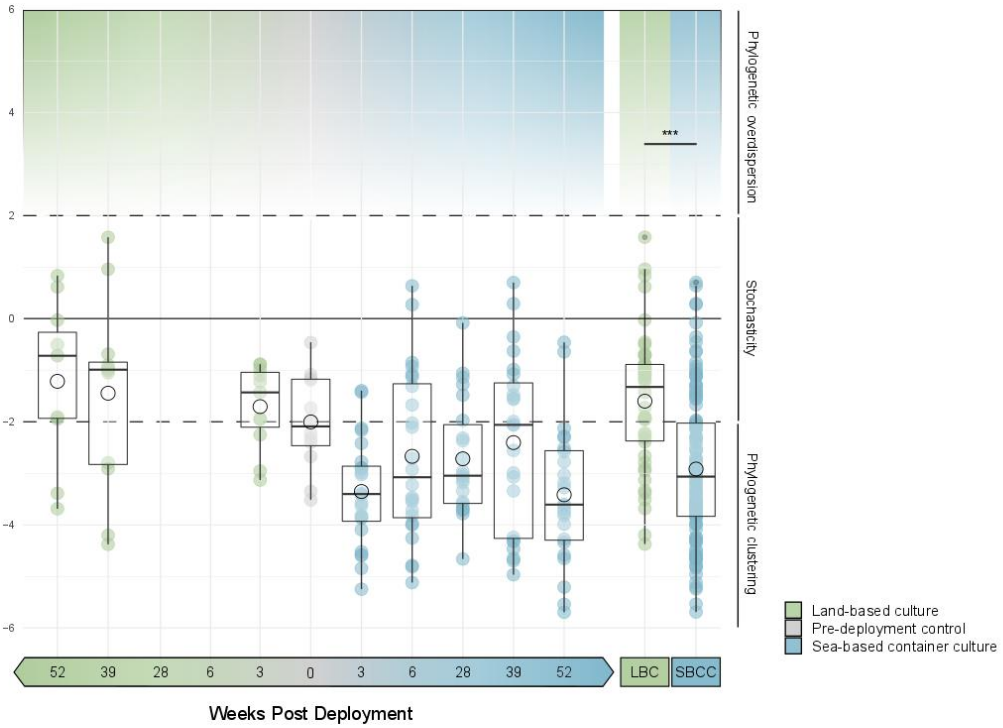

Figure 5.4. Standard effect size of mean-nearest taxon index (ses.MNTD) indicating phylogenetic clustering of sequence variants. Standard deviation of mean-nearest taxon index (MNTD) from random model. Ses.MNTD values > 2 indicate phylogenetic overdispersion of taxa, 2 < & > -2 indicate stochastic distribution across phylogeny, < -2 indicate phylogenetic clustering. Green = land-based culture (LBC). Blue = sea-based container culture (SBCC). Environmental comparison 'LBC' (including day 0) and 'SBCC' represent combined data of all corresponding groups. Boxes labelled with groups that are significantly different. *** p <= 0.001.

5.3.3 The presence of an enteric virus correlates with changes to the bacterial gut microbiome

Histological analysis of a group of LBC animals showed intranuclear inclusions, a characteristic sign of viral infection, in the HP of six out of the nine animals tested. PCR amplification of the viral *DNA polymerase* gene of the recently characterised nudivirus, HgNV (Holt *et al.*, 2019) produced positive signal for the virus in all six HP tissue samples. Individuals infected with HgNV harboured a less diverse bacterial gut microbiota compared to uninfected lobsters (Figure 5.5A). Furthermore, gut bacterial richness of infected individuals was more variable than those tested negative for HgNV infection (Figure 5.5B). Although there are compositional differences when comparing the average profiles of infected vs uninfected animals, for example, the genera *Marinifilum* and *Spirochaeta* are present in the gut of uninfected animals, but not in virus-infected animals (Figure 5.5C), there aren't any clear associations when comparing individuals. Generally, however *Photobacterium* spp. are consistently more dominant in virus-infected animals (Figure 5.5C). The unassigned genus again corresponds to the uncultured Alphaproteobacteria isolated likely belonging to the Sphingomonadaceae.

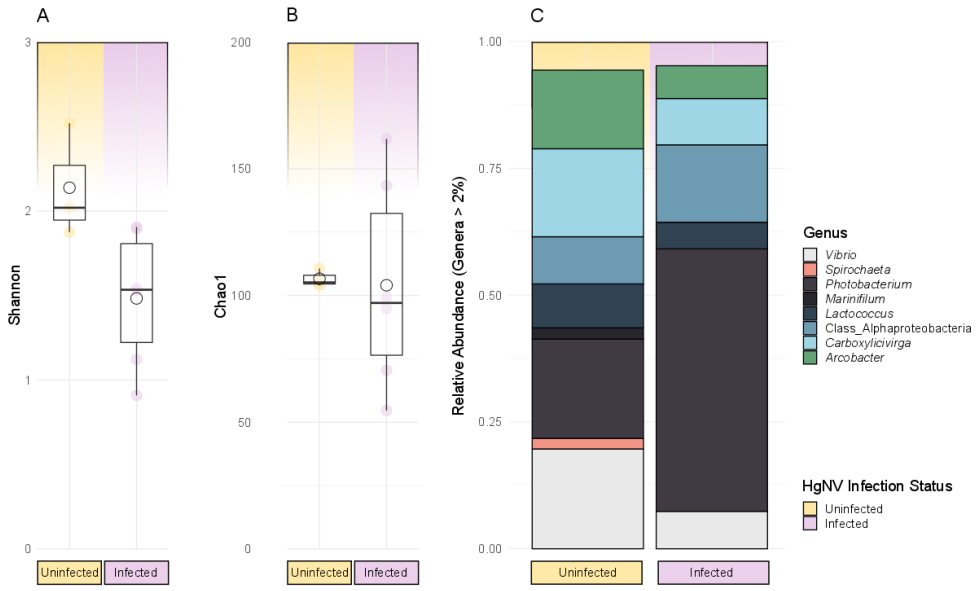

Figure 5.5 Changes to gut microbiota in the presence of Homarus gammarus nudivirus (HgNV). A: Shannon's measure of species diversity across healthy and infected individuals sampled at 104 weeks. B: Chao1 estimate of species richness. C: Bacterial genera representing more than 2 % of entire 16S community. Yellow = HgNV-negative samples. Pink = HgNV-positive samples. Genera coloured according to key.

