Environmental influences on the epidemiology of fibropapillomatosis in green turtles (*Chelonia mydas*) and consequences for management of inshore areas of the Great Barrier Reef

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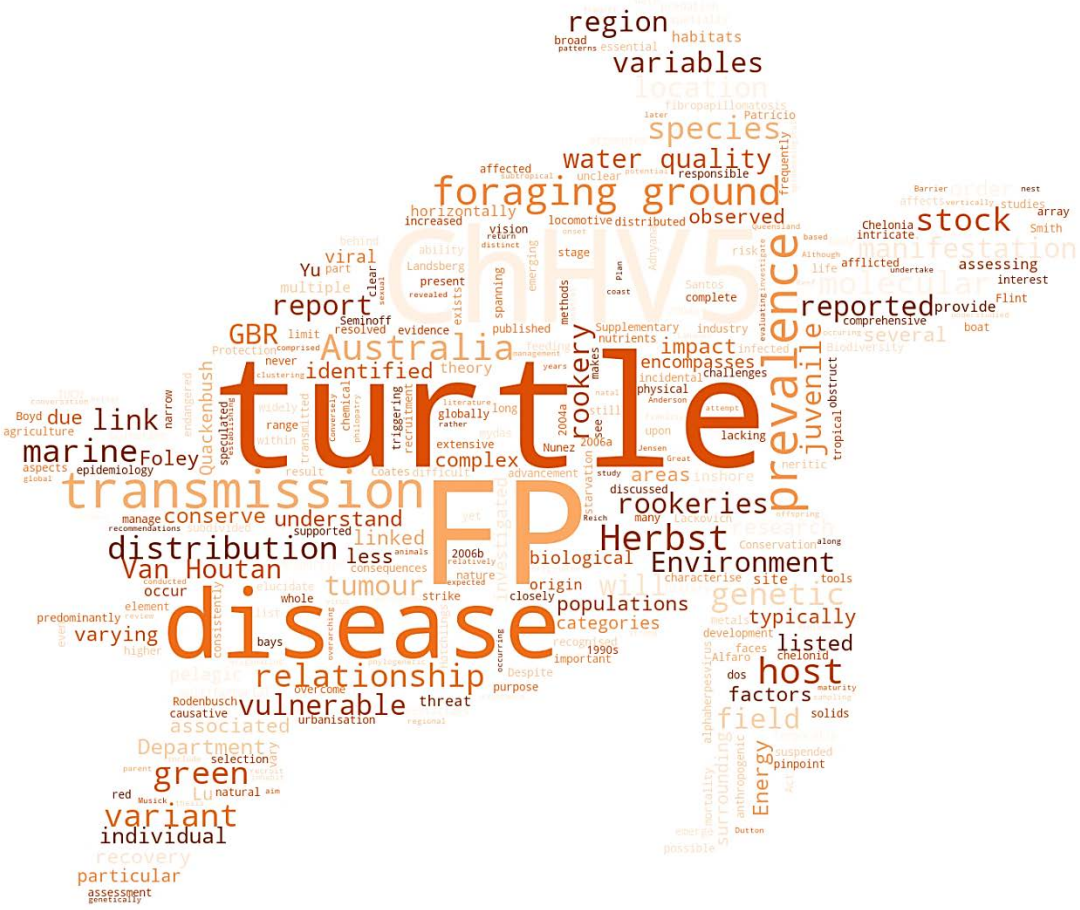

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Declaration of Ethics

The research presented and reported in this thesis was conducted with the approval of the James Cook University Research Ethics Committee and in accordance with the National Statement on Ethical Conduct in Human Research, 2007; Australian Code for the Care and Use of Animals for Scientific Purposes, 2007; and the Queensland Animal Care and Protection Act, 2001.
The proposed research methodology received clearance from James Cook University Experimentation Ethics Committee (A1501 and A1971). This research was conducted under permits granted by the Department of Environment and Science (WISP06619309 and WISP13754613) and the Great Barrier Reef Marine Park Authority (G10/33220.1 and G36593.1).

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Statement of Contribution of Others

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List of Abbreviations

BLAST	Basic Local Alignment Search Tool
bp	Base pair
CB	Cockle Bay
CCL	Curved Carapace Length
CFPHV	Chelonid fibropapilloma-associated herpesvirus
ChHV1	chelonid alphaherpesvirus 1
ChHV2	chelonid alphaherpesvirus 2
ChHV3	chelonid alphaherpesvirus 3
ChHV4	chelonid alphaherpesvirus 4
ChHV5	chelonid alphaherpesvirus 5
ChHV6	chelonid alphaherpesvirus 6
CmPV1	Chelonia mydas papillomavirus
CS	Coral Sea
DES	Department of Environment and Science
DIN	Dissolved inorganic nitrogen
D-loop	mtDNA d-loop control region
DNApol	DNA polymerase
FP	Fibropapillomatosis
FPHV	fibropapillomatosis-associated herpesvirus
FPTHV	fibropapilloma-associated turtle herpesvirus
F-Sial	Sialyltransferase
gB	glycoprotein B
GBR	Great Barrier Reef
GBRMPA	Great Barrier Reef Marine Park Authority
GBRWHA	Great Barrier Reef World Heritage Area
GI	Green Island
ICTV	International Committee on Taxonomy of Viruses
IUCN	The International Union for Conservation of Nature
JCU	James Cook University
LI	Low Isles
MSA	Mixed stock analysis
mtDNA	mitochondrial DNA
NC	New Caledonia
NCBI	National Center for Biotechnology Information
nGBR	northern Great Barrier Reef
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
sGBR	southern Great Barrier Reef
TEM	Transmission Electron Microscopy
TSS	Total suspended solids
WQI	Water quality index

Summary

The epidemiology of fibropapillomatosis (FP) in marine turtles is a global conservation concern and, until now, there has been a paucity of data surrounding this disease in Australia. An understanding of FP is critical to the effective management of all marine turtles, but particularly the endangered green turtle (*Chelonia mydas*) as this species is predominantly affected by FP. Green turtles have a complex life-history which includes a high degree of site fidelity; once recruited into a foraging area turtles will typically remain there, despite damage or destruction to their habitat. It is this trait, coupled with their long-lived nature, that has led them to be recognised as sentinels of marine ecosystem health; this species is particularly susceptible to the detrimental effects of environmental change. With high prevalence of FP consistently linked to regions associated with reduced water quality, the relationship between this disease and the environment is of increasing interest. A herpesvirus (chelonid alphaherpesvirus 5; ChHV5) has been identified as the likely aetiological agent of this disease. The distribution of variants of this virus appear to differ by region, indicating a site-specific element to viral transmission. These elements of the disease highlight the need for investigation of FP, ChHV5 and the environmental factors which may be facilitating disease manifestation in all regions where this disease has been reported.

The overarching aim of this thesis was to address the information gap for FP in green turtles in Australian waters and to provide recommendations for management of inshore areas of the GBR. This thesis clarified the spatial distribution and prevalence of FP along the Queensland coast, which encompasses the Great Barrier Reef (GBR). A potential relationship between FP prevalence and water quality on the GBR was also evaluated. To test for horizontal or vertical transmission of chelonid alphaherpesvirus 5 (ChHV5), a relationship between host genetic stock and viral variant was also investigated. Both inshore and offshore sites were studied throughout the thesis, yet the inshore study sites are under a greater influence of water that originates from the adjacent catchment; flood plumes rarely extend further than mid-shelf reefs and non-flood river outflow almost exclusively affects inshore areas. As such, the focus of this thesis is on the inshore areas of the GBR.

This thesis provides the first comprehensive report of FP prevalence in Australia. The spatial distribution and prevalence of FP on the GBR was characterised using 25,645 records from 15 sites along the Queensland coast. A total of 791 turtles with FP tumours were recorded in this dataset. The results of this study show that FP prevalence varies between sites and years, with juvenile turtles being the most frequently affected by FP. Survey method had a significant influence on the apparent FP prevalence value at each site; surveys which explicitly targeted FP detected higher

numbers of individual turtles with FP and therefore generated higher prevalence rates than general population surveys. This study highlighted shortcomings in both methods with respect to FP detection, and this must be considered when interpreting results and developing future marine turtle surveys.

A relationship between a subset of this FP prevalence data and water quality at these sites was assessed using water quality indices (WQIs). Sub-indexes for dissolved inorganic nitrogen (DIN), total suspended solids (TSS), pesticides and metals were developed for each study site using published data from a range of sources and enhanced using expert opinion. These WQI scores were also aggregated without weights to create an overall water quality index for each study site. A total of 18,380 records of individual capture records of green turtles, including 264 records of FP across 14 sites along the GBR, were used in conjunction with the WQIs. Despite the analysis of this expansive dataset, a significant relationship between FP prevalence and WQI rankings at each site could not be quantified or established. This investigation is the first attempt at creating WQIs based on data from published reports and peer-reviewed publications to compare with FP prevalence data along the Queensland coast. Unfortunately, due to the different methods used to capture and record data this information could not be used as 'fit-for-purpose' and it proved impossible to bridge between differing methods. However, this result does have significant implications for management as it highlights the importance of designing water quality monitoring programs and data capture in the GBR so these can be used across multiple disciplines in a more integrated way.

It has long been postulated that turtles first encounter the infectious agent of FP through horizontal transmission at their foraging grounds, and reports of the site-specific distribution of ChHV5 variants appear to support this theory. However, this theory has never been assessed by studying the genetic origin of the host turtle. Turtles frequenting a given foraging site usually represent genetic stock from multiple rookeries, with genetic stocks reflecting the region of origin. For example, turtles originating from rookeries in the southern GBR are genetically similar, yet genetically distinct from those originating from rookeries in the northern GBR. If ChHV5 transmission was occurring vertically from parent to offspring, then phylogenetic clustering of ChHV5 would be expected to be based on host genetic stock, rather than on sampling location. Conversely, if ChHV5 transmission is occurring horizontally at foraging grounds, a link between viral variant and host origin would be less likely.

To facilitate an investigation of host genetic origin and the ChHV5 variant each turtle was infected with, we first developed an assay which targets a longer fragment of mitochondrial DNA than used in previous studies. This assay was validated through a mixed stock analysis (MSA) of 278 turtles across three foraging grounds spanning more than 330 km: Cockle Bay, Green Island and Low Isles. The

MSA utilised the mtDNA sequences generated in this study to estimate the relative proportion of genetically-distinct breeding populations found at each foraging ground. Haplotype and nucleotide diversity was also assessed. A total of 35 haplotypes were identified across all sites, 13 of which had not been found previously in any rookery. The results showed that the northern GBR (nGBR), Coral Sea (CS), southern GBR (sGBR) and New Caledonia (NC) stocks supplied the bulk of the turtles at all three sites, with small contributions from other rookeries in the region. Stock contribution shifted gradually from north to south, although sGBR/CS stock dominated at all three sites. The major change in composition occurred between Cockle Bay and Low Isles. Our findings, together with other recent studies in this field, show that stock composition shifts with latitude as a natural progression along a coastal gradient. This phenomenon is likely to be the result of ocean currents influencing both post-hatchling dispersal and subsequent juvenile recruitment to diverse coastal foraging sites.

In addition to serving as a method validation, the results of the MSA improved our knowledge of the spatial ecology of green turtles on the GBR, which is fundamental to their effective conservation. The findings from this study were then combined with those of previous studies to provide a tool to estimate the main relative stock contributions at as yet unsampled foraging grounds. Such a tool may allow managers to target their efforts more effectively.

Following the development of the mtDNA assay for identification of host genetic origin, a relationship between host genetic origin and ChHV5 variant was investigated. This thesis presents improved molecular assays developed for detection of ChHV5, in combination with a robust molecular and phylogenetic analysis of ChHV5 variants. This approach utilised a multi-gene assay to detect ChHV5 in all FP tumors sampled from 62 marine turtles found at six foraging grounds along the Great Barrier Reef. Six distinct variants of ChHV5 were identified and the distribution of these variants was associated with host foraging ground. However, no association between host genetic origin and ChHV5 viral variant was found. These findings support the hypothesis that marine turtles undergo horizontal transmission of ChHV5 at foraging grounds and are unlikely to be contracting the disease at rookeries, either during mating or vertically from parent to offspring. As a consequence, management of this disease should be focused on green turtle foraging grounds.

Publications and Presentations throughout doctoral studies

Publications

Jones, K., Ariel, E., Burgess, G. and Read, M. 2016. A review of fibropapillomatosis in Green turtles (*Chelonia mydas*). *The Veterinary Journal.* Volume 212, p. 48–57

Jones K, Jensen M, Burgess G, Ariel E. 2018. Closing the gap: Mixed stock analysis of three foraging populations of green turtles (*Chelonia mydas*) on the Great Barrier Reef. PeerJ. 6(e5651). doi: https://doi.org/10.7717/peerj.5651

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Cárdenas DM, Cucalón RV, Medina-Magües LG, **Jones K,** Alfaro-Núñez A, Alemán RA, Cárdenas WB (2019) Fibropapillomatosis in the South-East Pacific region: first case report in Ecuador and its inclusion in a global phylogenetic analysis. Journal of Wildlife Diseases. Volume 55, Issue 1, pp00 - 000

Presentations

Conference Presentations

Jones, K. 2017. Closing the gap: Mixed stock analysis of three foraging populations of green turtles (*Chelonia mydas*) on the Great Barrier Reef. *Proceedings from the 4th Annual Sea Turtle Health and Rehabilitation Workshop*. 5-7th September, Townsville, Australia.

Jones, K. 2017. Sea Turtles 101: Managing turtles in the clinic and the field. *Australian Veterinary Students Conference*. 22nd - 28th January 2017, Townsville, Australia.

Jones, K. 2015. Fibropapillomatosis on the Great Barrier Reef: Directions of future research. *Proceedings from the international summit on fibropapillomatosis of marine turtles: Global status, trends and population impacts.* 11-14th June 2015, Honolulu, Hawaii.

Limpus, C., **Jones, K.** and Chaloupka, M. 2015. Fibropapilloma disease in marine turtles: Eastern Indian Ocean – south western Pacific Ocean. *Proceedings from the international summit on fibropapillomatosis of marine turtles: Global status, trends and population impacts*. 11-14th June 2015, Honolulu, Hawaii.

Conference Posters

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Mashkour, N., Burgess, G., **Jones, K.,** Elliman, J., Owens, L., and Ariel, E. 2017. Green sea turtle (*Chelonia mydas*) papillomavirus in the northern Great Barrier Reef, Australia. *Proceedings from the 10th International Symposium on Viruses of Lower Vertebrates*, 4 -7 June 2017, Budapest, Hungary

Table of Contents

Statement of Access Declaration ii
Statement of Sources Declaration ii
Electronic Copy Declaration iii
Declaration of Ethics iii
Acknowledgements iv
Statement of Contribution of Others vii
List of Abbreviations viii
Summary ix
Publications and Presentations throughout doctoral studies xii
Publications xii
Presentations xii
Conference Presentations xii
Conference Posters xiii
List of Figures xvii
List of Tables xx
Chapter One: General Introduction 1
Chapter Two: A review of fibropapillomatosis in green turtles (*Chelonia mydas*) 4
Background and aims of this chapter 4
Introduction 5
Disease presentation 6
Epidemiology of fibropapillomatosis in marine turtles 8
Disease prevalence and impact 8
Aetiology of fibropapillomatosis in marine turtles 10
Infectious nature of fibropapillomatosis 10
Chelonid alphaherpesvirus 5 12
Variants of chelonid alphaherpesvirus 5 14
Environmental factors 16
Direction of future research 23
The aims of this chapter were addressed as follows: 25
Publications and presentations arising from this study 28
My contributions to this study: 28

Chapter Three: Spatial distribution of fibropapillomatosis in marine turtles on the Great Barrier Reef 29
Backgrounds and aims of this chapter 29
Introduction 30
Materials and Methods 32
Study Sites 32
Data collection 35
Results 37
Grouped data 37
Age-class data subset 41
Discussion 43
The aims of this chapter were addressed as follows: 47
Publications and presentations arising from this study 47
My contributions to this study 47
Chapter Four: Investigating the relationship between water quality and prevalence of fibropapillomatosis in green turtles (*Chelonia mydas*) on the Great Barrier Reef 49
Backgrounds and aims of this chapter 49
Introduction 50
Materials and Methods 53
FP Prevalence Data and Study Site Selection 53
Development of Water Quality Indices (WQI) 57
Statistical analysis 69
Results 70
Grouped dataset 70
Individual data subset 74
Discussion 75
The aims of this chapter were addressed as follows: 82
Publications and presentations arising from this study 82
My contributions to this study 83
Chapter Five: Closing the gap: Mixed stock analysis of three foraging populations of green turtles (*Chelonia mydas*) on the Great Barrier Reef 84
Background and aims of this chapter 84
Introduction 86
Materials and Methods 89
Study Sites 89

Sample Collection 90
DNA extraction and Polymerase Chain Reaction (PCR) 90
Characterisation of mtDNA haplotypes and mixed stock analysis (MSA) 91
Results 92
Mixed stock analysis 94
Discussion 98
The aims of this chapter were addressed as follows: 102
Publications and presentations arising from this study 103
My contributions to this study: 103
Chapter Six: Molecular evidence of horizontal transmission of chelonid alphaherpesvirus 5 at green turtle (*Chelonia mydas*) foraging grounds in Queensland, Australia 104
Background and aims of this chapter 104
Introduction 105
Materials and Methods 107
Sample origin 107
Sample Collection 109
DNA extraction, Primer Design and Polymerase Chain Reaction (PCR) 109
Phylogenetic analysis 112
Results 113
Phylogenetic analysis 115
Discussion 124
The aims of this chapter were addressed as follows: 130
Publications and presentations arising from this study 131
My contributions to this study 131
Chapter Seven: General Discussion 132
References 138
Appendices: List of Supplementary Figures 160
Appendices: List of Supplementary Tables 161
Appendix One: Supplementary Files from Chapter Two 162
Appendix Two: Supplementary Files from Chapter Three 169
Appendix Three: Supplementary Files from Chapter Four 172
Appendix Four: Supplementary Files from Chapter Six 182
Appendix Five: Publications arising during candidature 186

List of Figures

Figure 2.1. The complex life history of green turtles. Adapted from (Lanyon et al., 1989). 5
Figure 2.2. The plastron and hind flippers of a green turtle severely affected by fibropapillomatosis, highlighting the diverse range of tumour appearance. Image provided by A/Prof Ellen Ariel. 7
Figure 2.3. A Minimum Evolution phylogenetic tree of *Alphaherpesvirinae* based on full length DNA polymerase sequence retrieved from GenBank (Accession numbers provided in tree). Bootstrap values for each node are provided (1000 replicates). The analysis involved 27 nucleotide sequences resulting in a total of 2593 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013)..... 14
Figure 3.1.Marine turtle capture sites along the Queensland coastline, with more detailed site maps in inset. Sites include: Warul Kawa (A); Clack Reef, The Howick Group, Cape Flattery (B); Ollera, Toolakea, Cockle Bay and Cleveland Bay (C); Upstart Bay and Edgecumbe Bay (D); Shoalwater Bay (E); Heron Island (F); Gladstone (G); Sandy Strait (H); and Moreton Bay (I). The Great Barrier Reef World Heritage Area is indicated by the hatched areas. 33
Figure 3.2. Retrospective prevalence rates of fibropapillomatosis in marine turtles at foraging grounds along the Queensland coastline. The difference between FP prevalence recorded by general population surveys (blue) and turtle health surveys (orange) is also in indicated. 39
Figure 3.3. The locations of two distinct “hotspots” of fibropapillomatosis: Cockle Bay (A) and Brisk Bay, within Edgecumbe Bay (B). The hotspots, indicated as red circles, were found to contain a high number of green turtles with fibropapillomatosis, while nearby areas hosted few, if any, turtles with FP..... 40
Figure 3.4. Annual age class distribution of fibropapillomatosis between turtles at western Shoalwater Bay (A), Heron Island (B) and Moreton Bay (C). Data collected during general population surveys between 1987 and 2014, with juvenile (blue), sub-adult (orange), adult (grey) and all turtles (yellow) are represented. 42
Figure 4.1. Proposed boundaries of the Great Barrier. The area inside the red line is the boundary of the GBRWHA while the entire area shaded yellow is the proposed Greater GBR management area, including the GBR catchment area, the GBRWHA, Torres Strait and Hervey Bay. Map prepared by J. Waterhouse, TropWATER. Data for the GBR provided by the Great Barrier Reef Marine Park Authority (Brodie and Pearson, 2016) 54
Figure 4.2. Connectivity map for green turtles. The genetic break reflects the approximate boundary where turtles residing north and south are more likely to be part of the northern and southern GBR breeding stocks respectively. A third genetic stock breeds on Coral Sea islands and disperses into the GBR (Johnson et al., 2018) 55
Figure 4.3. Marine turtle capture sites along the Queensland coastline, with more detailed site maps in inset. Sites include: Warul Kawa (A); Clack Reef, The Howick Group, Cape Flattery (B); Ollera, Toolakea, Cockle Bay and Cleveland Bay (C); Upstart Bay and Edgecumbe Bay (D); Shoalwater Bay (E); Heron Island (F); Gladstone (G); Sandy Strait (H). The Great Barrier Reef World Heritage Area is indicated by the hatched areas..... 56
Figure 4.4. General structure of an index (Sutadian et al., 2016)..... 57
Figure 4.5. An overview of the water quality index structure in the present study..... 58
Figure 4.6. A jittered plot of dissolved inorganic nitrogen (DIN) (A), total suspended solids (TSS) (B), pesticides (C) and metals (D) sub-index scores against fibropapillomatosis (FP) prevalence. In each plot, turtle health survey results are shown in black and general population surveys in red..... 71

Figure 4.7. A jittered plot of the overall water quality index (WQI) score scores against fibropapillomatosis (FP) prevalence. Turtle health survey results are shown in black and general population surveys in red. 72
Figure 5.1. Green turtle foraging sites at Low Isles, Green Island and Cockle Bay were sampled for genetic analysis in the present study. These three sites filled a large geographic gap that existed in prior sampling by Jensen et al. (2016). Broken-line ellipses indicate breeding areas of the following source populations: northern GBR (nGBR), Coral Sea comprised of Coringa-Herald group (CS(a)) and Chesterfield group (CS(b)), southern Great Barrier Reef (sGBR), and New Caledonia (NC). Blue lines provide a simplified representation of ocean currents in the region of interest: NQC = North Queensland Current, EAC = East Australian Current, SEC = South Equatorial Current. 89
Figure 5.2. For green turtle aggregations at selected foraging grounds in the Great Barrier Reef (GBR) and southern Queensland, Australia, the proportional contributions of three important genetic sources showed a notable relationship with latitude. The southern GBR (sGBR) and Coral Sea (CS) stocks were combined for this figure to allow comparison with Jensen et al. (2016) and are denoted in orange. The nGBR stock (northern GBR) is represented in blue and 'Other' stocks, represented in black; hatched areas in all three cases represented 95% confidence intervals. The 'Other' group comprises the remaining 22 stocks in this region (see Jensen et al. (2016)) and were combined because these stocks were found to contribute a small proportion of the turtles at each study site. Data for Low Isles, Green Island and Edgecumbe Bay from the present study and data for all other sites from Jensen et al. (2016). 97
Figure 6.1. Samples (n=62) were collected from six locations along the Queensland coast of Australia; Brisbane (n=7), Gladstone (n=4), Airlie Beach (n=1), Bowen (n=27), Townsville (n=22), and Cairns (n=1). Five of these sites are located within the Great Barrier Reef (GBR) Marine Park, whilst Brisbane is located just south of the GBR boundary (indicated by hatched area). 108
Figure 6.2. Phylogenetic tree using the Maximum Likelihood method generated from the aligned 2565bp ChHV5 glycoprotein B (gB) gene. The analysis involved 79 nucleotide sequences. Bootstrap values are indicated as a number on each branch and were calculated from 1000 replications. Individual samples are identified with source location, haplotype, scientific name, tag number, and sample collection year. Sequences retrieved from the GenBank database originating from the Pacific and Altantic are named in the same way, with the accession number in the place of the tag number, and no haplotype information included as it was unknown. 117
Figure 6.3. Condensed phylogenetic tree showing the positions of the distinct Australian Variants relative to published sequences. This tree was constructed using the Maximum Likelihood method generated from the aligned 2565bp ChHV5 glycoprotein B (gB) gene. The analysis involved 29 nucleotide sequences. Bootstrap values are indicated as a number on each branch and were calculated from 1000 replications. Sequences retrieved from the GenBank database are indicated with source location, scientific name, accession number, and sample collection year. Host haplotype was used to determine the origin composition of each Australian variant in this study, expressed here as a proportion with colour reflecting host origin region; Orange = Coral Sea (CS)/southern Great Barrier Reef (sGBR)/New Caledonia (NC), Pink = NC, Red = Unknown, Purple = north-east Borneo/Sulu Sea, Green= northern Great Barrier Reef (nGBR)/NC and Yellow = nGBR. 120
Figure 6.4. Phylogenetic tree using the Maximum Likelihood method generated from the aligned 963bp ChHV5 Sialyltransferase (F-sial) gene. The analysis involved 60 nucleotide sequences. Bootstrap values are indicated as a number on each branch and were calculated from 1000

replications. Sequences retrieved from the GenBank database are indicated with the accession number, the source turtle's scientific name, and sample collection location and year. 122

List of Tables

Table 3.1. Human influence on the 15 marine turtle capture sites along the Queensland coast. Sites include: Warul Kawa, Clack Reef, Howick Group, Cape Flattery, Ollera, Toolakea, southern Cleveland Bay, Cockle Bay, Upstart Bay, Edgecumbe Bay, western Shoalwater Bay, Gladstone, Heron Island, Sandy Strait, and Moreton Bay. 34
Table 3.2. The distribution of capture records used for analysis, including study site, survey type, species and number of turtles captured, capture type and time period that the records span. Sites are listed in approximately north to south order, and are divided green (*Chelonia mydas*), hawksbill (*Eretmochelys imbricata*) and loggerheard (*Caretta caretta*) turtle records. 36
Table 3.3. Prevalence rates of fibropapillomatosis in marine turtles at foraging grounds along the Queensland coastline. Values greater than zero are highlighted in bold. 38
Table 4.1. The study sites used in the present study, the main river influences acting on these sites and other considerations about these sites including land-use in the region. All distances are approximate and are reported to the nearest 10 km. 59
Table 4.2. Sub-index scores for DIN exposure at the 14 sites examined in this study. Sites are listed in approximately north to south order. 63
Table 4.3. Sub-index scores for TSS exposure at the 14 sites examined in this study. Sites are listed in approximately north to south order. 65
Table 4.4. Sub-index scores for pesticide exposure at the 14 sites examined in this study. Sites are listed in approximately north to south order. 65
Table 4.5. Sub-index scores for metal exposure at the 14 sites examined in this study. Metals for each site were ranked on a global and local (Great Barrier Reef only) and ultimately on a scale which considered both of these results in addition to expert opinion. Sites are listed in approximately north to south order. 68
Table 4.6. FP prevalence and, sub-index scores for DIN, TSS, pesticide and metal exposure at the 14 sites examined in this study. The FP prevalence values have been separated by survey method, and values greater than zero are highlighted in bold. An aggregated score which reflected the overall water quality at each site (by considering the four parameters) is also shown. Sites are listed in approximately north to south order. 69
Table 5.1. Green turtle demographics from three sampled Great Barrier Reef foraging grounds. The total number of turtles sampled per site (n), and number of juvenile (J), sub-adult (SA) and adult (A) turtles within each site are shown. Curved-carapace length (CCL) mean and range are also provided. 90
Table 5.2. Haplotype frequencies of green turtles sampled at Cockle Bay (CB), Green Island (GI) and Low Isles (LI) along the Great Barrier Reef, Australia. 93
Table 5.3. Sample size (n), number of haplotypes (H) and estimates (± SD) of haplotype (h) and nucleotide (π) diversity for 3 *Chelonia mydas* foraging sites on the Great Barrier Reef, Australia. 94
Table 5.4. Results (mean % ± 95% confidence intervals in parentheses) from the Bayesian mixed stock analysis (MSA) (Pella and Masuda, 2001) for Cockle Bay, Green Island and Low Isles Green Turtles (both individually and by region). MSA was calculated using 25 regional breeding stocks as possible sources, but for simplicity only the 4 main contributors are listed—nGBR: northern Great Barrier Reef; sGBR: southern Great Barrier Reef CS: Coral Sea and NC: New Caledonia. The combined contributions of the remaining 21 stocks are compiled into the 'Other' category. Model 1 = uniform priors; Model 2 = weighted priors 95

Table 6.1. Primer sequences used to target ChHV5 genes of interest (glycoprotein B; gB, sialyltransferase; F-sial and DNA polymerase; DNApol) and a green turtle (*C. mydas*) mtDNA gene (D-loop). F = forward, R = reverse. 110
Table 6.2. PCR thermocycling protocols for the newly described primers used in this study. 111
Table 6.3. Summary of haplotype distribution in green turtles in the present study, including the regions of origin and capture location. 114
Table 6.4. Number of positive detections of three ChHV5 target genes in FP tumour samples using Polymerase Chain Reaction (PCR). 115
Table 6.5. Distribution of chelonid alphaherpesvirus 5 (ChHV5) variants among marine turtles with fibropapillomatosis from six inshore areas in Queensland, Australia. 118
Table 6.6. Summary of variants observed in this study, including number of turtles infected with a particular chelonid alphaherpesvirus 5 (ChHV5) variant (n) and the defining characteristics of these variants. All differences and identity percentages are calculated relative to the full-length glycoprotein B reference sequence available from Hawaii (HQ878327). 119
Table 6.7. Nucleotide sequence analysis of sequences obtained from FP tumour samples collected from marine turtles (n). All differences and identity percentages are calculated relative to the full-length reference sequence available from Hawaii (HQ878327). 124

Chapter One:
General Introduction

The green turtle (*Chelonia mydas*) is recognised as endangered under the IUCN red list assessment (Seminoff, 2004). In Australia, this species is listed as vulnerable under the *Environment Protection and Biodiversity Conservation Act 1999* (Department of the Environment and Energy, 2017). In order to manage and conserve this vulnerable species, it is essential to understand all the threats it faces. Green turtles can be afflicted by the disease fibropapillomatosis (FP); a globally distributed disease which has been reported in all species of marine turtles, but which predominantly affects the green turtle (Jones et al., 2016). Despite being identified in 1938 (Smith and Coates, 1938) and being the subject of a number of studies from the 1990s to present, many aspects of the epidemiology of this disease are still unclear.

The impact of FP on individual turtles and at the population level have been widely discussed (Chaloupka et al., 2009; Chaloupka et al., 2008b; Ene et al., 2005; Flint et al., 2010b; Foley et al., 2005; Herbst, 1994). The tumours which characterise the disease may limit or obstruct the vision, feeding and locomotive ability of affected turtles (Jones et al., 2016) and as a result, these turtles are at increased risk of predation, starvation and boat-strike. The consequences of tumours on infected individuals can vary, with both mortality (Chaloupka et al., 2008b) and complete recovery (Machado Guimarães et al., 2013) being reported. The impact of FP on green turtle populations is less clear, with reports on prevalence varying both spatially and temporally (Jones et al., 2016). In Australia, incidental reports on FP prevalence have been published (see Appendix: Supplementary Table 1), but a comprehensive report on disease distribution is lacking.

The prevalence of FP in other regions has been linked to water quality, with higher FP prevalence typically observed in foraging grounds adjacent to catchments associated with urbanisation, agriculture and/or industry (Adnyana et al., 1997; Chaloupka et al., 2009; dos Santos et al., 2010; Foley et al., 2005; Herbst, 1994; Van Houtan et al., 2010). While it is possible that FP manifestation is multifactorial (Herbst, 1994; Jones et al., 2016), several studies have attempted to elucidate factors which may be responsible for triggering tumour development (Aguirre et al., 1994a; Arthur et al., 2008a; Arthur et al., 2006a; Arthur et al., 2006b; Keller et al., 2014; Landsberg et al., 1999; Van Houtan et al., 2010; Van Houtan et al., 2014). To date, any causative element behind this relationship has not been identified and this may be due to the complex nature of water quality; this field encompasses a range of physical, chemical and biological properties which can be natural or anthropogenic. While a considerable range of these water quality variables exists, only a selection is

typically investigated for a particular purpose (Boyd, 2015). The variables of interest can be subdivided into the categories of nutrients, suspended solids, pesticides and metals. Each of these fields is broad and intricate, and it is important to narrow down the variables within these categories to those that are likely to influence the manifestation of FP in green turtles.

The challenges surrounding research on this disease have, in part, been overcome with the advancement of molecular tools. While chelonid alphaherpesvirus 5 (ChHV5) has been consistently associated with FP using molecular methods (Alfaro-Nunez et al., 2014; Lackovich et al., 1999; Lu et al., 2000b; Lu et al., 2003; Nigro et al., 2004a; Nigro et al., 2004b; Page-Karjian et al., 2015; Page-Karjian et al., 2012; Quackenbush et al., 2001; Quackenbush et al., 1998; Rodenbusch et al., 2014; Yu et al., 2001; Yu et al., 2000), the factors surrounding viral transmission and disease manifestation have not yet been resolved. As FP is most frequently reported in juvenile turtles and has not been observed in pelagic juveniles, it has long been speculated that ChHV5 is horizontally transmitted upon recruitment to neritic bays (Herbst, 1994). This theory is supported by molecular evidence, with the distribution of genetic variants of ChHV5 being closely linked to foraging grounds (Ene et al., 2005; Herbst et al., 2004; Patrício et al., 2012). However, this theory has never been investigated by assessing a link with host genetic stock.

Marine turtles have a complex life-history, spanning multiple habitats, which makes it difficult to pinpoint the stage and location that ChHV5 transmission occurs. Hatchlings emerge from rookeries in tropical and subtropical regions where they then undertake a pelagic existence. Several years later, they recruit into inshore foraging grounds as juveniles (Reich et al., 2007). The animals at these foraging grounds are comprised of turtles from multiple regional rookeries (Anderson et al., 2013; Dutton et al., 2014; Jensen et al., 2016; Jones et al., 2018; Lahanas et al., 1998). These turtles have strong site fidelity to both the foraging ground they inhabit and the rookery from which they originated; turtles will attempt to return to this rookery to breed and nest at the onset of sexual maturity (Musick and Limpus, 1997). Due to this natal philopatry, turtles originating from rookeries in a particular region are genetically distinct stocks. Transmission of ChHV5 may occur at the rookery, the foraging ground, or in transit between these habitats. If ChHV5 transmission is occuring vertically from parent to offspring, then phylogenetic clustering of ChHV5 would be expected to be based on host genetic stock rather than sampling location. Conversely, if ChHV5 transmission is occurring horizontally at foraging grounds, a link between viral variant and host origin would be less likely. Assessing distribution patterns of the virus, and any relationship between ChHV5 variant and host genetic stock, may provide an indication as to which of these locations, if any, is the site of transmission.

Although FP is listed as a threat to marine turtles in the Recovery Plan for Marine Turtles in Australia (Department of the Environment and Energy, 2017), a review of the literature revealed that FP in Australia is relatively understudied. The overarching aim of this thesis is to include Australia in the global conversation about this disease, and to provide recommendations for management of inshore areas of the GBR in order to conserve this vulnerable species. This will be achieved by establishing the spatial distribution and prevalence of FP along the Queensland coast, which encompasses the Great Barrier Reef (GBR) and evaluating a potential relationship between FP prevalence and water quality on the GBR. A molecular epidemiological study will also be conducted to investigate any relationship between host origin and ChHV5 variant to better understand ChHV5 transmission.

Chapter Two:
A review of fibropapillomatosis in green turtles (*Chelonia mydas*)

Background and aims of this chapter

In 1994, the first literature review on fibropapillomatosis (FP) in marine turtles was published (Herbst, 1994). This review described all that was known about FP at the time of publication and highlighted the many knowledge gaps surrounding this disease. Following that literature review was over 20 years of studies, many of which aimed to address those knowledge gaps. This thesis chapter will review all available literature on FP in marine turtles, including the body of work that has emerged in recent years. Part of this chapter was published in 2016 as a review on fibropapillomatosis in green turtles. The chapter has since been updated and expanded for inclusion in this thesis. An additional section introduces the current knowledge on water quality parameters to form the basis for investigating a possible link to FP prevalence.

The overarching aim of this chapter is to review the available literature to assess the status of fibropapillomatosis (FP) in marine turtles globally. This will be conducted by addressing the following aims:

1. Describe the disease presentation of FP
2. Provide an epidemiological background of FP
3. Describe the likely aetiological agent of FP
4. Identify knowledge gaps in our understanding of this disease and suggest directions for future research
5. Set out the research questions and aims of this thesis

Introduction

The green turtle (*Chelonia mydas*) is one of seven species of marine turtle and is internationally recognised as endangered by the International Union for the Conservation of Nature (Seminoff, 2004). Eleven discrete population segments of green turtles have been identified, each of which is considered biologically and ecologically significant (NMFS and USFWS, 2014). Green turtles also hold great cultural significance to many indigenous peoples and are of economic importance, playing a significant role in ecotourism (Dobbs, 2001; Gulko, 2004). This species has a global distribution and a complex life history, occupying a range of habitats. Hatchling turtles have a pelagic existence and recruit into neritic waters at the age of 3-5 years (Reich et al., 2007). With the exception of migration for breeding, turtles typically remain in these foraging environments, which are commonly associated with seagrass meadows or coral reefs, for the remainder of their life (Musick and Limpus, 1997) (Figure 2.1).

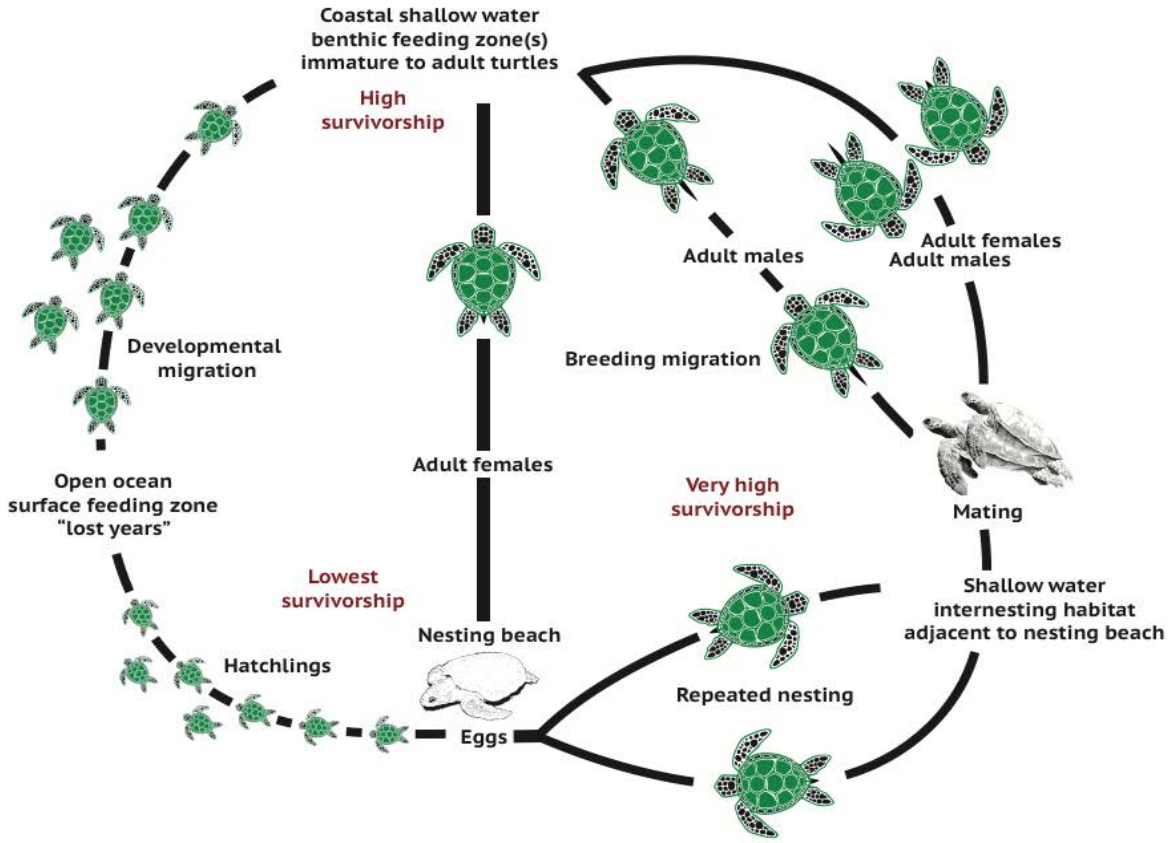

Figure 2.1. The complex life history of green turtles. Adapted from (Lanyon et al., 1989).

Green turtles are exposed to a number of threats including ingestion of marine debris, degradation, urbanisation and pollution of nesting habitats and foraging areas, nest and hatchling depredation by wild, feral and domestic animals, boat strike, traditional hunting and egg harvest, the impacts of climate change on the marine and terrestrial environment, and entanglement in fishing nets and

lines (Bjorndal, 1995; Herbst and Klein, 1995a). Conservation efforts which aim to abate many of these threats have assisted in the recovery of some of the major green turtle populations (Chaloupka et al., 2008a; Van Houtan et al., 2010). However, outbreaks of disease are also contributing to morbidity and mortality in this already vulnerable species (Chaloupka et al., 2008b; Flint et al., 2010b; Foley et al., 2005).

Fibropapillomatosis (FP) is a disease that has now been reported in every species of marine turtle; green (Smith and Coates, 1938), loggerhead (*Caretta caretta*) (Harshbarger, 1991), Kemp's ridley (*Lepidochelys kempii*) (Barragan and Sarti, 1994), hawksbill (*Eretmochelys imbricata*) (D'Amato and Moraes-Neto, 2000), olive ridley (*Lepidochelys olivacea*) (Aguirre et al., 1999), flatback (*Natator depressus*) (Limpus et al., 1993), and leatherback (*Dermochelys coriacea*) (Huerta et al., 2002) turtles. This disease is of greatest concern in green turtles as FP has only reached a panzootic status in this species (Williams et al., 1994). The disease is a neoplastic condition which may lead to the growth of tumours on the skin, oral cavity, shell, eyes and internal organs of the affected turtle, which in severe cases reduces the probability of survival (Flint et al., 2010a; Herbst, 1995; Work et al., 2004). The disease was first identified in a green turtle with multiple wart-like tumours on display at the New York Aquarium, although the turtle originated from Key West, Florida (Smith and Coates, 1938). Despite being described in 1938 (Lucke, 1938; Smith and Coates, 1938), FP did not reach epizootic proportions until the 1980s (Herbst et al., 2004; Herbst, 1994) and has now been reported from every major ocean basin that green turtles inhabit (Herbst, 1994). This review will cover the epidemiology and proposed aetiology of FP in green turtles, with considerable emphasis on the primary candidate for the aetiological agent, chelonid alphaherpesvirus 5 (ChHV5).

Disease presentation

Fibropapillomatosis can be identified in marine turtles by the presence of single or multiple benign fibroepithelial tumours. The characteristic tumours are easily noticed and are pathognomonic for FP, often limiting or obstructing the vision, feeding and locomotive ability of the affected turtle (Flint et al., 2010a; Herbst, 1994; Herbst, 1995; Work et al., 2004). Cutaneous tumours are typically present on the external soft tissue of the turtle, but may grow on the carapace, plastron (Balazs and Pooley, 1991; Brooks et al., 1994; Herbst, 1994; Jacobson et al., 1989; Smith and Coates, 1938) and cornea of affected turtles (Brooks et al., 1994; Flint et al., 2010a). These tumours can be observed on all visceral organs (Foley et al., 2005; Herbst, 1994; Work et al., 2004) and are thought to develop during later stages of the disease (Herbst et al., 1999; Wyneken et al., 2006). However, as most visceral tumours are observed during post mortem investigations, the data available on the prevalence of this type of tumour is skewed. Individual tumours can range from 0.1 to 30 cm in

diameter and can be sessile or pedunculated. The appearance of these tumours can vary from smooth to verrucous and the colour is dependent on the pigment at the site of origin (Herbst, 1994) (Figure 2.2).

Figure 2.2. The plastron and hind flippers of a green turtle severely affected by fibropapillomatosis, highlighting the diverse range of tumour appearance. Image provided by A/Prof Ellen Ariel.

Myxofibromas, fibrosarcomas, papillomas, fibromas and fibropapillomas have all been found to be associated with FP (Norton et al., 1990; Work et al., 2004). Three of these tumours are thought to be linked with different stages of tumour development (Herbst, 1994; Kang et al., 2008). The early development phase is associated with papilloma tumours; proliferation of epidermal cells, with little or no involvement of the dermal layer. The chronic phase of tumour development is marked by the presence of fibromas, with proliferation of the dermal layer, while the epidermal layer remains normal. Fibropapillomas represent the intermediate phase of tumour development and consist of characteristics of both the papillomas and fibromas (Herbst, 1994; Kang et al., 2008).

Histological studies on FP tumours have observed orthokeratotic hyperkeratosis and varying degrees of epidermal hyperplasia. Key features observed in FP tumours include cytoplasmic vacuolation and

ballooning degeneration of superficial epidermal cells (Adnyana et al., 1997; Herbst, 1994; Jacobson et al., 1991; Jacobson et al., 1989).

Hematologic and biochemical signs of immunosuppression, chronic stress, and chronic inflammation such as anaemia, lymphocytopenia, neutrophilia, monocytosis, hypoproteinaemia and hyperglobulinaemia have been observed in turtles with clinical signs of FP (Aguirre et al., 1995; dos Santos et al., 2010; Page-Karjian et al., 2014; Work et al., 2001). Although it is still unclear whether the immunosuppression occurs as a result of or as a precursor to FP development, it has been suggested that immunosuppression occurs as a result of FP (Work et al., 2001). While further study is essential to confirm the relationship between immunosuppression and FP infection, it is clear that immunosuppression leaves turtles with FP tumours susceptible to secondary infections and opportunistic pathogens (dos Santos et al., 2010; Stacy et al., 2008; Work et al., 2003; Work et al., 2001). Impacts of such secondary infections, combined with FP in marine turtles, are a major cause for concern in an already vulnerable species.

Epidemiology of fibropapillomatosis in marine turtles

Fibropapillomatosis typically occurs in marine turtles inhabiting neritic tropical and sub-tropical areas (Adnyana et al., 1997; Ene et al., 2005; Herbst, 1994; Work et al., 2004). This disease is most frequently observed in juvenile turtles; FP has also been reported in sub-adults and less commonly in adults (Adnyana et al., 1997; Ene et al., 2005; Herbst, 1994; Herbst and Klein, 1995b; Page-Karjian et al., 2014; Patrício et al., 2012; Work et al., 2004). This apparent age differentiation in certain locations may indicate that affected juveniles perish from the population altogether or recover with acquired immunity that protects them as adults (Van Houtan et al., 2010). Alternatively, it is possible that these adults were never exposed to this disease. There are no reports of this disease in pelagic post hatchlings or new recruits that have recently taken up residence in inshore foraging habitats (Herbst, 1994). Gender is not thought to be a contributing factor, as no significant difference has been observed in prevalence between males and females (Work et al., 2004).

Disease prevalence and impact

Smith and Coates (1938) reported a prevalence of 1.5% in the Florida Keys region. The disease was not documented in the area again until the 1980s, where the prevalence was then reported to range between 20-60% throughout the subsequent decade. The early to mid-1990s saw FP emerge in the Eastern Pacific, Hawaiian Islands, Indonesia and Australia. New reports of incidences of FP in other regions continue to emerge (Cardenas et al., 2018; Li et al., 2017; Mejía-Radillo et al., 2019; Reséndiz et al., 2016). As this disease has reached epizootic status in several locations globally, it is now considered a panzootic (Williams et al., 1994). Due to the conspicuous presentation of this condition,

any prior presence would have been noticed in a region where it currently occurs. The incidence of turtles with FP tumours as a percentage of total turtles captured is reported in Appendix 1 (Supplementary Table 2.1). Although age class is a risk factor, not all reports of FP prevalence have been corrected by demographic proportions and future reports would benefit from making this distinction.

The prevalence of this disease varies both spatially and temporally (see Appendix 1: Supplementary Table 2.1). The sporadic reports of this disease over time, in combination with a lack of oral history prior to the 1980s, indicate that FP is a globally emerging disease (Duarte et al., 2012; Greenblatt et al., 2005b). In several cases, a significantly different prevalence of this disease in nearby regions has been observed. In Florida, a prevalence of approximately 50% was observed in green turtle aggregations in the Indian River region. However, less than 1 km away at the Sabellariid worm reef, FP was not observed at all (Herbst, 1994). At Pala'au, Molokai, FP was not observed at all until 1985, with the prevalence increasing from 1% in 1987 to 60.7% in 1995 (see Appendix 1: Supplementary Table 2.1). A shift in FP prevalence at two closely monitored sites in Puerto Rico has been observed in recent years; FP prevalence began decreasing at Puerto Manglar and increasing at Tortuga Bay in 2009 (Patrício et al., 2011). In Australia, FP has been reported in a number of locations since it was first observed in Queensland in the early 1970s (Limpus et al., 2016).

The contribution of this disease to morbidity and mortality in affected turtles has also been widely discussed (Chaloupka et al., 2009; Chaloupka et al., 2008a; Ene et al., 2005; Flint et al., 2010b; Foley et al., 2005; Herbst, 1994; Patrício et al., 2016). A study on green turtles at Palaau, Hawaii found that this population was already recovering from previous overharvesting at the time of the FP outbreak in this region. The FP prevalence in this region has also been in decline since the mid-1990s (Chaloupka et al., 2009). Studies on regions in Australia (Flint et al., 2010b), Puerto Rico (Patrício et al., 2016; Patrício et al., 2011) and Florida (Hirama and Ehrhart, 2007) have all concluded that FP is not a significant factor in mortality of turtles. Conversely, a study conducted on data accumulated over 21 years from Hawaii implicated FP as the primary cause of strandings (Chaloupka et al., 2008b). Despite some conflicting conclusions, the overwhelming consensus is that FP does not significantly impact the survival of turtle populations. However, Hamann et al. (2010) highlighted that understanding and managing this disease is a priority research area for sea turtle conservation. Without a more complete understanding of the fundamental elements of this disease, FP cannot be discounted as a threat to the survival of this species.

Aetiology of fibropapillomatosis in marine turtles

Research to date suggests that FP is associated with a herpesvirus infection (Herbst et al., 1995; Lackovich et al., 1999; Quackenbush et al., 2001; Quackenbush et al., 1998). Although this virus was recently cultured in vitro, Koch's postulates have not been fulfilled (Work et al., 2017). Molecular techniques (Lackovich et al., 1999; Quackenbush et al., 2001; Quackenbush et al., 1998) have proven a strong association between FP and a herpesvirus and, according to the criteria established by (Hill, 1965), the relationship seems to be that of cause and effect. Chelonid alphaherpesvirus 5 (ChHV5), which belongs to the subfamily *Alphaherpesvirinae*, genus *Scutavirus*, is now the primary focus of research in this area (Davison and McGeoch, 2010). However, there are still some uncertainties surrounding the transmission of the virus, the circumstances that lead to tumour development and the role of environmental factors in the development of this disease.

Infectious nature of fibropapillomatosis

The epizootic nature of FP and the significant variation in the prevalence of FP between different populations of marine turtles, even between nearby localities, led to speculation that FP was primarily caused by an infectious agent.

Herbst et al. (1995) successfully transferred FP between animals by using cell-free tumour extracts from turtles with tumours to inoculate young captive-reared turtles that were theoretically naive to FP. All turtles in 3/4 experimental groups developed FP tumours. Control animals, which were housed in the same facility and conditions as the experimental turtles, did not develop FP during the same study period. The tumour extracts used in this experiment were filtered through a 0.45 µm syringe tip filter to prevent most pathogens, other than viruses, from being transferred. These findings support the case for the role of a viral agent in FP transmission in marine turtles.

Although in their initial description of FP, Smith and Coates (1938) did not identify any viral elements in histological examination of FP tumours, modern theories have focused on viruses as the primary aetiological agent of FP. A range of viruses are capable of producing neoplasms such as those seen in green turtle FP. As a result, papillomavirus (Herbst, 1994), papova-like virus (Lu et al., 2000a), retrovirus (Casey et al., 1997) and herpesviruses (Herbst et al., 2004; Herbst, 1994; Jacobson et al., 1991; Quackenbush et al., 1998) have all been proposed as potential candidates for the aetiological agents of FP in marine turtles.

Current research suggests that FP is associated with ChHV5 infection. Early molecular studies tested a range of tissues from turtles both with and without FP tumours and all concluded that while ChHV5 could be detected in tumour biopsies from turtles with FP, the virus was rarely detected in

normal skin samples from the same turtles (Lackovich et al., 1999; Quackenbush et al., 1998). Samples from turtles without FP tumours did not react in any of the PCR assays conducted in these early studies (Lackovich et al., 1999; Lu et al., 2000b; Quackenbush et al., 1998). These results support a strong association between the presence of ChHV5 and the presence of FP tumours.

Quackenbush et al. (2001) were the first researchers to successfully amplify ChHV5 from skin samples collected from turtles without FP tumours. Although only a subset of samples from turtles without FP tumours reacted in the assay, the results showed that the virus may be present in turtles despite a lack of clinical signs of disease. More recently, ChHV5 sequences have been amplified from skin samples of turtles without FP tumours with greater success (Alfaro-Nunez et al., 2014; Alfaro-Núñez et al., 2016; Alfaro-Núñez and Gilbert, 2014; Page-Karjian et al., 2017; Page-Karjian et al., 2012). Page-Karjian et al. (2017) also amplified ChHV5 DNA in kidney, heart and nerve samples of turtles without FP tumours. These results indicate that early or latent infection with ChHV5 is more common than previously thought. The prevalence of turtles with FP tumours may be small relative to the number of turtles infected with ChHV5. Therefore, an absence of FP tumours does not imply absence of ChHV5 infection. As latency is a typical feature of herpesviruses (Fields et al., 2013), such results are to be expected. The improved sensitivity and specificity of the assays used in these studies have revealed a feature of the disease that was undetectable using earlier assays.

If disease presentation is not dependent on viral infection alone, other factors contributing to tumour development must be considered. An interaction between host, pathogen and the environment (García-Sastre and Sansonetti, 2010) which tips the balance in favour of tumour development may be at play. Differences in host immunity may be preventing certain turtles from mounting an immune response to the virus (Griffin et al., 2010). Studies on other viral infections have shown that variants of a virus can have different levels of virulence, and as such disease presentation and severity may differ with each variant (Berumen et al., 2001; Kaashoek et al., 1996; Laegreid et al., 1993; Yunis et al., 2004; Zhang et al., 2001). It is possible that the development of FP tumours is dependent on which viral variant a turtle is infected with. It is also possible that turtles infected with the virus only develop tumours when the viral load surpasses a certain threshold. While the relationship between viral titre and tumour development has not been resolved for ChHV5, this relationship has been described in other viral infections (Brodie et al., 1992; Haralambus et al., 2010; Islam et al., 2006; Ladekjær-Mikkelsen et al., 2002; Liu et al., 2000; Nsubuga et al., 2008; Olvera et al., 2004; Quintana et al., 2001; Ravazzolo et al., 2006; Rosell et al., 2000; Rovira et al., 2002; Zhang et al., 2000). The consistent association of high viral load and tumour development provides support for the theory that this may be the case for ChHV5. Alternatively, FP may be the

result of a hyper-vigorous immune response that leads to ballooning degeneration of epithelial cells and eventual neoplastic transformation and tumour growth.

Chelonid alphaherpesvirus 5

Nomenclature and taxonomy

There are currently six herpesviruses documented in chelonids, named chelonid alphaherpesvirus 1 to 6 (ChHV1-6). Chelonid alphaherpesvirus 1, 5 and 6 are described in marine turtles whilst the others have been reported in freshwater turtles (Tidona and Darai, 2011). In the absence of sequence data, ChHV1, ChHV2, ChHV3 and ChHV4 remain unrecognised by the International Committee on Taxonomy of Viruses (ICTV) and their taxonomic place is unclear (Davison and McGeoch, 2010). With respect to the marine turtle herpesviruses, ChHV1 is described in association with grey patch disease (Haines et al., 1974; Rebell et al., 1975), ChHV5 is associated with FP and ChHV6 is known to be associated with lung-eye-trachea disease (Coberley et al., 2002; Coberley et al., 2001; Curry et al., 2000; Jacobson et al., 1986).

Chelonid alphaherpesvirus 5 (ChHV5) (Davison and McGeoch, 2010; Davison et al., 2015) is now the more commonly used name for this virus. However, it should be noted that previous studies have used a range of names for this virus. These names include: Green turtle herpesvirus, green turtle fibropapillomatosis-associated herpesvirus, fibropapillomatosis-associated herpesvirus (FPHV), fibropapilloma-associated turtle herpesvirus (FPTHV), fibropapilloma-associated marine turtle herpesvirus, chelonid fibropapilloma-associated herpesvirus (CFPHV) and chelonid herpesvirus 5 (ChHV5). This review refers to the virus as ChHV5.

Histological investigations of FP tumours showed indications of herpesvirus infection and subsequent studies using electron microscopy concluded that the virus-like particles that were observed were likely to belong to the family Herpesviridae based on location, size and morphology (Herbst et al., 1995; Jacobson et al., 1991; Jacobson et al., 1989).

More recent studies utilising a range of molecular techniques have confirmed herpesviral elements are present in FP tumours (Lackovich et al., 1999; Lawrance et al., 2018; Lu et al., 2000a; Lu et al., 2000b; Monezi et al., 2016; Nigro et al., 2004a; Nigro et al., 2004b; Quackenbush et al., 2001; Quackenbush et al., 1998; Yu et al., 2001; Yu et al., 2000). Phylogenetic analysis of the ChHV5 genes DNA polymerase and DNA binding protein sequences revealed that ChHV5 clusters closely with, but separate to, other members of the *Alphaherpesvirinae* subfamily (Greenblatt et al., 2005b; McGeoch and Gatherer, 2005). Davison and McGeoch (2010) targeted the single-stranded DNA-binding protein, glycoprotein B, the major capsid protein, DNA polymerase and two subunits of the DNA

packaging terminase (genes UL29, UL27, UL19, UL30, UL15 and UL28, respectively). The resulting Bayesian phylogenetic tree shows that ChHV5 exists as an out-group, clearly separate from the current genera. A Minimum Evolution phylogenetic tree of *Alphaherpesvirinae* based on full length DNA polymerase sequence further supports this result (Figure 2.3). Consequently, it has been proposed that ChHV5 be placed in its own genus (Davison and McGeoch, 2010). The proposed genus, *Scutavirus*, sits within the *Alphaherpesvirinae* subfamily of *Herpesviridae*.

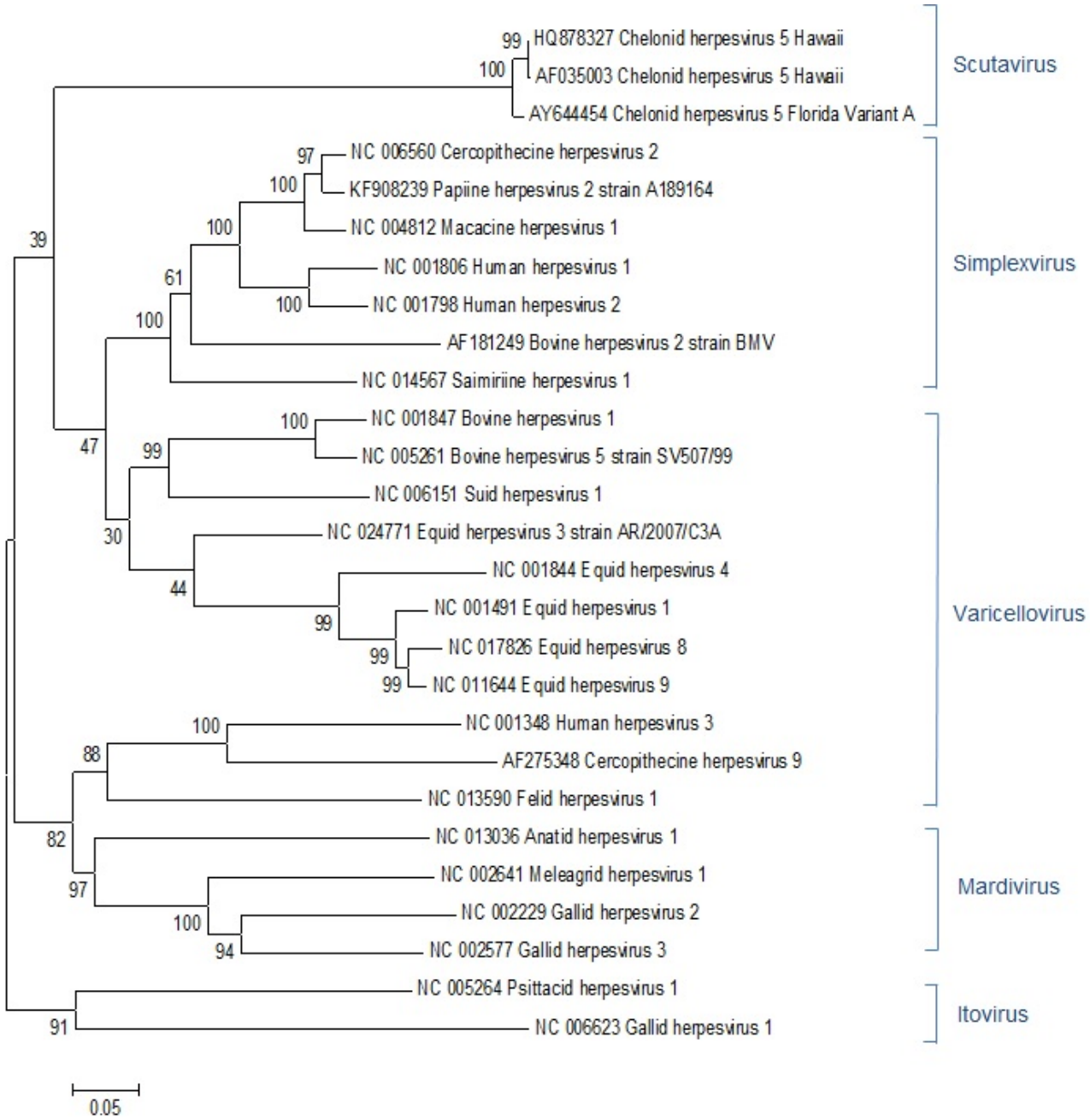

Figure 2.3. A Minimum Evolution phylogenetic tree of *Alphaherpesvirinae* based on full length DNA polymerase sequence retrieved from GenBank (Accession numbers provided in tree). Bootstrap values for each node are provided (1000 replicates). The analysis involved 27 nucleotide sequences resulting in a total of 2593 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Variants of chelonid alphaherpesvirus 5

Based on nucleotide sequence diversity, four viral variants of ChHV5 have been recorded in waters around Florida. At present, they are known as A, B, C and D (Ene et al., 2005) and they are distinct from a Hawaiian variant (Herbst et al., 2004). A recent study using high-throughput sequencing and long-range PCR products amplified from tumour tissue further confirmed that these variants share similarity, but also distinct differences based on sampling region (Morrison et al., 2018).