5.4 Discussion

Our results highlight the high degree of plasticity of the gut microbiota of the European lobster and demonstrate how environment, age (during early life) and infection status with a specific virus can correlate to differences in bacterial community composition. Individuals raised in SBCCs are associated with a more diverse gut microbiome, which may confer subsequent benefits to the health and growth of their hosts. Rearing lobsters in a more microbially rich and diverse natural marine environment, as opposed to a land-based system, likely

encourages the selection and colonisation of a more diverse gut community. Therefore, SBCC could potentially benefit production of this and other species.

Biological community assembly can be governed by both stochastic and deterministic processes (Dumbrell *et al.*, 2009; Dana Ofiteru *et al.*, 2010; Langengeder and Szekely, 2011; Stegen *et al.*, 2012). Stochastic processes include those pertaining to passive dispersal and ecological drift, *i.e.* random loss and gain, whereas deterministic colonisation refers to environmental selection governed by the relative differences in ecological fitness of its inhabitants (Stegen *et al.*, 2012; Burns *et al.*, 2016). Phylogenetic clustering of a bacterial community indicates a greater degree of environmental filtering and a non-random association with its environment, in this case the gut, as closely related species are predicted to be more ecologically similar and therefore subject to a greater degree of competition. There are conflicting results as to how important deterministic processes are in the establishment of gut communities in zebrafish of increasing age (Burns *et al.*, 2016; Yan *et al.*, 2016) yet we did not observe any temporal trends in lobsters held in either LBC or SBCCs. However, 52 weeks is a relatively short proportion of the typical life span of a healthy lobster. Our results suggest that LBC animals generally relied on stochastic means of gut assembly throughout their development indicating random dispersal of potential bacteria colonisers can account for considerable variations in gut community. Indeed, variation within LBC groups is greater than sea-based groups at corresponding time points and stochasticity has been demonstrated to induce heterogeneity in bacterial gut samples of *Caenorhabditis elegans* (Vega and Gore, 2017). Sea-based lobster gut samples typically harboured a more phylogenetically clustered bacterial community compared to LBC animals of the same age, suggesting that gut communities of SBCC lobsters were more

deterministically assembled; *i.e.* there are more factors limiting the random assemblage of bacteria in the gut. Previous studies have shown that the invertebrate gut microbiota tends to be distinct from that of the host rearing water (Harris, 1993; Meziti, Mente and Kormas, 2012; Xiong *et al.*, 2015; Zhang *et al.*, 2016). The gut and its ingested substrates may therefore support the positive selection of relatively rare bacterial lineages from the complex surrounding water column. Despite the majority of sea-based samples from each of the time points indicating phylogenetic clustering, many corresponded to ses.MNTD values of greater than -2, therefore the degree of environmental filtering is likely influenced by individual traits which vary within a population; such as growth capacity (Xiong *et al.*, 2017) or health state of the host (Wu *et al.*, 2018). It is also worth noting that animals were sampled in a random manner with respect to moulting stage. The moult cycle may impact the presence of specific microbes and therefore contribute to inter-sample variability (Mente *et al*., 2016).

The *Vibrio* genus, belonging to the phylum Proteobacteria, is commonly reported as the dominant genus of invertebrate digestive tracts (Harris, 1993) and is ubiquitous within many water column samples (Thompson *et al.*, 2016). Several *Vibrio* spp. are infamous for causing disease in humans, however many also pose risks to marine invertebrates. *Vibrio harveyi,* for example*,* can infect and disrupt the epidermal tissue of the digestive tract and can limit the production of penaeid shrimp (Austin and Zhang, 2006), *V. parahaemolyticus* encoding toxic *Photorhabdus* insect-related (Pir) binary toxins can cause Acute Hepatopancreatic Necrosis Disease (AHPND) and result in large production losses in shrimp aquaculture (Lee *et al.*, 2015) and *V. owensii* DY05 can cause mass mortalities of ornate spiny lobster (*Panulirus ornatus*) (Goulden *et al.*, 2012). Many species, however, are commensal and thought to be opportunistic

in their nature (Aguirre-Guzmán, Vázquez-Juárez and Ascencio, 2001). Non-pathogenic strains may have the potential to be employed as a probiotic. The addition of both *V. alginolyticus* and *V. gazogenes*, have resulted in a reduction of several pathogenic *Vibrio* spp. in the guts of *L. vannamei* (Thompson *et al.*, 2010). *Vibrio* spp. may also confer benefits by producing extracellular chitinases (Ceccaldi, 1989; Suginta *et al.*, 2000) which could aid in the digestion of prey and could also break down the host exuvia after ecdysis, routinely ingested to promote calcification of the new carapace. The *Aliivibrio* genus, erected to differentiate *A. fischeri* from other *Vibrio*, contains mainly salmonid pathogens associated with low water temperatures (Beaz-Hidalgo *et al.*, 2010). More notably, *A. fischeri* and its association with the Hawaiian bobtail squid (*Euprymna scolopes*) has become a model system for quorum sensing and host-microbe symbiosis, whereby the bacterial quorum initiates bioluminescence in the host (Miyashiro and Ruby, 2012). It should be noted that the V4 region of the rRNA SSU is not capable of fully differentiating between different species of the *Vibrio* and *Aliivibrio* genera as numerous database entries for both genera shared 100 % sequence identity.

"Candidatus Hepatoplasma crinochetorum" is a monophyletic species of Mollicute first isolated from the hepatopancreas of the terrestrial isopod, *Porcellio scaber* (Wang *et al.*, 2004). The presence of the symbiont in isopods was positively correlated with survival on low-quality food suggesting a beneficial endosymbiosis between the two organisms (Fraune and Zimmer, 2008). As terrestrial isopods feed on low-nutrient, decaying plant matter, an association from which they can better sequester nutrition from their ingesta should be evolutionary beneficial. It is hypothesised that symbiotic relationships such as this may have facilitated the expansion of isopods to terrestrial environments as no

such bacteria were found in the hepatopancreas of isopods from the marine environment (Wang, Brune and Zimmer, 2007). An ESV representative of the "*Candidatus* Hepatoplasma" assignment, isolated from lobster, was identical to that isolated from Norway lobster (*Nephrops nervegicus*) (Meziti, Mente and Kormas, 2012) and 96 % identical to a clone isolated from the high intertidal/sublittoral isopod *Ligia occidentalis* (Eberl, 2010).

Spongiimonas is a Gram-negative, aerobic genus within Flavobacteriaceae that has been isolated from a marine sponge (Yoon, Jang and Kasai, 2013). Our ESVs annotated as *Spongiimonas* are equally similar to several uncultured bacteria isolated from the guts of *Nephrops norvegicus* (Meziti, Mente and Kormas, 2012). Flavobacteriaceae is a large family of Bacteroidetes, many of which are responsible for several important fish diseases (Loch and Faisal, 2015). Flavobacteriaceae have been isolated from lesions of lobsters infected with epizootic shell disease (ESD), a cuticular disease causing erosion of the carapace in American lobster (*Homarus americanus*) (Tlusty *et al.*, 2007). Although the exact etiological agent(s) of ESD are unknown, and wild European lobsters seem to be unaffected by this disease, American lobsters displaying signs of ESD have been found in Norwegian waters (Davies and Wootton, 2018) and phenotypic signs of the infection can make the animal unmarketable.

The *Carboxylicivirga* and *Arcobacter* genera make up substantial proportions of 39- and 52-week LBC animals. The ESV assigned to *Carboxylicivirga* is highly similar to *Roseobacter* clones associated with harmful algal blooms (KY277569, KY277241) and those found in the gut of *N. norvegicus* and the mud crab *Scylla paramamosain* (Li *et al.*, 2012). However very little information is known about the role of this relatively new genus in the environment or in any host species from which it has been isolated (Yang *et al.*, 2019). Several *Arcobacter* species

have been isolated from both the marine environment (Zhang *et al.*, 2015; Park *et al.*, 2016) and shellfish samples (Collado *et al.*, 2009; Figueras *et al.*, 2011; José *et al.*, 2011; Levican *et al.*, 2012). Furthermore, several species are recognised as emerging human pathogens and can be associated with gastrointestinal disease (Lerner, Brumberger and Preac-Mursic, 1994; Figueras *et al.*, 2014). The ESV assigned to *Arcobacter* isolated from lobster guts are identical to those isolated from the guts of abalone (LC180340), sea cucumber (JX170271) as well as sequences isolated from the water column itself (GU584643, EU142059).

The unclassified ESVs limited to the LBC system and later assigned to Sphingomonadaceae. and Saprospiraceae could both represent biofouling species derived from the recirculating system. Sphingomonadaceae, a family of Alphaproteobacteria, have been identified in the guts of oriental river prawn (*Macrobrachium nipponense)* (C. Chen *et al.*, 2017) but also isolated from fouled membranes of water filtration systems (Vries *et al.*, 2018) and Saprospiraceae, a family of Bacteroidetes, have been isolated from shrimp rearing water (Cardona *et al.*, 2016; Zarul *et al.*, 2018) and recirculating systems (Brailo *et al.*, 2019; Li *et al.*, 2019) likely explaining their association with hatchery individuals. The Sphingomonadaceae ESV was also identical to a sequence derived from the guts of reared *N. norvegicus* (JN092211) (Meziti, Mente and Kormas, 2012).

In addition to direct causal links between particular bacterial species and disease, several studies claim that changes in bacterial diversity of the gut correlates to host health and the incidence of, particularly enteric, disease (Donaldson, Lee and Mazmanian, 2017). Bacteria within the lumen of the gut may contribute to host health in several ways; (1) as attachment sites within the gut are ultimately finite, the presence of a commensal community may limit the colonisation and

subsequent proliferation of potentially pathogenic microbes, in a process described as colonisation resistance (Lawley and Walker, 2013); (2) through the production of antimicrobial peptides (AMPs), members of this community can subsequently affect the abundance of other colonisers and therefore have the potential to antagonise pathogens (Garcia-Gutierrez *et al.*, 2019); (3) and by stimulating the host immune system, this community can influence host tolerance to other microbes in the gut (Lakshmi, Viswanath and Gopal, 2013). Antagonistic potential within a diverse gut perhaps increases the chances of resistance to a new pathogen and could reduce the susceptibility to incoming pathogens, preventing the establishment of infection. A reduction in diversity and subsequent compromise to colonisation resistance and its inherent redundancy could allow the proliferation of enteric pathogens such as HgNV. Although HgNV replicates in the nuclei of the hepatopancreatic epithelial cells, nudiviruses colonise the host via the digestive tract, relying on entry through the intestinal epithelia. A lower prevalence of the virus was detected in SBCC animals compared to LBC control groups (Holt *et al.*, 2019). A possible explanation for this is that sea-based animals have a significantly more diverse gut microbiome and the incidence of viral disease is dependent on the degree of the gut's resistance to its colonisation and subsequent infection. It should be noted however that sample size of infected versus healthy individuals was relatively low (n = 9), and this should be treated as preliminary data. Furthermore, we cannot discern between cause or effect within this infection model. Alternatively, an infection such as HgNV and associated compromise to host immunity may lead to a reduction in host selection pressures within the gut and lead to the observed variations in richness and diversity of the microbiota. Previous studies have indicated that a disease state may lessen the importance of deterministic assembly of the gut microbiota and

instead induce stochasticity as trade-offs divert resources to immune function and other host processes (Zhu *et al.*, 2016). Indeed, shrimp infected with AHPND demonstrate more stochastic means of assembly unlike healthy animals of the same age (W. Y. Chen *et al.*, 2017; Wu *et al.*, 2018). Possibly owing to a small sample size of HgNV infected individuals at distinct time points, we did not observe significant differences in ses.MNTD values corresponding to infection state.

As a knock-on effect of disease inducing differential abundance of the microbiota, if particular taxa in the microbiome are more adept at nutritional breakdown, there may be subsequent effects on the growth of the host which are detrimental to production. *Peneaus monodon* nudivirus (PmNV), previously referred to as *P. monodon* baculovirus (MBV) branches as a sister lineage to HgNV and has been noted to suppress growth rates in aquaculture (OIE, 2017). Experimental designs utilising gnotobiotic organisms, or those with a predefined microbiota, may help clarify these complex interactions and may discern between cause and effect. We indeed observed significant size variation between lobsters of the same age. Although genetic variation and differential food intake was not controlled in this experiment, we hypothesised that size variation in cohabiting animals can be influenced by individual variation of the gut microbiota and its ability to utilise available foodstuff, as keystone taxa are associated with digestive enzyme activity and growth of the host (Tzuc *et al.*, 2014). However, there are no significant variations in bacterial richness and diversity when comparing different sized animals of the same age, or indeed all samples after age-discriminatory taxa are predicted with a random forest model and removed from the entire dataset.