In Florida, Variant A is the most prevalent in the region, yet there is variation in relative prevalence of variants at each site. Co-infection with variants A and B was also found in one green turtle (Ene et al., 2005). Perhaps even more significantly, different species of marine turtle shared the same variant if they were present in the same locality (Ene et al., 2005; Herbst et al., 2004). More recently, the same trend was observed in foraging turtles in Brazil, with six variants of ChHV5 identified (Rodenbusch et al., 2014). Reports of regional variation in ChHV5 DNA sequences are beginning to increase (Ariel et al., 2017; Lawrance et al., 2018; Monezi et al., 2016), with the combined results of these studies indicating a strong geographic role in the transmission of the virus.

In 2012, ChHV5 was examined using samples from a variety of locations in order to create a global phylogeography of the virus. Four phylogeographical groups of ChHV5 were identified: eastern Pacific, western Atlantic/eastern Caribbean, mid-west Pacific and Atlantic (Patrício et al., 2012). The results of the study showed that the viral variant is similar between nearby foraging grounds while distant regions are considerably divergent. The study by Patrício et al. (2012) also found that sympatric species of marine turtle were infected with the same viral variant, further supporting the results of Herbst et al. (2004) and Ene et al. (2005). These findings indicate that individual turtles are likely to be infected with the virus through horizontal transmission in neritic bays (Patrício et al., 2012).

Co-evolution of virus and host

Herbst et al. (2004) suggested that the virus diverged prior to the separation of avian and mammalian alphaherpesviruses. This would mean that ChHV5 became specific to marine turtles approximately 300 million years ago (mya). In addition, it was estimated that the two most divergent clades were separated approximately 1.6-4.0 mya. These results led to speculation that the rise of the Isthmus of Panama (3.1-3.5 mya) was responsible for the divergence as it prevented genetic

exchange between these clades. Patrício et al. (2012) found that the most recent common ancestor of the currently known variants of this virus existed 193-430 years ago. This estimate is considerably more recent than that of Herbst et al. (2004). However, both studies demonstrate that ChHV5 has evolved with marine turtles and in either case, it is likely ChHV5 has undergone region specific co-evolution with its host.

While further research is needed to resolve the time of divergence, there is one clear conclusion: It is not a new virus, or even recent mutations in an old virus, that is causing tumours to develop. This evidence further supports the theory that the recent emergence of FP is linked to modern day extrinsic environmental factors promoting tumour development.

Genome organisation

The herpesvirus genome is divided into two unique regions, one composed of a unique long (UL) sequence and the other region is composed of a unique short (US) sequence. These unique sequences are flanked by repeat sequences. The number, position and direction of these sequences can vary, and as a result there are multiple types of herpesvirus genome structures. Current literature lists between four and six known herpesvirus genome types. Fauquet et al. (2005) recognises four herpesvirus genome types (denoted Type 1-4), while Pellet and Roizmann (2007) describe six different genome types (denoted Type A-F).

The entire genome of ChHV5 was described by Ackermann et al. (2012). The extensive sequence data generated from this study showed a clear division of the genome into UL and US regions. Inverted repeat sequences (IRS) were also found to flank the US sequence. This configuration is consistent with ChHV5 having a type D genome (Ackermann et al., 2012).

Ackermann et al. (2012) also described four genes that are atypical for an alphaherpesvirus genome. Two members of the C-type lectin-like domain superfamily (F-lec1, F-lec2), an orthologue to the mouse cytomegalovirus M04 (F-M04) and a viral sialyltransferase (F-sial) were all found to be present in the ChHV5 genome (Ackermann et al., 2012). While the products of these genes may not be critical for viral replication, each one has a potential role in pathogenesis or immune deviation (Ackermann et al., 2012). Orthologues to these genes have been described in other viral families and host cells (Markine-Goriaynoff et al., 2004; Neilan et al., 1999; Voigt et al., 2001; Wilcock et al., 1999). However, until now, none of these genes has ever been reported in the genome of an alphaherpesvirus. Two of these atypical genes (F-sial and F-M04) were found to be expressed in the FP tumours and it has been suggested that these genes may play a role in FP pathogenesis (Ackermann et al., 2012). The presence of these atypical genes, in addition to unambiguous

recombination events between ChHV5 samples from Hawaii and Florida, were confirmed in a recent study (Morrison et al., 2018).

Transmission of chelonid alphaherpesvirus 5

As this disease has not been observed in pelagic juveniles, it is thought that turtles are exposed to ChHV5 upon recruitment to neritic zones, indicating horizontal transmission (Ene et al., 2005; Herbst et al., 2004; Patrício et al., 2012). These new recruits may be exposed to several stressors associated with migration, adaptation to a new environment, and changes in population density, diet and pathogen exposure, which may all combine to reduce the efficacy of the immune system and make these juveniles more susceptible to infection (Ritchie, 2006) with ChHV5 and development of FP. It is also possible that these stressors combine to enhance transmission or elicit herpesviral recrudescence in latently infected turtles (Ritchie, 2006) leading to the development of FP tumours. Alternatively, direct transmission may be occurring between co-habiting turtles via interactions such as mating and aggression.

Considering FP as an infectious disease, researchers have speculated on the means of transmission and possible vectors. Marine turtles host a range of parasites and correlations have been made between parasite load and individual health. Spirorchid trematodes (Aguirre et al., 1994b; Aguirre et al., 1998b; Jacobson et al., 1991; Jacobson et al., 1989; Norton et al., 1990; Williams et al., 1994), coral reef cleaner fish (Booth and Peters, 1972; Losey et al., 1994; Lu et al., 2000c), saddleback wrasse (*Thalassoma duperrey*) (Lu et al., 2000c) and marine leeches (*Ozobranchus* spp.) (Greenblatt et al., 2004) have all been proposed as potential vectors of ChHV5. Significantly higher viral loads were detected in marine leeches when compared with the other parasites examined (Greenblatt et al., 2004) and they are currently the leading candidate for a mechanical vector. Although *Ozobranchus* leeches are the most likely candidates for transmission vectors of ChHV5, their exact role has not yet been confirmed. This is partly due to the possible latent state of the virus and involvement of other co-factors in disease expression of FP (Greenblatt et al., 2004).

Other marine turtle epibiota, including bladder parasites (*Pyelosomum longicaecum*), barnacles (*Platylepas* spp.), amphipods of the skin and oral cavity (order *Talitroidea*) and blood flukes of the genera *Carretacola*, *Hapalotrema* and *Laeredius* have been ruled out as potential vectors (Greenblatt et al., 2004).

Environmental factors

Animals are highly reliant on the environments they exist in for the provision of essential ecosystem services, such as food and shelter. Environmental change, be it natural or anthropogenic, can occur

under a variety of circumstances and are of particular concern to the conservation and management of threatened species. Documented cases of population crashes of threatened species, as the result of an environmental change, are not uncommon. For example, anthropogenic climate change is threatening a range of species by altering ecosystems at a faster rate than species are able to adapt to. Reptiles with temperature-dependant sex determination are at risk of skewed sex ratios and population crashes under increasing ambient temperatures (Mitchell and Janzen, 2010). Marine turtle populations in the northern Great Barrier Reef are already showing signs of feminisation, with one genetic stock being extremely biased towards female; over 99% of juvenile and subadult turtles were found to be female (Jensen et al., 2018). In a separate case, a pesticide spill at Lake Apopka, Florida was linked to a population decline in the then endangered American alligator (*Alligator mississippiensis*). The particular organochlorides involved are able to act as oestrogens, and their significant rise in concentration in the lake had irreversible effects on the gonads of both the male and female alligators; making steroidogenesis impossible and normal sexual maturation unlikely (Guillette et al., 1994). Extreme weather events are also a source of natural environmental change. A category five cyclone which crossed the north Queensland coast of Australia in 2011 caused widespread damage to the benthic communities of the Great Barrier Reef (GBR) and a major loss of seagrass in the region (GBRMPA, 2011). Green turtles, which feed on seagrass, subsequently stranded in unprecedented numbers; strandings in the year following this event more than tripled compared with the strandings recorded the previous year (Meager and Limpus, 2012). This mass stranding event was likely a result of starvation, following the destruction of the seagrass meadows in the region (Bell and Ariel, 2011).

Marine turtles are particularly susceptible to changes in their environment as they are long-lived animals with a complex life history (Aguirre and Lutz, 2004). A marine turtle will access a range of habitat types during its lifetime, but exhibits a high degree of site fidelity once recruited into their chosen foraging area. Mature female turtles are known to return to the natal area from which they originated as hatchlings in order to lay their eggs (Limpus, 2008). Due to this site fidelity, marine turtles are likely to persist in, or return to, their chosen localities despite unfavourable changes to the environment. As a result, any damage to or destruction of these sites could have extremely detrimental effects on populations that rely on them (GBRMPA, 2014a; Hawkes et al., 2009; Poloczanska et al., 2010).

It has been suggested that environmental factors may play a role in the development of FP (Adnyana et al., 1997; Aguirre and Lutz, 2004; Chaloupka et al., 2009; dos Santos et al., 2010; Herbst, 1994; Herbst and Klein, 1995a; Van Houtan et al., 2014). Moreover, the presence of chemical

contaminants may be part of a multifactorial problem that leads to FP (Herbst, 1994). Early proponents of a possible relationship between degraded water quality and the presence of FP proposed that chemical contaminants present in the water acted as immunotoxins or were causing damage at the cellular or genetic level (Herbst, 1994). Indirect disturbances to the immune system may occur if the chemical contaminants create a disruption of neuroendocrine function (Anderson et al., 1984; Arkoosh et al., 1994; Colborn et al., 1993; Dean et al., 1990; Dunier, 1994; Zeeman and Brindley, 1981). Herbst (1994) demonstrated that a positive correlation exists between the prevalence of FP in green turtle populations in foraging grounds adjacent to regions associated with agriculture, industry and urban development. Subsequent studies have observed the same correlation (Adnyana et al., 1997; dos Santos et al., 2010; Foley et al., 2005; Van Houtan et al., 2010). Although initial reports in Puerto Rico documented the same relationship, this trend was reversed after several years; the prevalence of FP at the more pristine site is now considerably higher than at the site which is subjected to high levels of human activity (Page-Karjian et al., 2012; Patrício et al., 2011). Researchers attempted to quantify this relationship in Hawaii by developing an information-rich index of eutrophication from the analysis of 82 different watersheds. The results showed a strong association between FP rates, nitrogen-footprints and macroalgae consumed by turtles (Van Houtan et al., 2010). Different quantification studies were also undertaken in waters around Brazil and found that green turtles residing in areas with degraded water quality had a higher prevalence of FP. However, this study based the assessment of water quality on the presence of benthic macrophytes and nutrient levels; pollution and the presence of chemical contaminants were not considered (dos Santos et al., 2010). A recent study detected high concentrations of copper and lead in the blood of marine turtles severely affected by FP compared to turtles without FP tumours (da Silva et al., 2016). However, only very low concentrations of persistent organic pollutants (Keller et al., 2014; Sánchez-Sarmiento et al., 2017) and selected trace metals and organic pollutants (Aguirre et al., 1994a) have been detected in turtles with FP tumours in other studies. Although these results suggest that the pollutants examined do not significantly contribute to FP development, it is possible that further investigations will uncover a relationship between this disease and other environmental contaminants (Keller et al., 2014).

Water temperature may also be a factor in tumour development and growth rate. It is possible that warmer water temperatures during summer promote tumour growth, resulting in tumours of a debilitating size by autumn (Herbst, 1994; Herbst et al., 1995). This seasonal trend has been observed in Florida, where a higher rate of FP was observed in turtles that strand in winter (Herbst, 1994). However, no seasonal trends have been observed in Hawaii (Murakawa et al., 2000), which

may be because there is less seasonal fluctuation in water temperature in this region (Foley et al., 2005).

Natural biotoxins have also been implicated as a co-factor involved in FP development. Landsberg et al. (1999) identified a correlation between high-risk FP areas in the Hawaiian Islands and the prevalence of the dinoflagellate *Prorocentrum,* a species that produces okadaic acid, a known tumour promoter (Cohen et al., 1990; Haystead et al., 1989; Huynh et al., 1997; Suganuma et al., 1988). Similarly, tissue concentrations of lyngbyatoxin, produced by the filamentous cyanobacteria *Lyngbya majuscula*, have been correlated with the presence of FP tumours in dead green turtles (Arthur et al., 2008a; Arthur et al., 2006a; Arthur et al., 2006b). However, this species constituted less than 2% of total dietary intake and therefore it was considered that any biotoxins would likely be at low concentrations in the turtles (Arthur et al., 2008a). If the dietary items containing these biotoxins form a natural component of the diet of green turtles and the amount being consumed was not altered, these toxins should have no influence on the development of FP.

An increased concentration of arginine in the diet of green turtles as a result of feeding on invasive macroalgae blooms has also been linked to an increasing prevalence of FP (Van Houtan et al., 2010). Arginine is a regulator of immune activity (Peranzoni et al., 2007) and is known to promote herpesviruses and contribute to tumour formation (Mannick et al., 1994). This amino acid is also a major component of glycoproteins on the viral envelope of herpesviruses (Van Houtan et al., 2010; Van Houtan et al., 2014). The results of a subsequent study found an association between eutrophication and arginine content of macroalgae, with the intake of arginine in turtles at eutrophied sites being up to 14 times the background level. This increased arginine content may metabolically promote ChHV5, leading to FP tumour development (Van Houtan et al., 2014). Although the conclusions from this study were subsequently challenged (Work et al., 2014), the epidemiological link between the prevalence of disease and feeding ecology found in Van Houtan et al. (2014) provides strong support that environmental factors play a role in the development of this disease. However, the environmental factors leading to the bloom of macroalgae may be causing the development of FP tumours directly, and the algal blooms may not be involved in tumour development at all. If this is the case, it is difficult to link cause and effect.

Despite there being a strong positive correlation between the prevalence of FP in green turtle populations and areas with degraded water quality, it is difficult to identify one specific causal contaminant or a combination of such working synergistically to the detriment of the turtles. Water quality is a complex field, with any physical, chemical, or biological property that influences the suitability of water for natural ecological systems or use by humans being considered a water quality

variable (Boyd, 2015). While an wide range of these variables exists, only a selection is typically investigated for a particular purpose (Boyd, 2015). The variables of interest can be subdivided into the categories of nutrients, suspended solids, pesticides and metals. Each of these categories is broad and intricate, and it is important to narrow down the variables within these categories to those that could have a biological link with FP manifestation.

Nutrients

The growth and survival of organisms is dependent on a wide array of chemical elements and compounds. In aquatic ecosystems, nitrogen and phosphorus are the most in demand, as their availability is limited relative to the needs of the organisms (Boyd, 2015). However, increased nitrogen and phosphorus in a body of water can encourage the growth of algae and aquatic weeds. Oxygen shortages also ensue as a result of the death and decomposition of these plants (Ansari and Gill, 2013). Macroalgae and phytoplankton blooms cause heavy shading and light attenuation which in turn impedes the photosynthetic process in benthic plants (Walker et al., 1999). Eutrophication of waterways, due to increased nutrient levels, can alter plant communities and change food web relationships. In the marine environment, eutrophication can result in seagrass loss (Ansari and Gill, 2013; Walker et al., 1999), which may have subsequent detrimental impacts on species which rely on the presence of seagrass, such as green turtles and dugongs.

Both nitrogen and phosphorus can exist in dissolved and particulate forms, with dissolved inorganic forms of nitrogen and phosphorus being of greatest concern as they are immediately and fully bioavailable for algal growth (Waterhouse et al., 2017). Particulate forms of these elements typically become bioavailable over longer time frames, and dissolved organic forms usually have limited and delayed bioavailability (Furnas, 2013). Dissolved inorganic nitrogen (DIN) has been linked to runoff from fertiliser use whereas dissolved inorganic phosphorus has a reduced association with fertiliser; phosphorus binds to soil so there is a reduced amount available in dissolved form. As a result, there is consensus that increased nitrogen inputs are of greater interest than phosphorus inputs (Waterhouse et al., 2017), with DIN frequently used as an indicator of anthropogenic influences on water quality (Waterhouse et al., 2017). With respect to marine turtles, it is possible that increased DIN levels could promote algal growth and in turn limit seagrass growth through light attenuation.

Suspended solids

Seagrass growth may also be affected by enhanced levels of suspended sediments, which can decrease water clarity. Decreased water clarity reduces photic depth (the depth light can penetrate the water column), with this loss of light affecting photosynthetic organisms like seagrass (Fabricius et al., 2014; Fabricius et al., 2016; Petus et al., 2016). Due to the risk that excess suspended solids

pose to marine ecosystems, the total suspended solids (TSS) in the environment is a well-studied water quality variable (Waterhouse et al., 2017). A reduction in seagrass coverage as a result of increased TSS has the potential to negatively impact those species (including green turtles) which rely on seagrass for forage.

Pesticides

Pesticide is a broad term which is used to describe a huge range of chemical compounds, which may be organic or inorganic, and are designed to kill, repel, attract and mitigate organisms which are nuisance to humans and their activities (Singh, 2012). A range of pests can be targeted by pesticides, and they can be divided into categories as a result (Singh, 2012); major categories include herbicides, insecticides, fungicides and rodenticides. Pesticides are typically associated with agricultural land use. With approximately 76 per cent of the land in GBR catchments being used for agriculture (Smith et al., 2012b), pesticides are a key water quality parameter of interest. Although our understanding of the spatial exposure of pesticides in the marine area is limited at present, pesticides pose the highest risk to ecosystems closest to the source (Waterhouse et al., 2017). That is, ecosystems closely associated with agricultural activities (such as rivers) are exposed to the highest concentrations of pesticides, followed by coastal ecosystems located near river mouths.

There are several possible ways that pesticides may pose a direct or indirect risk to marine turtles. The direct risk (albeit a small one) is from pesticides designed to kill animals, such as insecticides or rodenticides. However, an indirect risk from herbicides destroying seagrass is much more likely. Several studies have investigated the relationship between pesticides and turtles (García-Besné et al., 2015; Innis et al., 2008; Keller et al., 2014; Monagas et al., 2008; Novillo et al., 2017; Salvarani et al., 2018; Sánchez-Sarmiento et al., 2017; Sánchez-Sarmiento et al., 2016; Tremblay et al., 2017), with two of these studies specifically investigating the relationship between FP and pesticide concentrations in turtle blood (Keller et al., 2014) and tissue (Sánchez-Sarmiento et al., 2017) samples. However, both studies concluded that pesticides were unlikely to be of concern as a co-factor in FP development, due to a lack of consistent differences between turtles with and without FP (Keller et al., 2014; Sánchez-Sarmiento et al., 2017). However, there is a multitude of pesticides entering marine ecosystems with the potential to affect turtles, especially indirectly, and as yet the role of pesticides in FP development cannot be excluded.

Metals

There are a wide range of metals and metalloids available in the environment that are essential to plants and animals, primarily as trace elements. However, even these essential trace elements can have toxic effects on aquatic organisms at high concentrations (Boyd, 2015). Concentrations and bioaccesibilty of these metals varies depending on the element itself, local sources and

environmental conditions (Boyd, 2015; Villa et al., 2017). Elevated concentrations of metals can occur as a result of a range of anthropogenic or natural activities including coastal dredging, agricultural and industrial runoff, urbanisation and floods (Villa et al., 2017). However, the majority of instances of trace element toxicity in aquatic animals and humans typically result from anthropogenic pollution (Boyd, 2015).

In polluted areas, trace elements are typically found in elevated concentrations in the water and sediment, but also in green turtle forage such as algae and seagrasses (Talavera-Saenz et al., 2007). Elevated metal concentrations in the environment have also been linked to impaired immune function (Grillitsch and Schiesari, 2010), mass marine turtle strandings (Flint et al., 2015) and even FP development (da Silva et al., 2016). Marine turtles resident in a foraging site heavily influenced by agricultural activities were also found to have elevated levels of cobalt in their blood, with concentrations ranging from 4 to 25 times that of reference intervals generated within the same study (Villa et al., 2017). However, interpreting these results is challenging as an understanding of trace metal toxicity in marine turtles is lacking; species-specific toxicokinetic processes which determine the trace element differences among various tissues are poorly described for reptiles (Villa et al., 2017).It is therefore extremely difficult to narrow down specific trace elements of interest to marine turtles, yet their potential role in FP development cannot be ignored.

Direction of future research

The longevity of marine turtles, coupled with their close association with inshore habitats and seagrass meadows and coral reefs in these habitats, has led to the proposal that they may act as sentinel indicators of marine ecosystem health (Aguirre and Lutz, 2004). Gaining a better understanding of the health and prevalence of diseases in marine turtle populations provides a critical link between ecosystem health and turtle health. Effective management of both the habitat and the species that rely on it is critical for effective species conservation. As FP has been found to be associated with turtles resident in areas exposed to poor water quality (dos Santos et al., 2010; Herbst, 1994; Van Houtan et al., 2010; Van Houtan et al., 2014), FP prevalence may be a vital indicator for assessing ecosystem health in inshore marine habitats. To be of real value to researchers and managers alike, this monitoring of populations should occur over long time periods (>10 years), as this will allow researchers to more accurately establish disease prevalence, corrected by demographic proportions.

Many of the marine environments inhabited by turtles are also utilised by humans and consequently, research into the epidemiology of this disease could be mutually beneficial for green turtles, other species in these ecosystems and humans alike (Aguirre and Lutz, 2004; Flint et al., 2010b). However, it is important to consider the challenges surrounding establishing any link between water quality and FP. Studies on toxicity usually focus on chemicals that are persistent in the environment or can bio-accumulate. Damage occurring at the genetic level as a result of a toxin may occur as a consequence of transient exposure and as such, future studies would need to be expanded to include transient chemicals that could have this effect on green turtles. The practicality of such investigations is daunting considering the vast marine environment and the known and unknown possible causes of FP (Herbst, 1994; Herbst and Klein, 1995a). One way that potential links between FP and anthropogenic contaminants might be identified is to develop a monitoring program that records and compares contaminant residue levels, genetic changes and viral load in blood and/or tissue samples collected from turtles with and without FP tumours over a wide geographic area and across multiple seasons. Such a program could be integrated into existing turtle monitoring activities. Controlled laboratory studies in a closed experimental system may be needed to conclusively evaluate the roles of various environmental factors in FP development (Herbst and Klein, 1995a). Alternatively, results from both field and laboratory based studies may work synergistically to fully resolve this relationship.

This literature review has also highlighted that while FP prevalence and its relationship to water quality is well-studied in other regions, studies in Australia are lacking. Future studies should aim to

address this in order to better understand this disease and use this information to refine existing management of marine turtles in Australia.

Whether the development of FP tumours is a result of a single agent or the interaction between multiple factors is yet to be determined. It is clear that it is an infectious disease with a strong link to ChHV5. In addition, the strong influence of different geographic regions on the prevalence of FP and each of the viral variants indicate that FP is geographically specific (Ene et al., 2005; Herbst et al., 2004; Patrício et al., 2012; Rodenbusch et al., 2014). However, there has only been a preliminary study of this distribution in Australia (Ariel et al., 2017). Future research should aim to better characterise the distribution of ChHV5 variants in Australia. It could also be possible to investigate any relationship between the genetic stock of the host turtle, and the ChHV5 variant that it is infected with. The results of such a study may clarify whether vertical or horizontal transmission of this virus is occurring.

Molecular studies targeting ChHV5 in samples from turtles show that the virus is present in turtles with and without FP tumours (Alfaro-Núñez and Gilbert, 2014; Page-Karjian et al., 2012; Quackenbush et al., 2001). Future molecular studies targeting ChHV5 should consider these results and screen all samples for ChHV5, not only those from turtles with FP tumours. Biosecurity and potential zoonosis should always be considered by those handling marine turtles in both field and captive situations. However, future research should prioritise understanding the triggers for tumour development.

There are many aspects of FP in marine turtles that are yet to be resolved and future research needs to target those information gaps which will ultimately aid in managing the disease. Understanding how ChHV5 is transmitted between turtles and between regions is a key priority. Molecular epidemiology is a useful tool for revealing genetic differences in this virus between regions; possible relationships between host lineage and viral variant and the genes responsible for pathogenesis and viral replication. Molecular investigations on ChHV5 from different regions are essential to improve our understanding of the epidemiology and pathogenesis of this virus, which will in turn inform the management and conservation of a vulnerable species, the green turtle.

The aims of this chapter were addressed as follows:

1. **Describe the disease presentation of FP**

   FP is characterised by single or multiple benign fibroepithelial tumours which typically grow on external soft tissue but may also grow on the carapace, plastron and cornea. These tumours can also grow on the viscera, but such tumours can only be detected in live turtles by means of imaging technology.

2. **Provide an epidemiological background of FP**

   FP is a globally distributed disease which is typically found in turtles inhabiting tropical and sub-tropical waters. It has been documented in every species of marine turtle, but appears to affect green turtles more frequently. Juvenile turtles are the most common age-class affected by the disease, followed by sub-adults. The disease is rarely observed in adults. Disease prevalence varies both spatially and temporally, and there are contradictory reports about its influence on morbidity and mortality of turtles. Reports of population-level mortality as a result of FP also vary, with some reports of significant declines as a result of FP at a particular time (E.g. Hawaii; Chaloupka et al. (2008b)), while reports in other regions indicate this disease being of lower concern (E.g. Florida; Hirama and Ehrhart (2007)).

3. **Describe the likely aetiological agent of FP**

   Chelonid alphaherpesvirus 5 (ChHV5) has been consistently associated with FP infection and this has led the scientific community to believe that it is the likely aetiological agent of the disease. Mechanisms of transmission are as yet unknown, but it is probable that turtles become infected upon recruitment into their foraging areas from the pelagic life stage. Genetic similarities, and differences, have been reported globally. Based on this, it appears that viral variant distribution varies by location, even locally within a region (E.g. Florida), yet strains separated by significant distances still share high genetic similarity (E.g. Florida and Hawaii).

   Mechanisms which trigger tumour development remain unclear, but a significant association between reduced water quality and high FP prevalence has been consistently reported around the world. This suggests that there are environmental co-factors involved in disease manifestation.

4. **Identify knowledge gaps in our understanding of this disease and suggest directions for future research**

Despite a body of research, there are many gaps in our knowledge of FP. Although studies from Australia have been described in this chapter, they are limited in number compared to the bodies of work published from other regions. Obtaining a clearer understanding of the status of FP in Australia and how it affects the host population of green turtles is crucial to the effective management of this vulnerable species. The following areas appear to be of particular importance:

- Improving our understanding of the distribution and prevalence of FP in Australia.
- Determining whether there is a link between reduced water quality and prevalence of FP.
- Improving our understanding of genetic variation in ChHV5 in Australia through better characterisation of Australian viral variants. The results of a recent study suggested that viral variant distribution was linked to the foraging ground of sampling (Ariel et al., 2017), but further investigation is needed to confirm this.
- Determining whether ChHV5 strains are geographically unique and try to identify if viral variant distribution is linked to host genetic stock to better understand viral transmission pathways.

5. Set out the research questions and aims of this thesis

This thesis aimed to better understand FP in Australia, and provide recommendations for management of foraging areas of the Great Barrier Reef by addressing specific aims in separate chapters. These chapters, and their associated aims, are as outlined on the following page:

Chapter	Aims
Chapter Two	• Describe the disease presentation of FP • Provide an epidemiological background of FP • Describe the likely aetiological agent of FP • Identify knowledge gaps in our understanding of this disease and suggest directions for future research • Set out the research questions and aims of this thesis
Chapter Three	• Investigate FP prevalence at a range of locations spanning the Queensland coastline
Chapter Four	• Develop water quality indices for parameters of interest to green turtles at locations along the GBR • Investigate any link between water quality and FP prevalence on the GBR
Chapter Five	• Develop, optimise and validate a PCR assay which targets green turtle mtDNA control region sequences • Generate and use mtDNA control region sequences and MSA to quantify the stock composition of green turtles at three foraging areas located between Edgecumbe Bay and the Howick Group • Use our new data and data from previously sampled foraging areas to assess the correlation between stock composition and latitude of foraging areas in Eastern Australian waters.
Chapter Six	• Improve the resolution of the current phylogeny of ChHV5 in Australia by generating a more robust, sequence data set than has previously been used, including a larger sample size and increased geographical locations • Assess the relationship between host genetic stock and viral variant in order to clarify the mechanisms of viral transmission.

Publications and presentations arising from this study

- **Jones, K.**, Ariel, E., Burgess, G., & Read, M. (2016). A review of fibropapillomatosis in Green turtles (Chelonia mydas). *The Veterinary Journal, 212*, 48-57. doi: 10.1016/j.tvjl.2015.10.041

My contributions to this study:

- I reviewed the published literature, wrote extensive notes on each paper and collated the resulting information
- I collected FP prevalence data from the published literature and collated it into one table (Supplementary Table 2.1)
- I, under the advice of my supervisor, collected published DNA polymerase sequences from the Alphaherpesvirus subfamily. I then edited these sequences as needed, and used them to generate the phylogenetic tree in this chapter (Figure 2.3).
- I assisted in the production of Figure 2.1
- I prepared the image, including adding the scale-bar, for use in Figure 2.2
- I drafted the chapter and edited it as advised by my supervisors
- I drafted the manuscript and managed the process of journal submission and review

Chapter Three:
Spatial distribution of fibropapillomatosis in marine turtles on the Great Barrier Reef

Backgrounds and aims of this chapter

The literature review in Chapter Two highlighted that although FP is a globally distributed disease, studies on the status of the disease in Australia are limited. Incidental reports of FP from various locations were identified (see Appendix One: Supplementary Table 2.1), but a comprehensive understanding of the spatial distribution of this disease was lacking. This study aims to improve our knowledge of the distribution and prevalence of FP on the Queensland coast, which encompasses the Great Barrier Reef (GBR), by mining existing databases and undertaking surveys in the field. The specific aims were as follows:

1. Characterize FP prevalence at a range of locations spanning the Queensland coastline

Introduction

Despite their status as a flagship species for ecosystem health, marine turtles can be afflicted by diseases that are not well understood. Fibropapillomatosis (FP) is a neoplastic condition which has been reported in all species of marine turtles, but it predominantly affects the endangered green turtle (*Chelonia mydas*) (Jones et al., 2016). This disease is easily identifiable due to the growth of tumours on the soft tissue, carapace, plastron and/or cornea. The tumours may limit or obstruct the vision, feeding and locomotive ability of affected turtles (Jones et al., 2016) and as a result, these turtles are at increased risk of predation, starvation and boat-strike. Turtles with FP are also immunosuppressed and are therefore vulnerable to secondary infection (dos Santos et al., 2010; Stacy et al., 2008; Work et al., 2003; Work et al., 2001). The consequences of tumours on infected individuals can vary, with both mortality (Chaloupka et al., 2008b) and complete recovery (Machado Guimarães et al., 2013) being reported. Yet the factors influencing the infection, clinical presentation and prognosis for a turtle with FP are poorly understood.

Whilst the impact on individual turtles is clear, there is some uncertainty surrounding the impact of this disease at the population level. Although FP tumours are easily recognisable, obtaining accurate data on FP prevalence within a population is challenging (Hargrove et al., 2016; Rossi et al., 2016). Population surveys, which rigorously sample specific populations at regular and defined intervals and record those turtles encountered with FP tumours, are the best means of establishing an accurate FP prevalence. Marine turtle population surveys are challenging, requiring permissible weather and tidal conditions, uniquely-skilled personnel and suitable vessels and equipment. Reported FP prevalence values from these surveys provide an indication of FP presence, but there is still some uncertainty surrounding the true prevalence within populations. For example, an absence of FP records at a location could not be considered as confirmation of an absence of FP, so false negative reports are expected, but less so for false positive reports of FP, which means that there is a general under estimation of prevalence during surveys. The reliability of the prevalence value is influenced by the rigour of the surveys that generated the data; the number of turtles captured, the number of surveys per year, areas targeted, personnel involved, methods for recording data recording, methods for assessing and recording FP incidence are all variables that will influence the reliability of FP prevalence values and therefore hamper comparisons between sites and seasons.

Despite these challenges, incidental data on FP prevalence has been widely reported globally, with high prevalence often linked to areas associated with reduced water quality and high human influence (Jones et al., 2016) (also see Appendix 1: Supplementary Table 2.1). The first report of FP in a foraging population of marine turtles concluded that low rates of this disease in foraging populations is a natural condition of wildlife (Smith and Coates (1938). The reports of FP which have

followed since have largely echoed this sentiment. However, prevalence of this disease varies both spatially and temporally (Jones et al., 2016), with unexplained spikes and reductions in prevalence throughout the 1990s. At Kāne'ohe Bay (Oahu, Hawaii) between 1989 and 1991, the prevalence ranged from 49-92%. Subsequent reports found FP to be the primary cause of marine turtle strandings and mortality over a 26 year period in Hawaii (Chaloupka et al., 2008b), yet FP prevalence has been declining in this region since the mid 1990's (Chaloupka et al., 2009). As the cause of this variation is unclear, researchers have considered a number of factors which may be involved in FP development. For example, water temperature has been suggested to be a contributing factor, with warmer water believed to promote tumour growth (Herbst, 1994; Herbst, 1995). While a seasonal trend consistent with this theory has been observed in Florida (Herbst, 1994), it has not been observed in Hawaii (Murakawa et al., 2000). Such cases highlight the complex nature of FP manifestation and the need to develop a complete understanding of the fundamental elements of this disease. Without such an understanding, FP cannot be discounted as an ongoing threat to marine turtles (Jones et al., 2016).

Although understanding and managing this disease is a priority research area for marine turtle conservation (Hamann et al., 2010), there are no studies dedicated to describing the distribution and prevalence of this disease in Australia. Currently, reports of FP in Australia are largely incidental data included in other studies (Bell, 2003; Bell et al., 2019; Flint et al., 2015; Flint et al., 2010b; Glazebrook and Campbell, 1990; Hamann et al., 2006; Limpus et al., 1993; Limpus et al., 2005; WWF-Australia, 2018). While FP is listed as a threat in the Recovery Plan for Marine Turtles in Australia (Department of the Environment and Energy, 2017), no specific threat abatement plan is proposed. Moreover, this disease was grouped into the 'Diseases and pathogens' category to assess the risk of Australian green turtle genetic stocks to different threatening processes. Overall, the risk of threat to these stocks was determined to range from no long-term effect to moderate, while the likelihood of such an event occurring was denoted to be unknown in almost all cases (Department of the Environment and Energy, 2017). It is clear that our limited understanding of FP in Australia precludes the ability to make informed management decisions, especially considering that FP prevalence is relatively unpredictable.

This study aims to improve our knowledge of the distribution and prevalence of FP in green turtles along the Queensland coast, which encompasses the Great Barrier Reef (GBR). Here we intend to characterize FP prevalence, determined by tumour presence, at a range of sites spanning this coastline using retrospective data from established databases with a view to better inform management of this vulnerable species.

Materials and Methods

Study Sites

Queensland is Australia's second largest state, with an area of roughly 1.8 million square kilometres. Approximately 5 million people live in Queensland, with a high proportion of the population (close to 72%) residing in the southeast corner of the state (Queensland Government Statistician's Office, 2019), near Brisbane (Figure 3.1). The remaining portion (approximately 28%) of the population are spread at variable densities across the state, but are largely coastal (Queensland Government Statistician's Office, 2019).

Marine turtle capture data was obtained from 15 sites spread along the Queensland coast, with the most distant sites being separated by more than 2300km. Of these 15 sites, 12 are within the GBR World Heritage Area (Figure 3.1). This huge expanse of coast encompasses study sites both close to and distant from the coast, in addition to catchments which support remote, rural and urban communities with varying human population densities. To reflect this, the estimated resident population numbers for these regions (Queensland Government Statistician's Office, 2019) were combined with expert opinion (which considered the aforementioned variables) to reflect the human influence on each study site (Table 3.1).

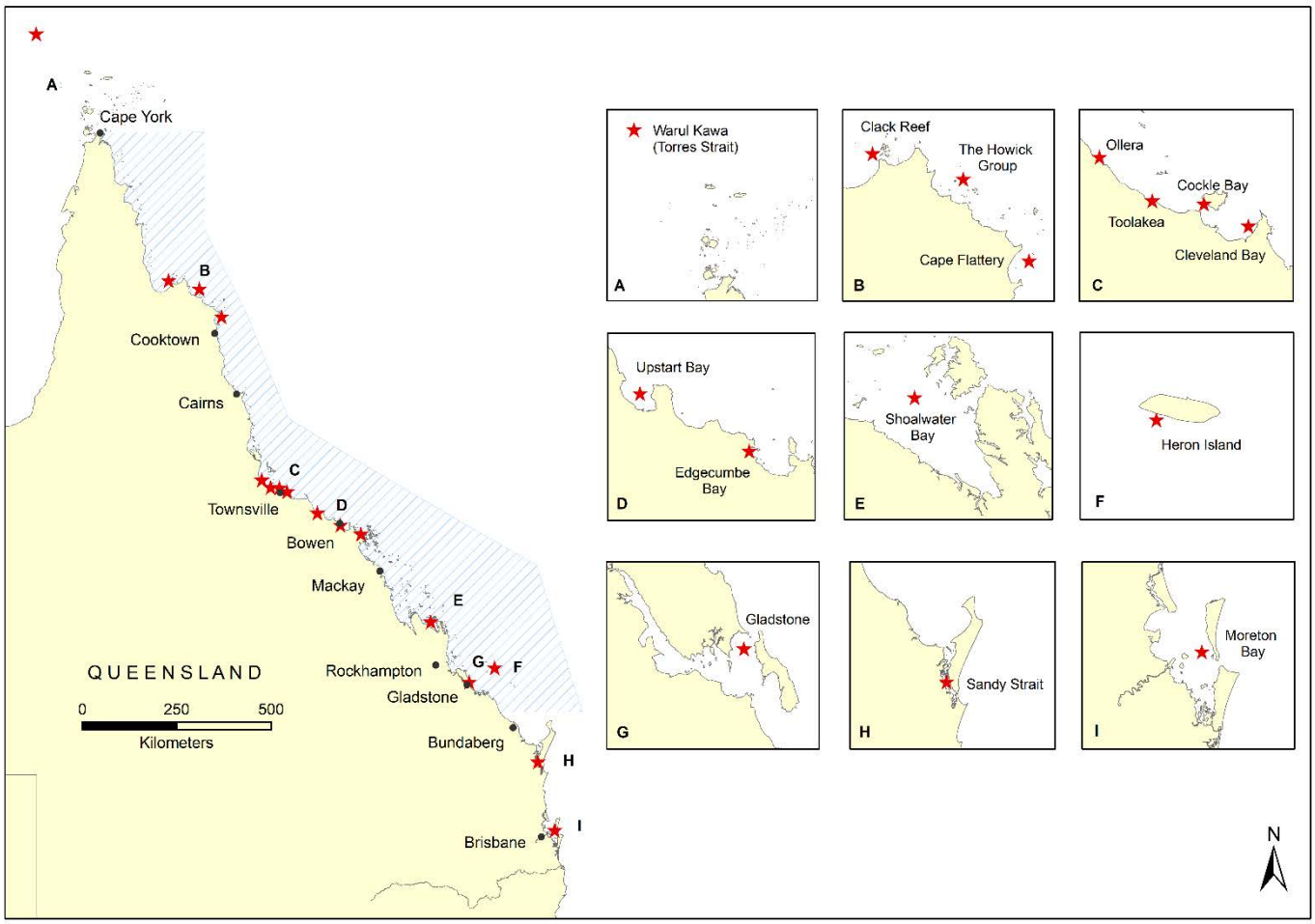

Figure 3.1. Marine turtle capture sites along the Queensland coastline, with more detailed site maps in inset. Sites include: Warul Kawa (A); Clack Reef, The Howick Group, Cape Flattery (B); Ollera, Toolakea, Cockle Bay and Cleveland Bay (C); Upstart Bay and Edgecumbe Bay (D); Shoalwater Bay (E); Heron Island (F); Gladstone (G); Sandy Strait (H); and Moreton Bay (I). The Great Barrier Reef World Heritage Area is indicated by the hatched areas.

Table 3.1. Human influence on the 15 marine turtle capture sites along the Queensland coast. Sites include: Warul Kawa, Clack Reef, Howick Group, Cape Flattery, Ollera, Toolakea, southern Cleveland Bay, Cockle Bay, Upstart Bay, Edgecumbe Bay, western Shoalwater Bay, Gladstone, Heron Island, Sandy Strait, and Moreton Bay.

Study site	Human population density of nearest town
Warul Kawa	Extremely Low
Clack Reef	Low
Howick Group	Low
Cape Flattery	Low
Ollera	Moderate
Toolakea	Moderate
Cockle Bay	High
Southern Cleveland Bay	High
Upstart Bay	Moderate
Edgecombe Bay	Moderate
Western Shoalwater Bay	Low
Gladstone	High
Heron Island	Low
Sandy Strait	High
Moreton Bay	Extremely High

Data collection

Retrospective turtle capture data from a range of sites was extracted from the TURT DATA database, operated by Queensland Turtle Research (Department of Environment and Science; DES) and James Cook University's Turtle Health Research (THR) database. Two additional records of marine turtle population surveys at Warul Kawa in the Torres Strait were obtained from the Torres Strait Regional Authority (TSRA).

The Queensland Turtle Research project commenced in 1968 while James Cook University's Turtle Health Research (THR) team has been studying marine turtle health in Queensland since 2011, often in collaboration with DES. Foraging turtle surveys conducted by THR are more targeted to understanding turtle health, rather than the general and extensive population surveys conducted by DES and TSRA. General population surveys are often conducted over two weeks with hundreds of turtles captured, whereas the turtle health surveys are typically conducted more sporadically as one day fieldtrips several times a year, with smaller numbers of turtles captured. Considering the varying degree of survey intent and extent, the data collected was broken down into two categories: Data generated from 1) Extensive general population surveys and 2) Turtle health focussed surveys.

The final dataset contained data from turtles captured during foraging population surveys either by turtle rodeo technique (Limpus and Reed, 1985) or beach jumping, with recaptured turtles being removed from the dataset to ensure each turtle was only counted once. Upon capture, the curved carapace length (CCL) is measured with flexible tape (±2mm) and used to determine age-class; juvenile (CCL <65.0 cm), sub-adult (CCL 65.0-90.0 cm) and adult (>90.0 cm) (Limpus and Chaloupka, 1997; Limpus et al., 1994a). A total of 23,423 green turtle capture records were included in the final dataset. Capture records from 412 hawksbill (*Eretmochelys imbricata*) and 1810 loggerheard (*Caretta caretta*) turtles were also included, resulting in a final dataset of 25,645 records (Table 3.2).

Table 3.2. The distribution of capture records used for analysis, including study site, survey type, species and number of turtles captured, capture type and time period that the records span. Sites are listed in approximately north to south order, and are divided green (*Chelonia mydas*), hawksbill (*Eretmochelys imbricata*) and loggerheard (*Caretta caretta*) turtle records.

Study site	Survey type	Species	Number of captures	Capture method	Time period spanned
Warul Kawa	General population	Green	325	Beach jump	2016-2017
	General population	Hawksbill	5	Beach jump	2016-2017
Clack Reef	General population	Green	1126	Rodeo jump	1987-1997
Howick Group	General population	Green	3850	Rodeo jump	1996-2016
Cape Flattery	General population	Green	45	Rodeo jump	2000
Ollera	Turtle health	Green	58	Beach jump	2011-2018
Toolakea	Turtle health	Green	117	Beach jump	2011-2017
Cockle Bay	General population	Green	444	Rodeo jump	2002-2016
	Turtle health	Green	138	Rodeo jump	2011-2018
	Turtle health	Hawksbill	9	Rodeo jump	2011-2018
Southern Cleveland Bay	General population	Green	108	Rodeo jump	2014-2016
Upstart Bay	General population	Green	430	Rodeo jump	2012-2016
	Turtle health	Green	63	Rodeo jump	2012-2014
Edgecumbe Bay	General population	Green	1386	Rodeo jump	2000-2016
	Turtle health	Green	541	Rodeo jump	2011-2017
	Turtle health	Loggerhead	3	Rodeo jump	2011-2017
Western Shoalwater Bay	General population	Green	6124	Rodeo jump	1987-2012
Gladstone	General population	Green	338	Rodeo jump	2011-2014
Heron Island	General population	Green	3204	Rodeo jump	1989-1999
	General population	Loggerhead	675	Rodeo jump	1989-1999
	General population	Hawksbill	360	Rodeo jump	1989-1999
Sandy Strait	General population	Green	83	Rodeo jump	1996-2011
Moreton Bay	General population	Green	5043	Rodeo jump	1990-2014
	General population	Loggerhead	1132	Rodeo jump	1990-2014
	General population	Hawksbill	38	Rodeo jump	1990-2014

The records in Table 3.2 were used to determine the prevalence of FP at 15 sites along the Queensland Coast. Available data was used to conduct further analysis of the proportion of FP amongst age classes of green turtles. An annual breakdown of juvenile, sub-adult and adult turtles captured at three sites (western Shoalwater Bay, Heron Island and Moreton Bay) between 1987 and 2014 was generated. Within each age-class, the number of turtles with FP was compared to the total number captured for a particular year, with the results expressed as a percentage.

Generalised linear models were used to investigate factors in the dataset which influenced FP prevalence. As the response variable (FP prevalence) is a proportion derived from the turtle counts,

a logistic regression model was used to investigate factors which may influence FP prevalence. Significant overdispersion was accounted for by using the quasibinomial family to model data dispersion. As the number of independent data points (study sites) was small relative to the number of potential explanatory variables, and several explanatory variables included some missing values, it was not possible to evaluate a model including all explanatory variables. We therefore examined the association of each explanatory variable with FP prevalence separately. Those variables which appeared to show an association were then examined in a combined model. All analyses used R (R Core Team, 2018), via the glm() function in the stats package to fit the models, and the Anova() function in the car package to execute analyses of deviance.

Results

Grouped data

A total of 25,645 records were used to determine FP prevalence and trends at 15 sites along the Queensland coast. Within this dataset, 791 turtles with FP tumours were recorded. Data collected for hawksbill and loggerhead turtles are reported in Table 3.3, but small sample sizes prevented further analysis and conclusions. Prevalence of FP in green turtles at study sites ranged from nil to 11.6% (Table 3.3). High frequencies of FP were found at Cockle Bay, Edgecumbe Bay and Moreton Bay (Table 3.3). Cockle Bay was recorded as having the highest prevalence recorded overall (11.6%), determined from turtle health surveys over 8 years, while the second highest report (10.5%) was from that of a general population survey at Moreton Bay, determined from general population surveys conducted over 25 years (Table 3.3). Prevalence was unevenly distributed among study sites and no evidence of a latitudinal north-south cline in increasing prevalence was observed.

Table 3.3. Prevalence rates of fibropapillomatosis in marine turtles at foraging grounds along the Queensland coastline. Values greater than zero are highlighted in bold.

Study site	Survey Type	Prevalence of FP (%)	Number of turtles with FP tumours	Total Captured	Species
Warul Kawa	General Population	**3.4**	**11**	325	Green
	General Population	0.0	0	5	Hawksbill
Clack Reef	General Population	**0.1**	**1**	1126	Green
Howick Group	General Population	0.0	0	3850	Green
Cape Flattery	General Population	0.0	0	45	Green
Ollera	Turtle Health	0.0	0	58	Green
Toolakea	Turtle Health	0.0	0	117	Green
Cockle Bay	General Population	**0.7**	**3**	444	Green
	Turtle Health	**11.6**	**16**	138	Green
	Turtle Health	0.0	0	9	Hawksbill
Southern Cleveland Bay	General Population	**1.9**	**2**	108	Green
Upstart Bay	General Population	**1.6**	**7**	430	Green
	Turtle Health	0.0	0	63	Green
Edgecumbe Bay	General Population	**0.7**	**10**	1386	Green
	Turtle Health	**7.9**	**43**	541	Green
	Turtle Health	**50.0**	**1**	2	Loggerhead
Western Shoalwater Bay	General Population	**1.6**	**99**	6124	Green
Gladstone	General Population	**3.0**	**10**	338	Green
Heron Island	General Population	**0.3**	**10**	3204	Green
	General Population	**1.3**	**9**	675	Loggerhead
	General Population	0.0	0	360	Hawksbill
Sandy Strait	General Population	**3.6**	**3**	83	Green
Moreton Bay	General Population	**10.5**	**527**	5043	Green
	General Population	**3.4**	**38**	1132	Loggerhead
	General Population	**2.6**	**1**	38	Hawksbill

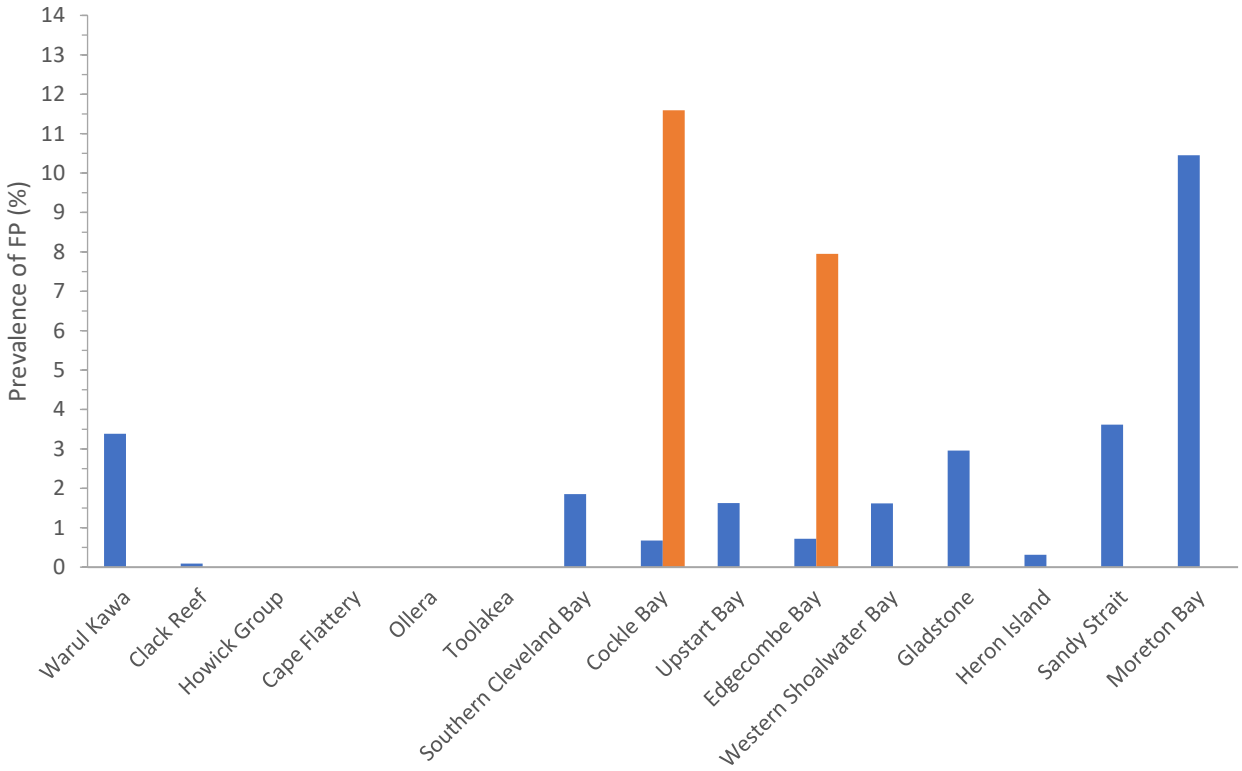

Figure 3.2. Retrospective prevalence rates of fibropapillomatosis in marine turtles at foraging grounds along the Queensland coastline. The difference between FP prevalence recorded by general population surveys (blue) and turtle health surveys (orange) is also in indicated.

Sites associated with high FP prevalence were also associated with moderate to extremely high human density. However, FP was not detected at Ollera or Toolakea despite these sites being associated with moderate human density. Despite being in proximity to extremely low human density, Warul Kawa was found to have a comparable FP prevalence with Gladstone.

Although not quantifiable, an incidental finding of the turtle health survey methods was the identification of two “hotspots” of FP. A narrow section of Cockle Bay and Brisk Bay, a small bay within Edgecumbe Bay, were found to contain a high number of turtles with FP (Figure 3.3). All turtles with FP tumours at these sites were captured within these hotspots. This is in contrast to the general population surveys at the same sites, which captured turtles from across the entirety of inshore areas of these sites, resulting in lower prevalence rates (Figure 3.2).

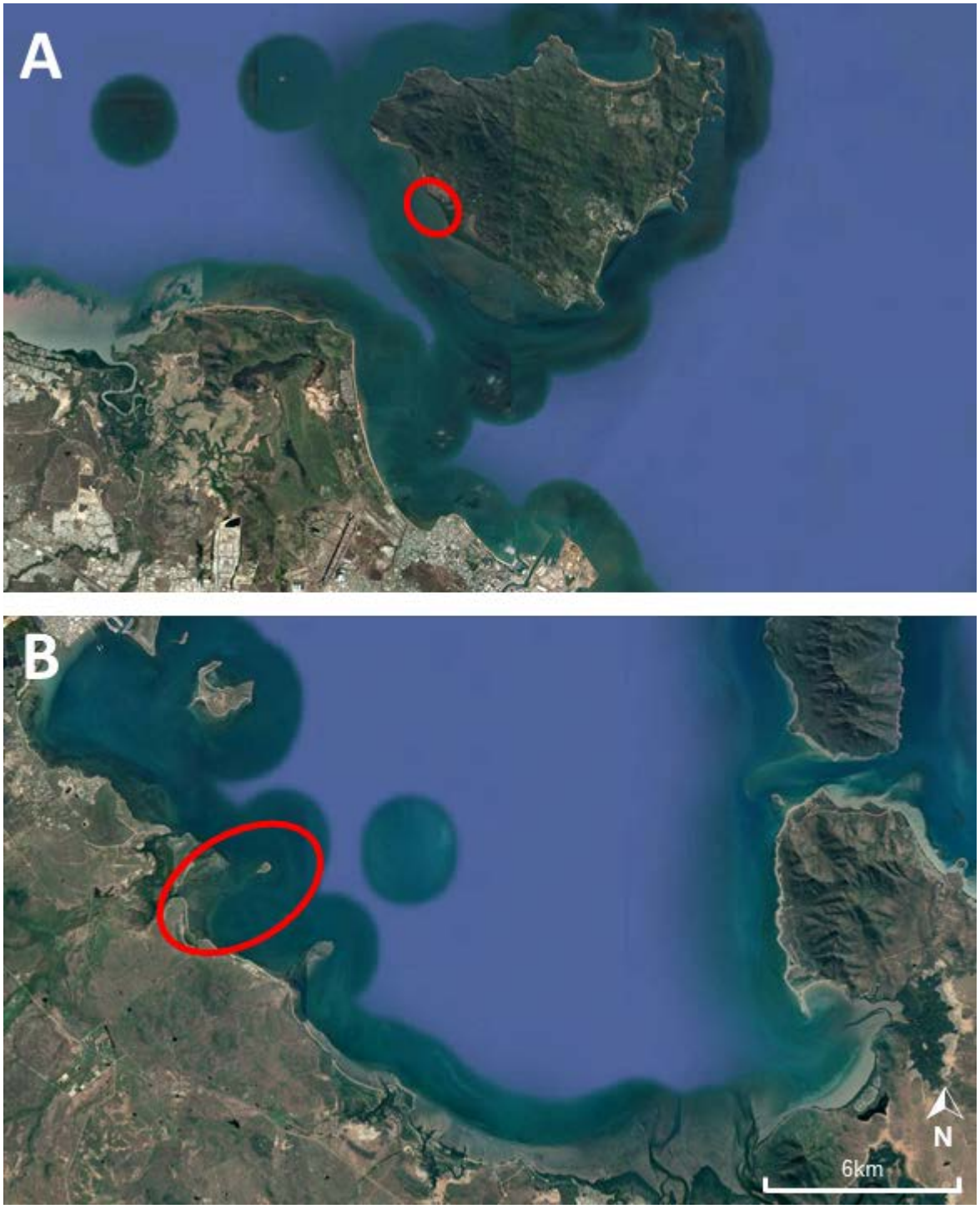

Figure 3.3. The locations of two distinct "hotspots" of fibropapillomatosis: Cockle Bay (A) and Brisk Bay, within Edgecumbe Bay (B). The hotspots, indicated as red circles, were found to contain a high number of green turtles with fibropapillomatosis, while nearby areas hosted few, if any, turtles with FP.

Statistical analysis

The explanatory variables examined individually were study site, human density of nearest town to site, survey method, the average age class of the turtles at each study site (average age class), and the median year of the study undertaken at each study site (median year) (see Appendix 2: Supplementary Table 3.1). Of these, only survey method (Likelihood Ratio (LR) $\chi 2 = 10.778$, df=1, $p = 0.001027$) and median year (LR $\chi 2 = 5.5173$, df = 1, $p = 0.01883$) showed significant association with FP prevalence. General population surveys gave much lower estimates of FP prevalence than turtle health surveys (odds ratio 0.13), and FP prevalence tended to increase with the chronological year of the survey. However, this second effect disappeared when both variables were included in the model, indicating that the apparent temporal trend was probably due to the health surveys only being undertaken after 2012.

Despite the database containing over 25,000 capture records across 15 study sites, further statistical analysis regarding factors influencing FP prevalence was restricted due to insufficient data and varying temporal scales.

Age-class data subset

From the available data, an annual age class breakdown of FP affliction was generated for three sites from the General Population surveys (western Shoalwater Bay, Heron Island and Moreton Bay). This subset of data was collected over bigger temporal scales, with higher numbers of individual turtles, which allowed for a better separation of trends. No comparable dataset could be obtained from the Turtle Health survey methods.

At western Shoalwater Bay, the average prevalence of FP for all green turtles was 1.9%, while among juveniles, sub-adults and adults the prevalence was 4.2%, 1.9% and 0% respectively. At Moreton Bay, while 12.5% of all turtles were affected by FP, the breakdown among age-classes was 15.2% of juveniles, 15.7% of sub-adults and 2.3% of adults. No juvenile turtles with FP were recorded at Heron Island (Appendix Two: Supplementary Table 3.2). Statistical analysis of the effect of age class and sites showed that both were strongly associated with FP prevalence (p <2e-16 for both variables). At all of these sites, although prevalence varies annually, juvenile and sub-adult turtles were the age class with the highest proportion of FP (Figure 3.4). A significant interaction between interaction between age class and study site ($p = 0.01678$) was also detected, suggesting that the effect of age-class is not consistent between the sites. However, further exploration of this was precluded by limited data.

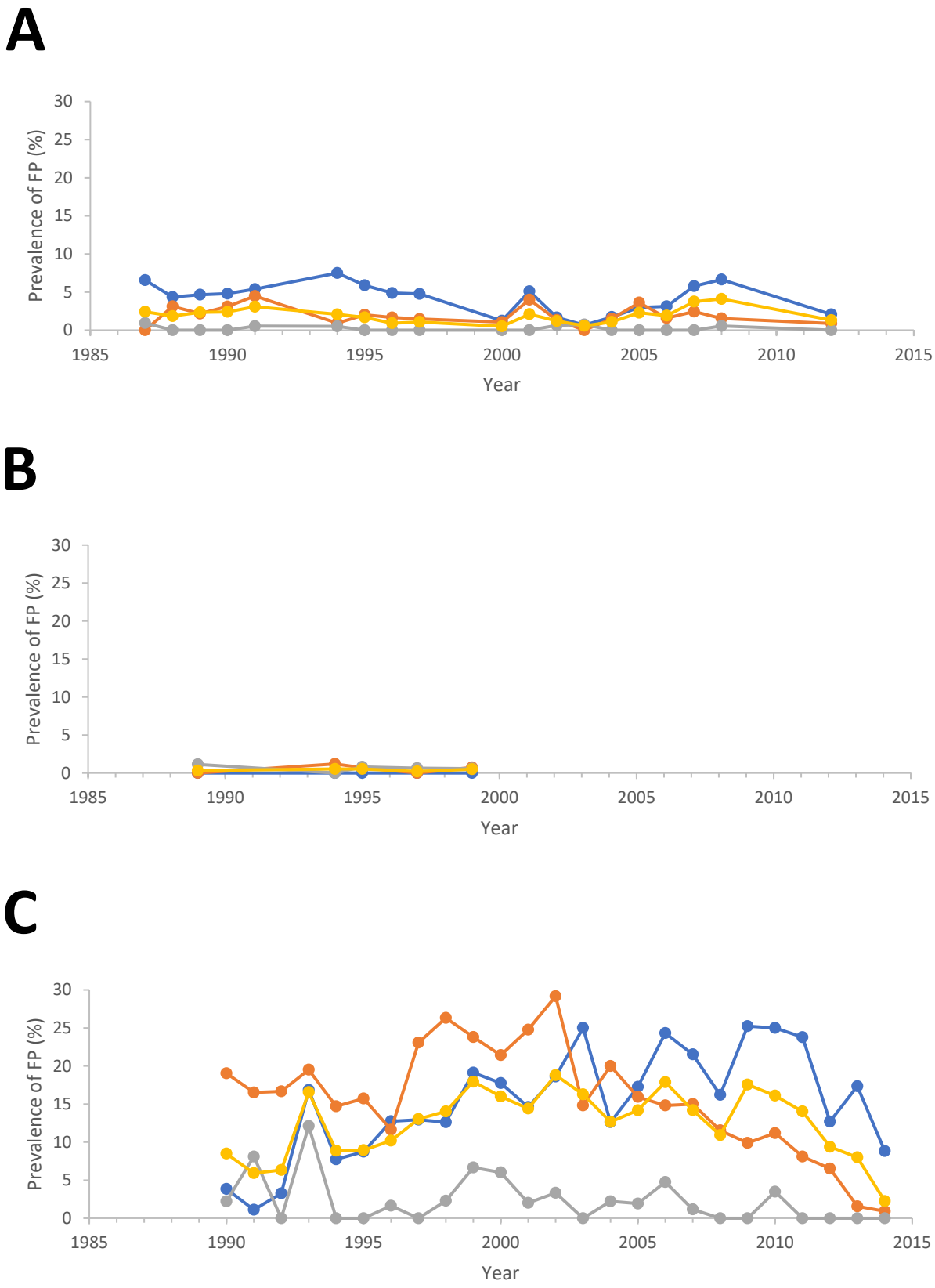

Figure 3.4. Annual age class distribution of fibropapillomatosis between turtles at western Shoalwater Bay (A), Heron Island (B) and Moreton Bay (C). Data collected during general population surveys between 1987 and 2014, with juvenile (blue), sub-adult (orange), adult (grey) and all turtles (yellow) are represented.

Discussion

This study provides the first comprehensive description of FP prevalence in marine turtles in Australia. While the FP prevalence in three species (green, loggerhead and hawksbill turtles) is provided, the data for FP in hawksbills and loggerheads is lacking compared to that for green turtles. Some datasets for these species were quite expansive for loggerheads (Moreton Bay and Heron Island) and hawksbills (Heron Island) and as such, the low FP prevalence is likely a reflection of the lower incidence of this disease in these species, rather than data availability. However, the limited data prevented further analysis of trends for such species, highlighting the need for further investigation. The remainer of this discussion will focus on the prevalence of FP in green turtles.

Here we report that prevalence varies between species, sites and years, with juvenile turtles being the most frequently affected by FP. This disease was rarely observed in adults, while sub-adults were moderately affected at some sites. These age-class results are consistent with other reports of FP around the world (Adnyana et al., 1997; Ene et al., 2005; Herbst, 1994; Herbst and Klein, 1995a; Page-Karjian et al., 2014; Patrício et al., 2012; Work et al., 2004). However, it is interesting to note that despite a significant number of juvenile turtles (n=1047) at Heron island, FP was never recorded in a juvenile turtle at this site.

High prevalence of FP in green turtles were found at Cockle Bay, Edgecumbe Bay and Moreton Bay (Table 3.3), and within Cockle Bay and Edgecumbe Bay two distinct hotpots were also identified (Figure 3.3). All turtles with FP were captured within these hotspots, yet other turtles without FP were also present in these areas. It is unclear whether these hotspots are the site of FP infection, or a refuge for infected turtles. Seeing that the THR team were specifically interested in unhealthy animals, their capture efforts were focused on these hotspots identified during general surveys. The presence of such hotspots within a large region highlights that although areas appear similar, both environmentally and in proximity to human habitation, there may be some unique character(s) that make them stand apart from the rest of that region. However, the cause of this is unclear as yet.

While seasonal variation cannot be discounted by this study, the study sites span several climate zones (Equatorial, Tropical and Subtropical; Bureau of Meteorology (2019)). The wide variation in climate zones in this study would have revealed a latitudinal trend between FP presence and climate if it were present. Yet, although water temperature has been theorised to play a role in tumour development (Herbst, 1994; Herbst, 1995), no latitudinal north-south cline in increasing prevalence was identified in this study. These results indicate that it is unlikely that water temperature has a role in tumour development in this region. However, this suggestion is based on the assumption that water temperate is directly correlated with climate zone, which may not be the case. An analysis

between water temperature and FP prevalence was beyond the scope of this study, but future research would benefit from such an investigation.

Significant differences in rates of FP prevalence were observed between survey methods, with turtle health surveys detecting FP at much higher rates than general population surveys. Population surveys target all age-classes of turtles at a location to better understand and describe the demographics of the population. The longevity and expansive nature of such surveys may mean that turtles with small tumours may go undetected. Conversely, the turtle health surveys in this study target juvenile turtles as they are most likely to be afflicted with health issues of interest to researchers. These surveys are conducted on a smaller scale than the general population surveys and each turtle captured is thoroughly checked for tumours, with all details recorded. As FP predominantly affects juvenile turtles, the turtle health survey data is biased towards detecting FP whilst the detection rate in the general population surveys may be lower. This inherent bias, coupled with the variation in methods and temporal scales, make it difficult to draw accurate conclusions from this dataset. While the general population surveys are arguably more reliable for drawing conclusions on populations as a whole, the turtle health survey data highlights that FP is present in higher numbers at certain specific sites and can be detected if a targeted approach is used (Figure 3.2). For example, at Edgecumbe Bay the general population surveys reported a prevalence of 0.7%, while turtle health surveys reported a prevalence of 7.9%. However, the general population survey and turtle health survey at this site differed in sample size (n=1386 and n =541 respectively), temporal scale (17 years and 8 years respectively) and survey method. Thus, it is difficult to compare the two values. Yet, the number of individual turtles captured with FP reported by the turtle health survey was more than four times greater than that of the general population survey (n=43 and n=10 respectively). It is likely that the true prevalence lies somewhere between those reported from each survey type.

A correlation between high FP prevalence and sites associated with reduced water quality have been consistently reported (Adnyana et al., 1997; Chaloupka et al., 2009; dos Santos et al., 2010; Foley et al., 2005; Herbst, 1995; Jones et al., 2016; Van Houtan et al., 2014). Whilst this general trend was also observed in the present study, some findings do not fully support this. Sites with nil or very low FP prevalence, like that of Heron Island, are all associated with low human activity. Conversely, sites with high FP prevalence, such as Moreton Bay, are heavily influenced by humans due to proximity to large cities and river mouths (McPhee, 2017). However, relatively similar prevalence rates were reported for both Warul Kawa in the Torres Strait (3.4%) and Gladstone (3.0%). These sites are distinctly different with respect to human influence. Warul Kawa is an uninhabited remote island located in northwestern Torres Strait, with no nearby urbanisation, industrialisation or agriculture

(Torres Strait Regional Authority, 2017). The nearest large river system is the Fly River, but based on current flow and oceanography the plume of this river has little influence on Warul Kawa (Wolanski et al., 2013). Conversely, Gladstone is a major port city on the Queensland coast. A range of industries are supported by the region, including agriculture, tourism, commercial fishing, a coal-fired power station and the processing and transport of aluminium, magnesium, coal and various petroleum products (Flint et al., 2015). Disease outbreaks and catastrophically high mortality rates in wildlife (including fish, sharks, rays, crabs, shellfish, turtles, dolphins and dugong) have been reported to coincide with the expansion of the port (Flint et al., 2015; Landos, 2012). An independent investigation linked these mortality events to the dredging associated with the expansion of the port (Landos, 2012). However, the findings of this report were later challenged, with heavy rain and associated flooding of rivers flowing into Gladstone cited as an alternative explanation (Gladstone Ports Corporation, 2013). Regardless of the cause, during this event marine turtle strandings spiked dramatically, yet prevalence of FP did not (Flint et al., 2015). If FP manifestation is indeed linked to reduced water quality, it is surprising that FP prevalence did not increase during this mortality event. That green turtles sampled at Gladstone and Warul Kawa had similar FP prevalence rates, despite drastically different levels of human influence, raises further questions about this theory. However, as a general trend between human influence and FP prevalence was observed in this study, this unexpected finding may simply be an anomaly or, may be due to factors that we have not yet identified.

The age-class results of the present study indicate that juvenile turtles are the most likely to be affected by FP, followed by sub-adult turtles. Adult turtles were rarely reported to be affected by FP. These results are consistent with other reports globally (Adnyana et al., 1997; Ene et al., 2005; Herbst, 1994; Herbst and Klein, 1995a; Page-Karjian et al., 2014; Patrício et al., 2012; Work et al., 2004). Such a trend raises questions regarding what factors of this stage in the lifecycle of green turtles may increase their susceptibility to FP. Upon recruitment to inshore areas from their pelagic existence, green turtles in Australia undergo an ontogenetic shift in diet (Arthur et al., 2008b). It may be possible that the dietary shift from an omnivorous to herbivorous diet in new recruits is associated with the increase in susceptibility to FP. As green turtles consume both macroalgae and seagrass (Brand-Gardner et al., 1999; Read and Limpus, 2002), either or the combination of both could be contributing to this susceptibility. However, studying a possible relationship between seagrass or algae and FP prevalence would be challenging, as coverage of this fodder varies significantly due to season and weather conditions (including flood events). These factors hamper attempts to assess any correlation between seagrass or algal distribution and FP prevalence on a broad scale. Moreover, small scale die-offs of seagrass at specific times and locations could have

disastrous impacts on turtles. In 2011 in north Queensland, high river discharge due to high rainfall and several tropical cyclones had a devasting impact on the distribution and abundance of seagrass (Bell and Ariel, 2011). Marine turtles, subsequently stranded in unprecedented numbers; strandings in the year following this event more than tripled the recorded strandings from the previous year (Meager and Limpus, 2012). Considering this, long-term monitoring programs should investigate any links between seagrass presence and FP prevalence.

This study highlighted some significant limitations in marine turtle surveys which should be addressed in order to better manage these vulnerable species. Here, we demonstrate that there were significant differences in reported FP prevalence between survey methods. Future research would benefit from the adoption of targeted and consistent survey methods for sampling marine turtles and determining FP prevalence data. This would improve the reliability of the reported FP prevalence, which will allow managers to make informed decisions regarding conservation efforts and the implementation of management measures. It should be noted that this study, consistent with others around the world, is based on the presence or absence of FP tumours. Future research would benefit from serological surveys of wild populations which test for current infection of, or past exposure to, the likely aetiological agent of FP (chelonid alphaherpesvirus 5 (ChHV5)).

The results of this study indicate that while the correlation between FP prevalence and water quality is not linear, the trend does exist. At present, it is unclear what is influencing this correlation. It may be that it is not reduced water quality as a whole, but a particular water quality variable (such as metals or nutrients) which could be driving this correlation. Future research should aim to resolve this by examining individual water quality variables of interest to determine if this is the case. Understanding this relationship will allow for informed management of this vulnerable species.

The aims of this chapter were addressed as follows:

1. **Characterize FP prevalence at a range of locations spanning the Queensland coastline**

   This study is the first comprehensive report of FP prevalence in Australia. Retrospective data for 15 sites along the Queensland coast was obtained from three established databases. A total of 25,645 records were used to determine FP prevalence and trends at 15 sites along the Queensland coast. Within this dataset, 791 turtles with FP tumours were recorded. Here we report that prevalence varies between sites and years, with juvenile turtles being the most frequently affected by FP. We also report that survey method has a significant influence on the apparent FP prevalence value at each site. That is, surveys which explicitly target FP detect higher numbers of individual turtles with FP, and therefore generate higher prevalence rates than general population surveys. This study highlighted shortcomings in both methods with respect to FP detection, and this must be considered when interpreting results.

   High FP prevalence was loosely correlated with human density, but this trend was not linear. These results raised questions about what factors, on a finer scale, could be influencing FP prevalence at marine turtle foraging grounds.

Publications and presentations arising from this study

- **Jones, K.** 2015. Fibropapillomatosis on the Great Barrier Reef: Directions of future research. *Proceedings from the international summit on fibropapillomatosis of marine turtles: Global status, trends and population impacts.* 11-14th June 2015, Honolulu, Hawaii.
- Limpus, C., **Jones, K.** and Chaloupka, M. 2015. Fibropapilloma disease in marine turtles: Eastern Indian Ocean – south western Pacific Ocean. *Proceedings from the international summit on fibropapillomatosis of marine turtles: Global status, trends and population impacts*. 11-14th June 2015, Honolulu, Hawaii.
- **Manuscript in progress:**

  The following manuscript combines Chapter Three and Chapter Four of this thesis:

  Jones, K., Limpus, C., Brodie, J., Jones, R., Shum, E., Read, M. and Ariel, E. 2019. Investigating the relationship between water quality and prevalence of fibropapillomatosis in green turtles (*Chelonia mydas*) on the Great Barrier Reef. *In progress.*

My contributions to this study

- I generated the ethics application and associated fieldwork to collect FP prevalence data from a subset of the sites investigated in this study (referred to in this chapter as the data from the JCU Turtle Health Database)

- I attended fieldtrips and liaised with other researchers whose data was supplied for analysis in this study, to ensure I understood their research methodology and that each could be cohesively compared.
- I contributed to the International Summit on FP of Marine Turtles (Global Status, Trends and Population Impacts) in Hawaii in 2015, and as result I am now a member of the International FP Working Group.
- I worked in person with researchers at the Department of Environment and Science and the Torres Strait Regional Authority to collect and clean the datasets for analysis
- I managed the datasets obtained
- I attended workshops which focussed on the use of the R-Studio program which aided in the statistical analysis
- Under advisement from an experienced statistician, I prepared the input file for statistical analysis and interpreted the results
- I drafted the chapter and edited it as advised by collaborators and supervisors

Chapter Four:
Investigating the relationship between water quality and prevalence of fibropapillomatosis in green turtles (*Chelonia mydas*) on the Great Barrier Reef

Backgrounds and aims of this chapter

The results of Chapter Three indicated that high FP prevalence was associated with higher human density. However, some sites with significantly different levels of human influence had comparable FP prevalence values. These results sparked further investigation regarding water quality and specific water quality variables which may be driving the correlation reported in other regions. Moreover, although a link between FP prevalence and reduced water quality has been observed in other regions, this relationship has never been investigated in Australia. This study aims to fill this knowledge gap, and better inform management, by assessing the relationship between FP prevalence and water quality on the GBR. Specifically, this study aims to:

1. Develop water quality indices for parameters of interest to marine turtles at 14 sites along the GBR
2. Investigate any link between water quality and FP on the GBR

Introduction

Marine turtles face many challenges, with six of the seven species of marine turtle being considered threatened in some capacity, and flatback turtles listed as data deficient (IUCN, 2019). These animals have a complex life-history which includes exhibiting a high degree of site fidelity; once recruited into a foraging area turtles will typically remain there (Musick and Limpus, 1997). As marine turtles are likely to remain in their chosen foraging area, irrespective of damage or destruction to these areas, they are particularly susceptible to the detrimental effects of environmental change. It is this trait, coupled with their long-lived nature, that has led them to be recognised as sentinels of marine ecosystem health (Aguirre and Lutz, 2004).

Fibropapillomatosis (FP) is a neoplastic condition which can afflict all species of marine turtle, but predominantly affects the green turtle (*Chelonia mydas*). This disease has been reported in every major ocean basin that turtles are known to inhabit (Herbst, 1994) and is characterised by the presence of benign tumours which can be present on the soft tissue, carapace, plastron, cornea and/or viscera (Jones et al., 2016). However, despite ongoing research, there are many knowledge gaps surrounding FP. For example, the prevalence of the disease varies both spatially and temporally (Jones et al., 2016), with the reason for this evading researchers.

Despite these knowledge gaps in our understanding of FP, a consistently observed element of the epidemiology of this disease is a link between FP prevalence and water quality, with high FP prevalence often reported in areas associated with reduced water quality (Adnyana et al., 1997; Chaloupka et al., 2009; dos Santos et al., 2010; Foley et al., 2005; Herbst, 1995; Jones et al., 2016; Van Houtan et al., 2014). Typically, these sites are associated with high anthropogenic influences like agriculture, urbanisation and/or industrialisation.

While it is possible that FP manifestation is multifactorial (Herbst, 1994; Jones et al., 2016), several studies have attempted to elucidate factors which may be responsible for triggering tumour development. A recent study detected high concentrations of copper and lead in the blood of marine turtles severely affected by FP compared to turtles without FP tumours (da Silva et al., 2016). Other studies have described very low concentrations of persistent organic pollutants(Keller et al., 2014; Sánchez-Sarmiento et al., 2017) and selected trace metals and organic pollutants (Aguirre et al., 1994a) in turtles with FP tumours, but the concentrations were not significantly different between turtles with and without FP tumours. A strong link between FP prevalence, nitrogen-footprints and macroalgae consumed by green turtles has been demonstrated, with an invasive macroalgae bloom increasing the concentration of arginine in the diet of turtles in Hawaii (Van Houtan et al., 2010). Subsequently, a link was established between eutrophication and arginine

content of macroalgae, with the intake of arginine in turtles at eutrophied sites being up to 14 times the background level (Van Houtan et al., 2014). However, the conclusions from this study were subsequently challenged based on a lack of inferential framework and compelling evidence (Work et al., 2014). Correlations between FP prevalence and presence of natural biotoxins have also been suggested and investigated (Arthur et al., 2006a; Landsberg et al., 1999). A subsequent study discounted this correlation as the particular algae containing the biotoxin was found to comprise only 2% of turtle diet (Arthur et al., 2008a). It is worth noting that the studies linking natural biotoxins to FP development all found a link between blooms of algae or cyanobacteria but had difficulty proving a cause-and-effect of this relationship. It may be possible that it is not the bloom, but the environmental factors which promoted the bloom, that are also triggering FP expression.

Despite the challenges of determining the prevalence of FP within populations, clear "hot spots" have been identified along the Queensland coast (see Chapter Two). These areas with a high FP prevalence indicate that the cause is localised, and this allows us to investigate potential triggers and/or co-factors in disease manifestation.

Water quality is a complex field, with any physical, chemical, or biological property that influences the suitability of water for natural ecological systems or use by humans being considered a water quality variable (Boyd, 2015). These variables can be natural or anthropogenic, and may work alone or in synergy to influence water quality. While an extensive array of these variables exist, typically only a smaller sub-section is investigated for a particular purpose (Boyd, 2015). The variables of interest can be subdivided into the categories of nutrients, suspended solids, pesticides and metals. Each of these categories is broad and intricate, and it is important to narrow down the variables within each category to those that could have a biological link with FP manifestation. In this study, we have chosen to investigate water quality variables which are likely to have a direct, or indirect, effect on turtles. Metals and pesticides have the potential to have a direct toxic effect on turtles, while dissolved inorganic nitrogen (DIN) and total suspended solids (TSS) may detrimentally affect seagrass growth (a key food source for green turtles (Read and Limpus, 2002)) and indirectly effect the health and presence of green turtles in their foraging grounds.

Water quality on the Great Barrier Reef (GBR) has been studied extensively and reported through a range of long-term monitoring programs from several institutions (Brodie and Waterhouse, 2012). The water quality parameters of greatest concern to the GBR are typically enhanced levels of suspended sediments, excess nutrients and pesticides (mainly PSII herbicides). These substances enter the GBR lagoon through discharge from adjacent catchments, and are therefore at their peak

concentrations during the summer wet season (December-April), transported by periods of high rainfall and river discharge (Waterhouse et al., 2017).

Due to the complexities associated with determining the concentrations of water quality parameters spatially and temporally, many studies rely on modelling and water quality indices as proxies. Water quality indices are designed to convert selected water quality parameters into a dimensionless number for a particular location and time. This number transforms an otherwise complex concept into a simple and easily understandable value, which can then be compared between locations and years to monitor changes (Sutadian et al., 2016) and the effectiveness of management arrangements to improve water quality.

Although a link between FP prevalence and reduced water quality has been observed in other regions (Adnyana et al., 1997; dos Santos et al., 2010; Foley et al., 2005; Herbst, 1994; Van Houtan et al., 2010) and this link is acknowledged in the 2017 Marine Turtle Recovery Plan released by the Australian Government (Department of the Environment and Energy, 2017), this relationship has never been investigated in Australia. This study aims to fill this knowledge gap and better inform management by assessing the relationship between FP prevalence and water quality on the GBR. Specifically, this study aims to 1) develop water quality indices for parameters of interest to marine turtles and 2) investigate any link between water quality and FP on the GBR.

Materials and Methods

To explore the potential relationship between FP prevalence and water quality on the GBR, information on water quality parameters likely to have an influence on marine turtles were extracted from the published literature. This information, coupled with expert opinion, was used to generate indices of water quality for various sites along the GBR. These indices were then compared with FP prevalence in green turtles at the same sites.

FP Prevalence Data and Study Site Selection

This study utilised the retrospective FP prevalence data collected in Chapter Two. In summary, marine turtle capture records from 15 sites along the Queensland coast were extracted from established databases and used to determine the prevalence of FP at each site. These sites were Warul Kawa (Torres Strait), Clack Reef, The Howick Group, Cape Flattery, Ollera, Toolakea, Cockle Bay, Cleveland Bay, Upstart Bay and Edgecumbe Bay, Shoalwater Bay, Heron Island, Gladstone, Sandy Strait, and Moreton Bay (see Figure 2.1 in Chapter Two). The spread of study sites extended more than 2300km with 12 study sites located within the GBR World Heritage Area (GBRWHA); and one site each located in the Torres Strait, Sandy Strait Marine Park and Moreton Bay Marine Park.

While the GBRWHA area encompasses most of the area of the GBR, the boundary excludes some large areas of contiguous ecosystems which are ecologically connected (Brodie and Pearson, 2016; Johnson et al., 2018). The Torres Strait and Hervey Bay are two such sites as they share a common northern and southern boundary with the GBRWHA respectively. As such, a more comprehensive management province for the GBR has been proposed which includes these areas and the overall catchment of this region (Brodie and Pearson, 2016). The proposed management province, referred to as the “Greater GBR” is shown in Figure 4.1. This area is also significant to the spatial ecology of green turtles, which extends beyond the current boundary of the GBRWHA into the same contiguous ecosystems described by Brodie and Pearson (2016) (Figure 4.2; Johnson et al. (2018)).

Figure 4.1. Proposed boundaries of the Great Barrier. The area inside the red line is the boundary of the GBRWHA while the entire area shaded yellow is the proposed Greater GBR management area, including the GBR catchment area, the GBRWHA, Torres Strait and Hervey Bay. Map prepared by J. Waterhouse, TropWATER. Data for the GBR provided by the Great Barrier Reef Marine Park Authority (Brodie and Pearson, 2016)

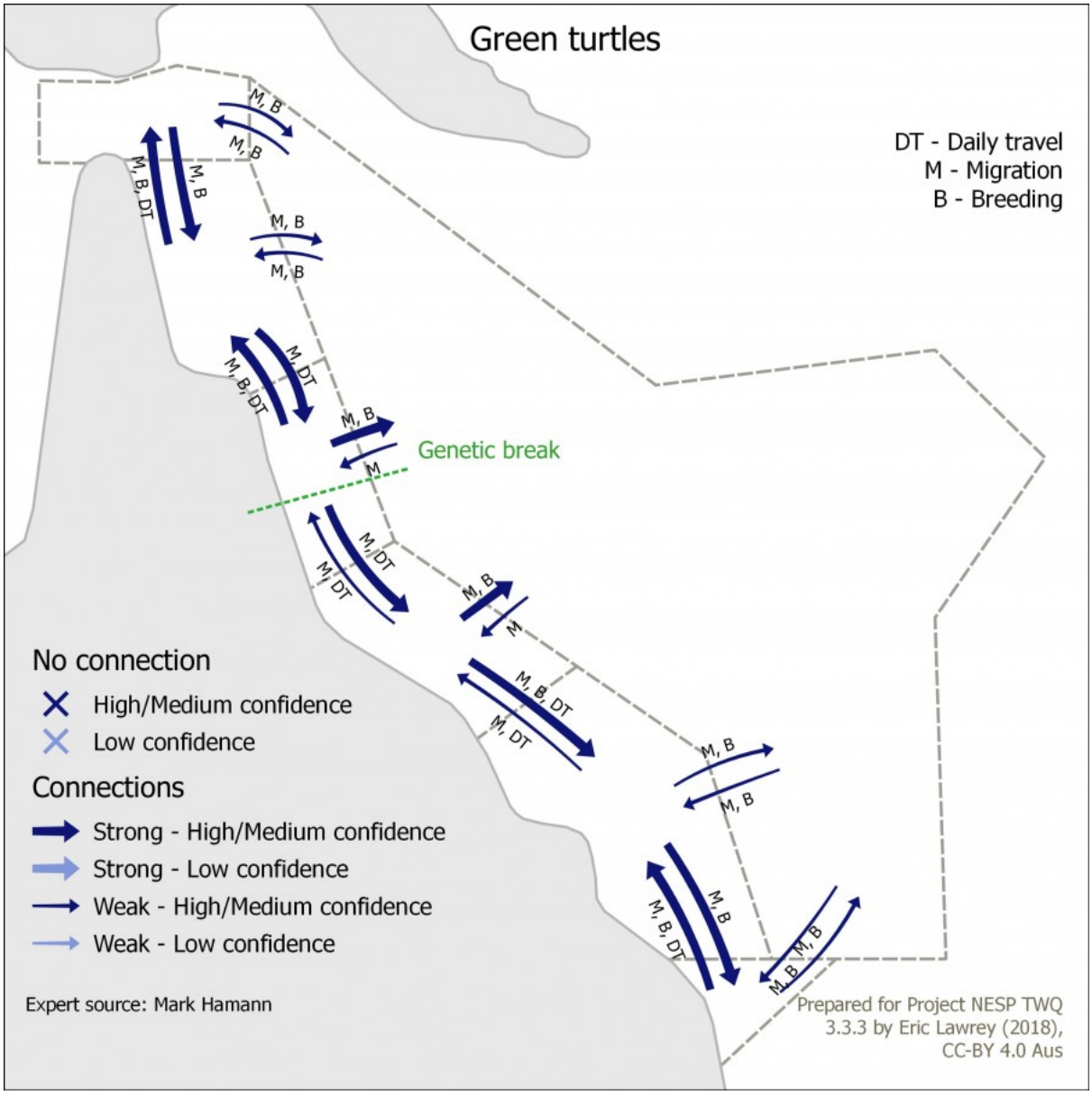

Figure 4.2. Connectivity map for green turtles. The genetic break reflects the approximate boundary where turtles residing north and south are more likely to be part of the northern and southern GBR breeding stocks respectively. A third genetic stock breeds on Coral Sea islands and disperses into the GBR (Johnson et al., 2018)

Due to this connectivity of ecosystems, these sites are typically studied and managed together. Considering this, further analysis in the present study was restricted to only those sites located within the Greater GBR in order to align with available water quality data and all sites from Chapter Two (except Moreton Bay) were therefore included in the present study, making the total number of study sites 14 (Figure 4.3).

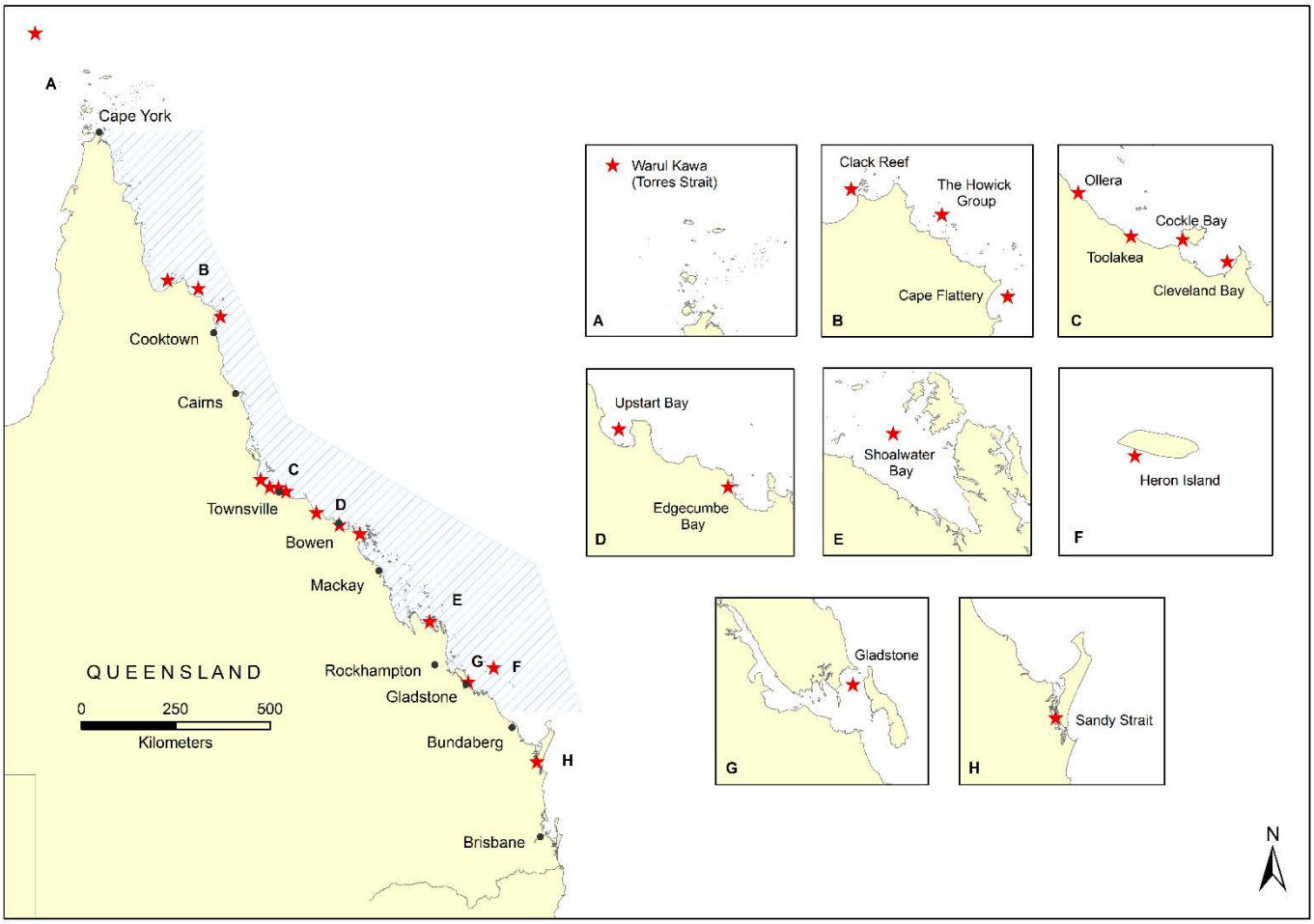

Figure 4.3. Marine turtle capture sites along the Queensland coastline, with more detailed site maps in inset. Sites include: Warul Kawa (A); Clack Reef, The Howick Group, Cape Flattery (B); Ollera, Toolakea, Cockle Bay and Cleveland Bay (C); Upstart Bay and Edgecumbe Bay (D); Shoalwater Bay (E); Heron Island (F); Gladstone (G); Sandy Strait (H). The Great Barrier Reef World Heritage Area is indicated by the hatched areas.

In addition to the FP prevalence data described in Chapter Two, a further subset of these data were used for the present study. The individual capture records for turtles in the Turtle Health Research Database were used to create a more data-rich file for statistical analysis. This subset comprised 1028 capture records of green turtles at five of the study sites: Ollera (n=63), Toolakea (n=147), Cockle Bay (n=139), Upstart Bay (n=63) and Edgecumbe Bay (n=616). As with the original dataset, recaptures were excluded from this subset in order to ensure accuracy in prevalence values. Two different datasets of FP prevalence were analysed alongside the WQIs: 1) The dataset described in Chapter Two with one prevalence value for a grouped range of years and 2) The dataset described in the present study with individual capture records for turtles at a subset of the sites described in the first dataset. As such, the results of these analysis will be presented in two separate sections to correspond to these datasets.

Development of Water Quality Indices (WQI)

Although there is no standardised method of developing water quality indices (WQI) (Sutadian et al., 2016), the general steps outlined by (Abbasi and Abbasi, 2012) were followed to develop the WQI in the present study. That is:

1. Selection of parameters
2. Obtaining sub-index values
3. Establishing weights to denote relative importance and influence on the final index value
4. Aggregation of sub-indices to produce a final index

These steps are highlighted in Figure 4.4.

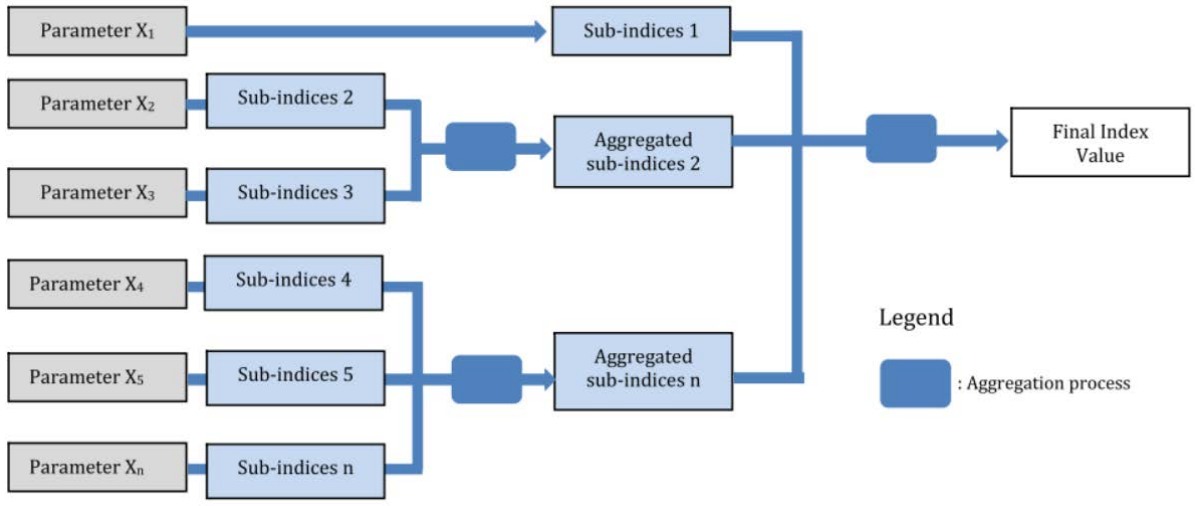

Figure 4.4. General structure of an index (Sutadian et al., 2016)

Water quality parameters of interest to this study were selected following an extensive literature search. The general parameters selected were nutrients, suspended solids, pesticides and metals. Both DIN and TSS were selected sub-indices of nutrients and suspended solids respectively as they are well described indicators of water quality which are frequently used in the region (Waterhouse et al., 2017). Moreover, these parameters have direct links to seagrass health and growth and are therefore of importance to green turtles. For example, an increase of DIN at a locality may result in a loss of seagrass due to eutrophication of other marine plant species and light attenuation affecting photosynthesis of the bottom-dwelling seagrass. For pesticides and metals, the parameter selection was less clear as little is known about the effects of either on marine turtle health. Due to the exploratory nature of this study, we elected to examine pesticides and metals as parameters with no sub-indices to determine if there was any relationship with either of these parameters as a whole (Figure 4.5). None of the parameters were weighted as data availability limited such depth of analysis.

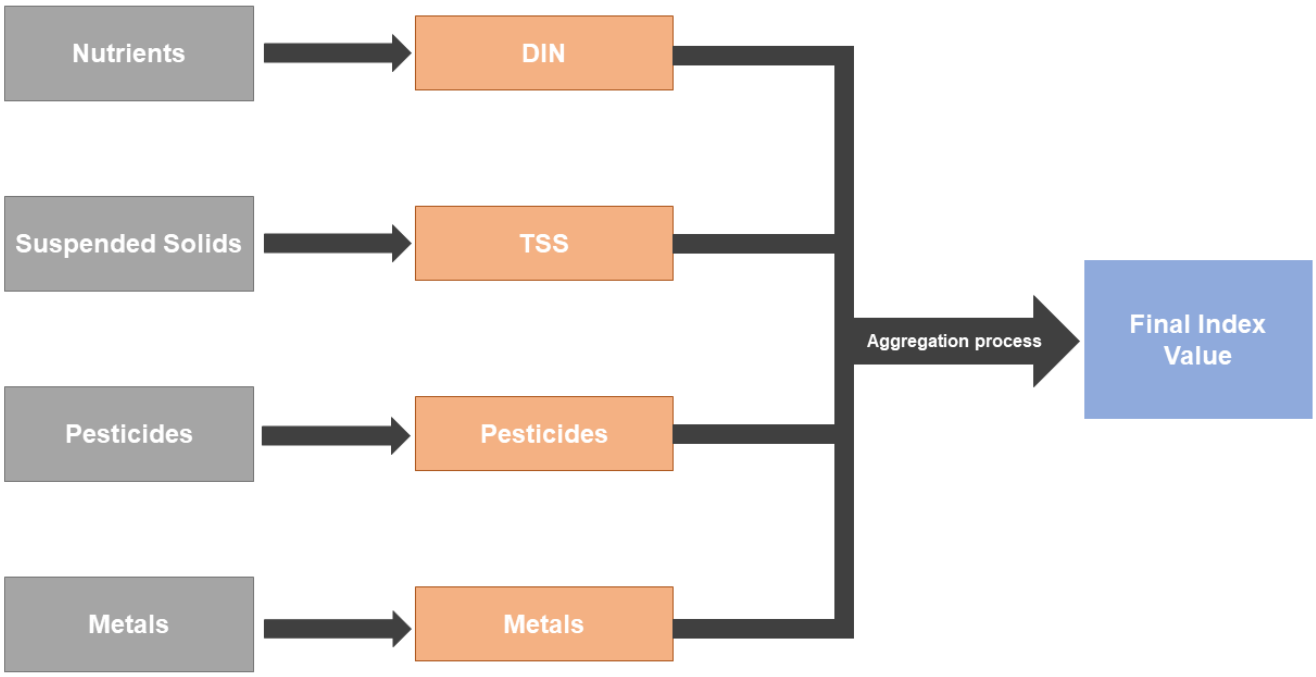

Figure 4.5. An overview of the water quality index structure in the present study.

Each sub-index was developed using a combination of available data in the published literature, and expert opinion where needed. Consistent with other WQI development studies in Australia (Jahan and Strezov, 2017; Ladson et al., 1999), all data obtained was then scored on a 5-point scale. In the present study the lowest score (1) indicated low levels of exposure to a particular parameter while the hightest score (5) indicated high levels of exposure. Proximity to sources of parameters of interest were considered, including proximity to river mouths and land-based activities conducted within the catchment associated with particular river systems (E.g. cropping and grazing). However, distinct river plumes formed during high river discharge events typically move northward along the Queensland coast (Waterhouse et al., 2017) and as such, rivers to the south of a study site are often more influential than the ones to the north. This was also considered when developing the sub-indices. The study sites in this study, their likely river influences and associated activities are outlined in Table 4.4. The approximate distances to the likely river influences were determined from a measurement of the shortest, straight distance between river mouths and the study site. As such, they do not account for obstructions, such as headlands, in this path. An outline of the relative positions of the study sites and main river influences is provided in Supplementary Figures 4.1-4.8 in Appendix 2.

Table 4.4. The study sites used in the present study, the main river influences acting on these sites and other considerations about these sites including land-use in the region. All distances are approximate and are reported to the nearest 10 km.

Site	Main river influences	Distance to main river influence	Descriptors
Warul Kawa	Fly River	230 km to the Fly River	Warul Kawa is an uninhabited remote island located in the northwest Torres Strait, with no nearby urbanisation, industrialisation or agriculture. The Fly River plume predominantly flows northeast (Wolanski et al., 2013) and, therefore, has little influence on this island. There are also two rivers to the northeast of Warul Kawa (Wassi Kussa and Mai Kussa) but these are both small with limited flow.
Clack Reef	Normanby River	40 km to the Normanby River	Clack Reef is located adjacent to the mouth of the Normanby River, which is associated with grazing and a high sediment load (Brodie et al., 2017; Howley, 2015).
Howick Group	Endeavour River	110 km to the Endeavour River	The Howick Group is a group of remote mid-shelf, uninhabited islands and reefs which are located distant to the nearest population centre and offshore from the Endeavour and Annan Rivers. The Endeavour catchment has low to moderate levels of agriculture, development and one small town (Cooktown) with likely low pressures from nutrient, sediment, and pesticide loads, or water regime changes and habitat alterations (Bell et al., 2019).
Cape Flattery	Endeavour River and Annan River	50 km to the Endeavour River 60 km to the Annan River	Cape Flattery shares similar characteristics with the Howick Group, but is located closer to the Endeavour and Annan Rivers. The Annan River is less developed than the Endeavour with little cropping and no major towns. However, it has previously been associated with mining activity and aquaculture.
Ollera	Ollera Creek, Bohle River, Ross River, Haughton Rive, Barratta Creek and Burdekin River	0 km to Ollera Creek 40 km to Bohle River 60 km to Ross River 90 km Haughton River 100km to Barratta Creek 120km to Burdekin River	A beach located on Halifax Bay adjacent to the mouth of Ollera Creek, which drains significant sugarcane cultivation in this region. The Bohle River drains some urban areas and light industry of Townsville. Influence from all other rivers is reduced due to distance.

Toolakea	Bluewater Creek, Ross River, Bohle River, Haughton River, Barratta Creek and Burdekin River	0 km to Bluewater Creek 30 km to Ross River 10 km to Bohle River 60 km Haughton River 80 km to Barratta Creek 110km to Burdekin River	Located close to Ollera and therefore shares similar characteristics. Toolakea is also influenced by Bluewater Creek which drains moderate urban areas.
Cockle Bay	Ross River, Haughton River, Barratta Creek, Burdekin River	10 km to Ross River 40 km Haughton River 50 km to Barratta Creek 100 km to Burdekin River	A small bay located on the southwest side of Magnetic Island, a small island approximately 8km offshore from the city of Townsville. Townsville is a large urban and industrial hub, and Cockle Bay is influenced by the activities occurring within and adjacent to Townsville. Townsville hosts one of the world's largest zinc refineries, a copper refinery, nickel refinery and a large port. The Ross River drains a large proportion of Townsville and therefore has heavy urban and light industrial influences. The Ross is also heavily hydrologically modified with one large dam and three weirs providing barriers to water flow.
Southern Cleveland Bay	Ross River, Haughton River, Barratta Creek, Burdekin River	10 km to Ross River 30 km Haughton River 40 km to Barratta Creek 80 km to Burdekin River	Cleveland Bay is an embayment adjacent of the city of Townsville, located south to Cockle Bay and Townsville City. Due to this location, this study site shares the same considerations as Cockle Bay.
Upstart Bay	Burdekin River	0 km the Burdekin	The catchment adjacent to Upstart Bay is dominated by agricultural and legacy mining activities (Bartley et al., 2014). The Burdekin River, which significantly influences Upstart Bay, is Australia's largest, most intensively developed agricultural floodplain (Davis et al., 2013). This river is the largest contributor of suspended sediment of all rivers in the GBR catchment, and also discharges large loads of nitrogen, phosphorus and pesticides (Davis et al., 2013). Molongle Creek, a small creek that also flows into Upstart Bay, drains some areas if intensive horticulture. There is limited horticulture to the south of this site.
Edgecumbe Bay	Gregory River and Don River	10 km to Gregory River 20 km to Don River	Influence from the Gregory River which drains a sugarcane region. Reduced influence from the Don River, which is also associated with some horticulture, due to north-flowing plume. Human populations in the Proserpine basin in proximity to Edgecumbe

			Bay are low, with the small urban centres of Proserpine and Airlie Beach situated to the south (Brodie et al., 2014).
Western Shoalwater Bay	Styx River and Fitzroy River	50 km to Styx River 140 km to Fitzroy River	Shoalwater Bay is irregularly influenced by the Fitzroy River, located to the south, which is associated with grazing. Very rarely influenced by the Mary River and Burnett Rivers. The bay itself is a designated military training ground, but is distant to urban and industrial influences.
Gladstone	Calliope River, Auckland Creek, Boyne River and Fitzroy River	10 km to Calliope River 10 km to Auckland Creek 20 km to Boyne River 30km to Fitzroy River	Gladstone supports a major port (Port Curtis) and is influenced by the Boyne River, Calliope River and Auckland Creek. This region supports a number of agricultural and industrial activities, including the processing and transport of magnesium, aluminium, coal and petroleum products, a local fishing industry, tourism and a coal-fired power station (Flint et al., 2015). Limited influence from the Fitzroy River, which is associated with grazing.
Heron Island	Fitzroy River, Burnett River and Mary River (all rarely)	110 km Fitzroy River 150 km to Burnett River 240 km to Mary River	Located offshore from Gladstone, Heron Island is influenced (extremely rarely) from the Fitzroy River, Burnett River and Mary River. An event in 1991 saw a flood plume extend to Heron Island (Brodie and Mitchell, 1991), yet other than this event this location is relatively untouched by mainland influences. The island itself supports an ecotourism resort, sewage treatment plant, research station and field station.
Sandy Strait	Mary River	0 km to Mary River	Most significantly influenced by the Mary River, which drains a region associated with sugarcane cultivation, beef-grazing, dairy grazing and other smaller areas of cropping farms (mainly horticulture). This area is most significantly associated with tourism and commercial fishing industries, with some influence from urban and industrial sources.

Nutrients: Dissolved In-organic Nitrogen (DIN)

The demonstrated usefulness of DIN as a nutrient indicator, coupled with the availability of data for this parameter (Waterhouse et al., 2017), led to its selection as a sub-index in the present study. The modelled likelihood of exposure to anthropogenic DIN along the GBR was assessed in Waterhouse et al. (2017) using three different spatial layers. These layers accounted for wet season and annual influences on DIN, as both conditions have roles in determining DIN levels at particular locations. The wet season influence was calculated using a long-term dataset (2003-2016) while the annual influence was calculated using the difference between current (2011 to 2014) and the estimated pre-development annual average concentrations of nutrients (measured as Chlorophyll *a*) in the water column. The combination of these spatial layers provides an indication of where the greatest probability of being exposed to DIN-enriched waters is likely to be. The results of this study were scored on a 6-point scale (Waterhouse et al., 2017). As this information was readily available, current and considered a long-term dataset, we used the modelled likelihood of exposure of GBR ecosystems to anthropogenic DIN results for the present study. To align with all other parameters, these results were transformed to a 5-point scale (with a score of 5 indicating the highest DIN exposure) and rounded to the nearest whole number for our analysis. Where scores were between values due to the conversion, anthropogenic river load data and the targets set for individual rivers were used to determine the final score (Brodie et al., 2017).The resulting DIN sub-indices are reported in Table 4.5. Note that although DIN exposure was not modelled for the Torres Strait in Waterhouse et al. (2017), DIN concentration is uniformly low throughout the Torres Strait and there are no significant anthropogenic sources of DIN (Waterhouse, 2013). Considering this, Warul Kawa was given a score of 1 for DIN. Similarly, DIN in the Cape York area is typically low and as such, this study site was also given a score of one.

Table 4.5. Sub-index scores for DIN exposure at the 14 sites examined in this study. Sites are listed in approximately north to south order.

Study site	Score
Warul Kawa	1
Clack Reef	1
Howick Group	1
Cape Flattery	1
Ollera	2
Toolakea	2
Cockle Bay	3
Southern Cleveland Bay	3
Upstart Bay	3
Edgecumbe Bay	2
Western Shoalwater Bay	1
Gladstone	5
Heron Island	1
Sandy Strait	3

Suspended Solids: TSS

Similar to DIN, data availability and demonstrated usefulness of TSS as an indicator of suspended solids (Waterhouse et al., 2017) led to its selection for use in the present study. The likelihood of exposure of GBR ecosystems to anthropogenic TSS was also modelled by Waterhouse et al. (2017) using four spatial layers which considered wet season and annual influences. The wet season influence was calculated using models that predicted dispersion of end of catchment TSS loads and suspended sediment exposure in the wet season. The annual influence was calculated using the difference between the current (2011-2014) annual light attenuation and pre-development scenarios. The combination of these spatial layers provides an indication of where the greatest probability of being exposed to TSS-enriched waters is likely to be from river discharges (Waterhouse et al., 2017). As with DIN, these data were readily available, current and considered a range of factors. As such, we used the modelled likelihood of exposure of GBR ecosystems to anthropogenic TSS results from Waterhouse et al. (2017) for the present study. These results were transformed form a 6-point scale to a 5-point scale (with a score of 5 indicating the highest TSS exposure) and rounded to the nearest whole number for our analysis. Where scores were between values due to the conversion, anthropogenic river load data and the targets set for individual rivers were used to determine the final score (Brodie et al., 2017). The resulting TSS sub-indices are reported in Table 4.6. Note that although TSS exposure was not modelled for the Torres Strait in Waterhouse et al. (2017), anthropogenic TSS concentration is relatively low throughout the Torres Strait. The Fly River is a large contributor of anthropogenic sources of TSS (Waterhouse et al., 2018), but this plume has little influence on Warul Kawa because prevailing water currents take the plume away from this site (Wolanski et al., 2013); this excludes an area very close to the PNG coast to the west of the Fly delta (Waterhouse et al., 2018). Considering this, Warul Kawa was given a score of 1 for TSS.

Table 4.6. Sub-index scores for TSS exposure at the 14 sites examined in this study. Sites are listed in approximately north to south order.

Study site	Score
Warul Kawa	1
Clack Reef	2
Howick Group	1
Cape Flattery	2
Ollera	3
Toolakea	3
Cockle Bay	4
Southern Cleveland Bay	4
Upstart Bay	5
Edgecombe Bay	2
Western Shoalwater Bay	2
Gladstone	4
Heron Island	1
Sandy Strait	3

Pesticides

Unlike DIN and TSS, data which models the risk of exposure to pesticides for GBR ecosystems is not readily available. It is expected that future research will benefit from the e-Reef hydrodynamic models which can be used to map the risk of pesticides to coral and seagrass. However, at present, these methods for modelling pesticide exposure are still being developed (Waterhouse et al., 2017). Considering this, a combination of available data (Bentley, 2012; Grant, 2018; Kennedy et al., 2012a; Kennedy et al., 2012b; Lewis et al., 2009; Shaw et al., 2010) and expert opinion was used to develop the water quality sub-indices for this parameter. Due to the complexities of pesticides, including the huge array of types and target species, the lack of knowledge surrounding their effects in turtles and the exploratory nature of this study, pesticides were treated as an aggregate, and not separated further into categories. The expert opinion considered land-use in the catchment (and therefore associated pesticide use) in addition to river influence. This section considered the river of interest to be those which are known to be associated with heavier pesticide use, and therefore in some cases this may not be the river of influence identified in Table 4.4. Scores were determined using a 5-point scale, with a score of 5 indicating the highest risk of pesticide exposure. These scores, and their justifications, are provided in Table 4.7.

Table 4.7. Sub-index scores for pesticide exposure at the 14 sites examined in this study. Sites are listed in approximately north to south order.

Study site	Considerations	Score
Warul Kawa	Far removed from likely pesticide sources; Daintree River (860 km) and Mapi River (320 km). Very little pesticide use associated with the Fly River.	1
Clack Reef	Far removed from likely pesticide sources; Daintree River (280 km). Limited pesticide use in the nearest river due to banana farms being distant to the river mouth; Normanby River (40 km).	1
Howick Group	Far removed from likely pesticide sources; Daintree River (200 km). Limited pesticide use in main river influence; Endeavour River (110 km).	1
Cape Flattery	Far removed from likely pesticide sources; Daintree River (150 km). Limited pesticide use in other rivers with influence.	1
Ollera	Main source of pesticides is Ollera Creek itself, as this waterway drains a region associated with significant sugarcane cultivation and discharges. The Haughton River (90 km), Barratta Creek (100 km) and Burdekin River (150 km) are also distant sources. The Herbert River (50 km) also drains a significant sugarcane region. However, with the plume from the Herbert flowing north, there is likely to only be a small influence from this river.	3
Toolakea	The most significant influence on this site is Bluewater Creek, which drains some urban and residential regions and discharges directly into Toolakea. Much less sugarcane cultivation than Ollera. The Haughton River (60 km), Barratta Creek (80 km), Burdekin River (120 km) and Herbert River (80 km) are also distant pesticide sources.	3
Cockle Bay	Main source of pesticides are the Burdekin River (100 km), Haughton River (40 km) and Barratta Creek (60 km). Significant sugarcane cultivation (and therefore pesticide use) in this region. This location is not under the influence of the Herbert.	4
Southern Cleveland Bay	Same considerations as Cockle Bay with some variation in distance to influence: Burdekin River (80 km), Haughton River (20 km) and Barratta Creek (30 km).	4
Upstart Bay	Reduced influence from Barratta Creek and limited pesticides draining out of the Burdekin due to high river banks. Some horticulture at Molongle Creek and limited horticulture to the south.	5
Edgecumbe Bay	Influence from the Gregory River (10 km) which drains a sugarcane region. Reduced influence from the Don River (20 km), which is also associated with some horticulture, due to north-flowing plume.	3
Western Shoalwater Bay	Carmilla Creek (70 km) drains a sugarcane region but is a very small waterway. The Pioneer River (150 km) is known to carry or discharge significant pesticide loads, but the plume flows north and is of little influence on this location. The Fitzroy River (140 km), located to the south, has lower pesticide loads but does contain Tebuthiuron due to grazing in the region.	2
Gladstone	Influenced by Boyne River (20 km), Calliopie River (10 km) and Auckland Creek (20 km) which are all low in pesticides. Limited influence from the Fitzroy River (50 km) which contains Tebuthiuron due to grazing. Waterways known to be higher in pesticides (Baffle Creek (110 km), Kolan (130 km) and Burnett River (150 km)) are some distance away with very limited influence here.	3
Heron Island	Influenced extremely rarely from the Fitzroy River (100 km), Burnett River (150 km) and Mary River (240 km).	1
Sandy Strait	Most significantly influenced by the Mary River (10 km), which drains a region associated with sugarcane cultivation and other small cropping farms. Some forestry utilising Simazine in this region.	4

Metals

Available data from the published literature was collated into a database for the purpose of this study. Due to the limited knowledge of the effects of metals and metalloids on marine turtles, and this study's interest in seagrass, only metal concentrations that were obtained from seagrass samples were considered. Data used in this analysis were separated by element, study site, with the units, source species and associated reference noted (Denton et al., 1980; Dight and Gladstone, 1993; Haynes, 2001; Haynes and Johnson, 2000; WWF-Australia, 2018). After all the available published data were incorporated into our database, each data-point was scored according to Govers et al. (2014) as this was a global study which considered available metal concentrations from seagrass samples from across the world. For each metal, the range of concentrations described by Govers et al. (2014) was converted to a 5-point scale and our data were scored according to this scale. For example, copper was described in a range of 0-100ug/g dry weight. This was broken down into the following 5-point scale as follows: (1) 0-200ug/g dry weight, (2) 200-400 ug/g dry weight, (3) 400-600 ug/g dry weight, (4) 600-800 ug/g dry weight and (5) 800-100 ug/g dry weight. However, when this international scoring method was applied to seagrasses from the GBR, the samples from all sites came out to be 1, suggesting that the available data for metals in seagrass on the GBR is of a lower concentration than other locations around the world. As this international scoring method did not allow for our study sites to be separated, a local scoring based on the available GBR data was needed for this study. We therefore developed a 5-point scale which encompassed the range of the data collected from the published literature. Our data were then scored based on the method described above. Although the metals were separated by element for data collection and initial scoring, the metal scores were ultimately aggregated for further analysis. This is due to both our limited understanding of metal toxicity in marine turtles and the exploratory nature of this study which made the analysis of individual elements unsuitable. As such. The results of both of these scoring methods are reported in Table 4.8. However, the available metals data from the literature for many of the sites used in this study is patchy. The resulting scorings were found to contain data gaps, or data that did not represent the true nature of metals at sites. For example, the Torres Strait scored the same as Cleveland Bay (2), despite the Torres Strait being removed from metal sources, while Cleveland Bay is located close to Townsville, which hosts one of the world's largest zinc refineries, a copper refinery and a nickel refinery (Table 4.1). Therefore, to improve resolution/ accurateness of the scoring, expert opinion which considered the available data and the characteristics for each study site, was used to make a final sub-index (Table 4.8).

Table 4.8. Sub-index scores for metal exposure at the 14 sites examined in this study. Metals for each site were ranked on a global and local (Great Barrier Reef only) and ultimately on a scale which considered both of these results in addition to expert opinion. Sites are listed in approximately north to south order.

Study site	Global score	Local score	Local score weighed using expert opinion
Warul Kawa	1	2	1
Clack Reef	1	2	1
Howick Group	1	2	1
Cape Flattery	1	3	1
Ollera	1	2	2
Toolakea	1	2	2
Cockle Bay	1	3	4
Southern Cleveland Bay	1	2	4
Upstart Bay	1	2	2
Edgecombe Bay	1	2	2
Western Shoalwater Bay	1	2	1
Gladstone	1	2	4
Heron Island	1	1	1
Sandy Strait	1	3	3

Aggregated Scores

Each sub-index for each site was aggregated to form a final index value reflecting the overall water quality at each site. The aggregation consisted of averaging the sub-index scores and rounding to the nearest whole number. No weights were applied to any of the sub-indices as data availability 1) precluded decisions regarding differing importance of parameters and, 2) because many studies favour equal weights on parameters to reduce bias (Sutadian et al., 2016). As the index combining both data sources and expert opinion allowed the most discrimination among sites, only this metal sub-index was incorporated into the final index value (Table 4.8). The final dataset, including the FP prevalence values and the sub-index and aggregated scores for each study site, are outlined in Table 4.9.

Table 4.9. FP prevalence and, sub-index scores for DIN, TSS, pesticide and metal exposure at the 14 sites examined in this study. The FP prevalence values have been separated by survey method, and values greater than zero are highlighted in bold. An aggregated score which reflected the overall water quality at each site (by considering the four parameters) is also shown. Sites are listed in approximately north to south order.

	FP Prevalence		Water quality indices (WQIs)				
Study site	**General Population**	**Turtle Health**	**DIN**	**TSS**	**Pesticides**	**Metals**	**Overall**
Warul Kawa	**3.4**		1	1	1	1	1
Clack Reef	**0.1**		1	2	1	1	1
Howick Group	0.0		1	1	1	1	1
Cape Flattery	0.0		1	2	1	1	1
Ollera		0.0	2	3	3	2	3
Toolakea		0.0	2	3	3	2	3
Cockle Bay	**0.7**	**11.6**	3	4	4	4	4
Southern Cleveland Bay	**1.9**		3	4	4	4	4
Upstart Bay	**1.6**	0.0	3	5	5	2	4
Edgecombe Bay	**0.7**	**7.9**	2	2	3	2	2
Western Shoalwater Bay	**1.6**		1	2	2	1	2
Gladstone	**3.0**		5	4	3	4	4
Heron Island	**0.3**		1	1	1	1	1
Sandy Strait	**3.6**		3	3	4	3	3

Statistical analysis

The FP prevalence datasets were analysed alongside the WQI developed for each site in order to determine if there is a relationship between water quality and FP prevalence on the GBR. As the response variable (FP prevalence) is a proportion derived from the turtle counts, logistic regression was used to investigate whether any of the WQI were associated with FP prevalence. Significant overdispersion was accounted for by using the quasibinomial family to describe residual dispersion.

For both the Grouped dataset (from Chapter Two) and Individual dataset (this Chapter), the association between DIN, TSS, pesticides, metals and the overall WQI was examined, both separately and together. Because some of the WQI indices had missing values, the datasets examined each WQI separately differed. Earlier analysis of the grouped data (Chapter 2) demonstrated that turtle age class and survey method both influenced the recorded FP prevalence, so these explanatory variables were also included where available.

Results

Grouped dataset

This study analysed 18,380 individual capture records of green turtles, including 264 records of FP across 14 sites along the GBR, in conjunction with WQIs developed from published data and expert opinion. Despite the analysis of this expansive dataset, there was no clear trend between FP prevalence and WQI rankings at any of the sites. Sites with high FP prevalence had moderate to high scores for both the sub-indices and aggregated score, but typically these sites were ranked moderately. For example, Cockle Bay scores ranged from 3-4 for all sub-indices and parameters despite having a high FP prevalence (determined by the FP survey method). Edgecumbe Bay, which also had a high FP prevalence, had WQI scores which ranged from 2-3. Gladstone and Warul Kawa had comparable FP prevalence rates (3.4% and 3.0% respectively), yet vastly different WQI scores; Warul Kawa scored 1 for the aggregated score and all sub-indices while Gladstone scored from 3-5. The variable results for the sub-index and Overall WQI scores were plotted in Figure 4.6 and Figure 4.7 respectively.