More samples are needed to analyse HgNV infection in relation to microbiome depletion and to test the significance of these preliminary changes. If HgNV colonisation and infection is dependent on microbes in the gut, variability of the gut microbiota in early stage animals may account for the differential ability of HgNV to infect individuals within a population and subsequently influence its abundance in older animals. If this is the case, the seeding of bacteria within the gut of juvenile lobsters (e.g. by ensuring they are fed a diverse bacterial diet) in a land-based system, or pre-conditioning exposure to the natural environment prior to release, could facilitate the establishment of more robust and/or healthy gut in later life stages. The application of metagenomic and/or transcriptomic analysis will further aid in differentiating the functional potential of the European lobster gut and any environmental-dependent impacts on metabolic processes of the host. Together, this information could be used in the design of novel and appropriate probiotic supplements to better cultivate this species.

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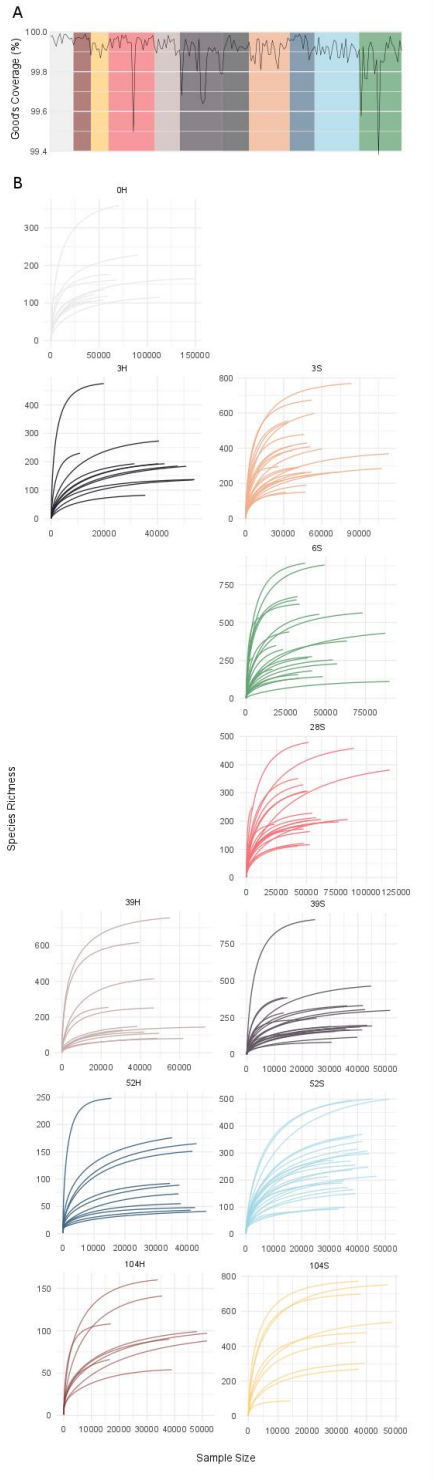
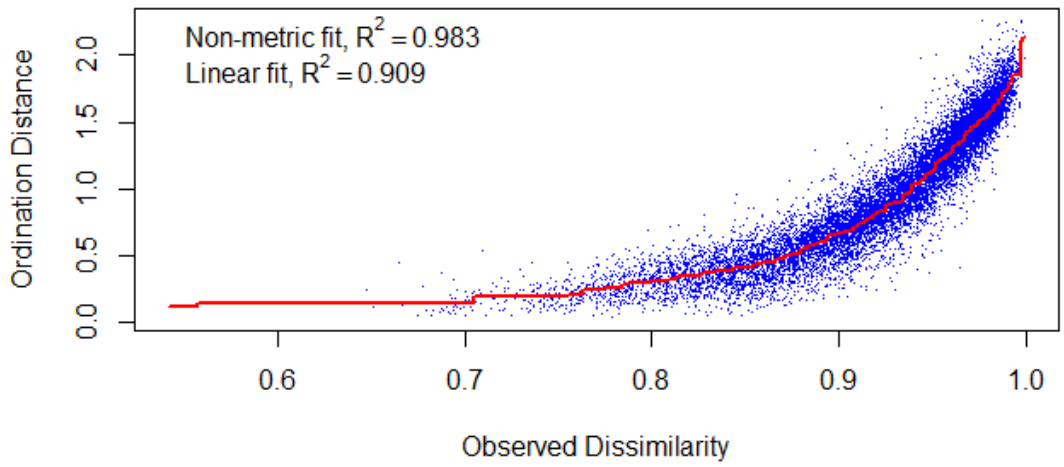
5.8 Supplementary material



Supplementary Figure 5.1 Sequencing coverage across all samples. Estimations of community saturation across all individuals. A: Good's coverage estimates. B: Rarefaction of increasing sequencing effort. Plots coloured according to sample group.



Supplementary Figure 5.2 Shepard plot indicating fit of NMDS. Shepard plot indicating fit of observed dissimilarity to ordination distance

Sample Group	Abundance	Phylum	Class	Order	Family	Genus
39SBCC	0.27	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio
6SBCC	0.21	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio
28SBCC	0.19	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio
52SBCC	0.18	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio
3SBCC	0.06	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio
3SBCC	0.04	Proteobacteria	Gammaproteobacteria	Francisellales	Francisellaceae	Allofrancisella
52LBC	0.2	Epsilonbacteraeota	Campylobacteria	Campylobacterales	Arcobacteraceae	Arcobacter
39LBC	0.18	Epsilonbacteraeota	Campylobacteria	Campylobacterales	Arcobacteraceae	Arcobacter
28SBCC	0.35	Tenericutes	Mollicutes	Entomoplasmatales	Entomoplasmatales_ Incertae_Sedis	Candidatus_Hepatoplasma
39SBCC	0.31	Tenericutes	Mollicutes	Entomoplasmatales	Entomoplasmatales_ Incertae_Sedis	Candidatus_Hepatoplasma
52SBCC	0.25	Tenericutes	Mollicutes	Entomoplasmatales	Entomoplasmatales_ Incertae_Sedis	Candidatus_Hepatoplasma
6SBCC	0.19	Tenericutes	Mollicutes	Entomoplasmatales	Entomoplasmatales_ Incertae_Sedis	Candidatus_Hepatoplasma
3SBCC	0.12	Tenericutes	Mollicutes	Entomoplasmatales	Entomoplasmatales_ Incertae_Sedis	Candidatus_Hepatoplasma
0LBC	0.02	Tenericutes	Mollicutes	Entomoplasmatales	Entomoplasmatales_ Incertae_Sedis	Candidatus_Hepatoplasma
52LBC	0.32	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiliaceae	Carboxylicivirga
39LBC	0.22	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiliaceae	Carboxylicivirga
52LBC	0.18	Proteobacteria	Alphaproteobacteria	Class_Alphaproteobacteria	Class_Alphaproteobacteria	Class_Alphaproteobacteria
39LBC	0.14	Proteobacteria	Alphaproteobacteria	Class_Alphaproteobacteria	Class_Alphaproteobacteria	Class_Alphaproteobacteria
39LBC	0.02	Bacteroidetes	Bacteroidia	Chitinophagales	Saprospiraceae	Family_Saprospiraceae
0LBC	0.03	Proteobacteria	Alphaproteobacteria	Rhodovibrionales	Kiloniellaceae	Kiloniella
6SBCC	0.17	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Photobacterium
3SBCC	0.14	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Photobacterium
3LBC	0.13	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Photobacterium