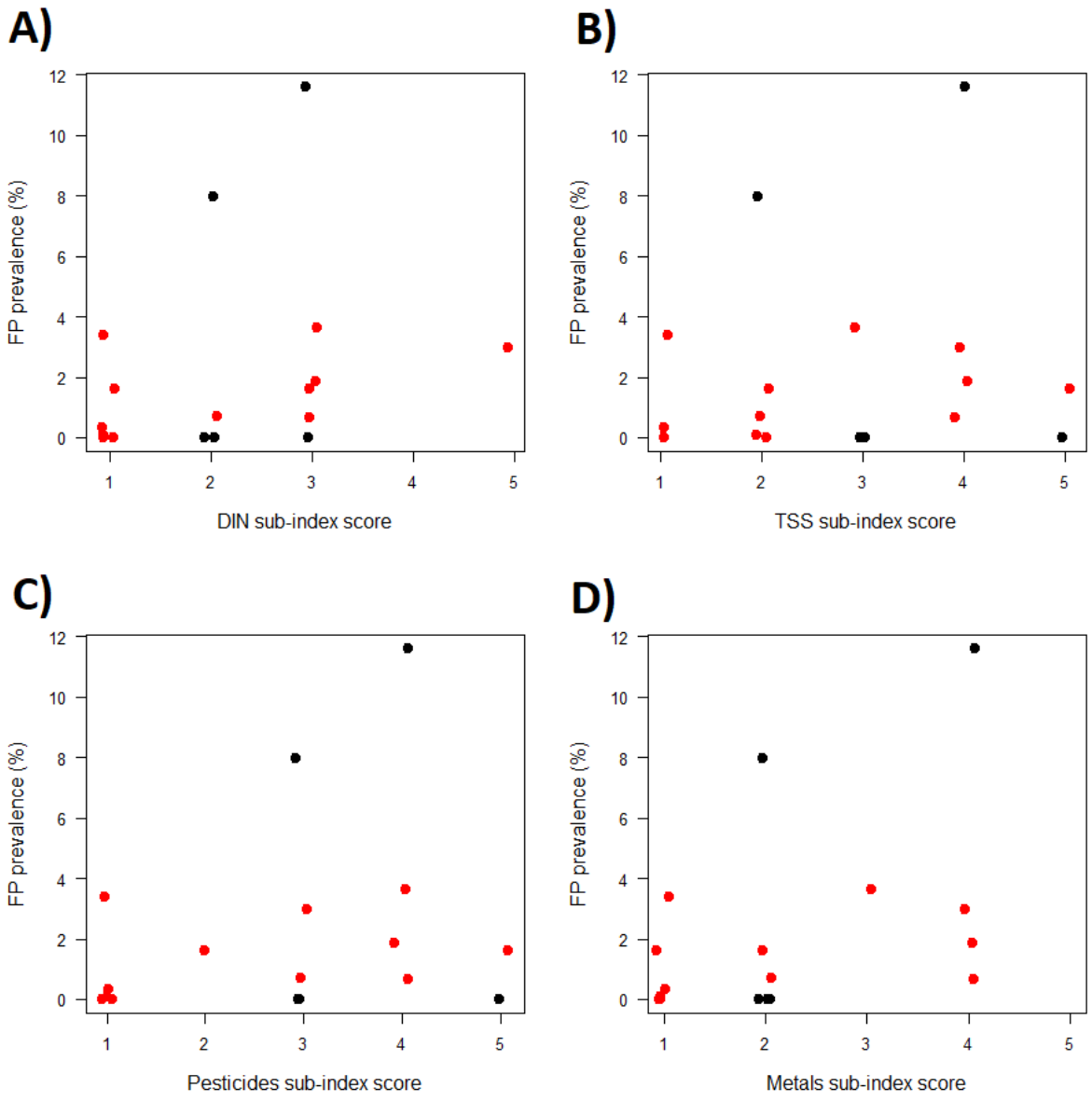

Figure 4.6. A jittered plot of dissolved inorganic nitrogen (DIN) (A), total suspended solids (TSS) (B), pesticides (C) and metals (D) sub-index scores against fibropapillomatosis (FP) prevalence. In each plot, turtle health survey results are shown in black and general population surveys in red.

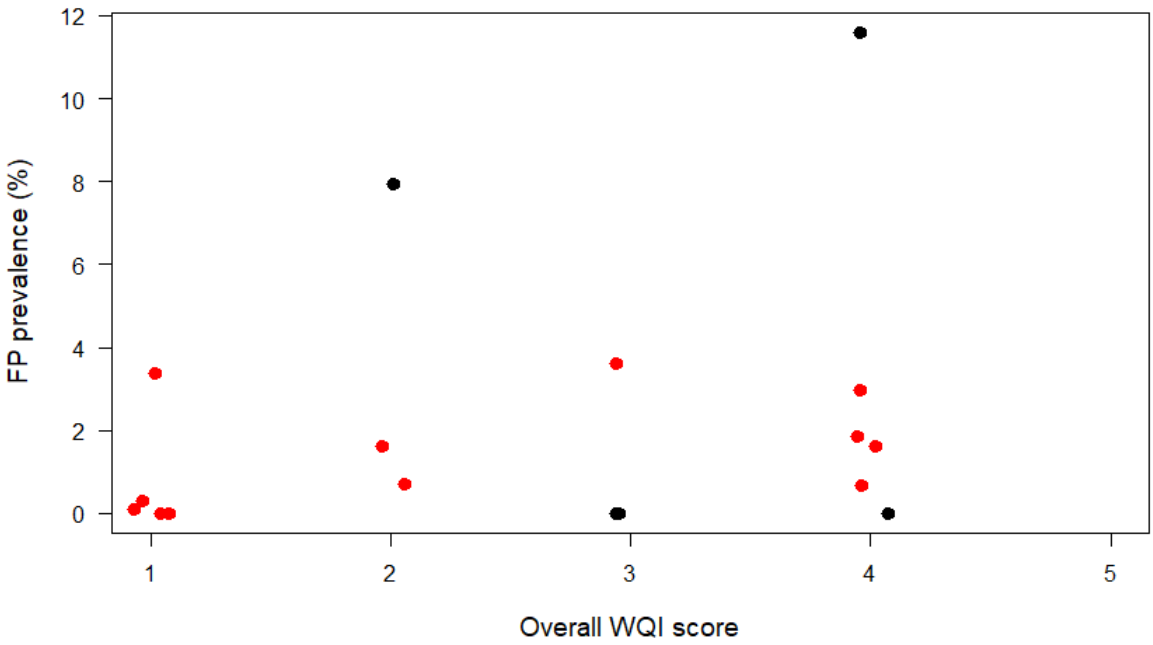

Figure 4.7. A jittered plot of the overall water quality index (WQI) score scores against fibropapillomatosis (FP) prevalence. Turtle health survey results are shown in black and general population surveys in red.

Overall, sites with high aggregated scores included those with moderate (Southern Cleveland Bay, Upstart Bay and Gladstone) and high FP prevalence (Cockle Bay). Sites with moderate scores had no (Ollera and Toolakea) and moderate (Sandy Strait) FP prevalence. This was also true of sites with low WQI scores that had no (Cape Flattery and the Howick Group), low (Clack Reef and Heron Island), moderate (Warul Kawa) and high (Edgecumbe Bay) FP prevalence.

These variable trends can also be observed when examining the results at a sub-index level. With respect to DIN, high scores (and therefore high DIN exposure) were correlated with FP prevalence but the trend wasn't linear. For example, Gladstone scored the highest for DIN but had a moderate FP prevalence. Low DIN scores didn't necessarily correlate with low, or no, FP; Warul Kawa scored low for DIN but had a moderate FP prevalence. The site scoring the highest rating for TSS was Upstart Bay, yet this site had low prevalence of FP. Sites with low scores for TSS, like that of Warul Kawa and Heron Island, were linked with moderate to low FP prevalence respectively. Sites with moderate TSS were linked with both no documented FP (Ollera and Toolakea) and moderate FP (Sandy Strait).

While there was a trend between high pesticide scores and moderate to high FP prevalence (Upstart Bay and Cockle Bay respectively), there was considerable variation in FP prevalence at sites with moderate to low pesticide scores. Sites with moderate pesticide scores included those with no documented FP (Ollera and Toolakea), moderate FP (Gladstone) and high FP (Edgecumbe Bay). Similarly, sites with low pesticide scores included those with no documented cases of FP (Howicks Group and Cape Flattery), low FP prevalence (Clack Reef and Heron Island) and moderate FP (Warul Kawa).

Similar to pesticides, a trend between high metal scores and sites with moderate to high FP (Cleveland Bay, Gladstone and Cockle Bay) was observed, and low metal scores were observed for sites with no (Howick Group), low (Clack Reef and Heron Island) and moderate FP (Warul Kawa and Western Shoalwater Bay). In the case of this sub-index, moderate scores were linked with sites with moderate FP (Upstart Bay and Sandy Strait).

These variable trends were examined further using statistical analysis (see Appendix 3: Supplementary Table 4.1). Both the survey method and median year were determined to have a significant effect on FP prevalence, as consistent with Chapter Two. Although a plot of the metals sub-index suggests a weak trend (Figure 4.6), it does not achieve statistical significance. None of the other analyses of either the sub-indices (DIN, TSS or Pesticides) or the Overall WQI suggested any association with FP prevalence. As further testing on the variables with significant effects was already conducted in Chapter Two, no further analysis was conducted here.

Thus, the huge variation in trends between the water quality sub-indices/Overall WQI and FP prevalence was also confirmed through statistical analysis wherein no statistically significant relationship between FP prevalence and the WQIs developed for this study were identified.

Individual data subset

This subset of data contained 917 individual capture records of green turtles from five sites along the GBR, including 59 individual FP records. The capture sites included Ollera, Toolakea, Cockle Bay, Upstart Bay and Edgecumbe Bay. These data were sourced from the JCU Turtle Health Research Database, with the survey method being turtle health focussed. This data subset was generated for statistical analysis as it contained detailed information of each capture event, including date, curved carapace length, and age class. This data-rich subset allowed for 917 datapoints instead of the five available for the Grouped Dataset. For example, to align with the other available data, 541 individual records were grouped into one overall datapoint for Edgecumbe Bay for all prior analysis (Grouped Dataset). The present subset allowed all 541 to be considered individually. However, statistical analysis had to exclude the 2009 and 2010 datasets at Edgecumbe Bay as they contained only records of FP turtles and were skewing the data towards 100% prevalence rates which weren't an accurate representation of the populations at the time.

As the same WQI were used for this dataset and the grouped dataset the trends observed remain the same, yet the statistical analysis varied. Both age class ($p<0.001$) and site ($p<0.001$) appeared to be significantly associated with FP. We then wanted to test what it was about site that may be responsible for this association. Thus, the WQI and sub-index values were used in this analysis. Unfortunately, statistical analysis was precluded due to limited data. As this dataset consisted of five study sites instead of 14, variation within the WQI and sub-index values was reduced. For example, only Cockle Bay scored a 4 in the pesticide sub-index, with all others scored as a 2. Similarly, all sites were scored as a 2 for the DIN sub-index. Similarly, the overall WQI for the five sites in this subset were ranked as either a 3 (Ollera, Toolakea and Edgecumbe Bay) or a 4 (Cockle Bay and Upstart Bay) (Table 4.9). This made it impossible to trace variation in FP to sub-indices and/or WQI. Particularly for the DIN sub-index, which had to be excluded as contrasts can only be applied to factors with 2 or more levels and DIN was 3 for all sites. We still attempted to test the combined effects of the remaining explanatory variables using a logistic regression model. However, there were too many variables for the data and the model was unable to be fitted. We then ran the same analysis, excluding one variable at a time. In each test, there appeared to be some significant influence on one of the sub-indices, with the significantly associated sub-index varying depending on the test. However, in every case, all variables were extremely confounded and therefore no further analysis could be undertaken.

Discussion

This study investigated whether there was a link between water quality and the prevalence of FP in green turtles foraging on the GBR. Water quality data from the published literature proved unsuitable for direct comparison with FP prevalence. To combat this, WQIs for each study site were developed using published data augmented by expert opinion to overcome data gaps and deficiencies. An extensive dataset was developed which contained 18380 individual green turtle records, including 264 records of FP, across 14 sites along the GBR. A WQI for each study site was developed based on available data and expert opinion. However, no clear trend or statistical relationship between FP prevalence and the WQI developed in this study was determined, regardless of whether the Grouped dataset or Individual subset of data was analysed. It is unclear whether this is a result of limitations of our data (be it FP prevalence, water quality or both) being insufficient for this type of analysis. It is also possible that FP prevalence may be influenced by a single particular water quality variable, like that of a specific pesticide, the effect of which has been lost when these variables were grouped into the broader categories. Similarly, any correlation with water quality may be due to a combination or interaction of variables which is beyond the power of this study to identify. Finally, the variable trends observed between the WQI and FP prevalence may be an indication that no strong correlation between these factors exists.

The challenging nature of generating accurate and reliable disease prevalence data from a species group like marine turtles is discussed in Chapter Two. Briefly, the ability to carry out fieldwork is highly weather-dependent, which often precludes the ability to conduct rigorous sampling at defined intervals. Instead, fieldwork is undertaken opportunistically, when weather conditions are suitable. Moreover, some survey methods are biased towards detecting FP whilst others are biased against it (Chapter Two). The result is an indication of FP prevalence at a particular site, with varying temporal scales and survey methods. These variations may have played a role in the inability to observe any trends or relationship between water quality and FP prevalence.

Similarly, obtaining accurate water quality data for the variables of interest to this study was incredibly challenging. Water quality on the GBR has been studied extensively, yet there is debate about effective management in this area (Brodie and Waterhouse, 2012). While a number of long-term water quality monitoring programs exist, each is run according to the individual objectives of each organisation. That is, the organisations conducting these programs range from academic research groups to state and federal government, with the methods used varying by target outcomes (Brodie and Waterhouse, 2012). Some programs analyse water samples directly, whilst others extrapolate from concentrations of variables isolated from sediment, seagrass, coral, invertebrates and/or vertebrates. Many studies rely on modelling of particular variables to draw

conclusions about water quality on large scales, such as river discharge (Brodie et al., 2009; Davis et al., 2017; Kroon et al., 2012; Smajgl et al., 2009). Reports on these studies are as variable as the methods, temporal scales and outputs of the different programs. Some studies report results in academic journals, while others are released in government reports such as the Reef Report Cards though the Great Barrier Reef Marine Park Authority. These reports are difficult to interpret as, in addition to the variation in methods and results, there are no set guidelines or minimum standards which can be used to assess the status of water quality on the GBR (Brodie and Waterhouse, 2012). To date, there are no standardised indicators (such as coral cover) of satisfactory ecosystem health (Brodie and Waterhouse, 2012), let alone more specific and comparable measures. Moreover, these data are not centralised in any way; therefore, studies wishing to utilise long-term water quality data, like that of the present study, must collect available data from various peer-reviewed articles and government reports which span various departments and levels of government. The data provided in such reports are often specific to a particular study, but are then relied on to make generalised conclusions about water quality at particular sites. Furthermore, some long-term monitoring programs do not make their data publicly available. Such challenges further compound the difficulty in managing conditions that could be induced by environmental factors and renders this enormous dataset unsuitable for teasing out potential links between FP and degraded water quality on the GBR.

With respect to the present study, the available water quality data was used to develop WQI for each site, but data gaps were often encountered. Many sites along the GBR are understudied, making it even more difficult to make conclusions about certain regions with confidence. This is particularly true of the Cape York region, where Clack Reef, the Howicks and Cape Flattery are located. While there is reasonable evidence to conclude that the eastern Cape York catchments present a relatively low risk to adjacent coastal and marine ecosystems, other basins in the catchment pose a probable risk to ecosystems in the southern Cape York region from degraded water quality (Waterhouse et al., 2017). This region has been flagged as one which requires further investigation and management of water quality threats (Waterhouse et al., 2017), yet three study sites in the present study are located in this region. The limited understanding of these study sites would likely have influenced the accuracy of the WQI produced for these sites. In addition to data gaps and limited data, some anomalies in the available data skewed some sub-indices away from the reality of the site. This was particularly true in the case of metals where, at Warul Kawa for example, three relatively high concentrations of metals isolated from seagrass increased the overall metals score to 2; single reports of moderate to high concentrations of nickel, copper and cadmium were found (Dight and Gladstone, 1993), yet all other metal concentrations at this site were extremely

low. Similarly, despite being located adjacent to Townsville which hosts one of the world's largest zinc refineries, a copper refinery and nickel refinery, Cockle Bay scored lower than would be expected when based on the available data alone. These anomalies in the available data highlight the need to include expert opinion, which was used to address gaps in the dataset as well as correct these irregularities. While this is still an accepted method of developing WQI (Sutadian et al., 2016), it should be acknowledged that the lack of available data may have limited the accuracy of the WQI developed in this study. This may have had flow on implications for our difficulty in being able to analyse the data. Moreover, the data that was available was too sparse to make conclusions about water quality during particular years or time periods, despite temporal variation in water quality being a certain feature of the ecosystem. This is particularly true for study sites which were affected by heavy flooding during particular years, which was not able to be captured in our dataset. FP prevalence is known to vary temporally (Jones et al., 2016), yet this feature of the disease was not mirrored by the WQI used. This may have also limited our ability to observe any relationship between these factors.

It is also possible that any relationship between FP prevalence and water quality is caused by a single variable whose effect has been lost by grouping it into the broader category. For example, a certain pesticide may be responsible for the link, but as all pesticides were grouped into a single sub-index value for pesticides, the trend has been confounded. Such an effect is certainly true for other endangered reptiles, like that of the then-endangered American alligator. In the 1980s, a decline in the alligator (*Alligator mississippiensis*) population at Lake Apopka in Florida (whilst other populations in America's southeast were increasing) was a cause for concern. The region supports an extensive agricultural industry and a sewage treatment plant and as a result, contaminants were already known to be present in the lake. A widespread pesticide spill in 1980, containing dicofol, dichlorodiphenyltrichloroethane (DDT) and its metabolites, led to a rise in the concentrations of chemical contaminants in the lake. These organochlorides are able to act as oestrogens and researchers speculated that the population crash was a result of reproductive failure. The estrogenic activity could alter embryonic development and reproductive function of adult alligators (Guillette et al., 1994). Further research on this alligator population revealed that the female alligators had much higher levels of plasma estradiol-17β concentrations than would be expected. These females also showed abnormal ovarian morphology, with polyovular follicles and polynuclear ooctyes also present in large numbers (Guillette et al., 1994). The juvenile male alligators had lower than expected testosterone levels coupled with poorly organised testes and abnormally small penis size (Guillette et al., 1996; Guillette et al., 1994). The irreversible effects on the gonads of both the male and female alligators in Lake Apopka have made steroidogenesis impossible and normal sexual

maturation unlikely (Guillette et al., 1994). This population crash was linked to two highly similar pesticides and their metabolites. It is worth considering that if researchers at the time had developed a WQI using all pesticides found in the region, whether the effects of dicofol and DDT would have been identified. However, it is also worth noting that this was a localised population crash which allowed researchers to focus sampling at that particular location. Conducting such a study for turtles affected with a globally distributed disease such as FP would be logistically impossible; however local investigations of parameters in an area of high FP prevalence may hold the key to identify abnormal presence or levels of some of these contaminants.

It has long been speculated that FP development is multi-factorial, with disease manifestation requiring specific co-factors to stimulate tumour growth and expression (Greenblatt et al., 2004; Herbst, 1994; Jones et al., 2016). Although a body of work has attempted to identify what some of these co-factors may be, the results remain inconclusive. Environmental factors such as natural biotoxins (Arthur et al., 2006b; Landsberg et al., 1999), eutrophication and dietary intake (Van Houtan et al., 2014), seasonality (Herbst, 1994), persistent organic pollutants (Keller et al., 2014) and trace metal and organic pollutants (Aguirre et al., 1994a) have all been investigated with varying results. The health status of individual turtles, including stress and immunosuppression (Work et al., 2001), have also been investigated.

While the correlation between FP prevalence and water quality has been reported consistently (Adnyana et al., 1997; dos Santos et al., 2010; Foley et al., 2005; Herbst, 1994; Van Houtan et al., 2010), evidence indicating that one particular factor is involved in FP manifestation is still lacking. Several of these factors may be interacting in a way that supports disease development and there may be additional factors involved, which have not yet been identified. While future studies should aim to clarify this, the practicality of such investigations is daunting considering the vast marine environment and expansive possible contributors to FP (Herbst, 1994; Herbst and Klein, 1995a). Such interactions were not considered in the WQI developed in this study, and it is therefore possible that any causal relationship was missed as a result.

Despite trends between degraded water quality and FP reported in published studies, the two factors have not been definitively linked. It is therefore possible that a quantifiable relationship does not exist. For example, in Puerto Rico, the positive correlation between high FP prevalence and reduced water quality was reported (Patrício et al., 2011). However, after several years the trend reversed; the prevalence of FP at the more pristine site became considerably higher than at the site which is subjected to high levels of human activity (Page-Karjian et al., 2012). Similar results are reported in the present study, wherein Warul Kawa and Gladstone share a similar FP prevalence

despite being vastly different with respect to human influence and water quality. Such results indicate that the correlation between water quality and FP prevalence isn't as strong as previously reported. These results also provide support to the theory that any correlation may be caused by a specific water quality variable, or that the correlation is multifactorial.

In addition to the limitations of the FP prevalence data and WQI development already discussed, this study was subject to further limitations. Due to the exploratory nature of this study, and the expansive number of water quality variables that could be tested for, this study focussed on the hypothesis that water quality variables which have a significant influence on seagrass would also have a significant impact on green turtles, as they rely on seagrass for forage. That is, a reduction in seagrass health and coverage as a result of either excess nutrients, suspended solids or pesticides could result in emaciated and stressed marine turtles, which may in turn play a role in FP manifestation. The role of metals is less well understood, but was included as it is a significant water quality parameter. Despite the hypothesis being centred on seagrass, seagrass coverage was not included as a variable itself due to the difficulties in obtaining accurate data (See Chapter Two).

The WQI developed in this study were also unable to consider seasonality, despite possible seasonal influences in FP prevalence (Herbst, 1994) and definite seasonal influence on water quality from discharge events into the GBR (Waterhouse et al., 2017). While the distinct wet and dry season climate of the GBR results in most pollutants being delivered to the GBR lagoon during high river discharge events associated with the summer wet season (December-April) (Waterhouse et al., 2017), the WQI developed for the present study was unable to consider this due to data limitations that were beyond the reliable estimation of expert opinion.

There are a huge range of water quality variables that were not considered in the development of the WQI for each study site. A recent study identified a range of xenobiotics in individual marine turtle blood, the chemical profiles of which clustered together by site (Heffernan et al., 2017). Interestingly, the chemical profiles from turtles at the control site and a site with high agricultural influence clustered together, whilst a site with urban and industrial influence clustered as distinctly different (Heffernan et al., 2017). The cause for this remains unclear, but this developing field may be key in better understanding marine turtle health, including FP, as some may have incidental toxicity with a direct impact on turtles. However, as this field is just developing, xenobiotics could not be considered in the WQI developed in this study.

The inconclusive results of this study have implications for marine turtles and the GBR as a whole. As all species of marine turtle are threatened, with one being listed as data deficient (IUCN, 2019), it is imperative that we understand all of their threatening processes, including those that influence

and/or contribute to FP. The present study focused on the endangered green turtle and a poorly understood disease which predominantly affects this species. Yet despite the combination of one of the most extensive datasets used to study FP in marine turtles to date, in addition to specifically designed WQI for 14 sites spanning the coastline of the GBR, we were unable to quantify any relationship between these factors. This was due to a range of limitations, including data gaps, varying temporal scales and methods in both the FP prevalence and water quality datasets. The inability to identify any correlation between the WQIs and FP prevalence may also be attributed to the design of the WQIs, which did not account for temporal variation in water quality. Moreover, several of the water quality variables which were included in the WQIs were also highly confounded, which restricted our ability to narrow down the cause of any correlations detected. Access to a single long-term water quality monitoring dataset for the expanse of the GBR may have alleviated these limitations, yet no such dataset exists (Brodie and Waterhouse, 2012). Thus, this study can inform management as it highlights significant deficiencies in the current monitoring of water quality on the GBR which need to be addressed.

One of the conclusions from this study is that there is a lack of integration between some of the research and monitoring programs conducted within the GBR. These programs have largely been designed to address and report on specific issues, locations or management initiatives; which often precludes the ability to access and use data from different programs. This is evident for those studies focussing on water quality, but also for seagrass and species like that green turtle that rely on them for food. The Reef 2050 Long-Term Sustainability Plan (Reef 2050 Plan) responds to the challenges facing the GBR and presents actions to protect its values, health and resilience, while allowing ecologically sustainable use (Great Barrier Reef Marine Park Authority and Queensland Government, 2015). This plan was developed by the Australian and Queensland Governments, together with its partners, including Traditional Owners, industry, scientists and communities. A key part of the Reef 2050 Plan is the Reef 2050 Integrated Monitoring and Reporting Program (RIMReP), which is a coordinated and integrated monitoring, modelling and reporting program for the GBR and its adjacent catchment. One of the key outcomes of RIMReP is to drive the coordination, alignment and integration of existing monitoring, modelling and reporting programs. Such integration would capitalise on existing program investment, provide value for money, improve efficiency and avoid duplication of effort. The inconclusive results from the present study underpin the value of RIMReP and highlight the need for integration and cross-disciplinary studies.

Future research would benefit from targeted and consistent survey methods for sampling marine turtles and FP prevalence data (see Chapter Two). Future studies should consider simultaneous water quality sampling alongside marine turtle surveys at a selection of locations with either high or

low FP prevalence. This would allow direct comparison of FP prevalence and water quality using consistent methods and temporal scales. It may also be valuable to investigate specific genetic stock-level impacts of FP, as recommended by Department of the Environment and Energy (2017). This would require genetic testing of the host turtle affected with FP and the viral strain affecting an individual turtle at their foraging grounds. Despite the challenge, such a study may reveal genetic stocks which can then be targeted for management. Consistent and centralised water quality monitoring along the expanse of the GBR is also an essential component of effective management and needs to be addressed in the future.

The aims of this chapter were addressed as follows:

1. **Develop water quality indices for parameters of interest to marine turtles at 14 locations along the GBR**

   Sub-indexes for dissolved inorganic nitrogen (DIN), total suspended solids (TSS), pesticides and metals were developed for each study site using published data from a range of sources and expert opinion. These scores were also aggregated without weights to create an overall water quality index for each study site.

2. **Investigate any link between water quality and FP on the GBR**

   This study utilised 18380 records of individual capture records of green turtles, including 264 records of FP across 14 sites along the GBR, in conjunction with WQIs developed from a range of published data and expert opinion. Yet, despite this expansive dataset, a relationship between FP prevalence and WQI rankings at each site could not be quantified or established. The analysis was challenged by a range of limitations, including data gaps, varying temporal scales and methods in both the FP prevalence and water quality datasets. The water quality datasets were further restricted by their inability to account for temporal variation and several of the water quality variables were confounded, preventing statistical analysis.

   This investigation is the first attempt at creating WQI based on a multitude of published reports and peer-reviewed publications to compare with FP prevalence data and it proved impossible to bridge between differing methodologies. However, this result does have significant implications for management as it highlights substantial deficiencies in the current monitoring of water quality on the GBR which need to be addressed.

Publications and presentations arising from this study

- **Jones, K.** 2015. Fibropapillomatosis on the Great Barrier Reef: Directions of future research. *Proceedings from the international summit on fibropapillomatosis of marine turtles: Global status, trends and population impacts.* 11-14th June 2015, Honolulu, Hawaii.
- **Manuscript in progress:**

  The following manuscript combines Chapter Three and Chapter Four of this thesis:

  Jones, K., Limpus, C., Brodie, J., Jones, R., Shum, E., Read, M. and Ariel, E. 2019. Investigating the relationship between water quality and prevalence of fibropapillomatosis in green turtles (*Chelonia mydas*) on the Great Barrier Reef. *In progress.*

My contributions to this study

- I generated the ethics application and associated fieldwork to collect FP prevalence data from a subset of the sites used in this study (referred to in this chapter as the data from the JCU Turtle Health Database)
- I attended fieldtrips and liaised with other researchers whose data was supplied for analysis in this study, to ensure I understood their research methodology and that each could be cohesively compared.
- I worked in person with researchers at the Department of Environment and Science and the Torres Strait Regional Authority to collect and clean the datasets for analysis
- I managed the datasets obtained
- I collected and collated available water quality data from a range of published sources into a central database
- Under advice from a water quality expert, I generated the sub-indices and aggregated water quality index
- I attended workshops which focussed on the use of the R-Studio program which aided in the statistical analysis
- Under advice from an experienced statistician, I prepared the input file for statistical analysis and interpreted the results
- I drafted the chapter and edited it as advised by collaborators and supervisors

Chapter Five:
Closing the gap: Mixed stock analysis of three foraging populations of green turtles (*Chelonia mydas*) on the Great Barrier Reef

Background and aims of this chapter

The literature review in Chapter Two highlighted that an understanding of ChHV5 transmission pathways was needed in order to better manage FP. The theory that marine turtles become infected with ChHV5 upon recruitment into inshore foraging grounds is widely supported, but has never been investigated using molecular methods. Turtles frequenting a given foraging site usually represent genetic stock from multiple rookeries, with genetic stocks reflecting the region of origin. For example, turtles originating from rookeries in the southern GBR are genetically similar and distinct from those originating from rookeries in the northern GBR. If ChHV5 transmission was occuring vertically from parent to offspring, then phylogenetic clustering of ChHV5 would be expected to be based on host genetic stock rather than sampling location. Conversely, if ChHV5 transmission is occurring horizontally at foraging grounds, a link between viral variant and host origin would be less likely.

We set out to assess whether there was a link between the genetic stock of the host turtle and the viral variant it possessed. In order to do so, we needed a means of identifying host genetic stock. Mixed stock analysis (MSA) in marine turtles estimates host genetic stock using haplotype frequencies, and we aimed to apply this method to the turtles in this study. Upon review of the literature, a recent study which conducted MSA at several foraging grounds along the GBR was found to detect a dramatic shift of stock contributions between two foraging grounds (Edgecumbe Bay and the Howick Group) (Jensen et al., 2016). However, these foraging grounds were separated by more than 700km. We found that this provided a unique opportunity to improve resolution of genetic stock contribution in this spatial gap and provide a solid foundation for testing a link between host genetic stock and viral strain. The overarching aim of this chapter was to validate a method for host stock identification for use in Chapter Six.

The specific aims of the present study were to:

1. Develop, optimise and validate a PCR assay which targets green turtle mtDNA control region sequences
2. Generate and use mtDNA control region sequences and MSA to quantify the stock composition of green turtles at three foraging areas located between Edgecumbe Bay and the Howick Group
3. Use our new data and data from previously sampled foraging areas to assess the correlation between stock composition and latitude of foraging areas in Eastern Australian waters

Introduction

Migratory marine mega vertebrates are often long lived and utilize a variety of habitats that span wide spatio-temporal scales. Humpback whales (*Megaptera novaeangliae*), for example, utilize distinctly separate feeding and breeding grounds and undergo seasonal migrations between these areas which can span thousands of kilometres (Acevedo et al., 2007; Clapham, 1996; Oña et al., 2017). The same is true of various species of sharks, rays, tuna, marine mammals and marine turtles (Lascelles et al., 2014). Species with complex life history patterns pose challenges to the understanding of population dynamics and the connectivity between breeding and non-breeding areas (Godley et al., 2010). Due to their wide-ranging movements, marine migratory species are exposed to different threats at their foraging and breeding habitats, and are further exposed to additional pressures as they migrate between these habitats (Jensen et al., 2016; Lascelles et al., 2014). These species often pass through the waters of multiple nations or areas beyond national jurisdiction (Lascelles et al., 2014) and as a result, monitoring, managing and ultimately conserving such species is challenging (Hamann et al., 2010; Jensen et al., 2016). In 2014, 48% of all marine migratory species were found to be threatened (critically endangered, endangered or vulnerable), near threatened or data deficient, with marine turtles being the most threatened group (Lascelles et al., 2014). A sound understanding of the spatial ecology of these species is essential to developing effective conservation strategies (Cooke, 2008), as it allows for the identification of key habitats and the likely sources of threatening processes.

The green turtle (*Chelonia mydas*) is recognised as endangered under the IUCN red list assessment (Seminoff, 2004). In Australia, this species is listed as vulnerable under the *Environment Protection and Biodiversity Conservation Act 1999* (Department of the Environment and Energy, 2017). Green turtles have a circumglobal distribution, are long-lived, highly migratory, and have a complex life history which spans a diverse range of habitats (Limpus, 2008). After emerging from tropical and subtropical sandy beaches hatchling green turtles take on a pelagic existence, recruiting into benthic, inshore foraging grounds as juveniles several years later (Reich et al., 2007). Foraging areas are often shared by turtles sourced from multiple regional rookeries (Anderson et al., 2013; Dutton et al., 2014; Lahanas et al., 1998). At the onset of sexual maturity, some 20-30 years later, green turtles migrate back to their natal nesting regions to breed and nest (Musick and Limpus, 1997).

Using mtDNA, Australian green turtles can be divided into nine genetically distinct breeding stocks: southern Great Barrier Reef (sGBR), Coral Sea (CS), northern GBR (nGBR), Gulf of Carpentaria, Coburg Peninsula, Ashmore Reefs/Browse Island, Scott Reef, the Northwest Shelf and Cocos "Keeling" Island (Dethmers et al., 2006; FitzSimmons and Limpus, 2014; Jensen et al., 2016; Limpus, 2008). In addition, Australian waters are in close proximity to multiple internationally important

stocks in neighbouring countries such as those nesting in Aru (Indonesia), Papua New Guinea and New Caledonia. Each of these stocks can be considered as a demographically independent population (Waldman, 2005), and as such, understanding how turtles from these stocks share regional foraging grounds is critical to the effective management of threats to this vulnerable species.

The Great Barrier Reef (GBR) region in Australia is home to some of the largest nesting and foraging green turtle populations in the world. Breeding green turtles of nGBR and sGBR stocks nest on several islands at the latitudinal extremes of the GBR (Dethmers et al., 2006; FitzSimmons and Limpus, 2014; Jensen et al., 2016). While very little nesting takes place along the central part of the reef, turtles from both breeding stocks share foraging areas located along the entire GBR (Limpus, 2008) and beyond into New South Wales and northern Australia. Foraging grounds along the GBR are discontinuous and irregularly spaced, likely reflecting the patchy nature of resources relevant for turtles. For research and monitoring purposes, GBR foraging grounds are defined by their geographical location, e.g. a bay or a cluster of neighbouring reefs. These foraging grounds typically support overlapping adult and juvenile age classes. Long-term mark-recapture studies have demonstrated that all size classes have strong fidelity to a single foraging ground with little movement between surrounding foraging grounds (Limpus and Chaloupka, 1997; Musick and Limpus, 1997). As such, GBR foraging grounds are considered to host independent foraging populations wherein the genetic composition is mixed.

Both traditional mark-recapture analysis (flipper tagging) and molecular methods (mixed stock analysis; MSA) have been used to describe the distribution of foraging green turtles along the GBR (Jensen et al., 2016; Limpus, 2008). The MSA method uses genetic markers measured in several source populations (rookeries) and a single mixed population (a foraging ground) to estimate the proportional contribution of each source to the mixed population (Bolker et al., 2007). This technique provides an effective tool to assess the connectivity between foraging and breeding grounds for migratory species like marine turtles, whose intricate life history complicates monitoring efforts. Major green turtle rookeries across the Indo-Pacific have been genetically characterised using the mtDNA control region, with 25 genetically differentiated stocks or Management Units (MUs) identified to date (Dutton et al., 2009; Dutton et al., 2014; Jensen et al., 2016; Nishizawa et al., 2014; Read et al., 2015). These MUs provide a comprehensive reference of source populations that can be used in MSA to determine the breeding stock origin of green turtles at regional foraging grounds along the GBR and elsewhere (Dethmers et al., 2006; FitzSimmons and Limpus, 2014; Jensen et al., 2016; Limpus, 2008).

Studies based on traditional flipper tagging, genetic data, or a combination of these tools have shown that foraging areas along the GBR mainly receive turtles originating from three stocks; the nGBR, the sGBR and the Coral Sea (CS) (Jensen et al., 2016; Limpus, 2008). In addition to these dominant breeding stocks, small proportions of turtles foraging in these locations are supplied by more distant rookeries (Jensen et al., 2016; Limpus, 2008). The composition of stocks at foraging grounds along the GBR also alters with latitude; northern foraging grounds are mostly populated with turtles originating from the nGBR breeding stock whilst sGBR and CS stocks are more prominent in southern foraging grounds (Jensen et al., 2016).

This latitudinal variance can be observed on a broad scale (north to south, as above) and also on a finer scale (between specific foraging grounds). A major shift in the stock composition between the more northerly Howick Group of islands and the more southerly Edgecumbe Bay (Figure 5.1) has been described using a combination of MSA and flipper-tag returns (Jensen et al., 2016). While foraging turtles at Edgecumbe Bay were predominantly from the sGBR and CS stocks, turtles at the Howick Group were a mixture of sGBR, CS and nGBR stock. However, there was a large geographic gap in the sampling of foraging grounds between the Howick Group and Edgecumbe Bay spanning six degrees of latitude and approximately 700 km. Assessing the stock composition at foraging grounds within this spatial gap would further refine our knowledge of the latitude at which the composition of green turtles shifts from predominantly sGBR to predominantly nGBR turtles. Furthermore, closing this knowledge gap and combining these results with already published data may provide a means of assessing the relationship between stock composition and latitude. If such a relationship exists, it may provide a means to predict stock composition at other un-sampled foraging grounds in this region. Therefore, in this study, we 1) generated and used mtDNA control region sequences and MSA to quantify the stock composition of green turtles at three foraging areas located between Edgecumbe Bay and the Howick Group, and 2) used our new data and data from previously sampled foraging areas to assess the correlation between stock composition and latitude of foraging areas in Eastern Australian waters.

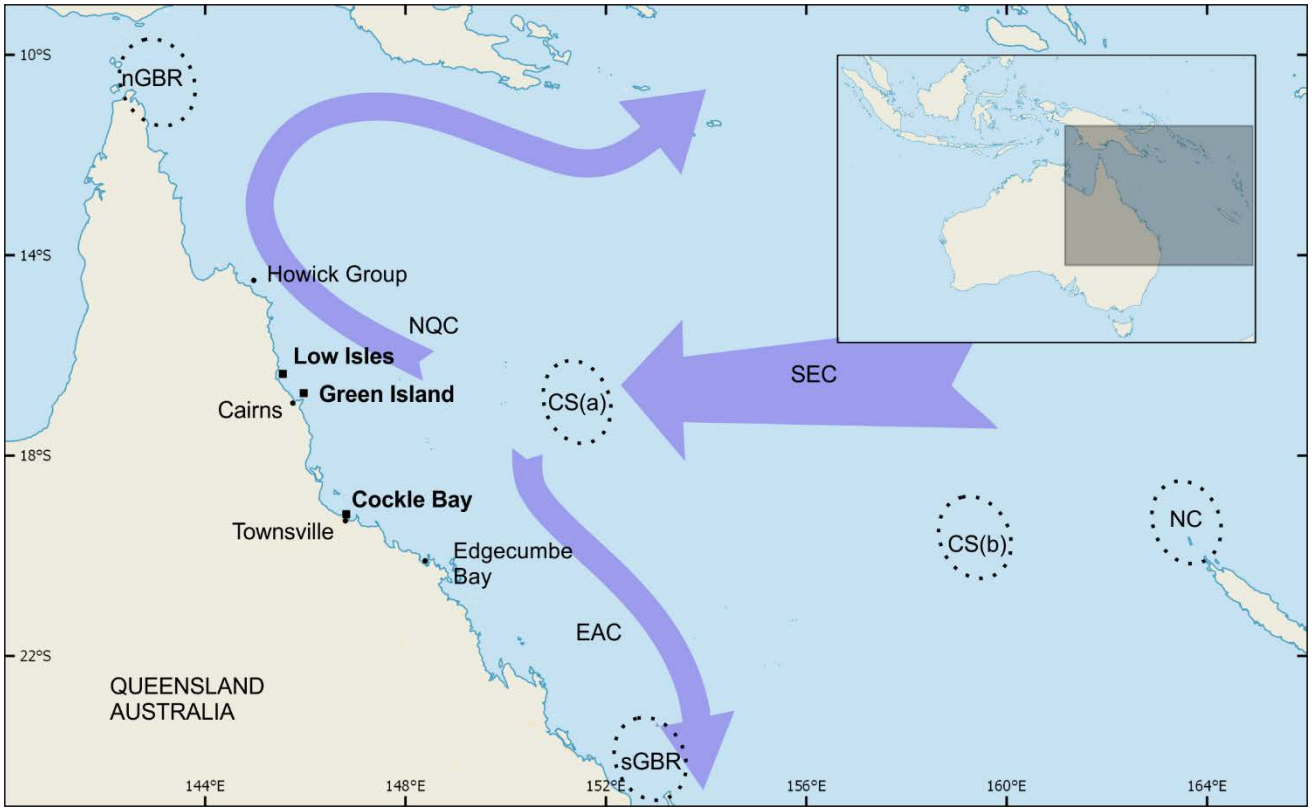

Figure 5.1. Green turtle foraging sites at Low Isles, Green Island and Cockle Bay were sampled for genetic analysis in the present study. These three sites filled a large geographic gap that existed in prior sampling by Jensen et al. (2016). Broken-line ellipses indicate breeding areas of the following source populations: northern GBR (nGBR), Coral Sea comprised of Coringa-Herald group (CS(a)) and Chesterfield group (CS(b)), southern Great Barrier Reef (sGBR), and New Caledonia (NC). Blue lines provide a simplified representation of ocean currents in the region of interest: NQC = North Queensland Current, EAC = East Australian Current, SEC = South Equatorial Current.

Materials and Methods

Study Sites

Green turtles were sampled during separate projects at three foraging grounds within the Great Barrier Reef, Queensland, Australia (Figure 5.1). The sites listed below are in north to south order.

Low Isles (LI) (16° 22' S, 145° 33' E), situated 15 km off the mainland of North Queensland, comprises two small islands on a shallow coral reef. Turtles at this site were sampled between June 2010 and November 2011.

Green Island (GI) (16° 45' S, 145° 58' E), a coral cay located in the northern GBR region approximately 27 km offshore from Cairns, Queensland was sampled in October 2012.

Cockle Bay (CB) (19° 10' S, 146° 49' E) is a small bay of Magnetic Island, located approximately 8 km offshore from Townsville, the largest tropical city in Australia. Sampling of this site was conducted in August and November of 2012.

Sample Collection

Turtles at all three sites were captured by rodeo method (Limpus and Reed, 1985). Captured turtles were flipper-tagged with a unique alpha-numeric inscribed titanium tag (Stockbrands Company, Pty. Ltd., Perth, Western Australia), and had their curved carapace length (CCL ± 1 mm) measured using a flexible tape measure. Skin samples (approx. 5 x 5 mm) were collected using a sterilised scalpel for each turtle. At CB and GI the skin samples were taken from the neck and stored in a 20% DMSO solution saturated with NaCl. At LI the samples were collected from the trailing edge of the front flipper and stored in 90% ethanol. Samples from a total of 278 turtles were collected (see Table 5.1 for details).

Table 5.1. Green turtle demographics from three sampled Great Barrier Reef foraging grounds. The total number of turtles sampled per site (n), and number of juvenile (J), sub-adult (SA) and adult (A) turtles within each site are shown. Curved-carapace length (CCL) mean and range are also provided.

Foraging Ground	n	Size class	Mean CCL(cm)	Range of CCL(cm)
Low Isles (LI)	147	114 J; 33 SA; 0A	55.2	39.7 – 80.2
Green Island (GI)	57	52 J; 5 SA; 0A	50.4	47.0 - 84.4
Cockle Bay (CB)	74	58 J; 12 SA; 4A	54.5	40.2-103.9

Sample collection at Cockle Bay and Green Island was conducted under scientific research permit G12/35326.1 by an appointed conservation officer under the Nature Conservation Act during population monitoring. Sample collection at Low Isles was conducted under James Cook University Ethics Approval A1474 and scientific research permits G10/33206.1, G10/33897.1 & WISP06563509.

DNA extraction and Polymerase Chain Reaction (PCR)

Cockle Bay and Green Island

DNA from the CB and GI samples was extracted using the Promega Wizard® SV Genomic DNA Purification System according to the manufacturer's instructions. An extra 10µL of proteinase K was used per reaction. Final DNA concentration was obtained by spectrophotometric analysis, using the ratios of absorption at 260nm versus 280nm to determine DNA purity.

The primers ChM-Dloop-960 F (5'-AAC TAT AAC CTT CCT AGA-3') and ChM-Dloop-960 R (5'-TGT AAG TAT CCT ATT GAT T-3') were designed to target a 960bp region of the mtDNA d-loop control region in green turtles. These primers were designed in AlleleID v7 using an alignment of 15 published

green turtle sequences. These primers were optimised in conventional polymerase chain reaction (PCR) using a gradient of 50°C-60°C.

PCRs were carried out in 20µL reactions consisting of 10µL GoTaq Green Hot Start Master Mix (Promega), 0.8µM of each primer, ~80 ng of template DNA and nuclease free water to 20µL.

The PCR protocol consisted of a 5 min denaturation step (94°C) followed by 35 cycles of: 10 s at 94°C, 15 s at 54°C, and 30 s at 72°C and a final extension step of 5 min at 72°C. PCR products were visualised on a 1.2% agarose gel. Following assay optimisation, PCR products were visualised in real time using 20µL reactions consisting of 10µL GoTaq qPCR Master Mix (Promega), 0.8µM of each primer, ~80 ng of template DNA and nuclease free water to 20µL. The qPCR protocol consisted of a 2 min denaturation step (95°C) followed by 45 cycles of: 10 s at 95°C, 30 s at 51°C, and 30 s at 72°C. These products were then sent to Macrogen (Macrogen Inc., Seoul, Korea) for purification and sequencing using both the forward and reverse primers to initiate sequencing. A consensus sequence was subsequently generated and used in further analysis.

Low Isles

The DNA extraction from LI samples was performed using a salting out procedure, based upon Sunnucks and Hales (1996). Genomic DNA concentration and quality of the LI samples was evaluated through gel electrophoresis in the presence of GelGreen (Biotium).

Partial mtDNA d-loop control region (760bp) was amplified using the primers LTEi9 (5'GAATAATCAAAAGAGAAGG 3') and H950 (5'GTCTCGGATTTAGGGGTTT 3') (Abreu-Grobois et al. 2006). PCR was performed in a 25µl reaction containing 1 x NH_4 Buffer, 1.5mM MgCl, 0.25 mM dNTPs, 0.4µM of each primer, 1 Unit of BioTaqTM polymerase and ~10ng DNA. The PCR protocol consisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of 45 s at 94°C, 45 s at 52°C, and 1 min at 72°C and a final extension step of 5 min at 72°C. PCR samples were purified and sequenced by Macrogen (Macrogen, Inc., Seoul, Korea) using ABI Dye terminator chemistry on an ABI 3730 sequencer.

Characterisation of mtDNA haplotypes and mixed stock analysis (MSA)

All sequences obtained were assembled in Geneious v7.1.5 (Kearse et al. 2012) and confirmed to be the correct target using the database of the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were trimmed to ~770 bp to allow comparison with known green turtle haplotypes in the published literature.

These sequences were then compared with known haplotypes and assigned existing names accordingly. Any sequences from three or fewer turtles which did not match any known haplotypes

were re-sequenced to a total of three replicates, in order to avoid sequencing error. Where possible, new template DNA was generated from the original sample. Once confirmed, these new haplotypes were named following the nomenclature for Pacific green turtles using the prefix CmP (Jensen et al., 2016).

Haplotype frequency at each site was recorded and haplotype (h) and nucleotide diversity (π) (Nei, 1987) were estimated using Arlequin version 3.5.2.2 (Excoffier et al., 2005).

To estimate the proportional contributions of stocks to the three foraging areas, MSA was conducted using a Bayesian approach in the software program Bayes (Pella and Masuda, 2001). The mtDNA haplotype frequencies of 25 genetically distinct green turtle breeding stocks across the Indo-Pacific (see Supplementary Table 1 in Jensen et al. (2016)) were used as a baseline. As MSA estimates the proportional contributions of stocks to one feeding ground at a time, each study site was analysed independently. Each analysis consisted of 4 independent chains with different starting points. Each chain was run for a total of 50 000 steps discarding the first 25 000 steps as burn-in. To determine whether all chains had converged we used the Gelman and Rubin shrink factor diagnostic (shrink factor < 1.2) (Pella and Masuda, 2001). The analysis was conducted with both uniform priors (Model 1) and weighted priors (Model 2). In model 2 the priors were weighted according to the nesting population size associated with each stock. The results were summarised for both individual stocks and regional estimates grouping the sGBR and CS (sGBR/CS) as well as 21 stocks that all contributed <5% (other).

Results

The sequence data of a 770bp fragment of the d-loop control region was obtained from 278 individual turtles across three foraging sites. A total of 35 haplotypes were identified, 13 of which had never been observed at a rookery (orphan haplotypes). Eight of this subset were previously undescribed; one at Cockle Bay (CmP80.4), four at Green Island (CmP234.1, CmP235.1, CmP236.1 and CmP237.1), and three at Low Isles (CmP145.1, 166.2 and CMP211.1). The remaining five haplotypes had been described in previous studies, but had also not yet been observed at a rookery (CmP34.1, CmP55.1, CmP119.1, CmP165.1 and CmP200.1) (Table 5.2). These orphan haplotypes occurred at low frequencies (2.7-10.5%) and comprised only 6.5% of the total number of turtles sampled. The most common haplotype observed was CmP47.1 at all three sites; CB (73%), GI (67%) and LI (53%) (Table 5.2). Haplotype and nucleotide diversity both increased from south to north along the GBR, although CB and GI share similar nucleotide diversity (Table 5.2).

Table 5.2. Haplotype frequencies of green turtles sampled at Cockle Bay (CB), Green Island (GI) and Low Isles (LI) along the Great Barrier Reef, Australia.

Haplotype Name	Accession Number	Reference	Location		
			Cockle Bay (CB)	Green Island (GI)	Low Isles (LI)
CmP20.1	AB819806	Hamabata et al. (2014)	-	2	1
CmP20.2	KF311744	Dutton et al. (2014)	-	-	1
CmP22.1	KF311747	Dutton et al. (2014)	1	-	1
CmP40.1	KF311750	Dutton et al. (2014)	-	-	2
CmP44.1	KF311751	Dutton et al. (2014)	4	3	10
CmP44.2	KF311752	Dutton et al. (2014)	-	-	1
CmP47.1	KF311753	Dutton et al. (2014)	54	38	78
CmP49.1	AB819808	Hamabata et al. (2014)	1	-	-
CmP57.2	KJ502567	Jensen et al. (2016)	-	-	3
CmP65.1	KF311756	Dutton et al. (2014)	-	-	1
CmP68.1	KJ502591	Jensen et al. (2016)	-	-	1
CmP77.1	KF311759	Dutton et al. (2014)	-	-	1
CmP80.1	KF311760	Dutton et al. (2014)	8	6	19
CmP81.1	KJ502610	Jensen et al. (2016)	-	-	2
CmP84.1	KJ502630	Jensen et al. (2016)	1	-	1
CmP85.1	KF311761	Dutton et al. (2014)	2	1	3
CmP91.1	KF311762	Dutton et al. (2014)	-	-	2
CmP98.1	FJ917199	Dutton et al. (2009)	-	-	6
CmP168.1	KJ502617	Jensen et al. (2016)	-	-	1
CmP169.1	KJ502608	Jensen et al. (2016)	1	-	-
CmP180.1	KJ502640	Jensen et al. (2016)		-	2
CmP193.1	KJ502635	Jensen et al. (2016)	-	1	1
		Total	72	51	137
Orphan Haplotypes					
CmP34.1	KJ502581	Jensen et al. (2016)	-	1	-
CmP55.1	KJ502596	Jensen et al. (2016)	-	1	4
CmP80.4	MH004276	This study	1	-	-
CmP119.1	KJ502611	Jensen et al. (2016)	-	-	1
CmP145.1	MH004277	This study	-	-	1
CmP165.1	KJ502582	Jensen et al. (2016)	1	-	-
CmP166.2	MH004278	This study	-	-	2
CmP200.1	KJ502586	Jensen et al. (2016)	-	-	1
CmP211.1	MH004283	This study	-	-	1
CmP234.1	MH004279	This study	-	1	-
CmP235.1	MH004280	This study	-	1	-
CmP236.1	MH004281	This study	-	1	-
CmP237.1	MH004282	This study	-	1	-
		Total	2	6	10
		Cumulative total	**74**	**57**	**147**

Table 5.3. Sample size (n), number of haplotypes (H) and estimates (± SD) of haplotype (h) and nucleotide (π) diversity for 3 *Chelonia mydas* foraging sites on the Great Barrier Reef, Australia.

Foraging site	n	H	h	π
Cockle Bay	74	10	0.4572 ± 0.0694	0.013573 ± 0.006930
Green Island	57	12	0.5476 ± 0.0772	0.012378 ± 0.006384
Low Isles	147	26	0.6970 ± 0.0396	0.019210 ± 0.009563

Mixed stock analysis

The MSA showed that the nGBR, CS, sGBR and New Caledonia (NC) stocks supplied the bulk of the turtles at all three sites (> 91.6% overall) (Table 5.4). Small contributions were also made by other more distant green turtle rookeries in the region, but together they made up ~8% at each site. Both Model 1 (uniform priors) and Model 2 (weighted priors) yielded similar results (Table 5.4), and for the purpose of simplicity, we only discuss results from Model 2 from hereon. Given the uncertainty surrounding small contribution estimates we grouped rookeries with <5% estimated mean contribution into 'Other'. We were unable to run the MSA for individual age classes due to insufficient sample sizes.

Table 5.4. Results (mean % ± 95% confidence intervals in parentheses) from the Bayesian mixed stock analysis (MSA) (Pella and Masuda, 2001) for Cockle Bay, Green Island and Low Isles Green Turtles (both individually and by region). MSA was calculated using 25 regional breeding stocks as possible sources, but for simplicity only the 4 main contributors are listed—nGBR: northern Great Barrier Reef; sGBR: southern Great Barrier Reef CS: Coral Sea and NC: New Caledonia. The combined contributions of the remaining 21 stocks are compiled into the 'Other' category. Model 1 = uniform priors; Model 2 = weighted priors

		Cockle Bay		Green Island		Low Isles	
	Stock	**Model 1**	**Model 2**	**Model 1**	**Model 2**	**Model 1**	**Model 2**
Individual	**nGBR**	7.5 (1.7-15.8)	8.0 (2.0-16.4)	1.5 (0.0-10.1)	3.7 (0.0-13.3)	14.7 (8.7-21.7)	15.0 (8.9-22.3)
	CS	2.5 (0.0-29.4)	0.6 (0.0-5.4)	50.4 (0.9-92.8)	48.6 (0.0-95.0)	60.1 (20.3-78.1)	60.0 (19.7-79.0)
	sGBR	79.7 (53.8-91.3)	82.8 (68.9-92.9)	33.7 (0.0-85.2)	38.4 (0.0-90.5)	10.8 (0.0-50.4)	11.8 (0.0-52.0)
	NC	7.3 (0.0-19.4)	6.9 (0.0-19.8)	9.4 (0.0-23.6)	6.1 (0.0-22.0)	6.0 (1.5-13.2)	6.3 (1.5-13.7)
	Other	3 (0.1-8.7)	1.7 (0.0-6.5)	5.0 (0.3-12.8)	3.2 (0.0-11.6)	8.4 (3.9-14.0)	6.9 (2.8-12.3)
Regional	**nGBR**	7.5 (1.7-15.8)	8.0 (2.0-16.4)	1.5 (0.0-10.1)	3.7 (0.0-13.3)	14.7 (8.7-21.7)	15.0 (8.9-22.3)
	sGBR/CS	82.2 (70.1-91.7)	83.3 (70.8-93.0)	84.1 (70.2-94.3)	87.0 (73.2-96.4)	70.9 (61.8-79.1)	71.8 (62.6-80.0)
	NC	7.3(0.0-19.4)	6.9 (0.0-19.8)	9.4 (0.0-23.6)	6.1 (0.0-22.0)	6.0 (1.5-13.2)	6.3 (1.5-13.7)
	Other	3.0 (0.1-8.6)	1.9 (0.0-6.5)	5.1 (0.4-12.9)	3.1 (0.0-11.6)	8.4 (4.0-14.0)	6.9 (2.8-12.3)

The contribution of NC stocks was approximately equal at all three sites (Table 5.2), and in all cases was above that which would be considered a small contribution. However, the nGBR, CS and sGBR stock contributions shifted between sites. Turtles at CB, the most southerly site, predominantly originated from sGBR stocks (82.8%, 95% CI 68.9-92.9%), with small contributions from nGBR (8.0%, 95% CI=2.0-16.4%) and CS (0.6%, 95% CI= 0.0-5.4%) stocks, respectively. The CS stock was dominant at both GI and LI (approximately 50% to 60%, respectively). As a general trend, the contributions of nGBR stock increased from south to north, whilst the sGBR stock contributions simultaneously decreased. The most dramatic shifts in nGBR stock contributions were observed between GI and LI; nGBR contributions increased from 3.7% (95% CI = 0.0-13.3%) at GI to 15.0% (95% CI = 8.9-22.3%) at LI and the sGBR contributions decreased from 38.4% (95% CI = 0.0-90.5%) at GI to 11.8% (95% CI = 0.0-52.0%) at LI. Interestingly, nGBR stock contributions were lower at GI than CB, despite GI being situated more northerly.

The results also indicate a shift in CS stock contributions from CB to GI, which are separated by approximately 280km. While the CS contribution is low at CB (0.6%), it makes up the majority of turtles at GI (48.6%, 95%CI = 0.0-95.0) and LI (60.0%, 95% CI = 19.7-79.0). In comparison, the contribution of sGBR is highest at CB (82.8%, 95% CI = 68.9-92.9%), medium at GI (38.4%, 95% CI = 0.0-90.5%) and lowest at LI (11.8%, 95% CI = 0.0-52.0%) (Table 5.4).

These results, combined with previously published reports, were plotted on a chart which shows the stock composition shifting along a latitudinal gradient (Figure 5.2). It is possible that these data could be used to predict stock compositions at sites along this gradient that have not been previously sampled.

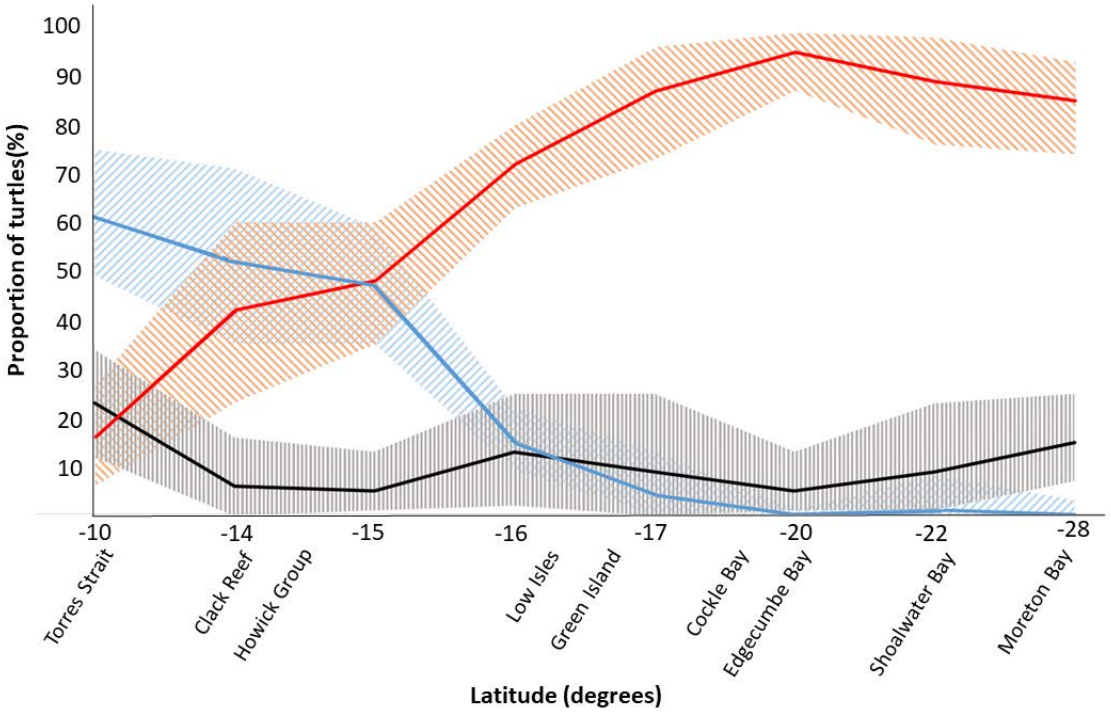

Figure 5.2. For green turtle aggregations at selected foraging grounds in the Great Barrier Reef (GBR) and southern Queensland, Australia, the proportional contributions of three important genetic sources showed a notable relationship with latitude. The southern GBR (sGBR) and Coral Sea (CS) stocks were combined for this figure to allow comparison with Jensen et al. (2016) and are denoted in orange. The nGBR stock (northern GBR) is represented in blue and 'Other' stocks, represented in black; hatched areas in all three cases represented 95% confidence intervals. The 'Other' group comprises the remaining 22 stocks in this region (see Jensen et al. (2016)) and were combined because these stocks were found to contribute a small proportion of the turtles at each study site. Data for Low Isles, Green Island and Edgecumbe Bay from the present study and data for all other sites from Jensen et al. (2016).

Discussion

Previous studies indicated that foraging grounds along the GBR are dominated by the nGBR, sGBR and Coral Sea genetic stocks and that the proportions of those stocks change gradually from north to south (Dethmers et al., 2006; Jensen et al., 2016). However, a 700 km unsampled gap separated foraging grounds of predominantly nGBR stocks (the Howick Group) and foraging grounds further south where only a small proportion of nGBR turtles were observed (Edgecumbe Bay) (Jensen et al., 2016) (Figure 5.1). This sampling gap precluded informed management regarding the stocks that might be impacted at foraging grounds along the central part of the GBR and was therefore the focal area of our study.