52LBC	0.12	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Photobacterium
39LBC	0.11	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Photobacterium
0LBC	0.05	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Photobacterium
52SBCC	0.03	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Psychrilyobacter
52SBCC	0.04	Proteobacteria	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas
39LBC	0.02	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Roseovarius
28SBCC	0.04	Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella
52LBC	0.03	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Spirochaeta
3LBC	0.2	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Spongiimonas
28SBCC	0.14	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Spongiimonas
52SBCC	0.12	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Spongiimonas
6SBCC	0.06	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Spongiimonas
39SBCC	0.05	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Spongiimonas
3SBCC	0.04	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Spongiimonas
52LBC	0.02	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Spongiimonas
28SBCC	0.02	Cyanobacteria	Oxyphotobacteria	Synechococcales	Cyanobiaceae	Synechococcus
0LBC	0.79	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
52SBCC	0.18	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
3SBCC	0.42	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
28SBCC	0.14	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
39SBCC	0.12	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
39LBC	0.09	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
6SBCC	0.17	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
52LBC	0.07	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
3LBC	0.57	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio

Supplementary Table 5.1 Percentage abundances from average bacterial profiles of all animals sampled over 52 weeks.

Chapter 6

General Discussion

6.1 Addressing aims and hypotheses

This body of work provides significant advances in lobster pathology research as well as the first comprehensive characterisation of the lobster bacterial gut microbiota using an extensive HTS approach.

In comparing the gut microbiota of sea-based and land-based animals, we hypothesised that SBCC might support the colonisation of a more diverse gut community which, based on patterns emerging in other animals, albeit mostly vertebrates, might benefit the health of the host. This hypothesis proved to be true. Sea-based container culture does indeed produce animals with a significantly more species-rich and species-diverse gut microbiota compared to those maintained within a hatchery system. Previous studies suggest that diet is a large determinant of community composition within the gut (Meziti, Mente and Kormas, 2012; Ringø *et al.*, 2016). This may well explain the change in diversity observed between the two culture environments. Individuals in SBCC have access to a wider source of nutrition compared to those in the hatchery. Although we were unable to determine what this diet consisted of, molecular and visual surveys of biofouling material indicate a wide range of potential prey species. Furthermore, the development of the 'crusher claw', which is also linked to dominance (van der Meeren, 2014), suggests that SBCC lobsters are actively predating, as opposed to solely relying on filter feeding. The crusher claw requires adequate substrate and nerve activity for its development and this physiology does not develop in the majority of hatchery-based animals (Wickins, 1986; Govind, 1992).

There is a greater degree of environmental filtering in terms of gut colonisation in SBCC animals compared to LBC. The gut microbiotas of individuals in SBCC

were significantly more phylogenetically clustered compared to those in the hatchery. Therefore, despite an increase in bacterial load in the gut, communities are being assembled more deterministically, potentially indicating that LBC does not support the assemblages of appropriate gut communities. The hatchery is a more controlled environment in terms of the availability of potential colonisers in the water column and the range of dietary substrates to support microbial growth within the gut (feed remained a constant throughout the one-year sample period). The integration of a more microbially rich environment, such as the use of a BioFloc system, and a more varied diet characteristic of life in the natural environment might encourage a more deterministic assembly that is more beneficial to the host.

The random assembly of the gut microbiota increases with age in the hatchery therefore initial LBC to reach juvenile stages for stock enhancement may not be detrimental to colonisation in early stages. In our aim to track the composition of the gut as the host ages, we hypothesised that the determinism of community assembly might shape differential abundances of bacteria. However, we did not observe significant changes in relation to ses.MNTD values or indeed alpha diversity trends. Although time did significantly cluster samples, the composition of the dominant taxa was relatively stable in the SBCC. In LBC, however, gut composition underwent a significant change in later stages and profiles became richer in *Carboxylicivirga* and *Arcobacter* species, which were not evident in sea-based samples. The *Candidatus* Hepatoplasma genus, however, was evident in all SBCC samples and, with suggestions of providing nutritional support in isopods (Fraune and Zimmer, 2008), may prove to be a beneficial inhabitant of the lobster gut and a possible target for probiotic development. Further characterisation of this association might benefit lobster production as other

studies have shown improvement in *H. gammarus* culture with the addition of pro- and prebiotics (Daniels *et al.*, 2010, 2013). Like many other aquatic invertebrate gut systems and previous insights into the European lobster gut (Daniels *et al.*, 2010), Proteobacteria dominated our communities. Proteobacteria are ubiquitous in the marine environment. The lack of adaptive immune system in invertebrates might mean that the gut microbiota is less tightly regulated and is more heavily influenced by the bacterial taxa residing in the water column. Zebrafish experimentally lacking adaptive immunity develop a more similar gut microbiota compared to wild-type individuals suggesting a potential role of the adaptive immunity is to serve as an ecological filter within the gut (Stagaman *et al.*, 2017).

An overarching aim of this project was to investigate pathogenesis in the European lobster and find targets to compare disease association with gut community changes. We were successful in discovering two novel lobster pathogens: the oomycete *Halioticidia noduliformans* and *Homarus gammarus* nudivirus (HgNV). Both of which might limit lobster production. *H. noduliformans* can cause mortality in larvae and eggs, affecting the fecundity of the brood stock and although we could not associate HgNV with mortalities, HgNV was linked to a reduction in growth. A similar phenomenon is observed in its closest relative infecting black tiger shrimp, PmNV (OIE, 2017). We did not observe *H. noduliformans* in SBCC or LBC lobsters earmarked for gut analysis and therefore this limited the gut comparison to HgNV. However, the oomycete-specific probing of environmental samples identified several potential pathogens of crustaceans/lobsters that should be monitored in future work; including species of the genus *Lagenidium*, several novel isolates belonging to the Saprolegniales and several sequences grouped within the *Haliphthoros/Halocrusticida* clade. In order to avoid age/culture environment-discriminating taxa affecting the HgNV

infected vs. uninfected comparisons, we also limited this comparison to one time point. We showed that the incidence of HgNV correlates to significant changes in the gut microbiome, in terms of gut diversity. However, as discussed, we could not discern between cause and effect and it should be noted that sample size of infected individuals was low (n=6). As we begin to work more with HgNV and explore its impact on the lobster, future studies should include greater numbers for more robust comparisons. The shrimp review demonstrates how the same pathogen can illicit inconsistent compositional changes in the gut across multiple studies. It would seem that pathogenesis in penaeid shrimp is not associated with specific changes in terms of taxa and their abundance, although more studies with repeated disease comparisons are needed. Instead, a more plausible idea is that pathogenesis results in dysbiosis which subsequently results in spurious alterations to the gut community and sometimes negative effects on the host. If this is the case, several key symptoms that often define diseases of aquatic organisms, such as lethargy and reduced growth, could be a result (at least in part) of the pathogen's impact on the host gut microbiome.