Due to the high degree of genetic similarity between the CS and sGBR stocks, the MSA estimates for these stocks are surrounded by high uncertainty. In order to address this, we combined the summary statistics of these genetically similar stocks. However, Read et al. (2015), who also utilised MSA to study turtles in the Indo-pacific region, reported summary statistics for individual stocks. To make our results comparable with both the Jensen et al. (2016) and Read et al. (2015) studies, we present the summary statistics for both individual stocks, as well as the combined CS/sGBR stock (Table 5.4).

Our results show that gradual changes in stock contribution occur between CB and LI. The combined sGBR/CS stock foraging at CB and GI made up a smaller proportion (83-87%) at these sites compared to the proportion observed at the more southerly Edgecumbe Bay (95%) (Jensen et al., 2016). This proportion decreased further at the more northern LI (72%). The contrary was evident for the nGBR stock that declined from making up half of the juvenile turtles foraging at the Howick group (Jensen et al., 2016) to 15% at LI and decreasing further at GI and CB (4% and 8%, respectively) to 0% at Edgecumbe Bay. In addition, we found that all three study sites (LI, GI and CB) were comprised of a small portion (6-7%) of turtles from the New Caledonia stock, which is derived from rookeries more than 1800 km away. These findings are consistent with both tag-recovery data and MSA results from other studies, suggesting that New Caledonia turtles use multiple feeding grounds along the Great Barrier Reef (Jensen et al., 2016; Read et al., 2015; Read et al., 2014). Interestingly, whilst CS stock was found to contribute a large proportion of the turtles at our study sites, this stock was found to contribute only a small proportion of turtles foraging in New Caledonia (Read et al., 2015).

The shift in the composition of regional stocks at foraging areas along the Great Barrier Reef may in part be explained by ocean currents, as has been suggested for mixed stocks of marine turtles in in other regions (Blumenthal et al., 2009; Carreras et al., 2006; Lahanas et al., 1998; Luke et al., 2004). The three foraging grounds sampled in our study (LI, GI and CB) are geographically situated near an

area of variable currents (Choukroun et al., 2010) associated with the South Equatorial Current dividing into the south-flowing East Australian Current and the north flowing North Queensland Current (Figure 5.1). Such a split is likely to influence the dispersal of new recruits approaching the Australian east coast following their oceanic phase as they move towards their neritic foraging areas. The high proportion of CS stock observed at our study sites on the GBR compared to the low proportion of this stock observed at New Caledonia (Read et al., 2015) further supports this theory. However, the mechanisms of how these new recruits settle at neritic foraging areas are not known and would be a worthy avenue for future research.

While the vast majority of sampled turtles came from rookeries within the GBR region, Coral Sea and New Caledonia, a small proportion of turtles came from more distant rookeries. The latter stocks were grouped collectively into the 'Other' category. However, the distribution of specific haplotypes at regional rookeries reveal their likely origin. For example, CmP20.1 is common throughout Micronesia, CmP22.1 in the Marshall Islands and CmP65.1 has only been found in American Samoa and French Polynesia (see Dutton et al. (2014) and Hamabata et al. (2014)). In this study, these haplotypes were infrequently found; two turtles at GI and one turtle at LI were found to be CmP20.1, while CmP22.1 was found once at both CB and LI. One turtle at CB was found to be CmP65.1, making this the first known record of this haplotype on the GBR. These rare long-distance dispersal events are supported by tag returns from turtles as far as the Marshall Islands foraging along the GBR (Limpus et al., 2009).

We identified 13 orphan haplotypes across all three sites and encountered them more frequently in the more northerly sites. These haplotypes were distributed as CB:2, GI:6 and LI:6, with one orphan haplotype (CmP55.1) present at both GI and LI. Eight of these haplotypes were previously undescribed whilst five others had been described in previous studies, but had not yet been observed at a rookery (Jensen et al., 2016). Orphan haplotypes at GI were found to comprise nearly 11% of all turtles sampled. These orphan haplotypes indicate that some of the known rookeries may require larger sample sizes to accurately capture the haplotype composition. It is also possible our study sites may have received turtles from unidentified and unsampled rookeries that might exist in south-east Asia or the south-western Pacific. While these orphan haplotypes highlight the need for additional sampling of green turtle rookeries in the region, it is encouraging that they only comprise a small percentage (<6.5%) of the total data set.

The Chm-dloop 960 primer set described here is specific to green turtles and can be used to obtain a longer (960bp) fragment of the d-loop control region, thereby allowing for an improved resolution. Many of the haplotypes in this study are shared between a number of stocks (e.g. CmP80.1 is found

in the nGBR, sGBR, Coral Sea and New Caledonia stocks). However, when analysing the longer fragment of mtDNA, this haplotype could be consistently split into two distinct haplotypes and potentially add resolution to the stock structure of those populations. Therefore, future studies may benefit from using this assay, or preferably designing primers that target the entire d-loop region. In particular, this increased resolution may aid in resolving any uncertainty in separating the sGBR and CS stocks in the MSA. Moreover, such work may allow researchers to more reliably distinguish the region of origin for particular haplotypes (for example, tracing a certain haplotype back to one stock instead of four).

As marine turtles have a complex life history, it is important that conservation strategies target the full range of life stages and habitats used by these turtles. In order to effectively manage threats to green turtles, we must understand the size of the stocks and the factors that are threatening them (Hamann et al., 2010). The identification of individual green turtle stocks present on the GBR has greatly improved the monitoring and management of this species by allowing a more targeted approach. Each stock is considered to be a separate management unit that is demographically independent, hence a decline in one stock would not be replenished by another (Dobbs, 2001; Waldman, 2005). As a result of unsustainable commercial harvesting of green turtles in the southern GBR in the early to mid 1900s (Limpus, 2008), the sGBR stock presumably declined. While the sGBR populations are presently recovering (Chaloupka et al., 2008a; Department of the Environment and Energy, 2017; GBRMPA, 2014b; Limpus, 2008), the pressure from historical consumptive use may have affected the distribution of this stock or the composition of different age classes. Similarly, the nGBR stock has demonstrated a plateau and there is the potential for a decline in population size due to decreased hatchling success at Raine Island (Chaloupka et al., 2008a; GBRMPA, 2014b; Limpus et al., 2003). This may already be reflected in the results from the present study, and it is likely that the nGBR contributions to these foraging grounds will decrease further in the future, increasing the urgency for effective conservation strategies which target threats to this stock. Our work confirms that threats to green turtles which occur in GBR foraging areas north and south of Low Isles will predominantly affect the nGBR stock and sGBR/CS stocks respectively. In the present study, we also show that the CS stock likely contributes significant proportions of turtles at both LI and GI with approximately half of the GI green turtles identified as CS stock. This alone indicates that in order to effectively protect green turtles residing in this region of the GBR, we must extend monitoring and conservation efforts to include the CS rookeries because there are currently no monitoring data available for the Coral Sea, making it difficult to know the status of this stock.

The GBR supports a large number of foraging marine turtles, yet monitoring has only occurred at a small number of sites because monitoring programs (and associated studies such as ours) in this

region are often logistically challenging to establish and maintain, requiring both considerable funding and uniquely skilled persons. Our data provides confirmation and improved resolution to show the current latitudinal spread of haplotypes of turtles inhabiting the GBR. Turtles at foraging sites north of the Howick Group are more likely to originate from the nGBR stock while turtles foraging south of LI appear more likely to come from CS and/or sGBR stock. These distribution patterns could potentially be influenced by declines or increases in nesting success at the major rookeries in the future and should therefore be regarded as a representation of the current situation. However, the steady shift in stock composition highlighted in this chapter (Figure 5.2) may provide a means to predict the stock composition at other un-sampled foraging grounds in this region in order to make more informed management decisions while circumventing the need to sample and assess additional locations. Continued monitoring of these stocks will allow managers to develop targeted management plans and effectively conserve this iconic species.

The aims of this chapter were addressed as follows:

1. **Develop, optimise and validate a PCR assay which targets green turtle mtDNA control region sequences**

   A primer set designed to target a 960bp fragment of d-loop region of green turtle mtDNA was designed for this study. This assay generates a resulting amplicon that is longer than previously used for these studies. We found that this allowed for improved resolution of haplotype identification. However, to compare with those already characterised we had to trim our sequences down to 770bp. This resulted in sequences we knew to be distinctly different to appear the same once trimmed. To address this, a re-characterisation of already published sequences is needed by either using this PCR assay, or the entire d-loop region.
   A total of 35 haplotypes were identified through use of this assay, eight of which were newly described in this study.

2. **Generate and use mtDNA control region sequences and MSA to quantify the stock composition of green turtles at three foraging areas located between Edgecumbe Bay and the Howick Group**

   The haplotype frequencies identified at each site were used in an MSA to estimate relative stock contributions. The results suggest that the northern GBR (nGBR), Coral Sea (CS), southern GBR (sGBR) and New Caledonia (NC) stocks supplied the bulk of the turtles at all three study sites, and the relative contributions of these stocks varied among sites. At the most southern site (Cockle Bay) turtles predominantly originated from the sGBR stock. This contribution was significantly decreased at the more northerly Green Island where the main contribution came from CS stock. At the most northerly site (Low Isles), the contribution from CS stock was further increased. The suggested that the dramatic shift in stock contributions which was previously reported (Jensen et al., 2016), was actually more of a gradual shift along a coastal gradient.
   It should be noted that the analysis was also conducted with the sGBR and CS stock grouped as a region, as there was some debate about whether the MSA could reliably distinguish between them. During this analysis, the trend of the shifting contributions was lost. This highlights the need for improved resolution in haplotype and subsequent genetic stock identification, which again may be addressed by using longer fragments of DNA from assays such as the one described in the present study.

3. **Use our new data and data from previously sampled foraging areas to assess the correlation between stock composition and latitude of foraging areas in Eastern Australian waters**

   Noting the trend of a gradual shift in stock contributions along the Queensland coast, data was kindly shared from the authors of Jensen et al. (2016) in order to assess whether this pattern was continuous. Figure 5.2 highlights the latitudinal spread of the main genetic stocks on the Great Barrier Reef and could be used to estimate stock contributions at as yet unsampled foraging grounds. This could be hugely beneficial to managers, who can make stock-level management decisions based on this estimation, without the need for intensive sampling and laboratory analysis.

Publications and presentations arising from this study

- **Jones K**, Jensen M, Burgess G, Ariel E. 2018. Closing the gap: Mixed stock analysis of three foraging populations of green turtles (*Chelonia mydas*) on the Great Barrier Reef. *PeerJ*. 6:e5651https://doi.org/10.7717/peerj.5651
- **Jones, K.** 2017. Closing the gap: Mixed stock analysis of three foraging populations of green turtles (*Chelonia mydas*) on the Great Barrier Reef. *Proceedings from the 4th Annual Sea Turtle Health and Rehabilitation Workshop*. 5-7th September, Townsville, Australia.

My contributions to this study:

- I conceived and co-designed the primers for the PCR assays with my supervisor
- I optimised the PCR assays
- I performed the laboratory work, including both DNA extraction and PCR
- I managed the datasets obtained
- I assembled, trimmed and, where appropriate, edited the resulting sequence data
- I, under the advice of my supervisor, analyzed the data and interpreted the results
- I submitted the newly described sequences to GenBank and obtained Accession Numbers
- I drafted the chapter and edited it as advised by collaborators and supervisors
- I drafted the manuscript and managed the process of journal submission and review

Chapter Six:
Molecular evidence of horizontal transmission of chelonid alphaherpesvirus 5 at green turtle (*Chelonia mydas*) foraging grounds in Queensland, Australia

Background and aims of this chapter

Chapter Two identified a gap in the understanding of ChHV5 transmission pathways and emphasised the need for research in this area in order to better inform management decisions. Chapter Five provided a validated method for green turtle genetic stock identification, which facilitates an investigation into a link between ChHV5 strain and genetic stock, and thereby explore the possibility of vertical transmission from parent to offspring. Based on a larger sample size and improved molecular techniques compared to previous reports (Ariel et al., 2017), this study expands the phylogenetic knowledge of ChHV5 in Australia and assesses any relationship between host genetic stock and viral variant.

In order to inform management decisions and improve conservation outcomes for green turtles, this study aimed to improve our understanding of ChHV5 phylogeny and transmission along the GBR through the following aims:

1. Improve the resolution of the current phylogeny of ChHV5 in Australia by generating a more robust sequence data set than has previously been used, including a larger sample size and increased geographical locations
2. Assess the relationship between host genetic stock and viral variant in order to clarify the mechanisms of viral transmission.

Introduction

Fibropapillomatosis (FP) is a marine turtle disease, characterised by the growth of benign tumours on the skin, eyes, shell, oral cavity and/or viscera. This disease has been reported in every species of marine turtle but predominantly affects the endangered green turtle (*Chelonia mydas*) (Jones et al., 2016). Although benign, FP tumours are physically debilitating as their positioning can impair vision, feeding and locomotion (Flint et al., 2010a; Herbst, 1994; Work et al., 2004), leaving the affected turtle with increased vulnerability to predation, starvation and boat-strike. Turtles with FP are also typically chronically stressed (Aguirre et al., 1995) and immunosuppressed (Aguirre et al., 1995; Work et al., 2001) and are therefore susceptible to secondary infections and opportunistic pathogens. FP has a global distribution, with prevalence rates varying spatially and temporally (Jones et al., 2016). Such variance in disease prevalence creates a unique challenge for environmental managers; a more solid understanding of this disease is critical the development of informed management plans.

Although the causative agent of FP is yet to be confirmed, studies have consistently reported a link between FP tumours and the presence of a herpesvirus (Herbst et al., 1995; Jacobson et al., 1991; Jacobson et al., 1989). As this virus could not be cultured in vitro until recently (Work et al., 2017), there has been an increase in studies utilizing molecular methods to better understand this herpesvirus (Alfaro-Nunez et al., 2014; Lackovich et al., 1999; Lu et al., 2000b; Lu et al., 2003; Nigro et al., 2004a; Nigro et al., 2004b; Page-Karjian et al., 2015; Page-Karjian et al., 2012; Quackenbush et al., 2001; Quackenbush et al., 1998; Rodenbusch et al., 2014; Yu et al., 2001; Yu et al., 2000). These studies have added to the body of evidence linking a turtle-specific herpesvirus, known as chelonid alphaherpesvirus 5 (ChHV5), and FP. As such, ChHV5 is now generally accepted as the likely causative agent of this disease.

Genetic variation of ChHV5 is an emerging field, wherein four distinct clades of ChHV5 have been described globally (eastern Pacific, western Atlantic and eastern Caribbean, Midwest Pacific, and Atlantic (Patrício et al., 2012). Samples collected from turtles from a particular region tends to cluster into the associated phylogeographic group; i.e. samples from Brazil cluster into the Atlantic (Rodenbusch et al., 2014) and samples from Ecuador into Pacific (Cardenas et al., 2018). Variation in ChHV5 has also been described at more local levels; four variants in Florida (Ene et al., 2005) and six variants in Brazil (Rodenbusch et al., 2014). A geographic influence on the distribution of these variants has been reported in both Brazil (Rodenbusch et al., 2014), Florida (Ene et al., 2005), Hawaii (Herbst et al., 2004) and most recently, Australia (Ariel et al., 2017). Characteristically, turtles at a particular foraging ground are infected with the same viral variant, which is distinct from variants

found at other foraging locations within a particular region (Ariel et al., 2017; Ene et al., 2005; Greenblatt et al., 2005a; Herbst et al., 2004; Patrício et al., 2012; Rodenbusch et al., 2014).

Marine turtles have a complex life-history, spanning multiple habitats, which makes it difficult to pinpoint the stage and location that ChHV5 transmission occurs. Hatchlings emerge from rookeries in tropical and subtropical regions where they then undertake a pelagic existence. Several years later, they recruit into inshore foraging grounds as juveniles (Reich et al., 2007). The animals at these foraging grounds are comprised of turtles from multiple regional rookeries (Anderson et al., 2013; Dutton et al., 2014; Lahanas et al., 1998). These turtles have strong site fidelity to both the foraging ground they inhabit and the rookery from which they originated; turtles will attempt to return to this rookery to breed and nest at the onset of sexual maturity (Musick and Limpus, 1997). Due to this natal philopatry, turtles originating from rookeries in a particular region are genetically distinct stocks. Transmission of ChHV5 may occur at the rookery, the foraging ground, or in transit between these habitats. Assessing distribution patterns of the virus may provide an indication as to which of these locations, if any, is the site of transmission.

If ChHV5 transmission is vertical, occurring at rookeries from parent to offspring, a homogeneous distribution of genetic variance of ChHV5 at each rookery would be expected (Ene et al., 2005). In such a situation, a link between viral variant and turtle origin (genetic stock) would also be expected, regardless of sampling location. Conversely, if ChHV5 transmission is occurring horizontally at foraging grounds, a homogeneous distribution of genetic variance of ChHV5 at each foraging ground (and heterogeneous distribution over multiple foraging grounds) would be observed (Ene et al., 2005). In this case, a link between viral variant and host origin would be less likely. The heterogeneity in viral variant distribution observed in previous studies, coupled with high FP prevalence in juvenile/immature turtles (Jones et al., 2016), has led to the hypothesis that ChHV5 transmission occurs upon recruitment into inshore foraging grounds after the pelagic phase in the marine turtle life-cycle (Ene et al., 2005; Jones et al., 2016; Patrício et al., 2012). Whilst this hypothesis is widely accepted, a molecular link between viral variant and host origin has never been investigated using molecular methods.

Although a global understanding of FP and ChHV5 is emerging, Australia is an understudied region. The Great Barrier Reef (GBR) supports some of the largest green turtle rookeries and foraging populations in the world (Chaloupka et al., 2008a; Limpus, 2008) and relies heavily on the presence of green turtles for ecotourism (Dobbs, 2001; Gulko, 2004). Turtles with FP have been observed at multiple locations on the GBR since the 1970's (Hargrove et al., 2016) yet, to date, only two molecular studies on ChHV5 have generated and analyzed sequence data from samples collected in

Australia (Ariel et al., 2017; Quackenbush et al., 2001). A geographic influence on viral variant distribution along the north Queensland coast was recently reported (Ariel et al., 2017), but a link between viral variant and host origin was not assessed. Moreover, the presence and distribution of ChHV5 along the entire coast of the GBR has not been investigated and a solid understanding of FP and ChHV5 on the GBR is yet to be established. As a result, marine turtle management plans are unable to detail an effective means of managing this threat.

In order to inform management decisions and improve conservation outcomes for *C. mydas* and other vulnerable turtle species, this study aims to improve our understanding of ChHV5 along the GBR through the following objectives: Firstly, this study will improve the resolution of the current phylogeny of ChHV5 in Australia by generating a more robust sequence data set than has previously been used, including a larger sample size and increased geographical locations. Secondly, the relationship between host genetic stock and viral variant will be assessed in order to clarify the mechanisms of viral transmission.

Materials and Methods

Sample origin

A total of 59 green turtles, two loggerhead (*Caretta caretta*) turtle and one green/hawksbill (*Eretmochelys imbricata*) hybrid turtle were sampled across five locations along the GBR. The majority of samples used in this study were collected opportunistically from turtles with FP tumours, captured using the rodeo capture technique (Limpus and Reed, 1985) at various foraging grounds along the GBR (Figure 6.5). The remaining tumour samples were collected during necropsy and others were donated (see Appendix Three: Supplementary Table 6.1). The final dataset consisted of turtles from waters near Brisbane (n=7), Gladstone (n=4), Airlie Beach (n=1), Bowen (n=27), Townsville (n=22), and Cairns (n=1). These turtles were predominantly juveniles, with an age class breakdown for the green turtles of 53 juveniles, five sub-adults and one adult. Both loggerheads were immature (Limpus et al., 1994b). The green/hawksbill hybrid (QA47488) was believed to be immature, based on ranges for both hawksbill (Limpus, 1992) and green turtles (Limpus et al., 1994a).

All live turtles were sampled under permits from James Cook University Animal Ethics Committee (A1501 and A1971), Department of Environment and Science (WISP06619309 and WISP13754613) and Great Barrier Reef Marine Park Authority (G10/33220.1 and G36593.1).

Figure 6.5. Samples (n=62) were collected from six locations along the Queensland coast of Australia; Brisbane (n=7), Gladstone (n=4), Airlie Beach (n=1), Bowen (n=27), Townsville (n=22), and Cairns (n=1). Five of these sites are located within the Great Barrier Reef (GBR) Marine Park, whilst Brisbane is located just south of the GBR boundary (indicated by hatched area).

Sample Collection

All live turtles were flipper-tagged with a unique alpha-numeric inscribed titanium tag (Stockbrands Company, Pty. Ltd., Perth, Western Australia), and had their curved carapace length (CCL ± 2 mm) measured using a flexible tape measure. Tumour samples were collected with a paired skin sample from the trailing edge of the front flipper of each turtle. Tissue samples were collected using fresh, sterile, disposable scalpel blades and stored in cryovials containing 90% ethanol. Samples were stored at 4°C prior to DNA extraction.

DNA extraction, Primer Design and Polymerase Chain Reaction (PCR)

DNA was extracted using the Promega Wizard® SV Genomic DNA Purification System according to the manufacturer's instructions with the exception of an additional 10µL of proteinase K used per reaction. Final DNA concentration was obtained by spectrophotometric analysis (Implen Nanophotometer), using the ratios of absorption at 260nm versus 280nm to determine DNA purity.

Primers were designed to target the full-length sequence of three genes within the ChHV5 genome; 1) glycoprotein B (gB), 2) sialyltransferase (F-sial) and 3) DNA polymerase (DNApol). The DNApol gene has been used extensively to determine the presence or absence of ChHV5 (Ene et al., 2005; Greenblatt et al., 2005a; Lu et al., 2000b; Lu et al., 2000c; Lu et al., 2003; Page-Karjian et al., 2012; Patrício et al., 2012; Quackenbush et al., 1998; Rodenbusch et al., 2014; Yu et al., 2001; Yu et al., 2000) due to the highly conserved nature of the gene (Monezi et al., 2016; Origgi et al., 2015). Conversely, the gB gene codes for glycoproteins which are located on the surface of the virion and therefore in contact with the host immune system, increasing selection pressure. This antigenic nature of gB has led to sequence variability, making it an ideal candidate gene for phylogenetic studies (Bender et al., 2007; Coberley et al., 2002; Origgi et al., 2015). Moreover, Ariel et al. (2017) demonstrated that this gene is effective in determining ChHV5 phylogeny in Australia. The F-sial gene is atypical of herpesviruses and poorly understood, but has been suggested to play a role in ChHV5 pathogenesis (Ackermann et al., 2012).

We also designed and optimised a set of four overlapping primers pairs for gB. Although each of these overlapping primer pairs could be used individually to detect and sequence fragments of ChHV5, it was considered as one assay for the purpose of this study (referred to herein as gB FullOverlap 1-4). All primer sets were designed to include the start and stop codons within the resulting amplicon; primers targeting these regions were placed outside of the target genes so that the resulting sequences could be trimmed to the open reading frame (ORF). The gB primer pairs outside the ORF were designed using an alignment of two ChHV5 gB sequences available from GenBank (National Center for Biotechnology Information; NCBI, Bethesda, Maryland), while primers pairs within the ORF were designed from an alignment of 17 ChHV5 gB sequences. The F-Sial and

DNApol primer sets were similarly designed from an alignment of two ChHV5 F-Sial sequences and two ChHV5 DNApol sequences respectively. All primers were designed using AlleleID version 7.7 (Premier Biosoft International, Palo Alto, California) and optimised in conventional PCR using a gradient of 50-60°C (Table 6.1); conventional PCR was selected based on the long amplicon length.

Table 6.1. Primer sequences used to target ChHV5 genes of interest (glycoprotein B; gB, sialyltransferase; F-sial and DNA polymerase; DNApol) and a green turtle (*C. mydas*) mtDNA gene (D-loop). F = forward, R = reverse.

Primers	Sequence (5′ → 3′)	Length (bp)	Target gene	Reference
gB-2873 F	AGTGTCCCTTGGTAGTTG	2873	Complete gB	This study
gB-2873 R	GCAATAACGAAATCATAAAGTGTA	2873	Complete gB	This study
gB-Part1-752 F	AGGAGAATCTTTGGTGGC	752	Partial gB	This study
gB-Part1 752 R	AAGTCGTAAGGATAAGGAGATTT	752	Partial gB	This study
gB-Part2 780 F	AATGGGTGTGGGAAAGAG	780	Partial gB	This study
gB-Part2 780 R	CCGAGTTAATGTGTTGCC	780	Partial gB	This study
gB-Part3 855 F	CGCTGCGGGTAGTGAATT	855	Partial gB	This study
gB-Part3 855 R	CAACGATCCCATTGAGCA	855	Partial gB	This study
gB-Part4 786 F	AACTGGTCAACGATCTGAA	786	Partial gB	This study
gB-Part4 786 R	GGCTCGAATGCAATAACG	786	Partial gB	This study
F-Sial-1104 F	AAAAGATGTACTTGGTATTTGTGT	1104	Complete F-Sial	This study
F-Sial-1104 R	GCTAATGACGTTACGACTTTT	1104	Complete F-Sial	This study
DNApol-3670 F	AAAACTCGCAAAGAAAAGTATC	3670	Complete DNApol	This study
DNApol-3670 R	ATAAGCGGTTTGTCATCAG	3670	Complete DNApol	This study
ChM-Dloop-960 F	AACTATAACCTTCCTAGA	960	mtDNA d-loop control region	(Jones et al., 2018)
ChM-Dloop-960 R	TGTAAGTATCCTATTGATT	960	mtDNA d-loop control region	(Jones et al., 2018)

PCRs for the F-Sial-1104 and gB FullOverlap 1-4 primer sets were carried out in 20µL reactions consisting of 10µL GoTaq® Green Hot Start Master Mix (Promega), 0.8µM of each primer, ~80ng of template DNA and nuclease-free water to 20µL. PCRs for the gB-Full-2873 and DNApol-3670 primer sets had the same component volumes but utilised GoTaq® Long PCR Master Mix (Promega) due to the target amplicon length. The thermocycling conditions for all primer sets are outlined in Table 6.2.

Table 6.2. PCR thermocycling protocols for the newly described primers used in this study.

		Primer Set							
		gB FullOverlap 1-4		F-Sial-1104		gB-2873		DNApol-3670	
Step	**Cycles**	**Temperature**	**Time**	**Temperature**	**Time**	**Temperature**	**Time**	**Temperature**	**Time**
Initial denaturation	1	95°C	2 min	95°C	2 min	94°C	2 min	94°C	2 min
Denaturation		95°C	10 s	95°C	10 s	94°C	30 s	94°C	30 s
Annealing	35	60°C	15 s	59°C	15 s	60°C	30 s	60°C	30 s
Extension		72°C	30 s	72°C	30 s	72°C	3 min	72°C	4 min
Final Extension	1	72°C.	5 min	72°C.	5 min	72°C.	10 min	72°C	10 min

All tumour samples collected from green turtles were also used a PCR to amplify a 960bp fragment of the mtDNA d-loop control region using the ChM-Dloop-960 primers and associated conventional PCR protocol described in Jones et al. (2018).

PCR products were visualised on a 1.2% agarose gel and sent to Macrogen (Macrogen Inc., Seoul, Korea) for purification and bi-directional sequencing.

The gB Overlap 1-4, F-sial-1104 and ChM-Dloop-960 raw sequences were imported into Geneious v7.1.5 (Kearse et al., 2012) and assembled for each individual using reference sequences: F-UL27 of HQ878327, F-Sial of HQ878327 and the CmP47.1 haplotype (KF311753.1) respectively. These sequences were then edited where appropriate and trimmed to the ORF. The resulting consensus sequence was then extracted and confirmed to be the correct target using the database of the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Although it has been noted that phylogenetic clustering is distributed the same way whether a 6801 bp fragment of the viral genome, a 2486 bp fragment of gB or a 483 bp DNA polymerase fragment are used (Herbst et al., 2004; Patrício et al., 2012; Rodenbusch et al., 2014), these studies involved samples which were collected from locations separated by large geographic distances. As a result, gB was selected as a target gene for the more fine-scale Australian viral variant characterization and F-Sial was investigated due to its possible role in pathogenesis. The DNApol assay was used as a confirmatory assay only, and the PCR products were not sequenced.

Phylogenetic analysis

Each ChM-Dloop-960 sequence generated here was compared with known green turtle haplotypes (Dutton et al., 2014; Jensen et al., 2016; Jones et al., 2018) in order to determine the haplotypes of the individual turtles used in this study. The assigned haplotype was then included in the sequence description for both the gB and F-sial sequences.

For gB, a total of 79 sequences including 58 which were generated in this study, were aligned using Geneious v7.1.5. Only full-length sequences were used, so the final dataset consisted of 2565 positions. This dataset was then imported into Molecular Evolutionary Genetics Analysis Version X (MEGAX; Kumar et al. (2018)) for evolutionary analysis. Following a model test, the evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The rate variation among sites was modelled with a gamma distribution. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved

79 nucleotide sequences. There were a total of 2565 positions in the final dataset, and all sites were used.

Upon characterisation of the Australian ChHV5 variants, a single consensus sequence was generated to represent each variant. These representative sequences were aligned with the 21 available reference sequences used for the previous tree, resulting in a final dataset of 29 distinct nucleotide sequences and 2565 positions. A simplified phylogenetic tree was constructed to show the position of these variants relative to the available reference sequences. This tree was constructed as above.

For F-sial, 58 sequences generated from this study and two reference sequences were aligned in Geneious 7.1.5. The analysis was therefore comprised of a total of 60 nucleotide sequences. Only full-length sequences were used, so the final dataset consisted of 963 positions. This dataset was then imported into MEGAX (Kumar et al., 2018). Following a model test, the evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969).

Results

All green turtle samples amplified in the Dloop-960 assay whilst the loggerhead and hybrid (green turtle/hawksbill) samples did not. This assay is specifically designed to target green turtle mtDNA, indicating that the hybrid turtle was likely maternally hawksbill. Analysis of sequence data generated from 59 samples from individual turtles that reacted in this assay revealed that most (74.6%) belong to the CmP47.1 haplotype (Table 6.3). This is the most common haplotype found on the GBR, typically found in rookeries in the Coral Sea, southern GBR and New Caledonia (Dutton et al., 2014; Jensen et al., 2016). The remaining 13.6% of individuals were found to belong to CmP80.1 which is also found in the same regions as CmP47.1. Other turtles were found to be haplotypes typically found to originate from the northern GBR (nGBR) region (CmP98.1, 1.7%) and New Caledonia (CmP85.1, 3.4%; CmP44.2, 1.7%). CmP44.1, a haplotype found in both the nGBR and New Caledonia regions, was found in one individual (1.7%). A haplotype known to originate in the Borneo/Sulu Sea region was found in one individual (CmP57.1, 1.7%) whilst another was found to be CmP34.1 (1.7%), a haplotype of as yet unknown origins. The geographic distribution of these haplotypes among study sites varied, with multiple haplotypes identified at each study site where more than one turtle was sampled (Table 6.3). This distribution and haplotype frequency is consistent with previous reports (Jensen et al., 2016; Jones et al., 2018). These results were included in the sequence descriptions of the relevant turtles for all other sequences generated in this study.

Table 6.3. Summary of haplotype distribution in green turtles in the present study, including the regions of origin and capture location.

Haplotype	n	Percentage	Region/s of Origin	Observed locations in this study
CmP47.1	44	74.6	Coral Sea Southern GBR New Caledonia	Townsville Bowen Airlie Beach Gladstone Brisbane
CmP80.1	8	13.6	Coral Sea Southern GBR New Caledonia	Townsville Bowen Brisbane
CmP98.1	1	1.7	Northern GBR	Cairns
CmP85.1	2	3.4	New Caledonia	Bowen Gladstone
CmP44.2	1	1.7	New Caledonia	Townsville
CmP44.1	1	1.7	Northern GBR New Caledonia	Bowen
CmP57.1	1	1.7	Borneo Sulu Sea	Townsville
CmP34.1	1	1.7	Unknown (Orphan haplotype)	Townsville

All FP tumour samples amplified in at least one of the assays, confirming the presence of ChHV5 in all 62 samples (Table 6.4). None of the paired skin samples amplified in any ChHV5 assay, with the exception of that from turtle QA42923. Of the 62 tumour samples tested, 58 samples reacted in the gB Overlapping 1-4 assay, the FSial-1104 assay and DNApol-3670 assay (Table 6.4). ChHV5 DNA was detected in 93.5% of samples in each assay, and in 100% of samples overall.

Table 6.4. Number of positive detections of three ChHV5 target genes in FP tumour samples using Polymerase Chain Reaction (PCR).

		Target Genes		
Location	**n**	**DNA Polymerase**	**Glycoprotein B**	**Sialyltransferase**
Cairns	1	1	1	1
Townsville	22	21	20	20
Bowen	27	25	27	26
Airlie Beach	1	1	1	1
Gladstone	4	3	2	3
Brisbane	7	7	7	7
Total	62	58	58	58

Phylogenetic analysis

Glycoprotein B (gB)

From the nucleotide and phylogenetic analysis of the 58 sequences from this study and 21 available sequences from the NCBI database we show that Australian ChHV5 grouped into four main clusters: a Queensland cluster, north Queensland cluster, Bowen cluster and Brisbane cluster (Figure 6.2). Both the Queensland and north Queensland clusters have been previously reported (Ariel et al., 2017) whilst the Bowen and Brisbane clusters are newly identified in this study. These results highlight a strong geographic link to viral variant distribution along the Queensland coast. The results of this study have also allowed us to better characterize the variants of ChHV5 present in Australia into six clear variants, with subdivisions based on nucleotide differences from the characterised variants. These variants have been named numerically in a hierarchical form, based on whether the variants are first, second or third order clades; second order clades were named to one decimal point (e.g. Australian Variant 2.3) and third order clades were named to three decimal places (e.g. Australian Variant 2.3.1).

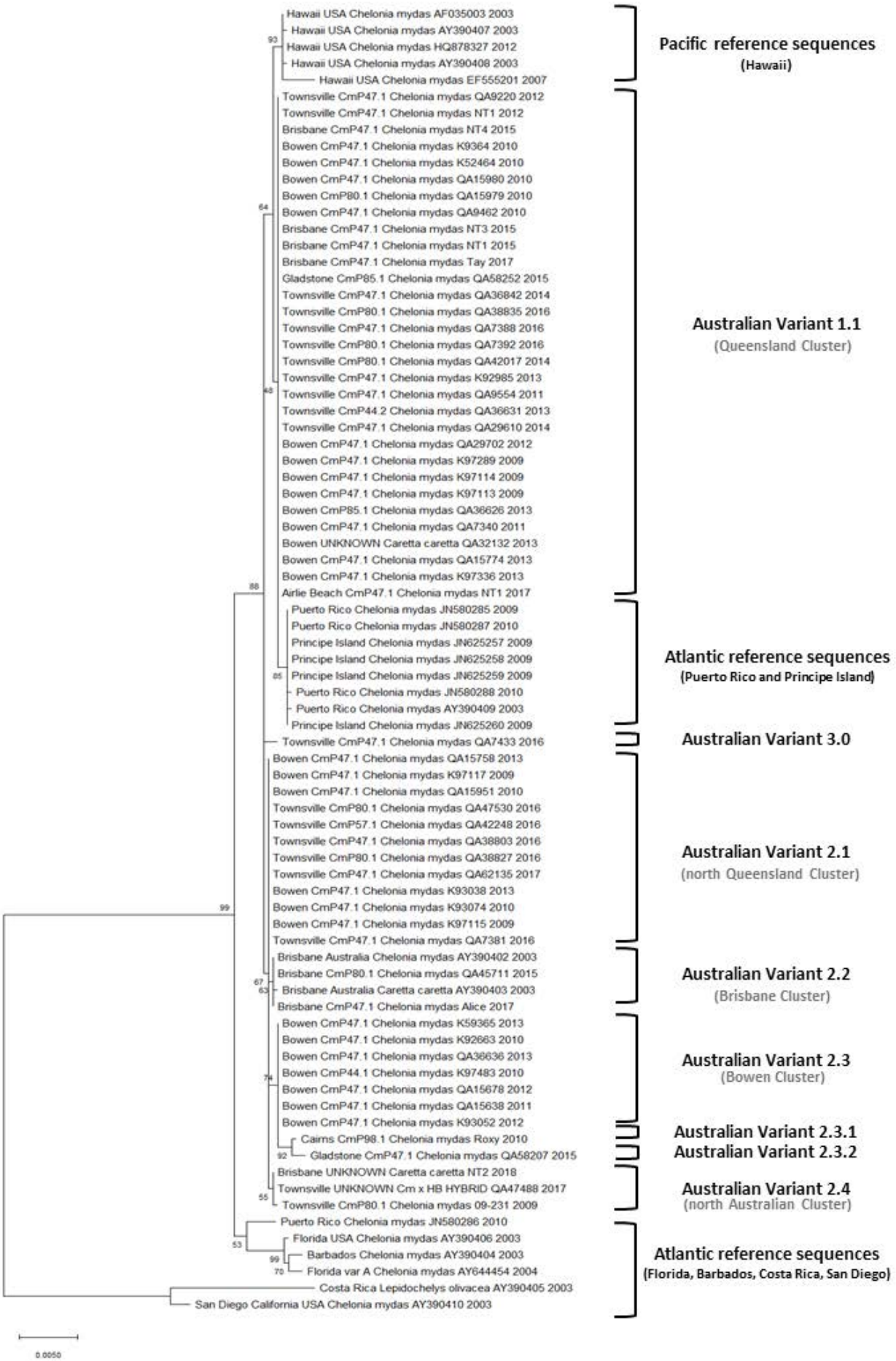
Hawaii USA Chelonia mydas AF035003 2003
Hawaii USA Chelonia mydas AY390407 2003
Hawaii USA Chelonia mydas HQ878327 2012
Hawaii USA Chelonia mydas AY390408 2003
Hawaii USA Chelonia mydas EF555201 2007
Townsville CmP47.1 Chelonia mydas QA9220 2012
Townsville CmP47.1 Chelonia mydas NT1 2012
Brisbane CmP47.1 Chelonia mydas NT4 2015
Bowen CmP47.1 Chelonia mydas K9364 2010
Bowen CmP47.1 Chelonia mydas K52464 2010
Bowen CmP47.1 Chelonia mydas QA15980 2010
Bowen CmP80.1 Chelonia mydas QA15979 2010
Bowen CmP47.1 Chelonia mydas QA9462 2010
Brisbane CmP47.1 Chelonia mydas NT3 2015
Brisbane CmP47.1 Chelonia mydas NT1 2015
Brisbane CmP47.1 Chelonia mydas Tay 2017
Gladstone CmP85.1 Chelonia mydas QA58252 2015
Townsville CmP47.1 Chelonia mydas QA36842 2014
Townsville CmP80.1 Chelonia mydas QA38835 2016
Townsville CmP47.1 Chelonia mydas QA7388 2016
Townsville CmP80.1 Chelonia mydas QA7392 2016
Townsville CmP80.1 Chelonia mydas QA42017 2014
Townsville CmP47.1 Chelonia mydas K92985 2013
Townsville CmP47.1 Chelonia mydas QA9554 2011
Townsville CmP44.2 Chelonia mydas QA36631 2013
Townsville CmP47.1 Chelonia mydas QA29610 2014
Bowen CmP47.1 Chelonia mydas QA29702 2012
Bowen CmP47.1 Chelonia mydas K97289 2009
Bowen CmP47.1 Chelonia mydas K97114 2009
Bowen CmP47.1 Chelonia mydas K97113 2009
Bowen CmP85.1 Chelonia mydas QA36626 2013
Bowen CmP47.1 Chelonia mydas QA7340 2011
Bowen UNKNOWN Caretta caretta QA32132 2013
Bowen CmP47.1 Chelonia mydas QA15774 2013
Bowen CmP47.1 Chelonia mydas K97336 2013
Airlie Beach CmP47.1 Chelonia mydas NT1 2017
Puerto Rico Chelonia mydas JN580285 2009
Puerto Rico Chelonia mydas JN580287 2010
Principe Island Chelonia mydas JN625257 2009
Principe Island Chelonia mydas JN625258 2009
Principe Island Chelonia mydas JN625259 2009
Puerto Rico Chelonia mydas JN580288 2010
Puerto Rico Chelonia mydas AY390409 2003
Principe Island Chelonia mydas JN625260 2009
Townsville CmP47.1 Chelonia mydas QA7433 2016
Bowen CmP47.1 Chelonia mydas QA15758 2013
Bowen CmP47.1 Chelonia mydas K97117 2009
Bowen CmP47.1 Chelonia mydas QA15951 2010
Townsville CmP80.1 Chelonia mydas QA47530 2016
Townsville CmP57.1 Chelonia mydas QA42248 2016
Townsville CmP47.1 Chelonia mydas QA38803 2016
Townsville CmP80.1 Chelonia mydas QA38827 2016
Townsville CmP47.1 Chelonia mydas QA62135 2017
Bowen CmP47.1 Chelonia mydas K93038 2013
Bowen CmP47.1 Chelonia mydas K93074 2010
Bowen CmP47.1 Chelonia mydas K97115 2009
Townsville CmP47.1 Chelonia mydas QA7381 2016
Brisbane Australia Chelonia mydas AY390402 2003
Brisbane CmP80.1 Chelonia mydas QA45711 2015
Brisbane Australia Caretta caretta AY390403 2003
Brisbane CmP47.1 Chelonia mydas Alice 2017
Bowen CmP47.1 Chelonia mydas K59365 2013
Bowen CmP47.1 Chelonia mydas K92663 2010
Bowen CmP47.1 Chelonia mydas QA36636 2013
Bowen CmP44.1 Chelonia mydas K97483 2010
Bowen CmP47.1 Chelonia mydas QA15678 2012
Bowen CmP47.1 Chelonia mydas QA15638 2011
Bowen CmP47.1 Chelonia mydas K93052 2012
Cairns CmP98.1 Chelonia mydas Roxy 2010
Gladstone CmP47.1 Chelonia mydas QA58207 2015
Brisbane UNKNOWN Caretta caretta NT2 2018
Townsville UNKNOWN Cm x HB HYBRID QA47488 2017
Townsville CmP80.1 Chelonia mydas 09-231 2009
Puerto Rico Chelonia mydas JN580286 2010
Florida USA Chelonia mydas AY390406 2003
Barbados Chelonia mydas AY390404 2003
Florida var A Chelonia mydas AY644454 2004
Costa Rica Lepidochelys olivacea AY390405 2003
San Diego California USA Chelonia mydas AY390410 2003
0.0050
Pacific reference sequences
(Hawaii)
Australian Variant 1.1
(Queensland Cluster)
Atlantic reference sequences
(Puerto Rico and Principe Island)
Australian Variant 3.0
Australian Variant 2.1
(north Queensland Cluster)
Australian Variant 2.2
(Brisbane Cluster)
Australian Variant 2.3
(Bowen Cluster)
Australian Variant 2.3.1
Australian Variant 2.3.2
Australian Variant 2.4
(north Australian Cluster)
Atlantic reference sequences
(Florida, Barbados, Costa Rica, San Diego)

Figure 6.2. Phylogenetic tree using the Maximum Likelihood method generated from the aligned 2565bp ChHV5 glycoprotein B (gB) gene. The analysis involved 79 nucleotide sequences. Bootstrap values are indicated as a number on each branch and were calculated from 1000 replications. Individual samples are identified with source location, haplotype, scientific name, tag number, and sample collection year. Sequences retrieved from the GenBank database originating from the Pacific and Altantic are named in the same way, with the accession number in the place of the tag number, and no haplotype information included as it was unknown.

Australian Variant 1.1 is the most common variant of ChHV5 in Australia, found in turtles along the expanse of the Queensland coast. This variant was found at almost all study sites in the present study, which is consistent with previous descriptions (Ariel et al., 2017). Here, 51.7% of all study turtles (n=31) were found to be infected with this variant (Figure 6.2). This variant is distinct and conserved, with all 31 samples clustering in this clade sharing 100% identity. A single consensus sequence representing Australian Variant 1.1 was generated for further analysis.

Australian Variant 2.1 is found only in turtles from the North Queensland region (sites Townsville and Bowen) and is therefore also consistent with previous descriptions (Ariel et al., 2017). In this study, we report 12 turtles infected with this variant of ChHV5. All Variant 2.1 sequences share 100% similarity, indicating that this variant is also highly conserved. A single consensus sequence representing Australian Variant 2.1 was generated for further analysis.

Australian Variant 2.2 is found only in Brisbane as yet, and shares 100% similarity with the Australian green turtle reference sequence (AY390402) (Figure 6.2). This variant differs from the loggerhead reference sequence (AY390403) by only one nucleotide. However, this is a non-synonymous substitution which alters the amino acid sequence of the resulting protein. Both of these reference sequences (AY390402 and AY390403) were generated from tumour samples from turtles in Moreton Bay (Brisbane), which is consistent with our results. A single consensus sequence representing Australian Variant 2.2 (excluding AY390403) was generated for further analysis.

Australian Variant 2.3 is found exclusively in turtles from Bowen (n=7) and is highly conserved; all sequences in this sub-clade share 100% similarity (Figure 6.2). A single consensus sequence representing Australian Variant 2.3 was generated for further analysis. Two distinct sequences (Variant 2.3.1 and Variant 2.3.2) comprise a subgroup which diverged from Variant 2.3. These sequences share a high similarity with Variant 2.3 (99.8%) yet are unique; Variant 2.3.1 and Variant 2.3.2 differ from Variant 2.3 by 0.2%, but also from each other by 0.2%. Moreover, some of these nucleotide substitutions are non-synonymous, resulting in one amino acid change in Variant 2.3.1 and four amino acid changes in Variant 2.3.2.

Australian Variant 2.4 is found in turtles from both Townsville and Brisbane. However, this variant was previously reported as the northern Australian variant, having been found in turtles from Townsville, Cairns and Western Australia (Ariel et al., 2017). Within this group, two of the sequences (Townsville QA47488 and Brisbane NT2) were identical while the one obtained from Townsville (09-231) differed by one nucleotide. This change, however, was synonymous and therefore the consensus sequence of this variant which was generated for further analysis is an accurate representative of this variant. Interestingly, this variant has a six base pair (bp) deletion that it shares with strains reported from Hawaii and was the most similar to Hawaiian sequences in the alignment. However, this similarity is not reflected in Figure 6.2, which suggests this variant is most closely related to Variant 2.1.

Australian Variant 3.0 is a clear outlier, distinct from all other samples analyzed in this study. Only one turtle from Townsville (QA7433) was infected with this viral variant, which has not been reported prior to this study. Of the Australian variants, this variant shares the highest similarity with Australian Variant 2.1 (99.8% identity) with all nucleotide substitutions being synonymous.

The frequency distribution of the ChHV5 variants among study sites in this study (Table 6.5) indicates that there is a strong link between viral variant and foraging ground, but that viral distribution within a foraging ground is not strictly homogenous.

Table 6.5. Distribution of chelonid alphaherpesvirus 5 (ChHV5) variants among marine turtles with fibropapillomatosis from six inshore areas in Queensland, Australia.

	Variant							
Location	**1.1**	**2.1**	**2.2**	**2.3**	**2.3.1**	**2.3.2**	**2.4**	**3.0**
Cairns	-	-	-	-	1	-	-	-
Townsville	11	6	-	-	-	-	2	1
Bowen	14	6	-	7	-	-	-	-
Airlie Beach	1	-	-	-	-	-	-	-
Gladstone	1	-	-	-	-	1	-	-
Brisbane	4	-	2	-	-	-	1	-
Total	31	12	2	7	1	1	3	1

We compared the consensus sequences of these variants with a Hawaiian reference sequence (HQ878327) as it is both a well described (Ackermann et al., 2012) and the most geographically close to the GBR that is currently available. Whilst all Australian variants shared a high similarity with HQ878327 (Table 6.6), Variant 2.4 was the most similar as it shared 99.8% identity. It is interesting to

note that this shared identity included a six bp which was not observed in any other Australian variants. This deletion appears to be uniquely Hawaiian, as it has not yet been observed in any other location. This deletion also accounted for a consistently observed difference between the Australian variants and HQ878327; all Australian variants, compared to the Hawaiian sequences, had six additional nucleotides resulting in two supplementary amino acids in the protein sequence.

Table 6.6. Summary of variants observed in this study, including number of turtles infected with a particular chelonid alphaherpesvirus 5 (ChHV5) variant (n) and the defining characteristics of these variants. All differences and identity percentages are calculated relative to the full-length glycoprotein B reference sequence available from Hawaii (HQ878327).

Variant	n	Nucleotide Substitutions	Identity (%)	Non-synonymous substitutions
Variant 1.1	31	9	99.6	2
Variant 2.1	12	11	99.6	2
Variant 2.2	2	12	99.5	2
Variant 2.3	7	13	99.5	2
Variant 2.3.1	1	17	99.3	3
Variant 2.3.2	1	19	99.3	5
Variant 2.4	3	2.6	99.8	1
Variant 3.0	1	13	99.5	2

These consensus sequences were used to create a condensed phylogenetic tree highlighting the host haplotype origin composition of these variants (Figure 6.3). No apparent close relationship with turtle origin was found, as most ChHV5 variants were found in turtles from mixed origins. Only two variants were found to be from one origin only: Variant 2.2 and Variant 3.0 were both only found in samples originating from CS/sGBR/nNGR. However, both of these variants are comprised of small sample numbers (n=2 and n=1 respectively). Similarly, the sublineages (Variant 2.3.1 and Variant 2.3.2) were each comprised of only 1 individual, limiting conclusions as to host origins. All variants comprised of 3 or more individuals (Table 6.6) were isolated from individuals of mixed origins.

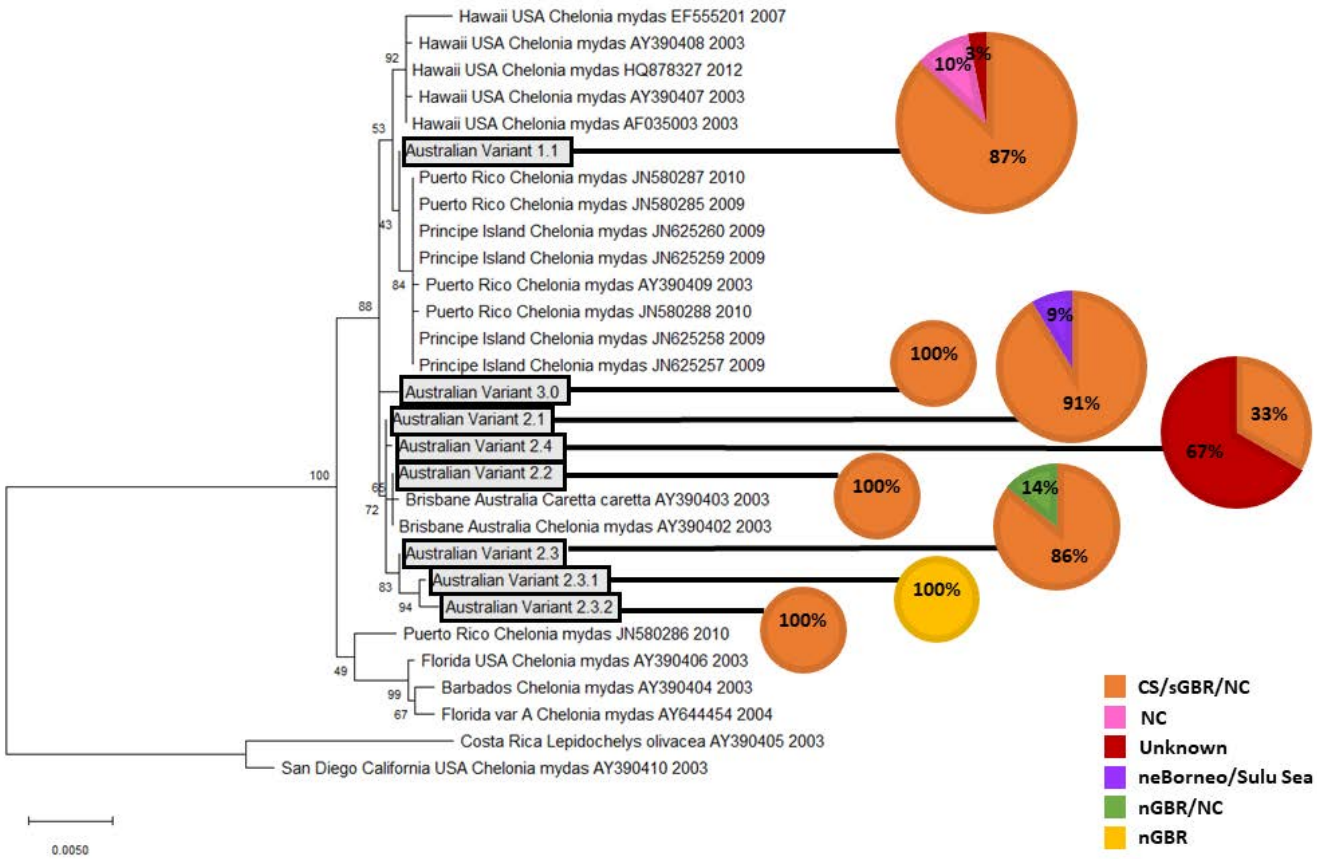

Figure 6.3. Condensed phylogenetic tree showing the positions of the distinct Australian Variants relative to published sequences. This tree was constructed using the Maximum Likelihood method generated from the aligned 2565bp ChHV5 glycoprotein B (gB) gene. The analysis involved 29 nucleotide sequences. Bootstrap values are indicated as a number on each branch and were calculated from 1000 replications. Sequences retrieved from the GenBank database are indicated with source location, scientific name, accession number, and sample collection year. Host haplotype was used to determine the origin composition of each Australian variant in this study, expressed here as a proportion with colour reflecting host origin region; Orange = Coral Sea (CS)/southern Great Barrier Reef (sGBR)/New Caledonia (NC), Pink = NC, Red = Unknown, Purple = north-east Borneo/Sulu Sea, Green= northern Great Barrier Reef (nGBR)/NC and Yellow = nGBR.

Sialyltransferase (F-sial)

From the nucleotide and phylogenetic analysis of the 58 sequences from this study, two available full-length sequences from the NCBI database and eight published sequences (Morrison et al., 2018), we show that the F-sial gene from Australian ChHV5 is highly conserved. Of the 58 sequences in this study, 52 were distinctly different from the Hawaiian reference sequences yet shared 100% similarity with each other. One sequence from Townsville (09-231) was found to be identical to the Hawaiian sequences whilst two other sequences only differed from the Hawaiian sequence by one nucleotide. Despite these minor substitutions, all sequences in the alignment shared 99.2% identity and this is reflected in the resulting phylogenetic tree (Figure 6.4). However, the highly conserved nature of these sequences indicate that this gene plays an important role in ChHV5 function.

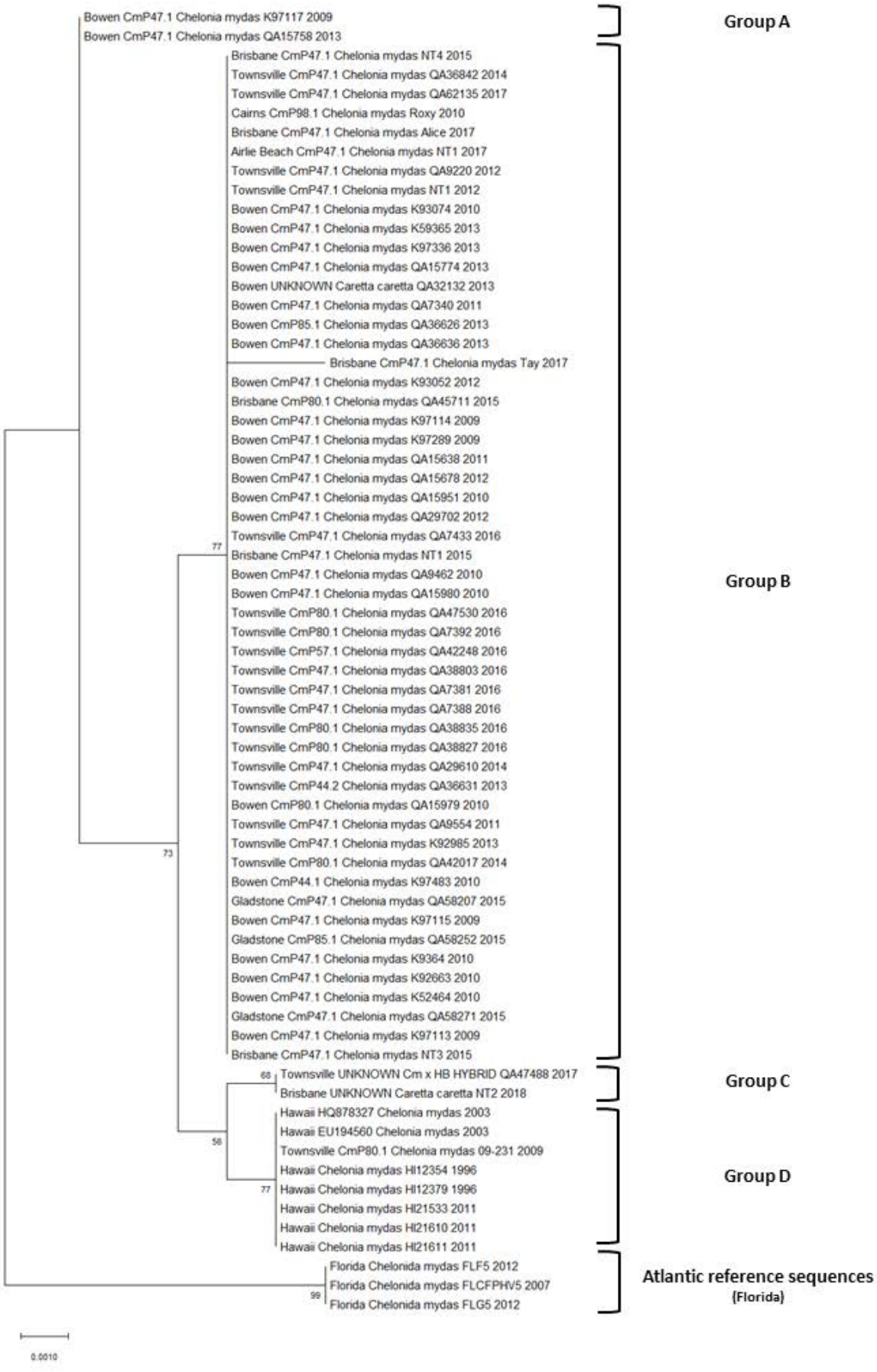
Bowen CmP47.1 Chelonia mydas K97117 2009
Bowen CmP47.1 Chelonia mydas QA15758 2013
Brisbane CmP47.1 Chelonia mydas NT4 2015
Townsville CmP47.1 Chelonia mydas QA36842 2014
Townsville CmP47.1 Chelonia mydas QA62135 2017
Cairns CmP98.1 Chelonia mydas Roxy 2010
Brisbane CmP47.1 Chelonia mydas Alice 2017
Airlie Beach CmP47.1 Chelonia mydas NT1 2017
Townsville CmP47.1 Chelonia mydas QA9220 2012
Townsville CmP47.1 Chelonia mydas NT1 2012
Bowen CmP47.1 Chelonia mydas K93074 2010
Bowen CmP47.1 Chelonia mydas K59365 2013
Bowen CmP47.1 Chelonia mydas K97336 2013
Bowen CmP47.1 Chelonia mydas QA15774 2013
Bowen UNKNOWN Caretta caretta QA32132 2013
Bowen CmP47.1 Chelonia mydas QA7340 2011
Bowen CmP85.1 Chelonia mydas QA36626 2013
Bowen CmP47.1 Chelonia mydas QA36636 2013
Brisbane CmP47.1 Chelonia mydas Tay 2017
Bowen CmP47.1 Chelonia mydas K93052 2012
Brisbane CmP80.1 Chelonia mydas QA45711 2015
Bowen CmP47.1 Chelonia mydas K97114 2009
Bowen CmP47.1 Chelonia mydas K97289 2009
Bowen CmP47.1 Chelonia mydas QA15638 2011
Bowen CmP47.1 Chelonia mydas QA15678 2012
Bowen CmP47.1 Chelonia mydas QA15951 2010
Bowen CmP47.1 Chelonia mydas QA29702 2012
Townsville CmP47.1 Chelonia mydas QA7433 2016
77
Brisbane CmP47.1 Chelonia mydas NT1 2015
Bowen CmP47.1 Chelonia mydas QA9462 2010
Bowen CmP47.1 Chelonia mydas QA15980 2010
Townsville CmP80.1 Chelonia mydas QA47530 2016
Townsville CmP80.1 Chelonia mydas QA7392 2016
Townsville CmP57.1 Chelonia mydas QA42248 2016
Townsville CmP47.1 Chelonia mydas QA38803 2016
Townsville CmP47.1 Chelonia mydas QA7381 2016
Townsville CmP47.1 Chelonia mydas QA7388 2016
Townsville CmP80.1 Chelonia mydas QA38835 2016
Townsville CmP80.1 Chelonia mydas QA38827 2016
Townsville CmP47.1 Chelonia mydas QA29610 2014
Townsville CmP44.2 Chelonia mydas QA36631 2013
Bowen CmP80.1 Chelonia mydas QA15979 2010
Townsville CmP47.1 Chelonia mydas QA9554 2011
Townsville CmP47.1 Chelonia mydas K92985 2013
73
Townsville CmP80.1 Chelonia mydas QA42017 2014
Bowen CmP44.1 Chelonia mydas K97483 2010
Gladstone CmP47.1 Chelonia mydas QA58207 2015
Bowen CmP47.1 Chelonia mydas K97115 2009
Gladstone CmP85.1 Chelonia mydas QA58252 2015
Bowen CmP47.1 Chelonia mydas K9364 2010
Bowen CmP47.1 Chelonia mydas K92663 2010
Bowen CmP47.1 Chelonia mydas K52464 2010
Gladstone CmP47.1 Chelonia mydas QA58271 2015
Bowen CmP47.1 Chelonia mydas K97113 2009
Brisbane CmP47.1 Chelonia mydas NT3 2015
68
Townsville UNKNOWN Cm x HB HYBRID QA47488 2017
Brisbane UNKNOWN Caretta caretta NT2 2018
Hawaii HQ878327 Chelonia mydas 2003
Hawaii EU194560 Chelonia mydas 2003
Townsville CmP80.1 Chelonia mydas 09-231 2009
56
Hawaii Chelonia mydas HI12354 1996
77
Hawaii Chelonia mydas HI12379 1996
Hawaii Chelonia mydas HI21533 2011
Hawaii Chelonia mydas HI21610 2011
Hawaii Chelonia mydas HI21611 2011
Florida Chelonida mydas FLF5 2012
Florida Chelonida mydas FLCFPHV5 2007
99
Florida Chelonida mydas FLG5 2012
0.0010
Group A
Group B
Group C
Group D
Atlantic reference sequences
(Florida)

Figure 6.4. Phylogenetic tree using the Maximum Likelihood method generated from the aligned 963bp ChHV5 Sialyltransferase (F-sial) gene. The analysis involved 60 nucleotide sequences. Bootstrap values are indicated as a number on each branch and were calculated from 1000 replications. Sequences retrieved from the GenBank database are indicated with the accession number, the source turtle's scientific name, and sample collection location and year.

Distinct clustering of these sequences into four main groups was observed (Figure 6.4). However, unlike the gB sequences generated in this study, the F-Sial sequences did not allow for viral variant characterization due to the significant similarity between these sequences. As such, these groups are named arbitrarily as Group A, B, C and D.

Group A consisted of two samples which were both obtained from tumours on green turtles in Bowen and is most similar to samples collected from Florida. Group B was comprised by the majority of samples (91.3%) in this study from a mixture of all study sites. All but one sample in this group share 100% identity. A sample collected from a green turtle in Brisbane (Tay) clustered in this group but differs from the other samples by two nucleotides; these nucleotide substitutions are both non-synonymous. Group C is comprised of two samples, one from a loggerhead and one from a green/hawksbill hybrid, and is most closely related to samples collected from Hawaii. Group D is almost exclusively comprised of samples collected from Hawaii, with the exception of one turtle from Townsville (09-231) which is identical to these Hawaiian sequences.

Although the significant similarity between the F-sial sequences prevented them from clustering in the same pattern as the gB sequences, there were some commonalities between the two phylogenetic trees. For example, the gB sequence of Australian Variant 2.4 shares a six bp deletion with sequences obtained from Hawaii. The same samples that comprise Variant 2.4 in Figure 6.2 cluster most closely with Hawaiian sequences in Figure 6.4; comprising both Group C and Group D.

As with the gB sequences, we compared the consensus sequences of these variants with a Hawaiian reference sequence (HQ878327). Whilst all Australian sequences shared a high similarity with HQ878327 (Table 6.7), the sequence in Group D was the most similar as it shared 100% identity. Of the 58 sequences produced in this study, 55 had a distinct amino acid change (relative to the Hawaiian reference sequences) at position 201. This indicates that this substitution is a characteristic of Australian viral variants.

Table 6.7. Nucleotide sequence analysis of sequences obtained from FP tumour samples collected from marine turtles (n). All differences and identity percentages are calculated relative to the full-length reference sequence available from Hawaii (HQ878327).

Cluster	n	Nucleotide Substitutions	Identity (%)	Non-synonymous substitutions
Group A	2	4	99.7	2
Group B	53	5	99.5	3
Group C	2	2	99.8	0
Group D	1	0	100	0

Discussion

This study describes improved molecular assays developed for detection of ChHV5 and subsequent phylogenetic analysis. This, combined with the largest sample size of individual turtles with FP tumours and national geographic spread to date, allowed for a thorough investigation of a link between host genetic origin and ChHV5 variant, which corroborated the probability of horizontal transmission of the virus at foraging sites.

Previous molecular studies of ChHV5 have targeted multiple genes and because detection rate is not 100% for any assay, it has been suggested that a combination of assays should be used to increase sensitivity of detection (Alfaro-Núñez and Gilbert, 2014). The molecular assays developed here target F-sial, DNA polymerase and gB genes with a higher rate of detection on an individual assay basis than previously reported and can be used to amplify and sequence complete genes with Sanger sequencing, making them suitable for both ChHV5 detection and phylogenetic studies.

The F-sial gene is atypical of herpesviruses and poorly understood, but has been suggested to play a role in ChHV5 pathogenesis (Ackermann et al., 2012). In this study, the F-sial gene was found to be highly conserved, with 89.6% of sequences obtained sharing 100% identity. Of the remaining sequences, two were highly similar to the HQ878327 reference sequence (Group C) and one was identical to the HQ878327 reference sequence (Group D). Although this high level of similarity between sequences did not allow for fine-scale separation of variants, it indicates that this gene is highly conserved. Thus, these results are consistent with the Ackermann et al. (2012) theory that F-Sial is likely to have an important role in pathogenesis.

While none of the assays described in this study were 100% effective in detecting ChHV5 presence alone, ChHV5 presence was confirmed in all 62 individual wild-captured marine turtles with FP tumours by a combination of the 3 assays (Table 6.4). This variability in detection is consistent with

results reported in previous studies targeting ChHV5 (Alfaro-Núñez and Gilbert, 2014; Ariel et al., 2017; Page-Karjian et al., 2012; Rodenbusch et al., 2014). However, the rate of ChHV5 detection in each of the three assays in this study is much higher than previously reported and the sample size is comparatively larger. Here, DNApol-3670, gBOverlap1-4 assay and FSial-1104 each detected ChHV5 in 93.5% of 62 samples representing 62 individual turtles. Comparable rates of ChHV5 detection in FP tumour samples from primary PCR assays range from low (0-22.2%; n=38 samples, Page-Karjian et al. (2012)) and mid-range (62.1-78.8%; n=66, (37 turtles), Alfaro-Núñez and Gilbert (2014); 67%; n=22 samples (22 turtles), Ariel et al. (2017)) to high (100%, n=29 samples (18 turtles), Quackenbush et al. (1998); 95%, n=20 samples (20 turtles) Quackenbush et al. (2001)) in smaller sample sizes than this study. Nested PCR's targeting ChHV5 in FP tumours have reported mid-range detection rates (60.5-86.9%, Page-Karjian et al. (2012); 63.2%,Lawrance et al. (2018)). This wide variation in detection rates highlights the need for a standardised ChHV5 assay, which will allow for more accurate comparisons of detection rates and resulting sequences of this globally distributed virus. It is important to note that the different methods of detection have likely contributed to this variability. In the present study, longer fragments of DNA were targeted for the phylogenetic analysis and therefore conventional PCR was selected. Future studies would benefit from screening the same samples in a qPCR assay, which would target a smaller amplicon, in order to accurately compare detection rates of these methods.

Although ChHV5 is frequently detected in FP tumour samples, the variable rate of ChHV5 detection in FP tumour samples is yet to be explained. It is possible that FP development is more complex than ChHV5 presence alone, and there is potential for multifactorial influences on disease manifestation (Herbst et al., 2008); these may include environmental co-factors and/or presence of other infectious agents working alone or in synergy with ChHV5 (Jones et al., 2016). Papillomaviruses in marine turtles have been reported and genetically characterized (Herbst et al., 2009), with a recent study reporting the presence of a papillomavirus (CmPV1) in tumour samples collected from Australian green turtles; further studies are underway to investigate the link between FP and papillomavirus presence (Mashkour et al., 2018).

The increased sample size and geographic spread, represented by six sampling locations spanning a distance of 1380km along the Queensland coast, enabled the identification and description of five main clusters of viral sequence relative to sampling location: Queensland, north Queensland, north Australian, Bowen and Brisbane (Figure 6.2). The Queensland, north Queensland and north Australian clusters of ChHV5 viral variants are previously reported, and our results are consistent with what is known about these clusters (Ariel et al., 2017). The Queensland cluster includes the most common viral variant observed in both studies, which is found at many locations along the

Queensland coast, whilst the north Queensland cluster contains variants that are only found in north Queensland (Townsville and Bowen). The north Australian cluster, distinct from the north Queensland Cluster, was previously reported to be comprised of viral variants obtained from FP tumours on turtles from Townsville, Cairns and Western Australia (Ariel et al., 2017). In the present study, two samples from Townsville and one sample collected in Brisbane was found to also contain this variant of ChHV5. These results are consistent with the idea that this variant is predominantly found in locations from northern Australia, but can also be found in locations great distances away (Ariel et al., 2017). However, limited sample sizes of this particular cluster in both studies prevent a conclusive understanding of the distribution of this variant. The Brisbane and Bowen clusters have not been previously reported, although the Brisbane sequences obtained in this study cluster with published sequences from Brisbane (AY390402 and AY390403).

These results suggest that there is a close relationship between ChHV5 variant and foraging ground, further supporting the theory that turtles are infected at foraging grounds, rather than rookery (Ariel et al., 2017; Ene et al., 2005; Herbst, 1994; Patrício et al., 2012; Rodenbusch et al., 2014). However, these results also indicate that viral variant distribution is not strictly homogenous at each foraging ground. For example, turtles from Bowen were found to be infected with either Variant 1.1 (51.9%), Variant 2.3 (25.9%) or Variant 2.1 (22.2%). This is consistent with ChHV5 variant distribution in foraging grounds in Florida, where multiple variants were detected within site but the frequency of each variant differed between sites (Ene et al., 2005). Here, we also report one variant that is common amongst almost all study sites and observed most frequently within the study (Variant 1.1). Such a trend has also been reported in Florida (Variant A) (Ene et al., 2005) and Brazil (Variant 4) (Rodenbusch et al., 2014) and may reflect turtle migration patterns. Whilst turtles typically remain in a foraging ground following recruitment, small-scale movements and seasonal shifts in foraging areas have been recorded on the Queensland coast (Shimada et al., 2016). These movements could allow for exposure to other viral variants, and may explain why ChHV5 is not strictly homogenous at each location.

Green turtle haplotype frequencies at rookeries around the world form the basis for estimates of which genetic stock a particular haplotype belongs to. Such methods can be used in bioinformatic programs to determine the genetic stock composition of turtles at a particular foraging ground (Mixed Stock Analysis) (Dutton et al., 2014; Jensen et al., 2016; Jones et al., 2018). Turtles frequenting a given foraging site usually represent genetic stock from multiple rookeries, although there is a trend for southern GBR haplotypes to occur at higher frequency in the more southern foraging sites and vice versa for the northern GBR stock (Jensen et al., 2016; Jones et al., 2018).

These host haplotypes and their associated genetic stock were used in the present study to reflect the origin of the host turtle.

This study used molecular methods to assess the relationship between turtle origin and viral variant with phylogenetic clustering of ChHV5 being closely linked to foraging grounds (sampling location) rather than host haplotype. As such, no close association between turtle origin and viral variant could be identified in the present study. These results lend weight to the theory of horizontal transmission of this virus at foraging sites, rather than vertical transmission at rookeries. Figure 6.2 shows that the phylogenetic clustering in this study was strongly linked to sampling location, whilst Figure 6.3 demonstrates that each variant found in this study was isolated from turtles with a mixture of origins. However, definitive conclusions are limited as many haplotypes have been linked to multiple source regions. Most turtles in this study (76.3%) were found to belong to the CmP47.1 haplotype. This is the most common haplotype found on the GBR and has been observed in rookeries in the southern GBR, Coral Sea and New Caledonia. At present, researchers are unable to decipher which one of these three regions an individual turtle may have originated from using molecular methods. Therefore, it not yet possible to know whether all of the CmP47.1 turtles originated exclusively from the southern GBR, Coral Sea or New Caledonia, or a mixture of these regions. It has been suggested that increasing the length of mtDNA targets may allow for further differentiation of known haplotypes and more reliable identification of the region of origin for particular haplotypes (Jones et al., 2018). The use of full mitochondrial genomic sequence or microsatellite markers to determine turtle haplotypes should be investigated in future studies. Despite the current limitations in establishing turtle origin by haplotype alone, the results of this study demonstrate that there is no close link between haplotype and viral variant.

Variant nomenclature was determined based on clade position in the gB phylogenetic tree (Figure 6.2), in a similar fashion to the hierarchical system used for avian influenza virus (Brown et al., 2009; Donis et al., 2008; Smith et al., 2012a). Prior to this study, Australian variants were referred to as “clusters” based on geographic location (Ariel et al., 2017), while other studies utilised letters to denote different variants (Ene et al., 2005; Rodenbusch et al., 2014). Lettering systems preclude classification of sublineages, and are often unable to indicate similarity while numerical systems recognize similarity between variants and sublineages. For example, Variant 2.1 and Variant 2.2 are closely related and Variant 2.3.1 is a sublineage of Variant 2.3. Here, we have adopted this system for Australian variants. However, a reclassification of all known ChHV5 variants was unable to be undertaken in this study due significant variation in published gene selection and sequence length. Past variants have been determined using a partial, or complete, sequences from a range of genes. A systematic reclassification of ChHV5 using one complete gene, similar to that undertaken for

Newcastle disease virus (Diel et al., 2012), would remove any ambiguities in the current phylogeny of this virus. We recommend ChHV5 gB as it is useful in both broad and small-scale phylogenic analyses. A numerical numbering system was not applied to F-sial as the highly conserved nature of the gene prevented fine-scale variant characterization, but this may change as research in this field progresses.

Research on FP and ChHV5 as a causative agent is challenging as it relies on opportunistic sampling of turtles with FP tumours and thus, sample sizes are often limited. While this study has used the largest number of individual FP affected turtles to date, the sample size is still small and sampling more extensively along the GBR would greatly improve our ability to analyse and understand this disease. This study was also somewhat limited by some inconsistency between bioinformatic programs. Australian Variant 2.4 shares a six-base deletion with published Hawaiian sequences and nucleotide analysis highlights that this variant is most closely related to these Hawaiian sequences. However, this was not accounted for in the resulting phylogenetic tree (Figure 6.2), despite selecting for the use of all sites in the alignment. A range of phylogenetic trees were constructed, including Neighbour-Joining, Minimum Evolution, Maximum Likelihood and Bayesian trees. Yet none of these trees reflected the similarity between these sequences, despite this deletion being repeatedly observed. This highlights limitations in some algorithms used by these programs, wherein deletions are treated as gaps and are ignored by the analysis. Such deletions may be biologically important, and a means of ensuring their inclusion in phylogenetic analysis should be targeted. However, while its position in Figure 6.2 is slightly inaccurate, nucleotide analysis of Australian Variant 2.4 confirms that it is a unique and distinct group of sequences.

As a whole, there are still many gaps in our understanding in the biology ChHV5 and is relationship to FP. Future research on ChHV5 should aim to better understand the functional consequences of the variation observed in ChHV5 sequences. Investigations linking viral variant to disease presentation or severity would be interesting, yet challenging due to the complex nature of the disease and possible differing timelines; turtles might be captured in the early or late stage of disease development and therefore observations might be due to disease progression rather than viral variant. However, identification of a genetic link to ChHV5 pathogenicity and/or FP presentation may be possible. This disease presents differently around the world; turtles with oral tumours are common in Hawaii yet oral tumours have rarely been observed in Australian turtles (Hargrove et al., 2016). This cause for this may be due to genetic variation of ChHV5 and should be investigated in future studies. Additionally, the results presented here, coupled with those of previous studies (Ackermann et al., 2012), suggest that F-Sial may play a strong role in ChHV5 pathogenicity and as such, it is worthy candidate for further investigation.

While discomfort and risk to survival for individual turtles affected by FP is widely accepted, the effects of this disease on populations is less clear. Spatial and temporal variation in disease prevalence is consistently reported (Jones et al., 2016), yet a mechanism behind such variation has not been determined. The unpredictable nature of FP prevalence has so far precluded effective management plans, and researchers must endeavor to understand this disease and its associated etiological agent(s) in order to effectively conserve this vulnerable species. Here, we present a molecular epidemiological study which supports the theory that ChHV5 transmission occurs at marine turtle foraging grounds, with no close relationship to host origin. These results enable informed management decisions regarding marine turtles, as they highlight that managing FP along the Queensland coast, including the GBR, requires focus on foraging grounds.

The aims of this chapter were addressed as follows:

1. **Improve the resolution of the current phylogeny of ChHV5 in Australia by generating a more robust sequence dataset than has previously been used, including a larger sample size and increased geographical locations**

   The improved molecular assays developed for detection of ChHV5 described in this study allowed for higher rates of ChHV5 detection. The subsequent phylogenetic analysis was therefore data-rich which, combined with the large sample size and geographic spread, allowed for improved resolution of the current phylogeny in Australia. Some results of this study confirmed what was already known about ChHV5 variant distribution in Australia; the Queensland, north Queensland and north Australian clusters appeared to cluster as previously described in the present study (Ariel et al., 2017). Yet, this study also described two new clusters which have not been previously identified (Bowen and Brisbane). The number of ChHV5 sequences isolated from tumours on individual turtles allowed us to characterise these variants beyond the clustering of previous studies. For example, because the Queensland cluster contained 31 identical sequences, each originating from individual turtles, it provided confidence that this is a distinct viral variant (Australian Variant 1.1).
   An additional finding when addressing this aim was that the F-Sial gene is not an ideal candidate for fine-scale phylogenetic analysis as it is highly conserved. This supports the theory suggested by Ackermann et al. (2012), that this gene is likely to play a role in viral pathogenesis and future studies would benefit from investigating further.
   Additionally, we were able to identify that the viral distribution at each foraging ground is not strictly homogeneous. For example, turtles from Bowen were found to be infected with either Variant 1.1, Variant 2.3 or Variant 2.1, with most being infected with Variant 1.1. These results are consistent with what is described for Florida and Brazil and as such, this study has been able to include Australia in the global conversation about ChHV5 variant distribution.

2. **Assess the relationship between host genetic stock and viral variant in order to clarify the mechanisms of viral transmission**

   The close association between ChHV5 variant distribution and foraging ground corroborates the probability of horizontal transmission of the virus at foraging sites. However, this study further assessed whether there was a relationship between host genetic origin and ChHV5 variant. The consensus sequences of the variants described in this study were used to create a condensed phylogenetic tree highlighting the host haplotype origin composition of these variants (Figure 6.3).

No apparent close relationship with turtle origin was found, as most ChHV5 variants were found in turtles from mixed origins; at study sites where more than one turtle was sampled, the genetic origin of the turtles was mixed while ChHV5 variant distribution at each study site was much more homogenous. These results negate any link between host genetic origin and viral variant, and further support the theory that horizontal transmission of ChHV5 is occurring at foraging grounds.

Publications and presentations arising from this study

- **Jones, K.**, Burgess, G., Budd, A.M., Huerlimann, R., Mashkour, N. and Ariel, E. 2018. Molecular evidence of horizontal transmission of chelonid alphaherpesvirus 5 at green turtle (Chelonia mydas) foraging grounds in Queensland, Australia. Under Review.

My contributions to this study

- I generated the permit applications for JCU Animal Ethics, the Great Barrier Reef Marine Park Authority and the Department of Environment and Science (Department of Environment and Heritage Protection at the time of approval). I also ensured the methods in these permits were followed and amended when needed. I also ensured that all reports regarding these permits were provided when required.
- I took part in extensive field operations in order to survey and sample marine turtles at Cockle Bay, Edgecumbe Bay, Gladstone and Moreton Bay. This included becoming proficient in the turtle rodeo technique, collection of morphometric data and samples and in my later years of study these trips also included boat driving.
- After the first year of my PhD studies, I also co-ordinated many of these field trips
- I collected the all of the samples and associated data used in this study (excluding those which were donated from collaborators or collected prior to the commencement of my PhD studies)
- I conceived and co-designed the primers for the PCR assays with my supervisor
- I optimised the PCR assays
- I performed the laboratory work, including both DNA extraction and PCR
- I managed the datasets obtained
- I assembled, trimmed and, where appropriate, edited the resulting sequence data
- I analyzed the data and interpreted the results
- I submitted the newly described sequences to GenBank and obtained Accession Numbers
- I drafted the chapter and edited it as advised by collaborators and supervisors
- I drafted the manuscript and managed the process of journal submission and review

Chapter Seven:
General Discussion

The endangered green turtle faces many threats, and it is imperative that we understand each threat in order to better conserve this vulnerable species. The Recovery Plan for Marine Turtles in Australia lists FP as a threat to marine turtles (Department of the Environment and Energy, 2017) and as such, knowledge on this disease in Australia is crucial to improving our management of our marine turtle species and stocks. This thesis aimed to address this by establishing the spatial distribution and prevalence of FP along the Queensland coast, and investigating potential epidemiological factors in order to better inform the management of green turtles.

This thesis provides the first comprehensive report of FP prevlance in Australia (Chapter Three). A total of 25,645 records were used to determine FP prevalence and trends at 15 locations along the Queensland coast. Within this dataset, 791 turtles with FP tumours were recorded. Survey method was found to have a significant influence on the apparent FP prevalence value at each site. That is, surveys which explicitly target FP detect higher numbers of individual turtles with FP, and the resulting prevalence rates are therefore much higher than those reported from general population surveys. It is likely that the true FP prevalence lies somewhere in between the values reported from the different survey methods, as both methods have shortcomings with respect to FP detection. Future studies should consider this and develop a consistent sampling method to accurately detect and document FP. As FP is a globally distributed disease, this is an important future development for not only Australia, but the broader marine turtle research community. As recommended by the International FP Working Group, the minimum data collection for FP should include: individual identification, standard measurements of the host turtle (length and weight), presence/absence of tumours, tumour severity, body condition, oral examination, method of capture, and effort (Hargrove et al., 2016). A standardised method such as this, used globally, would allow for more accurate comparisons of FP prevalence data and considerably improve our knowledge of this disease.

Despite the challenges in establishing accurate FP prevalence values, the results of this study substantiated reports on FP prevalence in other regions. The spatial and temporal variance in FP prevalence reported here is consistent with prevalence reports from other regions (see Appendix 1: Supplementary Table 2.1). This study also showed that juvenile turtles are the age-class most frequently affected by FP, supporting that found in other reports of FP from around the world (Adnyana et al., 1997; Ene et al., 2005; Herbst, 1994; Herbst and Klein, 1995a; Page-Karjian et al., 2014; Patrício et al., 2012; Work et al., 2004). High FP prevalence was loosely correlated with higher

human activity adjacent to catchments, with some exceptions. These results raised questions about which factors, on a finer scale, could be influencing FP prevalence at inshore foraging grounds.

An association between FP prevalence and water quality has frequently been reported, with high FP prevalence often reported in areas associated with reduced water quality (Adnyana et al., 1997; Chaloupka et al., 2009; dos Santos et al., 2010; Foley et al., 2005; Herbst, 1995; Jones et al., 2016; Van Houtan et al., 2014). Typically, these locations are associated with adjacent catchments with high anthropogenic influences like agriculture, urbanisation and/or industrialisation. While the results of Chapter Three largely support this, there were some sites with comparable prevalence values yet vastly different human influences (e.g. Warul Kawa in remote Torres Strait versus the heavily industrialised regional city of Gladstone). While such a result may simply be an anomaly, there are other reports of FP prevalence rates which challenge the idea that FP is closely associated with areas of high human influence. For example, in Puerto Rico, the positive correlation between high FP prevalence and reduced water quality was reported (Patrício et al., 2011). However, after several years the trend reversed; the prevalence of FP at the more pristine site became considerably higher than at the site which was subjected to high levels of human activity (Page-Karjian et al., 2012). These reports sparked two questions. The first being whether there is any correlation between FP prevalence and water quality on the GBR. The second, if such a correlation exists, is it a result of water quality as a whole or is it due to one or more water quality parameters that have a specific action on FP? If any correlation could be traced to an individual water quality parameter, such as pesticides, it may account for locations having a high FP prevalence, despite a reduced influence from human activities.

To investigate whether there is a relationship between water quality and FP (Chapter Four), sub-indexes for dissolved inorganic nitrogen (DIN), total suspended solids (TSS), pesticides and metals were developed for each study site using published data from a range of sources and expert opinion. These scores were also aggregated without weights to create an overall water quality index (WQI) for each study site. Both the sub-indexes and WQIs for each site were compared with the FP prevalence data from Chapter Three.

Despite analysing a comprehensive dataset, a relationship between FP prevalence and WQI rankings at each site could not be quantified or established. The analysis was challenged by a range of limitations, including missing data, varying temporal scales and methods in both the FP prevalence and water quality datasets. The water quality datasets used to develop the WQIs did not account for temporal variation, which further restricted their use. Moreover, several of the water quality variables were confounded. Although the combination of these limitations prevented in-depth

statistical analysis, this result does have significant implications for green turtle management. Chapter Four highlights substantial deficiencies in the current monitoring of water quality on the GBR, which should be addressed in order to better inform the management of green turtles in the GBR, with obvious flow-on benefits to associated species and their supporting habitats.

Having characterised the spatial distribution and prevalence of FP in Australia (Chapters Three) and explored any potential links to water quality (Chapter Four), it was then essential to determine the distribution of ChHV5. This virus has been consistently associated with FP tumours (Alfaro-Nunez et al., 2014; Lackovich et al., 1999; Lu et al., 2000b; Lu et al., 2003; Nigro et al., 2004a; Nigro et al., 2004b; Page-Karjian et al., 2015; Page-Karjian et al., 2012; Quackenbush et al., 2001; Quackenbush et al., 1998; Rodenbusch et al., 2014; Yu et al., 2001; Yu et al., 2000). While molecular studies in this field are increasing, Chapter Two highlighted that such studies in Australia are limited. This thesis aimed to characterise the distribution of ChHV5 variants in Australia, and assess whether there was a relationship between ChHV5 variant and host genetic stock in individual turtles. The overarching aim of this component of the thesis was to better understand viral transmission. Developing a means of identifying host genetic stock was the crucial first step in achieving these aims.

An assay targeting a 960bp fragment of d-loop region of green turtle mtDNA was designed in Chapter Five. This assay targets a longer fragment of mtDNA than previous studies and was found to improve the resolution of haplotype identification. The frequency of haplotypes identified in Chapter Five were used to estimate genetic stocks of green turtles at three foraging grounds (Low Isles, Green Island and Cockle Bay) on the GBR using Mixed stock analysis (MSA). The results of the MSA suggest that the northern GBR (nGBR), Coral Sea (CS), southern GBR (sGBR) and New Caledonia (NC) stocks supplied the bulk of the turtles at all three study sites, and the relative contributions of these stocks varied among sites. At the most southern site (Cockle Bay) turtles predominantly originated from the sGBR stock. sGBR contribution was significantly decreased at the more northern Green Island, where the main contribution was from CS stock. At the most northerly site (Low Isles), the contribution from CS stock further increased. These results suggest that the dramatic shift in stock contributions which was previously reported between these sites (Jensen et al., 2016), is actually more of a gradual shift along a coastal gradient. These results serve as a validation of the PCR assay designed in Chapter Five.

The results of Chapter Five have wide-reaching management implications for this vulnerable species. On their own, the results can be used to inform management as they highlight the source regions of the green turtles at these foraging grounds, and their relative contributions. However, by combining these results with those from past studies (Jensen et al., 2016), an estimation of the latitudinal

spread of the main genetic stocks on the GBR was developed. Such a model could be used to estimate stock contributions at as yet unsampled foraging grounds. This could be hugely beneficial to managers, who can make stock-level management decisions based on this estimation, without the need for intensive sampling and laboratory analysis. It should be noted that in order to compare with haplotypes already characterised in this region, the 960bp sequences generated in this study were trimmed down to 770bp. This resulted in sequences known to be distinctly different appearing the same once trimmed. To address this, a re-characterisation of already published sequences is needed by either using this PCR assay, or the entire d-loop region. This would improve the resolution of haplotype determination which may specific haplotypes to be linked to nesting regions with greater confidence.

Following the validation of the PCR assay for host genetic stock identification in Chapter Five, a means of determining which ChHV5 variant turtles were infected with was established in Chapter Six. While several assays were developed, the results of an assay targeting the complete sequence of the glycoprotein B gene in the ChHV5 was found to be the most effective in determining the distribution of the viral variants. This assay improves upon those currently used for the detection of ChHV5 and allows for higher rates of ChHV5 detection. The phylogenetic analysis of the sequences obtained was therefore data-rich. This, combined with the large sample size and geographic spread, allowed for improved resolution of the current phylogeny in Australia. Some results of this study (Chapter Six) confirmed what was already known about ChHV5 variant distribution in Australia; the Queensland, north Queensland and north Australian clusters appeared to cluster as previously described (Ariel et al., 2017). Yet, the current study also described two new clusters which have not been previously identified (Bowen and Brisbane). The number of ChHV5 sequences isolated from tumours on individual turtles allowed us to characterise these variants beyond the clustering of previous studies. For example, because the Queensland cluster contained 31 identical sequences, each originating from individual turtles, it provided confidence that this is a distinct viral variant (Australian Variant 1.1). This study also found that the viral distribution at each foraging ground is not strictly homogeneous. For example, turtles from Bowen were found to be infected with either Variant 1.1, Variant 2.3 or Variant 2.1, with most being infected with Variant 1.1. Such findings are consistent with what is described for Florida (Ene et al., 2005) and Brazil (Rodenbusch et al., 2014).

Phylogenetic analysis of complete sequences of the F-Sial gene from the ChHV5 genome in Chapter Six revealed that this gene is not an ideal candidate for fine-scale phylogenetic analysis as it is highly conserved. This supports the theory that this gene is likely to play a role in viral pathogenesis (Ackermann et al., 2012) and future studies would benefit from investigating this further.

The assays developed and validated in Chapters Five and Six were combined to generate sequence data and assess whether there was a relationship between host genetic stock and the viral variant they were infected with (Chapter Six). The overarching aim of Chapter Six was to better understand transmission pathways. The theory that turtles become infected with ChHV5 upon recruitment into their foraging grounds is widely supported (Ene et al., 2005; Herbst, 1994; Patrício et al., 2012), but has never been investigated using molecular methods. If ChHV5 transmission was occuring vertically from parent to offspring, then phylogenetic clustering of ChHV5 would be expected to be based on host genetic stock rather than sampling location. Conversely, if ChHV5 transmission is occurring horizontally at the foraging ground, a link between viral variant and host origin would be less likely.

The results of the study in Chapter Six showed that phylogenetic clustering was closely linked with sampling location, indicating that viral transmission is most likely occurring at the foraging ground. Following characterisation of viral variants, the host origins of the turtles from which these sequences were generated were assessed. All viral variants which were identified in more than three turtles were found in turtles from mixed origins, negating a link between viral variant and host origin. These results provide additional evidence for the theory of horizontal transmission of the virus at foraging grounds.

However, this study relied on linking individual haplotypes to a region of origin, despite many haplotypes being linked to multiple source regions. For example, CmP47.1 is the most common haplotype found on the GBR, and has been recorded at rookeries in the southern GBR, Coral Sea and New Caledonia. At present, it is impossible to determine which one of these three regions an individual turtle may have originated from using these methods. In practice, the frequencies of these haplotypes are used to estimate genetic stock contributions in an MSA (see Chapter Five). As no trend between host genetic stock and ChHV5 viral variant was found in the present study, the inability to determine which region individual haplotypes originated from was not a limitation to the present study. However, if such a trend was observed, it would have been impossible to determine the source region of particular viral variants. For instance, if all 31 turtles infected with Variant 1.1 were found to be the CmP47.1 haplotype, thereby it being likely they were infected with the variant at rookeries, we would have no means of knowing whether transmission was occurring in the southern GBR, Coral Sea, New Caledonia or a combination of these rookeries. As such, it is clear we need to improve methods of identifying genetic stocks from samples of individual turtles. This may require longer mtDNA fragments, full mitochondrial genomic sequence, or microsatellites markers.

The overarching aim of this thesis was to improve our understanding of FP in Australia and provide recommendations for management of inshore areas of the Great Barrier Reef. This project

characterised the spatial distribution and prevalence of FP on the GBR, and highlighted deficiencies in both population and water quality monitoring which need to be addressed in the future in order to better assess this threat to green turtles. A means for identifying host genetic haplotype was designed, validated and used to conduct an MSA at three foraging grounds of the GBR. This project also improved the resolution of ChHV5 variant distribution on the GBR, which corroborated current theories that viral transmission occurs horizontally at foraging grounds, allowing managers to focus their management of this disease to foraging grounds. Recommendations based on the results of this project have been made to direct both population monitoring programs and future research in this field. The results of this project, and subsequent recommendations, have significant implications for the management of inshore areas in which vulnerable green turtles resides.

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Appendices:

List of Supplementary Figures

Supplementary Figure 4.1. All study sites and rivers discussed in the present study. 173

Supplementary Figure 4.2. The location of Warul Kawa and its largest river influence, the Fly River. 174

Supplementary Figure 4.3. The location of Clack Reef, the Howick Group of islands and Cape Flattery. The river mouths of the Normanby River, Endeavour River, Annan River and Daintree River are also shown. 175

Supplementary Figure 4.4. The location of Ollera, Toolakea, Cockle Bay and southern Cleveland Bay. The river mouths of Ollera Creek, Bluewater Creek, Burdekin River, Ross River, Haughton River and Barratta Creek are also shown. 176

Supplementary Figure 4.5. The location of Upstart Bay and Edgecumbe Bay. The river mouths of the Burdkein River, Molongle Creek, Don River and Gregory River are also shown. 177

Supplementary Figure 4.6. The location of Shoalwater Bay. The river mouths of the Carmila Creek and Styx River are also shown. 178

Supplementary Figure 4.7. The location of Gladstone and Heron Island. The river mouths of the Fitzroy River, Calliope River, Auckland Creek and Boyne River are also shown. 179

Supplementary Figure 4.8. The location of Sandy Strait. The river mouths of Baffle Creek, Kolan River, Burnett River and Mary River are also shown. 180

Appendices:
List of Supplementary Tables

Supplementary Table 2.1. The prevalence of fibropapillomatosis in green (*C. mydas*), loggerhead (*C. caretta*), Olive Ridley (*L. olivacea*), hawksbill (*E. imbricata*), leatherback (*D. coriacea*), Kemp's Ridley (*L. kempii*) and flatback turtles (*N. depressus*), according to year of observation and location. This report is consistent as of the year of publication (see Jones et al. (2016)). 162

Supplementary Table 3.1. Statistical analysis of the grouped and individual datasets in Chapter Three, including models and associated results. 169

Supplementary Table 3.2. Annual age class breakdown of turtles with fibropapillomatosis at Moreton Bay, western Shoalwater Bay and Heron Island. All data was collected during general population surveys between 1987 and 2014, with age-class determined by curved carapace length (CCL) (Limpus et al., 1994, Limpus and Chaloupka, 1997) 170

Supplementary Table 4.1. Statistical analysis of the grouped and individual datasets in Chapter Four, including models and associated results. 181

Supplementary Table 6.1. The origin of samples used in this study, including location, turtle tag number, curved carapace length (CCL), weight and sample collection year. Whether the sample was collected from a live turtle, or during a necropsy and/or donated ([d]) is also noted. Polymerase Chain Reaction (PCR) results where the presence (+) or absence (−) of chelonid alphaherpesvirus 5 (ChHV5) in FP tumour samples collected from turtles with different capture locations and host haplotype is also reported. All samples were collected from green turtles, excluding two sample from loggerheads (*) and one from a green/hawksbill hybrid (**). 183

Appendix One: Supplementary Files from Chapter Two

Supplementary Table 2.1. The prevalence of fibropapillomatosis in green (*C. mydas*), loggerhead (*C. caretta*), Olive Ridley (*L. olivacea*), hawksbill (*E. imbricata*), leatherback (*D. coriacea*), Kemp's Ridley (*L. kempii*) and flatback turtles (*N. depressus*), according to year of observation and location. This report is consistent as of the year of publication (see Jones et al. (2016)).

Locality	Species	Prevalence of FP (%)	Sample period	Reference
Western Atlantic/Eastern Caribbean				
Florida, United States of America				
Volusia County	*C. mydas*	6	1980-1998	Foley et al. (2005)
Mosquito Lagoon	*C. mydas*	0	1975-1981	Ehrhart (1991); Ehrhart et al. (1986)
	C. mydas	29	1985	Ehrhart (1991); Ehrhart et al. (1986)
	C. mydas	1.6	1990	Ehrhart (1991); Ehrhart et al. (1986)
Brevard County	*C. mydas*	10.1	1980-1998	Foley et al. (2005)
Trident Submarine Basin	*C. mydas*	0	1993-2007	Hirama and Ehrhart (2007)
Indian River	*C. mydas*	11.7	1980-1998	Foley et al. (2005)
	C. mydas	20-61	1982-1990	Ehrhart (1991)
	C. mydas	20	1993	Ehrhart and Redfoot (1995)
	C. mydas	63	1998-1999	Hirama and Ehrhart (2002)
	C. mydas	28-72	1984-2000	Hirama and Ehrhart (2007)
	C. mydas	8-32.9	1988-2006	Eaton et al. (2008)
	C. mydas	52.2	1982-2013	Cope et al. (2013)
	C. caretta	5.1	1982-2013	Cope et al. (2013)
Nearshore Reef	*C. mydas*	14.8	1998-1999	Hirama and Ehrhart (2007)
Wabasso Beach	*C. mydas*	0	1988-1993	Ehrhart (1991)
St. Lucie County	*C. mydas*	12.8	1980-1998	Foley et al. (2005)
Martin County	*C. mydas*	15.3	1980-1998	Foley et al. (2005)
Palm Beach County	*C. mydas*	12.8	1980-1998	Foley et al. (2005)
Lake Worth Lagoon	*C. mydas*	63	2005-2007	de Maye et al. (2008)

Broward County	*C. mydas*	1.1	1980-1998	Foley et al. (2005)
Miami-Dade	*C. mydas*	20.6	1980-1998	Foley et al. (2005)
Atlantic Coast	*C. mydas*	10	1980-1990	Teas (1991)
Monroe County	*C. mydas*	51.6	1980-1998	Foley et al. (2005)
Florida Bay	*C. mydas*	70	1990-1993	Herbst (1994)
	C. mydas	69.2	1991	Schroeder and Foley (1995)
	C. mydas	62	1990-1996	Schroeder et al. (1998)
Florida Keys	*C. mydas*	1.5	1938	Smith and Coates (1938)
	C. mydas	20-60	1980-1990	Teas (1991)
Cape Sable	*C. mydas*	<1%	1938	Lucke (1938)
Collier County	*C. mydas*	23	1980-1998	Foley et al. (2005)
Lee County	*C. mydas*	39	1980-1998	Foley et al. (2005)
Charlotte County	*C. mydas*	27	1980-1998	Foley et al. (2005)
Sarasota County	*C. mydas*	49	1980-1998	Foley et al. (2005)
Manatee County	*C. mydas*	67	1980-1998	Foley et al. (2005)
Hillsborough County	*C. mydas*	71	1980-1998	Foley et al. (2005)
Pinellas County	*C. mydas*	53.5	1980-1998	Foley et al. (2005)
Pasco County	*C. mydas*	67	1980-1998	Foley et al. (2005)
Hernando County	*C. mydas*	50	1980-1998	Foley et al. (2005)
Citrus County	*C. mydas*	18	1980-1998	Foley et al. (2005)
Gulf Coast	*C. mydas*	50	1992 (First observed in 1985)	Teas (1991)
Bermuda	*C. mydas*	0	1968-1993	Herbst (1994)
Bahamas				
Inagua	*C. mydas*	0	1974-1993	Herbst (1994)
Nicaragua				
Cabezas Port	*C. mydas*	<5	1993	Herbst (1994)
Panama				
Chiriqui Lagoon, Bocas del Toro	*C. mydas*	35	1989-1993	Herbst (1994)
Puerto Rico	*C. mydas*	17	1988-1992 (First observed in 1987)	Teas (1991)
Manglar Bay	*C. mydas*	0	1997	Patrício et al. (2011)
	C. mydas	0	1998	Patrício et al. (2011)

	C. mydas	9	2000	Patrício et al. (2011)
	C. mydas	29	2001	Patrício et al. (2011)
	C. mydas	75	2002	Patrício et al. (2011)
	C. mydas	79	2003	Patrício et al. (2011)
	C. mydas	50	2004	Patrício et al. (2011)
	C. mydas	25	2005	Patrício et al. (2011)
	C. mydas	12	2006	Patrício et al. (2011)
	C. mydas	64	2000-2006	Velez-Zuazo et al. (2010)
	C. mydas	4	2007	Patrício et al. (2011)
	C. mydas	30.5	2004-2007	Page-Karjian et al. (2012)
	C. mydas	0	2008	Patrício et al. (2011)
	C. mydas	4	2009	Patrício et al. (2011)
	C. mydas	3	2010	Patrício et al. (2011)
Culebrita	*C. mydas*	<1	2000-2006	Velez-Zuazo et al. (2010)
Culebrita, Tortuga Bay	*C. mydas*	0	1997-2004	Patrício et al. (2011)
	C. mydas	2	2005	Patrício et al. (2011)
	C. mydas	6	2006	Patrício et al. (2011)
	C. mydas	0	2007	Patrício et al. (2011)
	C. mydas	0	2008	Patrício et al. (2011)
	C. mydas	33	2009	Patrício et al. (2011)
	C. mydas	6	2010	Patrício et al. (2011)
	C. mydas	17	2009-2011	Patrício et al. (2014)
Barbados				
Barclay's Park	*C. mydas*	90	1990 (First observed in 1982-1983)	Gamache and Horrocks (1991)
Cayman Islands	*C. mydas*	66	1980-1991	Wood and Wood (1993)
Cuba	*C. mydas*	0.6	1983-1996	Moncada and Prieto (2000)
Brazil				
Trindade Island	*C. mydas*	1.1	1989-1990	Baptistotte et al. (2005)
	C. mydas	0.09	1994-1995	Baptistotte et al. (2005)
	C. mydas	0.34	1995-1996	Baptistotte et al. (2005)
State of Espirito Santo	*C. caretta*	1.3	1994-1995	Baptistotte et al. (2005)
States of Pernambuco, Alagoas and Sergipe	*L. olivacea*	12.5	1996-1997	Baptistotte et al. (2005)

Itaipu coastal region	*C. mydas*	30	2008-2010	Machado Guimarães et al. (2012)
Eastern Pacific				
Mexico				
La Escobilla Beach	*L. olivacea*	1.45	1997	Vasconcelos et al. (2000)
Mexiquillo Beach	*D. coriacea*	1 case	1997	Huerta et al. (2002)
Rancho Nuevo	*L. kempii*	1 case	1993	Barragan and Sarti (1994)
Costa Rico				
Ostional	*L. olivacea*	6-10	1997	Aguirre et al. (1999)
	L. olivacea	10	1997	Quiros et al. (2000)
Tortuguero	*C. mydas*	2.1	1998	Troëng (1998)
Mid-west Pacific				
Hawaiian Islands, United States of America	C. mydas	47-69	1982-1998	Murakawa et al. (2000)
	C. mydas	28	1982-2003	Chaloupka et al. (2008b)
Kiholo Bay	*C. mydas*	0	1987-1990	Balazs and Pooley (1991)
Punalu'u Bay	*C. mydas*	1	1976-1993 (First observed in 1984)	Balazs and Pooley (1991); Herbst (1994)
Pala'au, Molokai	*C. mydas*	0	1982-1985	Balazs and Pooley (1991); Herbst (1994)
	C. mydas	1 case	1985	Balazs and Pooley (1991)
	C. mydas	1-53	1987-1993	Balazs and Pooley (1991); Herbst (1994)
	C. mydas	4.8	1988	Balazs et al. (1998)
	C. mydas	9.8	1989	Balazs et al. (1998)
	C. mydas	17.2-25.6	1990	Balazs et al. (1998)
	C. mydas	23.1	1991	Balazs et al. (1998)
	C. mydas	53	1992	Balazs et al. (1998)
	C. mydas	47	1992-1993	Balazs et al. (1998)
	C. mydas	39	1993	Balazs et al. (1998)
	C. mydas	41	1994	Balazs et al. (1998)
	C. mydas	60.7	1995	Balazs et al. (1998)
	C. mydas	42.2-55.9	1996	Balazs et al. (1998)
Kāne'ohe Bay, Oahu	*C. mydas*	49-92	1989-1991 (First observed in 1958)	Balazs and Pooley (1991)
	C. mydas	43.9	1989-1997	Balazs et al. (2000)
Waikiki Beach, Oahu	*C. mydas*	9	1990-1993	Balazs and Pooley (1991)

French Frigate Shoals	*C. mydas*	7-12	1988-1992	Balazs and Pooley (1991); Herbst (1994)
	C. mydas	15.3	1999	Pepi et al. (2005)
Pearl/Hermes Reef	*C. mydas*	0	1982-1987	Balazs and Pooley (1991)
Midway Island	*C. mydas*	0	1969-1978 (First observed in 1990)	Balazs and Pooley (1991)
Punalu'u	*C. mydas*	0.01	1984-1994	Balazs et al. (1994)
Southwest Pacific				
Queensland, Australia				
Gulf of Carpentaria	*C. mydas*	0	2001-2002	Hamann et al. (2006)
	E. imbricata	0	2001-2002	Hamann et al. (2006)
Crab Island	*N. depressus*	5 cases	1991	Limpus et al. (1993)
Torres Strait	*C. mydas*	0	1977-1980	Glazebrook and Campbell (1990)
Heron Island and Wistari Reefs	*C. mydas*	0	1988-1990	Limpus and Miller (1994)
	C. caretta	1-2	1988-1990	Limpus and Miller (1994)
	E. imbricata	0	1988-1990	Limpus and Miller (1994)
Clack Island Reef	*C. mydas*	0	1988-1990	Limpus and Miller (1994)
	C. caretta	0	1988-1990	Limpus and Miller (1994)
	E. imbricata	0	1988-1990	Limpus and Miller (1994)
Hazelwood Island Reef	*C. mydas*	0	1989	Limpus and Miller (1994)
	E. imbricata	0	1989	Limpus and Miller (1994)
Green Island Reef	*C. mydas*	0	1988-1990	Limpus and Miller (1994)
	E. imbricata	0	1988-1990	Limpus and Miller (1994)
Lucinda	*C. mydas*	0	2003	Bell (2003)
Bowen	*C. mydas*	0	1989	Limpus and Miller (1994)
	C. mydas	0	1989	Limpus and Miller (1994)
Abbot Point	*C. mydas*	0	2003	Bell (2003)
Hay Point	*C. mydas*	0	2003	Bell (2003)
Shoalwater Bay	*C. mydas*	2	1988	Limpus and Miller (1994)
	C. mydas	2	1989	Limpus and Miller (1994)
	C. caretta	0	1990	Limpus and Miller (1994)
	C. mydas	3	1990	Limpus and Miller (1994)
	C. mydas	0.5	2000	Limpus et al. (2005)
	C. mydas	2.1	2001	Limpus et al. (2005)

	C. mydas	1.2	2002	Limpus et al. (2005)
	C. mydas	0.6	2003	Limpus et al. (2005)
	C. mydas	1.1	2004	Limpus et al. (2005)
	C. mydas	0.5	1970-2010	Flint et al. (2010a)
Repulse Bay	*C. mydas*	0	1988 (First observed in 1989)	Limpus and Miller (1994)
	C. mydas	3	1989	Limpus and Miller (1994)
	C. mydas	22	1990	Limpus and Miller (1994)
Moreton Bay	*C. mydas*	8	1988-1990	Limpus and Miller (1994)
	C. caretta	1	1988-1990	Limpus and Miller (1994)
	C. mydas	7.9	1990-1992	Limpus et al. (1994a)
	C. mydas	16	1998	Aguirre et al. (1998a)
	C. caretta	6	1998	Aguirre et al. (1998a)
	C. mydas	1.6	2006-2009	(Flint et al., 2010b)
Western Australia, Australia				
Baba Head, Shark Bay	*C. mydas*	<1	1996	Raidal and Prince (1996)
New South Wales, Australia				
Julia Rock Aquatic Reserve	*C. mydas*	14.3	2002	Speirs (2002)
Bali, Indonesia	*C. mydas*	21.5	1994	Adnyana et al. (1997)
	E. imbricata	0	1994	Adnyana et al. (1997)
Atlantic				
The Gulf of Guinea, West Africa				
Corisco Bay	*C. mydas*	27	1998	Formia et al. (2007)
		17	1999	Formia et al. (2007)
		19	2000	Formia et al. (2007)
		17	2003	Formia et al. (2007)
		22	2004	Formia et al. (2007)
		10	2005	Formia et al. (2007)
		14	2006	Formia et al. (2007)
Principe Island	*C. mydas*		2009	Loureiro and Matos (2009)
	Juveniles	32		Loureiro and Matos (2009)
	Subadults	36		Loureiro and Matos (2009)
	Adults	0		Loureiro and Matos (2009)

Turks and Caicos Islands	*C. mydas*	13	2008-2010	Stringell et al. (2011)
Pointe Indienne and Loango Bay	*C. mydas*	15	2009	Girard et al. (2013)
	C. mydas	8	2012	Girard et al. (2013)
Indian Ocean				
Seychelles	*C. mydas*	0	1981-1992	Herbst (1994)
Aldabra Island	*C. mydas*	0	1981-1992	Herbst (1994)

Appendix Two: Supplementary Files from Chapter Three

Supplementary Table 3.1. Statistical analysis of the grouped and individual datasets in Chapter Three, including models and associated results. All models were run as a generalised linear models.

Dataset	Effect tested	Model	Result
Grouped	Study site on FP prevalence	Proportion of FP ~ Study site, family=quasibinomial(),data = FPgrouped, weights=Total	No significant relationship determined
	Survey method on FP prevalence	Proportion of FP ~ Survey method, family=quasibinomial(),data = FPgrouped, weights=Total	Significant difference between survey method and FP prevalence, with turtle health surveys finding distinctly higher FP prevalence rates (p <0.001027).
	Average age-class on FP prevalence	Proportion of FP ~ Average age-class, family=quasibinomial(),data = FPgrouped, weights=Total	No significant relationship determined
	Median year on FP prevalence	Proportion of FP ~ Median Year, family=quasibinomial(),data = FPgrouped, weights=Total	The median year of each survey had a significant effect on FP prevalence (p = 0.001027)
	Combined effects of the explanatory variables which appeared to be significant (Survey method and median year) on FP prevalence	Proportion of FP ~ Survey method + Median Year, family=quasibinomial(),data = FPgrouped, weights=Total	While the significant effect of the median year dropped off in this analysis, survey method was still significant (p = 0.0455).
Age-class breakdown	Age-class on FP prevalence	Proportion of FP ~ Age-class*Study site +Age class, family=quasibinomial(), weights = Total	Age class has a significant effect on FP prevalence (p <2e-16), as does study site (p <2e-16). There is some interaction between age class and study site (p = 0.01678).

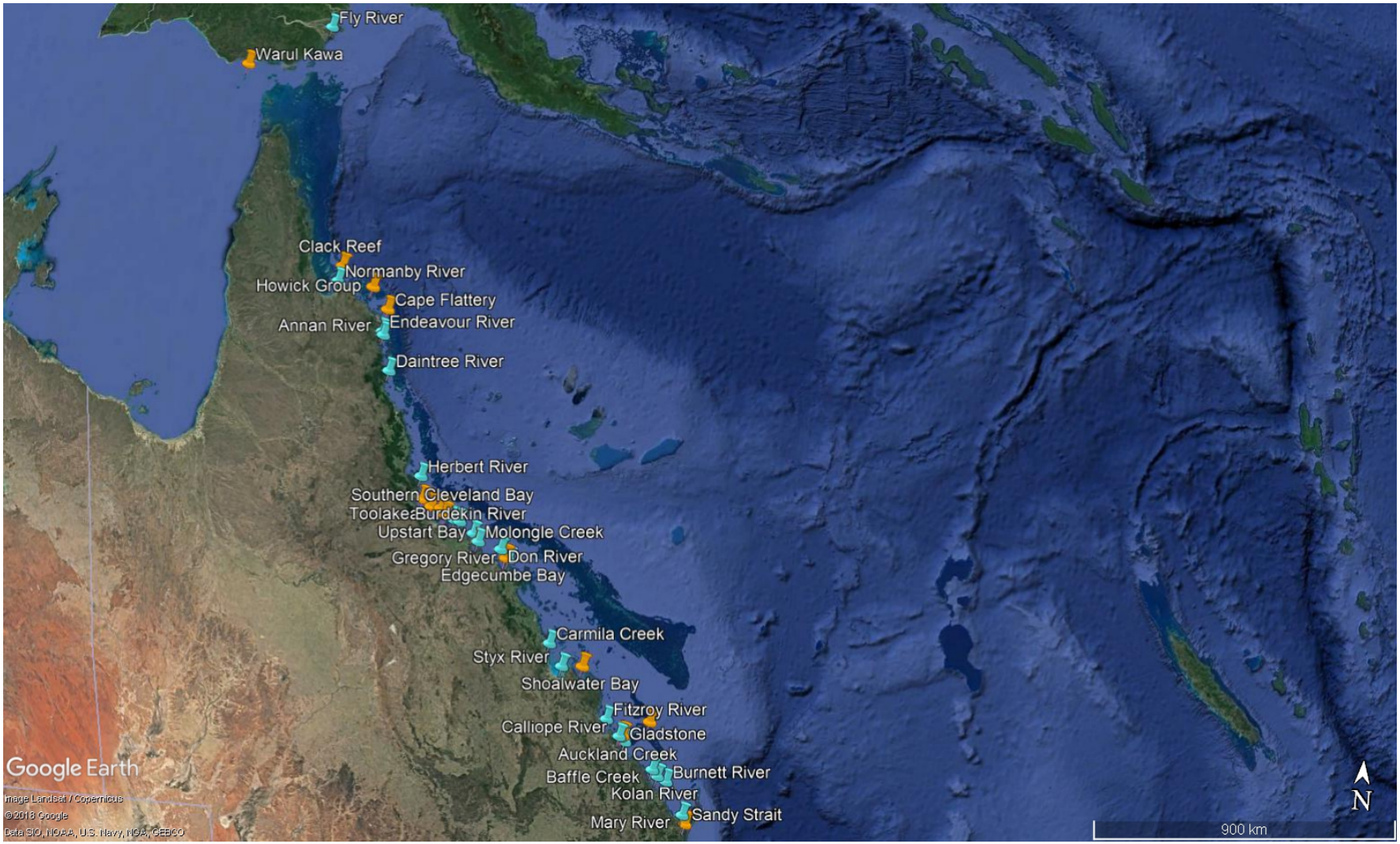
Supplementary Table 3.2. Annual age class breakdown of turtles with fibropapillomatosis at Moreton Bay, western Shoalwater Bay and Heron Island. All data was collected during general population surveys between 1987 and 2014, with age-class determined by curved carapace length (CCL) (Limpus et al., 1994, Limpus and Chaloupka, 1997)

		Juvenile Turtles			Sub-adult turtles			Adult turtles			Overall Total		
Site	Year	Total captured	Number with FP tumours	FP Prevalence (%)	Total captured	Number with FP tumours	FP Prevalence (%)	Total captured	Number with FP tumours	FP Prevalence (%)	Total captured	Number with FP tumours	FP Prevalence (%)
Moreton Bay	1990	130	5	3.85	84	16	19.05	45	1	2.22	259	22	8.49
	1991	270	3	1.11	115	19	16.52	37	3	8.11	422	25	5.92
	1992	153	5	3.27	54	9	16.67	14	0	0.00	221	14	6.33
	1993	95	16	16.84	41	8	19.51	33	4	12.12	169	28	16.57
	1994	168	13	7.74	34	5	14.71	1	0	0.00	203	18	8.87
	1995	206	18	8.74	89	14	15.73	63	0	0.00	358	32	8.94
	1996	157	20	12.74	86	10	11.63	61	1	1.64	304	31	10.20
	1997	170	22	12.94	91	21	23.08	69	0	0.00	330	43	13.03
	1998	103	13	12.62	95	25	26.32	87	2	2.30	285	40	14.04
	1999	115	22	19.13	63	15	23.81	45	3	6.67	223	40	17.94
	2000	169	30	17.75	98	21	21.43	83	5	6.02	350	56	16.00
	2001	260	38	14.62	113	28	24.78	99	2	2.02	472	68	14.41
	2002	172	32	18.60	48	14	29.17	30	1	3.33	250	47	18.80
	2003	40	10	25.00	27	4	14.81	19	0	0.00	86	14	16.28
	2004	198	25	12.63	65	13	20.00	45	1	2.22	308	39	12.66
	2005	168	29	17.26	69	11	15.94	52	1	1.92	289	41	14.19
	2006	111	27	24.32	54	8	14.81	42	2	4.76	207	37	17.87
	2007	144	31	21.53	80	12	15.00	86	1	1.16	310	44	14.19
	2008	179	29	16.20	78	9	11.54	91	0	0.00	348	38	10.92
	2009	313	79	25.24	91	9	9.89	97	0	0.00	501	88	17.56
	2010	328	82	25.00	152	17	11.18	172	6	3.49	652	105	16.10

	2011	206	49	23.79	74	6	8.11	112	0	0.00	392	55	14.03
	2012	244	31	12.70	46	3	6.52	72	0	0.00	362	34	9.39
	2013	98	17	17.35	64	1	1.56	63	0	0.00	225	18	8.00
	2014	68	6	8.82	109	1	0.92	133	0	0.00	310	7	2.26
Western Shoalwater Bay	1987	61	4	6.56	39	0	0.00	107	1	0.93	207	5	2.42
	1988	69	3	4.35	32	1	3.13	116	0	0.00	217	4	1.84
	1989	129	6	4.65	92	2	2.17	121	0	0.00	342	8	2.34
	1990	167	8	4.79	129	4	3.10	204	0	0.00	500	12	2.40
	1991	130	7	5.38	134	6	4.48	190	1	0.53	454	14	3.08
	1994	80	6	7.50	108	1	0.93	197	1	0.51	385	8	2.08
	1995	85	5	5.88	99	2	2.02	234	0	0.00	418	7	1.67
	1996	41	2	4.88	60	1	1.67	227	0	0.00	328	3	0.91
	1997	42	2	4.76	68	1	1.47	168	0	0.00	278	3	1.08
	2000	81	1	1.23	95	1	1.05	222	0	0.00	398	2	0.50
	2001	98	5	5.10	75	3	4.00	207	0	0.00	380	8	2.11
	2002	180	3	1.67	75	1	1.33	160	1	0.63	415	5	1.20
	2003	150	1	0.67	63	0	0.00	136	1	0.74	349	2	0.57
	2004	232	4	1.72	67	1	1.49	162	0	0.00	461	5	1.08
	2005	234	7	2.99	83	3	3.61	120	0	0.00	437	10	2.29
	2006	256	8	3.13	63	1	1.59	153	0	0.00	472	9	1.91
	2007	294	17	5.78	82	2	2.44	131	0	0.00	507	19	3.75
	2008	316	21	6.65	65	1	1.54	180	1	0.56	561	23	4.10
	2012	339	7	2.06	114	1	0.88	157	0	0.00	610	8	1.31
Heron Island	1989	94	0	0.00	126	0	0.00	88	1	1.14	308	1	0.32
	1994	93	0	0.00	167	2	1.20	116	0	0.00	376	2	0.53
	1995	112	0	0.00	142	1	0.70	123	1	0.81	377	2	0.53
	1997	177	0	0.00	154	0	0.00	158	1	0.63	489	1	0.20
	1999	94	0	0.00	136	1	0.74	173	1	0.58	403	2	0.50

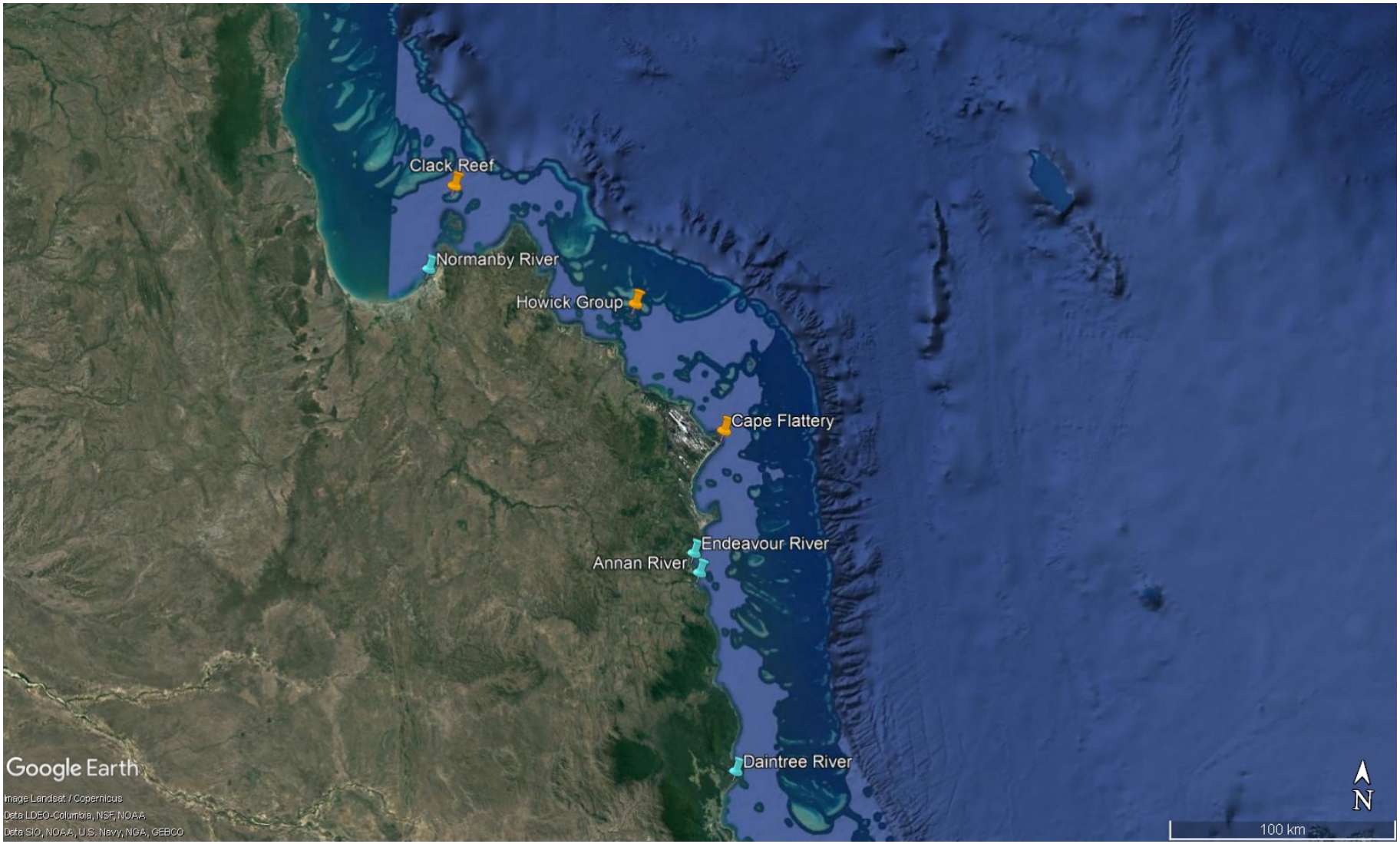
Appendix Three: Supplementary Files from Chapter Four

Chapter Four involved 14 study sites along the Queensland coast. Many of these sites are influence by multiple rivers. An outline of the relative positions of the study sites and main river influences is provided in the following pages of this Appendix.