We could not link changes to the gut microbiota to explain differential growth rates. We hypothesised that these changes might alter an individual's ability to sequester nutrition from its diet. Sample size might again impact results, as size comparisons were made within individual groups. However, we did not observe an effect when comparing the entire dataset after age-discriminating taxa were removed. It should be noted that size data was not available for all samples. This begs the question, is the gut microbiome important for lobster health and optimal functioning? And if so, is this something that can be managed? Based on the range of gut profiles that exist within this dataset and within those spread throughout the literature, it would appear that there is no typical gut profile for a

single species. Gut communities are heavily influenced by environmental and host-related factors which result in a high degree of intraspecies variability within a single population. The idea of a ‘core microbiome’ depends on the presence of specific taxa in every individual but a ‘core functional microbiome’ offers leniency in its recruitment of commensals. This makes more sense if selection is governed by ecological fitness. Functionality is shared across multiple taxa. If a niche within the gut is fulfilled, it does not matter which species of microbe is occupying that niche. It is perhaps the stochasticity of gut bacteria assembly in early stage animals that demonstrates an individual’s reliance on environmental consequence. This variation in early stages, and the pioneer effects of particular taxa during those stages, may well account for individual variation later on in life; not just in taxonomic composition but pathogen susceptibility and differential growth rates. The fact that this community may just be a consequence of its environment is actually of benefit to aquaculture. A culture environment can be defined and modified. In doing so, one could use controlled environmental modifications to shape the gut microbiome to maximise its benefit to the host and maximise the cultivation of the host species.

In order to reflect the true variability of the microbiome, we did not pool individual samples prior to sequencing. Multiple individuals are often pooled in an attempt to minimise individual variation and observe general profiles and trends between groups. Pooling is also used to overcome issues in terms of obtaining enough DNA from small individuals. In our case, pooling would mean that we would have been unable to compare individual health states with individual gut profiles. We worked with very small animals during the early stages of the production cycle (average CL of 4.98 mm ±0.45) and did encounter cases where we failed to obtain adequate concentrations of DNA. We overcame this by dissecting larval animals

under a microscope, post-fixation, allowing for redundancies in terms of sample numbers and fine-tuning our DNA extraction protocol.

6.2 16S profiling and lack of eukaryotic analysis

Although this dataset has provided valuable insight into the gut microbiota of the European lobster, it is important to recognise its limitations.

Our work here is largely limited to bacterial domain and this mirrors the majority of the literature describing other microbial communities within the gut. The collective lack of eukaryotic information within these communities is a widespread issue and one that will ultimately hamper our understanding of host-microbe interactions. The gut undoubtedly contains eukaryotic organisms however, it is difficult to separate these eukaryotic communities from host-derived sequences. Amplifying universal target genes, such as the 18S SSU, within these communities also amplifies the same genes within the host organism. As the volume of host material is far greater than that of the microbiota, the number of host-derived sequences often dwarf that of any from its associated microbial community.

We tried to mitigate this issue using two different techniques. The first involved the design and implementation of blocking primers, wherein one of the primers is redesigned so that it extends into a hypervariable region of sequence that is specific to the host organism. The primer is modified to include a three-carbon (C3) spacer on the 3’ end of the sequence. This modification prevents the polymerase from extending the primer sequencing during amplification. Thus, only one strand of the target gene belonging to the host organism is produced. These single-stranded oligos can then be removed, leaving amplicons generated

from non-host organisms. We also tried designing pseudo-universal target genes that excluded metazoa. However, metazoan organisms can be microscopic in scale and anti-metazoan primers may be preventing the amplification of genuine inhabitants of the gut, as well as host-derived sequences.

In both cases, we failed to generate substantial non-host profiles that were not largely (or in some cases, completely) dominated by lobster sequences but experimental evidence suggests that host-exclusion was indeed successful. Gut-associated eukaryotes may just be rare in European lobster guts. There is evidence of eukaryote symbionts in the gut of shrimp (Dai *et al.*, 2017). Remarkably, this study did not require host blocking to produce substantial numbers of non-host derived sequences and did so using homogenised digestive tissue. In our lobsters, the gut associated eukaryotes that were successfully amplified belonged to the phyla Orchrophyta in SBCC animals and Dinoflagellata and Ciliophora in LBC groups, possibly indicating a contrast in feeding strategies. Despite the lack of information derived from our efforts, we hypothesise that eukaryotic organisms make up a substantial proportion of the lobsters' diet and the lack of eukaryotes retrieved from these samples might be explained by the proximity of time since feeding.

Metagenomic sequencing can overcome the targeted limitations of amplicon sequencing, however it is often subject to the same downfall in that much of the output is dominated by the host genome. In our metagenomic sequencing attempts, host sequences made up over 99 % of the total number of reads. Those that were not derived from host tissue represented bacteria but lacked the numbers required to make accurate assertions.

In lieu of shotgun metagenomics, amplicon sequencing is sometimes used to provide functional annotation of the gut community using a software package called PICRUSt (Langille *et al.*, 2013). PICRUSt is a useful tool in providing insight into the transcriptional capacity of the microbiome but is highly dependent on reference genomes and comes with several limitations. The package generally works by assessing sample-weighted nearest-sequenced taxon index (NSTI) values to compare ESVs with reference genomes. The functional annotations of the most closely related genome are then adopted by the query ASV. This approach works well on well characterised communities such as the human gut, where the genomes of its inhabitants are often already sequenced but will not work well on poorly characterised communities such as the aquatic invertebrate gut. And if the NSTI cut-off is insufficient, the functional profile of samples from these communities will be incorrect. For this reason, we did not apply PICRUSt analysis to our dataset.