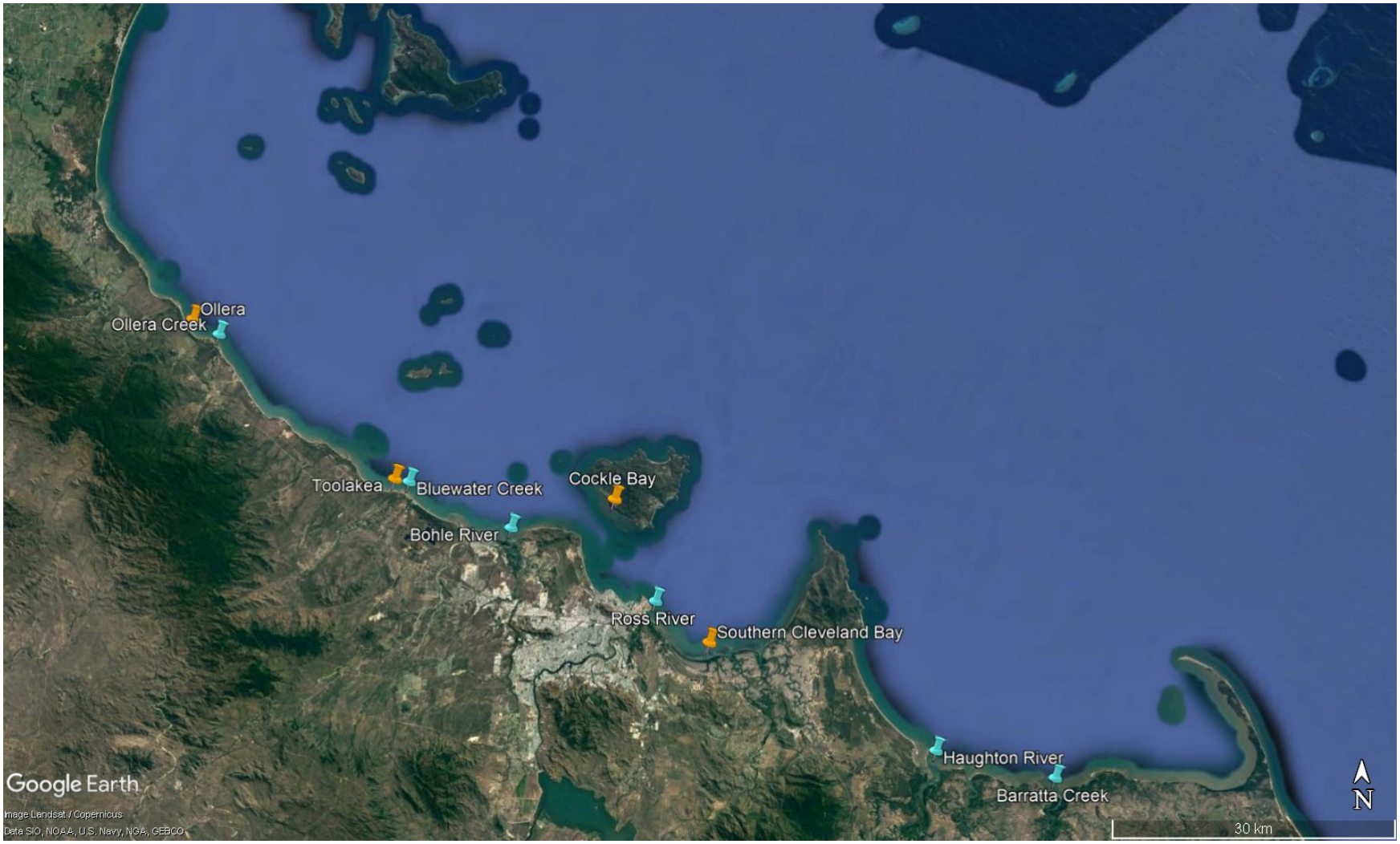


Supplementary Figure 4.1. All study sites and rivers discussed in the present study.

Supplementary Figure 4.2. The location of Warul Kawa and its largest river influence, the Fly River.

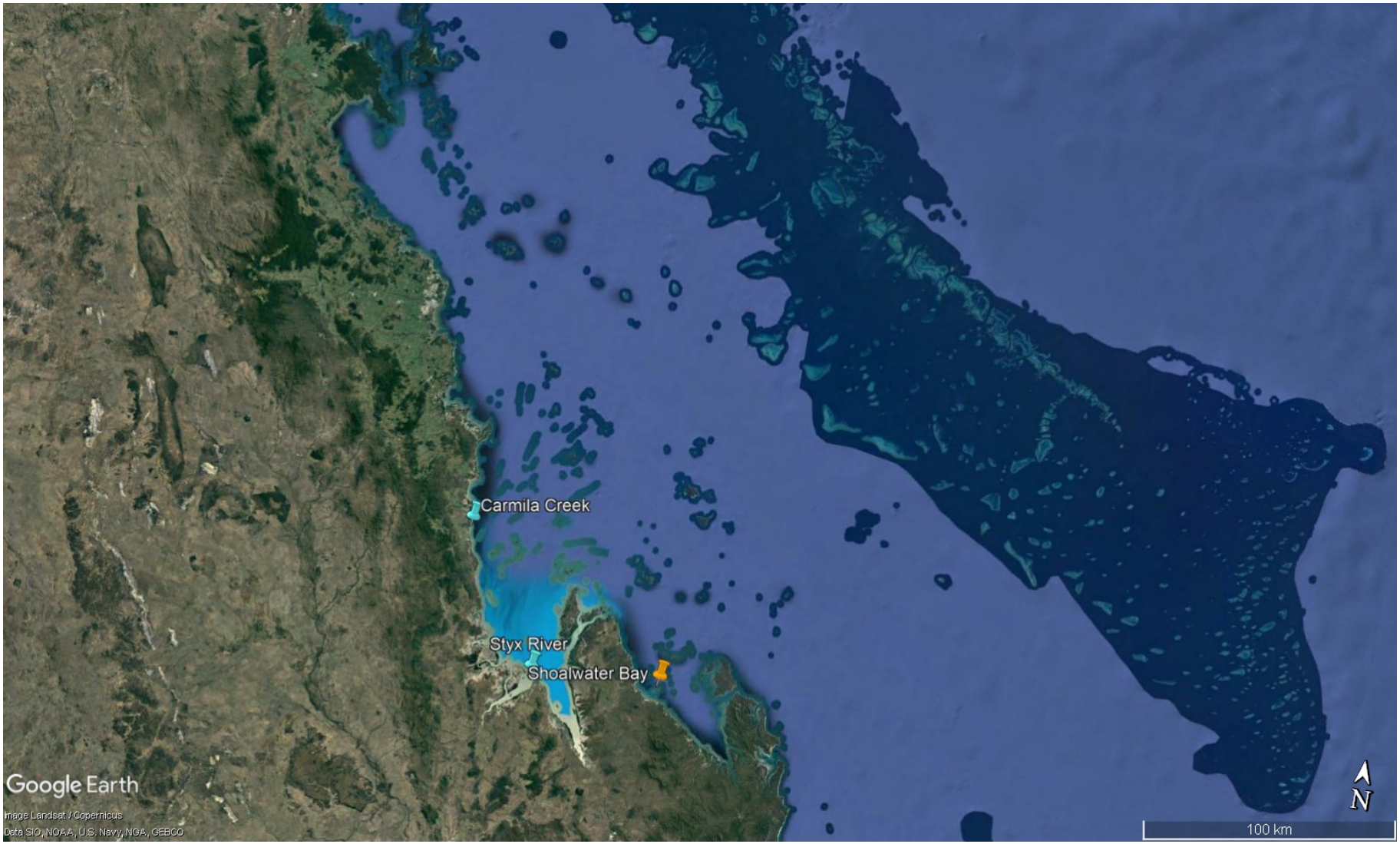


Supplementary Figure 4.3. The location of Clack Reef, the Howick Group of islands and Cape Flattery. The river mouths of the Normanby River, Endeavour River, Annan River and Daintree River are also shown.



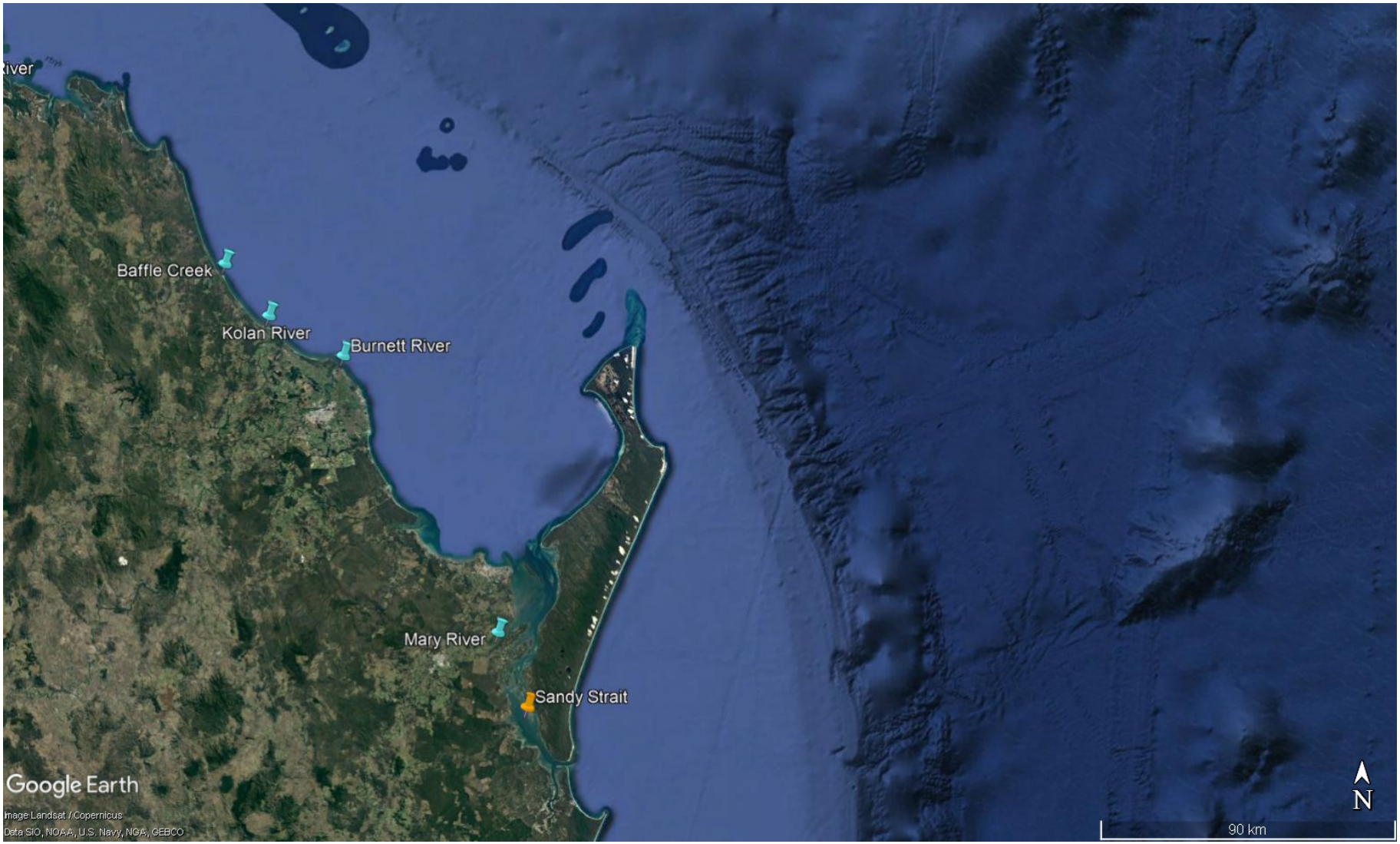
Supplementary Figure 4.4. The location of Ollera, Toolakea, Cockle Bay and southern Cleveland Bay. The river mouths of Ollera Creek, Bluewater Creek, Burdekin River, Ross River, Haughton River and Barratta Creek are also shown. Not pictured, but known to influence some of these locations, are the Herbert River and Burdekin River (see Supplementary Figure 4.1 for overview).

Supplementary Figure 4.5. The location of Upstart Bay and Edgecumbe Bay. The river mouths of the Burdkein River, Molongle Creek, Don River and Gregory River are also shown.



Supplementary Figure 4.7. The location of Shoalwater Bay. The river mouths of the Carmila Creek and Styx River are also shown.

Supplementary Figure 4.6. The location of Gladstone and Heron Island. The river mouths of the Fitzroy River, Calliope River, Auckland Creek and Boyne River are also shown.



Supplementary Figure 4.8. The location of Sandy Strait. The river mouths of Baffle Creek, Kolan River, Burnett River and Mary River are also shown.

Supplementary Table 4.1. Statistical analysis of the grouped and individual datasets in Chapter Four, including models and associated results.

Dataset	Effect tested	Model	Result
Grouped	DIN sub-index on FP prevalence	Proportion of FP ~ factor(DIN), family=quasibinomial(),data = FPgrouped, weights=Total	No significant relationship determined
	TSS sub-index on FP prevalence	Proportion of FP ~ factor(TSS), family=quasibinomial(),data = FPgrouped, weights=Total	No significant relationship determined
	Pesticide sub-index on FP prevalence	Proportion of FP ~ factor(Pesticides), family=quasibinomial(),data = FPgrouped, weights=Total	No significant relationship determined
	Metals sub-index on FP prevalence	Proportion of FP ~ factor(Metals), family=quasibinomial(),data = FPgrouped, weights=Total	No significant relationship determined
	Overall WQI on FP prevalence	Proportion of FP ~ factor(Overall WQI)), family=quasibinomial(),data = FPgrouped, weights=Total	No significant relationship determined
	Combined effects of these explanatory variables (in addition to those discussed in Chapter Two) on FP prevalence	Proportion of FP ~ Survey method + median Year + factor(DIN) + factor(TSS) + factor(Pesticides) + factor(Metals) + factor(Overall WQI), family=quasibinomial(),data = FPgrouped, weights=Total	Both the survey method and median year were determined to have a significant effect on FP prevalence (consistent with Chapter Two). All water quality sub-indices (DIN, TSS, Pesticides and Metals) in addition to the Overall WQI were determined to have no significant effect on FP prevalence using this model.
Individual	Age-class on FP prevalence	Proportion of FP ~ Study site +Age class, data= indiv.p, family=quasibinomial())	Age-class has a significant effect on FP prevalence (p=0.00000000004353)
	Study site on FP prevalence	Proportion of FP ~ Study site +Age class, data= indiv.p, family=quasibinomial())	Study site has a significant effect on FP prevalence (p=0.0001252)
	Combined effects of explanatory variables on FP prevalence	Proportion of FP ~ Year + Size Class + factor(TSS) + factor(Pesticides) + factor(Metals) + factor(Overall WQI)	Unable to fit model. The individual explanatory variables (suv-indices and overall WQI) for this dataset were tested as above but the model was unable to be fitted.

Appendix Four: Supplementary Files from Chapter Six

Supplementary Table 6.1. The origin of samples used in this study, including location, turtle tag number, curved carapace length (CCL), weight and sample collection year. Whether the sample was collected from a live turtle, or during a necropsy and/or donated ([d]) is also noted. Polymerase Chain Reaction (PCR) results where the presence (+) or absence (−) of chelonid alphaherpesvirus 5 (ChHV5) in FP tumour samples collected from turtles with different capture locations and host haplotype is also reported. All samples were collected from green turtles, excluding two sample from loggerheads (*) and one from a green/hawksbill hybrid (**).

Collection Details					Results			
Location	**Tag No.**	**CCL**	**Sample Year**	**Collection Type**	**Host Haplotype**	**DNApol**	**gB**	**Fsial**
Cairns	Roxy	47.6	2010	Live turtle	CmP98.1	+	+	+
Townsville	QA42923	43.6	2016	Live turtle	CmP34.1	+	-	-
Townsville	QA36631	59.6	2013	Live turtle	CmP44.2	+	+	+
Townsville	K92985	46.2	2013	Live turtle	CmP47.1	+	+	+
Townsville	QA15682	49.7	2013	Live turtle	CmP47.1	+	-	-
Townsville	QA29610	45.0	2014	Live turtle	CmP47.1	+	+	+
Townsville	QA36842	46.3	2014	Live turtle	CmP47.1	+	+	+
Townsville	QA38803	50.5	2016	Live turtle	CmP47.1	+	+	+
Townsville	QA62135	48.0	2017	Live turtle	CmP47.1	-	+	+
Townsville	QA7381	53.0	2016	Live turtle	CmP47.1	+	+	+

Townsville	QA7388	49.5	2016	Live turtle	CmP47.1	+	+	+
Townsville	QA7433	44.9	2016	Live turtle	CmP47.1	+	+	+
Townsville	QA9220	70.2	2012	Live turtle	CmP47.1	+	+	+
Townsville	QA9554	53.7	2011	Live turtle	CmP47.1	+	+	+
Townsville	TSV-NT1	Unknown	2012	Necropsy	CmP47.1	+	+	+
Townsville	QA42248	44.2	2016	Live turtle	CmP57.1	+	+	+
Townsville	09-231	57.1	2009	Live turtle	CmP80.1	+	+	+
Townsville	QA38827	44.0	2016	Live turtle	CmP80.1	+	+	+
Townsville	QA38835	50.3	2016	Live turtle	CmP80.1	+	+	+
Townsville	QA42017	48.6	2014	Live turtle	CmP80.1	+	+	+
Townsville	QA47530	48.1	2016	Live turtle	CmP80.1	+	+	+
Townsville	QA7392	50.2	2016	Live turtle	CmP80.1	+	+	+
Townsville	QA47488**	60.3	2017	Necropsy	N/A	+	+	+
Bowen	K97483	48.5	2010	Live turtle	CmP44.1	+	+	+
Bowen	K52464	54.0	2010	Live turtle	CmP47.1	-	+	+
Bowen	K59365	46.5	2013	Live turtle	CmP47.1	+	+	+
Bowen	K92663	44.7	2010	Live turtle	CmP47.1	+	+	+
Bowen	K93038	49.6	2013	Live turtle	CmP47.1	+	+	-
Bowen	K93052	42.5	2012	Live turtle	CmP47.1	+	+	+
Bowen	K93074	45.0	2010	Live turtle	CmP47.1	+	+	+
Bowen	K93640	47.9	2010	Live turtle	CmP47.1	+	+	+
Bowen	K97113	47.9	2009	Live turtle	CmP47.1	+	+	+

Bowen	K97114	50.5	2009	Live turtle	CmP47.1	+	+	+
Bowen	K97115	54.3	2009	Live turtle	CmP47.1	+	+	+
Bowen	K97117	45.0	2009	Live turtle	CmP47.1	+	+	+
Bowen	K97289	51.8	2009	Live turtle	CmP47.1	-	+	+
Bowen	K97336	25.7	2013	Live turtle	CmP47.1	+	+	+
Bowen	QA15638	48.6	2011	Live turtle	CmP47.1	+	+	+
Bowen	QA15678	76.2	2012	Live turtle	CmP47.1	+	+	+
Bowen	QA15758	49.4	2013	Live turtle	CmP47.1	+	+	+
Bowen	QA15774	48.7	2013	Live turtle	CmP47.1	+	+	+
Bowen	QA15951	45.4	2010	Live turtle	CmP47.1	+	+	+
Bowen	QA15980	44.0	2010	Live turtle	CmP47.1	+	+	+
Bowen	QA29702	44.4	2012	Live turtle	CmP47.1	+	+	+
Bowen	QA36626	44.8	2013	Live turtle	CmP47.1	+	+	+
Bowen	QA7340	45.3	2011	Live turtle	CmP47.1	+	+	+
Bowen	QA9462-1	44.5	2010	Live turtle	CmP47.1	+	+	+
Bowen	QA15979	47.2	2010	Live turtle	CmP80.1	+	+	+
Bowen	QA36636	47.6	2013	Live turtle	CmP85.1	+	+	+
Bowen	QA32132*	85.5	2013	Live turtle	N/A	+	+	+
Airlie Beach	AB-NT1	49.0	2017	Necropsy[d]	CmP47.1	+	+	+
Gladstone	QA34793	60.2	2015	Live turtle	CmP47.1	+	+	+
Gladstone	QA58252	70.1	2015	Live turtle	CmP47.1	+	-	-
Gladstone	QA58271	70.1	2015	Live turtle	CmP47.1	-	-	+

Gladstone	QA58207	60.3	2015	Live turtle	CmP85.1	+	+	+
Brisbane	Alice	45.0	2017	Necropsy[d]	CmP47.1	+	+	+
Brisbane	MB-NT1	46.3	2015	Necropsy	CmP47.1	+	+	+
Brisbane	MB-NT3	44.4	2015	Necropsy	CmP47.1	+	+	+
Brisbane	MB-NT4	43.1	2015	Necropsy	CmP47.1	+	+	+
Brisbane	Tay	52.4	2017	Necropsy[d]	CmP47.1	+	+	+
Brisbane	QA45711	52.1	2015	Live turtle	CmP80.1	+	+	+
Brisbane	MB-NT2*	98.0	2018	Necropsy[d]	N/A	+	+	+
				Total	62	58	58	58

Appendix Five: Publications arising during candidature

In this appendix, the following publications are provided:

Jones, K., Ariel, E., Burgess, G. and Read, M. 2016. A review of fibropapillomatosis in Green turtles (*Chelonia mydas*). *The Veterinary Journal.* Volume 212, p. 48–57

Jones K, Jensen M, Burgess G, Ariel E. 2018. Closing the gap: Mixed stock analysis of three foraging populations of green turtles (*Chelonia mydas*) on the Great Barrier Reef. PeerJ. 6(e5651). doi: https://doi.org/10.7717/peerj.5651

Ariel, E., Nainu, F., **Jones, K.,** Juntunen, K., Bell, I., Gaston, J., Scott, J., Tocini, S. and Burgess, G. W. 2017. Phylogenetic variation of chelonid alphaherpesvirus 5 in green turtle (*Chelonia mydas*) populations along the Queensland Coast, Australia. *Journal of Aquatic Animal Health*. Issue 3, p. 150-157

Cárdenas DM, Cucalón RV, Medina-Magües LG, **Jones K,** Alfaro-Núñez A, Alemán RA, Cárdenas WB (2019) Fibropapillomatosis in the South-East Pacific region: first case report in Ecuador and its inclusion in a global phylogenetic analysis. Journal of Wildlife Diseases. Volume 55, Issue 1, pp00 - 000

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Review

A review of fibropapillomatosis in Green turtles (*Chelonia mydas*)

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ABSTRACT

Despite being identified in 1938, many aspects of the pathogenesis and epidemiology of fibropapillomatosis (FP) in marine turtles are yet to be fully uncovered. Current knowledge suggests that FP is an emerging infectious disease, with the prevalence varying both spatially and temporally, even between localities in close proximity to each other. A high prevalence of FP in marine turtles has been correlated with residency in areas of reduced water quality, indicating that there is an environmental influence on disease presentation.

Chelonid herpesvirus 5 (ChHV5) has been identified as the likely aetiological agent of FP. The current taxonomic position of ChHV5 is in the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Scutavirus*. Molecular differentiation of strains has revealed that a viral variant is typically present at specific locations, even within sympatric species of marine turtles, indicating that the disease FP originates regionally. There is uncertainty surrounding the exact path of transmission and the conditions that facilitate lesion development, although recent research has identified atypical genes within the genome of ChHV5 that may play a role in pathogenesis. This review discusses emerging areas where researchers might focus and theories behind the emergence of FP globally since the 1980s, which appear to be a multi-factorial interplay between the virus, the host and environmental factors influencing disease expression.

Introduction

The Green turtle (*Chelonia mydas*) is one of seven species of marine turtle and is internationally recognised as endangered by the International Union for the Conservation of Nature (Seminoff, 2004). Eleven discrete population segments of Green turtles have been identified, each of which is considered biologically and ecologically significant (NMFS (National Marine Fisheries Service) and USFWS (US Fish and Wildlife Service), 2014). Green turtles also hold great cultural significance for many indigenous peoples and are of economic interest, playing a significant role in ecotourism (Dobbs, 2001; Gulko and Eckert, 2004). The species has a global distribution and a complex life history, occupying a range of habitats. Hatchling turtles have a pelagic existence and recruit into benthic inshore waters at the age of 3–5 years (Reich et al., 2007). With the exception of migration for breeding, turtles typically remain in these inshore environments, which are commonly associated with seagrass meadows or coral reefs, for the remainder of their life (Musick and Limpus, 1997) (Fig. 1).

Green turtles are exposed to a number of threats including ingestion of marine debris, degradation, urbanisation and pollution of nesting habitats and foraging areas, nest and hatchling depredation by wild, feral and domestic animals, boat strike, traditional hunting and egg harvest, the impacts of climate change on the marine and terrestrial environment, and entanglement in fishing nets and lines (Bjorndal, 1995; Herbst and Klein, 1995a; Lutz, 2002; Van Houtan et al., 2010). Conservation efforts which aim to abate many of these threats have assisted in the recovery of some of the major Green turtle populations (Chaloupka et al., 2008a). However, outbreaks of disease are also contributing to morbidity and mortality in this already vulnerable species (Foley et al., 2005; Chaloupka et al., 2008b; Flint et al., 2010b).

Fibropapillomatosis (FP) is a disease that has now been reported in every species of marine turtle: Green (Smith and Coates, 1938), Loggerhead (*Caretta caretta*) (Harshbarger, 1991), Kemp's Ridley (*Lepidochelys kempii*) (Barragan and Sarti, 1994), Hawksbill (*Eretmochelys imbricata*) (D'Amato and Moraes-Neto, 2000), Olive Ridley (*Lepidochelys olivacea*) (Aguirre et al., 1999), Flatback (*Natator depressus*) (Limpus et al., 1993), and Leatherback (*Dermochelys coriacea*) (Huerta et al., 2002) turtles. FP is of greatest concern in Green turtles as it has only reached a panzootic status in this species (Williams et al., 1994).

FP is a neoplastic condition which may lead to the growth of lesions on the skin, oral cavity, shell, eyes and internal organs of the affected turtle, which in severe cases reduces the probability of survival (Herbst, 1995; Work et al., 2004; Flint et al., 2010a). The disease was first identified in a Green turtle with multiple wart-like lesions on display at the New York Aquarium, although originally

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1090-0233/

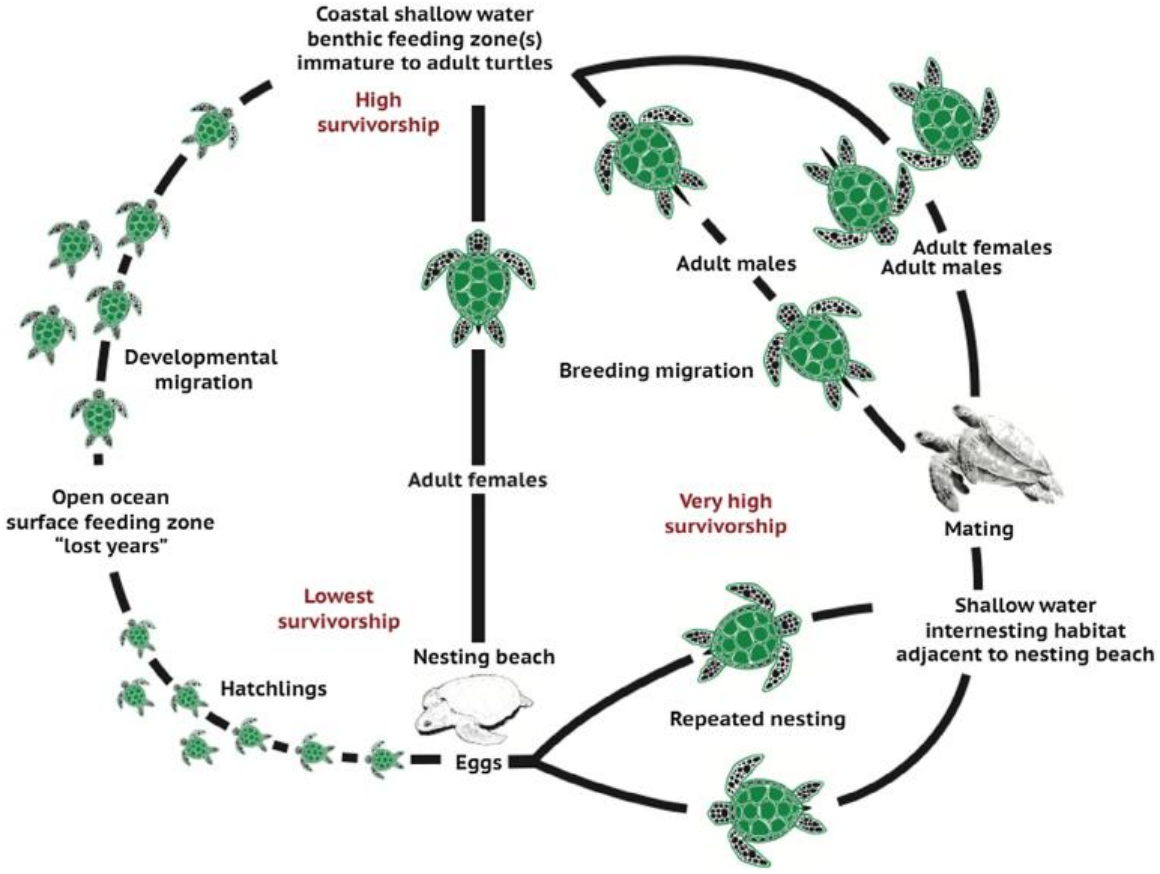

Fig. 1. The complex life history of Green turtles. Adapted from Lanyon et al. (1989).

from Key West, Florida (Smith and Coates, 1938). Despite being described in 1938 (Lucke, 1938; Smith and Coates, 1938), FP did not reach epizootic proportions until the 1980s (Herbst, 1994; Herbst et al., 2004) and has now been reported from every major ocean basin that Green turtles inhabit (Herbst, 1994).

This review covers the epidemiology and proposed aetiology of FP in Green turtles, with considerable emphasis on the primary candidate for the aetiological agent, chelonid herpesvirus 5 (ChHV5).

Disease presentation

FP can be identified in marine turtles by the presence of single or multiple benign fibroepithelial lesions. The characteristic lesions are easily noticed and are pathognomonic for FP, often limiting or obstructing the vision, feeding and locomotive ability of the affected turtle (Herbst, 1994, 1995; Work et al., 2004; Flint et al., 2010a). Cutaneous lesions are typically present on the external soft tissue of the turtle, but may grow on the carapace, plastron (Smith and Coates, 1938; Jacobson et al., 1989; Balazs and Pooley, 1991; Brooks et al., 1994; Herbst, 1994) and cornea of affected turtles (Brooks et al., 1994; Flint et al., 2010a). The lesions can be observed on all visceral organs (Herbst, 1994; Work et al., 2004; Foley et al., 2005) and are thought to develop during later stages of the disease (Herbst et al., 1999; Wyneken et al., 2006). However, as most visceral lesions are observed during post mortem investigations, the data available on the prevalence of this type of lesion are skewed. Individual lesions can range from 0.1 to 30 cm in diameter and can be sessile or pedunculated. The appearance of these lesions can vary from smooth to verrucous and the colour is dependent on the pigment at the site of origin (Herbst, 1994) (Fig. 2).

Myxofibromas, fibrosarcomas, papillomas, fibromas and fibropapillomas have all been found to be associated with FP (Norton et al., 1990; Work et al., 2004). Three of these lesions are thought to be linked with different stages of lesion development (Herbst, 1994; Kang et al., 2008). The early development phase is associated with papilloma lesions, proliferation of epidermal cells, with little or no involvement of the dermal layer. The chronic phase of lesion development is marked by the presence of fibromas, with proliferation of the dermal layer, while the epidermal layer remains

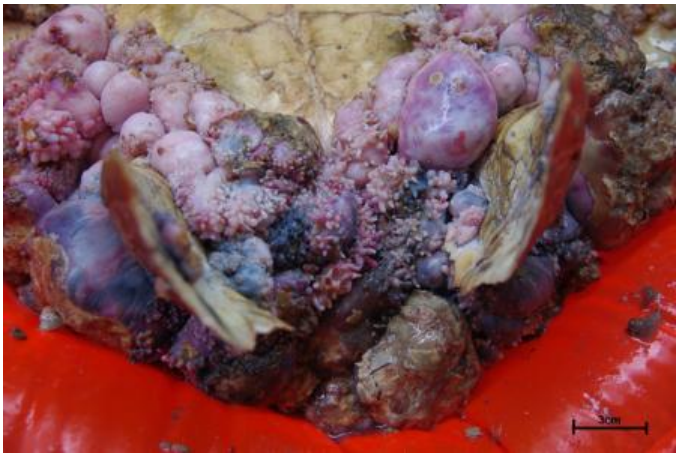

Fig. 2. The plastron and hind flippers of a Green turtle severely affected by fibropapillomatosis highlighting the diverse range of lesion appearance.

normal. Fibropapillomas represent the intermediate phase of lesion development and consist of characteristics of both the papillomas and fibromas (Herbst, 1994; Kang et al., 2008).

Histological studies on FP lesions have observed orthokeratotic hyperkeratosis and varying degrees of epidermal hyperplasia. Key features observed in FP lesions include cytoplasmic vacuolation and ballooning degeneration of superficial epidermal cells (Jacobson et al., 1989, 1991; Herbst, 1994; Adnyana et al., 1997).

Haematological and biochemical signs of immunosuppression, chronic stress, and chronic inflammation such as anaemia, lymphocytopenia, neutrophilia, monocytosis, hypoproteinaemia and hyperglobulinaemia have been observed in turtles with clinical signs of FP (Aguirre et al., 1995; Work et al., 2001; dos Santos et al., 2010; Page-Karjian et al., 2014). Although it is still unclear whether the immunosuppression occurs as a result of or as a precursor to FP development, it has been suggested that immunosuppression occurs as a result of FP (Work et al., 2001). While further study is essential to confirm the relationship between immunosuppression and FP infection, it is clear that immunosuppression leaves turtles with FP lesions susceptible to secondary infections and opportunistic pathogens (Work et al., 2001, 2003; Stacy et al., 2008; dos Santos et al., 2010). Impacts of such secondary infections, combined with FP in marine turtles, are a major cause for concern in an already vulnerable species.

Epidemiology of fibropapillomatosis in marine turtles

FP typically occurs in marine turtles inhabiting neritic tropical and sub-tropical areas (Herbst, 1994; Adnyana et al., 1997; Work et al., 2004; Ene et al., 2005). The disease is most frequently observed in juvenile turtles; FP has also been reported in sub-adults and less commonly in adults (Herbst, 1994; Herbst and Klein, 1995b; Adnyana et al., 1997; Work et al., 2004; Ene et al., 2005; Patrício et al., 2012; Page-Karjian et al., 2014). This apparent age differentiation in certain locations may indicate that affected juveniles perish from the population altogether or recover with acquired immunity that protects them as adults (Van Houtan et al., 2010). Alternatively, it is possible that these adults were never exposed to this disease.

There are no reports of FP in pelagic post hatchlings or new recruits that have recently taken up residence in inshore foraging habitats (Herbst, 1994). Sex is not thought to be a contributing factor, as no significant difference has been observed in prevalence between males and females (Work et al., 2004).

Disease prevalence and impact

Smith and Coates (1938) reported a prevalence of 1.5% in the Florida Keys region. The disease was not documented in the area again until the 1980s, where the prevalence was then reported to range between 20 and 60% throughout the subsequent decade. The early to mid-1990s saw FP emerge in the Eastern Pacific, Hawaiian Islands, Indonesia and Australia. As the disease reached epizootic status in several locations globally, it is now considered a panzootic (Williams et al., 1994). Due to the conspicuous presentation of FP, any prior presence would have been noticed in a region where it currently occurs. The incidence of turtles with FP lesions as a percentage of total turtles captured is reported in the Appendix: Supplementary Table S1. Although age class is a risk factor, not all reports of FP prevalence have been corrected by demographic proportions and future reports would benefit from making this distinction.

The prevalence of FP varies both spatially and temporally (see Appendix: Supplementary Table S1). The sporadic reports of the disease over time, in combination with a lack of oral history prior to the 1980s, indicate that FP is globally emerging (Greenblatt et al., 2005; Duarte et al., 2012). In several cases, a significantly different prevalence of the disease in nearby regions has been observed. In Florida, a prevalence of approximately 50% was observed in Green turtle aggregations in the Indian River region. However, less than 1 km away at the Sabellariid worm reef, FP was not observed at all (Herbst, 1994). At Pala'au, Molokai, FP was not observed at all until 1985, with the prevalence increasing from 1% in 1987 to 60.7% in 1995 (see Appendix: Supplementary Table S1).

A shift in FP prevalence at two closely monitored sites in Puerto Rico has been observed in recent years; FP prevalence began decreasing Puerto Manglar and increasing at Tortuga Bay in 2009 (Patrício et al., 2011). In Australia, FP has been reported in a number of locations since it was first observed in Queensland in the early 1970s (C. Limpus, personal communication).

The contribution of this disease to morbidity and mortality in affected turtles has also been widely discussed (Herbst, 1994; Ene et al., 2005; Foley et al., 2005; Chaloupka et al., 2008b, 2009; Flint et al., 2010b). A study on Green turtles at Palaau, Hawaii, found that this population was already recovering from previous overharvesting at the time of the FP outbreak in this region. The FP prevalence in this region has also been in decline since the mid-1990s (Chaloupka et al., 2009).

Studies on regions in Australia (Flint et al., 2010b), Puerto Rico (Patrício et al., 2011) and Florida (Hirama and Ehrhart, 2007) have all concluded that FP is not a significant factor in mortality of turtles. Conversely, a study conducted on data accumulated over 21 years from Hawaii implicated FP as the primary cause of strandings (Chaloupka et al., 2008b).

Despite some conflicting conclusions, the overwhelming consensus is that FP does not significantly impact the survival of turtle populations. However, Hamann et al. (2010) highlights that understanding and managing this disease is a priority research area for sea turtle conservation. Without a more complete understanding of the fundamental elements of this disease, FP cannot be discounted as a threat to the survival of this species.

Aetiology of fibropapillomatosis in marine turtles

Research to date suggests that FP is associated with a herpesvirus infection (Herbst et al., 1995; Quackenbush et al., 1998, 2001; Lackovich et al., 1999). Despite ongoing research, this virus cannot be cultured in vitro and therefore Koch's postulates have not been fulfilled (Herbst, 1994, 1995; Moore et al., 1997; Lu et al., 1999; Work et al., 2009). Molecular techniques (Quackenbush et al., 1998, 2001; Lackovich et al., 1999) have proven a strong association between FP and a herpesvirus and, according to the criteria established by Hill (1965), the relationship seems to be that of cause and effect. Chelonid herpesvirus 5 (ChHV5) is now the primary focus of research in this area and belongs to the subfamily *Alphaherpesvirinae*, genus *Scutavirus* (Davison and McGeoch, 2010). However, there are still some uncertainties surrounding the transmission of the virus, the circumstances that lead to lesion development and the role of environmental factors in the development of this disease.

Infectious nature of fibropapillomatosis

The epizootic nature of FP and the significant variation in the prevalence of FP between different populations of marine turtles, even between nearby localities, led to speculation that FP was primarily caused by an infectious agent.

Herbst et al. (1995) successfully transferred FP between animals by using cell-free lesion extracts from turtles with lesions to inoculate young captive-reared turtles that were theoretically naïve to FP. All turtles in 3/4 experimental groups developed FP lesions. Control animals, which were housed in the same facility and conditions as the experimental turtles, did not develop FP during the

same study period. The lesion extracts used in this experiment were filtered through a 0.45 μm syringe tip filter to prevent most pathogens, other than viruses, from being transferred. These findings support the case for the role of a viral agent in FP transmission in marine turtles.

Although in their initial description of FP, Smith and Coates (1938) did not identify any viral elements in histological examination of FP lesions, modern theories have focused on viruses as the primary aetiological agent of FP. A range of viruses are capable of producing neoplasms such as those seen in Green turtle FP. As a result, papillomavirus (Herbst, 1994), papova-like virus (Lu et al., 2000a), retrovirus (Casey et al., 1997) and herpesviruses (Jacobson et al., 1991; Herbst, 1994; Quackenbush et al., 1998; Herbst et al., 2004) have all been proposed as potential candidates for the aetiological agents of FP in marine turtles.

Current research suggests that FP is associated with ChHV5 infection. Early molecular studies tested a range of tissues from turtles both with and without FP lesions and all concluded that while ChHV5 could be detected in lesion biopsies from turtles with FP, the virus was rarely detected in normal skin samples from the same turtles (Quackenbush et al., 1998; Lackovich et al., 1999). Samples from turtles without FP lesions did not react in any of the PCR assays conducted in these early studies (Quackenbush et al., 1998; Lackovich et al., 1999; Lu et al., 2000b). These results support a strong association between the presence of ChHV5 and the presence of FP lesions.

Quackenbush et al. (2001) first successfully amplified ChHV5 from skin samples collected from turtles without FP lesions. Although only a subset of samples from turtles without FP lesions reacted in the assay, the results showed that the virus may be present in turtles despite a lack of clinical signs of disease. More recently, ChHV5 sequences have been amplified from skin samples of turtles without FP lesions with greater success (Page-Karjian et al., 2012; Alfaro-Núñez et al., 2014). These results indicate that early or latent infection with ChHV5 is more common than previously thought. The prevalence of turtles with FP lesions may be small relative to the number of turtles infected with ChHV5. Therefore, an absence of FP lesions does not imply absence of ChHV5 infection. As latency is a typical feature of herpesviruses (Fields et al., 2013), such results are to be expected. The improved sensitivity and specificity of the assays used in these studies have revealed a feature of the disease that was undetectable using earlier assays.

If disease presentation is not dependent on viral infection alone, other factors contributing to lesion development must be considered. An interaction between host, pathogen and the environment (García-Sastre and Sansonetti, 2010) which tips the balance in favour of lesion development may be at play. Differences in host immunity may be preventing certain turtles from mounting a response to the virus (Griffin et al., 2010). Studies on other viral infections have shown that variants of a virus can have different levels of virulence and as such, disease presentation and severity may differ with each variant (Laegreid et al., 1993; Kaashoek et al., 1996; Berumen et al., 2001; Zhang et al., 2001; Yunis et al., 2004).

It is possible that the development of FP lesions is dependent on which viral variant a turtle is infected with. It is also possible that turtles infected with the virus only develop lesions when the viral load surpasses a certain threshold. While the relationship between viral titre and lesion development has not been resolved for ChHV5, this relationship has been described in other viral infections (Brodie et al., 1992; Liu et al., 2000; Rosell et al., 2000; Zhang et al., 2000; Quintana et al., 2001; Ladekjær-Mikkelsen et al., 2002; Rovira et al., 2002; Olvera et al., 2004; Islam et al., 2006; Ravazzolo et al., 2006; Nsubuga et al., 2008; Haralambus et al., 2010). The consistent association of high viral load and lesion development provides support for the theory that this may be the case for ChHV5.

Chelonid herpesvirus 5

Nomenclature and taxonomy

There are currently six herpesviruses documented in chelonids, named chelonid herpesvirus 1 to 6 (ChHV1–6). Chelonid herpesvirus 1, 5 and 6 are described in marine turtles whilst the others have been reported in freshwater turtles (Tidona and Darai, 2011). In the absence of sequence data, ChHV1, ChHV2, ChHV3 and ChHV4 remain unrecognised by the International Committee on Taxonomy of Viruses (ICTV) and their taxonomic place is unclear (Davison and McGeoch, 2010). With respect to the marine turtle herpesviruses, ChHV1 is described in association with grey patch disease (Haines et al., 1974; Rebell et al., 1975), ChHV5 is associated with FP and ChHV6 is known to be associated with lung–eye–tracheal disease (Jacobson et al., 1986; Curry et al., 2000; Coberley et al., 2001, 2002).

Chelonid fibropapilloma-associated herpesvirus (CFPHV) or ChHV5 (Davison and McGeoch, 2010) is now the more commonly used name for this virus. However, it should be noted that previous studies have used a range of names for this virus (see Appendix: Supplementary Table S2). This review refers to the virus as ChHV5.

Histological investigations of FP lesions showed indications of herpesvirus infection and subsequent studies using electron microscopy concluded that the virus-like particles that were observed were likely to belong to the family Herpesviridae based on location, size and morphology (Jacobson et al., 1989, 1991; Herbst et al., 1995).

More recent studies using a range of molecular techniques have confirmed that herpesviral elements are present in FP lesions (Quackenbush et al., 1998, 2001; Lackovich et al., 1999; Lu et al., 2000a, 2000b, 2003; Yu et al., 2000, 2001; Nigro et al., 2004a, 2004b). Phylogenetic analysis of the ChHV5 genes DNA polymerase and DNA binding protein sequences revealed that ChHV5 clusters closely with, but separate to, other members of the *Alphaherpesvirinae* subfamily (Greenblatt et al., 2005; McGeoch and Gatherer, 2005). Davison and McGeoch (2010) targeted the single-stranded DNA-binding protein, glycoprotein B, the major capsid protein, DNA polymerase and two subunits of the DNA packaging terminase (genes UL29, UL27, UL19, UL30, UL15 and UL28, respectively). The resulting Bayesian phylogenetic tree shows that ChHV5 exists as an out-group, clearly separate from the current genera. A Minimum Evolution phylogenetic tree of *Alphaherpesvirinae* based on full length DNA polymerase sequence further supports this result (Fig. 3). Consequently, it has been proposed that ChHV5 be placed in its own genus. The proposed genus, *Scutavirus*, sits within the *Alphaherpesvirinae* subfamily of *Herpesviridae*.

Variants of chelonid herpesvirus 5

Based on nucleotide sequence diversity, four viral variants of ChHV5 have been recorded in waters around Florida. At present, they are known as A, B, C and D (Herbst et al., 2004; Ene et al., 2005). Variant A is the most prevalent in the region, yet there is variation in the relative prevalence of variants at each site. Co-infection with variants A and B was also found in one Green turtle (Ene et al., 2005). Perhaps even more significant, different species of marine turtle shared the same variant if they were present in the same locality (Herbst et al., 2004; Ene et al., 2005). This indicates a strong geographic role in the transmission of the virus.

In a recent study, ChHV5 was examined using samples from a variety of locations in order to create a global phylogeography of the virus. Four phylogeographical groups of ChHV5 were identified: eastern Pacific, western Atlantic/eastern Caribbean, midwest Pacific and Atlantic (Patrício et al., 2012). The results of the study showed that the viral variant is similar between nearby

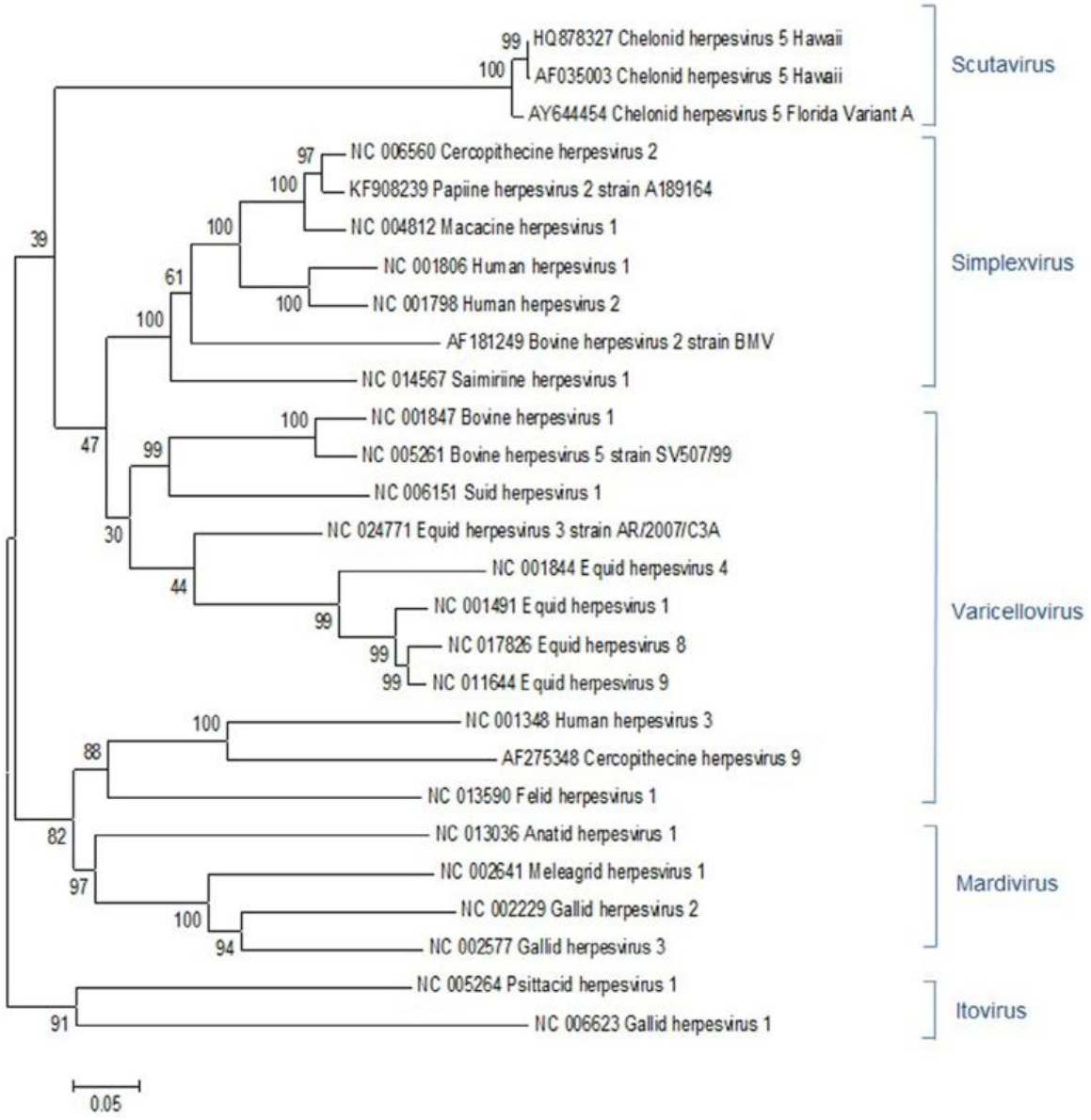

Fig. 3. A Minimum Evolution phylogenetic tree of *Alphaherpesvirinae* based on full length DNA polymerase sequence retrieved from GenBank (Accession numbers provided in tree). Bootstrap values for each node are provided (1000 replicates). The analysis involved 27 nucleotide sequences resulting in a total of 2593 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

foraging grounds while distant regions are considerably divergent. The study by Patrício et al. (2012) also found that sympatric species of marine turtle were infected with the same viral variant, further supporting the results of Herbst et al. (2004) and Ene et al. (2005). These findings indicate that individual turtles are likely to be infected with the virus through horizontal transmission in neritic bays (Patrício et al., 2012).

Co-evolution of virus and host

Herbst et al. (2004) suggested that the virus diverged prior to the separation of avian and mammalian alphaherpesviruses. This would mean that ChHV5 became specific to marine turtles approximately 300 million years ago (mya). In addition, it was estimated that the two most divergent clades were separated approximately 1.6–4.0 mya. These results led to speculation that the rise of the Isthmus of Panama (3.1–3.5 mya) was responsible for the divergence as it prevented genetic exchange between these clades. Patrício et al. (2012) found that the most recent common ancestor of the currently known variants of this virus existed 193–430 years ago. This estimate is considerably more recent than the work of Herbst et al. (2004) but both studies demonstrate that ChHV5 has evolved with marine turtles and, in either case, it is likely ChHV5 has undergone region specific co-evolution with its host.

While further research is needed to resolve the time of divergence, there is one clear conclusion; it is not a new virus, or even recent mutations in an old virus, that is causing lesions to develop. This evidence further supports the theory that the recent emergence of FP is linked to modern day extrinsic environmental factors promoting lesion development.

Genome organisation

The herpesvirus genome is divided into two unique regions: one composed of a unique long (UL) sequence and the other region is composed of a unique short (US) sequence. These unique

sequences are flanked by repeat sequences. The number, position and direction of these sequences can vary and as a result, there are multiple types of herpesvirus genome structures. Current literature lists between four and six known herpesvirus genome types. Fauquet et al. (2005) recognised four herpesvirus genome types (denoted Types 1–4), while Pellet and Roizmann (2007) described six different genome types (denoted Types A–F).

A recent study has described the entire genome of ChHV5 (Ackermann et al., 2012). The extensive sequence data generated from this study showed a clear division of the genome into UL and US regions. Inverted repeat sequences (IRS) were also found to flank the US sequence. This configuration is consistent with ChHV5 having a type D genome (Ackermann et al., 2012).

Ackermann et al. (2012) also described four genes that are atypical for an alphaherpesvirus genome. Two members of the C-type lectin-like domain superfamily (F-lec1, F-lec2), an orthologue to the mouse cytomegalovirus M04 (F-M04) and a viral sialyltransferase (F-sial), were all found to be present in the ChHV5 genome (Ackermann et al., 2012). While the products of these genes may not be critical for viral replication, each one has a potential role in pathogenesis or immune deviation (Ackermann et al., 2012). Orthologues to these genes have been described in other viral families and host cells (Neilan et al., 1999; Wilcock et al., 1999; Voigt et al., 2001; Markine-Goriaynoff et al., 2004). However, until now, none of these genes has ever been reported in the genome of an alphaherpesvirus. Two of these atypical genes (F-sial and F-M04) were found to be expressed in the FP lesions and it has been suggested that these genes may play a role in FP pathogenesis (Ackermann et al., 2012).

Transmission of chelonid herpesvirus 5

As this disease has not been observed in pelagic juveniles, it is thought that turtles are exposed to ChHV5 upon recruitment to neritic zones, indicating horizontal transmission (Herbst, 1994; Ene et al., 2005; Patrício et al., 2012). These new recruits may be exposed to several stressors associated with migration, adaptation to a new environment, and changes in population density, diet and pathogen exposure, which may all combine to reduce the efficacy of the immune system and make these juveniles more susceptible to infection (Ritchie, 2006) with ChHV5 and development of FP. It is also possible that these stressors combine to enhance transmission or elicit herpesviral recrudescence in latently infected turtles (Ritchie, 2006) leading to the development of FP lesions. Alternatively, direct transmission may be occurring between co-habiting turtles via interactions such as mating and aggression.

Researchers have speculated on means of transmission of FP as an infectious disease and possible vectors. Marine turtles host a range of parasites and correlations have been made between parasite load and individual health. Spirorchid trematodes (Jacobson et al., 1989, 1991; Norton et al., 1990; Aguirre et al., 1994b, 1998; Williams et al., 1994), coral reef cleaner fish (Booth and Peters, 1972; Losey et al., 1994; Lu et al., 2000c), saddleback wrasse (*Thalassoma duperrey*) (Lu et al., 2000c) and marine leeches (*Ozobranchus* spp.) (Greenblatt et al., 2004) have all been proposed as potential vectors of ChHV5. Significantly higher viral loads were detected in marine leeches when compared with the other parasites examined (Greenblatt et al., 2004) and they are currently the leading candidates for a mechanical vector. Although *Ozobranchus* leeches are the most likely candidates for transmission vectors of ChHV5, their exact role has not yet been confirmed. This is partly due to the possible latent state of the virus and involvement of other co-factors in disease expression of FP (Greenblatt et al., 2004).

Other marine turtle epibiota, including bladder parasites (*Pyelosomum longicaecum*), barnacles (*Platylepas* spp.), amphipods of the skin and oral cavity (order *Talitroidea*) and blood flukes of the genera *Carretacola*, *Hapalotrema* and *Laeredius* have been ruled out as potential vectors (Greenblatt et al., 2004).

Environmental factors

Marine turtles are particularly susceptible to changes in their environment as they are long-lived animals with a complex life history (Aguirre and Lutz, 2004). A marine turtle will access a range of habitat types during its lifetime, but exhibits a high degree of site fidelity once recruited into a near shore foraging area. Mature female turtles are known to return to the natal area from which they originated as hatchlings in order to lay their eggs (Limpus, 2008). Due to this site fidelity, marine turtles are likely to persist in, or return to, their chosen localities despite unfavourable changes to the environment. As a result, any damage to or destruction of these sites could have extremely detrimental effects on populations that inhabit them (Hawkes et al., 2009; Poloczanska et al., 2010; GBRMPA, 2014).

It has been suggested that environmental factors may play a role in the development of FP (Herbst, 1994; Herbst and Klein, 1995a; Adnyana et al., 1997; Aguirre and Lutz, 2004; Chaloupka et al., 2009; dos Santos et al., 2010; Van Houtan et al., 2014). Moreover, the presence of chemical contaminants may be part of a multifactorial problem that leads to FP (Herbst, 1994). Early proponents of a possible relationship between degraded water quality and the presence of FP proposed that chemical contaminants present in the water acted as immunotoxins or were causing damage at the cellular or genetic level (Herbst, 1994).

Indirect disturbances to the immune system may occur if the chemical contaminants create a disruption of neuroendocrine function (Zeeman and Brindley, 1981; Anderson et al., 1984; Dean et al., 1990; Colborn et al., 1993; Arkoosh et al., 1994; Dunier, 1994). Herbst (1994) demonstrated that a positive correlation exists between the prevalence of FP in Green turtle populations adjacent to regions associated with agriculture, industry and urban development. Subsequent studies have observed the same correlation (Adnyana et al., 1997; Foley et al., 2005; dos Santos et al., 2010; Van Houtan et al., 2010). Although initial reports in Puerto Rico observed the same relationship, this trend was reversed after several years; the prevalence of FP at the more pristine site is now considerably higher than at the site which is subjected to high levels of human activity (Patrício et al., 2011; Page-Karjian et al., 2012). Researchers attempted to quantify this relationship in Hawaii by developing an information-rich index of eutrophication from the analysis of 82 different watersheds. The results showed a strong association between FP rates, nitrogen-footprints and macroalgae consumed by turtles (Van Houtan et al., 2010). Different quantification studies were also undertaken in waters around Brazil and found that Green turtles residing in areas with degraded water quality had a higher prevalence of FP. However, this study based the assessment of water quality on the presence of benthic macrophytes and nutrient levels; pollution and the presence of chemical contaminants were not considered (dos Santos et al., 2010).

Only very low concentrations of persistent organic pollutants (Keller et al., 2014) and selected trace metals and organic pollutants (Aguirre et al., 1994a) have been detected in turtles with FP lesions. Although these results suggest that the pollutants examined do not significantly contribute to FP development, it is possible that further investigations will uncover a relationship between this disease and other environmental contaminants (Keller et al., 2014).

Water temperature may also be a factor in lesion development and growth rate. It is possible that warmer water temperatures during summer promote lesion growth, resulting in lesions of a debilitating size by autumn (Herbst, 1994; Herbst et al., 1995). This seasonal trend has been observed in Florida, where a higher rate of FP is observed in turtles that strand in winter (Herbst, 1994).

However, no seasonal trends have been observed in Hawaii (Murakawa et al., 2000), which may be because there is less seasonal fluctuation in water temperature in this region (Foley et al., 2005).

Natural biotoxins have also been implicated as a co-factor involved in FP development. Landsberg et al. (1999) identified a correlation between high-risk FP areas in the Hawaiian Islands and prevalence of *Prorocentrum*, a species that produces okadaic acid, a known tumour promoter (Suganuma et al., 1988; Haystead et al., 1989; Cohen et al., 1990; Huynh et al., 1997). Similarly, tissue concentrations of lyngbyatoxin A, produced by *Lyngbya majuscula*, have been correlated with the presence of FP lesions in dead Green turtles (Arthur et al., 2006, 2008). However, this species constituted less than 2% of total dietary intake and subsequently, any biotoxins would be at a low concentration in the turtles (Arthur et al., 2008). If the dietary items containing these biotoxins form a natural component of the diet of Green turtles and the amount being consumed was not altered, these toxins should have no influence on the development of FP.

An increased concentration of arginine in the diet of Green turtles as a result of invasive macroalgae blooms has also been linked to an increasing prevalence of FP (Van Houtan et al., 2010). Arginine is a regulator of immune activity (Peranzoni et al., 2008) and is known to promote herpesviruses and contribute to tumour formation (Mannick et al., 1994). This amino acid is also a major component of glycoproteins on the viral envelope of herpesviruses (Van Houtan et al., 2010, 2014).