Although metagenomes provide this functional information, they cannot indicate which genes are being expressed within the system. They merely describe the functional potential of the microbiome. Transcriptomic approaches, sequencing mRNA, are true indications of gene expression within a community. However, the generation of transcriptomes are expensive in that they require a large amount of sequencing effort, and starting material, which limits its application on small, individual samples. Ideally, a combination of sequencing approaches should be used to fully characterise the gut community: amplicon profiles to provide taxonomic descriptions, metagenomic profiles to assess functional capacity and the abundance of difficult to target organisms (such as viruses) and (meta)transcriptomics to map expression patterns.

6.3 The future for Lobster Grower and the lobster gut microbiome.

6.3.1 Detangling cause and effect

As discussed in Chapter 5, it is important to recognise that correlations between disease in the host and particular changes to the gut microbiome can be difficult to orientate. Does the incidence of a pathogen result in changes to the gut community, either through its impact on the host or direct competition with other microbes or, conversely, do changes to the gut microbiome allow the colonisation and subsequent infection of these pathogens? It is difficult to discern between the two and this was outside the scope of the current project. There are, however, ways in which we can separate correlation and causation. This involves the use and development of gnotobiotic organisms. Gnotobiotic organisms are those that contain a defined microbiota; this also includes those that are experimentally devoid of any microbes. In the case of the water flea (*Daphnia magna*) antibiotic treatment of eggs can produce germ-free larvae and supplementing the egg surface with a cocktail of microbial colonies can determine the composition of those that initially colonise the gut (Sison-mangus, Mushegian and Ebert, 2014). Owing to its destructive effects on the gut community, antibiotic treatment is a useful tool in understanding cause and effect. In the case of HgNV, would supplementing a germ-free gut with a more diverse bacterial community reduce an individual's susceptibility to a disease challenge? This is the next logical step for correlative gut comparisons like ours.

6.3.2 The application of long-read data

Short-read sequencing using 250 bp paired-end reads were long enough to cover the complete V4 hypervariable region of the SSU. These reads form a complete overlap, allowing generous trimming when removing low quality base calls.

However, there is a disparity in the power of independent hypervariable regions to classify taxa (Yang, Wang and Qian, 2016). The introduction of long-read sequencing platforms has encouraged the development of tools to compare the full length of the ribosomal SSU, incorporating information from all the hypervariable regions.

There are currently two main long-read sequencing platforms and approaches. NanoPore sequencing involves protein nanopores through an electrically resistant polymer membrane. A single strand of DNA is passed though the nanopore which disrupts the current passed through the membrane. Nucleotide combinations correspond to distinct changes in current as the read is sequenced (Jain *et al.*, 2016).

PacBio SMRT sequencing involves sequencing cells that contains zero-mode waveguides (ZMWs). Each one of these pores contains a DNA polymerase complex adhered to the bottom surface and this sequences a single DNA strand. During sequencing, phospholinked nucleotides are introduced which are labelled with a coloured fluorophore. Light is produced when one of these bases joins the polymerase complex reading the template DNA. To overcome long-read associated error reads, PacBio has developed circular consensus sequencing (CCS), whereby a consensus sequences is produced from re-sequencing circular DNA to generate a series of subreads (Rhoads and Au, 2015). As such, CCS could be useful in sequencing the full SSU sequence.

It is reported that the high error rate associated with long-read sequencing platforms requires the clustering of sequences to mitigate their inflation of the true biological diversity within the community (Schloss *et al.*, 2016; Calus, Ijaz and Pinto, 2018). However, combining PacBio circular consensus sequences (CSS),

which have a much lower error rate compared with Nanopore sequences, and the DADA2 pipeline provided more accurate depictions of the mock communities tested (Callahan *et al.*, 2019). Therefore, full-length 16S sequencing would have been useful in the determination of species and sub-species taxonomic resolution. With the development of new protocols for multiplexing long-read amplicons, we are currently investigating pooling samples for long-read sequencing in preparation for a publication comparing the two methodologies.

Long-read sequencing would have also been beneficial in sequencing the genome of HgNV. Many viruses, including HgNV, contain regions of repetitive sequence. When read length does not span these repetitive regions, assemblies often fail to generate a complete consensus sequence. This was true of HgNV in some instances however this was resolved by designing and sequencing specific amplicons that span these query regions.

6.3.3 Lobster farming contributing to aquatic protein production

Data from the lobster grower project clearly shows that sea-based containers can support a healthy population. Lobsters deployed at sea showed reduced mortality and increased growth rate in comparisons to hatchery controls. Furthermore, the health data and gut analysis from this thesis supports that ideology. Sea-based lobsters are less susceptible to pathogens like HgNV and have a more diverse gut community which could support a host during its culture. Improving productivity in crustacea aquaculture and developing a more sustainable method for farming will mean future crustacea aquaculture can play more of role in providing a healthy diet to a wider range of the global population.

Land-based aquaculture is receiving considerable attention. Re-circulating systems minimise environmental leaching in terms of escapees and effluent

(Martins *et al.*, 2010). However, these systems may require vast amounts of space and their conditions are often atypical of the natural environment, both in terms of the diet available to the stock and the chemical environment in which they are maintained, such as the accumulation of dissolved carbon dioxide (CO_2) (Tidwell, 2012).

One of the major benefits of this SBCC system is that it does not require any human-dependent feed input. Non-feed dependent species and systems are more sustainable (De Silva and Turchini, 2009). And although the time it takes for lobsters to reach commercially viable sizes may be longer in comparison to finfish, the absence of feed has massive implications on the carbon footprint of the production process. Crustacean fisheries account for a disproportionate amount of carbon emission (22 %) relative to their number of landings (Parker *et al.*, 2018). This is because, compared to finfish, there is a relatively low volume of catch per trip. Although many lobster fishermen will also fish for other crustacean species, the large vessels which make these trips can consume substantial amounts of fuel and subsequently emit large volumes of pollutants. The SBCC system developed for LG2 does not fall subject to these dependencies and could reduce carbon emissions associated with lobster fishing as lobsters require minor intervention after deployment.

6.3.4 The impact of climate change on aquaculture and pathogenesis

Increasing CO_2 levels in the atmosphere can lead to increasing sea temperatures, causing stratification of the water column; loss of sea ice and subsequent rise in sea levels; as well as ocean acidification through increased production of carbonic acid and free hydrogen ions. Ocean acidification translates to fewer carbonate ions available to marine calcifiers (Doney *et al.*, 2009). As

such, an increase in dissolved CO_2 can prevent proper shell formation in marine invertebrates (Doney *et al.*, 2009) and cause dissolution of the carapace (Chadwick *et al.*, 2019). Ocean acidification can also reduce chitin concentration in the exoskeleton of shrimp and cause internal acidosis (Mustafa, Kharudin and Yong Seok Kian, 2015) and reduce magnesium and calcium content of the carapace of *H. gammarus* (Arnold *et al.*, 2009). Furthermore, low alkalinity can reduce postmoult bicarbonate uptake and slow down calcification in juvenile *H. gammarus* (Middlemiss, Urbina and Wilson. 2015). In animals lacking an adaptive immune system, the physical barrier of the external carapace plays a huge role in supporting the overall health of the animal by preventing pathogenesis. Therefore, compromises to the integrity of this barrier or prolonged periods in the absence of this barrier, may allow pathogen entry and the establishment of infection in internal tissues and increased risk of mortality. A reduction in chitin concentration as result of ocean acidification may allow *H. noduliformans* to penetrate the exoskeleton of juvenile lobster more easily and increase its prevalence in wild populations and could even facilitate the infection of larger animals, which are currently resistant to carapace penetration. Acidosis and a reduction of chitin in the gut, may also alter its bacterial makeup and simplify HgNV's translocation of the gut barrier. Therefore, as the effects of climate change become more apparent, it is important to continue the monitoring of heath of aquatic animals and determine its impact on pathogenesis, an aspect of climate change currently receiving less attention.

Global warming and increasing sea water temperatures can increase the growth rate of pathogenic bacteria in the marine environment (Barange *et* al., 2018). Moreover, increasing temperatures are facilitating genetic mutations and greater gene transfer within bacterial populations as well as disease outbreaks in

previously unaffected areas (Barange *et al.*, 2018). Aquatic species often live within a strict temperature range. Increases in global sea temperature correspond to polar migration of parasites and pathogens. There are concerns, for example, with the unprecedented rate at which sea surface temperatures in the Gulf of Maine are increasing (warming three times faster than the global average), ESD will continue to spread Northward and affect the Maine American lobster fishery, which is a huge source of economic gain for the region (Maynard *et al.*, 2016). The temperature-dependent migration of pathogens may result in an increase in the number of susceptible hosts associated with a particular pathogen. Something that receives almost no attention, however, is the effect of warming sea temperatures on the gut microbiome. Microorganisms also operate within a sometimes-strict temperature range. Will these microbes adapt to future increases in temperature or will the catalogue of potential colonisers in the water column change and result in changes to the characteristic gut profile of a given species? Although near-future climate-induced increases in seawater temperature did not significantly affect community structure within the tissues of sea urchin (*Lytechinus variegatus)*, it did affect microbial community function resulting in changes in key metabolic categories (Brothers *et al.*, 2018). Therefore, even if climate change does not change the availability and composition of microbes associated with the gut, it may well impact how these microbes work in tandem with their host.

6.4 Manipulation of the gut to maximise cultivation success

To mitigate the increasing problem of bacterial disease outbreaks in aquaculture, antibiotic application has been commonplace and extensive; this includes

prophylactic applications which are particularly common in shrimp farming. Furthermore, commercial feed is often enriched with antibiotics. The extensive and prophylactic use of antibiotics in aquaculture has resulted in an increase in antibiotic resistant bacteria in the environment as well as in increase in antibiotic resistant pathogens (Cabello, 2006). Ignoring direct implications on future aquatic disease management, increased incidents of resistant microbes have significant implications on human healthcare. Seventy-six percent of antibiotics commonly used in aquaculture and agriculture are also important drugs in human medicine (Done, Venkatesan and Halden, 2015) therefore encouraging resistance through exposure to nontherapeutic doses in aquaculture (Sapkota *et al.*, 2007) may ultimately be dangerous for human healthcare as well.

Antibiotic application can also drastically change the bacterial landscape of the gut by reducing microbial diversity and increasing the proliferation of opportunistic pathogens (Dudek-Wicher, Junka and Bartoszewicz, 2018). The use of antibiotics may ultimately detriment the host by removing beneficial gut bacteria and their positive effect on the host animal. If HgNV is indeed associated with reduced bacterial diversity in the gut, routine application of antibiotics in a lobster culture system will result in an increase in the number of viral infections.

The characterisation of host-microbe interactions within the gut and better understanding of the beneficial effects of these microbes can support the development and application of microbial supplements, which could potentially substitute antibiotics in the future. Gut supplementation has previously proven successful in trials using European lobster (Daniels *et al.*, 2010, 2013). Larval lobsters fed a combination of probiotic *Bacillus* spp. and prebiotic mannan oligosaccharides (MOS) had significantly improved growth parameters such as weight gain and carapace length, perhaps partly explained by an increase in

microvilli length compared to the control group. Individuals fed the synbiotic preparation (synbiotics refer to the combination of probiotics and prebiotics) also showed significant improvements to post-larval condition and survival after 30 days (Daniels *et al.*, 2013). The DGGE analysis of the gut microbiota showed that bacterial diversity was similar in all groups however synbiotic application correlated to significantly reduced levels of *Vibrio* sequences. Although *Bacillus* spp. were shown to have positive effects on several growth and survival parameters, we did not observe substantial proportions of this genus in any group profile within our HTS dataset. Developing probiotic candidates from those isolated from the lobster gut itself may prove to be more beneficial in terms of its maintenance within the gut and its effect on the host and this should be explored further.

Prior identification of the gut community is vital in order to better understand the effects of gut supplementation and better predict the success of its application in a particular organism. Commercial preparations are often misidentified in terms of the taxa they contain (Huys *et al.*, 2006) and blind application of non-specific microbes may not confer significant benefit to the host. There is no guarantee that foreign bacteria can survive passage to the gut or maintain a quorum if it does survive. Therefore, characterisation of the gut microbiota beforehand can highlight potential native supplements, eliminating the uncertainty of both of these factors.

6.5 Closing remarks

The work presented in this thesis offers a substantial contribution to invertebrate pathology and lobster biology and management. The identification and

characterisation of lobster pathogens like *Halioticida noduliformans* and *Homarus gammarus* nudivirus (HgNV) is vital if SBCC and other culture systems are going to support the sustainable aquaculture of the species. The reference histology and diagnostic tools developed during this project should aid in assessing the potential impacts of the fishery. Furthermore, both bodies of work will hopefully encourage further study and the better understanding of the effects of these pathogens on the host lobster. The gut profiles and bacterial taxa highlighted during this project demonstrate how the way in which we culture aquatic species can dramatically impact the collection of microbes that colonise its gut, which might subsequently determine cultivation success. These data serve as important tools for the development of gut supplementation to improve the health of the host during its culture and offer a much-needed alternative to the use of broad-spectrum antibiotics in the maintenance of a healthy population.

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