The results of a subsequent study found an association between eutrophication and arginine content of macroalgae, with the intake of arginine in turtles at eutrophied sites being up to 14 times the background level. This increased arginine content may metabolically promote ChHV5, leading to FP lesion development (Van Houtan et al., 2014). Although the conclusions from this study were subsequently challenged (Work et al., 2014), the epidemiological link between the prevalence of disease and feeding ecology found in Van Houtan et al. (2014) provides strong support that environmental factors play a role in the development of this disease. However, the environmental factors leading to the bloom of macroalgae may be causing the development of FP lesions directly, and the algal blooms may not be involved in lesion development at all. If this is the case, it is difficult to link cause and effect.

Despite there being a strong positive correlation between the prevalence of FP in Green turtle populations and areas with degraded water quality, it is difficult to identify one specific causal contaminant or a combination of such working synergistically to the detriment of the turtles. Studies on toxicity usually focus on chemicals that are persistent in the environment or can bioaccumulate. Genetic damage as a result of a toxin may occur as a consequence of transient exposure and as such, future studies would need to be expanded to include transient chemicals that could have this effect on Green turtles. The practicality of such investigations is daunting considering the vast marine environment and the known and unknown possible causes of FP (Herbst, 1994; Herbst and Klein, 1995a).

One way that potential links between FP and anthropogenic contaminants might be identified is to develop a monitoring program that records and compares contaminant residue levels, genetic changes and viral load in blood and/or tissue samples collected from turtles with and without FP lesions over a wide geographic area and across several seasons. Such a program could be integrated into existing turtle monitoring activities. Controlled laboratory studies in a closed experimental system may be needed to conclusively evaluate the roles of various environmental factors in FP development (Herbst and Klein, 1995a). Alternatively, results from both field and laboratory based studies may work synergistically to fully resolve this relationship.

Direction of future research

The longevity of marine turtles, coupled with their close association with inshore habitats and seagrass meadows and coral reefs in these habitats, has led to the proposal that they may act as sentinel indicators of marine ecosystem health (Aguirre and Lutz, 2004). Gaining a better understanding of the health and prevalence of diseases in marine turtle populations provides a critical link between ecosystem health and turtle health. Effective management of both the habitat and the species that rely on it is critical for effective species conservation. As FP has been found to be associated with turtles resident in areas exposed to poor water quality (Herbst, 1994; dos Santos et al., 2010; Van Houtan et al., 2010, 2014), FP prevalence may be a vital tool in monitoring inshore marine habitats. Many of these marine environments are also utilised by humans and consequently, research into the epidemiology of this disease could be mutually beneficial for Green turtles, other species in these ecosystems and humans alike (Aguirre and Lutz, 2004; Flint et al., 2010b). Long term monitoring of populations will allow researchers to more accurately establish disease prevalence, corrected by demographic proportions.

Whether the development of FP lesions is a result of a single agent or the interaction between multiple factors is yet to be determined. It is clear that it is an infectious disease with a strong link to ChHV5. In addition, the strong influence of different geographic regions on the prevalence of FP and each of the viral variants indicate that FP is geographically specific (Herbst et al., 2004; Ene et al., 2005; Patrício et al., 2012). The results from molecular studies targeting ChHV5 in samples from turtles show that the virus is present in turtles with and without FP lesions (Quackenbush et al., 2001; Page-Karjian et al., 2012; Alfaro-Núñez et al., 2014). Future molecular studies targeting ChHV5 should consider these results and screen all samples for ChHV5, not only those from turtles with FP lesions. Biosecurity and potential zoonosis should always be considered by those handling marine turtles in both field and captive situations. However, future research should prioritise understanding the triggers for lesion development.

Conclusions

There are many aspects of FP in marine turtles that are yet to be resolved and future research needs to target those gaps which will ultimately aid in managing the disease. Understanding how ChHV5 is transmitted between turtles and between regions is a key priority. Molecular epidemiology is a useful tool for revealing genetic differences in this virus between regions; possible relationships between host lineage and viral strain and the genes responsible for pathogenesis and viral replication. Molecular investigations on ChHV5 from different regions are essential to improve our understanding of the epidemiology and pathogenesis of this virus which will in turn inform the management and conservation of a vulnerable species, the Green turtle.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.tvjl.2015.10.041.

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Table 1
The prevalence of fibropapillomatosis lesions in green (*Chelonia mydas*), loggerhead (*Caretta caretta*), Olive Ridley (*Lepidochelys olivacea*), hawksbill (*Eretmochelys imbricata*), leatherback (*Dermochelys coriacea*), Kemp's Ridley (*Lepidochelys kempii*) and flatback turtles (*Natator depressus*), according to year of observation and location.

Locality	Species	Prevalence of FP (%)	Sample period	Reference
Western Atlantic/Eastern Caribbean				
Florida, United States of America				
Volusia County	*C. mydas*	6	1980-1998	Foley et al., 2005
Mosquito Lagoon	*C. mydas*	0	1975-1981	Ehrhart, 1991; Ehrhart et al., 1986
	C. mydas	29	1985	Ehrhart, 1991; Ehrhart et al., 1986
	C. mydas	1.6	1990	Ehrhart, 1991; Ehrhart et al., 1986
Brevard County	*C. mydas*	10.1	1980-1998	Foley et al., 2005
Trident Submarine Basin	*C. mydas*	0	1993-2007	Hirama and Ehrhart, 2007
Indian River	*C. mydas*	11.7	1980-1998	Foley et al., 2005
	C. mydas	20-61	1982-1990	Ehrhart, 1991
	C. mydas	20	1993	Ehrhart and Redfoot, 1995
	C. mydas	63	1998-1999	Hirama and Ehrhart, 2002
	C. mydas	28-72	1984-2000	Hirama and Ehrhart, 2007
	C. mydas	8-32.9	1988-2006	Eaton et al., 2008
	C. mydas	52.2	1982-2013	Cope et al., 2013
	C. caretta	5.1	1982-2013	Cope et al., 2013
Nearshore Reef	*C. mydas*	14.8	1998-1999	Hirama and Ehrhart, 2007
Wabasso Beach	*C. mydas*	0	1988-1993	Ehrhart, 1991
St. Lucie County	*C. mydas*	12.8	1980-1998	Foley et al., 2005
Martin County	*C. mydas*	15.3	1980-1998	Foley et al., 2005
Palm Beach County	*C. mydas*	12.8	1980-1998	Foley et al., 2005
Lake Worth Lagoon	*C. mydas*	63	2005-2007	de Maye et al., 2007
Broward County	*C. mydas*	1.1	1980-1998	Foley et al., 2005
Miami-Dade	*C. mydas*	20.6	1980-1998	Foley et al., 2005
Atlantic Coast	*C. mydas*	10	1980-1990	Teas, 1991
Monroe County	*C. mydas*	51.6	1980-1998	Foley et al., 2005
Florida Bay	*C. mydas*	70	1990-1993	Herbst, 1994
	C. mydas	69.2	1991	Schroeder and Foley, 1995
	C. mydas	62	1990-1996	Schroeder et al., 1998
Florida Keys	*C. mydas*	1.5	1938	Smith and Coates, 1938
	C. mydas	20-60	1980-1990	Teas, 1991
Cape Sable	*C. mydas*	<1%	1938	Lucke, 1938
Collier County	*C. mydas*	23	1980-1998	Foley et al., 2005
Lee County	*C. mydas*	39	1980-1998	Foley et al., 2005
Charlotte County	*C. mydas*	27	1980-1998	Foley et al., 2005
Sarasota County	*C. mydas*	49	1980-1998	Foley et al., 2005
Manatee County	*C. mydas*	67	1980-1998	Foley et al., 2005
Hillsborough County	*C. mydas*	71	1980-1998	Foley et al., 2005
Pinellas County	*C. mydas*	53.5	1980-1998	Foley et al., 2005
Pasco County	*C. mydas*	67	1980-1998	Foley et al., 2005
Hernando County	*C. mydas*	50	1980-1998	Foley et al., 2005
Citrus County	*C. mydas*	18	1980-1998	Foley et al., 2005
Gulf Coast	*C. mydas*	50	1992 (First observed in 1985)	Teas, 1991
Bermuda	*C. mydas*	0	1968-1993	Herbst, 1994
Bahamas				
Inagua	*C. mydas*	0	1974-1993	Herbst, 1994
Nicaragua				
Cabezas Port	*C. mydas*	<5	1993	Herbst, 1994
Panama				
Chiriqui Lagoon, Bocas del Toro	*C. mydas*	35	1989-1993	Herbst, 1994
Puerto Rico	*C. mydas*	17	1988-1992 (First observed in 1987)	Teas, 1991
Manglar Bay	*C. mydas*	0	1997	Patrício et al., 2011
	C. mydas	0	1998	Patrício et al., 2011
	C. mydas	9	2000	Patrício et al., 2011
	C. mydas	29	2001	Patrício et al., 2011
	C. mydas	75	2002	Patrício et al., 2011
	C. mydas	79	2003	Patrício et al., 2011
	C. mydas	50	2004	Patrício et al., 2011
	C. mydas	25	2005	Patrício et al., 2011
	C. mydas	12	2006	Patrício et al., 2011
	C. mydas	64	2000-2006	Velez-Zuazo et al., 2010
	C. mydas	4	2007	Patrício et al., 2011
	C. mydas	30.5	2004-2007	Page-Karjian et al., 2012
	C. mydas	0	2008	Patrício et al., 2011
	C. mydas	4	2009	Patrício et al., 2011
	C. mydas	3	2010	Patrício et al., 2011

Culebrita	*C. mydas*	<1	2000-2006	Velez-Zuazo et al., 2010
Culebrita, Tortuga Bay	*C. mydas*	0	1997-2004	Patrício et al., 2011
	C. mydas	2	2005	Patrício et al., 2011
	C. mydas	6	2006	Patrício et al., 2011
	C. mydas	0	2007	Patrício et al., 2011
	C. mydas	0	2008	Patrício et al., 2011
	C. mydas	33	2009	Patrício et al., 2011
	C. mydas	6	2010	Patrício et al., 2011
	C. mydas	17	2009-2011	Patrício et al., 2014
Barbados				
Barclay's Park	*C. mydas*	90	1990 (First observed in 1982-1983)	Gamache and Horrocks, 1991
Cayman Islands	*C. mydas*	66	1980-1991	Wood and Wood, 1993
Cuba	*C. mydas*	0.6	1983-1996	Moncada and Prieto, 1998
Brazil				
Trindade Island	*C. mydas*	1.1	1989-1990	Baptistotte et al., 2005
	C. mydas	0.09	1994-1995	Baptistotte et al., 2005
	C. mydas	0.34	1995-1996	Baptistotte et al., 2005
State of Espirito Santo	*C. caretta*	1.3	1994-1995	Baptistotte et al., 2005
States of Pernambuco, Alagoas and Sergipe	*L. olivacea*	12.5	1996-1997	Baptistotte et al., 2005
Itaipu coastal region	*C. mydas*	30	2008-2010	Machado Guimarães et al., 2011
Eastern Pacific				
Mexico				
La Escobilla Beach	*L. olivacea*	1.45	1997	Vasconcelos et al., 2000
Mexiquillo Beach	*D. coriacea*	1 case	1997	Huerta et al., 2000
Rancho Nuevo	*L. kempii*	1 case	1993	Barragan and Sarti, 1994
Costa Rico				
Ostional	*L. olivacea*	6-10	1997	Aguirre et al., 1999
	L. olivacea	10	1997	Quiros et al., 2000
Tortuguero	*C. mydas*	2.1	1998	Troëng, 1998
Mid-west Pacific				
Hawaiian Islands, United States of America	C. mydas	47-69	1982-1998	Murakawa et al., 2000
	C. mydas	28	1982-2003	Chaloupka et al. 2008
Kiholo Bay	*C. mydas*	0	1987-1990	Balazs and Pooley, 1991
Punalu'u Bay	*C. mydas*	1	1976-1993 (First observed in 1984)	Balazs and Pooley, 1991; Herbst, 1994
Pala'au, Molokai	*C. mydas*	0	1982-1985	Balazs and Pooley, 1991; Herbst, 1994
	C. mydas	1 case	1985	Balazs and Pooley, 1991
	C. mydas	1-53	1987-1993	Balazs and Pooley, 1991; Herbst, 1994
	C. mydas	4.8	1988	Balazs et al., 1998
	C. mydas	9.8	1989	Balazs et al., 1998
	C. mydas	17.2-25.6	1990	Balazs et al., 1998
	C. mydas	23.1	1991	Balazs et al., 1998
	C. mydas	53	1992	Balazs et al., 1998
	C. mydas	47	1992-1993	Balazs et al., 1998
	C. mydas	39	1993	Balazs et al., 1998
	C. mydas	41	1994	Balazs et al., 1998
	C. mydas	60.7	1995	Balazs et al., 1998
	C. mydas	42.2-55.9	1996	Balazs et al., 1998
Kāne'ohe Bay, Oahu	*C. mydas*	49-92	1989-1991 (First observed in 1958)	Balazs and Pooley, 1991
	C. mydas	43.9	1989-1997	Balazs et al., 2000
Waikiki Beach, Oahu	*C. mydas*	9	1990-1993	Balazs and Pooley, 1991
French Frigate Shoals	*C. mydas*	7-12	1988-1992	Balazs and Pooley, 1991; Herbst, 1994
	C. mydas	15.3	1999	Pepi et al., 2005
Pearl/Hermes Reef	*C. mydas*	0	1982-1987	Balazs and Pooley, 1991
Midway Island	*C. mydas*	0	1969-1978 (First observed in 1990)	Balazs and Pooley, 1991
Punalu'u	*C. mydas*	0.01	1984-1994	Balazs et al., 1994
Queensland, Australia				
Gulf of Carpentaria	*C. mydas*	0	2001-2002	Hamann et al., 2006
	E. imbricata	0	2001-2002	Hamann et al., 2006
Crab Island	*N. depressus*	5 cases	1991	Limpus et al., 1993
Torres Strait	*C. mydas*	0	1977-1980	Glazebrook and Campbell, 1990
Heron Island and Wistari Reefs	*C. mydas*	0	1988-1990	Limpus and Miller, 1994
	C. caretta	1-2	1988-1990	Limpus and Miller, 1994
	E. imbricata	0	1988-1990	Limpus and Miller, 1994
Clack Island Reef	*C. mydas*	0	1988-1990	Limpus and Miller, 1994
	C. caretta	0	1988-1990	Limpus and Miller, 1994
	E. imbricata	0	1988-1990	Limpus and Miller, 1994
Hazelwood Island Reef	*C. mydas*	0	1989	Limpus and Miller, 1994
	E. imbricata	0	1989	Limpus and Miller, 1994
Green Island Reef	*C. mydas*	0	1988-1990	Limpus and Miller, 1994
	E. imbricata	0	1988-1990	Limpus and Miller, 1994
Lucinda	*C. mydas*	0	2003	Bell, 2003
Bowen	*C. mydas*	0	1989	Limpus and Miller, 1994
	C. mydas	0	1989	Limpus and Miller, 1994
Abbot Point	*C. mydas*	0	2003	Bell, 2003

Hay Point	*C. mydas*	0	2003	Bell, 2003
Shoalwater Bay	*C. mydas*	2	1988	Limpus and Miller, 1994
	C. mydas	2	1989	Limpus and Miller, 1994
	C. caretta	0	1990	Limpus and Miller, 1994
	C. mydas	3	1990	Limpus and Miller, 1994
	C. mydas	0.5	2000	Limpus et al., 2005
	C. mydas	2.1	2001	Limpus et al., 2005
	C. mydas	1.2	2002	Limpus et al., 2005
	C. mydas	0.6	2003	Limpus et al., 2005
	C. mydas	1.1	2004	Limpus et al., 2005
	C. mydas	0.5	1970-2010	Flint et al., 2010a
Repulse Bay	*C. mydas*	0	1988 (First observed in 1989)	Limpus and Miller, 1994
	C. mydas	3	1989	Limpus and Miller, 1994
	C. mydas	22	1990	Limpus and Miller, 1994
Moreton Bay	*C. mydas*	8	1988-1990	Limpus and Miller, 1994
	C. caretta	1	1988-1990	Limpus and Miller, 1994
	C. mydas	7.9	1990-1992	Limpus et al., 1994
	C. mydas	16	1998	Aguirre et al., 1998a
	C. caretta	6	1998	Aguirre et al., 1998a
	C. mydas	1.6	2006-2009	Flint et al., 2010c
Western Australia, Australia				
Baba Head, Shark Bay	*C. mydas*	<1	1996	Raidal and Prince, 1996
New South Wales, Australia				
Julia Rock Aquatic Reserve	*C. mydas*	14.3	2002	Speirs, 2002
Bali, Indonesia	*C. mydas*	21.5	1994	Adnyana et al., 1997
	E. imbricata	0	1994	Adnyana et al., 1997
Atlantic				
The Gulf of Guinea, West Africa				
Corisco Bay	*C. mydas*	27	1998	Formia et al., 2007
		17	1999	Formia et al., 2007
		19	2000	Formia et al., 2007
		17	2003	Formia et al., 2007
		22	2004	Formia et al., 2007
		10	2005	Formia et al., 2007
		14	2006	Formia et al., 2007
Principe Island	*C. mydas*		2009	Loureiro and D, 2009
	Juveniles	32		
	Subadults	36		
	Adults	0		
Turks and Caicos Islands	*C. mydas*	13	2008-2010	Stringell et al., 2011
Pointe Indienne and Loango Bay	*C. mydas*	15	2009	Girard et al., 2013
	C. mydas	8	2012	Girard et al., 2013
Indian Ocean				
Seychelles	*C. mydas*	0	1981-1992	Herbst, 1994
Aldabra Island	*C. mydas*	0	1981-1992	Herbst, 1994

Table 2
Variation in nomenclature of chelonid herpesvirus 5 (ChHV5).

Name	Reference
Green turtle fibropapillomatosis-associated herpesvirus	Herbst et al. (1996) Herbst et al. (1998) Quackenbush et al. (1998) Herbst et al. (1999) Herbst et al. (2001)
Green turtle herpesvirus [a]	Lu et al. (2000) Yu et al. (2000) Yu et al. (2001) Lu et al. (2003) Nigro et al. (2004a) Nigro et al. (2004b) McGeoch and Gatherer (2005)
Fibropapillomatosis-associated herpesvirus (FPHV)	Lackovich et al. (1999) Curry et al. (2000) Coberley et al. (2001a) Coberley et al. (2001b) Work et al. (2004) Chaloupka et al. (2009)
Fibropapilloma-associated turtle herpesvirus (FPTHV) or Fibropapilloma-associated marine turtle herpesvirus	Quackenbush et al. (2001) Greenblatt et al. (2004) Greenblatt et al. (2005a) Greenblatt et al. (2005b) Kang et al. (2008) Work et al. (2009) McGowin et al. (2011)
Chelonid fibropapilloma-associated herpesvirus (CFPHV) or Chelonid herpesvirus 5 (ChHV5)	Herbst et al. (2004) Ene et al. (2005) Flint et al. (2009) Flint et al. (2010) Herbst et al. (2008) Rossi et al. (2009) Davison and McGeoch (2010) dos Santos et al. (2010) Ariel (2011) Ackermann et al. (2012) Duarte et al. (2012) Page-Karjian et al. (2012) Patrício et al. (2012) Alfaro-Nunez et al. (2014) Alfaro-Núñez and Gilbert (2014) Page-Karjian et al. (2014) Rodenbusch et al. (2014)

[a] Used in reference to ChHV5.

Closing the gap: mixed stock analysis of three foraging populations of green turtles (*Chelonia mydas*) on the Great Barrier Reef

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ABSTRACT

A solid understanding of the spatial ecology of green turtles (*Chelonia mydas*) is fundamental to their effective conservation. Yet this species, like many marine migratory species, is challenging to monitor and manage because they utilise a variety of habitats that span wide spatio-temporal scales. To further elucidate the connectivity between green turtle rookeries and foraging populations, we sequenced the mtDNA control region of 278 turtles across three foraging sites from the northern Great Barrier Reef (GBR) spanning more than 330 km: Cockle Bay, Green Island and Low Isles. This was performed with a newly developed assay, which targets a longer fragment of mtDNA than previous studies. We used a mixed stock analysis (MSA), which utilises genetic data to estimate the relative proportion of genetically distinct breeding populations found at a given foraging ground. Haplotype and nucleotide diversity was also assessed. A total of 35 haplotypes were identified across all sites, 13 of which had not been found previously in any rookery. The MSA showed that the northern GBR (nGBR), Coral Sea (CS), southern GBR (sGBR) and New Caledonia (NC) stocks supplied the bulk of the turtles at all three sites, with small contributions from other rookeries in the region. Stock contribution shifted gradually from north to south, although sGBR/CS stock dominated at all three sites. The major change in composition occured between Cockle Bay and Low Isles. Our findings, together with other recent studies in this field, show that stock composition shifts with latitude as a natural progression along a coastal gradient. This phenomenon is likely to be the result of ocean currents influencing both post-hatchling dispersal and subsequent juvenile recruitment to diverse coastal foraging sites.

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INTRODUCTION

Migratory marine mega vertebrates are often long lived and utilise a variety of habitats that span wide spatio-temporal scales. Humpback whales (*Megaptera novaeangliae*), for example, utilise distinctly separate feeding and breeding grounds and undergo seasonal migrations between these areas which can span thousands of kilometres (*Acevedo et al., 2007*; *Clapham, 1996*; *Oña, Garland & Denkinger, 2017*). The same is true of various species of sharks, rays, tuna, marine mammals and marine turtles (*Lascelles et al., 2014*). Species with complex life history patterns pose challenges to the understanding of population dynamics and the connectivity between breeding and non-breeding areas (*Godley et al., 2010*). Due to their wide-ranging movements, marine migratory species are exposed to different threats at their foraging and breeding habitats, and are further exposed to additional pressures as they migrate between these habitats (*Jensen et al., 2016*; *Lascelles et al., 2014*). These species often pass through the waters of multiple nations or areas beyond national jurisdiction (*Lascelles et al., 2014*) and as a result, monitoring, managing and ultimately conserving such species is challenging (*Hamann et al., 2010*; *Jensen et al., 2016*). In 2014, 48% of all marine migratory species were found to be threatened (critically endangered, endangered or vulnerable), near threatened or data deficient, with marine turtles being the most threatened group (*Lascelles et al., 2014*). A sound understanding of the spatial ecology of these species is essential to developing effective conservation strategies (*Cooke, 2008*), as it allows for the identification of key habitats and the likely sources of threatening processes.

The green turtle (*Chelonia mydas*) is recognised as endangered under the IUCN red list assessment (*Seminoff, 2004*). In Australia, this species is listed as vulnerable under the *Environment Protection and Biodiversity Conservation Act 1999* (*Department of Environment Energy, 2016*). Green turtles have a circumglobal distribution, are long-lived, highly migratory, and have a complex life history which spans a diverse range of habitats (*Limpus, 2008*). After emerging from tropical and subtropical sandy beaches hatchling green turtles take on a pelagic existence, recruiting into benthic, inshore foraging grounds as juveniles several years later (*Reich, Bjorndal & Bolten, 2007*). Foraging areas are often shared by turtles sourced from multiple regional rookeries (*Anderson, Shaver & Karel, 2013*; *Dutton et al., 2014*; *Lahanas et al., 1998*). At the onset of sexual maturity, some 20–30 years later, green turtles migrate back to their natal nesting regions to breed and nest (*Musick & Limpus, 1997*).

Using mtDNA, Australian green turtles can be divided into nine genetically distinct breeding stocks: southern Great Barrier Reef (sGBR), Coral Sea (CS), northern GBR (nGBR), Gulf of Carpentaria, Coburg Peninsula, Ashmore Reefs/Browse Island, Scott Reef, the Northwest Shelf and Cocos "Keeling" Island (*Dethmers et al., 2006*; *Limpus, 2008*; *FitzSimmons & Limpus, 2014*; *Jensen et al., 2016*). In addition, Australian waters are in close

proximity to multiple internationally important stocks in neighbouring countries such as those nesting in Aru (Indonesia), Papua New Guinea and New Caledonia. Each of these stocks can be considered as a demographically independent population (*Waldman, 2005*), and as such, understanding how turtles from these stocks share regional foraging grounds is critical to the effective management of threats to this vulnerable species.

The Great Barrier Reef (GBR) region in Australia is home to some of the largest nesting and foraging green turtle populations in the world. Breeding green turtles of nGBR and sGBR stocks nest on several islands at the latitudinal extremes of the GBR (*Dethmers et al., 2006*; *FitzSimmons & Limpus, 2014*; *Jensen et al., 2016*). While very little nesting takes place along the central part of the reef, turtles from both breeding stocks share foraging areas located along the entire GBR (*Limpus, 2008*) and beyond into New South Wales and northern Australia. Foraging grounds along the GBR are discontinuous and irregularly spaced, likely reflecting the patchy nature of resources relevant for turtles. For research and monitoring purposes, GBR foraging grounds are defined by their geographical location, e.g., a bay or a cluster of neighbouring reefs. These foraging grounds typically support overlapping adult and juvenile age classes. Long-term mark-recapture studies have demonstrated that all size classes have strong fidelity to a single foraging ground with little movement between surrounding foraging grounds (*Limpus & Chaloupka, 1997*; *Musick & Limpus, 1997*). As such, GBR foraging grounds are considered to host independent foraging populations wherein the genetic composition is mixed.

Both traditional mark-recapture analysis (flipper tagging) and molecular methods (mixed stock analysis; MSA) have been used to describe the distribution of foraging green turtles along the GBR (*Jensen et al., 2016*; *Limpus, 2008*). The MSA method uses genetic markers measured in several source populations (rookeries) and a single mixed population (a foraging ground) to estimate the proportional contribution of each source to the mixed population (*Bolker et al., 2007*). This technique provides an effective tool to assess the connectivity between foraging and breeding grounds for migratory species like marine turtles, whose intricate life history complicates monitoring efforts. Major green turtle rookeries across the Indo-Pacific have been genetically characterised using the mtDNA control region, with 25 genetically differentiated stocks or Management Units (MUs) identified to date (*Dutton et al., 2009*; *Dutton et al., 2014*; *Jensen et al., 2016*; *Nishizawa et al., 2014*; *Read et al., 2015*). These MUs provide a comprehensive reference of source populations that can be used in MSA to determine the breeding stock origin of green turtles at regional foraging grounds along the GBR and elsewhere (*Dethmers et al., 2006*; *FitzSimmons & Limpus, 2014*; *Jensen et al., 2016*; *Limpus, 2008*).

Studies based on traditional flipper tagging, genetic data, or a combination of these tools have shown that foraging areas along the GBR mainly receive turtles originating from three stocks; the nGBR, the sGBR and the Coral Sea (CS) (*Jensen et al., 2016*; *Limpus, 2008*). In addition to these dominant breeding stocks, small proportions of turtles foraging in these locations are supplied by more distant rookeries (*Jensen et al., 2016*; *Limpus, 2008*). The composition of stocks at foraging grounds along the GBR also alters with latitude; northern

foraging grounds are mostly populated with turtles originating from the nGBR breeding stock whilst sGBR and CS stocks are more prominent in southern foraging grounds (*Jensen et al., 2016*).

This latitudinal variance can be observed on a broad scale (north to south, as above) and also on a finer scale (between specific foraging grounds). A major shift in the stock composition between the more northerly Howick Group of islands and the more southerly Edgecumbe Bay (Fig. 1) has been described using a combination of MSA and flipper-tag returns (*Jensen et al., 2016*). While foraging turtles at Edgecumbe Bay were predominantly from the sGBR and CS stocks, turtles at the Howick Group were a mixture of sGBR, CS and nGBR stock. However, there was a large geographic gap in the sampling of foraging grounds between the Howick Group and Edgecumbe Bay spanning six degrees of latitude and approximately 700 km. Assessing the stock composition at foraging grounds within this spatial gap would further refine our knowledge of the latitude at which the composition of green turtles shifts from predominantly sGBR to predominantly nGBR turtles. Furthermore, closing this knowledge gap and combining these results with already published data may provide a means of assessing the relationship between stock composition and latitude. If such a relationship exists, it may provide a means to predict stock composition at other un-sampled foraging grounds in this region. Therefore, in this study, we (1) generated and used mtDNA control region sequences and MSA to quantify the stock composition of green turtles at three foraging areas located between Edgecumbe Bay and the Howick Group, and (2) used our new data and data from previously sampled foraging areas to assess the correlation between stock composition and latitude of foraging areas in Eastern Australian waters.

MATERIALS AND METHODS

Study sites

Green turtles were sampled during separate projects at three foraging grounds within the Great Barrier Reef, Queensland, Australia (Fig. 1). The sites listed below are in north to south order.

Low Isles (LI)

(16°22′S, 145°33′E), situated 15 km off the mainland of North Queensland, comprises two small islands on a shallow coral reef. Turtles at this site were sampled between June 2010 and November 2011.

Green Island (GI)

(16°45′S, 145°58′E), a coral cay located in the northern GBR region approximately 27 km offshore from Cairns, Queensland was sampled in October 2012.

Cockle Bay (CB)

(19°10′S, 146°49′E) is a small bay of Magnetic Island, located approximately eight km offshore from Townsville, the largest tropical city in Australia. Sampling of this site was conducted in August and November of 2012.

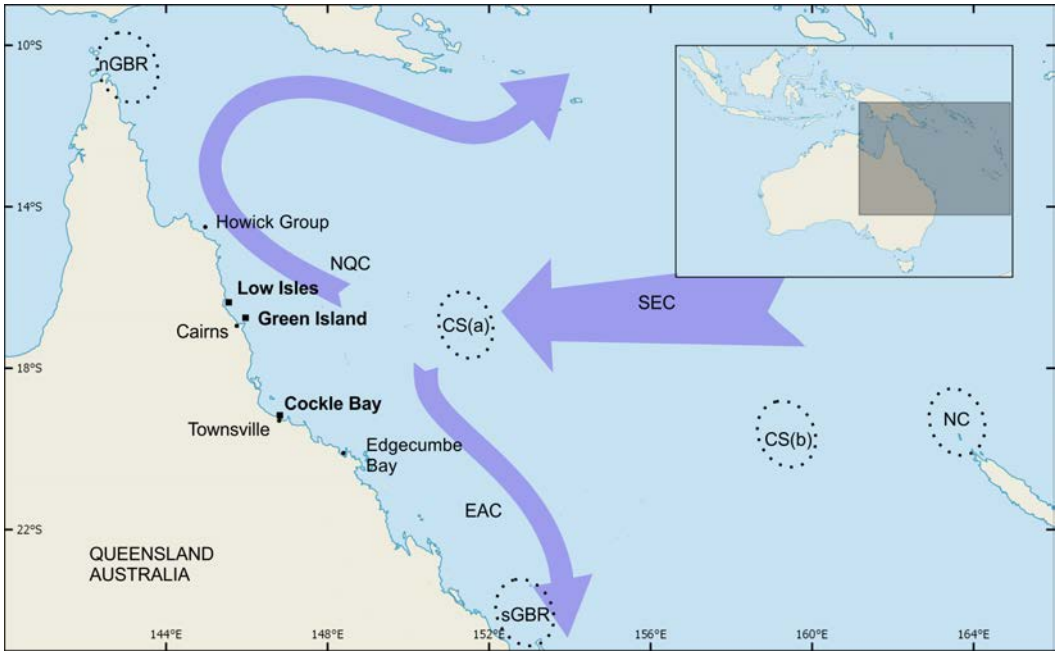

Figure 1 Green turtle foraging sites and genetic stocks of interest to this study. Green turtle foraging sites at Low Isles, Green Island and Cockle Bay were sampled for genetic analysis in the present study. These three sites filled a large geographic gap that existed in prior sampling by *Jensen et al. (2016)*. Broken-line ellipses indicate breeding areas of the following source populations: northern GBR (nGBR), Coral Sea comprised of Coringa-Herald group (CS(a)) and Chesterfield group (CS(b)), southern Great Barrier Reef (sGBR), and New Caledonia (NC). Arrows provide a simplified representation of ocean currents in the region of interest: NQC, North Queensland Current; EAC, East Australian Current; SEC, South Equatorial Current.

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Sample collection

Turtles at all three sites were captured by rodeo method (*Limpus & Reed, 1985*). Captured turtles were flipper-tagged with a unique alpha-numeric inscribed titanium tag (Stockbrands Company, Pty. Ltd., Perth, Western Australia), and had their curved carapace length (CCL $\pm$ 1 mm) measured using a flexible tape measure. Skin samples (approx. 5 $\times$ 5 mm) were collected using a sterilised scalpel for each turtle. At CB and GI the skin samples were taken from the neck and stored in a 20% DMSO solution saturated with NaCl. At LI the samples were collected from the trailing edge of the front flipper and stored in 90% ethanol. Samples from a total of 278 turtles were collected (see Table 1 for details).

Sample collection at Cockle Bay and Green Island was conducted under scientific research permit G12/35326.1 by an appointed conservation officer under the Nature Conservation Act 1992 during population monitoring. Sample collection at Low Isles was conducted under James Cook University Ethics Approval A1474 and scientific research permits G10/33206.1, G10/33897.1 & WISP06563509.

Table 1 Sample demographics. Green turtle demographics from three sampled Great Barrier Reef foraging grounds. The total number of turtles sampled per site (*n*), and number of juvenile (J), sub-adult (SA) and adult (A) turtles within each site are shown. Curved-carapace length (CCL) mean and range are also provided.

Foraging ground	*n*	Size class	Mean CCL (cm)	Range of CCL (cm)
Low Isles (LI)	147	114 J; 33 SA; 0A	55.2	39.7–80.2
Green Island (GI)	57	52 J; 5 SA; 0A	50.4	47.0–84.4
Cockle Bay (CB)	74	58 J; 12 SA; 4A	54.5	40.2–103.9

DNA extraction and Polymerase Chain Reaction (PCR)

Cockle Bay and Green Island

DNA from the CB and GI samples was extracted using the Promega Wizard® SV Genomic DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's instructions. An extra 10 µL of proteinase K was used per reaction. Final DNA concentration was obtained by spectrophotometric analysis, using the ratios of absorption at 260 nm versus 280 nm to determine DNA purity.

The primers ChM-Dloop-960 F (5′-AAC TAT AAC CTT CCT AGA-3′) and ChM-Dloop-960 R (5′-TGT AAG TAT CCT ATT GAT T-3′) were designed to target a 960 bp region of the mtDNA d-loop control region in green turtles. These primers were designed in AlleleID v7 using an alignment of 15 published green turtle sequences. These primers were optimised in conventional polymerase chain reaction (PCR) using a gradient of 50 °C–60 °C.

PCRs were carried out in 20 µL reactions consisting of 10 µL GoTaq Green Hot Start Master Mix (Promega, Madison, WI, USA), 0.8 µM of each primer, ∼80 ng of template DNA and nuclease free water to 20 µL.

The PCR protocol consisted of a 5 min denaturation step (94 °C) followed by 35 cycles of: 10 s at 94 °C, 15 s at 54 °C, and 30 s at 72 °C and a final extension step of 5 min at 72 °C. PCR products were visualised on a 1.2% agarose gel. Following assay optimisation, PCR products were visualised in real time using 20 µL reactions consisting of 10 µL GoTaq qPCR Master Mix (Promega, Madison, WI, USA), 0.8 µM of each primer, ∼80 ng of template DNA and nuclease free water to 20 µL. The qPCR protocol consisted of a 2 min denaturation step (95 °C) followed by 45 cycles of: 10 s at 95 °C, 30 s at 51 °C, and 30 s at 72 °C. These products were then sent to Macrogen (Macrogen Inc., Seoul, Korea) for purification and sequencing using both the forward and reverse primers to initiate sequencing. A consensus sequence was subsequently generated and used in further analysis.

Low Isles

The DNA extraction from LI samples was performed using a salting out procedure, based upon *Sunnucks & Hales (1996)*. Genomic DNA concentration and quality of the LI samples was evaluated through gel electrophoresis in the presence of GelGreen (Biotium, Fremont, CA, USA).

Partial mtDNA d-loop control region (760 bp) was amplified using the primers LTEi9 (5′GAATAATCAAAAGAGAAGG 3′) and H950 (5′GTCTCGGATTTAGGGGTTT 3′)

(*Abreu-Grobois et al., 2006*). PCR was performed in a 25 μL reaction containing 1 × NH_4 Buffer, 1.5 mM MgCl, 0.25 mM dNTPs, 0.4 μM of each primer, 1 Unit of BioTaqTM polymerase and ~10ng DNA. The PCR protocol consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 45 s at 94 °C, 45 s at 52 °C, and 1 min at 72 °C and a final extension step of 5 min at 72 °C. PCR samples were purified and sequenced by Macrogen (Macrogen, Inc., Seoul, Korea) using ABI Dye terminator chemistry on an ABI 3730 sequencer.

Characterisation of mtDNA haplotypes and mixed stock analysis (MSA)

All sequences obtained were assembled in Geneious v7.1.5 (*Kearse et al., 2012*) and confirmed to be the correct target using the database of the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were trimmed to ~770 bp to allow comparison with known green turtle haplotypes in the published literature.

These sequences were then compared with known haplotypes and assigned existing names accordingly. Any sequences from three or fewer turtles which did not match any known haplotypes were re-sequenced to a total of three replicates, in order to avoid sequencing error. Where possible, new template DNA was generated from the original sample. Once confirmed, these new haplotypes were named following the nomenclature for Pacific green turtles using the prefix CmP (*Jensen et al., 2016*).

Haplotype frequency at each site was recorded and haplotype (h) and nucleotide diversity (π) (*Nei, 1987*) were estimated using Arlequin version 3.5.2.2 (*Excoffier, Laval & Schneider, 2005*).

To estimate the proportional contributions of stocks to the three foraging areas, MSA was conducted using a Bayesian approach in the software program Bayes (*Pella & Masuda, 2001*). The mtDNA haplotype frequencies of 25 genetically distinct green turtle breeding stocks across the Indo-Pacific (see Table S1 in *Jensen et al. (2016)*) were used as a baseline. As MSA estimates the proportional contributions of stocks to one feeding ground at a time, each study site was analysed independently. Each analysis consisted of 4 independent chains with different starting points. Each chain was run for a total of 50,000 steps discarding the first 25,000 steps as burn-in. To determine whether all chains had converged we used the Gelman and Rubin shrink factor diagnostic (shrink factor <1.2) (*Pella & Masuda, 2001*). The analysis was conducted with both uniform priors (Model 1) and weighted priors (Model 2). In Model 2 the priors were weighted according to the nesting population size associated with each stock. The results were summarised for both individual stocks and regional estimates grouping the sGBR and CS (sGBR/CS) as well as 21 stocks that all contributed <5% (other).

RESULTS

The sequence data of a 770 bp fragment of the d-loop control region was obtained from 278 individual turtles across three foraging sites. A total of 35 haplotypes were identified, 13 of which had never been observed at a rookery (orphan haplotypes). Eight of this

subset were previously undescribed; one at Cockle Bay (CmP80.4), four at Green Island (CmP234.1, CmP235.1, CmP236.1 and CmP237.1), and three at Low Isles (CmP145.1, 166.2 and CMP211.1). The remaining five haplotypes had been described in previous studies, but had also not yet been observed at a rookery (CmP34.1, CmP55.1, CmP119.1, CmP165.1 and CmP200.1) (Table 2). These orphan haplotypes occurred at low frequencies (2.7–10.5%) and comprised only 6.5% of the total number of turtles sampled. The most common haplotype observed was CmP47.1 at all three sites; CB (73%), GI (67%) and LI (53%) (Table 2). Haplotype and nucleotide diversity both increased from south to north along the GBR, although CB and GI share similar nucleotide diversity (Table 3).

Mixed stock analysis

The MSA showed that the nGBR, CS, sGBR and New Caledonia (NC) stocks supplied the bulk of the turtles at all three sites (>91.6% overall) (Table 4). Small contributions were also made by other more distant green turtle rookeries in the region, but together they made up ∼8% at each site. Both Model 1 (uniform priors) and Model 2 (weighted priors) yielded similar results (Table 4), and for the purpose of simplicity, we only discuss results from Model 2 from hereon. Given the uncertainty surrounding small contribution estimates we grouped rookeries with <5% estimated mean contribution into 'Other'. We were unable to run the MSA for individual age classes due to insufficient sample sizes.

The contribution of NC stocks was approximately equal at all three sites (Table 2), and in all cases was above that which would be considered a small contribution. However, the nGBR, CS and sGBR stock contributions shifted between sites. Turtles at CB, the most southerly site, predominantly originated from sGBR stocks (82.8%, 95% CI [68.9–92.9]), with small contributions from nGBR (8.0%, 95% CI [2.0–16.4]) and CS (0.6%, 95% CI [0.0–5.4]) stocks, respectively. The CS stock was dominant at both GI and LI (approximately 50% to 60%, respectively). As a general trend, the contributions of nGBR stock increased from south to north, whilst the sGBR stock contributions simultaneously decreased. The most dramatic shifts in nGBR stock contributions were observed between GI and LI; nGBR contributions increased from 3.7% (95% CI [0.0–13.3]) at GI to 15.0% (95% CI [8.9–22.3]) at LI and the sGBR contributions decreased from 38.4% (95% CI [0.0–90.5]) at GI to 11.8% (95% CI [0.0–52.0]) at LI. Interestingly, nGBR stock contributions were lower at GI than CB, despite GI being situated more northerly.

The results also indicate a shift in CS stock contributions from CB to GI, which are separated by approximately 280 km. While the CS contribution is low at CB (0.6%), it makes up the majority of turtles at GI (48.6%, 95% CI [0.0–95.0]) and LI (60.0%, 95% CI [19.7–79.0]). In comparison, the contribution of sGBR is highest at CB (82.8%, 95% CI [68.9–92.9]), medium at GI (38.4%, 95% CI [0.0–90.5]) and lowest at LI (11.8%, 95% CI [0.0–52.0]) (Table 4).

These results, combined with previously published reports, were plotted on a chart which shows the stock composition shifting along a latitudinal gradient (Fig. 2). It is possible that this data could be used to predict stock compositions at sites along this gradient that have not been previously sampled.

Table 2 Haplotype frequencies. Haplotype frequencies of green turtles sampled at Cockle Bay, Green Island and Low Isles along the Great Barrier Reef, Australia.

Haplotype name	Accession number	Reference	Location		
			Cockle Bay (CB)	Green Island (GI)	Low Isles (LI)
CmP20.1	AB819806	*Hamabata, Kamezaki & Hikida (2014)*	–	2	1
CmP20.2	KF311744	*Dutton et al. (2014)*	–	–	1
CmP22.1	KF311747	*Dutton et al. (2014)*	1	–	1
CmP40.1	KF311750	*Dutton et al. (2014)*	–	–	2
CmP44.1	KF311751	*Dutton et al. (2014)*	4	3	10
CmP44.2	KF311752	*Dutton et al. (2014)*	–	–	1
CmP47.1	KF311753	*Dutton et al. (2014)*	54	38	78
CmP49.1	AB819808	*Hamabata, Kamezaki & Hikida (2014)*	1	–	–
CmP57.2	KJ502567	*Jensen et al. (2016)*	–	–	3
CmP65.1	KF311756	*Dutton et al. (2014)*	–	–	1
CmP68.1	KJ502591	*Jensen et al. (2016)*	–	–	1
CmP77.1	KF311759	*Dutton et al. (2014)*	–	–	1
CmP80.1	KF311760	*Dutton et al. (2014)*	8	6	19
CmP81.1	KJ502610	*Jensen et al. (2016)*	–	–	2
CmP84.1	KJ502630	*Jensen et al. (2016)*	1	–	1
CmP85.1	KF311761	*Dutton et al. (2014)*	2	1	3
CmP91.1	KF311762	*Dutton et al. (2014)*	–	–	2
CmP98.1	FJ917199	*Dutton et al. (2009)*	–	–	6
CmP168.1	KJ502617	*Jensen et al. (2016)*	–	–	1
CmP169.1	KJ502608	*Jensen et al. (2016)*	1	–	–
CmP180.1	KJ502640	*Jensen et al. (2016)*		–	2
CmP193.1	KJ502635	*Jensen et al. (2016)*	–	1	1
		Total	72	51	137
Orphan Haplotypes					
CmP34.1	KJ502581	*Jensen et al. (2016)*	–	1	–
CmP55.1	KJ502596	*Jensen et al. (2016)*	–	1	4
CmP80.4	MH004276	This study	1	–	–
CmP119.1	KJ502611	*Jensen et al. (2016)*	–	–	1
CmP145.1	MH004277	This study	–	–	1
CmP165.1	KJ502582	*Jensen et al. (2016)*	1	–	–
CmP166.2	MH004278	This study	–	–	2
CmP200.1	KJ502586	*Jensen et al. (2016)*	–	–	1
CmP211.1	MH004283	This study	–	–	1
CmP234.1	MH004279	This study	–	1	–
CmP235.1	MH004280	This study	–	1	–
CmP236.1	MH004281	This study	–	1	–
CmP237.1	MH004282	This study	–	1	–
		Total	2	6	10
		Cumulative total	**74**	**57**	**147**

Table 3 Haplpotype and nucleotide diversity. Sample size (n), number of haplotypes (H) and estimates ($\pm$ SD) of haplotype (h) and nucleotide (π) diversity for three *C. mydas* foraging sites on the Great Barrier Reef, Australia.

Foraging site	n	H	h	π
Cockle Bay	74	10	0.4572 ± 0.0694	0.013573 ± 0.006930
Green Island	57	12	0.5476 ± 0.0772	0.012378 ± 0.006384
Low Isles	147	26	0.6970 ± 0.0396	0.019210 ± 0.009563

Table 4 Mixed stock analysis results of 278 turtles from three foraging grounds along the Great Barrier Reef. Results (mean% ± 95% confidence intervals in parentheses) from the Bayesian mixed stock analysis (MSA) (*Pella & Masuda, 2001*) for Cockle Bay, Green Island and Low Isles Green Turtles (both individually and by region). MSA was calculated using 25 regional breeding stocks as possible sources, but for simplicity only the four main contributors are listed—nGBR, northern Great Barrier Reef; sGBR, southern Great Barrier Reef; CS, Coral Sea and NC, New Caledonia. The combined contributions of the remaining 21 stocks are compiled into the 'Other' category. Model 1, uniform priors; Model 2, weighted priors.

		Cockle Bay		Green Island		Low Isles	
	Stock	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
Individual	nGBR	7.5 (1.7–15.8)	8.0 (2.0–16.4)	1.5 (0.0–10.1)	3.7 (0.0–13.3)	14.7 (8.7–21.7)	15.0 (8.9–22.3)
	CS	2.5 (0.0–29.4)	0.6 (0.0–5.4)	50.4 (0.9–92.8)	48.6 (0.0–95.0)	60.1 (20.3–78.1)	60.0 (19.7–79.0)
	sGBR	79.7 (53.8–91.3)	82.8 (68.9–92.9)	33.7 (0.0–85.2)	38.4 (0.0–90.5)	10.8 (0.0–50.4)	11.8 (0.0–52.0)
	NC	7.3 (0.0–19.4)	6.9 (0.0–19.8)	9.4 (0.0–23.6)	6.1 (0.0–22.0)	6.0 (1.5–13.2)	6.3 (1.5–13.7)
	Other	3 (0.1–8.7)	1.7 (0.0–6.5)	5.0 (0.3–12.8)	3.2 (0.0–11.6)	8.4 (3.9–14.0)	6.9 (2.8–12.3)
Regional	nGBR	7.5 (1.7–15.8)	8.0 (2.0–16.4)	1.5 (0.0–10.1)	3.7 (0.0–13.3)	14.7 (8.7–21.7)	15.0 (8.9–22.3)
	sGBR/CS	82.2 (70.1–91.7)	83.3 (70.8–93.0)	84.1 (70.2–94.3)	87.0 (73.2–96.4)	70.9 (61.8–79.1)	71.8 (62.6–80.0)
	NC	7.3(0.0–19.4)	6.9 (0.0–19.8)	9.4 (0.0–23.6)	6.1 (0.0–22.0)	6.0 (1.5–13.2)	6.3 (1.5–13.7)
	Other	3.0 (0.1–8.6)	1.9 (0.0–6.5)	5.1 (0.4–12.9)	3.1 (0.0–11.6)	8.4 (4.0–14.0)	6.9 (2.8–12.3)

DISCUSSION

Previous studies indicated that foraging grounds along the GBR are dominated by the nGBR, sGBR and Coral Sea genetic stocks and that the proportions of those stocks change gradually from north to south (*Dethmers et al., 2006*; *Jensen et al., 2016*). However, a 700 km unsampled gap separated foraging grounds of predominantly nGBR stocks (the Howick Group) and foraging grounds further south where only a small proportion of nGBR turtles were observed (Edgecumbe Bay) (*Jensen et al., 2016*) (Fig. 1). This sampling gap precluded informed management regarding the stocks that might be impacted at foraging grounds along the central part of the GBR and was therefore the focal area of our study.

Due to the high degree of genetic similarity between the CS and sGBR stocks, the MSA estimates for these stocks are surrounded by high uncertainty. In order to address this, we combined the summary statistics of these genetically similar stocks. However, *Read et al. (2015)*, who also utilised MSA to study turtles in the Indo-pacific region, reported summary statistics for individual stocks. To make our results comparable with both the *Jensen et al. (2016)* and *Read et al. (2015)* studies, we present the summary statistics for both individual stocks, as well as the combined CS/sGBR stock (Table 4).

Our results show that gradual changes in stock contribution occur between CB and LI. The combined sGBR/CS stock foraging at CB and GI made up a smaller proportion

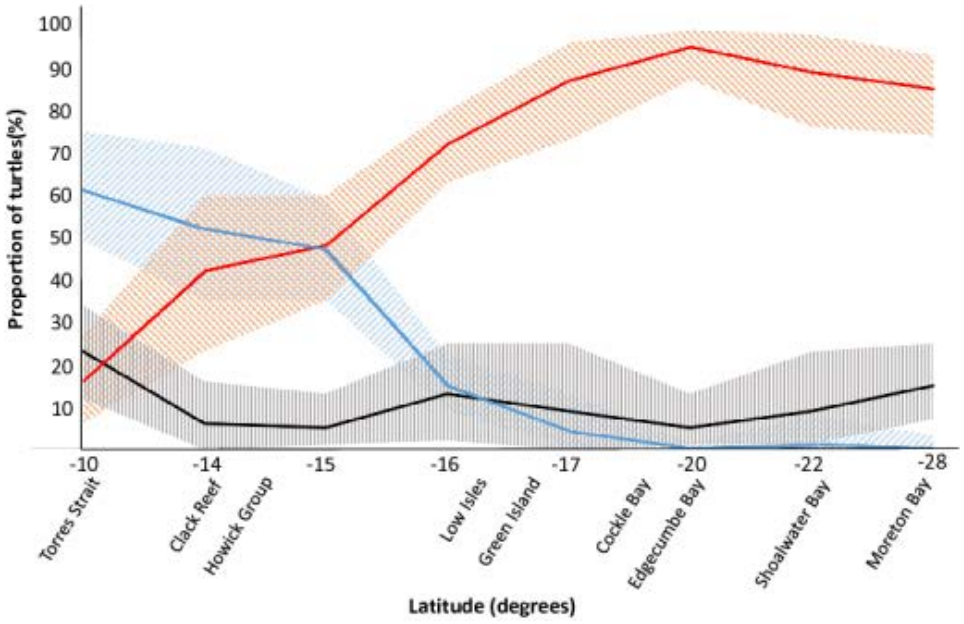

Figure 2 The latitudinal spread of the main genetic stocks on the Great Barrier Reef. For green turtle aggregations at selected foraging grounds in the Great Barrier Reef (GBR) and southern Queensland, Australia, the proportional contributions of three important genetic sources showed a notable relationship with latitude. The southern GBR (sGBR) and Coral Sea (CS) stocks were combined for this figure to allow comparison with *Jensen et al. (2016)* and are denoted in orange. The nGBR stock (northern GBR) is represented in blue and 'Other' stocks, represented in black; hatched areas in all three cases represented 95% confidence intervals. The 'Other' group comprises the remaining 22 stocks in this region (see *Jensen et al., 2016*) and were combined because these stocks were found to contribute a small proportion of the turtles at each study site. Data for Low Isles, Green Island and Cockle Bay from the present study and data for all other sites are from *Jensen et al. (2016)*.

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(83–87%) at these sites compared to the proportion observed at the more southerly Edgecumbe Bay (95%) (*Jensen et al., 2016*). This proportion decreased further at the more northern LI (72%). The contrary was evident for the nGBR stock that declined from making up half of the juvenile turtles foraging at the Howick group (*Jensen et al., 2016*) to 15% at LI and decreasing further at GI and CB (4% and 8%, respectively) to 0% at Edgecumbe Bay. In addition, we found that all three study sites (LI, GI and CB) were comprised of a small portion (6–7%) of turtles from the New Caledonia stock, which is derived from rookeries more than 1800 km away. These findings are consistent with both tag-recovery data and MSA results from other studies, suggesting that New Caledonia turtles use multiple feeding grounds along the Great Barrier Reef (*Read et al., 2014*; *Read et al., 2015*; *Jensen et al., 2016*). Interestingly, whilst CS stock was found to contribute a large proportion of the turtles at our study sites, this stock was found to contribute only a small proportion of turtles foraging in New Caledonia (*Read et al., 2015*).

The shift in the composition of regional stocks at foraging areas along the Great Barrier Reef may in part be explained by ocean currents, as has been suggested for mixed stocks of marine turtles in in other regions (*Blumenthal et al., 2009*; *Carreras et al., 2006*; *Lahanas*

et al., 1998; *Luke et al., 2004*). The three foraging grounds sampled in our study (LI, GI and CB) are geographically situated near an area of variable currents (*Choukroun et al., 2010*) associated with the South Equatorial Current dividing into the south-flowing East Australian Current and the north flowing North Queensland Current (Fig. 1). Such a split is likely to influence the dispersal of new recruits approaching the Australian east coast following their oceanic phase as they move towards their neritic foraging areas. The high proportion of CS stock observed at our study sites on the GBR compared to the low proportion of this stock observed at New Caledonia (*Read et al., 2015*) further supports this theory. However, the mechanisms of how these new recruits settle at neritic foraging areas are not known and would be a worthy avenue for future research.

While the vast majority of sampled turtles came from rookeries within the GBR region, Coral Sea and New Caledonia, a small proportion of turtles came from more distant rookeries. The latter stocks were grouped collectively into the 'Other' category. However, the distribution of specific haplotypes at regional rookeries reveal their likely origin. For example, CmP20.1 is common throughout Micronesia, CmP22.1 in the Marshall Islands and CmP65.1 has only been found in American Samoa and French Polynesia (see *Dutton et al., 2014*; *Hamabata, Kamezaki & Hikida, 2014*). In this study, these haplotypes were infrequently found; two turtles at GI and one turtle at LI were found to be CmP20.1, while CmP22.1 was found once at both CB and LI. One turtle at CB was found to be CmP65.1, making this the first known record of this haplotype on the GBR. These rare long-distance dispersal events are supported by tag returns from turtles as far as the Marshall Islands foraging along the GBR (*Limpus, Bell & Miller, 2009*).

We identified 13 orphan haplotypes across all three sites and encountered them more frequently in the more northerly sites. These haplotypes were distributed as CB:2, GI:6 and LI:6, with one orphan haplotype (CmP55.1) present at both GI and LI. Eight of these haplotypes were previously undescribed whilst five others had been described in previous studies, but had not yet been observed at a rookery (*Jensen et al., 2016*). Orphan haplotypes at GI were found to comprise nearly 11% of all turtles sampled. These orphan haplotypes indicate that some of the known rookeries may require larger sample sizes to accurately capture the haplotype composition. It is also possible our study sites may have received turtles from unidentified and unsampled rookeries that might exist in south-east Asia or the south-western Pacific. While these orphan haplotypes highlight the need for additional sampling of green turtle rookeries in the region, it is encouraging that they only comprise a small percentage (<6.5%) of our total data set.

The Chm-dloop 960 primer set described here is specific to green turtles and can be used to obtain a longer (960 bp) fragment of the d-loop control region, thereby allowing for an improved resolution. Many of the haplotypes in this study are shared between a number of stocks (e.g., CmP80.1 is found in the nGBR, sGBR, Coral Sea and New Caledonia stocks). However, when analysing the longer fragment of mtDNA, this haplotype could be consistently split into two distinct haplotypes and potentially add resolution to the stock structure of those populations. Therefore, future studies may benefit from using this assay, or preferably designing primers that target the entire d-loop region. In particular, this increased resolution may aid in resolving any uncertainty in separating the sGBR and CS

stocks in the MSA. Moreover, such work may allow researchers to more reliably distinguish the region of origin for particular haplotypes (for example, tracing a certain haplotype back to one stock instead of four).

As marine turtles have a complex life history, it is important that conservation strategies target the full range of life stages and habitats used by these turtles. In order to effectively manage threats to green turtles, we must understand the size of the stocks and the factors that are threatening them (*Hamann et al., 2010*). The identification of individual green turtle stocks present on the GBR has greatly improved the monitoring and management of this species by allowing a more targeted approach. Each stock is considered to be a separate management unit that is demographically independent, hence a decline in one stock would not be replenished by another (*Dobbs, 2001*; *Waldman, 2005*). As a result of unsustainable commercial harvesting of green turtles in the southern GBR in the early to mid 1900s (*Limpus, 2008*), the sGBR stock presumably declined. While the sGBR populations are presently recovering (*Chaloupka et al., 2008*; *Department of Environment Energy, 2016*; *GBRMPA, 2014*; *Limpus, 2008*), the pressure from historical consumptive use may have affected the distribution of this stock or the composition of different age classes. Similarly, the nGBR stock has demonstrated a plateau and there is the potential for a decline in population size due to decreased hatchling success at Raine Island (*Chaloupka et al., 2008*; *GBRMPA, 2014*; *Limpus et al., 2003*). This may already be reflected in the results from the present study, and it is likely that the nGBR contributions to these foraging grounds will decrease further in the future, increasing the urgency for effective conservation strategies which target threats to this stock. Our work confirms that threats to green turtles which occur in GBR foraging areas north and south of Low Isles will predominantly affect the nGBR stock and sGBR/CS stocks respectively. In the present study, we also show that the CS stock likely contributes significant proportions of turtles at both LI and GI with approximately half of the GI green turtles identified as CS stock. This alone indicates that in order to effectively protect green turtles residing in this region of the GBR, we must extend monitoring and conservation efforts to include the CS rookeries because there are currently no monitoring data available for the Coral Sea, making it difficult to know the status of this stock.

CONCLUSIONS

The GBR supports a large number of foraging marine turtles, yet monitoring has only occurred at a small number of sites because monitoring programs (and associated studies such as ours) in this region are often logistically challenging to establish and maintain, requiring both considerable funding and uniquely-skilled persons. Our data provides confirmation and improved resolution to show the current latitudinal spread of haplotypes of turtles inhabiting the GBR. Turtles at foraging sites north of the Howick Group are more likely to originate from the nGBR stock while turtles foraging south of LI appear more likely to come from CS and/or sGBR stock. These distribution patterns could potentially be influenced by declines or increases in nesting success at the major rookeries in the future and should therefore be regarded as a representation of the current situation. However, the

steady shift in stock composition highlighted in this paper (Fig. 2) may provide a means to predict the stock composition at other un-sampled foraging grounds in this region in order to make more informed management decisions while circumventing the need to sample and assess additional locations. Continued monitoring of these stocks will allow managers to develop targeted management plans and effectively conserve this iconic species.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Karina Jones conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Michael Jensen analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Graham Burgess, Lynne van Herwerden and Mark Hamann conceived and designed the experiments, contributed reagents/materials/analysis tools, approved the final draft.
- Johanna Leonhardt conceived and designed the experiments, performed the experiments, analyzed the data, approved the final draft.
- Julia Hazel conceived and designed the experiments, contributed reagents/materials/-analysis tools, prepared figures and/or tables, approved the final draft.
- Ian Bell contributed reagents/materials/analysis tools, approved the final draft, substantial fieldwork (data and sample collection).
- Ellen Ariel conceived and designed the experiments, contributed reagents/materials/-analysis tools, authored or reviewed drafts of the paper, approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

Sample collection was conducted under James Cook University Ethics Approval A1474.

Field Study Permissions

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Sample collection at Cockle Bay and Green Island was conducted under scientific research permit G12/35326.1 by an appointed conservation officer under the Nature Conservation Act during population monitoring. Sample collection at Low Isles was conducted under scientific research permits G10/33206.1, G10/33897.1 & WISP06563509.

Data Availability

The following information was supplied regarding data availability:

The newly described haplotypes were available at GenBank and all accession numbers have been listed in Table 2 (MH004276, MH004277, MH004278, MH004283, MH004279, MH004280, MH004281 and MH004282).

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ARTICLE

Phylogenetic Variation of Chelonid Alphaherpesvirus 5 (ChHV5) in Populations of Green Turtles *Chelonia mydas* along the Queensland Coast, Australia

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Abstract

Sea turtle fibropapillomatosis (FP) is a disease marked by the proliferation of benign but debilitating cutaneous and occasional visceral tumors, likely to be caused by chelonid alphaherpesvirus 5 (ChHV5). This study presents a phylogeny of ChHV5 strains found on the east coast of Queensland, Australia, and a validation for previously unused primers. Two different primer sets (gB-1534 and gB-813) were designed to target a region including part of

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the *UL27* glycoprotein B (gB) gene and part of *UL28* of ChHV5. Sequences obtained from FP tumors found on juvenile green turtles *Chelonia mydas* (<65 cm curved carapace length) had substantial homology with published ChHV5 sequences, while a skin biopsy from a turtle without FP failed to react in the PCRs used in this study. The resulting sequences were used to generate a neighbor-joining tree from which three clusters of ChHV5 from Australian waters were identified: north Australian, north Queensland, and Queensland clusters. The clusters reflect the collection sites on the east coast of Queensland with a definitive north–south trend.

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